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# Preparation of Basolateral Membrane Vesicles from Rat Enterocytes: Influence of Different Gradient Media

V. MILOVIC, J. STEIN, S. RUPPERT, S. ZEUZEM, W.F. CASPARY

*Laboratory of the Gastroenterology Division, Second Medical Department, Johann Wolfgang Goethe University, Frankfurt/Main, Germany*

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## Summary

An attempt was made to assess whether the choice of the gradient media could influence the yield of basolateral membrane vesicles isolated from the rat intestine as well as their functional characteristics. Crude membranes prepared in the same way were therefore centrifuged with 10 % Percoll, on a discontinuous sucrose gradient or on a continuous sorbitol gradient. The protein yield was significantly higher with the Percoll gradient than with sucrose and sorbitol gradient centrifugation (2.7±1.0 %; 0.4±0.1 %; 0.6±0.2 %, respectively). Enrichment in $Na^+,K^+$-ATPase was similar in all three preparations (8.50±2.34; 8.22±4.78; 8.20±2.08). However, contamination with brush border membranes was significantly higher after Percoll gradient centrifugation and negligible after the use of the other two gradient media. Transport of D-glucose in the BLM prepared by Percoll gradient centrifugation also indicated some contamination with functional brush-border membranes. An attempt to purify basolateral membrane vesicles after Percoll gradient centrifugation with $Ca^{2+}$ precipitation, however, reduced the protein yield to less than 1 %. We conclude that in the preparation of basolateral membrane vesicles from the rat enterocytes each of the gradient media may have certain advantages and disadvantages, which should be considered according to the purpose of the preparation.

**Key words**

Basolateral membrane – Enterocyte – Percoll – Rat – Sorbitol – Sucrose

## Introduction

Numerous procedures for the isolation of basolateral plasma membranes (BLM) of enterocytes have been developed over the past two decades (e.g. Fujita *et al.* 1972, Lewis *et al.* 1975, Murer *et al.* 1976, Dyer *et al.* 1990). However, their main disadvantages remain their long duration, low yields and poor reproducibility. A promising method, using the self-orienting Percoll gradient, as described by Scalera *et al.* 1980, seemed to offer numerous advantages over previously described procedures. However, this method also appeared to have several serious disadvantages – its questionable reproducibility related to even minor changes in Percoll concentration, the possibility of obtaining impermeable vesicles and considerable contamination with brush-border membranes (BBM).

In general, the methods for basolateral membrane vesicles (BLMV) isolation from enterocytes consist of homogenization, slow speed centrifugation to remove nuclei and non-disrupted cells, high speed centrifugation to collect smaller particles, and gradient centrifugation. The aim of this study was to assess whether the choice of the gradient media itself might influence the efficiency of BLM preparation. This was performed by processing the same crude membrane preparations on Percoll, sucrose and sorbitol gradients.

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## Material and Methods

*Membrane preparation*. Wistar male or female rats weighing 200–250 g were killed by cervical dislocation. The intestine was immediately removed and washed with ice-cold isotonic saline with addition of 2 mM benzamidine and 0.5 mM phentylmethylsulfonylfluoride (PMSF) to inhibit the proteases. Rat enterocytes were isolated by the method of Weiser (1973), after incubation with citrate and EDTA. Three rats were used for one preparation. Enterocytes were diluted (1:3, w/v) with a buffer (250 mM sucrose, 10 mM triethanolamine-HCl and 0.1 mM PMSF, pH=7.6) and homogenized for one minute (Braun, Melsungen, Germany) at 1200 rpm. The homogenate was then again diluted with the buffer (1:2, v/v). After centrifugation at 2500 *x g* for 15 min the pellet was discarded and the supernatant was centrifuged at 20 500 x *g* for 20 min. The white fluffy pellet was resuspended in an appropriate amount of the buffer (see below) and homogenized (Potter-Elvehjem glass-teflon homogenizer, 20 strokes/1200 rpm). This crude membrane fraction was processed as follows:

(A) In five preparations, crude membranes were resuspended in 35 ml buffer, mixed with 10 % Percoll (v/v) and spun at 47 800 x *g* at Sorvall SS-34 rotor for 60 min. Three millilitre fractions of the Percoll gradient were collected using a slow peristaltic pump and assayed for enzymes. Fluffy white band in the upper third of the gradient corresponded to the BLM fraction as described by Scalera *et al.* (1980).

