Plant Physiol. (1995) 107: 1257–1268

Magnesium Adenosine 5′-Triphosphate-Energized Transport of Glutathione-*S*-Conjugates by Plant Vacuolar Membrane Vesicles[1]

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By characterization of the uptake of glutathione-*S*-conjugates, principally dinitrophenyl-*S*-glutathione (DNP-GS), by vacuolar membrane vesicles, we demonstrate that a subset of energy-dependent transport processes in plants are not H^+-coupled but instead are directly energized by MgATP. The most salient features of this transport pathway are: (a) its specific, obligate requirement for MgATP as energy source; (b) the necessity for hydrolysis of the γ-phosphate of MgATP for uptake; (c) the insensitivity of uptake to uncouplers of the transtonoplast H^+ gradient (carbonylcyanide 4-trifluoromethoxyphenylhydrazone, gramicidin-D, and NH_4Cl); (d) its pronounced sensitivity to vanadate and partial inhibition by vinblastine and verapamil; (e) the lack of chemical modification of DNP-GS either during or after transport; (f) the capacity of *S*-conjugates of chloroacetanilide herbicides, such as metolachlor-GS, but not free herbicide, to inhibit uptake; and (g) the ability of vacuolar membrane vesicles purified from a broad range of plant species, including *Arabidopsis*, *Beta*, *Vigna*, and *Zea*, to mediate MgATP-dependent, H^+-electrochemical potential difference-independent DNP-GS uptake. On the basis of these findings it is proposed that the transport of DNP-GS across the vacuolar membrane of plant cells is catalyzed by a glutathione-conjugate transporter that directly employs MgATP rather than the energy contained in the transtonoplast H^+-electrochemical potential difference to drive uptake. The broad distribution of the vacuolar DNP-GS transporter and its inhibition by metolachlor-GS are consistent with the notion that it plays a general role in the vacuolar sequestration of glutathione-conjugable cytotoxic agents.

The prevailing working model for energy-dependent solute transport by plant cells is chemiosmosis (Mitchell, 1976; Spanswick, 1981). According to this scheme, the primary energizers for solute transport are pumps that mediate electrogenic H^+-translocation across the membranes concerned to generate a gradient of electrical potential ($\Delta\psi$) and chemical potential (ΔpH). Depending on the net charge on the solute-porter complex, the motive force for transport is believed to be derived from the ΔpH and/or $\Delta\psi$ components of the H^+-electrochemical potential difference ($\Delta\bar{\mu}_{H^+}$). For solutes that traverse the membrane in parallel with or in exchange for H^+, transport is driven by ΔpH; for solute-porter complexes that carry a net charge, transport is driven by $\Delta\psi$.

The notion of phosphoanhydride-energized H^+-translocation across the principal membranes of plant cells is irrefutable. Primary ATP-energized H^+ pumps and a PPi-energized H^+ pump in the case of the vacuolar membrane (Rea and Poole, 1993) have been demonstrated in the membranes bounding most of the compartments of plant cells, and in all cases the pumps concerned have been shown to be critical for establishment of the resting membrane potential and transmembrane pH gradients (Spanswick, 1981; Sze, 1985; Rea and Poole, 1993). What is not so clear, however, is whether essentially all energy-dependent secondary transport is H^+-coupled or whether other modes of energization might also be operative in plants. The literature on secondary transport in plants is large, but surprisingly few investigations have had a direct bearing on the molecular identity, mechanisms, and energetics of the elements involved. A broad range of transport processes have been shown to be H^+-coupled in plants by electrophysiological and biochemical criteria (see Bush, 1993, for recent review), but the range of transport processes examined has not been sufficiently exhaustive for the notion of alternate, more direct mechanisms of energy transfer to be excluded a priori.

Two recent developments are of potentially profound significance in this context. The first is the molecular cloning of two cDNAs, *MDR1* and *MDR2*, from the vascular plant *Arabidopsis thaliana* (K.T. Howitz, A. Menkens, J. Darling, E.J. Kim, A.R. Cashmore, and P.A. Rea, unpublished

[1] This work was supported by a grant from the Department of Agriculture (National Research Initiative Competitive Grants Program grant No. 9303007) awarded to P.A.R.

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Abbreviations: ABC transporter, ATP-binding cassette transport protein; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; CDNB, 1-chloro-2,4-dinitrobenzene; ΔpH, transmembrane pH difference; $\Delta\psi$, transmembrane electrical potential difference; $\Delta\bar{\mu}_{H^+}$, H^+-electrochemical potential difference, in mV (inside versus outside); DNP-GS, *S*-(2,4-dinitrophenyl)glutathione; FCCP, carbonylcyanide 4-trifluoromethoxyphenylhydrazone; FPLC, fast protein liquid chromatography; GSCT, glutathione-*S*-cinnamoyl transferase; I_{50}, concentration resulting in 50% inhibition; *MDR*, gene encoding multiple drug resistance ABC transporter (P-glycoprotein or putative P-glycoprotein homolog); metolachlor, 2-chloro-*N*-(2′-ethyl-6′-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide; NEM-GS, glutathione-*S*-*N*-ethylmaleimide; V-ATPase, vacuolar H^+-ATPase (EC 3.6.1.3); V-PPase, vacuolar H^+-translocating pyrophosphatase (EC 3.6.1.1).

data) and the independent isolation of a similar but nonidentical MDR-like gene (*atpg1*) from the same organism (Dudler and Hertig, 1992). All three genes encode polypeptides bearing remarkable sequence similarities to each other and to the human and mouse *MDR* gene products. Given that the MDR gene products of mammalian cells belong to a superfamily of ABC transporters (Higgins, 1992), most if not all of which utilize ATP as proximate energy source for active organic solute transport, the existence of genes encoding homologs in *Arabidopsis* implies that analogous, ATP-dependent, primary active transport functions remain to be discovered in plant cells. The second critical observation is that intact vacuoles isolated from *Hordeum vulgare* (barley) mediate MgATP-dependent, vacuolar H^+-ATPase-independent accumulation of glutathione-*S*-conjugates (Martinoia et al., 1993). This finding is seminal on two counts: not only does it implicate MgATP as a direct energy source for solute transport across plant membranes, but since most of the glutathione conjugates examined were herbicides, a transport pathway responsible for the sequestration of xenobiotics appears to have been identified.

As part of a long-term project directed at identification of energy-dependent solute transport processes in plants that are "ABC-like" in that they are driven by MgATP without the intervention of transmembrane H^+ gradients, we describe the functional characterization of the vacuolar *S*-glutathione conjugate transporter of *Vigna radiata* (mung bean). The studies we describe were performed on vacuolar membrane vesicles, instead of intact vacuoles, and employed the glutathione conjugate of CDNB, DNP-GS, instead of NEM-GS (cf. Martinoia et al., 1993), as a model compound.

The decision to use vacuolar membrane vesicles rather than intact vacuoles was based on their relative ease of handling and our eventual objective of identifying the *S*-conjugate transporter at the protein level. Although much progress has been made in understanding transport at the vacuolar membrane through the use of isolated intact vacuoles, vacuolar membrane vesicles are better suited for most biochemical investigations (Blumwald et al., 1987). Isolated vacuoles contain high concentrations of soluble proteins, inorganic and organic salts, and neutral compounds, which can complicate the evaluation of transmembrane energy gradients; they are extremely fragile and prone to disruption during the course of transport assays; and their membranes contain many loosely associated proteins that may complicate subsequent protein chemical analyses. Vacuolar membrane vesicles, by contrast, are essentially devoid of endogenous solutes, can be made to rapidly equilibrate with the bulk medium for experimental purposes, are mechanically robust, and, under appropriate conditions, can be stripped of loosely associated proteins (Blumwald et al., 1987). DNP-GS, rather than NEM-GS, was chosen as model compound to facilitate direct comparisons between the plant and mammalian glutathione conjugate transporters, the latter of which have been almost exclusively characterized with respect to DNP-GS, not NEM-GS, transport (reviewed by Zimniak and Awasthi, 1993).

