PESTICIDE BIOCHEMISTRY AND PHYSIOLOGY 28, 17–28 (1987)

Use of Tonoplast and Plasma Membrane Vesicles from Oat Root to Investigate Herbicidal Disruption of Proton Gradients

DENISE MARIE RATTERMAN AND NELSON E. BALKE

Department of Agronomy, University of Wisconsin, Madison, Wisconsin 53706

Received July 17, 1986; accepted November 26, 1986

Tonoplast-enriched (low-density) and plasma membrane-enriched (high-density) vesicle fractions were isolated from roots of oat (*Avena sativa* L.) seedlings. Membrane vesicles of both types were able to generate and maintain proton gradients in the presence of Mg^{2+} and ATP as determined by the quenching of quinacrine fluorescence. Proton gradient generation by the low-density vesicles was highly sensitive to inhibitors of tonoplast-type ATPase. Nitrate-resistant proton gradient generation by the high-density vesicles was only partially sensitive (15%) to vanadate (200 μM), an inhibitor of the plasma membrane ATPase. Oryzalin (0.1 mM) inhibited the initial rate of proton gradient formation and reduced the maximum proton gradient generated across both vesicle types by about 70%. Oxyfluorfen (0.1 mM) did not affect the production of proton gradients across the tonoplast-enriched vesicles but did accelerate the rate of decay of the gradient after a lag of about 3 min. Oxyfluorfen (0.1 mM) did not affect significantly the production and maintenance of proton gradients across the plasma membrane-enriched vesicles. Both herbicides produced concentration-dependent effects on proton gradients across the tonoplast-enriched vesicles. These results support the hypothesis that some herbicides can directly affect the functioning of plant cellular membranes.

INTRODUCTION

A number of herbicides have been shown to inhibit the uptake of minerals, sugars, or amino acids by plant cells (1–7). Inhibition of these transport processes may occur directly via the action of the herbicide on either the transport proteins or the membrane structure. Alternatively, the herbicide may interfere with transport indirectly by affecting the driving force for the process of transport.

A large body of work in the fields of bioenergetics and membrane transport has focused on the importance of ionic gradients maintained across cellular membranes and the utilization of these gradients as the driving forces for transport systems localized within the membranes. In plant cells, the proton gradients maintained across the plasma membrane and across the tonoplast are believed to be the major ionic gradients involved in the large electrical gradient across these membranes and in transport phenomena (8–10). Proton-translocating ATPases required for the maintenance of these proton gradients have been studied in a number of plant tissues (11). These studies have been enhanced by the development of techniques for the purification of vesicles of cellular membranes in a manner such that the isolated vesicles are able to maintain proton gradients formed by proton-translocating ATPases (12). The proton gradients thus formed have been monitored using fluorescent amines such as quinacrine, 9-aminoacridine, and acridine orange (13–15) and radiolabeled amines such as methylamine (16, 17), which distribute across a membrane according to the transmembrane pH gradient.

We have used these techniques of membrane vesicle isolation and proton gradient monitoring to investigate the effect of herbicides on the ability of isolated plasma membrane and tonoplast vesicles of oat root cells to develop and maintain proton gradients. In such a reduced system, possible secondary effects of a herbicide me-

0048-3575/87 $3.00

diated by effects on lipid metabolism or cellular ATP levels are avoided; thus only rapid, direct effects of the herbicide on the membranes are measured.

The two herbicides chosen for this study were oryzalin, a dinitroaniline, and oxyfluorfen, a diphenylether. Herbicides of both of these chemical families have been shown to inhibit mineral ion absorption by plant cells (2, 18). The mechanism of action of the dinitroanilines has not been clearly defined. Evidence suggests that they may act by interaction with tubulin protein causing disruption of microtubule assembly (19) and with membranes causing disruption of Ca^{2+} sequestration by mitochondria (20), uncoupling of respiration (21, 22), inhibitory uncoupling of photosynthesis (23, 24), and inhibition of plasma membrane enzymes (25). These effects on membranes have been observed in *in vitro* systems with I_{50} values in the range of 30 to 100 μM. Diphenylethers such as oxyfluorfen act primarily by peroxidative destruction of membranous structures and soluble enzymes following activation which requires light (26, 27). Destruction of membranes in cells can be observed after treatment with 1 μM herbicide and effects on membrane permeability in intact cells observed at 10 nM herbicide (27). Using the fluorescent probe quinacrine to monitor proton gradients, we have shown that these herbicides are able to alter the ability of membrane vesicles isolated from oat root to generate and maintain proton gradients.

MATERIALS AND METHODS

Plant material. Oat (*Avena sativa* L. cv. Dal) seeds were germinated on moist cheesecloth and the seedlings were grown in the dark at 26 ± 2°C over an aerated solution of 1 mM $CaSO_4$. On the fifth day, the apical 8–10 cm of the roots were harvested and used for vesicle isolation.

