Reduced cholesterol transmucosal transport in rats with inhibited mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function

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Abstract Absorption of cholesterol during inhibition of mucosal acyl CoA:cholesterol acyltransferase was studied in mesenteric lymph fistula rats with normal pancreatic function. The specific inhibitor used (Sandoz Compound 58-035; 3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide) greatly reduced cholesterol esterification in vitro and decreased lymphatic secretion of esterified cholesterol in vivo, but did not affect triglyceride metabolism by the gut in vitro or in vivo. During steady state lymphatic transport of cholesterol, unesterified cholesterol was increased and cholesteryl esters were decreased in whole lymph, lymph chylomicrons, and very low density lipoproteins. Incorporation of labeled cholesterol infused into the lumen into cholesteryl esters of lymph very low density lipoproteins was particularly suppressed. Labeled cholesterol incorporation into individual cholesteryl esters differed and was differentially affected when total cholesteryl ester synthesis was inhibited. The results support a major regulatory role for mucosal acyl CoA:cholesterol acyltransferase in cholesterol absorption and imply differences in the metabolism of endogenous and exogenous cholesterol by the intestinal mucosa.—**Bennett Clark, S., and A. M. Tercyak.** Reduced cholesterol transmucosal transport in rats with inhibited mucosal acyl CoA:cholesterol acyltransferase and normal pancreatic function. *J. Lipid Res.* 1984. **25:** 148–159.

Supplementary key words lymph • chylomicrons • very low density lipoproteins • intestine • lipids • absorption

Cholesterol absorbed by the small intestine is largely esterified before entering mesenteric lymph, incorporated into lymph lipoproteins (LP). The precise mechanism responsible for cholesterol esterification by the intestinal mucosa has been the subject of some debate recently. The traditional view, originally expressed by Swell et al. (1) and subsequently developed by Borja, Vahouny, and Treadwell (2) and by Gallo and co-workers (3–5), states that pancreatic cholesterol ester hydrolase (CEH) is absorbed into the mucosal cell cytosol where it catalyzes cholesteryl ester (CE) synthesis. This view was subsequently challenged by Norum and co-workers (6–11), by Bennett Clark (12), by Field, Cooper, and Erickson (13), and by Heller (14), all of whom suggested that esterification of cholesterol absorbed by the intestinal mucosa proceeds by a coenzyme A-dependent mechanism (ACAT), similar to cholesteryl ester formation in other tissues (15).

The question of which esterifying enzyme (CEH or ACAT) is regulatory for cholesterol absorption by the small intestine can, theoretically, be answered by in vivo experiments in which one, and only one, of the enzymes is specifically inactivated. If cholesterol absorption and esterification are reduced, then the enzyme whose action was prevented must play a regulatory role. Recently such an approach has been undertaken in a series of studies, some of which have been reported in abstract form (16–18). On the one hand, CEH activity was removed from pancreatic juice by precipitation with a specific IgG directed against purified CEH (4). Pancreatic fistula rats that received this juice showed greatly reduced cholesterol absorption into mesenteric lymph, to about one-fifth of control levels. However, the absorbed cholesterol was highly esterified (17), suggesting that while the absence of CEH may reduce the uptake of cholesterol into mucosal cells, esterification of cholesterol that has entered the mucosa probably occurs by an alternative mechanism. Absorption of small amounts of cholesterol with normal esterification in the absence of pancreatic juice has been clearly demonstrated also by Watt and Simmonds (19). The alternate experiments, which analyze the effect on cholesterol absorption and esterification of inhibiting mucosal ACAT, are the subject of the present report. The studies were conducted in mesenteric lymph fistula rats and the transfer of [^{3}H]cholesterol into lymph after either a gastric dose or during a duodenal infusion of

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; MGAT, monoglyceride acyltransferase; CEH, pancreatic cholesterol hydrolase; FA, fatty acid; CE, cholesteryl esters; UC, unesterified cholesterol; TG, triglyceride(s); DG, diglyceride(s); MG, monoglyceride(s); PL, phospholipid; LP, lipoprotein(s); CM, chylomicrons; VLDL, very low density lipoproteins; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high performance liquid chromatography.

cholesterol was monitored. Earlier, we reported (18) that cholesterol absorption determined by the dual-isotope method of Zilversmit and Hughes (20) was significantly reduced by administration of an ACAT inhibitor to intact rats.

MATERIALS AND METHODS

Animals

Two main in vivo approaches were used. In the first approach (Expt. 1), eleven male Sprague-Dawley rats weighing 280–310 g were provided with indwelling gastric and mesenteric lymphatic cannulas, under Nembutal anesthesia. All surgery was performed between 8–10 AM, in nonfasting rats that had been previously fed Purina rat chow ad libitum while on a normal light cycle. The rats were placed in restraint cages in a warm room and infused intragastrically with 0.85% NaCl–0.03% KCl at 2.6 ml/hr for 46–48 hr. Lymph flow was monitored continuously throughout the experiment. Gastric doses of the ACAT inhibitor (3-(decyldimethylsilyl)-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide, Sandoz Compound No. 58-035, 20 mg/kg) or the suspending vehicle without the drug (see below) were administered through the gastric cannula at 6 PM on the postoperative day, and again at 8 AM on the following day. Two hours later, each rat received a gastric dose of [^{3}H]cholesterol (10 mg, 2 μCi) and oleic acid (22 mg), emulsified in 2 ml of a solution of sodium taurocholate (10 mM) and bovine serum albumin (BSA) (10 mg/ml) in isotonic saline. Lymph for analysis was collected at 0°C for 1 hr before the second drug dose (basal) and 0–2, 2–4, 4–6, and 6–8 hr after the [^{3}H]cholesterol dose. The rats were then killed by cervical dislocation, the jejunum was thoroughly washed by flushing with cold saline and scraped off, and microsomes were prepared and frozen at −80°C.

