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Isolation of apical plasma membrane in rabbit gallbladder epithelium by Percoll density gradient centrifugation

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The apical membranes of rabbit gallbladder epithelial cells were isolated by treating the homogenate with Ca^{2+} or Mg^{2+} and centrifuging the suspension in Percoll gradient. In this way brush-border membranes were obtained with enrichment factors ranging between 10 and 20 and yields of 15–30%. A second method is described with which membranes were isolated, without any preliminary treatment, first by differential centrifugation, then with Percoll gradient; the final membrane enrichment was over 15, however the yield was very low (3%). Many possible enzymatic markers of the apical plasma membrane were investigated: L-γ-glutamyltransferase, alkaline phosphatase, leucine aminopeptidase, sucrase. The first appears to be that of choice. Apical membrane fraction could be also evidenced by autofluorescence or by labeling with *Lotus tetragonolobus* lectin. Preliminary experiments showed that apical plasma membranes isolated in this way form vesicles.

Introduction

Rabbit gallbladder is the site of a large transport of Na^+ salts and water [1] without overimposition of nonelectrolyte transfer [2]. The epithelial cells are all transporting cells with small differences between cells on the folds and in the pits [3,4]. The entry of Cl^- into the cell through the apical membrane is only due to cotransport with Na^+ without Cl^- conductive pathways [5,6]. On the other hand, the nature of this cotransport seems to be complex: at least two systems are present, a Na^+/H^+, Cl^-/HCO_3^- double exchange and a Na^+-Cl^- symport [7] which might coexist or alternatively substitute for each other in connection with homeostatic counterreactions.

Cell homogeneity, high rate ion transports and the presence of many Na^+-Cl^- cotransports are all features which make this substrate particularly suitable for studies on Na^+ and Cl^- transfers. Thus, isolating apical plasma membrane with the aim of investigating cotransports without the interference of homeostatic control of cytoplasm, is particularly interesting. In spite of the fact that apical plasma membranes have been isolated from many epithelia, so far no attempts have been made to isolate them from gallbladder, probably owing to the large number of animals required and the very poor characterization of the membrane markers.

The aim of this study was to investigate the

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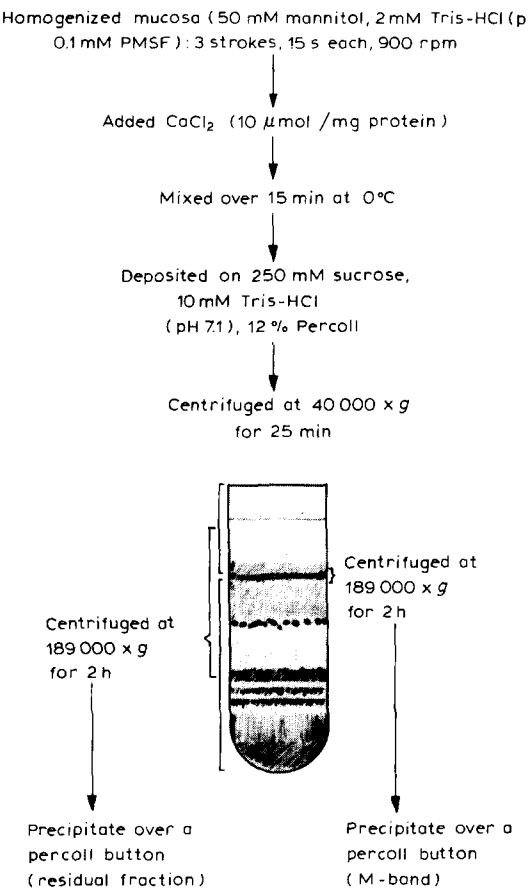
membrane markers and a method for plasma membrane isolation.

Materials and Methods

Gallbladders were excised from New Zealand rabbits purchased from Azienda Agricola Bernasconi, Valmorea (Como, Italy). Animals were killed by a blow on the head in the laboratory or by electric shock in the Bernasconi slaughterhouse. Gallbladders were washed free from bile at room temperature with physiological saline (soln. A) with the following composition (mM): 120.6 NaCl, 2.4 $CaCl_2$, 0.3 KH_2PO_4, 2.9 K_2HPO_4, 1.2 $MgSO_4$, 12.5 Na_2SO_4, 12.5 mannitol, 0.1 phenylmethylsulfonyl fluoride (PMSF) to inhibit proteinases (pH 7.4). The organs were then everted, tied at the neck, washed again and then used directly (laboratory) or kept in saline at 0°C. Mucosa was isolated by scraping gently at 0°C, then homogenized in a Potter-Braun apparatus (B. Braun Melsungen AG, F.R.G.) with 3 strokes (15 s each) at 900 rpm in saline (soln. B) with the following composition (mM): 50 mannitol, 2 Tris-HCl (pH 7.1), 0.1 PMSF. The final dilution gave 3–4 mg protein/ml.

