Efficient entrapment of large and small compounds during vesiculation of intestinal microvilli

F. S. VAN DOMMELEN, C. M. HAMER and H. R. DE JONGE

Department of Biochemistry I, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

An efficient method is described permitting the encapsulation of membrane-impermeable compounds at the interior of intestinal microvilli during vesicle formation. Rat intestinal epithelial cells were isolated by high-frequency vibration and exposed transiently to iso-osmotic medium containing 5 mM-EDTA. Vesiculation of microvilli was effected by freeze-thawing instead of mechanical fragmentation or hypo-osmotic lysis. Solutes to be entrapped were mixed with the extracellular medium before freezing in liquid N₂. Microvillous vesicles were isolated from thawed cell suspensions by Ca²⁺- or Mg²⁺-aggregation of contaminants and differential centrifugation. The yield, purity, orientation and transport properties of the vesicles were similar, or superior, to preparations described in the literature. A high loading efficiency was demonstrated for small impermeants (cyclic GMP, ATP, Arsenazo III) as well as proteins (albumin); in contrast, loading of isolated vesicles by hypo-osmotic shock was only partially effective (cyclic GMP, ATP) or ineffective (albumin). Entrapment of an ATP-regenerating system could partially block a Mg²⁺-dependent conversion of intravesicular ATP into ADP. No evidence was obtained for the contribution of a proton pump to the intrinsic Mg²⁺-ATPase of the vesicle. Potential applications of the vesicle-loading technique in studies of brush-border transport regulation by intramicrovillar factors are discussed.

INTRODUCTION

Membrane vesicles derived from intestinal or renal brush borders have found wide application as tools for analysis of transport systems localized at the luminal border of the epithelial cells (Hopfer, 1978; Murer & Kinne, 1980; Sachs *et al.*, 1980; Stevens *et al.*, 1984; Wright, 1984). A major obstacle in using the vesicle model to study transport regulation by intracellular factors is the spontaneous resealing of the brush-border membrane in a predominantly right-side-out orientation (Haase *et al.*, 1978). Externally added membrane-impermeable compounds possibly involved in transport modulation, e.g. cyclic nucleotides, ATP, calmodulin, protein kinases, are therefore unable to gain access to their regulatory sites at the interior of the microvillus membrane. Attempts in our laboratory to open the vesicles transiently or to change their orientation by mechanical or chemical methods either failed in restoring the integrity of the membrane (e.g. sonication, nitrogen cavitation, repeated freeze-thawing, alkali treatment) or led to incomplete loading, particularly in respect to macromolecules (hypo-osmotic shock; cf. Biber *et al.*, 1983; the present paper). An alternative approach, i.e. the encapsulation of compounds at the microvillus interior during the vesiculation process, appeared equally inefficient using standard techniques for tissue isolation (everted intestine; mucosal scrapings) and vesiculation (mechanical homogenization; cf. Kessler *et al.*, 1978; Hauser *et al.*, 1980). A drastic improvement of the loading efficiency could be reached by a modified procedure starting with isolated epithelial cells pre-exposed to iso-osmotic EDTA-containing medium and introducing a freeze-thaw cycle in liquid N₂ instead of mechanical rupture to provoke vesiculation of the microvilli. The yield, orientation,

membrane stability and transport properties (glucose overshoot) of vesicles obtained by this novel method was comparable with, or superior to, that of published procedures. Moreover, the use of concentrated cell suspensions considerably reduces the amount of costly impermeants (e.g. GTP, enzymes, pH or pCa indicators). Potential applications of the loading technique in studies of transport regulation at the level of intestinal brush-border-membrane vesicles (BBMV) are discussed.

MATERIALS AND METHODS

Isolation of intestinal epithelial cells

Male specific-pathogen-free Wistar rats weighing 200–230 g, fed *ad lib*, were used throughout the present study. Under light diethyl ether anaesthesia the small intestine was removed and rinsed twice with 20 ml of ice-cold saline (0.9% NaCl). All further steps were performed at 0–4 °C. Everted segments originating from the jejunum and ileum of two rats were mounted on metal rods attached to a vibration apparatus (Vibro-Mixer model E1 from Chemap. A.G., Hännedorf ZH, Switzerland) as described previously (Iemhoff *et al.*, 1970; Hülsmann *et al.*, 1974). Epithelial cells mainly derived from the villus region were released by vibration (30 min, frequency 50 Hz, amplitude 2 mm) in 400 ml of buffer A (10 mM-Tris/HCl/0.13 M-NaCl/5 mM-EDTA, pH 7.4). Alternatively, mucosal scrapings from rat jejunum or pig small intestine (obtained freshly from the Department of Experimental Cardiology of this University) were suspended in buffer A to about 5 mg of protein/ml and stirred gently for 30 min at 0–4 °C. Cells and cell sheets were finally collected by centrifugation (3 min, 270 g), washed three times with 40 ml of buffer B (10 mM-

Tris/HCl/0.3 M-mannitol, pH 7.2), and suspended in buffer B to a concentration of about 10 mg of protein/ml.