Vesicle isolation. Following the procedure of Churchill *et al.* (28), root tissue (30 g) was homogenized in 3 ml/g root tissue homogenization medium (HM; 250 mM mannitol, 25 mM Hepes–BTP[1] (pH 7.4), 3 mM EGTA, 1 mM DTT, 1 mM PMSF, and 0.5% (w/v) BSA) at 4°C with a chilled mortar and pestle. The homogenate was strained through four layers of cheesecloth and the material retained on the cheesecloth was washed with 1 ml HM/g root tissue and strained a second time. Combined filtrates were centrifuged at 13,000g for 15 min to remove unbroken cells, cell wall debris, and mitochondria. This supernatant was centrifuged at 60,000g for 30 min to obtain a microsomal pellet. Membrane vesicles of different densities were separated using a dextran discontinuous gradient. After resuspension in 2 ml of a resuspension medium (RM; 250 mM mannitol, 2.5 mM Hepes–BTP (pH 7.4), 1 mM DTT), the microsomal fraction was layered over a 5-ml layer of 6% (w/w) dextran (MW 70,000) that in turn was over a 30-ml layer of 15% (w/w) dextran. The gradient was centrifuged at 70,000g for 2 hr and vesicles accumulating at the 0–6% interface (low-density vesicles) and at the 6–15% interface (high-density vesicles) were collected separately, diluted to 25–35 μg protein/75 μl with RM, and stored on ice until used in assays. Vesicles were used on the same day they were isolated. All dextran solutions were prepared in 250 mM mannitol and 2.5 mM Hepes–BTP (pH 7.4). The amount of vesicles was quantitated by protein content as estimated by the Bradford method after solubilization in 0.1% Triton X-100 (29).

Quenching of quinacrine fluorescence. Formation of proton gradients across the vesicle membranes was monitored using the lipophilic fluorescent amine quinacrine. As the lumens of the vesicles become acidified, quinacrine accumulates inside the

[1] Abbreviations: BTP, bis-Tris propane [1,3-bis(Tris-(hydroxymethyl)-methylamino)propane]; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

vesicles and the fluorescence of the accumulated quinacrine is quenched (30). Vesicles (25–35 μg protein) were incubated at room temperature in a reaction mixture containing 1.5 m*M* ATP–BTP (pH 7.4), 1.5 m*M* $MgSO_4$, 10 m*M* Hepes–BTP (pH 7.4), 0.33 m*M* EGTA, 1.25 μ*M* quinacrine, 190 m*M* mannitol, and 50 m*M* KCl or KNO_3 with or without herbicide (0.1 m*M*) in a final volume of 1.5 ml (17). Proton gradient formation was initiated by the addition of the magnesium and potassium salts in a 30-μl volume. Fluorescence of quinacrine was monitored at 500 nm (4-nm slit width) after excitation at 420 nm (10-nm slit width) with a Perkin–Elmer 650-10S fluorescence spectrophotometer. Prior to initiation, fluorescence was set at 90% relative fluorescence. In those instances in which the herbicide being tested absorbed 420 or 500 nm light, relative fluorescence levels were set to the level observed for the control immediately after initiation (at final volume). Unless noted, quenching of quinacrine fluorescence by high-density vesicles, primarily plasma membrane, was monitored in the presence of 50 m*M* KNO_3 in order to eliminate the contribution of contaminating nitrate-sensitive proton-pumping ATPases located on the tonoplast (28). Decreases in quinacrine fluorescence not associated with development of proton gradients was monitored by omitting $MgSO_4$ from the initiation salt solution (blank). When herbicides were added, herbicides were in contact with the vesicles less than 35 sec before initiation of proton pumping. The final ethanol concentration in herbicide trials was 0.5%.

Initial slope (i.e., rate) of fluorescence quenching (*S*) was measured over the first minute of quench and was expressed as percentage relative fluorescence/min (Fig. 1). Maximum quench (*Q*) was calculated as the difference between the maximum relative fluorescence observed after initiation of proton pumping and the relative fluorescence observed during the plateau phase. Plateau phase was defined as the period during which changes in fluorescence were equal to that of the blank at the corresponding times. Rate of fluorescence rise (*R*) was measured between 5 and 6 min from the onset of quench and was expressed as percentage relative fluorescence/min. All *S*, *Q*, and *R* values were corrected for decreases of fluorescence observed in the corresponding blank.

Values reported are means ± SE as percentage of corresponding control of at least two experiments, each assayed in triplicate. In Tables 1 and 2, the corresponding control for the DIDS, oligomycin, and herbicide treatments contained 0.5% ethanol. Differences from control values were determined by Fisher's LSD comparison of untransformed means at $\alpha = 0.05$.

Chemicals. Technical grade oxyfluorfen (98.8%) and oryzalin (97.1%) were gifts of Rohm & Haas and Elanco/Eli Lilly, respectively. Herbicide stocks were prepared in absolute ethanol and stored at −15°C in glass tubes inside closed opaque containers until used. Vanadium-free sodium ATP (Sigma) was converted to the BTP salt using Dowex-50W cation exchange resin. Other chemicals were purchased from Sigma Chemical Co., St. Louis, Missouri.

RESULTS

ATP-dependent fluorescence quenching. Typical time courses of ATP-dependent quenching of quinacrine fluorescence by the two vesicle fractions are shown in Fig. 1. Onset of quenching by low-density vesicles occurred after a lag of 6 to 12 sec. Such lag phases, also observed with high-density vesicles, may reflect residual buffering capacity within the vesicles. Quenching was linear within the first minute of quench and a maximum level of quench was observed in 2.5 to 3.5 min. The plateau phase observed at maximum quench was maintained typically for 2 to 5 min after which a slow increase in fluorescence was observed, indicating that the

proton gradient would decay slowly. Addition of the proton ionophore gramicidin (2.5 μg/ml) at any point in the reaction resulted in an immediate increase of fluorescence to the level of the blank at the corresponding time (Fig. 1). This result indicated that the decrease in quinacrine fluorescence was associated with the development of proton gradients across the vesicle membranes which were collapsed by gramicidin. Other ionophores, such as nigericin (2 μ*M*) and CCCP with valinomycin (each 2 μ*M*) produced similar effects (results not shown). Similar time courses of quenching were observed when ATP, rather than $MgSO_4$, was used to initiate the reaction (data not shown). Ethanol (0.5%, v/v) did not affect *S* and *Q* values, but had a variable effect on *R* values (Table 1). Results of herbicide trials were therefore expressed relative to control time courses in which 0.5% ethanol was present.

