Biochimica et Biophysica Acta, 1152 (1993) 270–279

BBAMEM 76118

Dicarboxylate transport at the vacuolar membrane of the CAM plant *Kalanchoë daigremontiana*: sensitivity to protein-modifying and sulphydryl reagents

Mary Bettey and J. Andrew C. Smith *

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB (UK)

(Received 30 March 1993)
(Revised manuscript received 12 July 1993)

Key words: Dicarboxylate transport; Malate; Protein modification; Vacuole; Tonoplast; *(Kalanchoë)*

Malate is widespread as a charge-balancing anion in plant vacuoles and plays a central role in nocturnal CO_2 assimilation in crassulacean acid metabolism (CAM). To characterize the malate transport system at the vacuolar membrane of CAM plants, tonoplast vesicles were prepared from leaf mesophyll cells of the crassulacean plant *Kalanchoë daigremontiana*. Dicarboxylate uptake, assayed by a membrane-filtration method using [^{14}C]malate or [^{14}C]succinate, displayed saturation kinetics with apparent K_m values of 4.0 mM (malate) and 1.8 mM (succinate); competition experiments indicated that both anions were transported by the same system. Dicarboxylate uptake was stimulated severalfold by activation of the tonoplast H^+-ATPase or H^+-PP_iase, an effect inhibitable by ionophore. Passive (non-energized) dicarboxylate uptake was sensitive to the sulphydryl reagents *N*-ethylmaleimide and *p*-chloromercuribenzene sulphonate, as well as to a range of protein modifiers. In particular, inhibition by pyridoxal phosphate was completely substrate-protectable, and that by phenylglyoxal partially so, thus implicating at least one lysine residue and perhaps also an arginine residue in the substrate-recognition site of the transport protein. The involvement of one or more critical lysine residue was supported by analysis of the initial phase of inhibition by pyridoxal phosphate: this showed pseudo-first-order kinetics with a reaction order of 1.03 ± 0.13 and a K_d for substrate protection close to the apparent K_m for dicarboxylate uptake.

Introduction

Transport of ions across the vacuolar membrane (tonoplast) of plant cells is important in turgor maintenance, cytosolic homeostasis and sequestration of secondary metabolites [1,2]. Accumulation of organic acids is particularly important in plants for pH regulation and osmotic balance. Large ion fluxes may occur across the vacuolar membrane, such as in plants displaying crassulacean acid metabolism (CAM), where accumulation and remobilization of organic acids (predominantly malate) is central to the process of nocturnal carbon fixation [3,4].

Despite recent advances in characterizing ion-transport proteins and the genes that encode them in animal cells, none of the equivalent ion channels of the plant tonoplast has yet been identified. Recent reports have identified a number of integral membrane proteins that appear to be tonoplast-specific [5,6]. In CAM plants, some of the tonoplast polypeptides may be CAM-specific, possibly including transport proteins [7]. It is known that the tonoplast contains two electrogenic pumps, the H^+-ATPase and the H^+-PP_iase [8]. These pumps set up a protonmotive force, which provides a driving force for secondary transport of organic and inorganic ions, amino acids and carbohydrates [9]. Biochemical characterization of these other polypeptides at the tonoplast is now needed to identify those specifically involved in ion transport.

Malate is one of the most widespread anions accumulated in plant vacuoles, but its mechanism of transport across the vacuolar membrane is not yet properly understood. In CAM plants, malate accumulation is closely linked to nocturnal fixation of CO_2 by the cytosolic enzyme phospho*enol*pyruvate carboxylase, and vacuolar malate concentrations can increase by over 150 mM in the course of the night [3,4]. Previously it had been assumed that malate uptake occurred by

* Corresponding author. Fax: +44 865 275074.

