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Sealed Inside-Out and Right-Side-Out Plasma Membrane Vesicles

Optimal Conditions for Formation and Separation

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ABSTRACT

Plasma membrane preparations of high purity (about 95%) are easily obtained by partitioning in aqueous polymer two-phase systems. These preparations, however, mainly contain sealed right-side-out (apoplast side out) vesicles. Part of these vesicles have been turned inside-out by freezing and thawing, and sealed inside-out and right-side-out vesicles subsequently separated by repeating the phase partition step. Increasing the KCl concentration in the freeze/thaw medium as well as increasing the number of freeze/thaw cycles significantly increased the yield of inside-out vesicles. At optimal conditions, 15 to 25% of total plasma membrane protein was recovered as inside-out vesicles, corresponding to 5 to 10 milligrams of protein from 500 grams of sugar beet (Beta vulgaris L.) leaves. Based on enzyme latency, trypsin inhibition of NADH-cytochrome c reductase, and H+ pumping capacity, a cross-contamination of about 20% between the two fractions of oppositely oriented vesicles was estimated. Thus, preparations containing about 80% inside-out and 80% right-side-out vesicles, respectively, were obtained. ATPase activity and H+ pumping were both completely inhibited by vanadate (Ki ≈ 10 micromolar), indicating that the fractions were completely free from nonplasma membrane ATPases. Furthermore, the polypeptide patterns of the two fractions were close to identical, which shows that the vesicles differed in sidedness only. Thus, preparations of both inside-out and right-side-out plasma membrane vesicles are now available. This permits studies on transport, signal transduction mechanisms, enzyme topology, etc., using plasma membrane vesicles of either orientation.

A typical feature of biological membranes is the asymmetric arrangement of constituents across the lipid bilayer. This asymmetry is absolute for proteins: integral proteins, which span the membrane, expose different regions on either side of the membrane, whereas peripheral proteins are bound to either surface of the membrane. For lipids, the asymmetry is rather relative than absolute, such that each lipid species usually only shows some enrichment to either half of the bilayer. This asymmetric, transverse organization of membrane constituents forms the basis for all the vectorial activities exerted by biological membranes, and it is created through the asymmetric assembly of membranes (review, 22).

The most useful approach for characterizing the asymmetric properties of a membrane, including its vectorial activities, is to prepare sealed membrane vesicles of either orientation. With such preparations, each membrane surface can be probed selectively using impermeable agents, and transport in either direction can be measured as uptake into vesicles. The formation and subsequent separation of vesicles of opposite orientation was first achieved with the erythrocyte membrane through the pioneering work of Steck et al. (review, 30), and later with the mitochondrial inner membrane (review, 8) and the chloroplast thylakoid membrane (review, 2). The access to sealed membrane vesicles of either orientation made extensive studies on the topology of these membranes possible. With both the erythrocyte and the thylakoid membrane, aqueous two-phase partitioning was used to separate the oppositely oriented vesicles (2, 30). Indeed, two-phase partitioning should be a very suitable method in such cases, since it separates particles according to their surface properties (1) and vesicles of opposite orientation are expected to differ in this respect but not in size or density.

For the plant plasma membrane, the separation of inside-out (cytoplasmic side out) and right-side-out (apoplastic side out) vesicles was only recently achieved using either free-flow electrophoresis (7) or two-phase partitioning (21). We now report a number of essential improvements on the phase partition procedure, as well as a thorough characterization of the membrane fractions obtained.

MATERIALS AND METHODS

Plant Material

Four-week-old sugar beet plants (Beta vulgaris L.) were kindly supplied by Hilleshög AB, Sweden. Plants were maintained in soil in a greenhouse with supplementary light (23
W m⁻², 350–800 nm; Philips G/86/2 HPLR 400 W, The Netherlands). Leaves of 6- to 8-week-old plants were used.

