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1-Chloro-2,4-Dinitrobenzene-Elicited Increase in Vacuolar Glutathione-*S*-Conjugate Transport Activity[1]

Ze-Sheng Li, Rui-Guang Zhen, and Philip A. Rea*

Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104–6018

Unlike most other characterized organic solute transport in plants, uptake of the model compound *S*-(2,4-dinitrophenyl)glutathione (DNP-GS) and related glutathione-*S*-conjugates by vacuolar membranes is directly energized by MgATP. Here we show that exogenous application of the DNP-GS precursor 1-chloro-2,4-dinitrobenzene (CDNB) to seedlings of *Vigna radiata* (mung bean) increases the capacity of vacuolar membrane vesicles isolated from hypocotyls for MgATP-dependent DNP-GS transport in vitro. Our findings are 4-fold: (a) Pretreatment of seedlings with CDNB causes a progressive increase in MgATP-dependent DNP-GS uptake by vacuolar membrane vesicles, whereas the same range of CDNB concentrations causes only marginal stimulation when the compound benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine] is included in the pretreatment solution. (b) Increased DNP-GS uptake is accompanied by a proportionate and selective increase in $V_{max(DNP-GS)}$ but not in $K_{m(DNP-GS)}$ or $K_{m(MgATP)}$. (c) CDNB-enhanced DNP-GS uptake is not accompanied by a change in the density profile or sidedness of the vacuolar membrane fraction. (d) Basal and CDNB-enhanced DNP-GS uptake are indistinguishable in terms of their inhibitor profiles. On the basis of these findings, it is inferred that pretreatment with CDNB increases the amount or recruitment of functional transporter into the vacuolar membrane and that agents such as benoxacor antagonize the effects otherwise seen with CDNB in this sytem.

Two recent developments are of potentially profound significance for our understanding of energy-dependent solute transport across plant membranes. The first is the molecular cloning of two genes, *AtMDR1* and *AtMDR2*, from *Arabidopsis thaliana* (K.T. Howitz, A. Menkens, J. Darling, E.J. Kim, A.R. Cashmore, P.A. Rea, unpublished results) and the independent isolation of a similar but nonidentical gene (*ATPG1*) from the same organism (Dudler and Hertig, 1992). All three genes encode polypeptides bearing remarkable sequence similarities to each other and the human and rodent *MDR* gene products. When account is taken of the fact that the *MDR* gene products of mammalian cells belong to a superfamily of ABC transporters (Higgins, 1992), most if not all of which utilize MgATP as a direct energy source for active organic solute transport, the existence of genes encoding sequence homologs in Arabidopsis implies that analogous, ATP-dependent, primary active transport functions remain to be discovered in plants. The second critical development is the finding that intact vacuoles isolated from *Hordeum vulgare* (Martinoia et al., 1993) and vacuolar membrane vesicles purified from a broad range of plant species, including Arabidopsis, *Beta vulgaris*, *Vigna radiata*, and *Zea mays* (Li et al., 1995), mediate MgATP-dependent, $\Delta\bar{\mu}_{H^+}$-independent accumulation of glutathione-*S*-conjugates. Not only does this finding constitute the first unequivocal demonstration of the direct energization of organic solute transport by MgATP in plants, but from what is known of the functional characteristics of the transport pathway, participation of an ABC transporter is implicated. Hence, the results of both molecular cloning and transport studies have converged to disclose a subset of transporters that, contrary to the prevailing chemiosmotic model for transport in plants, use MgATP rather than the $\Delta\bar{\mu}_{H^+}$ as proximate energy source.

The vacuolar transporter bears a remarkable resemblance to the glutathione-*S*-conjugate transporters of membrane vesicles derived from erythrocytes (LaBelle et al., 1986), rat liver canaliculus (Akerboom et al., 1990; Kobayashi et al., 1990; Kitamura et al., 1990), and heart sarcolemma (Ishikawa, 1989). The transporters from all of these sources and plant vacuoles have an obligate requirement for energization by MgATP, are highly sensitive to inhibition by the phosphoryl transition state analog vanadate, and, with the exception of the transporter associated with erythrocytes, are moderately sensitive to inhibition by oxidized glutathione but not GSH (Li et al., 1995). If these similarities and the congruence of their kinetic parameters (table IV, Li et al., 1995) imply that the vacuolar and mammalian glutathione-conjugate transporters are functionally equivalent, it may follow that the vacuolar transporter also contributes to the detoxification of xenobiotics. Since the glutathione conjugation reaction, mediated by cytosolic GSTs, is considered to be instrumental in the

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* Corresponding author; e-mail parea@sas.upenn.edu; fax 1–215–898–8780.

Abbreviations: ABC transporter, ATP binding cassette transport protein; benoxacor, 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoazine; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-GS, *S*-(2,4-dinitrophenyl)glutathione; $\Delta\bar{\mu}_{H^+}$, H^+-electrochemical potential difference in mV (inside versus outside); ΔpH, transmembrane pH difference; GST, glutathione-*S*-transferase; *MDR*, gene encoding multiple drug resistance ABC transporter (P-glycoprotein or putative P-glycoprotein homolog); MPB, 3-(*N*-maleimidylpropionyl)biocytin; V-ATPase, vacuolar H^+-ATPase; V-PPase, vacuolar H^+-translocating inorganic pyrophosphatase.

detoxification of lipophilic electrophiles derived from both endogenous and exogenous sources (Ishikawa, 1992), it has been suggested that, through the concerted actions of GSTs and the plasma membrane glutathione-*S*-conjugate transporter, mammalian cells can confer a common structural determinant on and increase the water solubility of the toxins in question and thereby eliminate them from the cytosol by MgATP-dependent extrusion (Ishikawa, 1992). Thus, the existence of an ostensibly equivalent function in the vacuolar membrane of plant cells may denote an analogous process but one involving vacuolar sequestration instead of, or in addition to, extrusion via the plasma membrane.

