

Identification, Isolation, and Partial Characterization of a Fatty Acid Binding Protein from Rat Jejunal Microvillous Membranes

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Abstract

The mechanisms by which FFA are absorbed by the gut are unclear. To examine these processes, binding of [^{14}C]oleate to isolated rat jejunal microvillous membranes (MVM) was studied *in vitro*. When [^{14}C]oleate alone or compounded with bovine serum albumin at various molar ratios was incubated with MVM aliquots, binding was time- and temperature-dependent, inhibitable by addition of excess cold oleate, and decreased by heat denaturation or trypsin digestion of the membranes. When [^{14}C]oleate binding to heat denatured MVM, which increased continuously as a function of the free oleate concentration and was taken as a measure of nonspecific binding, was subtracted from total binding to native MVM, a curve suggestive of saturable specific binding was observed. In contrast to fatty acids, there was no specific binding of [^{14}C]taurocholate or [^{35}S]sulfolobomophthalein to jejunal MVM. After MVM solubilization with 1% Triton X-100, affinity chromatography over oleate-agarose and elution with 7 M urea yielded a single 40,000-mol-wt protein. This Sudan Black/periodic acid-Schiff-stain-negative protein co-chromatographed on Sephadex G-100 with [^{14}C]oleate, [^{14}C]palmitate, [^{14}C]arachidonate, and [^{14}C]linoleate, but not with the [^{14}C]oleate ester of cholesterol, [^{14}C]phosphatidylcholine, [^{14}C]taurocholate, or [^{35}S]sulfolobomophthalein. A rabbit antibody to the previously reported hepatic membrane fatty acid binding protein (FABP) gave a single line of immunologic identity between the FABPs of rat jejunum and rat liver membrane. It inhibited the binding of [^{14}C]oleate to native MVM but not heat denatured MVM, and, in immunohistochemical studies, demonstrated the presence of the FABP in the apical and lateral portions of the brush border cells of the jejunum, but not on the luminal surface of esophagus or colon. These data are compatible with the hypothesis that a specific FABP plays a role in fatty acid absorption from the gut.

Introduction

The mechanisms by which FFA enter the liver cell or are absorbed from the gut are incompletely understood. Although cellular uptake of FFA was long considered a passive, diffusional process, recent studies suggest (a) that fatty acids bind specifically to rat liver plasma membranes (LPM)¹ (1); (b) that such

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1. *Abbreviations used in this paper:* BSP, [^{35}S]sulfolobomophthalein; FABP, fatty acid binding protein; LPM, liver plasma membranes; MVM, microvillous membrane.

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binding is attributable to a specific, LPM-fatty acid binding protein (FABP), which has been partially characterized and is distinct from the cytosolic FABP of rat liver and small intestine (1,2); (c) that fatty acid uptake in various mammalian cells demonstrates saturation kinetics (3-7) and, in the specific case of the hepatocyte, also demonstrates energy dependence, an association with sodium transport, and inhibition by an antibody to LPM-FABP (8). The preliminary immunohistologic observation that a protein reactive with antibodies to LPM-FABP was present on the surface of rat jejunal mucosa raised the question of whether absorption of FFA from the gut may also be mediated by a specific intestinal membrane transport process. Since binding to plasma membranes represents the first step in any cellular uptake mechanism, the aim of this study was to characterize the binding of a representative fatty acid, oleate, to microvillous membranes (MVM) of rat jejunum and to isolate a putative FABP from these membranes.

Methods

Preparation of MVM. MVM-enriched fractions were prepared by a modification of the Ca^{2+} precipitation procedure (9, 10). Three male Sprague-Dawley rats (200 g body wt; Zentralinstitut für Versuchstiere, Hannover, FRG) were starved overnight. After decapitation the intestine was removed, rinsed three times with 20 ml ice-cold saline, and divided approximately into duodenum (first third), jejunum (second third), and ileum (final third). The lumen was opened by a longitudinal section, adherent fat tissue removed, and the mucosa was scraped off gently with a glass slide. Each mucosal scraping was suspended in 300 ml of 50 mM mannitol-2 mM Tris/HCl, pH 7.5, and homogenized in a Waring blender at full speed for 2.5 min. To each preparation solid CaCl_2 was added, while stirring, to a final concentration of 10 mM. After standing on ice for 15 min, the suspension was spun down at 4,500 rpm in a GSA rotor (Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont de Nemours, E. I. & Co., Inc./ Sorvall Instruments Div., Newtown, CT) for 15 min. The supernatant was then centrifuged at 13,000 rpm in the GSA rotor for 30 min. The pellets were resuspended in 80 ml of 100 mM mannitol, 1 mM Tris-Hepes (pH 7.5), 50 μl of 0.25 M MgCl_2 was added and the suspension evenly homogenized with three strokes of a loosely fitting motor driven Teflon pestle in a 30-ml Thomas homogenizer (Fisher Scientific Co., Allied Corp., Pittsburgh, PA). After centrifugation for 30 min at 15,000 rpm in an SS-34 rotor (Du Pont de Nemours, E. I. & Co., Inc.), the pellets were resuspended in 80 ml of 100 mM mannitol, 1 mM Tris-Hepes (pH 7.5) and left standing for 30 min on ice. After a final centrifugation at 15,000 rpm (in the SS-34 rotor) for 30 min the pellets were collected in 0.013 M Tris/HCl, pH 7.5, and frozen at -20°C until use. All operations were performed at 4°C .

MVM preparations were characterized by electron microscopy (11) and enzymatic markers. Sucrase activity was determined according to Fujita et al. (12); 5'-nucleotidase and glucose-6-phosphatase were assayed as previously described (13). Membrane preparations were analyzed by double immunodiffusion in agar (14) against antibodies to rat-ligandin (gift of Dr. A. W. Wolkoff, Albert Einstein College of Medicine, New York, NY), -transferrin, -albumin, and to whole rat serum proteins (all from Cappel Laboratories, Cochranville, PA), to detect cytoplasmic and serum protein contamination. Membrane protein was determined according to the method of Lowry et al. (15).

Membrane binding studies. [^{14}C]oleate (900 mCi/mmol; 99% radiochemically pure; New England Nuclear, Boston, MA) was mixed with known quantities of nonradioactive oleic acid (Sigma Chemical Co., St. Louis, MO) in chloroform, dried under an N_2 gas stream, and dissolved in 0.2 ml of 0.1 N NaOH at 37°C. After the addition of 0.15 M NaCl/0.02 M phosphate-buffered saline (PBS) and adjusting the pH to 7.6 the solution was used as described or after further addition of defatted (16) bovine serum albumin (BSA) (fraction V, Sigma Chemical Co.) to achieve oleate/albumin in ratios of 0.1:1–4:1. The freshly prepared [^{14}C]oleate solutions were, in all cases, clear, and exhibited no evidence of turbidity or precipitation.