In the second in vivo approach (Expt. 2), nine other rats received two indwelling duodenal cannulas and a cannula for mesenteric lymph collection. Postoperative maintenance was similar to that of Expt. 1 except that 2% glucose was added to the 2.7 ml/hr saline infusion during the 40–43 hr recovery period. On the experimental day a basal lymph collection was obtained between 5–6 AM. At 6 AM, an 8-hr duodenal infusion of the 58-035 suspension, (0.875 ml/hr = 17.5 mg 58-035/hr) or the suspending vehicle alone, was begun while the saline-glucose infusion was continued through the other duodenal cannula. Two hours later a [^{3}H]cholesterol-safflower oil emulsion (see below) replaced the saline-glucose infusion at the same rate; the cholesterol infusion rate was 1.23 mg/hr. The lipid emulsion was infused for exactly 6 hr, the rats were then killed by cervical dislocation, and the contents of the stomach, small intestine, and cecum were quantitatively recovered by repeated washing with ice-cold saline. Microsomes were prepared from scraped jejunal mucosa and frozen at −80°C. Lymph for analysis was collected at 0°C during the 1-hr basal period prior to beginning the drug infusion and for four subsequent 2-hr collection periods. Aliquots (5 ml) of the final lymph collection were separated further into chylomicrons (CM), VLDL, and an HDL fraction (see below).

Analytical

Lymph

Lymph was collected into calibrated ice-cooled tubes free of additives; the volumes were recorded and the lymph was defibrinated. Aliquots were taken immediately for liquid scintillation counting and for total lipid extraction (21). Where indicated, lymph aliquots were separated into CM, VLDL, and an HDL fraction as follows. Five ml of lymph (d 1.03 g/ml) was overlayered with 7 ml of d 1.006 g/ml NaCl solution in a 12-ml ultracentrifuge tube and centrifuged at 30,000 rpm for 30 min at 4°C in a swinging bucket rotor (SW41). The top 1 ml containing packed CM was harvested by tube slicing. The 6-ml cloudy layer immediately below the CM layer was designated VLDL, but probably also contained some small CM. The bottom layer, equal to the original lymph volume (5 ml), was treated with dextran sulfate-$MgCl_2$ to precipitate any remaining apoB-containing LP (22). The precipitate was removed by centrifugation at 4°C (15 min, 15,000 rpm, Sorvall SS34 rotor) and the clear supernatant was designated HDL. The precipitate contained negligible radioactivity and was not analyzed further. Aliquots of CM, VLDL, and HDL were taken for liquid scintillation counting and for total lipid extraction.

Enzymes

Microsomes were prepared at 0–4°C from jejunal mucosa by standard methods. Briefly, after thorough washing, the proximal half of the intestine distal to the ligament of Treitz was slit open on an ice-cooled glass plate and the mucosa was scraped off with a glass microscope slide. To remove mucus, the mucosa (ca. 2 g) was dispersed gently in 30 ml of isotonic saline and centrifuged briefly (10 min, 1000 rpm, Sorvall SS34 rotor). The loosely packed sediment was transferred to a Dounce homogenizing tube containing 18 ml of 0.25 M sucrose–10 mM Tris, pH 7.4, and the cells were broken by 10 passes with a loose plunger followed by 20 passes with a tightly-fitting plunger. The homogenate was centrifuged for 15 min at 7,000 rpm (Sorvall SS34) and microsomes were sedimented by centrifugation of the resulting supernatant at 40,000 rpm for 1 hr in a 70 Ti rotor. The microsomes were dispersed in 2 ml of pH 7.4 potassium phosphate buffer, 0.2 M, (KPB) to yield a protein concentration of

about 5 mg/ml; 0.5-ml aliquots were frozen at −80°C for up to 3 weeks. Because control and experimental rats were perfused in pairs, the time of storage at −80°C before microsomal enzymes were assayed could be counterbalanced across the two groups by performing all enzyme assays on the same day.

Acyl CoA cholesterol acyltransferase (ACAT). The incorporation of oleyl CoA into cholesteryl esters was followed, essentially as described by Norum, Helgerud, and Lilljequist (10). For rats that received the continuous duodenal infusion of [^{3}H]cholesterol (Expt. 2), nonradioactive oleyl CoA was used and cholesteryl ester (CE) formation was determined from the increase in ^{3}H-labeled CE that occurred during incubation of the [^{3}H]cholesterol-labeled microsomes. For the rats in Expt. 1 (gastric [^{3}H]cholesterol), whose microsomal cholesterol was inadequately labeled with ^{3}H, [^{14}C]oleyl CoA was used and the incorporation of [^{14}C]oleate into CE was determined. Similar microsomal ACAT specific activities, expressed as nanomoles of CE formed per min per mg microsomal protein (see Results), were found in control rats of Expt. 1 and Expt. 2, implying that the two methods for measuring ACAT were equivalent.

Monoglyceride acyl transferase (MGAT). The radioactive substrate method of Rodgers (23) was adopted except that [^{14}C]oleyl CoA replaced [^{14}C]palmitoyl CoA. Because the monoglyceride substrate used contained both 1- and 2-MG, radioactive di- and triglyceride products were summed to calculate MGAT specific activities (pmol of substrate incorporated per min per mg microsomal protein).

Pancreatic cholesterol ester hydrolase (CEH). Fresh homogenates of rat pancreas and jejunal mucosa were assayed for CEH activity by standard methods (24).[1] To determine the effect of adding 58-035 in vitro on CEH activity, various amounts of the drug, from 10 to 1000 μg suspended in 1.5% aqueous carboxymethyl cellulose or dissolved in DMSO were added to aliquots of fresh pancreatic homogenate; control flasks received equal volumes of the dispersing agents. To determine whether CEH absorbed into the mucosa (5) would be inhibited by the oral ingestion of 58-035, jejunal mucosa obtained from two rats that had received a gastric dose of 58-035 (20 mg/kg in 0.25 ml of 1.5% carboxymethyl cellulose) 2 hr earlier and from two control rats given 0.25 ml of the vehicle without drug was assayed for CEH activity.

Chemical

Lipids, extracted from lymph and tissue samples by the Folch procedure (21), were further separated by thin-layer chromatography (TLC), gas–liquid chromatography (GLC), and high performance liquid chromatography (HPLC) as described below. Lipid phosphorus was determined in aliquots of Folch extracts (chloroform–methanol 2:1) as described by Bartlett (25). Protein was determined according to Lowry et al. (26) and lymph triglycerides were quantitated by an enzymatic kit method (Sigma Chemical Co.). All liquid scintillation counting was performed in Aquasol (after evaporation of organic solvent when present) in a Beckman 2 channel liquid scintillation spectrometer (LS-250). Appropriate corrections were applied to transform cpm to dpm for single label (^{3}H or ^{14}C) or double label (^{3}H + ^{14}C) counting, as applicable (27).

