Ultrastructural immunolocalization of apolipoprotein B within human jejunal absorptive cells

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Abstract Apolipoprotein B (apoB) was localized by electron microscopy within absorptive cells of human jejunal biopsy specimens taken fasting and after micellar fat infusion. Nakane's double antibody immunoperoxidase technique was used to label apoB near open cut surfaces of 60-μm fixed tissue slices sectioned by a Ralph knife in a Vibratome. In fasting tissue, apoB label was found within structurally intact perimitochondrial rough endoplasmic reticulum (RER) and within Golgi cisternae of absorptive cells covering the tips of jejunal villi. After fat infusion, apoB label was found adjacent to very low density lipoproteins (VLDL) and chylomicrons within apical smooth endoplasmic reticulum (SER). Less label was seen within RER than in fasting absorptive cells, and RER-SER connections containing apoB label were occasionally seen. Expanded Golgi vesicles and cisternae contained VLDL, chylomicrons, and apoB label. Vesicles containing chylomicrons and apoB label were occasionally visualized bordering the lateral plasma membrane in a configuration suggesting exocytosis. Specific apoB label was regularly seen within intercellular spaces and capillaries, but the in vivo significance of this localization was problematical. These observations suggest that apoB is synthesized in RER, transfers to SER where it is incorporated into new VLDL and chylomicrons, and moves to Golgi cisternae and vesicles to be prepared for exocytosis through the plasma membrane.—**Christensen, N. J., C. E. Rubin, M.C. Cheung, and J. J. Albers.** Ultrastructural immunolocalization of apolipoprotein B within human jejunal absorptive cells. *J. Lipid Res.* 1983. **24:** 1229–1242.

Supplementary key words immunoperoxidase technique • fat absorption • lipoprotein synthesis • chylomicron • very low density lipoprotein

The small intestine absorbs fat very efficiently, and the ultrastructural pathway of absorbed lipid through intestinal epithelial cells has been well described (1–4). Interest is now focused on the regulation of fat absorption, and it is clear that the various apoproteins associated with lipid particles during their absorption play an important role. One of these proteins, apolipoprotein B (apoB), is the subject of this study. Following lipid absorption from the intestinal lumen into the absorptive cell, apoB is essential for the formation and transport of lipid particles out of the absorptive cells into the lacteals (5–8).

Many studies in recent years have explored the structure and function of the apoproteins involved in lipid metabolism. It is now well established that the intestine, as well as the liver, is a major site of synthesis of many of these apoproteins in rats (9–11) and in man (12–16).

Some quantitative work has been done; for example, Wu and Windmueller (10) have determined the relative contributions of liver and intestine to different plasma apoproteins in the rat. Their data for apoB show that in fat-fed animals, the intestine contributes approximately 16% of the circulating apoB. In a later study, these investigators (17) found that when dietary or biliary fat was withheld, the intestinal contribution to circulating apoB was reduced to about 5%, supporting the idea that this apoprotein plays an active role in the synthesis of transportable lipid particles.

These physiological and biochemical data tell us little about the intracellular synthesis and transport of lipoprotein particles within the intestinal epithelium. Attempts to explain these mechanisms have used such techniques as immunofluorescent localization of apoB in isolated rat (5, 6, 18) and human (7, 8) intestinal cells, localization by light microscopy of apoB in fixed human biopsy specimens using an immunoperoxidase technique (19), and analysis of in vitro lipoprotein synthesis by cell fractions (20, 21). Data from these studies indicate that intestinal cells in a fasting animal contain apoB. The quantity of this apoprotein in the intestine appears to rise after administration of fat (6, 7, 18, 19), although one study (13) demonstrated a drop in apoB within human intestinal epithelial cells 45 min after administration of fat.

There is a need to visualize these intracellular events more directly. The purpose of the present study was to localize apoB in human intestinal absorptive cells ob-

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Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein; apoB, apolipoprotein B; HRP, horseradish peroxidase; DAB, 3,3'diaminobenzidine; PBS, phosphate buffered saline; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum.

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tained after fasting and fat feeding using a technique that maintained their ultrastructural integrity.

METHODS

Source of tissue

Thirty-six normal human volunteers were involved in this study. Informed consent was obtained from each of these subjects (University of Washington Human Subjects Review Committee approval, April, 1978). After a 10-hr overnight fast, a hydraulic biopsy tube (22) was passed perorally into the small bowel of each volunteer and positioned fluoroscopically near the duodenojejunal junction. Three baseline fasting mucosal biopsy specimens were taken. A micellar solution of fatty acids was then infused through the biopsy port, after which additional biopsy specimens were taken. The composition of this micellar solution, the length of the infusion, and the rate of infusion were varied.

Sources of proteins and antisera

Antigen and antisera preparation. Low density lipoproteins (LDL) of density 1.030–1.050 g/ml were isolated from plasma of normolipidemic human donors as previously described (23). The preparations were then chromatographed over a 2.6 × 100 cm column containing Biogel A5M to remove contaminating albumin and high density lipoproteins. The purified LDL showed only B protein bands on 3% SDS polyacrylamide gel electrophoresis. This LDL was emulsified with Freund's complete adjuvant and injected into rabbits at an injection dose of 500 μg of LDL protein per rabbit. Booster doses of 250 μg of LDL protein were given to each rabbit at monthly intervals, and the rabbits were bled 7–14 days after each booster injection. Antisera obtained from individual rabbits were used for isolation of specific anti-B immunoglobulin.

Isolation of specific anti-B immunoglobulin. Immunoglobulins specific for B protein were isolated by passing rabbit anti-B sera through a 1.5 × 30 cm column containing LDL coupled to cyanogen bromide-activated Sepharose CL 4B (Pharmacia Fine Chemicals). The gel was then extensively washed with 0.01 M Tris, 0.15 M NaCl, pH 7.4, to remove all nonbinding proteins, and then with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.6, and with 0.1 M CH_3COONa, 0.5 M NaCl, pH 4.0, to remove nonspecifically bound proteins. Specific anti-B immunoglobulins were eluted with 0.2 M glycine at pH 2.5, immediately dialyzed against PBS, concentrated to 1-2 mg/ml, and stored at −70°C until further use. The specific anti-B immunoglobulin isolated reacted with human plasma and LDL with precipitin lines of identity, but did not react with high density lipoproteins and albumin when tested by the Ouchterlony gel diffusion technique.

Preparation of sheep anti-rabbit Fab'-HRP. The second antibody (sheep anti-rabbit Fab'-HRP) used in this study was most kindly provided by Dr. Joel Levine and Dr. Paul Nakane of the University of Colorado. It was prepared, affinity-purified, and conjugated to horseradish peroxidase according to the protocol described by Levine, Nakane, and Allen (24).

In this study, all antibodies were diluted in 0.01 M phosphate buffer, pH 7.2, in 0.15 M NaCl (phosphate buffered saline-PBS) containing 10% sucrose and 1% bovine serum albumin (BSA). The optimal antibody preparations were defined as the highest possible dilutions providing specific labeling, and these were determined experimentally. For anti-B (first antibody) this dilution was found to be 1:1000; for anti-rabbit Fab'-HRP (second antibody), 1:10.