Abbreviations: CAM, crassulacean acid metabolism; DTT, DL-dithiothreitol; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzene sulphonate; DEPC, diethyl pyrocarbonate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonate; $TPMP^+$, triphenylmethylphosphonium (bromide salt).

some form of 'carrier' or permease [10,11], but recent electrophysiological studies indicate that malate influx across the vacuolar membrane in CAM plants may occur via an anion-selective ion channel (Refs. 4 and 12, Pennington, A.J., Pantoja, O. and Smith, J.A.C., unpublished data). As yet, the kinetic properties and inhibitor sensitivity of this malate channel have not yet been characterized. Nor is it clear how this transport is related to a number of other tonoplast organic-anion transport systems that have been studied, such as citrate transport into tomato fruit tonoplast vesicles [13], citrate and malate transport into barley mesophyll vacuoles [14], and malate transport into vacuoles of *Catharanthus roseus* [15]. Dicarboxylate transport has also been studied at the peribacteroid membrane from N_2-fixing soybean root nodules [16], a membrane thought to share some similarities with the tonoplast of plant cells.

The aim of the present work was to explore further the properties of the malate-influx system at the tonoplast of CAM plants by assaying [^{14}C]dicarboxylate uptake into membrane vesicles using a rapid-filtration assay. Previous studies of tracer fluxes in such species have employed intact vacuoles, but membrane vesicles equilibrated with solutions of defined composition are more suitable for the study of transport kinetics. Using this direct assay system, we have investigated both the kinetics of dicarboxylate uptake into tonoplast vesicles of the CAM plant *Kalanchoë daigremontiana*, as well as the effect of inhibitors known to modify anion-recognition centres in proteins. The sensitivity of the transport system to such inhibitors could be useful in subsequent identification and purification of the transport protein using affinity labelling.

Materials and Methods

Plant material

Plants of *Kalanchoë daigremontiana* Hamet et Perrier de la Bathie were propagated vegetatively and raised in a heated glasshouse. They were grown in John Innes No. 3 compost and watered weekly with half-strength Hoagland solution No. 3 [17] supplemented with full-strength Long Ashton micronutrients [18]. Natural lighting was supplemented by high-pressure sodium-vapour lamps (Osram 400 W SON-T; GEC, London, UK) to provide a 14-h photoperiod. About 2 weeks before use, plants aged between 6 and 9 months were transferred to a growth room with illumination provided by a combination of fluorescent tubes and tungsten lamps (Philips TLD 50 W/83HF and Philips RS 60 W, respectively; Philips, Croydon, UK) for 14 h daily at a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} at mid-plant height. Air temperature was maintained at 25°C in the light and 14°C in the dark.

Isolation of tonoplast vesicles

Fully expanded leaves were harvested 1 to 2 h after the start of the dark period, when the leaf malic-acid content was close to its minimum value. Leaf material (100 g fresh weight, with midribs and margins removed) was homogenized in a commercial food blender in 250 ml of a buffer comprised of 450 mM mannitol, 3.0 mM $MgSO_4$, 10 mM EGTA, 0.5% (w/v) PVP-40 (polyvinylpyrrolidone, average molecular weight 40000), 100 mM Tricine (*N*-tris[hydroxymethyl]methylglycine) buffered to pH 8.0 with BTP (1,3-bis[tris(hydroxymethyl)methylamino]propane), and 10 mM DTT (DL-dithiothreitol). The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at $13000 \times g_{av}$, (Sorvall RC28S centrifuge, F28/36 rotor, 15 min, 4°C). The resulting supernatant was centrifuged at $80000 \times g_{av}$ (40 min, 4°C), and the pellet resuspended in a glycerol-containing medium based on that used by Pope and Leigh [19] comprised of 1.1 M glycerol, 1.0 mM EDTA, 10 mM Tricine (pH 8.0, BTP), and 2.0 mM DTT. After centrifuging at $80000 \times g_{av}$ (40 min, 4°C), the pellet was resuspended in 18 ml of the glycerol medium. This was layered onto a cushion of 6 to 8 ml of 23% (w/v) sucrose in glycerol medium which was centrifuged at $100000 \times g_{av}$ (70 min, 4°C). The interface was removed and diluted 1:1 with glycerol medium. Tonoplast vesicles were pelleted at $100000 \times g_{av}$ (30 min, 4°C) and resuspended in a resuspension medium comprised of 150 mM mannitol, 5.0 mM $MgSO_4$, 1.0 mM EDTA, 25 mM BTP (pH 7.5, Tricine) and 2.5 mM DTT.

