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Characterization of Passive Ion Transport in Plasma Membrane Vesicles of Oat Roots[1]

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ABSTRACT

The passive influx and efflux of inorganic ions across plasma membrane vesicles purified from extracts of *Avena sativa* roots were investigated. Uptake was measured by incubating the vesicles in a radioisotope for various times. The "loaded" vesicles were separated from the external solution by gel filtration. Efflux was measured by dialyzing the preloaded vesicles.

Ion transport was differentiated from superficial ion binding by (*a*) the time course of association of radioisotope with the vesicles; (*b*) the rate of loss of radioisotope from the vesicles; (*c*) the linear increase in isotope associated with the vesicles as the external concentration was increased; (*d*) the enhanced loss of radioisotope from the vesicles induced by Triton X-100; and (*e*) the low amount of isotope associated with the vesicles at low temperatures.

The plasma membrane vesicles were differentially permeable to the alkali cations with the order of decreasing permeation being $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. The relative transport of Rb^+, Na^+, and Cl^- across the plasma membrane vesicles was about 1.0:0.50:0.18. The permeability coefficient (P) for Rb^+ was estimated to be $0.29 \pm 0.15 \times 10^{-8}$ cm/sec.

ATP (and ADP) decreased the passive uptake of Rb^+ into the vesicles, however, this effect did not appear to be related to the ATPase of the plasma membrane.

Elucidation of the mechanism of ion transport across the plasma membrane and tonoplast of plant cells is complicated by the simultaneous transport of ions across both of these membranes as well as by the confounding influences of other aspects of metabolism. For these reasons, it would be desirable to investigate the transport of ions across a single type of membrane vesicle. This can be accomplished only by using isolated membrane vesicles. Such studies have been conducted with plasma membrane vesicles isolated from bacteria (11) and animal cells (2, 3), but similar studies have not been reported for specific plant membranes. However, there are reports concerned with ion fluxes in a mixture of membrane vesicles obtained from *Lemna* (21) and cotyledons of *Phaseolus* (12). The latter study was of particular interest since it was found that ATP had an effect on the K^+ content of the vesicles. However, it was not shown whether the effect of ATP was specific and whether it involved a physical effect or an energy transduction.

We have previously reported on the purification of plasma membrane vesicles from oat roots (9, 10), and in this paper we present data concerning the passive fluxes of ions into and out of these vesicles.

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MATERIALS AND METHODS

The procedure for the preparation of plasma membrane vesicles from roots of oat (*Avena sativa* L. cv. Goodfield) has been described (9).

Ion Uptake. Ion uptake into the vesicular space was determined by incubating the vesicles (200–400 μg protein) in 0.5 ml of reaction medium containing 17 mM tris-MES buffer, pH 6.5, 5 mM $MgSO_4$, 1 to 100 mM of an alkali-chloride with one of the ions being radioactive ($1–3 \times 10^6$ cpm/μmol), and 8% sucrose to maintain a stable osmolarity. Li^+ was determined with an atomic absorption spectrophotometer. Reactions were usually conducted at 30 C for 10 to 30 min. Uptake was stopped by passing the reaction mixture through a cold (6 C) Sephadex G-25 coarse column (1 × 20 cm) equilibrated with 5 mM tris-MES buffer, pH 6.5, and 5 mM $MgSO_4$. The eluting solution was the same as the equilibrating solution. Eluates were collected in 1-ml fractions and measured for radioactivity (4) and protein (14). Net ion uptake into the vesicles was expressed as nmol of ions retained/mg protein. Results presented are averages of at least two experiments.

Ion Efflux. The loss of ions from preloaded vesicles was investigated by measuring the diffusion of ions from a dialysis bag containing the preloaded vesicles. About 1.5 to 2 ml (approximately 100–200 μg protein/ml) of preloaded vesicles, collected as described above, were immediately placed into a dialysis bag (8 × 100 mm), and samples were removed for determination of total radioactivity and protein. The dialysis bag was placed in a test tube with 15 ml of 5 mM tris-MES buffer, pH 6.5, and 5 mM $MgSO_4$. The solution was stirred vigorously by a magnetic stirrer. At various time intervals, the bag was removed and placed into a fresh buffer solution. An aliquot of the dialysate was analyzed for radioactivity. Efflux experiments were conducted at room temperature (23 C), unless otherwise stated. Ion loss was expressed as a percentage of ions lost from the dialysis bag as a function of time.