Standard isolation method. Liquid $CaCl_2$ (1 M) was added to the homogenate to give a ratio of 10 μmol/mg protein; the suspension was mixed for 15 min at 0°C to cause aggregation of cellular fragments except microvilli [8]. 2 ml suspension was deposited on 25 ml soln. C (250 mM sucrose, 10 mM Tris-HCl (pH 7.1), 12% Percoll) in a 25 × 89 mm test-tube, with a 38.5 ml nominal volume. Test-tubes were centrifuged at 40000 × *g* (23000 rpm) on a Centrikon 2070 ultracentrifuge (Kontron AG, Analytical Division, Zurich, CH-8048) with a TFT 70,38 rotor for 25 min at 4°C. The liquid was then collected in 10 fractions by a peristaltic pump (Miniplus 2, Gilson, Villiers Le Bel, France) or the M-band (Fig. 1) vs. the entire residual fraction was collected with a syringe. The corresponding fractions or bands derived from many test-tubes were mixed and centrifuged at 189000 × *g* (50000 rpm) for 2 h to remove Percoll. The same centrifugation was performed on the homogenate mixed with soln. C. At the end a compact and glassy Percoll precipitate was obtained on which an easily separable precipitate of cellular fragments was present. The treated homogenate was taken as reference. Percoll was supplied by Pharmacia (Uppsala, Sweden).



Alternative isolation methods. Three alternative isolation methods were used:

(a) The same procedure as for the standard method was followed without previous treatment with Ca^{2+}.

(b) The homogenate without any treatment with Ca^{2+} was centrifuged at 3000 × *g* (6300 rpm) for 15 min. The supernatant was collected and centrifuged at 43000 × *g* (23800 rpm) for 20 min; the precipitate was resuspended in an equal volume of soln. B and again centrifuged at 43000 × *g* for 20 min. The new precipitate was resuspended in an equal volume of soln. B and purified with iso-

pycnic centrifugation in Percoll gradients as described for the standard method.

(c) The same procedure as for the standard method was followed, but the homogenate treatment was with Mg^{2+} instead of Ca^{2+}.

Assays. Proteins were determined with Bradford's method [9] using the Bio-Rad Protein Assay Kit; L-γ-glutamyltransferase (EC 2.3.2.2), alkaline phosphatase (EC 3.1.3.1), leucine aminopeptidase (EC 3.4.11), and sucrase (EC 3.2.1.26) activities were measured according to Persijn and Van der Slik [10], Hausamen et al. [11], Nagel et al. [12] and Semenza and Von Balthazar [13], respectively, using the corresponding assay kits purchased from Boehringer Mannheim GmbH (F.R.G.); ($Na^+ + K^+$)-ATPase (EC 3.6.1.3) was assayed according to Berner and Kinne [14] and Sohoner et al. [15]; acid phosphatase (EC 3.1.3.2), KCN-resistant NADH oxidoreductase (EC 1.6.99.2) and DNA were determined according to Hillmann [16], Wallach and Kanat [17], Sottocasa et al. [18], and Peters and Dahmus [19]. Cytochrome-*c* oxidase (EC 1.9.3.1) was assayed by the method of Smith [20].

L-γ-Glutamyltransferase assay on intact everted gallbladder sacs was performed on organs excised from animals just killed in the laboratory. The organs were bathed on the luminal side for 10 min with isosmotic soln. D (290 mosM, pH 7.5) with the following composition (mM): 123.2 glycilglycine, 14 Tris-HCl, 44.4 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$. The saline was bubbled at 37°C with 5% CO_2, 95% O_2. The substrate for L-γ-glutamyltransferase (L-γ-glutamyl-3-carboxy-4-nitroanilide) was present in the luminal saline with a 2.9 mM concentration; 1 ml sample of the solution bathing the tissue was collected after 5 min and at the end of the 10 min incubation. Finally, the tissue was carefully washed and scraped; the mucosa thus obtained was homogenized and tested for total enzymatic activity.

