Localization and origin of rat intestinal cholesterol esterase determined by immunocytochemistry

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Abstract Monospecific rabbit antisera to rat pancreas cholesterol esterase were employed in the unlabeled antibody enzyme method of immunocytochemistry in combination with the horseradish peroxidase–antihorseradish peroxidase complex to localize this pancreatic enzyme within the wall of rat small intestine. Intestinal rings were fixed in paraformaldehyde with satisfactory preservation of structure and retention of cholesterol esterase antigenic determinants. Fixed sections, 6 μm thick, were stained. In the light microscope, specific reaction product, represented by intense brown areas, was uniformly distributed within the absorptive cells but was notably absent from the microvillar membrane. Reaction product was also seen within the laminapropria and submucosa. In contrast, reaction product was absent from sections of proximal intestine surgically deprived of pancreatic juice for 72 hours. Furthermore, the intensity of staining in sections of normal intestine decreased with increasing distance from the pancreatic duct. These observations support the concept that "intestinal" cholesterol esterase is of pancreatic origin. This enzyme is localized within the cells as opposed to the absorptive surface.—**Gallo, L. L., Y. Chiang, G. V. Vahouny, and C. R. Treadwell.** Localization and origin of rat intestinal cholesterol esterase determined by immunocytochemistry. *J. Lipid Res.* 1980. **21:** 537–545.

Supplementary key words absorption · pancreas · unlabeled antibody enzyme method · cholesterol · intestine

During the intestinal absorption of cholesterol, 70–90% undergoes esterification. The efficiency of this esterification is a regulatory factor in cholesterol absorption (1–3).

A cholesterol esterifying enzyme, cholesterol esterase (EC 3.1.1.13), active in crude extracts of intestinal mucosa from rat (4, 5) and other species (6), and associated with isolated rat intestinal cells (7) can account for cholesterol flux from the intestine. There are conflicting reports concerning the localization and origin of this enzyme. With respect to localization, the brush border membranes (8) and soluble fraction (9) are reported to contain the major portion of the total esterifying activity in homogenates of rat intestinal mucosa when the conditions employed for cell fractionation are identical.

With respect to origin, the exocrine pancreas and intestine are candidates. Several lines of evidence suggest that the mucosal enzyme is derived from the cholesterol esterase secreted by the exocrine pancreas into the intestinal lumen. For example, the properties of cholesterol esterase from each tissue are identical with respect to bile salt requirements, pH optima (5), lack of requirement for high energy intermediates (10) and immunological identity (11). In addition, in experimental animals and humans, the removal of pancreatic secretion, either by pancreatectomy (5, 12–14) or surgical cannulation of the pancreatic duct (2) reduces mucosal cholesterol esterification and lymphatic transport, while oral supplementation with pancreatic tissue (13, 15) or secretion (2) restores both processes. Related to this, partially purified pancreatic cholesterol esterase restores esterifying activity to cells isolated from the intestine of rats surgically deprived of pancreatic juice (7). In contrast to these findings, it has been reported that prolonged cholesterol feeding to rats increases cholesterol esterifying activity in the mucosa with no change in pancreatic cholesterol esterase activity (16), and that the exclusion of pancreatic juice in rats has no effect on cholesterol absorption (17).

The aim of the present investigation was to determine the localization and source of mucosal cholesterol esterase by an immunological approach which employed specific anti-pancreatic cholesterol esterase sera.

Abbreviations: PBS, phosphate buffered saline; PAP, horseradish peroxidase–antihorseradish peroxidase; DAB, 3,3′diamino benzidine tetrahydrochloride.

MATERIALS AND METHODS

Enzyme preparation and purity

Pancreatic cholesterol esterase was solubilized and purified from rat pancreas (Pel Freez Biologicals) by the procedure reported earlier (18). Briefly, the enzyme was extracted from the tissue with digitonin and purified on hydroxylapatite and Sephadex G-200 chromatography columns. The purity of the final product was assessed by electrophoresis on 7.5% polyacrylamide gels prepared in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.1% sodium dodecyl sulfate (19). Protein was stained with 1.0% Coomassie blue dye.

The source of intestinal cholesterol esterase was a 100,000 *g* supernatant prepared from homogenates of mucosa scraped from the proximal intestine (9).