TLC. Unesterified and esterified cholesterol were separated on silica gel H using petroleum ether–diethyl ether–acetic acid 85:15:1.5. For ACAT assays, the CE band was scraped directly into counting vials. When further separation of CE's by HPLC was performed, the CE's were quantitatively extracted from the silica gel with 5 ml of methanol followed by two elutions with 5 ml of chloroform–methanol 2:1.

Neutral lipids were separated on silica gel H using a dual solvent system (28). For MGAT assays all fractions were scraped and counted directly (origin, FA, MG's, DG's, TG, and CE).

GLC. Total (TC) and unesterified cholesterol (UC) were quantitated as described earlier (29). Stigmasterol, added prior to lipid extraction, was used as an internal standard. Esterified cholesterol was calculated from the difference between the two determinations (TC − UC).

The FA composition of lipoprotein CE's was determined after hydrolysis of the CE fraction obtained by TLC followed by methylation (30) and separation of the FA methyl esters on a 6 ft 5% DEGS column at 170°C.

HPLC. Cholesteryl esters were separated by reverse-phase HPLC as described by Carroll and Rudel (31). To determine radioactivity, peaks were collected directly into counting vials and solvents were removed by evaporation under nitrogen prior to addition of Aquasol. Individual CE's were quantitated by integration of peak areas obtained at 213 nm, using calibration curves established separately for each CE species.

Emulsions

Compound 58-035. A 1.5% aqueous solution (vol/vol) of carboxymethyl cellulose containing 0.2% Tween 80 was prepared by stirring at room temperature until clear (2–3 hr). To 100 ml was added 2.0 g of 58-035 and the mixture was stirred vigorously (magnetic stirrer) for several days until a stable suspension was obtained.

[^{3}H]Cholesterol-albumin. Ten μCi of [1,2-^{3}H]cholesterol, 50 mg of cholesterol, and 125 μl of oleic acid were mixed in a Potter-Elvehjem homogenizing tube in a small amount

[1] Performed by Dr. Linda L. Gallo, George Washington University, Washington, DC.

of benzene which was then evaporated. To this was added 10 ml of a 10 mM sodium taurocholate-isotonic NaCl solution containing 100 mg of BSA. The mixture was homogenized repeatedly, alternating with vortexing for about 1 hr, until a homogeneous isotope distribution was obtained; it was stored at 15°C. Rats in Expt. 1 received a 2-ml gastric bolus of this emulsion, equivalent to 10 mg of cholesterol.

[^{3}H]Cholesterol–safflower oil. The following concentrations were infused intraduodenally to rats of Expt. 2: safflower oil TG, 16.1 μmol/ml; Tween 80, 5 mg/ml; sodium taurocholate, 2 mM; cholesterol, 0.45 mg/ml; [^{3}H]cholesterol, 0.2 μCi/ml. The cholesterol (labeled and carrier), bile salt, and Tween 80 were placed in a 500-ml beaker and solvents were removed under N_2. Isotonic NaCl (50 ml) was added and the mixture was stirred vigorously (magnetic stirrer) at room temperature until a clear solution was obtained (3–4 hr). A further 200 ml of saline and 6 ml of safflower oil were then added and this mixture was sonicated intermittently until a homogeneous stable emulsion was obtained. The emulsion was stored at 15°C and required only minimal shaking just before use to restore uniformity. At 2.7 ml/hr, the rats received 3.2 μmol of [^{3}H]cholesterol/hr (sp act 2.2×10^5 dpm/μmol) and 43.5 μmol of TG/hr.

Materials

[1,2-^{3}H]Cholesterol, [1-^{14}C]oleoyl coenzyme A, and Aquasol were purchased from New England Nuclear (Boston, MA). Sprague-Dawley rats were from Charles River Breeding Laboratories (Wilmington, MA). Sodium taurocholate, dextran sulfate (mol wt 500,000), oleoyl coenzyme A, monoglyceride, oleic acid, and fatty acid-poor BSA were from Sigma Chemical Company (St. Louis, MO). Cholesterol was from Nu-Chek-Prep (Elysian, MN). Carboxymethylcellulose was from Pfaltz & Bauer (Stamford, CT). Tween 80 and HPLC grade solvents were from Fisher Scientific (Pittsburgh, PA). Safflower oil was purchased from a commercial supplier. All other chemicals were of analytical grade.

Calculations

Statistical comparison between drug-treated and control groups were by Student's *t*-test, paired *t*-test, or analysis of variance with trend analysis, as appropriate (32).

RESULTS

Specificity of action of 58-035

Three independent approaches established that CE synthesis by the jejunal mucosa was inhibited by 58-035, but that TG formation was not affected. First, addition of 58-035 to jejunal microsomes in vitro produced a profound reduction in oleyl CoA incorporation into CE (ACAT activity), with 55% inhibition upon addition of 1.8 μg of 58-035 per mg microsomal protein (0.6 μg/ml medium). There was no reduction of oleyl CoA incorporation into DG and TG (MGAT activity) even when 20-fold greater concentrations of the drug were used (**Fig. 1**). In this experiment both enzymes were assayed simultaneously in the same sample of microsomes prepared from a rat fasted for 48 hr.

Secondly, microsomes obtained from rats 2 hr and 10 hr after a 20-mg oral dose of 58-035 had the same range of MGAT specific activities (33–55 pmol/min per mg protein at 2 hr vs 38–50 at 10 hr). These values were similar to those found in control rats that received only the vehicle (58 ± 5 for controls, compared with 50 ± 6 after 58-035; mean ± SE).

Thirdly, lymph fistula rats that received a duodenal infusion of safflower oil at 43.5 μmol TG/hr with cholesterol (Expt. 2) transported similar amounts of TG in mesenteric lymph in 6 hr, whether receiving 20 mg/hr of 58-035 or the vehicle alone (64 ± 8 vs 72 ± 6 percent

