Intestinal cholesterol esterase: intracellular enzyme or contamination of cytosol by pancreatic enzymes?

F. Jeffrey Field

Department of Internal Medicine, University of Iowa, University of Iowa Hospitals and Clinics, Iowa City, IA 52242

Abstract The location of cholesterol esterase in rabbit intestine was re-evaluated. In three different experiments that were designed to eliminate contaminating mucus and pancreatic enzymes from the lumen of the small intestine, it was observed that the activities of cholesterol esterase and amylase in intestinal cytosol and whole homogenate decreased in parallel fashion. After the mucus was carefully wiped from the intestinal mucosa prior to the preparation of cytosol, amylase and cholesterol esterase activities decreased sevenfold. The recovery of the total activity of both enzymes in the cytosol was approximately 15%. When the lumen of the small intestine was filled with phosphate buffer and incubated at 37°C for 20 min, cholesterol esterase and amylase activities in the cytosol prepared from this segment were further decreased. Moreover, the activities of amylase and cholesterol esterase were completely recovered from the lumen. Amylase and cholesterol esterase activities in the cytosol were eliminated if dithiothreitol was used as a mucolytic agent to prepare intestinal mucosa for the isolation of intestinal cells. In whole homogenates prepared from these intestinal segments, approximately 10–15% of the total cholesterol esterase activity remained. This activity, which could not be accounted for by pancreatic contamination, was associated with intestinal nuclei and cellular debris. Progesterone, ethinyl estradiol, and 25-hydroxycholesterol regulated microsomal acyl CoA:cholesterol acyltransferase activity and caused similar directional changes in the rate of cholesteryl ester synthesis in isolated intestinal cells. These same sterols, however, failed to affect cytosolic cholesterol esterase activity in vitro.—**Field, F. J.** Intestinal cholesterol esterase: intracellular enzyme or contamination of cytosol by pancreatic enzymes? *J. Lipid Res.* 1984. **25:** 389–399.

Supplementary key words cholesterol esterification • small intestine • amylase • acylcoenzyme A:cholesterol acyltransferase

Essentially all exogenous and endogenous cholesterol that is absorbed from the intestinal lumen into the absorptive cell is in the form of the unesterified sterol. When cholesterol is eventually secreted into the mesenteric lymphatic system as part of the core lipids in chylomicrons and very low density lipoproteins, approximately 80% of the cholesterol has been esterified. The presumed site for this esterification reaction is the intestinal absorptive cell (1). Two mechanisms have been described for the esterification of absorbed cholesterol in the intestine. It has previously been postulated that subunits of the pancreatic enzyme, cholesterol esterase, penetrate the brush border membrane of the enterocyte, aggregate to an active enzyme once inside the cell, and catalyze cholesterol esterification through a reversal of the hydrolytic reaction (2). Intestinal cholesterol esterase is located predominantly in the cytosol of the intestinal cell (3). More recently, using an immunocytochemical technique, the reaction product (cholesterol esterase antigenic determinants) was uniformly distributed within the intestinal absorptive cell, lamina propria, and submucosa. No antigens were found in the microvillar membrane (4). Conjugated trihydroxy-bile salts are an absolute requirement for the catalysis of cholesterol esterification by cholesterol esterase (5, 6). It is felt that the bile salts protect the enzyme from proteolytic degradation and act as a cofactor for the enzyme. Cholesterol esterase does not require energy (7) and fatty acids are esterified to cholesterol without first being esterified to their coenzyme A derivatives. The pH optimum for this cytosolic enzyme is 6.2, with less than 5% of the esterifying activity being present at pH 7.4 (5, 6).

In contrast to cholesterol esterase, acylcoenzyme A:cholesterol acyltransferase (ACAT), a microsomal enzyme, has been described in the intestine of humans (8), rats (9), guinea pigs (10), rabbits (11), and subhuman primates.[1] Intestinal ACAT has been localized to the endoplasmic reticulum, with highest specific activity being observed in the rough membranes (12). The enzyme is inhibited by bile salts (13). Energy is necessary for ACAT to catalyze cholesterol esterification (9). It is a requirement that fatty acids first be "activated" to their CoA-derivatives prior to esterification. The pH optimum for ACAT is 7.2 (9). Information is accumulating on the regulation of intestinal ACAT by fats, cholesterol and other sterols, particularly 25-hydroxycholesterol, β-sitosterol, progesterone, and ethinyl estradiol (12–14).

There is a marked disparity between these two mechanisms of cholesterol esterification in the intestine. Yet,

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase; TLC, thin-layer chromatography

[1] Armstrong, M., and F. J. Field. Unpublished data.

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it is still not clear which mechanism is primarily responsible for the esterification of cholesterol in the gut. The data presented in this report support the hypothesis that cholesterol esterase activity in intestinal cytosol is a contamination of this subcellular fraction by pancreatic enzymes which are present in the lumen during cell fractionation. Progesterone, ethinyl estradiol, and 25-hydroxycholesterol, sterols which regulate intestinal microsomal ACAT activity but which do not regulate cholesterol esterase activity in vitro, affect the rate of cholesterol esterification in isolated intestinal cells in the same direction that ACAT activity is affected. Thus, it is concluded that ACAT is more likely responsible for catalyzing cholesterol esterification in the intestine than cholesterol esterase.

MATERIALS AND METHODS

[1-^{14}C]Oleoyl coenzyme A, [4-^{14}C]cholesterol, [9,10-^{3}H]oleic acid, and [1,2-^{3}H(N)]cholesterol were purchased from New England Nuclear. Oleoyl coenzyme A, oleic acid, cholesterol, taurocholic acid, starch substrate, agarose Type VII, progesterone, maltose, β-NADH, and dithiothreitol were from Sigma. 1,3,5(10)-Estratrien-17-ethinyl-3,17-diol and 5-cholesten-3,25-diol were from Steraloids. Lipid standards were from Nu-Chek, Inc. All other reagents 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. Rabbits were fed Purina rabbit chow and water ad lib.