Encapsulation and vesiculation procedure

Compounds to be trapped at the vesicle interior were added to 0.5 ml of buffer B and mixed vigorously with 1 ml of the cell suspension in polyethylene tubes on a vortex mixer. After 30 s, the tube was immersed in liquid N₂ and kept frozen for at least 10 min. Subsequently, the tube content was thawed slowly in ice/water for 60–90 min and diluted 5-fold with ice-cold buffer B. Where indicated, 10 mM-theophylline was also included in this buffer. BBMV generated by the freeze-thaw procedure were separated from other membrane fragments by differential precipitation with 10 mM-CaCl₂ or -MgCl₂ and centrifugation (5 min, 3000 g) essentially as described by Kessler *et al.* (1978) and Hauser *et al.* (1980). BBMV were isolated from the supernatant by a second centrifugation step (30 min, 27000 g). Routinely, the crude vesicle pellet was resuspended in 40 ml of buffer C (10 mM-Hepes/Tris/0.1 M-mannitol, pH 7.5). The suspension was homogenized in a Potter-Elvehjem homogenizer and subjected again to the differential precipitation procedure. The final pellet was resuspended in buffer C to a concentration of 5–8 mg of protein/ml. For comparison, in some experiments the vesiculation was effected by mechanical homogenization (Virtis homogenizer, 2 min, full speed) instead of freeze-thawing. In order to distinguish adherence of compounds to the outer side of the vesicle membrane from encapsulation, parallel experiments were performed in which equal concentrations of the membrane impermeants were added to the medium immediately after freeze-thawing or mechanical homogenization. For the experiments shown in Fig. 2 (below), buffer D (10 mM-Tris/HCl/0.6 M-mannitol, pH 7.2) was used throughout the vesicle-isolation procedure.

Loading efficiency of the vesicles

The entrapment efficiency of low-*M_r* compounds (ATP, cyclic GMP, Arsenazo III) was measured by filtering 20 µl samples of vesicle suspension (about 100 µg of protein) through nitrocellulose filters (Sartorius, pore size 0.45 µm) and rinsing the filter three times with 2.5 ml of buffer C. Entrapment of cyclic [³H]GMP was measured by dissolving the filter in 10 ml of Instagel and counting the radioactivity in a liquid-scintillation counter. Other compounds were first released from the vesicle interior by immersing the filter in 0.5 ml of 5% (w/v) trichloroacetic acid and removal of vesicle protein by centrifugation (2 min, 20000 g). Samples of the supernatant (0.2 ml) were extracted three times with 1.0 ml of diethyl ether (water-saturated) to remove trichloroacetic acid and were neutralized by addition of 20 mM-Tris/HCl, pH 7.4. In the neutralized extract, ATP was measured by mixing 0.1 ml of sample or ATP standard with 1 ml of buffer containing 8 mM-Na₂HAsO₄, 5 mM-MgSO₄, 25 mM-glycylglycine, 1 mM-EGTA, pH 8.0, and 50 µl of luciferase (Lumac B.V., Bergheyk, Belgium) followed by measurements of chemiluminescence in a LKB 1250 Lumino-meter. ADP was first converted into ATP by incubating 0.1 ml of sample with 0.05 ml of the same buffer containing 10 µg of creatine kinase (0.25 unit) and 0.2 mM-creatine phosphate for 10 min at 37 °C. Under these conditions, the conversion of similar amounts of ADP standards appeared virtually complete. The amount of Arsenazo III was measured by monitoring the

absorbance of the Ca²⁺ dye at 660 nm in the presence of excess Ca²⁺ (10 mM) or EGTA (10 mM) on an Aminco-Chance DW-2 spectrophotometer. Standards of the same Arsenazo III batch were used for calibration. Unlabelled cyclic GMP was determined by a radioimmunoassay kit (The Radiochemical Centre, Amersham, Bucks., U.K.). The loading efficiency of BSA was determined by adding 0.5% Triton X-100 to about 200 µg of the vesicle suspension and removal of insoluble material by centrifugation in a Beckman Airfuge at 4 °C (10 min, 130000 g). The amount of BSA in the supernatant was quantified by densitometric scanning (Vitatron TLD 100) of Coomassie Blue-stained SDS/polyacrylamide gels (Laemmli, 1970) loaded with Triton extracts and BSA standards.

Loading of isolated vesicles by osmotic shock

Vesicle suspensions in buffer B (40–55 mg/ml of protein) were rapidly diluted 15-fold with a solution of compounds to be encapsulated in distilled water (30 °C). After prolonged incubation at 30 °C (5 min) to allow membrane resealing, the vesicle suspension was centrifuged for 30 min at 27000 g. The vesicle pellet was resuspended in 20 ml of buffer C and centrifugation was repeated. The final pellet was resuspended in buffer C and the encapsulation efficiency of compounds was measured as described above. In parallel experiments, vesicles were osmotically shocked first and compounds were added before the first centrifugation step.

Cyclic GMP binding to the vesicle interior

The maximal binding capacity of BBMV for cyclic GMP was determined by incubating 0.1 ml of the vesicle suspension (about 1 mg of protein) for 10 min at 30 °C in the presence of 10 mM-theophylline, 0.33 µM-cyclic [8-³H]GMP (15 Ci/mmol) and the membrane-perturbing antibiotic alamethicin needed to unmask latent cyclic GMP-binding sites at the vesicle interior (De Jonge, 1981; Van Dommelen & De Jonge, 1984) (50 µg/ml). Binding was arrested by addition of 2 ml of ice-cold 3.6 M (NH₄)₂SO₄ in buffer C, serving to prevent dissociation of cyclic GMP from its receptor during filtration (cf. Corbin & Døskeland, 1983), and vesicle protein was collected on nitrocellulose filters (0.45 µm pore size). The filter was washed four times with 5 ml of the stopping solution and radioactivity was counted and corrected for quenching as described by Corbin & Døskeland (1983). To correct for binding to the vesicle exterior, parallel experiments were carried out in the absence of alamethicin. Non-specific binding as determined by addition of 50 µM unlabelled cyclic GMP to the binding assay appeared negligible. In view of the high affinity of the cyclic GMP receptors in intestinal brush border for its ligand (*K_D* ~ 5 nM; De Jonge, 1981), the concentration of cyclic [³H]GMP used (0.33 µM) was fully saturating.

