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# ON THE PREPARATION AND SOME PROPERTIES OF CLOSED MEMBRANE VESICLES FROM HOG DUODENAL AND JEJUNAL BRUSH BORDER

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## SUMMARY

Closed and nearly spherical vesicles were obtained from both hog duodenum and jejunum after mucosa homogenization in the absence of EDTA and a series of fractional centrifugations. The vesicles were found to contain large amounts of two of the characteristic enzyme markers of the brush border membrane (aminopeptidase and alkaline phosphatase). They were seen by electron microscopy on thin sections or after negative staining to be composed of an apparently intact, 90–100 Å-thick membrane overlaid by the fuzzy coat and to be partly filled by a fibrous material tentatively identified with the cross-filaments of the microvilli. This filling was not removed by 5 mM EDTA or/and 1 M Tris unless the structure of the vesicles was largely destroyed. Very few empty vesicles were obtained at the end of these treatments.

The vesicles from hog duodenum and jejunum were observed to contain nearly 2 molecules of cholesterol for 1 molecule of phospholipids. Specific differences were noted between both types of vesicles at the level of their sugar composition and associated enzyme activities. For instance, the jejunal vesicles contained no sialic acid and no enterokinase. They contain, respectively, 2 and 4 times as much alkaline phosphatase and aminopeptidase as duodenal vesicles.

## INTRODUCTION

The membrane of the intestinal brush border is known to be involved in a number of active transport processes. It has been demonstrated with the aid of isolated bacterial membranes[1] that these processes can be successfully investigated *in vitro* when the intact membrane is obtained in the form of vesicles.

Several methods using specific enzyme markers [invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) and other glycoside hydrolases; alkaline phosphatase (EC 3.1.3.1); an aminopeptidase active on L-leucyl-β-naphthylamide* ] have already

* It is not yet clear whether this enzyme is a leucyl peptide hydrolase (EC 3.4.1.1) or an aminoacyl oligopeptide hydrolase (EC 3.4.4.2). Information about the specificity of the pure enzyme will be available in the near future.

been described for the isolation of the brush border membrane. After homogenization of the mucosa in 0.3 M sucrose, the membranes were found to sediment with the microsomal fraction[2,3], except in one case[4] where they could be characterized in several fractions, including the nuclei fraction. Further purification of the membrane was not pursued after homogenization in sucrose. By contrast, an important step forward was made when mucosa homogenization in 5 mM EDTA, either alone[5,6] or in admixture with 0.3 M sucrose[7], was recognized to generate large organelles designated "brush borders", composed of the microvillous plasma membrane, core and terminal web. These brush borders could be readily sedimented by low-speed centrifugation and freed from the fibrous core by sucrose or glycerol density gradient centrifugation after incubation in an hypotonic medium containing EDTA[6] or in 1 M Tris[5,8,9].

This latter technique using EDTA has so far been considered as quite effective for the purification of the brush border membrane. The membrane has been reported to be obtained in the form of sheets or empty vesicles of varying size and shape. The main purpose of this work is to show that vesicles can readily be obtained from both hog duodenal and jejunal brush border after homogenization in 0.25 M sucrose in the absence of EDTA. These vesicles are limited by an apparently intact membrane, overlaid with the fuzzy coat[10,11] and partly filled by a fibrous material apparently attached to the internal side of the membrane and perhaps identifiable with the cross-filaments of the microvilli[12,13].

## METHODS

### *Analytical techniques*

The membrane preparations were solubilized in a 20 mM sodium phosphate buffer (pH 7.2) containing 1% sodium dodecyl sulfate. The proteins were evaluated, either spectrophotometrically by 2 successive readings at 280 and 260 nm using Warburg's table[14] and factor F, or with the aid of the technique of Lowry *et al.*[15] by reference to bovine serum albumin. Results obtained with both techniques were in fair agreement.

Nucleic acids were precipitated by 10% cold trichloroacetic acid containing bovine serum albumin as coprecipitant, washed 3 times by 5% trichloroacetic acid and hydrolysed in 5% trichloroacetic acid at 90 °C for 20 min[16]. RNA was evaluated with the aid of the orcinol color reaction[17] by reference to purified yeast RNA (Type III, Sigma Chemical Co). DNA was determined colorimetrically with diphenylamine[18] by comparison with a calf thymus DNA sample obtained from Sigma Chemical Co.

Phosphorus was determined colorimetrically[19] in the total lipid fraction extracted according to Folch *et al.*[20]. The result was multiplied by a factor of 25 to obtain the phospholipid content of the fraction. Cholesterol was measured spectrophotometrically[21] in the same extracts.

Neutral sugars, amino sugars and sialic acid were determined, respectively, by the phenol–sulfuric acid method[22], the Elson–Morgan technique[23] modified by Professor J. Montreuil (personal communication) and the thiobarbituric acid reaction[24]. These methods were applied to membrane preparations from which sucrose serving for gradient centrifugations was carefully removed by passage through

Sephadex G-25 coarse equilibrated with a 10 mM Tris–HCl buffer (pH 7.3) containing 0.15 M NaCl and 10 mM $MgCl_2$ (Buffer A) and by a 48-h dialysis against several changes of Buffer A. Membrane recovery from the Sephadex column was nearly quantitative.

*Enzyme activity determinations*

Aminopeptidase activity was measured colorimetrically[7] with the substrate L-leucine β-naphthylamide (Cyclo Chemical) or spectrophotometrically[25] with the substrate L-alanine *p*-nitroanilide (Cyclo Chemical). In this latter case, the Tris–HCl buffer recommended by the authors was replaced by a 50 mM phosphate buffer at pH 7.0. Alkaline phosphatase was tested against solutions of *p*-nitrophenyl phosphate in a 0.1 M Tris buffer (pH 8.5) containing 0.4 M NaCl. Hydrolysis was followed spectrophotometrically at 410 nm. Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase (EC 1.9.3.1)) and NADPH:cytochrome *c* reductase (rotenone insensitive) (reduced-NADP:(acceptor) oxidoreductase (EC 1.6.2.3)) were also determined spectrophotometrically[26,27]. Cytochrome *c* and rotenone were obtained from Sigma and NADPH, from Boehringer.

Enzyme units were defined in each case as the amount of enzyme inducing the disappearance of 1 nmole of substrate (or the formation of 1 nmole of product) per min under the conditions of the assay.

