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Role of Pancreatic Cholesterol Esterase in the Uptake and Esterification of Cholesterol by Isolated Intestinal Cells[1] (39921)

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Pancreatic cholesterol ester hydrolase (CEH) (EC 3.1.1.13) catalyzes the hydrolysis of dietary cholesteryl esters in the intestinal lumen. The free cholesterol produced, together with the free cholesterol in the diet and in the bile, is taken up by the absorptive cells. Here it is re-esterified to cholesterol esters to the extent of 70–90%, and these cholesterol esters are transported into the lymph in chylomicrons or lipoproteins.

There are data which suggest that CEH associated with the intestinal mucosa is derived from that secreted into the lumen from the pancreas. Hernandez *et al.* (1) demonstrated that elimination of pancreatic secretions from the intestinal lumen reduced the level of cholesteryl ester in the lymph of dogs. Swell *et al.* (2) reported similarities in properties of the pancreatic and mucosal enzymes with respect to bile salt requirements, pH optima, and heat inactivation. Borja *et al.* (3) reported that diversion of pancreatic juice reduced mucosal cholesterol esterification and lymphatic transport in rats, while reinfusion of pancreatic juice restored both mucosal CEH activity and cholesterol absorption. Furthermore, Bell and Swell (4) described the maintenance of normal serum cholesterol levels in depancreatized humans by administration of oral doses of pancreatin. In the absence of pancreatin, serum cholesterol levels dropped markedly.

The aims of the present study were to determine: (i) if the component in pancreatic juice, essential for cholesterol absorption, is pancreatic CEH; and (ii) if cholesterol uptake by the intestinal cell is independent of CEH. Intestinal cells have been isolated from rats deprived of pancreatic and biliary secretions. The uptake and esterification of cholesterol have been compared in these cells after preincubation in the presence or absence of purified subunit or active forms of pancreatic CEH (5).

Materials. Male, albino rats (CFN strain) were obtained from Carworth Farms. Chemicals were obtained as follows: collagenase (Type III), bovine serum albumin (BSA Fr. V, fatty acid poor), oleic acid, and cholesterol from Sigma Chemical Co.; monoolein, sodium taurocholate, and thin-layer chromatography (tlc) standard lipid mixtures from Supelco; [U-^{14}C]glucose, L-[1-^{14}C]leucine, and [1-^{14}C]galactose, from ICN Pharmaceuticals; [1-^{14}C]oleic acid and [4-^{14}C]cholesterol from Amersham/Searle; liquid scintillation cocktails, LSC Complete and Hydromix, from Yorktown Research.

Methods. Animal preparation. Rats weighing approximately 200 g were anesthetized with pentabarbital. The common duct was cannulated as described by Borja *et al.* (3), and pancreatic juice and bile were diverted from the intestinal lumen for 72 hr prior to the study.

CEH purification. The CEH (subunit form) of rat pancreas was purified through the hydroxylapatite step in the procedure reported by Calame *et al.* (5). By this procedure, the CEH was approximately 150-fold purified over that in pancreas. The column fractions were pooled and concentrated by Diaflo filtration. Earlier studies (6) have shown that the subunit form of the enzyme can be converted into the active form by exposure to cholic acid or its conjugates. Thus, one-half of the concentrated Diaflo filtrate was incubated with 5.0 m*M* sodium taurocholate to induce subunit aggregation and formation of the enzymatically active CEH (mol wt, 400,000). Both the subunit and active forms of CEH were tested for their effect upon mucosal cholesterol esterification.

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Isolation of mucosal cells. Mucosal cells were prepared from control and common duct-cannulated rats by a modification of the collagenase dissociation technique of Yousef and Kuksis (7). This technique is based upon preloading absorptive cells with lipid, and subsequent flotation of these cells following enzymatic dissociation of the tissue. However, this procedure was not applicable for isolation of mucosal cells from bile-cannulated rats in which lipid absorption had been altered. Thus, in the present study, animals were not administered the lipid load prior to tissue dissociation. Collagenase digestion of the intestinal mucosa (7) was carried out in 20 m*M* Tris–HCl buffer, pH 7.4, containing 120 m*M* NaCl, 3 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$. The isolated cells were washed with the Tris–HCl buffer, collected by centrifugation four times, and then resuspended in either 1% BSA, 20 m*M* Tris–HCl buffer, pH 7.4, or in 0.154 *M* sodium phosphate buffer, pH 6.8, as indicated in individual experiments. Plastic labware was used in the preparation of the cells and in subsequent incubations. Cell viability was 90%, as determined by exclusion of vital stain (.03% trypan blue). Photomicrographs of isolated mucosal cells revealed no visible alterations in the plasma membranes, microvilli, and cell organelles.