Miscellaneous procedures

Transient accumulation of D-[1-³H]glucose inside BBMV in response to an NaSCN gradient (0.1 M-outside; 0 M-inside) was determined essentially as described by Kessler *et al.* (1978) as described by Van Dommelen & De Jonge (1984). Plots of glucose uptake as a function of time in BBMV prepared by the freeze-thaw procedure approached linearity for the first 0.1 min (cf. Braun *et al.*, 1984). Vesicles referred to as 'Ca-BBMV' and 'Mg/EGTA-BBMV' were prepared from fresh rat jejunal

Table 1. Characteristics of BBMVs obtained from rat small-intestinal epithelium by four different procedures

In each experiment, BBMVs were isolated from fresh jejunal-ileal segments of two rats by one of the following procedures specified in detail in the Materials and methods section: (1) cell isolation (vibration), freeze-thawing, Ca²⁺ precipitation (method A); (2) cell isolation (vibration), mechanical homogenization, Ca²⁺ precipitation (method B); (3) everted whole intestine, mechanical homogenization, Ca²⁺ precipitation (Ca-BBMV, method C); (4) everted intestine, mechanical homogenization in the presence of 5 mM-EGTA, Mg²⁺ precipitation (Mg/EGTA-BBMV, method D). Characteristics of Na⁺-dependent [³H]glucose uptake in response to a NaSCN gradient and sucrose activity were determined as indicated in the Materials and methods section. Glucose overshoot was defined as the ratio of peak levels of intravesicular glucose, [glucose]_{max}, and the equilibrium level following dissipation of the Na⁺ gradient after 30 min of incubation at 25 °C. The enrichment factor of sucrose was obtained by comparison of the specific enzyme activity in each preparation of BBMVs with mucosal scrapings (average value 0.11 unit/mg of protein). Values given represent averages ± S.E.M. for ten experiments.

Method of vesicle preparation	Yield (mg of protein/rat)	Sucrase activity (enrichment factor)	Preincubation Temp. . . .	Glucose transport (pmol/0.1 min per mg of protein)		Glucose overshoot:	
				1 min 25 °C	30 min 37 °C	[Glucose] _{max}	
						[Glucose] _{equilibrium}	
A (freeze-thaw)	9 ± 3	29 ± 6		34 ± 7	38 ± 5	18 ± 2	
B } (homogenization)	13 ± 5	18 ± 4		16 ± 5	14 ± 4	15 ± 4	
C }	13 ± 4	20 ± 5		27 ± 8	12 ± 6	21 ± 7	
D }	22 ± 7	14 ± 3		19 ± 4	14 ± 6	19 ± 4	

segments exactly as described by Kessler *et al.* (1978) and Hauser *et al.* (1980) respectively for rabbit intestine. Sucrase activity measured in 0.1% Triton X-100 was determined by the method of Forstner *et al.* (1968). Protein was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Materials

Cyclic [8-³H]GMP (15 Ci/mmol), [γ -³²P]ATP (3000 Ci/mmol) and D-[1-³H]glucose were obtained from The Radiochemical Centre. Creatine phosphate, ATP (disodium salt), purified enzymes and carboxyl cyanide *p*-trifluoromethoxyphenylhydrazone were purchased from Boehringer. Filipin came from Upjohn and was obtained through Polysciences, Arsenazo III came from Sigma. Kodak X-ray film (SB-5) was used for autoradiography. All other chemicals were of the highest purity available.

RESULTS

Characteristics of vesicles prepared by the freeze-thaw method

In Table 1 a comparison is made between intestinal brush-border-membrane vesicles isolated by four different procedures in respect to yield, enrichment of the brush-border marker enzyme sucrase and glucose-transport properties. As judged by sucrase measurements, the purity of BBMVs obtained by the freeze-thaw method is slightly improved as compared with procedures frequently used in other laboratories (Mg/EGTA- and Ca-BBMVs). All four vesicle preparations showed similar protein-staining patterns on SDS/polyacrylamide gels and electron-microscopic appearance (results not shown). The vesicle yield was not affected appreciably by isolation of the enterocytes before vesiculation (Table 1, methods A and B), confirming the quantitative release of epithelial cells by the vibration technique shown previously by histological examination (Iemhoff *et al.*, 1970). The transient accumulation of glucose driven by an inwardly directed Na⁺ gradient, a parameter reflecting both the activity of the Na⁺-glucose co-transport system and the salt permeability of the vesicle membrane (cf. Van Dommelen & De Jonge, 1984), is also at least equal to the values determined for Mg/EGTA- and Ca-BBMVs and clearly more resistant to preincubation at higher temperatures (30 min, 37 °C; Table 1).

Entrapment of low-M_r compounds during vesiculation

Among the various compounds tested in the vesicle-loading procedure, cyclic GMP added to the cells in combination with the phosphodiesterase inhibitor theophylline was found to accumulate at the vesicle interior about 60-fold above its extracellular level (0.33 μ M), confirming the presence of high-affinity cyclic GMP receptors in the intestinal microvilli (Table 2; cf. De Jonge, 1981). Recovery of cyclic [³H]GMP amounted to about 23 pmol/mg of vesicle protein, i.e. close to the cyclic GMP-binding capacity of intestinal BBMVs determined in the presence of the pore-forming antibiotic alamethicin (25 pmol/mg; Table 2). In contrast, the addition of cyclic [³H]GMP to the cellular medium subsequent to freeze-thawing or mechanical homogenization, or exposure of isolated vesicles to this compound resulted in a very poor loading efficiency (< 2 pmol/mg; Table 2). These data, taken together, led to the following conclusions: (1) the cyclic GMP receptors are situated