Incubations of [^{14}C]oleate (100–4,500 nM), either alone or bound to albumin in various molar ratios, with aliquots of 100 μg MVM protein were carried out in polypropylene tubes at a final volume of 440 μl PBS, pH 7.6. Heat denatured MVM were prepared by incubation for 16 h at 45°C; agglutination of membrane particles was avoided. Studies of [^{14}C]oleate binding to MVM included the following information: time course of binding; and assessment of temperature dependency at 4°, 23°, 37°, and 50°C, and of pH dependency by varying the pH of the incubation medium from 2.0 to 9.0 using the citrate-phosphate-borate/HCl buffer system (17). Saturability of binding was studied by incubation of membrane aliquots with increasing concentrations of [^{14}C]oleate (0.1–4.5 μM) either alone or bound to BSA in molar ratios of 0.1:1–4:1 (oleate/albumin). For each incubation with native membranes, a comparable study was conducted with heat denatured membranes to provide an estimate of nonspecific binding. Binding to native and heat denatured membranes was plotted as a function of the concentration of free oleate in the incubation mixture at equilibrium. This, in turn, was calculated by the stepwise equilibrium constant method of Wosilait and Nagy (18) using a computer program written by Dr. Luis M. Isola (Mount Sinai School of Medicine, New York, NY), and the dissociation constants for the oleate/BSA complex reported by Spector et al. (19). Heat sensitive (specific) binding was calculated as the difference between binding to native and heat denatured MVM. The effect of trypsin digestion on [^{14}C]oleate binding was determined by preincubation of 100 μg MVM protein aliquots (20 μl) with 200 μl of 0.148 mg/ml trypsin (Sigma Chemical Co.) in 1 mM Tris/HCl, pH 8.0, for 2 h at 37°C and subsequent incubation with 200 μl (1 nmol) free [^{14}C]oleate in aqueous solution at pH 8.0 and 4.0. In comparative studies binding of 0.5–9 μM [^{35}S]sulfobromophthalein (BSP) (Amersham Corp., Arlington Heights, IL; 100 mCi/mmol) and 0.5–230 μM [^{14}C]taurocholate (New England Nuclear, 43.3 mCi/mmol) to aliquots of 0.1 mg MVM protein was studied at 37°C.

Binding to MVM was assessed by a vacuum filtration assay (20). 0.2 ml of the incubation mixtures was rapidly vacuum-filtered on a Whatman GF/C glass filter (24 mm; Whatman Chemical Separation, Inc., Clifton, NJ) which was quickly washed with 20 ml ice-cold PBS. Washing with more than 20 ml did not change the amount of radioactivity remaining in the filter. The filter was placed in a scintillation vial, 10 ml Econofluor (New England Nuclear) was added, and radioactivity was determined in a liquid scintillation spectrometer (model 3380; Packard Instrument Co., Inc., United Technologies, Downers Grove, IL). Quench correction was determined by a channels ratio method. Recovery of a given amount of ^{14}C was independent of the presence of a filter, as determined by the radioactivity of an unfiltered 0.2-ml aliquot of the incubation mixture. Fatty acid absorption to the filters in the absence of MVM was very reproducible for any given set of experimental circumstances, and constituted 1–4%. The [^{14}C]oleate absorbed to the filters without MVM was routinely determined and subtracted from the experimental values. To ensure that the filters did not allow significant quantities of MVM to pass through, aliquots of 100 μg MVM protein were applied to filters and washed with 20 ml of PBS. Used as controls, identical aliquots were applied to other filters and placed directly into a test tube without washing. Protein content of both samples was found to be identical (0.1 ± 0.07 mg); even after trypsin digestion of MVM >90% of the protein was retained on the filter. Binding activity of jejunal basolateral membranes was not examined.

Effect of antibodies on [^{14}C]oleate binding to MVM. Aliquots of 0.1 mg MVM protein were incubated in 240 μl PBS for 1 h at 23°C with increasing concentrations of the IgG fractions (2 mg/ml) of a rabbit antibody to the fatty acid binding membrane protein isolated from rat liver (2) or of anti-rat fibronectin (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). Then 200 μl (1 nmol) of [^{14}C]oleate/albumin (1:1) was added and incubated for an additional 5 min. For evaluation of the influence of the antibody on nonspecific binding, heat denatured MVMs were also employed.

Preparation of an oleate agarose affinity column. Oleate-coupled agarose was prepared by incubation of 40 ml swollen, washed AH-Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) in 1.5 vol of a 0.1-M solution of the sodium soap of oleic acid at pH 10.0; 50 mg of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (Sigma Chemical Co.) per millimeter of Sepharose was added, and the mixture was rotated gently for 3 d at 37°C (2, 21). The product was washed extensively with 50% (vol/vol) ethanol, with ethanol/0.075 M sodium phosphate (1:1), pH 2.4, and with ethanol/0.05 N NaOH (1:1). Unreacted amino groups were blocked by acetylation with acetic anhydride at pH 7.0 for 1 h.

Isolation of an oleate binding protein from rat jejunal MVM. 500-mg MVM proteins were solubilized with 1% (vol/vol) Triton X-100 (Sigma Chemical Co.) as previously described (22). After centrifugation at 100,000 g for 60 min, residual detergent was removed from the clear supernatant by passage over a column of Bio-Beads SM2 (Bio-Rad Laboratories, Richmond, CA) (22). The solubilized proteins were then charged onto the oleate-agarose column and washed with PBS at 20 ml/h until no further protein (OD_{280}) appeared in the washes. Then the column was eluted with 7 M urea. All of the above procedures were carried out at 4°C. The eluate was concentrated by ultrafiltration (Diaflow membranes PM-10; Amicon Corp., Danvers, MA), and the urea dialyzed out with 1 mM NaHCO_3 , pH 7.6.