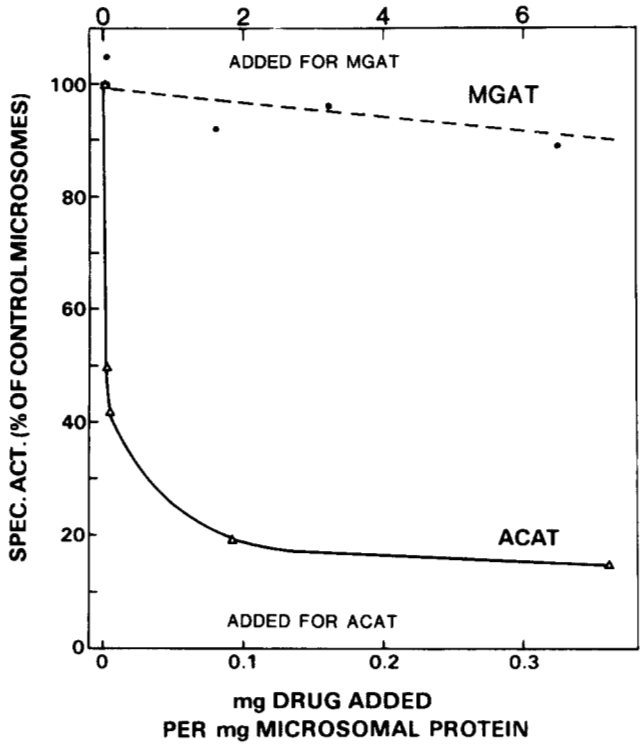

Fig. 1. Effect of adding 58-035 in vitro on ACAT and MGAT activities of jejunal microsomes. Microsomes were prepared from the jejunum of a 48-hr fasted rat. Compound 58-035 dissolved in 10 μl of DMSO was added to 300 μl of incubation mixture for MGAT and to 500 μl of incubation mixture for ACAT. Control incubations received 10 μl DMSO. Enzyme assays were performed as described in Methods. Reactions were initiated by the addition of [^{14}C]oleyl CoA, after a 2-min preincubation of all other components at 37°C.

of TG infused, $P > 0.05$). This TG transport rate compares favorably with previous studies (33). Hence, neither MG esterification by mucosal cells nor overall TG absorption into lymph was reduced by 58-035 under conditions that profoundly reduced mucosal ACAT activity (see Fig. 1 and below).

Compound 58-035 also had no effect on the CEH activity of rat pancreas or jejunal mucosa. CEH determined in a fresh homogenate of rat pancreas was unaltered by the in vitro addition of up to 0.2 mg of 58-035/ml of incubation medium. Also, the cytosolic CEH activity of jejunal mucosa obtained from two rats 2 hr after a gastric dose (20 mg/kg), although somewhat lower than in two controls, was within the normal range, whereas ACAT activity in the same homogenate was reduced to 24% and 46% of that found in the control rats.

Finally, 58-035 did not retard the solubilization of cholesterol from an emulsion into a micellar solution. In this experiment aliquots of [^{3}H]cholesterol-albumin were mixed with either the 58-035 suspension, the vehicle, or saline, in the proportions used in Expt. 1, and the mixtures were dialyzed against a 16 mM sodium taurocholate solution at 37°C. Samples of dialysate were removed at timed intervals up to 24 hr and their radioactivity was determined. Equilibration of label was achieved between 30–60 min in all three incubations, and, if anything, the presence of 58-035 accelerated the dialysis rate of [^{3}H]cholesterol slightly.

Absorption of a gastric bolus dose of cholesterol and jejunal ACAT activity after gastric administration of 58-035 (Expt. 1)

In the 8 hr following a gastric bolus dose of 10 mg of [^{3}H]cholesterol emulsified with oleic acid and albumin, 7.2 ± 1.2% of the label was recovered in the mesenteric lymph of five rats that had received 58-035, compared with 11 ± 0.4% in six controls ($P < 0.05$). The ^{3}H-labeled UC output into lymph was the same in both groups (2.8 ± 0.3% vs 2.2 ± 0.1% of the dose in 8 hr, respectively) whereas the ^{3}H-labeled CE output was halved after 58-035 (4.4 ± 1.0% vs 8.8 ± 0.3% of the dose in 8 hr; $P < 0.01$). When lymph was analyzed every 2 hr in four rats from each group, the reduction in ^{3}H-labeled CE output was found to be most marked in the earlier lymph collections obtained closest to the drug dose (**Table 1**).

When the mass of cholesterol present in lymph UC and CE was determined, the results were substantially the same (**Fig. 2**). CE mass output differed significantly between the two groups, especially in the 0–2 and 2–4-hr collection periods.

ACAT specific activity of jejunal microsomes prepared 10 hr after the drug dose was found to be significantly lower than control ACAT (0.8 ± 0.2 vs 0.3 ± 0.05 nmol/min per mg protein). In 13 other rats without lymph fistulas, microsomal ACAT was determined at 2 hr and 24 hr after an oral dose of 58-035. The specific activity was significantly lower at 2 hr (1.3 ± 0.1 vs 0.5 ± 0.2 nmol/min per mg protein; $P < 0.01$) but had returned to normal levels by 24 hr. These results establish an approximate duration of efficacy of an oral dose of 58-035 (>10 hr but <24 hr).

Cholesterol absorption and ACAT activity during continuous duodenal infusion of cholesterol and 58-035 (Expt. 2)

Whereas Expt. 1 was specifically designed to parallel other studies (2) that had assessed the effect of removal of CEH on the transport of a gastric cholesterol bolus dose into lymph, Expt. 2 utilized direct duodenal infusion in order to eliminate the experimental variation that results from differences in gastric emptying over time and between animals. In Expt. 2, a steady state of cholesterol transmucosal mass transport also was established, thus permitting a more detailed examination of the cholesterol

TABLE 1. Lymph cholesterol output after a gastric bolus of [^{3}H]cholesterol and oleic acid

	0–2 Hr	2–4 Hr	4–6 Hr	6–8 Hr	Total
	% of [^{3}H]cholesterol dose				
^{3}H-labeled UC					
Exper. (4)	0.6 ± 0.1[a]	0.9 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	2.8 ± 0.4
Control (4)	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	2.2 ± 0.1
P	NS	NS	NS	NS	NS
^{3}H-labeled CE					
Exper.	0.4 ± 0.1	1.2 ± 0.5	1.7 ± 0.6	1.4 ± 0.3	4.7 ± 1.3
Control	1.3 ± 0.4	2.7 ± 0.2	1.9 ± 0.3	2.5 ± 0.6	8.4 ± 0.3
P	<0.01	<0.01	NS	NS	0.05 < *P* < 0.1

Rats were given gastric doses of drug or vehicle, followed at time zero by a 10-mg dose of [^{3}H]cholesterol emulsified in oleic acid, albumin, and Na taurocholate, as described in Methods.
[a] Mean ± SE; number of rats in parentheses.