By employing DNP-GS as model compound for the investigation of glutathione conjugate uptake by purified vacuolar membrane vesicles, we have been able to confirm, extend, and complement the findings of Martinoia et al. (1993). Confirmatory data include demonstration of the strict dependence of glutathione conjugate transport on MgATP hydrolysis, its near complete insensitivity to $\Delta\bar{\mu}_{H^+}$ and its association with the vacuolar membrane fraction. New and complementary data include demonstration of the pronounced sensitivity of transport to inhibition by vanadate, vinblastine, and verapamil, the lack of a requirement for covalent modification of the transported species either during or after transport, and the apparent ubiquity of MgATP-dependent glutathione conjugate transport in vacuolar membrane fractions purified from a wide range of vascular plants. Our studies, in conjunction with those of Martinoia et al. (1993), unequivocally demonstrate an MgATP-energized, organic solute transport pathway in plants, which, contrary to the conventional view of tonoplast energization, is not driven by the transmembrane H^+-electrochemical potential difference.

MATERIALS AND METHODS

Plant Materials

Most of the experiments described were performed with *Vigna radiata* cv Berken (mung bean). Supplementary plant materials were *Arabidopsis thaliana* cv Columbia, *Beta vulgaris* (red beet), and *Zea mays*, Pioneer hybrid 3709 (corn). Seeds of *Vigna* or *Zea* were surface-sterilized, allowed to imbibe tap water for 48 h, and grown in a 1:1 mixture of vermiculite and perlite in the dark at 20 to 25°C. Etiolated hypocotyls of *Vigna* and the terminal 2 cm of root segments from *Zea* were harvested 3 to 4 d and 7 to 10 d after planting, respectively. Fresh red beets were purchased commercially. *Arabidopsis* root cultures were established by a combination of the procedures of Valvekens et al. (1988) and Koncz et al. (1992). Briefly, *Arabidopsis* seeds were sterilized in 0.05% (w/v) sodium hypochlorite containing 0.1% (v/v) Tween-20, washed exhaustively with sterile water, and placed on Petri dishes containing germination medium (Murashige and Skoog medium containing 0.5 g/L Mes, 0.9% [w/v] Difco Bacto agar, 20 g/L Suc, 0.4 mg/L vitamin B_1, 0.3 mg/L vitamin B_6, and 0.1 mg/L nicotinic acid). After germination for 1 d at 4°C followed by 3 to 4 d at 23 to 27°C, groups of two to five seedlings were transferred to 125-mL culture flasks containing 10 mL of germination medium minus agar and grown for 14 to 21 d with gentle shaking at 22°C in a 16-h light/8-h dark cycle before harvesting.

Preparation of Vacuolar Membrane Vesicles

Vacuolar membrane vesicles were purified from etiolated hypocotyls of *Vigna*, leaves and roots of *Arabidopsis*, and roots of *Zea* by a modification of the methods of Maeshima and Yoshida (1989) and Rea and Turner (1990), as detailed by Rea et al. (1992), and from *Beta* storage roots

as described by Rea and Turner (1990). For the experiment shown in Figure 4, 2 mL (7–8 mg of protein) of *Vigna* vacuolar membrane vesicles purified on a 10/23% (w/w) Suc step gradient (Rea et al., 1992) were subjected to further fractionation by centrifugation through a 30-mL linear 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.

All membrane preparations that were not used immediately were suspended in suspension medium, frozen in liquid nitrogen, and stored at −85°C.

Synthesis of DNP-GS and Metolachlor-GS

DNP-GS was synthesized from GSH and CDNB by a modification of the enzymatic procedure of Kunst et al. (1989). The reaction mixture consisted of 50 μmol of GSH and 50 μmol of CDNB dissolved in 5 mL of 50 mM Tris-Mes (pH 6.5) to which 5 units of glutathione-*S*-transferase were added. Conjugation was allowed to proceed for 1 h at room temperature, after which time the reaction mixture was applied to a 10-mL column packed with QAE-Sepharose. The column was successively washed with 3 bed volumes of Tris-Mes buffer (pH 6.5) and 2 bed volumes of distilled water before elution of bound DNP-GS with 2 bed volumes of 0.7 M formic acid. The eluate was dried by rotary evaporation at 35°C and the residue was redissolved in distilled water. The concentration of DNP-GS was estimated spectrophotometrically at 334 nm assuming $\epsilon_{334} = 9.6\ mM^{-1}\ cm^{-1}$ (Habig et al., 1974).

[^{3}H]DNP-GS was synthesized from [*glycine*-2-^{3}H]GSH (44 Ci/mmol) in a similar manner except that the antioxidant (DTT) in which the [^{3}H]GSH was shipped was removed by extraction with acidic ethyl acetate immediately before use. After adjustment of the specific radioactivity of the [^{3}H]GSH to 8.7 Ci/mmol by the addition of unlabeled GSH, it was conjugated with CDNB and purified as described above. The overall yield and purity of the resulting [^{3}H]DNP-GS was 60 to 63% and 92 to 96% as determined by radiometric analysis and reverse-phase FPLC, respectively.

GSSG and metolachlor-GS were synthesized by general base catalysis by the addition of 100 μmol of GSH, alone, to 50 mM Tris-Mes, pH 9.0, or the addition of both 100 μmol of GSH and 50 μmol of metolachlor to ethanolic (40%, v/v) 50 mM Tris-Mes, pH 9.0, respectively. After reaction for 3 to 4 h at 60°C, the resulting GSSG and metolachlor-GS were purified by reverse-phase FPLC.

Purification of GS Conjugates

All of the GS conjugates employed in these studies were purified by reverse-phase FPLC on a C_{18} column (PepRPC HR 5/5, Pharmacia-LKB, Inc.). The column was conditioned by multiple washes with pure acetonitrile and distilled water, and 0.5 mL of the GS conjugate, dissolved in distilled water, was injected. Elution was at a flow rate of 0.5 mL/min with a four-phase gradient: phase 1 (0–7 min), 0% acetonitrile; phase 2 (7–27 min), 0 to 50% (v/v) acetonitrile; phase 3 (27–32 min), 50 to 100% acetonitrile; phase 4 (32–37 min), 100% acetonitrile. The fractions (0.5 mL) were collected and analyzed by spectrophotometry, in the case of unlabeled conjugates, and/or liquid scintillation counting, in the case of radiolabeled conjugates. The conjugates were identified according to A_{334} (DNP-GS), A_{240} (GSSG), and A_{220} (metolachlor-GS) and the acetonitrile concentrations (%, v/v) at which they were eluted from the PepRPC column (32.5% [DNP-GS], 0.0% [GSSG], 37.5% [metolachlor-GS]). The purified conjugates were evaporated to dryness, redissolved in distilled water, and stored in the dark at −20°C immediately after chromatography and spectrophotometry.

To determine the fate of the ^{3}H label of [^{3}H]DNP-GS after 1 and 20 min of uptake by vacuolar membrane vesicles purified from *Vigna*, uptake was initiated and terminated as described in "Measurement of DNP-GS Uptake." The vacuolar membrane samples were subjected to membrane filtration and the filters were rinsed with three 1-mL aliquots of ice-cold wash medium and extracted with 50% (v/v) acetonitrile. The extracts from 8 to 10 cellulose nitrate membrane filters per treatment were dried by rotary evaporation, reconstituted in 600 μL of distilled water, centrifuged for 5 min at 14,000 rpm in an Eppendorf microfuge, and analyzed by reverse-phase FPLC as described above.

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 kinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol, 25 mM Tris-Mes buffer (pH 8.0), and 64.2 μM [^{3}H]DNP-GS (7.7 mCi/mmol). Uptake was initiated by the addition of 12 μL of vacuolar 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 procedure except that ATP and Mg^{2+} were omitted from the assay media.

