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ISOLATION AND ASSAY OF CORN ROOT MEMBRANE VESICLES WITH REDUCED PROTON PERMEABILITY

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Conditions promoting the formation of sealed membrane vesicles from corn roots with reduced proton permeability were examined using the probe 9-aminoacridine as a rapid indicator of pH gradient formation and dissipation. Plasma membrane vesicles isolated by differential and density gradient centrifugation were leaky to protons and rapidly equilibrated when exposed to artificially imposed pH gradients. The leaky plasma membrane vesicles showed reduced proton permeability when incubated with calcium or with excess phospholipids. However, these vesicles were unable to form ATP-induced pH gradients. Sealed vesicles isolated by discontinuous Ficoll gradient centrifugation of a microsomal fraction displayed reduced proton permeability and were osmotically active. In contrast to purified plasma membrane vesicles, the microsomal-derived vesicles were more suitable for studies of active proton transport.

Introduction

The plasma membrane of higher plant cells has been postulated to contain an electrogenic proton pump [1–4]. Accumulated biochemical evidence indicates that Mg^{2+}-dependent ATPase is associated with purified plasma membrane preparations [5–8] and this enzyme may serve as the plant cell counterpart to the $(Na^+ + K^+)$-ATPase of animal cell membranes [9].

Since the original finding that plant plasma membranes possessed ATP-hydrolysis activity [5], a substantial lag has existed between the biochemical characterization of this activity and the in vitro demonstration of its transport function. Recently, it has been possible to demonstrate ATP-induced pH [10–12] and electrical [10–12] gradient formation in membrane vesicle preparations enriched with Mg^{2+}-ATPase activity.

It was believed that the major difficulty hampering the detection of sustained ion gradients in plant plasma membrane fractions was the presence of unsealed vesicles [13]. Such leaky vesicles would make it difficult to detect active transport because the gradient would be rapidly dissipated by diffusion through the leakage pathway. For an accurate interpretation of transport phenomena, sealed membrane vesicles must be present. Sze [10] circumvented this problem by applying a technique used to isolate sealed vesicles from erythrocyte membranes [14]. This method relied on the differential permeability of a large dextran molecule to separate two populations of vesicles: a fraction in which the dextran could not penetrate (sealed) and a fraction which was leaky to dextran (unsealed). It was possible with the sealed popula-

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Abbreviations: Hepes, 4-(2-hydroxyethyl))-1-piperazineethanesulfonic acid; Mes, 4-morpholine-ethanesulfonic acid.

tion to demonstrate stimulation of ATP hydrolysis by proton ionophores. Furthermore, these vesicles were also capable of supporting ATP-induced pH and electrical gradients [11]. This isolation procedure, while satisfying the criterion for sealed vesicles, required the use of a microsomal fraction for vesicle preparation and was therefore limited by the ultimate percentage of plasma membranes comprising the final membrane vesicle population.

The formation of sealed vesicles is an essential step in isolating functionally active membranes. However, it is equally important to evaluate the passive proton permeability characteristics of plant cell plasma membranes, since these characteristics will ultimately limit both the rate and steady-state magnitude of the pH gradient formed.

This report expands on a prior communication [15] describing the use of 9-aminoacridine as a rapid indicator of relative proton permeability in plant membrane vesicles. The speed and sensitivity of this procedure permitted conditions promoting decreased passive proton permeability to be investigated.

Materials and Methods

Plasma membrane isolation. *Zea mays* L., cv. WF9-M017 was used for the isolation of corn root plasma membranes. Seeds were imbibed for 24 h, then germinated on wet paper towels in a plastic tray and kept in the dark at room temperature for 5 to 6 days. Seedlings were washed with tap water at day 3.

