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PURIFICATION OF MICROVILLUS MEMBRANE VESICLES FROM PIG SMALL INTESTINE BY IMMUNOADSORBENT CHROMATOGRAPHY

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Microvillus membrane vesicles from pig small intestine, isolated by hypotonic lysis, Mg^{2+} aggregation of contaminants and differential centrifugation, have been further purified by immunoadsorbent chromatography. The vesicles adhere to an immunoadsorbent prepared by coupling antibodies raised against three of the principal proteins of the brush border membrane (aminopeptidase, sucrase-isomaltase and lactase) to Sepharose 4B. After the contaminants are removed by washing, the adherent vesicles are released from the immunoadsorbent by applying shear forces. The purity of the immunoadsorbed vesicles has been established by electron microscopy and by measuring the activity of marker enzymes. The enrichment factor is 1.17 ± 0.02 for aminopeptidase and 0.70 ± 0.05 for 5′-nucleotidase. The contamination of the preparation before immunoadsorption constitutes 10% of the membrane protein and consists mainly of basolateral membrane fragments as judged from marker enzyme determinations and the lipid composition.

Introduction

Microvillus membrane vesicles from pig small intestine, isolated as previously described [1] by hypotonic lysis, Mg^{2+} aggregation of contaminants and differential centrifugation, are to some extent contaminated with basolateral membrane fragments. Preparation of pure microvillus membrane vesicles is a prerequisite for the study of protein-lipid interactions in membranes and in attempts to characterize the minor protein components of the membrane.

Procedures for purification of cells and cell membranes by affinity chromatography, using an immobilized ligand, have been described (see Ref. 2 for a review). Most work is carried out using lectins as affinity ligands, and lectins have especially been used to separate right-side-out from inside-out vesicles [3]. Affinity chromatography with antibodies, against characteristic surface antigens, as ligands, could be a specific method to isolate the desired structures. However, little work on immunoadsorbent chromatography of cell membranes has been done, mainly because of a lack of elution methods [2,4,5].

Microvillus membranes from rabbit intestine are known to be precipitated by specific antibodies against aminopeptidase [6], and precipitation of rat microvillus membranes by specific antibodies against the sugar hydrolases is also well established [7]. The hydrolases of the microvillus membrane protrude from the surface of the membrane and antibody binding to one of the enzymes has little effect on the binding to the others, indicating a degree of molecular freedom similar to that of the molecules in solution [7]. Therefore, a separation method based on the ability of microvillus membrane vesicles to adhere to antibodies against the hydrolases immobilized on Sepharose might be a procedure for further purification of microvillus

membranes. In this paper, we present evidence that pure preparations of right-side-out microvillus membrane vesicles can be obtained by this method.

Materials and Methods

Chemicals

Pig small intestines were kindly delivered by the Department of Experimental Pathology (Rigshospitalet, Copenhagen, Denmark). CNBr-activated Sepharose 4B, Con A-Sepharose and Protein A-Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). L-Alanyl-*p*-nitroanilide and Folin-Ciocalteau's reagent were from Merck (Darmstadt, F.R.G.), bovine serum albumin from Sigma Chemical Co. (St. Louis, U.S.A.) and the glucose reagent kit GLOX from Kabi Diagnostics (Stockholm, Sweden). Other substrates and the enzymes were delivered by Boehringer (Mannheim, F.R.G.). Incomplete Freund's adjuvant was obtained from Statens Seruminstitute (Copenhagen, Denmark). The organic solvents were of analytical reagent grade and were distilled prior to use. Standard phospholipids were obtained from Serdary Research Laboratories (London, Canada) and standard glycolipids from Supelco, Inc. (PA, U.S.A.). All other chemicals were of analytical grade.