Measurement of ATPase and PPase Activity

Plasma membrane ATPase activity was measured at pH 6.5 in reaction medium containing 3 mM Tris-ATP, 3 mM $MgSO_4$, 50 mM KCl, 0.05% (w/v) Triton X-100, 5 μM gramicidin-D, 30 mM Tris-Mes (pH 6.5), and the indicated concentrations of sodium vanadate. V-PPase activity was assayed at pH 8.0 in the same medium containing 0.3 mM

Tris-PPi and 1.3 mM $MgSO_4$ in place of 3 mM Tris-ATP and 3 mM $MgSO_4$. The assays were initiated by the addition of membrane protein and Pi was measured by the method of Ames (1966).

ATP- and PPi-dependent H^+-translocation by vacuolar membrane vesicles purified from *Vigna* were assayed fluorimetrically at 25°C using quinacrine as ΔpH indicator (Rea and Turner, 1990). Fluorescence was measured at excitation and emission wavelengths of 420 and 495 nm, respectively, at a slit width of 5 nm for both excitation and emission. The assay media contained 3 mM Tris-ATP (or 0.3 mM Tris-PPi), 50 mM KCl, 0.1% (w/v) BSA, 1 mM NaN_3, 4 μM quinacrine, 400 mM sorbitol, 20 mM Tris-Mes (pH 8.0), and the indicated concentrations of inhibitors. Intravesicular acidification was initiated by the addition of 3.0 mM $MgSO_4$ (for ATP-dependent H^+-translocation) or 1.3 mM $MgSO_4$ (for PPi-dependent H^+-translocation).

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

GSH, CDNB, and QAE-Sepharose were purchased from Fluka; AMP-PNP, ATP, creatine kinase (type I from rabbit muscle, 150–250 units/mg protein), creatine phosphate, FCCP, glutathione-*S*-transferase (from equine liver, 50–100 units/mg protein), gramicidin-D, PPi, verapamil, and vinblastine were from Sigma; cellulose nitrate membranes (0.45-μm pore size, HA filters) were from Millipore; acetonitrile, HPLC grade, was from Aldrich; BCS liquid scintillation cocktail was from Amersham; [^{3}H]glutathione [*glycine*-2-^{3}H]L-Glu-Cys-Gly (44 Ci/mmol) was from DuPont-NEN; and the PepRPC HR 5/5 column was from Pharmacia-LKB. Metolachlor was a gift from CIBA-Geigy (Greensboro, NC). All other reagents were of analytical grade and purchased from Fisher, Fluka, or Sigma.

RESULTS

MgATP-Dependent DNP-GS Uptake

Vacuolar membrane vesicles purified from plant tissues contain two predominant phosphohydrolase activities: V-ATPase (Sze et al., 1992) and V-PPase (Rea and Poole, 1993). At the outset of these investigations it was considered to be of strategic value to select a plant source with low V-ATPase and preferably high V-PPase activity for two reasons. First, by initiating the experiments with vacuolar membrane vesicles possessing low V-ATPase activity, complications attending the operation of a parallel ATP-dependent pathway might be diminished. Second, through the use of membrane vesicles capable of V-PPase-mediated (ATP-independent) electrogenic H^+-translocation, a system for rigorously testing the $\Delta\tilde{\mu}_{H^+}$ dependence of DNP-GS uptake might be provided. Thus, for most of the experiments described, vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata* were employed. High purity, transport-competent vacuolar membrane vesicles can be obtained in high yield from this tissue source (Maeshima and Yoshida, 1989; Rea et al., 1992), and V-PPase activity, whether measured as the rate of PPi hydrolysis or PPi-dependent H^+-translocation, exceeds V-ATPase activity by a factor of 3 to 6 (Rea et al., 1992).

The MgATP- and time dependence of [^{3}H]DNP-GS uptake by vacuolar membrane vesicles purified from *Vigna* is shown in Figure 1. Providing that both creatine phosphate and creatine kinase were included in the uptake medium, to ensure the regeneration of ATP, uptake was critically dependent on the provision of MgATP. Inclusion of MgATP increased the initial rate and extent of net uptake by between 500- and 700-fold and 15- and 18-fold, respectively. Hence, the initial rate of net [^{3}H]DNP-GS uptake was 3 to 4 nmol mg^{-1} min^{-1} in the presence of 3 mM MgATP and approximately 6 pmol mg^{-1} min^{-1} in its absence, and, whereas MgATP-energized vesicles were capable of establishing a steady-state DNP-GS concentration of 6 to 7 nmol/mg protein after 60 min, the corresponding value for vesicles incubated in the absence of MgATP was only 0.4 nmol/mg protein.

MgATP, not free ATP, was the active substrate for energization. Uptake after 20 min was more than 7-fold stimulated by the provision of both 3 mM ATP and 3 mM Mg^{2+} but approximated that measured in the absence of both ATP and Mg^{2+} when 3 mM ATP or 3 mM Mg^{2+} were provided singly (Table I).

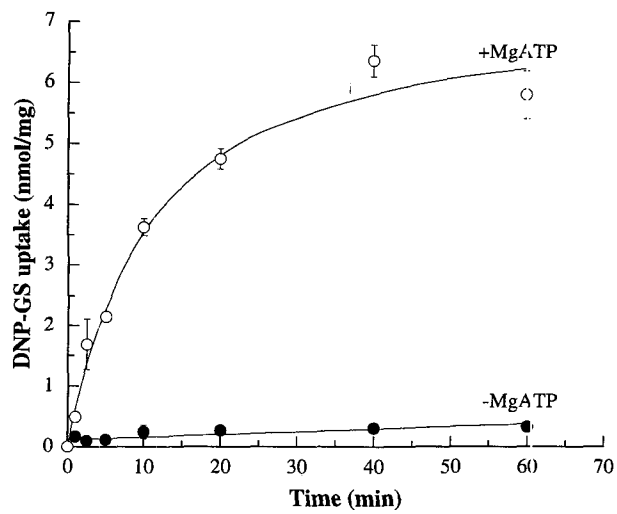

Figure 1. Time course of [^{3}H]DNP-GS uptake into vacuolar membrane vesicles purified from etiolated hypocotyls of *V. radiata*. Uptake was measured in the absence of MgATP (−MgATP) or in the presence of 3 mM MgATP (=3 mM Tris-ATP + 3 mM $MgSO_4$) in reaction media containing 64.2 μM [^{3}H]DNP-GS, 10 mM creatine phosphate, 16 units/mL creatine kinase, 50 mM KCl, 0.1% (w/v) BSA, 400 mM sorbitol, and 25 mM Tris-Mes buffer (pH 8.0) at 25°C. Values shown are means ± SE (n = 3).

Table I. *Effect of ATP, Mg^{2+}, potassium salts, and BSA on DNP-GS uptake by Vigna vacuolar membrane vesicle*

Uptake was for 20 min in uptake media containing 64.2 μM [^{3}H]DNP-GS and the concentrations and combinations of ATP, $MgSO_4$, KCl, KNO_3, and BSA indicated. Values shown are mean ± SE (n = 3–6).

Additions	DNP-GS Uptake
	nmol mg^{-1} protein (20 min)$^{-1}$
−ATP − $MgSO_4$	0.68 ± 0.02
−ATP + $MgSO_4$ (3 mM)	0.82 ± 0.05
+ATP (3 mM) − $MgSO_4$	0.89 ± 0.05
+ATP (3 mM) + $MgSO_4$ (3 mM)	3.53 ± 0.38
+ATP + $MgSO_4$ + KCl (50 mM)	5.36 ± 0.41
+ATP + $MgSO_4$ + KNO_3 (50 mM)	4.92 ± 0.11
+ATP + $MgSO_4$ + BSA (0.1%, w/v)	5.15 ± 0.28
+ATP + $MgSO_4$ + KCl + BSA	6.45 ± 0.78

Maximal MgATP-dependent uptake was dependent on the inclusion of both KCl and BSA in the uptake medium. The addition of KCl or BSA alone increased MgATP-dependent DNP-GS uptake from 3.5 ± 0.4 to 5.4 ± 0.3 and 5.2 ± 0.3 nmol mg^{-1} (20 min)$^{-1}$, respectively, and the two in combination resulted in a net uptake of 6.5 ± 0.8 nmol mg^{-1} (20 min)$^{-1}$ (Table I). Thus, to promote uptake and thereby facilitate kinetic measurements, all of the transport assays discussed from here on, including those shown in Figure 1, were performed using media containing both 50 mM KCl and 0.1% (v/v) BSA.