Preparation of microsomes

All rabbits were killed between 0800 and 1000 hr by cervical dislocation. Only the duodenum and proximal jejunum were used to prepare microsomes from scraped mucosa as previously described (12). Microsomes were prepared from isolated cells in the same manner.

Cytosol preparation

Three 20-cm segments of bowel were resected from the small intestine in the area of the ligament of Treitz. The three unmarked segments were placed in a common beaker filled with cold normal saline. The lumen of each segment was flushed twice with a total of 60 ml of cold saline. Each small intestinal segment was prepared differently prior to cytosol preparation. In one randomly selected segment, the intestine was cut longitudinally. Without further manipulation, the mucosa was gently scraped from the muscularis mucosae with a glass slide. In another segment, one of the following procedures was done. *a*) Prior to scraping the mucosa, the overlying mucus was carefully wiped off using a four-by-four gauze soaked with cold normal saline. *b*) The lumen of the bowel was filled with 0.154 M phosphate buffer (pH 6.2) and the ends were clamped. The bowel segment was placed in a beaker of normal saline at 37°C and oscillated for 20 min. The luminal buffer was drained and saved for analysis. The segment was then opened and the mucosa was gently scraped. *c*) The lumen was filled with a rinse consisting of 8 ml of cold normal saline containing 1 mM DTT. After 2 min of agitation, the contents were drained and saved for analysis. The lumen was then filled with Buffer A (15), 8 mM KH_2PO_4 (pH 6.2) containing 1.5 mM KCl, 96 mM NaCl, 27 mM Na citrate. The intestine was clamped at both ends, placed in a beaker of normal saline at 37°C, and oscillated for 20 min. Buffer A was drained and saved. The segment was then either cut open for mucosal scraping or isolated cells were prepared by a modified method of Weiser (15). To prepare cells, the lumen was filled with Buffer B, phosphate-buffered saline (pH 6.2) containing 1.5 mM EDTA and 0.5 mM dithiothreitol. The ends were clamped and the intestine was placed back in the oscillating beaker at 37°C. The mucosal cells were collected by draining the luminal contents into separate plastic centrifuge tubes and refilling the intestine with Buffer B. Exchanges were made every 6 min for a total of seven exchanges and all collected cells were used for cell fractionation. The cells were diluted with phosphate buffer (pH 6.2) and recovered by centrifugation at 2,000 rpm for 5 min. The cells were washed twice in this manner before homogenization.

In another experiment utilizing agar gel, the following procedure was performed (16). At the ligament of Treitz, a 12-cm segment of small intestine was excised and rinsed twice with 30 ml of cold normal saline. The lumen was then filled with 2% agar that was at 37°C. The segment was placed in a beaker filled with normal saline at 37°C. The gel was allowed to harden slowly by refrigerating the beaker at 4°C. When the saline reached a temperature of 15°C, the beaker was placed on ice and cooled further to 10°C. The intestine was removed and carefully cut longitudinally so as not to disrupt the gel cast. The cast was homogenized in 7 ml of 0.154 M phosphate buffer (pH 6.2) in a Potter-Elvehjem homogenizer. The slurry was centrifuged at 105,000 *g* for 90 min and the supernatant was taken for assays. The remaining intestinal mucosa was scraped with a glass slide as before.

These different mucosal preparations were fractionated by homogenizing the scraped mucosa or isolated cells in a Dounce homogenizer, five passes with a loose-fitting pestle. The buffer used was 0.154 M phosphate buffer (pH 6.2) except as stated in the text. The whole homogenate was centrifuged at 2,000 *g* for 10 min. The supernatant from this spin was centrifuged at 10,000 *g* for 20 min. Microsomes were prepared by centrifuging this

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supernatant at 105,000 *g* for 60 min. The resulting cytosol was decanted off and used for enzyme assays.

The luminal contents that were drained from the above preparations were centrifuged at 2,000 rpm for 10 min to pellet cells and feces. The supernatants were used for analysis.

Enzyme assays

Amylase assay. The assay was modified from the method described by Dahlqvist (17). Total volume of the assay was 0.2 ml containing cytosol, whole homogenate or luminal contents, 0.25–0.50 mg protein, phosphate-buffered saline (pH 6.9), and 0.1 ml of starch substrate (Sigma). The reaction was started by the addition of substrate following a 5-min pre-incubation at 37°C. The reaction was stopped after 5 min by adding 0.2 ml of 3,5-dinitrosalicylate (DNS). The assay tubes were then placed in a boiling water bath for 10 min. After cooling, 1 ml of water was added to each assay and the tubes were vortexed. Color was read on a Gilford spectrophotometer at 530 nm. Controls were also prepared for each assay. In control tubes, DNS was added before adding the substrate. The absorbances obtained from the control tubes and blanks (assays without protein) were subtracted from each assay. Standard curves were prepared using maltose as standard. Activities were expressed as μg of maltose formed mg^{-1} 5 min^{-1}.

Lactate dehydrogenase assay. Lactate dehydrogenase activity was measured using Sigma Kit No. 340-UV according to the spectrophotometric method of Wroblewski and La Due (18).

Cholesterol esterase assay. The assay was modified from the methods described by Watt and Simmonds (19) and Hyun et al. (6). The substrate mixture was prepared by drying down chloroform containing 52 μmol of [^{14}C]cholesterol, specific activity 40 dpm/nmol, and 160 μmol of oleic acid under a stream of nitrogen. Two ml of 0.154 M phosphate buffer (pH 6.2) containing 40 mg BSA, 102 μmol Na taurocholate, and 1.3 mmol NH_4Cl was added and homogenized in a Potter-Elvehjem homogenizer. The substrate mix was vortexed and kept on ice until used on the same day of preparation.