For some assays, a crude microsomal membrane fraction prepared from mesophyll-cell protoplasts was used, rather than the tonoplast fraction obtained by the bulk homogenization method above. Protoplasts were prepared [20] and were homogenized by drawing them gently through a metal cannula (0.8 mm i.d. × 40 mm) three times. The final homogenate was transferred to eppendorf tubes and spun in a Beckman Microfuge at $12000 \times g_{av}$ (4°C, 2 min) to pellet chloroplasts and mitochondria. The supernatant was spun in a Beckman TL 100 Tabletop Ultracentrifuge at 200000 $\times g_{av}$ (TLA 100–3 rotor, 15 min, 4°C), to pellet the membrane vesicles. The pellet was resuspended in resuspension medium to give a microsomal membrane fraction.

For preparation of purified tonoplast vesicles from this fraction, the microsomal preparation was layered onto a 25% (w/v) sucrose cushion containing 5 mM Hepes (pH 7.5, Tris), 1 mM DTT, and spun at 200000 $\times g_{av}$ (30 min, 4°C). The material at the interface was removed, diluted with resuspension medium, and pelleted again at $200000 \times g_{av}$, (15 min, 4°C). The pellet

(purified tonoplast vesicles) was then resuspended in resuspension medium and used in assays.

Anion uptake assays

Anion uptake assays contained 0.1 mM ^{14}C-labelled anion ([1,4-^{14}C]succinate, 4.11 GBq mmol^{-1}; [2,3-^{14}C]succinate, 4.0 GBq mmol^{-1}; or L-[U-^{14}C]malate, 1.9 GBq mmol^{-1}; all from Amersham International, Bucks, UK), 150 mM mannitol, 25 mM BTP (pH 7.5, Tricine), 0.2 mM sodium azide, 0.1 mM sodium orthovanadate, 3.0 mM $MgSO_4$, and 2.5 mM DTT. The assay was started by the addition of vesicles (final protein concentration in the assay was 100 to 200 μg ml^{-1} for microsomal vesicles, and 20 to 100 μg ml^{-1} for purified tonoplast vesicles). In the standard assays, a zero-time aliquot of the reaction medium was taken immediately after adding the vesicles and a second aliquot after incubation for 10 min at 25°C. These aliquots were immediately filtered under suction on Whatman 0.45 μm cellulose nitrate membrane filters, using a Hoefer filter manifold (Hoefer Scientific Instruments UK, Newcastle-under-Lyme, Staffs, UK), and rapidly washed with 5 ml of an ice-cold buffer containing 150 mM mannitol, 25 mM BTP (pH 7.5, Tricine), 0.1 mM succinate, and 1.0 mM DTT. Filters were then placed into scintillation vials with 4 ml scintillant (Optiphase Hisafe 3, Wallac, Milton Keynes, UK) and counted (when the filters had dissolved) on an LKB 1215 Rackbeta Liquid Scintillation Counter (LKB Wallac, Milton Keynes, UK). Counts were adjusted for counting efficiency using a standard quench curve. The zero-time aliquot was used to determine the amount of radiolabel non-specifically bound to the filter and vesicles.

Measurement of vesicle acidification

Rates of vesicle acidification on energizing the tonoplast H^+-ATPase or the H^+-PP_iase were used as described previously [21] to determine the relative permeability of the tonoplast to various charge-balancing anions. Vesicle acidification was monitored by following the fluorescence quenching of quinacrine (6-chloro-9-{[4-(diethylamino)-1-methylbutyl]amino}-2-methoxyacridine dihydrochloride) using a luminescence spectrometer (LS50, Perkin Elmer, Bucks, UK) as described previously [21].

Measurement of protein

Protein was measured by the method of Bradford [22] using bovine serum albumin as the standard.