Table 2. Loading efficiency of cyclic [^3H]GMP in intestinal brush-border membrane vesicles under various conditions

Villous cells isolated by the vibration technique, mucosal scrapings (occasionally pre-exposed to EDTA) or BBMVs originating from the jejunal-ileal segment of two rats were exposed to $0.33\ \mu\text{M}$ -cyclic [^3H]GMP ($15\ \text{Ci}/\mu\text{mol}$) plus $10\ \text{mM}$ -theophylline as described in the Materials and methods section (conditions specified in the Table). The amount of ^3H label associated with the vesicles (prepared by differential Ca^{2+} precipitation) was measured by liquid-scintillation counting. Replacement of cyclic [^3H]GMP by unlabelled cyclic GMP under conditions of the first experiment in the Table, followed by cyclic GMP measurements in BBMVs by radioimmunoassay, result in similar values as obtained by ^3H counting ($24.3\ \text{pmol}/\text{mg}$ of protein; $n = 2$), indicating that all radioactivity recovered in the vesicles was still associated with cyclic GMP. The accumulation factor for cyclic GMP was defined as the ratio of intravesicular levels of cyclic [^3H]GMP recovered and the concentration of extracellular or extravesicular [^3H]cyclic GMP added, taking $1.12\ \mu\text{l}/\text{mg}$ of protein as the intravesicular volume (glucose equilibrium space; average of 15 determinations). Values are means $\pm$ S.E.M.; n = number of experiments.

Starting preparation	Loading conditions		Cyclic [^3H]GMP recovered in BBMVs (pmol/mg of protein)	Accumulation factor (average)
	Cyclic [^3H]GMP addition	Pretreatment		
Isolated enterocytes	Prior to freeze-thawing	EDTA	23.4 ± 3.2 ($n = 5$)	63
	After freeze-thawing	EDTA	1.1 ± 0.6 ($n = 4$)	3
	Prior to mechanical homogenization	EDTA	18.5 ± 3.1 ($n = 3$)	50
	After mechanical homogenization	EDTA	1.8 ± 0.7 ($n = 3$)	5
Mucosal scrapings	Prior to freeze-thawing	EDTA	21.0 ± 3.2 ($n = 3$)	57
		EDTA omitted	4.7 ± 2.0 ($n = 3$)	13
Isolated vesicles	No other additions	—	1.5 ± 1.0 ($n = 5$)	4
	After exposure to alamethicin ($50\ \mu\text{g}/\text{ml}$)	—	25.5 ± 2.6 ($n = 5$)	69
	During one freeze-thaw cycle	—	5.6 ± 2.0 ($n = 3$)	15
	The same, after EDTA exposure ($5\ \text{mM}$, $30\ \text{min}$, 0°C)	—	2.1 ($n = 1$)	6

almost exclusively at the vesicle interior; (2) the vesicle membrane is virtually impermeable to submicromolar concentrations of cyclic GMP; (3) sealing of the vesicles is completed during the thawing or homogenization process; cyclic GMP added at a later stage is no longer encapsulated; (4) entrapment is not critically dependent on the vesiculation procedure used (freeze-thawing or mechanical homogenization); in contrast, the transient exposure of enterocytes (either during vibration or during stirring of mucosal scrapings) to iso-osmotic medium containing $5\ \text{mM}$ -EDTA (but not EGTA; results not shown) appeared a prerequisite for efficient loading. Omission of EDTA resulted in a 5-fold lower trapping efficiency (Table 2); (5) in contrast with cells, exposure of vesicles to EDTA and to one cycle of freeze-thawing in the presence of cyclic [^3H]GMP did not result in efficient loading (Table 2).

Similar conclusions could be drawn from ATP-loading experiments in the absence of Mg^{2+} (Table 3). In this case, the final level of ATP associated with the vesicles ($9.3\ \text{mM}$) was close to the equilibrium value expected in the absence of binding ($10\ \text{mM}$). Over 95% of the vesicular ATP could be released by alamethicin, suggesting that, in contrast with cyclic GMP, most of the ATP was not tightly bound to intravesicular receptors (results not shown).

A completely different picture emerged if MgATP instead of ATP alone was loaded into the vesicles (Table 3). In this case the final ATP level in BBMVs was reduced by about 50%. Additional measurements of ADP levels in the vesicles showed that a conversion of ATP into ADP could almost completely account for the loss of internal ATP during isolation (Table 3). The basal ADP levels in

the vesicles ($1.3\ \text{mM}$) were much higher than basal ATP levels, possibly owing to ADP sequestration by intravesicular actin.

Table 3 also demonstrates a further breakdown of MgATP upon incubation of BBMVs for 10–20 min at higher temperature (30°C). In contrast with observations in kidney membranes (Kinne-Saffran *et al.*, 1982), the Mg-ATPase activity in intestinal BBMVs was not inhibited by filipin, neither could it be stimulated by H^+ ionophore or NaHCO_3 , arguing against its possible origin as a proton pump.

Table 3 furthermore shows that, upon loading of lower levels of MgATP ($2\ \text{mM}$), its breakdown could be compensated for effectively by the additional encapsulation of creatine phosphate ($20\ \text{mM}$); the activity of endogenous creatine kinase in intestinal BBMVs apparently allowed complete regeneration of ATP from ADP and creatine phosphate [cf. De Jonge (1973) for measurements of creatine phosphate levels and creatine kinase activities in rat enterocytes].

Entrapment of the membrane-impermeable Ca^{2+} -indicator Arsenazo III, applicable as a receptor molecule for intravesicular Ca^{2+} levels (Van Dommelen & De Jonge, 1984), also appeared very effective (Table 3). Since alamethicin was able to provoke a quantitative release of Arsenazo III from the vesicles (not shown), binding to the vesicle interior could be excluded.