$\Delta\tilde{\mu}_{H^+}$-Independent DNP-GS Uptake

The uptake of [^{3}H]DNP-GS was independent of the transtonoplast $\Delta\tilde{\mu}_{H^+}$. Agents (gramicidin-D, FCCP, NH_4Cl) that dissipate the inside-acid H^+ gradient that would otherwise be established by the V-ATPase in the presence of 3 mM MgATP had very little or no effect on the extent of [^{3}H]DNP-GS uptake (Table II). Similarly, agents such as permeant anions (Cl^- and NO_3^-) and K-valinomycin, which act to diminish or abrogate establishment of an inside-positive membrane potential, also exerted negligible effect on net uptake (Table II). These data, in conjunction with the finding that provision of Mg^{2+} + PPi (Mg_2PPi), instead of MgATP, as energy source, did not stimulate [^{3}H]DNP-GS uptake (Table II), in spite of the capacity of the V-PPase of vacuolar membranes purified from *Vigna* for establishing a H^+ gradient of greater magnitude than that achieved by the V-ATPase (Rea et al., 1992), clearly eliminate any direct contribution from the transmembrane $\Delta\tilde{\mu}_{H^+}$ toward net uptake.

Concentration-Dependent DNP-GS Uptake

MgATP-dependent DNP-GS uptake approximated Michaelis-Menten kinetics with respect to the concentrations of energy source (MgATP) and transported species (DNP-GS) (Figs. 2 and 3). MgATP-dependent uptake increased as a simple hyperbolic function of both MgATP and DNP-GS concentration to yield K_m values of 51.3 ± 10.4 μM (MgATP) and 81.3 ± 41.8 μM (DNP-GS) and a V_{max} of 11.6 ± 2.1 nmol mg^{-1} (20 min)$^{-1}$ (DNP-GS).

Association of DNP-GS Uptake with the Vacuolar Membrane Fraction

MgATP-dependent [^{3}H]DNP-GS uptake co-purified with the vacuolar membrane fraction. When 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

Table II. *Effect of protonophores, ionophores, V-ATPase inhibitors, reduced and oxidized thiols, ATP analogs, and PPi on [^{3}H]DNP-GS uptake by Vigna vacuolar membrane vesicles*

For comparative purposes the rates of ATP- and PPi-dependent H^+ translocation by the same membranes, measured as the decrease in fluorescence of quinacrine (%F) against time, are shown. Conjugate uptake was measured in standard uptake medium containing 64.2 μM [^{3}H]DNP-GS *plus* the compounds indicated. Values shown are mean ± SE (n = 3–6). Fluorescence quenching of quinacrine (4 μM) was initiated by the addition of 3.0 mM $MgSO_4$ or 1.3 mM $MgSO_4$ to media containing 3.0 mM Tris-ATP or 0.3 mM Tris-PPi, 400 mM sorbitol, 50 mM KCl (except when substituted with KNO_3), 0.1% (w/v) BSA, 1 mM NaN_3, and 20 mM Tris-Mes (pH 8.0). FCCP, gramicidin-D, KNO_3, and NH_4Cl were added at concentrations of 2 μM, 5 μM, 50 mM, and 1 mM, respectively. ND, Not determined.

Additions	DNP-GS Uptake		H^+ Translocation
	nmol mg^{-1} protein (20 min)$^{-1}$	% Control	*% F min^{-1} mg^{-1} protein*
−ATP	0.79 ± 0.17	12.0	0
+ATP (control)	6.56 ± 0.71	100	259
+ATP + gramicidine-D (5 μM)	6.10 ± 0.20	93.0	0
+ATP + FCCP (5 μM)	5.96 ± 0.50	90.9	9
+ATP + NH_4Cl (1 mM)	6.03 ± 0.24	91.9	12
+ATP + valinomycin (100 μM)	5.76 ± 0.16	87.8	ND
+ATP + AMP-PNP (3 mM)	1.26 ± 0.07	19.2	ND
+ATP(−KCl) + KNO_3 (50 mM)	6.57 ± 0.50	100	0
+ATP + NaN_3 (1 mM)	5.93 ± 0.18	90.4	260
+ATP + Gly (1 mM)	6.11 ± 0.12	93.1	ND
+ATP + GSSG (1 mM)	3.27 ± 0.22	49.8	ND
+ATP + GSH (1 mM)	6.48 ± 0.10	98.8	ND
+ATP + DTT (0.5 mM)	6.24 ± 0.33	95.1	ND
−ATP + PPi (3 mM)	0.74 ± 0.09	11.3	656
−ATP + AMP-PNP (3 mM)	0.75 ± 0.06	11.4	ND

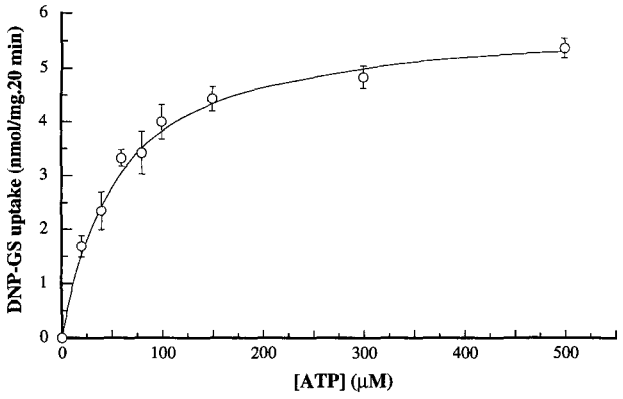

Figure 2. MgATP concentration dependence of net [^{3}H]DNP-GS uptake by *Vigna* vacuolar membrane vesicles. Uptake was allowed to proceed for 20 min in standard uptake medium containing 64.2 μM [^{3}H]DNP-GS and the indicated concentrations of MgATP. K_m(MgATP) = 51.3 ± 10.4 μM; V_{max} = 5.8 ± 0.4 nmol mg^{-1} (20 min)$^{-1}$. The line of best fit and kinetic parameters were computed by nonlinear least-squares analysis using the Michaelis-Menten equation (Marquardt, 1963). Values shown are mean ± SE (n = 3).

gradients, MgATP-dependent [^{3}H]DNP-GS uptake and K^+-stimulated PPase activity were found to co-sediment (Fig. 4). Whereas the small amount of vanadate-inhibitable ATPase activity (a plasma membrane marker) associated with membranes collected from the 10/23% Suc interface equilibrated at a higher Suc density, K^+-stimulated PPase activity and [^{3}H]DNP-GS uptake equilibrated at the same position and showed identical density-activity profiles (Fig. 4). Therefore, since K^+-activated PPase (V-PPase) activity is largely vacuolarly localized in this and most other plant tissues (Rea and Sanders, 1987; Rea and Turner, 1990;

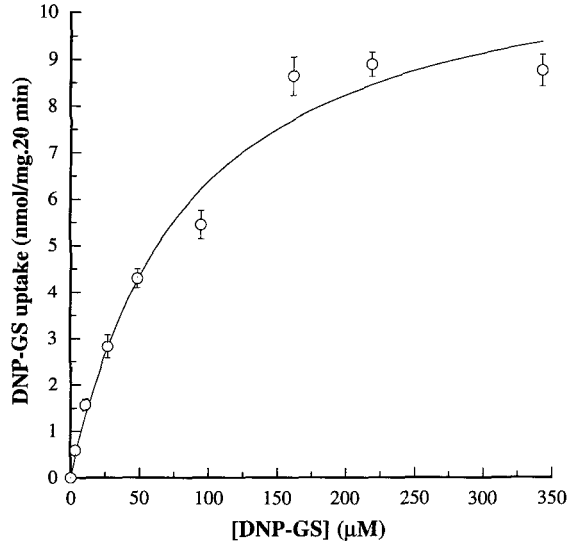

Figure 3. DNP-GS concentration dependence of net uptake by *Vigna* vacuolar membrane vesicles. Uptake was allowed to proceed for 20 min in standard uptake medium containing 3 mM MgATP and the indicated concentrations of [^{3}H]DNP-GS. K_m(DNP-GS) = 81.3 ± 41.8 μM; V_{max} = 11.6 ± 2.1 nmol mg^{-1} (20 min)$^{-1}$. Values shown are mean ± SE (n = 3).

