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Research Article

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Intestinal Surfactant-like Material

A Novel Secretory Product of the Rat Enterocyte

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Abstract

Surface-active phospholipid-containing particles are traditionally considered to be the product of type II pneumocytes. We now demonstrate membrane-bound lamellar cytoplasmic organelles in adult and suckling rat enterocytes that are densely reactive with phospholipid-staining reagents. These structures were seen in the basolateral space, within the intercellular junctions, and unraveling on the luminal surface, and were more abundant after fat feeding. Light scrapings of intestinal mucosa and luminal washings that contained these bodies, as evidenced by morphology and biochemical analysis, lowered surface tension in a pulsating bubble assay. Production by normal enterocytes of material with surfactant-like appearance and properties demonstrates that these structures are present in extrapulmonary epithelia, and extends the possible range of their function beyond gaseous exchange, e.g., solute exchange or lubrication on membrane surfaces.

Introduction

The hallmarks of pulmonary surfactant structure and function are a lamellar body with a substructure of predictable periodicity, membranous profiles uncoiling on the cell surface (1–4), lowering of surface tension, and a predominance of dipalmitoyl-phosphatidylcholine (5, 6). The nature of the mucosal cytoprotective barrier has extensively been studied in the stomach of dogs and rats, and a phospholipid-containing product identified in luminal contents was visualized on the surface of rat stomach (7, 8). Because the luminal contents showed surface-lowering activities, these findings suggested an analogy with pulmonary surfactant. This material has not, however, been identified as surfactant-like within cells other than type II pneumocytes. The production of such material in cells outside the lung would necessitate an expansion of the concept of surface-active particles on mucosal surfaces. In the small intestine, intraluminal vesicles (50–90 nm), smaller than

granules of pulmonary surfactant have been reported on the apical surface of the polarized mucosal cells, and were increased after feeding (9–12). We examined the intestine in the fasted and fat-fed rat and herein report in addition to these microvesicles, lamellar membranous structures morphologically and biochemically reminiscent of surfactant structures in the lung. These lamellar structures originated within intestinal cells, were secreted across the basolateral membrane, and migrated to the lumen.

Methods

Animals

150–180-g male Sprague-Dawley adults or 14-d-old suckling male rats were obtained from Sasco (Omaha, NE). Suckling animals were anesthetized immediately after removal from their mothers. 2 ml of corn oil or 2 ml of 1 M sucrose were given intragastrically without anesthesia to fasted adult rats. Rats were killed by ether anesthesia 30–180 min later.

Tissue sampling

Normal saline (10 ml) was passed through the upper half of small intestine and the washings were collected directly. The washings were centrifuged at 600 *g* for 10 min to remove particulate material, and the supernatant fraction was centrifuged at 105,000 *g* for 60 min. The resulting pellet was fixed in buffered 2.5% glutaraldehyde for 10 min and processed for electron microscopy. Alternatively, after washing, the mucosa was lightly scraped with filter paper (no. 3; Whatman Instruments, Clifton, NJ) to remove the gelatinous surface material. These filters were sonicated or vortexed briefly to remove the material from the paper. The scrapings were processed for electron microscopy or were fractionated in a continuous NaBr gradient from 0.49 to 1.46 M in Tris-saline buffer (pH 7.6) containing 5 mM CaCl₂, as used for the isolation of human pulmonary surfactant (13). A visible band of density 1.07–1.08 g/liter was formed after centrifugation for 16 h at 100,000 *g*, in rotor (model SW-41; Beckman Instruments, Fullerton, CA), identical to the conditions for isolating pulmonary surfactant. The scraped gelatinous surface material and/or its NaBr band and pellet were subjected to surface activity measurement (14), adding 0.2 μ mol/ml to 1 μ mol/ml of total phospholipid. These samples were also assayed for hydrolase activity or processed for electron microscopy. Brush borders were purified (15) and assayed for phospholipid content and hydrolase activity.

