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# Isolation of Heavy Endosomes from Dog Proximal Tubules in Suspension

**V. Marshansky[1], A. Fleser[1], J. Noël[1], S. Bourgoin[2], P. Vinay[1]**

[1]Nephrology Laboratory, Centre de Recherche Louis-Charles Simard, Hôpital Notre-Dame de Montréal; Groupe de Recherche en Transport Membranaire, Université de Montréal, Montréal, Québec H2L 4M1, Canada

[2]Centre de Recherche en Rhumatologie et Immunologie, Centre de Recherche du CHUL, Sainte-Foy, Québec G1V 4G2, Canada

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**Abstract.** During the preparation of a suspension of dog kidney proximal tubules by collagenase treatment, an uptake of FITC-albumin was demonstrated. This process is attributed to the activation of receptor-mediated endocytosis leading to the appearance of FITC-albumin into intracellular vesicular structures. The isolation of brush border membrane vesicles (BBMV) from the dog kidney proximal tubules in suspension by the magnesium precipitation technique leads to the copurification of a large population of endosomes. These endosomes were separated from BBM vesicles by a technique involving wheat-germ agglutinin. The enrichment in BBM markers and in bafilomycin-sensitive ATPase activity was comparable in endosomes and BBM vesicles. However, the acridine orange acidification assay showed a V-type ATPase-dependent acidification in endosomes but not in BBMV, demonstrating a different orientation of the proton pumps in these structures. SDS-PAGE analysis also showed significant differences in protein pattern of vesicles and endosomes. The most notable difference was the presence of 42–44 kDa and 20–24 kDa proteins in BBMV and their complete absence in endosomes. Western blot analysis identified these proteins as actin and RhoA, among other small proteins, respectively. Western blot experiments also demonstrated a different distribution of β-COP, β-adaptin, and RhoGDI in vesicles and endosomes. The morphological aspect (electron microscopy) and sedimentation of endosomes in a 50% Percoll gradient identified these structures as ''heavy endosomes'' (buoyant density $D = 1.036$ g/ml). Flow cytometry analysis of heavy endosomes purified from tubules isolated in presence of FITC-albumin showed the presence of FITC-albumin in up to 92% of these intracellular organelles. Western blot analysis using anti-FITC and anti-collagenase antibodies allowed quantification of the FITC-albumin and collagenase A in the purified endosomes. Our results indicate that heavy endosomes are formed during the preparation of the proximal tubules following activation of receptor-mediated endocytosis, probably by soluble proteins. The suspension of tubules thus offers a experimental tool to study the protein reabsorption and traffic of endosomal vesicles in the proximal tubules.

**Key words:** Kidney cortex — Proximal tubules — Wheat-germ agglutinin — Endosomes — BBM Vesicles — V-type $H^+$-ATPase — FITC-albumin — Collagenase A

## Introduction

Since its original description [45], suspensions of proximal tubules are widely used to study *in vitro* many aspects of the function of this nephron segment. We have used this preparation extensively to evaluate the contribution of the activity of membrane-bound ATPases such as the $Na^+$,$K^+$-ATPase and $H^+$-ATPase on the proximal cell ATP turnover [25, 29, 30, 46]. During these studies, we found that a large fraction of the cell energy expenditure was suppressed by bafilomycin i.e., was attributable to the activity of the V-type proton pump [29, 30]. Surprisingly, part of this activity was influenced by the intracellular sodium concentration ($Na_i$) (stimulated by an increment and inhibited by a decrement of $Na_i$) in a manner sensitive to amiloride [9]. This suggested that the pH gradient established by the $H^+$-pumps across vesicular endosomes was modulated by sodium/proton exchangers located on endosomes. This also suggested that a significant fraction of the proton pumps were located on intracellular endosomes in tubular suspensions.

*Correspondence to:* V. Marshansky

In this paper, we describe the spontaneous formation and accumulation of newly formed endosomes occurring during the preparation of proximal tubules. This phenomenon probably follows the stimulation of the mechanisms of protein transport induced by the exposition of the brush-border membrane to the albumin-rich collagenase digestion medium, triggering the activation of receptor mediated endocytosis. Both albumin and collagenase A proteins, present in extracellular medium during preparation of proximal tubules, were found within purified heavy endosomes. This demonstrates that heavy endosomes are not produced during the BBMV preparation but in intact cells through an active protein reabsorption process during the preparation of proximal tubules in suspension. The presence of this large population of endosomes may modify some physiological functions of isolated tubules and explain the reciprocal influence of $Na^+$,$K^+$-ATPase and $H^+$-ATPase activity in intact renal proximal cells [9].

To our knowledge there are no previous publications reporting the purification and characterization of endosomal fractions from proximal tubules in suspension. We report here a protocol that allows us to isolate ''heavy endosomes'' from dog kidney proximal tubules in suspension using a combination of magnesium precipitation and wheat-germ agglutinin negative selection techniques. We demonstrate that these endosomes are derived from BBM, but that their protein pattern differs from that of BBM in a specific fashion, especially in regard to actin, RhoA, RhoGDI and β-adaptin.

## Materials and Methods

### Preparation of Tubular Suspension and FITC-albumin Uptake Experiments

#### *Preparation of Tubules*

Cortical tubules (80–90% proximal) were prepared from slices of dog renal cortical tissue by collagenase digestion as previously described [42, 45]. The final suspension containing 60 mg wet wt per ml was kept at 4°C in Krebs Henseleit saline (KHS) fully gassed with 5% $CO_2$:95% $O_2$ until utilization. In some experiments, sucrose 300 mM was added to the digestion medium in order to increase the osmolarity to 600 mOsm/L.

#### *Fluorescence Microscopy of Tubules*

Nonfluorescent albumin (2 mg/ml) (Control) or fluorescein isothiocyanate-conjugated albumin (2 mg/ml) (FITC-albumin) were added into collagenase digestion medium during the preparation of proximal tubules. Immediately after their preparation, proximal tubules were fixed in 2% paraformaldehyde, 0.1M phosphate buffer (pH = 7.4). FITC-albumin fluorescence in the tubular suspension was examined on a ''Diaplan'' (Leitz Werzlar, W-Germany) inverted fluorescent microscope fitted with a regular objective (PLAN 40/0.65) or with an oil-immersion objective (PLAPO 63/1.40). Fluorescence images were taken with ''Wild MPS 45'' (Wild Heerbrugg Ltd., Switzerland) photoautomat on high-speed Kodak film (PJC 1600 ASA).

### Purification of BBMV and Heavy Endosomes

#### *Preparation of BBMV*

Pieces of superficial renal cortex freshly obtained from dog kidney or freshly prepared cortical tubules were homogenized in 10 ml of homogenization buffer (250 mM sucrose, 1 mM EDTA, 18 mM Tris-(hydroxymethyl)-aminomethane(Tris) adjusted to pH 7.4 with N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES)) per gram of tissue using a glass potter fitted with a Teflon pestle in the presence of protease inhibitors (0.1 μM aprotinin, 1 μM pepstatin A, 10 μM chymostatin and 100 μM phenylmethyl-sulfonyl fluoride (PMSF)). A sample of the homogenate was kept for enzymatic measurements. BBM vesicles (BBMV) were prepared from this homogenate by magnesium precipitation as previously described [1, 29]. In some cases the same renal tissue was used to prepare BBMV from cortex or tubules. The vesicles were kept in liquid nitrogen for no more than two months. This material was used to measure enzymatic activities and proton transport. An enrichment factor (E.F.) in specific BBM markers was calculated from the values obtained in BBMV and homogenate. Protein concentrations were measured with the BCA assay (Pierce, Rockford, IL) using albumin as standard.

#### *Separation of BBMV from Endosomes*

The wheat-germ agglutinin (WGA) negative selection technique described by T.G. Hammond and P.J. Verroust [12] was used to separate endosomes (E) from BBM vesicles (V). VE proteins 10 mg were added to 10 ml of resuspension buffer (150 mM KCl, 5 mM Tris-HEPES, pH 7.4) together with 1 mg WGA creating a protein to agglutinin ration of 10:1. This mixture was left overnight at 4°C with continuous stirring and centrifuged at 14.500 g for 3 min. The pellet contained BBMV and the supernatant contained endosomes which were pelleted by a second centrifugation at 48.000 g for 30 min. All centrifugations were performed at 4°C using a Beckman Model J2-21 high speed centrifuge and JA-20 rotor.

### Characterization of BBMV and Endosomes

#### *Enzymatic activities*

The $H^+$-ATPase activity was taken as the NEM- or bafilomycin (BAF)-sensitive moiety of the ATPase activity measured under optimal conditions at 37°C in presence of oligomycin (inhibition of the mitochondrial ATPase) and ouabain (inhibition of the $Na^+$,$K^+$-ATPase) using a continuous spectrophotometric assay [29]. The activity measured in BBMV suspensions prior to solubilization with a detergent was taken as that of vesicles exposing the ATP-binding sites of the $H^+$-ATPase to the medium i.e., under the configuration of endosomes and membrane sheets. The total $H^+$-ATPase activity was measured upon addition of the detergent (0.1% deoxycholate, concentration without effect on the enzymatic activity). The activity revealed by membrane solubilization was taken as that of BBMV hiding the ATP-binding sites in the intravesicular space i.e., closed according to the physiological polarity. In experiments involving comparison of ATPase activity and proton pumping, the $H^+$-ATPase hydrolytic activity was measured at the same temperature (22–24°C) used for endosomal acidification measurements. Note that the $H^+$-ATPase activity is 2–3 times lower at 22°C

than 37°C (*compare* Tables 1 and 2). The $Na^+,K^+$-ATPase activity was estimated as the ouabain-sensitive ATPase activity. BBM markers such as γ-glutamyl-transpeptidase (GGT) and alkaline phosphatase (AP) as well as a mitochondrial marker such as glutamate dehydrogenase were measured as previously described [29].

