Regulation of acyl CoA:cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells

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Abstract The regulation of rabbit intestinal acyl CoA:cholesterol acyltransferase (ACAT) by 25-hydroxycholesterol was studied. 25-Hydroxycholesterol significantly increased jejunal microsomal ACAT activity. The stimulation of ACAT activity by 25-hydroxycholesterol was inversely related to microsomal cholesterol content. In enterocytes, 25-hydroxycholesterol stimulated cholesteryl ester synthesis 6-fold. The esterification of cholesterol, taken up by enterocytes from liposomes or bile salt micelles, was increased by 25-hydroxycholesterol. This, however, did not affect the rate of uptake of cholesterol by the cells. In intestinal cells from rabbits fed cholesterol, the effect of 25-hydroxycholesterol on cholesteryl ester synthesis was 50% of that in cells prepared from animals fed no cholesterol. 25-Hydroxycholesterol stimulated the esterification of newly synthesized cholesterol. As new free cholesterol increased in enterocytes, 25-hydroxycholesterol lost its effect. Despite large amounts of newly synthesized cholesterol, oleic acid incorporation into cholesteryl ester was not increased. We conclude that 25-hydroxycholesterol increases intestinal ACAT activity. The effect of 25-hydroxycholesterol on ACAT is dependent upon the availability of cholesterol to the enzyme. At cholesterol concentrations below saturation, the oxygenated sterol has a stimulatory effect. If ACAT is saturated, 25-hydroxycholesterol has no effect. ACAT catalyzes the esterification of two separate pools of cholesterol within the enterocyte, i.e., newly synthesized cholesterol and membrane cholesterol. 25-Hydroxycholesterol increases the esterification rate of cholesterol in both pools.—**Field, F. J., and S. N. Mathur.** Regulation of acyl CoA:cholesterol acyltransferase by 25-hydroxycholesterol in rabbit intestinal microsomes and absorptive cells. *J. Lipid Res.* 1983. **24:** 1049–1059.

Supplementary key words cholesterol • cholesterol esterification • intestine • absorption

The oxygenated derivative of cholesterol, 25-hydroxycholesterol, is a very potent regulator of intracellular cholesterol metabolism (1–3). It has been shown to decrease cholesterol synthesis in intact cells by inhibiting the regulatory enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (2, 4–7). In human fibroblasts (3), rat hepatocytes (8, 9), and intestinal explants (10), it increases cholesteryl ester synthesis through an increase in the activity of the microsomal enzyme, acylcoenzyme A:cholesterol acyltransferase (ACAT). The effect of 25-hydroxycholesterol on hepatic ACAT activity was extensively studied by Drevon, Weinstein, and Steinberg (8). They observed that 25-hydroxycholesterol increased ACAT activity in both microsomes and intact hepatocytes. Protein synthesis was not required for this effect. They also found that by increasing cholesterol synthesis in the cells by adding mevalonolactone, cholesterol ester synthesis was greatly increased. The mechanism of the regulation of cholesteryl ester synthesis by 25-hydroxycholesterol is not known.

ACAT has been shown to catalyze the esterification of cholesterol in the intestine of many different animals (11–14) with highest activity found in the jejunum and proximal ileum of the small bowel (10, 13). In rabbit, the specific activity of ACAT is higher in intestinal crypt cells than in villous cells. With cholesterol feeding, however, the activity of the enzyme increases in villous absorptive cells with no change occurring in crypt cells (10). Dietary cholesterol as well as fats have been shown to regulate ACAT activity (10, 12, 13, 15). Recently, we have shown that dietary unsaturated fats, independently of luminal cholesterol, increase ACAT activity in the intestine. Dietary saturated fat does not produce any increase and actually blunts the increase of ACAT activity that is caused by dietary cholesterol (13).

The intestinal absorptive cell ultimately controls the amount of cholesterol entering the body, both of dietary cholesterol and biliary cholesterol. Most of the cholesterol that enters the lymph is esterified, suggesting that intracellular esterification of cholesterol plays an important role in the process of absorption. It has been postulated that ACAT is responsible for catalyzing the esterification of cholesterol in the intestine as it does in

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; TLC, thin-layer chromatography.

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other organs (10–15). In this communication we report the regulation of ACAT in the intestine by 25-hydroxycholesterol. The results confirm many of the observations made in hepatocytes as reported by Drevon et al., (8) and, in addition, suggest that the regulation of ACAT by 25-hydroxycholesterol is dependent upon cholesterol availability.

MATERIALS AND METHODS

[1-^{14}C]Oleoyl coenzyme A, [4-^{14}C]cholesterol, [9,10-^{3}H]oleic acid, [1,2-^{3}H(N)]cholesterol, [^{14}C]inulin, and [^{3}H]mevalonolactone were purchased from New England Nuclear. Oleoyl-coenzyme A, mevalonolactone, cycloheximide, dipalmitoyl phosphatidylcholine, oleic acid, and cholesterol were obtained from Sigma. 25-Hydroxycholesterol was purchased from Steraloids. All other chemicals were reagent grade.

Animals and diet

Male New Zealand white rabbits weighing 1.0 kg ± 0.5 kg were housed in a windowless room that was illuminated from 0700 to 1900 hr. The safflower-cholesterol diet was prepared by dissolving 10 g of cholesterol in 100 ml of oil. This was thoroughly mixed with 1 kg of Purina rabbit chow. The animals were provided food and water ad lib.