Characterization of the oleate binding MVM protein. The protein composition of the urea eluate was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11% gels (23). The protein bands were stained routinely with Coomassie Blue (Bio-Rad Laboratories), and representative gels were also stained with Sudan Black and periodic acid-Schiff-stain (24). 0.1-mg aliquots of the isolated oleate binding MVM protein were mixed with tracer doses of ultrasonically dispersed [^{14}C]oleate, [^{14}C]palmitate (600 mCi/mmol), [^{14}C]arachidonate (400 mCi/mmol), [^{14}C]linoleate (600 mCi/mmol), [^{14}C]oleate ester of cholesterol (56.6 mCi/mmol), [^{14}C]L- α -1-palmitoyl,2-oleyl phosphatidylcholine (57.0 mCi/mmol), all from New England Nuclear, and [^{14}C]taurocholate (43.3 mCi/mmol) or ^{35}S -BSP. Then the protein-ligand mixtures were subject to gel filtration over a column of Sephadex G-100 (Pharmacia, Inc.), equilibrated with 0.1 M NaCl/1.0 mM NaHCO_3 , pH 7.6, as previously described (2). Elution was carried out with the same buffer at 5 ml/h. Eluates, in 0.5-ml fractions, were monitored for protein at 280 nm and for radioactivity by liquid scintillation spectrometry as described above.

Purity and specificity of the isolated oleate binding MVM protein was further examined by double immunodiffusion in agar (14) with rabbit antisera against rat albumin, rat serum proteins, ligandin, and the oleate (2) and BSP/bilirubin (22) binding membrane proteins isolated from rat liver. Conversely, the antibody to the oleate binding membrane protein of rat liver was tested against the jejunal and liver fatty acid binding membrane proteins, the rat liver BSP/bilirubin binding membrane protein (22), whole rat serum, and concentrated rat liver as well as rat jejunal cytosols, prepared from the 100,000 g supernatants of the corresponding homogenates.

Immunofluorescence studies. Frozen sections of esophagus, stomach, jejunum, and colon, with an average thickness of 5 μm , were air dried. They were incubated for 30 min at room temperature with rabbit antibody against the oleate binding membrane protein isolated from rat liver, diluted 1:50, 1:100, and 1:200 with PBS, pH 7.4. Sections were washed with PBS for 30 min, then incubated with fluorescein isothiocyanate-conjugated swine anti-rabbit antiserum (Dako, Copenhagen, Denmark) diluted 1:20 or 1:50. After thorough washing with PBS for 60–90 min, sections were mounted with buffered glycerol.

Controls consisted of incubations with antiserum previously absorbed with the fatty acid binding MVM protein in equal dilution, incubations without immune serum, and incubations with preimmune serum. Sections were examined with a fluorescence microscope (Zeiss, Oberkochen, FRG) using filter combination PB 485, FT 510, LP 520, as previously described (2).

Results

Characterization of MVM fractions. In numerous randomly analyzed electron micrographs of the MVM fractions employed, only one kind of mostly vesiculated membrane was seen (Fig. 1). Some of the vesicles were filled with electron-dense material, which presumably originated from the core of the microvilli. Contaminating membranes from organelles other than brush borders could not be detected. The pictures resembled those previously published (9, 10). Determination of sucrase as a specific marker enzyme for MVM revealed 46.6 ± 1.6 U/mg protein sp act in the jejunal MVM fraction as compared with 6.7 ± 1.8 U/mg protein sp act in the homogenate. The 5'-nucleotidase was elevated from 4.3 ± 0.5 U/mg protein sp act in the homogenate to 23.4 ± 1.3 U/mg protein sp act in the jejunal MVM fraction. Contamination with microsomes was assessed by assaying glucose-6-phosphatase (0.163 ± 0.016 vs. 0.440 ± 0.131 U/mg protein sp act in the homogenate). Cytoplasmatic and serum protein contamination were not detectable by radial immunodiffusion of the MVM-enriched fraction against antibodies to rat ligandin, transferrin, albumin, and whole rat serum proteins. Antibody preparations against the intestinal cystolic FABP (25) were not available to us.

Membrane binding studies. Since fatty acids self-associate in aqueous solutions at concentrations exceeding 10^{-6} – 10^{-5} M, membrane binding studies were performed with [14 C]oleate

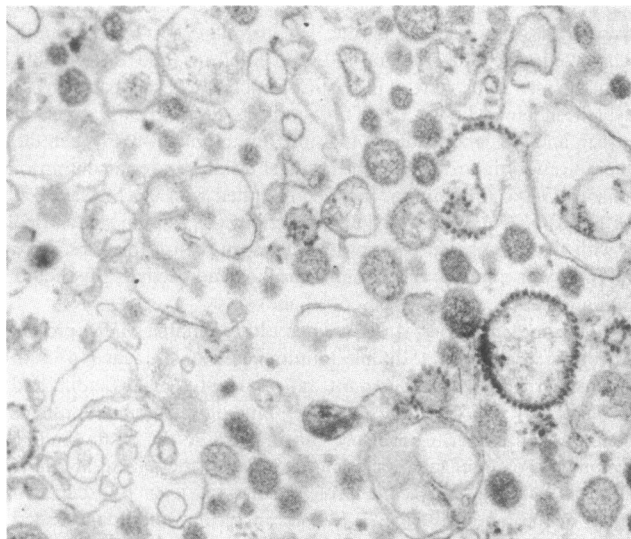


Figure 1. Electron microscopy of representative jejunal MVM preparation. Typical of reported MVM preparations (9, 10), the specimen contains a heterogeneous collection of vesicles that vary in shape and amount of electron-dense material within the cavity. There are some membrane fragments that did not vesiculate, and do not enclose a cavity. The surface of many of the vesicles appears rough, and of these, some had a cogwheel appearance, as previously described (9, 10). Some minimal microsomal contamination of the MVM preparation is suggested by both the electron microscopic appearance and the residual glucose-6-phosphatase activity (see text).

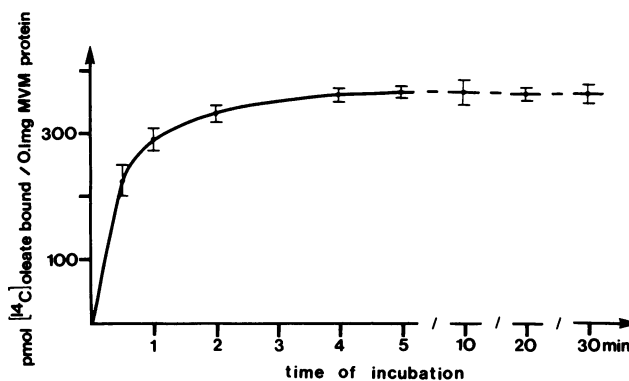


Figure 2. Time course of [14 C]oleate binding to MVM. $2.272 \mu\text{M}$ [14 C]oleate bound to albumin (1:1) was incubated at 23°C with aliquots of 0.1 mg MVM protein. Values are means \pm SD of three replicate experiments.

bound to BSA in various molar ratios. Thus, in accordance with the equilibrium binding constants of albumin-oleate complexes (18, 19), the free oleate concentration in the incubation medium could be varied by changing the molar ratio of oleate/albumin while maintaining concentrations of free oleate within the range of solubility. Additional incubation studies were performed with [14 C]oleate in protein-free aqueous solutions at concentrations lower than 4.6×10^{-6} M.