Autofluorescence. This was examined by an MPV2 microfluorimeter (E. Leitz Wetzlar GmbH, D-6330 Wetzlar, F.R.G.): the light source was a xenon high pressure lamp (75 W). The set of filters with maximal fluorescence was: excitation filter BP450-490, dichroic filter RKP510, barrier filter LP515. Fluorescence was examined both on histological preparations and on fractions obtained upon purification on Percoll gradient. Percoll particles were carefully washed away since they were fluorescent.

For histological preparations an everted gallbladder sac was fixed with a 10 mM phosphate buffer (pH 7.2) and 4% formaldehyde for 12–20 h, then washed with soln. E (mM: 2.7 KCl, 0.9 $CaCl_2$, 7.3 KH_2PO_4, 0.5 $MgCl_2$, 136.8 NaCl and 6.5 Na_2HPO_4) many times for 24 h. The tissue was then directly cut by a cryostat (10 μm thick sections). Some small intestine specimens taken at about 10 cm from the ileocaecal valve were prepared in the same way as controls. All the specimens were examined with the microfluorimeter (photomultiplier voltage: 1000 V; rectangular measure field: 5 × 20 μm; magnification ×500).

Liquid samples (200 μl) were poured into small pits (50 $mm^2 \times 2$ mm) and examined with the microfluorimeter (photomultiplier voltage: 800 V; circular measure field: 163 μm diameter).

Fluorescent lectin. Everted gallbladder sacs were incubated in the dark for 30 min on the luminal side in soln. E with 60 μg/ml final concentration of *Lotus tetragonolobus* fluorescent lectin (fluorescein isothiocyanate labeled; Sigma Chem. Co. St. Louis, MO, U.S.A.). Gallbladders were then washed with soln. E to remove the lectin excess; the epithelia were scraped off and processed following the standard isolation method. Fluorescence was measured as described above with the same filter combination.

Results

Plasma membrane enzymatic markers

Activities of some typical markers of intestinal and renal plasma membranes were determined in the crude homogenate of rabbit gallbladder epithelium scraped from remaining subepithelial layers of the organ wall. Measurements were made on gallbladders just excised from animals in the laboratory (controls) or preincubated at 0°C for 3–4 h, i.e. the time needed to collect and transport them from the slaughterhouse to the laboratory; in both cases they were everted and tied before scraping to avoid any contamination with liver remnants on the serosal side; at least 50 ml saline was used to wash each gallbladder.

The results obtained, compared with de-

TABLE I

SPECIFIC ACTIVITIES OF SOME PLASMA MEMBRANE MARKERS IN THE CRUDE HOMOGENATE OF RABBIT GALLBLADDER AND INTESTINE EPITHELIUM

Results are reported as means ± S.E. of the mean, expressed as mU/mg protein. The number of determinations is given in parentheses.

Enzyme	Gallbladder		Intestine
	Control [a]	Preincubated at 0°C	Control [a]
L-γ-Glutamyltransferase	27.0 ± 4.9 (4)	16.6 ± 3.2 (6)	59.2 ± 9.3 (4)
Alkaline phosphatase	11.5 ± 2.6 (5)	3.6 ± 1.0 (5) *	328 ± 22.2 (4)
Leucine aminopeptidase	12.4 ± 1.1 (4)	5.6 ± 0.3 (8) **	108.5 ± 22.7 (4)
Sucrase	0 (2)	0 (2)	48.0 ± 7.1 (4)
$(Na^+ + K^+)$-ATPase	145.3 ± 16.8 (6)	113.0 ± 13.7 (6)	142.5 ± 14.8 (4)

[a] Determination performed immediately after excision of the organ.
* $P < 0.05$; ** $P < 0.01$ vs. control gallbladder.

terminations made on controls in rabbit jejunum, are reported in Table I. It was found that: (i) preincubation at 0°C caused a general tendency to a decrease in enzymatic activity, which was significant only for alkaline phosphatase ($P < 0.05$) and leucine aminopeptidase ($P < 0.01$), (ii) the typical brush-border markers usually used exhibited no (sucrase) or negligible (alkaline phosphatase, leucine aminopeptidase) activity in gallbladder with respect to intestine, except for L-γ-glutamyltransferase whose activity was about 50% that in intestine, (iii) the usual basolateral membrane marker ($(Na^+ + K^+)$-ATPase) displayed a specific activity similar to that of intestine.