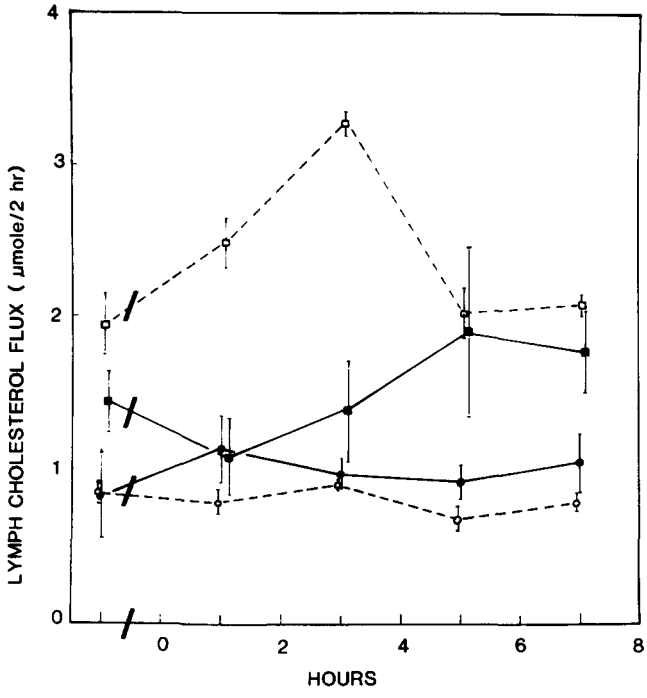

Fig. 2. Lymph cholesterol output after a gastric bolus (Expt. 1). Mesenteric lymph fistula rats were given a gastric bolus containing oleic acid, BSA, and 10 mg (25.8 μmol) of [^{3}H]cholesterol, as described in Methods, 2 hr after a gastric dose of 58-035 and/or 0.25 ml 1.5% carboxymethylcellulose-Tween 80. Lymph was collected at 0°C. A basal lymph collection immediately before the drug dose and four lymph collections after the lipid dose were analyzed and are shown above. Control UC, ○ - - - ○; exper. UC, ● - - - ●; control CE, □ - - - □; exper. CE, ■ - - - ■. Vertical lines represent one SE on each side of the mean.

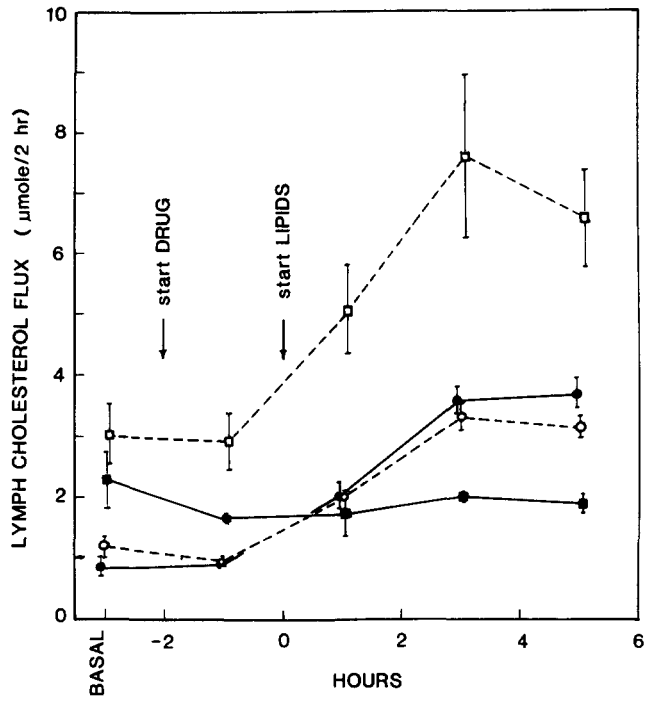

Fig. 3. Lymph cholesterol output during duodenal infusion (Expt. 2). Mesenteric lymph fistula rats were infused continuously for 6 hr with a safflower oil-[^{3}H]cholesterol emulsion, and with 58-035 or vehicle for 8 hr, as described in Methods. Lymph was collected at 0°C. Symbols as in Fig. 2.

distribution among lymph LP subfractions during inhibition of mucosal ACAT.

Table 2 summarizes the total cholesterol secreted in mesenteric lymph during the 6-hr lipid infusion, corrected for basal values determined immediately before beginning the drug (see also Fig. 3). Output of both cholesterol mass and label, respectively representing cholesterol from endogenous and exogenous sources, were significantly reduced by 58-035 infusion to less than half of control values. The reduced lymphatic cholesterol output was due to the complete absence of any increase in CE secretion during the TG-cholesterol infusion; indeed, compared with basal lymph, CE output in drug-treated rats actually decreased during lipid infusion. Total UC output, however, was normal.

In **Fig. 3,** the lymphatic outputs of UC and CE masses are depicted over time. Already in the first lymph collection after duodenal administration of 58-035 was begun (−2-0 hr), the CE output was less than the basal CE output. Hence, the drug inhibited esterification of endogenous cholesterol. Subsequent infusion of the [^{3}H]cholesterol-safflower oil emulsion produced no increase in lymph CE mass, unlike control rats in which the increase over basal CE output equalled more than half of the total cholesterol

TABLE 2. Lymph cholesterol output during continuous duodenal infusion of a [^{3}H]cholesterol-safflower oil emulsion

	^{3}H	Total Chol.[a]	UC[a]	EC[a]
	% of total infused[b]		*μmole/6 hr*	
Exper. (5)	16.5 ± 1.0[c]	29.6 ± 7.9	6.76 ± 0.48	−1.12 ± 1.19
Control (4)	37.4 ± 2.5	80.7 ± 15.4	5.04 ± 0.85	10.25 ± 2.24
P	<0.01	<0.05	NS	<0.001

[a] Net output = total cholesterol mass secreted in lymph in 6 hr minus 6 × basal secretion (see Figure 3).
[b] Total [^{3}H]cholesterol infused in 6 hr = 19.07 μmol.
[c] Mean ± SE; number of rats in parentheses.