**Preparation of Plasma Membranes**

Plasma membranes (predominantly right-side-out vesicles) were purified from a microsomal fraction (10,000–50,000 g pellet) of sugar beet leaves by partitioning in an aqueous polymer two-phase system as described earlier (reviews, 18, 20) with minor modifications. The homogenization medium was essentially as in Palmgren and Sommarin (25) and contained 330 mM sucrose, 50 mM Mops-BTP (pH 7.5), 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.2% (w/v) BSA (Sigma; protease free), 0.2% (w/v) casein (boiled enzymatic hydrolysate, Sigma type I), 0.6% (w/v) insoluble PVP. Lots of 125 g of leaves were homogenized in 275 mL, and the resulting microsomal fraction (about 100 mg of protein) was suspended in 330 mM sucrose, 5 mM K-phosphate (pH 7.8), 5 mM KCl, 1 mM DTT, 0.1 mM EDTA. This microsomal fraction was added to a phase system with a final weight of 36.0 g and a final composition of 6.5% (w/v) Dextran T500, 6.5% (w/v) polyethylene glycol 3350, 330 mM sucrose, 5 mM K-phosphate (pH 7.8), 5 mM KCl, 1 mM DTT, 0.1 mM EDTA (4°C). We routinely start with either 250 or 500 g of leaves and process two to four 36 g phase systems in parallel using the three-step batch procedure described previously (18, 20). The final upper phases containing the plasma membranes were diluted several-fold with 330 mM sucrose, 5 mM K-phosphate (pH 7.8), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and the plasma membranes were pelleted and resuspended to 15 to 20 mg mL⁻¹ protein in the same medium. The yield was 15 to 18 mg of protein per 125 g of leaves, and the preparations were free of Chl and also otherwise of high purity as determined by standard marker assays (cf. 11). The membranes (usually >90% right-side-out vesicles) were stored in liquid N₂ until further use.

**Formation of Inside-Out Vesicles from Right-Side-Out Vesicles**

The highly purified right-side-out plasma membrane vesicles were frozen and thawed to produce a mixture of inside-out and right-side-out vesicles. Typically, portions of 0.8 to 1 mL were frozen in liquid N₂ and thawed in water at 20°C a total of four times.

**Separation by Counter-Current Distribution**

The freeze/thawed plasma membranes (now being a mixture of oppositely oriented vesicles) were fractionated by phase partition using counter-current distribution (1) to produce one fraction enriched in inside-out vesicles, another fraction enriched in right-side-out vesicles, and two intermediate fractions as described earlier (21) (Fig. 1). Freeze/thawed plasma membranes (0.8 mL) from 125 g of leaves were added to a 7.2 g phase mixture to give an 8.0 g phase system with a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) polyethylene glycol 3350, 330 mM sucrose, 5 mM KCl, 1 mM DTT, 0.1 mM EDTA, 5 mM K-phosphate (pH 7.8; 4°C). The phase system was shaken and spun for 5 min at 1500g (swinging bucket centrifuge) to facilitate phase separation. About 90% of the upper phase was removed without disturbing the interface, and was added to a second tube containing fresh lower phase, as well as upper phase corresponding to the 10% upper phase not removed from tube 1. Fresh upper phase was added to tube 1, and mixing and centrifugation was repeated. Then, 90% of the upper phase in tube 2 was moved to a third tube containing fresh lower phase, the upper phase in tube 1 was moved to tube 2, and fresh upper phase was added to tube 1. The procedure was repeated once more to produce four tubes containing complete phase systems and plasma membrane vesicles. The contents of each tube (fractions 1–4 in Fig. 1) was diluted about 10-fold with 330 mM sucrose, 10 mM Mops-BTP (pH 7.5), 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF, and the plasma membranes were pelleted at 100,000g for 1 h. The pellets were gently resuspended...
in the same medium (minus DTT for the NADH-oxidoreductase assays) and immediately used in the different assays. The procedure is scaled up by either processing two or more 8 g phase systems in parallel or by using larger phase systems.

Inside-out vesicles were enriched in fraction 1, and right-side-out vesicles were enriched in fraction 4 (Fig. 1). Thus, the inside-out and right-side-out vesicles were separated by essentially repeating the phase partition step originally used to isolate the plasma membranes as right-side-out vesicles (see above). Almost identical phase compositions were used, and as in the previous step the right-side-out vesicles partitioned to the upper phase; by contrast, inside-out plasma membrane vesicles behaved as intracellular membranes and were therefore recovered in the lower phase + interface, which made the separation possible. Note, that the main difference between the two procedures is that in the latter procedure the wash phases are saved to produce the inside-out fraction and the two intermediate fractions, whereas the corresponding fractions are simply discarded in the former procedure. The counter-current distribution procedure is recommended in initial work, since all material is saved and accounted for, which makes it simpler to optimize separation.

