REGULAR STRUCTURES IN UNIT MEMBRANES

II. Morphological and Biochemical Characterization of Two Water-Soluble Membrane Proteins Isolated from the Suckling Rat Ileum

E. R. JAKOI, G. ZAMPIGHI, and J. D. ROBERTSON

From the Department of Anatomy, Duke University, Durham, North Carolina 27710

ABSTRACT

Specialized plasma membranes from the endocytic complex of ileal epithelial cells of suckling rats were isolated by differential flotation. Thin-section and negative-stain electron microscopy showed the luminal surfaces of these membranes to be covered by an ordered array of particles ~7.5 nm in diam joined together with ~14.5-nm separations in long rows. This particulate coating was released from the membrane surfaces by 10 mM $CaCl_2$ and recovered free of membranes after dialysis against 0.5 mM EGTA and high-speed centrifugation. Two proteins were resolved by gel filtration to be in the supernate: *n*-acetyl-β-glucosaminidase and a filamentous protein which attaches *n*-acetylglucosaminidase to the membrane surface thereby providing bidirectionality to the array of enzyme.

We believe that the filamentous protein has not been previously described. Therefore we have called it ligatin from the latin *ligare,* which translates "to bind together." Furthermore, we suggest that the membranes of the endocytic complex contain sites for the extracellular digestion of carbohydrate moieties in the maternal milk.

In newborn mammals during the suckling period, the plasma membrane of the ileal epithelial cells forms a highly invaginated complex consisting of tubular membranes located beneath the microvilli (42, 16, 5). The entire system is interconnected and open to the lumen but does not connect directly with the large central vacuole (42, 16). The luminal surface of these membranes is covered by an ordered array of particles ~7.5 nm in diam joined together with a center-to-center separation of ~14.5 nm to form long strips (42, 16, 34). These strips aggregate laterally in either a square or an oblique two-dimensional lattice which has been described by negative-stain (16, 34) and thin-section electron microscopy (42, 32). This system of invaginated plasma membranes has been called the endocytic complex (42).

Several authors (7, 14, 17, 19, 21) have reported that during the suckling stage of development (0–21 days of age), different disaccharidases are highly active in homogenates of epithelial scrapings from the ileum. This elevation of disaccharidase activity falls dramatically at weaning (14, 17, 19, 21) or upon the administration of corticosteroids (25, 23, 24, 20). Before 15–21 days of age, there is little or no digestion of food in

the stomach and the duodenum because neither the stomach (4) nor the pancreas (33) is secreting hydrolases. Thus, the milk ingested during this period is most likely digested in part by the lysosomal enzymes found in the ileal homogenates. It occurred to us that the array of particles on the surface of the differentiated plasma membranes might in fact be a lattice of enzymes involved in this digestive process. In particular, it seemed likely that disaccharidases might be located in the lattice. In this case, the surface of the membranes would contain sites for the extracellular digestion of at least the carbohydrate present in the maternal milk immediately before absorption through the membranes into the cytoplasm. In order to determine whether or not any specific enzymatic activity was associated with these membranes and in particular with the particulate coat covering their surfaces, the membranes were isolated and analyzed biochemically and structurally.

MATERIALS AND METHODS

Isolation of Endocytic Membranes

Suckling rat pups (9–11 days old, Sprague-Dawley, Madison, Wis.) were decapitated, and their ileums were excised, irrigated with ice-cold distilled water and scraped with a glass slide to remove the lumenal epithelium. The scrapings from 30 to 50 ileums were pooled in 20 ml of HEPES buffer (10 mM HEPES, Sigma Chemical Co., St. Louis, Mo., 1 mM sodium azide, adjusted to pH 7.6 with KOH) and kept in ice. All subsequent procedures were done at 5°C.

The ileal scrapings were gently homogenized (three strokes by hand) in a Potter-Elvehjem homogenizer with a Teflon pestle and centrifuged at 27,000 *g* for 10 min (Sorvall RC2B, SS34 rotor DuPont Instruments, Sorvall Operations, Newtown, Conn.). The pellet was resuspended in HEPES-buffered sucrose (specific gravity = 1.1560), layered into a 12-ml sucrose step gradient made up of equal quantities (3 ml) of the following sucrose densities: 1.2087, 1.1821, 1.1560 (sample), and 1.1315. Specific gravities were determined with a Bausch & Lomb refractometer (Bausch & Lomb Inc., Rochester, N. Y.). The preparation was centrifuged at 95,000 *g* for 45 min (Beckman L2 65B, SW41 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellet and lowest band, which contained cellular debris, were discarded. The second band from the top (at 1.1560 ρ) was removed and recentrifuged (95,000 *g*, 30 min) on a sucrose step gradient of 1.1821, 1.1560 (sample), and 1.1315 densities in a SW-41 tube. The pellet, consisting predominantly of mitochondrial membranes, was discarded. Material at the 1.1315/1.1560 interface (band 1) and at the 1.1560/1.1821 interface (band 2) was retained.

Release of the Particulate Array

Bands 1 and 2 were pooled, resuspended twice in HEPES buffer and centrifuged at 27,000 *g* for 20 min (Sorvall RC2B, SS34 rotor) to remove the sucrose. The pellet was resuspended in 10 mM $CaCl_2$ which released the particulate array. The Ca^{++}-treated pellet was then dialysed against a HEPES-EGTA buffer (1 mM HEPES, 0.5 mM EGTA, 14.4 mM 2-mercaptoethanol, adjusted to pH 7.6 with KOH) for 16–24 h with at least two changes of buffer. The dialysate was centrifuged at 110,900 *g* for 2 h (Beckman L5 75, 50 Ti rotor). The supernate was subjected further to gel filtration on Sephadex G-200 (0.9 × 90 cm column, Pharmacia Fine Chemicals, Uppsala, Sweden). The pellet was retained for electron microscope examination.

Protein Determination

Protein content of the various fractions was determined by the procedure of Lowry et al. (26), using bovine serum albumin (Sigma Chemical Co.) as the standard.

Enzymatic Activity Assays

Disaccharidase activities were assayed in each fraction. The disaccharidases and their substrates were as follows: β-galactosidase (β-GAL, 20 mM *p*-nitrophenyl-β-galactopyranoside, pH 3.5, Sigma Chemical Co.) (18), α-galactosidase (α-GAL, 10 mM *p*-nitrophenyl-α-galactopyranoside, Sigma Chemical Co.) (23), β-glucuronidase (β-GLU, 10 mM *p*-nitrophenyl-β-glucuronide, Sigma Chemical Co.) (23) and *n*-acetyl-β-glucosaminidase (NAG, 10 mM *n*-acetyl-*p*-nitrophenyl-β-glucosaminide, Sigma Chemical Co.) (23). Where indicated, activities were converted to units of micromoles *p*-nitrophenol released per hour, using a molar extinction coefficient of 1.24×10^4.

Alkaline phosphatase (AP) was determined by the method of Morton (31) and acid phosphatase (AcP) by the method of Gianetto and de Duve (12), using β-glycerophosphate as the substrate (15). Inorganic phosphate was determined by the method described by Taussky and Shorr (39). Where indicated, activities were converted to units of micromoles of inorganic phosphate released per hour, using a molar extinction coefficient of 2.67×10^3.