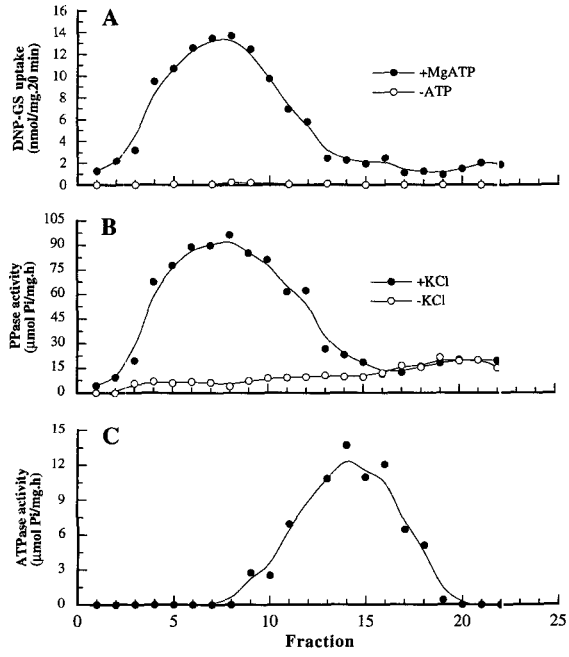

Figure 4. Distribution of MgATP-dependent [^{3}H]DNP-GS uptake (A), K^+-stimulated PPase activity (B), and vanadate-sensitive ATPase activity (C) after density gradient centrifugation of *Vigna* vacuolar membrane-enriched vesicles collected from a 10/23% (w/w) Suc interface. The vesicles were subjected to density gradient centrifugation on a linear 10% (w/w) (fraction 1) to 40% (w/w) (fraction 25) Suc gradient and aliquots of the fractions were assayed for [^{3}H]DNP-GS uptake in the presence (+MgATP) or absence of ATP (−ATP), for PPi hydrolytic activity in the presence (+KCl) or absence of 50 mM KCl (−KCl), and for vanadate (100 μM)-inhibited ATP hydrolytic activity as described in "Materials and Methods." Values shown are means of duplicate measurements.

Chanson and Pilet, 1988), it appears that the MgATP-dependent [^{3}H]DNP-GS uptake measured in these experiments was located on the vacuolar membrane, and possibly other endomembranes, but not on higher-density contaminants associated with the 10/23% interface.

Integrity of DNP-GS after Uptake

Chromatographic analysis of the ^{3}H label extracted from vacuolar membrane vesicles after 1 and 20 min of [^{3}H]DNP-GS uptake confirms that uptake is not accompanied by irreversible chemical modification of the conjugate. Extraction of vacuolar membrane vesicles with 50% (v/v) acetonitrile after uptake yielded ^{3}H-labeled material that eluted as a single peak, corresponding to 32.5% (v/v) acetonitrile when subjected to gradient elution from a C_{18} PepRPC reverse-phase column (Fig. 5). Since more than 90% of the extractable label was associated with this peak, which co-chromatographed with pure DNP-GS (see "Materials and Methods") irrespective of whether uptake was allowed to proceed for 1 or 20 min (Fig. 5) and extracts spiked with pure [^{3}H]DNP-GS showed identical chromatographic properties, complications that might arise from the action of γ-glutamylpeptidases or other enzymes capable of degrading or modifying *S*-glutathione conjugates during

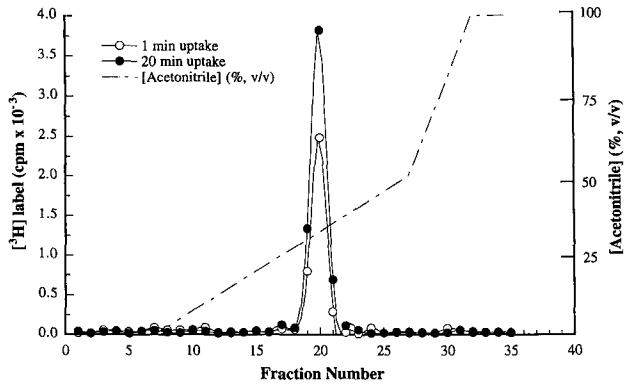

Figure 5. Reverse-phase FPLC of ^{3}H label after [^{3}H]DNP-GS uptake by *Vigna* vacuolar membrane vesicles. [^{3}H]DNP-GS uptake was allowed to proceed for 1 min (○) or 20 min (●). After the termination of uptake, the membrane filters (10 for 1 min uptake; 8 for 20 min uptake) were exhaustively washed and extracted with 50% (v/v) acetonitrile. The extracts were concentrated by rotary evaporation and reconstituted in 600 μL of distilled water and 200-μL aliquots were injected onto a PepRPC HR5/5 column. The column was run at 0.5 mL/min, 0.5-mL fractions were collected, and 100-μL aliquots of the fractions were assayed for ^{3}H label by liquid scintillation counting. The program parameters were: 0 to 7 min, 0% acetonitrile; 7 to 27 min, 0 to 50% (v/v) acetonitrile; 27 to 32 min, 50 to 100% acetonitrile; 32 to 37 min, 100% acetonitrile.

or after transport do not appear to have been operative. By the same token, the inability of unlabeled 1 mM Gly to inhibit [^{3}H]DNP-GS uptake (Table II) and the exclusive location of the ^{3}H label of DNP-GS on the Gly residue of the glutathione moiety (see "Materials and Methods") eliminates the possibility of extravesicular cleavage of [^{3}H]DNP-GS to [^{1}H]DNP-glutamyl-Cys and [^{3}H]Gly and uptake of the latter.

Inhibitors of DNP-GS Transport

The transport of DNP-GS by vacuolar membrane vesicles exhibited a distinctive inhibitor profile. DNP-GS uptake was extremely sensitive to inhibition by vanadate (I_{50} = 7.5 ± 3.9 μM) and moderately sensitive to inhibition by vinblastine (I_{50} = 26.3 ± 12.8 μM) and verapamil (I_{50} = 38.9 ± 12.9 μM) (Fig. 6). The significance of the effects of vinblastine and verapamil remains to be determined (see "Discussion"), but the susceptibility of DNP-GS transport to inhibition by the phosphoryl transition-state analog, vanadate, together with the inability of the nonhydrolyzable ATP analog, AMP-PNP, to support uptake (Table II), suggest that MgATP-dependent DNP-GS transport is contingent on ATP hydrolysis and formation of a phosphoenzyme intermediate.

GSH, DTT, and CDNB alone did not inhibit [^{3}H]DNP-GS uptake (Table II), whereas the two *S*-glutathione conjugates tested, GSSG and metolachlor-GS, did (Table II; Fig. 6C). The insensitivity of [^{3}H]DNP-GS uptake to the chloroacetanilide herbicide metolachlor alone but its pronounced sensitivity to metolachlor-GS (I_{50} = 150.0 ± 29.3 μM) suggest that the DNP-GS transporter identified is competent in the transport of *S*-conjugated chloroacetanilide but not free herbicide and is equivalent to the transport function delineated by Martinoia et al. (1993).