Corn root plasma membranes were isolated by a modification of the procedure described by Leonard and Hotchkiss [8]. Freshly cut corn roots (50 g) were chopped into a medium (150 ml) containing 0.25 M sucrose 3 mM Na_2EDTA, 2 mg/ml bovine serum albumin and 50 mM Tris-Mes, pH 8.0. The chopped roots were immediately ground with a cold mortar and pestle until a uniform brei was formed. The homogenate was filtered through Miracloth and the filtrate was centrifuged at $13000 \times g$ for 10 min. The brown mitochondrion-enriched pellet was discarded and the supernatant was centrifuged at $80000 \times g$ for 1 h to produce a microsomal pellet. The pellet was resuspended in 0.25 M sucrose and 10 mM Tris-Mes, pH 7.5 and layered above a 34% sucrose cushion. The membranes were centrifuged at $100000 \times g$ for 1 h, and a plasma membrane-enriched pellet was recovered. The plasma membranes were used immediately or frozen in liquid nitrogen for future use.

Fluorescence quenching and recovery measurements. Plasma membrane vesicles were suspended in a 1 ml medium containing 150 mM KCl, 0.5 mM Mes, pH 6.5, 5 μM 9-aminoacridine and 250 μg/ml membrane protein. Fluorescence quenching was initiated by the addition of enough base (NaOH) to change the external pH from pH 6.5 to 8.5. Both the final extent of fluorescence quenching (Q) and the initial rate of fluorescence recovery (IRR, expressed as the percent fluorescence recovery in the first 30 seconds) were determined. Total fluorescence was monitored in a Varian 634 spectrofluorometer, using an excitation wavelength of 422 nm. Emitted light was passed through a band pass filter with a window between approximately 460 and 650 nm.

Transport assays. Rapid filtration assays were modified slightly from the procedure described by Kaback [16]. A suspension of plasma membrane vesicles (greater than 21 mg/ml membrane protein) in 0.25 M sucrose and 5 mM Mes, pH 6.2, was added to an equal volume containing the same buffer but with the appropriate substrate and tracer (as indicated in the figure legends). At the indicated times, 100 μl of the reaction mixture was passed through a pre-wet, 0.45 μm millipore filter and washed with 3 ml of ice-cold buffer. The entire filtration procedure lasted 3–4 s.

Protein. Protein determinations were made using a modified Lowry procedure [17].

Electron microscopy. Isolated membrane fractions were fixed with 2.5% glutaraldehyde for 1 h in a medium containing 100 mM sodium cacodylate, pH 7.2 and 3 mM $CaCl_2$. The membranes were fixed in the presence of either 0.25 M or 1.0 M sucrose. The membranes were post-fixed with 1% OsO_4, 3 mM $CaCl_2$, 100 mM sodium cacodylate, pH 7.2 and sucrose at a concentration of either 0.25 M or 1.0 M. The vesicle suspension was washed as described above, dehydrated by sequential acetone additions, and infiltrated by dropwise addition of Spurr's resin [18]. The membrane pellets were thin sectioned and viewed under a Phillips EM300 electron microscope. Aver-

age diameters for vesicles fixed in either 0.25 M or 1.0 M sucrose were obtained by measuring 38 random vesicle diameters on each of 7 different negatives obtained at the indicated sucrose concentrations.

Results and Discussion

The quenching and subsequent recovery of 9-aminoacridine fluorescence in response to an applied interior acid pH gradient were used to monitor the apparent passive proton permeability of plasma membrane vesicles. The pH gradient probe 9-aminoacridine was chosen because it has been shown to measure the formation of interior acid pH gradients in isolated membrane vesicles rapidly and effectively [19–23]. Further, the observed fluorescence quenching can be related to the pH gradient by the simple equation [19,22]:

$$\Delta\text{pH} = \log[Q/(100-Q)] + \log(V_o/V_i) \qquad (1)$$

where Q is the measured percent quenching, V_i, V_o are the respective internal and external volumes