Entrapment of proteins at the vesicle interior

The association of BSA with BBMVs after its addition to a suspension of enterocytes before (b) or after (a) vesiculation by freeze-thawing is shown in Fig. 1.

Table 3. Loading efficiency of ATP, MgATP and Arsenazo III in intestinal BBMV: hydrolysis of intravesicular ATP by a Mg^{2+} -ATPase

Villous cells originating from jejunal-ileal segments of two rats and isolated by the vibration technique were exposed to ATP, MgATP, MgATP plus creatine phosphate (CP) or the Ca^{2+} indicator Arsenazo III before or after vesiculation by freeze-thawing. BBMVs were isolated by differential Ca^{2+} precipitation. After washing of the vesicles on nitrocellulose filters and removal of vesicle protein by trichloroacetic acid precipitation, ATP and ADP were determined in the supernatant by a luciferase assay, and Arsenazo III by spectrophotometric measurements as described in the Materials and methods section. The concentration of compounds in BBMVs were calculated on the basis of an intravesicular volume of $1.12 \mu\text{l}/\text{mg}$ of protein (cf. Table 2). Filipin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were added in ethanol solution (final ethanol concn. 0.5%). Values are means $\pm$ S.E.M.; n = number of experiments.

Addition before or after freeze- thawing	Compound entrapped	Period of incubation of vesicles at 30 °C (min)	Concentration recovered in BBMV (mM)		
			ATP	ADP	Arsenazo III
—	None	—	0.09 ± 0.01 ($n = 4$)	1.35 ± 0.03 ($n = 4$)	—
Before	ATP (10 mM)	—	9.30 ± 0.21 ($n = 3$)	1.40 ± 0.05 ($n = 2$)	—
Before	ATP (10 mM)	30	9.02 ± 0.20 ($n = 2$)	—	—
After	ATP (10 mM)	—	0.13 ± 0.04 ($n = 3$)	—	—
Before	MgATP (10 mM)	—	5.63 ± 0.45 ($n = 4$)	4.78 ± 0.24 ($n = 4$)	—
	MgATP (10 mM)	10	2.30 ± 0.23 ($n = 3$)	—	—
	MgATP (10 mM)	10*	2.25 ± 0.18 ($n = 2$)	—	—
	MgATP (10 mM)	20	1.50 ± 0.08 ($n = 3$)	8.60 ($n = 1$)	—
	MgATP (2 mM)	—	1.05 ± 0.06 ($n = 3$)	—	—
	MgATP (2 mM) plus CP (20 mM)	—	1.93 ± 0.08 ($n = 4$)	—	—
	MgATP (2 mM) plus CP (20 mM)	20	1.85 ± 0.10 ($n = 3$)	—	—
Before	Arsenazo III (0.5 mM)†	—	—	—	0.45 ± 0.02
After	Arsenazo III (0.5 mM)†	—	—	—	0.02 ± 0.01 ($n = 4$)

* Incubated in the presence of filipin ($10 \mu\text{g}/\text{ml}$); similar results were obtained in the presence of the H^+ ionophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ($20 \mu\text{M}$) and NaHCO_3 (10 mM) (not shown).

† A similar loading efficiency was obtained in pig BBMVs if mucosal scrapings were pre-exposed to EDTA-containing medium.

Albumin recovered under condition (a) could be removed completely by washing the vesicles twice in buffer C containing 0.5 M-KCl or by incubation with trypsin (results not shown). A similar treatment of vesicles (b) decreased the BSA content by about 15%, suggesting entrapment of the residual 85% at the vesicle interior. The average intravesicular concentrations of albumin entrapped by procedure (b) and corrected for external protein (Fig. 1a) amounted to $155 \pm 15\%$ ($n = 4$) of the extracellular concentration (20 mg/ml), indicating a partial binding of the protein to the vesicle interior.

The loading efficiency determined for several purified cytosolic enzymes (lactate dehydrogenase, pyruvate kinase) and corrected for binding to the vesicle exterior varied between 93 and 112% (results not shown).

Loading of isolated vesicles by freeze-thaw cycles or osmotic shock

Obviously a loading procedure based on transient opening and resealing of isolated vesicle membranes should offer some advantages compared with the cellular-loading technique if it would lead to a similar entrapment efficiency and full restoration of the vesicular transport properties. Therefore relatively mild membrane disruption procedures, i.e. osmotic shock and repeated freeze-thawing, were studied as alternative loading techniques for rat intestinal BBMVs. As shown in Table 2, one freeze-thaw cycle appeared insufficient to provoke trapping of cyclic GMP at the vesicle interior. Repeated cycles (up to five; Table 4) almost fully unmasked cyclic GMP receptor sites, but did not lead to permanent entrapment of ATP. A substantial leakiness of the BBMVs

is suggested by a 80% decrease in glucose-uptake rates (Table 4).

By contrast, exposure of BBMVs to hypo-osmotic shock (15-fold decrease in medium osmolarity) led to a partial entrapment of cyclic GMP (fractional saturation of receptors about 25–30%) and ATP (15–30%), but resulted in a low loading efficiency for albumin (< 15%). Transient opening of part of the vesicle population (~ 30–40%) was suggested too by the protein-phosphorylation experiment shown in Fig. 2. As noted previously (De Jonge, 1981), exposure of BBMVs to $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the absence of detergent or alamethicin was extremely low in accordance with a predominantly right-side-out orientation of the vesicle population and intramicrovillar localization of protein kinases and substrate proteins. As determined by densitometric scanning of the autoradiograph shown in Fig. 2, the phosphorylation of most microvillar proteins, including autophosphorylation of cyclic GMP-dependent protein kinase (86 kDa; De Jonge, 1981) was increased about 7-fold by addition of alamethicin. For comparison, osmotic shocking (15-fold) in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cyclic nucleotide provoked a 2–3-fold increase of ^{32}P incorporation (Fig. 2). Resealing of the vesicle membrane was indicated by a full restoration of its glucose-transport properties (Table 4) and the return of ^{32}P incorporation to basal levels after osmotic shock (result not shown).