Immunocytochemical methods

The double antibody immunolocalization technique used in this study was based on procedures devised by Nakane (25) and Levine et al. (24). Biopsy specimens were mounted mucosal side up on monofilament mesh and immersed in a 2% formaldehyde-picric acid fixative (26) at room temperature for 24 hr. The tissue was then washed with several changes of 0.01 M PBS containing 10% sucrose over an 8-hr period at 4°C. The final wash of the day was continued for an additional 48 hr at 4°C. The washed biopsy specimens were placed briefly in PBS without sucrose and removed from the mesh. They were then mounted in 5% agar. The agar was trimmed to a cubical shape, and attached to a glass platform specimen-holder with cyanoacrylate glue. The mucosal specimen was then cut into 60-μm slices at right angles to its luminal surface using an Oxford Vibratome adapted to accommodate a Ralph glass knife (27) (**Fig. 1**). The slices were placed singly on albuminized slides and attached by air drying for at least 1/2 hr to ensure adherence throughout subsequent washing. The slices were rehydrated in three 10-min washes of PBS containing 10% sucrose. Subsequent washes with this buffer throughout the procedure were carried out according to this schedule (three 10-min periods). Excess buffer was wiped from around each slice and 25 μl of first antibody was applied; the slices were incubated for 16–18 hr with this antibody in a wet chamber at room temperature. After washing in buffer, 25 μl of the labeled second antibody was applied to each slice; they were incubated 3–4 hr with this antibody in a wet chamber at room temperature, and again washed. The slides were postfixed for 1 hr in 4% formaldehyde in 0.1 M

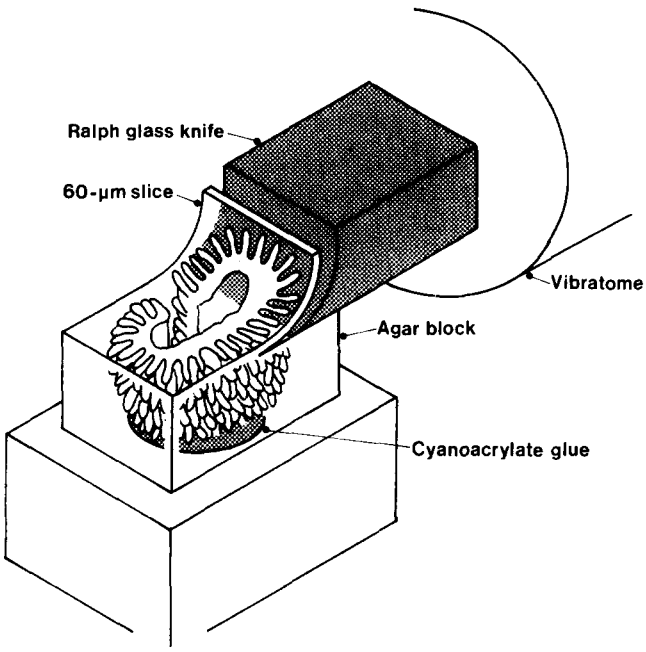


Fig. 1. Diagrammatic representation of sectioning of the fixed biopsy specimen by the Vibratome. The tissue is oriented as shown and mounted in an agar block which is attached to the specimen holder with cyanoacrylate glue. The biopsy specimen and glass knife are immersed in 0.01 M phosphate buffered saline; when the 60-μm slice floats free of the underlying block, it is picked up with a camel-hair brush and transferred to an albuminized slide.

cacodylate buffer, pH 7.4 (containing 0.05% $CaCl_2$ and 10% sucrose), and washed in buffer. The slides were incubated for 30 min in a solution of 21 mg% DAB, 10% sucrose, in 0.1 M Tris buffer, pH 7.6 (28). H_2O_2 was then added to obtain a final concentration of 0.005%, and the sections were incubated an additional 5 min. After three 10-min washes in plain PBS (no sucrose), the slides were fixed for 1 hr in 2% OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.4. To enhance staining, potassium ferrocyanide (50 mg) was added during the last 5 min of OsO_4 fixation. The slices were dehydrated in a graded series of ethanol (70%, 95%, 100%), and embedded in Luft's Epon (29) by inverting an Epon-filled gelatin capsule over each slice (**Fig. 2a**). After 24 hr at 37°C, the capsules were polymerized for 48 hr at 60°C and snapped off after the slides were heated gently over a flame.

Specificity controls

On various control slices, one of the following was substituted for the first antibody: 1:1000 normal (nonimmune) rabbit IgG; 1:1000 anti-B pre-incubated for 24 hr with an excess of LDL; or buffer (PBS, 10% sucrose, 1% BSA) containing no antibodies. In addition, antibodies were eliminated completely from the treatment of some slices; of these, half underwent the DAB-H_2O_2 reaction and half did not. Vibratome slices were also prepared and processed from rectal biopsies as an additional control for method and antibody specificity.

Electron microscopy

The unreacted side of the tissue slice was the presenting surface of the epon block. To reach the labeled side of the tissue it was necessary to section almost through the whole thickness of the tissue slice (Fig. 2b). Antibodies penetrated into the reacted surface only via portions of cells that were cut open during Vibratome sectioning. Furthermore, intracellular penetration even through these cut surfaces was extremely limited. One-μm sections were examined by light microscopy to locate villi with cut edges exposing the interior of cells containing brown reaction product. The block was trimmed to include such areas. Sections (80–90 nm) were made using a diamond knife on a Reichert OMU2 ultramicrotome. These sections were placed on parlodion-carbon-coated copper grids, lightly stained with Reynolds' lead citrate (30), and examined with an AEI Corinth 500 electron microscope. Considerable searching was required to find labeled "open" cells.

Three main areas of the epithelium of the upper one-third of each villus were compared in experimental and control sections: cell apices, Golgi regions, and inter-

