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Separation of Two Types of Electrogenic H^+-Pumping ATPases from Oat Roots[1]

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ABSTRACT

Microsomal vesicles of oat roots (*Avena sativa* var Lang) were separated with a linear dextran (0.5–10%, w/w) or sucrose (25–45%, w/w) gradient to determine the types and membrane identity of proton-pumping ATPases associated with plant membranes. ATPase activity stimulated by the H^+/K^+ exchange ionophore nigericin exhibited two peaks of activity on a linear dextran gradient. ATPase activities or ATP-generated membrane potential (inside positive), monitored by SCN^- distribution, included a vanadate-insensitive and a vanadate-sensitive component. In a previous communication, we reported that ATP-dependent pH gradient formation (acid inside), monitored by quinacrine fluorescence quenching, was also partially inhibited by vanadate (Churchill and Sze 1983 Plant Physiol 71: 610–617). Here we show that the vanadate-insensitive, electrogenic ATPase activity was enriched in the low density vesicles (1–4% dextran or 25–32% sucrose) while the vanadate-sensitive activity was enriched at 4% to 7% dextran or 32% to 37% sucrose. The low-density ATPase was stimulated by Cl^- and inhibited by NO_3^- or 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid (DIDS). The distribution of Cl^--stimulated ATPase activity in a linear dextran gradient correlated with the distribution of H^+ pumping into vesicles as monitored by [^{14}C]methylamine accumulation. The vanadate-inhibited ATPase was mostly insensitive to anions or DIDS and stimulated by K^+. These results show that microsomal vesicles of plant tissues have at least two types of electrogenic, proton-pumping ATPases. The vanadate-insensitive and Cl^--stimulated, H^+-pumping ATPase may be enriched in vacuolar-type membranes; the H^+-pumping ATPase that is stimulated by K^+ and inhibited by vanadate is most likely associated with plasma membrane-type vesicles.

The primary active transport process in higher plants is thought to be an electrogenic transport of protons (21, 25). The proton motive force generated by proton pumping can provide the driving force for transport of cations, anions, amino acids, sugars, and hormones according to Mitchell's chemiosmotic hypothesis (19). Electrophysiological studies suggested that electrogenic proton pumps were localized in the plasma membrane (H^+ extrusion) (21, 25) and probably the tonoplast (H^+ uptake) (16). The importance of such proton pumps to solute transport, as well as the regulation of growth and development, has made the study of proton transport an exciting research topic. Until recently, there was no direct evidence for a membrane constituent that pumped H^+. Within the last few years, evidence for electrogenic, H^+-pumping ATPases has appeared from our laboratory (4–6, 27–31) and several other laboratories (1, 7, 8, 10, 17, 18, 23, 24, 26, 32).

H^+-pumping ATPases have been identified in nonmitochondrial membranes of several plant tissues. These transport ATPases exhibit three types of activities in sealed microsomal vesicles: (a) ionophore-stimulated ATPase activity (24, 27, 28), (b) ATP-dependent generation of a membrane potential (positive inside the vesicle) (1, 23, 26, 30), and (c) ATP-dependent pH gradient formation (acid inside) (1, 6, 8, 10, 18, 26, 29, 31, 32).

Although several laboratories have demonstrated a vanadate-resistant, H^+-pumping ATPase (8, 10, 18, 32), one report using oat roots (26) and our studies with sealed microsomal vesicles from tobacco callus or oat roots have suggested the presence of at least two types of H^+-pumping ATPases, one sensitive and one insensitive to vanadate (5, 6, 28, 31). This paper demonstrates partial separation of two types of electrogenic, H^+-pumping ATPases using a linear dextran or sucrose gradient. The two types of ATPases can be distinguished by their relative densities, K^+ or Cl^- sensitivities and sensitivity to inhibitors. The Cl^--stimulated, proton pump appears to be enriched in vacuolar membranes and the K^+-sensitive, proton pump is enriched in plasma membrane-type vesicles. Preliminary reports of these results have been presented (5, 31).

MATERIALS AND METHODS

Plant Material. Oat (*Avena sativa* L. var Lang) seedlings were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$. After 5 to 6 days of growth, the apical tips (3–4 cm) of the roots were harvested. Lang oats were generously provided by the Agronomy Department of Kansas State University.

Isolation and Separation of Sealed Microsomal Vesicles. Sealed microsomal vesicles were prepared as described by Churchill and Sze (6) using a 6% (w/w), 10%, or 12% dextran cushion. In one case, a two-step dextran gradient of 6% and 15% was used to separate vesicles at the 0/6% and 6/15% dextran interfaces.

Microsomal vesicles (60,000*g* pellet) were sometimes separated with a linear dextran gradient (0.5–10%) as described before (6). Dextran rather than sucrose was chosen for most experiments as we found sucrose gradients did not improve membrane separation. Furthermore, we found slightly higher activities of pH gradient and membrane potential generation in vesicles prepared with dextran than with sucrose. Many of the dextran gradient tubes were intentionally overloaded with microsomal vesicles to permit membrane potential, pH gradient, and ATPase assays from the same gradient tube. Furthermore, vesicles were centrifuged for 2 h, and not to equilibrium, because vesicles often

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became leaky and failed to show active transport after a lengthy preparation time. These procedures resulted in partial separation of activity peaks. Data presented are from one representative experiment of at least three experiments.

The relative density of fractions collected from the dextran gradients was determined with a refractometer calibrated in Brix units (or sucrose per cent). The readings of relative sucrose equivalence in per cent were useful in determining the linearity of the gradient. Relative densities of vesicles are generally expressed as per cent dextran. Standard solutions of various dextran concentrations (w/w) were made in 250 mM mannitol and 2.5 mM Hepes-BTP[3] at pH 7.3 and their relative sucrose equivalents in per cent were determined to be directly proportional to the dextran concentration. A 10% dextran (average mol wt, 70 kD) solution made in 250 mM mannitol and 2.5 mM Hepes-BTP at pH 7.3 has a density of about 1.06 g/cc at 20°C.