DISCUSSION

Sodium and chloride transport systems at the apical membrane of the enterocyte have been implicated as

Fig. 1. Encapsulation of bovine serum albumin in intestinal BBMV

Villous cells originating from the jejunum and ileum of two rats and isolated by the vibration technique were exposed to BSA (20 mg/ml) before (lane *b*) or after (lane *a*) vesiculation by freeze-thawing. BBMV were isolated by differential Ca^{2+} precipitation, extracted with 0.5% Triton X-100 and analysed by SDS/polyacrylamide-gel electrophoresis and Coomassie Blue staining of the gel (see the Materials and methods section). The amount of BSA entrapped into the vesicles was quantified by densitometric scanning of the 68 kDa region of the gel and comparison with known quantities of BSA standards run in parallel lanes (not shown).

major targets for intracellular signals (cyclic nucleotides, Ca^{2+}) regulating trans-epithelial ion and water absorption and secretion (Frizzel *et al.*, 1979; De Jonge & Lohmann, 1985). Specific proteins at the interior of the microvilli recently identified as phosphate acceptors for endogenous cyclic GMP-, cyclic AMP-, Ca^{2+} -calmodulin and Ca^{2+} -phospholipid-dependent protein kinases could play a

Fig. 2. Effect of alamethicin and osmotic shock on the protein phosphorylation profile in intestinal BBMV

BBMV isolated from jejunal-ileal enterocytes of rats by freeze-thawing and differential Mg^{2+} precipitation were resuspended in buffer D to a final protein concentration of 40 mg/ml. Membranes were diluted 15-fold into a protein phosphorylation mix containing 10 mM-Tris/HCl, 10 mM- β -glycerophosphate, 5 mM- MgSO_4 , 20 μM - $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ (10 Ci/mmol) and, where indicated, 1 μM -cyclic GMP, 5 μM -cyclic AMP and alamethicin (50 $\mu\text{g}/\text{ml}$). Under iso-osmotic phosphorylation conditions (—, alamethicin; left and centre panels), mannitol (0.6 M) was also included in the mixture. Phosphorylation was carried out for 30 s and 30 °C and terminated by addition of an SDS stopping solution and boiling as described previously (De Jonge, 1976). Phosphoproteins were separated on 7.5%-(w/v)-polyacrylamide gels (Laemmli, 1970) and gels were processed for drying and autoradiography (De Jonge, 1976). Molecular masses of vesicle proteins calibrated by co-electrophoresis of standard proteins (Weber & Osborn, 1969) are expressed in kDa. Phosphorylation of the 86 kDa protein represents autophosphorylation of endogenous cyclic GMP-dependent protein kinase (De Jonge, 1981). Phosphorylation of 60, 105, 135 and 180 kDa proteins is catalysed by an endogenous type II cyclic AMP-dependent protein kinase (De Jonge & Lohmann, 1985).

crucial role in signal transfer from hormone and bacterial toxin receptors on the cell membrane to ion transport systems in the brush-border membrane (De Jonge, 1981; De Jonge, 1984; De Jonge & Lohmann, 1985). Our initial attempts to reconstitute transport regulation by intracellular signals at the level of BBMV were hampered by the right-side-out orientation of the vesicle membrane and its impermeability to regulatory factors added to the vesicle exterior. Disruption of the membrane barrier by non-ionic detergents or pore-forming agents (alamethicin, digitonin) was essentially irreversible, precluding the subsequent analysis of transport systems. Among other methods used to induce transient opening of the vesicle membrane (e.g. sonication, freeze-thawing), exposure to

Table 4. Loading of isolated BBMVs by repeated freeze-thawing or osmotic shock

BBMV isolated from jejunal-ileal enterocytes by freeze-thawing and differential Mg^{2+} precipitation were resuspended in buffer C (5–8 mg/ml of protein) containing $0.33 \mu M$ cyclic $[8-^3H]GMP$ (15 Ci/mmol) plus 10 mM-theophylline or 10 mM-ATP. The vesicle suspension was repeatedly immersed in liquid N_2 and thawed in ice/water. Alternatively, a concentrated vesicle suspension in buffer B (40–55 mg of protein/ml) was rapidly diluted 15-fold in distilled water containing the same additives or 20 mg of bovine serum albumin/ml (osmotic shock) and incubated for 5 min at $30^\circ C$ to allow membrane resealing. In parallel experiments, vesicles were shocked first and compounds were added afterwards. Determination of cyclic $[^3H]GMP$ and ATP levels associated with the vesicles and of initial rates of $[^3H]$ glucose uptake were performed as described in the Materials and methods section. Entrapment of BSA was quantified after SDS/polyacrylamide-gel electrophoresis by the procedure described in the legend to Fig. 1. Values are means $\pm$ S.E.M.; n = number of experiments.