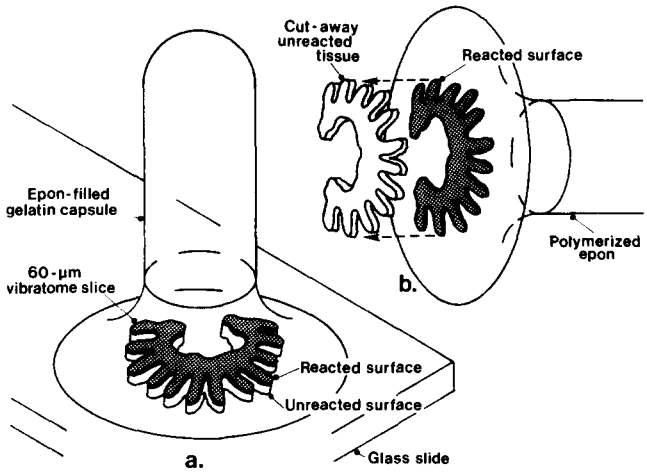
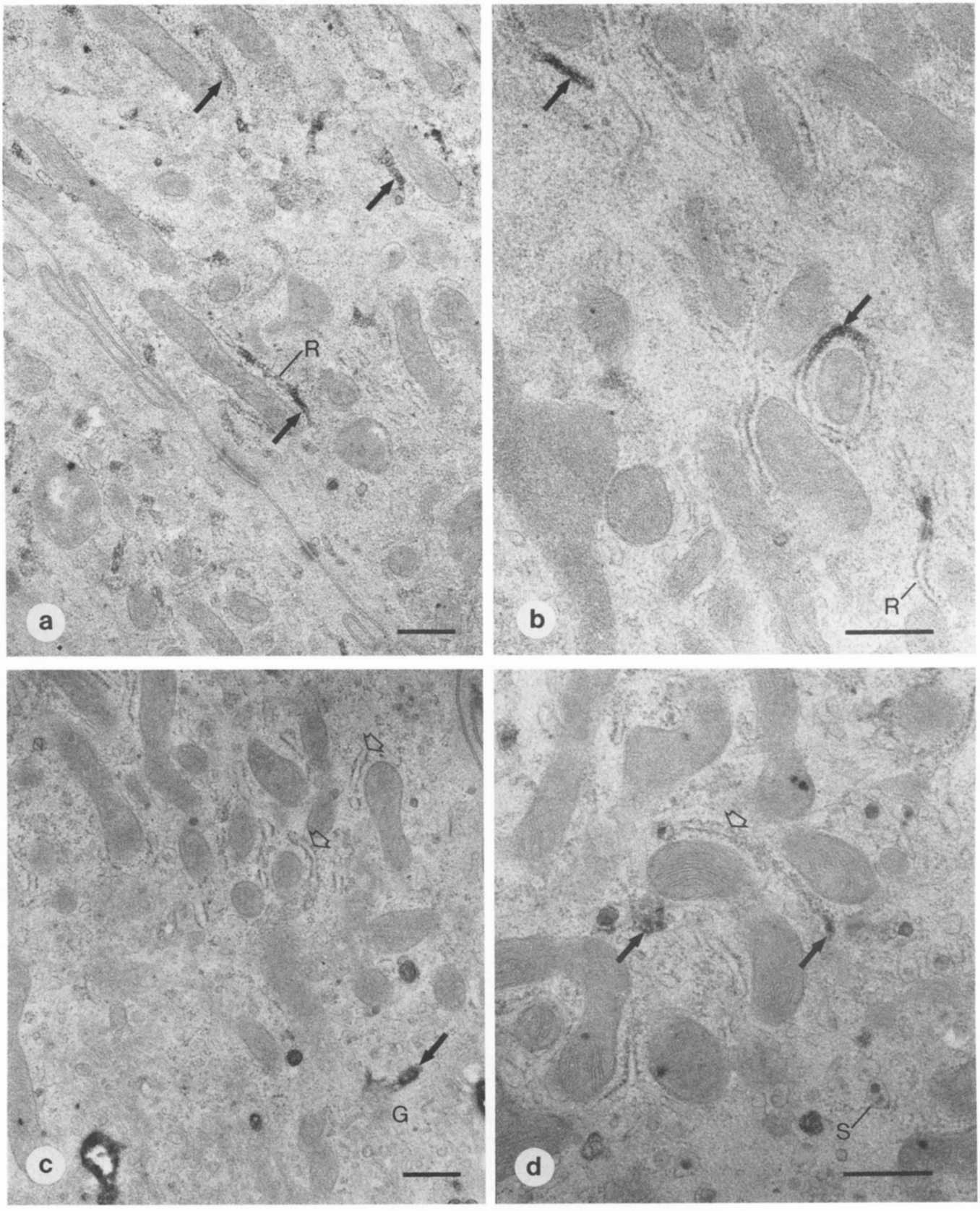


Fig. 2. a. Epon-filled gelatin capsule is inverted over the 60-μm Vibratome slice which has been processed for localization of apoB. The stippled area represents the reacted surface of the slice within the Epon block; this is the side incubated with antibodies. Antibodies penetrate into this side only a short distance, so that most of the slice is made up of unreacted tissue. b. After the polymerized Epon with its embedded slice is snapped off the glass slide, the unreacted side of the slice is the presenting surface. This unreacted tissue must be cut away and discarded in order to reach the limited part of the slice penetrated by antibodies (diagrammatically represented as the stippled reacted surface).



cellular spaces bordering on absorptive cell lateral plasma membranes. Control and experimental sections were compared blindly.

RESULTS

Fat infusion

Different fat infusion protocols led to variations in *1*) electron density of intracellular lipid; *2*) size of lipid particles; and *3*) retention of lipid particles throughout immunocytochemical processing, although the loss of some fat at the cut surface seemed to be unavoidable regardless of the protocol used.

The electron density of intracellular lipid was influenced by the composition of the infused fatty acid solution. Since unsaturated fats are readily fixed by OsO_4, the infusion of pure linolenate, with its three unsaturated bonds, produced chylomicrons so electron-dense that they were often indistinguishable from labeled apoB. Varying the proportions of the three fatty acids with unsaturated bonds used in this study (linolenate, linoleate, and oleate) resulted in lipoprotein particles of varying electron densities.

Retention of chylomicrons throughout washing was also affected by infusate composition. The addition of saturated palmitate, which is solid at room temperature, increased retention of these lipoprotein particles. Infusion rates and fat concentration in the infusate influenced lipid particle size. The larger the lipid particle, the less likely it was to be retained. A 45-min infusion of a 15 mM fatty acid solution produced very large lipid droplets which often washed out from the cut surfaces of cells. A 3–5-min infusion of the same solution followed by biopsy 20 min later produced small fat particles but it also eliminated any absorbed fat in the cell apices. Consideration of all these variables led to a fat-infusion protocol that produced small intracellular particles continuously throughout the experiment; these particles had a dark gray density easily distinguishable from labeled apoB. According to this protocol, a micellar solution of 7 mM oleate, 1.5 mM linolenate, 1.5 mM palmitate, 10 mM taurocholate, and 5 mM 1-monoolein was infused at 10 ml/min for 5 min, after which three biopsy specimens were taken. The infusion was resumed at 2 ml/min for 20 additional min, after which three final biopsy specimens were taken. This protocol mimics both normal postprandial concentrations of luminal fatty acids and exponential gastric emptying after a fatty meal.

Localization of apoB

In biopsy specimens taken from fasting subjects, apoB was localized within the rough endoplasmic reticulum (RER) (**Fig. 3a and b**) and Golgi cisternae (**Fig. 4a**) of absorptive cells in the upper third of the villus. ApoB label was particularly prominent within RER surrounding mitochondria and within the ends of Golgi cisternae.

Five minutes after the start of the fat infusion, RER labeling for apoB was greatly reduced (Fig. 3c and d). A comparison of Figs. 3a and b with 3c and d shows that the labeled apoB that filled some of the RER cisternae in the fasting cells was almost gone after 5 min of fat infusion. Nothing comparable to the labeled apoB found in fasting tissue was ever seen in fat-fed tissue.

More label was seen in profiles of smooth endoplasmic reticulum (SER) than was seen in fasting cell apices (**Fig. 5a**). This label within SER occasionally accompanied particles the size of the very low density lipoproteins (VLDL) and chylomicrons. Golgi cisternae were also labeled for apoB, especially the expanded ends of these cisternae and their associated vesicles which contained lipid particles in the size spectrum of VLDL and small chylomicrons (Fig. 4b and c). Empty spaces where larger chylomicrons had washed out during processing were also evident (Fig. 4c). The particles in absorptive cells at the tip of the villus were larger (Fig. 4c) than those in cells below the villous tip (Fig. 4b).

Twenty-five minutes after the start of the fat infusion, Golgi apoB labeling was obviously increased over that seen in fasting tissue (compare Fig. 4a and d). Chylomicrons accompanied by label were seen in the profiles of the SER (Fig. 5b and c) and within Golgi vesicles and expanded cisternae (Fig. 4d). Occasionally chylomicrons were seen in vesicles bordering intercellular spaces in configurations suggesting exocytosis (**Fig. 6**). Labeling in cell apices was restricted mainly to SER, often associated with VLDL and chylomicron-sized particles. Occasionally these labeled SER profiles were seen to connect with RER cisternae (Fig. 5b).