Quenching of quinacrine fluorescence by high-density vesicles (Fig. 1B) was also linear during the first minute of quench.

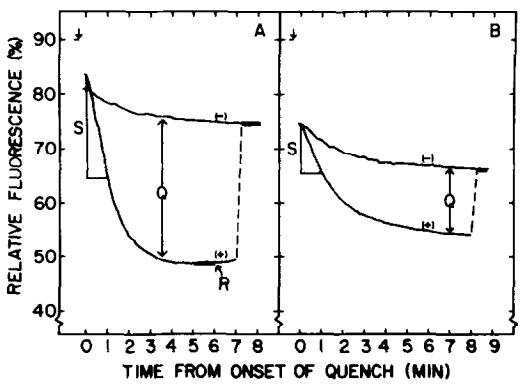

FIG. 1. *Time courses of ATP-dependent quenching of quinacrine fluorescence by low-density (A) and high-density (B) vesicles in the absence (−) and presence (+) of $MgSO_4$. Quenching was measured in the presence of 50 mM KCl for low-density vesicles and 50 mM KNO_3 for high-density vesicles. The arrow at −0.5 min indicates initiation of the reaction. Gramicidin (2.5 μg/ml) was added to the reaction mixtures containing $MgSO_4$ at 7 and 8 min from onset of quenching for the low- and high-density vesicles, respectively. Measurement of S, Q, and R as described under Materials and Methods are illustrated.*

Maximum quenching was observed in 6 to 7 min and was maintained for periods up to 45 min. Response to the addition of gramicidin was similar to that observed for the low-density vesicles. Ethanol (0.5%, v/v) did not affect *S* and *Q* values (Table 1). Positive *R* values were not observed in the absence or presence of ethanol.

Increasing the concentration of vesicles in the reaction mixture, with a constant quinacrine concentration of 1.25 μ*M*, increased the *S* and *Q* values for vesicle concentrations of less than 65 μg protein/1.5 ml (Fig. 2A). Above this concentration, increases in *S* and *Q* did not occur with increasing vesicle concentrations. A similar saturation of the probe response was observed with the high-density vesicles with a cutoff concentration of approximately 40 μg protein/1.5 ml (Fig. 2B). For all further investigations, vesicle concentrations of less than 35 μg protein/1.5 ml were used.

Membrane identity. Churchill *et al.* (28) have characterized the vesicle fractions obtained from oat roots and have suggested that the low-density vesicle fraction is enriched in vesicles of tonoplast origin and that the high-density vesicles are enriched in vesicles of plasma membrane origin. The effect of inhibitors on the vesicles we obtained from oat roots agreed well with their results.

Vanadate (0.2 m*M*), an inhibitor of the plasma membrane ATPase (31), did not affect the quenching of quinacrine fluorescence by low-density vesicles (Table 1), indicating the absence of plasma membrane contribution in this response. Combination of the presence of the tonoplast ATPase inhibitor nitrate (50 m*M*) and the absence of the tonoplast ATPase stimulator chloride completely eliminated ATP-dependent quenching of quinacrine fluorescence by the low-density vesicles. This result indicated that the tonoplast ATPase was primarily responsible for the quenching of quinacrine fluorescence by the low-density vesicles. It also suggested that replacement

TABLE 1

Effect of Solvent, Standard Inhibitors, and Herbicides on the Initial Rate (S), Maximum Quench (Q), and Rate of Fluorescence Rise (R) of ATP-Dependent Quenching of Quinacrine Fluorescence by Membrane Vesicles

	Percentage of corresponding control[a]				
	Low-density vesicles			High-density vesicles	
	S	*Q*	*R*	*S*	*Q*
Ethanol (0.5%, v/v)	94 ± 2	92 ± 2	190 ± 54	104 ± 4	101 ± 4
Vanadate (0.2 m*M*)	100 ± 5	94 ± 3	—	84 ± 6	85 ± 4
Nitrate (50 m*M*)[b]	6 ± 2	7 ± 2	25 ± 11	—	—
DIDS (0.01 m*M*)	12 ± 5	29 ± 7	20 ± 4	—	—
Oligomycin (0.2 μg/ml)	103 ± 5	99 ± 6	—	111 ± 6	108 ± 4
Oryzalin (0.1 m*M*)	34 ± 9	28 ± 7	146 ± 52	31 ± 16	26 ± 12
Oxyfluorfen (0.1 m*M*)	113 ± 5	115 ± 7	173 ± 13	107 ± 9	83 ± 7

Note. Quenching was measured in the presence of 50 m*M* KCl for low-density vesicle and 50 m*M* KNO_3 for high-density vesicles.

[a] Control for DIDS, oligomycin, oryzalin, and oxyfluorfen treatments contained 0.5% ethanol. Control for ethanol, vanadate, and nitrate treatments contained no solvent additions.