Since the epithelium scraping also removes connective tissue, contamination of marker enzymes by the subepithelial layers were checked. Whereas the subepithelial layer was richer than the epithelium both in alkaline phosphatase (about 24 times) and leucine aminopeptidase (about 5 times), only the epithelium contained a measurable activity of L-γ-glutamyltransferase. The specific activity of L-γ-glutamyltransferase in the bile was similar to that in the epithelium, so that only a negligible or null fraction of the former should contribute to the latter due to the frequent washing of the tissue.

In order to be certain that at least the major part of the L-γ-glutamyltransferase activity was localized in the apical membrane we exposed some everted gallbladder sac preparations just excised from the animal * to physiological saline containing the substrate of the reaction catalyzed by the enzyme (see Methods); the total activity thus measured was 50.5 ± 5.9 mU (four experiments). The epithelia of the same gallbladders were then scraped off and homogenized and the total activity determined again. It was 33.8 ± 8.2 mU, i.e. about 67% of the previous determination. Tissue losses during the scraping and the homogenization procedure must have been partially responsible for this smaller activity.

Autofluorescence (histological preparations)

When histological preparations of gallbladder epithelial cells were inspected by a microfluorimeter, they were found autofluorescent. Maximal autofluorescence was obtained by exciting the tissue with 450–490 nm wavelengths and measuring the emitted light at more than 515 nm wavelengths. Under these conditions the brush-border region had an emission of 33.1 ± 3.9 conventional units (c.u.) and the remaining cellular part of 14.1 ± 1.0 c.u. with 13 determinations in different cells. The ratio was 2.4 ± 0.2, a value significantly different from zero ($P < 0.01$).

Enterocyte cytoplasm was also fluorescent, in

* These everted sacs were perfectly functioning preparations accumulating saline at the high rate of 101.0 ± 7.0 $\mu l \cdot cm^{-2} \cdot h^{-1}$ (four experiments) as ascertained by gravimetrical measurements.

the same wavelength range; absolute values were not significantly different from gallbladder values although they tended to be lower. However, in this case the ratio between the brush border region and remaining cytoplasm was 1.1 ± 0.1 (19 determinations in different cells), a value which is not significantly different from 1.

Isolation of apical plasma membrane with Percoll gradients

Fig. 2 reports the results obtained from analysis of all the fractions, separated by isopycnic centrifugation with Percoll gradients, of the homogenated mucosa treated with Ca^{2+} (standard isolation method). Ten fractions were collected by a peristaltic pump from the bottom of the test-tube, the first of 7 ml, the tenth of 4 ml, the others of 2 ml as shown in the figure.

The enrichment factor of the enzymes used as markers of mitochondria (cytochrome-*c* oxidase), lysosomes (acid phosphatase), endoplasmic reticulum (KCN-resistant NADH oxidoreductase) and nucleus (DNA) was about 1–2 in the first fraction, then progressively declined to 0.3–1.3 (fractions 6, 7) and 0–0.7 (subsequent fractions). The greatest part of these markers was recovered in fraction 1 (60–70%), whereas in fractions 6, 7 recovery was minimal (2–4%) and in the subsequent fractions nearly negligible (less than 1%).

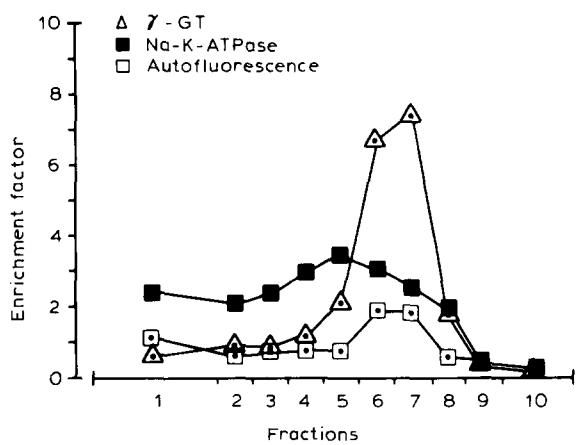


Fig. 2. Enrichment factors of several enzymatic markers in the different fractions obtained by isopyknic centrifugation on Percoll gradient; homogenate treated with Ca^{2+} (standard isolation method). γ-GT, L-γ-glutamyltransferase.