### *Proton Transport*

Acidification of the intravesicular space of intact vesicles was followed by measuring the changes in fluorescence of acridine orange at room temperature (22–24°C). Ice-cold vesicles (a mixture of BBMV and endosomes (VE)) were added in 2 ml acridine orange buffer (50 mM Tris-HEPES, 150 mM KCl, 5 mM $MgSO_4$, 2 mM ATP, 5 μM acridine orange adjusted at pH 7.4) to initiate proton transport. Fluorescence measurements were performed using a ''Deltascan Model RFM-2001'' spectrofluorimeter (Photon Technology International, South Brunswick, NJ) with excitation at 450 nm (slit width 1 nm) and emission at 525 nm (slit width 2 nm). Fluorescence was recorded using the Oscar™ software. To compare the acidification elicited by VE prepared from cortex or tubules, the same amount of proteins (100 μg) was added in each assay. Bafilomycin ($10^{-7}$ M) was used to inhibit the $H^+$-ATPase while CCCP ($10^{-5}$ M) and/or nigericin ($2 \times 10^{-5}$ M) were used to dissipate the proton gradient.

### *Determination of Endosomal Density in Percoll*

Endosomes were submitted to a 48.000 g centrifugation in 16% (30 min.) or 50% (60 min.) Percoll with tubes containing a mixture of density marker beads (Pharmacia Biotech™, Uppsala, Sweden). The density of the endosomal fraction was estimated from the position of marker beads of known density.

### *Electron Microscopy*

The heavy endosomal fraction was fixed with 2.5% glutaraldehyde, post-fixed with osmium tetroxide and processed for routine electron microscopy.

### *Small-Particle Flow Cytometry Analysis*

Flow cytometry analysis of heavy endosomes was performed on ''Becton Dickinson FACScan'' flow cytometer (Becton Dickinson, Canada). Data were collected as 5.000 event-list-mode files on a FACScan and were analyzed using LYSYS II software. Before flow cytometry, extravesicular fluorescein was quenched by addition of anti-FITC polyclonal antibodies in optimal quantity as determined in separate experiments.

### *SDS Polyacrylamide Gel Electrophoresis and Western Blotting*

Electrophoresis was performed with 12% SDS-Tris-glycine-polyacrylamide gels (SDS-PAGE) according to Laemmli [23]. Equal protein aliquots (70 μg protein) of BBM vesicles (V) and heavy endosomes (E) separated by the WGA negative selection technique were applied on gel and submitted to electrophoresis. Electrophoretic transfer cells (Hoeffer Scientific Instruments, Canberra Packard Canada, Mississauga, ON, Canada) were used to transfer proteins from the gels to Immobilon PVDF membranes (Millipore, Bedford, MA), as previously described [43]. Before immunoanalysis, nonspecific binding sites were blocked by exposing the membrane to 2% gelatin for 1 h at 37°C. The membranes were incubated with a first antibody against actin (1:2.500), RhoA (1:500), β-adaptin (1:500), β-COP (1:1.000), Rho GDI (1:2.000) and then exposed to peroxidase-conjugated anti-rabbit (1:10.000) or anti-mouse (1:15.000) IgG for 1 h at 37°C in presence of 2% gelatin. The membranes were then washed three times in Tris-buffered saline-Tween solution (25 mMTris-HCL, 190 mM NaCl, 0.15% Tween 20, pH 8.0) and covered with ECL detection reagents (Amersham Life Science, Oakville, ON, Canada) for 1 min at room temperature. Autoradiographs were obtained by exposing Kodak X-Omat film to membranes for 2–30 sec at room temperature. The same membranes were also stained with Coomassie Blue.

### *Identification and Quantification of FITC-Albumin and Collagenase A in Endosomes*

Direct identification of FITC-albumin and collagenase A loaded into heavy endosomes during the preparation of proximal tubules was performed by Western blot using polyclonal anti-FITC and anti-collagenase A antibodies, respectively. Electrophoresis was carried out using ''Mini-Protean II'' (Bio-Rad Laboratories Ltd., Hercules, CA) electrophoresis cell on 7.5% SDS-Tris-glycine-polyacrylamide gels (SDS-PAGE) according to Laemmli [23]. A graphite electroblotter system ''MilliBlot™'' (Millipore, Bedford, MA) was used to transfer proteins from the gels to Immobilon PVDF membranes (Millipore, Bedford, MA). Electroblotting of the proteins was carried out in transfer buffer (96 mM glycine, 20% (v/v) methanol, 10 mM Tris, pH 8.3). Before immunoanalysis, nonspecific binding sites were blocked by exposing the membrane to 5% (w/v) Carnation nonfat dry milk in TBS-Tween buffer (15 mM NaCl, 0.3% (v/v) Tween-20, 5 mM Tris-HCl, pH 7.0) overnight. Sheep anticollagenase (bacterial from *Clostridium histolyticum*) polyclonal antibody (Biodesign International, Kennebunk, ME) was used with 1:500 dilution (22 μg/ml) and rabbit anti-FITC polyclonal antibody (DAKO, Carpinteria, CA) was used with 1:10.000 dilution (7 μg/ml) in the TBS-Tween-albumin buffer (15 mM NaCl, 0.3% (v/v) Tween-20, 3% (w/v) BSA, 5 mM Tris-HCl, pH 7.0) and incubated with the blots for 1 h. After washing the blots four times for 10 min in TBS-Tween buffer, the membranes were incubated with a second antibody (dilution 1:10.000) in TBS-Tween-milk buffer for 1 hr. Mouse horseradish peroxidase-conjugates anti-sheep antibody (Sigma, St. Louis, MO) was used for detection of Collagenase A and donkey horseradish peroxidase-conjugated anti-rabbit antibody (Amersham Life Science, Oakville, ON, Canada) for detection of FITC-albumin, respectively. Membranes were then washed four times for 10 min each in TBS-Tween buffer and were covered with ECL detection reagents (Amersham Life Science, Oakville, ON, Canada) for 1 min. Luminograms were obtained by exposing Fuji RX film to membranes for 2–15 sec. All procedures were made at room temperature. Quantitative densitometry of the X-ray films from the luminograms was performed with a Molecular Dynamics model ''Personal SI'' scanning densitometer interfaced with a ''Power Macintosh 6100/60'' computer. Quantification of FITC-albumin and collagenase A loaded into heavy endosomes was made using collagenase A (from *Clostridium histolyticum;* Boehringer Mannheim, GmbH, W.-Germany) and FITC-albumin (Sigma, St. Louis, MO) calibration curves running simultaneously with the endosomal samples. Quantification analysis was made using ''IP Lab Gel'' software from ''Signal Analysis'', where density of ECL signal was expressed as Volume and computed as: $Volume = \Sigma(Ixy) - NB$, were *Ixy* is the pixel values within the region; *N* is the number of pixels in the region; and *B* is the background value.

## MATERIALS

Ouabain, NEM, oligomycin, nigericin, CCCP, bafilomycin, wheat-germ agglutinin, aprotinin, pepstatin A, chy-