Isolation of Decorated Strips by Isopycnic Centrifugation

Samples of the 10 mM Ca^{++}-treated membranes (0.5 ml) were layered onto a 10.5-ml linear HEPES-buffered sucrose gradient (specific gravity = 1.0621–1.1615 containing 5 mM $CaCl_2$) supported by a 1-ml cushion of 1.1821 ρ sucrose. The preparation was centrifuged for 20.4 h at 173,800 *g* (Beckman L2 65B, SW-41 rotor) and fractions (0.4 ml) were collected from the bottom of the tubes. The absorbance (280 nm) and the refractive

index (Bausch & Lomb refractometer) were determined for each fraction. Each fraction was analyzed for enzymatic activity as described above and negatively stained for electron microscope examination as described below after removal of sucrose with HEPES buffer.

Polyacrylamide Gel Electrophoresis

Electrophoresis was performed on samples containing 10–60 μg of protein according to the method of Fairbanks et al. (9). Polyacrylamide gels (7.6%, Eastman Kodak Co., Rochester, N. Y.) containing 1% sodium dodecyl sulfate (SDS, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) were run for 4.5 h at 5 mA/gel tube or until the tracking dye (pyronine Y) had migrated to the end of the gel. Gels were fixed and stained with either Coomassie brilliant blue (Canalco Inc., Rockville, Md.) or the periodic acid-Schiff procedure (PAS, basic fuchsin, J. T. Baker Chemical Co., Phillipsburg, N. J.; periodic acid, G. Frederick Smith Chemical Co., Columbus, Ohio) (9). The gels were scanned with a spectrophotometer (Beckman DU) equipped with a linear transport accessory (Gilford model 2410, Gilford Instrument Laboratories, Inc. Oberlin, Ohio) at 550 nm for Coomassie blue- and 560 nm for PAS-stained gels. Molecular weights were approximated by using molecular weight standards: ribonuclease A (bovine pancreas type IA, Sigma Chemical Co.), ovalbumin (grade III, Sigma Chemical Co.), bovine serum albumin (Sigma Chemical Co.), catalase (bovine liver, Sigma Chemical Co.), α-chymotrypsinogen (bovine pancreas type II, Sigma Chemical Co.).

Electron Microscopy

SECTIONING: The control and Ca^{++}-treated membrane pellets were fixed in a solution containing 5% glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.) in 10 mM HEPES buffer overnight at room temperature. Postfixation was carried out in 2% osmium tetroxide in 0.2 M cacodylate buffer pH 7.35 for 2 h. The pellets were dehydrated and embedded according to the modified Epon 812 technique described by Luft (27). Gray-to-silver sections were obtained with a Dupont diamond knife on a Reichert OMU-3 Ultramicrotome (C. Reichert, Sold by American Optical Corp., Buffalo, N. Y.) and mounted on carbon-coated 200-mesh grids. They were stained with a saturated solution of uranyl acetate in 0.1 M sodium acetate buffered to pH 5.0 and with lead according to the method of Sato (36). Observations were made with a Philips EM 301 at 80 kV with an objective aperture of 50 μm.

NEGATIVE STAINING: 1% phosphotungstic acid (PTA, Fischer Scientific Co., Pittsburgh, Pa.) was neutralized with sodium hydroxide. Zinc bacitracin (40–80 μg/ml PTA solution, Burroughs Welcome & Co., Research Triangle Park, N. C.) (13) was used as a wetting agent. The PTA solution was stored at 5°C and used within a 2-wk period maximally.

RESULTS

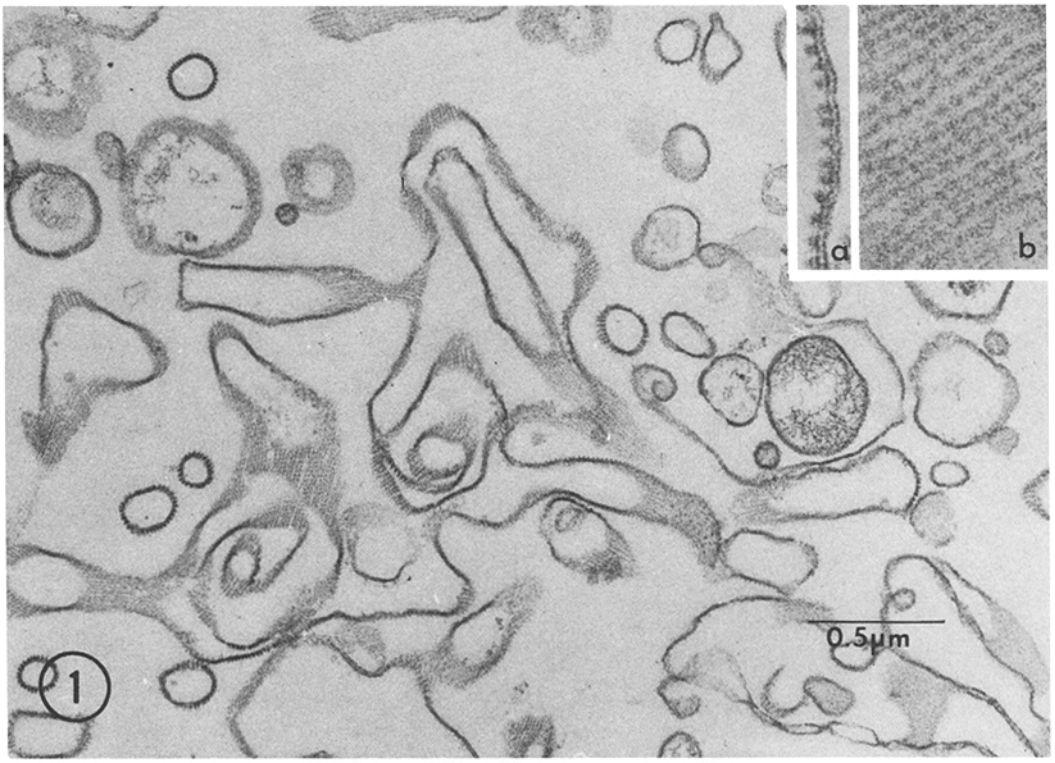
Morphology of the Endocytic Membrane Fraction

The purity of the endocytic membrane fractions was assessed by electron microscopy, using thin-sectioning and negative-stain techniques. Low power views of thin sections showed that the fractions contained primarily membranes with an insignificant amount of nonmembranous material (Fig. 1). As a result of the homogenization procedure the membranes were of different shapes and sizes. Most commonly observed were large, apparently closed cisternae often with smaller round profiles in their lumens, vesicles of different diameters, and dumbbell profiles. Flat sheets with free ends were seen occasionally. In cross sections at higher power, the membranes had a characteristic ~8-nm-thick trilaminar structure with one leaflet decorated with discrete particles about 7.5 nm in diam. As shown in Fig. 1 (inset *a*), the particles were spaced regularly at a period of ~14.5 nm. A tangential view is shown in Fig. 1 (inset *b*). Note that the ~7.5-nm particles were arrayed in an ordered two-dimensional lattice covering the surfaces of the membranes with a spacing of ~12 nm. The appearance in sections of the isolated endocytic membranes was identical to that of *in situ* membranes described elsewhere (42, 16, 34).

Negatively stained preparations of the isolated membranes confirmed the high degree of purity seen in sectioned material. No significant amount of mitochondrial, rough endoplasmic reticulum or nuclear membranes was observed. Yet there was some nonuniformity in the preparations. Most of the membranes were covered with particles arranged in a square pattern (Fig. 2), but some membranes had particles in irregular patterns (Fig. 2, arrows). These latter membranes at times were continuous with the endocytic membranes. We believe that the membranes with irregular patterns on their surfaces were extensions of the microvilli of the ileal epithelium.