Loading procedure	Concentration recovered in BBMVs				Glucose transport (NaSCN gradient) (pmol/0.1 min per mg of protein)
	Cyclic $[^3H]GMP$ (pmol/mg of protein)	ATP (mM)	BSA (mg/ml)		
None	1.5 ± 1.0 ($n = 5$)	0.09 ± 0.01 ($n = 4$)	—		34 ± 7 ($n = 10$)
Freeze-thaw cycles ($4 \times$)	20.3 ± 2.6 ($n = 5$)	0.81 ± 0.04 ($n = 3$)	—		7 ± 4 ($n = 4$)
Osmotic shock					
Compounds added before shock	8.6 ± 1.9 ($n = 4$)	2.3 ± 0.8 ($n = 4$)	3.1 ± 1.2 ($n = 3$)		32 ± 6 ($n = 4$)
Compounds added after shock	1.6 ± 0.5 ($n = 3$)	0.10 ± 0.03 ($n = 3$)	1.5 ± 0.6 ($n = 3$)		—

an osmotic shock appeared to be relatively the most effective. Intestinal BBMVs subjected to such a procedure showed perfect resealing and full restoration of their capacity to accumulate glucose in response to an Na^+ gradient. Osmotic shock resulted in a partial (25–30%) entrapment of small molecules (ATP, cyclic GMP), but a negligible loading of proteins, e.g. albumin. Osmotic shock of kidney BBMVs has been reported to result in a more efficient entrapment of small molecules (L-glucose, ATP; 60–90% equilibration; see Biber *et al.*, 1983); the loading efficiency of proteins was not documented. Such a difference in membrane rigidity between intestinal and kidney BBMVs is also suggested by our recent finding that treatment of intestinal Mg - or Ca -BBMVs with a chaotropic agent (0.5 M-KSCN), in complete contrast with kidney vesicles (Peerce & Wright, 1984), was unable to remove intravesicular cytoskeletal elements (H. R. De Jonge, unpublished work).

A much higher loading efficiency of intestinal BBMVs could be reached by mixing the impermeants with a villous-cell suspension just before vesiculation of the microvilli by freeze-thawing. This procedure even allowed cyclic GMP to saturate most of its high-affinity receptors at the inner surface of the vesicle membrane (cf. De Jonge, 1981). To obtain maximal entrapment efficiency, it appeared necessary to pre-expose isolated enterocytes or mucosal scrapings for at least 30 min to an EDTA-containing medium. The beneficial effect of this protocol is not fully understood, but might involve a labilization of the cellular membranes and tight junctions and/or a reorganization of the cellular cytoskeleton by Mg^{2+} and Ca^{2+} depletion. Interestingly, EDTA pretreatment and freeze-thawing of isolated BBMVs was unable to provoke extensive membrane opening and encapsulation, suggesting that the compounds do not simply invade the microvilli through EDTA-induced pores in the apical membrane.

Instead of villous cells isolated by the vibration technique, mucosal scrapings from rat or pig small intestine if pre-exposed to EDTA were also suitable as

starting material for the encapsulation experiments. Although mechanical homogenization was almost equally effective, we preferred to use a freeze-thaw cycle in view of its reproducibility, its effectiveness even with small volumes of loading buffer (< 1 ml) and the option to store the frozen cell suspension for indefinite time in liquid N_2 without damage to the transport properties of the vesicles (results not shown). It should be noted that published procedures for BBMVs isolation often start with a freeze-thaw step before mechanical homogenization without the realization that freeze-thawing alone may trigger the vesiculation process (cf. Kessler *et al.*, 1978; Hauser *et al.*, 1980). The principle of fracturing a lipid bilayer by rapid freezing (inducing ice-crystal formation), followed by fusion of the exposed hydrophobic cores during slow thawing, has been applied previously to increase the size and trapping capacity of small unilamellar liposomes (Kasahara & Hinkle, 1977; Pick, 1981). In these studies with preformed liposomes, however, comparable with our results with preformed intestinal BBMVs, the entrapment efficiency was always below 30% (Pick, 1981). In contrast, freeze-thawing of isolated enterocytes resulted in full equilibration of low- M_r compounds and proteins with the vesicle interior.

Encapsulation of compounds before vesicle isolation has the potential disadvantage that internalized molecules may become metabolized during the isolation process, even at $0^\circ C$. In the case of cyclic GMP, however, hydrolysis could be prevented by the additional entrapment of the phosphodiesterase inhibitor theophylline. In the case of ATP, its degradation to ADP by an endogenous Mg^{2+} -ATPase activity at the vesicle interior could be prevented by trapping ATP in the absence of Mg^{2+} and by using Ca^{2+} instead of Mg^{2+} aggregation in the isolation procedure. In studies correlating protein phosphorylation with vesicle transport, Mg^{2+} -dependent protein kinases in such ATP-loaded vesicles might be re-activated by addition of Mg^{2+} and a Mg^{2+}/Ca^{2+} ionophore, circumventing the need for a photoactivatable ATP analogue. Alternatively, ATP levels may be restored

by co-entrapment of creatine phosphate, exploiting the presence of endogenous creatine kinase in intestinal BBMV.

The Mg^{2+} -ATPase demonstrated inside intestinal BBMV prepared by differential Ca^{2+} precipitation did not bear any resemblance to the proton-pump described previously in cortical BBMV (Kinne-Saffran *et al.*, 1982) in that: (1) it is not activated by an H^+ ionophore or HCO_3^- ion; (2) it is not blocked by filipin; (3) its activity (~ 3 nmol/10 min per mg of protein; Table 3) is substantially higher (renal proton pump: 0.17 mg/5 min per mg of protein, i.e. 0.6 pmol/10 min per mg). Although its identity has still to be elucidated, potential candidates include the intrinsic Ca^{2+}/Mg^{2+} -ATPase activity of the 110 kDa cross-filaments (Collins & Borysenko, 1984), a Mg^{2+} -ATPase dissimilar to a proton pump described in erythrocyte membranes (Forgac & Cantley, 1984) and a phosphorylation-dephosphorylation cycle of membrane proteins driven by Mg^{2+} -dependent protein kinase and endogenous phosphatase, recently identified at the vesicle level (De Jonge, 1984; De Jonge & Lohmann, 1985).