Metabolic and transport activity. Galactose accumulation and $^{14}CO_2$ production from [U-^{14}C]glucose were assessed by techniques described by Kimmich (8). The incorporation of [1-^{14}C]leucine into trichloroacetic acid-precipitable protein was determined according to O'Doherty *et al.* (9). Protein was estimated by the procedure of Lowry *et al.* (10).

The uptake of oleic acid by the isolated cells and its incorporation into triglyceride were assessed by incubating 1 ml of cell suspension with 3 ml of a micellar preparation which contained 20 μmoles of sodium taurocholate, 2 μmole of [1-^{14}C]oleic acid (2 μCi), 1 μmole of monoolein, and 1 μmole of cholesterol in 0.154 *M* sodium phosphate buffer, pH 6.8, the total volume was 4.0 ml. Incubations were carried out at 37° in a shaker bath. At timed intervals, the cells were separated from the incubation medium by Millipore (0.3 μM) filtration under suction. Cells were washed with phosphate buffer and added, with the filter, to chloroform:methanol (2:1, v/v) for total lipid extraction (11). An aliquot of the total lipid extract was monitored for radioactivity and the remainder was fractionated into major lipid classes by thin-layer chromatography (tlc). Using a solvent system of hexane:acetone:acetic acid (89:11:4, v/v/v) and iodine staining, the bands corresponding to authentic lipid classes were scraped directly into liquid scintillant for radioactive counting. Radioactivity was determined in a Beckman LS-250 liquid scintillation spectrometer, and quenching was corrected for by external standardization.

The uptake of cholesterol and its incorporation into cholesteryl ester by the isolated cell preparation was measured by the same approach as above except that the micelle was prepared to contain [4-^{14}C]cholesterol (0.5 μCi) as the radioactive component.

Results. General characteristics of isolated intestinal cells. The metabolic and transport data, as well as microscopic examinations of isolated intestinal cells prepared from control and common duct-cannulated rats, were comparable and indicated retention of the structure and functions associated with normal intestinal tissue. These cells actively accumulated galactose, generated a concentration gradient in about 1 min, and attained a maximum in 5 min. Dinitrophenol, an inhibitor of active sugar transport (12) effectively blocked galactose accumulation in these cells.

Mucosal cell oxidation of glucose (40 nmole of CO_2 produced/hr/mg of protein) was linear during the 2-hr incubation period. These data were comparable to results obtained by others with intestinal rings (8) and isolated intestinal cells (8, 13).

The ability of the isolated cells to incorporate [1-^{14}C]leucine into TCA-precipitable protein is shown in Table I. Incorporation was linear for the 30-min time period studied and the levels were comparable to those previously reported by O'Doherty *et al.* (9).

When the isolated cell suspensions were incubated with a mixed micellar solution of lipid containing either radioactive oleic acid

TABLE I. UPTAKE AND INCORPORATION OF L-[1-^{14}C]LEUCINE, [1-^{14}C]OLEIC ACID, AND [4-^{14}C]CHOLESTEROL BY ISOLATED INTESTINAL CELLS.

Time (min)	L-Leucine[a]		Oleic acid[b]		Cholesterol[c]	
	Uptake (dpm)	Incorporation into protein (dpm/mg)	Uptake (%)	Incorporation into TG[d] (%)	Uptake (%)	Incorporation into CE[d] (%)
10	–	–	48.5	27.0	35.5	3.5
15	11,606	2,001	–	–	–	–
20	–	–	55.2	33.7	37.8	5.2
30	23,346	4,025	–	–	43.3	6.6

[a] Isolated intestinal cells (5.8 mg of protein) were incubated for 15 and 30 min at 37° with 2 μCi of L-[1-^{14}C]leucine in 20 m*M* Tris-HCl, pH 7.4, total volume 5.0 ml. Reactions were stopped with 10% TCA. Each value is the average of three experiments.