In all biopsy specimens, fasting and fat-fed, label was found in intercellular spaces, often filling these spaces

Fig. 3. Supranuclear portions of proximal jejunal absorptive cells near the tip of the villus. a. Fasting biopsy specimen. Note labeled apoB (solid arrows) within profiles of perimitochondrial rough endoplasmic reticulum (R). b. Higher magnification of fasting absorptive cells. Note that the length of some rough endoplasmic reticulum is filled with labeled apoB (solid arrows). c. After 5 min of exposure to micellar fat, note absence of apoB label in rough endoplasmic reticulum (hollow arrows) and apoB label (solid arrow) in Golgi (G). d. Higher magnification of absorptive cells after 5 min of exposure to fat. Rare apoB label (solid arrows) is seen within the tips of rough endoplasmic reticulum profiles and within some profiles of smooth endoplasmic reticulum (S). Again, note reduction in apoB label within rough endoplasmic reticulum (hollow arrow). Bars = 0.5 μm.

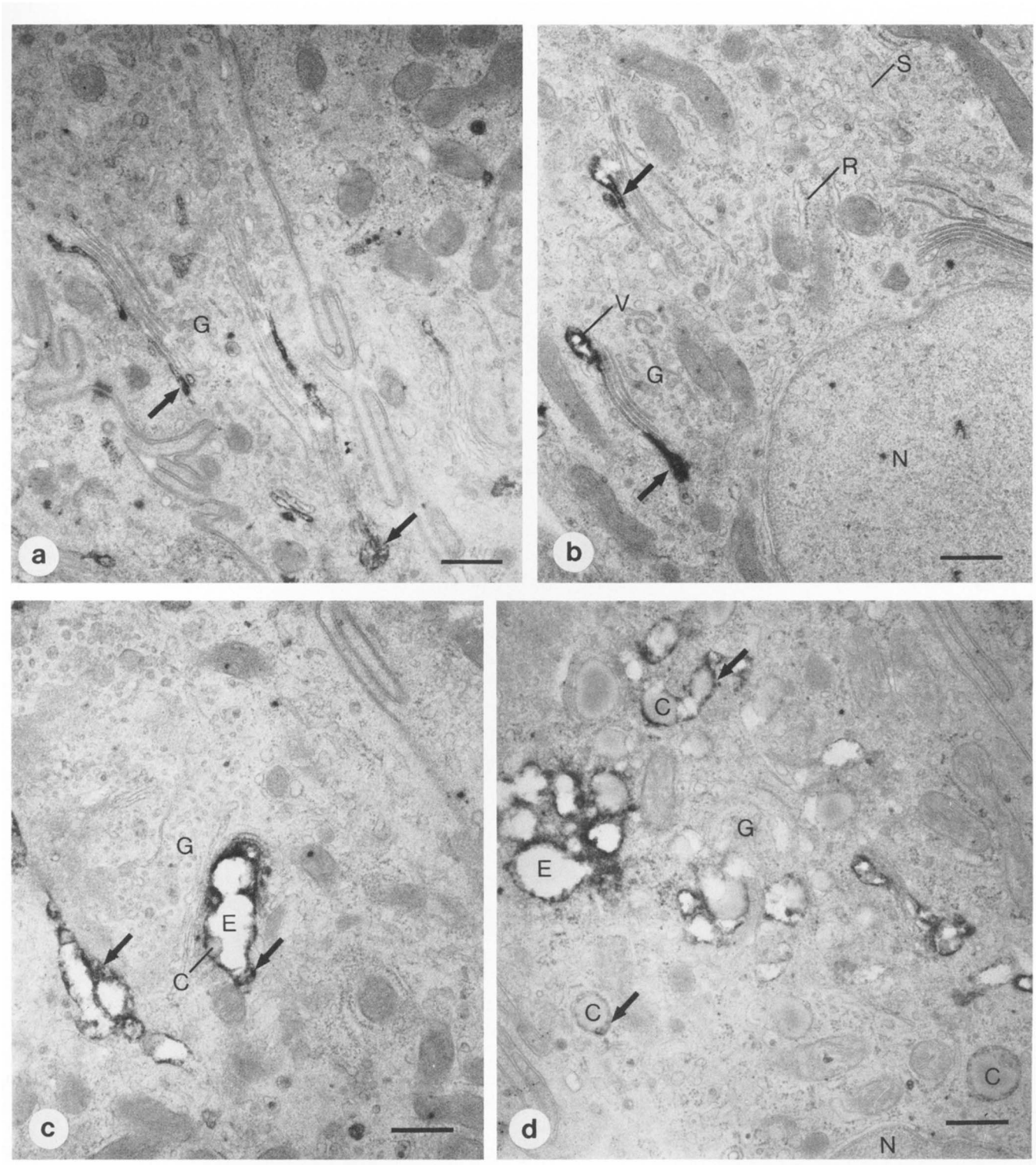


Fig. 4. Golgi regions of jejunal absorptive cells. a. Golgi cisternae (G) in fasting absorptive cell. Note labeled apoB (arrows) especially in ends of the cisternae. b. Golgi cisternae (G) in absorptive cell on the side of the villus a few cells down from its tip, 5 min after infusion of micellar fat. Note VLDL-sized negative images (V) in expanded ends of the cisternae accompanied by labeled apoB (arrows). Rough endoplasmic reticulum (R), smooth endoplasmic reticulum (S), nucleus (N). c. Five minutes after infusion of micellar fat, Golgi cisternae (G) in absorptive cells at the tip of the villus contain chylomicrons (C) that are larger than the VLDL seen in (b). Empty spaces (E) are evident where chylomicrons have washed out during processing. d. After 25 min of micellar fat infusion, expanded Golgi cisternae and vesicles (G) contain chylomicrons (C), empty spaces (E), and labeled apoB (arrows). Nucleus (N). Bars = 0.5 μm.