**H⁺ Pumping**

H⁺ uptake into the vesicles was monitored as the absorbance decrease at 495 nm of the ΔpH probe acridine orange (32). The assay medium was essentially as described earlier (25) and consisted of 20 μM acridine orange, 2 mM ATP-BTP, 4 mM MgCl₂, 10 mM Mops-BTP (pH 7.0), 140 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mg mL⁻¹ BSA (Sigma; A 0281, essentially fatty acid free), 2.5 μg mL⁻¹ valinomycin, and 50 to 100 μg mL⁻¹ membrane protein in a total volume of 1 mL. After 5 min preincubation at 20°C, the reaction was initiated by addition of MgCl₂. The rate of H⁺-accumulation was estimated from the initial slope of absorbance quenching (ΔA₄₉₅) of acridine orange.

**ATPase Assay (P, Release)**

ATPase activity determined from the released Pᵢ was measured according to Baginski et al. (4) as modified by Brotherus et al. (6). Two different assay media were used: (a) A medium essentially as in Gallagher and Leonard (10) containing 330 mM sucrose, 50 mM Mes-Tris (pH 6.0), 25 mM K₂SO₄, 3 mM MgSO₄, 3 mM ATP, 0.1 mM EDTA, 1 mM azide, 0.1 mM molybdate, and 10 μg protein in a final volume of 120 μL. The assay was run for 30 min at 25°C, and ±0.02% (w/v) Triton X-100 (Sigma; T 6878). (b) The same medium as used to assay H⁺ pumping (above), except that EDTA was omitted. This medium was used to measure vanadate inhibition of H⁺ pumping and ATPase activity in the same sample. Aliquots of 200 μL were withdrawn after 20, 140, and 260 s for Pᵢ determination, and H⁺ pumping was recorded simultaneously at 495 nm. The ATPase assay based on the release of ADP (below) could not be used for this purpose, since vanadate interfered with that assay.

**H⁺-ATPase Assay (ADP Release and H⁺ Pumping)**

H⁺ pumping and ATPase activity were monitored simultaneously in the same cuvette using the H⁺-ATPase assay described earlier (25). The concentration of ATP was kept constant by including 0.25 mM NADH, 1 mM phosphoenolpyruvate, 15 μg mL⁻¹ lactate dehydrogenase (Boehringer; 003565, solution in glycerol) and 30 μg mL⁻¹ pyruvate kinase (Boehringer; 005541, solution in glycerol) in the assay medium used for H⁺ pumping (above). Thus, ATP hydrolysis was coupled to oxidation of NADH (24), and H⁺ pumping and ATPase activity were monitored simultaneously by plotting the absorbance at 495 nm and 340 nm, respectively.

**1,3-β-D Glucan Synthase**

1,3-β-Glucan synthase activity was measured as incorporation of UDP-[³H]glucose into polyglucan according to Kauss et al. (14) with minor modifications (9). The assay medium contained 330 mM sucrose, 2 mM DTT, 2 mM spermine, 20 mM cellubiose, 0.2 mM CaCl₂, 2 mM UDP-[³H]glucose (20 GBq mol⁻¹), 50 mM Hepes-KOH (pH 7.25), and 1 μg of protein in a total volume of 100 μL. The activity was assayed ± 0.006% (w/v) of the detergent digitonin (Serva; 19550). After 30 min at 25°C the reaction was terminated by immersion of the test tubes in boiling water, and 95 μL aliquots were withdrawn and added to cellulose filters (Whatman 3 MM). Filters were dried and washed as described (14). Standards containing 50% of the radioactivity added to each sample were counted on wetted glass fiber filters (Whatman GF/F), since the radioactivity of the low-mol wt substrate was quenched on the cellulose filters (9). Radioactivity was determined by liquid scintillation counting using Ready Safe scintillation cocktail (Beckman).

**NADH-Acceptor Oxidoreductase**

NADH-acceptor oxidoreductase with either ferricyanide or Cyt c (Sigma; C 7752) as acceptor was assayed essentially as described earlier (3). NADH-ferricyanide reductase activity was measured as ∆(A₄₃₀⁻⁻⁻₄₅₀) using an Aminco DW 2 spectrophotometer operated in the dual beam mode. The assay was run at 25°C in 1 mL of 330 mM sucrose, 25 mM Hepes-KOH (pH 7.3), 0.25 mM NADH, 0.2 mM K₃[Fe(CN)₆], 40 μg protein, and ± 0.025% (w/v) Triton X-100. The reaction was initiated by the addition of NADH. Correction was made for nonenzymatic reduction of ferricyanide.