Tissue processing for electron microscopy

Tissues were fixed in 2% buffered paraformaldehyde for 1 h and stored in hypertonic sucrose at 4°C overnight. They were then processed for the various histochemical reactions and after dehydration, embedded in Spurr's plastic. 1- μ m-thick toluidine blue-stained sections were used to verify tissue orientation. Thin sections were viewed in an electron microscope (model CM10; Philips Electronics Instruments,

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Mahwah, NJ). Quantitative comparison of lamellar body number was done on electron micrographs from fasted and fed animals by counting a mean of 20 intercellular spaces (range 6 to 28) in 19 different preparations (from 4 different animals).

Histochemistry for phospholipids

The method of Kalina was slightly modified (16). Briefly, tissues were post-fixed in a mixture of 2% tannic acid in 0.1 M cacodylate buffer/3% glutaraldehyde in cacodylate buffer (1:2) for 2 h, followed by 1% OsO₄ in cacodylate buffer for 1 h.

Fragments of tissues fixed in glutaraldehyde were washed in 0.05 M Tris buffer pH 6.9 followed by Tris-iodoplatinate solution for 3 h. The iodoplatinate solution was composed of 10% hydrogen hexachloroplatinate IV, distilled water, 0.2 M Tris buffer (pH 6.9) and 6% potassium iodide in 1:16:16:33 proportions with a final pH 6.5–6.8. 0.3% sodium bisulfite was then added dropwise until the color changed to brownish yellow and further incubated for 1 h. Three washes in 1% sodium sulfate in 0.05 M Tris were followed by ammonium sulfide/Tris buffer/1% sodium sulfate. After dehydration in acetone, the tissues were processed for plastic embedding (7). As a control for this and the neutral lipid stains, sections from fasted animals were examined.

Histochemistry for neutral lipids

Tissues were postfixed with 2% OsO₄ in cacodylate buffer for 3 h. After a brief rinse in 70% alcohol, they were incubated in 2% *p*-phenylenediamine in 70% ethanol for 1 h, and dehydrated for plastic embedding (17).

Histochemistry for acid phosphatase

The method of Robinson and Karnovsky was used as modified by Neutra (18). Briefly, acid phosphatase activity was demonstrated after rinsing in cacodylate buffer (pH 7.2) and in 0.1 M sodium acetate buffer (pH 5.0) both containing 5% sucrose. Tissues were incubated in 2 mM cesium chloride (Alfa Products, St. Louis, MO), 5% sucrose, and 0.1% Triton X-100. After 30 min, β -glycerophosphate (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mM and incubated at 37°C for 1 h. To determine that the only phosphatase activity detected was acid phosphatase, the brush border area (containing alkaline phosphatase) was routinely examined for a reaction product.

Surface activity measurement

Light mucosal scrapings were obtained from the proximal intestine of a 7-h post-fat-fed adult rat. Intestinal scrapings were collected from each of six rats and analyzed separately. Part of the samples were subjected to NaBr gradient centrifugation. After lowering the density of the gradient purified band to < 1.03, the fraction was centrifuged at 64,000 *g* for 1 h, after which the supernatant was discarded and the pellet resuspended in 0.14 M NaCl and 5 mM CaCl₂. Both this resuspended pellet and the original light scrapings were subjected to surface activity measurements. Measurements of surface tension expressed in dynes/centimeter were determined at 37°C using the pulsating bubble technique using the Enhorning surfactometer model ASC serial no. 8, (International, Toronto, Ontario), which measures the pressure gradient across a liquid air interface of a bubble that pulsates. 20 μ l of the samples, equivalent to 0.2–1.0 μ mol/ml of phospholipid, was loaded on the surfactometer. Surface tension was calculated by the law of Young and Laplace (14).

Lipid extraction and analysis

Lipid in luminal scrapings, the band fraction of the NaBr gradients, the phosphatidylcholine spot isolated on thin layer chromatography, and brush borders were extracted using the method of Bligh and Dyer (19).