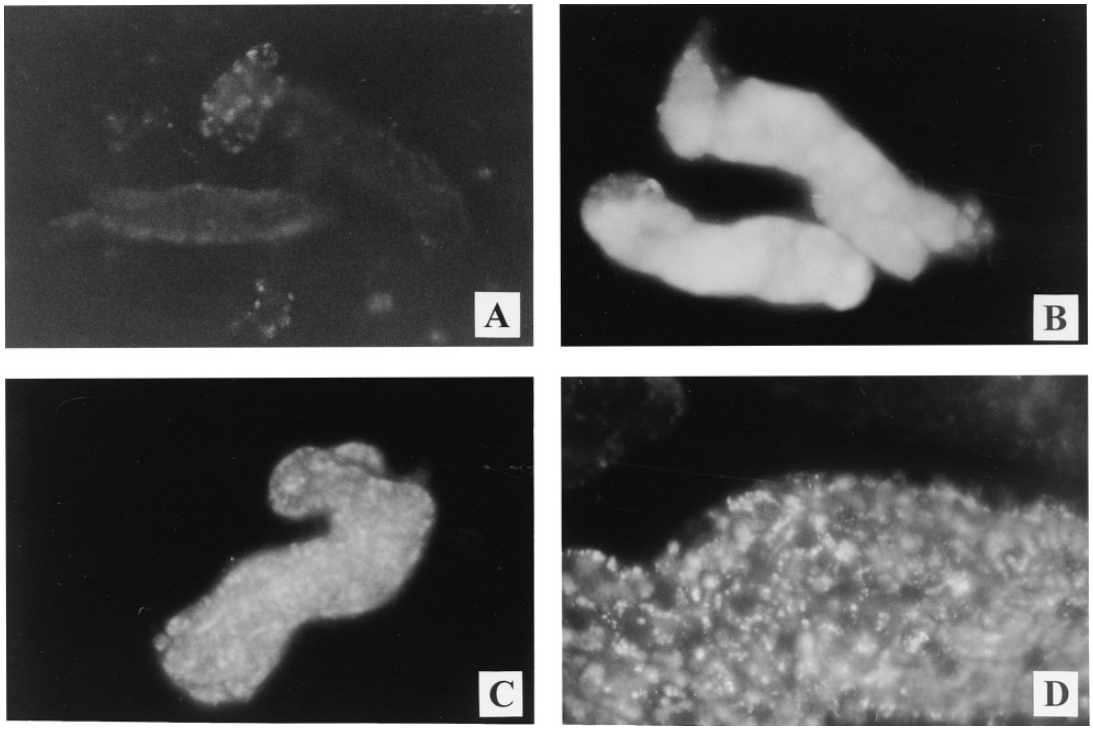


**Fig. 1.** Detection of FITC-albumin uptake during preparation of dog proximal tubules in suspension. FITC-albumin uptake by dog proximal tubules was demonstrated using inverted fluorescence microscopy. Bovine serum albumin (Control) (Panel *A*) or FITC-albumin (Panels *B, C* and *D*) were present at same concentration (2 mg/ml) in the collagenase digestion medium during preparation of proximal tubules from slices of dog kidney cortex. Fixation of proximal tubules and inverted fluorescence microscopy was made as described in Materials and Methods. The gain of the microscope (PLAN 40/0.65) and the image exposition (15 sec) were the same for Control (*A*) and FITC-albumin (*B*) and (*C*) photographs. Photograph (*D*) was made under immersion with microscope gain (PLAPO 63/1.40) and image exposition for 30 sec.

mostatin, phenylmethylsulfonyl fluoride and FITC-albumin were purchased from Sigma (St. Louis, MO). Enzymes and cofactors for enzymatic determinations were from Boehringer-Mannheim (GmbH, W.-Germany). All reagents for SDS-PAGE and Western blot were from Bio-Rad Laboratories (Hercules, CA). Acridine orange was of the highest purity and was provided by Molecular Probes (Eugene, OR). Polyclonal anti-FITC antibodies were provided by Molecular Probes (Eugene, OR) and by DAKO (Carpinteria, CA). Monoclonal anti-actin (clone AC-40), anti-β-COP (M3A5) and monoclonal antibody against β-adaptins (clone #100/1) were purchased from Sigma. Polyclonal anti-RhoA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rho-GDI antibodies were raised in rabbit using purified GST-Rho-GDI from *Escherichia coli*. The bacterial strain expressing Rho-GDI was kindly supplied by Dr. Allan Hall (University College, London, UK). Mouse horseradish peroxidase-conjugated anti-sheep antibody was purchased from Sigma (St. Louis, MO) while donkey horseradish peroxidase-conjugated anti-rabbit antibody and sheep horseradish peroxidase-conjugated anti-mouse antibody were purchased from Amersham Life Science (Oakville, ON, Canada).

### Statistical Analysis

All measurements are presented as means ± SEM. The data were analyzed using appropriate ANOVA analyses (SuperANOVA software, Abacus).

## Results

### Proximal Tubules are Involved in Protein Uptake

When cortical slices are incubated with FITC-albumin and bacterial collagenase A to digest the tissue, both proteins are progressively accumulated in proximal tubules. FITC-albumin uptake is demonstrated by the fluorescence observed in freshly prepared isolated tubules (Fig. 1, panels *B* and *C*). The fluorescence signal is not observed in control tubules incubated with nonfluorescent albumin (Fig. 1, panel *A*). High magnification analysis demonstrates the distribution of the fluorescence signal within intracellular vesicles (Fig. 1, panel *D*). This process could be attributed to the activation of receptor-mediated endocytosis by proximal tubules lead-

**Table 1.** Characterization of BBMV prepared from renal cortex or proximal tubules in suspension using magnesium precipitation technique

| | | | BBMV FROM CORTEX | | | BBMV FROM TUBULES | | |
|---|---|---|---|---|---|---|---|---|
| | | | MEAN ± SEM | *n* | E.F. | MEAN ± SEM | *n* | E.F. |
| A Total $H^+$-ATPase in BBMV | (V+E) | (+DOC) | 560 ± 50 | 21 | 7.5 | 560 ± 60 | 9 | 4.1 |
| B $H^+$-ATPase of endosomes | (E) | (−DOC) | 210 ± 20 | 21 | | 360 ± 40 | 9 | |
| C $H^+$-ATPase of BBM Vesicles | (V) | (A − B) | 350 | | | 200 | | |
| D $Na^+$, $K^+$-ATPase | | (+DOC) | 200 ± 30 | 21 | 0.5 | 330 ± 110 | 9 | 1.6 |
| E γ-Glutamyltranspeptidase | | (−DOC) | 5380 ± 40 | 21 | 7.5 | 3070 ± 300 | 9 | 5.1 |
| F Alkaline Phosphatase | | (−DOC) | 2270 ± 280 | 21 | 7.3 | 1130 ± 230 | 9 | 6.3 |
| G Glutamate Dehydrogenase | | (−DOC) | 80 ± 20 | 13 | 0.1 | 110 ± 20 | 3 | 0.1 |

Data are nmoles of substrates hydrolyzed per min and per mg of protein at 37°C, MEAN ± SEM (*n* = number of experiments).
The activities were measured in presence (+) or absence (−) of 0.1% deoxycholate (DOC).
E.F.: enrichment factor i.e., enrichment of enzymatic activity in BBMV over that measured in the tissue homogenates.

ing to the appearance of FITC-albumin into intracellular vesicular structures of endosomal origin.

## BBMV Prepared from the Renal Cortex or Tubules under Normoosmolar Condition

### *$H^+$-ATPase Activity*

BBMV prepared from the renal cortex using the standard magnesium precipitation technique are enriched sevenfold in brush border markers such as GGT and AP (Table 1). The markers of the basolateral membrane (BLM) and mitochondrial membrane were de-enriched with EF of 0,5 and 0,1, respectively. The $H^+$-ATPase activity is also enriched 7-fold. However, 38% of this activity is measured in absence of detergent i.e., is present either on vesicles oriented under the polarity of endosomes or on membrane sheets. The addition of 0.1% deoxycholate increases the $H^+$-ATPase activity by recruiting the $H^+$-ATPases located in BBM vesicles which account for 62% of the total activity (Table 1). Indeed the ATP-binding site of these $H^+$-ATPase is hidden in the intravesicular space of right-side-out sealed BBMV. The increment of bafilomycin-sensitive ATPase activity elicited by the addition of deoxycholate thus allows to calculate the fraction of the pumps which are located on genuine sealed BBM vesicles ($V_S$) and to estimate the contamination with open vesicles (or membrane sheets) ($V_o$) and/or intracellular endosomes ($E$).

When BBMV are prepared from freshly isolated proximal tubules, the overall $H^+$-ATPase activity observed is comparable to that demonstrated with BBMV prepared from renal cortex (Table 1). However, the activity recovered as $E$ and/or $V_o$ increased to 64% of the total, leaving only 36% of physiologically oriented $V_S$. The enrichment in BBM markers measured in absence of detergent is also lower than that observed with BBMV prepared from renal cortex. Thus the magnesium precipitation technique separates a mixture of $V_S$, $V_o$ and $E$, and this mix may differ according to circumstances.

## Proton Transport

To examine if the $H^+$-ATPase activity found in absence of detergent is due to the presence of open vesicles ($V_o$) or to sealed endosomes ($E$), the acidification of BBM vesicles prepared from renal cortex or tubules was examined in the presence of ATP (and of an ATP-regenerating system) by the acridine orange fluorescence technique. Since both sealed vesicles ($V_S$) and open vesicles ($V_o$) are not capable for acidification, from here we named them simple as BBM vesicles ($V$). The conditions insured that a identical amount of membrane ($V + E$) proteins were used in the assay. Upon addition of membranes prepared from the renal cortex, only a small quenching of fluorescence was observed indicating a modest endosomal acidification (Fig. 2, curve 1). This process was 100% bafilomycin-sensitive and oligomycin-insensitive confirming that a V-type proton pump was at play. Thus only a small amount of the membranes prepared from renal cortex ($V + E$) are present under the endosomal configuration ($E$). In contrast, when *VE* obtained from renal tubules were added, a steep acidification was observed (Fig. 2, curve 2). This process was fully ATP dependent, bafilomycin-sensitive and occurred in sodium-free medium. Thus the population of endosomes is larger in this preparation as seen above with the $H^+$-ATPase enzymatic activity measured in absence of detergent (Table 1). This indicates that the $H^+$-ATPase activity related to $V$ and $E$ is distributed differently in BBMV prepared from proximal tubules located in the renal cortex and from tubules isolated by the collagenase digestion technique suggesting a membrane redistribution (*see below*).