With the objective of further defining the participation of the vacuolar glutathione-*S*-conjugate transporter in the intracellular detoxification of xenobiotics, we describe studies directed at determining whether the activity of this translocase is modulated by exogenous application of CDNB to seedlings of *V. radiata* (mung bean). CDNB was selected as a model compound for these studies for several reasons: (a) The kinetics of uptake of the product of the *S*-conjugation of CDNB to glutathione, DNP-GS, by vacuolar membrane vesicles from *Vigna* are now well characterized and provide a firm basis for comparisons with mammalian glutathione-conjugate transporters (Li et al., 1995), which have been almost exclusively analyzed with respect to the transport of this compound (reviewed by Zimniak and Awasthi, 1993). (b) The only known metabolite of CDNB in animal cells is DNP-GS (Wareing et al., 1993). Providing that this is also true of plants, complications that might otherwise arise from the modification of CDNB before its *S*-conjugation should be small. (c) CDNB is an established and effective in vitro substrate for plant GSTs. (d) Due to its lipophilicity, CDNB readily permeates membranes by simple diffusion; access to cytosolic GSTs and, in turn, access of DNP-GS to the vacuolar membrane are assured.

Our studies using CDNB as an artificial elicitor show that the vacuolar glutathione-*S*-conjugate transporter satisfies the minimum requirement of an element involved in the detoxification of xenobiotics; namely, enhancement of its activity in response to exposure of the intact organism to the target compound.

MATERIALS AND METHODS

Plant Materials

All of the experiments described were performed using *Vigna radiata* cv Berken (mung bean). Seeds were surface-sterilized, allowed to imbibe with tap water overnight, and sown in seed trays containing a 1:1 mixture of vermiculite and perlite. The trays were watered to saturation with water or 10 μM benoxacor once a day for 2 d, after which time the seedlings were watered daily with benoxacor alone or benoxacor plus the indicated concentrations of CDNB. The seedlings were grown in the dark at 25°C and harvested 5 d after planting.

Extraction of GST

Ten-gram samples of etiolated hypocotyls were frozen and briefly ground in liquid nitrogen using a mortar and pestle. Fifty milliliters of extraction buffer (1 mM EDTA, 1 mM PMSF, 1 mM DTT, 50 mg/mL polyvinylpolypyrollidone, 50 mM Tris-Mes buffer [pH 7.5]) were added and, after further grinding, the crude homogenate was centrifuged at 20,000*g* for 25 min to remove cell debris. The supernatants were decanted and used immediately for the measurement of GST activity.

Measurement of GST Activity

GST was assayed in 1-mL reaction volumes containing 100 μL of enzyme extract, 1 mM CDNB, and 50 mM potassium phosphate buffer (pH 7.5). Reaction was initiated by the addition of 1 mM glutathione, and the formation of DNP-GS was monitored as the increase in A_{334}. GST specific activity was calculated as μmol mg^{-1} min^{-1} by assuming an ϵ_{334} for DNP-GS of 9.6 mM^{-1} cm^{-1} (Habig et al., 1974). Nonenzymic conjugation was estimated and subtracted by measuring the activity of neutralized extracts after their deproteinization with 2% (w/v) TCA.

Preparation of Vacuolar Membrane Vesicles

Vacuolar membrane-enriched vesicles were purified as described previously (Li et al., 1995). For the experiment shown in Figure 4, 2 mL of membrane vesicles (5–7 mg of protein) purified on a 10 to 23% (w/w) Suc step gradient (Rea et al., 1992) were subjected to further fractionation by centrifugation through a 30-mL 10 to 40% (w/w) Suc density gradient at 100,000*g* for 2 h. Successive 1-mL fractions were collected from the top of the centrifuge tube, diluted with suspension medium (1.1 M glycerol, 1 mM Tris-EGTA, 5 mM DTT, 5 mM Tris-Mes, pH 8.0), sedimented at 100,000*g*, and resuspended in 0.2 mL of suspension medium for subsequent assay.

Synthesis of [^{3}H]DNP-GS

[^{3}H]DNP-GS was synthesized from [*glycine*-2-^{3}H]glutathione and CDNB by a modification of the enzymatic procedure of Kunst et al. (1989). After the removal of antioxidant (DTT) by extraction with acidic ethyl acetate and adjustment of its specific activity from 44 Ci/mmol to 7.7 mCi/mmol by the addition of unlabeled glutathione, [^{3}H]glutathione was conjugated with CDNB and purified as described by Li et al. (1995).

Measurement of DNP-GS Uptake

Unless otherwise indicated, [^{3}H]DNP-GS uptake was measured at 25°C in 200-μL reaction volumes containing 3 mM ATP, 3 mM $MgSO_4$, 10 mM creatine phosphate, 16 units/mL creatine phosphokinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol, 25 mM Tris-Mes buffer (pH 8.0), and 66.0 μM [^{3}H]DNP-GS. Uptake was initiated by the addition of 12-μL membrane vesicles (30–40 μg of protein) and brief mixing of the samples on a vortex mixer, and was allowed

to proceed for 1 to 60 min. Uptake was terminated by the addition of 1 mL of ice-cold wash medium (400 mM sorbitol, 3 mM Tris-Mes, pH 8.0) and vacuum filtration of the suspension through prewetted Millipore HA cellulose nitrate membrane filters (pore diameter 0.45 μm). The filters were rinsed twice with 1 mL of ice-cold wash medium and air-dried, and radioactivity was determined by liquid scintillation counting in 5 mL of BCS liquid scintillation cocktail (Amersham). Nonenergized [^{3}H]DNP-GS uptake and extravesicular solution trapped on the filters were enumerated by the same procedures, except that ATP and Mg^{2+} were omitted from the uptake media.