Antisera preparation

Antisera to pancreatic cholesterol esterase were prepared as previously described (11). Briefly, cholesterol esterase (specific activity of 2,400 units/mg protein) which migrated as a single band on electrophoresis in polyacrylamide gel was employed for immunization of New Zealand, white female rabbits (R and H Rabbitry; 2 kg). For the primary immunization, each rabbit was injected intramuscularly with enzyme protein (240 μg) emulsified with Freund's complete adjuvant (Difco Laboratories; 0.5 ml). Intravenous booster immunizations (200 μg protein) followed at 3, 4, and 5 weeks. Antigen protein was estimated with Bradford's reagent (Biorad Laboratories) (20). After the fifth week, the rabbits were bled from the ear vein with the rabbit-bleeding apparatus (Bellco). A pool of antisera from three rabbits was prepared and stored at −20°C. Control sera drawn prior to immunization were similarly pooled and stored.

Immunological methods

Immunodiffusion and immunoelectrophoresis were carried out on agarose plates (Meloy Laboratories) (21). For immunodiffusion, the plate was coated with 1.0% agarose gel prepared in 0.2 M potassium phosphate buffer, pH 8.0, containing 0.1% sodium azide. Twenty microliters of pancreatic or intestinal cholesterol esterase solution (0.1–2.0 units) were added to the satellite wells and the central well contained 20 μl of control or antisera. For immunoelectrophoresis, the plate was coated with 1.0% agarose gel prepared in 0.025 M sodium barbital buffer, pH 8.6, containing 0.1% sodium azide. The wells contained 5 μl of antigen which was subjected to electrophoresis for 2 hr at 10°C with a voltage of 6 V/cm. After electrophoresis, 100 μl of control or antiserum was added to the troughs. In both methods, immunodiffusion was allowed to proceed for 24 hr at 4°C, and the plates were washed for 3 days with several changes of phosphate buffered saline (PBS) followed by distilled water. Protein staining was described above.

Tissue preparation

Standard conditions for tissue preparation were followed (22). Male rats (Charles River Breeding Laboratories, Inc.; 150–200 g), either normal or common duct-cannulated (72 hr) as described previously (2), were killed by decapitation. The section of intestine between the entry of the common duct and the ileum was removed. At distances of 2.5, 25, and 50 cm below common duct entry, a cross-sectional ring of intestine 1.0 cm in length was excised and rinsed briefly with cold physiological saline. Each segment was fixed for 4 hr at 4°C in 4% paraformaldehyde (purified; Fisher Scientific Co.), then rinsed overnight with 0.01 M PBS, pH 7.6, at 4°C. These conditions of fixation were optimal for retention of antigenicity with satisfactory preservation of tissue structure. The fixed tissues were dehydrated by sequential placement for 30 min in each concentration of a graded series of ethanol solutions (80%, 95%, 100%, and 100%; Fisher Scientific Co.) and then wet in xylene (histological grade; Fisher Scientific Co.) for 90 min. Next, the tissues were infiltrated and cast in melted paraffin contained in truncated embedding molds (Scientific Products Co.) by lowering the segment into the paraffin with the luminal opening longitudinal to the base of the mold. The paraffin blocks were solidified by brief placement in water at 4°C and stored at that temperature until sectioned. Cross-sections of each segment, 6 μm in thickness, were cut with the microtome (American Optical Co., Model 820) and floated from a 37°C water bath onto gelatin-coated slides. Deparaffinization of the sections was accomplished by sequential immersion for approximately 3 min per immersion in 100% xylene × 3; 100% ethanol × 1; 95% ethanol × 2; 80% ethanol × 1; and distilled water × 1.

Unlabeled antibody enzyme method

The deparaffinized sections of intestine were analyzed for cholesterol esterase by the unlabeled antibody enzyme method (23). In the initial experiments, the method was standardized for the specific antisera and tissue antigen. The optimal dilutions of the specific rabbit antisera, peroxidase–antiperoxidase (PAP) complex and goat antirabbit IgG sera were

established by block titration and working dilutions determined to be 1/1000, 1/80, and 1/10, respectively, based upon the absence of non-specific reaction product in control sections. The optimum exposure time to the tissue was established for each dilution of reagent and was determined to be 48 hr, 5 min, and 30 min, respectively.

Two series of controls were included to insure the specificity of the reaction for cholesterol esterase. In the first, either the specific antisera, PAP complex, or the diaminobenzidine (DAB) plus hydrogen peroxide solution was replaced with PBS in the otherwise complete sequence, and in the second, the specific rabbit antisera was replaced with normal rabbit sera. In both series, non-specific reaction product was slight or nonexistent. Subsequently, intestinal sections treated with antisera were compared with adjacent sections from the same intestine treated with control sera.