[b] 50 m*M* KNO_3 replaced 50 m*M* KCl in the reaction mixture.

of KCl by KNO_3 in the reaction mixture could effectively eliminate tonoplast-type contribution to the measurements. DIDS (0.01 m*M*), another inhibitor of the tonoplast ATPase (11), caused a dramatic reduction of fluorescence quenching by this fraction.

With the high-density vesicles, vanadate (0.2 m*M*) only partially reduced the quenching of fluorescence (Table 1). Similar results were obtained whether the vanadate stock solutions were prepared with concentration and pH within the ranges suggested by Gallagher and Leonard (31) or prepared according to the method of Surowy and Sussman (32). This result indicated that this fraction, although enriched in plasma membrane vesicles, also contained membranes with a vanadate- and nitrate-resistant proton pump. Oligomycin (0.2 μg/ml, equivalent to 10 μg/mg protein) had little effect on either fraction, indicating the absence of tight submitochondrial vesicles of the proper orientation to accumulate protons.

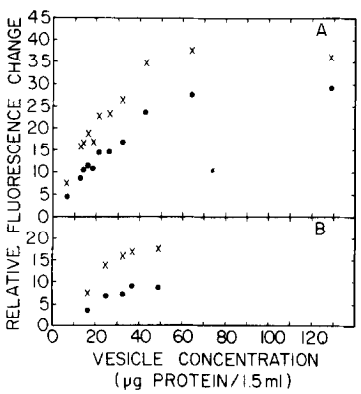

FIG. 2. *S* (●) *and Q* (x) *values for ATP-dependent quenching of quinacrine fluorescence by varying concentrations of low-density (A) and high-density (B) vesicles. S values are expressed as percentage relative fluorescence change/min; Q values are expressed as percentage relative fluorescence change. All reaction mixtures contained* $MgSO_4$. *Other assay conditions were as for Fig. 1.*

Effect of herbicides. Oryzalin (0.1 m*M*) caused a reduction of *S* and *Q* for both vesicle fractions (Fig. 3 and Table 1). Because oryzalin solutions absorbed 420 nm light, the adjustments described under Materials and Methods were used for all measurements involving oryzalin-treated vesicles. Upon the addition of gramicidin (2.5 μg/ml), fluorescence levels increased to the level of the unadjusted blank (without oryzalin) at the corresponding time (Fig. 3), indicating the appropriateness of the adjustments made. In the presence of oxyfluorfen

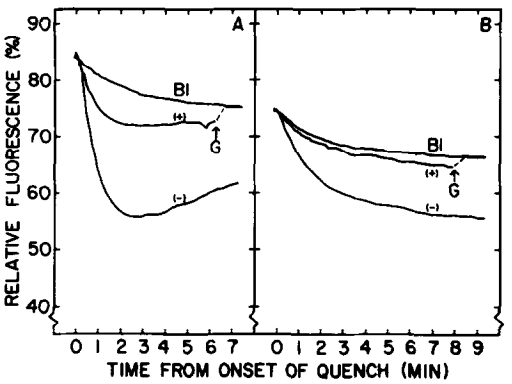

FIG. 3. *Representative time courses of ATP-dependent quenching of quinacrine fluorescence by low-density (A) and high-density (B) vesicles in the absence (−) and presence (+) of 0.1 mM oryzalin. Assay conditions were as for Fig. 2. Uppermost traces (Bl) are those of blanks without herbicide. The arrows indicate the addition of gramicidin (2.5 μg/ml) to the reaction mixture.*

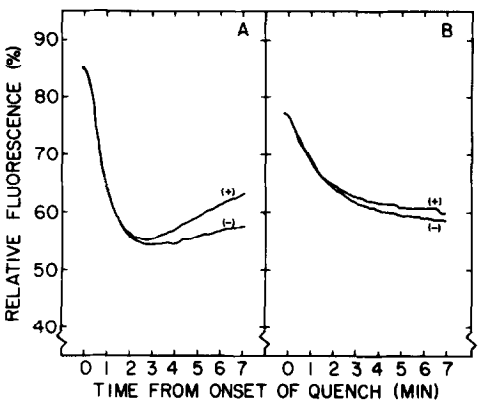

FIG. 4. *Representative time courses of ATP-dependent quenching of quinacrine fluorescence by low-density (A) and high-density (B) vesicles in the absence (−) and presence (+) of 0.1 mM oxyfluorfen. Assay conditions were as for Fig. 2.*

(0.1 m*M*), *S* and *Q* for the light-density vesicles were unaffected, but an enhanced rate of decay of the gradient was observed beginning at about 3 min (Fig. 4A). This enhanced decay was reflected in the increase of *R* (Table 1). With the high-density vesicles, oxyfluorfen did not cause a significant reduction of either *S* or *Q* (Table 1 and Fig. 4B). Oxyfluorfen, unlike oryzalin, did not affect the fluorescence of the reaction mixture at final volume.

The effect of different concentrations of oryzalin and oxyfluorfen on the production of proton gradients by the low-density vesicles was also determined. Only the highest concentration of oryzalin tested (0.1 m*M*) affected *S* as well as *Q* (Fig. 5 and Table 2). At 0.05 m*M*, oryzalin significantly affected *Q*. Oxyfluorfen increased *R* for low-density vesicles at 0.01 m*M*, 0.05 m*M*, and 0.1 m*M* (Fig. 6 and Table 2). No significant effect on *S* or *Q* were observed for any of the oxyfluorfen concentrations. The apparent changes in *Q* values depicted in Fig. 6 are not statistically significant (Table 2).

