A Possible Role for Rat Intestinal Surfactant-like Particles in Transepithelial Triacylglycerol Transport

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Abstract

To further examine whether surfactant-like particles (DeSchryver-Kecskemeti, K., R. Eliakim, S. Carroll, W. F. Stenson, M. A. Moxley, and D. H. Alpers. 1989. *J. Clin. Invest.* 84:1355–1361) were involved in the transepithelial transport of lipid, alkaline phosphatase activity and surfactant-like particle content were measured in apical mucosal scrapings, enterocytes, lamina propria, and serum after inhibition of chylomicron transport. Serum triacylglycerol levels were decreased 60–76% by Pluronic L-81, fenfluramine, and choline deficiency compared with fat-fed controls. 5 h after triacylglycerol feed, alkaline phosphatase activity in all three experimental groups was decreased compared with controls by 52–69% in mucosal scrapings and by 33–72% in serum. A parallel decline (60%) in alkaline phosphatase activity occurred in the lamina propria of Pluronic-treated animals. Total particle content (measured by an ELISA using antiserum against purified particle) after Pluronic treatment was decreased in mucosal scrapings, lamina propria, and serum by 16, 22, and 29% at 3 h and by 33, 40, and 8%, respectively, at 5 h after fat feeding. In contrast, particle content was increased in enterocytes by 29% 3 h and by 8% 5 h after fat feeding. By electron microscopy, enterocytes from Pluronic- and fenfluramine-treated animals exhibited a two- to threefold increase in large intracellular cytoplasmic lipid globules and the appearance of lamellae in apposition, with a marked decrease in the number of surfactant-like particles overlying the brush border. These changes, produced by inhibition of chylomicron transport, in the distribution of surfactant-like particles and particle-bound alkaline phosphatase are consistent with a role for these particles in transepithelial triacylglycerol transport across and out of the enterocyte. (*J. Clin. Invest.* 1994. 93:70–80.) Key words: intestinal alkaline phosphatase • surfactant-like particles • triacylglycerol transport

Introduction

The mechanism whereby dietary fat droplets traverse the enterocyte has been the object of considerable speculation in previous studies. Fat droplets were observed within the cisternae of the endoplasmic reticulum throughout the cell, giving rise to the speculation that these structures were formed from pinocytosis and released by reverse pinocytosis (1). The demonstration of the resynthesis of triacylglycerols within the enterocyte and the inability to identify the process of discharge of chylomicrons from the cell led to the rejection of this hypothesis (2).

Later work suggested that the pathway for triacylglycerol resynthesis and intracellular movement led through vesicles of smooth endoplasmic reticulum to fusion with Golgi vesicles, which fused with the basolateral membrane, discharging chylomicrons into the basolateral space (3). We have demonstrated recently that an intracellular membrane containing intestinal alkaline phosphatase (IAP)[1] surrounded lipid droplets in the region of the Golgi vesicles (4). These structures show some morphologic resemblance to a particle we have isolated from the enterocyte apical surface. This particle is enriched for intestinal alkaline phosphatase and has a chemical, physical, and morphological resemblance to pulmonary surfactant (5, 6). In addition, the particles are much more abundant after fat feeding (7) and have been found to contain relatively large amounts of IAP. When IAP was localized by immunoelectron microscopy within the enterocyte, it was found on membranes surrounding fat droplets (4). Moreover, Golgi vesicles contained lamellated structures that appeared to be the intracellular counterparts of the extracellular apical vesicles, and these intracellular structures contained IAP by immunoelectron microscopy (8). Although the morphology of these IAP-containing membranes within the cell suggested a relationship with the surfactant-like particles outside the cell, the morphologic features were not absolutely identical.

On the basis of these data we hypothesized that the particles formed intracellularly in response to fat feeding, surrounded the fat droplet, and played a role in, or were associated with, the intracellular movement of the droplet across the cell, with subsequent delivery of the fat and the particle to the extracellular environment (9). We have also postulated that the IAP appearing in the serum after fat feeding could derive from the secretion of the particle and could be liberated from the membrane by the action in serum of a glycosyl-phosphatidylinositol anchor-specific phospholipase D (10). If these hypotheses are correct, inhibition of fat absorption should lead to a decrease in surfactant-like particles and in particle-bound IAP on the apical cell surface, accompanied by a decrease in these parameters in the lamina propria and by a decrease in particles and membrane-free IAP in the serum.

There is evidence that chylomicrons and VLDL are packaged differently within the enterocyte (3, 11, 12). The difference in intercellular lipid packaging has been reinforced by

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Received for publication 8 September 1992 and in revised form 30 August 1993.

J. Clin. Invest.

0021-9738/94/01/0070/11 $2.00
Volume 93, January 1994, 70–80

1. *Abbreviations used in this paper:* IAP, intestinal alkaline phosphatase; SLP, surfactant-like particles.

experiments using the nonionic hydrophobic surfactant, Pluronic L-81. This compound, when given intraluminally by continuous infusion or by bolus injection, caused a decrease in chylomicron formation and transport (13). The formation and packaging of VLDL particles was not affected by Pluronic L-81 (14). Moreover, the pathway for triacylglycerol synthesis using fatty acids and α-glycerophosphate, characteristic of VLDL formation, was unaffected by Pluronic L-81 (15). Administration of the drug produces large droplets in the cytoplasm, apparently inhibiting intracellular movement between the endoplasmic reticulum and the Golgi vesicles (16). After the drug was stopped, the enterocyte was cleared rapidly of fat droplets, suggesting that the treatment was reversible and relatively nontoxic.

Chylomicron transport across the enterocyte has been altered by use of other inhibitors, although these have been less well studied than Pluronic L-81. Fenfluramine, a central nervous system appetite suppressant, blocks chylomicron transport into the serum in fat-fed rats (17). Choline deficiency affects transcellular movement of both VLDL and chylomicrons (18), leading to retention of lipid droplets within both the hepatocyte and the enterocyte.

To test the hypotheses stated above regarding the surfactant-like particles, we used choline deficiency to inhibit both VLDL and chylomicron formation and Pluronic L-81 or fenfluramine administration to preferentially inhibit chylomicron transport. IAP and an ELISA for particle proteins were measured as markers for the presence of the surfactant-like particle in the enterocyte, lamina propria, serum, and in the light mucosal scrapings from the apical surface of the enterocytes. Both particle content and IAP activity fell in apical scrapings, lamina propria, and serum, as predicted by the hypothesis that the surfactant-like particle is secreted from the cell after fat feeding. Moreover, after Pluronic L-81 or fenfluramine treatment, electron microscopy demonstrated fat droplet accumulation, accompanied by a striking redistribution of membranes resembling the surfactant-like particles. In addition, after Pluronic L-81 treatment, particle content as measured by ELISA rose by 29%. These data support the hypothesis that the particles could play a role in the transepithelial transport of dietary lipid.