B) In the further five preparations, crude membranes were resuspended in 4.5 ml buffer containing 50 % sucrose (w/v), 10 mM triethanolamine-HCl (pH 7.5) and 0.5 mM EDTA, and homogenized with 20 strokes at 1000 rpm in a glass-teflon homogenizer. This membrane suspension was placed at the bottom of a gradient consisting of 3.7 ml of 40 %, 2.7 of 30 % and 1.7 of 20 % sucrose (w/v) buffered with 5 mM triethanolamine-HCl and 0.5 mM EDTA (pH 7.5). The sucrose gradient was centrifuged at 150 000 x *g* for 90 min in the swing-out rotor (Beckman SW40 Ti, Beckman L7 ultracentrifuge). The white band at the 30/40 interface was collected, diluted with buffer (300 mM mannitol, 20 mM Hepes/Tris pH 7.5, 0.1 mM $MgSO_4$ and 0.02 % $NaN_3$ w/v) and collected at 100 000 x *g* for 30 min.

C) In five preparations, crude membranes were resuspended in 10 ml buffer (250 mM sorbitol, 20 mM Hepes/Tris, pH 7.5), homogenized with 20 strokes at 1000 rpm in a glass-teflon homogenizer, and layered to the top of a linear sorbitol gradient (35–55 % w/v, in 20 mM Hepes/Tris buffer, pH 7.5). The gradient was centrifuged in a swing-out rotor at 190 000 x *g* for 35 min (Beckman SW 40 Ti). The white band at the 35/40 interface was collected with a syringe, resuspended in the 15 ml mannitol buffer (the same as used in the procedure B) and centrifuged at 100 000 x *g* for 30 min (Sorvall FS-28H). The membranes were resuspended in 1 ml mannitol buffer.

In three further BLMV preparations, vesicles obtained from the Percoll gradient were processed as described by Orsenigo *et al.* (1985), i.e. resuspended in 250 mM sorbitol, 10 mM Hepes/Tris, pH 7.5; 0.1 ml 1 M $CaCl_2$ was added, and the suspension was stirred on ice for 15 min. The BLM suspension was then centrifuged at 3000 x *g* for 10 min and pellet was collected at 45 000 x *g* for 20 min.

*Purification of the vesicle preparation*. Purity of BLMV was assessed by the determination of marker enzyme activities. All assays are the result of duplicate measurements from 2–5 separate preparations. $Na^+,K^+$-ATPase, marker for basolateral membranes, was estimated by the method of Lewis *et al.* (1975); the presence of dicyclohexylcarbodiimide in the assay eliminated a contribution from mitochondrial ATPase, which is sensitive to dicyclohexylcarbodiimide. Alkaline phosphatase (AP) was measured by the method of Murer *et al.* (1976), leucine arylpeptidase (LAP), gamma-glutamyl transferase (gGT), lactate dehydrogenase (LDH) and acid phosphatase activities were assessed using commercial Merck kits. Protein was determined by its ability to bind Coomassie blue, using a commercial kit (Bio-Rad).

*Transport studies*. Uptake of radiolabelled D-glucose was determined by the rapid filtration technique, as described by Murer *et al.* (1974). One-hundred $\mu$g protein were added into 100 $\mu$l incubation solution at 37 °C. The incubation medium consisted of 10 mM D-glucose labelled with [$^3$H]-D-glucose, 100 mM mannitol, 150 mM KCl or NaCl and 20 mM Hepes-Tris, pH 7.4. After the desired intervals, the transport was terminated by abrupt dilution in 1 ml of ice-cold stop solution (100 mM mannitol, 20 mM Hepes/Tris, pH 7.2, 20 mM $MgSO_4$). After immediate filtration, filters were rinsed five times with 1 ml ice-cold stop solution and the remaining radioactivity was measured using liquid scintillation counting.