Species Distribution of Vacuolar DNP-GS Transport

Vacuolar DNP-GS transport appears to be of relatively broad distribution in vascular plants. The preparation of vacuolar membrane-enriched fractions from all of the plant tissues examined, in addition to *Vigna* hypocotyls, *Arabidopsis* leaf, *Arabidopsis* root, *Beta* storage root, and *Zea* root, yielded vesicles competent in MgATP-dependent [^{3}H]DNP-GS uptake (Table III). However, although a pro-

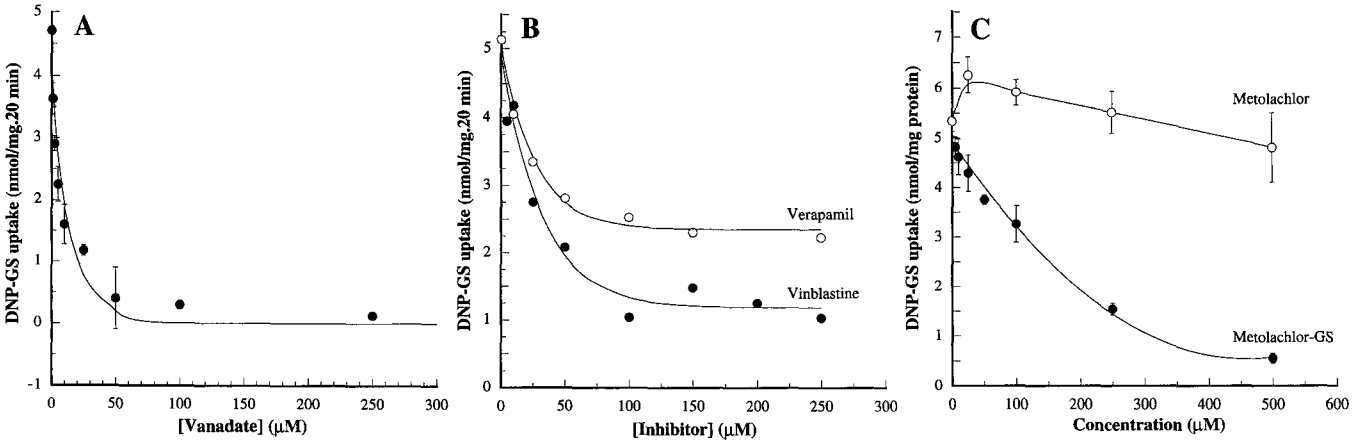

Figure 6. Inhibition of MgATP-dependent [^{3}H]DNP-GS uptake by *Vigna* vacuolar membrane vesicles by vanadate (A), vinblastine and verapamil (B), or metolachlor-GS (C). Uptake was measured for 20 min in standard uptake medium containing 64.2 μM [^{3}H]DNP-GS and the indicated concentrations of vanadate, vinblastine, verapamil, metolachlor, or metolachlor-GS. The lines of best fit and kinetic parameters were estimated by nonlinear least-squares analysis. I_{50}(vanadate) = 7.5 ± 3.9 μM; I_{50}(vinblastine) = 26.3 ± 12.8 μM (inclusive of uninhibitable component), 21.2 ± 10.3 μM (exclusive of uninhibitable component); I_{50}(verapamil) = 38.9 ± 12.9 μM (inclusive of uninhibitable component), 17.5 ± 5.8 μM (exclusive of uninhibitable component); I_{50}(metolachlor-GS) = 150.0 ± 29.3 μM. Values shown are mean ± SE (n = 3).

Table III. *[^{3}H]DNP-GS uptake by vacuolar membrane-enriched fractions prepared from leaves, roots, or hypocotyls of A. thaliana, B. vulgaris, V. radiata, and Z. mays*

Uptake was measured in standard uptake medium containing 64.2 μM [^{3}H]DNP-GS in the presence (+MgATP) or absence (−ATP) of 3 mM MgATP. Values shown are mean ± SE (n = 3).

Tissue Source	DNP-GS Uptake	
	+MgATP	−ATP
	nmol mg^{-1} protein (20 min)$^{-1}$	
Arabidopsis		
Leaf	2.95 ± 0.09	0.64 ± 0.03
Root	4.96 ± 0.65	1.21 ± 0.05
Beta		
Storage root	19.11 ± 1.24	0.91 ± 0.12
Vigna		
Hypocotyl	6.73 ± 1.42	0.98 ± 0.44
Zea		
Root	3.32 ± 0.27	1.22 ± 0.18

nounced stimulation of uptake was elicited by MgATP in all cases, the relative extents differed markedly among species. For instance, vacuolar membrane vesicles purified from *Beta* storage root showed a rate of 19.1 ± 1.2 nmol mg^{-1} (20 min)$^{-1}$, whereas the corresponding values for vesicles from *Arabidopsis* leaf and *Zea* root were only 3.0 ± 0.1 and 3.3 ± 0.3 nmol mg^{-1} (20 min)$^{-1}$, respectively. The choice of vacuolar membrane vesicles purified from *Vigna* hypocotyls, which exhibited an intermediate level of DNP-GS transport (6.7 ± 1.4 nmol mg^{-1} [20 min]$^{-1}$; Table III), for the bulk of the functional studies therefore appears to have been appropriate.

DISCUSSION

The experiments described demonstrate that uptake of the *S*-glutathione conjugate, DNP-GS, by vacuolar membrane vesicles purified from *V. radiata* is an MgATP-dependent process. Four primary observations implicate a direct requirement for MgATP rather than utilization of the energy contained in a pre-existent electrochemical gradient: (a) Abrogation of the development of an inside-acid pH gradient across the vacuolar membrane by uncouplers (FCCP, gramicidin-D, NH_4Cl) exerts little or no effect on either the rate or extent of DNP-GS uptake. (b) Provision of the V-PPase substrate, Mg_2PPi, in place of MgATP results in the establishment of an inside-acid transmembrane pH gradient greater than that established by the V-ATPase but fails to drive DNP-GS uptake. (c) Total inhibition of the capacity of the V-ATPase for H^+-translocation by KNO_3 does not inhibit MgATP-dependent DNP-GS uptake. (d) Agents (valinomycin, permeant anions) that dissipate the inside-positive membrane potential, otherwise generated by the V-ATPase and V-PPase, do not inhibit DNP-GS uptake.

Two features of the MgATP dependence of vacuolar DNP-GS uptake show that MgATP must be serving the role of energy source. First, the amounts of DNP-GS taken up are not explicable other than by invoking a significant energy input. Estimates of the internal volume of vacuolar membrane vesicles prepared in the same manner as those employed in the studies described here yield a value of 10 μL/mg membrane protein (Poole et al., 1985). Assuming a population constituted of an approximately 1:1 mixture of right-side-out and inside-out vesicles, as is the case for vacuolar membrane vesicles purified from plant sources by density gradient centrifugation (Rea et al., 1987; Zhen et al., 1994), accumulation ratios of 9.1, 15.2, 58.6, 20.6, and 10.2 can be enumerated for the vacuolar membrane vesicles purified from *Arabidopsis* leaf, *Arabidopsis* root, *Beta* storage root, *Vigna* hypocotyl, and *Zea* root, respectively, when uptake is measured for 20 min at an external DNP-GS concentration of 64.2 μM. Second, nonhydrolyzable ATP analogs, such as AMP-PNP, do not promote DNP-GS uptake, indicating a requirement for hydrolysis of the γ-phosphate of ATP rather than nucleoside triphosphate-mediated gating of an otherwise exergonic transport pathway.

The calculated accumulation ratios are commensurate with the measured ratios of MgATP-dependent:MgATP-independent DNP-GS uptake: namely, 4.6 (*Arabidopsis* leaf), 4.1 (*Arabidopsis* root), 21.0 (*Beta* storage root), 6.9 (*Vigna* hypocotyl), and 2.6 (*Zea* root). This not only supports the validity of the estimated accumulation ratios but, since the apparent intravesicular concentration of [^{3}H]DNP-GS achieved in the absence of MgATP approximates its concentration in the bulk medium, implies that MgATP-independent uptake is equilibrative.