Preparation of microsomes

All rabbits were killed between 0800 and 1000 hr by cervical dislocation. Microsomes from the jejunum and proximal ileum were prepared in a buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, pH 7.4, as previously described (13).

Isolated cell preparation

Approximately 25 cm of mid-gut from animals fed normal rabbit chow was used for cell isolation by a modification of the method described by Weiser (16). The intestinal segment was flushed thoroughly with ice-cold saline. The lumen was then filled with buffer A (8 mM KH_2PO_4, pH 7.3, containing 1.5 mM KCl, 96 mM NaCl, and 27 mM Na citrate), and the segment was clamped at both ends and placed in a beaker filled with normal saline at 37°C. The beaker was oscillated in a Dubnoff metabolic shaker for 20 min after which buffer A was discarded. The segment was then filled with buffer B (phosphate-buffered 0.9% NaCl containing 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.3) at 37°C. The mucosal cells were collected by draining the intestinal luminal contents into separate plastic centrifuge tubes at 6-min intervals. Exchanges 4 and 5 were used for the experiments. The cells were diluted with Krebs buffer without Ca^{2+} and recovered by centrifugation at 2,000 rpm for 5 min. The cells were washed twice with the Krebs buffer before use.

Preparation of [^{14}C]cholesterol–BSA emulsion

A [^{14}C]cholesterol–BSA emulsion was made according to the method of Stokke and Norum (17).

ACAT assay

Radiolabeled cholesterol as substrate. The method was modified from that of Haugen and Norum (11). The total volume of each assay was 0.2 ml consisting of 0.05 mg to 0.10 mg of microsomal protein in the buffered sucrose solution. Approximately 75,000 dpm of the BSA emulsion, containing 4.5 mg of BSA and [^{14}C]cholesterol (59.4 mCi/mmol) was added to the microsomal membranes and incubated at 37°C for 2 hr. After the 2-hr period, the reaction was started with 8.6 nmol of unlabeled oleoyl-CoA.

Radiolabeled oleoyl-CoA as substrate. The total volume of each sample was 0.2 ml consisting of 0.05 to 0.1 mg of microsomal protein, 0.5 mg of fatty acid-poor bovine serum albumin (unless otherwise stated), 8.6 nmol of [^{14}C]oleoyl-CoA with specific activity 12,360 dpm/nmol. Buffered sucrose solution was added to adjust the final volume to 0.2 ml. The assay mixture was incubated at 37°C for 5 min or for a predetermined time as stated in the text before the addition of the substrate [^{14}C]oleoyl-CoA to start the reaction.

The reaction from both methods was stopped at 2 min with 5 ml of chloroform–methanol 2:1 (v/v). Approximately 8,000–10,000 cpm of [^{3}H]cholesteryl oleate was added as an internal standard to calculate recoveries. One ml of 0.04 N HCl was added. The samples were vortexed and allowed to stand at 4°C overnight to separate the phases completely. The aqueous phase was then removed and the chloroform phase was dried under N_2. The residue was dissolved in 0.125 ml of chloroform and spotted on thin-layer chromatography plates layered with silica gel 60 H (E. Merck). The chromatograms were developed in hexanes–ethyl acetate 9:1 (v/v). Lipids were visualized by exposure of the chromatograms to iodine vapor, and the area corresponding to cholesteryl ester was scraped directly into liquid scintillation vials containing 10 ml of a Liquifluor (New England Nuclear)–toluene scintillation solution. Radioactivity was measured in a Beckman model LS8100-liquid scintillation counter. Quenching was monitored by an external standard. Efficiencies did not vary between samples and for ^{14}C the efficiency was approximately 75%. Recoveries ranged from 75 to 90%. Spillover of ^{3}H counts into the ^{14}C channel were calculated for each assay and subtracted from the total

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^{14}C counts. ACAT activity was expressed as pmol of cholesteryl ester formed min^{-1} mg^{-1} of microsomal protein. Specific activity of the substrate for the method with labeled cholesterol was determined by dividing the dpm added to the assay mixture by the amount of cholesterol in the microsomal membranes.

Preparation of cholesterol-containing liposomes

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

Enrichment of microsomal membranes with cholesterol

Increasing amounts of cholesterol, 0, 0.15, 0.30, 0.60 μmol, solubilized in DPPC liposomes were incubated with 3.0–5.0 mg protein of intestinal microsomal membranes at 37°C from 1–4 hr in a Dubnoff metabolic shaker. Fusion of intact liposomes with microsomes was minimized by inclusion of 1 mM EDTA (18) and 5 mg/ml bovine serum albumin (19) in the incubation mixture. Phospholipid content in the microsomal membranes per mg protein did not change over a 4-hr incubation, evidence that no net transfer of phospholipids took place. After the designated time of incubation, the microsomes were layered over a 20% sucrose solution and centrifuged at 106,500 *g* for 45 min. The supernatant was removed by aspiration and the microsomal pellet was resuspended in Tris buffer and again layered on top of a 20% sucrose solution. After centrifugation at 106,500 *g* for 45 min, the recovered microsomal pellet was resuspended in Tris buffer. This suspension was used to determine protein, sterol content, phospholipid content, and ACAT activity.