Thin layer chromatography. Phospholipids were measured using two dimensional chromatography with chloroform/methanol/acetic acid/water (50:25:8:4) for the first phase, and the same solvents

(50:7.5:8:2) in the second phase (20). Sample size varied from 50–100 μ l. Synthetic phospholipids (Sigma Chemical Co.) were run in parallel as controls.

Phosphorus determination. Individual spots were stained with a sulfuric acid/molybdenum blue spray (Sigma Chemical Co.), and scraped off the plates. The silica gel was heated to 180°C for 20 min in 70% perchloric acid, and assayed for phosphorus using ammonium molybdate (20). Individual phospholipids are reported as percent of total phospholipid (1 mol P per 1 mol phospholipid) measured on unfractionated samples.

Gas chromatography. Fatty acids extracted from the phosphatidylcholine spot on thin-layer chromatography were methylated with 14% BF₃ in methanol for 10 min at 95°C and the methyl esters were extracted into petroleum ether. Esters were separated in a chromatograph with a flame ionization detector (model 3760; Varian Instruments, Palo Alto, CA), using a silica column (model SP-2330; Supelco, Inc., Bellefonte, PA). Relative mass amounts were determined by an integrator (model 3390A; Hewlett Packard Instruments, Palo Alto, CA). Authentic fatty acid methyl esters were used to identify retention times.

Enzyme assays. Brush border disaccharidases, alkaline phosphatase activity, and protein content were measured as previously described (21).

Results and Discussion

Small intestinal mucosa stained with tannic acid, known to have an affinity for phospholipid-rich surfactant-like material in the lung (16), showed an almost continuous coating over the brush border of the fat-fed adult rat intestine (Fig. 1 *a*). Fragments of similar material were found also in the lumen. In fasted animals, the material coating the brush border was so scant as to almost escape notice. Iodoplatinate, another reagent with phospholipid affinity (7), produced a similar staining pattern in fasted and fat-fed animals (data not shown). In the intestine of the 14-d-old suckling rat, an animal on a high-fat diet, the tannic acid-staining material was more abundant and a dense staining was seen throughout the apical portion of the cells, including the brush border itself and a luminal fuzzy coat (Fig. 1 *b*). Electron microscopy revealed much more abundant membranous structures over the brush border in these animals. (Compare Fig. 3, *b* and *c* to *a*). This material was also abundant in the area below the brush border and in the intercellular space. The presence of these phospholipid-staining membranes thus seems associated with fat feeding.

The ultrastructure of this material was examined further. Variably coiled lamellar structures were present on the surface of the villous pole of the adult fat-fed enterocyte (Fig. 2 *a*). After tannic acid staining internal periodicity became apparent, measuring 4 nm for both the dark band and the lucent spaces (Fig. 2 *b*). After fat feeding, the earliest recognizable intracellular form of this lamellar body appeared close to mitochondria within the matrix of membrane-bound cytoplasmic organelles (Fig. 2 *c*, *left*). The amount of membranous material increased until the fully mature granule was packed with stacks of dense membranes (Fig. 2 *c*, *left* and *right*). Thus, granules were found both intra- and extracellularly in the fat-fed adult and fed 14-d-old suckling animals. When reagents with an affinity for neutral lipids were used, such as imidazole or *p*-phenylenediamine (17), there was only slight enhancement of part of the granule without staining of the lamellar structures, consistent with a minor neutral lipid component in these structures (data not shown). The size of the lamellar bodies was variable, but was in the range of multivesicular