NADH-Cyt c reductase activity was measured similarly using 40 μM Cyt c as acceptor instead of 0.2 mM K₃[Fe(CN)₆], and with 0.4 μM antimycin A (Sigma; A 2006) and 1 mM KCN present in the assay medium. The activity was recorded as ∆(A₃₅₀⁻⁻⁻₄₅₀), and was determined ±0.015% (w/v) Triton X-100.

The extinction coefficients used were 1 and 19 mm⁻¹ cm⁻¹ for ferricyanide and Cyt c, respectively.

**SDS-PAGE**

SDS-PAGE was run on gradient gels (concentration of monomers, 10–22%; crosslinking, 2.7%; 5% stacking gel; gel
dimensions—175-160·1.5 mm) in the buffer system of Laemmli (17). The samples were solubilized at 80°C for 10 min, and gels were run for 15 h at 12°C and 15 mA per gel. Silver staining was essentially as described by Guevara et al. (13).

Protein

Protein was measured essentially as described by Bearden (5), with BSA as a standard.

RESULTS AND DISCUSSION

Formation of Inside-Out Vesicles

Plasma membrane preparations of high purity (about 95%) are easily obtained by partitioning in aqueous polymer two-phase systems (reviews, 18, 20). These preparations, however, mainly contain sealed, right-side-out (apoplastic side out) vesicles (19). This was demonstrated by assaying the plasma membrane ATPase in the absence and presence of the detergent Triton X-100. Since the active site of the ATPase is located on the inner, cytoplasmic surface of the plasma membrane, the activity associated with sealed, right-side-out vesicles is only measured in the presence of detergent. Thus, the proportion of right-side-out vesicles may be calculated from the ratio of latent activity (difference in activity measured ± detergent) to total activity (activity measured + detergent). Using this method to determine vesicle orientation, plasma membrane preparations from most materials, including the sugar beet leaves used in this study, contain about 90% right-side-out vesicles. Thus, for these to be a suitable starting material for the separation of inside-out and right-side-out vesicles, part of the right-side-out vesicles in the plasma membrane preparation need to be turned inside-out. We therefore looked at a number of treatments (vigorously pottering in different media, hypotonic shock, sonication, freeze/thawing) that could be expected to cause vesicle breakage and revesiculation. As markers for inside-out vesicles we used: (a) ATP-dependent H+-pumping, (b) nonlatent activity of the ATPase, and (c) nonlatent activity of NADH-ferricyanide reductase, an activity also associated with the cytoplasmic surface of the plasma membrane (3, see below). The first marker is crucial, since the nonlatent activities could at least theoretically be due to leaky right-side-out vesicles or membrane sheets, whereas only sealed, inside-out vesicles may support ATP-dependent H+-pumping. Two of the treatments investigated, sonication and freeze/thawing, significantly increased the proportion of inside-out vesicles, as determined by their H+-pumping activity and by their nonlatent NADH-ferricyanide reductase activity (data not shown). Sonication simultaneously caused a decrease in total NADH-ferricyanide reductase activity indicating some damage to the membrane. Freezing in liquid N2 and thawing in a waterbath at 20°C (which should minimize the formation of concentration gradients during the process) did not inhibit NADH-ferricyanide reductase activity and we therefore chose to optimize this procedure.

The composition of the freeze/thaw medium was important (Fig. 2). Addition of 50 mM KCl to the basic medium (330 mM sucrose, 5 mM K-phosphate [pH 7.8], 1 mM DTT, 0.1 mM EDTA) gave an approximate twofold increase in H+-pumping after one freeze/thaw cycle compared to no addition. NaCl at the same concentration caused a similar increase, whereas MgCl2 (6 and 12 mM) was less efficient. Omission of sucrose (hypotonic medium) caused a decrease in H+-pumping. The effect of KCl was concentration-dependent (Fig. 2, g–j), and 50 mM was chosen for further experiments.