*Centrifugation*

A Sorvall Superspeed refrigerated centrifuge Model RC 2B equiped with a SS 34 angle rotor (8 × 50 ml) was employed for low-speed centrifugations. High-speed centrifugations were carried out in a Spinco-Beckman preparative centrifuge Model L2 65 B with the 50 Ti angle rotor. The 60 Ti angle rotor was used for larger volumes. Gradient centrifugations were also performed in a Spinco-Beckman centrifuge equiped in this case with a swinging bucket rotor SW 27. The *g* values mentioned below correspond to the acceleration calculated for the bottom (Sorvall) or the top (Spinco-Beckman) of the tubes.

*Further treatment of the brush border vesicles*

The brush border vesicles contained in Fraction II (see Table I and text) were sedimented by high-speed centrifugation and the resulting pellet was suspended by gentle stirring (protein concentration of the suspension 5 mg/ml) in one of the following solutions: (a) 10 mM Tris–HCl buffer at pH 7.3; (b) 1 M Tris–HCl buffer at pH 7.3; (c) 5 mM EDTA at pH 7.0. Incubations were performed at 4 °C for 30 min. In a 4th series of assays designated by the letter d, a solution 2 M in Tris and 5 mM in EDTA was added dropwise to incubated suspension c so that the final Tris concentration was 1 M for a constant 5 mM EDTA concentration. Incubation was pursued under these conditions for an additional period of 30 min.

*Electron microscopy*

Samples for electron microscopy were prepared by fixation of a pellet with 1% $OsO_4$ in acetate–veronal buffer (pH 7.2), containing 2.4 mM $CaCl_2$ and 0.06 M NaCl. Fixation was continued for 15 h at 25 °C. The pellet was block-stained with uranyl acetate (0.5% in acetate–veronal buffer), dehydrated in ethanol and embedded

in Epon. All sections were doubly stained (uranyl acetate and lead citrate) and finally examined in an electron microscope Siemens Elminskop I operated at 80 kV with a double condenser and 50 $\mu$m objective aperture.

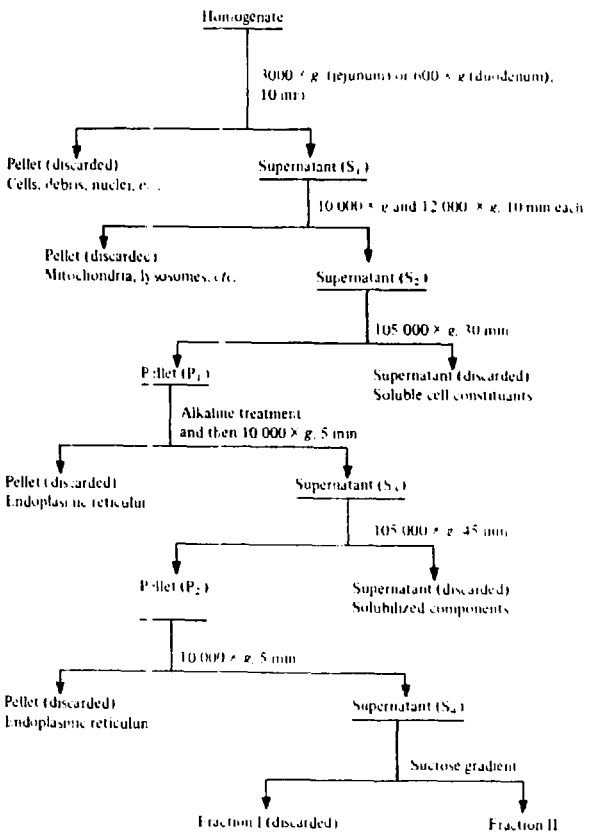
Negatively stained material was prepared as follows: a small drop of membrane suspension was placed on the carbon-coated grid of the microscope and left for a few seconds. The excess of liquid was removed by touching the side of the grid with filter paper. Then, the grid was wetted with a few drops of uranyl acetate (1% in water) and immediately air dried.

## RESULTS

### *Preparation of membrane vesicles*

#### *Mucosa homogenization and subsequent centrifugation of the homogenates*

The homogenizations and centrifugations described below were all carried out at 4 °C. Hog jejunum or duodenum (3 or 20, respectively) were removed at the slaughterhouse immediately after the death of the animals, freed from most attached mesenteric and adipose tissue, washed with ice-cold Buffer A, placed on cold glass plates and opened longitudinally with scissors. The mucosa was gently scraped off with a microscope slide and the collected scrapings (about 200 g) were suspended by mild magnetic stirring in 6 times (jejunum) or 4 times (duodenum) their weight of a 10 mM Tris–HCl buffer (pH 7.3) containing 1 mM $CaCl_2$, 10 mM $MgCl_2$ and 0.25 M sucrose. The suspensions obtained from jejunum or duodenum were homogenized by 2 or 1 stroke, respectively, of a motor-driven Teflon–glass homogenizer (clearance, $5.5 \cdot 10^{-3}$–$7.5 \cdot 10^{-3}$ inch) operated at 300 rev./min. After several filtrations through gauze to remove a part of the mucus, the resulting fluids were further homogenized by one additonal up and down stroke of the pestle and the final homogenates were fractionated by several centrifugations under varying conditions. A complete flow sheet of the fractionation procedure is given in Scheme 1.




Scheme 1. Flow sheet of the purification of the membrane vesicles originating from hog duodenal and jejunal mucosa.

Some observations on the composition and enzyme content of the fractions are presented in Tables I and II. Aminopeptidase and alkaline phosphatase were used as specific markers of the brush border membrane[28,29] whereas mitochondria and endoplasmic reticulum (rough or smooth) were characterized, respectively, by cytochrome *c* oxidase[30] and NADPH:cytochrome *c* reductase[31]. The absorbance ratio at 280 and 260 nm expressed the relative content of the solutions in proteins and nucleic acids. DNA was regarded as an index of the nuclei content of the fractions whereas RNA could originate from rough endoplasmic reticulum, polysomes and

TABLE I

COMPOSITION AND ENZYME CONTENT OF VARIOUS FRACTIONS FROM HOG JEJUNUM MUCOSA

The separation of the fractions is described in Table I. For each enzyme, the first figure indicates the number of units found in the corresponding fraction per 100 units in the homogenate. The second figure in parentheses gives the specific activity (number of enzyme units per mg protein) of the enzyme in the fraction. The figures are the average of 6 assays for aminopeptidase, alkaline phosphatase and proteins; of 2 assays for cytochrome oxidase, cytochrome reductase and DNA. The indicated $A_{280}/A_{260}$ ratio values are averaged from 9 experiments. *A*, absorbance.