*Materials*. [$^3$H]-D-glucose (2.9 Ci/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). The membrane filters (Millipore HAWP02500, cellulose nitrate, 0.45 $\mu$m pore size, 2.5 cm diameter) were obtained from Millipore (Eschborn, Germany). ATP used for the assay of $Na^+,K^+$-ATPase was vanadium-free and purchased from Boehringer, (Mannheim). Percoll was obtained from Pharmacia (Uppsala, Sweden). Sucrose and sorbitol were purchased from Sigma (St.Louis, USA). All other chemicals were of the highest analytical grade and used without further purification.

## Results

The total volume of about 45 ml after Percoll gradient centrifugation was divided into 15 aliquots of 3 ml each; the rise in protein concentration in fractions 3–5 corresponded to the whitish band near the top of the tube (data not shown). $Na^+$, $K^+$-ATPase was also distributed mainly in the upper six fractions (Fig. 1). However, the concentration of marker enzymes of BBMs was also high in the upper part of the gradient, overlapping with fractions rich in $Na^+$,$K^+$-ATPase (Fig. 2).

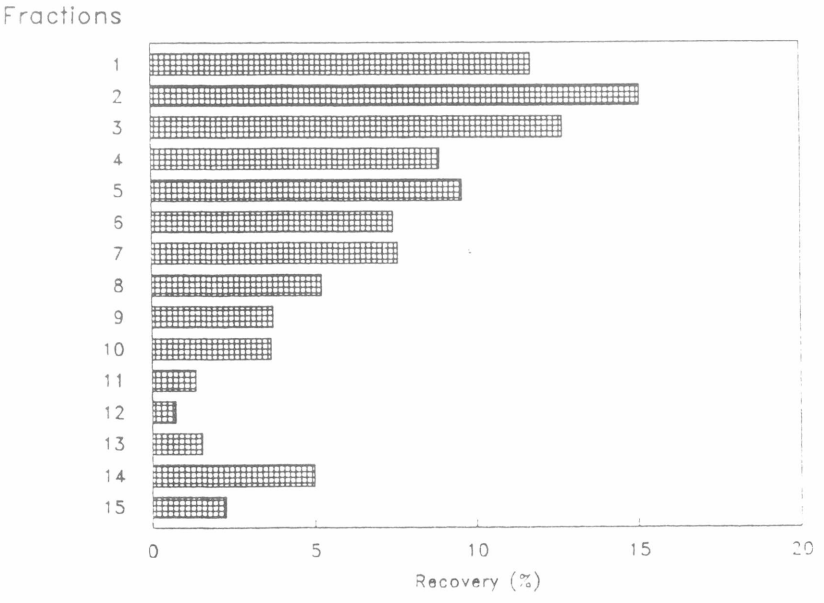


**Fig. 1**
Distribution of $Na^+$,$K^+$-ATPase across Percoll gradient. Values are the mean from two duplicate measurements.