RESULTS

Uptake and Loss of $^{86}Rb^+$. Plasma membrane vesicles were first incubated in a reaction mixture containing 1 mM RbCl (^{86}Rb), and after 30 min at 30 C, the entire reaction mixture was passed through a Sephadex G-25 column (Fig. 1). The vesicles eluted in the void volume, and a peak of radioactivity coincided with the protein peak. The free (external) radioactivity appeared just prior to the complete elution of the protein. As a routine practice, the first 10 tubes were collected, and both radioactivity and protein were determined. In order to exclude any contamination by the free radioactivity, only the samples containing similar specific radioactivities (*i.e.* cpm/mg protein) were used for the expression of results in subsequent experiments.

The association of $^{86}Rb^+$ with the peak of protein appeared to represent an entry of ions into the vesicular space as contrasted to a surface binding since the time required for an apparent equilibrium was quite long (Fig. 2), and the amount of radioac-

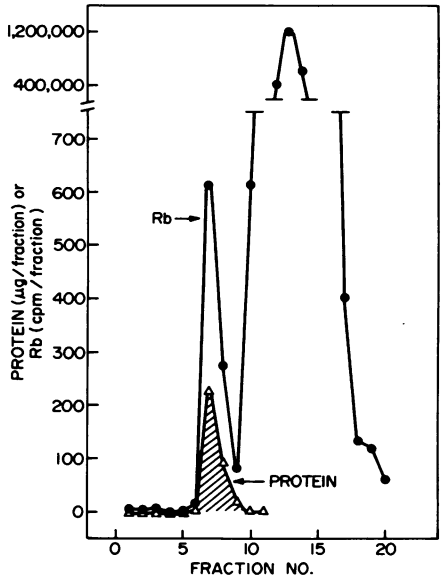

FIG. 1. Elution profile of the separation of plasma membrane vesicles from free radioactive $^{86}Rb^+$ using a Sephadex G-25 column. Shaded area indicates the amount of membrane vesicles expressed as μg protein.

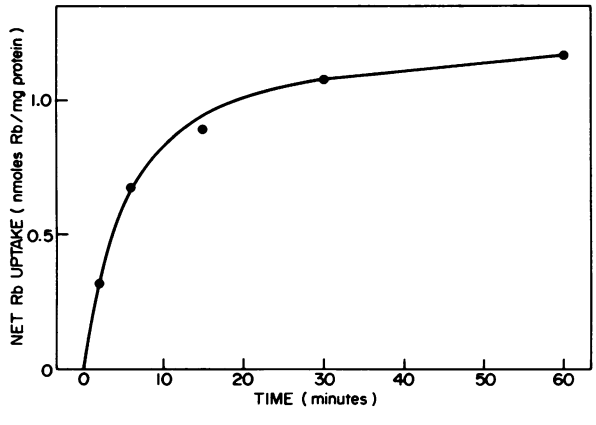

FIG. 2. Time course of Rb^+ uptake into plasma membrane vesicles. Reaction medium consisted of 17 mM tris-MES, pH 6.5, 5 mM $MgSO_4$, 8% sucrose, and 1 mM RbCl.

tivity associated with the vesicles increased linearly as the external concentration was increased (Fig. 3). If surface binding was responsible for the associated radioactivity, the reaction would have been completed in a few seconds and the sites should have become saturated as the external concentration was raised. Thus, we will refer to the radioactivity associated with the vesicles as a net uptake, since the vesicles were originally devoid of the ions. The basis for the term net is that not all of the ions that entered stayed in, *i.e.* ion loss was also occurring during the vesicle loading period and during the time the vesicles were being separated on the column, which was about 7 to 10 min.