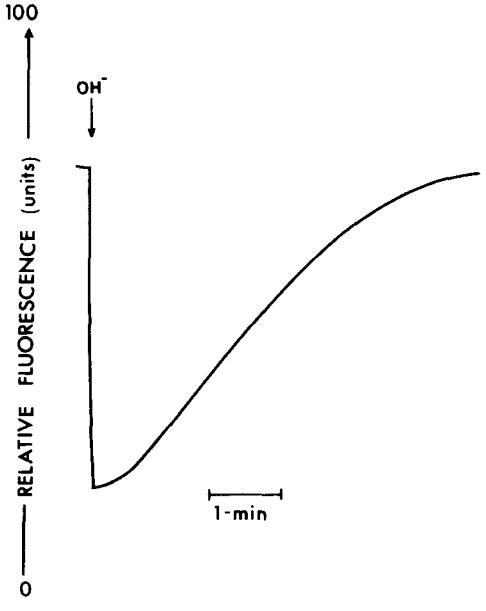

Fig. 1. Diagram depicting the fluorescence quenching and recovery of 9-aminoacridine in response to an applied pH gradient. Fluorescence quenching was initiated by the addition of base (NaOH) to a 1 ml suspension of plasma membranes suspended in 0.5 mM Mes, pH 6.5, 150 mM KCl and 5 μM 9-aminoacridine.

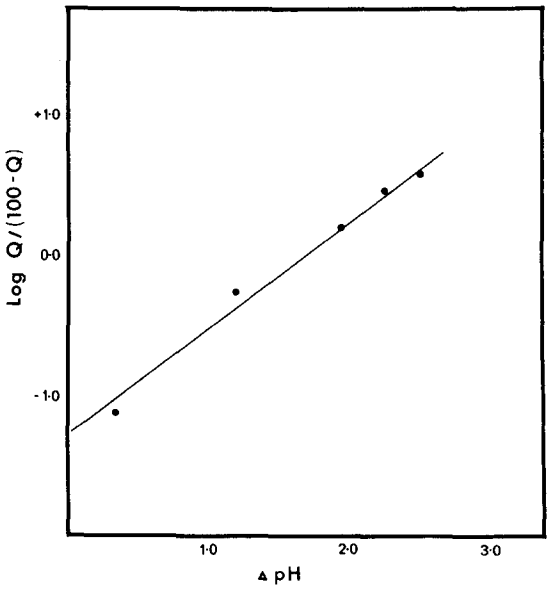

Fig. 2. The distribution of 9-aminoacridine molecules as a function of the pH gradient formed across plasma membrane vesicles. The fluorescence quenching of 9-aminoacridine $[Q/(100-Q)]$ was assumed to be equivalent to the distribution of protonated 9-aminoacridine molecules (see Eqn. 1). A 1 ml suspension of plasma membrane vesicles was pre-equilibrated in a medium containing 0.5 mM Mes, pH 6.0, 150 mM KCl, 5 μM 9-aminoacridine and 200 μg membrane protein. The external pH was raised by the addition of small quantities of NaOH to obtain the indicated pH gradients.

and $[Q/(100-Q]$ represents the effective distribution of amine cation between the internal and external volumes.

When corn root plasma membrane vesicles were subjected to a 2-unit pH gradient by raising the external pH, the result was an immediate quenching of fluorescence, followed by a rapid fluorescence recovery (Fig. 1). It appeared that protons rapidly equilibrated across the membrane, thereby causing a redistribution of 9-aminoacridine molecules and a restoration of relative fluorescence. It was presumed therefore that the vesicles were leaky to protons.

Since the fluorescence quenching and subsequent recovery in response to an imposed pH gradient was easily measured, this system could serve as an effective screening device for isolating membrane vesicles with reduced proton permeability. Such vesicles would be desirable for investigating active proton transport due to the electrogenic proton pump.

In order for 9-aminoacridine to serve as a reliable pH gradient probe, it was important to demonstrate that the cationic form of the molecule could not traverse plant plasma membranes. Since this was one of the basic assumptions used to derive eqn. 1, a plot of the pH gradient versus $\log[Q/(100-Q)]$ should be linear. Any deviation from linearity would indicate that the non-protonated monoamine was not the only permeant species. Fig. 2 indicates that such a plot produced a straight line and confirmed the validity of the assumption for this system.