Cytosol, whole homogenate, or luminal contents, containing 0.50 to 2.0 mg of protein, were made up to 1.8 ml with phosphate buffer (pH 6.2). The reaction was started by adding 0.2 ml of the substrate mixture. After 2 hr at 37°C the assays were stopped by adding 6 ml of chloroform–methanol 2:1 (v/v). A blank containing cytosol heated to 60°C for 15 min was run for each analysis. [^{3}H]Cholesteryl oleate, 15,000 cpm, was added as an internal standard. Lipid standards were also added as carriers and for TLC identification. One ml of 0.04 N HCl was added and the tubes vortexed and kept overnight at 4°C to ensure separation of the phases. The water phase was aspirated and the chloroform phase was taken to dryness under nitrogen. The residue was taken up in 0.125 ml of chloroform and spotted on TLC plates layered with Silica Gel 60H (E. Merck). The plates were developed with a solvent system containing hexane–ethyl acetate 9:1 (v/v). Lipids were visualized by exposure of the chromatograms to I_2 vapor, and the area corresponding to cholesteryl ester was scraped directly into liquid scintillation vials containing 10 ml of a Liquifluor–toluene scintillation solution. Radioactivity was measured in a Beckman model LS8100-liquid scintillation counter. Quenching was monitored by an external standard. Efficiency for ^{14}C was 75% and did not vary significantly between assays. Recoveries ranged from 75 to 90%. Spillover of ^{3}H counts into the ^{14}C channel were calculated for each assay, as well as ^{14}C counts from the blank. These were subtracted from the total ^{14}C counts of each assay. Cholesterol esterase activity was expressed as nmol of cholesteryl oleate formed mg^{-1} hr^{-1}.

Acylcoenzyme A:cholesterol acyltransferase assay. Microsomal ACAT activity was determined as described previously (12), using [^{14}C]oleoyl CoA (18,370 dpm/nmol) and endogenous microsomal cholesterol as substrates. Total volume of the assay was 0.2 ml and the reaction time was 4 min.

Measurement of [^{3}H]oleic acid incorporation into cholesteryl oleate in isolated intestinal cells

Isolated cells were prepared as described previously. The cells were diluted with Krebs buffer (pH 7.4) without Ca^{2+} and recovered by centrifugation at 2,000 rpm for 5 min. The cells were washed twice with Krebs buffer before use. The determination of cholesteryl ester synthesis in these cells has been described (13).

Protein analysis

Protein was determined by the method of Lowry et al. (20) with bovine serum albumin as the standard.

RESULTS

Cholesterol esterase activity in small intestine

Parameters of the cholesterol esterase reaction were first characterized. The reaction was linear for 4 hr in a protein concentration range from 0.5 to 3.0 mg. The pH optimum was between 6.0 and 6.3 and no enzyme activity was observed in the absence of bile salts. Unless otherwise stated, the cholesterol esterase assay was performed at pH 6.2 for 2 hr at 37°C. Protein concentrations ranged from 0.5 mg to 2.0 mg.

The subcellular fractionation of intestinal mucosa is a difficult task. Significant cross-contamination of subcel-

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lular organelles occurs. This is thought to be due to the abundant mucus which is found in the lumen of the small intestine. The elimination of mucus prior to cell fractionation is usually incomplete (21). Some investigators have observed that by first removing the mucus by gently wiping it off, or using a mucolytic agent such as dithiothreitol, or by preparing isolated cells prior to cell fractionation results in the preparation of more pure fractions of subcellular organelles (22, 23). Because of the abundance of mucus in the intestinal lumen and the problem it causes with cell fractionation in the intestine, it was postulated that pancreatic enzymes might adsorb to this adherent layer of mucus and contaminate the subcellular fractions during preparation.

Three different experiments were performed to eradicate or decrease the mucus found in the lumen of the small intestine. Amylase activity was used as an internal check for pancreatic enzyme contamination of the intestinal cytosol during its preparation. Lactate dehydrogenase, a cytosolic enzyme, was used as a marker for intracellular enzyme activity.

In these experiments, three segments of small bowel in proximity to the ampulla of Vater were used. The three segments were in continuity with each other and therefore would be bathed by similar amounts of pancreatic and biliary secretions. Because the activity of cholesterol esterase in the intestine does appear to decrease with increasing distance from the ampulla, the three intestinal segments that were excised from the animal were randomly selected from a common beaker. Changes which occur in cholesterol esterase and amylase activities from the different intestinal segments, therefore, could not be attributed to its location from the ampulla.