Enzymatic Characterization of the Isolated Membranes

To assess how much microvillar contamination may have accompanied the endocytic membrane fractions, the activity of AP was measured since this enzyme is located in the ileal microvilli of suckling rats (21, 10, 8). Approx. 222% of the specific activity (Table I) of AP found in the whole



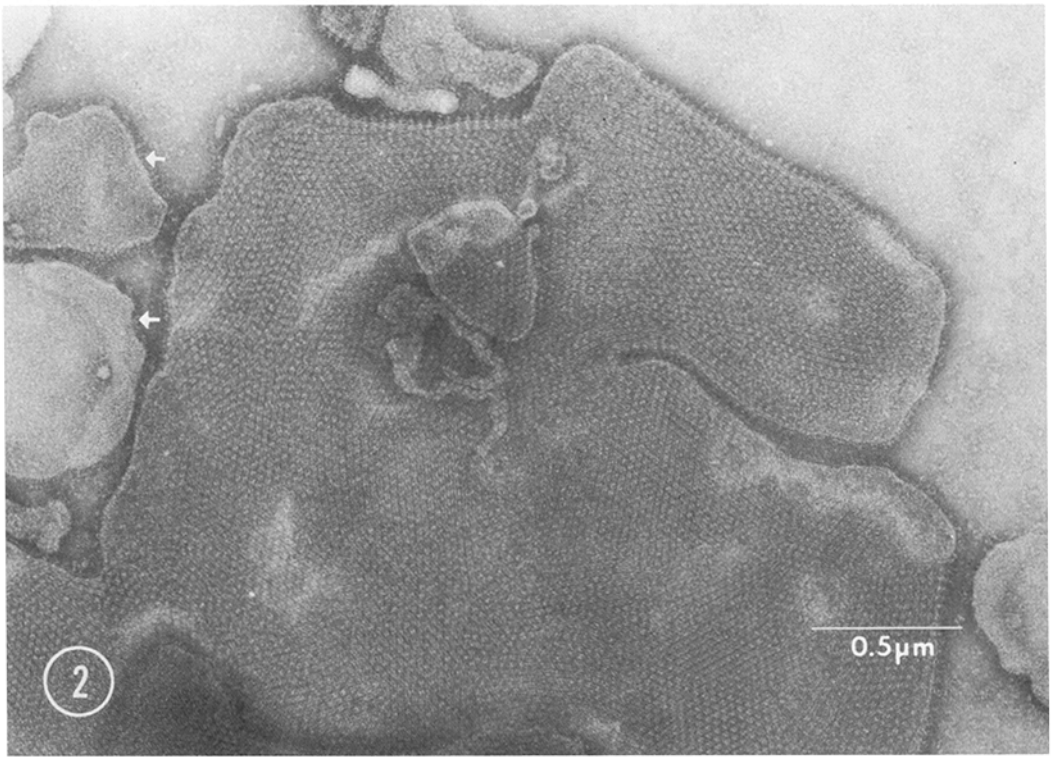


FIGURE 1 Thin section of the isolated endocytic membrane pellet. Transverse views show discrete particles attached to one of the membrane surfaces. A higher power view of a transverse section is shown in *inset a*. Tangential views show particles attached in rows covering the entire membrane surface. A higher power *en face* view is shown in *inset b*. × 36,000. *Inset a*, × 182,000. *Inset b*, × 182,000.

FIGURE 2 Negatively stained preparation of the isolated endocytic membranes. The membrane surfaces are covered by a particulate array which retains the same lattice spacings seen *in situ*. Some microvillar membrane contamination (arrows) is also seen in these fractions. The microvillar membranes are at times continuous with the particulate-coated membranes. × 38,000.

homogenate was present in the isolated membrane fractions, which supports the suggestion of a microvillar contamination obtained from the negatively stained preparations of these membranes.

Koldovsky et al. (19, 18, 22) have reported that β-GAL, NAG, β-GLU, and α-GAL were highly active in homogenates of the epithelial scrapings from suckling rat ileums. We assayed our membrane fractions specifically for these disaccharidases in order to determine whether they were sequestered in the endocytic complex of the ileum. We also assayed for AcP which has been histochemically located in the microvilli and in the supranuclear vacuole of ileal epithelial cells during the suckling period (41, 7). Low specific activities of α-GAL and β-GLU were detected in the isolated membrane fractions as well as in the whole homogenate (Table I). Similarly, a low specific activity of AcP was found indicating a 33% enrichment of this enzyme in these fractions. Some β-GAL activity was also present at 35% of its total specific activity in the whole homogenate (Table I). NAG was the predominant disaccharidase activity associated with the isolated membranes. Approx. 98% of NAG specific activity in the total homogenate was retained in this fraction. Thus, apparently four enzymatic activities are associated with the membrane fraction containing the endocytic membranes: AP, AcP, β-GAL, and NAG.

Morphological Studies of the Calcium-Treated Membranes

In order to see which of the enzymes comprise the particulate array, the membranes were treated with 10 mM $CaCl_2$ in a partially successful effort to release the particles. As shown in Fig. 3, two types of membranes resulted from this treatment: *(a)* partially denuded membranes retaining the particulate coat along one edge (Fig. 3 *inset a*), and *(b)* completely denuded membranes displaying only the characteristic trilaminar unit membrane structure (Fig. 3 *inset b*). Also present were large, amorphous aggregates not seen in the untreated pellets.

Negatively stained preparations, on the other hand, showed many membranes with the array of particles still present (Fig. 4). Some of these lattices were identical to those seen in untreated membranes, but in some instances the pattern no longer completely covered the membrane surfaces. We estimate that approx. 40% of the membranes appeared either to have no lattice, i.e., denuded membranes, as compared to the untreated material or to be partially denuded (Fig. 4). This finding is consistent with a partial release of the particulate array in this fraction. In support of this interpretation, we observed linear aggregates of particles in the background, free from the membranes of negatively stained preparations (Figs. 4 and 5). These "decorated strips," although no longer associated with the membrane surface, had particles which retained the ~14.5-nm periodicity (Fig. 5, *inset*) that was observed in the rows of particles on the intact membrane surface. This tendency for the H-shaped particles to remain attached in rows, although free from the membrane, was also observed in crude membrane preparations as described in the other two papers of this series (16, 34).