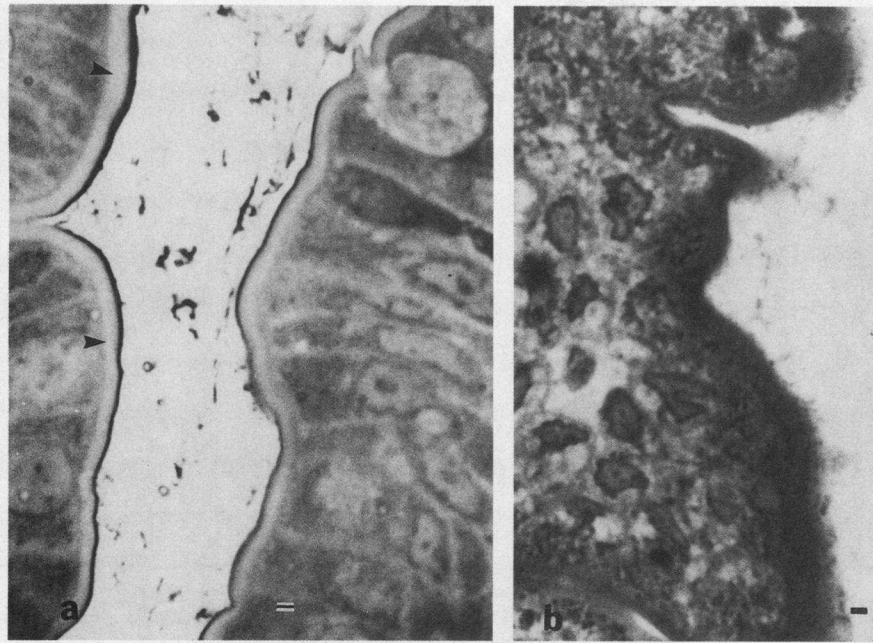


Figure 1. Light micrographs of adult fat-fed (a) and 14 d-old (b) suckling rat jejunum after tannic acid treatment. The dense staining membranous material overlays the mucosal surface. The arrow points to the unstained brush border in adult intestine. Bar, 10 μ m.

bodies (50–200 nm). Fat feeding in the adult rat increased the number of intercellular granules by \sim 20-fold, as assessed by quantitative observations of multiple sections.

Lamellar bodies in type II pneumocytes appear to derive from multivesicular bodies (1–4), organelles related to the lysosomal and acid vesicle compartments, and the lamellar bodies stain positively for acid phosphatase. Ultrathin sections from fat fed adult enterocytes were examined by histochemical staining for acid phosphatase activity, using β -glycerophosphate as substrate (18). These sections demonstrated that the coiled structures were surrounded by a positive reaction, whether the bodies were within the cell, in the intercellular space, or in the lumen (Fig. 2 d).

The luminal forms of the lamellar bodies had varied morphologies. In the fat-fed adult, the predominant form encountered included the tightly coiled “granule” (Fig. 2 b), and various stages of uncoiling, with segments of membranes consisting of a double leaflet of finite length (Fig. 3 a). In no place were the membranes seen to form a continuous barrier over the microvilli, consistent with their derivation by uncoiling from granules. No exocytosis from the apical surface was observed. Other luminal forms, observed almost exclusively in the suckling rat, were a lattice-like structure (Fig. 3 b) and a fragmented tubular myelin-like configuration (Fig. 3 c). These more complex and fragmented forms result in a higher density of membranes and may account for the increased phospholipid staining seen by light microscopy (Fig. 1 b). The square lattice form of pulmonary surfactant (1–4) was not encountered. These results demonstrate extensive morphologic similarity with the multiple forms of pulmonary surfactant.

These novel findings prompted us to examine further the biochemical characteristics of the particle. Because these lamellar bodies were so numerous over the apex of the enterocyte and in the lumen, we isolated them by light scraping with filter paper or by collecting luminal washings (13). The 100,000-g pellet of luminal washings contained large amounts of uncoiled bilaminated membranes (Fig. 3 d). The NaBr gra-

dient fraction derived from the washings shows similar structures (data not shown). Centrifugation of either luminal washings or light mucosal scrapings in a NaBr gradient produced a visible band of membranes at $d = 1.07$ – 1.08 g/liter, as reported for pulmonary surfactant (5, 13).