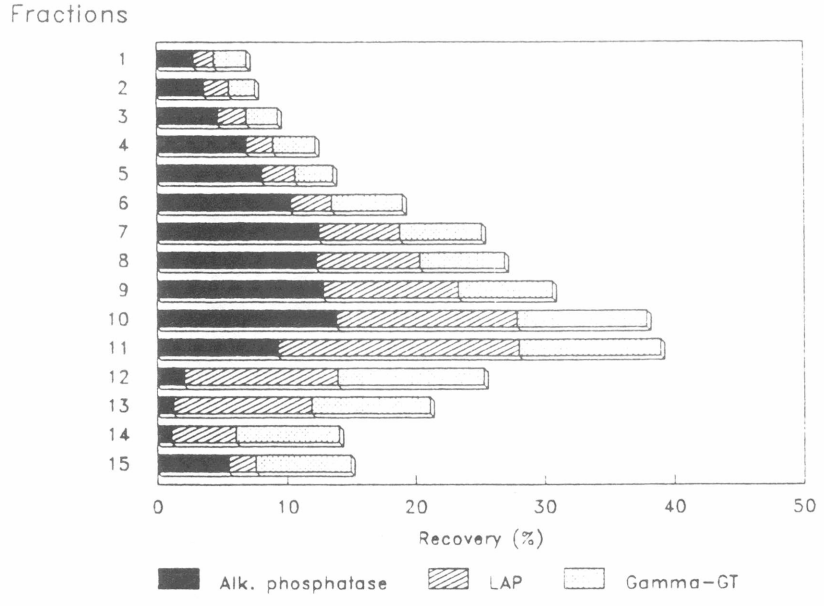


**Fig. 2**
Distribution of marker enzymes for BBM across Percoll gradient. Values are the mean from two duplicate measurements.

Sucrose and sorbitol gradients were also divided into fractions and all bands and interfaces were collected. $Na^+$,$K^+$-ATPase and BBM markers were also estimated in each fraction. It is shown in Fig. 3 that the $Na^+$,$K^+$-ATPase activity on the sucrose gradient was the highest mainly at 30 %/40 % interface. Furthermore, $Na^+$,$K^+$-ATPase activity on the sorbitol gradient was located mainly within the white band near the top, while BBM marker enzymes were distributed mainly through 40 %/45 % band and the 45 % layer (Fig. 4).

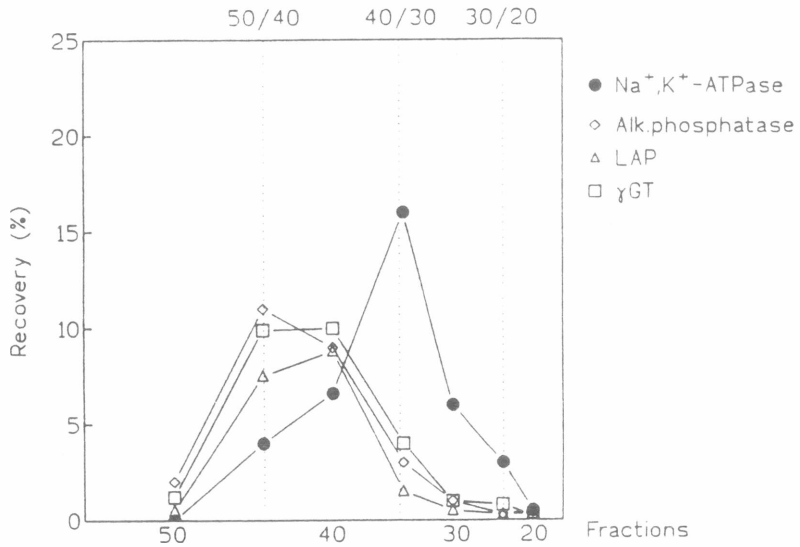


**Fig. 3**
Distribution of marker enzymes for BLM and BBM across the discontinuous sucrose gradient. Corresponding concentrations of sucrose are shown on the abscissa with proportional distance from the bottom of the centrifugation tube. Values are the mean from two duplicate measurements.