Methods

Animals. Male Sprague-Dawley rats, 140–160 g, were obtained from Sasco (Omaha, NE). They were fasted overnight before the beginning of the acute experiments. Conscious rats were fed by gavage using an infant feeding tube. Some rats were force fed 2 ml of corn oil alone (2.28 mmol triacylglycerol), some with 2 ml of normal saline, and others with 2 ml of corn oil containing 97 mg of Pluronic L-81 (19), provided by Patrick Tso (Louisiana State University Medical School, Shreveport, LA). Some animals were given intraperitoneal fenfluramine (A.H. Robins Co., Inc., Richmond, VA) (20 mg/kg body wt) 2 h before force feeding with 2 ml of corn oil. To identify a correlation between triacylglycerol absorption and particle secretion the 2-ml dose of corn oil was chosen because lower doses do not lead to a reproducible increase in particle appearance in serum or in light mucosal scrapings (7). For experiments using choline deficiency, growing rats were fed for 2 wk with a choline-deficient diet (containing 20% lard) or the same diet with choline added (United States Biochemical Corp. Cleveland, OH), so that at the end of the test period their weight was about 150 g, comparable with that of the rats used for the acute experiments. These animals were killed at 8 a.m. without overnight fasting or an acute fat feeding. All protocols were approved by the Committee on Animal Research at Washington University School of Medicine.

Isolation of tissue samples. At designated times after fat feeding, animals were anesthetized with barbital sodium, the thorax was opened, blood was withdrawn by cardiac puncture, and a blunt needle was inserted through the left ventricle into the ascending aorta. Through this needle the animal was perfused with normal saline until the liver was completely blanched. Using this technique, contamination of intestinal tissue scrapings and homogenates by serum was minimized, as monitored using rocket immunoassay of rat albumin as a serum marker (20). The intestine was removed, and divided in two, and the proximal half was perfused with 10 ml of chilled normal saline. The bowel was opened longitudinally, and the mucosa was scraped lightly with paper (No. 3; Whatman Inc., Clifton, NJ), as described previously, yielding a preparation suspended in 2 ml of 10 mM Tris buffer, pH 7.4, enriched in surfactant-like particles (6). Then, the underlying mucosa was removed from the muscle layer by scraping with glass slides. In other experiments, the enterocytes were removed totally by a 1-h treatment with EDTA, as described previously (20). Sections of remaining tissues were examined histologically to confirm that all cells had been removed. The underlying lamina propria was removed by scraping with glass slides. The enterocytes were recovered, washed, and homogenized, and the cytosolic fraction was separated as described previously (20).

Biochemical analyses. Serum was assayed for total triacylglycerols (21) and for IAP activity (6). In some experiments, the fraction of total serum alkaline phosphatase due to IAP was determined by immunoelectrophoresis or immunoprecipitation (22). IAP assays in all tissue fractions were performed in duplicate. The presence of Pluronic L-81 or fenfluramine, at concentrations of up to 5 mg/ml, had no effect on the assay for alkaline phosphatase activity. Mucosa was homogenized as a 3% solution in 10 mM Tris, pH 7.4, with 5 mM $MgCl_2$, and the total volume of the mucosal scraping was recorded and assayed for IAP activity (22). Total activity (the sum of enterocytes and lamina propria) was calculated from the product of activity $\times$ volume. Samples of the mucosal scrapings were separated on a NaBr gradient, d = 1.04–1.17, to isolate the surfactant-like particles (d = 1.07–1.08) (6, 23). In the control and Pluronic L-81–treated animals, over 80% of the total IAP activity in the scraping that was recovered in the gradient was in the surfactant-like particle, with the rest in the brush border fraction at the bottom of the gradient (d = 1.17) (6). Choline deficiency in rats has been reported to result in irregular microvilli with fragmentation and blebs forming on the outer surface (24). Consistent with this finding was a much smaller percentage of the total IAP activity in the scraped samples that migrated with a d = 1.07–1.08 (see Results). The mean±SD of samples from control and treated animals were compared by a two-tailed Student's t test, either alone or after one-way ANOVA.

ELISA for surfactant-like particle. Particle was purified from light mucosal scrapings as described previously (6). After dialysis to remove NaBr, the particle was injected with Freund's adjuvant into New Zealand white rabbits. The resulting antiserum recognized four major proteins on Western blot, (116, 97, 66, and 45 kD). None of these proteins were detected in preparations of purified brush border or basolateral membranes. To eliminate the small amount of reaction against IAP and sucrase-isomaltase, the antiserum was adsorbed with rat brush border membranes (1 mg/ml for 60 min at 22°C). Antiserum also was raised against each major protein component isolated by SDS-PAGE, eluted, and reelectrophoresed so only a single protein was present by silver staining. Antiserum raised in rabbits against each individual protein recognized all four major proteins, suggesting that they were either closely related or aggregates of one or two proteins. Amino-terminal sequencing of proteins transferred to polyvinyldifluoride membranes revealed that the four proteins showed great similarity (6 of 18 residues are the same and are in the same positions) but the sequences were not identical.

Because the four major proteins offered multiple epitopes for binding, a two-antibody sandwich ELISA was selected (25), using antibodies against the whole particle and β-galactosidase bound to the second antibody as the detection system. Purified particle was used for the standard curve. Linearity was established between the ranges of 5 and

110 ng of particle protein. For assay of serum 0.5–2 μl was used. For assay of tissue 0.5–1.0 μl of a 3:1 homogenate (3 parts buffer:1 part tissue) was used. Assays were performed in duplicate using two different concentrations on the linear portion of the curve. Variation in samples was 4±0.6%. These results were essentially reproduced by using an antigen-capture assay with antibody bound to the solid phase, biotinylated particle as the marker, and competition with unlabeled particle to produce a standard curve. Because the slope of the curve with the two-antibody sandwich assay was steeper, this latter assay was chosen for routine use. Neither human serum or intestinal homogenate nor rabbit serum contained proteins cross-reacting in Western blots with the antiserum against the rat particle. These tissues also did not produce reactivity in the rat particle ELISA. The assay identified rat particle protein only in fractions 3 and 4 of a NaBr gradient, found previously to be the fractions containing the surfactant-like particle by morphologic and biochemical parameters (6).

Morphology. Samples of intestine from mid-duodenum and mid-jejunum were removed at the time of killing and were fixed for 2 h in 4% buffered *p*-formaldehyde and 2% glutaraldehyde (5). Tissues were treated with the mordant, tannic acid (2%), to enhance phospholipid identification. Portions were osmicated, as described previously (5). 1-μm sections were cut for orientation, and, after thin sectioning, grids were examined in an electron microscope (model 201; Philips Electronic Instruments Co., Mahwah, NJ). For immunoelectron microscopy, LR gold-embedded sections (Polysciences Inc., Warrington, PA) were incubated with antiserum against rat IAP as described earlier (4), with modifications adopted from the method of Berryman and Rodewald (26). These consisted of an additional postfixation step of en bloc staining with 2% uranyl acetate to further stabilize membrane phospholipids before dehydration, for which acetone was used. After overnight incubation of grids with the primary antibody at 4°C and abundant buffer rinses, the grids were floated on 10-nm gold-labeled anti-rabbit IgG (Janssen Pharmaceutica, Beerse, Belgium) for 1 h at room temperature and subsequently were postosmicated.