Isolation of microvillus membranes

All procedures were performed at 4°C. Microvillus membranes were prepared from 50 g frozen inverted pig intestine by a modification of the method of Kessler et al. [8] as previously described [1]. Aliquots of the mucosa cell homogenate were taken for marker enzyme determinations and stored at −80°C. The pellet from the 28000 × *g* centrifugation step, containing the microvillus membrane vesicles, was homogenized in 20 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl in a Potter-Elvehjem homogenizer, and the resulting suspension was centrifuged at 800 × *g* for 15 min. The supernatant which contained the microvillus membrane vesicles was pelleted at 28000 × *g* for 30 min. The pellet was suspended in the desired volume of 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and used as starting material in the immunoadsorbent chromatography.

Preparation of the antisera

A microvillus membrane preparation solubilized and partially purified by Con A-Sepharose chromatography was used as antigen: Con A-Sepharose eluate [9]. Crossed immunoelectrophoresis of the Con A-Sepharose eluate against a microsomal antiserum prepared from pig intestinal mucosa showed only three precipitates identified as aminopeptidase, sucrase-isomaltase and lactase [10]. Rabbits were immunized with the antigen (protein concentration 0.25 mg/ml), mixed with an equal volume of incomplete Freund's adjuvant. Over a period of 6 weeks, 200 µl of antigen were injected intercutaneously with intervals of 2 weeks [11]. 1 week after the last injection the rabbits were bled for 40 ml. The immunization was continued with a booster injection every sixth week and 40 ml blood were collected 1 week after each injection. Anti-aminopeptidase was prepared as described previously [9]. The IgG fraction was isolated from the antisera by chromatography on Protein A-Sepharose [12].

Preparation of the immunoadsorbent

The isolated IgG was coupled to CNBr-activated Sepharose 4B, according to the protocol delivered by the manufacturer, using 0.1 M Na_2CO_3, pH 8.3, containing 0.5 M NaCl as coupling buffer. The IgG concentration was about 2 mg/ml and the ratio between the antibody solution and the settled gel was 2:1 (v/v). About 90% of the IgG was bound under the conditions used.

Immunoadsorbent chromatography

Normal procedure. The chromatography was performed on a 2.2 × 20 cm column packed with 60 ml immunoadsorbent. The gel was equilibrated in 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and this buffer was used in all the chromatographic steps. 25 ml of the microvillus membrane preparation, diluted with buffer to the desired concentration, were applied to the column by pumping (40 ml/h). When the membrane suspension had drained into the gel the outlet was closed and the column left overnight at 4°C for incubation. Membranes not adhered to the gel were washed out of the column with 60 ml buffer by pumping (40 ml/h) and three fractions of 20 ml

were collected. Dissociation of the adherent vesicles was achieved by suspending the settled gel in 25 ml buffer and gently shaking the column. After shaking for 30 s the column was rapidly drained by pumping (20 ml/min) and the eluate was collected. To displace the released vesicles, trapped in the void volume of the column, another 20 ml of buffer were flushed through the settled gel and the eluate collected. The two eluates containing the adherent vesicles were combined. To ensure complete removal of membrane vesicles from the beads the whole dissociation procedure was repeated.

The chromatography was followed by measuring the aminopeptidase activity in the fractions (Fig. 1). The combined eluates containing the adherent membranes (fractions 5 and 6, Fig. 1), and the eluate containing the nonadherent membranes (fraction 2, Fig. 1) together with the control suspension (an aliquot of the preparation, kept at 4°C during the complete column cycle) were sedimented for 30 min at 25000 × *g*. The pellets were resuspended in a small volume of 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and aliquots were taken for protein determinations and for measuring marker enzymes which were assayed immediately or on samples which had been stored at −80°C.

Purification of the nonadherent fraction. By repeated chromatography of the nonadherent fraction, the microvillus membrane preparation could be depleted of vesicles able to adhere to the immunoadsorbent. Microvillus membranes corresponding to 70 U aminopeptidase was loaded to the column and after 10 h of incubation the elution was started. The first washing fraction was applied to the column immediately after elution of the adherent vesicles and the binding procedure repeated. After five cycles, the last nonadherent fraction, the combined adherent fractions and the control membrane preparation were sedimented, resuspended in a small volume of buffer, and aliquots were taken for marker enzyme determinations and the rest was used for lipid extraction.