Measurement of cholesterol esterification in isolated enterocytes

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

Isolated intestinal cells were incubated in oxygenated Krebs buffer (without calcium) and 50 μM [^{3}H]oleic acid–BSA solution (sp act 70,000 dpm/nmol). Each dish contained 5 mg of BSA, and the total volume was 1 ml. 25-Hydroxycholesterol was added in 10 μl of 95% ethanol (control dishes contained 10 μl of 95% ethanol) at a specified time. The incubation was continued for 1 hr. Incorporation of [^{3}H]oleic acid into cholesteryl-[^{3}H]oleate was linear for 1 hr in the presence or absence of 25-hydroxycholesterol in these cells. The cell suspensions were collected, diluted with buffer, and kept on ice. The cells were washed twice by resuspending them in excess buffer and centrifuging them at 2,000 rpm for 10 min. The cell pellet was extracted twice with 2 ml of chloroform–methanol 1:1 (v/v). Two ml of chloroform and 1.3 ml of 0.04 N HCl were added to the lipid extract. The chloroform phase was taken to dryness under nitrogen. The residue was dissolved in 125 μl of chloroform and plated on TLC plates coated with silica gel 60 H. The plates were developed in hexane–diethyl ether–methanol–acetic acid 85:20:1:1 (v/v). Cholesteryl esters, triglycerides, fatty acids, and phospholipids were visualized with iodine vapor and scraped into counting vials containing 10 ml of toluene-liquifluor (New England Nuclear). Recoveries were determined by scraping the entire plate and dividing the dpm recovered by the total dpm in the tube before plating. Quenching was determined by an external standard. There was no significant variation in the efficiency between samples.

To determine mevalonolactone incorporation into cholesteryl ester, [^{3}H]mevalonolactone at various concentrations was added to dishes containing the isolated cells. 25-Hydroxycholesterol was added in 10 μl of 95% ethanol. The conditions of the incubation were the same as those described above for oleic acid incorporation. Following the 1-hr incubation, the cells were processed as before.

The esterification of exogenous cholesterol was determined by solubilizing [^{14}C]cholesterol in either dipalmitoyl phosphatidylcholine vesicles or a sodium taurocholate micellar solution. [^{14}C]Cholesterol (59.4 mCi/mmol) and 751 nmol of unlabeled cholesterol dissolved in chloroform were added to 75 μmol of sodium taurocholate dissolved in 95% ethanol. This solution was dried under a stream of nitrogen. Two ml of Krebs-bicarbonate buffer was added and vigorously vortexed until the solution was clear. Another ml of buffer was added, vortexed, and kept at 37°C until use. To each dish containing the isolated intestinal cells was added 50 nmol of cholesterol with sp act of 9,625 dpm/nmol. The final taurocholate concentration was 5 mM. The incubation was continued for 1 hr. The assay was processed as described before except that [^{3}H]cholesteryl oleate (approximately 8,000 cpm) was added to calculate recoveries.

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Cholesterol uptake by isolated cells

Cholesterol uptake was determined by a modification of the method of Thomson and O'Brien (20). The total volume of the incubation mix was 1 ml. It consisted of Krebs buffer, 5 mg of bovine serum albumin, and [^{3}H]cholesterol solubilized in sodium taurocholate so that the final concentrations of cholesterol and taurocholate were 50 μM and 5 mM, respectively. The sp act of cholesterol was 15,960 dpm/nmol. [^{14}C]Inulin, approximately 10,000 cpm, was also added to each dish to correct for nonspecific adherence. The total protein of the isolated cells per dish was 3–5 mg. The dishes were incubated at 37°C in 95% O_2, 5% CO_2 for 1 hr. The [^{3}H]cholesterol-bile salt solution was added at specified intervals; however, all cells were incubated for a full hour. 25-Hydroxycholesterol, 10 μg, was added to the experimental dishes in 10 μl of 95% ethanol. A zero time point was calculated by adding the radiolabeled cholesterol solution to a dish and immediately stopping the reaction by diluting with cold Krebs buffer. After incubation, the cells were transferred to 12-ml conical centrifuge tubes and placed on ice. The cells were then washed three times by resuspending the cells in 6 ml of Krebs buffer and centrifuging at 2,000 rpm for 10 min. After the final centrifugation, the pelleted cells were dried under a stream of nitrogen. One ml of 0.75 N NaOH was added and the tubes were heated at 90°C for 20 min. The samples were transferred to scintillation vials containing 10 ml of Aquasol. The rate of uptake was calculated by first determining the fraction of [^{14}C]inulin counts present. This percentage (approximately 5%) represented nonspecific adherence of the inulin probe. This was subtracted together with the cpm from the zero time point, from each sample. [^{3}H] spillover into the [^{14}C] channel was corrected for and radioactivity was determined using an external standard with correction for quenching of the two isotopes. The rate of uptake was expressed as nmol of cholesterol taken up/mg protein per time period.

Chemical analysis

Lipids were extracted from microsomes using chloroform–methanol 2:1 (v/v). Free cholesterol was determined by gas–liquid chromatography with cholestane as an internal standard to determine recoveries. Cholesterol from Applied Science was used as a standard. Gas–liquid chromatography was performed on a Hewlett-Packard 5840 A Gas Chromatograph with a 3-ft glass column containing 3% OV-1 on 80/100 Supelcoport. Microsomal phospholipid content was estimated according to the procedure of Raheja et al. (21) using dipalmitoyl phosphatidylcholine as the standard. Protein was determined by the method of Lowry et al. with bovine serum albumin as the standard (22).

Statistical analysis

The paired Student's t test was used to determine P values.