| *Fraction* | *Amino-peptidase* | *Alkaline phosphatase* | *Proteins* | *DNA* | *Cytochrome oxidase* | *Cytochrome reductase* | $A_{280}/A_{260}$ |
|---|---|---|---|---|---|---|---|
| Homogenate | 100 (126) | 100 (118) | 100 | 100 | 100 (35) | 100 (5.5) | 0.705 |
| Supernatant $S_1$ | 86 (350) | 78 (285) | 31 | — | 10 (9.2) | 76 (7.7) | 0.773 |
| Supernatant $S_2$ | 57 (310) | 48 (256) | 22 | 0 | — | 56 (8.5) | 0.775 |
| Supernatant $S_3$ | 51 (1280) | 45 (1190) | 4.8 | — | — | — | 0.965 |
| Supernatant $S_4$ | 33 (1610) | 33 (1469) | 2.6 | — | 0.3 (3.6) | 1.8 (1.7) | 1.20 |
| Fraction II | 25 (2100) | 24 (1900) | 1.5 | 0 (also no RNA) | 0.05 (1.0) | 0.25 (1.2) | 1.43 |

TABLE II

COMPOSITION AND ENZYME CONTENT OF VARIOUS FRACTIONS FROM HOG DUODENAL MUCOSA

For details, see legend of Table I. The $A_{280}/A_{260}$ ratio values were averaged in this case from 13 assays, from 9 assays for aminopeptidase, alkaline phosphatase and proteins and from 3 assays for cytochrome oxidase, cytochrome reductase, DNA and RNA.

| *Fraction* | *Amino-peptidase* | *Alkaline phosphatase* | *Proteins* | *DNA* | *RNA* | *Cyto-chrome oxidase* | *Cyto-chrome reductase* | $A_{280}/A_{260}$ |
|---|---|---|---|---|---|---|---|---|
| Homogenate | 100 (62) | 100 (83) | 100 | 100 | 100 | 100 (126) | 100 (8.3) | 0.680 |
| Supernatant $S_1$ | 60 (107) | 63 (142) | 34 | 0.5 | — | — | — | 0.855 |
| Supernatant $S_2$ | 50 (127) | 58 (165) | 29 | — | 19 | 8 (17.7) | 86 (19.5) | 0.750 |
| Supernatant $S_3$ | 31.5 (237) | 48 (400) | 9.7 | — | — | — | — | 0.810 |
| Supernatant $S_4$ | 21 (306) | 27 (507) | 4.3 | — | 8.4 | 3 (88) | 10 (20.6) | 0.830 |
| Fraction II | 14.7 (552) | 17.3 (888) | 1.7 | 0 | 2.5 | 0.3 (29) | 1 (5.8) | 0.930 |

free ribosomes. No specific marker for the Golgi membranes was employed. However, this type of membrane could be expected to be easily eliminated in the light fraction of the sucrose gradient because of its high lipid content[32,33] (low density).

Mucus, intact cells, debris and nuclei were first spun down at $3000 \times g$ (jejunum) or $600 \times g$ (duodenum) for 10 min. Supernatant ($S_1$) was centrifuged at $10000 \times g$ for 10 min and then at $12000 \times g$ for an additional period of 10 min, to give a supernatant ($S_2$) containing the largest part of the brush border markers and practically no nuclei.

This latter material was then sedimented at $105000 \times g$ for 30 min to give a pellet ($P_i$) which was submitted to a mild alkaline treatment similar to that described by Meldolesi *et al.*[16] for discharging soluble proteins occluded within the lumen of several types of vesicles prepared from pancreas acinar cells. For this purpose, the pellet ($P_1$) was suspended by gentle hand homogenization in a buffer similar to Buffer A except for an increased NaCl molarity (0.17 M). The resulting suspension was diluted 3.5-fold with a 0.2 M Tris–HCl buffer (pH 7.8) 10 mM in $MgCl_2$. The mixture, in which the proportions were so adjusted as to give a final volume half that of supernatant ($S_2$) was immediately centrifuged at $10000 \times g$ for 5 min to separate a white viscous pellet composed of aggregated endoplasmic reticulum. A 45-min centrifugation at $105\,000 \times g$ sedimented a brush border membrane-containing pellet ($P_2$) which was resuspended by hand homogenization in 10 ml of the modified Buffer A. The suspension was finally centrifuged at $10000 \times g$ for 10 min to separate a second crop of aggregated reticulum and a supernatant ($S_4$).

*Sucrose density gradient centrifugation*

All sucrose solutions contained 10 mM $MgCl_2$ and 10 mM Tris–HCl buffer (pH 7.3). Supernatant $S_4$ was adjusted at $43 \pm 0.1\%$ sucrose (w/w) (1.50 M) by dropwise addition of a 55% sucrose solution and the following step gradient was set up in each tube of a Spinco SW 27 rotor (the first figure indicates the volume and the second in parentheses, the sucrose concentration): 4 ml (55%), 8 ml (44%), 8 ml (43%, in which the membranes were suspended), 8 ml (42%), 6 ml (39%). The remaining empty space of the tubes was filled with a 10% sucrose solution.

Two typical distribution diagrams, one for duodenum and the other for jejunum, after a 5 h centrifugation at 22000 rev./min and 1 °C of Fraction $S_4$ are reproduced in Fig. 1. In both cases, the bulk of the suspended material was observed to partition

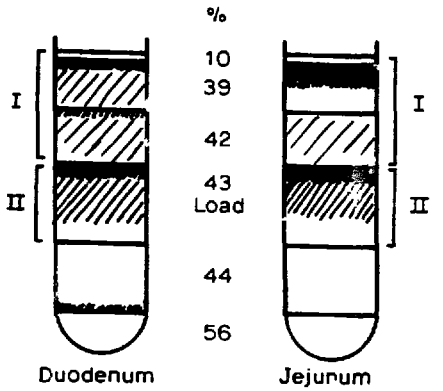



Fig. 1. Distribution of the membranous material after centrifugation in a sucrose step gradient. The sucrose concentration of the layers is given in %. The word "load" designates the 43% layer containing the supernatant ($S_4$) material at the beginning of the assays.

between the 43%, 42% and 39% layers. The denser material migrating into the 44% layer in the case of duodenum was probably an aggregated endoplasmic reticulum contaminant. It was especially abundant when the white viscous pellets mentioned above poorly separated in previous steps.

The centrifuged material was pooled to give two fractions, I and II, as indicated in Fig. 1. Both were characterized by analysis as shown in Table II. Before analysis, the material composing Fraction II was diluted by 5 vol. of Buffer A, collected by a 45-min centrifugation at $105000 \times g$ and taken up in a small volume of Buffer A (approximate protein concentration, 10 mg/ml).

The additional purification effect brought about by the gradient centrifugation can be evaluated in Table III. Fraction II contains the majority of the brush border enzyme markers and only 60–65% of the total proteins of supernatant $S_4$.