The $(Na^+ + K^+)$-ATPase enrichment factor (Fig. 2) was 2.1–2.4 in fractions 1 and 2; it then increased progressively to reach a peak of 3.5 in fraction 5, after which it fell to between 0.4–0.5 in fractions 9 and 10. The largest recoveries were in fractions 1 and 2 (65% overall) and fractions 4 and 5 (20% overall), whereas they fell to less than 10% overall in fractions 6, 7 and were nearly negligible elsewhere.

The L-γ-glutamyltransferase enrichment factor (Fig. 2) was less than 1 in the first three fractions, then sharply increased to exhibit a maximum of 6.7–7.4 in fractions 6 and 7; it fell sharply in the subsequent fractions to 0.2–0.4. Only 20% was recovered in fraction 1 but nearly 50% was in fractions 6 and 7.

Analysis of autofluorescence revealed only one peak which was localized in fractions 6 and 7 like that of L-γ-glutamyltransferase; the enrichment factor of the peak was 1.9, a value similar to the ratio found between fluorescence in brush border and cytoplasm in the histological preparations.

A visual inspection of the test-tube revealed the presence of many thick bands; the topmost band seemed to be of homogeneous material, was about 4 mm thick (the central zone being denser) and was localized at about 5.7 cm from the bottom (Fig. 1). It corresponded to about 2 ml fluid which was part of fractions 6 and 7. This band (microvillar band, M-band) was withdrawn carefully by a syringe to minimize fluid stirrings and analyzed. The results are reported in Table II and showed that M-band contained the L-γ-glutamyltransferase bound cellular fragments in a much more purified condition with respect to the overall fractions 6 and 7; L-γ-glutamyltransferase enrichment was improved (11.8 vs. about 7); also purification was improved with respect to $(Na^+ + K^+)$-ATPase (0.6 vs. about 2.8), cytochrome-*c* oxidase (0.1 vs. about 0.5) and DNA (0.4 vs. about 1.1). The improvement was considered attributable to two different causes: (i) M-band was much more restricted than fractions 6 and 7, (ii) stirring due to peristaltic pump lessened differences between fractions.

The final overall recoveries of over 90% showed that only little material and activity was lost during isolation. Partial recoveries in M-band indicated that about 30% L-γ-glutamyltransferase was

TABLE II

ANALYSIS OF M-BAND COMPARED WITH THE HOMOGENATE (STANDARD ISOLATION METHOD)

Specific activities are expressed as mU/mg protein, except for cytochrome-*c* oxidase (s^{-1} per mg protein) and DNA (μg/mg protein). Results are reported as means ± S.E. of the mean, the number of determinations is given in parentheses.

	L-γ-Glutamyl-transferase	$(Na^+ + K^+)$-ATPase	Cytochrome-*c* oxidase	KCN-resistant NADH oxidoreductase	Acid phosphatase	DNA
(a) Homogenate						
Specific activity	22.8 ± 3.4 (10)	226.0 ± 22.5 (10)	395.0 ± 28.4 (9)	1114.0 ± 234.0 (4)	22.7 ± 1.5 (5)	110 ± 7.1 (5)
(b) M-band						
Specific activity	247.4 ± 30.2 (10) **	141.0 ± 20.0 (10) *	34.5 ± 4.0 (9) **	316.0 ± 57.0 (4) **	21.0 ± 1.1 (5)	45.6 ± 6.3 (5) **
Enrichment factor	11.8 ± 1.3 (10)	0.6 ± 0.05 (10)	0.1 ± 0.01 (9)	0.3 ± 0.02 (4)	0.9 ± 0.06 (5)	0.4 ± 0.02 (5)
Yield (%)	29.2 ± 4.1 (5)	1.7 ± 0.5 (5)	0.2 ± 0.1 (4)	0.9 ± 0.2 (4)	2.2 ± 0.05 (5)	0.9 ± 0.2 (5)
(c) Overall recovery (%)	91.6 ± 6.0 (5)	93.0 ± 4.0 (5)	104.0 ± 6.0 (4)	92.5 ± 6.0 (4)	95.6 ± 4.0 (5)	93.0 ± 6.0 (5)

* $P < 0.05$; ** $P < 0.01$.

present whereas the other activities were negligible.