TABLE 3. Lymph chylomicron and VLDL lipid composition during steady state lymph cholesterol transport

	TG	PL	UC	EC	PL/UC	TG/EC	TG + EC/PL
	mole %					*molar ratio*	
Chylomicrons							
Exper. (5)	88.4 ± 0.5[a]	8.3 ± 1.0	2.9 ± 0.1	1.0 ± 0.1	2.8 ± 0.2	92.1 ± 6.3	11.4 ± 1.1
Control (4)	86.1 ± 1.0	8.6 ± 0.8	2.0 ± 0.3	3.5 ± 0.3	4.5 ± 0.3	25.4 ± 2.2	10.8 ± 1.1
P	NS	NS	<0.05	<0.001	<0.01	<0.001	NS
VLDL							
Exper.	79.4 ± 1.4	13.3 ± 0.9	4.0 ± 0.3	3.3 ± 0.4	3.3 ± 0.4	35.7 ± 11.3	6.3 ± 0.6
Control	72.4 ± 1.5	15.2 ± 1.2	3.3 ± 0.4	9.2 ± 1.4	4.8 ± 0.3	8.4 ± 1.2	5.5 ± 0.5
P	NS	NS	NS	<0.01	<0.01	<0.05	NS

[a] Mean ± SE; number of rats in parentheses.

infused (see also Table 1). At all time points UC mass outputs were the same in the two groups and both groups showed the increase in UC output expected with the onset of chylomicron secretion. In Fig. 3 it can also be seen that both UC and CE outputs reached a steady state in both groups after 2 hr. At the same time, TG secretion became constant in both groups at 78–108% of the TG infusion rate (data not shown), in agreement with earlier reports (33). The distribution of lipids among the individual lymph lipoprotein fractions was therefore determined in the steady state (4–6 hr) collection period, in order to establish the relative fluxes of cholesterol into CM and VLDL.

Chylomicron and VLDL composition during lymphatic steady state cholesterol transport

The data are shown in **Table 3.** Major differences were found between the two groups of rats, both in the surface and in the core compositions of both CM and VLDL. As expected, the CE concentration was markedly reduced by 58-035 in both particles. In contrast, the UC concentration was elevated compared with controls. Because PL was unchanged, both CM and VLDL in the drug-infused rats were enriched about 50% with UC relative to PL over control particles.

Despite the profound decrease in CE content, the sizes of both CM and VLDL were unchanged as judged by the molar core: surface ratios (TG + CE/PL). This is to be expected because in all cases by far the bulk of the particle core was TG, which was substantially unchanged by the drug.

The major FA's present in CE's of CM and VLDL are shown in **Table 4.** There were no differences between the groups, but CM differed significantly from VLDL in both groups. Chylomicron CE's were relatively enriched in linoleate and deficient in arachidonate compared with VLDL, suggesting different origins for the CE's of the two particles. It appears that FA derived from the infused TG, which was largely linoleate, contributed more to CM than to lymph VLDL CE's.

Incorporation of cholesterol infused into the duodenum into lymph and lymph lipoproteins

The incorporation of [^{3}H]cholesterol into lymph UC and CE as a function of infusion time is shown in **Fig. 4.** Whereas the incorporation rates into UC were the same in both groups, and also did not differ from the ^{3}H in-

TABLE 4. Major fatty acids of chylomicron and VLDL cholesteryl esters during steady state cholesterol absorption

	16:0	16:1	18:0	18:1	18:2	20:4
			% by weight			
Chylomicrons						
Exper. (4)	14.2 ± 2.8[a]	2.1 ± 0.5	7.0 ± 0.4	24.7 ± 2.1	37.7 ± 4.6[b]	3.7 ± 1.3[b]
Control (4)	15.0 ± 2.0	2.0 ± 0.2	5.1 ± 0.6	28.5 ± 2.3	43.8 ± 5.1[b]	2.5 ± 2.5[b]
VLDL						
Exper. (5)	18.3 ± 0.8	2.5 ± 0.1	8.9 ± 1.3	20.9 ± 1.6	26.0 ± 3.2	17.1 ± 1.7
Control (4)	18.1 ± 2.4	3.9 ± 1.0	7.1 ± 0.8	21.9 ± 2.2	28.1 ± 3.6	13.5 ± 1.4
Safflower oil[c]	6.4	0	3.1	13.4	77.5	trace

[a] Mean ± SE; number of rats in parentheses.
[b] Significantly different from VLDL.
[c] The FA composition of safflower oil, infused with [^{3}H]cholesterol, is included for comparison.

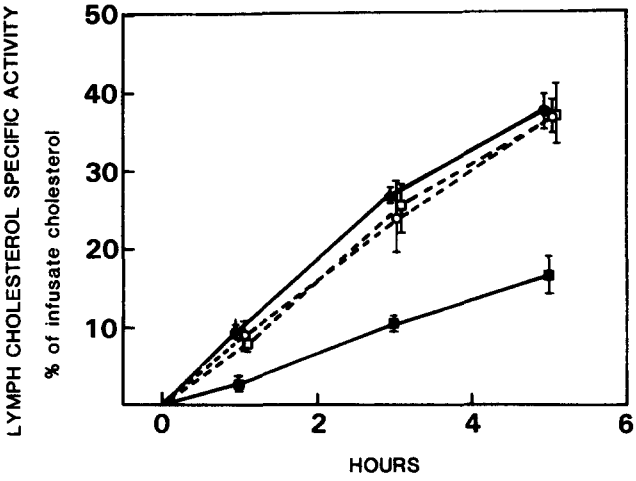

Fig. 4. [^{3}H]Cholesterol specific activity of lymph cholesterol during duodenal infusion. Symbols as in Fig. 2.

corporation rate into CE in control rats, ^{3}H incorporation into CE's of the drug-infused rats was distinctly slower. This suggests that infusion of 58-035 prevented the esterification of labeled (exogenous) cholesterol but did not affect the absorption and secretion of cholesterol that was not esterified. It is clear from Figs. 3 and 4 that an isotopic steady state was not reached in the time taken to achieve a mass steady state. Thus it was not possible to determine the absolute incorporation rates of the infused cholesterol into lymph LP's. Analysis of CM and VLDL from the 4–6-hr lymph collection nevertheless revealed some important, qualitative differences. In control rats the specific activities of UC and CE in both CM and VLDL were all similar (Table 5). In the drug-infused rats, however, CE of both CM and VLDL were of lower specific activity than UC in the same particle and this difference was much more apparent in VLDL, again suggesting that mesenteric lymph CM and VLDL CE's may be derived in part from different sources.