Morphologic quantitation. Electron micrographs of low power (at a magnification of 2,500) were used for counting the numbers of intracellular fat globules, as well as lamina propria, intracellular and luminal/brush border particles. 42 different experimental specimens derived from three animals in each group were thus evaluated in a coded manner and were scored (0–4+) for the abundance of these features. The results are expressed as means of the relative scores. High magnification (29,000) electron micrographs were used in all instances to check morphology and also to further demonstrate relationships between cytoplasmic organelles. The quantitation evaluated the relative numbers of lamellar stacks present within cells, apposed or not to fat globules, without separating out the actual number of bilayer profiles attached to each individual fat globule.

Results

Serum triacylglycerol. Acute corn oil feeding increased serum triacylglycerol levels, peaking at 5 h after the feed to more than three times the fasting level (Table I). At 5 h after feeding with Pluronic L-81, the level was below fasting, and with fenfluramine it was only slightly above the fasting level. Similarly, the chronic fat-fed choline-deficient animals had triacylglycerol levels near the fasted controls (Table I). These data demonstrate that the three experimental models altered fat transport across the enterocyte and into the serum.

IAP activity in tissue fractions and in serum after acute fat feeding. After 2 ml of corn oil, the IAP activity (units per gram mucosa) in total mucosal homogenates increased significantly (1.4-fold), consistent with our earlier report (7). Values for the total homogenate include activity from both lamina propria and enterocyte, but brush border IAP, which accounts for > 95% of total activity, is the major determinant of enterocyte

Table I. Effect of Inhibitors of Triacylglycerol Absorption on Serum Triacylglycerols after Fat Feeding in Rats

Lipid addition to diet	Inhibitor added	Hours after feed	Serum triacylglycerols
			mg/dl
None	None	0	94.5±25.8 (10)
Corn oil (2 ml)		5	320.8±182 (8)*
Corn oil (2 ml)	Pluronic L-81	1	90.6±27.4 (4)
		3	52.3±11.8 (4)
		5	76.2±36.2 (7)
Corn oil (2 ml)	Fenfluramine	5	134.2±76.7 (7)
20% lard	None	NA	214.7±93 (5)*
20% lard	Choline deficiency	NA	124.3±68 (5)

* $P < 0.05$ vs control.
Experiments using Pluronic L-81 and fenfluramine followed an acute feeding of 2 ml corn oil, after overnight fasting. Experiments using choline deficiency–sampled blood at 9 a.m. after 2 wk of feeding on a diet containing 20% lard. Triacylglycerols were assayed as described in Methods. Number of animals is given in parentheses. Data are the mean±1 SD. NA, not applicable, as animals were killed in the morning without fasting.

IAP activity (27). Treatment with Pluronic L-81 along with fat feeding for 5 h increased homogenate IAP specific activity by only 1.1-fold, not significantly increased from fasting control values, but significantly lower than 5 h fat-fed controls (Table II). Fenfluramine treatment prevented any increase in tissue homogenate IAP activity (Table II).

The total IAP activity in the mucosal scrapings showed a more dramatic pattern. Fat feeding increased the activity by 3.4-fold after 5 h (Table II), similar to previous experiments (7). These differences were statistically significant when compared with fasting levels, as was found previously (7). Pluronic L-81 and fenfluramine treatment with fat feeding produced increases of only 1.7- and 1.4-fold, respectively, a decrease of over 70% of the fat-induced increment (Table II). Analysis of samples from each of the experimental points showed that in scrapings from control and Pluronic L-81–treated animals the percentage of total IAP activity in the mucosal scrapings that was in the surfactant-like particle on NaBr gradients was > 90% (Fig. 1). In fenfluramine-treated animals the brush borders were apparently more fragile, and relatively more brush border was recovered in the mucosal scraping. Only a mean of 48% of IAP activity from nine animals was recovered from gradients in the particle, the remainder being in fraction 12 (brush borders) (Fig. 1). Because of this variation, particles were isolated on NaBr gradients from each of the experimental scrapings. Using data from isolated particles, it is clear that Pluronic L-81 completely blocked production of apical particles after a fat feed; the inhibition after fenfluramine was nearly complete (Table II).

Identical effects of the two drugs were seen on the increase in serum alkaline phosphatase activity, in that fat feeding in control animals increased total phosphatase activity by 2.5-fold by 5 h, an increase that was nearly obliterated by the two treatments (Table II). Moreover, the peak increment occurred in control animals at 5 h after feeding, as with the rise in serum triacylglycerol levels (Table I) and in light apical mucosal scrapings (Table II). Over 70% of the total serum activity after

Table II. Effect of Inhibitors of Fat Absorption on Intra- and Extracellular Rat Intestinal Alkaline Phosphatase

Time after feed	Drug added	Alkaline phosphatase activity							
		Intestinal homogenate		Light mucosal apical scrapings				Serum	
				Total		Particle			
h		*U/g mucosa*	*fold increase*	*total U*	*fold increase*	*total U*	*fold increase*	*U/ml*	*fold increase*
0	—	51.3±10.3 (9)		5.6±2.2 (18)		4.5±3.1 (7)		0.24±0.52 (18)	
3	—			16.1±4.3 (6)‡				0.54±0.12 (6)‡	
5	—	71.8±19.8 (17)‡	1.4	19.7±9.5 (15)‡	3.4	15.4±5.2 (7)‡	3.4	0.6±0.15 (18)†	2.5
1	P	40.7±8.2 (6)		8.6±2.2 (6)				0.27±0.09 (9)	
3	P	48±17.1 (9)		6.4±3.2 (6)				0.25±0.1 (9)	
5	P	57.3±14.2 (15)	1.1	9.5±3 (15)*	1.7	4.1±1.1 (7)	0.9	0.32±0.09 (15)*	1.3
5	F	47.2±19.4 (9)	0.9	8.1±2.2 (9)*	1.4	7.9±4.3 (7)*	1.8	0.34±0.09 (9)*	1.4

* $P < 0.05$ compared with 5-h fat-fed control. ‡ $P < 0.05$ compared with 0-h fat-fed control.
2 ml of corn oil with or without Pluronic L-81 (P) was fed by gastric tube, and samples were collected as described in Methods. Some animals were treated with intraperitoneal fenfluramine (F) 2 h before feeding. Data are recorded as the mean±SD. Alkaline phosphatase activity in intestinal mucosal homogenate was all due to IAP. Apical scrapings were assayed before (total) and after (particle) separation on NaBr gradients. The increase in total serum alkaline phosphatase activity after fat feeding was > 70% due to IAP (22). The numbers in parentheses refer to the number of animals. The fold increase is compared with zero time (2 ml saline-fed controls).

fat feeding was due to IAP, when assayed by immunoelectrophoresis.

Choline deficiency. Choline deficiency did not produce any evidence of weight loss, nor a significant change in total mucosal IAP activity (Table III). Because choline deficiency led to more fragile microvilli, only the results of the gradient-purified particles could accurately assess the IAP activity in surfactant-like particles. Choline deficiency produced a 58% reduction in total IAP activity in surfactant-like particles recovered in the mucosal scrapings and a parallel decrease (37%) in immunoreactive IAP activity in the serum.