Binding of [14 C]oleate to aliquots of 0.1 mg MVM protein was rapid. When $2.272 \mu\text{M}$ [14 C]oleate/albumin (1:1) was incubated at 23°C , maximal binding was attained by 3 min (Fig. 2). Thus, in the subsequent binding studies an incubation time of 5 min was used.

Binding of [14 C]oleate ($2.272 \mu\text{M}$; oleate/albumin = 1:10) to MVM as a function of temperature was maximal at 37°C (Fig. 3). Influence of different pH values was determined by incubation of $2.272 \mu\text{M}$ [14 C]oleate free in aqueous solution so as to avoid concomitant changes in the affinity of oleate for albumin. The overall binding of [14 C]oleate showed a maximum at pH 4 (Fig. 4). At more physiologic pH values between 6.8 and 8.0, binding was relatively constant. Binding increased below pH 6.8 and decreased above 8.0. Because of the unusual

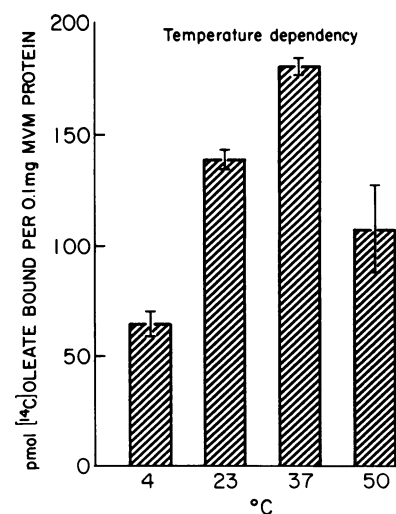


Figure 3. Influence of temperature on binding of [14 C]oleate to MVM. In these studies, $2.272 \mu\text{M}$ [14 C]oleate as a 1:10 complex with BSA was used.

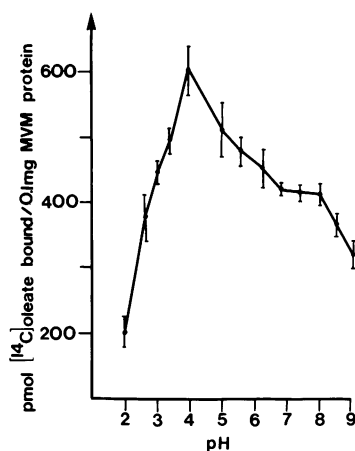


Figure 4. Effect of pH on binding of [^{14}C]oleate to MVM. $2.272\ \mu\text{M}$ [^{14}C]oleate free in aqueous solution was incubated with aliquots of $0.1\ \text{mg}$ MVM protein in $440\ \mu\text{l}$ citrate-phosphate-borate-/HCl buffer pH 2–9. Values are means \pm SD of three replicate experiments.

shape of this binding curve, an additional study was performed. Pretreatment of MVM with trypsin did not significantly change binding of [^{14}C]oleate ($583.3 \pm 12.7\ \text{pmol}/0.1\ \text{mg}$ MVM protein) as compared with native MVM (602.1 ± 11.7) at pH 4.0. At pH 8.0, however, trypsin reduced binding by 80.3%, from $391.9 \pm 14.9\ \text{pmol}$ to $77.2 \pm 7.8\ \text{pmol}/0.1\ \text{mg}$ MVM protein. These different binding characteristics suggest differences in the mechanism of oleate-MVM interaction at these pH values. The data suggest the possibility that at low pH, the largely nonionized oleic acid molecule binds preferentially to membrane lipids, whereas at physiologic pH, the fatty acid anions bind to a protein binding site.

When $0.455\ \mu\text{M}$ [^{14}C]oleate/albumin (1:1) was incubated in the presence of a 1,000-fold excess of cold oleate/albumin (1:1), membrane binding of [^{14}C]oleate was inhibited by 98.2%, from 67.4 ± 3.9 to $1.24 \pm 0.14\ \text{pmol}/0.1\ \text{mg}$ MVM protein. Similarly, incubation of $0.455\ \mu\text{M}$ [^{14}C]oleate/albumin (1:10) with heat denatured MVM led to an 89% reduction of binding compared with that observed with native MVM under similar conditions (59.2 ± 6.0 to $6.5 \pm 1.0\ \text{pmol}/0.1\ \text{mg}$ MVM protein).

In Fig. 5, the quantity of membrane-bound oleate observed in the presence of $4.54\ \mu\text{M}$ [^{14}C]oleate at various oleate/albumin molar ratios was plotted as a function of the calculated free oleate concentration in each incubation. When total [^{14}C]oleate bound to native MVM was corrected for binding

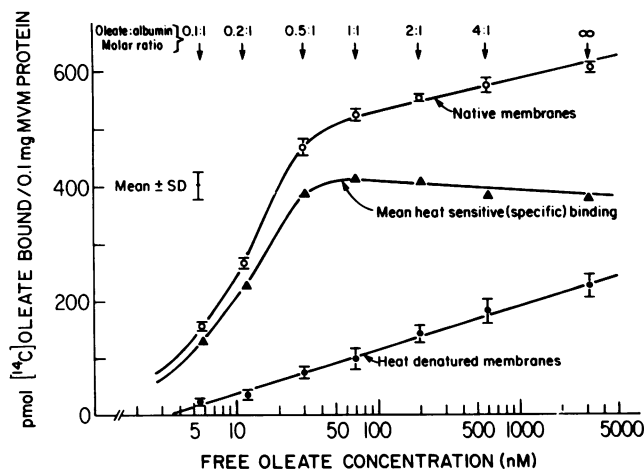


Figure 5. Influence of calculated free oleate concentration on binding of [^{14}C]oleate to native and heat denatured jejunal MVM. See text for experimental details.

to heat denatured membranes, as an estimate of nonspecific binding, a curve representative of heat sensitive, specific binding was obtained. This curve indicates that heat sensitive binding is saturable, with a dissociation constant (K_d) = $1.6 \times 10^{-8}\ \text{M}$ and a maximal binding capacity of $460\ \text{pmol}/0.1\ \text{mg}$ MVM protein estimated by Scatchard analysis (26).