DISCUSSION

Our results demonstrate that membrane vesicles isolated from oat roots can be used to investigate direct effects of herbicides on the ability of plasma membrane and tonoplast to maintain proton gradients. Both membrane fractions contain sufficient numbers of sealed vesicles for the generation and maintenance of proton gradients to be monitored. The quenching of quinacrine fluorescence by the vesicles in both the presence and the absence of herbicides reflect the development of proton gradients, not herbicide-probe interactions, as deter-

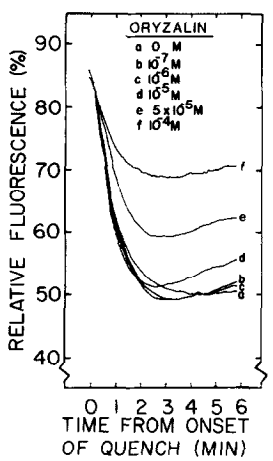

FIG. 5. *Representative time courses of ATP-dependent quenching of quinacrine fluorescence by low-density vesicles in the presence of various concentrations of oryzalin.*

mined by the reversal of quenching upon addition of the proton ionophore gramicidin at any stage in the reaction (Figs. 1, 3).

The majority of the investigations into effects of herbicides on the transport functions of membranes had used tissue segments or intact cells. In some cases the rapidity of the response (25) suggested that the site of action of the herbicide was at the plasma membrane. The use of isolated cellular membranes, instead of whole cells, offers several advantages for discerning such direct effects of herbicides on a membrane. In this reduced system, the composition of the medium bathing the vesicles can be controlled. This control is particularly important with respect to the concentration of ATP available to the proton-pumping ATPase. Because oryzalin can act as an uncoupler of respiration (21), in intact cells it may alter the ability of cellular membranes to maintain proton gradients due to a reduction of cytoplasmic ATP levels. The use of membrane vesicles revealed that oryzalin can have an additional, direct effect on the plasma membrane and tonoplast (Fig. 3, Table 1). Also, the controlled ionic environment present during the fluorescence assays eliminates indirect effects of the herbicide resulting from altered sequestration of particular ions such as Ca^{2+}, as has been observed for a number of herbicides including oryzalin (20).

Also eliminated by the use of isolated membrane vesicles are indirect effects on membrane function due to an inhibition of the biosynthesis of component lipids or proteins. However, a herbicide may still possess the ability to alter the lipid composition of the vesicles by direct action as suggested for the effect of 2,4-dinitrophenol on the membranes of barley roots (33).

An additional advantage of using isolated vesicles is the ability to separate effects on the plasma membrane from effects on the tonoplast. Herbicidal inhibition of transport by cells could be caused by disruption of proton gradients maintained across either or both of these membranes. The two may respond differently to a specific herbicide. Using two vesicle fractions, we were able to test for differential action on the tonoplast and the plasma membrane.

Tonoplast and plasma membrane responses are separated by two means in our studies. The first of these is the separation of vesicle types based on their inherent densities, using a dextran discontinuous gradient. Secondly, because of possible contamination of the high-density (plasma membrane-enriched) vesicles by tonoplast vesicles, the assays utilizing high-density vesicles were performed with KNO_3 substituted for KCl in the reaction mixture. KNO_3 was shown to eliminate proton gradient generation across low-density vesicles (Table 1). There was no evidence for a contribution by sealed plasma membrane vesicles when fluorescence quenching was measured in the low-density fraction.

There are some limitations to the use of vesicles as a model system for membranes of intact cells. Factors that are important in the regulation of the proton pumps may not be present in the reduced system, because they might be soluble, loosely associated with the membrane, or susceptible to degradation during the isolation procedures. Other components, such as antioxidants, that may mitigate the response of intact membranes to direct effects of herbicides (27) may be eliminated similarly from the system. Also, membrane composition can be altered by the action of lipases and proteases during isolation procedures. The presence of fatty acids due to the degradation of lipids will affect proton permeability of membranes (34).

Another limitation is the inability to obtain vesicle fractions containing only one type of membrane. The development of techniques for the isolation of organelles, relatively free of contamination by other membranes, has been invaluable to the

TABLE 2

Effect of Various Concentrations of Oryzalin and Oxyfluorfen on the Initial Rate (S), Maximum Quench (Q), and Rate of Fluorescence Rise (R) of ATP-Dependent Quenching of Quinacrine Fluorescence by Low-Density Vesicles