Measurement of V-PPase Activity

V-PPase (EC 3.6.1.1) activity was measured in reaction medium containing 0.3 mM Tris-PPi, 1.3 mM $MgSO_4$, 50 mM KCl, 5 μM gramicidin-D, and 30 mM Tris-Mes (pH 8.0). The assays were initiated by the addition of membrane protein, and Pi was measured by the method of Ames (1966).

Determination of Membrane Orientation

Vacuolar membrane vesicle orientation was estimated using the membrane-impermeant, V-PPase-reactive Cys reagent MPB as probe. This compound selectively reacts with Cys^{634}, located in putative cytosolic loop X of the V-PPase, to irreversibly inhibit the enzyme (Zhen et al., 1994). Measurement of V-PPase activity in the presence of substrate-permeabilizing detergent (Triton X-100) before treatment with MPB provides a measure of total ("inside-out" plus "right-side-out") activity; measurement of activity in the presence of Triton X-100 after treatment with MPB provides a measure of MPB-insensitive, inside-out activity; and measurement of activity in the absence of Triton X-100 before treatment with MPB provides a measure of right-side-out activity. The inclusion or omission of detergent from the V-PPase assay medium before and after treatment with MPB thereby enables enumeration of the relative proportion of right-side-out and inside-out vesicles.

The standard mixture for reaction with MPB contained MPB (0–1 mM), 0.3 mM Tris-PPi, and 30 mM Tris-Mes (pH 8.0). Membrane protein (5 μg) was added, and the mixture was incubated for 10 min at 0°C. Reaction was terminated by the addition of 2.5 mM DTT, and aliquots of the mixture were assayed for V-PPase activity in the presence or absence of 0.05% (w/v) Triton X-100 (Zhen et al., 1994).

Protein Estimations

Protein was estimated by a modification of the method of Peterson (1977).

Computations

All data were fitted by nonlinear least squares analysis (Marquardt, 1963) using the Ultrafit nonlinear curve-fitting package from BioSoft (Ferguson, MO).

Commercial Chemicals

Glutathione and CDNB were purchased from Fluka; ATP, creatine phosphokinase (type I from rabbit muscle, 150–250 units/mg protein), creatine phosphate, carbonylcyanide-4-trifluoromethoxy-phenylhydrazone, gramicidin-D, PPi, verapamil, and vinblastine were purchased from Sigma; cellulose nitrate membranes (0.45-μm pore diameter, HA filters) were purchased from Millipore; [^{3}H]glutathione ([*glycine*-2-^{3}H]-L-Glu-Cys-Gly, 44 Ci/mmol) was purchased from DuPont-NEN; and BCS liquid scintillation cocktail was purchased from Amersham. Analytical grade benoxacor was a kind gift from CIBA-Geigy (Greensboro, NC). All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

RESULTS

Effect of CDNB on Hypocotyl Growth and GST Activity

In preparation for the transport studies, it was necessary to establish the concentration range over which CDNB is toxic for seedlings of *V. radiata* and gain an indication of the involvement of *S*-conjugation in its metabolism. Thus, seedlings were treated with a broad range of CDNB concentrations and tested for both growth retardation and changes in extractable GST activity. The results are summarized in Figures 1 and 2.

Pretreatment of seedlings for 3 d (d 2–5 after the start of imbibition) caused a monotonic decrease in hypocotyl elongation with increase in CDNB concentration (Fig. 1A). Whereas untreated hypocotyls achieved a length of 9.4 ± 0.1 cm after a total of 5 d of growth, hypocotyls from seedlings treated with 5 mM CDNB during the final 3 d of the growth period achieved a final length of 3.9 ± 0.3 cm. Similarly, although the effect was less pronounced than that seen for hypocotyl elongation, mean seedling fresh weight was diminished by approximately 30% from 0.49 to 0.34 g as a result of pretreatment with 5 mM CDNB (Fig. 1B).

Parallel measurements of extractable CDNB-dependent GST demonstrated that treatment with CDNB elicited a moderate elevation of activity (Fig. 2). Activity increased to a maximum of 0.24 ± 0.02 μmol mg^{-1} min^{-1} after treatment with 3 mM CDNB, versus 0.1 ± 0.02 μmol mg^{-1} min^{-1} for untreated controls, and then returned to the control level as the concentration of CDNB increased to values in excess of 3 mM.