Specimen preparation for electron microscopy. After three rechromatography cycles as described above, the three separate adherent fractions, the nonadherent fraction and the control membrane preparation were concentrated as described. Aliquots were taken for marker enzyme determinations and the rest of the preparation was used for electron microscopy.

Assays

Aminopeptidase (EC 3.4.11.2) activity was determined with L-alanyl-*p*-nitroanilide as substrate [9] at 37°C by use of the Reaction Rate Analyzer (LKB Products AB, Stockholm, Sweden). One unit of aminopeptidase is defined as the activity hydrolyzing 1 μmol substrate per min. 5'-Nucleotidase (EC 3.1.3.5) activity was measured at 37°C and pH 7.4 with 0.1 mM AMP as substrate and with the addition of 30 mM phosphate in order to distinguish it from alkaline phosphatase as described by Gratecos et al. [13]. $(Na^+ + K^+)$-dependent ouabain-sensitive ATPase (EC 3.6.1.3) was measured in a coupled assay [13]. After recording the total ATPase activity for 3 min, oubain was added to a concentration of 0.5 mM and the new activity recorded. The difference in the slope between the two lines is a measure of the oubain-inhibited activity. Sucrase (EC 3.2.1.48) activity was measured according to the method of Dahlqvist [14]. The liberated glucose was quantified by the glucose reagent kit, GLOX. Protein was determined according to the method of Lowry et al. [15] using crystalline bovine albumin as standard.

Lipid analysis

A total lipid extract was obtained by chloroform/methanol extraction of the membrane fractions by the method of Suzuki [16] as described earlier [1]. Separation of the lipid extracts into individual lipids was performed by thin-layer chromatography in the solvent system chloroform/methanol/2-propanol/0.25% KCl/ethyl acetate (30:9:25:6:18, v/v), developed three times, and the quantitation of individual phospholipids and glycolipids was performed on silica gel spots [1].

Electron microscopy

Suspensions of the membrane preparations were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, by standing at 4°C for 18 h. The fixed membranes were pelleted and post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, at 4°C for 1.5 h. After rinsing in buffer, the specimens were dehydrated through a series of

graded ethanol solutions and embedded in Araldite according to the method of Luft [17]. Sections of silver to golden colors were cut on an LKB Ultratome III and stained with uranyl acetate and lead citrate [18] and examined in a Philips 201 C electron microscope.

Results and Discussion

The results will be presented and discussed under two subheadings. The first concerns the methodology, the second the properties of the purified microvillus membrane vesicles.

Methodology

Properties of the immunoadsorbent. When a microvillus membrane suspension was incubated on the immunoadsorbent prepared from antiserum raised against aminopeptidase, sucrase-isomaltase and lactase, a fraction of the membranes adhered to the beads (Fig. 1). The contaminating material was removed by washing, together with excess microvillus membranes. The adherent fraction was recovered from the immunoadsorbent by applying mechanical force.