Repeating the freeze/thaw cycle increased both the H+-pumping capacity and the non-latent ATPase activity (Fig. 3) measured simultaneously in the same sample using an H+-ATPase assay (25). The cumulative effect of repeated freeze/thawing suggests that only a minor proportion of the vesicles were broken and resealed in each cycle. Little additional effect was found after three freeze/thaw cycles, and four cycles were used for further experiments.
Freezing and thawing of animal plasma membranes has been reported to cause increased ATPase activity by 'unmasking' of latent ATP binding sites (23). The fact that H+ pumping and ATPase activity increased in parallel in the sugar beet plasma membranes (Fig. 3) indicates that the effect of freezing and thawing was not only 'exposure' of new ATP binding sites but was coupled to the formation of sealed, inside-out vesicles from right-side-out vesicles.

On homogenization of plant tissue, a right-side-out orientation of the resulting plasma membrane vesicles is usually strongly favored. This is shown by a high recovery (70-80%) of plasma membrane markers in preparations which contain predominantly right-side-out vesicles (11, 15, 18, 20). However, the proportion of inside-out vesicles obtained seems to be dependent on the method used for homogenization (7), and the composition of the homogenization medium is also likely to affect vesicle orientation. For the erythrocyte membrane a right-side-out orientation seems to be favored by: (a) a higher negative net charge density of the outer surface compared to the inner one, which affects membrane curvature, and (b) remaining cytoskeleton anchored to the inner surface also affecting curvature (30, and references therein). The same factors might favor the formation of right-side-out plasma membrane vesicles with plant material. Thus, free-flow electrophoresis of plasma membrane vesicles (7) suggests that the cytoplasmic surface has a lower net charge density than the apoplastic one at neutral pH, and fibrous material which might be remnants of the cytoskeleton has been observed in right-side-out vesicles (S Widell, C. Larsson, unpublished results). The increased formation of inside-out vesicles on freeze/thawing at higher KCl concentration (Fig. 2) may be due to screening of charges on the membrane surfaces, thus reducing the charge difference between the inner and outer surface. The results obtained with MgCl2 (Fig. 2) do not support this conclusion, however, but Mg2+ may have additional effects on the membrane which counteract its screening effects. Nevertheless, the composition of the freeze/thaw medium is likely to affect both the probability of vesicle breakage upon freeze/thawing, and the probability for resealing with a certain orientation after breakage.

Separation of Vesicles of Opposite Sidedness

The optimal composition of the two-phase system for separation of inside-out and right-side-out vesicles was determined by partitioning the freeze/thawed plasma membranes in a series of phase systems with increasing polymer concentration (Fig. 4). At 6.2% (w/w) of both polymers only about 20% of both the H+ pumping and the non-latent NADH-ferricyanide reductase activity were partitioned to the upper phase compared to about 70% of the latent NADH-ferricyanide reductase activity, indicating a good separation of inside-out and right-side-out vesicles. When purified plasma membranes were subjected to counter-current distribution at this polymer concentration two peaks of material were observed provided the vesicles had been freeze/thawed (Fig. 5). Increasing the number of freeze/thaw cycles increased the amount of material recovered in fraction 1 with a parallel decrease in fraction 4. Material partitioning mainly to the interface + lower phase (= stationary phase) would be recovered in frac-

**Figure 4.** Effect of polymer concentration on the partitioning of inside-out and right-side-out plasma membrane vesicles in an aqueous polymer two-phase system. The phase system contained 330 mM sucrose, 5 mM K-phosphate (pH 7.8), 0.1 mM EDTA, and equal concentrations of Dextran T 500 and polyethylene glycol 3350 as indicated. Markers for inside-out vesicles were H+ pumping (▲) and nonlatent NADH-ferricyanide reductase (○), and for right-side-out vesicles latent NADH-ferricyanide reductase (●). Assays were performed on aliquots withdrawn from the phases after separation and dilution.

**Figure 5.** Effect of the number of freeze/thaw cycles on protein distribution after counter-current distribution of plasma membrane vesicles. Plasma membranes were loaded in tube 1 and three transfers of the upper phase were made keeping the interface + lower phase stationary (see Fig. 1). The plasma membranes were either used fresh (○), or subjected to 1 (●), 3 (■), or 4 (▲) freeze/thaw cycles before loading.