TABLE III

COMPARATIVE COMPOSITION OF FRACTIONS I AND II

The letters J and D designate jejunum and duodenum, respectively. The figures (expressed in percentages of the load except for the $A_{280}/A_{260}$ ratio) are averaged from 6 or 9 assays for jejunum and duodenum, respectively.

| *Fraction* | *Proteins* | | *$A_{280}/A_{260}$ ratio* | | *Aminopeptidase* | | *Alkaline phosphatase* | |
|---|---|---|---|---|---|---|---|---|
| | *J* | *D** | *J* | *D* | *J* | *D* | *J* | *D* |
| I | 35 | 26 | 1.07 | 0.92 | 15 | 25 | 20 | 29 |
| II | 65 | 60 | 1.3 | 0.92 | 85 | 75 | 80 | 71 |

* The total did not amount to 100 in this case because of the presence of an appreciable quantity of protein in the heavier fraction passing into the 44% layer (Fig. 1).

A joint examination of the results presented in Tables I–III shows what was really achieved during the fractionation procedure. With jejunal mucosa (Table I), all of the nuclei and 90% of the mitochondria were already removed by the first low-speed centrifugation. The remainder of the mitochondria and nearly all of the endoplasmic reticulum were eliminated by the other conventional and gradient centrifugations finally leading to Fraction II. The 2 markers of the brush border membrane used in the present work, aminopeptidase and alkaline phosphatase, were purified 17-fold when compared with the crude homogenate, with an overall yield of 25%.

When applied to duodenal mucosa (Table II), the same procedure led to somewhat less satisfactory results. The RNA and cytochrome reductase content of the final preparations was consistent with a slight but persistant contamination by endoplasmic reticulum. Hence, before being analyzed as reported in Table IV, the preparations derived from duodenum were further purified by means of a centrifugation in a continuous sucrose gradient. The corresponding technique will be described later (Louvard, D., Maroux, S. and Baratti, J., unpublished).

*Electron microscopy*

Representative pictures at ×120000 and ×180000 magnification of thin sections of the material constituting the 105000 × *g* pellet of Fraction II are presented in Figs 2 and 4. Typical views of the same material after negative staining are reproduced in Fig. 3. The general impression of homogeneity given by the chemical and enzymatic determinations referred to above is seen to be confirmed. Micrographs after negative staining definitely established that the preparations were essentially composed of nearly spherical vesicles with an average diameter of 0.10 $\mu$m (duodenum) or 0.15 $\mu$m (jejunum). Micrographs related to thin sections further indicated that the vesicles were closed and limited by an apparently intact, trilamellar membrane with a 90–100-Å thickness. The external side of the membrane was observed to be overlaid with the fuzzy coat[10,11].

Another point of interest was that most vesicles seemed to be partly filled by an electron-opaque, fibrous material which could be observed in Fig. 4 to be bound to the internal side of the membrane. This latter observation is consistent with its tentative identification with the cross-filaments of the microvilli[12,13]. The longitudinal core-filaments of the microvilli have been reported not to be directly attached to the membrane[12,13]. Moreover, because of their rigidity, their size and their implantation into the terminal web, they can hardly be expected to be confined in small vesicles, except if their structure is partly destroyed. In this case, it might be assumed that short pieces of the core-filaments exist in the vesicles and that they are bound to the membrane by the interposition of cross-filaments.

*Attempts to remove the fibrous filling*

In an attempt to remove the fibrous material described above, vesicles originating from duodenum were incubated with various solutions (a–d). The composition of the solutions is given in *Analytical techniques*. These solutions are known to free the brush borders from their fibrous core[6,8,9]. The resulting suspensions were centrifuged at high speed in a sucrose gradient similar to that already employed to obtain Fraction II. Representative drawings indicating the effect of the incubations on the final distribution of the particles in the gradient are reproduced in Fig. 5.

The first drawing designated "None" (no treatment) in Fig. 5 shows that during this second centrifugation, the bulk of untreated Fraction II remained in the 43% layer whereas a faint trace of turbid material migrated into the 22% layer. This distribution was not appreciably altered after hypotonic treatment (a) in a 10 mM Tris–HCl buffer. By contrast, anyone of the other treatments (b–d) induced the appearance of appreciable quantities of lighter material mostly concentrated in the 39% layer with a slight turbidity in the 42% layer. This proved that a substantial part of the original vesicles have been modified to some extent by these latter treatments.

In order to discern more clearly the nature of these modifications, the content of Tube c (5 mM EDTA) was fractionated with the aid of a fraction collector. Alkaline phosphatase and aminopeptidase activities were found to be strongly reduced in the lighter material of the 39% layer. This result was not unexpected since both activities are known to require $Zn^{2+}$, part of which is probably chelated by EDTA. More significant observations were made by electron microscopy of thin sections of the material related to the 43 and 39% layer (Figs 6A and 6B, respectively). The first