In addition, it was demonstrated that the initial fluorescence (prior to an imposed pH gradient) could be completely recovered by rapid equilibration of the pH gradient with NH_4Cl or with nigericin plus potassium. These results suggest that fluorescence quenching was the result of 9-aminoacridine molecules redistributing across the vesicle membranes in response to a pH gradient. Thus, 9-aminoacridine behaved as a reliable pH gradient probe in accordance with its behavior in other systems [20,22,24].

The fluorescence quenching and recovery procedure rapidly provided useful information about proton permeability and it enabled conditions to be determined which reduced the proton leak of plasma membrane vesicles. Relative proton permeability was related to the initial rate of fluorescence recovery (IRR) and was expressed as the percent recovery of quench in the first 30 s. Furthermore, it was predicted from Eqn. 1 that if the external volume was held constant and a uniform pH gradient was applied to different samples from the same vesicle population, then changes in fluorescence quenching would reflect differences in internal volume. Both parameters, the IRR and total quench, were monitored for each group of membranes in response to a constant pH gradient.

Decreasing proton leaks

1. Sucrose gradient-derived vesicles

It was possible that membrane leakiness was a result of stripping away divalent cations in the isolation procedure (the divalent cation chelator, EDTA, was used for vesicle preparation). Since divalent cations are important for maintaining membrane structure [25], their role in restoring membrane integrity was determined. The addition of calcium to isolated vesicles above 1 mM caused a significant decrease in the relative proton permeability (Fig. 3). Magnesium and manganese could substitute for calcium, but only at higher concentrations. Vesicle volume was unaffected by the divalent cation treatments.

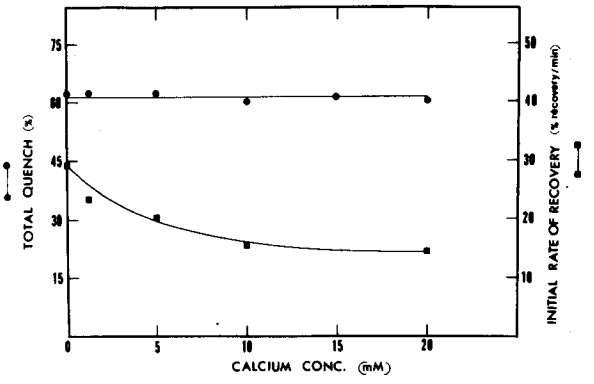

Fig. 3. The effect of calcium on fluorescence quenching and recovery in plasma membrane vesicles. Plasma membrane vesicles were suspended in a 1 ml volume containing 0.5 mM Mes, pH 6.5, 150 mM KCl, 5 μM 9-aminoacridine and 200 μg membrane protein. The vesicles were incubated with $CaCl_2$ at the indicated concentrations for 1 h at 4°C. Fluorescence quenching and recovery was initiated by the addition of NaOH.

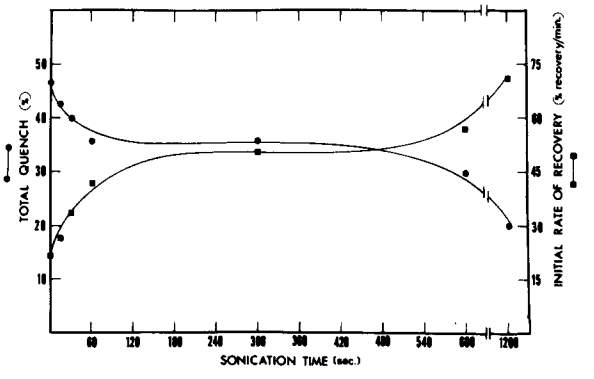

Fig. 4. The influence of sonication time on the fluorescence quenching and recovery of plasma membrane vesicles. Plasma membrane vesicles suspended in 5 mM Mes, pH 6.2 and 150 mM KCl were sonicated in a bath-type sonifier under nitrogen. 100-μl aliquots consisting of 100 μg membrane protein were removed at the indicated times and added to 0.9 ml containing 150 mM KCl and 5 μM 9-aminoacridine. Fluorescence quenching was initiated by the addition of NaOH.