To examine participation of the GST-dependent pathway further, seedlings were also treated with benoxacor, which is known to elevate GST activity in some plant species (Viger et al., 1991). The results demonstrate that although benoxacor exerts little or no protection of hypocotyl elongation and seedling fresh weight gain from the inhibitory action of CDNB (Fig. 1), and when applied alone has only a minor effect on extractable GST activity, increasing it from 0.09 ± 0.003 to 0.13 μmol mg^{-1} min^{-1}, it does nonetheless markedly promote the CDNB-elicited increase in GST activity (Fig. 2). Simultaneous treatment of seedlings with benoxacor (10 μM) and CDNB increased GST activity

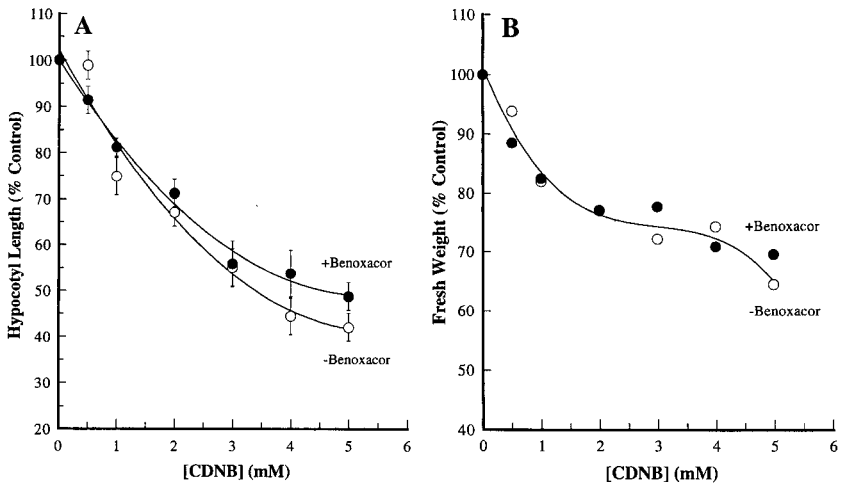

Figure 1. Inhibition of hypocotyl length (A) and seedling fresh weight (B) by CDNB. Seedlings were grown in the presence or absence of 10 μM benoxacor for 2 d and then treated with the indicated concentrations of CDNB plus or minus benoxacor for an additional 3 d before harvesting. After determining seedling fresh weight, their hypocotyls were excised and measured. Values shown are means ± SE (n = 7–12).

from a value of approximately 0.12 μmol mg^{-1} min^{-1} at 0 mM to 0.38 μmol mg^{-1} min^{-1} at 4 mM CDNB, and the diminution of activity otherwise seen with CDNB concentrations in excess of 3 mM in the absence of benoxacor was alleviated (Fig. 2).

It is therefore apparent that CDNB is toxic for seedlings of *Vigna*, that intermediate concentrations of this compound elicit an elevation of extractable GST activity, and that benoxacor promotes the CDNB-elicited increase in GST activity.

Enhancement of DNP-GS Transport by Pretreatment with CDNB

Having broadly defined the effective concentration ranges for CDNB and benoxacor with respect to growth retardation and elevation of tissue GST activity, the effects of these agents on vacuolar DNP-GS transport were examined after subjecting seedlings to identical treatments.

Pretreatment of seedlings with CDNB elicited a concentration-dependent increase in the in vitro capacity of vacuolar membrane vesicles prepared from hypocotyls for MgATP-dependent DNP-GS uptake (Fig. 3). Net uptake of [^{3}H]DNP-GS increased by more than 3-fold, from 6.59 ± 0.50 nmol mg^{-1} (20 min)$^{-1}$ in untreated controls to a value of 21.36 ± 1.45 nmol mg^{-1} (20 min)$^{-1}$ in membranes from hypocotyls exposed to 3 mM CDNB. However, contrary to the results obtained for extractable GST activity (Fig. 2), inclusion of 10 μM benoxacor in the pretreatment medium diminished, rather than enhanced, the stimulatory effect of CDNB on vacuolar DNP-GS transport. Ten micromolar benoxacor alone had a slight stimulatory effect, increasing [^{3}H]DNP-GS uptake from 6.59 ± 0.36 to 8.60 ± 0.87 nmol mg^{-1} (20 min)$^{-1}$, but pretreatment with CDNB plus 10 μM benoxacor resulted in an increase of only about 1.8-fold, from 8.60 ± 0.87 nmol mg^{-1} (20 min)$^{-1}$ minus CDNB to 16.14 ± 0.60 nmol mg^{-1} (20 min)$^{-1}$ plus 3 mM CDNB (Fig. 3).

Kinetics of DNP-GS Uptake

Increased DNP-GS uptake by vacuolar membrane vesicles prepared from CDNB- or CDNB-plus-benoxacor-pretreated hypocotyls was largely attributable to an increase in the V_{max} for transport (Table I). Irrespective of the pretreatment, MgATP-dependent DNP-GS uptake approximated Michaelis-Menten kinetics with respect to both DNP-GS and MgATP concentration. However, $V_{max(DNP\text{-}GS)}$ and $V_{max(MgATP)}$ were