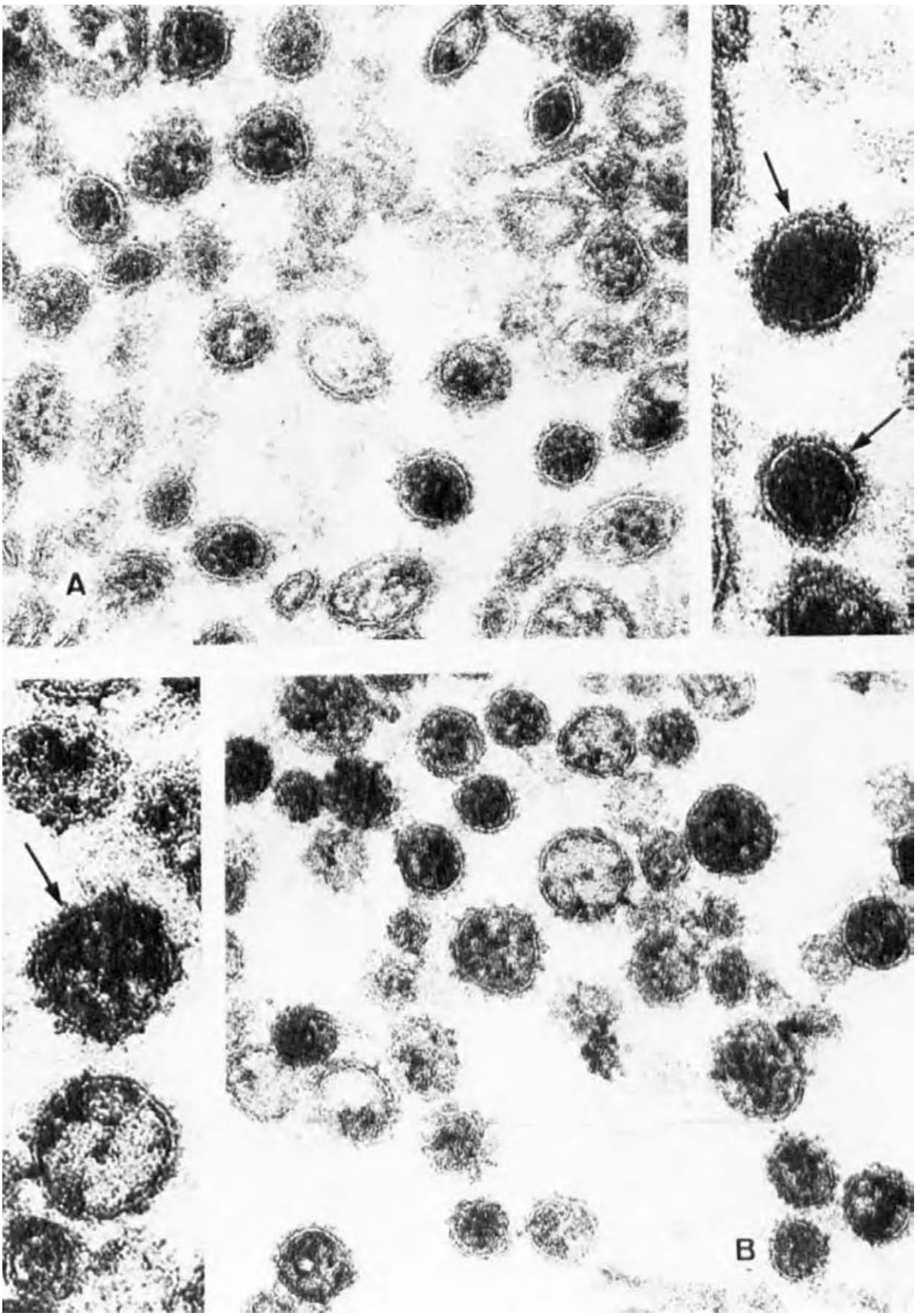



Fig. 2. Representative section through Fraction II pellet. (A) Jejunum. (B) Duodenum. Magnification × 120000 in the main pictures and × 180000 in the two corresponding inlets. The trilamellar structure of the limiting membrane of the vesicles is clearly visible. Arrows indicate the fuzzy coat.

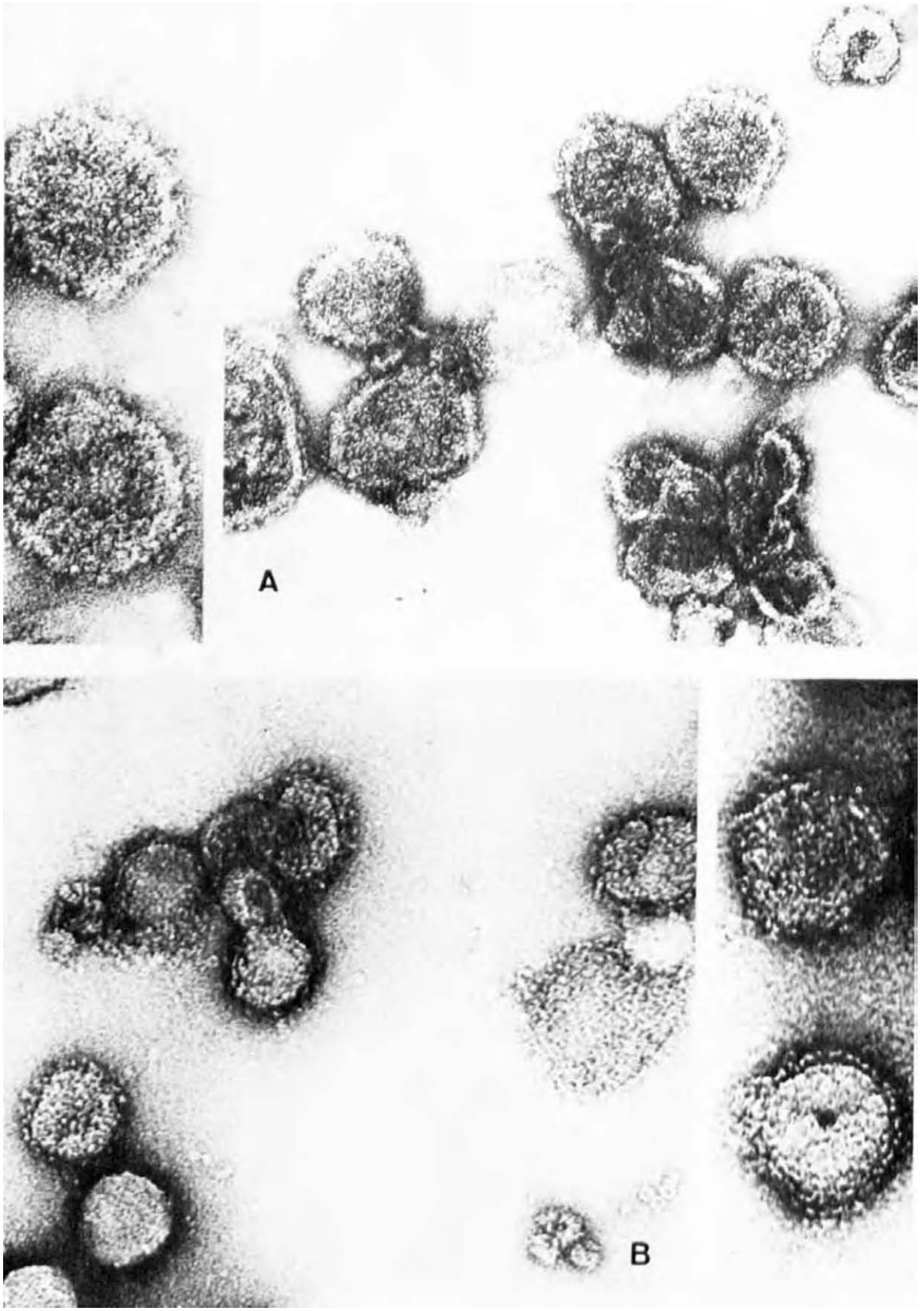



Fig. 3. Negative staining of Fraction II. (A) Jejunum. (B) Duodenum. Magnification ×120000 and ×180000 in the two inlets. The rough aspect of the external side of the vesicle membranes is identifiable to the knobs already described for brush border preparations[34] and believed to contain a part of the membrane enzymes.