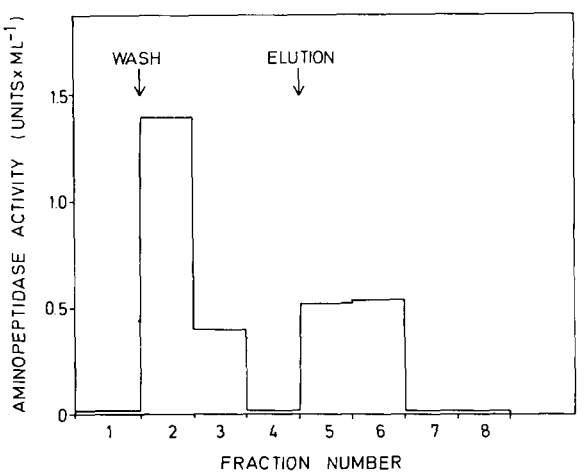

Fig. 1. Immunoadsorbent chromatography, elution profile. The column (bed volume 60 ml) was incubated as described in Materials and Methods with membranes corresponding to 58 U aminopeptidase. The chromatography was followed by measuring the aminopeptidase activity in the fractions. After the nonadherent membranes were washed out of the column, the adherent vesicles were released by resuspension and gentle shaking of the beads. The released vesicles were then recovered by rapid elution.

A monovalent antiserum as anti-aminopeptidase, coupled to Sepharose, could not retain any membranes although the antiserum in solution was able to precipitate the vesicles (data not shown). The increased number of binding sites created by the polyvalent antiserum seems to be a necessity for the adherence of the vesicles.

The immunoadsorbent columns were able to bind 0.4 U membrane-bound aminopeptidase, corresponding to about 0.2 mg membrane protein/mg settled gel, under conditions where maximum binding was obtained. The overall recovery of aminopeptidase in the adherent fraction plus the nonadherent fraction, compared to the amount applied to the column, was always close to 100%. The capacity of the immunoadsorbent slowly decreased by about 10% per month. Attempts to reactivate the columns by treatment with 0.1 M Na_2CO_3 buffer (pH 10.2), 0.1 M sodium citrate buffer (pH 2.3) or 2 M guanidinium chloride (pH 7.2) did not restore the original capacity, and the decrease was probably due to denaturation and/or proteolytic degradation of the antibodies.

Factors affecting the binding. The adherence of the vesicles to the antibody-coated beads was time dependent. Incubation was routinely carried out overnight, approx. 18 h, to ensure equilibrium. Longer incubation times did not increase the amount of adherent vesicles. When the incubation time was zero and the washing of the column was started immediately after the microvillus membrane suspension had drained into the column, only very slight binding was observed. Incubation times of 1 and of 3 h resulted in 58 and 67%, respectively, of the maximum yield obtained after 18 h of incubation.

When a fixed amount of immunoadsorbent beads was incubated with various amounts of microvillus membranes, distinct adsorption of microvillus membrane vesicles to the gel was observed. Fig. 2 shows the results of a typical experiment. The column must be overloaded to ensure maximum binding. Incubation of the immunoadsorbent (bed volume 60 ml) with 100 U membrane-bound aminopeptidase, corresponding to about 50 mg membrane protein, was necessary to ensure maximum yield of the adherent fraction.

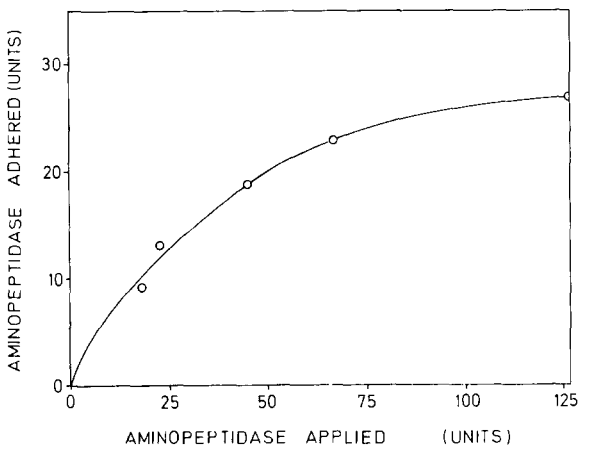

Fig. 2. Immunoadsorption of microvillus membranes. Various amounts of membranes, quantitated as aminopeptidase activity, were applied to the column (bed volume 60 ml) and incubated as described in Materials and Methods. After incubation for 18 h the adherent vesicles were released and the aminopeptidase activity assayed.