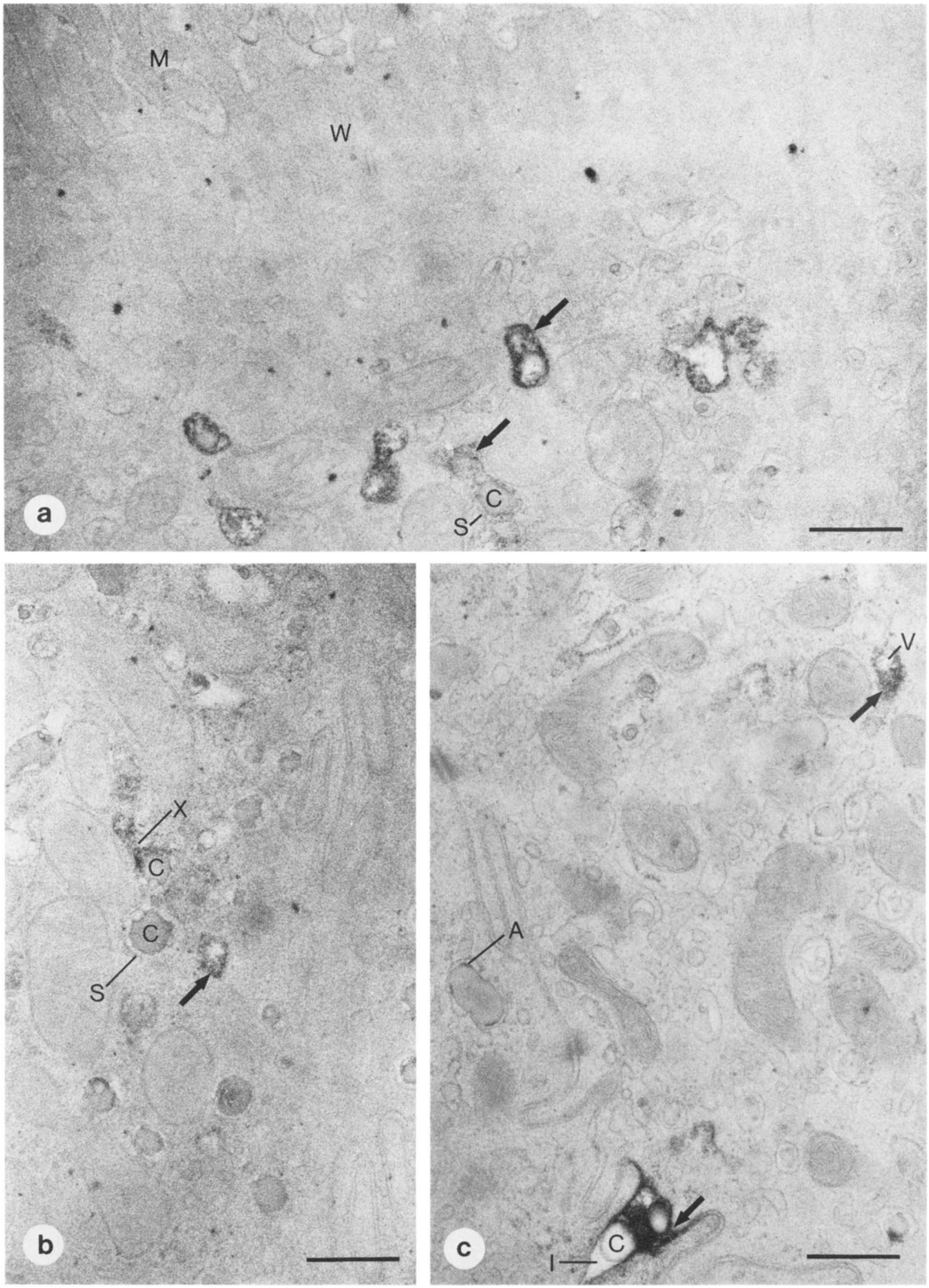


Fig. 5. Apical portions of proximal jejunal absorptive cells, 5 min (a) and 25 min (b and c) after infusion of micellar fat. a. Chylomicrons (C) are visible within profiles of smooth endoplasmic reticulum (S) accompanied by labeled apoB (arrows). Microvilli (M), terminal web (W). b. No lead citrate stain. Again, note chylomicrons (C) within smooth endoplasmic reticulum (S), at times accompanied by labeled apoB (arrow). Labeled perimitochondrial endoplasmic reticulum can be seen connecting (X) with smooth endoplasmic reticulum. c. Note VLDL particle (V) within profiles of endoplasmic reticulum with associated labeled apoB (arrow). Chylomicron (C), accompanied by labeled apoB (arrow), is seen in the intercellular space (I). Electron-dense lipid artifact is evident (A). Bars = 0.5 μm.

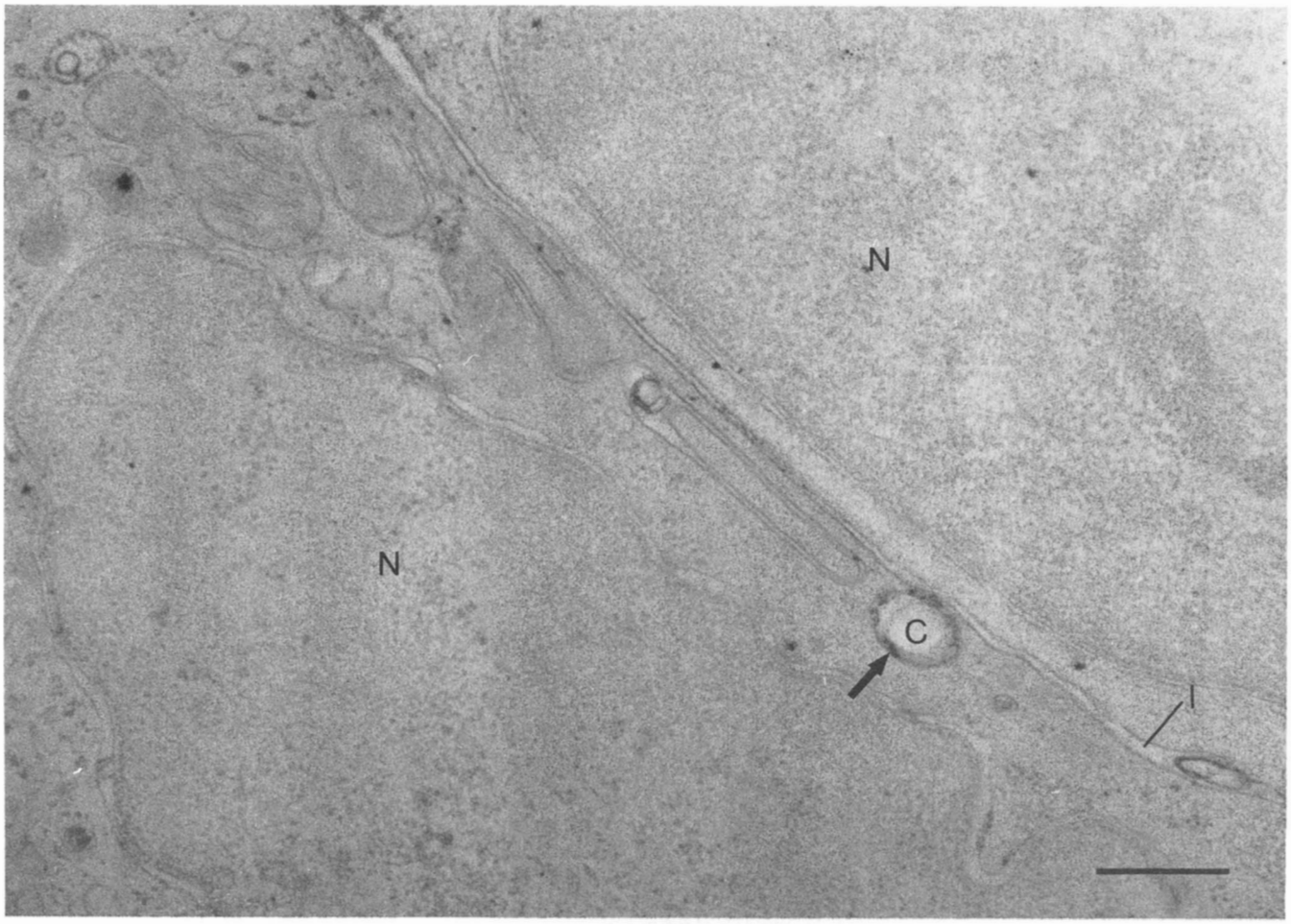


Fig. 6. Chylomicron (C) with associated labeled apoB (arrow) in the process of exocytosis via the lateral plasma membrane of jejunal absorptive cells into the intercellular space (I) after 25 min of micellar fat infusion. Nuclei (N). Bar = 0.5 μm.

and coating the exterior of lateral plasma membranes of the absorptive cells (Fig. 5c and 7b). Label was also seen in capillary lumina and on the luminal plasma membrane of capillary endothelial cells. Pinocytotic vesicles within the endothelial cell cytoplasm near the luminal plasma membrane were often filled with label (**Fig. 7a**).

Label was consistently seen at the ragged open surface of sliced cells on the reacted side of all experimental vibratome slices. This "edge effect" was present at all cut edges in thin sections made from tissue processed for localization of apoB (**Fig. 8a**).

Controls

No intracellular label was found in any of the control sections (Fig. 8b).