	Percentage of control		
	S	*Q*	*R*
Ethanol control	100 ± 6	100 ± 2	100 ± 26
Oryzalin 10^{-7} *M*	102 ± 4	101 ± 6	135 ± 8
10^{-6} *M*	93 ± 10	90 ± 8	118 ± 16
10^{-5} *M*	99 ± 12	92 ± 10	160 ± 40
5×10^{-5} *M*	89 ± 11	78 ± 7	211 ± 58
10^{-4} *M*	57 ± 8	49 ± 11	61 ± 11
$LSD_{0.05}$	25	22	89
Ethanol control	100 ± 4	100 ± 6	100 ± 6
Oxyfluorfen 10^{-7} *M*	101 ± 6	105 ± 5	125 ± 26
10^{-6} *M*	102 ± 7	106 ± 4	141 ± 35
10^{-5} *M*	99 ± 8	108 ± 5	277 ± 33
5×10^{-5} *M*	94 ± 8	99 ± 5	214 ± 32
10^{-4} *M*	96 ± 4	104 ± 7	234 ± 40
$LSD_{0.05}$	18	14	44

study of the effects of herbicides on organellar membranes (35, 36). Marker enzyme studies (28) demonstrated that the low-density fraction from oat roots contained ER and Golgi membranes as well as tonoplast and that the high-density fraction had vesicles of plasma membrane and Golgi origin. Only those membranes with proton-translocating ATPases or some other mechanism for translocating H^+ will contribute to the measurements of proton gradient generation, however. The almost complete elimination of fluorescence quenching by the low-density vesicles in the presence of inhibitors of the tonoplast-type proton pump (Table 1) indicates that the proton gradients were across tonoplast membranes. With the high-density vesicles, the lack of complete inhibition of quenching by both nitrate (50 m*M*, no chloride present) and vanadate (0.2 m*M*) suggests that proton gradients across vesicles containing a nitrate- and vanadate-insensitive proton pump contribute to quenching. This component may be vesicles of Golgi origin as has been characterized in corn coleoptile microsomes (37).

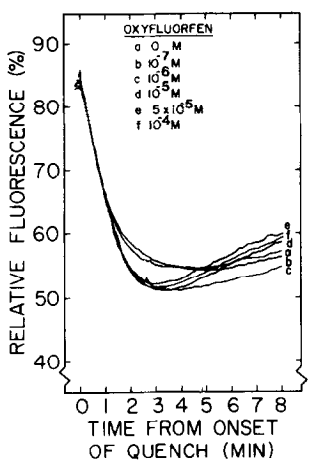

FIG. 6. *Representative time courses of ATP-dependent quenching of quinacrine fluorescence by low-density vesicles in the presence of various concentrations of oxyfluorfen.*

A concern in the design of experiments utilizing any membrane vesicles is indicated by the saturation of the probe response observed for both vesicle types (Fig. 2). It is clear that vesicle-to-quinacrine concentration ratios must be kept low to retain the sensitivity necessary to detect changes in the population of sealed ves-

icles. As well, response to herbicide may be dependent on the effective herbicide concentration within the membranes rather than the concentration of herbicide in the medium. In this case, the response to 0.1 m*M* herbicide might differ when vesicle concentrations differ. Inhibition levels correlating with inhibitor/mg protein ratios rather than inhibitor concentration has been observed for oligomycin (38). In light of this we chose to use similar vesicle concentrations in all assays (25–35 μg protein/1.5 ml).

Because the generation of proton gradients across membrane vesicles was monitored for a population of membrane vesicles rather than a single vesicle, it is important to know if the membrane vesicle population was homogeneous. Knowing this, one would be able to interpret the significance of *S*, *Q*, and *R* with respect to the generation of a proton gradient across a vesicle of the population. Because the evidence indicates that vesicles of tonoplast origin are the major contributor to the ATP-dependent quenching of quinacrine fluorescence by low-density vesicles, one could consider this as a homogeneous population of vesicles. In such an instance, a reduction of quenching would reflect directly the reduction of the proton gradient across a vesicle of this population.

Herbicides may act to disrupt proton gradients maintained across a vesicle membrane by inhibiting the proton pumping necessary to maintain the gradient or by increasing the permeability of the membrane to protons. The latter effect may be caused by specific protonophorous activity or by more general disruption of membrane structure. In order to evaluate the type of effect a herbicide has on a membrane it is necessary to separate effects on proton pumping from effects on proton permeability. In our studies, the addition of a herbicide to the membrane vesicles prior to initiation of proton pumping allowed observation of effects of the herbicide on both the development of the proton gradient and on the magnitude of the equilibrium proton gradient. Although the probe quinacrine responds to the net proton gradient across the vesicle membranes, the initial rate of quench (*S*) would reflect largely the rate of proton influx (pumping into the vesicle lumen) because proton efflux at the initially low proton gradient would be low in comparison to the rate of proton pumping (13, 39). The efflux of protons would increase as the proton gradient across the vesicle membrane increased to an equilibrium level, which would be achieved when proton efflux equalled influx. The magnitude of the equilibrium proton gradient, measured by *Q*, would be a function of both pump activity and the permeability of the membrane to protons. Thus, a comparison of the relative effects of a herbicide on *S* and *Q* might indicate whether the compound acted to inhibit proton pumping or to increase proton efflux from the vesicles.

The similar reduction of both *S* and *Q* of proton gradient development across the low-density vesicles by oryzalin (0.1 m*M*) (Fig. 3A) could be explained in two ways: (1) a rapid inhibition of proton pumping, which would in turn reduce the equilibrium proton gradient formed, or (2) a rapid dissipation of the proton gradient being formed even in the initial phase of proton gradient formation. As the concentration of oryzalin was decreased, the effect on *S* was lost (Fig. 5). This result is consistent with the hypothesis that oryzalin increases proton efflux from the vesicle and that at 0.1 m*M* it is effective at low proton gradients. This result needs to be confirmed by determining the effect of oryzalin on ATPase activity.