Results

Kinetics of dicarboxylate uptake by tonoplast vesicles

Tonoplast vesicles isolated from mesophyll tissue of *Kalanchoë daigremontiana* were assayed for uptake of

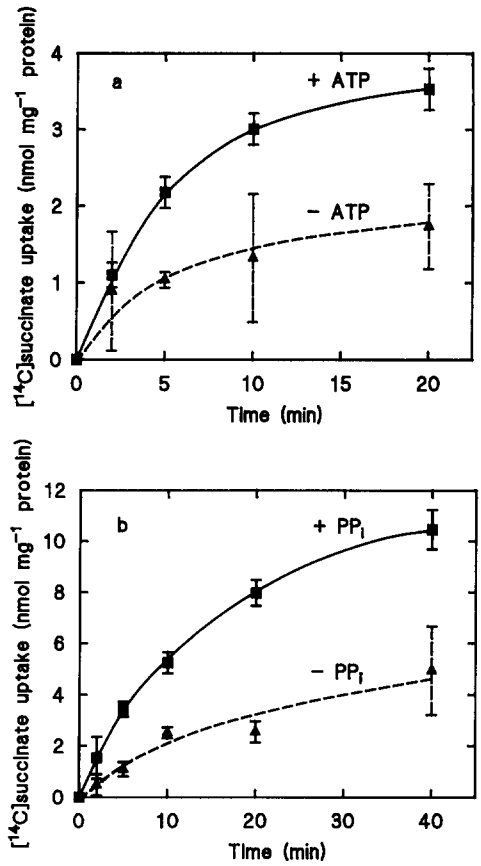

Fig. 1. Time-course of [^{14}C]succinate uptake by tonoplast vesicles from *Kalanchoë daigremontiana*. Tonoplast vesicles were prepared by a combination of differential and density gradient centrifugation from bulk homogenates of leaf mesophyll tissue. (a) Uptake assays were conducted at pH 8.0 in the presence or absence of 3 mM ATP at 25°C. (b) Uptake assays were conducted at pH 8.0, with 7.5 mM $MgSO_4$ and 90 mM KOH (adjusted to pH 8.0 with Tricine) in the presence or absence of 0.5 mM Na_4PP_i. Data are expressed as means ± standard deviations ($n = 3$).

[^{14}C]succinate or [^{14}C]malate using a membrane-filtration assay. The rate of dicarboxylate uptake gradually decreased with incubation time (Fig. 1). Significant dicarboxylate uptake occurred in the absence of ATP or PP_i, presumably driven partly by a concentration gradient of dicarboxylate and partly by a residual transmembrane electrical potential difference. Non-energized uptake of dicarboxylate was reduced to 69% of the control rate (mean of five separate experiments) in the presence of 10 mM $TPMP^+$ (triphenylmethylphosphonium, a permeant lipophilic cation), consistent with a contribution from a residual membrane potential to the driving force for dicarboxylate uptake. Uptake was stimulated by ATP (Fig. 1a) or PP_i (Fig. 1b). Energization of the tonoplast H^+-ATPase or the H^+-PP_iase by substrate sets up an inside-positive membrane potential, which can provide the driving force for passive dicarboxylate uptake by an electrophoretic mechanism [21,23]. Stimulation of dicarboxylate uptake by PP_i was abolished by the ionophore carbonylcyanide *m*-chloro-

TABLE I

Comparison of rates of dicarboxylate uptake and malate-dependent vesicle acidification in crude microsomal membranes and purified tonoplast

The microsomal fraction was prepared by homogenization of mesophyll cell protoplasts; this fraction was further purified on a discontinuous sucrose density gradient to yield a tonoplast fraction, as described in Materials and Methods. Uptake of [^{14}C]succinate was measured over 10 min at 25°C. Vesicle acidification was assayed by the fluorescence quenching of quinacrine on energizing the vesicles with 0.5 mM PP_i in the presence of 50 mM malate as described in Materials and Methods; rates are expressed as % change in fluorescence (F, arbitrary units)min^{-1}(mg protein)$^{-1}$.