In all sections, experimental and control, some lipid particles appeared to have patchy labeling on their surfaces. These electron-dense areas were present even in sections that had had no antibody treatment and no DAB-H_2O_2 reaction. They resulted from an interaction between the aldehyde and OsO_4 fixatives, since they were absent in sections fixed with OSO_4 alone. The problem was reduced if the tissue was post-fixed after antibody treatment with formaldehyde instead of gluteraldehyde. As the study progressed, it became apparent that this artifact was actually rarely found at the level of the slice penetrated by antibodies. At the cut surface where labeled apoB was found, the fat that remained after washing was usually free of the artifact, although exceptions could be found (Fig. 5c). The artifact was most conspicuous deeper in the vibratome slice, where there was no apoB label anyway; as this became clear, the problem became less perplexing. A comparison of specific apoB label with the "fat artifact" is seen in **Fig. 9.** Unlike true apoB label, the artifact was an actual component of the lipid particle, and could be differentially "bleached" out by treating grids ready for electron microscopy with 0.25% H_2O_2 for 15 min before staining the sections with lead.

Traces of the "edge effect" described above were

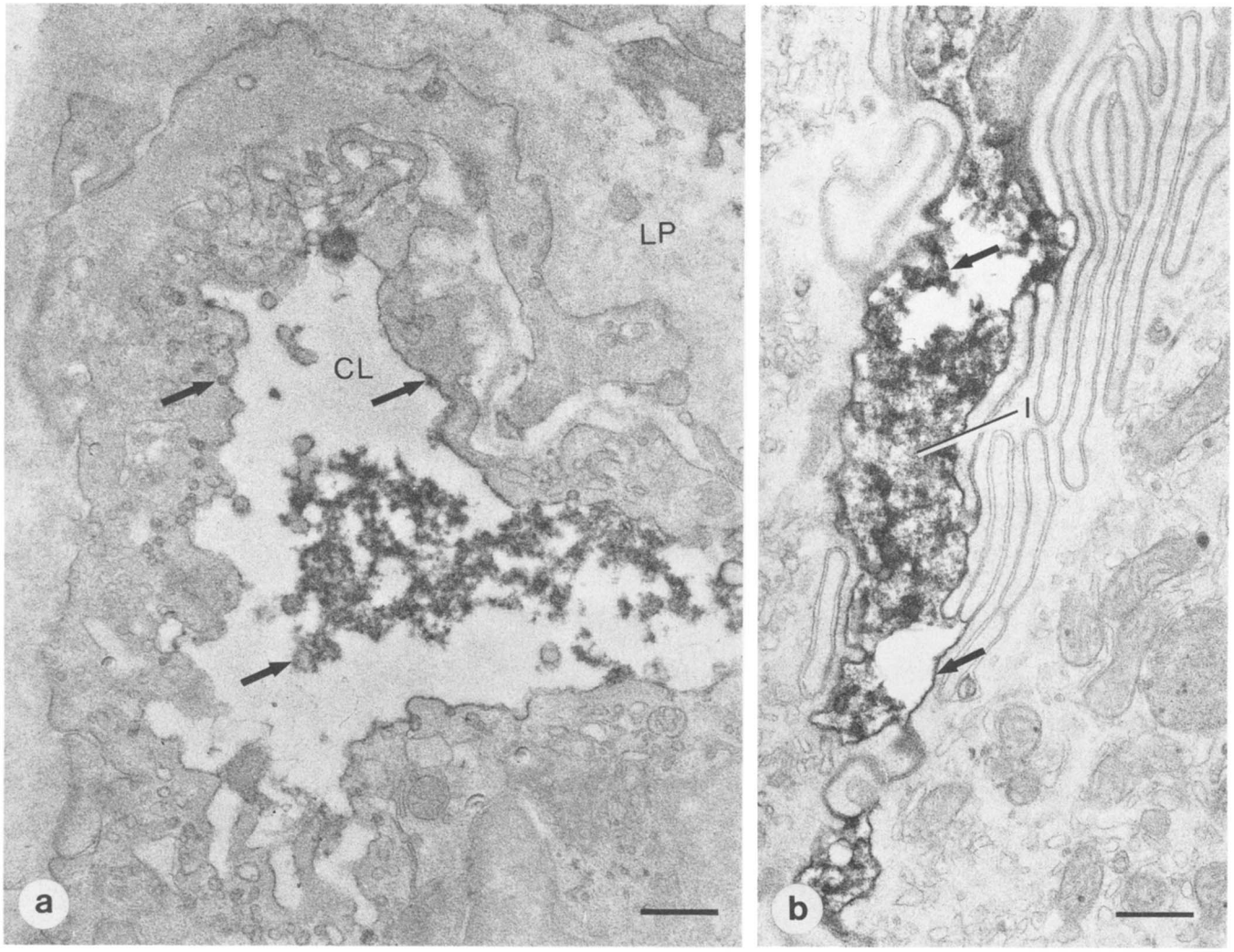


Fig. 7. ApoB-labeled capillary and intercellular space. a. Capillary within lamina propria (LP). Note apoB label (arrows) within the capillary lumen (CL), coating the luminal plasma membrane of the endothelial cell, and within pinocytotic vesicles in the cytoplasm of the endothelial cell. b. Intercellular space (I) between two adjacent jejunal absorptive cells. Note apoB label (arrows) within this space and coating the plasma membrane of the absorptive cells. Bars = 0.5 μm.

seen in some of the control slices (normal rabbit IgG, anti-B pre-incubated with LDL, or plain buffer substituted for specific first antibody). Small amounts of this "edge effect" were also seen in the rectal tissue controls processed for localization of apoB.

DISCUSSION

Methodology

Successful immunocytochemistry at the ultrastructural level requires a compromise between quality of fixation on the one hand and availability of tissue antigen for reaction with antibody on the other. If the tissue is thoroughly fixed, antigenic sites within cells not only will be cross-linked by fixation themselves, but also will be buried within cross-linked tissue components impenetrable to antibodies. However, if fixation is poor, and antibodies penetrate the disrupted cells easily, questions arise about the possibility that antigens have migrated from their true physiologic location. If artificial breaks and discontinuities in membranes and cytosol are large enough to allow antibodies free access to cell interiors, these same breaks could allow diffusion of cellular contents, extracellular components, and possibly even reaction product (oxidized DAB) (31). Taylor (32) has shown, for example, that markedly different results can be obtained by varying tissue preparation methods in experiments localizing calcium-binding protein in chick duodenum by immunofluorescence. Because of the prolonged buffer washes and incubation periods used routinely in enzyme-labeled antibody techniques,