In one experiment some everted gallbladders were taken as controls and some others were treated on the luminal side with *Lotus tetragonolobus* fluorescent lectin as described in Methods. Autofluorescence in the controls and total fluorescence in the lectin-treated gallbladders were measured both in the homogenate and at the M-band; the respective enrichment factors were calculated and were 1.7 and 6.8. The first value agreed roughly with that found for fractions 6 and 7 autofluorescence; the second value was the expression of the enrichment of total fluorescence and should increase if the autofluorescence could be carefully subtracted from total fluorescence. The L-γ-glutamyltransferase enrichment factor in this case was 11.2 for M-band: lectin-labeled cellular fragments were enriched together with those labeled by L-γ-glutamyltransferase.

Attempted improvements of the isolation method

Three different methods were used in order to avoid treating the homogenate with Ca^{2+} which may partially alterate membrane function [21–25].

(a) The isolation procedure in Percoll gradient was repeated without previously treating the homogenate with Ca^{2+}. The result was negative as the peak of L-γ-glutamyltransferase enrichment factor (fraction 4 from the bottom), which was 13.4, coincided with those for $(Na^+ + K^+)$-ATPase and cytochrome-*c* oxidase (10 and 3.8, respectively). Thus plasma and mitochondrial membranes were isolated altogether.

(b) After differential centrifugations without treating the homogenate with Ca^{2+} (see Methods), the last pellet was resuspended and purified by isopycnic centrifugation in Percoll gradient. The preliminary differential centrifugations were similar to those used by Malathi et al. [26] on the assumption that the fragile gallbladder brush border is not isolated entirely but reduced into small microvillar vesicles by the homogenization procedure performed. Based on the supposition that the highest visible band observed corresponded to the M-band, it was withdrawn by syringe and analyzed. Purification of the L-γ-glutamyltransferase bound fragments was modest at the end of the differential centrifugations; however, it increased after purification with Percoll to levels better in some aspects than those obtained with the standard method (enrichment factors were: L-γ-glutamyltransferase, 17.7; $(Na^+ + K^+)$-ATPase, 0.2; cytochrome-*c* oxidase, 0.3; KCN-resistant NADH oxidoreductase, 1.0; acid phosphatase, 2.0; DNA, 1.0). Moreover, M-band in this case contained about 55% of L-γ-glutamyltransferase activity run along the Percoll gradient. Nevertheless the outcome of this method was negative inasmuch as the preliminary differential centrifugations caused the loss of a large amount of activity so that recovery in the M-band with respect to the initial homogenate was only 3% in spite of the fact that the final overall recoveries for all the enzymes in all fractions were nearly 100%.

(c) The standard method was followed, but Mg^{2+} was used instead of Ca^{2+} for the homo-

TABLE III

ANALYSIS OF M-BAND COMPARED WITH THE HOMOGENATE (TREATMENT WITH Mg^{2+})

Specific activities are expressed as mU/mg protein except for cytochrome-*c* oxidase (s^{-1} per mg protein). Results are reported as means ± S.E. of the mean, the number of determinations is given in parentheses.

	L-γ-Glutamyl-transferase	$(Na^+ + K^+)$-ATPase	Cytochrome-*c* oxidase	KCN-resistant NADH oxido-reductase	Acid phosphatase
(a) Homogenate					
Specific activity	21.2 ± 3.0 (5)	209.0 ± 36.4 (5)	656.0 ± 31.0 (5)	1204 (1)	24.0 (1)
(b) M-band					
Specific activity	373.0 ± 30.1 (5) **	148.2 ± 12.0 (5)	71.6 ± 6.2 (5) **	563 (1)	27.4 (1)
Enrichment factor	18.3 ± 1.8 (5)	0.77 ± 0.1 (5)	0.1 ± 0.0 (5)	0.5 (1)	1.1 (1)

** $P < 0.01$.

genate treatment; L-γ-glutamyltransferase, ($Na^+ + K^+$)-ATPase and cytochrome-*c* oxidase were tested in five experiments, the other markers in only one (Table III). Purification of the L-γ-glutamyltransferase bound fragments was even better than that obtained with Ca^{2+}. The small recoveries of all markers in the M-band ($< 1\%$) with the exception of L-γ-glutamyltransferase (about 15%) is also interesting; however, the yield of L-γ-glutamyltransferase bound fragments was half that obtained using Ca^{2+} treatment. The high specific activity of cytochrome-*c* oxidase obtained with this method could be explained by the presence of Mg^{2+}. Although the oxidase per se is not activated by Mg^{2+}, this could occur in the complex membrane fragments; in fact we found such an activation in some parallel experiments on mitochondria fragments deriving from both intestine and gallbladder.