Membrane preparation	Rate of [^{14}C]succinate uptake (nmol/min per mg protein)	Rate of malate-dependent vesicle acidification (%F/min per mg protein)
Microsomes	0.36	339
Tonoplast	1.48	817

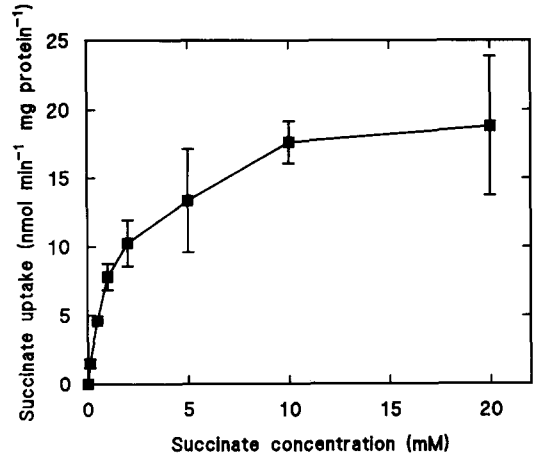

Fig. 2. Concentration-dependence of succinate uptake by tonoplast vesicles from *Kalanchoë daigremontiana*, assayed under the standard conditions described in Materials and Methods. Total succinate concentration was varied between 0.1 and 20 mM, with the concentration of labelled succinate maintained at 0.1 mM. Data are expressed as means ± standard deviations (n = 3).

phenylhydrazone (CCCP) (data not shown), indicating that the stimulation is due to some component of the protonmotive force. Assays were routinely carried out in the presence of 0.2 mM azide and 0.1 mM orthovanadate to inhibit any H^+ transport driven by contaminating F- or P-type ATPases, respectively, present in this membrane preparation (although the activity of these enzymes in the tonoplast fraction prepared as described here is minimal) [24]. Also, ATP-stimulation of dicarboxylate uptake was completely abolished by nitrate (data not shown), a diagnostic inhibitor of the vacuolar-type H^+-ATPase [25].

For routine assays, purified tonoplast vesicles were obtained after discontinuous sucrose-gradient centrifugation of a mixed microsomal membrane fraction. To test whether dicarboxylate transport activity in the absence of ATP or PP_i was specifically associated with the tonoplast, the uptake activity of these two fractions was compared. The sucrose-gradient purification step increased the specific activity of succinate transport approximately 4-fold (Table I). In comparison, the rate of vesicle acidification driven by the tonoplast H^+-PP_iase (in the presence of malate as balancing anion) was increased by a factor of 2.4 (Table I). Thus dicarboxylate transport activity appeared to be associated largely, if not exclusively, with the tonoplast fraction from mesophyll cells of *K. daigremontiana*.

When examined with respect to substrate concentration, passive (non-energized) succinate uptake by the tonoplast vesicles showed saturation kinetics (Fig. 2). Non-linear regression analysis revealed an apparent K_m for succinate of 1.8 ± 0.42 mM (best fit ± S.E.). An equivalent experiment with [^{14}C]malate gave an apparent K_m for malate of 4.0 ± 1.2 mM (data not shown).

To determine whether succinate and malate uptake occurred via the same transport system, tonoplast vesicles were incubated with [^{14}C]succinate or [^{14}C]malate in the presence of competing unlabelled anions (Table II). Unlabelled succinate and unlabelled malate inhibited uptake of labelled succinate to approximately the same extent; similarly, the two unlabelled anions inhibited the uptake of labelled malate almost equally. This suggests that succinate and malate share a common uptake system, a conclusion further supported by the fact that the non-transported anion maleate [21] had little effect on the transport of either anion.