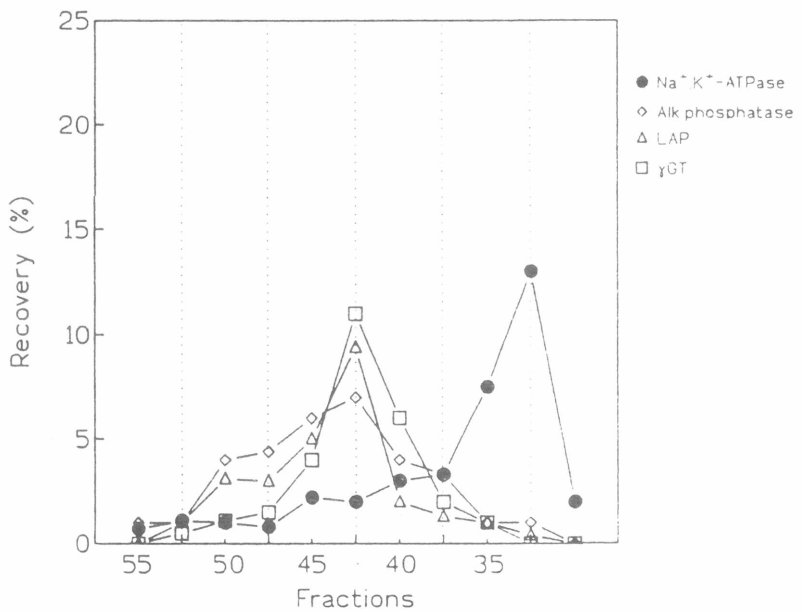


**Fig. 4**
Distribution of marker enzymes for BLM and BBM across continuous sorbitol gradient. Values are the mean from two duplicate measurements.

Activities of marker enzymes in the fractions labelled as BLMV from all three gradients are shown in Table 1. From the Percoll gradient, upper four to five 3 ml fractions were used, optically corresponding to the described white band. From the sorbitol gradient it was a white band near the top of the gradient whereas from the sucrose gradient it was the 30 %/40 % band. There was a significant enrichment in $Na^+,K^+$-ATPase obtained by all three procedures; however, contamination with BBMV in the Percoll gradient was present. BLMV from the other two gradients were, on the contrary, satisfactorily purified, although the protein yields were lower than on Percoll.

The functional assessment of BLMV by the ability to transport D-glucose served as evidence for a contamination with functional BBMV in BLMV obtained from the Percoll gradient (Fig. 5a). An overshoot of 35 % was noted when potassium in the incubation medium was replaced with sodium. A similar overshoot was absent in BLMV from the other two gradient media (Fig. 5b and 5c). However, the velocity of D-glucose uptake into BLMV from sorbitol gradient was about 25 % higher than into those from the sucrose gradient.

**Table 1**
Enzyme recoveries and enrichment factors in small intestinal crude membranes and BLM fractions compared to the initial homogenate (mean ± S.D. from five preparations).

| Fractions | | C | BLM/P | BLM/Su | BLM/So |
|---|---|---|---|---|---|
| Proteins | (R %) | 11.4 ± 1.8 | 2.7 ± 1.0 | 0.4 ± 0.1 | 0.6 ± 0.2 |
| $Na^+,K^+$-ATPase | (SA in homogenate 11.86 ± 4.32) | | | | |
| | (R %) | 22.2 ± 8.5 | 13.5 ± 7.4 | 18.8 ± 3.2 | 19.0 ± 5.3 |
| | (E) | 2.88 ± 1.17 | 8.50 ± 2.34 | 8.22 ± 4.78 | 8.20 ± 2.08 |
| AP | (SA in homogenate 472.4 ± 78.9) | | | | |
| | (R %) | 29.9 ± 9.8 | 8.9 ± 3.6 | 3.1 ± 1.4 | 3.5 ± 1.2 |
| | (E) | 1.89 ± 0.89 | 2.41 ± 1.15 | 0.56 ± 0.22 | 1.54 ± 1.17 |
| LAP | (SA in homogenate 13.26 ± 3.22) | | | | |
| | (R %) | 32.2 ± 8.8 | 2.0 ± 0.8 | 1.1 ± 0.4 | 2.0 ± 1.1 |
| | (E) | 1.56 ± 0.78 | 1.78 ± 0.29 | 0.24 ± 0.09 | 0.62 ± 0.30 |
| LDH | (SA in homogenate 3940 ± 1872) | | | | |
| | (R %) | 16.3 ± 1.9 | 0.93 ± 0.15 | 0.13 ± 0.08 | 0.16 ± 0.12 |
| | (E) | 0.78 ± 0.32 | 0.40 ± 0.29 | 0.15 ± 0.06 | 0.27 ± 0.16 |
| gGT | (SA in homogenate 5.47 ± 1.87) | | | | |
| | (R %) | 24.9 ± 9.1 | 6.9 ± 2.6 | 2.7 ± 0.6 | 2.2 ± 0.4 |
| | (E) | 2.27 ± 0.54 | 1.68 ± 0.51 | 0.37 ± 0.11 | 1.06 ± 0.19 |
| Acid phosphatase | (SA in homogenate 61.99 ± 15.11) | | | | |
| | (R %) | 31.2 ± 5.3 | 2.4 ± 1.2 | 0.6 ± 0.4 | 0.6 ± 0.1 |
| | (E) | 1.99 ± 1.03 | 0.78 ± 0.35 | 0.22 ± 0.09 | 0.27 ± 0.13 |