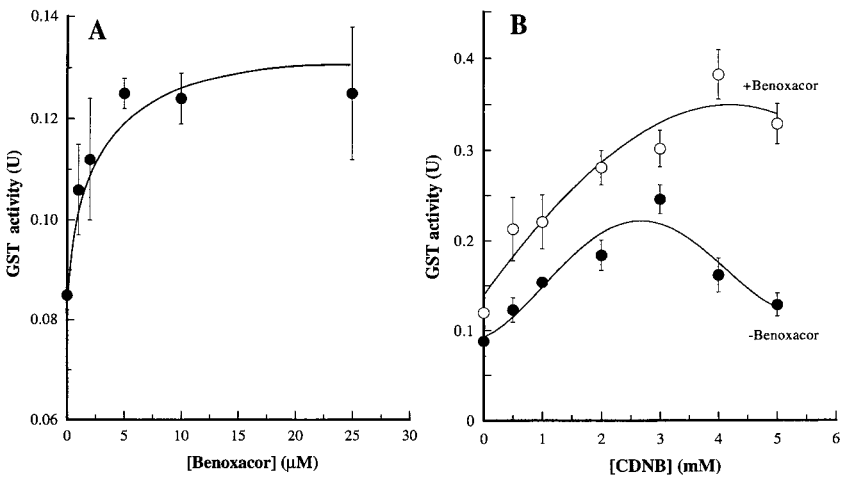

Figure 2. Effect of CDNB and benoxacor on extractable hypocotyl GST activity. A, Effect of benoxacor alone. B, Effect of CDNB plus (+Benoxacor) or minus benoxacor (−Benoxacor). Seedlings were treated with 10 μM benoxacor for 5 d and harvested (A) or grown in the presence or absence of benoxacor for 2 d and then treated with CDNB plus or minus benoxacor for an additional 3 d before harvesting the hypocotyls (B). Extractable GST activity was assayed as described in "Materials and Methods." Values shown are means ± SE (n = 3–6). U, Enzyme unit.

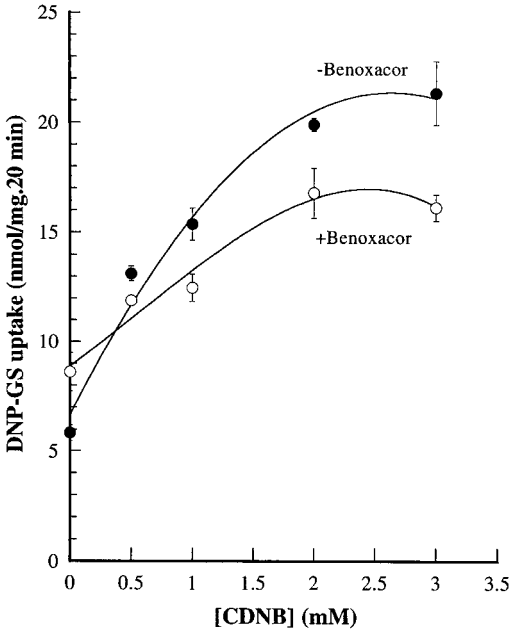

Figure 3. Effect of CDNB and benoxacor pretreatment on [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from hypocotyls. The seedlings were pretreated with CDNB (−Benoxacor) or benoxacor plus CDNB (+Benoxacor) as described in Figures 1 and 2. After harvesting the hypocotyls, vacuolar membrane vesicles were purified and assayed from MgATP-dependent [^{3}H]DNP-GS uptake. Uptake was measured in standard uptake medium (see "Materials and Methods") containing 66.0 μM [^{3}H]DNP-GS. Values shown are means ± SE (n = 3).

proportionately increased by pretreatment with CDNB and/or benoxacor. $V_{max(DNP\text{-}GS)}$, when measured at an MgATP concentration of 3 mM, was increased from 11.1 ± 1.3 nmol mg^{-1} (20 min)$^{-1}$ for membranes from untreated controls to values of 35.2 ± 3.3, 12.0 ± 1.0, and 17.0 ± 2.0 nmol mg^{-1} (20 min)$^{-1}$, respectively, for membranes prepared from CDNB- (3 mM), benoxacor- (10 μM), and CDNB-plus-benoxacor-pretreated hypocotyls. The corresponding values of $V_{max(MgATP)}$, measured with 66.0 μM [^{3}H]DNP-GS, were 4.2 ± 0.8, 12.7 ± 2.5, 3.5 ± 0.8, and 6.4 ± 0.9 nmol mg^{-1} (20 min)$^{-1}$. Both $K_{m(DNP\text{-}GS)}$ and $K_{m(MgATP)}$ were, by contrast, relatively invariant, independent of the pretreatment regime, and poised at values of between 68 and 78 μM and 116 and 183 μM, respectively.

Inhibitor Sensitivity of Basal and CDNB-Enhanced DNP-GS Transport

Both basal and CDNB-stimulated [^{3}H]DNP-GS uptake were independent of the transmembrane $\Delta\bar{\mu}_{H^+}$. Agents (gramicidin-D, carbonylcyanide-4-trifluoromethoxy-phenylhydrazone, NH_4Cl) that dissipate the inside-acid pH gradient that would otherwise be established by the V-ATPase (EC 3.6.1.3) in the presence of MgATP negligibly affected [^{3}H]DNP-GS uptake (Table II). Similarly, V-ATPase inhibitors (KNO_3) or provision of the V-PPase substrate Mg_2PPi (Mg^{2+} + PPi), instead of MgATP as an energy source, did not support [^{3}H]DNP-GS uptake by membranes from either control or CDNB-pretreated hypocotyls (Table II). In conjunction with the finding that vanadate, verapamil, and vinblastine—three type-specific inhibitors of the vacuolar glutathione-*S*-conjugate transporter (Li et al., 1995)—inhibited basal and CDNB-enhanced transport with similar efficacies (Table III), these data indicate that both basal and CDNB-enhanced uptake were catalyzed by the same transporter and were unaffected by the transmembrane $\Delta\bar{\mu}_{H^+}$ established by either the V-ATPase or V-PPase.