Each experiment was performed on intestine from five different rats. Each of the three segments (at 2.5, 25, 50 cm) taken per intestine was sectioned and the sections were stained in triplicate. Within the experimental group of normal rats, location of the immunocytochemical product in all animals, except as noted in the results, and the staining gradient along the intestines were consistent. The only variation among intestines was in staining intensity. Within the control group of normal rats and within the common-duct cannulated rats, immunocytochemical product was consistently absent except as noted in the results.

Throughout the method all washes were made and reagents diluted with 0.01 M PBS, pH 7.6 except for the several sera which were diluted in 0.01 M PBS containing 1.0% normal goat sera, incubations were carried out on a microscope slide with sufficient reagent (5 drops) to completely cover the tissue section, and all incubations were performed in a humidity chamber at 4°C.

The staining sequence was as follows: tissue sections were covered with 3% normal goat sera (Cappel Laboratories); this has been reported to minimize non-specific staining (23). After 30 min, the sections were drained. Those designated as experimental were treated with antisera (1:1000 dilution) and those designated as control with control sera (1:1000 dilution) for 48 hr. The sera were removed by three successive 2-min immersions in PBS. Next, the sections were incubated for 30 min with 10% goat antirabbit IgG sera (Cappel Laboratories) and again washed by three successive 2-min immersions in PBS. The PAP complex (1:80 dilution) (23) was applied for 5 min and then removed by the washing procedure described above. The sites of immunological reaction were located by exposure of the sections for 5 min to a freshly-prepared solution of 0.05% DAB (Polysciences, Inc.) and 0.01% H_2O_2 (certified A.C.S., Fisher Scientific Co.). These reactants were removed by immersion in double distilled water for 10 min. The sections were stained for 90 sec in Harris's hemotoxylin (histological grade, Fisher Scientific Co.), and mounted in Permount (Fisher Scientific Co.).

Microscopy and photography

Each section was viewed under the Zeiss photomicroscope III and photographed on Kodachrome 25 film under the 40× and 100× oil immersion objectives to give final magnifications of 160× and 400×, respectively.

RESULTS

Antisera specificity

Antisera to highly purified pancreatic cholesterol esterase in either immunodiffusion or immunoelectrophoresis formed one major precipitin arc with both the purified and the crude enzyme preparation, thus revealing no immunological relationship with other proteins in the extract. When this antisera (anti-rat pancreas cholesterol esterase) was tested in the immunodiffusion assay with intestinal cholesterol esterase, cross-reactivity occurred with a pattern of precipitin lines indicative of complete identity between the cholesterol esterases isolated from the two sources. The immunodiffusion pattern is shown in **Fig. 1** and is representative of the pattern seen with crude cholesterol esterase preparations from twelve intestines. No cross-reaction occurred between cholesterol esterase from either source and normal rabbit sera, nor between enzyme from either source and antisera which had been adsorbed with either purified pancreatic cholesterol esterase or crude enzyme from intestine or pancreas (not shown).

Cholesterol esterase localization

To facilitate the interpretation of the light micrographs which follow, a schematic representation of a cross-section of small intestine is shown in **Fig. 2.** The box designated as I represents the area photographed in Fig. 3 and 5A, and that designated as II represents the area photographed in Fig. 4 and 5B.

In the light micrographs (**Fig. 3** and **4**) of sections of small intestine excised 2.5 cm below entry of the common duct and treated with antisera, brown reaction product was seen. Heavy reaction product was consistently visualized (Fig. 3A) within mature absorptive cells (villus tips and halfway down the villus) but

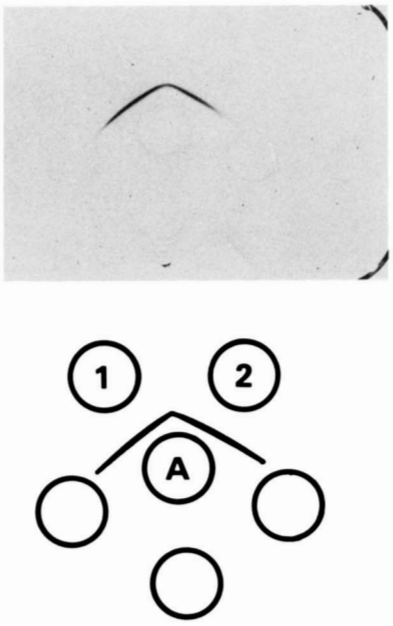

Fig. 1. Immunodiffusion pattern showing cross-reactivity of anti-rat pancreas cholesterol esterase (A) with purified pancreatic cholesterol esterase (1) and crude intestinal cholesterol esterase (2).