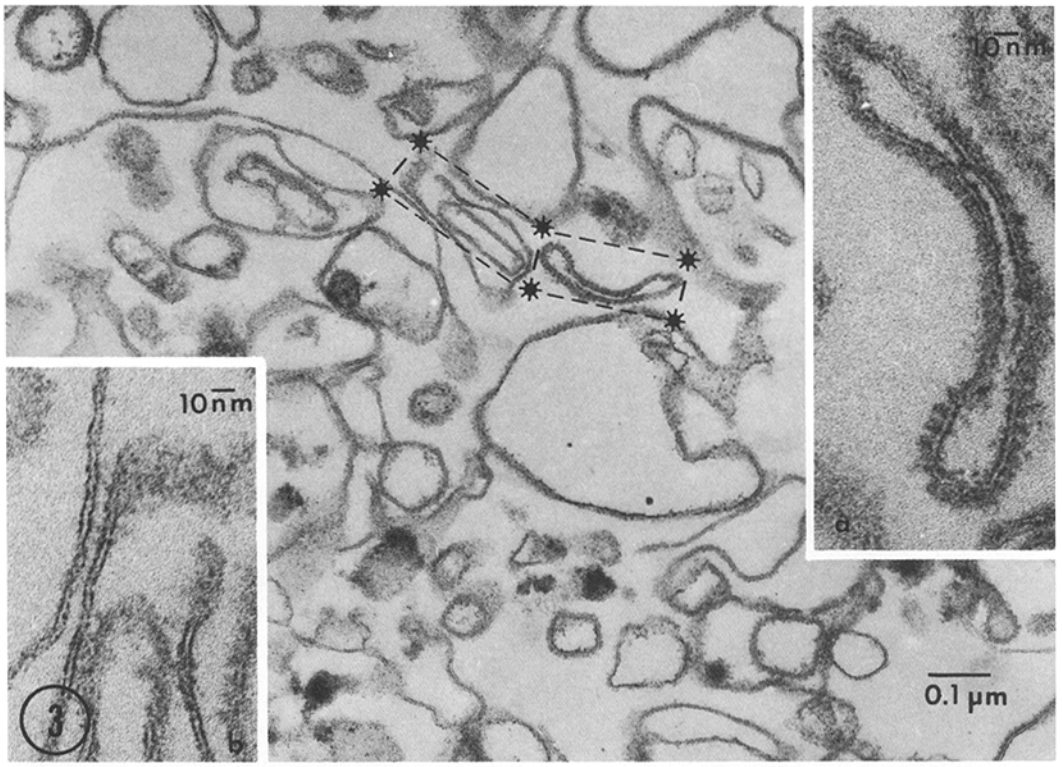
In addition to the decorated strips, individual particles were seen scattered about on the grids. These particles measured ~10.5 nm in diam (Fig. 6). They seemed to consist of four domains, each ~3 nm in diam, which made up the four corners of a square. This appearance clearly results from a pool of stain located in the center of each of the particles but which may not completely penetrate through the particle. Although these square particles were consistently seen in the Ca^{++}-treated material, only a few of them were seen in any one preparation. The squares were reminiscent of the particles seen edge on while still attached to the

TABLE I

Distribution of Phosphatase and Disaccharidase Activities During Subfractionation of the Suckling Rat Ileal Epithelium

Fraction	Sp act* (μmol substrate split/mg protein h^{-1})					
	β-GAL	NAG	α-GAL	β-GLU	AP	AcP
Whole homogenate	34 ± 27	51 ± 28	8.1 ± 0.8	11 ± 0.7	63 ± 38	18 ± 0.6
Supernate	12 ± 0.1	16 ± 9.0	10 ± 0.2	11 ± 0.2	44 ± 9.4	20 ± 1.6
Band 1 + 2	12 ± 6.0	50 ± 14	6.4 ± 0.6	6.0 ± 0.8	140 ± 53	24 ± 7.2

* Mean and the standard deviation are given.


10 nm
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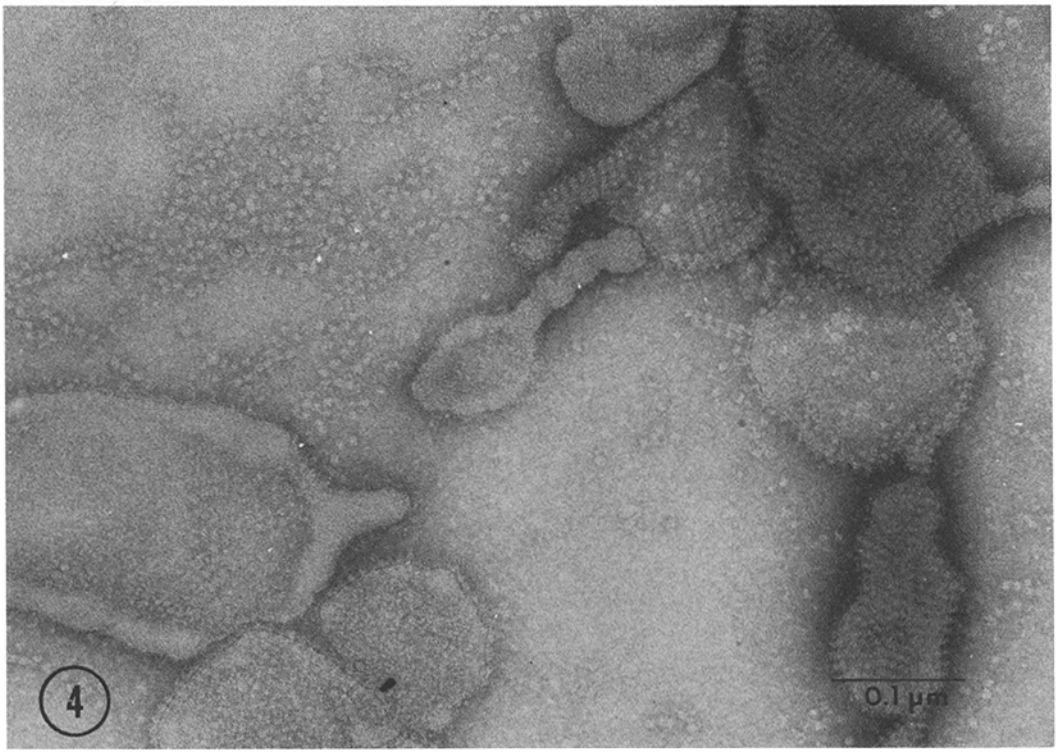
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membrane surface, as described in the following paper of this series (34), but they were somewhat larger.

The particulate array was also released from the membrane surface by decreasing the pH of the membrane suspension to 4.4. Negatively stained preparations of this material showed the presence of a few short decorated strips only two to three particles in length. In addition, single and tangled bundles of indefinitely long filaments ~3 nm in diam were seen (Fig. 7, single arrows, and Fig. 8). Occasionally a few of the particles were observed in close association with the thin filaments (Fig. 7, double arrow).

Purification of the Decorated Strips and Denuded Membranes

Isopycnic centrifugation of the Ca^{++}-treated endocytic membranes through a linear sucrose gradient (in the range of 1.0620–1.1615 ρ) resolved two populations of membranes: one which had retained a lattice and banded at 1.1560 ρ, and another consisting of membranes which were free of particles and banded at 1.0900 ρ. In addition to the denuded membranes in the lighter fraction, there were also numerous short decorated strips. This lighter fraction contained predominantly NAG activity with only a small amount of β-GAL activity and no AP activity (Fig. 9). Most of the β-GAL activity and all of the AP activity were in the heavier fraction (Fig. 9). In this heavier fraction (1.1560 ρ), no recognizable decorated strips were found by negative-stain examination.