The data presented above suggest that, as with LPM, binding of [^{14}C]oleate to jejunal MVM exhibits the characteristics of specific binding. In contrast to the results with [^{14}C]oleate, incubation of [^{35}S]BSP in the concentration range 0.5 – $9.1\ \mu\text{M}$, and of [^{14}C]taurocholate in the concentration range 0.5 – $230\ \mu\text{M}$, under experimental conditions identical to those used for [^{14}C]oleate, did not show any evidence for specific binding to jejunal MVM.

Isolation and characterization of an FABP from MVM.

The urea eluate of the oleate agarose affinity chromatography column yielded $1.25 \pm 0.3\ \text{mg}$ (SD) protein from the original $500\ \text{mg}$ solubilized MVM protein mixture. Compared with the multiplicity of bands it revealed in the initial solution of solubilized membrane proteins, SDS-PAGE showed only a single band of $\sim 40,000$ mol wt in the concentrated urea eluate from the oleate-agarose columns (Fig. 6). Rod gels of this oleate binding membrane protein did not stain with either Sudan Black or periodic acid-Schiff-stain. When the oleate binding MVM protein was co-chromatographed over Sephadex G-100 with various organic anions and other hydrophobic membrane constituents, binding to it was demonstrated by all of the various FFA tested (Fig. 7). However, the oleate ester of cholesterol and oleate-containing phosphatidylcholine did not migrate with the protein. [^{35}S]BSP and [^{14}C]taurocholate also were eluted separately from the protein. These data suggest that the oleate binding MVM protein serves as a general free FABP, and that its affinity spectrum is not limited solely to oleate. Moreover, there appears not to be any significant degree of nonspecific binding to lipid membrane constituents other than FFA.

In double immunodiffusion studies, the isolated fatty acid binding MVM protein showed no immunologic activity to antibodies against rat albumin, whole rat serum, ligandin, or the hepatocyte membrane BSP/bilirubin binding protein (22). However, the rabbit antibody against the LPM-FABP (2) showed a single precipitin line of identity between the LPM-FABP, the FABP isolated from jejunal MVM, and the solubilized MVM protein mixture. No reactivity was observed between this antibody and rat albumin, whole rat serum, the rat liver BSP/bilirubin binding protein, and concentrated rat liver—as well as jejunal—cytosol (each of which contains a cytosolic FABP).

Antibody inhibition of oleate binding to MVM. When $2.272\ \mu\text{M}$ [^{14}C]oleate/albumin (1:1) was incubated at 23°C with native MVM in the presence of IgG fractions of antifibrinogen, binding remained constant; in the presence of increasing concentrations of anti-LPM-FABP binding progressively decreased (Fig. 8). Binding to heat denatured MVM remained unchanged even in the presence of $400\ \mu\text{g}$ of the IgG fraction of LPM-FABP. Antibody inhibition of specific binding (i.e., binding to native MVM minus binding to heat denatured MVM) ranged from 25 to 75% under these conditions.

Immunofluorescence studies. In frozen sections of rat jejunal mucosa, incubation with the rabbit anti-LPM-FABP before fluorescein-isothiocyanate-conjugated swine anti-rabbit antiserum revealed marked fluorescence of villi and crypts that was limited to the apical parts of brush border cells and to the

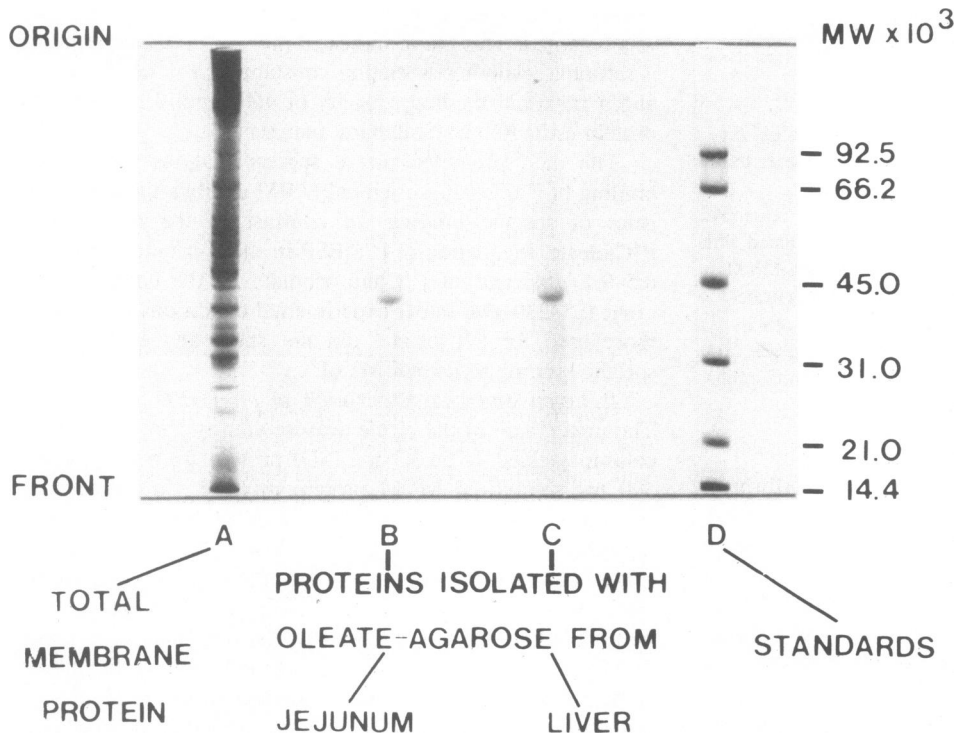


Figure 6. Affinity chromatography of detergent-solubilized proteins from rat jejunal MVM. Figure presents SDS polyacrylamide gel patterns of total solubilized membrane proteins (column A), and a single band representing the protein (40,000 mol wt) eluted from oleate-agarose (column B). For comparison the 40,000-mol-wt oleate binding membrane protein isolated from rat liver is shown in column C. Molecular weight standards are in column D. MW, mol wt.

lateral cell border in the region of the tight junctions. Goblet cells in the crypts did not stain (Fig. 9, A and B). No fluorescence appeared in any of the control sections. Similar staining, albeit less intense, was observed in the proximal ileum. Frozen sections of the esophagus did not show specific fluorescence. The surface staining observed (Fig. 9 C) was considered to be nonspecific fluorescence because of the keratin of the stratified squamous epithelium, and was equally seen in control sections. In the stomach, faint specific immunofluorescence was found on the surface of some parietal cells, while

the remaining mucosal epithelial cells did not show any staining. The mucosa of the colon also did not exhibit specific fluorescence. However, in the submucosa, prominent staining of macrophages was observed (Fig. 9 D).