RESULTS

Effect of 25-hydroxycholesterol on microsomal ACAT activity

Microsomal membranes prepared from intestines of rabbits fed normal rabbit chow were incubated with 25-hydroxycholesterol at a concentration of 10 μg/ml for 1 hr at 37°C. ACAT activity was measured subsequently by adding [^{14}C]oleoyl-CoA. ACAT activity was increased from 205 ± 50 pmol of cholesteryl oleate formed/mg per min in control incubations to 400 ± 75 after adding the oxygenated sterol (n = 12, $P < 0.001$, data not shown). This stimulatory effect of 25-hydroxycholesterol was concentration-dependent reaching maximum stimulation at a concentration exceeding 2 μg/ml. Subsequent experiments were done with 8–10 μg/ml of 25-hydroxycholesterol to ensure its maximal effect. The stimulation of ACAT activity by the oxygenated sterol occurred within 5 min after its addition and remained at this level for at least 3 hr. For consistency, 1 hr incubations with 25-hydroxycholesterol were used in subsequent experiments.

Table 1 shows the effect of 25-hydroxycholesterol on intestinal microsomal ACAT activity at different concentrations of oleoyl-CoA. Despite variability observed at low rates of cholesterol esterification, in five separate experiments, the oxygenated sterol caused similar increases in ACAT activity at concentrations of the fatty acyl-CoA below and at V_{max} for the enzyme.

Drevon et al. (8) and Erickson et al. (6) have shown that 25-hydroxycholesterol is esterified in hepatocytes by ACAT. The increase in ACAT activity observed with 25-hydroxycholesterol is not due to the synthesis of 25-hydroxycholesteryl-[^{14}C]oleate. In our thin-layer chromatography system, the ester of 25-hydroxycholesterol migrates slower than cholesteryl ester in the region of triacylglycerols. The synthesis of 25-hydroxycholesteryl-[^{14}C]oleate, therefore, would not contribute to the dpm of cholesteryl-[^{14}C]oleate generated in our assay. **Table 2** shows the effect of 25-hydroxycholesterol on ACAT activity when the radiolabel is on cholesterol. Using this method, it is assumed that the added [^{14}C]cholesterol equilibrates with the endogenous microsomal cholesterol pool. The activity of the enzyme is measured in the presence or absence of 25-hydroxycholesterol by initiating the reaction with unlabeled oleoyl-CoA, 43 μM. With the label on cholesterol, the rate of cholesterol esterification in the presence and

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TABLE 1. Effect of 25-hydroxycholesterol on intestinal microsomal ACAT activity at different concentrations of oleoyl-CoA

	ACAT Activity[a]		
	25-Hydroxycholesterol		
Oleoyl-CoA	0	10 μg/ml	% Increase
μM	*pmol/mg per min*		
2.1	17 ± 6	22 ± 8	29
4.2	21 ± 4	31 ± 7	48
8.4	63 ± 17	84 ± 26	33
21.0	105 ± 13	140 ± 14	33
42.0	107 ± 15	153 ± 17	43

Microsomes were incubated for 1 hr at 37°C with or without 25-hydroxycholesterol. Total volume was 0.2 ml and 25-hydroxycholesterol was added in 5 μl of 95% ethanol. Five μl of ethanol was added to control incubations. The reaction was started immediately after the 1-hr incubation by adding [^{14}C]oleoyl-CoA at the concentrations listed. The assay time was 2 min.

[a] Means ± SE (n = 5).

absence of 25-hydroxycholesterol is 418 pmol/mg per min vs 304, respectively. The oxygenated sterol increased ACAT activity to about the same extent as when oleoyl-CoA contained the label. If 25-hydroxycholesterol was being esterified to any great extent, one would have expected less [^{14}C]cholesteryl oleate being formed in the presence of the oxygenated sterol as the unlabeled oleoyl-CoA would have been utilized for the synthesis of 25-hydroxycholesteryl oleate. Because this did not happen, it suggests that the stimulation of ACAT activity by 25-hydroxycholesterol is not due to the esterification of this oxygenated sterol.

Intestinal microsomal membranes prepared from rabbits fed normal rabbit chow were incubated for 4 hr at 37°C with increasing concentrations of cholesterol solubilized in dipalmitoyl phosphatidylcholine (DPPC) liposomes. After removing the liposomes and thoroughly washing the membranes, the effect of 25-hydroxycholesterol on ACAT activity was determined. Approximately 40% of the microsomal protein was recovered following the two washings. It was observed that ACAT activity increased 4-fold secondary to the 4-hr incubation and the minor purification step of the washings. The results are shown in **Table 3.** Cholesterol content of the microsomal preparations ranged from 29 to 66 μg/mg protein. ACAT activity progressively increased (0.82 to 2.31 nmol cholesteryl oleate formed/mg per min) as membranes were enriched with cholesterol. However, the effect of 25-hydroxycholesterol to further increase ACAT activity became less. When membrane cholesterol reached 49 μg/mg protein, 25-hydroxycholesterol had no stimulatory effect.

Effect of 25-hydroxycholesterol in isolated intestinal cells

The effect of 25-hydroxycholesterol, 10 μg/ml, on the incorporation of [^{3}H]oleic acid into cholesteryl ester, triglyceride, fatty acid, and phospholipid by isolated jejunal absorptive cells was determined (**Table 4**). In 1 hr, incorporation of [^{3}H]oleic acid into cholesteryl ester increased by almost 600% in the dishes containing 25-hydroxycholesterol. This was seven times greater than the effect observed in microsomal membranes. By contrast, the oxygenated sterol had no significant effect on the fatty acid uptake and triglyceride synthesis. Phospholipid synthesis, however, decreased slightly in the presence of 25-hydroxycholesterol.