Phospholipid analysis of the NaBr gradient band fraction derived from light mucosal scrapings revealed that $> 90\%$ of phospholipid was either lysophosphatidylcholine or phosphatidylcholine (Table I). Fatty acid analysis of phosphatidylcholine extracted from the NaBr gradient fraction from the light scraping reveals a composition of 14:0 myristate, 0.9%; 16:0 palmitate, 42.2%; 18:0 stearate, 31.9%; 18:1 oleate, 21.5%; 18:2 linoleate, 3.4% ($n = 3$). Thus 75% of all the fatty acids in the vesicle phosphatidylcholine are saturated. This implies that at least 50% of the vesicle phosphatidylcholine molecules contain saturated fatty acids at both the sn-1 and sn-2 portions. In comparison phosphatidylcholine from rat lung surfactant contains 14:0 myristate, 2%; 16:0 palmitate, 80%; 16:1 palmitoleic, 1%; 18:0 stearate, 2%; 18:1 oleate, 6%; and 18:2 linoleate, 6% (5). Rat lung surfactant phosphatidylcholine thus has a higher percentage of palmitate than the intestinal vesicles; nonetheless, the phosphatidylcholine from the intestinal vesicles contains a higher percentage of saturated fatty acids in general and palmitate in particular than intestinal brush border membrane (22). Moreover, phosphatidylcholine accounts for only 50% of the phospholipid of rat intestinal basolateral membranes (23). The presence of lysophosphatidylcholine in light scrapings is probably due to the action of luminal pancreatic phospholipase A₂, and does not necessarily reflect the intracellular phospholipid composition of the membranes. The phospholipid profile with the high saturated phospholipid content strongly resembles pulmonary surfactant (1–4).

The gradient purified band of the light scraping also contained the brush border hydrolases alkaline phosphatase (7.6 U/mg protein) and maltase (0.23 U/mg protein). These specific activities were markedly different from those in isolated brush borders (1.43 U and 4.04 U/mg protein, respectively).

