Biochimica et Biophysica Acta 903 (1987) 449–464
Elsevier

BBA 73707

Study of sidedness and tightness to H^+ of corn root plasmalemma vesicles: preparation of a fraction enriched in inside-out vesicles

Jean-Pierre Grouzis, Rémy Gibrat, Jacqueline Rigaud and Claude Grignon

Biochimie et Physiologie Végétales, INRA (C.N.R.S. UA 573), ENSAM, Montpellier (France)

(Received 10 March 1987)

Key words: ATPase, Mg^{2+}-; Inside-out vesicle; Proton transport; Lysophosphatidylcholine; Triton X-100; (Corn root plasmalemma)

The latency of the Mg^{2+}-ATPase of corn root plasmalemma was measured using Triton X-100, Zwittergent 3-14, sodium dodecyl sulfate and lysophosphatidylcholine. Lysophosphatidylcholine was the only permeabilizing agent which did not inhibit the enzyme. It gave a higher latency than the other surfactants. The sidedness of plasmalemma vesicles obtained after sucrose gradient purification was estimated from: (i) the Mg^{2+}-ATPase latency; (ii) the fraction of Mg^{2+}-ATPase resistant to trypsin attack; (iii) the effect of the specific binding of concanavalin A on the microelectrophoretic mobility of the vesicles; and (iv) the separation of the vesicles by chromatography on a Sepharose-Con A column. These four kinds of independent data were consistent. They led to the conclusion that this membrane preparation contained only sealed vesicles (impermeable to MgATP), the majority of which (approx. 65%) were oriented right-side out. Addition of 0.25 M sucrose in the same way inhibited the MgATP hydrolysis measured with or without lysophosphatidylcholine, so that the latency of the activity remained unchanged. The inhibition was higher in the presence of 0.25 M inositol and trehalose, again without modifying the latency. Finally, this preparation contained no significant proportion of tightly sealed active vesicles, as indicated by the absence of quinacrine quenching after addition of MgATP. Tightly sealed vesicles, displaying quinacrine quenching, were obtained according to De Michelis and Spanswick ((1986) Plant Physiol. 81, 542–547). The sidedness in this vesicle preparation was the same as the previous one (approx. 65% right-side out). Washing with Triton X-100 described by Clement et al. ((1986) Physiol. Vég. 24, 25–35) was applied. The simultaneous measurements of quinacrine quenching and Mg^{2+}-ATPase latency suggested a general inversion of the vesicle sidedness (approx. 65% of inside-out vesicles) after 0.1% treatment with (w/v) Triton X-100. Furthermore, the total Mg^{2+}-ATPase activity was recovered in spite of protein loss of approx. 60%, and the specific activity increased by approx. 150% in the presence of lysophosphatidylcholine. In the absence of lysophosphatidylcholine, coupling of Mg^{2+}-ATPase activity with H^+ transport was indicated by a 30% increase of MgATP hydrolysis upon addition of gramicidin or NH_4Cl.

Introduction

In vitro studies of H^+ transports by plasmalemma ATPase are particularly difficult to carry out in cereals, probably due to leakiness of the membrane vesicles [1,2]. With respect to accessi-

Abbreviations: Bistris propane, 1,3-bis(tris(hydroxymethyl)-methylamino)propane; Mes, 4-morpholineethanesulfonic acid; SDS, sodium dodecyl sulfate; lysoPC, lysophosphatidylcholine.

Correspondence: C. Grignon, Biochimie et Physiologie Végétales, INRA (CNRS UA 573), ENSAM, 34060, Montpellier cedex, France.

0005-2736/87/$03.50 © 1987 Elseviers Science Publishers B.V. (Biomedical Division)

bility for MgATP to the catalytic site, several kinds of vesicles may be distinguished in membrane preparations [3,4]: (i) unsealed fragments and leaky vesicles with catalytic sites freely accessible for MgATP; (ii) sealed vesicles, impermeable to MgATP but not to small ions; (iii) tightly sealed vesicles impermeable to MgATP and to small ions. Furthermore, the vesicles may be oriented right-side out (catalytic site inside the vesicle) or inside out (catalytic site exposed to the external medium). In native preparations, MgATP hydrolysis is only found with leaky vesicles, and sealed and tightly sealed vesicles with inside-out orientation. Only tightly sealed vesicles with inside-out orientation are capable of sustaining a pH gradient. Statistical distribution of vesicle orientation is generally estimated from the so-called latency of Mg^{2+}-ATPase activity, i.e., the fraction of the enzyme not reacting with MgATP in native preparations, but revealed by addition of a permeabilizing agent (generally Triton X-100 [5]). Most of the published studies have used membrane preparations isolated by differential centrifugation and sucrose gradients. These native preparations exhibit higher Mg^{2+}-ATPase activity than those obtained from phase partitioning [6]. Determinations of the sidedness of plasmalemma vesicles are scarce, but indicate a higher proportion of inside-out vesicles (approx. 50%) when obtained by a sucrose gradient [1,3,7] than by phase partition (approx. 10% inside out vesicles [5,8,9]). The values of the sidedness of vesicles obtained by a sucrose gradient has been claimed to be overestimated, regarding probable vesicles bursts due to hypoosmotic conditions under the classical conditions of ATPase assays (such bursts could lead to confusion of leaky rightside-out vesicles with true inside-out vesicles) [9]. On the other hand, purification by phase partitioning leads to a more homogeneous sidedness, but, unfortunately, such vesicles are unsuitable for studying transport properties of Mg^{2+}-ATPase because of their right-side-out orientation. Furthermore, the small basal Mg^{2+}-ATPase activity of these preparations was reported to be insensitive to ionophores and uncouplers [9]. From this, the authors concluded that these preparations did not contain tightly sealed vesicles. De Michelis and Spanswick [10] recently described a differential centrifugation method to obtain plasmalemma tightly sealed vesicles from corn root that show MgATP-dependent H^+ transport. No estimation of the sidedness of the vesicles was given. We report here a detailed analysis of the estimation and significance of the latency of corn root plasmalemma sealed and tightly sealed vesicles prepared by centrifugation. We show that the latency gives the true inside-out fraction, irrespective of the application of isoosmotic conditions. Finally, we conclude that washing with Triton X-100, as used by Clement et al. [11], increase the proportion of inside-out vesicles, probably by causing a general inversion of the vesicles, and improves their tightness to H^+. This method also increases the stimulation of corn plasma Mg^{2+}-ATPase by uncouplers.

Materials and Methods

Membrane preparation. Corn seeds (*Zea mays* L., var INRA 508) were surface-sterilized for 15 min with 3% calcium hypochlorite, soaked in water and germinated for 3 days on damp paper towels. Two methods were used to prepare plasma membrane vesicles. Sealed vesicles were prepared from excised roots as described by Leonard and Hotchkiss [12] except that β-mercaptoethanol was replaced by 2.5 mM DL-dithiothreitol and 1 mM phenylmethylsulfonyl fluoride was added to the homogenization medium. The $80\,000 \times g$ pellet was purified on a sucrose gradient. The final plasma membrane-enriched fraction was collected at the 34–45% (w/v) interface. It was resuspended in 1 mM Tris-Mes buffer (pH 6.5), 0.25 M sorbitol, 20% (v/v) glycerol, 1 mM DL-dithiothreitol. Tightly sealed vesicles were prepared according to De Michelis and Spanswick [10]. Briefly, this method differs from the former on the following points: the grinding medium contained several protecting agents (bovine serum albumin, glycerol, ATP, $MgSO_4$), EDTA and Tris-Mes were replaced by EGTA and Bistris propane-Mes, respectively, and the $80\,000 \times g$ pellet was washed with the grinding medium plus 100 mM KI. The washed $80\,000 \times g$ pellet was resuspended in a resuspension medium and was used without a further purification step. We applied this method without any modification except that Bistris propane-Mes

was replaced by Bistris propane-Cl. Both kinds of membrane fraction were stored in liquid N_2.

Enzymic assays. The specific Mg^{2+}-ATPase activity was measured as described in Gibrat et al. [13]. Inorganic phosphate was assayed with the method of Ames [14]. SDS (0.75% w/v) was added to prevent any interference with high concentrations of Triton X-100. Proteins were estimated according to Schaffner and Weissman [15]. In the presence of 50 mM KCl, MgATP hydrolysis by both sealed and tightly sealed vesicles were inhibited 75% by 250 μM vanadate, and inhibited 2% by 2 $\mu g \cdot ml^{-1}$ oligomycin. Inhibition was 10% (sealed vesicles) or 22% (tightly sealed vesicles) by 100 mM KNO_3 and 23% (sealed vesicles) or 5% (tightly sealed vesicles) by 100 μM molybdate.

Tryptic digestion of membranes. Membranes were incubated for different times at 22°C with trypsin (Boehringer; 0.13 mg per mg of membrane proteins) in 50 mM Tris-Cl (pH 7.5) and 0.25 M sucrose. Proteolytic digestion was stopped by addition of a 2.5-fold excess of trypsin inhibitor from soybean (Boehringer). In control experiments, the inhibitor was added prior to trypsin.