In the first experiment, an attempt was made to mechanically remove the mucus from the small intestine (**Table 1**). The mucus was either wiped from the mucosa with gauze and cold saline or it was removed by filling the intestinal lumen with phosphate buffer, incubating the segment in normal saline at 37°C with shaking, and then draining the luminal contents prior to scraping the mucosa. One intestinal segment (I) served as a control. In this segment, the mucosa was scraped without any previous manipulation. If it is true that pancreatic cholesterol esterase adheres to the intestinal mucus, then contamination of the cytosol by this enzyme would occur during preparation. The effects of removing the mucus prior to measuring cholesterol esterase activity would therefore be most pronounced in this subcellular fraction. Protein content, amylase activity, cholesterol esterase activity, and lactate dehydrogenase activity were determined in intestinal cytosol. The results are shown in Table 1. The intestinal cytosol designated as (I) theoretically contained total protein and total enzyme activities as no attempt was made to remove luminal contaminants prior to preparing the cytosol. Thus, recoveries from this control segment were designated at 100%. Amylase activity was observed in the cytosol prepared from this control segment, 82.4 μg maltose formed per mg of cytosolic protein 5 min^{-1}. This suggests that pancreatic enzymes do contaminate intestinal cytosol if prepared in this way. The activity of cholesterol esterase in this fraction, 1.5 nmol of cholesteryl ester formed per mg of cytosolic protein hr^{-1}, is similar to the activities of this enzyme that have been described in earlier reports, i.e., 0.65 nmol mg^{-1} hr^{-1} (19), 1.60 (5), and 2.67 (3). Lactate dehydrogenase activity, expressed as nmol of NADH oxidized per mg of cytosolic protein min^{-1}, was 102 in the cytosol prepared from this segment. In the intstinal segment first wiped clean of contaminating mucus prior to cytosol preparation (II), amylase activity decreased sixfold to 13.9 with a recovery of only 16%. Recoveries were determined by dividing the total activity of the enzyme in the cytosol prepared from the experimental segments by the total activity of the enzyme measured in the cytosol prepared from the control segment (I). The specific activity of cholesterol esterase in II decreased in parallel fashion along with amylase activity. It decreased to a specific activity that was sevenfold lower than control (0.22 vs 1.5) and a recovery of only 14%. On the other hand, lactate dehydrogenase specific activity did not change significantly and all the activity was recovered in the cytosol from this segment, 126%. In the cytosol that was prepared from the intestinal segment that was first filled with phosphate buffer (IV), a more marked decrease in amylase and cholesterol esterase specific activities occurred. Amylase activities were not detected in the cytosols prepared from the intestines of three animals. In cytosols prepared from the intestines of two animals, cholesterol esterase activities were not detected. Because of this, the standard errors for amylase, 8.2 ± 5.4, and cholesterol esterase activities, 0.14 ± 0.08, were large. The specific activity of lactate dehydrogenase did not change appreciably and the percent recovery in the cytosol from this segment was 84%. In the buffer that was drained from the lumen of this segment, specific activities of amylase and cholesterol esterase increased in parallel, 1,273 U and 43 U, respectively. The total activities of both these enzymes measured in the lumen accounted for the total activities observed in the cytosol prepared from the control segment, 122% and 226%. Remaining contamination of cytosol by amylase activity in IV (6%) accounted for the activity of cholesterol esterase also detected in this cytosol (6%). The fact that amylase activity measured in the lumen could account for the total enzyme activity suggests that amylase is a good extracellular marker.

Although intestinal cholesterol esterase is thought to be of pancreatic origin and found predominantly in intestinal cytosol (3, 4), an earlier report observed that a

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TABLE 1. Protein, amylase, cholesterol esterase, and lactate dehydrogenase activities in the cytosol and lumen of the small intestine

		Protein[a]	Amylase[b]	Cholesterol Esterase[c]	Lactate Dehydrogenase[d]
I	Cytosol: no manipulation, mucosa scraped	139 ± 2.0 (100%)[e]	82.4 ± 12 (100%)	1.5 ± 0.3 (100%)	102 ± 11 (100%)
II	Cytosol: mucosa wiped, sponged, and scraped	130 ± 12 (96%)	13.9 ± 3.7 (16%)	0.22 ± 0.1 (14%)	140 ± 21 (126%)
III	Luminal contents	11 ± 3.5 (8%)	1273 ± 594 (122%)	43 ± 8.1 (226%)	22 ± 10 (2%)
IV	Cytosol: mucosa scraped from III	89 ± 11 (64%)	8.2 ± 5.4 (6%)	0.14 ± 0.08 (6%)	134 ± 5.0 (84%)

Three 20-cm segments of small bowel from the ligament of Treitz were removed. The lumen of each segment was rinsed twice with 30 ml of cold normal saline. Without further manipulation, one segment was cut longitudinally and the mucosa was carefully scraped and cytosol was prepared as described in Methods. In another segment, the bowel was cut longitudinally, the mucosa was gently wiped with gauze, rinsed with cold normal saline, and sponged. The mucosa was scraped and cytosol was prepared. The lumen of the third segment was filled with 10 ml of 0.154 M phosphate buffer (pH 6.2) and incubated at 37°C in normal saline for 20 min. The buffer was drained and saved. The segment was then cut and the mucosa was scraped and cytosol was prepared. The data are the means and SE from five rabbits. Assays were done in duplicate, except for amylase which was done in triplicate.

[a] Milligrams cytosol or luminal protein.
[b] Micrograms maltose formed/mg cytosol or luminal protein per 5 min.
[c] Nanomoles cholesteryl ester formed/mg cytosol or luminal protein per hr.
[d] Nanomoles NADH oxidized/mg cytosol or luminal protein per min.
[e] Percent recovery.

third of the activity in the intestine was associated with intracellular membranes (3). To test if this membrane-associated activity of cholesterol esterase could be changed by removing intestinal mucus prior to cell fractionation, the same experiment was carried out. This time, however, amylase, cholesterol esterase, and lactate dehydrogenase activities were measured in whole homogenates. The results of these experiments are shown in **Table 2.** Again, the whole homogenate designated as (I) was from a control segment and theoretically contained total protein and total enzyme activities. The specific activity of cholesterol esterase in the whole homogenate of the control segment was 3.4 nmol of cholesteryl ester formed per mg of protein hr^{-1}. The higher specific activity of cholesterol esterase in the whole homogenate as compared to the cytosolic fraction, 1.9, (Table 1) has been noted before (3). As expected, the specific activities of amylase and lactate dehydrogenase were lower in the whole homogenate than in the cytosolic fraction (Table 1). In the intestinal segment in which the mucosa was first wiped and sponged prior to preparing the whole homogenate (II), the specific activities of both amylase and cholesterol esterase decreased in parallel from 57 and 3.4 observed in the control whole homogenate (I) to 15 and 1.0, respectively. The recoveries of both enzymes were essentially the same, approximately 25%. As in the previous experiment, the total activity of amylase found in the lumen essentially accounted for the total amylase activity found in the whole homogenate, 1,304 U (81%). The specific activity of cholesterol esterase increased 17-fold in the lumen with a recovery of 58%. The cholesterol esterase activity in the whole homogenate from this segment (IV) still contained 15% of the total cholesterol esterase activity. Only 2% of this activity can be considered contamination by pancreatic enzymes. This suggests that approximately 10–15% of the cholesterol esterase activity that is measured in the whole homogenate may not be secondary to pancreatic contamination and is associated with an intracellular membrane fraction. After further subcellular fractionation in a phosphate buffer containing 0.25 M sucrose at pH 6.2, it was observed that 98% of the remaining cholesterol esterase activity in the whole homogenate was associated with nuclei and cell debris. Only 2% of the remaining activity was present in either the mitochondrial or microsomal fraction.