As an alternative to the procedure starting with isolated enterocytes, encapsulation during vesiculation might also be promoted by exposing isolated intact brush borders to a chaotropic agent (KSCN; Hopfer *et al.*, 1983) in the presence of the compounds of interest. Since membrane sealing is dependent on a drastic lowering of the salt concentration, this loading procedure may require rather large quantities of compounds. Moreover, the chaotropic salt may not only separate the cytoskeleton from the brush-border membrane but could also remove or inactivate peripheral membrane proteins involved in transport regulation (protein kinases, calmodulin). Although not explored in the present study, this approach might be used advantageously in case low residual amounts of endogenous regulators are required to study the effect of purified exogenous compounds on vesicle transport.

The vesicle-loading procedure described may find a wide application in studies of transport regulation at the microvillus level. Potential applications include: encapsulation of ATP and membrane-impermeable pH and pCa probes (e.g. carboxyfluorescein; quin-2), allowing a search for functional proton and Ca^{2+} pumps in the apical membrane; loading of lucigenin, a fluorescent probe quenched by anions, to study Cl^- -transport systems in the vesicle membrane; loading of MgGTP to study regulation of guanylate cyclase in BBMV (cf. Van Dommelen & De Jonge, 1984); encapsulation of ATP, cyclic nucleotides, purified protein kinases, kinase inhibitors or calmodulin, enabling the study of transport regulation by protein and phospholipid phosphorylation reactions; loading of antibodies against membranal or cytoskeletal proteins to define their role in transport; entrapment of metabolic enzymes and cofactors preventing the accumulation of a solute at the *trans*-side of the vesicle membrane and extending the period of linear solute uptake.

This research was supported in part by the Foundation of Medical Research (FUNGO), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO). We are indebted to Miss A. C. Hanson and Miss M. I. Wieriks for expert secretarial assistance.

REFERENCES

- Biber, J., Malström, K., Scalera, V. & Murer, H. (1983) *Pflügers Arch.* **398**, 221–226
- Braun, H. J., Birkenhäger, J. C. & De Jonge, H. R. (1984) *Biochim. Biophys. Acta* **774**, 81–90
- Collins, J. H. & Borysenko, C. W. (1984) *J. Biol. Chem.* **259**, 14128–14135
- Corbin, J. D. & Døskeland, S. O. (1983) *J. Biol. Chem.* **258**, 11391–11397
- De Jonge, H. R. (1973) *Biochem. Pharmacol.* **22**, 2659–2677
- De Jonge, H. R. (1976) *Nature (London)* **262**, 590–593
- De Jonge, H. R. (1981) *Adv. Cyclic Nucleotide Res.* **14**, 315–333
- De Jonge, H. R. (1984) *Kroc Found. Ser.* **17**, 263–286
- De Jonge, H. R. & Lohmann, S. H. (1985) *Ciba Found. Symp.* **112**, 116–138
- De Jonge, H. R. & van Dommelen, F. S. (1981) *Cold Spring Harbor Conf. Cell Prolif.* **8**, 1313–1332
- Forgac, M. & Cantley, L. (1984) *J. Membr. Biol.* **80**, 185–190
- Forstner, G. G., Sabenin, S. M. & Isselbacher, K. J. (1968) *Biochem. J.* **106**, 381–390
- Frizzel, R. A., Field, M. & Schultz, S. G. (1979) *Am. J. Physiol.* **236**, F1–F8
- Haase, W., Schäfer, A., Murer, H. & Kinne, R. (1978) *Biochem. J.* **172**, 57–62
- Hauser, H., Howell, K., Dawson, R. M. C. & Bowyer, D. E. (1980) *Biochim. Biophys. Acta* **602**, 567–577
- Hopfer, U. (1978) *Am. J. Physiol.* **234**, F89–F96
- Hopfer, U., Crowe, T. D. & Tandler (1983) *Anal. Biochem.* **131**, 447–452
- Hülsmann, W. C., van den Berg, J. W. O. & de Jonge, H. R. (1974) *Methods Enzymol.* **32**, 665–673
- Iemhoff, W. G., van den Berg, J. W. O., de Pijper, A. M. & Hülsmann, W. C. (1970) *Biochim. Biophys. Acta* **215**, 229–241
- Kasahara, M. & Hinkle, P. C. (1977) *J. Biol. Chem.* **252**, 7384–7390
- Kessler, M., Acuto, O., Storelli, C., Murer, H., Müller, M. & Semenza, G. (1978) *Biochim. Biophys. Acta* **506**, 136–154
- Kinne-Saffran, E., Beauwens, R. & Kinne, R. (1982) *J. Membr. Biol.* **64**, 67–76
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Murer, H. & Kinne, R. (1980) *J. Membr. Biol.* **55**, 81–95
- Peerce, B. E. & Wright, E. M. (1984) *J. Biol. Chem.* **259**, 14105–14112
- Pick, U. (1981) *Arch. Biochem. Biophys.* **212**, 186–194
- Sachs, G., Jackson, R. J. & Rabon, E. C. (1980) *Am. J. Physiol.* **238**, G151–G164
- Stevens, B. R., Kaunitz, J. D. & Wright, E. M. (1984) *Annu. Rev. Physiol.* **46**, 417–433
- Van Dommelen, F. S. & De Jonge, H. R. (1984) *Adv. Cyclic Nucleotide Res.* **17**, 303–313
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Wright, E. M. (1984) *Am. J. Physiol.* **246**, F363–F372