Fig. 1A shows the effect of 25-hydroxycholesterol concentration on cholesteryl ester synthesis in isolated cells. As observed in microsomal membranes, the stimulatory effect was concentration-dependent at low concentrations. Dependence on concentration was lost,

TABLE 2. Effect of 25-hydroxycholesterol on cholesteryl ester synthesis in intestinal microsomes using cholesterol or oleoyl-CoA as radiolabeled substrate

[^{14}C]Cholesterol	[^{14}C]Oleoyl-CoA (43 μM)	25-OH Cholesterol (8 μg/ml)	Cholesteryl Ester Synthesis	
			pmol/mg per min	
+	−	−	304 ± 25	
				38%
+	−	+	418 ± 44	
−	+	−	197 ± 11	
				34%
−	+	+	265 ± 19	

Microsomes were incubated for 2 hr at 37°C with [^{14}C]cholesterol-BSA or buffer containing an equivalent amount of BSA (4.5 mg/assay). 25-Hydroxycholesterol was added in 5 μl of 95% ethanol. The same volume of ethanol without 25-hydroxycholesterol was added to control tubes. Incubations were continued for another hour before adding labeled or unlabeled oleoyl-CoA to start the reaction. Cholesteryl ester synthesis was then determined as described in Methods. Results are the mean ± SE of five determinations.

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TABLE 3. Effect of 25-hydroxycholesterol on ACAT activity in intestinal microsomes enriched with cholesterol

Cholesterol	ACAT Activity[a] 25-Hydroxycholesterol 0	10 μg/ml	% Increase	P Value
μg/mg protein	*nmol/mg per min*			
29 ± 4	0.82 ± 0.05	1.28 ± 0.07	56	<0.001
38 ± 5	1.29 ± 0.12	1.61 ± 0.14	12	<0.001
49 ± 8	1.74 ± 0.13	1.85 ± 0.16	7	>0.10
66 ± 2	2.31 ± 0.17	2.30 ± 0.17	0	

Microsomes were incubated with increasing concentrations of cholesterol-containing dipalmitoyl phosphatidylcholine liposomes at 37°C for 4 hr. The microsomes were washed as described in Methods. 25-Hydroxycholesterol was added in 5 μl of 95% ethanol to the microsomes and incubated for 1 hr at 37°C. ACAT activity was measured as previously described. Each incubation was carried out in duplicate and ACAT assays were done in triplicate.

[a] Mean ± SEM (N = 3).

however, between 4 and 8 μg/ml. The effect of time on stimulation of cholesteryl ester synthesis by 25-hydroxycholesterol is shown in Fig. 1B. In contrast to the experiments performed with microsomal membranes (where the effect of 25-hydroxycholesterol was rapid), the effect of 25-hydroxycholesterol was time-dependent over 1 hr.

Protein synthesis was not required for the stimulatory effect of 25-hydroxycholesterol on ACAT activity in isolated intestinal cells. The addition of 0.1 mM cycloheximide at the start of the 1-hr incubation had no effect on the increase in cholesteryl ester synthesis due to the addition of 25-hydroxycholesterol (data not shown). At this concentration of cycloheximide, leucine incorporation into precipitable protein was inhibited by 95%.

It has been shown previously that feeding a diet of 1% cholesterol to rabbits increases microsomal ACAT activity in the small intestine (9, 12). We, therefore, wanted to determine if 25-hydroxycholesterol could enhance the esterification rate of cholesterol taken up by isolated intestinal cells. The results are shown in **Table 5.** [^{14}C]Cholesterol, solubilized in either DPPC-liposomes or taurocholic acid micelles, was added to a suspension of isolated intestinal cells. The effect of 25-hydroxycholesterol on the incorporation of this radiolabeled cholesterol into intracellular cholesteryl ester was determined. Approximately three times more cholesterol was esterified when it was added in bile salt micelles as compared to liposomes. The rate of esterification of the absorbed cholesterol, however, was significantly increased 2-fold from liposomal cholesterol and 1.5-fold from micellar cholesterol by the addition of 25-hydroxycholesterol.

Because 25-hydroxycholesterol can increase the esterification rate of exogenous (luminal) cholesterol, we investigated the effect of this polar sterol on cholesterol

TABLE 4. Effect of 25-hydroxycholesterol on oleic acid incorporation into cholesteryl ester, triglyceride, fatty acid, and phospholipid in isolated intestinal cells

25-Hydroxycholesterol	Lipid Class: Cholesteryl Ester	Triglyceride	Fatty Acid	Phospholipid
	nmol/mg per hr			
0	0.070 ± 0.013	4.13 ± 0.52	0.207 ± 0.023	0.960 ± 0.083
10 μg/ml	0.432 ± 0.035 ($P < 0.001$)	4.01 ± 0.50 (N.S.)	0.221 ± 0.018 (N.S.)	0.846 ± 0.060 ($P = 0.02$)

Isolated intestinal cells were incubated for 1 hr at 37°C with 95% O_2, 5% CO_2 in 1 ml of Krebs-bicarbonate buffer without calcium. The incubation media contained [^{3}H]oleic acid, 50 μM, with sp act 70,000 dpm/nmol. 25-Hydroxycholesterol, 10 μg in 10 μl of 95% ethanol, was added to experimental dishes. The control dishes contained 10 μl of 95% ethanol without the sterol. After 1 hr, the cells were harvested, washed, and extracted with chloroform–methanol (1:1 (v/v). Chloroform was added to the combined extracts to make chloroform–methanol 2:1 (v/v). Water was added to separate the phases and the chloroform phase was taken to dryness. Chloroform was added to the residue and the lipid classes were separated by thin-layer chromatography as described in Methods.