The effect of oxyfluorfen on the generation of proton gradients across the tonoplast vesicles was distinctly different from that of oryzalin and indicates that either oxyfluorfen acts to disrupt the proton gradient only after a large proton gradient is generated or that its action involves an ini-

tial lag period. The rate of decay of the proton gradient across oxyfluorfen-treated tonoplast vesicles (Fig. 4A) was not rapid enough to rule out the possibility that the effect was due to inhibition of the proton pump after such a lag period.

With the high-density vesicles the presence of a mixed, rather than homogeneous, population of vesicles complicates the interpretation of the quenching data because, analogous to the vesicle concentration studies, the complete elimination of proton gradients across some subpopulation of the total vesicle population would result in a partial reduction of both S and Q. The large reduction of S and Q by oryzalin (Fig. 3B) indicates that all types of vesicles capable of pumping protons in the high-density fraction were affected. Similar to the result with tonoplast vesicles, this may be due to the rapid inhibition of proton pumping or the rapid dissipation of the proton gradient. This second possibility is supported by the lack of inhibition of ATPase activity by oryzalin in plasma membrane-enriched vesicles from oat root observed by Watson and co-workers (40).

Oryzalin appears to be nonspecific in its ability to disrupt proton gradients across membranes. This conclusion is supported by its uncoupling activity on isolated mitochondria (21, 22). Because of this ability to disrupt the proton gradients maintained across various cellular membranes, oryzalin could inhibit a wide variety of transport processes. The differential activity of oxyfluorfen on the tonoplast and plasma membrane vesicles suggests that the tonoplast may be more susceptible to oxyfluorfen action. Alternatively, components involved in the activation of oxyfluorfen may be present in the low-density but not the high-density fractions.

Overall, these results demonstrate that the measurement of proton gradients generated across vesicles of tonoplast and plasma membrane can be used to identify herbicides capable of disrupting proton gradients across these membranes. Additional experiments could be designed to discriminate between possible mechanisms of disruption suggested by the evaluation of the effects on S, Q, and R. Also, such experiments might add insight into the mechanism of how herbicides interact with cellular membranes to reduce mineral ion absorption and content of plant cells (7).

ACKNOWLEDGMENTS

This material is based upon work supported by a National Science Foundation Graduate Fellowship to D.M.R., with additional support by the Graduate School and the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

REFERENCES

1. J. P. Blein, Action of some herbicides on growth, respiration, plasmalemma integrity and proton extrusion of *Acer pseudoplatanus* cells. I. Substituted ureas, *Pestic. Biochem. Physiol.* **16,** 179 (1981).
2. J. P. Blein, Action of some herbicides on growth, respiration, plasmalemma integrity, and proton extrusion of *Acer pseudoplatanus* cells. II. Amides, diphenyl ethers, nitriles, phenols, triazines, and uracils, *Pestic. Biochem. Physiol.* **17,** 156 (1982).
3. G. Ferrari, S. Nardi, G. Cacco, and G. Dell'Angola, Sulfate transport of excised roots as an index of genotype response to herbicides, *Physiol. Plant.* **52,** 29 (1981).
4. E. Bergmannova and L. Taimr, The effect of chlorfenprop-methyl on ^{32}P uptake, transport and metabolism in *Avena fatua* L. and *Hordeum vulgare* L., *Weed Res.* **20,** 1 (1980).
5. B. Truelove, L. R. Jones, and D. E. Davis, Light and prometryne effects on leucine uptake and incorporation, *Weed Sci.* **21,** 24 (1973).
6. J. D. Mann, L. S. Jordan, and B. E. Day, A survey of herbicides for their effect upon protein synthesis, *Plant Physiol.* **40,** 840 (1965).
7. N. E. Balke, Herbicide effects on membrane functions, *in* "Weed Physiology" (S. O. Duke, Ed.), p. 113, CRC, Boca Raton, 1984.
8. R. M. Spanswick, Electrogenic ion pumps, *Annu. Rev. Plant Physiol.* **32,** 267 (1981).
9. R. T. Leonard, Membrane-associated ATPases and nutrient absorption by roots, *in* "Advances in Plant Nutrition" (P. B. Tinker and A. Lauchi, Eds.), Vol. 1, p. 209, Praeger, New York, 1984.
10. R. J. Poole, Electrogenic transport at the plasma membrane of plant cells, *in* "Membranes and

Transport" (A. N. Martonosi, Ed.), Vol. 2, p. 651, Plenum, New York, 1982.