was absent (Fig. 4A) from the lower villus and crypts. A faint line of reaction product was occasionally seen (two of five intestines) on the microvillar surface of the mature cells. The lamina propria and submucosa of these same sections of antisera-treated tissue (Fig. 4A) consistently contained heavy staining. In the adjacent sections treated with control sera, little or no reaction product was associated with the absorptive cells (Fig. 3B), lamina propria, or submucosa (Fig. 4B).

In the same experiment, when sections of small intestine excised at 25 cm and 50 cm below entry of the common duct were treated with antisera, the brown immunocytochemical product, a measure of cholesterol esterase content, was consistently present within the absorptive cells, lamina propria, and submucosa, but was visually estimated to be one-fourth and one-tenth as intense, respectively, as that in the 2.5 cm sections.

Further, the serosal surface from each region of intestine, whether treated with control or antisera, occasionally stained. Such staining was considered non-specific.

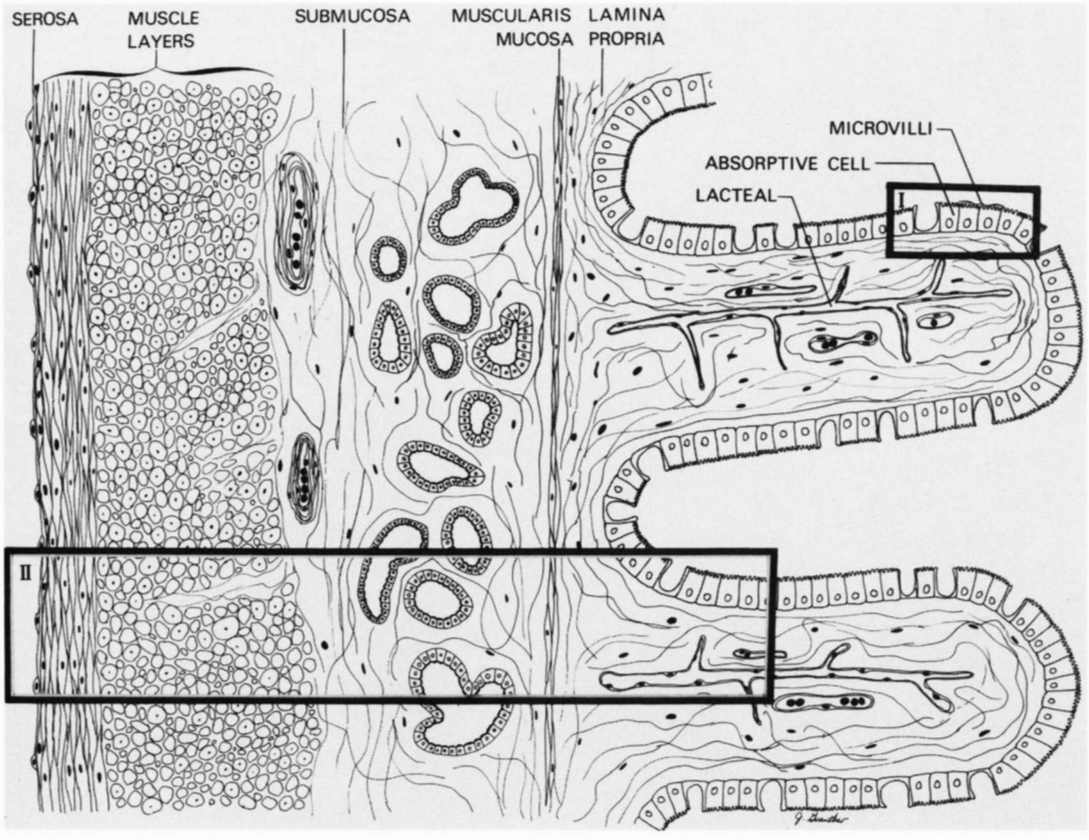

Fig. 2. Schematic representation of a cross-section of rat intestine: (I) represents the area seen in Figs. 3 and 5A; (II) the area seen in Figs. 4 and 5B.