*C – crude membranes; BLM/P/Su/So – BLM prepared on Percoll, sucrose and sorbitol gradient; (SA) – specific activity in homogenate, expressed as mU/ml/mg protein; (R %) – recovery: (E) – specific activities relative to the activities found in homogenate (enrichment factor); the results are means ± S.D. from five preparations.*

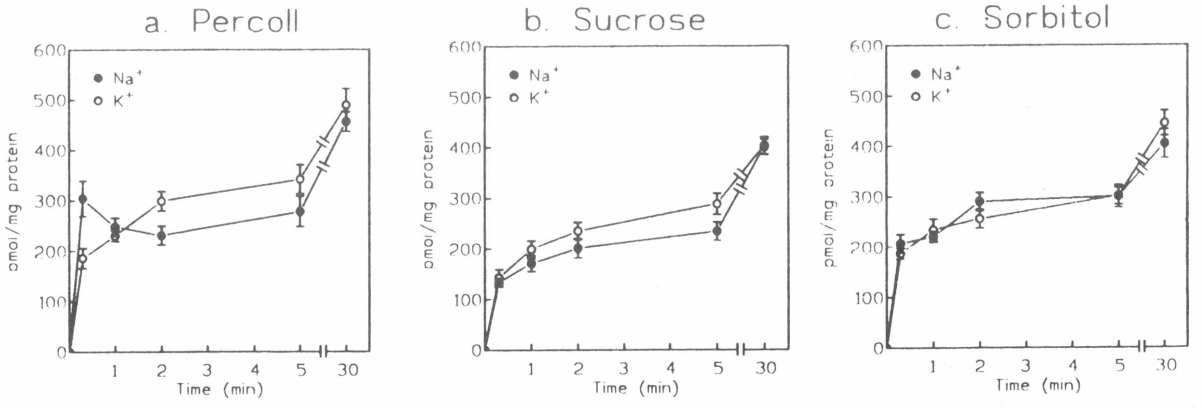


**Fig. 5**
D-glucose transport across BLMV isolated on Percoll (a), sucrose (b) and sorbitol gradient (c). Content of the incubation medium is given in the Methods section. Values are mean ± S.E.M. from three separate preparations.

The attempt to purify our BLMV from the Percoll gradient by $Ca^{2+}$ precipitation, as previously described by Orsenigo *et al.* 1985, reduced the protein yield to 0.65±0.12 % (mean±S.E.M. from three preparations); $Na^+,K^+$-ATPase showed an enrichment of 7.9±1.4, and contamination with LAP as BBM marker was 0.9±0.2. Therefore, divalent cation precipitation decreased the amount of material and did not improve the procedure sufficiently.

## Discussion

Preparation of isolated membrane vesicles from the enterocytes provided a possibility to study transport processes independently of other cellular events. Numerous methods have been developed for the isolation of brush-border membranes, using divalent cation precipitation. The addition of $CaCl_2$ or $MgCl_2$ in adequate proportion to the homogenized enterocytes leads to agglomeration of the membranous material. Brush-border membranes do not form agglomerates with calcium or magnesium, being protected by glycocalyx (Bjorkman and Brigham 1990), and can be easily collected from the supernatant.