### BBMV Prepared from Tubules Incubated under Hyperosmolar Condition

To determine when this membrane redistribution between BBM and endosomes had occurred, we prepared tubules in a collagenase digestion buffer enriched with 300 mM sucrose to increase the osmolarity to 600 mOsm/L. Tubules prepared using the conventional procedure were simultaneously isolated from pieces of the same renal cortices. BBMV were prepared from the intact renal cortex and from both preparations of tubules.

Figure 2 (curve 3) demonstrates that *VE* obtained from tubules in suspension prepared under hyperosmolar condition present a modest acidification comparable to that observed with *VE* isolated from renal cortex. In contrast, BBMV prepared from tubules separated under the conventional protocol showed a steep acidification i.e., a large endosomal population as described above. Thus hyperosmolarity has prevented the membrane redistribution to occur. These experiments indicate that the new endosomes found in tubules are formed during the collagenase digestion procedure.

### Separation of BBMV and Endosomes

To characterize these newly formed endosomes it was necessary to separate them from the BBMV. To do this we took advantage of the specific selection produced by wheat-germ agglutinin [5, 12]. This lectin allows to aggregate and precipitate BBM vesicles ($V_S$) present under the right-side-out configuration as well as open vesicles (or membrane sheets) ($V_o$) (both named here simple as BBM vesicles (*V*)) but not endosomes (*E*). Figure 3 demonstrates that two membrane populations can be separated from the tubules-derived BBMV (*V* + *E*). A population showing steep acidification (*E*) and a population incapable of acidification (*V*) in presence of ATP were purified. The acidification observed with the mixture *V* + *E* was modest. The initial rate of acidification was linearly related to the amount of *V* and *E* proteins present in the assay (Fig. 4). This observation allowed to calculate a tenfold purification of endosomal vesicles by the negative selection technique. A comparable $H^+$-ATPase activity was demonstrated in both populations of vesicles after solubilization of the membranes with 0.1% DOC (Table 2).

### Enzymatic Characterization

Table 2 presents the enrichment in two BBM markers, GGT and AP, found in BBM vesicles before (*V* + *E*) and after separation of vesicles (*V*) and endosomes (*E*) by WGA technique. It can be seen that these three fractions present the same activity of both markers, indicating the origin of endosomes from brush-border membrane.

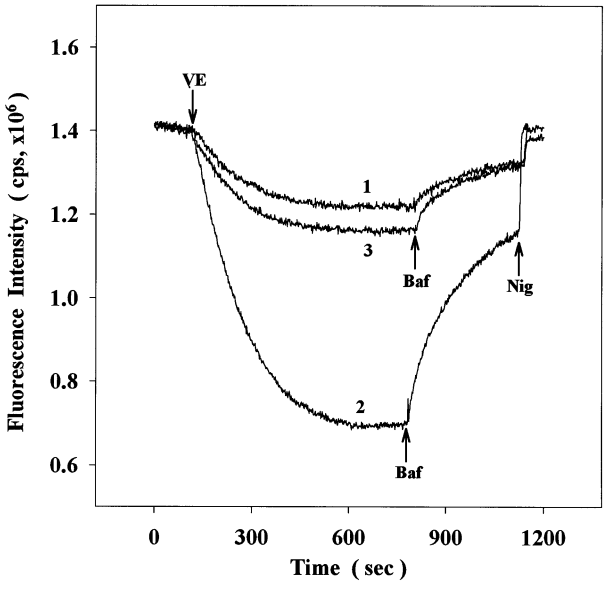


**Fig. 2.** Endosomes in BBMV prepared from cortex or tubules. Spontaneous ATP-dependent acidification observed in BBMV (VE) prepared from renal cortex (1) or tubules isolated from the same cortex under normo-osmolar (2) or hyperosmolar (3) conditions. The effect of $10^{-7}$M bafilomycin (Baf) and $2 \times 10^{-5}$M nigericin (Nig) is indicated with arrows.

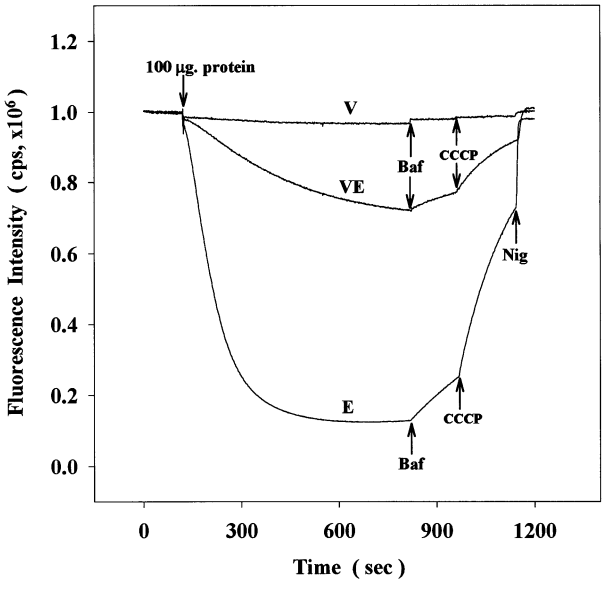


**Fig. 3.** Separation of endosomes from BBMV. The spontaneous ATP-dependent acidification observed in BBMV (100 µg, VE) isolated from tubules in suspension is presented. The acidification observed in endosomes (100 µg, E) or BBMV (100 µg, V) purified from the mixed VE population by wheat-germ agglutination is shown. The effect of $10^{-7}$M bafilomycin (Baf), $10^{-5}$M CCCP and $2 \times 10^{-5}$M nigericin (Nig) is indicated with arrows.

### Sedimentation of Endosomes

When the endosomes separated by WGA negative selection technique were centrifuged in a 16% Percoll gradient, they migrated in a low position corresponding to heavy endosomes [13]. The buoyant density was estimated as 1.036 g/ml using 50% Percoll.

### Morphological Aspect of Endosomes

Figure 5 presents the morphological aspect of endosomes purified by WGA negative selection technique seen by electron microscopy. The endosomal preparation demonstrates numerous vesicular and tubular bodies with no contamination with identifiable cellular organelles. No clathrin-coated vesicles could be detected in endosomal fraction. The morphological aspect of this preparation is extremely similar to that reported by Hammond et al. [14] and described as ''heavy endosomes'' isolated from the rat renal cortex.

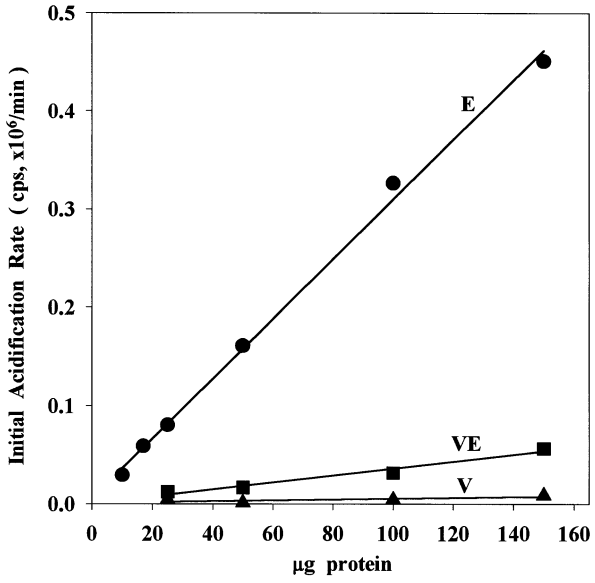


**Fig. 4.** Acidification in endosomes and BBMV. Initial rate of acidification observed with different concentrations of V, E, and VE proteins. A 10-fold purification of endosomes is obtained by the agglutination procedure.

### SDS-PAGE and Western Blot Analysis

Figure 6 presents the SDS-PAGE gel obtained with three consecutive and independent preparations of vesicles (*V*) and endosomes (*E*) isolated from BBMV (*VE*) preparations. This allows to demonstrate that the protein distribution pattern observed with vesicles is similar in all vesicular preparations studied, and contrast with the pattern observed with endosomes. The apparent molecular weight of each band was estimated using calibrated molecular weight standards. It is clear that two major proteins or groups of proteins are found associated with vesicles (Fig. 6 *C* and *F*) that are lacking or greatly diminished in endosomes. The apparent molecular weight of these proteins are 42–44kDa for (*C*) and 20–24 kDa for (*F*) respectively. On the other hand, several proteins with molecular weight of 70 kDa (*A*), 60 kDa (*B*) and 30 kD (*D*), which could be the subunits of $V_1$ complex of $H^+$-ATPase, are present in similar quantities in vesicular and endosomal fractions (Fig. 6). The rest of the protein pattern is also similar between *V* and *E*. To further understand this difference between vesicles and endosomes, immunoblots were performed using antibodies raised against different proteins susceptible to be associated with these elements.