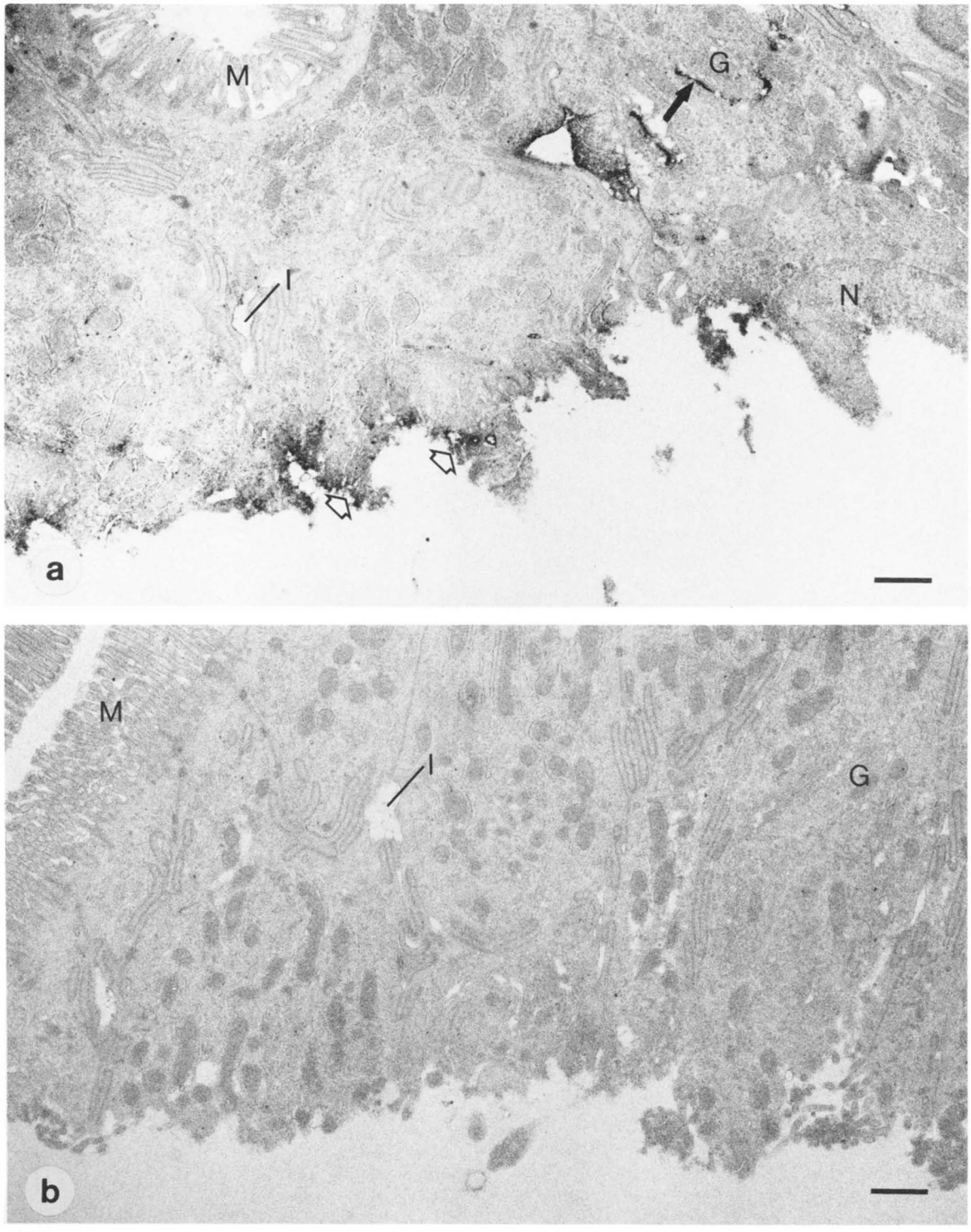


Fig. 8. Low-power comparison of specifically labeled and control sections, apex of fasting jejunal absorptive cells, ragged cut edge of vibratome slice. a. Note specific apoB label (black arrows) in Golgi (G). Hollow arrows indicate nonspecific "edge effect" found in all slices processed for localization of apoB. Nucleus (N), microvilli (M), intercellular space (I). b. Control section in which anti-B pre-incubated with an excess of LDL was substituted for specific first antibody. Note absence of any label, even at ragged edge. Golgi (G), microvilli (M), intercellular space (I). Bars = 1.0 μm.

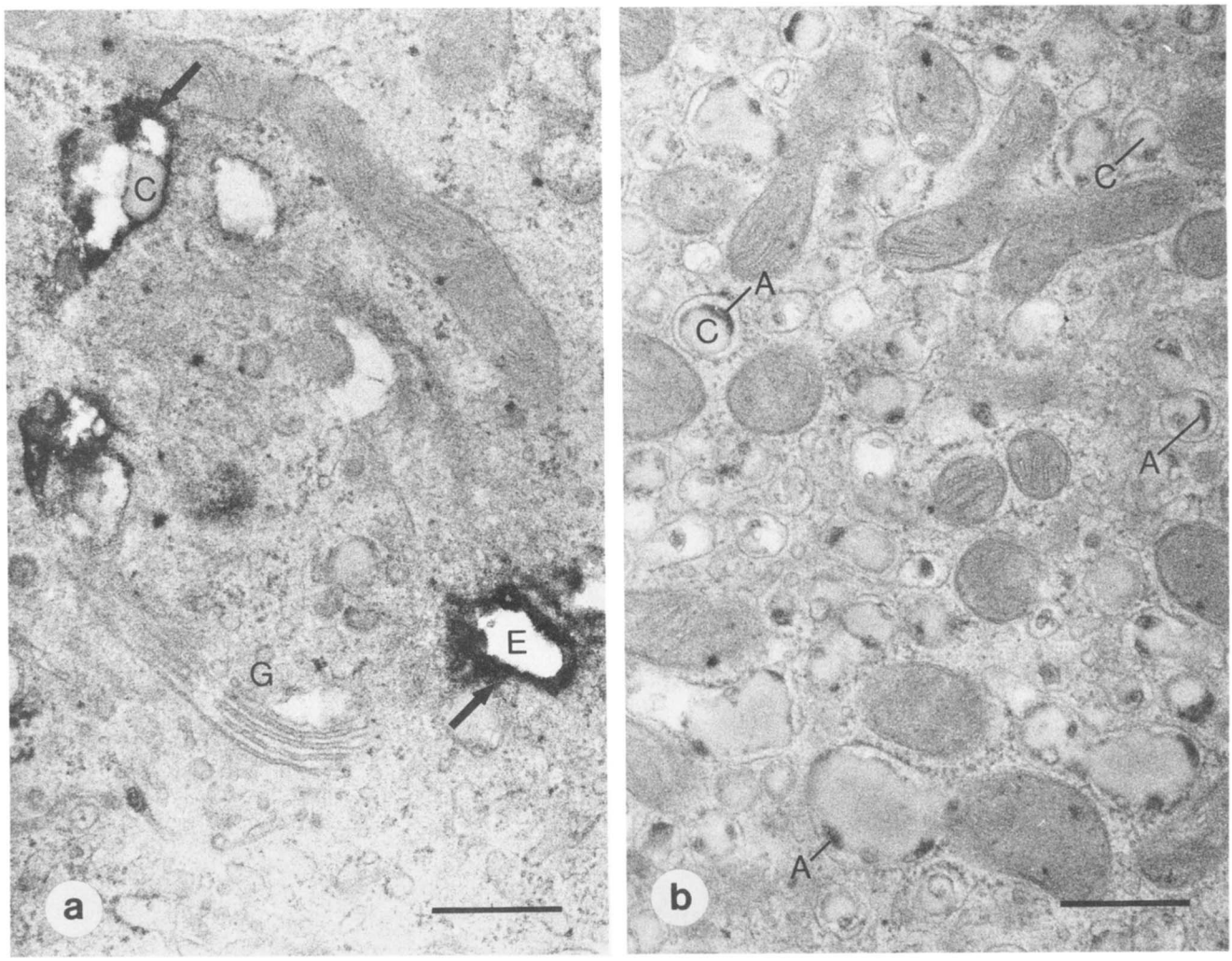


Fig. 9. Absorptive cells 25 min after micellar fat infusion. Comparison of a) specifically labeled apoB in cell near cut surface of Vibratome slice, and b) nonspecific staining artifact on lipid particles deep in the Vibratome slice where antibodies have not penetrated. a. Note labeled apoB (arrows), found mostly at expanded ends of Golgi cisternae (G); compare to artifact (A) in b). Intact chylomicron (C), empty space where chylomicrons have been washed out (E). b. Note nonspecific staining artifact (A) on chylomicrons (C). Bars = 0.5 μm.

it is not surprising to find that diffusable substances have been translocated during the process. Such problems suggest that caution is advisable in interpreting results of immunolocalization procedures.