IAP and surfactant-like particle content in isolated enterocytes and lamina propria. If IAP were a reliable marker for the surfactant-like particles, the results in Tables II and III would be consistent with the conclusion that both particles and attached enzyme were secreted in decreased amounts after treatments that blocked triacylglycerol secretion. To confirm this interpretation we measured the particle content directly by ELISA after Pluronic L-81 treatment, and extended the tissue fractionation to include measurement of IAP activity and particle content in isolated enterocytes and in the underlying lamina propria, as well as in the compartments measured previously, apical scrapings and serum. Table IV shows the results of total IAP activity in intestinal mucosal fractions. As in Table II, corn oil feeding led to an increase (twofold) in total IAP activity, blocked nearly completely by Pluronic L-81. The compartments that accounted for this difference were the lamina propria and the apical mucosal scrapings, not the enterocytes. However, in enterocytes the brush border IAP accounts for > 95% of total activity (27). Thus, a change in intracellular surfactant-like particle (SLP)-associated IAP activity would not be detected by analysis of total cellular IAP activity. IAP activity in the lamina propria constituted the largest proportion of total mucosal IAP activity. Recovery of IAP activity from all three fractions was 85–100%. Thus, the EDTA treatment and washings required for separation of these fractions did not lead to a significant loss of enzyme.

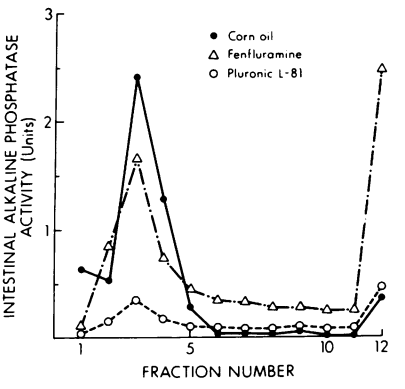

Figure 1. Representative NaBr gradient separation of surfactant-like particles from fat-fed control and Pluronic L-81– and fenfluramine-treated rats. Rats were fed 2 ml of corn oil. 5 h after feeding, they were killed, their proximal intestines were washed, the mucosa was lightly scraped, and an aliquot of the scraping was applied to the top of continuous NaBr gradient (0.49–1.46 M) and centrifuged at 100,000 *g* for 16 h at 4°C. 1-ml fractions were collected and assayed in duplicate for alkaline phosphatase activity.

We examined the same tissues for SLP protein content at 3 and 5 h after fat feeding. IAP activity peaks in enterocytes and apical washings at 5–7 h after fat feeding (7). This delayed peak is due to prolonged brush border synthesis after fat feeding (28). In contrast, SLP protein content in the enterocyte peaks at 3 h after fat feeding, 2 h before the peak of SLP content appears in apical scrapings, and both SLP protein content and IAP activity peak at 3 h in the lamina propria (Yamagishi, F., and D.H. Alpers, unpublished observations). Surfactant-like particle protein content of the fractions containing membrane-bound IAP without brush border enzyme (light mucosal scrapings and lamina propria) was decreased from corn oil–fed controls and paralleled changes in IAP activity (compare Table V with Tables II and IV). 3 and 5 h after corn oil feeding, respectively, the surfactant-like particle protein content of lamina propria and apical scrapings peaked and was significantly increased over control. This increase was inhibited by Pluronic L-81. These data reflect the inhibition of IAP activity in apical scrapings and lamina propria (Tables II and IV). In the enterocyte cytoplasm the content rose 64% after 3 h, and SLP content was increased by nearly 50% after Pluronic L-81 treatment, a

Table III. Effect of Choline Deficiency on Intra- and Extracellular Intestinal Alkaline Phosphatase

Conditions	Weight increase	Intestinal alkaline phosphatase activity		
		Intestinal homogenate	Particle purified from light mucosal scrapings	Serum
	g	*U/g mucosa*	*total U*	*U/ml*
Control	13.2±0.6	27.5±2.3	3.32±0.87	0.33±0.052
Choline deficient	15.1±0.8	20.8±7	1.41±0.11*	0.21±0.064*

* $P = <0.05$ vs. control±SD.

Animals ($n = 5$) were maintained for 2 wk on a choline-free diet or on the same diet with choline supplement (control). After weighing and killing, tissues were collected as described in Methods. Because with choline deficiency light mucosal scrapings removed many more brush border fragments than in control tissues, all scrapings were separated by NaBr density gradient, and the peak of IAP activity at $d = 1.07$–1.08 was calculated for total IAP activity. The IAP activity in serum was determined by rocket immunoelectrophoresis, as described previously (22).

significant difference compared with corn oil alone (546 vs 423 μg/ml, a 29% overall increase). By 5 h after fat feeding, the difference was no longer significant between controls and Pluronic-treated animals. Within the enterocyte, SLP protein content is a reflection of particle content, whereas IAP activity is not, because of the predominance of brush border IAP. Particle protein was increased in serum by ~ 15% at 3 and 5 h after fat feeding, but the difference was not significant.

Using the data from Tables IV and V, the specific activity of IAP per milligram of SLP protein was determined at 3 and 5 h after feeding for lamina propria and apical scrapings (Table VI). When the calculation (IAP activity per milligram of surfactant-like particle protein) was made, a two- to threefold increase in IAP activity per milligram of surfactant-like particle protein was found in lamina propria and apical scrapings after fat feeding. Values in the tissue fractions were comparable at both 3 and 5 h in fat-fed animals. This increase was inhibited by Pluronic L-81 treatment. The mucosal fractions from control and Pluronic L-81–treated animals contained IAP that was largely, but not completely, bound to surfactant-like particle membranes (see Fig. 1). In calculating specific activity in the tissue fractions, the assumption was made that all of the IAP was bound to the surfactant-like particle and that only a small proportion became soluble during isolation of the tissue fraction. It is possible that some IAP activity could have been solubilized and diffused from the mucosal fraction examined, but such a loss cannot be estimated.

Morphology. Previously, we have described lamellar bodies as having morphological similarities with the extracellular surfactant-like particles of the lung (5). The surfactant-like particle contains IAP as identified by immunolabeling (4). These particles were increased after fat feeding and were seen intracellularly not associated with other organelles (Fig. 2 *a, arrows*), within the microvillar intervillous spaces, and on the luminal surface (Fig. 2 *b, arrow*). The location of the SLP within the microvillous area is the result of routine, not necessarily optimal, cutting incidences, as these studies were intended to demonstrate intracellular particle. These particles arise from the enterocyte, not the goblet cell (5). In the absence of Pluronic L-81, the particles are not associated with the large cytoplasmic lipid droplets (Fig. 2 *b*). Pluronic L-81–treated animals exhibited within the apical portions of the enterocyte large, retained, cytoplasmic lipid globules, ~ 20% of which in the entire cell had abundant lamellae in apposition (Fig. 2 *c, arrows*). When only the outer quarter of the cell near the apical membrane was examined, as in Fig. 2 *c*, nearly half of the globules showed abundant lamellae in apposition. A parallel absence of surfactant-like particles overlying the brush border was consistent with retention within intracellular domains.