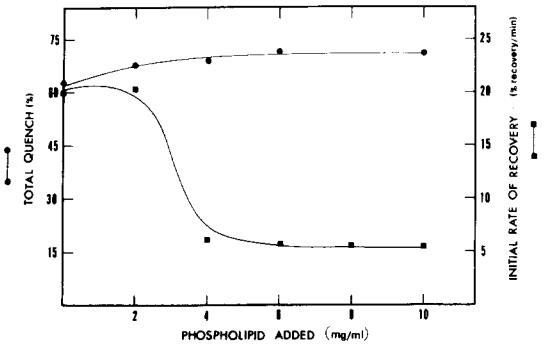

Fig. 5. The effect of excess phospholipids on fluorescence quenching and recovery of plasma membrane vesicles. Sonicated plasma membrane vesicles (1 mg/ml protein) were incubated in the presence of sonicated phospholipids (at the indicated concentration), 1 mM $CaCl_2$ and 5 mM Hepes, pH 8.3. The suspension was sonicated for 1 min under nitrogen at 37°C, and then placed on ice for 10 min. The vesicle suspension was layered on a 5% Ficoll cushion and centrifuged at $100000 \times g$ for 1 h. This procedure separated impermeable liposomes from plasma membranes. The vesicle pellets were resuspended in 0.5 mM Mes, pH 6.5, 150 mM KCl and adjusted for equal protein. Prior to fluorescence quenching and recovery assays, the vesicles were sonicated for 5–10 s. Fluorescence quenching was initiated by adding NaOH to a membrane suspension containing 0.5 mM Mes, pH 6.5, 150 mM KCl, 5 μM 9-aminoacridine and 100 μg/ml membrane protein.

Membrane vesicles were sonicated to determine whether a reduction in vesicle size would result in the production of tight vesicles. The effect of sonication was to cause an apparent increase in the rate of proton leakage and decrease the relative vesicle volume (Fig. 4). However, since proton equilibration is a passive diffusion process, smaller vesicles with the same leak as large vesicles would show faster equilibration times.

The most effective way to isolate a population of vesicles with low proton permeability was to incubate membranes with excess phospholipids (Fig. 5). It was expected that fusion of liposomes (possessing relatively low proton permeabilities) with membranes would produce vesicles with reduced proton leaks. Thus, conditions which promoted membrane fusion (i.e., high external pH and high calcium concentration) did also reduce the apparent proton leak of plasma membrane vesicles.

The basic protocol called for the addition of sonicated phospholipids (crude soybean lipids) to sonicated plasma membranes and incubation with 1.0 mM calcium at 37°C. The membrane suspension was sonicated and then centrifuged through a 5% Ficoll cushion to separate liposomes from plasma membranes (plasma membranes were pelleted). Sonicated membranes increased the effectiveness of the fusion process, possibly by increasing the curvature of vesicles which promoted a necessary close contact of membrane lipids needed for fusion [26]. Calcium was maintained at 1 mM, since higher concentrations resulted in significant membrane aggregation.

The sharp decline of IRR in Fig. 5 could be shifted to the right by increasing the amount of plasma membrane lipids. In order to ensure separation of proton-impermeable liposomes from plasma membrane vesicles, the denser plasma membrane vesicles were pelleted through a 5% Ficoll pad. The separation was confirmed by labeling the liposomes with fluorescamine [27]. Greater than 95% of the fluorescence was recovered above the Ficoll layer, but a small amount penetrated the pad. The small recovery of fluorescence in the pellet probably indicated liposome fusion with plasma membranes. The slight increase in internal volume, as determined by an increase in total quenching, also supported this assumption.

The use of divalent cations and elevated temperature to produce vesicles with low proton leaks was contrary to the conditions necessary to produce sealed erythrocyte plasma membrane vesicles. It was more consistent with the protocol used to reseal erythrocyte ghosts [14]. However, the decreased proton permeability reported here probably resulted from membrane fusion, since it could not be accounted for by either calcium or elevated temperature effects alone. In fact, elevated temperature significantly increased the rate of proton equilibration in plasma membrane vesicles (data not shown).