Procedures for the separation of basolateral membrane vesicles were time consuming, with low yields and requiring ultracentrifugation. They were suitable for analytical rather than preparative purposes. Percoll gradient centrifugation seemed to offer an useful alternative to ultracentrifugation techniques. However, even in the initial report of Scalera *et al.* (1980), some drawbacks were reported – reproducibility was highly dependent on minor changes in Percoll concentration, even in different batches; there was also a marked contamination with BBM. In the consequent study of Orsenigo *et al.* (1985), a further disadvantage was found – less permeable vesicles, probably because of the presence of calcium in the intravesicular space.

The aim of this study was to assess whether the choice of the gradient medium might influence the yield and transport properties of BLMV from the rat enterocyte. Although homogenization of the isolated enterocytes might also be a crucial step in vesicle preparation (Murer *et al.* 1974, Brasitus and Keresztes 1983), we prepared crude membranes from the rat enterocytes in the same manner. Therefore, only the choice of the gradient medium was a factor that was different in these preparations.

Similarly to previous reports, our centrifugation with Percoll showed significant overlapping of enzyme markers for BLM and BBM throughout the gradient. The problem could not be overcome by purifing BLMV with calcium precipitation because the enrichment in $Na^+$, $K^+$-ATPase did not improve significantly, although LAP as a BBM marker was substantially reduced. Furthermore, D-glucose transport into BLMV isolated on the Percoll gradient showed a sodium-dependent overshot of 35 %, indicating contamination with functional BBMV.

BLMV from both sucrose and sorbitol gradient were better purified and showed acceptable transport properties for D-glucose. Nevertheless, their yield remained low, and ultracentrifugation was necessary for their preparation. However, BLMV from the sorbitol gradient showed a higher velocity of D-glucose uptake than BLMV from the sucrose gradient. It was previously shown (Del Castillo and Robinson 1982) that 60 % of basolateral membranes in the preparation using Percoll consisted of inside-out oriented vesicles and 40 % membrane sheets; different vesicle/sheet ratio may be the reason for different transport properties of our two preparations. Furthermore, sucrose has been widely used as a gradient medium because of its biological inertness, low cost and stable nature (Ridge 1978). However, sucrose solutions have high osmotic strength and solutions more concentrated than 9 % (w/v) are hypertonic, showing great viscosity at higher concentrations (Rickwood 1989). Sorbitol as a gradient medium is less viscous and less dense than the equivalent sucrose solution. Our experience with the linear sorbitol gradient in simultaneous preparations of rabbit intestinal BBMV and BLMV (Stein *et al.* 1993) showed that, in addition to the successful separation of the two membranes, enzyme activities were better preserved in the presence of sorbitol than on our preparations using the sucrose gradient.

The enrichment of $Na^+,K^+$-ATPase in all three gradient media did not exceed 10. This might be due to the enzyme assay used – in the method of Lewis *et al.* (1975) dicyclohexylcarbodiimide was used to inhibit mitochondrial ATPase; our enzyme enrichment resembles, therefore, only the activity of $Na^+,K^+$-ATPase originating from BLM. $Na^+,K^+$-ATPase enrichment of over 10, described in earlier studies, may therefore present a sum of activities of mitochondrial and basolateral ATPases and indicate that the BLM preparations are contaminated with mitochondria.

We conclude that the gradient media themselves may influence the yield and functional quality of rat intestinal BLMV preparations. If the rat intestinal BLMV are to be prepared, sucrose and sorbitol gradients provide a low yield but high purification; the Percoll gradient gives a higher yield, but the contamination with BLMV might influence the results of the transport studies. An appropriate method should be found for further purification of BLMV prepared on the Percoll gradient. Finally, the equipment required and duration of the three procedures should be considered when selecting the most suitable method.

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**Reprint Requests**
Dr. J. Stein, Abteilung für Gastroenterologie, Zentrum der Inneren Medizin, Johann Wolfgang Goethe Universität, Theodor Stern Kai 7, D-60590 Frankfurt a. Main, Germany.