Inhibitor sensitivity of dicarboxylate uptake

The sensitivity of enzyme function to amino-acid-modifying reagents can be used to identify residues

TABLE II

Effect of competing unlabelled anions on the uptake of [^{14}C]succinate and [^{14}C]malate into tonoplast vesicles

Tonoplast vesicles from a bulk homogenization preparation were assayed for succinate or malate uptake in the presence of 1 mM competing unlabelled anion. Competing anions were present as the BTP salt (pH 7.5). Results are expressed as percentage of control uptake (1.10 nmolmin^{-1}(mg protein)$^{-1}$ for succinate uptake, 2.36 nmolmin^{-1}(mg protein)$^{-1}$ for malate) and are given as means ± standard deviations (n = 3).

Added anion	Uptake of [^{14}C]labelled anion (% of control)	
	[^{14}C]succinate	[^{14}C]malate
Control	100.0 ± 13.1	100.0 ± 14.2
1 mM succinate	46.1 ± 7.1	27.4 ± 2.1
1 mM malate	35.9 ± 2.3	17.4 ± 1.1
1 mM maleate	121.2 ± 5.8	81.6 ± 3.4

that have an essential role in the enzyme mechanism [26]. This approach can also be applied to solute transport processes to identify amino-acid residues of functional importance in the translocation mechanism [27–29]. Passive (non-energized) dicarboxylate uptake was