The observed shift of material from fraction 4 to fraction 1 is thus consistent with the formation of inside-out vesicles from right-side-out ones upon freeze/thawing (Figs. 2 and 3), as well as with the partitioning of inside-out vesicles to the interface + lower phase (Fig. 4). The yield of inside-out vesicles (fraction 1) after four freeze/thaw cycles was 15 to 25% of total plasma membrane protein, corresponding to 5 to 10 mg of protein from 500 g of leaves.
We have earlier (34) suggested that inside-out plasma membrane vesicles partition to the interface + lower phase based on the dual distribution of plasma membrane markers, and in analogy with inside-out erythrocyte membranes. This has now been confirmed by the separation of inside-out and right-side-out vesicles by phase partitioning (21) and the present work. Thus, in addition to the fact that the plasma membrane vesicles formed on homogenization of the plant material are usually mainly right-side-out, this is yet another reason that plasma membrane preparations obtained by two-phase partitioning contain mainly right-side-out vesicles; the inside-out vesicles are simply lost during the purification procedure. However, there seem to be some exceptions. For example, freshly prepared plasma membranes from oat roots show only about 70% latency of the ATPase activity (19), and sometimes even less, and they also support high rates of H+ pumping (M Palmgren, unpublished results). Thus, there may be cases where the phase composition used for the original purification does not resolve inside-out and right-side-out vesicles. With the plasma membranes from sugar beet leaves a lower polymer concentration can be used for the separation of inside-out and right-side-out vesicles than is needed to separate right-side-out plasma membranes from intracellular membranes (6.2 and 6.5% [w/w], respectively, in otherwise identical phase systems), and with many materials identical phase systems should do. However, with some materials, such as oat root, a much higher polymer concentration (or Cl− concentration [18, 20]) may be needed, and optimal conditions should be determined for each material as demonstrated in Figure 4.

**Determination of Sidedness**

To determine the proportions of inside-out and right-side-out vesicles in fractions 1 to 4 (see Figs. 1 and 5), the activity of markers for the cytoplasmic surface (ATPase, 1,3-β-glucan synthase, NADH-ferricyanide reductase and NADH-Cyt c reductase; see 33 for a detailed discussion for sidedness) were assayed ± detergent (Fig. 6). Nonlatent activities (markers for inside-out vesicles) were enriched in fraction 1, whereas the activities in fractions 3 and 4 were highly latent, indicating a high proportion of right-side-out vesicles. Intermediate values were found for fraction 2. H+ pumping, a more definite marker for sealed, inside-out vesicles, correlated well with the nonlatent ATPase activity (Fig. 6, top, left). However, latent activities were also observed in fraction 1, and for the ATPase this indicated a contamination by about 40% right-side-out vesicles.

The use of enzyme latency to assess vesicle orientation assumes that the detergents used do not have any other effect than to permeabilize the vesicles. That this assumption is not entirely valid is illustrated by Figure 7 (top). Thus, when the ATPase activity was assayed in another medium than that used in Figure 6 (see legends) no latent activity was observed in fraction 1. Rather, the Triton X-100 concentration optimal to reveal the latent activity in fractions 3 and 4 (0.025% [w/v]) inhibited the activity in fraction 1 with about 30%. In addition, the latencies obtained with fractions 3 and 4 were lower indicating that also the latent activities were inhibited (cf. Figs. 6 and 7). That the vesicles were permeabilized by the detergent is shown by the collapse of the H+ pump (Fig. 7, bottom), which was complete already at 0.015% (w/v) Triton X-100. Thus, the vesicles were permeable to H+ at a slightly lower detergent concentration than that needed to permeabilize them to MgATP. This is consistent with the differences in size and charge of the two species. The latencies obtained with fractions 1 and 4 are summarized in Table 1. The disagreements are obvious, particularly for fraction 1, and suggests that the detergents used (Triton X-100 and digitonin) may be either slightly stimulatory or inhibitory depending on the activity investigated and the assay conditions used. To find an ideal detergent for determination of enzyme latency we have recently screened a large number of detergents regarding their effect on the ATPase activity in the H+-ATPase assay (26). From this investigation Brij 58 seems to be ideal, since it neither inhibits nor stimulates the ATPase activity, and a concentration of 0.01% (w/v) can be used routinely with protein concentrations up to 50 μg mL−1. Using this detergent a latency of about 20% was obtained with the ATPase in fraction 1, whereas freshly prepared plasma membranes showed a latency of about 90% (26).