In order to assess the magnitude of the ion loss, the vesicles were labeled in $^{86}Rb^+$ as described above, separated on the Sephadex column, and then placed into dialysis bags. The loss of radioactivity from the vesicles into buffer is shown in Figure 4. The half-time for the loss was about 20 min. If only $^{86}Rb^+$ was placed in the dialysis bag (*i.e.* no vesicles), the half-time for diffusion was about 5 to 6 min. When 1% Triton X-100 was placed in the dialysis bag with the preloaded vesicles, the half-time for ion loss was about 10 min. In this case, the initial rate of $^{86}Rb^+$ loss from the bag was about 4 times greater than the rate of loss from the untreated vesicles. Although it is not shown in this experiment, it was found that Triton X-100 did not affect the rate of loss of free $^{86}Rb^+$ from the dialysis bag. The increased rate of loss of $^{86}Rb^+$ that was associated with the vesicles, when treated with the nonionic detergent, confirms the previous conclusion that the ions had actually entered the vesicles. Thus, with the two procedures described, it is possible to estimate both the passive influx and the passive efflux of ions across the vesicle membrane.

Effect of Temperature and pH on the Passive Uptake of $^{86}Rb^+$. Temperature was found to affect both the uptake and loss of $^{86}Rb^+$ across the plasma membrane. At 5 C, the net passive uptake of $^{86}Rb^+$ was about 2.5 times lower than at 30 C (Table I). At a higher temperature, the net uptake was also less than at 30 C. This indicated that some membrane damage had probably occurred at the higher temperature and that $^{86}Rb^+$ efflux was increased. The effect of temperature on $^{86}Rb^+$ efflux is shown in Figure 5. As was the case for uptake, passive efflux was decreased markedly by a lower temperature. The half-time of $^{86}Rb^+$ loss at 8 C was about 4 times longer than at 27 C. The decrease in the passive permeability properties of the plasma membrane at cold temperatures is undoubtedly responsible, at least in part, for the decreased flux observed *in situ* as the temperature is decreased (20).

Because of the marked effect of temperature on $^{86}Rb^+$ efflux (Fig. 5), the separation of the preloaded vesicles on the Sephadex column was performed in a cold room at 6 C. Since the

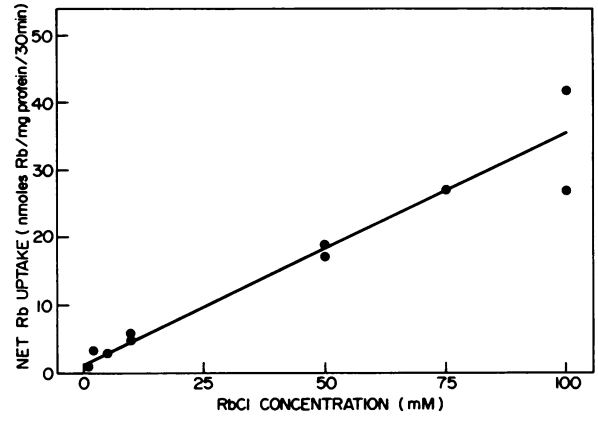

FIG. 3. Net uptake of Rb^+ into plasma membrane vesicles as a function of external concentration of RbCl. Reaction mixture was the same as that of Figure 2 except RbCl concentration was varied from 1 to 100 mM. Incubation time was 30 min.

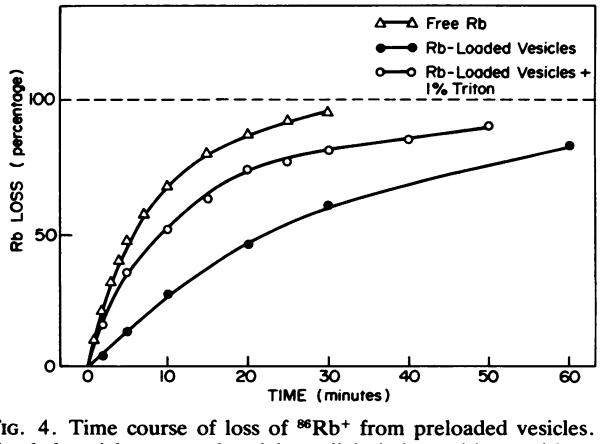

FIG. 4. Time course of loss of $^{86}Rb^+$ from preloaded vesicles. The preloaded vesicles were placed in a dialysis bag with or without 1% Triton X-100. Only free $^{86}Rb^+$ was placed in the bag in one treatment. Buffer solution outside of the dialysis bag contained 5 mM tris-MES, pH 6.5, and 5 mM $MgSO_4$. The experiment was conducted at room temperature (23 C).