The formation of mixed membrane vesicles by fusing liposomes with plasma membranes was also similar to the freeze-thaw-sonication reconstitution method developed for the incorporation of transport proteins into liposomes with larger internal volumes [28]. This technique used a freeze-thaw cycle to promote membrane fusion and thus eliminated the need for calcium and alkaline pH.

2. Ficoll-gradient-derived vesicles

The relative proton permeability of the plasma membrane vesicles was reduced significantly by the addition of excess lipid (Fig. 5) but the process required small sonicated vesicles which were undesirable for transport studies, since they were not well trapped by the filters in our rapid filtration system. Furthermore, these vesicles failed to form ATP-induced pH gradients, as monitored by 9-aminoacridine fluorescence quenching.

Recently, Sze [10] applied a technique designed for separating sealed erthrocyte vesicles from unsealed vesicles [14] to tobacco suspension culture membranes. This procedure relied on dextran gradient centrifugation and separated sealed and unsealed vesicles on the basis of their different buoyant properties. This assumed that osmotically inactive dextran molecules could not penetrate sealed membrane vesicles but could enter unsealed vesicles. The application of this principle has also been successfully used to isolate impermeable vesicles from gastric mucosa [21].

This technique was applied to corn root membranes by layering a microsomal fraction (80000 $\times g$ pellet) above a discontinuous Ficoll gradient (5% and 10% Ficoll) and centrifuging. The result was a suspension containing three main membrane regions: the region above the 5% Ficoll, the 5/10% Ficoll interface and a large pellet. A membrane junction enriched with Mg^{2+}-dependent ATPase activity was removed from the 5/10% interface and assayed.

The membrane vesicles removed from the Ficoll interface had a very low passive permeability relative to those which penetrated the 10% Ficoll layer (Table I). There was, however, a measurable proton leak and it was important to confirm whether the vesicles were actually sealed.

It was reasoned that if the membrane vesicles were osmotically active this would provide evidence for the presence of sealed vesicles. Light scattering was used to measure differences in vesicle volume in response to applied osmolarities. This analysis indicated that decreasing osmolarity increased the magnitude of 90° light scattering (Fig. 6). It was not possible to determine whether the vesicles behaved as true osmometers and

TABLE I

PROTON EQUILIBRATION OF FICOLL GRADIENT-DERIVED MEMBRANE VESICLES

A microsomal pellet prepared as described in the Materials and Methods was resuspended in 10 mM Mes, pH 6.5 and 0.25 M sucrose. The membranes were layered on a discontinuous Ficoll gradient consisting of 5 and 10% Ficoll 400, prepared in a buffer containing 0.25 M sucrose and 5 mM Mes, pH 6.5. The gradients were centrifuged at 60000× g for 2 h and membranes were removed from both the 5/10% interface and the pellet. Both sets of membranes were washed by centrifugation in a medium containing 0.25 M sucrose and 0.5 mM Mes, pH 6.5. The membranes were resuspended in the same medium, adjusted for equal protein and assayed for pH gradient-induced fluorescence quenching and recovery, as described in Fig. 1. The initial rate of recovery (IRR) was expressed as the percent quench recovery in 1 min. The values indicate duplicate assays.

Fraction	Total quench (%)	IRR (%Q/min)	$T_{1/2}$ (min)
5/10% interface	66	8.3	6.02
Pellet	66	23.8	2.10