To confirm that nonlatent activities were due to inside-out vesicles and latent activities due to right-side-out vesicles we used trypsin digestion of NADH-Cyt c reductase activity; the rationale being that the activity associated with inside-out vesicles would be abolished, whereas the activity of right-side-out vesicles would be revealed by a subsequent addition of trypsin inhibitor and Triton X-100. The nonlatent NADH-Cyt c reductase activity was almost totally inhibited by trypsin in both fractions 1 and 4, whereas most of the latent activity...
contamination of about 20% is indicated between these pumping the vesicles (3), whereas vesicles in different cuvette (25) were determined with fractions (cf. Fig. 6 and Table I).

Table I. Percent Latencies of Enzyme Markers for the Cytoplasmic Surface of the Plasma Membrane

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fraction 1 (%)</th>
<th>Fraction 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase (assay medium a)</td>
<td>41 ± 4 (n = 3)</td>
<td>87 ± 6 (n = 3)</td>
</tr>
<tr>
<td>ATPase (H⁺-ATPase medium)</td>
<td>-45 ± 11 (n = 4)</td>
<td>54 ± 4 (n = 2)</td>
</tr>
<tr>
<td>1.3-β-Glucan synthase</td>
<td>51 ± 7 (n = 6)</td>
<td>85 ± 2 (n = 4)</td>
</tr>
<tr>
<td>NADH-FeCN reductase</td>
<td>34 ± 6 (n = 16)</td>
<td>85 ± 6 (n = 16)</td>
</tr>
<tr>
<td>NADH-Cyt c reductase</td>
<td>26 ± 6 (n = 8)</td>
<td>76 ± 6 (n = 8)</td>
</tr>
</tbody>
</table>

remained although the levels of remaining activity were widely different (Fig. 8), confirming the assumptions above. Since NADH-Cyt c reductase is inhibited by Triton X-100 above the 0.015% (w/v) optimal for determination of latent activity (3), whereas this concentration is probably not sufficient to permeabilize the vesicles to Cyt c (cf. Fig. 7), the remaining latent activities would be underestimates of the proportions of right-side-out vesicles. Taking this into account, a cross contamination of about 20% is indicated by this experiment.

Another, relative measure of the proportions of inside-out vesicles in fractions 1 and 4 is given by the ratio of H⁺ pumping between these two fractions. This ratio was 5.0 ± 0.4 (n = 5) (cf. Figs. 6 and 7) which fits well with a cross contamination of 20%.

Characterization of the H⁺-ATPase Activity

Both the H⁺ pumping and the ATPase activity of the inside-out plasma membrane vesicles (fraction 1) were completely inhibited by vanadate (Ki ≈ 10 μM at 100 μg protein mL⁻¹; Fig. 9), an inhibitor of the plasma membrane H⁺-ATPase (10, 31). Inhibition of the ATPase activity lagged slightly behind at higher vanadate concentrations. This is consistent with the presence of also a Ca²⁺-ATPase in the plant plasma membrane (12, 28); an activity which is also inhibited by vanadate but with a higher Ki (12) and which constitutes only a minor proportion of total plasma membrane ATPase activity (12, 28, 29). Molybdate, which inhibits vanadate-sensitive acid phosphatases (10, 29), and azide, which inhibits mitochondrial ATPase (10, 29) did not affect ATPase activity in fraction 1 (data not shown). In the presence of K⁺, H⁺ pumping was stimulated by the K⁺ ionophore valinomycin, and the H⁺ gradient was collapsed by nigericin (0.1 μg mL⁻¹; data not shown), which catalyzes the electroneutral exchange of H⁺ for K⁺ (27). These properties are consistent with previous findings that inside-out, plant plasma membrane vesicles accumulate H⁺ through an electroneutral H⁺-ATPase, which is sensitive to vanadate but not to molybdate or azide (reviews, 29, 31).
Addition of BSA (fatty acid free) to the assay medium often more than doubles the rate of H⁺ accumulation leaving the ATPase activity essentially unaffected (26). To measure maximum H⁺ pumping capacity, BSA was therefore always included in the assay medium. The effect of BSA is to bind fatty acids which may act as uncouplers. The fatty acids are probably produced by endogenous phospholipase activity, which may explain the often observed ‘leakiness’ of plasma membranes, particularly on ageing (26).