No difference in the binding of the vesicles or in the dissociation of the adherent vesicles from the beads was observed with changes in the incubation medium. Homogenization of the microvillus membrane pellet (see Materials and Methods) in 12 mM Tris-HCl + 0.3 M mannitol (pH 7.1), 10 mM Hepes-Tris + 0.1 M mannitol (pH 7.4) or 50 mM Tris-HCl + 0.15 M NaCl (pH 7.4) and subsequent immunoadsorbent chromatography in the same media all gave the same result.

The binding of the vesicles to the immunoadsorbent beads could be abolished by applying shear forces during the incubation. Even when the beads were stirred only mildly, just to prevent them from settling, no binding was observed during an 18 h incubation period.

Membranes from a crude mucosa cell homogenate also adhere to the immunoadsorbent and membranes with the same specific aminopeptidase activity as obtained with the microvillus membrane preparation could be isolated. However, more investigations, e.g., electron microscopy, are needed to ensure the quality of this preparation.

Specificity of the binding. To exclude nonspecific binding to the Sepharose beads the separation procedure was carried out with beads prepared from nonspecific rabbit IgG coupled to CNBr-activated Sepharose 4B. No significant binding could be detected even after 18 h of incubation. Therefore, the adherence of microvillus membrane vesicles was due to specific binding to the antibodies.

Dissociation of the adherent vesicles. At the end of the incubation period the adherent vesicles were released by resuspension of the gel and gentle shaking. We found no rebinding during elution, and no further vesicles were released when the whole elution procedure was repeated. This elution method is nonspecific and, as described for immunoadsorbent chromatography of antibody-bearing cells [19,20], releasing by elution with the antigen, acting as a competitive inhibitor of the binding, is more reliable [2] as it implies true biospecificity. However, this method is not general applicable as the membrane surface compounds are not generally available in soluble form.

Elution with denaturating agents, a high concentration of chaotropic ions or with strongly acid or alkaline buffers, normally used to dissociate soluble proteins from the complex, is useless when working with membranes as this might damage the lipid bilayer. In our laboratory, low-ionic-strength buffers have been successfully used for the elution of different detergent-solubilized, brush border enzymes [21]. Thus, aminopeptidase, sucrase-isomaltase and lactase were eluted from their respective immunoadsorbent columns with 2 mM Tris-HCl, pH 8.0, or 1 mM phosphate, pH 7.4 [9,22,23]. This extremely mild elution procedure had no releasing effect on immunoadsorbed microvillus membrane vesicles.

The necessity of using mechanical forces for the dissociation could imply that secondary interactions between membrane structures and the Sepharose matrix occur after the binding step, as suggested by Brunner et al. [24]. They found that plasma membranes from thymocytes specifically adhered to Con A-Sepharose could not be eluted with α-methylmannoside, but shearing forces (stirring of the gel) were necessary to dissociate the lectin-bound membranes. Our observation that the eluant normally used to release the three solubilized enzymes from their respective immunoadsorbents was without releasing effect on the vesicles supports the assumption of secondary interactions.

Rechromatography. Rechromatography of a

nonadherent fraction resulted in binding of another portion of vesicles, ending up with a new nonadherent fraction enriched in membranes not able to bind to the immunoadsorbent. After several cycles of rechromatography a fraction without ability to bind to the immunoadsorbent could be isolated. This fraction represents the impurity in the microvillus membrane preparation.

Rechromatography of a once adherent fraction resulted in rebinding to the immunoadsorbent, indicating that the sites participating in the binding of the vesicles to the immunoadsorbent were not impaired during binding and the subsequent elution.

Properties of the purified microvillus membrane vesicles

Purification of right-side-out microvillus membrane vesicles by immunoadsorbent chromatography using an antibody raised against three of the principal brush border hydrolases was followed by measuring marker enzymes, determination of lipid composition and electron microscopy.

Enzyme markers. The purity of the starting material, a microvillus membrane preparation, was as described previously [1]. The only impurity in the preparation as judged from measurements of marker enzymes was basolateral membrane fragments.