Table I. *Effect of Temperature on Net Uptake of Rb^+ into Plasma Membrane Vesicles of Oat Roots*

The reaction mixture contained 17 mM tris-MES, pH 6.5, 5 mM $MgSO_4$, 8% sucrose, and 10 mM RbCl.

Temperature, C	Net Rb^+ Uptake (nmol/mg protein·30 min)
5	3.80
30	9.92
58	2.16

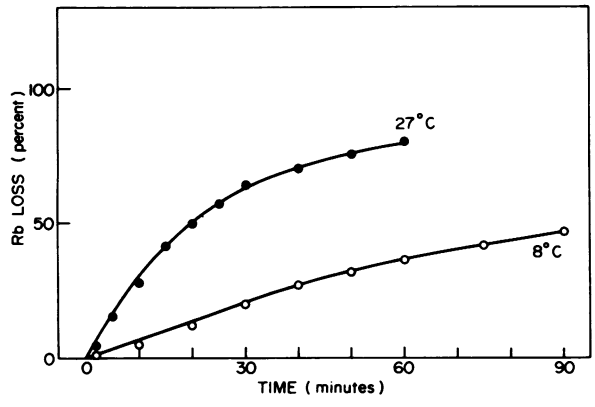

FIG. 5. Effect of temperature on $^{86}Rb^+$ efflux from preloaded vesicles. Experimental conditions were the same as in Figure 4.

Table II. *Effect of pH on Net Uptake of Rb^+ into Plasma Membrane Vesicles of Oat Roots*

The reaction mixture contained 17 mM tris-MES at various pH values, 5 mM $MgSO_4$, 8% sucrose, and 1 mM RbCl. Incubation temperature was 30 C.

pH	Net Rb^+ Uptake (nmol/mg protein·30 min)
5.0	0.59
6.5	1.23
8.0	1.16

separation required less than 10 min, the amount of $^{86}Rb^+$ lost from the vesicles during the elution was less than 10% (Fig. 5).

Table II shows the effect of pH on net $^{86}Rb^+$ uptake into the plasma membrane vesicles. Although a large pH range was not studied, the net uptake was greater at pH 6.5 than at 5 or 8. The significantly lower uptake at pH 5 indicates that negatively charged groups on, or within, the membrane can influence the rate of diffusion of Rb^+ across the membrane.

Net Uptake of Other Ions. In order to assess whether the isolated membrane had retained semipermeability properties, the entry of several ions into the vesicles was determined. The net uptake of the five alkali cations, at a cation concentration of 50 mM, is shown in Table III, and the time course of entry of Rb^+, Na^+, and Cl^- into the vesicles is shown in Figure 6. The passive uptake of the various ions into the vesicles was indeed different. For the alkali cations, the relative amounts of entry were $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. From the time course (Fig. 6), the influx, as well as the apparent equilibrium levels, of Rb^+, Na^+, and Cl^- were very different. The apparent impermeable nature of the membrane vesicles to Cl^-, as compared to Rb^+, was particularly surprising since the salt was RbCl in both treatments. The mechanism for maintaining the balance of charges has not been determined, but it must have been due to either the loss of a cation (possibly protons) or the uptake of another anion (MES^- and SO_4^{2-} were the only other external anions present). Regardless of the basis for the charge balance, these results illustrate clearly that the isolated plasma membrane vesicles were differentially permeable to inorganic cations and anions.