In the method of intestinal cell isolation as described by Weiser (15), dithiothreitol is used as a mucolytic agent. The following experiment utilizes this technique to investigate the mucolytic effect of dithiothreitol on amylase, cholesterol esterase, and lactate dehydrogenase activities in intestinal cytosol. Cytosols were prepared from isolated cells or from mucosa that was scraped from intestines that were first prepared for cell isolation according to the method of Weiser. The results are shown in **Table 3.** The intestinal cytosol designated as (I) is from a control segment. In another segment of bowel, the lumen was filled with a rinse solution containing normal saline and 1 mM dithiothreitol. After 2 min of agitation, this solution was drained (II) and the lumen was filled with Buffer A

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TABLE 2. Protein, amylase, cholesterol esterase, and lactate dehydrogenase activities in the lumen and whole homogenate of small intestine

		Protein[a]	Amylase[b]	Cholesterol Esterase[c]	Lactate Dehydrogenase[d]
I	Whole homogenate: no manipulation, mucosa scraped	340 ± 47 (100%)[e]	57 ± 15 (100%)	3.4 ± 1.8 (100%)	59 ± 22 (100%)
II	Whole homogenate: mucosa wiped, sponged, and scraped	293 ± 46 (86%)	15 ± 8 (23%)	1.0 ± 0.3 (25%)	59 ± 16 (86%)
III	Luminal contents	12 ± 3 (4%)	1304 ± 531 (81%)	56 ± 32 (58%)	65 ± 11 (4%)
IV	Whole homogenate: mucosa scraped	218 ± 20 (64%)	2 ± 1 (2%)	0.77 ± 0.2 (15%)	54 ± 16 (58%)

Three 20-cm segments of small bowel from the ligament of Treitz were removed. The lumen of each segment was rinsed twice with 30 ml of cold normal saline. Without further manipulation, one segment was cut longitudinally and the mucosa was carefully scraped and whole homogenate was prepared as described in Methods. In another segment, the bowel was cut longitudinally, the mucosa was gently wiped with gauze, rinsed with cold normal saline, and sponged. The mucosa was scraped and whole homogenate was prepared. The lumen of the third segment was filled with 10 ml of 0.154 M phosphate buffer (pH 6.2) and incubated at 37°C in normal saline for 20 min. The buffer was drained and saved. The segment was then cut and the mucosa was scraped and whole homogenate was prepared. The data are the means and SE from four rabbits. Assays were done in triplicate, except for lactate dehydrogenase which was done in duplicate.

[a] Milligrams whole homogenate or luminal protein.

[b] Micrograms maltose formed/mg whole homogenate or luminal protein per 5 min.

[c] Nanomoles cholesteryl ester formed/mg whole homogenate or luminal protein per hr.

[d] Nanomoles NADH oxidized/mg whole homogenate or luminal protein per min.

[e] Percent recovery.

TABLE 3. Protein, amylase, cholesterol esterase, and lactate dehydrogenase activities in the lumen and cytosol of scraped intestinal mucosa or isolated cells

		Protein[a]	Amylase[b]	Cholesterol Esterase[c]	Lactate Dehydrogenase[d]
I	Cytosol: no manipulation, mucosa scraped	148.5 ± 17 (100%)[e]	95.5 ± 1.8 (100%)	1.9 ± 0.3 (100%)	124 ± 43 (100%)
II	Luminal contents: rinse	3.4 ± 0.9 (2.3%)	2177 ± 501 (52%)	107 ± 51 (129%)	ND[f]
III	Luminal contents: buffer	2.6 ± 0.4 (1.8%)	1167 ± 180 (21%)	22.8 ± 9 (21%)	12 ± 5 (0.2%)
IV	Cytosol: mucosa scraped	100.3 ± 18 (67.5%)	ND	ND	151 ± 43 (82%)
V	Cytosol: isolated cells	31.4 ± 8.0	ND	ND	151 ± 54

Three 20-cm segments of small bowel from the ligament of Treitz were removed. The lumen of each segment was rinsed twice with 30 ml of cold saline. Without further manipulation, one segment was cut longitudinally and the mucosa was carefully scraped and cytosol was prepared as described in Methods. The lumen of the second segment was filled with 8 ml of rinse solution (see Methods) and agitated for 2 min at room temperature. The rinse was drained and saved. The lumen was then filled with buffer A (see Methods) and incubated at 37°C in normal saline for 20 min. The buffer was drained and saved. The segment was then cut, mucosa was scraped, and cytosol was prepared. The same procedure was done on the third segment, except, after the buffer was drained, isolated absorptive cells were prepared as described in Methods. The cells were homogenized and cytosol was prepared. The data are the means and SE from four rabbits. Assays were done in duplicate, except for amylase which was done in triplicate.

[a] Milligrams cytosol or luminal protein.

[b] Micrograms maltose formed/mg cytosol or luminal protein per 5 min.

[c] Nanomoles cholesteryl ester formed/mg cytosol or luminal protein per hr.

[d] Nanomoles NADH oxidized/mg cytosol or luminal protein per min.

[e] Percent recovery.

[f] ND, none detected.

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containing 8 mM KH_2PO_4 (pH 6.2), 1.5 mM KCl, 96 mM NaCl, and 27 mM Na citrate. The intestine was incubated at 37°C for 20 min with shaking, after which time this solution was drained (III). At this point, cytosol was either prepared from scraped mucosa (IV) or isolated cells (V) as described in Methods.