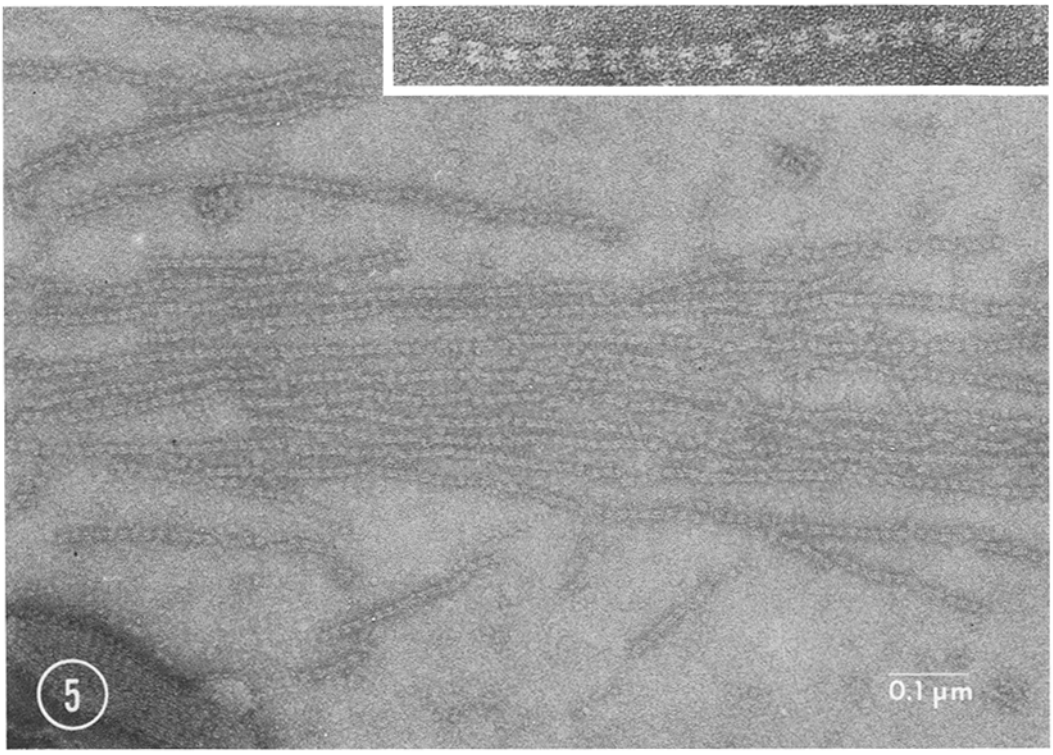
When the Ca^{++}-treated membranes were centrifuged in isopycnic conditions but in the absence of Ca^{++}, no decorated strips were resolved in the lighter fraction (1.0900 ρ) although denuded membranes were present. Under this condition, only a small amount of NAG activity was detected in the lighter fraction, but more abundant activity was noted in the heavier fraction (1.1560 ρ) which contained membranes covered with lattices (Fig. 10). Furthermore, NAG activity had remained at the top of the gradients although no membranes were present in this fraction.

Column Chromatography of the Particulate Material

Since isopycnic banding did not separate the decorated strips from the denuded membranes, rate centrifugation was employed. In order to insure a complete removal of the membranes (denuded and coated), it was necessary to decrease the length or size of the decorated strips so as to decrease their sedimentation coefficient. This was done by dialysis of the Ca^{++}-treated membrane pellet against EGTA as described in Materials and Methods. EGTA dissociated the decorated strips into individual particles which were capable of recombination into the decorated strips upon the addition of $CaCl_2$. The dialyzed membranes were pelleted by centrifugation at 110,900 *g* for 2 h, and the supernate was applied to a Sephdex G-200 column. Usually, 40 μg of protein were applied to the column which represented a 30% release of total membrane protein. The elution profile obtained is shown in Fig. 11. A large peak containing NAG activity eluted in the void volume. Negatively stained preparations of this material did not show any symmetrically organized material nor any membrane fragments. An insignificant amount of β-GAL activity was also seen. AP and AcP activities were absent. If the 110,000 *g* supernate was from membranes dialyzed against a higher ionic strength (5 mM HEPES, 0.5 mM EGTA pH 7.9) than that cited in Materials and Methods and if $CaCl_2$ was added to this supernate before chromatography, then symmetrical images of NAG were seen by negative-stain electron microscopy. Uranyl oxalate and PTA revealed not

FIGURE 3 Thin section of the Ca^{++}-treated membrane pellet. Two types of membranes are seen: *(a)* membranes which retain the particulate coat along one edge *(inset a)*, and denuded membranes which display only the trilaminar unit membrane structure *(inset b)*. Also present are large amorphous aggregates not seen in the untreated pellets. × 78,000. *Insets* × 248,000.

FIGURE 4 Negatively stained preparation of the Ca^{++}-treated membrane pellet. Both denuded and particulate-coated membranes are seen. However, not all of the membrane surfaces which retained the particulate coating are completely covered. Also seen in the background are aggregates of particles free from the membranes. × 106,000.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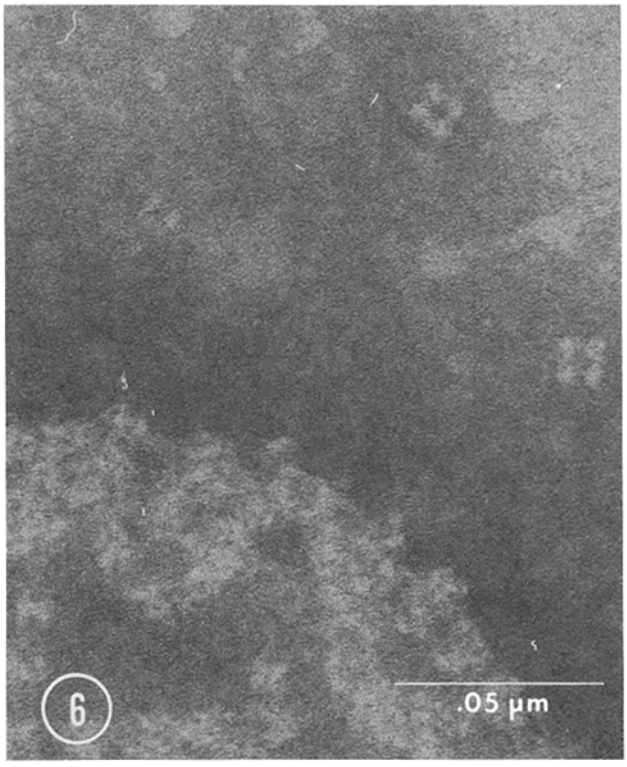
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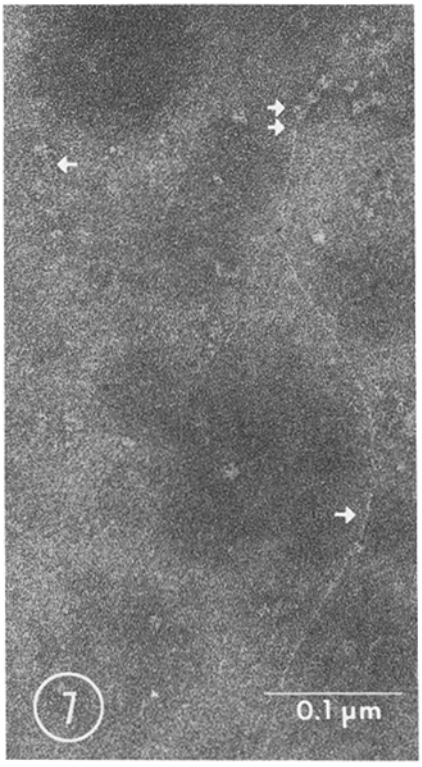
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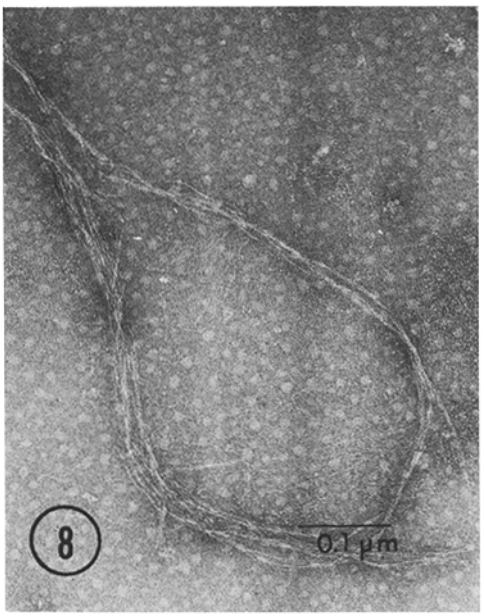


FIGURE 8 Negatively stained preparation of the filaments released from the endocytic membranes by pH 4.4. The filaments are ~3 nm in diam and of varying lengths. They are seen in tangled bundles as well as individual filaments. × 115,000.

only 10 nm squares (Fig. 12 *a–d*) but also 10 nm-diam doughnuts (Fig. 12 *e–g*) and 15 nm × 10 nm barrels (Fig. 12 *h–j*).