Determination of Enzyme Activities, Membrane Potential, and pH Gradient Generation. ATPase activity was determined by Pi release (11) at 36°C and the various forms of activities are expressed as in Sze (28). Activities of NADH Cyt *c* reductase (cyanide insensitive) or Cyt *c* oxidase were measured by following the reduction or oxidation of Cyt *c* (11), respectively. UDPase activity was determined in the presence of Triton (4, 20). Membrane potential ($\Delta\psi$) generation (inside positive) was determined by ATP-dependent thiocyanate (^{14}C or ^{35}S) uptake into vesicles using a filtration method described previously (30). The pH gradient (ΔpH) formation (inside acid) was determined by ATP-dependent [^{14}C]methylamine uptake into vesicles using the direct filtration procedure described (6). Both transport assays were conducted at room temperature (21°C). Protein concentration was estimated by the Lowry method (14) after precipitation with 10% TCA using BSA as a standard.

Chemicals. Nigericin was a generous gift from J. Berger and J. W. Westley of Hoffmann-La Roche. K-iminodiacetate was formed by titrating a known quantity of KOH to pH 6.75 with iminodiacetate (Sigma). Sodium ATP was purchased from Boehringer-Mannheim and converted by Dowex ion exchange and bis-tris-propane titration to ATP-BTP. [^{14}C]Methylamine and [^{14}C]thiocyanate were obtained from New England Nuclear and Amersham, respectively. Most other biochemicals were obtained from Sigma.

RESULTS

Vanadate Sensitivity of ATPase Activity and Membrane Potential Formation. Initially we examined the effect of vanadate on ATPase activity and generation of a membrane potential in sealed microsomal vesicles of oat roots to determine whether the vesicle population included one or more types of ATPases. ATPase activity of sealed microsomal vesicles was partially inhibited by sodium orthovanadate at relatively high concentrations (Fig. 1). Only about 20% of the total MgATPase or MgKCl-ATPase activity was inhibited by 200 μM vanadate, and about 35% of the KCl-stimulated MgATPase was inhibited. The increase in inhibition of KCl-stimulated MgATPase activity by vanadate is due to specific vanadate inhibition of a K^+-stimulated MgATPase activity as explained later.

Generation of a membrane potential, inside positive, was similarly inhibited by sodium orthovanadate (Fig. 2). ATP-dependent thiocyanate uptake, measured in the absence of Cl^-

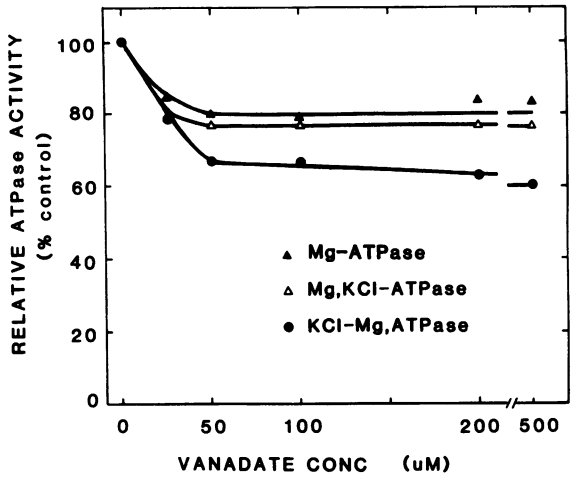

FIG. 1. Effect of vanadate concentration on MgATPase, MgKCl-ATPase, and KCl-stimulated MgATPase activities in sealed microsomal vesicles from oat roots. The reaction medium consisted of 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, vesicles from a 6% dextran interface and vanadate at concentrations from 25 to 500 μM. Specific activities of control MgATPase and MgKCl-ATPase were 8.3 ± 2.0 and 13.9 ± 3.4 μmol Pi/mg protein·h, respectively. Results shown are the mean of 4 to 6 experiments.

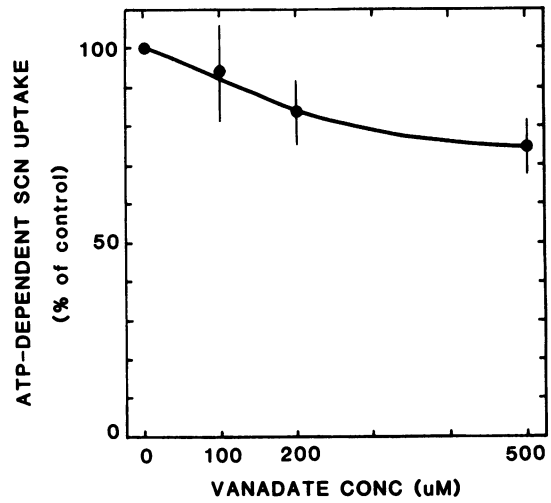

FIG. 2. Effect of vanadate concentration on ATP-dependent thiocyanate uptake into sealed microsomal vesicles from oat roots. Reaction mixture consisted of 25 mM Hepes-BTP at pH 6.75, 175 mM mannitol, 0.1 mM [^{35}S]SCN (1–1.5 μCi/ml), 10 mM $MgSO_4$, microsomal vesicles from a 10% interface with or without 3 mM ATP-Tris. Triplicate samples were taken after 10-min incubation at 21°C. Results shown are the mean of 4 to 6 experiments. Error bars are SE.