Microelectrophoretic measurements. The electrophoretic mobility of single vesicles was measured with a Rank Brothers Mark II apparatus fitted out with an ultra-thin walled cylindrical glass cell [16]. This allows an ultramicroscope illumination and, thus, very small particles (e.g., 0.1-μm latex beads or single plasmalemma vesicles) appear as brilliant points against a dark field. The medium (used at 25°C) contained 25 mM Tris adjusted to pH 6.5 with Mes (Tris is a univalent cation at this pH) and 1 mM Na_2-EDTA. The mean electrophoretic mobility of 30 vesicles was used to calculate the zeta potential with the help of the Helmholtz-Smoluchovsky relation [17]. The electrostatic charge density was calculated from the zeta potential using the classical Gouy-Chapman relation [18,19]. In order to study the distribution of the electrostatic parameters, the individual mobility, zeta potential and charge density of 100 vesicles were determined. The statistical analysis of the data was performed with Ross's Maximal Likelihood Program [20], using a program distributed by Statistics Department, Rothamsted Experimental Station, Herts, U.K.

Affinity chromatography on Sepharose-Con A. Sucrose was remove from the membrane suspension by a 1-h dialysis against 25 mM Tris-Mes (pH 6.5), 0.14 M KCl and 1 mM DL-dithiothreitol. Membranes were mixed with Sepharose-Con A at room temperature according to Resch et al. [21]. The fraction of nonbound membranes was eluted with the dialysis medium (fraction P_1). The bound membranes were extracted by adding 0.1 M α-methyl-D-mannoside, waiting for 2 min and then eluting with the dialysis medium plus 0.1 M α-methyl-D-mannoside (fraction P_2).

Transport assays. Quinacrine quenching was used according to De Michelis and Spanswick [10]. Membrane vesicles (50–100 $\mu g \cdot ml^{-1}$) were incubated for 20 min at 26°C in 50 mM Bistris propane-Cl (pH 6.5), 10 μM quinacrine, 100 mM KCl or KNO_3 and 5 mM Bistris propane-ATP. The Mg^{2+}-ATPase reaction was started by adding $MgSO_4$ (5 mM final concentration). Fluorescence was measured with a Jobin-Yvon JY3-CS apparatus, using excitation/emission wavelengths of 423/500 nm. No quenching was observed with sealed vesicles, in contrast to tightly sealed vesicles. The initial rate of quenching was linear with protein concentration (up to 200 $\mu g \cdot ml^{-1}$) as was the total extent of quenching (up to 150 $\mu g \cdot ml^{-1}$). Thus, the quenching parameters (initial rate and total extent of quenching) could be expressed in specific units (percent quenching $\cdot min^{-1} \cdot mg^{-1}$ protein and percent quenching $\cdot mg^{-1}$ protein). Half-time of the quenching after inhibition of Mg^{2+}-ATPase by 30 mM EDTA (free acid, adjusted to pH 6.5 with Bistris propane) was determined to monitor the H^+ permeability according to Lew and Spanswick [22]. In the presence of 0.1 μM valinomycin, the specific initial rate of quinacrine quenching was inhibited 20% by 100 mM KNO_3 (as compared to 100 mM KCl), and oligomycin had no significant effect. Vanadate was less efficient in inhibiting quinacrine quenching (1 mM for 85% inhibition in 100 mM KNO_3) than MgATP hydrolysis (250 μM for 85% inhibition), perhaps because of the intravesicular location of the inhibition site [23] or delayed action time [10]. The results are given as means of 3–6 independent repetitions and the confidence limits are 95%.

Results

Effect of surfactants on the MgATP hydrolysis of sealed vesicles: definition of the latency

The effect of increasing amounts of Triton X-100 on MgATP hydrolysis is shown in Fig. 1A. The stimulation was maximum at 250 μM Triton X-100 (0.015%, w/v), and decreased at higher concentrations. When lysoPC was used, a plateau of stimulation was observed when the concentration of the surfactant was in the range of 50–200 μM (Fig. 1C). In these experiments, almost all proteins could be pelleted at 100000 × *g* for 60 min at the surfactant concentration which gave the maximum of stimulation (data not shown). Thus, neither Triton X-100 nor lysoPC apparently solubilized the membranes. At 100 μM lysoPC, the addition of increasing amounts of Triton X-100 did not induce additional stimulation, but rather an inhibition (Fig. 1B, continuous line). This decrease was probably due to a direct effect of Triton X-100 on the enzyme. Correcting the curve of Fig. 1A for the inhibitory effect of Triton X-100 gave the calculated curve of Fig. 1B (dotted line), which shows the stimulating effect of Triton X-100 alone, and is qualitatively and quantitatively similar to the one obtained with lysoPC alone (Fig. 1C). The effects of Zwittergent 3-14

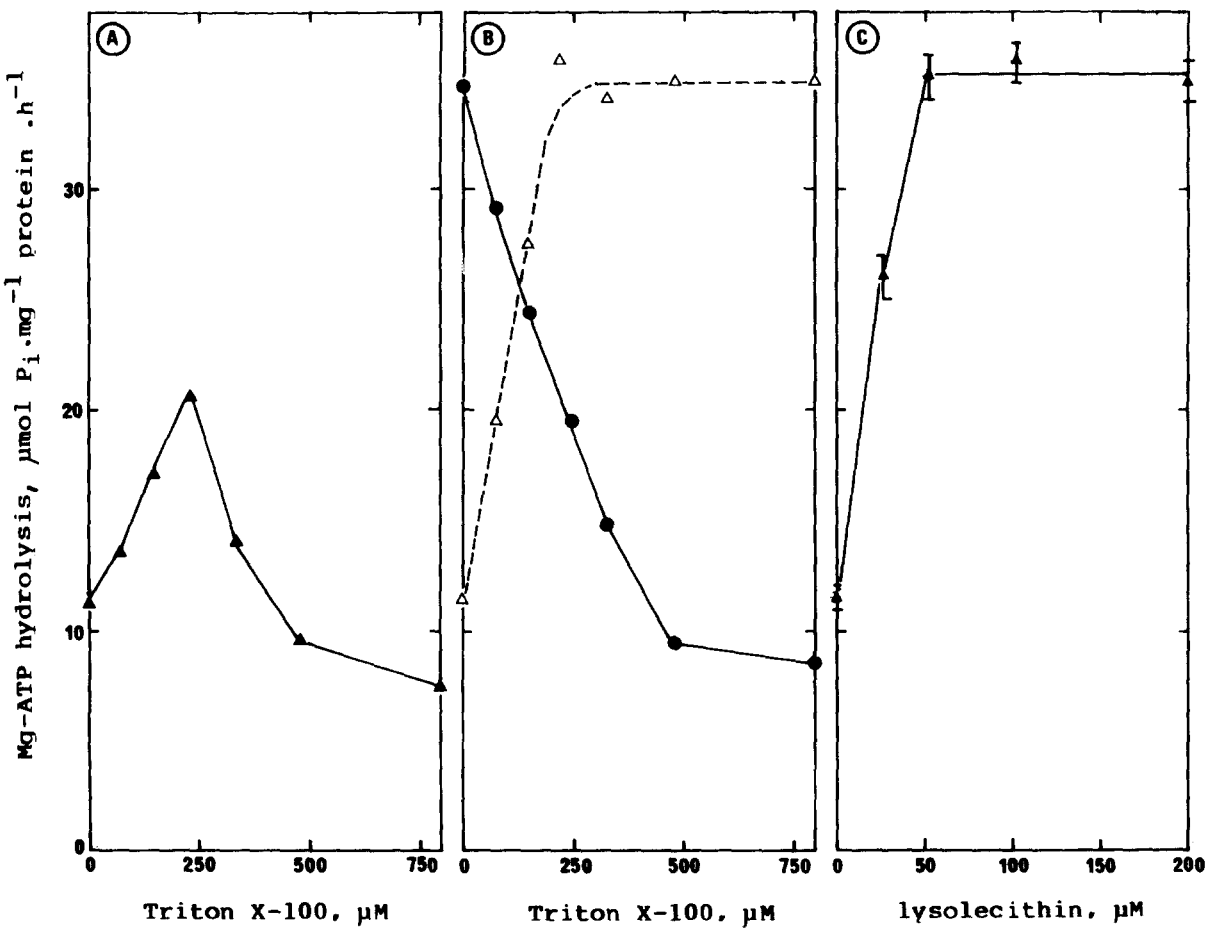


Fig. 1. Effect of Triton X-100 and lysoPC on MgATP hydrolysis by sealed vesicles. The assay medium contained 25 mM Tris-Mes (pH 6.5), 3 mM $MgSO_4$, 50 mM KCl, 3 mM Tris-ATP and surfactants. (A) Triton X-100; (B) Triton X-100 plus 100 μM lysoPC (continuous line); (C) lysoPC: incubation was for 20 min at 30 ° C. The dotted curve in B was calculated as follows: the amount of inhibition was determined for each detergent concentration on curve B (continuous line) and added to the corresponding value on curve A. Lysolecithin, lysoPC.

and SDS were compared to those of lysoPC in the same way as described for Triton X-100. Again, false optima were observed, which resulted from antagonist actions of the surfactants (Figs. 2 and 3). From these results, it was concluded that lysoPC appears to be a more suitable permeabilizing agent than the three other surfactants, and was used in the following experiments. The percent latency was calculated as $100 \cdot (v_{lyso} - v)/(v_{lyso})$, where v and v_{lyso} are the activities measured in the absence and in the presence of 100 μM lysoPC, respectively.