As seen in Table I, a reproducible increase in the activity of the aminopeptidase and a decrease in the activity of 5′-nucleotidase could be obtained, which indicates that the adherent fraction represents a purified microvillus fraction. The adherent fraction was, however, still enriched in 5′-nucleotidase activity compared to the homogenate, as the enrichment factor of about 6.3 [1] was only lowered by a factor of 0.7. However, as stated by Colas and Maroux [6] for rabbit enterocyte membranes, 5′-nucleotidase is, although mainly located in the basolateral membrane, an enzyme shared by the two types of membrane. The ouabain-sensitive $(Na^+ + K^+)$-ATPase is strictly localized to the basolateral membrane [6] and is therefore a better marker enzyme for this membrane. However, this enzyme was completely inactivated after 18 h at 4°C. Attempts to measure the ouabain-sensitive K^+-dependent phosphatase activity, an activity exhibited by the $(Na^+ + K^+)$-ATPase [25], shows, in contrast to the findings of Colas and Maroux [6] on rabbit enterocyte membranes, that also this activity was inactivated during the incubation. When the incubation time was cut to 1.5 h, resulting in a halving of the yield of the adherent fraction, it was possible to use the ouabain-sensitive $(Na^+ + K^+)$-ATPase as marker enzyme. Under this condition, the control suspension had a 50% decrease in activity. The nonadherent fraction was enriched by a factor of 2 in ouabain-sensitive $(Na^+ + K^+)$-ATPase activity compared to the control, whereas the activity in the adherent fraction was zero, indicating that this fraction was essentialy free from basolateral membrane contamination.

Of the other digestive enzymes known to be localized in the brush border, sucrase-isomaltase was in some cases also measured. We found that the adherent fraction was enriched to the same extent as the aminopeptidase, whereas the nonadherent fraction show a decrease in the specific activity of this enzyme similar to that of the aminopeptidase.

Lipid composition. The nonadherent fraction was enriched in membranes with a protein composition different from that of the adherent fraction as shown by marker enzyme measurements; but also differences in lipid composition could be demonstrated in experiments were the nonadherent fraction was rechromatographed until all vesicles able to adhere to the beads were removed. After five cycles of rechromatography a membrane fraction without any ability to bind to the immunoadsorbent was isolated (pure nonadherent fraction). This membrane fraction constitutes about 10% of the

TABLE I

ENRICHMENT FACTORS OF THE FRACTIONS FROM THE IMMUNOADSORBENT CHROMATOGRAPHY

The columns were incubated with 50 U membrane-bound aminopeptidase as described in Materials and Methods. The values are the ratio between the specific activity of the control suspension and the fractions, and are the mean ± S.E. of eight experiments.

Fraction	Aminopeptidase	5′-Nucleotidase
Adherent	1.17 ± 0.02	0.70 ± 0.05
Nonadherent	0.87 ± 0.02	1.24 ± 0.04

TABLE II

LIPID COMPOSITION OF THE DIFFERENT MEMBRANE FRACTIONS OBTAINED BY IMMUNOADSORBENT CHROMATOGRAPHY OF A MICROVILLUS MEMBRANE PREPARATION

The values, expressed as % of total lipid, are the mean of double determinations from two different preparations.

Fractions	Control	Adherent	Pure nonadherent
Cholesterol	16	15	21
Phosphatidylethanolamine	13	13	16
Phosphatidylinositol	5	6	7
Phosphatidylserine	5	5	6
Phosphatidylcholine	16	13	22
Sphingomyelin	6	6	14
Monohexosylceramide	6	7	5
Dihexosylceramide	21	21	8
Pentahexosylceramide	12	14	2

total protein in the microvillus membrane preparation and consists mainly of basolateral membranes as judged from the activity of marker enzymes. Thus, enrichment factors of 0.09 for aminopeptidase and 2.7 for 5′-nucleotidase (mean of two experiments) were obtained in comparison to the control. The finding that aminopeptidase is still present in this fraction, although in quantities too small to allow binding to the immunoadsorbent, is in accordance with the results of Colas and Maroux [6]. They found that basolateral membranes from rabbit enterocytes purified by gradient centrifugation contain small amounts of all the characteristic digestive enzymes of the brush border membrane.