Eluting late from the Sephadex column was a large protein peak (20–30 μg) which if globular would have a mol wt of approx. 20,000. The material in this fraction did not contain identifiable structures when examined by negative-stain methods. However, upon addition of 20 mM $CaCl_2$, bundles and single thin (~3 nm) filaments of indefinite length were found with the electron microscope by the negative-stain method (Fig. 13). If either a Sephadex G-200 column or a Sepharose 2B column was developed in a phosphate buffer or in the presence of $CaCl_2$, then the 3 nm diam filaments were found in the void volume. These thin filaments were also seen to aggregate into larger bundles or tactoids after $CaCl_2$ addition to a column fraction which had been frozen but not concentrated (Fig. 14).

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis of NAG and the filament fractions are shown in Fig. 15. The

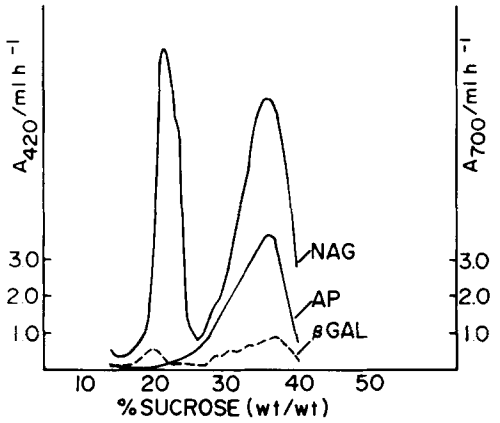


FIGURE 9 Sedimentation profile of Ca^{++}-treated membranes after an isopycnic centrifugation on a linear 15–40% (wt/wt) sucrose gradient in the presence of $CaCl_2$. Left ordinate is the sp act of NAG (–) and β-GAL (---) expressed as A_{420} units per milliliter sample per hour. Right ordinate is the sp act of AP (–) expressed as A_{700} units per milliliter sample per hour.

FIGURE 5 Decorated strips released from the membrane surface by $CaCl_2$ and negatively stained with 1% PTA containing bacitracin. Each decorated strip consists of individual ~7.5 nm particles joined together with a center-to-center separation of ~14.5 nm (*inset* enlargement). × 104,000. *Inset* × 314,000.

FIGURE 6 Negatively stained preparation of the Ca^{++}-treated membrane pellet shows square particles ~10.5 nm in diam. The particles appear to consist of four globular domains which make up the four corners of the square, but this appearance may result from a pool of stain located in the center of the particle. × 546,000.

FIGURE 7 Negatively stained preparation of the endocytic membrane pellet exposed to pH 4.5. Indefinitely long, individual filaments (~3 nm in diam) are seen (single arrow). In addition, there are a few short decorated strips only 2–3 particles in length. Occasionally, a few of the particles appear to be associated with the thin filaments (double arrows). × 183,000.

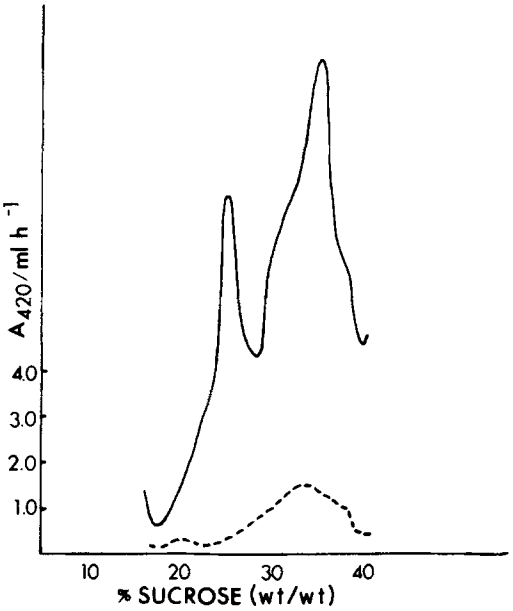


FIGURE 10 Sedimentation profile of $CaCl_2$-treated membranes after an isopycnic centrifugation on a linear 15–40% (wt/wt) sucrose gradient. Left ordinate is the sp act of NAG (–) and β-GAL (---) expressed as A_{420} units per milliliter sample per hour.

NAG fraction from the Sephadex G-200 column consistently migrated as a doublet with apparent mol wt of 110–115,000 and 100–105,000 (Fig. 15 *a*, arrows). Both bands were PAS positive (Fig. 15 *b*). Also present in the NAG fraction were two minor proteins with apparent mol wt of 80,000 and 70,000 which were not PAS positive.

Although 60 μg of filamentous protein were applied to a SDS-polyacrylamide gel, no Coomassie blue band was resolved. The failure of these gels to stain well with Coomassie blue was consistent in several repeated experiments. One explanation for the absence of staining of these gels is that the filamentous protein is a glycoprotein. The heavily glycosylated glycoproteins do not readily bind Coomassie blue (9) but do react in the PAS staining technique. However, neither did the gels containing the filament material stain with PAS in the region of the faint Coomassie blue band, nor were any additional bands resolved in the gels.

DISCUSSION

By morphological criteria, a homogeneous population of membranes has been obtained which *in situ* comprise the endocytic complex of the suckling rat ileum. However, these membranes are not all identical in several respects, perhaps both structurally and functionally. They were resolved into two fractions by differential flotation: one obtained at a density of 1.1315/1.1560 and the other at 1.1560/1.1821. In addition, the membranes responded differently to $CaCl_2$ treatment. Approx. 40% of the combined endocytic membrane fractions release their surface lattice when exposed to $CaCl_2$. The other 60% appear to be either unaffected or only slightly so as seen in minor lateral shifts in the spacings between the rows of particles. Yet, no apparent correlation was seen between sensitivity to Ca ions and density (data not shown). The primary effect of $CaCl_2$ on the membranes was not simply due to a change in ionic strength, since 1 M KCl and 0.5 M $(NH_4)_2SO_4$ did not release the particles (data not shown). Furthermore, the strips of particles when released had a low density, approx. equal to that of the stripped membranes themselves (1.0900 ρ). This suggests to us that the particulate array is released as a complex with lipid and thus that the Ca^{++} effect may be primarily involved in the lipid-lipid interactions of these membranes. If the effect of Ca^{++} is to produce a disturbance in the lipid-lipid interactions, then the differences in density of the two populations of membranes need not corre-

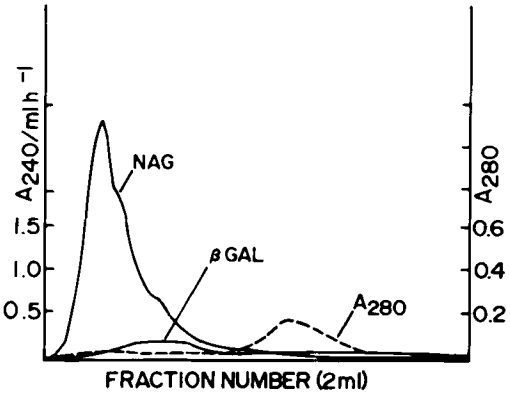


FIGURE 11 Elution profile from a Sephadex G-200 separation of the protein components of the decorated strips. Left ordinate is the sp act of NAG (–) and β-GAL (–) expressed as A_{420} units per milliliter sample per hour. The right ordinate is protein concentration expressed as A_{280} units (---). Essentially two proteins are resolved: one excluded from the Sephadex G-200 bed, NAG, and one retarded by the Sephadex G-200 bed shown by its A_{280} peak. The β-GAL activity is consistently present but always as a minor enzymatic activity and therefore is considered to be a contaminant.