Latency of the Mg^{2+}-ATPase activity of sealed and tightly sealed vesicles

Mg^{2+}-ATPase activity measurements were complicated by the presence of phosphatases, as indicated by *p*-nitrophenylphosphate hydrolysis (approx. 4.2 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein). MgATP hydrolysis was inhibited 23% (sealed vesicles) or 5% (tightly sealed vesicles) by 100 μM sodium molybdate, an inhibitor of acid phosphatases (Table I). The relative inhibition of MgATP hydrolysis upon addition of 250 μM orthovanadate (an inhibitor of phosphatases and plant plasmalemma ATPases [24]) was approx. 75% in the absence of molybdate, and slightly less in the presence of 100 μM molybdate. The residual activity was insensitive to subsequent additions of molybdate (data not shown) and vanadate (Fig. 4). The addition of 100 μM lysoPC did not significantly modify the percentages of inhibition of MgATP hydrolysis by

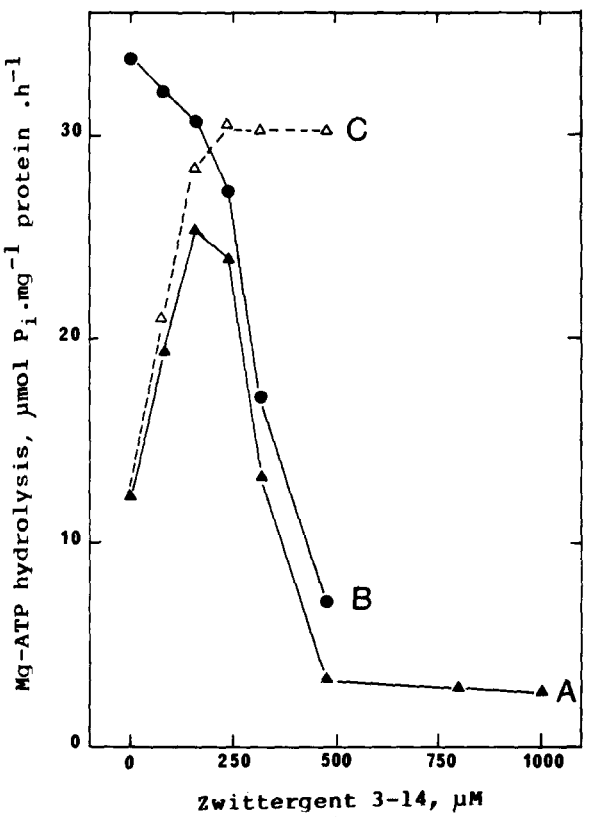


Fig. 2. Effect of Zwittergent 3-14 and lysoPC on MgATP hydrolysis by sealed vesicles. The assay medium was similar to that in Fig. 1, except for surfactants. Curve A, Zwittergent 3-14; curve B, Zwittergent 3-14 plus 100 μM lysoPC; curve C, activating effect of Zwittergent 3-14, calculated by correcting curve A for the inhibitory effect of Zwittergent 3-14 (curve B) (see legend to Fig. 1).

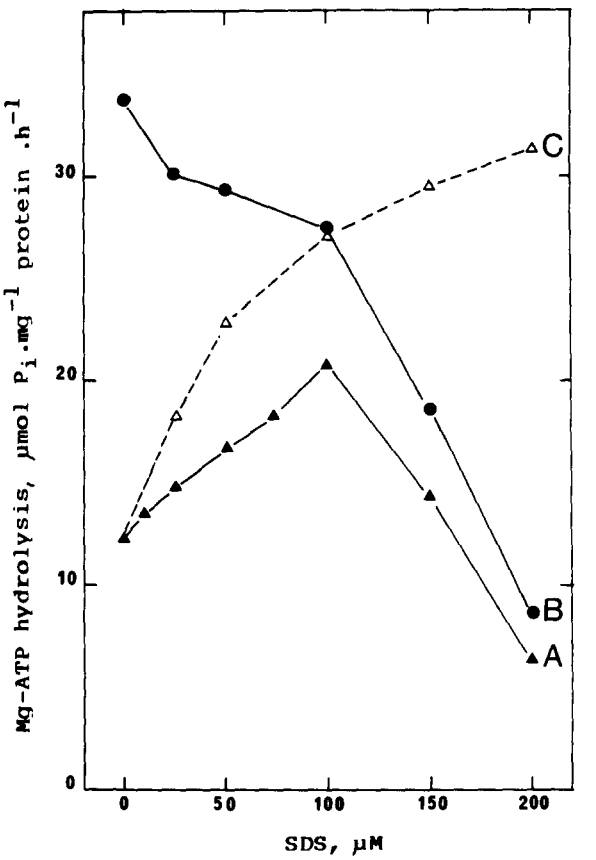


Fig. 3. Effect of SDS and lysoPC on MgATP hydrolysis by sealed vesicles. The assay medium was similar to that in Fig. 1, except for surfactants. Curve A, SDS; curve B, SDS plus 100 μM lysoPC; curve C, activating effect of SDS, calculated by correcting curve A for the inhibitory effect of SDS (curve B) (see legend to Fig. 1).

TABLE I

EFFECTS OF MOLYBDATE AND VANADATE ON MgATP HYDROLYSIS

Sealed (S) or tightly sealed (TS) vesicles were incubated for 20 min at 30 °C in 25 mM Bistris propane-Cl, (pH 6.5), 3 mM Bistris propane-ATP, 3 mM $MgSO_4$ and 50 mM KCl, in the presence or absence of 100 μM lysoPC. When indicated, the medium was supplemented with 100 μM sodium molybdate (Mo) or 250 μM sodium vanadate (Va). Latency is calculated as $100 \cdot (v_{lyso} - v)/v_{lyso}$), where v_{lyso} is the activity in the presence of lysoPC.

Parameters	Without lysoPC				100 μM lysoPC			
	none	Mo	Va	Mo+Va	none	Mo	Va	Mo+Va
S vesicles								
v(μmol·h^{-1}mg^{-1} protein)	14.3	11.1	3.3	3.4	40.8	33.5	10.6	10.2
Inhibition (%)	0	23	77	69	0	18	74	75
Latency (%)	–	–	–	–	65	67	67 [b]	67 [b]
							64	67
TS vesicles								
v(μmol·h^{-1}·mg^{-1} protein)	20.0	19.0	5.6	5.8	65.3	62.6	16.1	15.6
	22.1 [a]	20.9 [a]						
Inhibition (%)	0	5	72	70	0	4	75	75
Latency (%)	–	–	–	–	69	70	65	63
					66 [a]	67 [a]	67 [ab]	68 [ab]

[a] In the presence of 2 μM gramicidin.
[b] Calculated from the vanadate-sensitive activity.

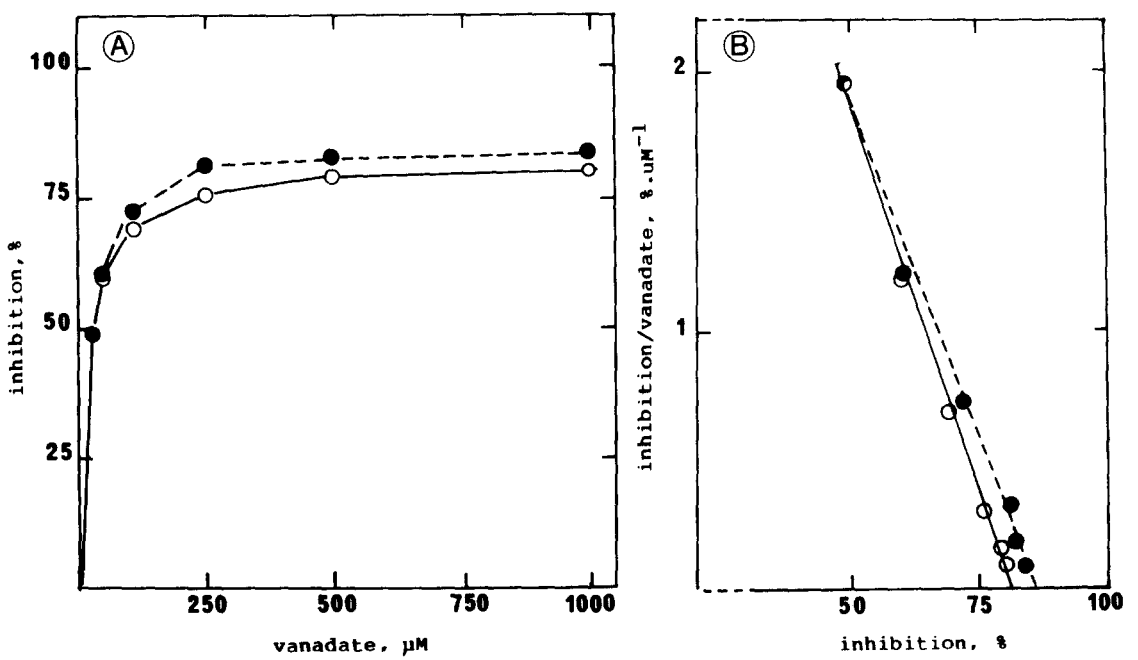


Fig. 4. (A) Effect of orthovanadate on the inhibition of MgATP hydrolysis by sealed vesicles. The assay medium contained 25 mM Tris-Mes, 3 mM $MgSO_4$, 3 mM Tris-ATP, 50 mM KCl and sodium orthovanadate at the indicated concentrations. Assays were performed at 30 °C in the absence (open symbols) and presence (solid symbols) of 100 μM lysoPC. (B) Scatchard plot.

the two inhibitors (Table I). Various triphosphonucleotides were used as substrates in the presence of 100 μM lysoPC (Table II). The total hydrolytic activity of sealed and tightly sealed vesicles was poorly specific, but vanadate specifically inhibited the hydrolysis of MgATP. These results indicate that, in the presence of sodium molybdate, the decrease in MgATP hydrolysis upon addition of vanadate (denoted as ΔVa) only represents the inhibition of the Mg^{2+}-ATPase. The above-defined latency of this Mg^{2+}-ATPase activity was approx. 67% for sealed vesicles (Table I). In tightly sealed vesicles, Mg^{2+}-ATPase activity increased slightly upon addition of 2 μM gramicidin (approx. 10%). In this case, the uncoupled Mg^{2+}-ATPase activity must be taken into account when estimating the true latency (68%). Finally, the latency of the Mg^{2+}-ATPase activity and the latency of the total MgATP hydrolysis were nearly the same.