Figure 7 presents the SDS-PAGE pattern of *V* and *E* (Lane 1) and shows the Western blot analysis using anti-actin (Lane 2) and anti-RhoA antibodies (Lane 3). Lane 2 demonstrated that the 42–44 kDa protein is actin. The Western blot confirms that actin is present in BBM vesicles but is not detectable in endosomal fractions. Figure 7 (Lane 3) demonstrates that BBM vesicles are

**Table 2.** Separation of endosomes (E) and vesicles (V) by wheat-germ agglutinin negative selection technique from BBMV (VE) prepared from proximal tubules in suspension

| | | VE | | V | | E | |
|---|---|---|---|---|---|---|---|
| | | MEAN ± SEM | E.F. | MEAN ± SEM | E.F. | MEAN ± SEM | E.F. |
| $H^+$-ATPase | (−DOC) | 81 ± 7 | 3,4 | 55 ± 13 | 2,3 | 123 ± 18 | 5,2 |
| $H^+$-ATPase | (+DOC) | 195 ± 29 | 6,1 | 166 ± 55 | 6,5 | 159 ± 62 | 6,4 |
| γ-Glutamyltranspeptidase | (+DOC) | 4498 ± 296 | 6,8 | 4377 ± 836 | 7,2 | 4499 ± 996 | 7,5 |
| Alkaline Phosphatase | (+DOC) | 912 ± 156 | 12,5 | 628 ± 179 | 7,2 | 760 ± 119 | 10,3 |

Data are nmoles of substrates hydrolyzed per min and per mg of protein; MEAN ± SEM ($n = 3$).
(V-type) $H^+$-ATPase activity was measured as bafilomycin (1 µM) sensitive ATPase using an incubation medium (Material and Methods) and experimental conditions (T = 22°C) similar to that used for in acridine orange experiments.
The activities were measured in presence (+) or absence (−) of 0.1% deoxycholate (DOC).
E.F.: enrichment factor i.e., enrichment of enzymatic activity in V, E or VE suspensions over that measured in the tissue homogenates.

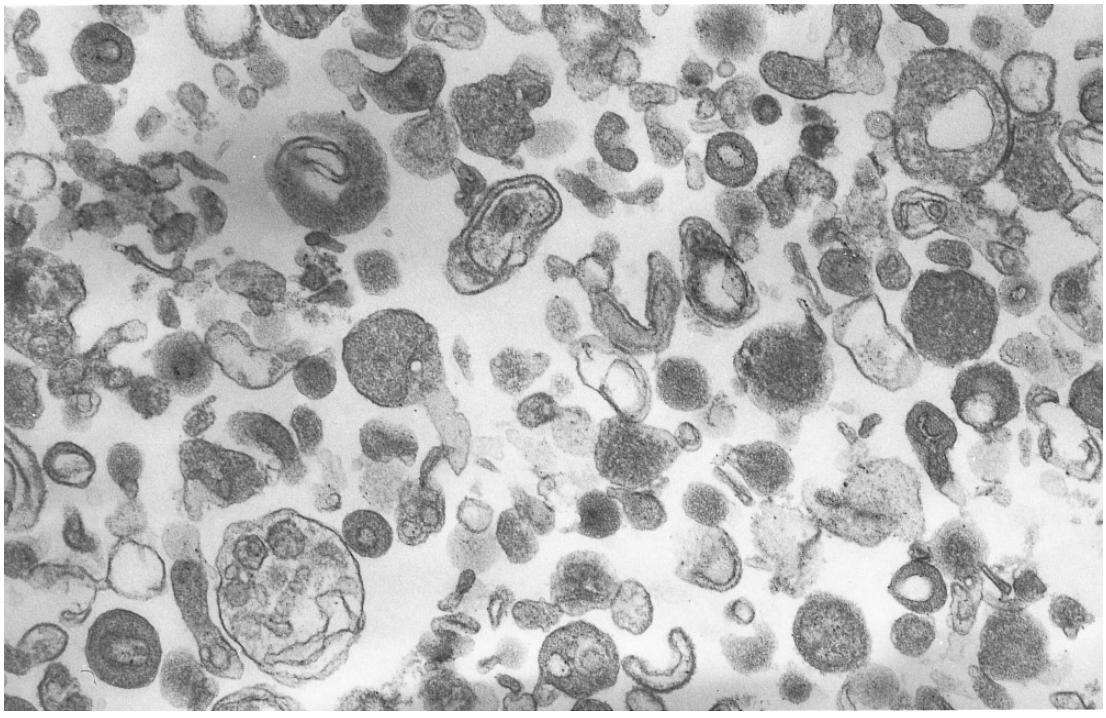

**Fig. 5.** Morphological aspect of endosomes. Electron micrograph of heavy endosomes purified from dog kidney proximal tubules in suspension using wheat-germ agglutinin negative selection. Magnification ×44.100.

closely associated with RhoA, a small G protein involved in regulating of actin polymerization. RhoA is especially abundant in vesicular but greatly diminished in the endosomal pool. Note that anti-RhoA antibodies identifies two immunoreactive bands thought to be the processed (lower band) and unprocessed (upper band) isoforms of RhoA proteins. Interestingly, the slower migrating band of RhoA is drastically reduced in endosomes, while the band with higher mobility on gel electrophoresis is slightly diminished compared to BBMV. The GDP/GTP dissociation inhibitor for Rho-related proteins Rho-GDI is present in vesicles but barely detectable in endosomal preparations (Figure 7, Lane 6), suggesting that a significant amounts of RhoA is complexed to Rho-GDI in BBMV.

Figure 7 also presents a comparable analysis made using β-adaptin (Lane 4) and β-COP (Lane 5) antibodies. Western blot analysis with anti-β-COP demonstrated the presence of this protein in vesicular but not in endosomal fractions. The β-adaptin antibody recognized two different proteins of approximately 110–100 kDa and 75–70 kDa associated with the vesicular preparations. Only the 75–70 kDa protein is present in endosomal preparations.

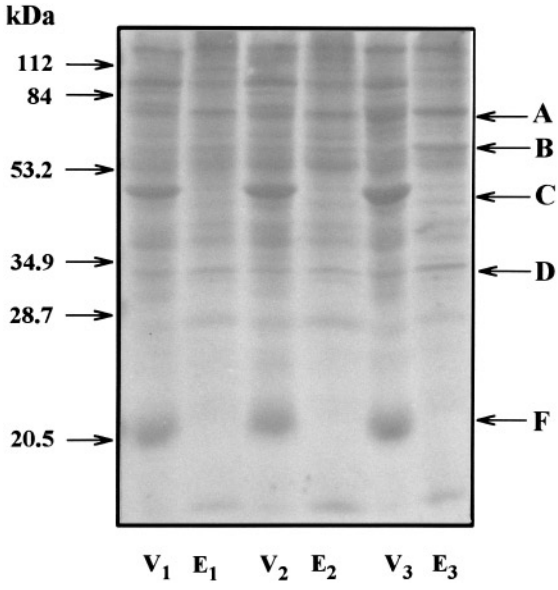


**Fig. 6.** SDS-PAGE of BBM vesicles and endosomes. SDS-PAGE analysis of protein content in three independent preparations of BBM vesicles ($V_1$, $V_2$, $V_3$) and heavy endosomes ($E_1$, $E_2$, $E_3$) from dog kidney proximal tubules in suspension. Coomassie Blue staining of total proteins is made.

### Presence of Extracellular Proteins in Heavy Endosomes

Small-particle flow cytometry analysis of heavy endosomes prepared from dog proximal tubules in suspension using the combination of magnesium precipitation and wheat-germ agglutinin negative selection techniques is shown in Fig. 8. Overlaid histograms demonstrate the particle by particle fluorescence analysis of heavy endo-

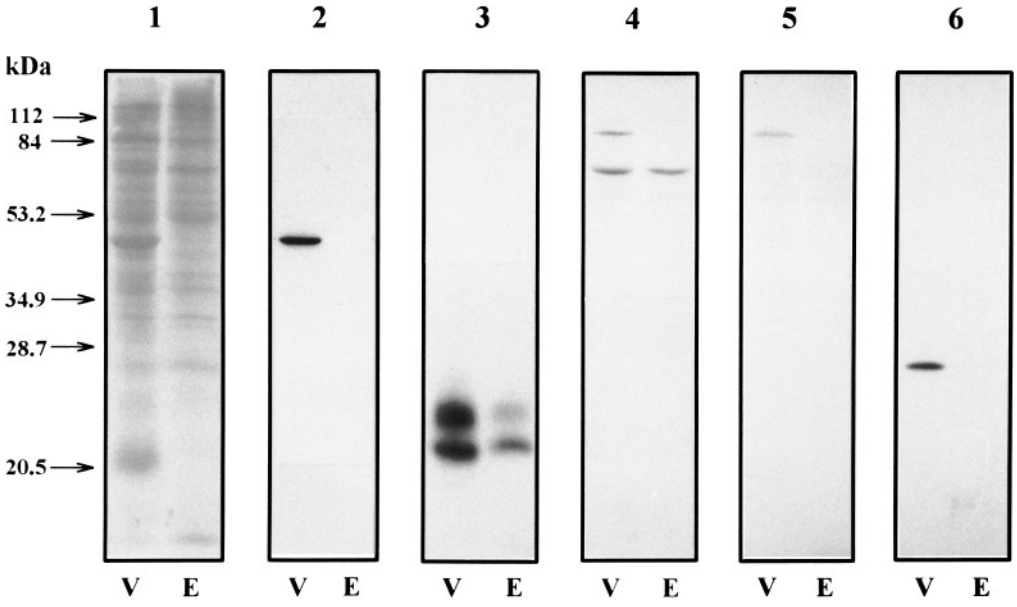


**Fig. 7.** Western blot analysis of BBM vesicles and endosomes. Coomassie Blue staining of total proteins (lane 1) and Western blot analysis of the proteins of BBM vesicles (V) and heavy endosomes (E) using anti-actin (lane 2), anti-RhoA (lane 3), anti-β-adaptin (lane 4), anti-β-COP (lane 5) or anti-RhoGDI (lane 6) antibodies.

somes purified from proximal tubules exposed during their preparation either to nonfluorescent albumin (Control) or to fluorescein isothiocyanate-conjugated albumin (FITC-albumin). The histograms showed no overlap. Moreover, the statistical analysis shows the presence of FITC-albumin entrapped molecules in up to 92% of the heavy endosomes purified from tubules exposed to FITC-albumin, indicating homogeneity of the endosomal fraction.