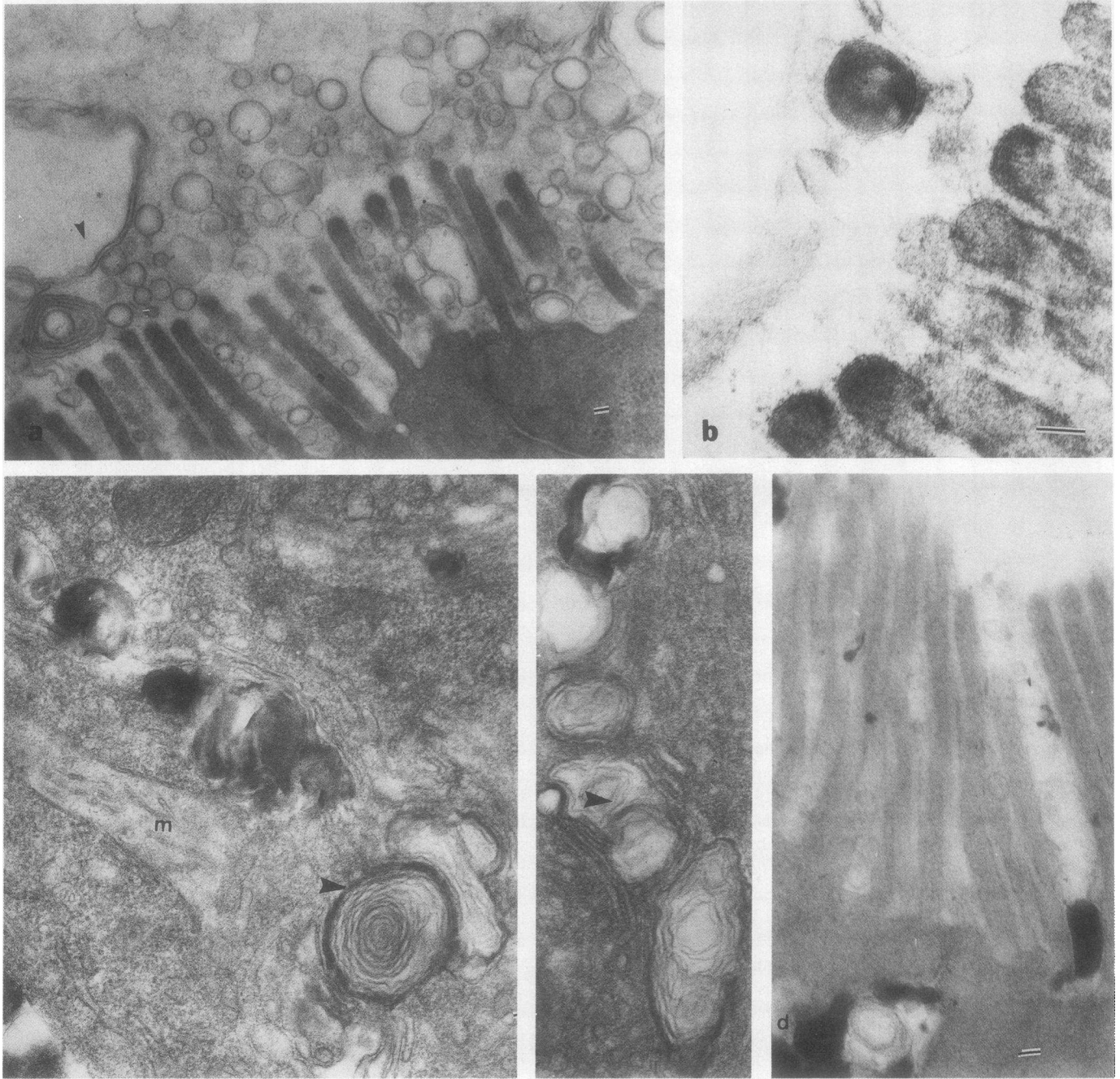


Figure 2. Electron micrographs of lamellar bodies in adult 7-h fat-fed rat jejunum. (a) Apical portion of enterocyte stained with uranyl acetate and lead citrate. (b) Large apical granule with internal periodicity (tannic acid). (c) Earliest form of intracellular lamellar bodies (arrows) which seem to be forming within a membrane bound organelle (tannic acid). m, mitochondria. (d) Acid phosphatase stain showing a reaction product surrounding lamellar structures in the intercellular and intervillous spaces. The brush border is nonreactive. Bar, 0.1 μm .

Thus, the composition of the purified membranes from mucosal scrapings is inconsistent with an origin from either plasma membranes or brush borders.

Both mucosal scrapings and NaBr gradient band fractions derived from those scrapings were tested for their ability to lower surface tension (14). Minimum surface tension had a mean value of 19.8 ± 0.17 ($n = 6$) dyn/cm (range 16.7 to 22.7) when light mucosal scrapings with a phospholipid content ranging from 0.2 to 1.0 μmol were used. Moreover, the gradient-purified material retained a similar degree of surface ac-

tivity (Fig. 4). This value was not as low as that obtained using an optimum concentration of rat pulmonary surfactant (0–5 dyn/cm). The bubble surfactometer requires concentrations of $\sim 1.5 \mu\text{M}$ phospholipid to demonstrate very low surface tensions with natural pulmonary surfactant (24). The intestinal material had to be sonicated or vortexed vigorously to remove it from the filter paper used for gentle scraping of the mucosa, a treatment that may diminish slightly the surface tension-lowering capacity of pulmonary surfactant (5, 6, 25). These experiments demonstrated that surface active properties resembling