Vacuolar Membrane Localization of Both Basal and Enhanced DNP-GS Uptake

Whether it is measured with membrane vesicles prepared from control or CDNB-pretreated hypocotyls, MgATP-dependent [^{3}H]DNP-GS uptake co-purifies with the vacuolar membrane fraction (Fig. 4). When vacuolar membrane-enriched vesicles collected from the 10:23% (w/w) interface of Suc step gradients (see "Materials and Methods") were subjected to further fractionation on linear 10 to 40% (w/w) Suc density gradients, the density profiles for MgATP-dependent DNP-GS uptake were found to be identical for both control membranes and membranes iso-

Table I. *Summary of kinetic parameters for MgATP-dependent [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from untreated hypocotyls (Control) or hypocotyls pretreated with 10 μM benoxacor (Benoxacor), 3 mM CDNB (CDNB), or both (Benoxacor + CDNB) as described in Figure 1*

The MgATP-dependence of net [^{3}H]DNP-GS uptake was measured in standard uptake medium containing 66.0 μM [^{3}H]DNP-GS and 0 to 300 μM MgATP. The DNP-GS concentration dependence of net uptake was measured in uptake medium containing 3 mM MgATP and 0 to 185 μM [^{3}H]DNP-GS. Values shown are means ± SE (n = 3).

Treatment	MgATP		DNP-GS	
	K_m	V_{max}	K_m	V_{max}
	μM	nmol mg^{-1} (20 min)$^{-1}$	μM	nmol mg^{-1} (20 min)$^{-1}$
Control	132.2 ± 48.1	4.2 ± 0.8	72.1 ± 19.2	11.1 ± 1.3
Benoxacor	116.3 ± 53.1	3.5 ± 0.8	67.5 ± 12.3	12.0 ± 1.0
CDNB	183.4 ± 65.4	12.7 ± 2.5	76.1 ± 15.3	35.2 ± 3.3
Benoxacor + CDNB	137.9 ± 39.4	6.4 ± 0.9	77.6 ± 20.0	17.0 ± 2.0

Table II. *Comparison of effects of uncouplers, V-ATPase inhibitors, and the V-PPase substrate, Mg_2PPi (3.0 mM $MgSO_4$ + 3.0 mM Tris-PPi), on [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from control hypocotyls (Control) or hypocotyls pretreated with CDNB (CDNB)*

The pretreatment conditions were as in Figure 1. Uptake was measured in uptake medium containing 66.0 μM [^{3}H]DNP-GS. Values shown are means ± SE (n = 3–5). Numbers in parentheses are [^{3}H]DNP-GS uptake expressed as percent activity obtained in standard uptake medium containing 3 mM MgATP.

Additions	DNP-GS Uptake	
	Control	CDNB
	nmol (20 min)$^{-1}$	
+ATP (3 mM)	6.59 ± 0.46 (100)	17.62 ± 0.38 (100)
−ATP	0.84 ± 0.06 (12.7)	0.67 ± 0.05 (3.8)
−ATP + Mg_2PPi	0.90 ± 0.13 (13.7)	0.81 ± 0.08 (4.6)
+ATP + gramicidin-D (5 μM)	6.71 ± 0.26 (101.8)	18.94 ± 0.33 (107.5)
+ATP + FCCP (5 μM)	6.38 ± 0.22 (96.8)	17.48 ± 0.75 (99.2)
+ATP + NH_4Cl (1 mM)	5.90 ± 0.10 (89.5)	15.31 ± 0.08 (86.9)
+ATP + KNO_3 (50 mM)	6.56 ± 0.24 (99.5)	17.39 ± 0.41 (98.7)
+ATP + NaN_3 (1 mM)	6.71 ± 0.11 (101.8)	18.76 ± 0.37 (106.5)

lated from CDNB-treated hypocotyls. Whereas the maximum activities achieved with membranes from 3 mM CDNB-pretreated hypocotyls exceeded those of the controls by more than 3-fold, both activities peaked at the same position on density gradients and exhibited identical profiles. Density shifts and/or the appearance of subsidiary activity peaks were not evident after CDNB pretreatment, indicating that enhanced DNP-GS uptake did not result from the elaboration or activation of transport by a subpopulation of membrane vesicles distinct from those obtained from control hypocotyls. From these results and those from previous studies indicating a vacuolar membrane location for the glutathione-*S*-conjugate transporter on the basis of its co-sedimentation with the V-PPase (Li et al., 1995), it is inferred that both the control and CDNB-elicited activities are specifically associated with this membrane.

Pretreatment with CDNB Does Not Influence Membrane Vesicle Orientation

A potential complication of the studies described is that CDNB pretreatment alters membrane vesicle orientation. For example, treatment of seedlings with CDNB might influence the lipid composition of the vacuolar membrane and in so doing increase the disposition of the vesicles derived from this membrane for adopting a right-side-out orientation. This could cause an apparent increase in the capacity for MgATP-dependent DNP-GS transport without an increase in the intrinsic activity or amount of transporter by simply increasing the proportion of vesicles in which the MgATP-binding site(s) of the transporter is extravesicularly oriented. Evidence against this possibility was provided by the demonstration that the V-PPase of vacuolar membrane vesicles from control and CDNB-pretreated hypocotyls exhibited identical susceptibilities to inhibition by the membrane-impermeant maleimide MPB.

Table III. *Summary of sensitivities of MgATP-dependent [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from untreated hypocotyls (Control) or hypocotyls pretreated with 3 mM CDNB (CDNB) to inhibition by vanadate, vinblastine, and verapamil*

Pretreatment conditions were as in Figure 1. Uptake was measured for 20 min in standard uptake medium containing 66.0 μM [^{3}H]DNP-GS and 0 to 300 μM vanadate, verapamil, or vinblastine. The concentrations of inhibitor required for 50% inhibition of MgATP-dependent [^{3}H]DNP-GS uptake (I_{50} values) were estimated by least squares analysis after fitting the data to a single negative exponential. Values shown are means ± SE (n = 3–5).