Discussion

The mechanism by which FFA are absorbed from the intestinal lumen is uncertain, although, as with their entry into mammalian cells in general, their initial entry into intestinal epithelial

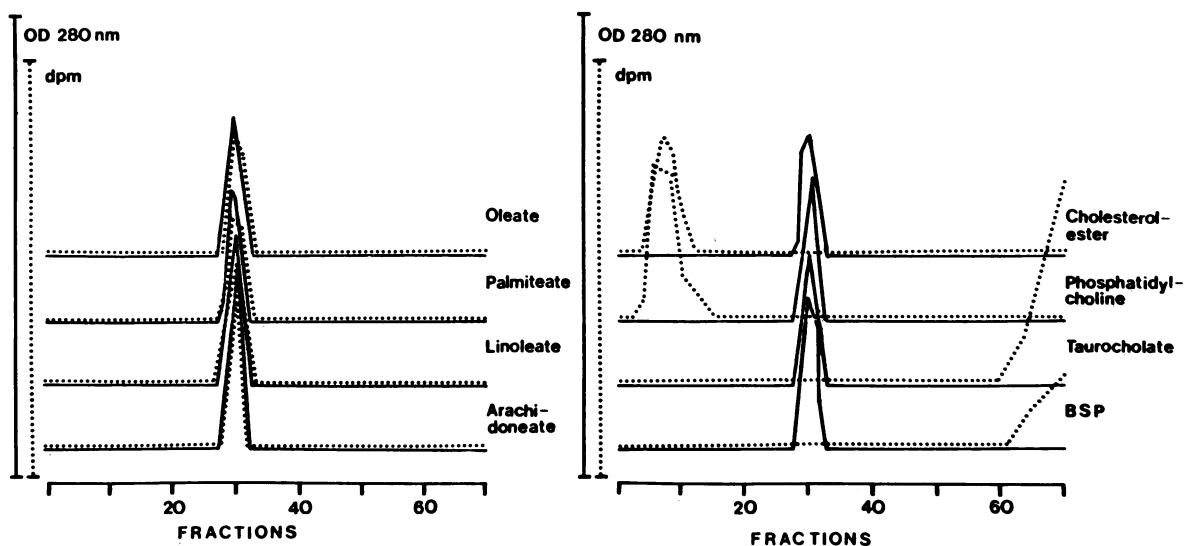


Figure 7. Co-chromatography on Sephadex G-100 of 0.1-mg aliquots of the isolated oleate binding MVM protein with tracer doses of ultrasonically dispersed [^{14}C]oleate, [^{14}C]palmitate, [^{14}C]arachidonate, [^{14}C]linoleate, [^{14}C]oleate ester of cholesterol, [^{14}C]phosphatidylcholine, and [^{14}C]taurocholate or [^{35}S]BSP. Only long chain FFA were eluted together with the protein peak. The oleate ester of cholesterol and phosphatidylcholine appeared in the void volume (up to fraction 19), and taurocholate and BSP in the effluent of the column starting at fraction 63. None was associated with protein. See text for experimental details.

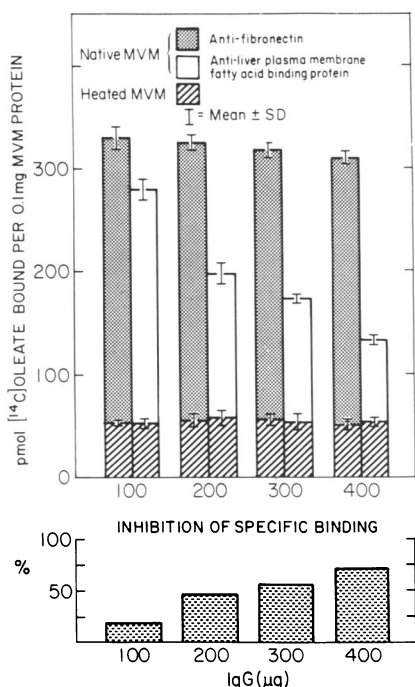


Figure 8. Inhibition of the binding of [¹⁴C]oleate to MVM by the rabbit antibody to the rat liver fatty acid binding membrane protein. In paired studies aliquots of 0.1 mg of both native and heat denatured MVM protein were incubated with 2.272 µM [¹⁴C]oleate/albumin (1:1) at 23°C in the presence of increasing concentrations of the IgG fractions of either antifibronectin or anti-LPM-FABP. With increasing concentrations of the anti-LPM-FABP, [¹⁴C]oleate binding to native MVM progressively decreased, while that to heat denatured MVM was unaffected. Binding to both native and denatured MVM in the presence of antifibronectin remained unchanged. Inhibition of specific [¹⁴C]oleate binding (i.e., binding to native minus binding to heat denatured MVM) ranged from 25 to 75% in these studies.