The ^{3}H label of [^{3}H]DNP-GS constitutes a reliable measure of the distribution of the transported species. Interpretative problems associated with the metabolism of DNP-GS, specifically diminution of its effective intravesicular concentration and overestimation of the chemical potential against which it must be transported, are excluded by the results of the chromatographic analyses of the ^{3}H label from [^{3}H]DNP-GS, which show that this compound is not subject to irreversible covalent modification either during or soon after uptake.

The DNP-GS transporter of plant vacuolar membranes bears a close functional resemblance to the DNP-GS transporter of mammalian cells. Membrane vesicles derived from human erythrocytes (LaBelle et al., 1986), rat liver canaliculus (Kitamura et al., 1990; Kobayashi et al., 1990; Akerboom et al., 1991), and heart sarcolemma (Ishikawa, 1989) mediate DNP-GS transport and, as shown here for the vacuolar transporter, require MgATP as direct energy source, are highly sensitive to inhibition by vanadate, and, with the exception of the transporter associated with erythrocytes, are moderately sensitive to inhibition by GSSG but not GSH (Table IV). In view of these similarities and the congruence of their kinetic parameters [K_m(MgATP), K_m(DNP-GS), V_{max}(DNP-GS); Table IV], the vacuolar and mammalian DNP-GS transporters may be functionally equivalent. Therefore, an important corollary follows: the vacuolar DNP-GS transporter may play a role in the detoxification of xenobiotics. Since the glutathione conjugation reaction, mediated by cytosolic glutathione-*S*-transferases, is instrumental in the detoxification of lipophilic electrophiles derived from both exogenous and endogenous sources, it has been suggested that, through the concerted actions of glutathione-*S*-transferases and the plasma

Table IV. *Comparison of functional characteristics of DNP-GS transporters of plant vacuolar membrane, rat liver canalicular membrane, rat heart sarcolemma, and human erythrocyte plasma membrane*

Property	Value			
	Vigna vacuolar membrane[a]	Rat liver canalicular membrane[b]	Rat heart plasma membrane[c]	Human erythrocyte plasma membrane[d]
K_m(MgATP)	51.3 ± 10.4 μM	50 μM	30 μM	1 mM
K_m(DNP-GS)	81.3 ± 41.8 μM	71 ± 13 μM	20 μM	290 μM
V_{max}(DNP-GS)	0.6 ± 0.1 nmol mg^{-1} min^{-1}	0.3 ± 0.1 nmol mg^{-1} min^{-1}	0.04 nmol mg^{-1} min^{-1}	0.2 nmol mg^{-1} min^{-1}
Inhibition by				
Vanadate	7.5 ± 3.9 μM (I_{50})	Inhibited	30 μM (I_{50})	50 μM (I_{50})
GSSG	1 mM (I_{50})	440 μM (K_i)	18% inhibited by 100 μM	Not inhibited
GSH	Not inhibited	Not inhibited	Not inhibited	Not inhibited
Vinblastine	26.3 ± 12.8 μM (I_{50})	Not inhibited	Not inhibited	Not determined
Verapamil	38.9 ± 12.9 μM (I_{50})	Not inhibited	Not determined	Not determined

[a] This study. [b] Akerboom et al. (1991). [c] Ishikawa (1989). [d] LaBelle et al. (1986).

membrane DNP-GS 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). The existence of an apparently equivalent function in the vacuolar membrane of plant cells could denote an analogous process but one involving vacuolar sequestration instead of, or in addition to, extrusion via the plasma membrane (Fig. 7).

Numerous studies have established the existence of glutathione-S-transferase families comprising isozymes possessing varying degrees of substrate specificity for particular herbicides (Fuerst and Gronwald, 1986; Kreuz, 1993). Of the herbicides known to be S-conjugated to glutathione by glutathione transferases, the best characterized are the chloroacetanilides. These compounds are initially metabolized through conjugation with glutathione by nucleophilic displacement of chlorine from the chloracetyl side chain (Fig. 7), and acquired resistance to the chloracetanilides is associated with induction of the appropriate glutathione transferase (Kreuz, 1993). Thus, the studies performed by Martinoia et al. (1993) and ourselves, demonstrating that the vacuolar DNP-GS transporter is susceptible to inhibition by and competent in the transport of chloroacetanilide-glutathione conjugates (metolachlor-GS in this study) are at least consistent with participation of this transport pathway in vacuolar sequestration of detoxified (conjugated) herbicide.

It is, of course, improbable that the primary function of the plant DNP-GS transporter is the vacuolar sequestration of herbicides unless compounds like them are to be found endogenously or in the natural habitat. The capacity of the vacuolar DNP-GS transporter for herbicide conjugate transport is undoubtedly of agronomic importance but does not explain the existence of the transporter in the first place. Whereas leukotriene C_4 has been found to be transported by the mammalian DNP-GS transporter with a K_m of 0.2 μM, which is 1 order of magnitude lower than for any other glutathione conjugate studied, making it a candidate endogenous substrate (Ishikawa et al., 1990), no equivalent endogenous compound is known for the vacuolar enzyme. The range of possibilities could nevertheless be broad. Glutathione-S-transferase isozymes that conjugate the olefinic double bond of *trans*-cinnamic acid (Fig. 7) and presumably related compounds have been reported in a wide range of plant tissues (Dean et al., 1991; Edwards and Dixon, 1991), and when account is taken of the capacity of Cyt P450s in conferring the requisite electrophilicity on otherwise unreactive compounds for subsequent S-conjugation with glutathione, the spectrum of potential substrates is extended further.

The sequestration of "natural herbicides," allelochemicals, and/or pathogen-related compounds (Fig. 7) is a strong possibility in view of the allelopathic properties of phenolic compounds, including cinnamic acid and its derivatives (Li et al., 1993), and the inferred involvement of glutathione-S-transferases and cinnamic acid in plant-pathogen interactions. The glutathione-S-transferase responsible for glutathione conjugation of *trans*-cinnamic acid (GSCT) is activated by *p*-coumaric acid and 7-hydroxycoumarin (Dean and Machota, 1993), both of which are themselves allelochemicals (Li et al., 1993), and GSCT is induced after the exposure of cell-suspension cultures to fungal elicitors (Dean and Machota, 1993). Notwithstanding our ignorance of the true physiological meaning of these reactions, the possibility that GSCT catalyzes the conversion of either exogenous (allelopathic) or endogenous (pathogen-elicited) *trans*-cinnamic acid, or related compounds, into a form that is more readily transported into and stored within the vacuole through direct mediation of the vacuolar DNP-GS transporter is intriguing.

Before ascribing too strict an equivalence between the mammalian and vacuolar DNP-GS transporters, a final cautionary note is perhaps warranted. There is one striking and potentially poignant difference between the mammalian and plant enzymes. Whereas DNP-GS transport by the enzymes from rat liver (Ishikawa, 1990; Akerboom et al., 1991) and heart (Ishikawa, 1990) is not affected by vinblastine or verapamil, even at concentrations in excess of 200 μM, the vacuolar DNP-GS transporter is inhibited by both of these compounds (Table IV). Although the finding that the inhibitions exerted by vinblastine and verapamil, unlike those caused by vanadate, are partial (Fig. 6) may reflect the participation of more than one transporter, the alkaloid sensitivity of DNP-GS uptake is nonetheless more akin to that of another MgATP-dependent transporter, the