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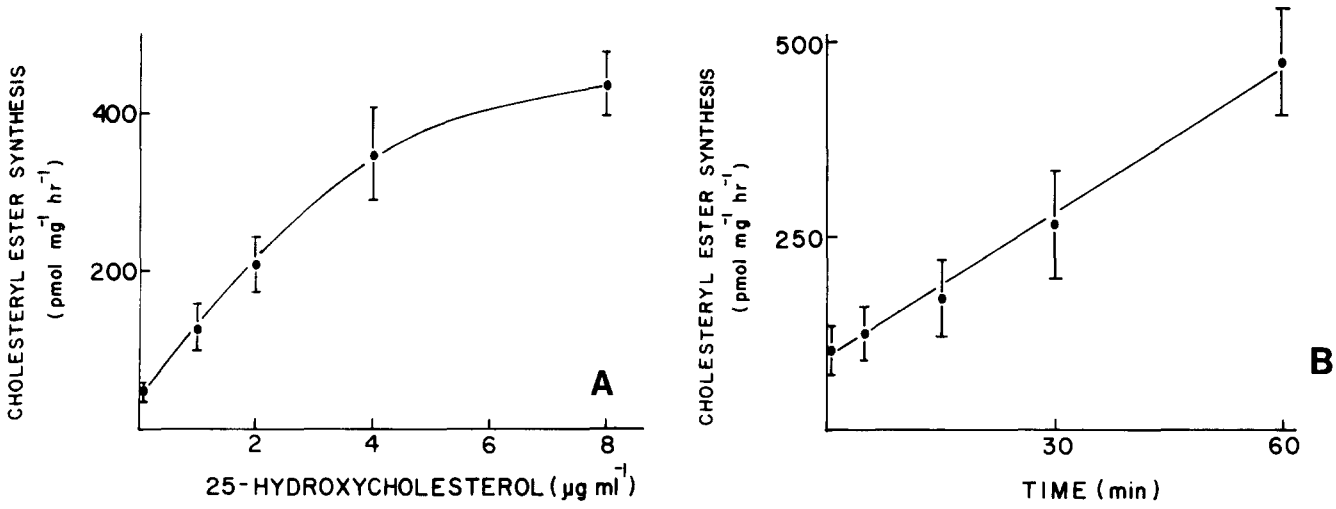

Fig. 1. Effect of 25-hydroxycholesterol on [^{3}H]oleic acid incorporation into cholesteryl oleate in isolated intestinal cells. Intestinal cells were incubated for 1 hr at 37°C in Krebs buffer without calcium and 50 μM of [^{3}H]oleic acid (sp act 70,000 dpm/nmol) in a CO_2 incubator. 25-Hydroxycholesterol was added in 10 μl of 95% ethanol (control dishes contained 10 μl of 95% ethanol). Incubation dishes were processed as described in Methods. A, 25-Hydroxycholesterol was added at the concentrations indicated at the start of the incubations. B, 25-Hydroxycholesterol, 10 μg/ml, was added to the incubations at the times indicated.

uptake by absorptive cells. [^{3}H]Cholesterol was solubilized in taurocholic acid and added to a suspension of isolated jejunal absorptive cells to make the final concentration of cholesterol and taurocholate 50 μM and 5 mM, respectively. [^{14}C]Inulin, 10,000 cpm, was added as an extracellular marker. 25-Hydroxycholesterol at a concentration of 10 μg/ml was added to the experimental dishes. At timed intervals, the cells were harvested and the total number of counts in the intracellular lipid fraction was determined. The rate of uptake of cholesterol was linear for 10 min. In the presence and absence of 25-hydroxycholesterol, the rate of uptake was 163 pmol/mg per min vs 218, respectively (mean of three animals). This difference was not significant.

Isolated cells were then prepared from animals fed a 1% cholesterol diet. Intestinal microsomal cholesterol content and ACAT activity are significantly increased by this dietary modification (13). The effect of 25-hydroxycholesterol on oleic acid incorporation into cholesteryl ester was then determined in these cells. **Table 6** shows these results and compares the results obtained with intestinal cells prepared from animals fed normal rabbit chow (Table 4). In cells prepared from animals fed the cholesterol diet, cholesteryl oleate synthesis was increased 4-fold compared to cells prepared from ani-

TABLE 5. Effect of 25-hydroxycholesterol on the esterification of cholesterol solubilized in liposomes or bile salt micelles by isolated intestinal cells

	Number of Dishes	Cholesteryl Ester Synthesis[a]	
		Liposomes	Bile Salt Micelles
		pmol/mg per hr	
Buffer	10	5.2 ± 0.42	15.2 ± 1.5
Buffer + 25-hydroxycholesterol	10	11.0 ± 0.91[b]	21.6 ± 2.0[c]

Isolated intestinal cells were incubated at 37°C in 95% O_2, 5% CO_2 in 1 ml of Krebs-bicarbonate buffer without calcium, and 50 μM [^{14}C]cholesterol solubilized in dipalmitoyl phosphatidylcholine liposomes or 5 mM taurocholic acid with or without 25-hydroxycholesterol (10 μg) for 1 hr. The cells were harvested, washed, and extracted twice with chloroform–methanol 1:1 (v/v). Chloroform was added to make the chloroform–methanol ratio 2:1 (v/v). Water was added to separate the phases and the chloroform phase was dried. After adding a small amount of chloroform, the lipid classes were separated by TLC.

[a] Mean ± SEM.