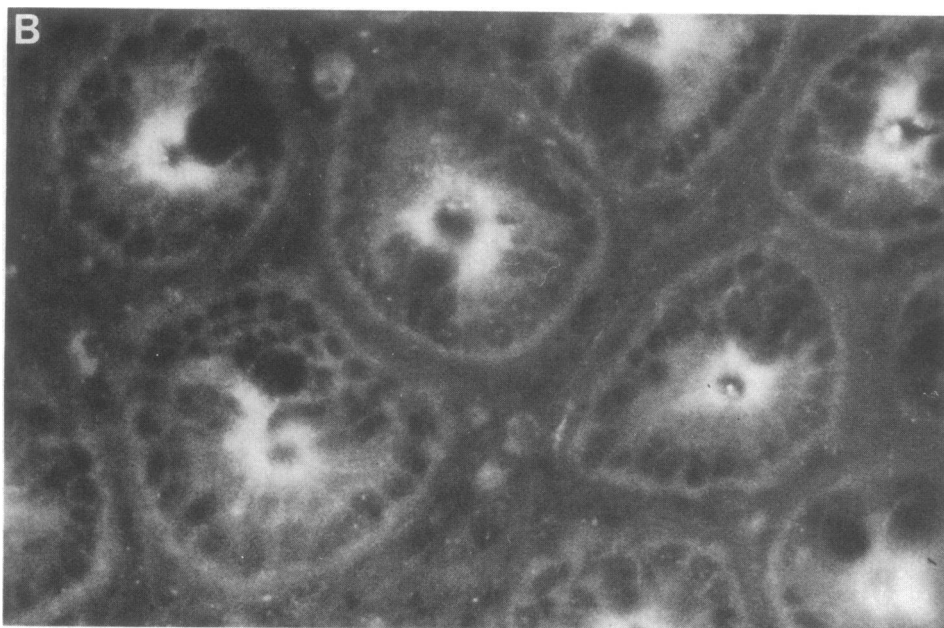
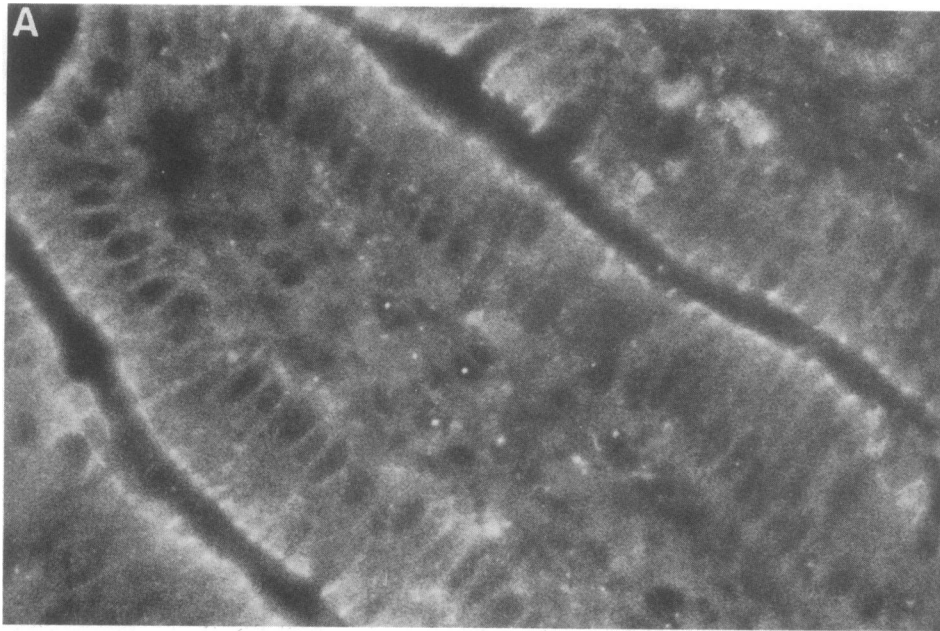
cells has often been described as a passive diffusional process (27). However, more recent studies of a variety of cell types (3, 4, 6, 7), and particularly of adipocytes (5), suggest the existence of a saturable uptake mechanism. An adipocyte membrane protein of $M_r = 85,000$ that has an apparent role in fatty acid uptake has been identified by the labeling of membranes with [³H]4,4'-diisothiocyanostilbene-2,2'-disulfonate (28). Moreover, data from our own laboratory suggesting that a component of the hepatocellular uptake of FFA was carrier-mediated and involved a specific FABP in the sinusoidal plasma membrane (2, 8), raised the possibility that a similar membrane transport process also mediated their intestinal uptake. The goals of this study, therefore, were to characterize the binding to jejunal MVM of a representative FFA, [¹⁴C]oleate, and to seek in such MVM a membrane FABP similar to that recently isolated and characterized from the sinusoidal plasma membranes of rat liver (2).

The binding of [¹⁴C]oleate to MVM was, in fact, shown to be time-, temperature-, and pH-dependent, to be inhibitable and reversible by incubation in the presence of excess cold oleate, and to be markedly reduced by incubation with trypsin-digested membranes. Binding was shown to have two components: one, heat resistant, with characteristics of nonspecific binding; and the other, saturable and heat sensitive, with characteristics suggestive of specific binding. In these binding

studies, oleate was presented to the MVM in the form of complexes with albumin rather than as aggregates or micelles, as may occur in the intestinal lumen. The incubations therefore represented a nonphysiologic interaction between MVM and the oleate/protein complexes. This disadvantage was offset by the ability to accurately calculate the free oleate concentration in the incubation mixtures, and consequently, to characterize the binding relationship more precisely. Given the extensive binding observed between oleate and MVM in the presence of alternative competing binding sites on albumin, it is most unlikely that MVM would not similarly bind FFAs within the micelle-containing environment of the jejunum.

The binding characteristics observed suggested that specific binding of oleate to MVM was due to an intrinsic membrane protein. This suggestion was pursued by application of an affinity chromatography technique previously successfully employed to demonstrate an FABP in rat liver plasma membranes (2). MVM do, in fact, contain a single 40,000-mol-wt protein with a high affinity for fatty acids, lacking either lipid or carbohydrate components. While the protein's physicochemical similarity to the LPM-FABP was noted, the identity of the two membrane FABPs was confirmed by double immunodiffusion in agar against the antibody to the LPM-FABP. Although we have not yet examined adipocytes, it is possible that the 85,000-mol-wt membrane protein with a fatty acid transport function identified in these cells by Abumrad et al. (28) is a dimeric complex of the 40,000-mol-wt protein present in hepatocytes and jejunal enterocytes, since the adipocyte protein was identified with membrane-labeling reagents that may serve as bifunctional cross-linking agents. The jejunal membrane FABP does not share immunologic determinants with either ligandin or the cytosolic FABPs previously described in liver (29) and small intestine (25).

The isolated oleate-binding MVM protein revealed in co-chromatography studies an affinity for all long chain fatty acids tested, but not for other classes of organic anions or other membrane constituents such as esterified cholesterol and phosphatidylcholine. This suggested that the protein might serve specifically as a general acceptor for FFAs in the gut. Its significance in the binding of fatty acids to jejunal mucosa was further demonstrated by the specific inhibition of [¹⁴C]oleate binding to MVM by the antibody to the LPM-FABP. Immunohistochemical studies of various parts of the rat gastrointestinal tract revealed the presence of this protein in the upper ileum, but predominantly in the jejunum, the major site of the fatty acid absorption. Consistent with an absorptive function it was localized principally to the apical portion, and to a lesser extent at the lateral cell border in the region of the tight junctions of the brush border cells of the villi. Some staining of the basolateral membranes may, conceivably, reflect a role for the protein in transport of fatty acids out of the epithelial cell for subsequent disposition. An analogous situation occurs in the hepatocyte with respect to the membrane BSP/bilirubin binding protein, which is localized principally to the sinusoidal (basolateral) plasma membrane, but is also identifiable by immunofluorescence in the canalicular (apical) membrane (22). Presence of the MVM-FABP in crypt cells is somewhat unexpected, unless the fatty acid transport system is a primitive mechanism that appears very early in the differentiation and maturation of jejunal enterocytes. There are currently no data to confirm this hypothesis. In contrast to jejunum and ileum, there was no specific staining at the luminal surface of esophagus



and colon, tissues that are not known to be involved in absorption of fatty acids. However, staining was also observed in some parietal cells of the stomach and in the macrophages of the colon. These cells are known to have high energy requirements, but the specific function of a possible membrane FABP in these cells remains to be determined.