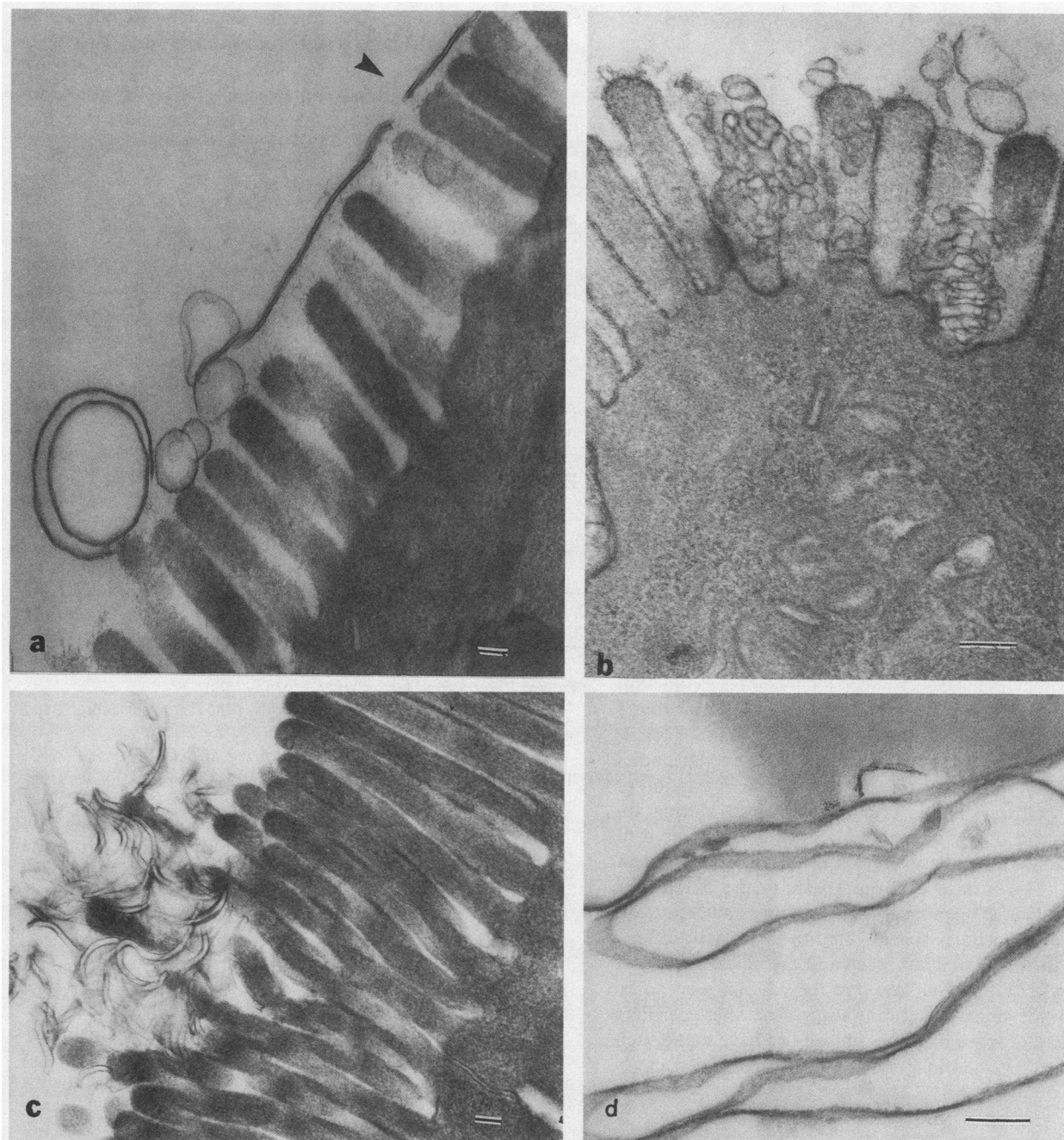


Figure 3. Electron micrographs of various luminal forms of lamellar bodies stained with tannic acid. (a) Fat-fed adult intestine. The bilaminated membrane covering the enterocyte has a finite length and corresponds to an uncoiled granule. Part of another membrane is also seen (arrow). (b and c) 14-d-old suckling rat intestine. Luminal forms of the membranous material include lattice-like (b) and fragmented tubular myelin-like (c) configurations. (d) Electron micrograph of the 100,000 g pellet of luminal washings. Bilaminated membranes partially unfolded were observed. Similar structures were seen in the purified gradient band fractions. Bar, 0.1 μm .

pulmonary surfactant were present in particles produced by the enterocyte. These results are in agreement with previous findings published using crude intestinal scrapings that reduced this coefficient of friction by 85% (20).