Immunodetection analysis using anti-FITC antibody also demonstrates the presence of FITC-albumin in heavy endosomes prepared from proximal tubules previously exposed to FITC-albumin (Fig. 9, Panel *A*, Lane $E_{FITC}$). This anti-FITC-antibody is highly specific and does not recognize albumin itself (Fig. 9, Panel *A*, Lane Alb) nor other endosomal proteins (Fig. 9, Panel *A*, Lane $E_{Cont}$). Surprisingly, it was found that FITC-albumin loaded into heavy endosomes is appeared in two forms (Fig. 9, Panel *A*, Lane $E_{FITC}$). Both the intact protein of 75 kDa and its proteolytic fragment of 55 kDa are present in approximately the same quantities in three different and independent preparations of endosomes (Fig. 9, Panel *B*, Lanes $E_1$, $E_2$, $E_3$). Similarly, another extracellular protein used during preparation of proximal tubules in suspension, bacterial collagenase A was found to be present into purified heavy endosomes (Fig. 10, Panel *A*, Lane E) but not into purified BBM vesicles (Fig. 10, Panel *A*, Lane V). Preparation of *Clostridium histolyticum* collagenase A from Boehringer-Mannheim appeared in three isoforms: main polypeptide of 120 kDa and two minor polypeptides of 110 kDa and 100 kDa

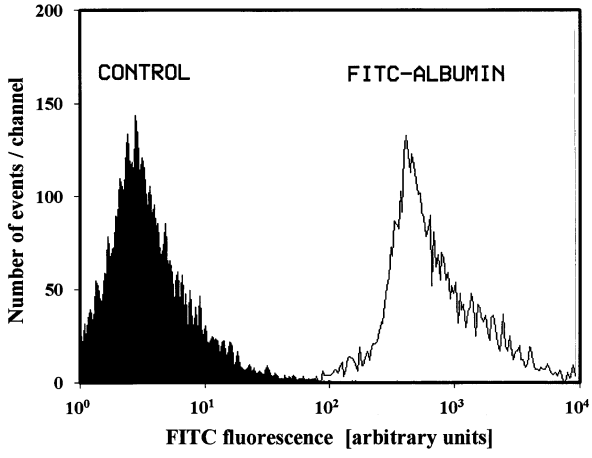


**Fig. 8.** Flow cytometry analysis of endosomal vesicles. Small-particle flow cytometry analysis of heavy endosomes prepared from dog proximal tubules in suspension using combination of magnesium precipitation and wheat-germ agglutinin negative selection techniques. Overlaid histograms demonstrate the particle by particle fluorescence analysis of heavy endosomes purified from proximal tubules prepared in the presence of nonfluorescent albumin (Control) or fluorescein isothiocyanate-conjugated albumin (FITC-albumin). Each histogram comprises 5.000 events. A representative result of three independent endosomal preparations is shown.

(Fig. 10, Panels *A* and *B*, Lanes Collagenase A). All three isoforms of collagenase A were detected in purified heavy endosomes (Fig. 10, Panel *B*, Lanes $E_1$, $E_2$, $E_3$). Western blot quantitative ECL detection technique allowed us to estimate the amount of FITC-albumin (Fig.

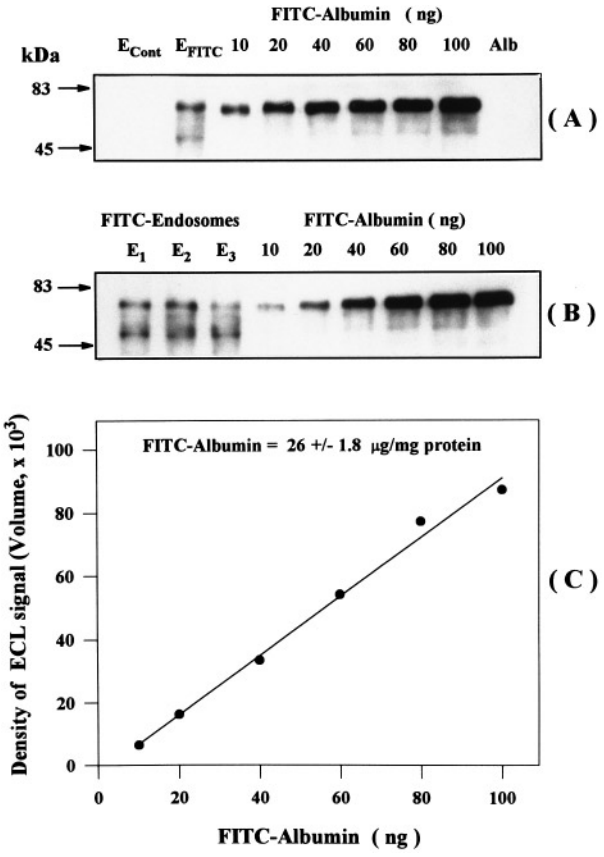


**Fig. 9.** Detection and quantification of FITC-albumin loaded into endosomal vesicles. Western blot detection and quantification of FITC-albumin into endosomal vesicles using polyclonal anti-FITC antibodies. (*A*) SDS-PAGE and Western blot analysis of heavy endosomes (2 μg protein in each line) purified from proximal tubules prepared in the presence of 2 mg/ml nonfluorescent albumin (Control) ($E_{Cont}$) or in the presence of 2 mg/ml fluorescein isothiocyanate-conjugated albumin (FITC-albumin) ($E_{FITC}$). In the same gel 10; 20; 40; 60; 80; 100 ng of FITC-albumin and 100 ng of nonfluorescent albumin (Alb) were run; (*B*) SDS-PAGE and Western blot analysis of heavy endosomes (2 μg protein in each line) purified from three different preparations of proximal tubules prepared in the presence of 2 mg/ml of FITC-albumin ($E_1$, $E_2$, $E_3$). In the same gel 10; 20; 40; 60; 80; 100 ng of FITC-albumin were run; (*C*) Estimation of FITC-albumin content into heavy endosomes by quantitative ECL Western blots using FITC-albumin standard curve. The points plotted in the figure indicate the means of duplicate determinations.

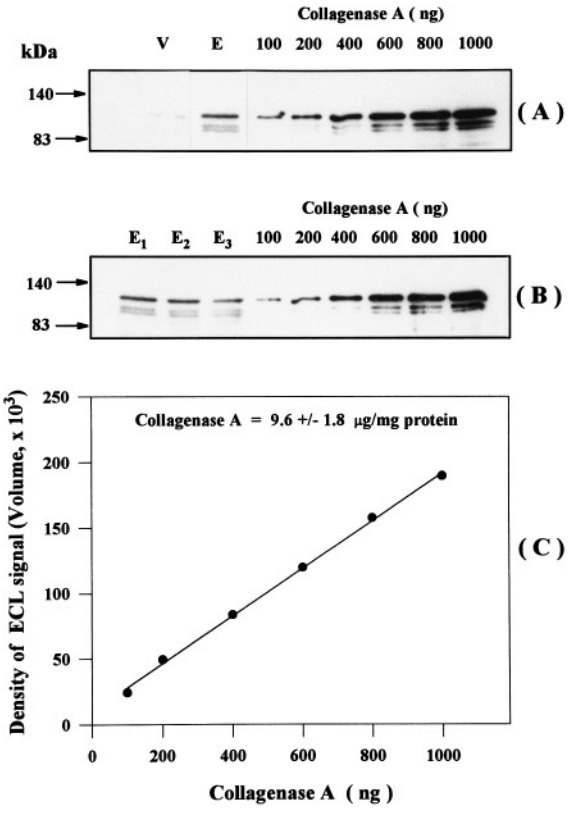


**Fig. 10.** Detection and quantification of collagenase A loaded into endosomal vesicles. Western blot detection and quantification of collagenase A into endosomal vesicles using polyclonal antibacterial collagenase A antibodies. (*A*) SDS-PAGE and Western blot analysis of BBM vesicles (V) and heavy endosomes (E) (20 μg protein in each line) purified from proximal tubules in suspension. In the same gel 100; 200; 400; 600; 800; 1000 ng of bacterial collagenase A were run; (*B*) SDS-PAGE and Western blot analysis of three different preparations of heavy endosomes ($E_1$, $E_2$, $E_3$) (20 μg protein in each line). In the same gel 100; 200; 400; 600; 800; 1000 ng of bacterial collagenase A were run; (*C*) Estimation of bacterial collagenase A content into heavy endosomes by quantitative ECL Western blots using collagenase A standard curve. The points plotted in the figure indicate the means of duplicate determinations.