These localizations were scored at various times after Pluronic L-81 or fenfluramine treatment, and the data are summarized in Fig. 3. At all times tested, treatment with Pluronic L-81 led to a marked decrease in surfactant-like particles at the apical surface and an increase in cytoplasmic fat globules. At 3 h after fat feeding, the relative value of intracellular surfactant-like particles was slightly lower in control (relative value 2.4±0.2) than in Pluronic-treated intestine (2.7±0.1, $P < 0.05$). Moreover, 20% of all the particles seen were around or

Table IV. Yield of Alkaline Phosphatase from Intestinal Mucosal Fractions before and after Corn Oil Feeding

Condition	Total homogenate (observed)	IAP activity			Total (calculated)
		Cells	Lamina propria	Apical scraping	
		U	*U*	*U*	
Control	54.6±8.4	22.6±2.2	22.3±4.8	5.6±0.98	50.5 (94%)
5-h corn oil–fed	114±21†	20.8±1.4	72.2±15.6‡	20.7±4.6*	114‡ (100%)
5-h corn oil–fed Pluronic L-81*	66±12.7	19.2±2.1	19.2±4.8	9.03±4.3	56.4 (85%)

Animals ($n = 6$) were treated and assayed as described in Table II. * $P < 0.05$; ‡ $P < 0.001$ calculated from the t test with pooled variance after one way ANOVA, with comparison with the control value. Data are the mean±SD. The percentage in parentheses refers to the IAP activity recovered in the cells, lamina propria, and apical scraping, compared with the IAP activity in total homogenate.

Table V. SLP Protein Content before and 3 and 5 h after Corn Oil Feeding

Tissue fraction	Control	SLP protein content			
		Corn oil		Corn oil plus PL-81	
	0 h	*3 h*	*5 h*	*3 h*	*5 h*
Enterocyte cytoplasm	258±40	423±84‡	310±91*	546±93*	335±46
Light mucosal scraping (apical)	132±43	152±51	217±35*	128±28‡	146±33‡
Lamina propria (basolateral)	114±37	180±27‡	160±30*	104±21‡	97±13‡
Serum	41.4±10	46.3±14	47.6±11.0	42.1±6	43.6±7

After intragastric feeding of 2.0 ml of corn oil, with or without 97 mg of Pluronic L-81 (PL-81), animals were killed after anesthesia and removal of serum; tissue fractions were isolated and SLP protein content was measured as described in Methods. Total volume for enterocytes was ~ 3 ml, for scrapings ~ 5 ml, and for lamina propria ~ 10 ml. For an $n = 6$ (same animals as Table IV for 5-h point), P values were calculated from the t test with pooled variance after one-way ANOVA, with comparison with the control value (corn oil vs control, or corn oil plus PL-81 vs corn oil). Data refer to the mean±SD.

* $P < 0.05$; ‡ $P < 0.01$. Corn oil–fed samples were compared with control, and Pluronic L-81 samples with corn oil–fed animals.

apposed to lipid droplets, and these were clustered in the upper quarter of the cell (Fig. 2 *c*). By 5 h after fat feeding, the number of particles in the Pluronic-treated samples had declined by 12% (relative value 1.9±0.2 vs control 2.4±0.1), but still 20% of the particles were apposed to lipid droplets. In control specimens and after Pluronic treatment, presumably because flow to extracellular sites was diminished (see Fig. 2 *c*). Lamina propria particles also decreased after both Pluronic-81 or fenfluramine treatments (data not shown).

At 3 h after fat feeding, the Pluronic-treated tissue showed a small but significant increase (11%) in abundance of total intracellular particles. As about 20% of the lipid globules in the entire cell were surrounded by the dense lamellae of apposed surfactant-like particles (see Figs. 2 *c* and 4 *a*) only after Pluronic L-81 treatment, and as the globule-associated particles were all very multilamellar (Fig. 2 *c*), the total intracellular particle content appeared to be even more increased after Pluronic L-81. The thickness of the fat globule–apposed lamellae varied from stacks in a caplike fashion to a circumferential apposition of lamellar stacks. However, the variation was such that quantitation by morphology was not possible. Occasional lipid globules had half or more of their surface occupied by lamellae. 15% of the lipid globules were surrounded by membrane, but 85% showed a caplike distribution. Similar changes were seen after fenfluramine treatment. Fig. 4 *a* demonstrates a higher power view of the lamellar bodies apposed to the enlarged fat globules, and Fig. 4 *b* is the corresponding IAP immunolabeled particle. The gold particles were unevenly distributed on the retained fat globule, consistent with the cap arrangement on the corresponding nonlabeled particle. This localization of the enzymic marker for these particles, IAP, is consistent with the fact that the intracellular lamellar bodies represent an intracellular form of the surfactant-like particles.

Table VI. Specific Activity of Rat IAP in the SLP

Tissue fraction	IAP-specific activity in SLP		
	Control	Fat-fed	Pluronic L-81
		U/mg SLP protein	
Lamina propria	19.56±4.3	52±6.4 (*3 h*)	24±5.2 (*3 h*)
		45.1±6.4 (*5 h*)	19.7±2.8 (*5 h*)
Apical scraping	9.4±2.3	24.7±2.1 (*3 h*)	11.7±3.2 (*3 h*)
		21.2±3.9 (*5 h*)	13.7±4.1 (*5 h*)

The values provided are the mean±SD of the six individual animals reported in Tables II, IV, and V for the 0-, 3-, and 5-h points. The 3-h IAP lamina propria data, not included in Tables II and IV, are reported here. The IAP activity in each fraction was assumed to achieve a theoretical value of 100% IAP bound to membrane. The percentage of IAP activity in the detergent-rich phase of a Triton X-114 partition assay (10) ranged from 64 to 88% in the samples from apical scrapings and lamina propria. Thus, most of the IAP in the recovered fractions was particle-bound.

Discussion

The enterocyte secretes both VLDL and chylomicrons into the lymph after fat feeding (12). Whether or not these lipoproteins represent separate populations within the cell, they are recognized morphologically as lipid droplets surrounded by what appears to be smooth endoplasmic reticulum (29). The process of discharge of these droplets occurs quickly, but occasionally reverse exocytosis is observed (3, 13). Outside the cell, the droplets are devoid of membranous coating (3, 13). No evidence has been found for a connection between the endoplasmic reticulum and the exterior of the cell that would be involved in this secretory process. Thus, the Golgi apparatus has been implicated in lipid secretion from the enterocyte.