In the cytosol prepared from the scraped mucosa (IV), neither amylase nor cholesterol esterase activity was detected. Specific activity of lactate dehydrogenase was unchanged (151 U) and recovery of its activity was virtually complete. It is unlikely, therefore, that the elimination of cholesterol esterase activity observed in the cytosol prepared from this segment was secondary to cell breakage and the subsequent release of enzyme during the rinse or incubation processes. In the lumen which consists of the rinse solution (II) and buffer (III), the specific activities of amylase and cholesterol esterase again increased in parallel. Complete recovery of total enzyme activities from the lumen (recovery of total protein was 73.5%) suggested that amylase and cholesterol esterase were located extracellularly. Very little to no lactate dehydrogenase activity was recovered from the lumen (II and III) suggesting that very little cell damage occurred during incubations of the small intestine. In cytosol prepared from isolated cells (V), results were similar to those found in cytosol prepared from scraped mucosa. Amylase and cholesterol esterase activities were again not detected. The prior preparation of intestinal mucosa for intestinal cell isolation, as described by Weiser (15), completely eliminated contamination of the intestinal cytosol by pancreatic amylase and cholesterol esterase activities. In this experiment, approximately 10% of cholesterol esterase activity was observed in whole homogenates that could not be attributed to pancreatic contamination. The activity that remained in the whole homogenate of isolated cells was again found predominantly (94%) in the nuclei and cell debris.

In another experiment, the lumen of a 12-cm segment of bowel resected at the ligament of Treitz was filled with 2% agar at 37°C (16). The agar was allowed to solidify slowly by decreasing the temperature of the surrounding saline bath. The gel cast was removed, homogenized in phosphate buffer (pH 6.2), and centrifuged at 105,000 *g* for 90 min. The supernatant was decanted for analysis. The remaining intestinal mucosa was scraped and cytosol was prepared. The results are shown in **Table 4.** The specific activities of amylase and cholesterol esterase in the supernatant from the gel cast (I), were both increased fourfold over the activities found in the cytosol (98.8 vs 364 and 0.9 vs 3.9, respectively). The specific activity of cholesterol esterase in the cytosol was half of the expected activity that was observed in control segments as indicated in Tables 1 and 3. Data from this experiment support data from the previous experiments, suggesting that procedures to eradicate mucus from the small intestine lead to a decrease in contamination of intestinal cytosol with pancreatic enzymes. Because cholesterol esterase and amylase activities in the cytosol are eradicated in parallel fashion, it suggests both are extracellular enzymes that contaminate the cystol during preparation.

In other experiments carried out in exactly the same way as described for Tables 1–3, except that the sucrose-containing phosphate buffer was adjusted to pH 7.4, instead of 6.2, the activity of acyl coenzyme A:cholesterol acyltransferase (ACAT) in microsomes and whole homogenates was measured. The results of these experiments are shown in **Table 5.** As expected, ACAT activities in intestinal whole homogenates and microsomes were not changed by the same manipulations which were shown to eliminate pancreatic contamination and intestinal mu-

TABLE 4. Protein, amylase, cholesterol esterase, and lactate dehydrogenase activities in the cytosol and lumen of the small intestine

	Protein[a]	Amylase[b]	Cholesterol Esterase[c]	LDH[d]
I Agar gel supernatant	12.8 ± 1.8	364 ± 58	3.9 ± 0.8	3.5 ± 0.4
II Cytosol: mucosa scraped from I	29.5 ± 6.0	98.8 ± 7.7	0.9 ± 0.1	3.3 ± 0.6

A 12-cm segment of small bowel from the ligament of Treitz was removed. The lumen was rinsed twice with 30 ml of cold normal saline. The lumen was filled with 2% agar at 37°C. The segment of bowel was placed into a beaker of normal saline at 37°C. The beaker was refrigerated at 4°C, until the saline reached a temperature of 15°C. The beaker was then placed on ice until the temperature reached 10°C. The intestine was removed and carefully cut longitudinally so that the gel cast was not disrupted. The gel cast was put into 7 ml of 0.154 M phosphate buffer (pH 6.2) and homogenized in a motor-driven Potter-Elvehjem homogenizer. This slurry was centrifuged at 105,000 *g* for 90 min and the supernatant was taken for protein determination and enzyme assays. The intestinal mucosa was scraped and cytosol was prepared as described in Methods. The data are the means and SE from four rabbits. Assays were done in duplicate, except for amylase which was done in triplicate.

[a] Milligrams.
[b] Micrograms maltose formed/mg protein from cytosol or agar gel cast per 5 min.
[c] Nanomoles cholesteryl ester formed/mg protein per hr.
[d] Nanomoles NADH oxidized/mg protein per min.

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TABLE 5. Acylcoenzyme A:cholesterol acyltransferase activity in whole homogenates and microsomes from small intestine

		Acylcoenzyme A:Cholesterol Acyltransferase Activity[a]	
		Whole Homogenate	Microsomes
I	No manipulation: mucosa scraped	61 ± 2 (100%)[b]	317 ± 25 (100%)
II	Mucosa, wiped, sponged, and scraped	67 ± 3 (95%)	284 ± 25 (90%)
III	Isolated cells	84 ± 13	277 ± 50

Three 20-cm segments of small bowel from the ligament of Treitz were removed. The lumen of each segment was rinsed twice with 30 ml of cold normal saline. Without further manipulation, one segment was cut longitudinally and the mucosa was carefully scraped and whole homogenate and microsomes were prepared. In another segment, the bowel was cut longitudinally, the mucosa was gently wiped with gauze, rinsed with cold normal saline, and sponged. The mucosa was scraped and whole homogenate and microsomes were prepared. In the last segment, whole homogenate and microsomes were prepared from isolated intestinal cells as described in Methods. The buffer used was 0.154 M phosphate buffer containing 0.25 M sucrose at a pH 7.4. The data are the means and SE from three rabbits. Assays were done in triplicate.