(30), qualitatively reflected MgATPase activity. Thiocyanate uptake was inhibited 18% by 200 μM vanadate (Fig. 2) similar to MgATPase activity (Fig. 1). The difference in the concentration dependence of vanadate inhibition between MgATPase and SCN^- uptake could be caused by the following reasons: (a) SCN^- uptake is not linearly related to ATP hydrolysis (30); and (b) SCN^- uptake and ATP hydrolysis do not necessarily reflect activity from the same population of vesicles, as explained in the following section. These results are qualitatively similar to our findings with the microsomal vesicles of tobacco callus (28–30) and indicated to us that microsomal membranes of oat roots included at least two types of electrogenic ATPases, one sensitive and one insensitive to vanadate.

Separation of Two Types of Electrogenic ATPases on Dextran

[3] Abbreviations: BTP, bis-tris propane (1,3-bis[tris(hydroxymethyl) methylamino]propane); CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl)-*N*,*N*′-tetraacetic acid; IDA, iminodiacetate; $\Delta\psi$, membrane potential; ΔpH, pH gradient; vanadate, sodium orthovanadate.

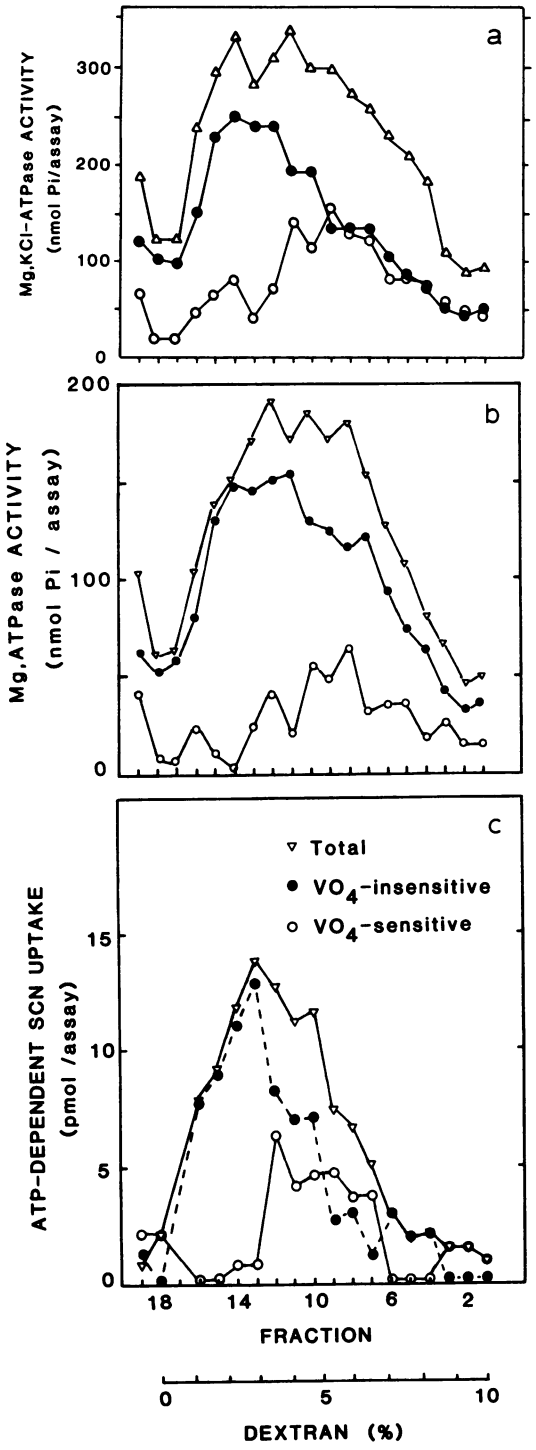

FIG. 3. Dextran gradient distribution of vanadate-insensitive and vanadate-sensitive electrogenic ATPase activities of microsomal vesicles from oat roots. a, Distribution and sensitivity to vanadate of MgKCl-ATPase activity in microsomal vesicles separated with a dextran gradient as in Churchill and Sze (6). Vanadate-insensitive MgKCl-ATPase activity (●) was detected in the presence of vanadate. Vanadate-sensitive ATPase activity (○) was the difference between the vanadate-insensitive and the total MgKCl-ATPase activity (△). Reaction mixtures contained 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, 50 mM KCl, 2 μM nigericin, and 50 μl enzyme with or without 200 μM vanadate. Reactions were incubated for 40 min at 36°C. Means of three experiments are shown. b, Distribution and sensitivity to vanadate of MgATPase activity in microsomal vesicles separated with a linear dextran gradient. Reaction mixtures were as described in (a) except KCl and nigericin were absent. c, Distribution and sensitivity to vanadate of ATP-dependent [^{14}C]thiocyanate uptake into microsomal vesicles from oat roots. Reaction media contained 25 mM Hepes-BTP at pH 6.75, 175 mM mannitol, 16 μM [^{14}C]thiocyanate, 5 mM $MgSO_4$, 3 mM ATP-Tris with or without 200 μM vanadate. Reactions incubated at 21°C.

Gradient. To separate the two types of electrogenic ATPases, we assumed that a vanadate-sensitive ATPase is completely inhibited by 200 μM vanadate and that ATP hydrolysis detected in the presence of the inhibitor is the vanadate-insensitive ATPase activity. The difference between the total ATPase and vanadate-insensitive ATPase activities is the vanadate-sensitive activity. Using this analysis, partial separation of the two types of activities was obtained with a linear dextran gradient. The vanadate-insensitive and the vanadate-sensitive MgKCl-ATPases were distributed at 1% to 4% and 4% to 8% dextran, respectively (Fig. 3a). MgATPase activity could also be resolved into two types of vanadate sensitivities. A vanadate-insensitive MgATPase was enriched at 2% to 4% dextran and a vanadate-inhibited Mg-ATPase exhibited peak activity around 4% to 7% dextran (Fig. 3b). Similar results were obtained with KCl-stimulated Mg-ATPase activities (data not shown). These results suggested that a vanadate-sensitive and a vanadate-insensitive ATPase could each exhibit MgATPase and salt-stimulated ATPase activities.