Inhibitor	I_{50}	
	Control	CDNB-treated
	μM	
Vanadate	2.93 ± 2.57	3.17 ± 2.66
Vinblastine	59.92 ± 36.80	66.59 ± 47.23
Verapamil	81.71 ± 50.95	91.93 ± 53.08

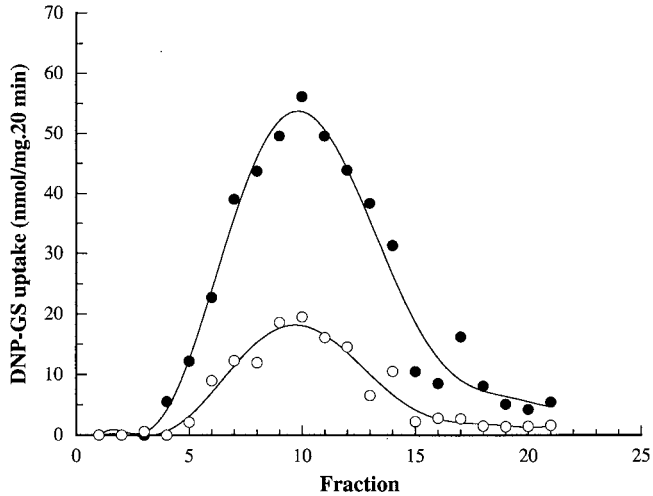

Figure 4. Distribution of MgATP-dependent [^{3}H]DNP-GS uptake after linear density gradient centrifugation of vacuolar membrane vesicles collected from a 10:23% (w/w) Suc interface. Vesicles from control (○) and CDNB-pretreated hypocotyls (●) were subjected to density gradient centrifugation on linear 10% (w/w) (fraction 1) to 40% (w/w) (fraction 25) Suc gradients, and aliquots of the fractions were assayed for [^{3}H]DNP-GS uptake in standard uptake medium. Values shown are means of duplicate measurements.

Vacuolar membrane vesicles isolated from plant sources by Suc density gradient centrifugation typically consist of an approximately 1:1 mixture of right-side-out and inside-out vesicles (Rea and Turner, 1990). As a result, three factors determine the percentage inhibition of a vacuolarly localized enzyme, such as the V-PPase, after treatment with an irreversible inhibitor. These are: (a) the membrane orientation of the modifiable residues of the enzyme; (b) the relative permeability of the membrane to the inhibitor; and (c) how the activity remaining after treatment with inhibitor is measured, specifically, whether activity is assayed in the presence or absence of agents, such as detergents, that permeabilize the membrane to substrate. In the case of a randomly oriented population of vesicles, in which the MPB-reactive residue of the V-PPase (Cys^{634}) has an extravesicular orientation in right-side-out vesicles (Zhen et al., 1994), the maximum theoretical inhibition by MPB will be 100% when V-PPase activity is measured in the absence of detergent but only 50% when it is measured in its presence.

This is what was found experimentally, irrespective of whether the vacuolar membrane vesicles were from control or CDNB-pretreated hypocotyls (Fig. 5). Inhibition by MPB approached a limit of 90% when detergent was omitted from the V-PPase assay medium, but assay in the presence of an Mg_2PPi-permeabilizing concentration of Triton X-100 (Zhen et al., 1994) revealed that approximately 50% of the initial hydrolytic activity was not inhibited by MPB. Approximately 50% of the MPB-reactive Cys^{634} residues of the V-PPase therefore appear to have a cytosolic (native) orientation and about 50% have a lumenal orientation in vacuolar membrane vesicles from both control and CDNB-treated hypocotyls, indicating that CDNB pretreatment has little or no effect on the usual 1:1 orientation of the final vesicle preparation.

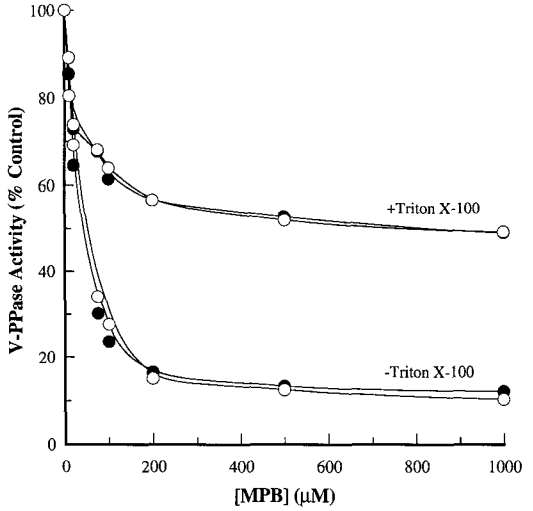

Figure 5. Comparison of the effects of MPB on inhibition of V-PPase of vacuolar membrane vesicles purified from control (○) and CDNB-pretreated hypocotyls (●). Vacuolar membrane vesicles were treated with the indicated concentrations of MPB for 10 min at 0°C. After termination of the reaction with maleimide by the addition of 2.5 mM DTT, aliquots of the suspension were assayed for V-PPase activity in the absence (−Triton X-100) or presence (+Triton X-100) of 0.05% (w/v) Triton X-100 as described in "Materials and Methods."

DISCUSSION

These studies show that pretreatment of seedlings of *V. radiata* with CDNB increases the capacity of vacuolar membrane vesicles isolated from hypocotyls for MgATP-dependent, $\Delta\bar{\mu}_{H^+}$-independent DNP-GS uptake. This finding, in combination with the demonstrated toxicity of CDNB for hypocotyl growth, provides a clear demonstration of the susceptibility of the vacuolar glutathione-*S*-conjugate transporter to modulation by xenobiotics in the intact organism. The increase in vacuolar DNP-GS transport seen upon exposure of seedlings to CDNB is precisely what would be expected if the vacuolar sequestration of glutathione-*S*-conjugates does indeed represent a xenobiotic detoxification mechanism or a component thereof.