[b] $P < 0.001$ vs buffer.

[c] $P < 0.02$ vs buffer.

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TABLE 6. Effect of 25-hydroxycholesterol on oleic acid incorporation into cholesteryl ester in isolated cells prepared from animals fed a high cholesterol diet

Diet	Cholesteryl Oleate Synthesis		
	25-Hydroxycholesterol		
	0	10 μg/ml	Increase
	nmol formed/mg per hr		
Normal chow	0.070 ± 0.013	0.432 ± 0.035	6.2-fold
Safflower oil + 1% cholesterol	0.302 ± 0.037	1.00 ± 0.081	3.3-fold

Isolated intestinal cells prepared from animals fed normal rabbit chow or 1% cholesterol were incubated for 1 hr at 37°C in 95% O_2, 5% CO_2 in 1 ml of Krebs-bicarbonate buffer without calcium. The incubation media contained [^{3}H]oleic acid, 50 μM, with sp act 70,000 dpm/nmol. 25-Hydroxycholesterol, 10 μg in 10 μl ethanol, was added to experimental dishes. The control dishes contained 10 μl of 95% ethanol without the sterol. After 1 hr, the cells were harvested, washed, and extracted twice with chloroform–methanol 1:1 (v/v). Chloroform was added to the combined extracts to make chloroform–methanol 2:1 (v/v). Water was added to separate the phases and the chloroform phase was taken to dryness. Chloroform was added to the residue and the lipid classes were separated by TLC.

mals fed normal chow. The stimulatory effect of 25-hydroxycholesterol on cholesteryl oleate synthesis, however, was reduced by 50% in the cells prepared from animals fed the cholesterol diet compared to control animals.

Isolated cells were driven to synthesize free cholesterol by adding mevalonolactone to the incubation medium in increasing concentrations. The esterification of this newly synthesized cholesterol was measured by measuring the incorporation of radiolabeled mevalonolactone into cholesteryl ester. Cholesterol esterification was also measured by the incorporation of oleic acid in cholesteryl ester as described before. **Table 7** shows the results of [^{3}H]mevalonolactone incorporation into cholesteryl ester in the presence or absence of 25-hydroxycholesterol. Without added mevalonolactone, only a small amount of newly synthesized cholesterol was esterified. With the addition of 25-hydroxycholesterol, however, the rate of esterification did increase significantly. As the concentration of mevalonolactone was

TABLE 7. Effect of 25-hydroxycholesterol on [^{3}H]mevalonolactone or [^{3}H]oleic acid incorporation into cholesteryl ester in isolated intestinal cells at different mevalonolactone concentrations

Unlabeled Mevalonolactone	pmol [^{3}H]Mevalonolactone Incorporation into Unesterified Cholesterol	25-Hydroxycholesterol (μg/ml)			
		0	10	0	10
		pmol [^{3}H]Mevalonolactone Incorporation		pmol [^{3}H]Oleic Acid Incorporation[a]	
mM		*cholesteryl ester formed mg^{-1} hr^{-1}*			
0	11	0.86 ± 0.07	1.22 ± 0.1[b]	46	182
0.01	36	4.5 ± 0.65	5.8 ± 0.77[c]	47	182
0.10	365	104 ± 21	125 ± 23[d]	48	202
1.0	848	682 ± 114	708 ± 128[e]	48	202
10.0	1348	1622 ± 293	1609 ± 413	32	187

Isolated intestinal cells were incubated for 1 hr at 37°C in 95% O_2, 5% CO_2 in 1 ml of Krebs-bicarbonate buffer without calcium containing [^{3}H]mevalonolactone at the concentrations listed (sp act ranged from 1152 cpm/pmol to 0.458 cpm/pmol) or [^{3}H]oleic acid, 50 μM, (sp act 70,000 dpm/nmol). 25-Hydroxycholesterol was added in 10 μl 95% ethanol 30 min after the start of the incubation. The cells were processed as described in Methods.

[a] One representative experiment of four; values are means of duplicate dishes.
[b] $P < 0.001$ vs control (0 μg 25-hydroxycholesterol ml^{-1}, paired *t*-test).
[c] $P < 0.005$ vs control.
[d] $P = 0.02$ vs control.
[e] $P > 0.10$ vs control.

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increased, the amount of newly synthesized cholesterol that was esterified greatly increased, but the effect of 25-hydroxycholesterol to further increase the esterification of this newly formed cholesterol diminished. As observed in microsomes enriched in free cholesterol, 25-hydroxycholesterol loses its stimulatory effect when the enzyme already has maximal activity due to expanded cholesterol substrate pool.

Table 7 also shows the results of [^{3}H]oleic acid incorporation into cholesteryl ester as mevalonolactone concentration in the medium is increased. The data are in contrast to that seen with radiolabeled mevalonolactone. Despite the many-fold increase in synthesis of free cholesterol within the cells, the incorporation of [^{3}H]oleic acid into cholesteryl ester was virtually unchanged. The previously observed increase in [^{3}H]oleic acid incorporation into cholesteryl ester occurred when 25-hydroxycholesterol was added. At the highest concentration of mevalonolactone (10.0 mM), 25-hydroxycholesterol still increased oleic acid incorporation into cholesteryl ester 6-fold.