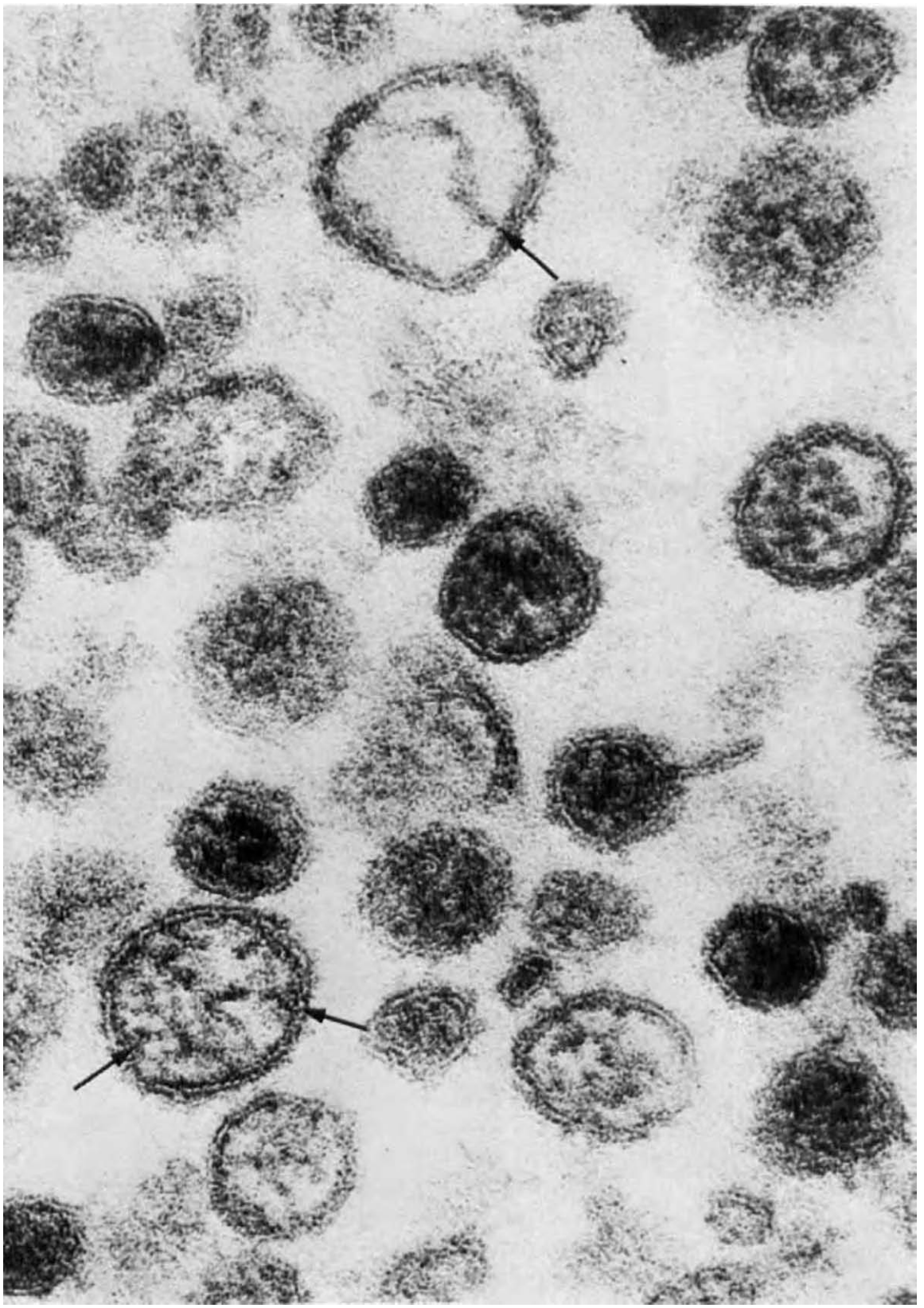


Fig. 4. Section through the pellet originating from Fraction II (duodenum). Magnification × 180000. Binding of the fibrous core to the internal side of the membrane is indicated by arrows.

was observed to be composed of normal vesicles still containing the fibrous core. By contrast, a few empty vesicles and also membrane sheets were visible in the second, with fibres (indicated by arrows) now seen to be outside and apparently independent from the membrane. At this stage, an additional centrifugation would have probably separated the membrane and the core, as it was previously reported for the intact brush borders[5,6,8,9]. However, the morphological aspect of the vesicles was observed to be drastically affected so that experimentation in this direction was discontinued.

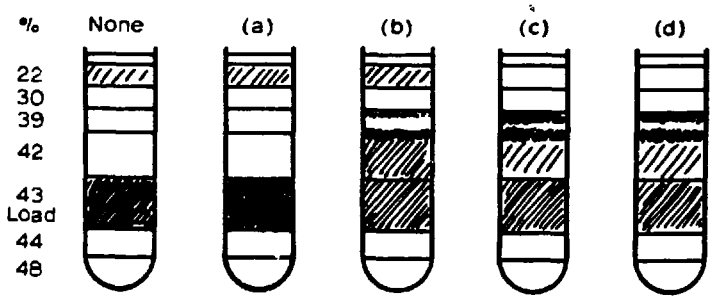



Fig. 5. High-speed centrifugation in a sucrose gradient of the brush border vesicles after incubation with: (a) hypotonic 10 mM Tris-HCl buffer (pH 7.3); (b) 1 M Tris-HCl buffer (pH 7.3); (c) 5 mM EDTA (pH 7.0); (d) 5 mM EDTA in 1 M Tris-HCl buffer (pH 7.3). The blank is marked "none".

Figs 7A and 7B illustrate the morphology of unfractionated vesicles after hypotonic shock or 1 M Tris treatment. They confirm that the hypotonic shock has practically no effect on the vesicles. After incubation with 1 M Tris, a number of apparently intact vesicles are still visible. These vesicles, spherical or elongated, are still filled. But, some empty vesicles and membrane sheets are also present.

*Chemical composition of the vesicles*

It was of interest to quantitate the content of these nearly homogeneous vesicle preparations in some important constituents such as phospholipids, cholesterol and sugars. Results obtained in this respect are indicated in Table IV. They will be discussed in the following section.

## DISCUSSION

The absorbing cells of intestinal mucosa are characterized by the presence of a brush border controlling the passage of dietary products after intraluminar digestion and of electrolytes. The morphology of this brush border is fairly constant along the small intestine, although the role played during absorption by the three parts of the intestine, namely duodenum, jejunum and ileum, is quite different. For instance, fats have been reported to be absorbed by the duodenum and the proximal region of the jejunum[35] whereas sugars[36] and amino acids[37] are absorbed essentially in the jejunum. Bile salts have been shown to be largely reabsorbed by the ileum[38]. It is of interest to try to interpret these important processes in terms of chemical composition, structure and enzyme content of the corresponding brush border membranes.