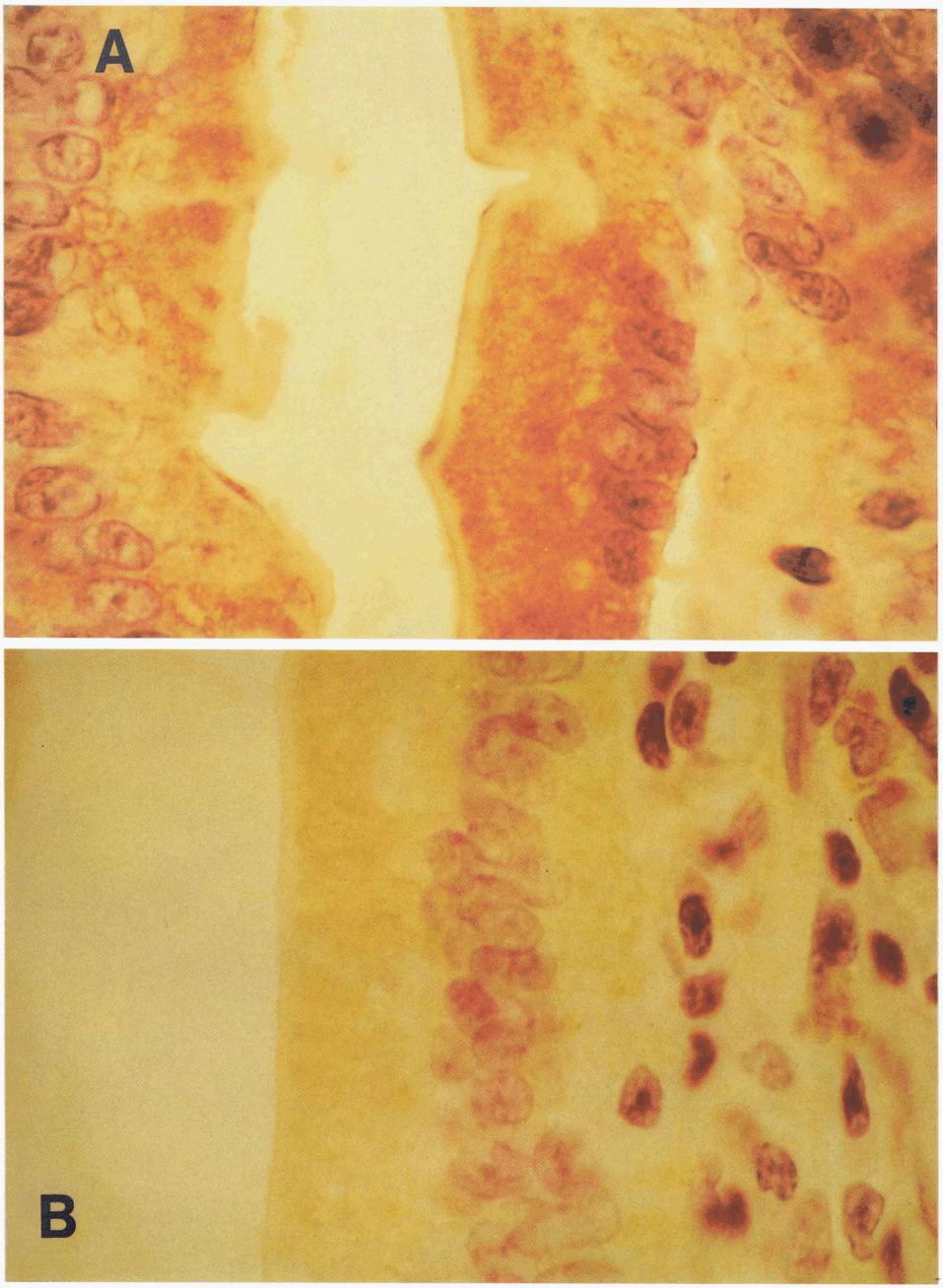

Fig. 3. Photomicrographs of cholesterol esterase staining in absorptive cells of intestinal sections excised at 2.5 cm below the common duct of normal rats and treated with (A) specific antisera and (B) control sera. Magnification 400×. See Fig. 2, area I for reference.