Golgi membranes have been found to contain lipid droplets, which are enriched in dietary fatty acids (30). In addition, figures similar to lamellar bodies in our preparations (5) have been found after fat feeding associated with nascent Golgi lipoproteins (29). When lipoproteins were isolated from the Golgi membranes, they were found to be enriched with protein compared with secreted chylomicrons (30). Moreover, phosphatidylcholine represented 60% of the phospholipid in the Golgi lipoproteins, as compared with 44% in microsomal membranes (30), although dipalmitoyl phosphatidylcholine, the major phospholipid in intestinal surfactant-like particles (5, 6), is not the dominant form in Golgi membranes. Some phospholipids, however, (at least phosphatidylethanolamine) are transported independently of protein to the cell surface (31). Thus, it is not clear that it is the Golgi membrane alone that

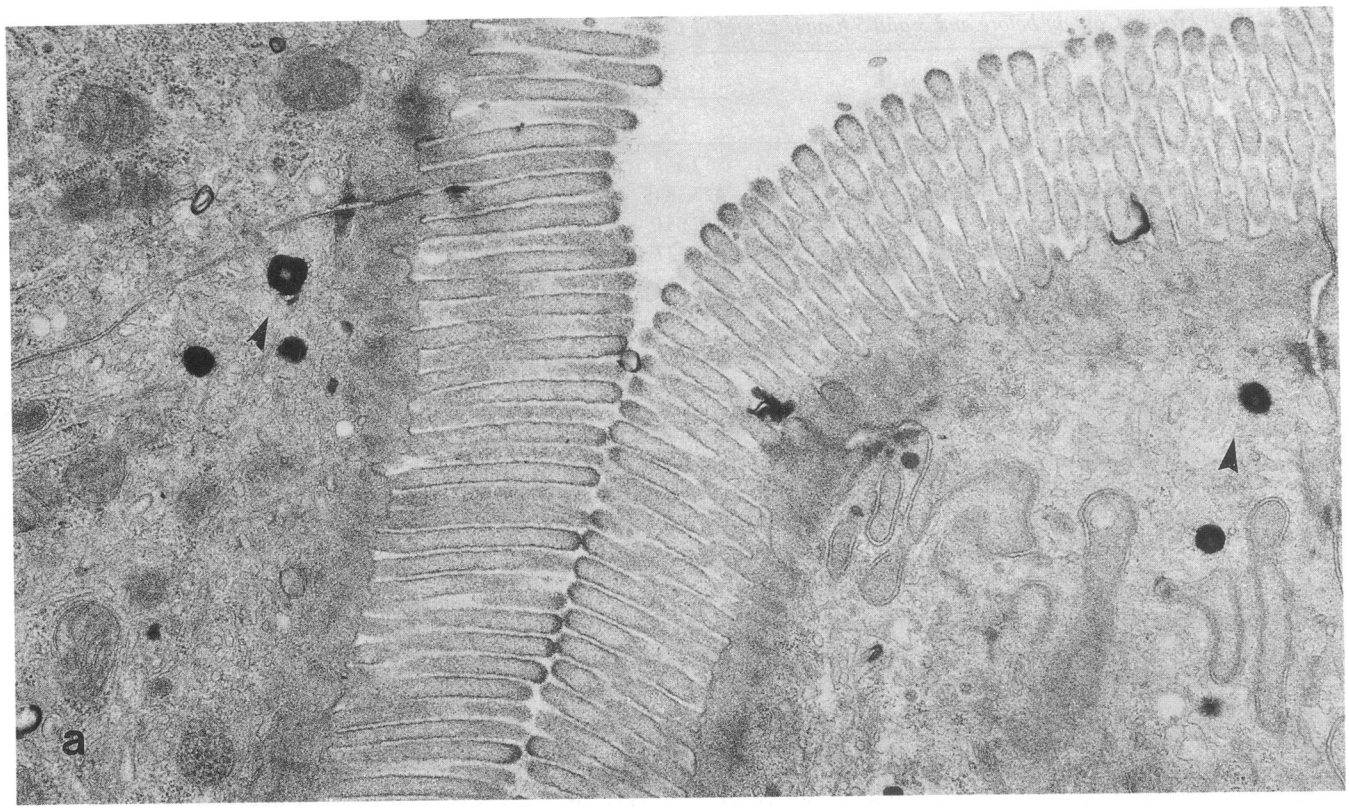

Figure 2. (*a*) Intestine 5 h after fat feeding, showing electron-dense surfactant-like particles in the apical portion of two enterocytes (*arrows*). ×13,700. (*b*) Intestine 5 h after fat feeding with numerous electron-dense surfactant-like particles (*arrow*) within or on the brush border. ×6,200. (*c*) In the presence of Pluronic L-81 5 h after fat feeding, particles remained intracellularly, partially, or completely surrounding apical fat droplets (*arrows*).

accompanies the secreted lipoprotein inside (or outside) the cell. In addition, phosphatidylcholine turnover is also increased during fat absorption (32). These findings are consistent with the presence of a protein and phospholipid-enriched membrane surrounding the fat droplets, possibly derived from but not necessarily identical with usual Golgi membranes. These characteristics could be provided by the surfactant-like particles.

If these particles were associated with a final common pathway for both VLDL and chylomicron secretion occurring before or near Golgi processing vesicles, inhibitors should affect secretion of both lipid particles equally. In fact, Pluronic L-81 preferentially blocks chylomicron secretion (14) by interfering with the movement of chylomicrons from the endoplasmic reticulum to the Golgi membrane (16). Thus, particle formation might occur in a pre-Golgi compartment. We have observed previously that the enterocyte contains whorled structures near the Golgi membrane (5) that surround fat droplets after fat feeding and contain immunoreactive IAP (4). We have hypothesized that these membranous structures might be important in fat transport. We further suggested that these structures might be the source of the surfactant-like particles that are isolated from the apical surface of the enterocyte (8, 9).

These experiments provide some further support for this hypothesis. Three inhibitors that act by different mechanisms each lower serum triacylglycerol and IAP activity after fat ingestion and lead to a decrease in the amount of surfactant-like particles recovered from the apical surface of the mucosa. Intestinal alkaline phosphatase activity is a marker for the apical particles, because nearly all IAP activity in the isolated particles is present in membrane-bound form, provided that perfusion of the animal reduces serum contamination as a source of glycosyl-phosphatidylinositol anchor-specific phospholipase D (33). In addition, secreted IAP activity recovered both in serum and in apical mucosal scrapings increases in parallel after fat feeding and after the administration of secretagogues (7). The enterocyte data for IAP activity are not useful for understanding SLP kinetics because of the large brush border component of total enterocyte IAP (27). After inhibition of chylomicron transport, the decrease of particle secretion onto the apical surface and into the lamina propria, as suggested by IAP activity, was confirmed using an ELISA for particle proteins (Tables V and VI). These data also demonstrate that after fat feeding the specific activity of the particle for IAP increases, and this increase is prevented by Pluronic L-81 feeding. This observation shows that the composition of the particle can be altered. Moreover, it is consistent with the role of the particle as a precursor for the increased IAP that appears in the serum after fat feeding.