When microsomal vesicles were separated with a linear dextran gradient (0.5–10%), ATP-dependent SCN^- uptake was distributed broadly (Fig. 3c) similar to the distribution of total Mg-ATPase activity (Fig. 3b). In the presence of 200 μM vanadate, SCN^- accumulation was detected around 1% to 4% dextran suggesting that a vanadate-insensitive electrogenic ATPase was enriched in vesicles of low density. Inhibition of SCN^- uptake by vanadate was found in vesicles distributing between 4% to 7% dextran. These results taken together suggested that ATP-dependent SCN^- uptake reflected electrogenic pumping by MgATPases (30) and that both types of ATPases could generate electrical gradients.

It is important to note that even when one is specifically measuring an electrogenic, H^+-pumping ATPase activity, ATP hydrolysis and ATP-dependent transport (either SCN^- or methylamine uptake) do not necessarily reflect activity from the same population of vesicles. When ATP is accessible, only tightly sealed vesicles are capable of active transport, yet both sealed and relatively leaky vesicles can hydrolyze ATP. Since sealed vesicles are lighter in density than leaky ones (27), it is not surprising to find a broad distribution of ATPase activities in density gradients, with the distribution of SCN^- uptake localized in the low density region (Fig. 3, b and c). Parts b and c of Figure 3 are directly comparable as SCN^- uptake was measured in the absence of Cl^- which dissipates the membrane potential (30). The deviations in the ATPase and SCN^- uptake assays do not allow us to conclude at this time whether there are several vanadate-sensitive ATPases distributed in different membrane types. The data, however, demonstrate two types of electrogenic ATPases enriched in membranes of different densities.

Partial separation of two types of ATPases was also obtained with a linear sucrose gradient of 25% to 45% (w/w) (Fig. 4). The vanadate-insensitive MgKCl-ATPase was enriched in vesicles at 25% to 32% sucrose and the vanadate-inhibited ATPase demonstrated peak activity around 32% to 37% sucrose. The results show that both dextran and sucrose gradients were similarly effective in separating the two types of ATPases from oat roots. To maintain osmotic stability of the sealed vesicles, we chose to use dextran instead of sucrose gradients.

We have previously shown that quinacrine fluorescence quenching reflected H^+ pumping into vesicles from both types of ATPases (6). H^+ pumping, as monitored by quinacrine fluorescence quenching in microsomal vesicles from tobacco callus

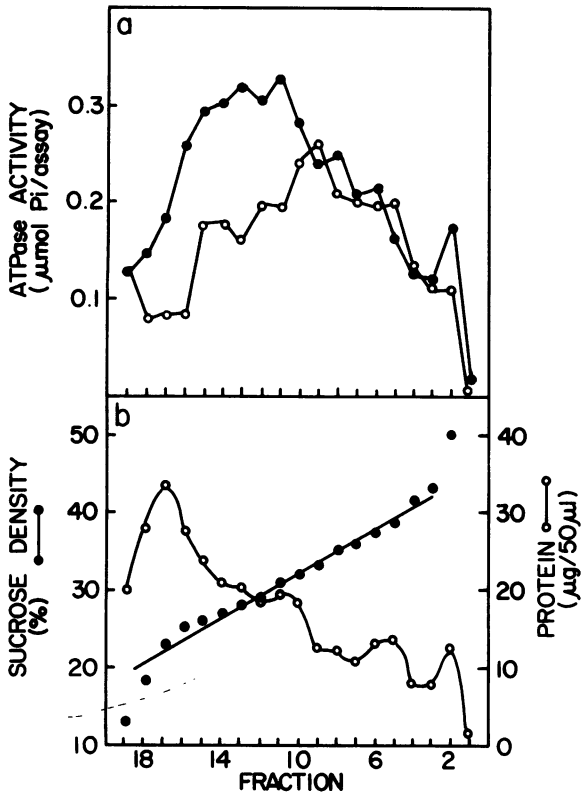

FIG. 4. Separation of ATPases from microsomal vesicles of oat roots with a linear sucrose gradient. a, Separation of a vanadate-insensitive and vanadate-sensitive MgKCl-ATPase. Microsomal vesicles resuspended in 2.0 ml of 250 mM sucrose and 2.5 mM Hepes-BTP at pH 7.3 were separated with a linear sucrose gradient (containing 30 ml of 25% to 45% sucrose in 2.5 mM Hepes-BTP, pH 7.2) layered over a 2.5 ml cushion of 60% sucrose. Reaction mixtures were made to final concentrations of 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, 50 mM KCl, 75 μl membrane fraction with or without 200 μM vanadate and incubated for 45 min at 35°C. Vanadate-sensitive ATPase activity (○) was calculated from the difference of total MgKCl-ATPase (not shown) and vanadate-insensitive ATPase activities (●). b, Sucrose and protein concentrations of membrane fractions.

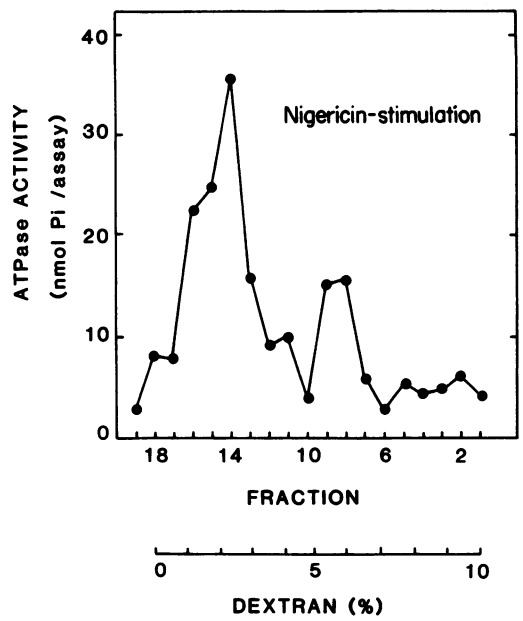

FIG. 5. Distribution of nigericin-stimulated ATPase activity in oat root microsomal vesicles separated with a linear dextran gradient. Nigericin stimulation was calculated by subtracting MgKCl-ATPase activity without nigericin from MgKCl-ATPase activity in the presence of nigericin. Reaction media contained 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, 3 mM ATP-Tris, and 50 μl membrane fraction. When present, nigericin was 5 μM and KCl was 50 mM. Mean results of 3 experiments shown.