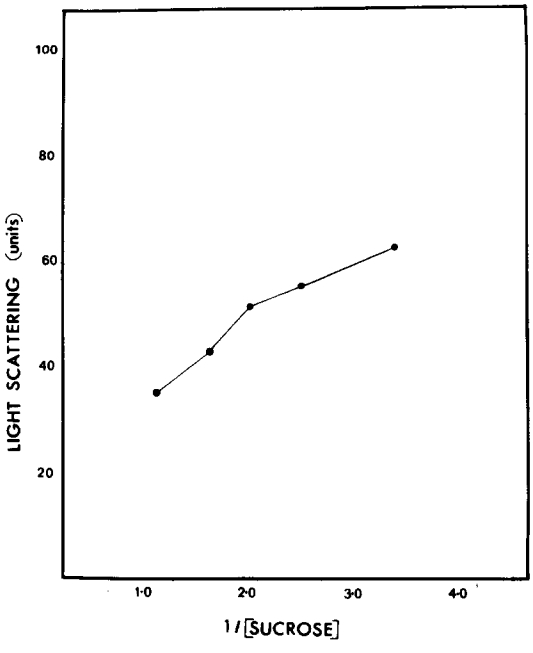

Fig. 6. The effect of external osmolarity on the light scattering properties of plasma membrane vesicles. A suspension of plasma membrane vesicles in 5.0 mM Mes, pH 6.5 and 200 μg/ml membrane protein were incubated at room temperature for 1 h at the indicated external sucrose concentrations. The light scattering properties of vesicles were determined by right angle scattering at a wavelength of 550 nm.

conformed to a Boyle-Van't Hoff relationship. It may, however, be assumed that differences in light scattering were the result of volume changes [29].

Electron microscopy was used to demonstrate that size differences could be quantified when vesicles were suspended in different osmolarities. Membranes were fixed in the presence of either 0.25 M or 1.0 M sucrose and then thin-sectioned for electron microscopy. The average diameter for vesicles suspended in 0.25 M sucrose was 0.376 ± 0.143 μm, while vesicles suspended in 1.0 M sucrose had an average size diameter of 0.242 ± 0.104 μm.

If the vesicles obeyed a Boyle-Van't Hoff relationship, then volume changes would be proportional to changes in the external osmolarity. Thus, it was predicted that vesicles suspended in 0.25 M sucrose would have 4 times the volume of vesicles suspended in 1.0 M sucrose. Since the ratio of volumes varies as the $(\text{radius})^3$, such a ratio could be obtained from the average diameter of vesicles determined by electron microscopy (assuming the vesicles were predominantly spherical). The vesicles suspended in 0.25 M sucrose occupied 3.75 times the volume of vesicles suspended in 1.0 M sucrose. This result indicates that the vesicles were osmotically active. It was further presumed that the osmotically active vesicles were sealed.

Transport studies with sealed membrane vesicles

The transport properties of the Ficoll-derived vesicles were analyzed by measuring a potassium permeability coefficient and a potassium- and valinomycin-induced diffusion potential.

Passive potassium permeability was determined by rapid filtration (see Methods) using ^{86}Rb as a potassium analog. The uptake of ^{86}Rb reached saturation in 25 min (Fig. 7) and the half-time for equilibration of this ion was 3.98 ± 0.08 ($n = 3$) min.

A permeability coefficient of $6.97 \cdot 10^{-7}$ cm/s was determined for these vesicles [30]. This value was within the range previously described for oat root and tobacco suspension culture plasma membrane vesicles [10,13] and similar to that for membrane vesicles from shoots of *Suaeda maritima* [31]. However, since the vesicle population was not completely homogeneous (i.e., other intracellular membranes were present in small amounts), the indicated permeability coefficient does not solely reflect the plasma membrane.