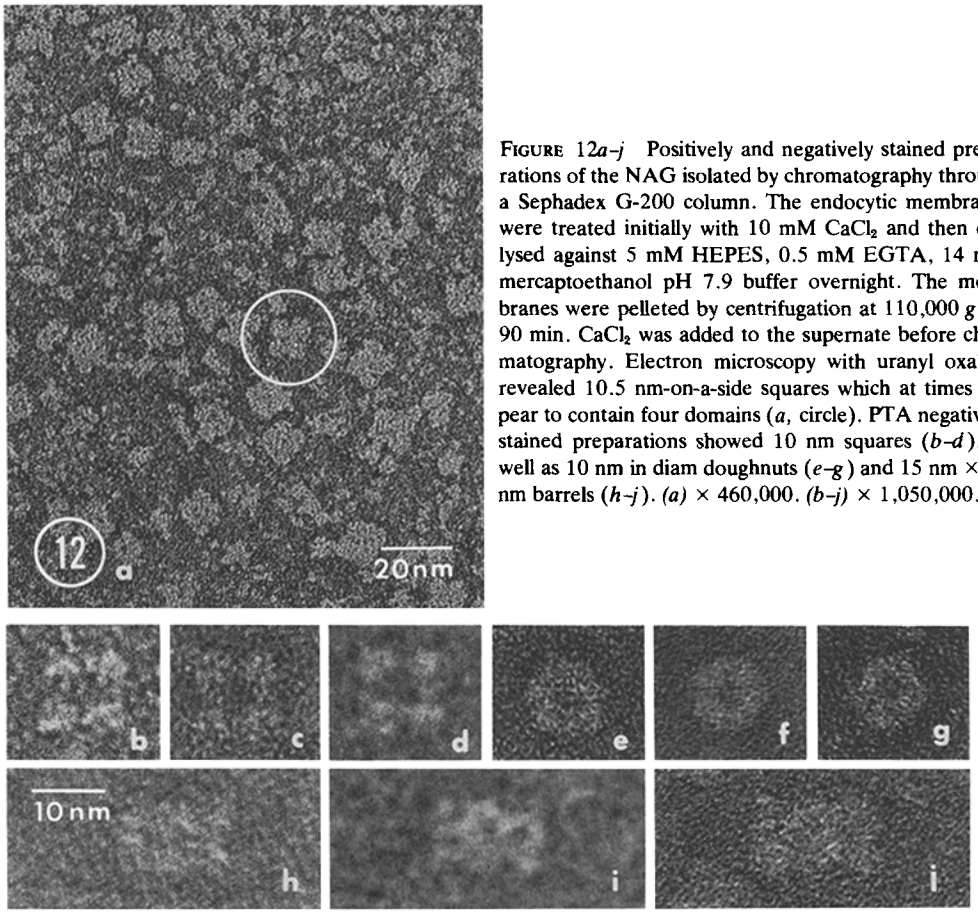
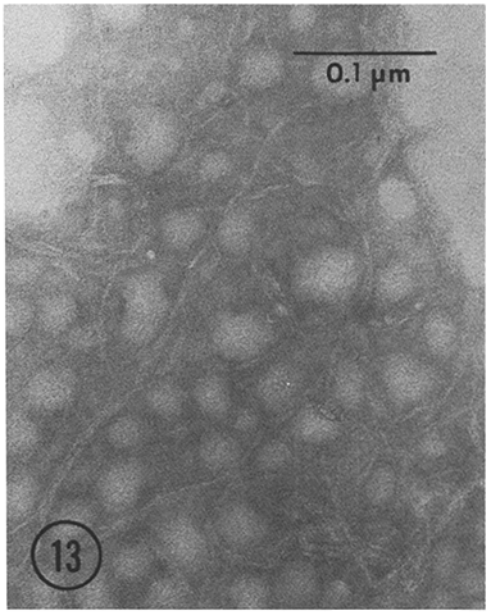


FIGURE 12*a–j* Positively and negatively stained preparations of the NAG isolated by chromatography through a Sephadex G-200 column. The endocytic membranes were treated initially with 10 mM $CaCl_2$ and then dialysed against 5 mM HEPES, 0.5 mM EGTA, 14 mM mercaptoethanol pH 7.9 buffer overnight. The membranes were pelleted by centrifugation at 110,000 *g* for 90 min. $CaCl_2$ was added to the supernate before chromatography. Electron microscopy with uranyl oxalate revealed 10.5 nm-on-a-side squares which at times appear to contain four domains (*a*, circle). PTA negatively stained preparations showed 10 nm squares (*b–d*), as well as 10 nm in diam doughnuts (*e–g*) and 15 nm × 10 nm barrels (*h–j*). *(a)* × 460,000. *(b–j)* × 1,050,000.

late directly with the Ca^{++} effect but, rather, the lipids of the Ca^{++}-sensitive membranes must be different in some way from the insensitive ones. The release mechanism is most likely not due to a conformational change within the filament-NAG complex exclusive of its lipids, since these proteins are common to the membranes which release their pattern and to those which do not. It should be noted that the release of the lattice occurred at 5–10 mM $CaCl_2$ and that higher concentrations of Ca^{++} had no additional effect.

The particulate array was comprised of NAG and a second protein capable of polymerizing in the presence of Ca^{++}. The NAG bound to this filamentous protein had an apparent mol wt of 100,000 daltons, which is consistent with the molecular weight reported for NAG isolated from pinto bean meal (2), from hen oviduct (38), and from human spleen (35). The apparent doublet of 100,000 and 110,000 daltons for NAG from the endocytic membranes was highly reproducible with a constant ratio of 1:1. This suggests that two NAG's are present, with one slightly modified either in charge or in carbohydrate content. Such a change may be related to the attachment of the enzyme to the filamentous protein. Usually, NAG is a soluble enzyme sequestered in the lysosomes of the cell (35). However, in the human spleen, two forms of NAG ("A" and "B") occur. Both forms are found in the lysosomal fraction, but form A is also thought to be membrane bound (35). Form A is a glycoprotein while form B is not. By gel filtration, both A and B are approx. 100,000 daltons.

The H or square shapes revealed by negatively stained preparations of the membranes and decorated strips are probably not the monomeric forms of NAG. Under certain conditions, these symmetrical shapes were lost, yet enzymatic activity was retained. Although the square appearance of



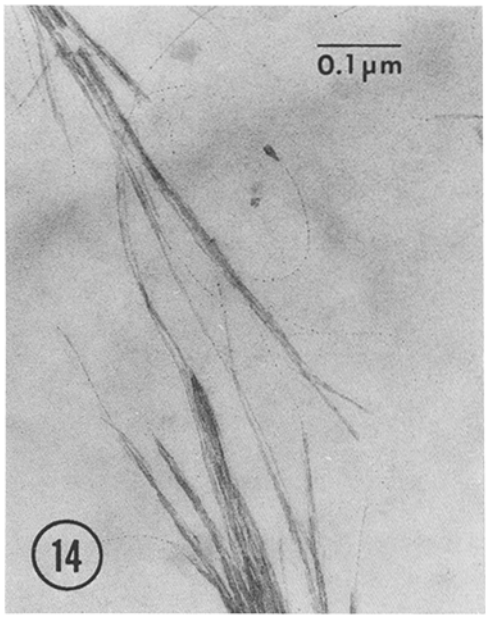


FIGURE 13 Negatively stained preparation of the filament fraction after chromatography through a Sephadex G-200 column and the readdition of $CaCl_2$. The filaments are ~3 nm in diam and of varying lengths. × 192,000.