While the estimated K_d of both the LPM- (1, 2) and MVM-FABP/oleate complexes is smaller than that of the oleate/albumin complex (19), providing a possible explanation for movement of FFA from albumin to the plasma membrane, it is also smaller than the K_d of the oleate/cytosolic FABP complex (30). Hence, in an equilibrium system, the relative binding affinities of oleate for albumin, membrane, and cytosolic FABPs would appear to favor accumulation of ligand on the membrane. Similar relative affinities for albumin, membrane binding protein (22, 31), and ligandin (29) also exist for BSP

and bilirubin. Thus, for both BSP/bilirubin and FFA, transfer from membrane to cytosol presumably reflects a nonequilibrium state resulting from metabolic depletion of the cytosolic ligand pool.

Although specific membrane binding proteins appear to play a role in the hepatocellular uptake of BSP and bilirubin (22, 31), conjugated bile acids (32), and certain organic anions such as sulfate (33), the physiologic significance of a specific FABP in hepatocytic plasma membranes and brush border MVM also has yet to be established. Nevertheless, its anatomic location principally in intestinal sites of fatty acid uptake, as well as in liver and myocardium (2), its high affinity for FFA, and the inhibition of fatty acid binding to MVM by antibodies to the protein all argue circumstantially for a significant physiologic role. Observations suggesting that an identical protein in the sinusoidal plasma membrane is involved in the

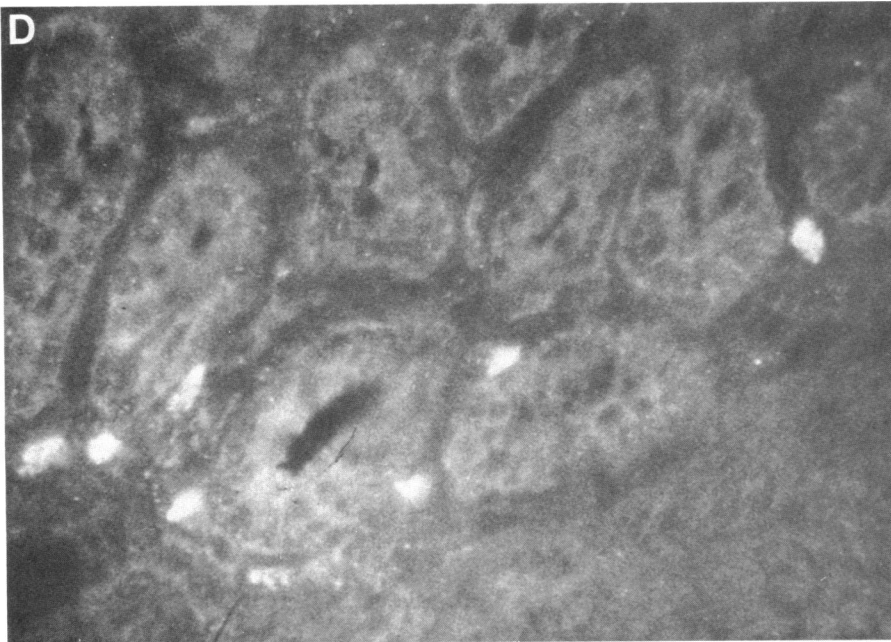
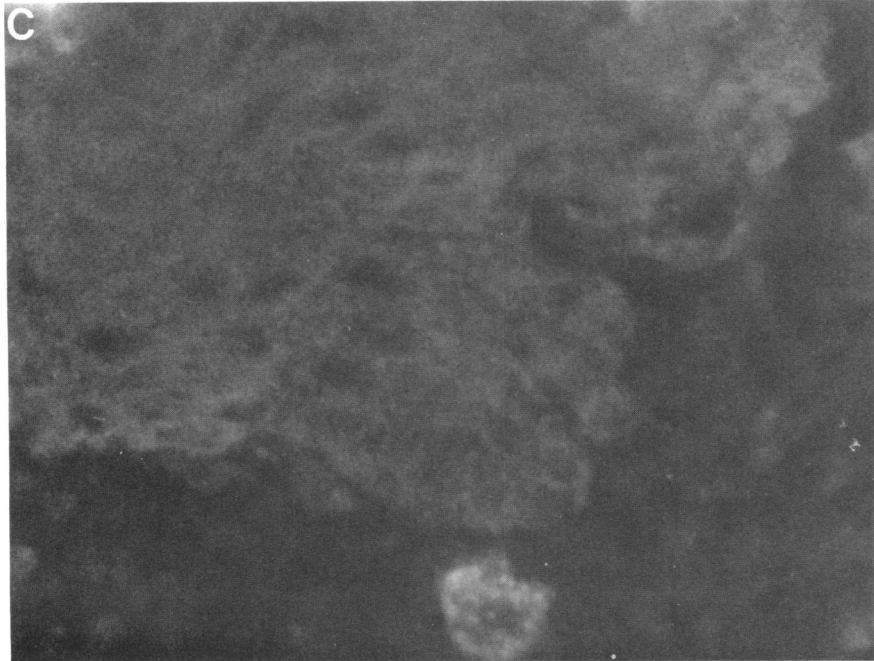


Figure 9. Immunofluorescence studies of different sections of the rat gastrointestinal tract with rabbit antibody to the oleate binding membrane protein from rat liver. In the jejunal mucosa marked fluorescence of villi (A) and crypts (B) is limited to the apical parts of the brush border cells and to the lateral cell border in the region of the tight junctions. Goblet cells in the crypts did not stain. In control sections with antigen-absorbed antibody no fluorescence appeared. Sections of the esophagus do not show specific staining. Nonspecific fluorescence at the surface is due to keratin (C). The colonic mucosa does not exhibit specific immunofluorescence. However, in the submucosa, staining of macrophages is observed (D).

carrier-mediated uptake of FFA by hepatocytes also suggest an analogous function in fatty acid uptake by enterocytes. The implications for regulation of fatty acid metabolism by a carrier-mediated membrane fatty acid transport system are substantial and indicate the need for further studies.

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