9, Panels *B* and *C*) and bacterial collagenase A (Fig. 10, Panels *B* and *C*) entrapped into heavy endosomes. In three independent endosomal preparations the presence of 26 ± 1.8 μg/mg. protein of FITC-albumin (Fig. 9, Panel *C*) and 9.6 ± 1.8 μg/mg. protein of bacterial collagenase A (Fig. 10, Panel *C*) was demonstrated.

## Discussion

A small population of endosomes with composition very similar to the BBM exists in proximal tubular cells of renal cortex in the region immediately adjacent to the BBM. These endosomes are co-isolated with BBM vesicles prepared by the conventional magnesium precipitation technique [29], probably indicating their physicochemical resemblance with the BBM.

In fact, three membrane fractions associated with $H^+$-ATPase activity are isolated upon preparation of BBM vesicles from dog renal cortex using the classical magnesium precipitation technique: (i) BBM vesicles closed under the physiological polarity presenting their proton pumps with the ATP binding sites facing the intravesicular space i.e., the ATPase activity and vesicular acidification is unresponsive to ATP addition. This ATPase activity represents 62% of the total $H^+$-ATPase

activity but is only revealed after solubilization of the membranes with a detergent [29] or following inversion of the $H^+$-ATPase polarity using a short cholate treatment [28, 29]. (ii) Membrane sheets or open BBMV giving rise to a spontaneous enzymatic $H^+$-ATPase activity before solubilization but to no acidification signal. (iii) Sealed endosomes exposing the ATP-binding sites to the incubation buffer and therefore detected both by $H^+$-ATPase activity assay without solubilization (together with sheets) and/or by following endosomal acidification with acridine orange fluorescence. These endosomes contain however only a fraction (<38%) of the total $H^+$-ATPase activity found in renal cortex BBMV preparation. The presence of such endosomes have been noted before in preparations of BBM vesicles isolated from rat kidney cortex using the magnesium precipitation technique [39].

In proximal tubules in suspension these endosomes accumulate considerably in the cytoplasm of the proximal cells and lead to the appearance of fluorescence signal into intracellular vesicular structures as shown in this study. These endosomal vesicles can be clearly seen by electron microscopy examination of isolated proximal tubules which contain numerous intracellular vesicles [42]. We believe that these endosomes are formed upon stimulation of protein transport occurring during the preparation of the tubular suspension. Indeed, dog proximal tubules in suspension transport albumin through receptor-mediated endocytosis, as demonstrated by electron microscopy immunogold detection of albumin in endosomes immediately adjacent to the BBM or by lysosomial accumulation of albumin-Evan's blue complexes (*unpublished data*). This process can be stopped in reversible fashion by exposing the tubules to an hyperosmolar environment.

When BBMV are prepared from freshly isolated dog proximal tubules in suspension, the overall $H^+$-ATPase activity observed is comparable to that demonstrated with BBMV prepared from renal cortex. However, the $H^+$-ATPase activity recovered with endosomes and/or membrane sheets now increases to 64% of the total activity. To examine if this activity measured in absence of detergent is due to the presence of membrane sheets (open vesicles) or to sealed endosomes, the acidification of BBM vesicles ($V + E$) prepared from renal cortex or tubules was examined by the acridine orange fluorescence technique. Upon addition of membranes prepared from the renal cortex, only a small quenching of fluorescence was observed indicating a modest endosomal acidification. In contrast, when *VE* obtained from renal tubules were added, a steep acidification was observed. This process was fully ATP-dependent and bafilomycin-sensitive. Thus BBMV prepared from proximal tubules in suspension are largely enriched with endosomes.

To determine when this endosomal formation had occurred, we prepared tubules in a collagenase digestion medium enriched with 300 mM sucrose to increase the osmolarity to 600 mOsm/L, a procedure known to inhibit receptor-mediated endocytosis by blocking the formation of clathrin-coated pits [15, 31]. BBMV prepared from tubules exposed to hyperosmolar conditions present a modest ATP-driven acidification, of magnitude comparable to that observed with *VE* isolated from renal cortex. Thus hyperosmolarity has prevented the membrane redistribution to occur between BBM and endosomes. These experiments indicate that the endosomes found in tubules are formed during the preparation of the proximal tubules in suspension, probably following activation of receptor-mediated endocytosis by soluble proteins during the collagenase digestion procedure.

It is possible to purify these newly formed endosomes (and/or to obtain endosomes-free BBMV) by using the wheat-germ agglutinin negative selection technique. This procedure is capable to agglutinate right-side-out BBM vesicles and open vesicles because of the presence of N-acetylglucosamine residues on the external leaflet of the membrane [48, 49], allowing the precipitation of large BBMV-agglutinin complexes by centrifugation and leaving endosomes in the supernatant. This technique was developed [5] for the separation of two membrane fractions of cardiac sarcolemma and was recently used to separate the clathrin-coated vesicles [12]. In our case, the wheat-germ agglutinin negative selection technique allowed the purification of endosomes (*E*) from BBM vesicles (*V*) (here is mixture of sealed BBM vesicles ($V_S$) and open vesicles ($V_o$)). To estimate the proportion of endosomes in a BBMV preparation from proximal tubules in suspension, the ratio between the endosomal proteins (separated by WGA treatment) *vs.* total proteins was used. In a typical preparation, from ≈15 gram (wet weight) of proximal tubules in suspension, ≈20 mg protein of *VE* could be obtained after magnesium precipitation from which ≈18 mg protein of BBM vesicles and/or membrane sheets (*V*) and ≈2 mg protein of endosomes (*E*) can be purified. Our results indicate that ≈10% ($n = 5$) of proteins of a tubule-derived BBMV preparation (*VE*) are on endosomes. This quantification is in perfect agreement with the ten-fold enrichment in endosomes estimated by the acridine orange acidification assay. Centrifugation in 16% or 50% Percoll [13], together with a tube containing standard density beads, allowed us to demonstrate that these endosomes are ''heavy endosomes'' with a buoyant density of 1.036 g/ml similar to that of BBM vesicles (*not shown*). The morphological aspect of endosomes purified by WGA negative selection technique was examined by electron microscopy. Vesicular and tubular bodies with no clathrin-coated vesicles or other cellular organelles were seen. The morphological aspect of this preparation is similar to that reported by Hammond et al. [14]

and described as ''heavy endosomes'' isolated from the rat renal cortex.

The nature of these endosomes should be discussed. They could be seen as: (i) physiological structures present in the cell before the BBMV preparation and acting as a reservoir of proton pumps and co-isolated with BBMV; (ii) a small population of BBM vesicles closed under the reverse configuration during the preparation of vesicles; (iii) lysosomes, Golgi, microsomes normally occurring in the cell and co-isolated with BBMV.

In the latter case, the membrane of these endosomes should differ from the BBM in protein composition as well as in markers. The activities of BBM markers such as AP and GGT were comparable in solubilized *E* and *V*, indicating that these endosomes originate from the proximal brush-border membrane. Furthermore, comparable $H^+$-ATPase activity was demonstrated in BBM vesicles (*V*) and endosomes (*E*) after solubilization of the membranes with 0.1% DOC.

As expected however, the proton pumps had opposite orientation on *E* and *V* membranes. This could represent an increased tendency to form sealed BBM vesicles closed under the reverse, inside-out, orientation. However, there is a strong indication that this is not the case and that these endosomes are formed before the preparation of the BBM vesicles by the magnesium precipitation technique. Indeed, the formation of endosomes was suppressed by preparing the tubules under conditions of hyperosmolarity. Receptor-mediated endocytosis and protein transport is a process known to be sensitive to osmolarity, an hypertonic medium blocking the formation of clathrin-coated pits [4, 15]. It is probable that exposure of BBM to proteins of the incubation buffer during the tubules preparation is responsible for the activation of receptor-mediated endocytosis and explaining the formation of these new endosomes. Alternatively, these endosomes may represent a mechanism incorporating the BBM inside cells and reducing transepithelial transport because the cells were exposed to nonphysiological conditions during the preparation of tubules.

That these membrane structures are not BBM vesicles closed under reverse orientation is further confirmed by the selective association of *V* with specific proteins. Indeed, SDS-PAGE and immunodetection demonstrated the presence of actin and RhoA in BBM vesicles (*V*). The actin is completely absent in endosomes (*E*), clearly distinguishing both types of vesicles. Actin is a very important structural protein for brush-border membranes [7, 36] as a main protein of microvilli. Each microvillus contains a bundle of 20 to 30 actin microfilaments anchored in the plasma membrane at the tip of the microvillus. Actin is thus a marker of brush-border membrane. It must be present in BBM vesicles but probably not or much less in endosomal structures. The clear separation observed here between actin-rich BBMV and actin-poor endosome with comparable GGT and AP activities confirms this view. However phalloidin binding still identifies some actin in heavy endosomes prepared from renal cortex [14].