FIGURE 14 Negatively stained preparation of the filament fraction after chromatography through a Sephadex G-200 column and the addition of $CaCl_2$. This material was frozen (−20°C) and not concentrated before electron microscopy was done. The filaments have aggregated into bundles or tactoids of varying lengths. No individual filaments were seen. × 114,000.

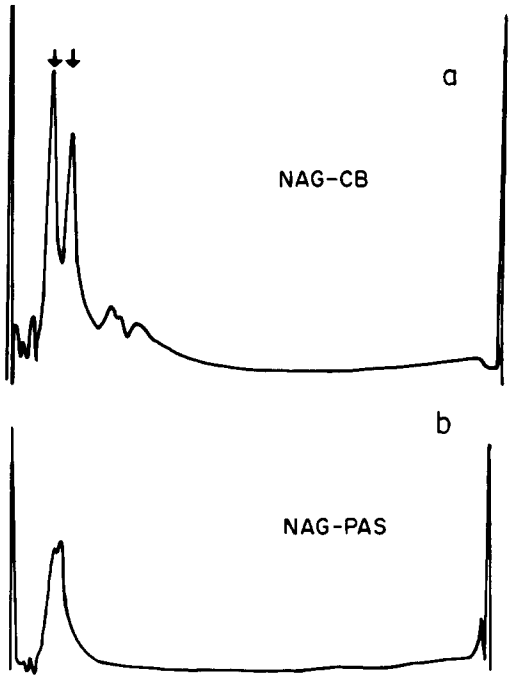


FIGURE 15 Densitometry traces of SDS-polyacrylamide gels of NAG. The two traces show the migration of NAG (30 μg) in SDS-polyacrylamide gels stained either with Coomassie blue (*a*, 550 nm) or with PAS (*b*, 560 nm). NAG migrates as a doublet with apparent mol wt of 110–115,000 and 100–105,000 (arrows). Both NAG bands are PAS positive *(b)*. Two minor polypeptide chains are also seen in the NAG gels stained with Coomassie blue. Of these two bands with mol wt of 80,000 and 70,000, only the 80,000 dalton polypeptide is consistently present.

the bound particle (presumably NAG) suggests that there are four internal domains which appear globular by negative stain, we do not believe that these squares are complexes of four globular monomers since NAG was at all times voided from the Sephadex G-200 column. Any globular protein of 600,000 or less molecular weight should be retarded by the Sephadex bed. Therefore, the monomeric form of NAG cannot be globular, but its exact shape remains unknown. The doughnut and barrel images of the isolated NAG are most likely multiple aggregates of this enzyme, as are the squares. However, at this time we do not know the number of monomers contained within these forms.

The interaction of NAG with the filament is probably an ionic one since NAG was dissociated from the filament at its isoelectric point. If the monomer of the filament is globular, then its nom-

inal mol wt would be 20,000 by gel filtration, a value also compatible with its amino acid composition (our unpublished data). Our failure to resolve any significant bands for this protein on the SDS-polyacrylamide gels by either Coomassie blue or PAS may be due to either an inadequate fixing of this protein in the gels or a failure of this protein to enter the gels. We are currently trying to resolve this phenomenon in various ways.

The filamentous protein was not actin, on the basis of several criteria: *(a)* although actin is known to polymerize with Mg^{++} (30), polymerization of the filament was clearly dependent on Ca^{++} and was affected nonreproducibly by Mg^{++}. *(b)* Actin can be decorated with heavy meromyosin to show the characteristic arrowhead structure (30), but the filament did not respond to this treatment. *(c)* Actin can be separated as a 48,000 dalton protein on SDS-polyacrylamide gels with a strong Coomassie blue reaction (37). In comparison, the filament protein on SDS-polyacrylamide gels responded poorly to Coomassie blue. Similarly, we suggest that the filament was not spectrin (6, 11) simply on the basis of its electrophoretic properties on SDS-polyacrylamide gels and its ability to polymerize into filaments in the presence of calcium ions.

Although the filament from the endocytic membranes may serve as a site for attachment of NAG to the membrane surface, it does not seem to be identical with the protein nectin isolated from prokaryotes (3, 1). Nectin binds adenosine triphosphatase (ATPase) to the plasma membrane of *Streptococcus faecalis* (3). Nectin was released from the membrane surface by removing Mg^{++} through chelation with EDTA and reassociated with the membrane in the presence of Mg^{++} (3). The filament in these experiments was released from the membrane surface in the presence of Ca^{++}, and Mg^{++} had no effect. Furthermore, nectin has an apparent mol wt of 37,000 by gel filtration (3, 1). The filament monomer in these studies had an apparent mol wt, by gel filtration, of 20,000.

One other "attachment protein," the oligomycin-sensitivity conferring protein (OSCP), has been reported (29, 28, 40). OSCP binds ATPase to the inner mitochondrial membrane surface (29, 28, 40). It is extracted lipid-free from the cristal membranes of the mitochondrion with ammonium hydroxide (29, 40). The apparent mol wt of OSCP estimated by either gel filtration (29) or SDS-polycrylamide gel electrophoresis (29) is approx. 18,000 daltons. OSCP migrates as a discrete band on SDS-polyacrylamide gels and gives a strong Coomassie blue reaction (29). Furthermore, OSCP will form tetrads 12 nm on a side (28), but larger aggregates of this protein have not been reported. Therefore, on the basis of its physical chemical properties and the manner in which it is removed from the mitochondrion, OSCP does not seem to be the filament protein isolated from the suckling rat ileum. We believe that the filament protein is a protein not previously described. Therefore, we have named it "ligatin" from the latin *ligare,* which translates as "to bind together."

The OSCP tetramers resemble closely in shape and size the ~10.5 nm square particles seen in negatively stained preparations of the Ca^{++}-treated endocytic membranes. OSCP is released from bovine heart (29) and yeast (40) mitochondria by alkaline pH and therefore seemed an unlikely candidate for the square particles seen in our preparations. However, to ascertain that this was so, we isolated mitochondria from rat liver and treated them with $CaCl_2$. Negative-stain electron microscopy of the Ca^{++}-treated mitochondria did not show any square particles as was expected. We believe that the ~10.5-nm square particles are images of NAG.

CONCLUSION

The particulate array on the endocytic membranes has been found to consist of NAG bound to the membrane surface via a protein, ligatin, capable of polymerizing in the presence of $CaCl_2$. NAG cleaves the β-linked *n*-acetylglucosamine groups from glycoproteins, glycolipids, and mucopolysaccharides (35). The membranes of the endocytic complex are therefore sites for the extracellular digestion of the carbohydrate moieties in the maternal milk. The structural organization of the complex, consisting of tortuous channels of plasma membrane enfolded within the apex of the cells from the bases of the microvillus border, provides a highly increased absorptive area for the uptake of these carbohydrate products. The lumens of these channels maintain direct communication with the extracellular gut lumen and thus provide continuous access to undigested substrate. The confined and presumably relatively stagnant environment within these cisternal and tubular structures may help maintain a local homeostasis for a specialized lysosomal digestion of foodstuffs external to the epithelial cell cytoplasm. The movement of milk through this convoluted system may be simply one of passive diffusion down a

concentration gradient, or active intracellular contractile phenomena may be involved.

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