The lipid composition of the purified microvillus membranes and the impurity was compared to that of the original microvillus membrane preparation (Table II). The pure nonadherent fraction had a higher cholesterol and phospholipid content and a lower glycolipid content than the adherent fraction, the lipid composition of which was very similar to that of the original microvillus membrane preparation. The increase in phospholipid content of the nonadherent fraction was mainly due to a doubling of the amount of choline-containing phospholipids, especially the sphingomyelin content which was almost tripled. The decrease in glycolipid content was due to a drastic decrease in the amount of dihexosylceramides and pentahexosylceramides.

The difference in the lipid composition of the adherent and pure nonadherent fractions agrees with the difference in gross lipid composition of rat and mouse microvillus and basolateral membrane lipids [26,27]. The lipid composition together with the enrichment factors of the marker enzymes implies that the adherent fraction represents the microvillus region of plasma membrane, and the pure nonadherent fraction consists mostly of basolateral membranes.

The extremely low content of fucose-containing pentahexosylceramides [1] in the pure nonadherent fraction in comparison to the purified microvillus membrane fraction may indicate that these complex glycolipids are not part of the basolateral membrane, but may be recognized as marker lipids of the microvillus part of the pig intestine plasma membrane.

Electron microscopy. The adherent and nonadherent fractions were examined by electron microscopy by thin sectioning (Fig. 3). As described in Materials and Methods, the nonadherent fraction was rechromatographed in order to deplete it of membranes able to bind to the immunoadsorbent. After three chromatographic cycles, the enrichment factor for the final nonadherent fraction was 0.57 for aminopeptidase and 2.19 for 5′-nucleotidase as compared to the control. The enrichment factors for the three successive adherent fractions were equal and the same as those obtained for the purified microvillus fraction. No morphological difference between the three fractions was observed and therefore electron micrographs of only one of these fractions are given.