Discussion

In a preliminary report some of us described attempts to isolate the entire brush border of rabbit gallbladder epithelium [27] by using methods successful for intestine: the outcome was negative probably owing to the scarce density and to the fragility of microvilli in gallbladder compared with intestine. Conversely, in the same study the isolation of apical membrane fragments was successful following the method described by Malathi et al. [26] based on precipitation with Ca^{2+}. However, uncertainties due to the marker used (alkaline phosphatase), its relatively low enrichment factor (6.5) and its very small yield (5.9%) in the fraction that should contain apical membranes led us to look for alternative possibilities. They are described in this paper, which shows that a satisfactory enrichment (> 10) with a sufficient yield ($> 15\%$) can be obtained only with methods based on Ca^{2+} or Mg^{2+} precipitation followed directly by separation of cellular fragments on Percoll gradients.

Among the possible markers tested only L-γ-glutamyltransferase seemed suitable. It exhibited a specific activity comparable (although lower) to that of intestine and the possibility of contamination was low. Moreover, it was mainly or solely localized at the apical membrane, as for intestine and proximal tubule [28–30]. The localization is clearly indicated by the comparison between the enzyme activity developed by the epithelium exposed to the reagent only on the apical side and the enzyme activity of the overall epithelium scraped and homogenized. Water transport in the organs was normal; thus no damaged sites were present and it was unlikely that the luminal reagent could cross the epithelium in bulk towards the basolateral side. Since the substrate of the enzyme is hydrosoluble and has a molecular weight of 346.3 it is unlikely to enter cells either through the lipidic moiety or watery pores. Since the reagent remains in the lumen the L-γ-glutamyltransferase activity measured should be only that at the apical membrane. Direct cytochemical localization based on electronmicroscopy is difficult and in fact it was unsuccessful, since the available method [31] was at the limits of a good resolution even for cells with higher enzymatic activities.

Autofluorescence analysis confirmed the correct localization showing a peak in the brush border region (histological preparation) and in the M-band, corresponding to the L-γ-glutamyltransferase peak (Percoll gradient). The cause of this fluorescence is difficult to define. Ileal enterocytes also exhibit cytoplasmic autofluorescence with the same features and magnitude as that of gallbladder, without, however, showing a higher brush-border emission. Thus most of the cytoplasmic autofluorescence could be due to a factor shared by both cell types, e.g. flavoproteins which fluoresce in the wavelength range higher than 520 nm in response to an incident blue light [32]. Flavoproteins are mainly contained in mitochondria and in fact analysis of gallbladder epithelial fragments on Percoll gradients showed a diffuse distribution of both fluorescence and cytochrome-*c* oxidase from fraction 1 to fraction 5 with a tendency in both cases to an increase toward fraction 1. The brush-border (and possibly part of the cytoplasmic) autofluorescence of gallbladder must have another explanation. Evidence has been produced that biliary pigments in the hydrophobic unconjugated form can enter the lipidic moiety of the apical plasma membrane passively and diffuse into the cytoplasm [33] where they should be distributed in the hydrophobic phases of intracellular membranes. The same pos-

sibility exists for deconjugated, high pK_a bile acids which, in the unionized form, can enter (chenodeoxycholic acid, pK_a 6.4, more than cholic acid, pK_a 5.5) [34]. In this respect biliverdin and deoxycholic acid (pK_a 6.58), present in rabbit bile, could be important. Biliverdin maintained at room temperature or at least at a temperature over 0°C shows a maximum emission at 500 nm with an excitation band at $\lambda_{max} = 470$ nm [35]. In the same way, glycocholic and taurocholic acids exhibit a similar fluorescence with a maximal emission at over 520 nm λ with an excitation at 450–490 nm. On this basis brush-border autofluorescence and possibly part of the cytoplasmic autofluorescence in gallbladder, are likely to be due to diffusion into the cell at least of the unconjugated biliverdin and biliary acids.

Further evidence that L-γ-glutamyltransferase is a marker of the apical membrane was provided by the experiment with lectin derived from *Lotus tetragonolobus*. This lectin was demonstrated to bind selectively to apical membranes of gallbladder epithelium without any reactivity for cytoplasm and basolateral membranes [36].

Finally, it is interesting that preliminary experiments on Na^+/H^+ transport measured by acridine orange fluorescence quenching, indicate that membranes isolated in this way form vesicles.

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