Cholesterol esterase origin

In the light micrographs of sections of intestinal segments excised from common duct-cannulated rats (diversion of pancreatic secretion) and treated with antisera, no reaction product was visible within the absorptive cells, (**Fig. 5A**) lamina propria, or submucosa (Fig. 5B) of the five intestines. These sections are indistinguishable from those treated with control sera (not shown; see normal controls in Fig. 3B, 4B for comparison). However, inconsistent staining occurred on the serosal surface as previously noted.

Sections shown in Fig. 5 are from intestinal segments excised at a distance of 2.5 cm below entry of the common duct, i.e., the area of intestine in normal rats which stained most intensely for cholesterol esterase in the previous studies. However, reaction

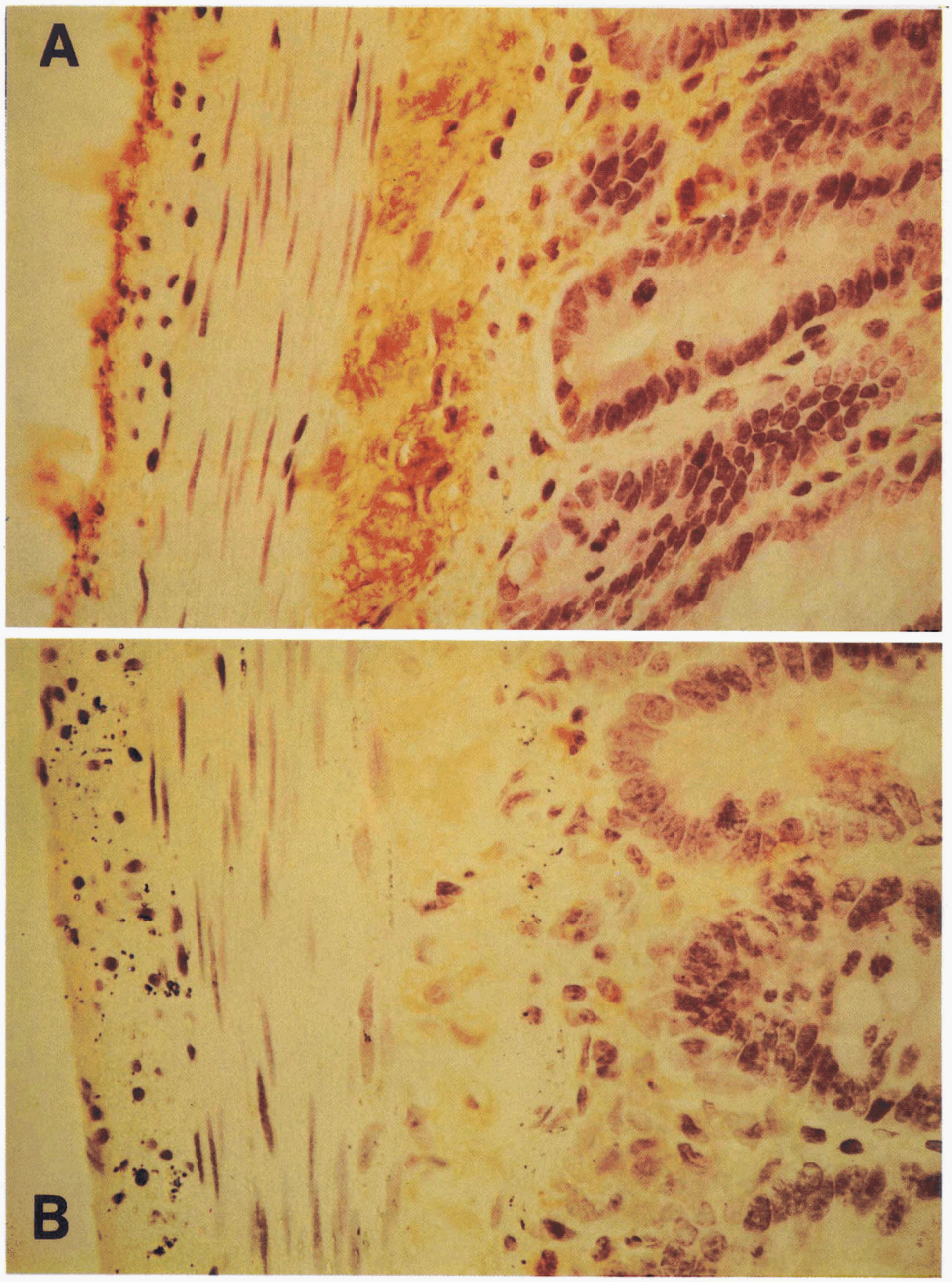

Fig. 4. Photomicrographs of cholesterol esterase staining in the lamina propria and submucosa of intestinal sections excised at 2.5 cm below the common duct of normal rats and treated with (A) specific antisera and (B) control sera. Magnification 160×. See Fig. 2, area II for reference.

product was absent from intestine excised from the two lower sites in the cannulated rats.