Effect of ATP on $^{86}Rb^+$ Transport. The plasma membrane vesicles contain ATPase activity, and we have previously presented evidence that implicates this enzyme in cation transport (5, 8, 13). It was, therefore, of interest to determine whether ATP would alter the transport of Rb^+ across the plasma membrane vesicles. Figure 7 shows that ATP decreased the net uptake of $^{86}Rb^+$ into the plasma membrane vesicles. A similar reduction in K^+ content of vesicles isolated from cotyledons of *Phaseolus* was reported by Lai and Thompson (12). The effect of ATP in our studies, however, was not specific; ADP was as effective as ATP (Fig. 7). This indicates that the nucleotide effect was not related to an energy transduction because the

Table III. *Net Cation Uptake into Plasma Membrane Vesicles*

The reaction mixture was the same as in Table I except the concentration of alkali chlorides was 50 mM. Incubation temperature was 30 C.

Cation	Net Cation Uptake (nmol/mg protein·30 min)	Uptake relative to K^+
K^+	18.9	1.00
Rb^+	17.7	0.94
Cs^+	16.1	0.80
Na^+	13.4	0.71
Li^+	12.1	0.65

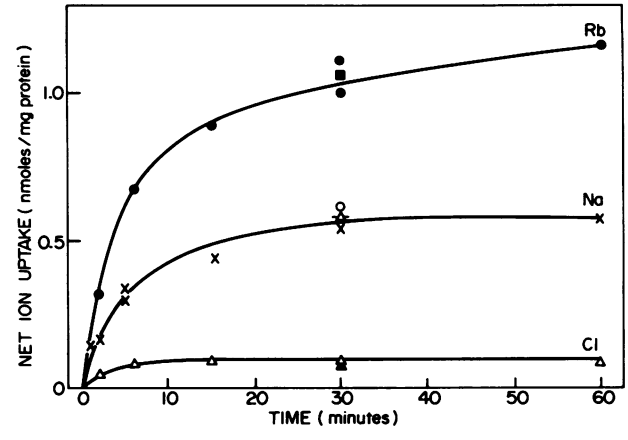

FIG. 6. Time course of net uptake of Rb^+, Na^+, and Cl^- into plasma membrane vesicles. Concentration of RbCl or NaCl in the reaction medium was 1 mM. The Cl^- data were obtained with 1 mM RbCl. Experimental conditions were the same as in Figure 2. Different symbols indicate measurements were made on separate plasma membrane preparations: Rb (●, ■), Na (○, ☆, ×), Cl (△, ▲).

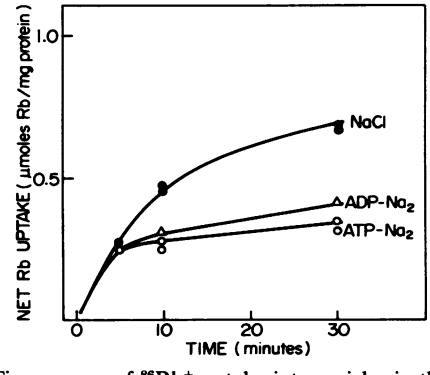

FIG. 7. Time course of $^{86}Rb^+$ uptake into vesicles in the absence of nucleotides and in the presence of ATP and ADP. Reaction mixture was the same as Figure 2 except for the presence of 5 mM ADP-Na_2 or 5 mM ATP-Na_2. The reaction mixture of the control vesicles contained 10 mM NaCl.

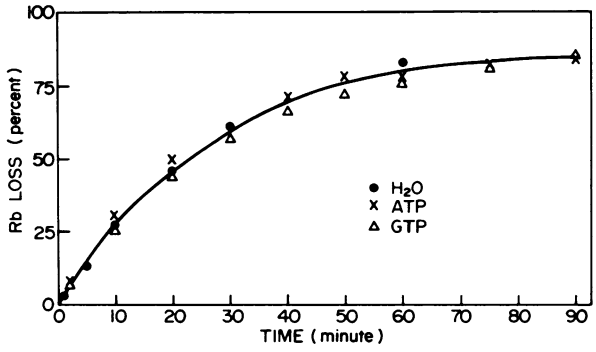

FIG. 8. Effect of ATP and GTP on $^{86}Rb^+$ loss from preloaded vesicles. Buffer solution outside dialysis bag consisted of 5 mM tris-MES, pH 6.5, 5 mM $MgSO_4$, and 10 mM RbCl. ATP and GTP concentration was 1.7 mM, and the nucleotides were placed inside and outside of the bag. The experiment was performed at 18 C.