**Polypeptide Pattern**

The polypeptide patterns of inside-out (fraction 1) and right-side-out (fraction 4) plasma membrane vesicles are close to identical (Fig. 10). This was expected, since the inside-out vesicles were formed from the right-side-out ones (Figs. 2, 3, and 5) and the main difference should be their sidedness. However, some minor differences are evident.

The inside-out vesicles are depleted in some polypeptides (of 82, 80, 57, 34, 32, and 14.5 kD), which are found in the freeze/thaw supernatant. These polypeptides probably represent soluble proteins trapped within right-side-out vesicles during homogenization of the leaves, although it can not be excluded that some are peripheral plasma membrane proteins. The 57 and 14.5 kD polypeptides (which dominate in the supernatant) are most probably the large and small subunit, respectively, of ribulose-1,5-bisphosphate carboxylase/oxygenase, the most abundant soluble protein in leaves.

Three polypeptides (of 73, 44, and 20 kD) are clearly enriched in the inside-out vesicles. This may reflect some heterogeneity within the isolated plasma membrane population. For instance, in vivo there may be a lateral heterogeneity in the plasma membrane, which upon homogenization of the tissue gives rise to vesicles of slightly different composition. There may also be a difference between plasma membrane vesicles derived from different cell types in the tissue. Thus, the polypeptide patterns of plasma membranes obtained from barley leaves and roots show some minor differences (16). As soon as a heterogeneity is present, this could affect both the probability of vesicle breakage upon freeze/thawing and the probability to form an inside-out vesicle upon revesiculation, and thus give rise to the observed differences in polypeptide pattern. However, we do not observe a difference with all materials, and the polypeptide patterns of fractions 1 and 4 from cauliflower inflorescences were indistinguishable (data not shown).

**CONCLUDING REMARKS**

The rationale of the present work was to use the pure right-side-out plasma membrane vesicles readily obtained by two-
phase partitioning as the starting material for inside-out vesicles. In this way, preparations of plasma membrane vesicles of both orientations are obtained which are essentially free of contaminating membranes.

It should be noted, that if only inside-out vesicles are to be prepared (e.g. for studies on H⁺ pumping) a more direct procedure may be used: The lower phase of the phase system containing freeze/thawed plasma membranes may simply be extracted with a number of fresh upper phases leaving the inside-out vesicles enriched in the lower phase + interface. Similarly, if only right-side-out vesicles are needed, the original plasma membrane preparation is usually more enriched in these vesicles than the fraction of right-side-out vesicles obtained with the present procedure. However, for a direct comparison of the properties of inside-out and right-side-out plasma membrane vesicles, a comparison of the fractions obtained after freeze/thawing and subsequent phase partitioning is probably more valid, since these fractions have been subjected to identical treatments.

To accurately determine the proportions of inside-out and right-side-out vesicles in different fractions has been a constant problem. This seems to be solved by using the detergent Brij 58 for determination of ATPase latency (26). A more rapid and convenient assay for sidedness is to determine the latency of the NADH-ferricyanide reductase using Triton X-100, which, however, gives an underestimation of the percentage of inside-out vesicles.

During the development of the present procedure it turned out to be very important to include a number of protective agents (DTT, EDTA, BSA, casein, PMSF, insoluble PVP) in the different steps to retain high activities (data not shown). In particular, the inside-out vesicles easily lost activity. It seems that the exposure to the medium of active sites located on the cytoplasmic surface makes these sites very susceptible to inactivation compared to the sites hidden inside right-side-out vesicles. This was particularly pronounced for the ATPase, for H⁺ pumping and for the 1,3-β-glucan synthase. For H⁺ pumping, lost activity could be restored by addition of fatty acid free BSA which binds released fatty acids (26).

Taken together, our results indicate that preparations of about 80% inside-out and 80% right-side-out plasma membrane vesicles, respectively, are obtained with the present procedure. Thus, preparations ideal for studies on plasma membrane transport, signal transduction mechanisms, enzyme topology, etc., are now available. Using these fractions, the donor and acceptor sites of the plasma membrane-bound NADH-acceptor oxidoreductase have recently been localized to the cytoplasmic surface (3), and so have the activator sites for Ca²⁺, spermine and cellulbiose of the 1,3-β-glucan synthase (9).

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LITERATURE CITED