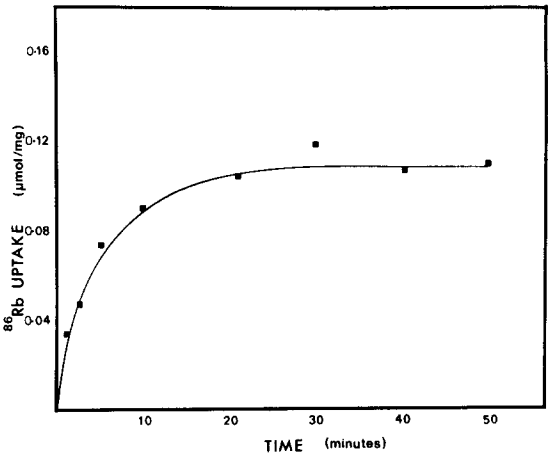

Fig. 7. Time-course for the uptake of ^{86}Rb into sealed membrane vesicles. Plasma membrane vesicles were added to a 1 ml medium containing 0.25 M sucrose, 1 mM KCl, 5 mM Mes, pH 6.2, ^{86}Rb (0.5 μCi) and 2 mg/ml membrane protein. Uptake was determined (0.5–50 min) after the vesicle addition by rapidly filtering 100 μl of the above medium at the indicated times and washing with 5 ml of ice-cold 0.25 M sucrose in 5 mM Mes buffer, pH 6.2. Each time point represents the average of three replicates.

The permeant anion ^{35}SCN has been used to detect interior positive membrane potentials [32].

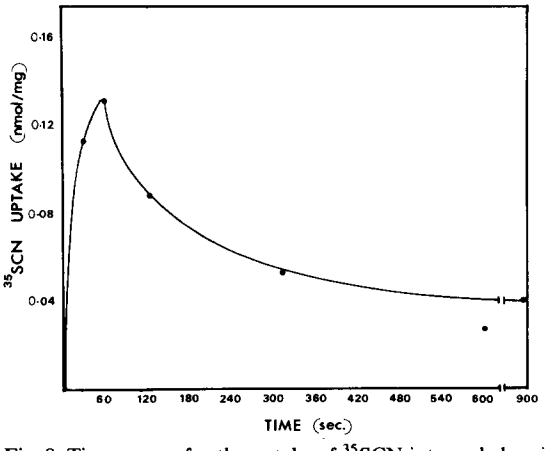

Fig. 8. Time-course for the uptake of ^{35}SCN into sealed vesicles following an applied interior positive membrane potential. Plasma membrane vesicles suspended in 0.25 M sucrose were diluted into a 1 ml medium containing 0.25 M sucrose, 10 mM KCl, 5 μg valinomycin, 2 μM ^{35}SCN (0.4 μCi), 5 mM Mes, pH 6.2. The final membrane protein concentration was 2 mg/ml. Uptake was determined (30–900 s) after vesicle additon by rapid filtration. Each time point represents the average of three replicates.

It was of interest to demonstrate that ^{35}SCN acts as a reliable potential probe with corn root membrane vesicles. Uptake of ^{35}SCN was determined by rapid filtration without washing. This ensured that any ^{35}SCN trapped by the vesicles would not be released during the wash phase.

The addition of valinomycin and potassium to membrane vesicles resulted in the generation of an interior positive membrane potential as indicated by an immediate uptake of label (Fig. 8). ^{35}SCN eventually leaked out to a diffusional level once the potential was dissipated. Thus, ^{35}SCN appeared to be an effective membrane potential probe. These results also support the earlier conclusion that the membrane vesicles must be sealed.

Conclusion

The pH gradient probe 9-aminoacridine was used as an effective indicator of relative proton permeability in vesicles derived from the same membrane population. It was determined that membrane vesicles isolated by differential and density gradient centrifugation rapidly equilibrated artificially induced pH gradients. The vesicles could be made less leaky to protons by incubation with divalent cations or by the addition of excess phospholipids. Unfortunately, these plasma membrane-enriched vesicles failed to form ATP-induced pH gradients. It was uncertain whether this was due to the relatively high proton permeability still apparent in these vesicles or to an alteration of the ATPase during the incubation periods.

An alternative procedure for preparing Mg^{2+}-ATPase-enriched vesicles using a microsomal membrane fraction and Ficoll gradient centrifugation, also resulted in the recovery of vesicles with reduced proton permeability. However, in contrast to the sucrose-gradient derived plasma membrane vesicles, these microsomal-derived vesicles were capable of sustaining ATP-induced pH gradients (Bennett, A., personal communication). In fact, vesicles isolated by a similar procedure involving dextran gradient centrifugation readily form ATP-induced pH [12] and electrical gradients [11]. These results suggest that vesicles active in electrochemical proton gradient formation can be recovered from microsomal membrane fractions by the use of flotation gradients.

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