Table IV. *Effect of Various Inhibitors on Net Rb^+ Uptake into Plasma Membrane Vesicles in the Presence and Absence of ATP*

The reaction mixture was the same as in Table I. The concentration of RbCl was 1 mM and of ATP (Na salt) was 5 mM. DCCD: *N,N'*-dicyclohexylcarbodiimide. mCl-CCP: carbonylcyanide *m*-chlorophenyl hydrazone.

Additives	Net Rb^+ Uptake	
	-ATP	+ATP
	(nmol/mg protein·10 min)	
H_2O	0.510	0.208
Ethanol (1%)	0.560	0.209
DCCD (0.1 mM)	0.523	0.238
Oligomycin (5 µg/ml)	0.596	0.235
mCl-CCP (10 µM)	0.494	0.250
$CaSO_4$ (1 mM)	0.600	0.310

plasma membrane vesicles of oat roots have very low ADPase activity (9).

The effect of the nucleotides was on ion entry rather than on ion loss. The lack of effect of either ATP or GTP on $^{86}Rb^+$ loss is illustrated in Figure 8. In this experiment, the $^{86}Rb^+$-loaded vesicles were dialyzed in a solution containing 10 mM RbCl alone or in the presence of 1.7 mM ATP or 1.7 mM GTP.

The effect of various agents known to affect membrane permeability and energy transduction on the net uptake of $^{86}Rb^+$, in the absence and presence of ATP, is shown in Table IV. None of the inhibitors had an appreciable effect on the uptake of $^{86}Rb^+$. Unfortunately, these results did not clarify why ATP (and ADP) decreased the net uptake of $^{86}Rb^+$ into the plasma membrane vesicles. However, they strongly suggest that the effect is not associated with the functioning of the ATPase, since two of the inhibitors, *N,N'*-dicyclohexylcarbodiimide (13) and Ca^{2+} (1), are known to inhibit the plasma membrane ATPase.

DISCUSSION

We have used gel filtration and dialysis for measuring the uptake and loss of ions across plasma membrane vesicles isolated from oat roots. Both methods gave very reproducible results. We explored the possibility of using Millipore filtration or centrifugation to separate the ion-loaded vesicles from the external solutions, but neither of these methods was as satisfactory as the gel filtration. One limitation that was common to both the gel filtration and dialysis procedures was the amount of membranes required relative to the amounts available. For example, from 50 g fresh weight of oat roots, about 0.5 to 1.0 mg of plasma membrane protein can be obtained. The amount of membrane protein required per sample, when measuring uptake, was 100 to 200 µg and, when measuring efflux, was 200 to 400 µg. Thus, the number of treatments and replicates that could be tested from a single membrane isolation was somewhat restricted.

Ion fluxes, as contrasted to superficial ion binding, occurred across the vesicular membrane. This was evident from the fairly slow increase in $^{86}Rb^+$ content of the vesicles when the latter was placed into a $^{86}Rb^+$ solution (Fig. 2), the linear increase in Rb^+ associated with the vesicles as the external Rb^+ concentration was increased (Fig. 3), and the enhanced rate of loss of Rb^+ from Rb^+-loaded vesicles that was induced by Triton X-100 (Fig. 4). None of these results would be expected from a simple exchange of Rb^+ onto negative sites of the membrane. Hence, it can be concluded that Rb^+ actually traversed the membrane and entered the vesicular space. The decreased uptake (Table I) and loss (Fig. 5) of $^{86}Rb^+$ caused by low temperatures would also be consistent with this interpretation.