Effects of trypsin on the Mg^{2+}-ATPase activity of sealed vesicles

When tryptic attack and Mg^{2+}-ATPase assay were performed in the presence of lysoPC, 95% of the activity was lost after 30 min (Fig. 5, curve B). To determine the sensitivity of non-latent activity to trypsin attack, the digestion was performed in the absence of lysoPC. In contrast to the expected result, the non-latent activity was not totally eliminated (Fig. 5, curve C). This could result from incomplete digestion. This hypothesis seems improbable, since the digestion was almost complete in the presence of lysoPC. Alternatively, trypsin could increase the permeability of some

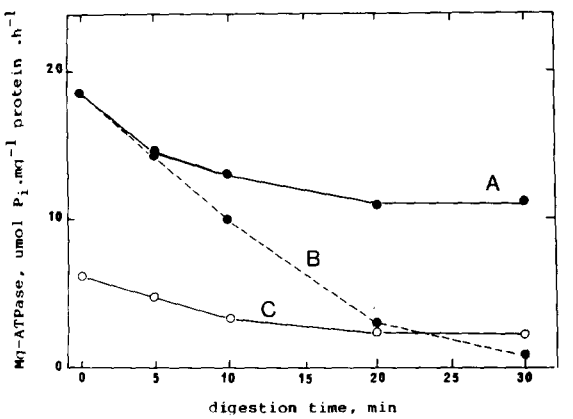


Fig. 5. Effect of trypsin attack on the Mg^{2+}-ATPase activity of sealed vesicles. Sealed vesicles were treated with 0.13 mg trypsin per mg protein at 22 °C in 50 mM Tris-HCl (pH 7.5) and 0.25 M sucrose, with or without lysoPC. Trypsin digestion was stopped by addition of 2.5-fold excess of trypsin inhibitor. Mg^{2+}-ATPase hydrolysis was determined at 30 °C in a medium containing 25 mM Tris-Mes (pH 6.5), 3 mM $MgSO_4$, 3 mM Tris-ATP, 50 mM KCl, 0.25 M sucrose, and 100 μM sodium molybdate, with or without 100 μM lysoPC. Mg^{2+}-ATPase activity was calculated as the difference between the activities measured in the presence and in the absence of 250 μM vanadate. Curve A, digestion without lysoPC, assay with 100 μM lysoPC; curve B, digestion and assay with 100 μM lysoPC; curve C, no lysoPC applied.

TABLE II

SELECTIVITY OF TRIPHOSPHONUCLEOTIDES HYDROLYSIS BY SEALED AND TIGHTLY SEALED VESICLES AND EFFECT OF WASHING WITH TRITON X-100

Vesicles were incubated for 20 min at 30 °C in 25 mM Bistris propane-Cl (pH 6.5), 3 mM Bistris propane-ATP or 3 mM sodium trinucleotide phosphates, 3 mM $MgSO_4$, 50 mM KCl, 100 μM sodium molybdate, 100 μM lysoPC and, when indicated, 250 μM sodium vanadate. TPN hyd, triphosphonucleotide hydrolysis activity; ATPase, vanadate sensitive activity. The activities (v) are expressed in $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein. S and TS, sealed and tightly sealed vesicles, respectively; n.d., not determined.

Substrate	S vesicles				TS vesicles				TS, Triton X-100 washed			
	TPN hyd		ATPase		TPN hyd		ATPase		TPN hyd		ATPase	
	v	%	v	%	v	%	v	%	v	%	v	%
ATP	34.0	100	25.0	100	56.9	100	46.5	100	151.9	100	129.9	100
CTP	7.2	21	3.0	12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GTP	n.d.	n.d.	n.d.	n.d.	16.7	29	1.9	4	12.7	8	0.0	0
UTP	11.0	32	0.8	3	27.2	48	4.3	9	28.4	19	3.2	3
ITP	16.0	47	4.7	19	15.5	27	0.0	0	8.1	5	0.0	0

vesicles to MgATP, and unmask a fraction of latent activity, as shown for canine (Na^+K^+)-ATPase [25]. In other experiments, lysoPC was omitted in the digestion medium, but added in the Mg^{2+}-ATPase assay (Fig. 5, curve A). In this case, only 37% of the activity was eliminated, indicating that 63% of the catalytic sites was inaccessible to trypsin in the absence of the permeabilizing agent. Finally, the latency of the Mg^{2+}-ATPase determined in parallel runs and without trypsin attack was 63% (Table III).

Effect of concanavalin A on the statistical distribution of the surface charge (sealed vesicles)

The membrane fraction was resuspended in 25 mM Tris-Mes buffer (pH 6.5), containing 1 mM Na_2-EDTA. The microelectrophoretic mobilities of the vesicles were measured and used to calculate the surface charge density. Addition of concanavalin A depolarized the vesicle surface (Fig. 6). The kinetic analysis of this effect suggested a high-affinity first-order binding (dissociation constant: 0.36 μM) and probably a minor component

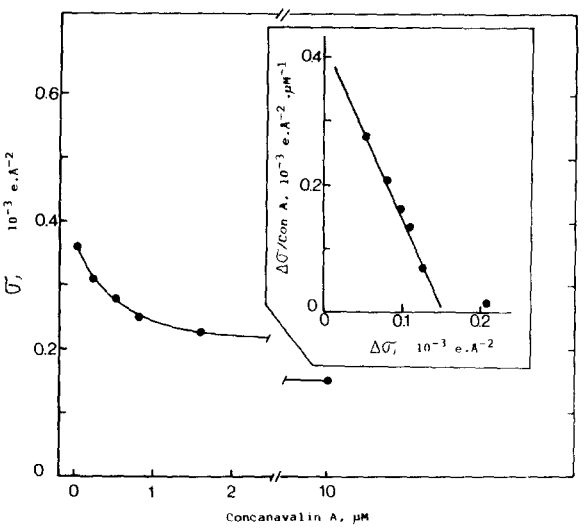


Fig. 6. Surface charge density of sealed vesicles as a function of concanavalin A concentration. The assay medium contained 25 mM Tris-Mes (pH 6.5), 1 mM Na_2-EDTA and concanavalin A as indicated. Inset: variation of the surface charge density induced by concanavalin A, shown as Scatchard plot. The surface charge σ was calculated from the electrostatic potential determined from microelectrophoretic mobility. Each point is the mean of 30 vesicles.

TABLE III

EFFECT OF TRYPSIN ATTACK ON MgATP HYDROLYSIS AND Mg^{2+}-ATPase ACTIVITY

MgATP hydrolysis by sealed vesicles was determined at 30 °C in a medium containing 25 mM Tris-Mes (pH 6.5), 3 mM $MgSO_4$, 3 mM Tris-ATP, 50 mM KCl, 0.25 M sucrose and 100 μM sodium molybdate. Mg^{2+}-ATPase activity was calculated as the difference between the activities measured in the presence and in the absence of 250 μM vanadate. + LPC: lysoPC (100 μM) was present during the measurement; − LPC, without lysoPC; Digested: vesicles were treated for 20 min with 0.13 mg trypsin per mg protein at 22 °C in 50 mM Tris-HCl (pH 7.5), 0.25 M sucrose. Trypsin digestion was stopped by addition of a 2.5-fold excess of trypsin inhibitor and MgATP hydrolysis was measured in the presence of lysoPC; Nondigested: as digested, except that trypsin inhibitor was added prior to trypsin. Activities are given as $\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein. Confidence limits are for $P = 0.05$ (six repetitions). n.d., not determined.

Measurements	Nondigested		digested	
	activity	% inaccessible	activity	% Inaccessible
MgATP hydrolysis − LPC	9.6	–	n.d.	–
MgATP hydrolysis + LPC	27.5	65 ± 5	17.8	65 ± 5
Mg^{2+}-ATPase − LPC	5.5	–	n.d.	–
Mg^{2+}-ATPase + LPC	16.1	66 ± 4	10.2	63.3

corresponding to a low-affinity mechanism (Fig. 6, inset).