Table I. *Effect of Valinomycin, CCCP, and Nigericin on ATPase Activities of Sealed Microsomal Vesicles from Oat Roots*

Reaction media consisted of 30 mM Tris-Hepes at pH 6.5, 3 mM $MgSO_4$, 3 mM ATP-Tris, ±50 mM KCl, 2 μM ionophores (valinomycin, CCCP, or nigericin), and a final ethanol concentration of 0.5%. Vesicles were obtained from a 10% dextran interface. Values in parentheses are standard errors. Results are the average of 7 experiments.

Addition	ATPase Specific Activity			Relative Activity
	Mg	MgKCl	ΔKCl	ΔKCl
	μmol Pi/mg protein·h			
Control	14.3 (1.2)	21.7 (1.3)	7.4	100
Valinomycin	14.1 (1.2)	22.5 (1.4)	8.4	113
CCCP	14.4 (1.3)	22.9 (1.5)	8.5	115
Valinomycin + CCCP	15.4 (1.7)	27.3 (2.6)	11.9	161
Nigericin	14.9 (1.7)	25.6 (2.3)	10.7	145

(29) or oat roots (6), was partially inhibited by vanadate at high concentrations (200 μM) suggesting that a vanadate-sensitive as well as a vanadate-insensitive ATPase were translocating protons.

Ionophore-Stimulated ATPase Activities. At least two peaks of ionophore-stimulated ATPase activities could be distinguished on a linear dextran gradient (Fig. 5). A large peak of nigericin-stimulated ATPase activity was found between 1% to 3% dextran and a smaller peak of activity was detected around 5.5% to 7% dextran. Gramicidin alone or CCCP combined with valinomycin (not shown) gave similar results, though stimulation by CCCP plus valinomycin was usually higher than by nigericin alone (Table I). CCCP or valinomycin alone showed a relatively small stimulatory effect on ATPase activity of the sealed microsomal vesicles (Table I), similar to results with tobacco callus (27). Ionophore-stimulated ATPase activity reflects ATPase that is capable of pumping ions (mainly protons) (27, 28) and thus its distribution on a dextran gradient represents the distribution of sealed vesicles that can hold ATP-dependent ion gradients. This idea was supported by the similar distributions of nigericin-stimulated ATPases (Fig. 5) and ATP-dependent thiocyanate uptake (Fig. 3c) which could be resolved into two distinct peaks when fewer membrane vesicles were loaded per dextran gradient. As previously explained, total ATPase activity includes ATP-hydrolyzing activity from leaky vesicles that are not related to H^+ pumping. Thus, the distribution of ionophore-stimulated ATPases (Fig. 5) was similar but not identical to the distribution of vanadate-insensitive and vanadate-sensitive ATPases on a dextran gradient (Fig. 3a). These results are consistent with the idea that at least two types of ATPases are pumping H^+.

Membrane Identity. Preliminary experiments were conducted to identify the membranes with H^+-pumping ATPases. Microsomal vesicles separated with a continuous dextran gradient were analyzed for cyanide-insensitive NADH-Cyt *c* reductase (ER marker) (11), MgUDPase in the presence of Triton (a Golgi marker) (20), vanadate-sensitive ATPase (a plasma membrane marker) (9), and Cyt *c* oxidase (a mitochondria marker) (11). The increasing order of relative membrane densities in a dextran gradient was ER < Golgi < plasma membrane < mitochondria (Figs. 3a and 6) similar to the relative distribution of subcellular membranes in sucrose gradients (11).

Though the separation of subcellular membranes was poor, a

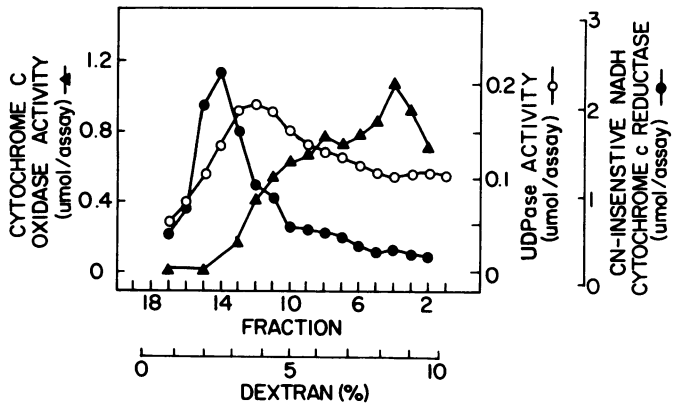

FIG. 6. Distribution of cyanide-insensitive NADH-Cyt *c* reductase, UDPase, and Cyt *c* oxidase activities of oat root microsomal vesicles separated with a linear dextran (0.5–10%) gradient. NADH-Cyt *c* reductase and Cyt *c* oxidase assays were conducted at 21°C (11). UDPase activity was measured at 36°C (4, 20).

few tentative conclusions were drawn from these results: (a) a vanadate-sensitive H^+-pumping ATPase was enriched in plasma membrane and Golgi vesicles (compare Figs. 3, a and c, 5, 6); (b) vanadate-insensitive H^+-pumping ATPase could be associated with the tonoplast as well as ER and Golgi (Figs. 3, a and c, 5, 6). This conclusion is based on the low density of the vanadate-insensitive ATPase as well as its similar properties to isolated tonoplast ATPase (6, 33). We have shown that electrogenic, H^+ pumping from oligomycin-sensitive (mitochondrial) ATPase is minimal (6, 30).