[b, c] Isolated intestinal cells (11.0 mg of protein) were incubated with a micellar preparation (see text) which contained either [b]2 μmole of [1-^{14}C]oleic acid (2 μCi) or [c]1 μmole of [4-^{14}C]cholesterol (0.5 μCi).

[d] Incorporation is expressed as a percentage of uptake.

or cholesterol, the precursors were taken up from the media and esterified by the cells (Table I). Approximately one-third of the fatty acid taken up by the cells appeared in triglycerides. This yield of triglyceride is comparable with that recorded for intestinal slices (14) and with that present in intestinal cells isolated from rat intestine after duodenal infusion of a micellar preparation containing radioactive oleic acid.[2]

Effect of purified pancreatic CEH on cholesterol esterification in isolated intestinal cells. Isolated intestinal cells, prepared from common duct-cannulated rats, were preincubated for 30 min with different concentrations of the subunit or active form of pancreatic CEH. A 1-ml aliquot of cell suspension was preincubated either in 1.5 ml of 50 m*M* phosphate buffer, pH 6.2, which contained the purified CEH, or in buffer alone. After preincubation, the cells were centrifuged, washed three times with buffer (6 ml/wash), and then incubated with a micellar solution containing [4-^{14}C]cholesterol. After 30 min, the cells and incubation media were separated by Millipore filtration. The cells were washed with 4 ml of nonradioactive micellar solution and then three times with buffer (4 ml/wash). The cellular lipids were extracted and analyzed. The data in Table II show that cells isolated from the intestines of rats deprived of pancreatic secretion and bile exhibited a very low level of esterification of labeled cholesterol, which averaged 1.5%. However,

[2] Gallo, L. L., Newbill, T., and Vahouny, G. V., unpublished data.

TABLE II. EFFECT OF PANCREATIC CEH ON CHOLESTEROL ESTERIFICATION AND UPTAKE BY ISOLATED INTESTINAL CELLS FROM COMMON DUCT-CANNULATED RATS.[a]

Preincubation condition	Cholesterol uptake (%)	Esterification (%)
Phosphate buffer	43.3 ± 8.4	1.5 ± 0.5
CEH Subunits		
(a) 400 units[b]	31.3 ± 1.8	6.4 ± 1.2
(b) 800 units	32.3 ± 2.7	5.9 ± 1.0
(c) 890 units	39.3 ± 2.3	9.1 ± 1.6
CEH active enzyme		
(a) 400 units	32.2 ± 3.0	6.8 ± 1.3
(b) 800 units	32.0 ± 2.3	6.7 ± 0.4
(c) 890 units	38.0 ± 3.8	8.7 ± 2.0

[a] Isolated intestinal cells (6.0–8.0 mg protein) were preincubated with either CEH subunits or the active enzyme and then incubated with micellar [4-^{14}C]cholesterol as described in the text. Each value is the average of six cell preparations run in triplicate ± SE.

[b] 1 unit CEH = 1 μmole of cholesterol esterfied per hr.

when these cells were preincubated with subunits or active CEH before incubation with the micellar solution, the level of esterification of labeled cholesterol ranged from 5.9 to 9.1%. Compared to the controls, this represents a fourfold to sixfold increase in esterification of the labeled cholesterol, depending on the level of enzyme used.

Discussion. The design of the present experiments was based upon two earlier studies in this laboratory. The first (3) was the finding that common-duct fistulas in rats produced a marked depletion of CEH activity in the intestinal mucosa and an almost complete elimination of the absorption of

exogenous cholesterol. The second (5) was the development of a reproducible procedure for the purification of pancreatic CEH. These developments allowed for a more critical assessment of the effect of the purified pancreatic enzyme on the ability of intestinal cells prepared from rats with common-duct fistulas to take up and esterify "exogenous" cholesterol.

The isolated intestinal cells used in this study were depleted of CEH activity and of the ability to aggregate the CEH subunit to active CEH (no bile salt). Experimentation time was well within the 2-hr period over which glucose oxidation was linear and cell viability remained over 80%.

Preincubation of enzyme-depleted cells with either form of CEH (active or subunit) led to equivalent increases in the level of cholesterol esterification. Also, preincubation with either level of CEH activity, 400 or 800 units, was equally effective in the cellular esterification of cholesterol. This observation may reflect a limitation in the ability of the cells to take up CEH or an inhibition of the intracellular esterification reaction by cholesteryl ester which is not being released by the cells. These levels of enzyme activity correspond to that found in 4–8 ml of rat pancreatic juice, and are within the physiological range found *in vivo* (3).