The superior preservation of ultrastructure achieved by the method used in this study permits very limited penetration of antibodies. While this makes the search for labeled "open" cells extremely tedious, intracellular label, once found, can be accepted as an accurate localization of apoB with more confidence than is possible when ultrastructure is poorly preserved. On the other hand, extracellular label within intercellular spaces and capillaries is much easier to find. Topographically, both of these are open spaces on the surface of the vibratome slice with full access to the exterior. Antibodies do not have to go through a fixed cytoplasmic matrix to penetrate intercellular spaces and capillaries, so it is not surprising that label was found extending deeper into the slice via these open spaces.

Because of this very accessibility, however, label found in capillaries and intercellular spaces must be interpreted with caution, given the evidence cited earlier that substances may be moved around during immunocytochemical processing. It may well be that these spaces serve as natural traps for cellular contents, extracellular components, or reaction product washed from the surface of the slice during processing.

Localization of apoB

The ultrastructural localization of apoB in intestinal absorptive cells appears to parallel that reported by Alexander, Hamilton, and Havel (33) in rat liver. These

investigators proposed the following sequence for biosynthesis of VLDL, based on single-antibody immunoperoxidase techniques with frozen sections and fixed slices prepared with the Farquhar tissue chopper: *a*) triglyceride-rich particles originate in SER; *b*) at SER-RER junctions, particles receive apoproteins; *c*) specialized tubules take particles to the Golgi apparatus where they are concentrated in secretory vesicles; *d*) the vesicles move to the sinusoidal surface and exocytosis occurs.

The results of the present study are compatible with such a sequence in absorptive cells. In fasting tissue, RER cisternae contain label, suggesting that apoB is produced by the protein-synthesizing machinery of the cells even in the absence of absorbed dietary lipid. After the infusion of a micellar solution of fatty acids, small chylomicrons and VLDL-sized particles are seen within the profiles of the SER, some of which connect to RER, and these particles are accompanied by label. This would suggest that, as triglycerides are resynthesized from absorbed fatty acids in the SER, apoB is delivered from the RER to be incorporated into the resulting lipoprotein particles.

Specific label is seen accompanying lipid particles within SER and Golgi, but not on or within these particles. As was mentioned earlier, patchy electron-dense areas on some of the lipid particles were found to be artifactual, resulting from OsO_4-aldehyde interaction. An obvious concern is that this artifact might obscure specific label on lipid particle surfaces. However, visualization of apoB label on lipid particles themselves is highly unlikely. Analysis by Zilversmit (34) of chylomicron composition in dogs, rats, and man showed that these particles contain only enough protein to cover 10–20% of their surface. According to Imaizumi, Fainaru, and Havel (35), apoB represents only about 10% of that protein, which leads to the conclusion that, at most, 2% of the chylomicron surface is composed of apoB. The chance that this 2% would be included in a thin section of a spherical particle about 120 nm in diameter is very slight. The fact that chylomicrons and VLDL are accompanied by label within the cisternae of the SER suggests that apoB may be incorporated into the chylomicron at that point.

A consistent and somewhat puzzling finding is the reduction in RER labeling after fat infusion. A study by Glickman, Kilgore, and Khorana (5), in which protein synthesis inhibitors were administered to rats, showed that after 15 min of maximal lipid challenge, apoB immunofluorescence in absorptive cells was gone and lipid droplets were accumulating within the cells. This was taken to indicate the depletion of a preformed pool of apoB. It is possible that in the present study, in which the lipid challenge was by no means maximal, the preformed pool of apoB was still being utilized after 25 min of infusion, and further protein synthesis had not yet begun.

The relationship of this "performed pool" of apoB to its increased synthesis in response to fat is a matter of debate, and seemingly conflicting data exist on this subject. Rachmilewitz, Albers, and Saunders (13), measuring protein content in human intestinal biopsy specimens, found a reduction in apoB content in specimens taken 45 min after the intraduodenal administration of corn oil. In a more recent light-microscopy study, Green et al. (19) showed that immunoperoxidase labeling of apoB in human absorptive cell apices decreased after administration of corn oil, except at the extreme villous tip, data in basic agreement with the results of the present study. In the study of Green et al. (19), the investigators reported an apparent rise in overall apoB labeling, demonstrating that the label was redistributed to intercellular spaces in the villous epithelium. This provides a possible explanation for the data of Rachmilewitz et al. (13), because in this study apoB was quantitated in epithelial cells after they had been separated from underlying tissue and washed. This process may have removed the extracellular apoB found by Green et al. (19) to be in intercellular spaces. These two studies, however, seem to provide evidence which differs from that reported earlier by Glickman et al. (7), who demonstrated an increase in apoB immunofluorescence within isolated epithelial cells from human biopsy specimens 60 min after lipid infusion into the duodenum. In all three of these studies data from fat-fed specimens were compared with similar measurements in fasting specimens. Standardization of time sequences and amount of lipid infused might help to resolve this issue.

In the present study, Golgi labeling is present in fasting tissue, perhaps reflecting the processing of endogenous and biliary fat (36, 37). There is a striking increase in the amount of Golgi labeling after fat infusion, suggesting that apoB has arrived there with the lipid particles seen in Golgi cisternae and vesicles.

Label is also found in intercellular spaces and capillaries. Since this label is found only in slices incubated with specific first antibody and not in control sections, it indicates the presence of apoB. It is logical to assume that apoB is to be found in intercellular spaces accompanying chylomicrons out of the absorptive cells, and one would expect to find label in capillaries due to the presence in plasma of apoB. As was discussed above, however, there is a distinct possibility that apoB itself or reaction product could have been washed to these open spaces during processing.

The reliability of intercellular and capillary localization of apoB is also called into question by the fact that the pattern of labeling is the same regardless of the specific first antibody used. In other studies of human

jejunal mucosa in progress in this laboratory, rabbit anti-human apoA-I, apoA-II, ferritin, and transferrin all produce intercellular and capillary labeling indistinguishable from one another and from that obtained with anti-B.

The reaction product referred to above as "edge effect" is certainly nonspecific, as it is found at all ragged cut surfaces of open cells in both experimental and some control slices. It may represent the diffusion of antigen or reaction product, or it may be the result of nonspecific binding of second antibody to the cut surface of the vibratome slice.

One additional point merits discussion. Recent work from several investigators indicates that apoB is heterogeneous (38–43), and that essentially two major forms exist, one of higher molecular weight than the other. The evidence suggests that apoB made by the intestine is the form having the lower molecular weight. Additional variant forms are thought to be derived from the higher molecular weight variety (38, 43).

Although plasma LDL, the antigen used in this study to prepare the first antibody, does not contain any lower molecular weight ("intestinal") apoB (38), it is evident from the results obtained in this study that "intestinal" apoB must share enough immunogenic determinants with the higher molecular weight variant to be recognized by antibodies against this latter species.

In conclusion, the immunocytochemical method used in this study preserves the ultrastructural integrity of intestinal absorptive cells, increasing the dependability of the morphological observations described in this paper. Synthesis of apoB by the intestinal cell is suggested by the presence of labeled RER in cell apices. Chylomicrons and VLDL are accompanied by label from the time they are first observed in the apical SER until they reach the basolateral cell membranes via the Golgi. These immuno-electron microscopic observations support current concepts of the role of apoprotein B in VLDL and chylomicron formation in human jejunal absorptive cells.

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