11. H. Sze, H^+-Translocating ATPases: Advances using membrane vesicles, *Annu. Rev. Plant Physiol.* **36,** 175 (1985).
12. H. Sze and K. A. Churchill, Mg^{2+}/KCl-ATPase of plant plasma membranes is an electrogenic pump, *Proc. Natl. Acad. Sci. USA* **78**(9), 5578 (1981).
13. A. B. Bennett and R. M. Spanswick, Optical measurements of ΔpH and $\Delta\psi$ in corn root membrane vesicles: Kinetic analysis of Cl^- effects on a proton-translocating ATPase, *J. Membr. Biol.* **71,** 95 (1983).
14. G. F. E. Scherer, Subcellular localization of H^+-ATPase from pumpkin hypocotyls (*Cucurbita maxima* L.) by membrane fractionation, *Planta* **160,** 348 (1984).
15. F. Rasi-Caldogno, M. C. Pugliarello, and M. I. De Michelis, Electrogenic transport of protons driven by the plasma membrane ATPase in membrane vesicles from radish, *Plant Physiol.* **77,** 200 (1985).
16. I. J. Mettler, S. Mandala, and L. Taiz, Characterization of *in vitro* proton pumping by microsomal vesicles isolated from corn coleoptiles, *Plant Physiol.* **70,** 1738 (1982).
17. K. A. Churchill and H. Sze, Anion-sensitive, H^+-pumping ATPase in membrane vesicles from oat roots, *Plant Physiol.* **71,** 610 (1983).
18. D. L. Bucholtz and T. L. Lavy, Alachlor and trifluralin effects on nutrient uptake in oats and soybeans, *Agron. J.* **71,** 24 (1979).
19. S. D. Strachan and F. D. Hess, The biochemical mechanism of action of the dinitroaniline herbicide oryzalin, *Pestic. Biochem. Physiol.* **20,** 141 (1983).
20. C. Hertel and D. Marme, Herbicides and fungicides inhibit Ca^{2+} uptake by plant mitochondria: A possible mechanism of action, *Pestic. Biochem. Physiol.* **19,** 282 (1983).
21. B. Wang, S. Grooms and R. E. Frans, Response of soybean mitochondria to substituted dinitroaniline herbicides, *Weed Sci.* **22**(1), 64 (1974).
22. D. E. Moreland and S. C. Huber, Inhibition of photosynthesis and respiration by substituted 2,6-dinitroaniline herbicides. III. Effects on electron transport and membrane properties of isolated mung bean mitochondria, *Pestic. Biochem. Physiol.* **11,** 247 (1979).
23. D. E. Moreland, F. S. Farmer, and G. G. Hussey, Inhibition of photosynthesis and respiration by substituted 2,6-dinitroaniline herbicides. I. Effects on chloroplast and mitochondrial activities, *Pestic. Biochem. Physiol.* **2,** 342 (1972).
24. S. J. Robinson, C. F. Yocum, H. Ikuma, and F. Hayashi, Inhibition of chloroplast electron transport reactions by trifluralin and diallate, *Plant Physiol.* **60,** 840 (1977).
25. R. L. Travis and W. G. Woods, Studies on the mechanism of action of dinitramine: Effect on soybean root plasma membrane, *Plant Physiol.* **60,** 54 (1977).
26. K. J. Kunert, C. Homrighausen, H. Bohme, and P. Boger, Oxyfluorfen and lipid peroxidation: Protein damage as a phytotoxic consequence, *Weed Sci.* **33,** 766 (1985).
27. G. L. Orr and F. D. Hess, Proposed site(s) of action of new diphenyl ether herbicides, *in* "Biochemical Responses Induced by Herbicides" (D. E. Moreland, J. B. St. John, and F. D. Hess, Eds.), p. 131, American Chemical Society, Washington, DC, 1982.
28. K. A. Churchill, B. Holaway, and H. Sze, Separation of two types of electrogenic H^+-pumping ATPases from oat roots, *Plant Physiol.* **73,** 921 (1983).
29. G. O. Gogstad and M. B. Krutnes, Measurement of protein in cell suspensions using the Coomassie brilliant blue dye-binding assay, *Anal. Biochem.* **126,** 355 (1982).
30. H. C. Lee and J. G. Forte, A study of H^+ transport in gastric microsomal vesicles using fluorescent probes, *Biochim. Biophys. Acta* **508,** 339 (1978).
31. S. R. Gallagher and R. T. Leonard, Effect of vanadate, molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots, *Plant Physiol.* **70,** 1335 (1982).
32. T. K. Surowy and M. R. Sussman, Immunological cross-reactivity and inhibitor sensitivities of the plasma membrane H^+-ATPase from plants and fungi, *Biochim. Biophys. Acta* **848,** 24 (1986).
33. P. C. Jackson and J. B. St. John, Effects of 2,4-dinitrophenol on membrane lipids of roots, *Plant Physiol.* **70,** 858 (1982).
34. D. W. Deamer, Proton permeability in biological and model membranes, *in* "Intracellular pH: Its Measurement, Regulation, and Utilization in Cellular Functions" (R. Nuccitelli and D. W. Deamer, Eds.), p. 173, Liss, New York, 1982.
35. D. E. Moreland and W. P. Novitzky, Interference by herbicidal inhibitors of electron transport with phosphorylation and permeability properties of chloroplast membranes, *Z. Naturforsch,* **39c,** 329 (1983).
36. C. Gauvrit, Action of the herbicides nebruron and siduron on membrane permeabilities in potato tuber mitochondria, *Physiol. Plant* **48,** 448 (1980).

37. A. Chanson and L. Taiz, Evidence for an ATP-dependent proton pump on the Golgi of corn coleoptiles, *Plant Physiol.* **78,** 232 (1985).
38. M. I. De Michelis, F. Rasi-Caldogno, and M. C. Pugliarello, On the inhibiting effect of oligomycin on Mg:ATP-dependent ΔpH and $\Delta\psi$ in microsomal vesicles from radish, *Plant Sci. Lett.* **36,** 111 (1984).
39. J. M. Wolosin and J. G. Forte, Kinetic properties of KCl transport at the secreting apical membrane of the oxyntic cell, *J. Membr. Biol.* **71,** 195 (1983).
40. M. C. Watson, P. G. Bartels, and K. C. Hamilton, Action of selected herbicides and Tween 20 on oat (*Avena sativa*) membranes, *Weed Sci.* **28**(1), 122 (1980).