DISCUSSION

These experiments confirm, in general, the findings of others regarding the effect of 25-hydroxycholesterol on ACAT activity in fibroblasts (3) and hepatocytes (8). However, three important new observations were made: *1*) 25-hydroxycholesterol increases the esterification of exogenous cholesterol solubilized in either phospholipid liposomes or bile salt micelles that is taken up by intestinal absorptive cells; *2*) the uptake of cholesterol by isolated intestinal cells is not affected by 25-hydroxycholesterol; and *3*) as microsomal cholesterol content increases, the stimulatory effect of 25-hydroxycholesterol on ACAT activity diminishes.

25-Hydroxycholesterol increases ACAT activity in intestinal microsomes. Since the oxygenated sterol can increase the activity of ACAT in a cell-free preparation, it suggests that 25-hydroxycholesterol has a direct effect upon the enzyme or affects the membrane in close proximity to the enzyme. This data is in agreement with results obtained from hepatic microsomal membranes (8). The very rapid change of ACAT activity produced by 25-hydroxycholesterol makes it unlikely that a derivative or breakdown of 25-hydroxycholesterol is the cause for its effect. Only a small amount of the oxygenated sterol (0.5 μg/ml) is necessary to significantly increase the activity of ACAT.

Experiments with isolated cells produced data consistent with our findings in microsomal membranes. 25-Hydroxycholesterol increases cholesteryl ester synthesis but does not affect synthesis of triacylglycerols or uptake of fatty acids. This suggests that the effect of 25-hydroxycholesterol is specific for ACAT. In contrast to microsomal membranes, however, the effect of the oxygenated sterol on enzyme activity in cells is dependent upon time over 1 hr. This difference is not surprising. The response of ACAT activity to 25-hydroxycholesterol in isolated cells depends upon a number of variables including its uptake by the cell, its transport to the microsomal membrane, and finally its modulation of the enzyme.

The passive absorption of cholesterol into intestinal cells is dependent upon a concentration gradient of free cholesterol across the plasma membrane of the cell (23). By enhancing cholesteryl ester synthesis within absorptive cells by 25-hydroxycholesterol, one might suspect an increase in the free cholesterol gradient across the membrane. Thus, uptake of cholesterol from the lumen into the cell may be increased. Our results show that the rate of cholesterol taken up by cells exposed to 25-hydroxycholesterol is no different from cells that were not exposed to the oxygenated sterol. This suggests that 25-hydroxycholesterol, despite its ability to increase cholesteryl ester synthesis 6-fold, does not affect the uptake of luminal cholesterol into the cells.

Mitropoulos et al. (24, 25) have shown that the regulation of hepatic cholesterol esterification depends upon the availability of a membrane cholesterol pool that is in close proximity to the enzyme. This substrate pool for hepatic ACAT is apparently different from the pool regulating HMG-CoA reductase (24). In order for 25-hydroxycholesterol to regulate both enzymes reciprocally, which it does in intact cells, the oxygenated sterol could affect a common molecule in both pathways, i.e., cholesterol. When cholesterol-enriched microsomes were incubated with 25-hydroxycholesterol, the effect of the polar sterol was inversely related to the amount of cholesterol that was added to the membrane (Table 3). When the free cholesterol pool in isolated intestinal cells was expanded by adding increasing concentrations of mevalonolactone, the effect of 25-hydroxycholesterol on the esterification of this newly synthesized cholesterol was inversely related to the amount of mevalonolactone added and therefore free cholesterol content (Table 7). Similarly, the effect of 25-hydroxycholesterol on ACAT activity was greatly diminished in intestinal cells prepared from animals fed a diet high in cholesterol compared to cells prepared from animals fed normal chow (Table 6). The regulation of ACAT activity in microsomes and intact cells by 25-hydroxycholesterol is related to the amount of free cholesterol that is available to the enzyme. As the activity of ACAT is increased by the addition of substrate, the oxygenated sterol loses its stimulatory effect.

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We have observed that protein synthesis is not necessary for 25-hydroxycholesterol to be effective. There are two mechanisms, however, that we cannot exclude. 25-Hydroxycholesterol may interact with the microsomal membrane in such a way that more cholesterol is made available to the substrate pool for ACAT. To our knowledge, there is no evidence for this in the literature, but we suspect the possibility exists. The other possibility is that the substrate and effector sites on ACAT are structurally similar so that, in the presence of excess cholesterol, 25-hydroxycholesterol cannot bind to its effector site. It must be dissimilar, however, to the site where acyl-CoA interacts with the enzyme as the stimulatory effect of 25-hydroxycholesterol on ACAT activity was independent of the oleoyl-CoA concentration (Table 1).

When [^{3}H]oleic acid was used as the tracer for measuring cholesteryl ester synthesis in enterocytes, the addition of increasing concentrations of mevalonolactone (Table 7) did not increase cholesteryl ester synthesis. The stimulatory effect of 25-hydroxycholesterol was present and equal at all mevalonolactone concentrations. This suggests that there are possibly two pools of cholesterol that are utilized as substrate by ACAT within the enterocyte. One pool is the newly synthesized cholesterol from mevalonolactone. The second pool is the cholesterol that is a structural part of the membrane. The incorporation of [^{3}H]oleic acid into cholesteryl ester utilizes cholesterol in this membrane pool in proximity to ACAT. The newly synthesized cholesterol does not readily enter this membrane pool, at least within the 1-hr period of observation. This pool of newly synthesized cholesterol, however, is esterified. Similar results to these were obtained in fibroblast extracts (3). It was shown that the addition of increasing concentrations of exogenous cholesterol to fibroblast extracts did not affect the rate of oleic acid incorporation into cholesteryl ester. Yet, the exogenous cholesterol was esterified. This supports our observations and suggests that two pools of free cholesterol exist within the enterocyte.

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