DISCUSSION

Since studies from our laboratory have repeatedly shown that mucosal cholesterol esterase activity is dependent upon pancreatic secretion, we have considered this enzyme to be derived from pancreas and to serve as a regulatory factor in the absorption of luminal cholesterol.

In previous localization studies with mucosal esterase, cell fractions of mucosal homogenates were assayed for activity. In separate studies, the brush border membranes (8) and the soluble fraction (9) were each reported to contain 60–70% of the total activity. These differences may represent fractionation

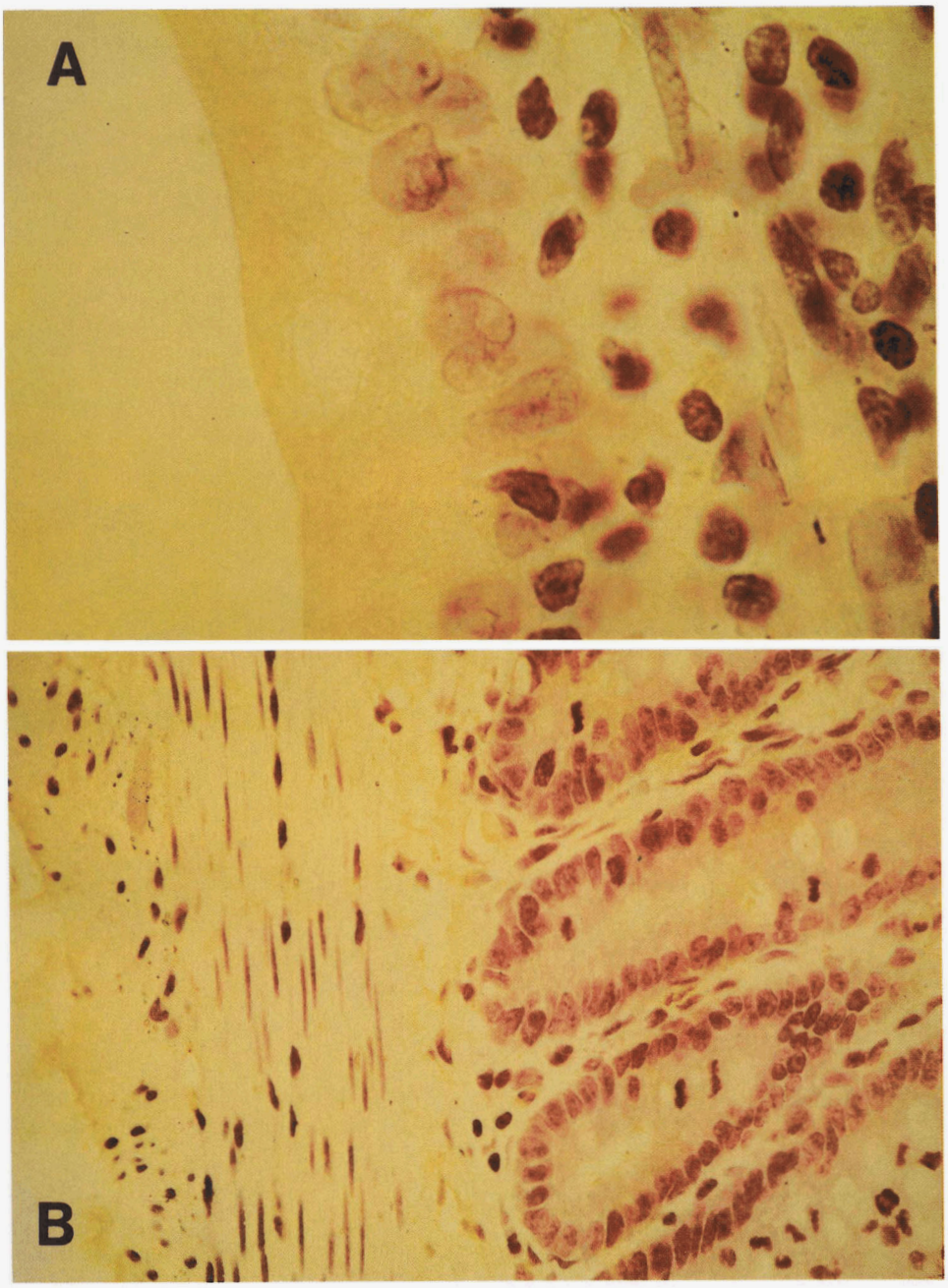

Fig. 5. Photomicrograph of (A) absorptive cells (400×) and (B) lamina propria and submucosa (160×) in intestinal sections excised at 2.5 cm below the common duct of cannulated rats (72 hr) and treated with specific antisera. Note the absence of cholesterol esterase staining. See Fig. 2, areas I and II for reference.

artifacts. Inasmuch as the enzyme is taken up by mucosal cells and is not inherent in a particular organelle, loose association with, and easy disassociation from, organelles seem likely. The current immunocytochemical localization study, devoid of fractionation pitfalls, reveals reaction product, representative of cholesterol esterase sites, clearly concentrated within the absorptive cells in all cases and occasional faint staining in the brush border membrane. At 2.5 cm below entry of the common duct, the staining is dense and fills the cell interior. However, at distances further removed from the common duct or with higher dilutions of antisera, the staining is concentrated in an intense band just below the terminal web. This area corresponds to the location of the smooth endoplasmic reticulum. While the resolution with the present method does not allow a definitive subcellular localization, it is tempting to speculate that choles-

terol esterase activity is associated with this organelle or within its tubular network. This is in keeping with the concept that lipid re-esterifications, which represent the first step in the intestinal processing of fat and in lipoprotein formation, occur at this site (24, 25).

Examination of the total population of absorptive cells which line the villus reveals cholesterol esterase in the cells of the villus tips and mid-villus region but absent from the cells of the lower villus region and crypts. This observation correlates with the known maturation of absorptive cells during migration from crypts to tips and with the occurrence in mature absorptive cells of the monoglyceride pathway of triglyceride resynthesis (26).