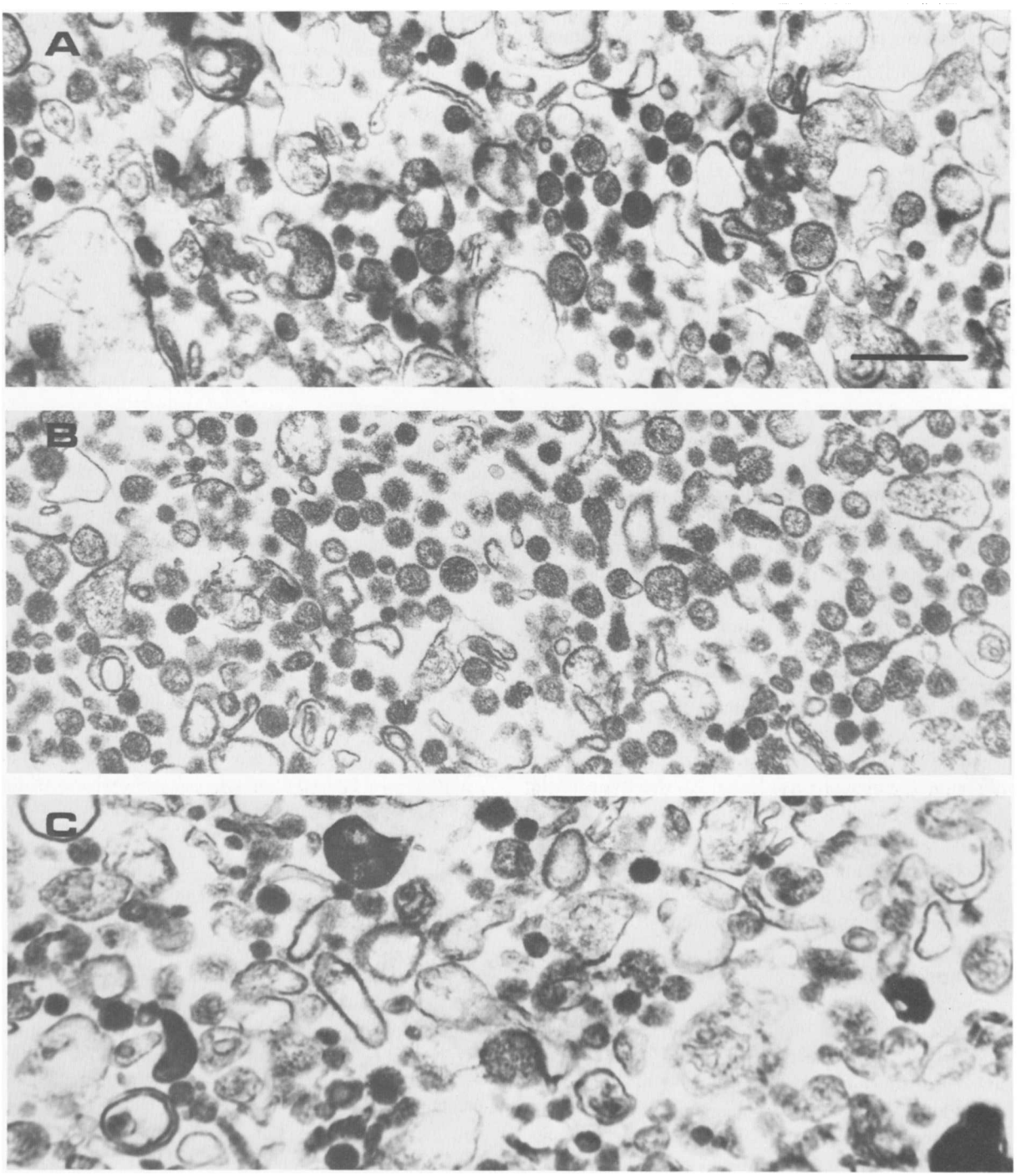

Fig. 3. Electron micrographs of fractions obtained by immunoadsorbent chromatography of a microvillus membrane preparation from pig small intestine. (A) Control microvillus preparation, (B) the adherent fraction, (C) the nonadherent fraction obtained after three successive cycles of rechromatography. The bar represents 0, 5 μm.

No difference could be observed between the control suspension (Fig. 3A) and a microvillus membrane vesicle preparation fixed immediately after its isolation. As already observed [1], the preparation consisted mostly of spherical vesicles filled with fibrous material, but also structures without, or partly filled with, fibrous material as well as elongated structures were seen.

The adherent fraction (Fig. 3B) consisted almost entirely of filled or partly filled microvillus membrane vesicles easily recognized by their size and density [6] in contrast to the nonadherent fraction (Fig. 3C) where the amount of filled vesicles was considerably lower. However, after only three cycles of rechromatography, there were still microvillus membrane vesicles present in the final nonadherent fraction in agreement with the reduced but still significant aminopeptidase activity.

General conclusions. By means of immunoadsorbent chromatography, a fraction of the microvillus membrane preparation is able to adhere to the antibody-coated beads, whereas the preparation contains structures unable to adhere. From marker enzyme activities, morphology and lipid composition we conclude that the fraction able to adhere represents a purified microvillus membrane vesicle fraction, virtually free from contamination with other membrane structures.

As the antibody used in the immunoadsorbent chromatography is raised against the hydrophilic form of three of the principal proteins of the brush border, the purified vesicles we obtain are vesicles which have antigens localized on the outside of the vesicles, and must therefore represent right-side-out vesicles with respect to the original brush border.

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