The specific activities of the individual CM cholesteryl esters determined by HPLC and liquid scintillation counting in CM are shown in **Table 6.** Significant differences were found between the two groups. In control rats, cholesteryl linoleate was about twice as intensely labeled as cholesteryl arachidonate, oleate, palmitate, or stearate which were all of similar specific activity. After 58-035, cholesteryl stearate, though present (see Table 4), was not labeled. Cholesteryl linoleate was, relatively, even more intensely labeled than in control CM, with a 2.3-fold greater specific activity than that of cholesteryl palmitate (compared with 1.8-fold in controls). These findings suggest either a more rapid turnover of cholesteryl linoleate than of other CE's in both groups of rats, or a preferential esterification of exogenous (labeled) cholesterol with linoleate. After ACAT inhibition by 58-035, the distinction between the different FA substrates appears further exaggerated.

Generally similar results were found for VLDL except that all CE's were less intensely labeled than in CM's and significant labeling of cholesteryl stearate was detected in both groups of rats (data not shown).

ACAT activity and composition of microsomes of jejunal mucosa

Table 7 shows that duodenal infusion of 58-035 profoundly reduced the ACAT specific activity of jejunal microsomes, to one-fifth of control levels. ACAT was assayed by following the conversion of the ^{3}H-labeled UC of the microsomes themselves to ^{3}H-labeled CE, in the presence of unlabeled oleyl CoA. Microsomes contained only small amounts of ^{3}H-labeled CE initially (1.1 ± 0.3 vs 2.8 ± 0.4% of total ^{3}H present in CE in drug-treated and control rats, respectively) and in both groups less than 2% of the total microsomal cholesterol was esterified during the incubation period. Microsomes from the two groups had similar compositions (UC/protein ratios) and were obtained in similar yields. The microsome ^{3}H-labeled UC specific activity also was the same in both groups and was similar to that of mesenteric lymph ^{3}H-labeled UC at 4–6 hr (see Fig. 4). As stated earlier, no change in the activity of another microsomal enzyme, MGAT, was found after infusing the drug. These results suggest that the action of 58-035 in inhibiting ACAT was quite specific and also that the drug did not interfere with the equil-

TABLE 5. Relative incorporation of [^{3}H]cholesterol into esterified and unesterified cholesterol of lymph lipoproteins during steady state cholesterol transport

	Lymph	CM	VLDL	HDL (Range)	*P* CM vs VLDL
	CE dpm/μmol ÷ UC dpm/μmol				
Exper. (5)	0.42 ± 0.04[a]	0.72 ± 0.06	0.26 ± 0.02	0.06–0.80	<0.001
Control (4)	0.98 ± 0.19	0.96 ± 0.03	0.85 ± 0.04	0.06–0.37	=0.05
P	<0.01	<0.02	<0.001	NS	

[a] Mean ± SE; number of rats in parentheses.

TABLE 6. [^{3}H]Cholesterol incorporation into individual cholesteryl esters of chylomicrons during steady state cholesterol transport

	CE Fatty Acid					
	16:0	16:1	18:0	18:1	18:2	20:4
	dpm/nmol					
Exper. (3)	77 ± 6[a]	0	0	97 ± 3	178 ± 7	61 ± 8
Control (3)	130 ± 14	0	128 ± 8	139 ± 14	237 ± 6	129 ± 23
P	<0.05		<0.001	<0.05	<0.01	<0.05

[a] Mean ± SE; number of rats in parentheses.

ibration of UC among luminal, mucosal, and lymph compartments.

DISCUSSION

The present experiments have established that when the activity of mucosal ACAT is blocked by administration of a specific inhibitor such as compound 58-035, the secretion of total cholesterol into mesenteric lymph is markedly inhibited. The results in lymph fistula rats confirm and extend an earlier observation in intact rats (18) in which absorption of a 2-mg gastric dose of cholesterol was halved after administration of 58-035, as determined by the dual-isotope method of Zilversmit and Hughes (20). The rats used in all of these experiments had normal pancreatic function. Moreover, normal mucosal CEH activity was found in two rats treated with the drug.[1] Both mucosal ACAT inhibition and reduced lymphatic secretion of CE occurred rapidly (<2 hr after a gastric dose of 58-035). The drug, although poorly absorbed,[2] was nevertheless detected by HPLC in lipid extracts of jejunal microsomes (data not shown). The present experiments establish that inhibition of mucosal ACAT activity is rapidly reflected in a reduced lymphatic secretion of esterified cholesterol, while unesterified cholesterol is not lowered. The overall findings clearly implicate mucosal ACAT as a rate-controlling enzyme in the absorption of both exogenously and endogenously derived cholesterol, and in particular in the lymphatic transport of esterified cholesterol.

Elimination of pancreatic CEH activity in a separate series of experiments (16, 17) also reduced cholesterol absorption. In those studies a large amount of [^{14}C]cholesterol (25 mg) was administered as a duodenal bolus. The amount of label that appeared in lymph in 6 hr was equivalent to 0.4 mg in rats lacking active CEH, compared with 2 mg in controls. However, >80% of the label in lymph nevertheless appeared in CE. This result is in agreement with Watt and Simmonds (19) who found that absorption of small amounts of cholesterol (<0.3 mg/hr infused intraduodenally for 8 hr) into lymph and the extent of esterification were unaffected by pancreatic juice diversion.

The combined results may be reconciled if CEH and ACAT are considered to function "in series," perhaps in the manner postulated by Bhat and Brockman (34). According to this concept, CEH would act predominantly by promoting cholesterol uptake into mucosal cells, although small amounts of cholesterol clearly can be taken up in the absence of CEH. ACAT remains the major enzyme regulating cholesterol esterification within mucosal cells, prior to secretion into lymph. Blocking ACAT could reduce overall cholesterol uptake from the lumen if intermediate compartments became saturated with cholesterol when "removal" by esterification is prevented.

Inhibition of mucosal ACAT was found to have differential effects on lymph CE derived from endogenous (unlabeled) or exogenous [^{3}H]cholesterol. During steady cholesterol mass secretion in lymph (4–6 hr lymph collection in Expt. 2) cholesterol was 34 ± 2% esterified compared with 67 ± 3% in control rats. In the same lymph sample, 18 ± 1% of the ^{3}H-label was found in CE,

TABLE 7. Composition and ACAT specific activity of jejunal microsomes

	UC/Prot. Ratio	UC Sp Act[a]	ACAT[b]
	nmol/mg	*% of infusate*	*nmol/min per mg*
Exper. (5)	153 ± 11[c]	36.9 ± 5.6	0.26 ± 0.12
Control (4)	152 ± 7	41.0 ± 5.0	1.26 ± 0.07
P	NS	NS	<0.001

Microsomes were prepared from jejunal mucosa of rats of Expt. 2, as described in Methods.