The enterocyte content of SLP protein rose 64% by 3 h after triacylglycerol feeding, and this was increased another 50% to 2.1-fold over the 0 h control value (29% over 3 h fat-fed animals). After Pluronic L-81 treatment, enterocyte SLP protein rose by 111% over the 0 h control value, significantly different from corn oil feeding alone (Table V). These data and the decreased SLP content in apical scrapings and lamina propria

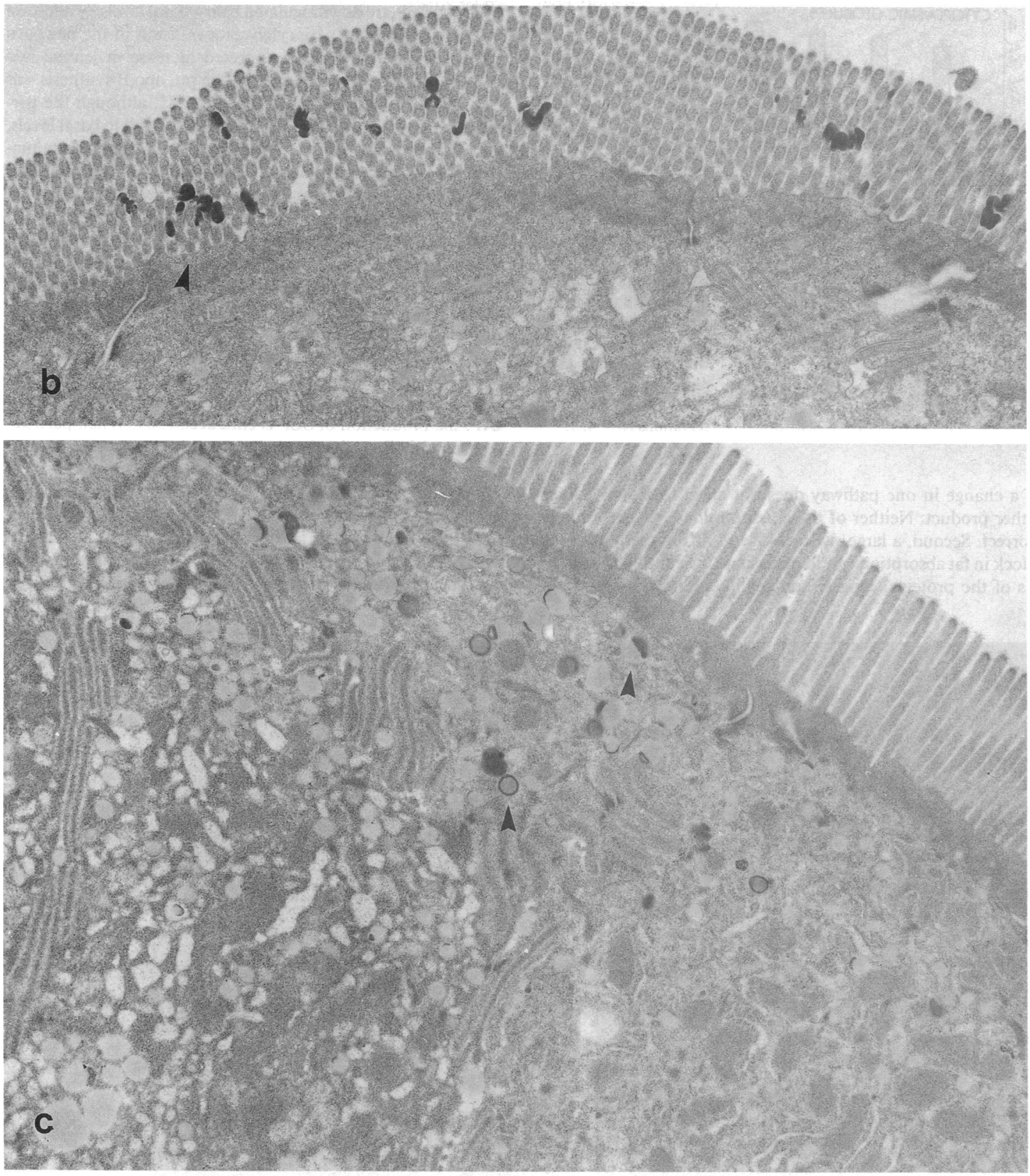

Figure 2 (Continued)

are consistent with a block in SLP production and secretion caused by Pluronic L-81. There are several reasons why the intracellular increase in SLP content is not as dramatic as might be expected. First, despite superficial resemblance, the experiments described here are not an exact example of the classical blocked metabolic pathway. In fact, we suggest that two pathways may be converging: the synthesis and packaging of the lipid droplet itself, and the production of its surrounding membrane, which we suggest may be the SLP, at least in part. In such a dual pathway, a block in one path (e.g., lipid secretion by Pluronic L-81) will produce a comparable rise in membrane content only if the two processes are completely linked, and/or

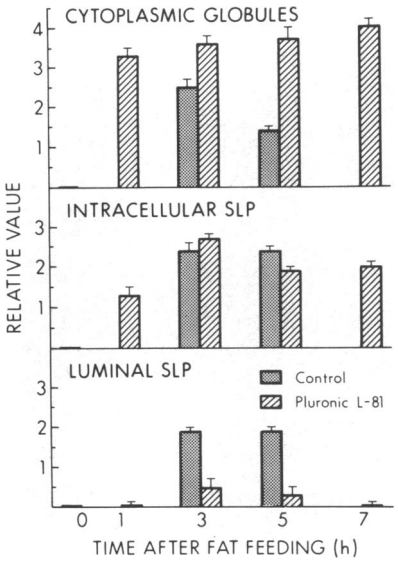

Figure 3. Quantitation of SLP by electron microscopy was performed as described in Methods. Luminal surfactant-like particles dramatically decreased after Pluronic L-81 treatment, while the sum of their intracellular counterparts (cytoplasmic SLP and in apposition to the retained fat globules) was somewhat increased. Data represent the mean±1 SEM for each set of observations (n = 42).

if a change in one pathway does not affect formation of the other product. Neither of these assumptions may be entirely correct. Second, a large rise in IAP and SLP protein after the block in fat absorption would necessitate the continued synthesis of the proteins. Jejunal apo B-48 content, synthesis, and mRNA levels are downregulated below basal levels by addition of Pluronic L-81 to a triacylglycerol infusion in the newborn piglet (34). Moreover, the expected increase in jejunal apo A-IV synthesis was attenuated. In the rat, apo B synthesis was not affected significantly by Pluronic L-81, although the percentage of total protein synthesized as apo B fell to basal levels, as in the piglets (35). These results suggest that similar attenuation might occur for other proteins such as IAP and SLP, an hypothesis that can be tested in further experiments.

Third, surfactant-like particles are produced in fasting rats (5–7), so they do not require triacylglycerol absorption for their production. In addition, the ability to increase SLP content after fat feeding is quite limited (Table V), consistent with the observation that the number of fat particles after fat feeding increases much less than their volume (35). This observation would be expected if the amount of membrane particles were limiting. Finally, some evidence exists that the production of SLP may be regulated. When Caco-2 cells are transfected with IAP, the production of SLP is enhanced (36). When particle-associated IAP production is impaired by Pluronic L-81, it is possible that SLP production itself is thereby decreased.