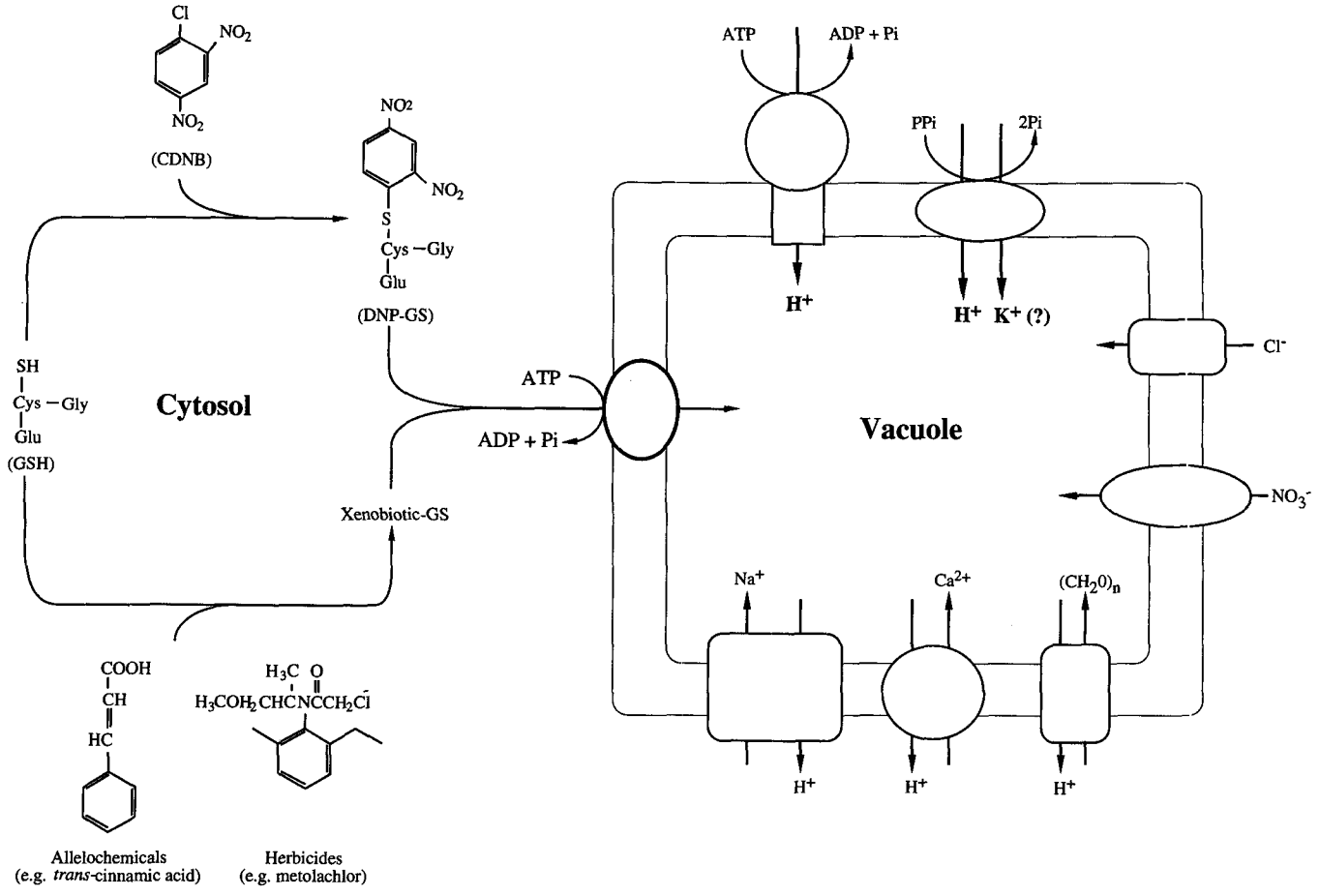

Figure 7. Schematic diagram depicting participation of DNP-GS transporter in vacuolar sequestration of *S*-glutathione conjugates synthesized in the cytosol by glutathione *S*-transferases. Shown is the conjugation of CDNB with GSH to generate DNP-GS, the conjugation of xenobiotics (allelochemicals [e.g. *trans*-cinnamic acid] and chloroanilide herbicides [e.g. metolachlor]) to generate xenobiotic-GS, and MgATP-dependent transport of both classes of conjugate into the vacuole via the DNP-GS transporter. Of the various solute transporters known to reside in the plant vacuolar membrane, a limited selection of which are shown, only the DNP-GS transporter is directly energized by MgATP and not by the prevailing $\Delta\tilde{\mu}_{H^+}$ established by the concerted activities of the V-ATPase and V-PPase.

mammalian P-glycoprotein, than to that of the DNP-GS transporters of rat liver and heart.

Mammalian P-glycoprotein, one of the most studied ABC transporters, is responsible for drug extrusion and the resistance of cancer cells to various chemotherapeutic agents (Endicott and Ling, 1989; Juranka et al., 1989). Its homologs include certain bacterial permeases (Ames, 1986), the yeast STE6 transporter responsible for the secretion of a mating factor (Kuchler et al., 1989; McGrath and Varshavsky, 1989), the *PDR5* gene encoding multidrug resistance in yeast (Balzi et al., 1994), the products of the *pfmdr* genes conferring resistance to antimalaria drugs in *Plasmodium* (Foote et al., 1989; Wilson et al., 1989), and the human cystic fibrosis gene product, CFTR (Kerem et al., 1989; Riordan et al., 1989).

Four features of the mammalian P-glycoprotein are pertinent in the present context: (a) its utilization of MgATP as direct energy source (Mimmack et al., 1989); (b) its capacity to mediate transport of a broad range of cytotoxic lipophilic cationic drugs, including vinblastine, vincristine, daunomycin, actinomycin D, colchicine, and puromycin (Cornwell et al., 1987; Safa et al., 1987; Horio et al., 1988); (c) the observation that agents that reverse drug resistance, such as verapamil and quinidine, also appear to be substrates for the transporter and compete for transport sites with cytotoxic drugs (Horio et al., 1988); and (d) its pronounced sensitivity to vanadate. Half-maximal inhibitions have been reported for vanadate concentrations ranging from 1.5 to 12 μM (Horio et al., 1988; Doige et al., 1992; Al-Shawi and Senior, 1993). Thus, although it has been argued that DNP-GS transport by mammalian cells is not catalyzed by P-glycoprotein (Ishikawa, 1990), the vacuolar DNP-GS transporter does nonetheless exhibit functional attributes characteristic of the P-glycoprotein of animal cells. It is therefore tempting to speculate that the vacuolar glutathione conjugate transporter is intermediate in function between the mammalian DNP-GS transporter and P-glycoprotein.

Studies directed at elucidating the molecular identity of any DNP-GS transporter are still in their infancy (see review by Zimniak and Awasthi, 1993), but it may be instructive to note that the three plant MDR-like clones isolated to date from *Arabidopsis* (*atpg1*, Dudler and Hertig, 1992; and *MDR1* and *MDR2*, K.T. Howitz, A. Menkens, J. Darling, E.J. Kim, A.R. Cashmore, and P.A. Rea, unpublished data) all bear the hallmark of typical eukaryotic (P-glycoprotein-like) ABC transporter genes. They contain two ATP-binding cassettes; hydropathy analyses of the sequences linking the ATP-binding cassettes reveal a pattern consistent with six transmembrane α helices and an overall hydropathy profile reminiscent of that of mouse MDR1; and comparisons between the deduced sequences of the polypeptides specified by *Arabidopsis MDR1*, *MDR2*, and *atpg1* and other ABC proteins suggest that the *Arabidopsis* clones are more closely related to mammalian P-glycoprotein than any other member of the ABC superfamily.

In the light of the apparent functional intermediacy of the vacuolar DNP-GS transporter versus the mammalian DNP-GS transporter and mammalian P-glycoprotein and the existence of multiple *MDR* homologs in plants, it is now crucial to determine if, as its functional characteristics suggest, the vacuolar glutathione conjugate carrier is indeed an ABC transporter and, if so, whether it is encoded by one or more of the plant *MDR* genes identified to date or others that remain to be found.

ACKNOWLEDGMENTS

We wish to thank Dr. Janice MacFarland (CIBA-GEIGY) for the kind gift of metolachlor and her enthusiasm for this work, our colleagues Eugene J. Kim and Rui-Guang Zhen for useful discussions and assistance with some aspects of the project, and John Chan for advice on *Arabidopsis* root culture.

Received October 11, 1994; accepted December 2, 1994.

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