[a] Picomoles cholesteryl oleate formed/mg microsomal or whole homogenate protein per min.

[b] Percent recovery.

cus. Thus, the activity of microsomal ACAT, which has also been shown to catalyze the esterification of cholesterol in the small intestine, is not changed by procedures that affect luminal contents prior to cell fractionation.

Table 6 shows the results of the regulation of cholesterol esterification in isolated intestinal cells by progesterone, ethinyl estradiol, and 25-hydroxycholesterol. For comparison, the effects of these sterols on microsomal ACAT and cholesterol esterase activities are shown. To determine cholesterol esterase activity, cytosol was prepared from mucosal scrapings of control intestine without prior manipulation. Both progesterone and ethinyl estradiol decreased microsomal ACAT activity (second column). They had no effect, however, on the in vitro assay for cytosolic cholesterol esterase activity. In isolated intestinal cells, these hormones significantly decreased the rate of synthesis of cholesteryl oleate from oleic acid (column 3). 25-Hydroxycholesterol significantly increased microsomal ACAT activity without affecting cholesterol esterase activity. In the isolated cells, the oxygenated sterol increased the rate of cholesteryl ester synthesis sixfold.

DISCUSSION

The data presented in this report strongly suggest that pancreatic cholesterol esterase and amylase adsorb to a mucus layer that covers the small intestinal mucosa. These enzymes contaminate intestinal cytosol during the routine

TABLE 6. The effect of progesterone, ethinyl estradiol, and 25-hydroxycholesterol on cytosolic cholesterol esterase activity, microsomal ACAT activity, and cholesteryl ester synthesis in isolated intestinal cells

	Cytosolic Cholesterol Esterase Activity[a]	Microsomal ACAT Activity[b]	Oleic Acid Incorporation into Cholesteryl Oleate in Isolated Cells[c]
Control	2.46 ± 0.52	315 ± 42	0.067 ± 0.005
Progesterone (10 μg/ml)	2.35 ± 0.49	150 ± 36[d]	0.044 ± 0.003[d]
Ethinyl estradiol (10 μg/ml)	2.16 ± 0.43	55 ± 10[e]	0.050 ± 0.003[f]
25-Hydroxycholesterol (10 μg/ml)	2.19 ± 0.38	567 ± 50[e]	0.418 ± 0.082[d]

Progesterone, ethinyl estradiol, and 25-hydroxycholesterol (10 μg/ml) were added to the assays in 95% ethanol (1% of the total volume). Control incubations contained an equal volume of ethanol but without the sterol. For cholesterol esterase and ACAT assays, the sterols were added 30 min before starting the reaction with labeled substrate. In the isolated cell preparation, the sterols were present during the entire 1-hr incubation of cells with labeled oleic acid. The number of animals for: cholesterol esterase, N = 6, assays done in triplicate; ACAT, N = 4, assays done in triplicate; isolated cells, ten dishes.

[a] Nanomoles cholesteryl ester formed/mg cytosol protein per hr.

[b] Picomoles cholesteryl oleate formed/mg microsomal protein per min.

[c] Nanomoles cholesteryl oleate formed/mg cell protein per hr.

[d] $P < 0.001$ vs. control.

[e] $P < 0.01$ vs. control.

[f] $P < 0.005$ vs. control.

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preparation of this subcellular fraction. This was tested using three different methods to remove the mucus layer prior to cell fractionation. In the first method, intestinal mucus was mechanically removed by carefully wiping the mucosa. The second method utilized dithiothreitol as a mucolytic agent according to the technique described by Weiser (15) to prepare intestinal mucosa for cell isolation. In the third method, liquified agar gel was used to adsorb the contaminating enzymes from the lumen of the small intestine. By all three methods, amylase activity, as an indicator of pancreatic contamination, was profoundly decreased in the subsequently prepared small intestinal whole homogenates and cytosols. Moreover, the changes observed in the activity of cholesterol esterase closely paralleled the changes that were observed in amylase activity. This suggests that as pancreatic contamination of the intestinal lumen is eliminated, by whatever means, cytosolic cholesterol esterase is also eliminated. Thus, cholesterol esterase is a pancreatic contaminant of intestinal cytosol when it is prepared in the usual fashion. Further proof that cholesterol esterase and amylase were extracellular and not intracellular prior to cell fractionation was that the total activities of both enzymes in the cytosol were recovered from the lumen of the small intestine. It was not unexpected that 10–15% of the cholesterol esterifying activity remained in the whole homogenate following the different methods to eradicate luminal contamination. In an earlier report, approximately a third of cholesterol esterase activity was recovered in microsomes and nuclei plus cell debris after intestinal subcellular fractionation (3). In agreement with that previous study, 60% of cholesterol esterase activity was observed in the cytosol in this study. Thus, it may have been expected that 30–40% of the cholesterol esterase activity might have remained associated with membrane fractions in the whole homogenate. The fact that we observed only 10–15% suggests that a part of cholesterol esterase activity that is associated with various intracellular membranes may also be secondary to contamination.