RhoA is also diminished in endosomes as compared to BBMV. RhoA protein regulates the assembly of actin filaments [33] and organization of actin cytoskeleton [6, 21]. It also regulates the actomyosin system required for cell motility [41], normal cell morphology [27] and smooth muscle contraction [20]. Tissue-specific differences in the expression of RhoA and Rho-regulating factors [10] indicate that the rabbit kidney is an abundant source of these proteins. In rats, RhoA is especially found in kidney, brain, spleen, lung, thymus, liver and small intestine [3, 44]. Recently [3] an extensive study of Rho and Rho-related small GTP-binding proteins was made in fractions obtained from rat kidney cortex. It was demonstrated that RhoA and CDC42 proteins are localized in brush-border membranes in association with cytoskeletal elements. RhoA protein was found in higher quantity in the cytosol than in the membranes. The cytoplasmic form of RhoA is thought to represent a precursor form since it migrates at a slightly higher molecular weight than the mature membrane-bound form [3]. In our study, RhoA was found mainly in connection with BBMV, and much less with endosomes. The fact that two bands are detected as immunoreactive to RhoA antibody in BBMV may indicate that both cytoplasmic and membrane-bound forms are present in BBMV.

There are two recognized classes of coated transport vesicles [34]. The first class is clathrin-coated vesicles (CCV) which are involved in receptor-mediated endocytosis. The major coat constituents of CCV are clathrin triskeletons and adaptor complexes which are known to provide the interaction between clathrin coat and components of the membrane [22, 35]. The β-adaptins exist as $\beta_1$ and $\beta_2$ isoforms and form part of adaptor complexes from trans-Golgi network (AP-1) and from plasma membrane (AP-2) respectively. It is generally accepted that β-adaptins are essential elements of adaptor complexes since these proteins mediate the interaction of their adaptors with clathrin trimers and regulate process of coating/decoating of CCV.

The second class of transport vesicles, referred to as ''nonclathrin-coated vesicles'' or ''COP-coated vesicles'' has been shown to mediate vesicular traffic along exocytic pathway [8, 32]. The coat constituents of COP-coated vesicles includes four stoichiometric subunits: α-COP (160 kDa), β-COP (110 kDa), γ-COP (98 kDa), δ-COP (61 kDa) and several smaller subunits of 36 kDa, 35 kDa, 20 kDa. These proteins are also present in cytosol as a large multimeric precursor termed a ''coatomer'', which represents about 0.2% of total

soluble bovine brain cytosol proteins [47]. Gel filtration of unfractionated cytosol indicates that β-COP resides exclusively in the coatomer complex.

A remarkable difference between BBM vesicles and endosomes is disclosed by β-adaptins antibodies which identified two proteins of approximately 110–100 kDa and 75–70 kDa in our BBMV preparation (*V*). However, the 110–100 kDa protein thought to be $\beta_2$-adaptin, based on its molecular weight and immunoreactivity, is lacking in endosomal preparation (*E*). This result suggest that the endosomes isolated here are not associated with clathrin.

Surprisingly, Western blot analysis with anti-β-COP demonstrated the presence of this protein in vesicular but not in endosomal fractions. The presence of β-COP protein in our BBM vesicles may be due to: (i) contamination of BBM vesicles with Golgi membranes covered with β-COP proteins, (ii) trapping the cytoplasmic form of β-COP within the intravesicular space during cellular disruption and preparation of BBM vesicles. These proteins would be absent from the endosomes because they are formed before the disruption of the cellular structure.

Contamination of BBM vesicles with ''COP-coated vesicles'' of Golgi origin is not likely. Indeed this hypothesis does not explain the presence of this protein in BBM vesicles (*V*) and its complete absence in endosomes (*E*). Furthermore, both *V* and *E* fractions migrated as a single band (D = 1.036 g/ml) during determination of buoyant density by centrifugation in 50% Percoll gradient: no contamination with other vesicles was observed. The buoyant density of COP-coated vesicles prepared from rabbit liver Golgi was determined as 1.18 g/ml [40]. Vesicles with such different buoyant densities should have been easily separated and detected in our experiments.

To verify the second hypothesis, Western blot experiments using anti-RhoGDI antibody were performed. Rho-GDP-dissociation inhibitor (RhoGDI) is known to be exclusively a cytoplasmic protein [44]. In kidney cortex, RhoGDI was detected only in the cytosol [3] complexed with RhoA and CDC42. Our results demonstrate that this cytoplasmic protein is present in BBM vesicles, probably trapped into the intravesicular space. This protein is completely lacking in preformed endosomes.

These results have three implications: (i) that the proteins found in the SDS-PAGE may not originate only from membrane but also from cytoplasmic proteins enclosed in BBM vesicles during their formation; (ii) these results also suggest different mechanisms and different time-frames for the formation of BBM vesicles and endosomes; (iii) that no contamination by COP-coated vesicles of Golgi origin is observed in our endosomal preparation.

Our results show that the large endosomal population observed in isolated proximal tubules arise from the stimulation of protein reabsorption in tubules, presumably because of the presence of proteins in the extracellular medium. Two different experimental approaches, Western blot analysis and small-particle flow cytometry analysis, were used to demonstrate the presence of extracellular proteins in purified heavy endosomes.

First, immunochemical analysis indicated that both albumin and collagenase A, originally present in extracellular medium during preparation of proximal tubules, were found in purified heavy endosomes. The appearance of a 55 kDa albumin fragment together with the intact albumin polypeptide in endosomes could be explained either (1) due to a nonspecific proteolytic action on albumin of some proteases contaminating collagenase A preparation and not digestion of albumin by collagenase A itself or (2) due to specific cleavage of albumin by endosomal proteases. The specific proteolysis of mannosylated bovine serum albumin and parathyroid hormone by membrane-associated cathepsin D has been described previously in endosomes isolated from rabbit alveolar macrophages [2].

Collagenase A was not found in purified BBM vesicles. This demonstrates further that heavy endosomes are not formed during the BBM vesicles preparation but rather in intact cells during the preparation of tubules through an active protein reabsorption process. However, no preferential reabsorption of albumin in comparison with bacterial collagenase A by proximal tubules was demonstrated. Indeed the albumin/collagenase A ratio ($R_{end}$ = 2.7) (26 μg/9.6 μg per mg. endosomal protein) was similar in purified heavy endosomes to the albumin/collagenase A ratio ($R_{med}$ = 2.5) (2 mg/0.8 mg per ml of medium) present in extracellular digestion medium during preparation of proximal tubules. This result demonstrates the similar reabsorption rate of albumin and collagenase A proteins.

Finally, small-particle flow cytometry analysis technique allows us to demonstrate the presence of FITC-albumin entrapped molecules in up to 92% of purified heavy endosomes. This result indicates high homogeneity of endosomal fraction since up to 92% of endosomal vesicles were derived from the receptor-mediated endocytosis pathway. Recently [26], using Rab4 and Rab5 small GTPases as a marker proteins, the heavy endosomes were identified as early endosomes derived from proximal tubules receptor-mediated endocytosis pathway. Also, a complete analysis of regulation of acidification process of early endosomes purified from dog kidney proximal tubules in suspension has been made [26].

Endosomal fractions derived from intact renal cortex were used previously to study receptor-mediated endocytosis in the kidney. Heavy endosomes are usually prepared from homogenates of renal cortex by density centrifugation in 16% Percoll [37, 38, 39]. The bottom of

this gradient contains heavy endosomes overlayed with brush-border membrane vesicles but clearly separated from BLM [13]. Thus heavy endosomes and BBM vesicles are of similar buoyant density explaining the unavoidable contamination of heavy endosomes with BBMV [24, 38]. Recently, the heavy endosomes were separated from BBMV through potassium treatment and centrifugation [14]. The identification of clathrin elements, gp280 and gp330 lead to the conclusion that heavy endosomes may originate from intermicrovillar clefts. Light endosomes were also recently fractionated from intact renal cortex [13].

The preparation of endosomes from intact rabbit renal cortex was also reported by R.W. Gurich and D.G. Warnock [11]. They used a linear sucrose density gradient. However, the analysis of the buoyant density of these endosomes made by T.G. Hammond and P.J. Verroust [13] strongly suggest that this technique isolates clathrin-coated vesicles, which can be further purified by a wheat germ agglutinin negative selection technique [12]. A different technique was used by S.A. Hilden and N.E. Madias [16, 17, 18, 19] to purify an endosomal fraction from rabbit renal cortex using differential centrifugation and magnesium treatment. The enzymatic characterization studies demonstrate the exclusion of lysosomes, Golgi apparatus, mitochondria, endoplasmic reticulum, luminal and basolateral membranes from this preparation [16, 17].

To our knowledge there are no previous publications reporting the purification and characterization of endosomal fractions from proximal tubules in suspension. Here we describe the isolation and characterization of heavy endosomes from dog kidney proximal tubules in suspension using a combination of magnesium precipitation and wheat-germ agglutinin negative selection techniques. In contrast to the works presented above, our technique is simple, efficient and provides a large amount of heavy endosomes of known proximal origin.

Thus, the suspension of tubules offers a model where the formation of endosomes can be controlled: stimulated by proteins and inhibited not only by osmolarity, but also by bafilomycin and other maneuvers altering endosomal acidification (*unpublished observations*). It is a convenient experimental tool to study the regulation of endocytotic vesicle traffic in proximal tubules. It is possible to isolate easily large amounts of these newly formed endosomes, opening the way to their characterization and to the detailed study of the mechanisms regulating the insertion/de-insertion of these endosomes in the brush-border membrane.

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