Sensitivities to K^+, Cl^-, NO_3^- Vanadate, and DIDS. To determine the sensitivities of microsomal ATPases to salts, we examined the effect of various K anions on ATPase activities in vesicles separated with a linear dextran gradient. Vesicles of low density (2–5% dextran) were sensitive to anions (Fig. 7). Cl^- stimulated and NO_3^- inhibited the MgATPase activity. Sulfate appeared to have either no or a slight inhibitory effect. The ATPase associated with vesicles that distributed between 5.0% to 9% dextran was less sensitive to anions and appeared to be stimulated by K^+.

To determine whether the anion-stimulated ATPase was pumping H^+, the distribution of chloride-stimulated ATPase activity was compared with the distribution of ATP-dependent [^{14}C]methylamine uptake in vesicles separated with a linear dextran gradient. Chloride-stimulated ATPase activity was determined from the difference between KCl-stimulated and K_2SO_4-stimulated ATPase activities. Figure 8 shows that the distributions of chloride-stimulated ATPase and proton pumping activities were similar. We have shown that ATP-dependent methylamine uptake reflected mainly proton pumping by a vanadate-insensitive ATPase (6). Taken together, these results suggested that a chloride-stimulated, vanadate-insensitive ATPase corresponded to an electrogenic proton pump, similar to findings made recently by other laboratories (7, 10, 17).

To understand the salt and inhibitor properties of the two ATPases, the effect of K^+ or Cl^- on ATPase activity was studied separately by using KIDA or BTP-Cl, respectively. IDA, an impermeant anion, has no dissipating effect on generation of membrane potential (4) and did not stimulate generation of a pH gradient (6). Bis-tris propane is considered an impermeant cation and has little stimulatory effect on ATPase activity (4). Thus, KIDA-stimulated MgATPase activity should reflect activity due to stimulation by K^+ and BTP-Cl-stimulated MgATPase activity represented that mainly from a Cl^--sensitive enzyme.

The two types of ATPases could be partially separated with a two-step dextran gradient. Table IIB shows that specific MgKCl-ATPase activity in the 0/6% dextran interface was 21% vanadate-

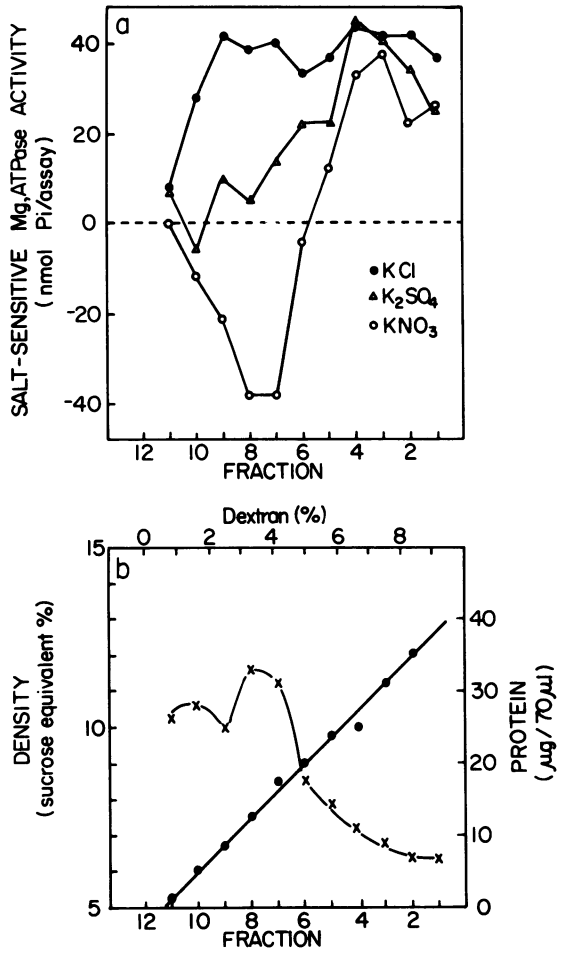

FIG. 7. Distribution of KCl-, K_2SO_4-, or KNO_3-sensitive MgATPase activity in oat root microsomal vesicles separated with a linear dextran gradient (0.5–10%). Reaction mixtures contained 30 mM Hepes-BTP at pH 6.75, 3 mM $MgSO_4$, and 3 mM ATP-Tris. KCl, K_2SO_4, or KNO_3 was present as 50 mM K^+. MgATPase activity is set to zero. Results from one representative experiment are expressed as KCl (●)-, K_2SO_4 (Δ)-, or KNO_3 (○)-sensitive MgATPase activity. Lower panel, protein concentration and relative density profile of membrane fractions.

sensitive whereas the activity in the 6/15% dextran interface was 62% vanadate-sensitive. The specific activity of vanadate-sensitive MgATPase or Mgsalt-ATPase activity in the 6/15% dextran interface was enriched 6- to 8-fold over that in the 0/6% dextran interface. For example, vanadate-sensitive MgATPase activity in the 6/15% and 0/6% interface were 8.1 and 1.3 μmol Pi/mg·h, respectively. Thus, the vanadate-insensitive and vanadate-sensitive, salt-stimulated ATPases were relatively enriched in the 0/6% and 6/15% dextran interface, respectively (Table II). These results agree with those obtained from linear dextran gradients (Fig. 3A). Table II, A and B also shows that vanadate completely inhibited the KIDA-stimulated MgATPase activity. In both dextran interfaces, 200 μM vanadate inhibited 97% to 99% of the K^+-stimulated ATPase. The Cl^--stimulated ATPase is mostly insensitive to the inhibitor, showing about 0% to 15% vanadate sensitivity. Whether the vanadate-sensitive Cl^--ATPase reflects a separate enzyme or is part of the K^+-ATPase is not clear. KCl-stimulated ATPase reflected activity from at least two enzymes and thus it showed partial vanadate sensitivity. However, the K^+- and Cl^--stimulated ATPases were enriched in the 6/15% and 0/6% interface, respectively.