The statistical distributions of the surface charge densities of 100 vesicles are shown in Fig. 7. In the absence of concanavalin A, the mean surface charge density was $361 \cdot 10^{-6} \pm 5 \cdot 10^{-6}$ elementary charge per Å^2 (confidence limits for $P = 0.05$). In the presence of 0.4 μM concanavalin A, the surface charge density was $280 \cdot 10^{-6} \pm 8 \cdot 10^{-6}$ elementary charge per Å^2. Finally, 5 mM α-methyl-D-glucopyranoside and 5 mM α-methyl-D-mannoside were added to 0.4 μM concanavalin A, as specific inhibitors of its binding. This treatment partially reversed the surface depolarization by concanavalin A $(351 \cdot 10^{-6} \pm 7 \cdot 10^{-6}$ elementary charge per Å^2).

The data were fitted to normal distributions, or linear combinations of normal distributions, and the quality of the fits was evaluated using chi-squared tests (Table IV). In the absence of the lectin (Fig. 7A), it is highly probable that there was a single population with a normal distribution. In the presence of the lectin (Fig. 7B), the best fit was obtained for two mixed populations,

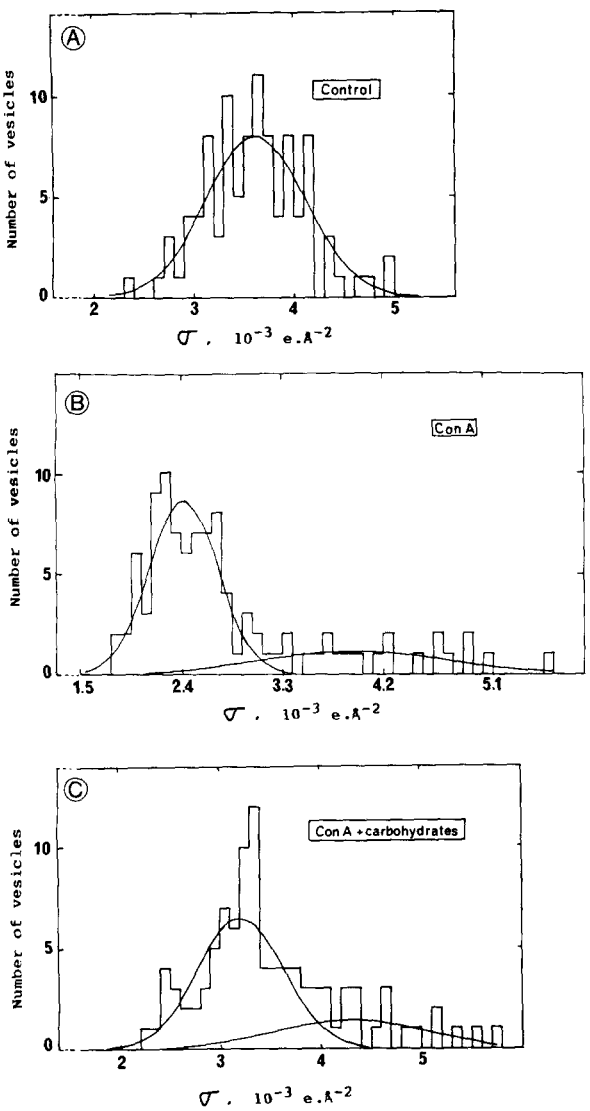


Fig. 7. Distribution of the surface charge density of sealed vesicles. The surface charge density, σ, of 100 vesicles was determined as indicated in Fig. 6, in 25 mM Tris-Mes (pH 6.5), 1 mM Na_2-EDTA. A, control; B, in the presence of 0.4 μM concanavalin A; C, in the presence of 0.4 μM concanavalin A plus 5 mM α-methyl-D-glucopyranoside plus 5 mM α-methyl-D-mannoside. Bar graphs, observed. Smooth curves, calculated normal distribution (best fit to the observed ones), with parameters as follow (means and S.D., 10^{-6} elementary charge per A^{-2}): A, mean = 361, S.D. = 49; B, mean = 240, S.D. = 30, and mean = 390, S.D. = 83; C, mean = 320, S.D. = 43, and mean = 430, S.D. = 76.

each normally distributed with means equal to $390 \cdot 10^{-6} \pm 29 \cdot 10^{-6}$ elementary charge per Å^2 (26% of the vesicles) and $240 \cdot 10^{-6} \pm 4 \cdot 10^{-6}$ elementary charge per Å^2 (74%), respectively. When the sugars were added to concanavalin A, the two mixed normal populations led again to the best fit (Fig. 7C). The minor population was not significantly distinct from that observed in the absence of the sugars ($430 \cdot 10^{-6} \pm 57.10^{-6}$ elementary charge per Å^2, 31% of the vesicles). The major population had a mean of $320 \cdot 10^{-6} \pm 10 \cdot 10^{-6}$ elementary charge per Å^2. Thus, the surface depolarization induced by concanavalin A corresponded to an unspecific binding for approx. 30% of the vesicles, and to a specific binding (partially reversed by the methyl hexoses) for 70% of the vesicles.

Affinity chromatography of sealed vesicles

When sealed vesicle suspensions were layered on a Sepharose-con A column, one fraction (P_1) was not retained (Fig. 8). It contained 33 ± 3% of the total layered proteins (Table V). Thus, 33% of the vesicles in the initial suspension did not expose their lectin-binding sites to the external medium. Another fraction (P_2) was eluted by 0.1 M α-methyl-D-mannoside, but its protein content represented only a minor part of the proteins retained on the column.

Effects of carbohydrates on MgATP hydrolysis and H^+ transport

MgATP hydrolysis by sealed vesicles was approx. 20% decreased by 0.25 M sucrose, but the latency was not modified (67%, Table VI). No quinacrine quenching could be observed, with or without sucrose. With tightly sealed vesicles, MgATP hydrolysis was again approx. 20% decreased by 0.25 M sucrose, 30% by 0.25 M inositol and the latencies were not affected (approx. 65%). Quinacrine quenching indicated that H^+ was accumulated in the tightly sealed vesicles (Fig. 9A). Values of the initial rate of quenching (Tables VI and VII) were in good agreement with that obtained by De Michelis and Spanswick [10] under the same conditions. The initial rate was decreased 16% and the maximum total extent of quenching was increased 20% by 0.25 M sucrose (Table VI). Trehalose effects on the MgATP hydrolysis and on the H^+ transport were qualitatively similar to those of sucrose, but approx. 2-fold higher. Both sucrose and trehalose increased the half-time of

TABLE IV

DISTRIBUTION OF THE SURFACE CHARGE DENSITY OF SEALED VESICLES

The surface charge density of 100 vesicles was determined, as indicated in Fig. 6, in 25 mM Tris-Mes (pH 6.5), 1 mM Na_2-EDTA and, when indicated, 0.4 μM concanavalin A (Con-A), or 0.4 μM concanavalin A plus 5 mM α-methyl-D-glucopyranoside plus 5 mM α-methyl-D-mannoside (+S). Observed parameters M, S.D., *N* and S.E. are mean, standard deviation, number of vesicles, standard error of the mean or S.D., respectively. Theoretical distributions were calculated as normal populations, or sum of two normal populations, 1 and 2, with parameters M1, SD.1, *N*1, and *M*2, S.D.2, *N*2. The analysis was performed by use of a Maximal Likelihood Program (see text). The reported values are those which gave the best fit to the observed data.

Treatment	Observed parameters			Parameters used for fitting						Chi-squared	
	M ± S.E.	S.D. ± S.E.	*N*	M1	S.D.1	*N*1	M2	S.D.2	*N*2	Obs	$P = 0.05$
Native	361 ± 5	49 ± 4	100	361	49	100	–	–	0	7.7	14.1
Con-A	280 ± 8	84 ± 6	100	280	84	100	–	–	0	62.2	19.7
Con-A				390	83	26	240	30	74	5.1	15.5
Con-A + S	351 ± 7	73 ± 5	100	351	73	100	–	–	0	21.9	19.7
Con-A + S				430	76	28	320	43	72	10.1	15.5

the pH gradient after stopping the active H^+ transport. Mg^{2+}-ATPase uncoupling was weak (10% stimulation by 2 μM gramicidin or 5 mM NH_4Cl) and was not affected by carbohydrates (Table VI).