The differential permeability of the isolated plasma membrane vesicles to various ions (Table III, Fig. 6) was surprisingly similar to that reported for the plasma membrane *in situ*. For example, it is generally recognized that the plasma membrane of plant cells is more permeable to cations than to anions. In the case of the vesicles used here, the initial rates for the passive entry of Rb^+, Na^+, and Cl^- were 0.15, 0.075, and 0.025 nmol/mg protein/min, respectively, when the external ion concentrations were 1 mM. The relative values were Rb^+ (1.0), Na^+ (0.5), and Cl^- (0.18). These relative values are similar to the relative permeability coefficients for K^+ (1.0), Na^+ (0.68), and Cl^- (0.038) flux across the plasma membrane of cells of the oat coleoptile (18). Permeability coefficients for various ions crossing the plasma membrane of oat root cells have not been determined. However, based on the efflux of Rb^+ from the vesicles (Fig. 4) one can estimate the permeability coefficient for this ion using the equation

$$P = \left(\frac{r}{3t}\right) \ln 2,$$

where r is the radius of the vesicle and t is the half-time for ion loss. This expression is derived from Ficks' first law of diffusion (16) and assumes no driving forces other than activity gradients, thus any contribution due to membrane electrical potentials is not considered. Vesicle diameters were determined from electron micrographs and found to be 0.3 ± 0.15 µm (10) and the $t_{1/2}$ from Figure 5 was 20 min. Based on these values, the P_{Rb^+} was calculated to be 0.29 ± 0.15 × 10^{-8} cm/sec. For *in situ* plasma membrane of plant cells, the P_{K^+} is in the range of 0.8 to 22.0 × 10^{-8} cm/sec (18, 19). Thus, the permeability coefficient of Rb^+ for the isolated plasma membrane vesicles of oat roots is somewhat lower than the permeability coefficient of K^+ for the *in situ* plasma membrane of various cells. This difference could be due to the lower permeability coefficient of the counterion.

The plasma membrane vesicles also exhibited differential permeability to the alkali cations (Table III). This was not surprising since artificial bilayer membranes made from phosphatidylserine, phosphatidylglycerol (17), or a chloroform-methanol solution of egg phosphatidylcholine and tetradecane (15) exhibit considerable selectivity to alkali cations. In the latter system, relative permeabilities to the alkali cations, expressed as cation transference numbers, were $K^+ > Cs^+ > Na^+ > Rb^+ > Li^+$ (15). And, the self-diffusion rates of Na^+, K^+, and Rb^+ measured separately through phosphatidylserine vesicles (17) followed the series $K^+ > Rb^+ > Na^+$ (1.0:0.82:0.01). The degree of discrimination was influenced by proteins, Ca^{2+}, cholesterol, and pH. Thus, the specificity of the plasma membranes to cation flux is probably determined in part by the particular phospholipids present.

The effect of ATP on Rb^+ uptake across the vesicular membrane appeared to be physical rather than related to an energy transfer. This conclusion was based on the finding that ADP was

as effective as ATP, and that *N,N'*-dicyclohexylcarbodiimide and Ca^{2+}, which inhibit the ATPase, did not alter the ATP effect on Rb^+ permeation. The previous report of an ATP effect on ion fluxes in a membrane preparation from plants (12) may also have been due to a physical effect since the specificity for ATP was not checked. One possible reason for the failure to demonstrate an ATP-driven ion flux in the plasma membrane vesicles is that the membrane preparation probably consisted of some vesicles that were completely sealed, others that were only partially sealed, and still others that were completely unsealed. Also, the sealed vesicles were likely to be oriented both as right side- and inside-out vesicles, and either ATP or Rb^+ may not have been on the appropriate sides of the membrane. The problem of demonstrating and studying an ATP-driven transport in a vesicle preparation is not insurmountable, however. For example, it has been shown recently, using techniques similar to those reported here but with different experimental conditions, that Na^+ and K^+ transport in vesicles prepared from egg lecithin can be coupled to ATP via the purified (Na^+ + K^+) -ATPase of the plasma membrane of canine brain gray matter (6) and the rectal gland of *Squalus acanthias* (7).

In summary, we have shown that the isolated plasma membrane vesicles of oat roots can be employed for investigating the passive influx and efflux of inorganic ions. The isolated membranes are differentially permeable and similar in this respect to the plasma membrane *in situ*.

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