[a] Microsomal cholesterol specific activity in dpm/μmol expressed as a percentage of infusate cholesterol dpm/μmol.

[b] Reaction was initiated by addition of oleyl CoA as described in Methods. ACAT activity was calculated from the net esterification of [^{3}H]cholesterol present in the microsomes, during the 2-min incubation.

[c] Mean ± SE; number of rats in parentheses.

[2] Personal communication, Dr. John Heider, Sandoz Inc., East Hanover, NJ.

compared with 66 ± 5% in controls. The corresponding lymphatic fluxes of exogenous and endogenous esterified cholesterol were, respectively, 1.6 ± 0.2 μmol/2 hr (compared with 4.0 ± 0.6 in controls) and 0.3 ± 0.03 μmol/2 hr (compared with 2.4 ± 0.4 for controls). That is to say, the flux of unlabeled cholesteryl esters was reduced to 40% by ACAT inhibition whereas the flux of ^{3}H-labeled (exogenously derived) cholesteryl esters was reduced much more, to 12% of control ^{3}H-labeled CE flux. In contrast, when CEH was removed by pancreatic juice diversion in rats with normal ACAT, a mean of 51% of the total cholesterol mass in lymph, but 85% of the label, were esterified.[3] The results suggest that ACAT of mucosal cell microsomes preferentially catalyzes the esterification of cholesterol taken up from the lumen. Whether a further distinction is made between the different sources of luminal cholesterol (ingested, biliary, or secreted by the mucosa into the lumen) cannot be determined by the present experiments because an isotopic steady state was not achieved in lymph. Such studies are presently underway.

The precise mode of action of compound 58-035 is not yet known. It is clear, however, that the effect is not one of nonspecific mucosal toxicity, for the following reasons. Seven ad libitum fed, intact rats that received the drug (20 mg/kg) daily for 5 days gained the same weight as eight controls (data not shown). The yield and composition (UC/protein ratio) of jejunal microsomes obtained from these rats, and also from those described in the present report (see Table 2), were the same as controls. No gross histological abnormalities were found. The activity of another microsomal enzyme (MGAT) was unchanged by 58-035. More importantly, the rate of TG secretion into lymph, which is a very sensitive index of mucosal function, also was normal. Because MGAT and ACAT share a common substrate (fatty acyl CoA) yet showed completely different responses to the drug, it is likely that 58-035 binds either to the microsomal cholesterol that forms the substrate for the ACAT reaction, or perhaps inactivates the enzyme active site itself.[4]

It is of interest that complete inactivation of ACAT did not occur, even after 8 hr of continuous luminal perfusion of the drug. Either the kinetics of drug absorption are such that a saturating concentration in mucosal cell microsomes is never achieved, or a portion of the enzyme activity is not accessible to inactivation. Curiously, in a previous study (35), the minimum levels of CE found in lymph chylomicrons during mucosal ACAT inhibition by duodenal lecithin infusion also was 15% of the total cholesterol present, a value very close to that reached in the present studies. Possibly the common denominator to both methods of inhibiting mucosal ACAT lies in an alteration in the physical properties of the microsomal membranes, potentially leading to a reduction in membrane fluidity and/or migration of cholesterol substrate molecules to the enzyme active site.

The fatty acid compositions of CM and lymph VLDL cholesteryl esters also suggest that CEH is less likely than ACAT to have been the mediator of lymph cholesteryl ester formation, in both the control and the experimental rats of the present study. Linoleate is a preferred substrate for CEH (36), yet the fraction of linoleate present in CM and VLDL CE's was markedly reduced compared with the fatty acid composition of the infused safflower oil triglycerides. Rat mucosal ACAT, on the other hand, shows a slight preference for palmitate and oleate over linoleate (6), and both these FA's were incorporated in large amounts into CM and VLDL CE's (see Table 4). The CE-FA composition data in Table 4 also imply that the origins of CM and VLDL CE's differ at least in part and hence that the major portion of the $d < 1.006$ g/ml particles designated "VLDL" were not merely "small CM." This inference is also clear from the data in Table 5, which show significant differences in the relative incorporation of labeled and unlabeled cholesterol into CE's of CM compared with VLDL, particularly in the experimental group but also in controls. The CE's of lymph VLDL nevertheless would have been mainly of intestinal origin because rats lack an exchange protein to transfer CE's from lipoproteins that may have entered the lymph from plasma (37), and because the contribution of plasma VLDL to intestinal lymph has been shown to be negligible, at least in fasting lymph fistula rats (38).

Chylomicrons and VLDL secreted by the intestine during inhibition of ACAT were greatly enriched in unesterified cholesterol (see Table 3), reaching PL/UC molar ratios as low as 2.3 in CM from one of the rats. The potential implications for metabolism are considerable. TG-rich lipoproteins in mesenteric lymph are normally relatively depleted of UC, but can acquire more UC from plasma LP's after entering the bloodstream (39). Also, during CM metabolism by lipoprotein lipase, an HDL fraction is generated from the CM surface. Normally, this HDL is depleted of UC and probably acquires UC from tissues (40). In this way, CM's derived from the intestine probably help to deplete peripheral tissues of excess cholesterol. However, these processes will be markedly blunted if CM's that enter the plasma are al-

[3] Bennett Clark, S., and L. L. Gallo. Unpublished results.

[4] Since these studies were performed and analyzed, a report by Heider et al. of a related compound, (n-(1-oxo-9-octadecenyl)-DL-tryptophan(Z)ethyl ester) has appeared (*J. Lipid Res.* 1983. **24:** 1127–1134). This compound was shown to be an apparent competitive inhibitor of microsomal ACAT in jejunum of cholesterol-fed rabbits and to inhibit cholesterol absorption in intact rabbits.

ready rich in UC. Thus the net result of blocking cholesterol absorption by inhibiting intestinal ACAT on overall cholesterol homeostasis may not be particularly beneficial, although development of hyperlipidemia in cholesterol-fed rabbits has been prevented.[2] Other metabolic changes that CM undergo, such as apoprotein binding and exchange, the possible action of LCAT on CM surface remnants, TG lipolysis, and transfers and exchanges of PL's and CE's among LP classes probably all will be influenced by the composition of the CM surface. Many further studies are needed to understand fully the metabolic consequences of modifying CM composition by means of inhibiting intestinal ACAT.

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