Effect of washing tightly sealed vesicles with Triton X-100

Tightly sealed vesicles were incubated for 10 min at 0 °C in the resuspension medium of De Michelis and Spanswick [10], with increasing Triton X-100 concentrations or without Triton X-100 (reference treatment). Thereafter, membranes were sedimented and pellets were washed with the resuspension medium to eliminate Triton X-100 and sedimented again. The total Mg^{2+}-ATPase activity was recovered in both cases (97 ± 5%). Triton X-100 treatment increased both the Mg^{2+}-ATPase activity and the initial rate of quenching in the presence of 0.1 μM valinomycin (Fig. 10). The optimum concentration of Triton X-100 treatment was 0.1% (ratio Triton X-100/protein = 2), for which the Mg^{2+}-ATPase activity and the initial rate of quenching were 4-fold higher as compared to the reference treatment. Protein loss was approx. 35% in the reference treatment and 60% in that Triton X-100 concentration. The increase of

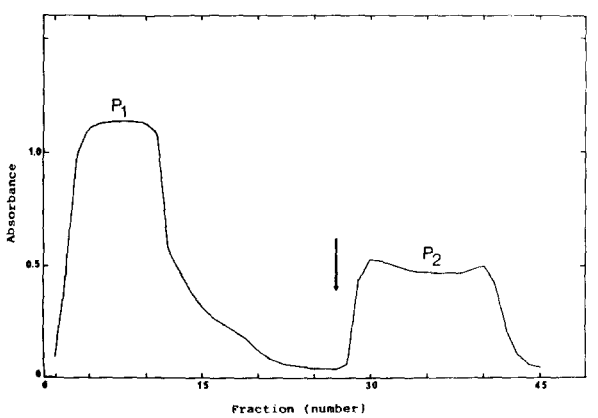


Fig. 8. Fractionation of sealed vesicles on Sepharose-con A affinity columns. The vesicles were resuspended in 25 mM Tris-Mes (pH 6.5), 0.14 M KCl, 1 mM DL-dithiothreitol, mixed with Sepharose-Con A and eluted with the same buffer (fraction P_1). Fraction P_2 was eluted with the same buffer supplemented with 0.1 M α-methyl-D-mannoside (arrow). The curve is the absorbance at 280 nm vs. fraction number (0.6 ml fractions).

TABLE V

FRACTIONATION OF SEALED VESICLES ON SEPHAROSE-Con A AFFINITY COLUMNS

The vesicles were resuspended in 25 mM Tris-Mes (pH 6.5), 0.14 M KCl, 1 mM DL-dithiothreitol mixed with Sepharose-Con. A and eluted with the same buffer. The eluted fractions were pooled, sedimented and assayed for proteins (recovered protein).

Parameters	Repetitions						
Initial protein (μg)	143	143	143	400	465	600	867
Recovered (μg)	41	43	45	140	173	210	311
Recovered (% initial)	29	30	31	35	37	35	36

TABLE VI

EFFECTS OF CARBOHYDRATES ON MgATP HYDROLYSIS, SIDEDNESS AND H^+ TRANSPORT PROPERTIES OF SEALED AND TIGHTLY SEALED VESICLES

Vesicles were incubated at 26 °C for 20 min in 2 ml of medium containing 50 mM Bistris propane-Cl (pH 6.5), 5 mM Bistris propane-ATP, 10 μM quinacrine, 100 mM KCl and carbohydrates as indicated. H^+ transport was monitored with quinacrine quenching. Reaction was started by addition of $MgSO_4$ (final concentration of 5 mM). Quenching decay was initiated by addition of 30 mM Bistris propane-EDTA. MgATP hydrolysis was measured under the same conditions as quinacrine quenching, except that quinacrine was omitted and 2 μM gramicidin or 100 μM lysoPC were present when indicated. Latency is calculated as $100 \cdot (v_{lyso} - v)/v_{lyso}$, where v_{lyso} is the activity in the presence of lysoPC. S and TS represent sealed and tightly sealed vesicles, respectively. n.d., not determined.

Added carbohydrate (0.25 M)	MgATP hydrolysis ($\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein)			Latency (%)	Quinacrine quenching		
	none	gramicidin	lysoPC		initial rate (% $min^{-1} \cdot mg^{-1}$ protein)	total extent (% mg^{-1} protein)	decay $T_{1/2}$ (min)
S vesicles							
None	5.8	5.8	17.8	67	0	0	–
Sucrose	4.7	4.8	14.4	67	0	0	–
TS vesicles							
None	9.3	10.1	31.0	67	50	336	9
Sucrose	7.4	8.2	23.2	65	42	408	10
Inositol	6.3	7.0	21.7	68	n.d.	n.d.	n.d.
Trehalose	5.3	5.9	17.4	66	30	412	13

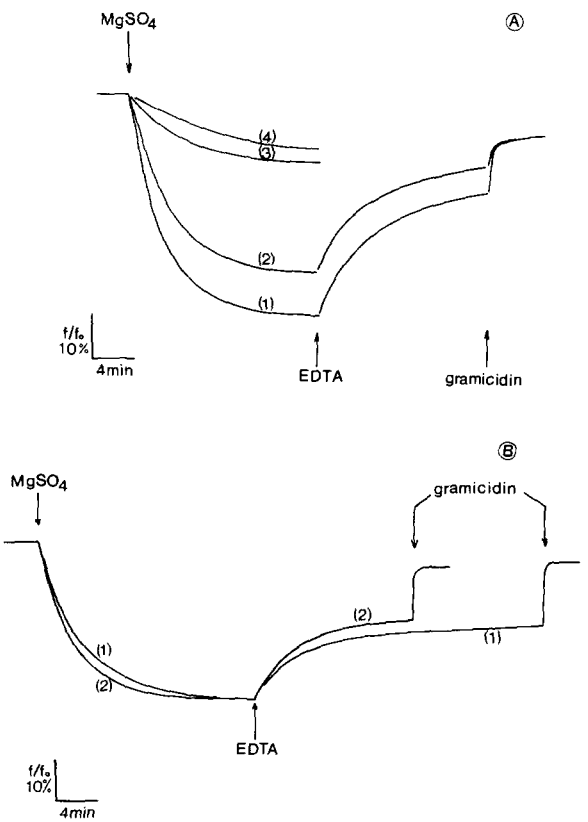


Fig. 9. MgATP-dependent H^+ transport by tightly sealed vesicles. Tightly sealed vesicles were incubated at 26 °C for 20 min in 2 ml of medium containing 50 mM Bistris propane-Cl (pH 6.5), 5 mM Bistris propane-ATP, 10 μM quinacrine, and 100 mM potassium salt (see below). When indicated, 1 mM sodium vanadate or 0.1 μM valinomycin were present. The reaction was started by addition of $MgSO_4$ (final concentration of 5 mM). Arrows indicate addition of Bistris propane EDTA (30 mM final, pH 6.5) or gramicidin (2 μM final). (A) Percent of quenching of the initial fluorescence by control tightly sealed vesicles (200 μg protein), in the presence of valinomycin. 1, KCl; 2, KNO_3; 3, KCl plus vanadate; 4, KNO_3 plus vanadate. (B) Tightly sealed vesicles washed in 0.1% Triton X-100 (60 μg protein). The procedure for washing and elimination of Triton X-100 is described in the text. 1, KCl; 2, KCl plus valinomycin.

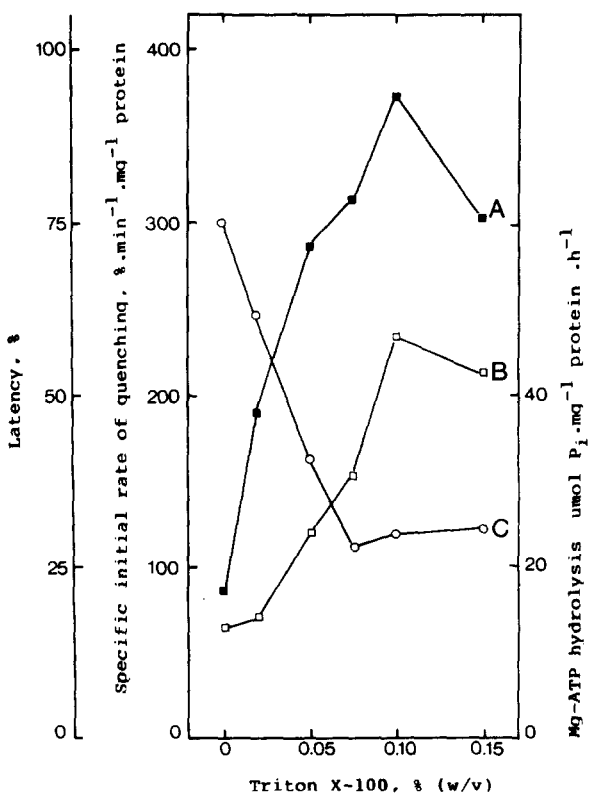
the specific Mg^{2+}-ATPase activity (approx. 160%, Table VII) in the presence of lysoPC was entirely due to the elimination of membrane proteins. Furthermore, Triton X-100 treatment increased the specificity of triphosphonucleotide hydrolysis (Table II) and slightly increased the maximum inhibition of Mg^{2+}-ATPase hydrolysis by vanadate (I_{max}) from 76% to 85% (Fig. 11). However, in the absence of lysoPC, the increase in Mg^{2+}-ATPase

TABLE VII

EFFECTS OF TRITON X-100 WASHING ON MgATP HYDROLYSIS, SIDEDNESS AND H^+ TRANSPORT PROPERTIES OF TIGHTLY SEALED VESICLES

Vesicles were washed with 0.1% Triton X-100 and the surfactant was eliminated (see text). Unwashed and washed vesicles were incubated at 26 ° C for 20 min in 2 ml of medium containing 50 mM Bistris propane-Cl (pH 6.5), 5 mM Bistris propane-ATP, 10 μM quinacrine, 100 mM KCl and 0.1 μM valinomycin when indicated. H^+ transport was monitored with quinacrine quenching. Reaction was started by addition of $MgSO_4$ (final concentration of 5 mM). The quenching decay was initiated by addition of 30 mM Bistris propane-EDTA. MgATP hydrolysis was measured under the same conditions as quenching, except that quinacrine was omitted and 2 μM gramicidin and 100 μM lysoPC were present as indicated. Latency is calculated as $100 \cdot (v_{lyso} - v)/v_{lyso}$, where v_{lyso} is the activity in the presence of lysoPC. n.d., not determined.