The comparable effect on cholesterol esterification with the two forms of CEH raises the question as to the site of this enzymatic activity. Under physiological conditions, the intestinal cells should be exposed predominantly to the active form of pancreatic CEH since the subunit form would mix with bile salts in the common duct. It seems unlikely that this large protein would penetrate the plasma membrane of absorptive cells. For this reason, it has been suggested (15) that the active enzyme may act at the brush border surface and may even facilitate the movement of cholesterol into the cell via the esterification reaction. However, the localization studies of Gallo and Tradwell (16) suggested that mucosal CEH is a soluble fraction enzyme. This finding is indirectly supported by the present study. The cells took up and esterified cholesterol from a micelle at a pH of 6.8 (medium). It is unlikely that esterification could occur on the cell surface at this pH, for it has been determined that the optimum pH of CEH-esterifying activity *in vitro* is pH 6.2 and only sterol ester hydrolysis occurs at pH 6.8. Moreover, cholesteryl esters do not accumulate to any significant extent in the intestinal mucosa during active absorption (17). This lack of accumulation suggests that cholesteryl ester formation is a part of the terminal sequence of events in lipid absorption which is carried out in the basal or lateral portion of the cell. Therefore, it seems likely that cholesterol esterfication occurs just prior to its exit from the cell.

Cholesterol uptake by the absorptive cells was independent of the presence of pancreatic CEH, since comparable levels of radioactive cholesterol were associated with CEH-"deficient" and CEH-"rich" cells. Since isotopic equilibrium had not been reached in the 30-min incubation period, an increase in radioactive cholesterol would be expected in CEH-"rich" cells if uptake were CEH dependent, and a higher precentage of the extracted cholesterol should be esterified if cholesterol entrance depended upon a cell surface esterification reaction.

In accord with the present data and with earlier reports (1–4), it is suggested that the subunit form of the enzyme first penetrates the brush border membrane, aggregates to the active enzyme, and then catalyzes cholesterol esterification. Proteins similar in size to the CEH subunit, e.g. albumin and horseradish peroxidase (18), are known to enter intestinal cells. The availability of the subunit may be due to dissociation of the active enzyme in the microenvironment of the lumenal surface of the cell. Here, the concentration of cholic acid would be diluted since the secretion from the common duct would have mixed with the contents of the intestinal lumen. Dilution, *in vitro,* of the active CEH readily leads to its dissociation into subunits of mol wt 69,000. Such dissociation of the enzyme has been observed during Diaflo filtration and on Sephadex G-200 columns (6). Inside the cell, the subunit may polymerize in the presence of passively absorbed bile salts normally present in the biliary secretion. In our ex-

periments, the bile salt would be derived from the micelle to which the cell was exposed. These steps in cholesterol absorption are consistent with (a) the reported localization of the intestinal enzyme; (b) enzyme dependence on bile salt for activity; and (c) the failure of cholesteryl esters to accumulate in intestinal cells.

Further studies with the isolated cell system are directed toward elucidation of the processes which result in release of the esterified cholesterol from the cell. Preliminary experiments have indicated that these cells are capable of packaging and releasing lipoprotein-like particles.

Summary. Isolated intestinal mucosal cells from normal and common duct-cannulated rats have been prepared by collagenase dissociation. These cells displayed ultrastructural integrity and retained several metabolic characteristics typical of intact intestinal mucosa. These included galactose accumulation and its inhibition by dinitrophenol, glucose oxidation, incorporation of labeled leucine into cellular protein, and the uptake and esterification of fatty acid. The uptake of cholesterol by cells from common duct-cannulated rats was comparable to that in cells from control animals; however, esterification was only about 25% of that in control cells. Preincubation of the "defective" cells for 30 min with the purified subunit or active form of pancreatic sterol ester hydrolase had no effect on cholesterol uptake but resulted in a fourfold to sixfold increase in the ability of the cells to esterify cholesterol. These studies provide additional evidence for the essential role of pancreatic sterol ester hydrolase in the mucosal esterification of absorbed cholesterol prior to lymphatic transport.

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