Cholesterol esterifying activity in isolated intestinal cells was 67 pmol/mg cell protein per hr as determined by oleic acid incorporation into cholesteryl oleate (Table 6). ACAT activity in whole homogenates of these isolated cells was 84 pmol/mg per min (Table 5). Theoretically, then, microsomal ACAT could account for the total amount of cholesteryl ester formed in these cells. After adding progesterone, ethinyl estradiol, or 25-hydroxycholesterol to a suspension of isolated cells, the directional change of cholesterol esterifying activity was reflected by similar directional changes in microsomal ACAT activity (Table 6). Quantitatively, however, the changes observed correlate poorly between the two methods of measuring the rate of cholesterol esterification. In the microsomal preparation, the sterols were added directly to the incubation assay. The sterols were therefore in direct contact with the membranes and ACAT. In isolated cells, it is more complex. The sterols must penetrate the cell, traverse the cytosol, reach their target (endoplasmic reticulum), and regulate ACAT activity. Also, in whole cells, the sterols may affect other parameters of cholesterol esterification such as ATP production, coenzyme A synthesis, fatty acid CoA-ligase or substrate availability (14). Thus, judgement can only be made on the effects of these sterols on the relative rates of cholesterol esterification and not on the absolute rates. The point still remains that cholesterol esterase activity was unaffected by these sterols despite a significant change in the rate of cholesterol esterification in isolated cells.

There is an ongoing debate in the gastrointestinal literature regarding the absorption of intact protein molecules by intestinal mucosa (24–29). In the dog, Levitt et al. (28) concluded that the absorption of pancreatic amylase by the small intestine was negligible (<0.01%). Likewise, the absorption of intact pancreatic cholesterol esterase at an estimated molecular weight of 136,000, would be difficult to explain by accepted physiological mechanisms. It is possible, however, that small hydrolyzed peptides of these proteins could enter absorptive cells by a transport process or pinocytosis. This could explain why antigenic determinants of pancreatic cholesterol esterase were observed in absorptive cells, lamina propria, and the submucosa of the small intestine (4). There is no precedent in the literature, however, that provides evidence that small peptide fragments can reaggregate to a functional protein once absorbed by intestinal cells as suggested by Gallo et al. (2).

The pH optimum for cholesteryl ester synthesis and hydrolysis as catalyzed by cholesterol esterase is 6.2 and 8.0, respectively. Lombardo, Deprez, and Guy, (30) suggested that the existence of an equilibrium between synthesis and hydrolysis of cholesteryl esters in vivo would be impossible with this wide span of pH optima. Lin, Karvinen, and Ivy (31), while studying the effects of pancreatic juice on cholesterol absorption, concluded that pancreatic cholesterol esterase played no essential role in the absorption of dietary free cholesterol. The exclusion of pancreatic juice had no significant effect on the elimination of endogenous cholesterol in this study. Likewise, Watt and Simmonds (19) observed that cholesterol was absorbed and esterified with equal efficiency in rat intestine whether in the presence or absence of pancreatic enzymes. They found no correlation between coenzyme A-independent cholesterol esterifying activity in the lumen or mucosa of the intestine and the amount of cholesterol ester secreted into the lymph. The role of luminal pancreatic cholesterol esterase in free cholesterol absorption remains unsettled, however, as Gallo et al. (32) recently demonstrated a decrease in cholesterol absorption in the absence of luminal cholesterol esterase.

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In the major metabolic pathways of fatty acid metabolism, energy is required. Fatty acids are first acetylated to coenzyme A (33). Unlike triacylglycerol and phospholipid synthesis which require energy (33), the reaction catalyzed by cholesterol esterase does not (7). Intestinal enzymes that catalyze lipid esterifications are bound to the endoplasmic reticulum (34, 35). Cholesterol esterase is not. The presence of bile salts is not required for triacylglycerol or phospholipid synthesis (33). They are mandatory for the action of cholesterol esterase. It is obvious that the in vitro properties of cholesterol esterase differ remarkably from the properties of other enzymes which catalyze the esterification of lipids within the intestinal cell. Implicating cholesterol esterase as the enzyme responsible for catalyzing cholesterol esterification makes an uncomplicated process very complex. Must fatty acid:CoA ligase discriminate between fatty acids which are to be used for cholesteryl ester synthesis or triacylglycerol synthesis? Does the intestinal cell have two separate systems for esterification that differ radically in their energy requirement and consumption? Does the trihydroxy-bile salt concentration that is necessary for cholesterol esterase activity exist within the intestinal cell? Finally, are there two locations for chylomicron assembly within the enterocyte, one being the cytoplasm, the other the endoplasmic reticulum?

In distinct contrast to cholesterol esterase, the in vitro properties of intestinal ACAT conform to our understanding of esterification reactions that occur within intestinal mucosa. ACAT is a microsomal enzyme with highest specific activity found in the rough endoplasmic reticulum (12). The enzyme is found throughout the length of the small intestine with highest activity in the jejunum and proximal ileum (11, 12). The reaction catalyzed by ACAT is dependent on ATP and coenzyme A, and its pH optimum is 7.2 (9). ACAT activity is inhibited by bile salts (13). In contrast to the lack of correlation between cholesterol absorption and the presence of pancreatic enzymes in the intestinal lumen as observed by Watt and Simmonds (19), Bennett Clark (36) reported a strong correlation between the activity of intestinal ACAT and the mass of cholesteryl ester being secreted into the lymphatics. Recently, Heider, Pickens, and Kelly (37) reported that a competitive inhibitor of intestinal ACAT (57-118) causes an inhibition of cholesterol absorption. The results of the present study lend strong support that intestinal cytosolic cholesterol esterase is a contamination of pancreatic enzymes which adhere to mucus within the intestinal lumen. This, along with the growing body of evidence implicating the importance of ACAT in intestinal cholesterol metabolism, favors this enzyme as the one responsible for catalyzing the esterification of cholesterol in the small intestine.

I wish to thank Ron Salome for his skilled technical assistance and Ms. Ella Albright, Ms. Kathy Funk, and Ms. Julie Ostrem for typing the manuscript. I also wish to thank Dr. A. A. Spector for critical discussion of the manuscript. This work was supported in part by Grant #81-6-14 from the American Heart Association, Iowa Affiliate, Atherosclerosis, Specialized Center of Research #HL-14230 from the National Heart, Lung, and Blood Institute, and by Grant AM 29706 from National Institute of Arthritis, Metabolism, and Digestive Diseases.

Manuscript received 19 August 1983.

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