Treatment	MgATP hydrolysis				Quinacrine quenching		
	activity ($\mu mol \cdot h^{-1} \cdot mg^{-1}$ protein)		latency (%)	uncoupling (%)	initial rate (% $min^{-1} \cdot mg^{-1}$ protein)	total extent (% mg^{-1} protein)	decay $T_{1/2}$ (min)
	none	lysoPC					
Unwashed TS vesicles							
Control	10.6 ± 1.9	38.9 ± 6.3	73	–	51 ± 20	319 ± 51	8 ± 4
Valinomycin	11.6 ± 2.5	39.6 ± 4.5	71	9	75 ± 12	388 ± 26	n.d.
Gramicidin	12.1 ± 1.5	38.7 ± 3.9	70	14	0	0	–
Triton X-100 washed TS vesicles							
Control	48.1 ± 3.7	98.6 ± 4.0	51	–	277 ± 28	1140 ± 99	22 ± 7
Valinomycin	50.6 ± 3.8	101.5 ± 8.3	50	5	320 ± 23	1170 ± 187	n.d.
Gramicidin	63.1 ± 4.1	100.3 ± 8.2	37	31	0	0	–



specific activity due to washing with Triton X-100 was far too large (approx. 420% in the presence of 2 μM gramicidin) to be explained by the protein loss. This increase was responsible for the diminution of the latency of the MgATP hydrolysis, from approx. 70% to 37% after washing with Triton X-100, in the presence of 2 μM gramicidin (Fig. 10 and Table VII). Furthermore, washing with Triton X-100 increased the initial rate of quinacrine quenching in the same proportion as

Fig. 10. Effects of Triton X-100 washing on MgATP hydrolysis and H^+ transport by tightly sealed vesicles. The procedure for washing and elimination of Triton X-100 is described in the text. Tightly sealed vesicles washed with Triton X-100 at the indicated concentration were incubated at 26 ° C for 20 min in 2 ml of medium containing 50 mM Bistris propane-Cl (pH 6.5), 5 mM Bistris propane-ATP, 10 μM quinacrine, 0.1 μM valinomycin and 100 mM KNO_3. H^+ transport was monitored with quinacrine quenching. The reaction was started by addition of $MgSO_4$ (final concentration of 5 mM). MgATP hydrolysis was measured under the same conditions, except that valinomycin was replaced by 2 μM gramicidin and quinacrine was omitted. The latency of MgATP hydrolysis was determined with lysoPC. (A) Specific initial rate of quenching; (B) MgATP hydrolysis rate; (C) latency.

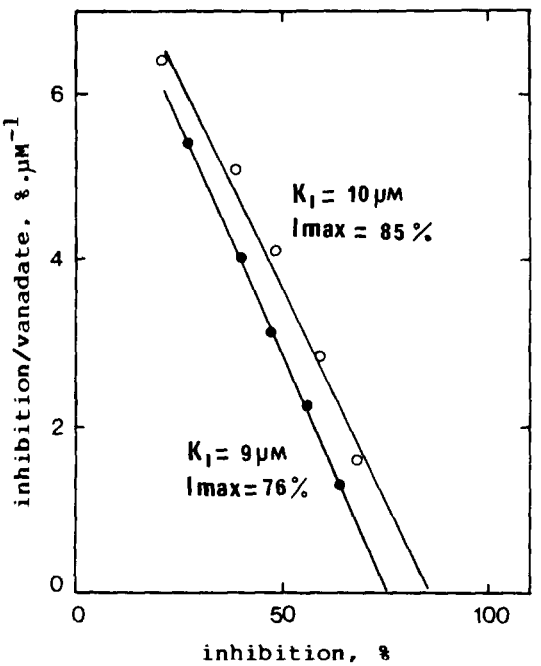


Fig. 11. Effect of orthovanadate on inhibition of MgATP hydrolysis by tightly sealed vesicles. The assay medium contained 25 mM Bistris propane-Mes (pH 6.5), 3 mM Bistris propane-ATP, 3 mM $MgSO_4$, 100 μM sodium molybdate, 50 mM KCl, 100 μM lysoPC and 0–50 μM sodium vanadate. The results are shown as Scatchard plots. Solid symbols, control; open symbols, washed with 0.1% Triton X-100 (see text). The inhibition parameters I_{max} and K_i were estimated by linear regression analysis.

the Mg^{2+}-ATPase hydrolysis in the absence of lysoPC. This proportion was 330% in the absence of valinomycin, and 450% when 0.1 μM valinomycin was added for relieving the electrical constraint (Table VII). Finally, the Triton X-100 washed vesicles exhibited both a stimulation (30%) of the Mg^{2+}-ATPase by addition of 2 μM gramicidin, and an increase of the half-time of the H^+ gradient (from approx. 8 min to 22 min, Table VII and Fig. 9B).

Discussion

Different latencies of the MgATP hydrolysis were obtained with Triton X-100, Zwittergent 3-14, SDS, and lysoPC (respectively, approx. 40%, 46%, 51% and 65%). The complex shape of the response of the activity to increasing concentrations of the three detergents suggests that both stimulating and inhibiting effects occurred (Figs. 1A–3A). In contrast to the detergents, lysoPC only stimulated the MgATP hydrolysis (Fig. 1C). Only the inhibiting effects, with distinct kinetics, remained observable when the detergents were added in the presence of 100 μM lysoPC (Fig. 1B, continuous line, and Figs. 2 and 3, curve A). It is possible to correct the curves of Figs. 1A–3A for these inhibiting effects, assuming that they are independent of the presence of lysoPC. Thus, it is possible to isolate the sole stimulating effects of the detergents (dotted lines in Figs. 1–3). This procedure gives the same maximum activity value and the same latency for each of the four permeabilizing agents. This unique stimulating effect contrasts with the different inhibiting effects. It is better explained by an identical indirect stimulating effect (the permeabilization of the vesicles), than by an identical direct effect. This conclusion in the absence of direct effect of lysoPC on the Mg^{2+}-ATPase is consistent with the similitude of the orientation values calculated from the latency and from the effects of trypsin and concanavalin A (see below). The inhibiting effect of the detergents is probably due to the fact that they bind to proteins [26,27]. This precludes their use for determining the maximum Mg^{2+}-ATPase activity in membrane vesicles, even if care is taken to choose surfactant concentrations yielding maximum activity [28]. LysoPC is suitable for determining the true latency, since it does not inhibit the Mg^{2+}-ATPase activity in the used concentration range, perhaps because its interaction with the membrane is restricted to the phospholipid moiety [29].

Since the catalytic site of the Mg^{2+}-ATPase is on the cytoplasmic face of the membrane, the latency represents the percentage of right-side-out vesicles impermeable to MgATP [5]. The data on the latency (Table I and III) indicate that sealed and tightly sealed preparations contain approx. 67% of such right-side-out vesicles and approx. 33% of inside-out vesicles, leaky right-side-out vesicles or unsealed membrane fragments. On the other hand, the percentage of Mg^{2+}-ATPase undigested by trypsin is in good agreement with the above proportion of right-side-out vesicles impermeable to MgATP (63%, Table III). This result indicates that the membrane fraction does not contain a significant proportion of right-side-out

vesicles leaky only to MgATP but not to trypsin. Furthermore, the fraction which passed straight through the Sepharose-Con A column (Table V), or which did not specifically bind the lectin in microelectrophoretic experiments (Table IV), contained approx. 33% of the total membranes. Taking into account the extra-cellular location of the lectin-binding site [30], one may conclude that this fraction does not contain a significant proportion of unsealed membrane fragments or right-side-out vesicles leaky to trypsin, but contains only true inside-out vesicles.

In summary, the percentage of Mg^{2+}-ATPase latency (66%), the percentage of non-digested enzyme (63%) in trypsin experiments, and the percentage of vesicles exhibiting specific binding of concanavalin A (70% and 67% from microelectrophoretic and chromatographic experiments, respectively) are close. It must be pointed out that the two former methods are based on a specific property of the plasmalemma vesicles (the Mg^{2+}-ATPase activity), and the two latter are based on a general property of all the vesicles of the preparation (interaction with concanavalin A). Nevertheless, these four kinds of independent data are consistent. This indicates that: (i) the preparation does not contain a significant proportion of unsealed membrane fragments; (ii) all right-side-out vesicles are impermeable to MgATP; (iii) approx. 65% of the membrane vesicles are right-side-out oriented.

The first conclusion is at variance with that of Randall and Ruesink [3] who detected a high proportion of unsealed fragments or leaky vesicles (approx. 70%) in a plasmalemma fraction from carrot protoplasts. Nevertheless, the mild homogenization of protoplasts used was likely to produce such large membrane fragments. Furthermore, these fragments were probably not eliminated because the membrane suspension was directly layered on a discontinuous gradient without differential centrifugation.