The two types of ATPases showed different sensitivities to DIDS. DIDS is an inhibitor of Cl^- transport into red cells (3), corn protoplasts (13), and *Chara* (12). We have shown that DIDS

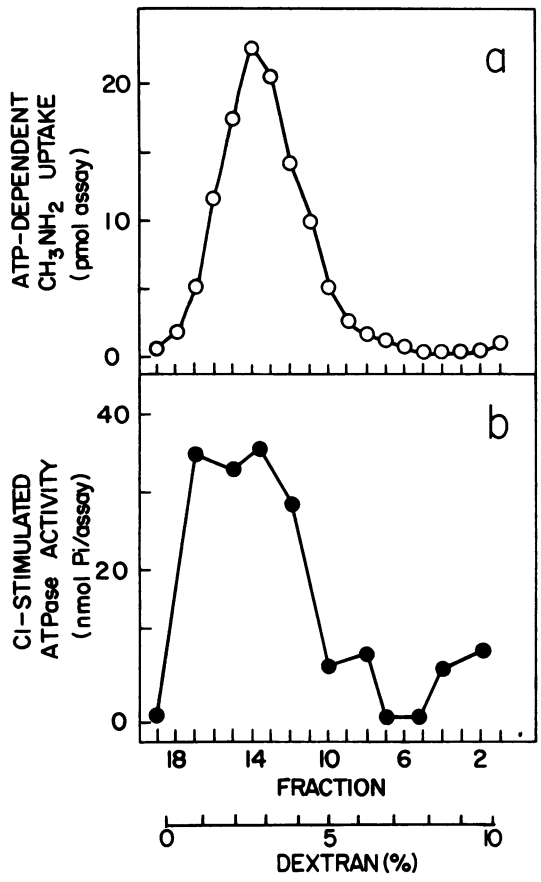

FIG. 8. Distribution of (a) methylamine uptake (O) and (b) Cl^--stimulated MgATPase activity (●) in microsomal vesicles from oat roots separated in a dextran gradient. Cl^--stimulated ATPase activity was calculated from the data presented in Figure 7 by subtracting the activity with K_2SO_4 from that with KCl. [^{14}C]Methylamine uptake was measured as described by Churchill and Sze (6).

inhibited nigericin-stimulated ATPase activity of tobacco callus (28) and ΔpH generation in vesicles from oat roots (6), but we were uncertain about its specificity. Our present results show that DIDS (2–5 μM) inhibited MgATPase activity in the presence or absence of vanadate (Table III), indicating that DIDS inhibition of ATPase activity was independent of Cl^- as reported previously (1). However, Cl^--stimulated ATPase was 32% inhibited by 10 μM DIDS which had little (10%) or no effect on K^+-stimulated ATPase activity (Table III). Partial DIDS inhibition of the MgATPase activity from the 6/12% dextran interface (Table III) was probably due to the presence of vanadate-insensitive ATPase (Table II). The concentration dependence of DIDS inhibition was similar for MgATPase, and Cl^--stimulated ATPase activity in the presence or absence of gramicidin (Table III, data not shown). These results suggested that DIDS may be a relatively specific inhibitor of a vanadate-insensitive ATPase. Incomplete inhibition by DIDS of Cl^--stimulated ATPase could be due to the multiple modes of Cl^- activation of the ATPase (6). A more detailed study of the anion-sensitive ATPase is reported in a separate paper.

DISCUSSION

At least two distinct types of electrogenic proton-pumping ATPases are found in microsomal (nonmitochondrial) vesicles of oat roots. The two types of ATPases can be separated partially with a linear dextran gradient of 0.5% to 10% or a linear sucrose gradient (25–45%). One type of ATPase is enriched in the low density vesicles found between 1% to 4% dextran and the other type is enriched in vesicles around 4% to 8% dextran. Evidence for two types of proton-pumping ATPases are the following: (a) nigericin-stimulated ATPase activities can be resolved on a linear dextran gradient into two major peaks (Fig. 5); (b) ATPase activity can be resolved into a peak of vanadate-insensitive activity and another peak of vanadate-sensitive activity (Figs. 3 and 4); (c) ATP-dependent membrane potential generation can be resolved into two types of activities (a vanadate-insensitive and a vanadate-sensitive activity) distributed similarly to the ATPase activities (Fig. 3c); (d) ATP-dependent proton pumping as monitored by methylamine accumulation is insensitive to vanadate and enriched in low-density vesicles (6); (e) proton pumping as determined by quinacrine fluorescence quenching is partially vanadate-inhibited (6, 29); and (f) an anion-sensitive ATPase is enriched in low density vesicles while a K^+-stimulated enzyme is enriched in vesicles of higher density (Table II; Fig. 6).

The two types of electrogenic, proton-pumping ATPases can be identified by the following properties: one ATPase, enriched in low density vesicles, is stimulated by Cl^-, inhibited by NO_3^- or DIDS, and insensitive to cations and vanadate. The other ATPase, enriched in vesicles at 4% to 8% dextran, is stimulated by K^+, relatively insensitive to anions, and inhibited by vanadate but not by DIDS.