A new procedure (Scheme 1) is described for the purification of the brush border membrane from homogenates of hog duodenum and jejunum mucosa. It consists in several low- and high-speed centrifugations, the last one being performed in a discontinuous sucrose gradient by the "sandwich" technique. Electron micro-

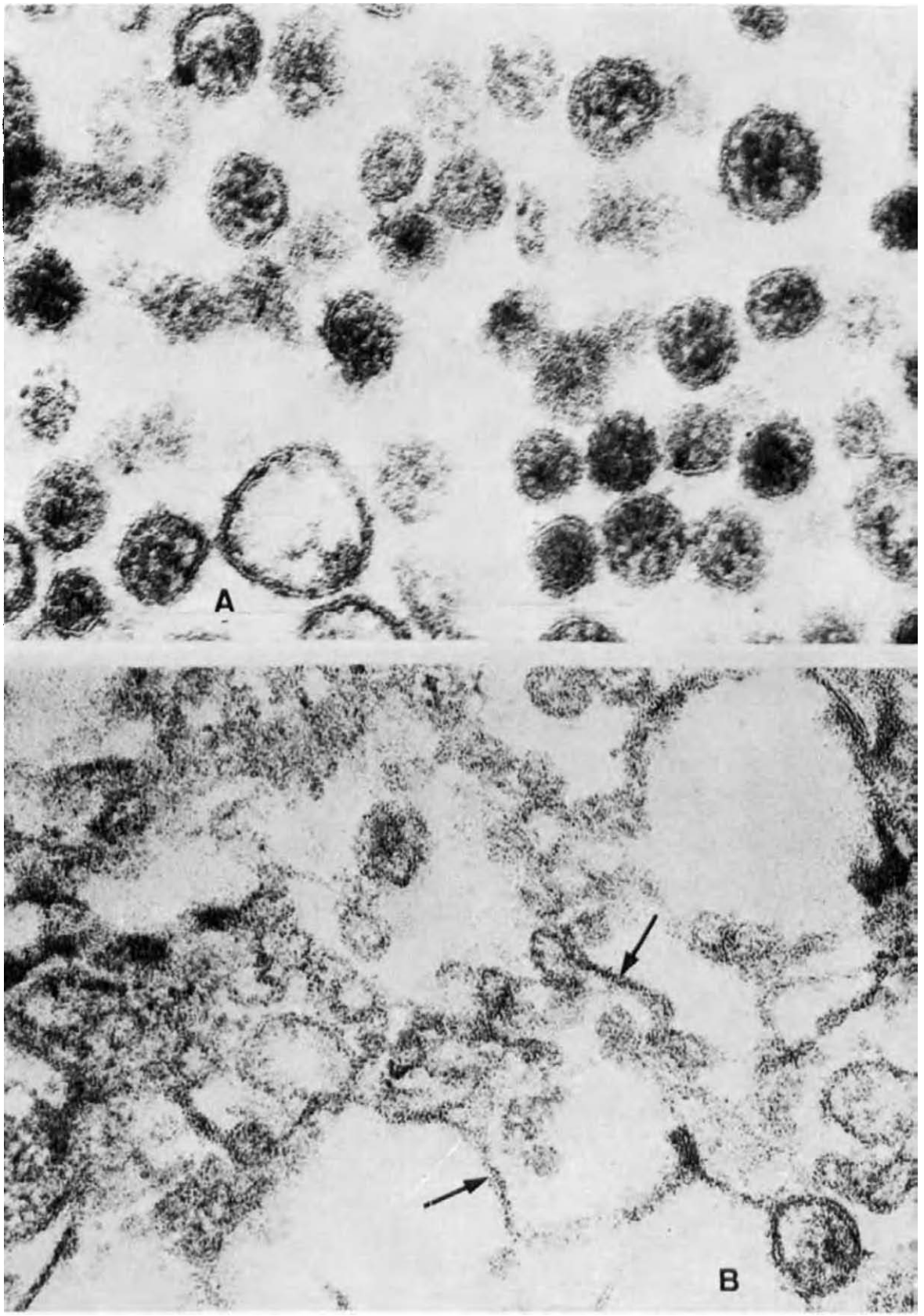



Fig. 6. Effect of EDTA treatment on the vesicles. Electron micrographs (magnification × 120000) on the material remaining in the 43% layer in Fig. 5 (A) or migrating into the 39% layer (B).