Of the various parameters examined, only one, the V_{max} for transport, was significantly increased by CDNB. Since pretreatment with CDNB had no discernible effect on vacuolar membrane density distribution or orientation, or influenced the $\Delta\bar{\mu}_{H^+}$-dependence or inhibitor sensitivity profile of DNP-GS uptake, it is concluded that pretreatment with CDNB elicits an increase in the amount or recruitment of functional transporter into the vacuolar membrane. The elaboration of a new, kinetically distinguishable transporter on another membrane need not be invoked.

The metabolism and detoxification of xenobiotics comprises three main components, designated phases I, II, and III (Ishikawa, 1992). In phase I, the compound is oxidized, reduced, or hydrolyzed to expose or introduce a functional group of the appropriate reactivity for phase II enzymes. In phase II, the activated derivative is conjugated with hydrophilic substances, such as glutathione, glucuronic acid, or Glc. In phase III, the conjugate is either excreted to the extracellular medium or sequestered in an intracellular compartment. From the studies reported here demonstrating xenobiotic-elicited enhancement of its activity, and the results of previous experiments revealing its wide distribution and capacity for the transport of a broad range of *S*-conjugates, including those derived from herbicides (Martinoia et al., 1993; Li et al., 1995), we conclude that the vacuolar glutathione-*S*-conjugate transporter, like its mammalian counterpart, has the characteristics of a phase III enzyme. It was for this reason and the need for interaction between cytosolic GST activity, cytosolic glutathione, and the vacuolar conjugate transporter that the effects of the dichloroacetamide safener benoxacor, an established modulator of phase II enzymes, were examined.

Benoxacor is commonly employed to protect cereal crops against the toxic effects of dichloroacetanilide herbicides. The safening action of benoxacor in these systems is attributed to increased GST-mediated *S*-conjugation of the herbicide with glutathione (Viger et al., 1991), which renders the herbicide less toxic (Miller et al., 1994). Accordingly, benoxacor has been shown to induce the expression of GST in monocots (Fuerst and Gronwald, 1986; Fuerst et al., 1993) and to diminish the levels of free dichloroacetanilides in seedlings (Kreuz et al., 1989; Viger et al., 1991). On the

basis of these findings, our experiments confirming that benoxacor similarly promotes xenobiotic-elicited elevations of GST activity in the dicot *Vigna*, and the ability of the vacuolar glutathione-*S*-conjugate transporter to mediate transport of the conjugated chloroacetanilide metolachlor (Martinoia et al., 1993; Li et al., 1995), we were interested to know if benoxacor also modulates vacuolar *S*-conjugate transport activity. Striking, therefore, was the finding that benoxacor attenuates the enhancing effect of CDNB on the transporter despite the increase in extractable GST activity. The reason for this reciprocal modulation of vacuolar DNP-GS transport against cytosolic GST activity upon exposure to benoxacor is not known, but we suggest that unconjugated CDNB, or some secondary consequence of its presence, is the intracellular signal for increased vacuolar DNP-GS transport activity. If so, the antagonism exerted by benoxacor is explicable in terms of diminution of the steady-state cytosolic concentration of CDNB through its increased rate of conjugation with glutathione as a result of the benoxacor-elicited increase in CDNB-enhanced GST activity.

Although it was not our intention to show that benoxacor is a CDNB antidote in *Vigna*, and indeed its lack of effect is demonstrated by the inability of this compound to alleviate the growth inhibition exerted by CDNB (Fig. 1), two corollaries may nonetheless follow from our studies of the interactions between CDNB and benoxacor. First, whereas GST activity is responsive to both benoxacor and CDNB, vacuolar DNP-GS transport appears to be primarily responsive to the latter, indicating a degree of bifurcation of the two regulatory pathways at least as far as benoxacor is concerned. Second, since the vacuolar glutathione-*S*-conjugate transporter is not known to transport unconjugated compounds (Li et al., 1995), its capacity to respond to unconjugated xenobiotics by increasing its transport capacity may constitute a mechanism for feed-forward regulation wherein increased transport capacity precedes an increase in cytosolic-*S*-conjugate concentration. End-product inhibition of GST and xenobiotic toxicity are thereby abrogated, while ensuring that only the conjugated form of the compound is sequestered in the vacuole.

During the course of these investigations and at the time of submission of this paper we were not aware of the work of Gaillard et al. (1994). These authors, however, have demonstrated that mesophyll vacuoles purified from *Hordeum vulgare* exhibit increased glutathione-*S*-conjugate (metolachlor-GS) transport after exposure of seedlings to the safener cloquintocet-hexyl but not benoxacor. Although these workers did not examine the influence of the unconjugated form of the transported species nor its interaction with safener with respect to the induction of vacuolar transport activity, the kinetic effects observed, like those reported here, were wholly explicable in terms of an effector-elicited increase in V_{max}. Notwithstanding the differences between *Hordeum* and *Vigna* with regard to the types of compound that elicit a response, the studies described by Gaillard et al. (1994) and those reported here nevertheless demonstrate the susceptibility of the glutathione-*S*-conjugate transporters of both monocots and dicots to modulation by xenobiotics by a mechanism that probably entails an increase in the amount of active pump associated with the vacuolar membrane.

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