Reaction product is also present in the lamina propria and submucosa. The significance of this finding is not immediately apparent but may represent cholesterol esterase in the lymph, since the central lacteal transcends the lamina propria and the smaller lymphatics merge in the submucosa. This suggestion is supported by the finding that a component in lymph from normal rats cross-reacts with anti-cholesterol esterase sera in the immunodiffusion assay with a pattern of partial identity.[1] Such a pattern implies that antigenic determinants have been lost from the esterase during its movement from the mucosa, where there is complete identity (Fig. 1), to lymph where there is partial identity. Based upon this evidence, we suggest that the lymphatics represent a route of clearance for cholesterol esterase. Lymph assayed for cholesterol esterase has been reported to be enzymatically inactive (27). We have recently confirmed this finding[2] and therefore consider the esterase of lymph physiologically insignificant as an esterifying enzyme.

This technique, that has demonstrated cholesterol esterase in the intestine of normal rats, has also demonstrated the absence of reaction product from the intestine of rats deprived of pancreatic juice by surgical cannulation of the common duct. The absence of biliary secretion, also diverted upon common duct cannulation, cannot account for the loss of immunocytochemical staining in the intestine. Biliary secretion does not cross-react with the anti-cholesterol esterase sera nor is it required for the immunoreactivity of the cholesterol esterase (11). This finding confirms the pancreatic contribution of cholesterol esterase to the intestine. Consistent with this finding is the observation that, in normal rats, staining intensity is greatest within intestinal cells near the opening of the pancreatic duct where enzyme concentration is greatest and diminishes along the intestine from the proximal to distal end.

Experiments are currently underway to determine both the subcellular localization of cholesterol esterase in absorptive cells with electron microscopy and the mechanism of entry of pancreatic cholesterol esterase into absorptive cells.

This work was supported in part by USPHS grants AM-17269 and HL-02033. The authors gratefully acknowledge the helpful and extensive advice of Dr. Ludwig Sternberger, Edgewood Arsenal, in the development of the immunocytochemical technique and for the generous gift of PAP complex. We gratefully acknowledge the photography of Mr. Dan Hogan, Pathology Department, The George Washington University School of Medicine and Health Sciences.

Manuscript received 21 August 1979 and in revised form 7 January 1980.

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