This work provides morphologic, biochemical, and functional evidence in the rat small intestine for a membranous lamellar body that has surfactant-like characteristics. The exact relationship of the smaller luminal vesicles reported previously (9–12) to these lamellar bodies is not clear at this time. They may be part of the dynamics of lamellar body

secretion or represent an entirely different mechanism, possibly related to the release of membrane bound proteins into the serum (26–29).

The material described on the intestinal surface seemed more adherent and/or more thickly deposited than in the lung. This abundance may explain its ease of preservation after tissue fixation compared with pulmonary surfactant (1–4). This observation would be consistent with the role of the lung in gaseous exchange, a process requiring a thin liquid diffusion phase, whereas abundant secreted intestinal lamellar bodies

Table I. Phospholipid Composition of Intestinal Extracellular Particles and Brush Borders

Type	Light scrape NaBr gradient	Lung surfactant (Harwood, reference 5)	Intestinal brush border
Lysophosphatidylcholine	33±5.7		2
Sphingomyelin	7±8		16
Phosphatidylinositol	3±2.8		14
Phosphatidylcholine/ saturated phospholipid	58±15/75%	70/85%	22
Phosphatidylserine	0		18
Phosphatidylethanolamine	0	5	28
Phosphatidylglycerol	ND	5-10	ND

NaBr gradient fractions of light mucosal scrapings and brush borders (15) were prepared and extracted for phospholipid analysis as described in Methods. Phospholipids are reported as molar percent of total phospholipid measured on unfractionated samples. Results represent the mean±SEM of three separate determinations.

might confer an advantage in the preferential absorption of certain lipids or other lipid soluble substances. On the other hand, lamellar structures seen on the surface of cells other than type II pneumocytes have been suggested by others to function as a protective or lubricating coating, or as a permeability barrier at the intercellular interface (30).

Additionally, the surface activity of these lamellar bodies might predict an important role for them in the regulation of paracellular pathways across occluding junctions during absorption. Instead of the relatively high pressures required to overcome surface forces between closely apposed lipid/protein membranes (31), surface active particles in the basolateral space might facilitate opening of tight junctions and expansion of the lateral paracellular spaces. That these bodies are seen abundantly in the basolateral space and the apical surface of enterocytes after fat feeding and that they do not seem to be derived from the apical membrane suggest that these particles may traverse the tight junctions. Indeed, we have made such observations (32). Preliminary data indicate that similar mate-

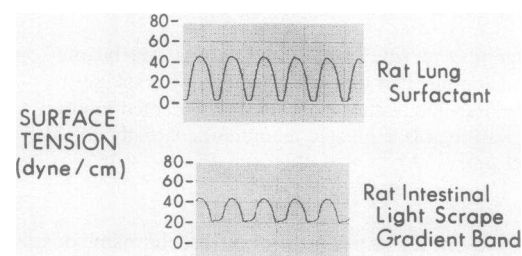


Figure 4. Typical tracing of surface lowering activity of rat lung surfactant (1.5 μ M phospholipid) and NaBr gradient purified mucosal scraping (0.2 μ M phospholipid). The visible band from a NaBr gradient, obtained from the proximal intestine of a 7-h post-fat-fed adult rat, was isolated as described in Methods. Rat lung surfactant was purified as described (34). 20 μ l of sample, equivalent to 0.2 μ mol/ml of phospholipid was analyzed. Note that the pulsating tracing similar to lung surfactant is reproduced by addition of the intestinal material.

rial is present in human intestine and the colon carcinoma-derived Caco-2 cell line. In fact, secretion of such a particle would be consistent with the basolateral and apical secretion of alkaline phosphatase observed from Caco-2 cells grown on filters (33). These results together suggest that these particles could act as a surface active agent between epithelial cells at a number of sites in the body.

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