The properties of the two types of electrogenic, proton-pumping ATPases suggest that the anion-sensitive and K^+-sensitive ATPases may be enriched in the tonoplast (references in Ref. 6; 32) and plasma membrane (9), respectively. Though dextran gradients did not permit a complete separation of microsomal membranes, the Cl^--stimulated, vanadate-insensitive ATPase activity was distributed in vesicles of low density (Figs. 3, 7, 8). Using a continuous sucrose gradient, the vanadate-insensitive and vanadate-sensitive ATPases were enriched in vesicles at 25% to 32% (1.10–1.13 g/ml) and 32% to 37% (1.13–1.16 g/ml) sucrose, respectively (Fig. 4). These membrane densities are in the range of densities characteristic to tonoplast (2) and plasma membranes on sucrose gradients (22). Our results of the vanadate-insensitive ATPase are similar to those from corn coleoptiles (17) and corn roots (7) where a Cl^--stimulated ATPase of low density was thought to originate from vacuolar membranes. However, our results suggest that a vanadate-insensitive ATPase may also be associated with the ER (10) while a vanadate-sensitive ATPase could be associated with Golgi membranes. These results would not be surprising since vacuolar membranes might originate from the ER, and plasma membranes might originate in part from fusion of Golgi vesicles to the cell membrane.

Our conclusion of two types of electrogenic H^+-pumping ATPases enriched in the vacuolar and plasma membrane of plant tissues is supported by Scherer's finding (24) where the distribution of ionophore-stimulated ATPases from pumpkin hypocotyl correlated with markers of the plasma membrane and tonoplast on a sucrose gradient.

Instead of using KCl as the salt, the K^+-stimulated MgATPase might be better studied by using K^+-iminodiacetate where the anion has little or no effect on ATPase, membrane potential or pH gradient activities (31). The Cl^--stimulated MgATPase can be more appropriately determined with BTP-Cl or choline-Cl, where the organic cations have little or no effect on ATPase, membrane potential, or pH gradient activities (6).

The differential sensitivities of the two types of ATPases to K^+ or Cl^- and to inhibitors (vanadate or DIDS) clearly suggest that the mechanism of ATP hydrolysis, proton pumping, and the molecular properties of the two enzymes differ. For example, vanadate inhibits phosphohydrolases that form a covalent phosphoenzyme intermediate in their reaction mechanism (15). Lack

Table II. *Vanadate, K^+, or Cl^- Sensitivity of ATPase Activities of Microsomal Vesicles from Oat Roots Isolated at the 0/6 and 6/15% Dextran Interface*

A. Specific activities of vanadate-sensitive and vanadate-insensitive ATPases.

Condition	MgSalt-ATPase			Salt-Stimulated ATPase		
	−Vanadate	+Vanadate	ΔVanadate	−Vanadate	+Vanadate	ΔVanadate
	μmol Pi/mg protein·h					
0/6% Dextran interface						
$MgSO_4$	6.69	5.41	1.28			
MgKIDA	8.42	5.47	2.95	1.73	0.06	1.67
MgBTPCl	10.67	8.78	1.89	3.98	3.37	0.61
MgKCl	11.22	8.93	2.29	4.53	3.52	1.01
6/15% Dextran interface						
$MgSO_4$	19.40	11.29	8.11			
MgKIDA	29.59	11.33	16.26	8.19	0.04	8.15
MgBTPCl	25.03	16.92	8.11	5.63	5.63	0
MgKCl	31.80	12.25	19.55	12.40	0.96	11.44

B. Relative activities of vanadate-sensitive and vanadate-insensitive ATPases in vesicles isolated from the 0/6% and 6/15% dextran interfaces. Reaction media contained 30 mM Hepes-BTP at pH 6.5, 3 mM $MgSO_4$, 3 mM ATP, 5 μg/ml oligomycin, ±200 μM sodium orthovanadate, and 50 mM salt (KCl, BTPCl, or KIDA). Results are mean of 3 experiments.

Condition	Relative Specific Activity			
	MgSalt-ATPase		Salt-Stimulated ATPase	
	Vanadate insensitive	Vanadate sensitive	Vanadate insensitive	Vanadate sensitive
	%			
0/6% Dextran interface				
$MgSO_4$	81	19		
MgKIDA	64	36	3	97
MgBTP-Cl	82	18	85	15
MgKCl	79	21	78	22
6/15% Dextran interface				
$MgSO_4$	58	42		
MgKIDA	41	59	1	99
MgBTPCl	67	33	100	0
MgKCl	38	62	8	92

Table III. *Effect of DIDS on ATPase Activities of Vesicles Isolated from the 0/6% and 6/12% Dextran Interface*

ATPase activities of vesicles isolated from the 0/6% dextran interface were measured in the presence of 100 μM vanadate, 25 mM Hepes-BTP at pH 6.7, 3 mM $MgSO_4$, 3 mM ATP-BTP, 100 μM ammonium molybdate, 0.2 mM NaN_3, with or without 50 mM choline chloride. ATPase activities of vesicles isolated from the 6/12% dextran interface were measured in mixtures containing 25 mM Hepes-BTP at pH 6.7, 3 mM $MgSO_4$, 3 mM ATP-BTP, 100 μM ammonium molybdate, 0.2 mM NaN_3, with or without 50 mM KIDA. Relative activities are the average of 3 or 5 experiments.

DIDS	Relative ATPase Activity			
	0/6% Dextran interface		6/12% Dextran interface	
	Mg	Cl^--stimulated	Mg	K^+-stimulated
μM	*% (μmol Pi/mg protein·h)*			
0	100 (11.0)	100 (3.2)	100 (14.1)	100 (4.7)
2	83	89	92	110
5	63	77	72	104
10	56	68	60	90

of vanadate inhibition could involve a reaction mechanism where a phosphorylated intermediate is not formed, as in mitochondrial/chloroplast/bacterial-type ATPases. Different types of electrogenic, proton-pumping ATPases could be one way of regulating proton pumping and maintaining homeostasis under changing cellular and environmental conditions.

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