The other conclusions are at variance with those of Berczi and Møller [9]. These authors claimed that relatively high proportions of inside-out vesicles generally obtained with classical centrifugation procedures are overestimated, because some of right-side-out vesicles are leaky, due to the hypoosmotic conditions used in the assays. Two points must be noted. Firstly, they measured an increase in Mg^{2+}-ATPase activity upon elimination of sucrose in the absence of surfactants and attributed it to bursting of the vesicles because they observed that the sugar diminished the latency. However, this decrease of latency was observed by comparing the activity without sucrose (in the absence of a surfactant) to the activity in the presence of both a surfactant and sucrose. Our data (Table VI) show that similar increases in Mg^{+}-ATPase are induced by sucrose elimination in the presence or in the absence of the permeabilizing agent. Thus, the increase of the activity is not attributable to vesicle bursting. The calculated latency remains constant and the effect of sucrose merely arises from a direct effect on Mg^{2+}-ATPase. This interpretation is supported by the fact that trehalose, at the same concentration as sucrose, was more efficient in inhibiting Mg^{2+}-ATPase (40% instead of 20%, Table VI), which cannot be explained by osmotic effects. Finally, the comparison of the initial quenching rates (Table VI) indicates that the H^{+} transport was higher in the absence of sugars, which is inconsistent with the hypothesis of vesicle bursting under hypoosmotic conditions.

The second point to be noted is that if we had used Triton X-100 instead of lysoPC for determination of the latency, we should have obtained approx. 50% right-side-out vesicles in the experiments of Fig. 1. Since the experiments with concanavalin A gave 30% inside-out vesicles, the discrepancy between these data would have led to the conclusion that 20% of the right-side-out vesicles were leaky to MgATP and trypsin.

In summary, the relatively low latency observed in membrane preparations obtained by centrifugation is not due to the presence of leaky vesicles, but actually reflects a high proportion of inside-out sealed vesicles. Triton X-100 has been used to determine the latency of various enzymic markers of cereal plasmalemma preparations obtained by centrifugations: Mg^{2+}-ATPase of corn root (this paper) and wheat root [9], UDPase [1], UDPG sterol-D-glucosyl transferase [7] and NDPase [31] of corn coleoptile. In all instances, the latency values obtained were approx. 50%, and the enzymic activities presented a sharp maximum when plotted against Triton X-100 concentration. The

results of Fig. 1 suggest that inhibition by Triton X-100 occurs in the same concentration range as permeabilization and renders this surfactant inadequate for determining the latency.

In contrast to the inhibition of Mg^{2+}-ATPase observed in the presence of both Triton X-100 and lysoPC (Fig. 1), pretreatment with the surfactant followed by its elimination did not modify the total activity of the pelleted fraction measured in the presence of lysoPC. Washing with Triton X-100 had two effects, viz. an elimination of membrane proteins leading to an increase of the specific Mg^{2+}-ATPase activity, and a strong reduction of the latency determined with lysoPC.

The increase in specific activity of MgATP hydrolysis (+167%, from the data of Table II for tightly sealed vesicles) was associated with an increase of the sensitivity of MgATP hydrolysis to vanadate and a higher specificity for MgATP as compared to other triphosphonucleotide (Fig. 11 and Table II). These results indicate that non-ATPase proteins were specifically eliminated by the pretreatment.

The reduction of the latency after washing with Triton X-100 did not result from the observed increase of Mg^{2+}-ATPase specific activity, but was due to a reorientation of a large proportion of the vesicles, from 30 to 63% (Table VII) or 70% inside-out vesicles (Fig. 10). Furthermore, Mg^{2+}-ATPase uncoupling by gramicidin or NH_4^+ was enhanced by washing with Triton X-100 (Table VII). Similar values for the initial rate of quinacrine quenching were obtained for vesicles washed or unwashed, when expressed per unit of Mg^{2+}-ATPase activity (respectively, 378 and 390% quenching $\cdot \mu mol^{-1}$ MgATP hydrolyzed, from the data of Table VII, in the presence of valinomycin). Since washing with Triton X-100 did not modify active H^+ transport by inside-out vesicles, it probably increased gramicidin-induced uncoupling by improving membrane tightness to H^+. This hypothesis is supported by the increase of the quenching half-time after stopping active H^+ transport (Fig. 10 and Table VII). Another reason for the enhancement of uncoupling by washing with Triton X-100 could be the simultaneous increases in the proportions of tightly sealed and inside-out vesicles (Fig. 10).

In summary, the results on the initial quenching rate and latency indicate that washing with Triton X-100 induces, in parallel, the stimulation of active H^+ transport and the enhancement of the frequency of inside-out vesicles. This demonstrates that this treatment does not create leaky vesicles, and suggests that it truly inverts the vesicle orientation from right-side out to inside out, as hypothesized by Clement et al. [11], giving new inside-out vesicles with the same H^+ transport properties as those that already existed before. The fact that the proportions of inside-out and right-side-out vesicles were almost exactly inverted (Fig. 10) suggests that Triton X-100 generally produces an inversion of the sidedness of vesicles. An alternative explanation would be that the orientations of the catalytic sites on each vesicle are partially randomized by Triton X-100. However, such a randomization would probably necessitate membrane solubilization [32]. Since membranes remained pelletable in the presence of 0.1% Triton X-100 (see Results and Ref. 33), this explanation seems doubtful.

Acknowledgments

The authors are greatly indebted to Suzette Astruc for her valuable assistance. This work was supported by a grant from the Ministère de la Recherche et de l'Industrie (Action Concertée No. 84 C 1 406).

References

1 Hartmann, M.A., Ehrhardt, E. and Benveniste, P. (1983) Plant Sci. Lett. 30, 227–238
2 Perlin, D.S. and Spanswick, R.M. (1980) in Plant Membrane Transport: Current Conceptual Issues (Spanswick , R.M., Lucas W.J. and Dainty, J., eds.), pp. 529–530, Elsevier/North-Holland, Amsterdam
3 Randall, S.K. and Ruesink, A.W. (1983) Plant Physiol. 73, 385–391
4 Sze, H. (1985) Ann. Rev. Plant Physiol. 36, 175–208
5 Larsson, C., Kjellbom, P., Widell, S. and Lundborg, T. (1984) FEBS Lett. 171, 271–276
6 Hodges, T.K. and Mills, D. (1986) Methods Enzymol. 118, 41–54
7 Quantin, E., Hartmann-Bouillon, M.A., Schuber, F. and Benveniste, P. (1980) Plant Sci. Lett. 17, 193–199
8 Körner, L.E., Kjellbom, P., Larsson, C. and Möller, I.M. (1985) Plant Physiol. 79, 72–79
9 Berczi, A. and Möller, I.M. (1986) Physiol. Plant. 68, 59–66
10 De Michelis, M.I. and Spanswick, R.M. (1986) Plant Physiol. 81, 542–547

11 Clement, J.D., Blein, J.P., Rigaud, J. and Scalla, R. (1986) Physiol. Vég. 24, 25–35
12 Leonard, R.T. and Hotchkiss, C.W. (1976) Plant Physiol. 58, 331–335
13 Gibrat, R., Grouzis, J.P., Rigaud, J. and Grignon, C. (1985) Biochim. Biophys. Acta 816, 349–357
14 Ames, B.N. (1966) Methods Enzymol. 8, 115–118
15 Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514
16 Bangham, A.D., Flemans, R., Heard, D.H. and Seaman, G.V.H. (1958) Nature 6, 642–644
17 Hunter, R.J. (1981) in Zeta Potential in Colloid Science (Hunter, R.J., ed.), pp. 386, Academic Press, New York
18 McLaughlin, S. (1977) Current Top. Membrane Transp. 9, 71–144
19 Gibrat, R., Romieu, C. and Grignon, C. (1983) Biochim. Biophys. Acta 736, 196–202
20 Ross, G.J.S. (1970) R. Statistical Soc. C, 19, 205–221
21 Resch, K., Schneider, S. and Szamel, M. (1981) Anal. Biochem. 117, 282–292
22 Lew, R.R. and Spanswick, R.M. (1985) Plant Physiol. 77, 352–357
23 Hager, A. and Biber, W. (1984) Z. Naturfosch. 39 c, 927–937
24 Gallagher, S.R. and Leonard, R.T. (1982) Plant Physiol. 70, 1335–1340
25 Forbush, B., III (1982) J. Biol. Chem. 257, 12678–12684
26 McIntosh, D.B. and Davidson, G.A. (1984) Biochemistry 23, 1959–1965
27 Nicholson, D.W. and McMurray, W.C. (1986) Biochim. Biophys. Acta 856, 515–525
28 Vara, F. and Serrano, R. (1982) J. Biol. Chem. 257, 12826–12830
29 Tokumura, A., Mostafa, M.H., Nelson, D.R. and Hanahan, D.J. (1985) Biochim. Biophys. Acta 812, 568–574
30 Nicholson, G.L. and Singer, S.J. (1974) J. Cell Biol. 60, 236–241
31 M'Voula-Tsieri, M., Hartmann-Bouillon, M.A. and Benveniste, P. (1981) Plant Sci. Lett. 20, 379–386
32 Karlish, S.J.D. and Stein, W.D. (1982) J. Physiol. (London) 328, 295–316
33 Anthon, G.E. and Spanswick, R.M. (1986) Plant Physiol. 81, 1080–1085