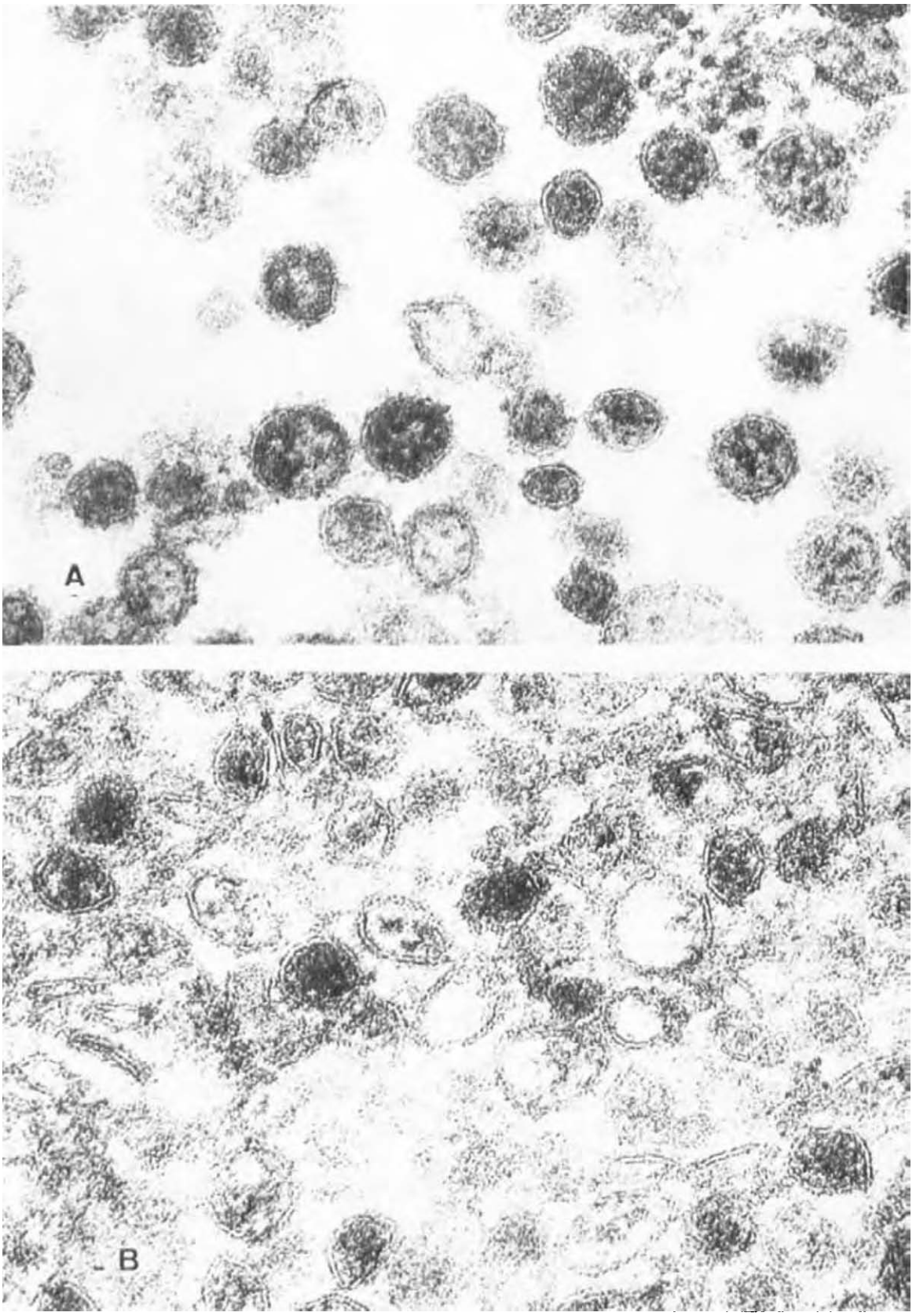



Fig. 7. Electron micrographs (magnification × 120000) on sections after incubation with 10 mM Tris (pH 7.3) (hypotonic shock) (A) or 1 M Tris (pH 7.3) (B).

TABLE IV

CHEMICAL COMPOSITION OF THE MEMBRANE VESICLES

The number of mg and μmoles of the constituents are given per 100 mg total proteins. The column marked "ratios" indicates the molar ratio of the amount of cholesterol over those of phospholipids found in the vesicles.

| *Origin of the vesicles* | *Phospholipids* | | *Cholesterol* | | *Ratios* | *Neutral sugars* | | *Amino sugars* | | *Sialic acid* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *mg* | *μmoles* | *mg* | *μmoles* | | *mg* | *μmoles* | *mg* | *μmoles* | *mg* | *μmoles* |
| Duodenum | 15 | 29.4 | 12.2 | 31.6 | 1.63 | 13.7 | 76 | 4.6 | 25 | 0.9 | 2.9 |
| Jejunum | 11.6 | 14.7 | 11 | 28.4 | 1.93 | 10.5 | 58 | 6.2 | 34 | 0.0 | 0.0 |

graphs after negative staining established that the final preparations were essentially composed of nearly spherical and closed vesicles with a diameter of 0.10–0.15 μm. Observations on thin sections further showed that the vesicles were limited by a trilamellar, 90–100-Å-thick membrane. A 90–100-Å thickness has already been reported for the membrane of the intact brush border[12].

Three points pertaining to this procedure are noteworthy: (a) No metal-chelating EDTA was used during mucosa homogenization and subsequent treatment of the homogenates. As a consequence, the brush border membrane spontaneously formed vesicles. The $Zn^{2+}$-requiring membrane markers, aminopeptidase and alkaline phosphatase, could be securely determined throughout the fractionation and found to be fully active in the vesicles. (b) The vesicles were filled by a fibrous material tentatively identified with the cross-filaments of the microvilli. This material could not be removed by the mild alkaline treatment used by Meldolesi *et al.*[16] for emptying pancreatic vesicles from their cytoplasmic content. EDTA and 1 M Tris generated a few empty vesicles and a majority of membrane sheets. Most fibrous material was not removed unless the structure of the vesicles was modified. The presence of fibres, probably proteic in nature, in our vesicle preparations explain why the purification of the main enzyme markers of the brush border membrane never exceeded 17-fold. (c) One of the difficulties encountered during the purification was to eliminate the last traces of endoplasmic reticulum. In this respect, aggregation of the reticulum by $Ca^{2+}$ [39] was found very helpful.

Finally, it is of interest to comment briefly on the analogies and specific differences existing between the chemical composition and enzyme content of the vesicles prepared, respectively, from duodenal and jejunal mucosa. Table IV indicates that the phospholipid content of both types of vesicles is approximately the same and also very similar to that normally found in other membranes. By contrast, both duodenal and jejunal membranes appear to be especially rich in cholesterol. In this respect, the results presented here are somewhat higher than those previously obtained with rat intestine[40]. They suggest that the brush border membrane contains 2 molecules of cholesterol for one molecule of phospholipid, whereas a 1:1 molar ratio is most frequently encountered for other membranes.

Table IV further shows that the neutral sugar–amino sugar molar ratio is twice as high in duodenal as in jejunal vesicles. The significance of this difference cannot

yet be evaluated. However, it is noteworthy that, in contrast with duodenal vesicles, jejunal vesicles contain no sialic acid. Enterokinase, an enzyme specific for duodenum contains 2% sialic acid, whereas this acid is not a constituent of aminopeptidase mostly found in jejunum (Maroux, S. and Louvard, D., unpublished results).

Further interesting differences between duodenal and jejunal vesicles were found at the level of their enzyme content. Enterokinase, recently shown to be responsible for the *in vivo* activation of pancreatic zymogens[41], has been observed, at least in the pig, to be exclusively restricted to duodenal vesicles (Louvard, D., Maroux, S. and Baratti, J., unpublished). By contrast, the results presented in Tables I and II prove that, always in the pig, jejunum contains approximately twice as much alkaline phosphatase and 4 times as much aminopeptidase as duodenum. This latter enzyme has very recently been obtained in a pure state and shown to constitute as much as 8% of the total proteins of the jejunal vesicles. The actual level of the enzyme in the membrane itself should be still higher since an appreciable proportion of the proteins in our vesicles is probably contributed by the fibrous core.

Hence, the enzymatic equipment of the 3 regions of the intestine appears to differ more than usually realized and to be well adapted to their biological function. It is tempting for instance to speculate that jejunal aminopeptidase is not only involved in the final hydrolysis of peptides but also in the active transport of the liberated amino acids through the membrane. Experiments are in progress in our laboratory to check this assumption.

From a more general point of view, it is hoped that vesicles such as these described in the present paper will be useful for future *in vitro* investigations of active transport processes at the level of the microvilli.

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