The present experiments also provide direct morphological evidence that particle secretion was interrupted by Pluronic L-81. The inhibition of fat transport produced an increase in intracellular lipid droplets (References 13, 24, and Figs. 2 *c* and 3). If the extracellular surfactant-like particles are related to the

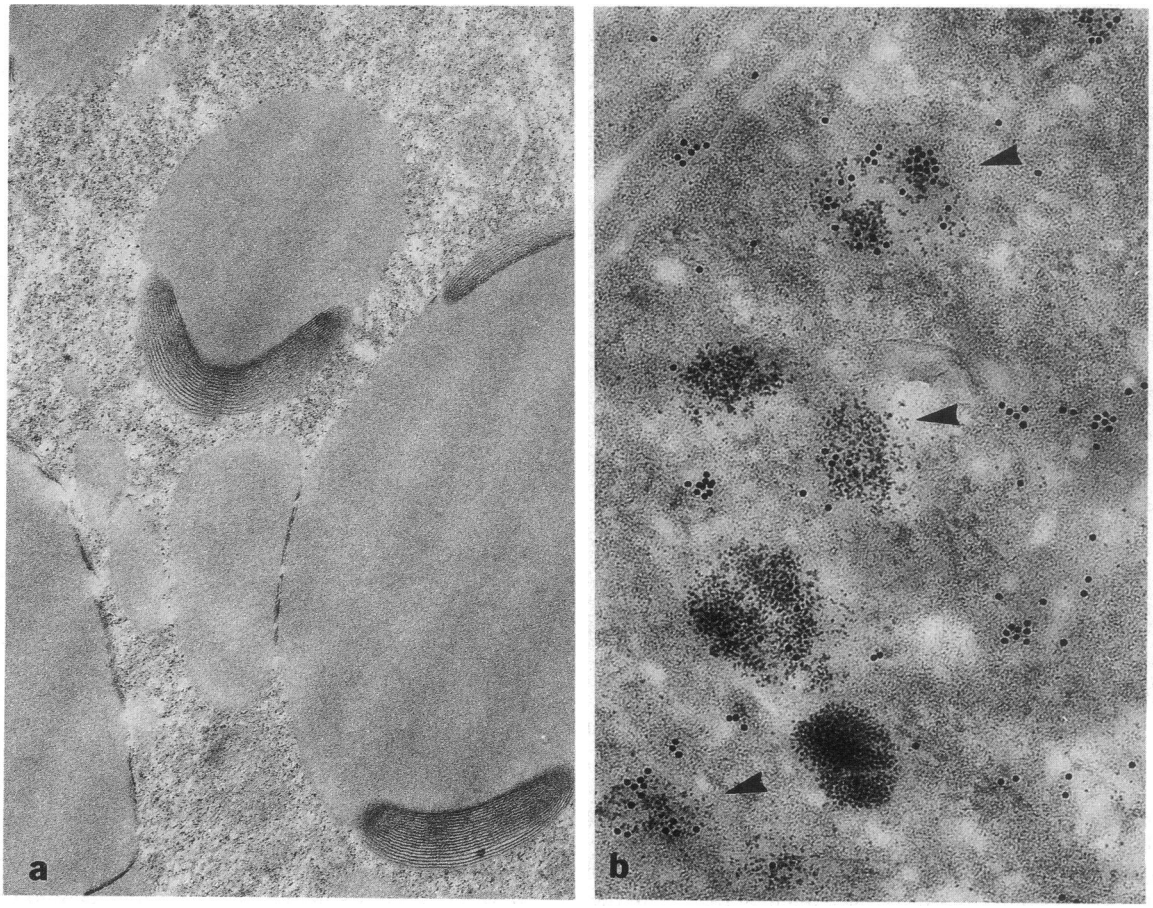

Figure 4. (*a*) Fenfluramine-treated animals showed similar retention intracellularly of both fat globules and surfactant-like particles, the latter often arranged in a caplike fashion. Sample was obtained 5 h after fat feeding. ×42,000. (*b*) Outlines of several electron-dense globules can be seen, and show IAP immunolabel concentrated at the periphery of the globule (*arrows*). ×29,000.

intracellular lamellar bodies, inhibition of fat transport should produce differences in the intracellular distribution of these structures, in addition to the decrease in recovery of particles from the cell surface. This predicted result was found, with the striking appearance of caps of lamellar figures adjacent to the lipid droplets in the Golgi region (Figs. 2 *c* and 4 *a*). This result was seen morphologically using both Pluronic L-81 and fenfluramine inhibition. The peripheral labeling of these droplets with antibody-decorating IAP (Fig. 4 *b*) is consistent with the relationship of these intracellular lamellar figures to isolated surfactant-like particles.

A role for surfactant-like particles in the transcellular movement of lipid droplets is consistent with the results of the present experiments and could help to explain a number of other observations. An inhibitor of IAP activity given in vivo to rats has been shown to decrease IAP activity in the mucosa, correlated with prevention of the absorption of linoleic acid into lymphatics (37). In addition, the surfactant-like particles are more abundant in proximal intestine of the rat (5), consistent with the relatively greater capacity for fat transport in proximal versus distal rodent gut (38). In fact, when large daily feedings (4.7 mmol) of lipid are given to rats, comparable with doses used in the present experiments (2.3 mmol), trophic mucosal effects were noted most in the mid gut (jejunum) (39). The observation that only a large bolus of fat stimulates surfactant-like particle release (7) may suggest that the mechanism could be related to the release of intestinal hormones from the mid and distal intestine, where fat absorption becomes prominent with large boluses, rather than from the effect of the lipid per se.

The large bolus of fat needed to demonstrate the effects on IAP and SLP may mean that the mechanism postulated here is significant only with high fat loads. Other explanations are possible, however. The methods used to detect the responses are insensitive, perhaps preventing detection of changes at lower fat loads. Alternatively, triacylglycerol residence time in the duodenum may be a factor. Most IAP in the rat intestine is found in the duodenum, and this is the source that accounts for most of the rise in serum IAP activity after fat feeding (40). Most SLP is also found in the apical scrapings from the proximal bowel, both fasting and after fat feeding (6). If residence time for fat were short in the duodenum, as seems likely after gastric feeding, then a large bolus of fat might be needed to maintain a steady flow of fat into the duodenum over the 5 h of the experiment.

The presence of a membrane with surface active properties might explain the very rapid discharge of the fat droplets from the cell. Thus, this hypothesis could also help to explain the fact that reverse exocytosis (involving plasma membrane vesicles) as a mechanism for fat droplet secretion has rarely been documented. Mansbach and Parthasarathy (41) demonstrated that 2–7% of total mucosal lipid appears to enter the lumen. Such movement could be mediated by surfactant-like particles. Intracellular mucosal lipid droplets were noted to sediment at greater density (1.026) than expected for VLDL or chylomicrons (< 1.006) (42), a result consistent with the presence in the cell of a protein-rich surfactant-like particle with a density of 1.07 surrounding or associated with the lipid droplet. The redistribution of surfactant-like particles around the lipid droplet after Pluronic L-81 treatment could also provide an explanation for the conversion of the large lipid cytoplasmic droplets to smaller chylomicrons after removal of Pluronic L-81 (43), an event that does not require triacylglycerol hydrolysis.

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