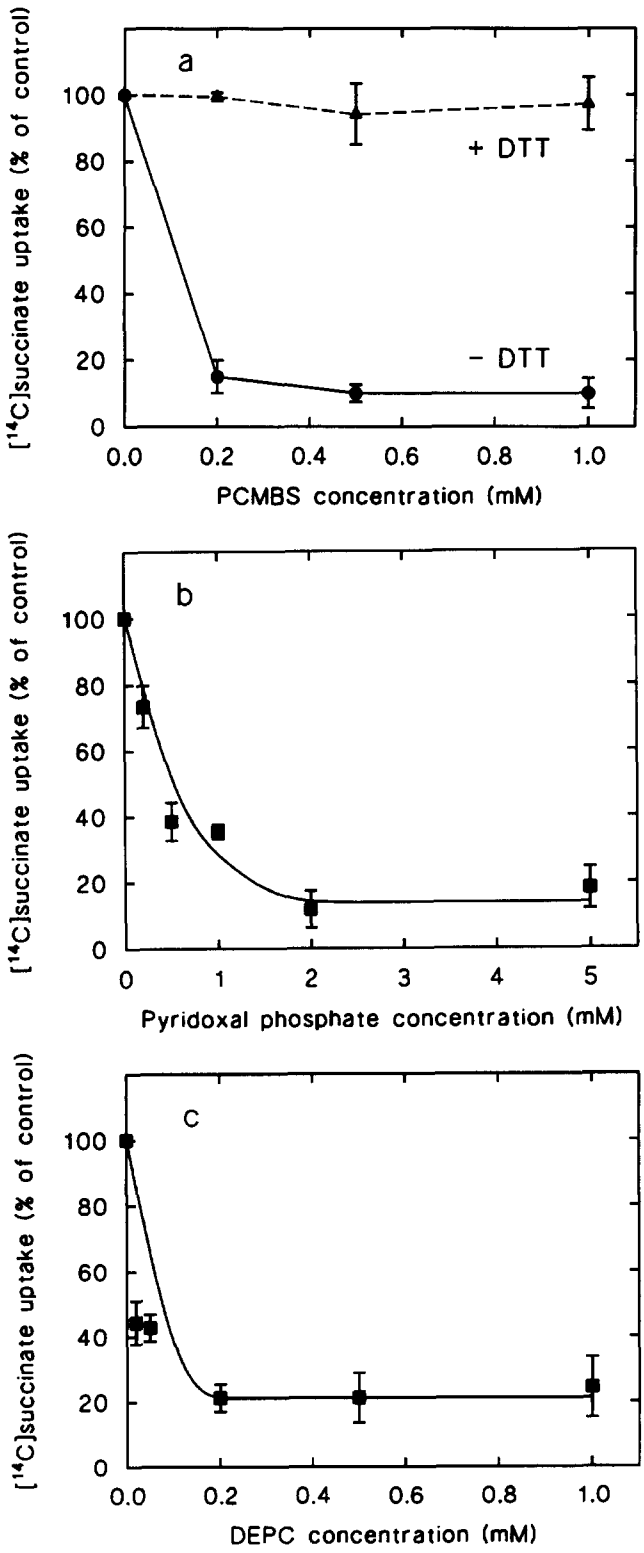

TABLE III

Effect of inhibitors on [^{14}C]succinate uptake by tonoplast vesicles

For measurements of the effect of *N*-ethylmaleimide (NEM) on dicarboxylate uptake, tonoplast vesicles from a bulk homogenization preparation were prepared in the absence of DTT in the final resuspension medium and were preincubated with different concentrations of NEM in the presence or absence of DTT for 30 min at room temperature. Control rates of uptake (in the absence of DTT) were 0.954 nmol min^{-1}(mg protein)$^{-1}$ for the minus DTT treatment, and 0.863 nmol min^{-1}(mg protein)$^{-1}$ for the plus DTT treatment. For the other inhibitors, membrane vesicles from the microsomal fraction of a protoplast preparation were preincubated with various concentrations of the inhibitor for 30 min at room temperature prior to assay. Control rates of uptake were 0.141 nmol min^{-1}(mg protein)$^{-1}$ for the DIDS experiment and 0.108 nmol min^{-1}(mg protein)$^{-1}$ for the phenylglyoxal experiment. Results are given as means ± standard deviations ($n = 3$).

Treatment	[^{14}C]Succinate uptake (% of control)
0 mM NEM	100.0 ± 16.3
1 mM NEM	81.6 ± 5.8
2 mM NEM	55.9 ± 0.6
5 mM NEM	35.2 ± 4.4
5 mM NEM + 5 mM DTT	83.2 ± 3.1
0 mM phenylglyoxal	100.0 ± 32.4
0.5 mM phenylglyoxal	24.1 ± 13.9
2 mM phenylglyoxal	14.8 ± 25.9
0 mM DIDS	100.0 ± 13.5
0.1 mM DIDS	49.6 ± 31.9
1 mM DIDS	4.3 ± 7.8

Fig. 3. Effect of protein-modifying reagents on [^{14}C]succinate uptake by tonoplast vesicles of *Kalanchoë daigremontiana*. (a) Effect of the sulphydryl reagent *p*-chloromercuribenzene sulphonate (PCMBS) and the protective effect of dithiothreitol (DTT). Microsomal vesicles from a protoplast preparation were prepared in the absence of DTT in the final resuspension medium, and were preincubated with different concentrations of PCMBS, in the presence or absence of 5 mM DTT, for 1 h on ice. After the preincubation time vesicles were pelleted at 200000 × g_{av} (20 min, 4°C) and resuspended in resuspension medium without DTT. Data are expressed as % of control value (means ± standard deviations, $n = 3$). The uptake rate in the control assays was 0.19 nmol min^{-1}(mg protein)$^{-1}$ in the absence of DTT, and 0.17 nmol min^{-1}(mg protein)$^{-1}$ in the presence of DTT. (b) Effect of a lysyl-specific reagent on uptake of [^{14}C]succinate into tonoplast vesicles of *Kalanchoë daigremontiana*. Vesicles from a bulk homogenization preparation were preincubated for 5 min at room temperature with various concentrations of pyridoxal phosphate; 5 mM sodium borohydride was then added to each tube to stabilize the reversible bond and reduce the Schiff base. Data are expressed as% of control value (means ± standard deviations, $n = 3$). The uptake rate in the control assays was 0.92 ± 0.07 nmol min^{-1}(mg protein)$^{-1}$. (c) Effect of a histidyl-specific reagent on [^{14}C]succinate uptake by tonoplast vesicles of *Kalanchoë daigremontiana*. Vesicles from a microsomal fraction of a protoplast preparation were preincubated with various concentrations of diethyl pyrocarbonate (DEPC) for 45 min on ice, prior to assay. Data are expressed as% of control value (means ± standard deviations, $n = 3$). The uptake rate in the control assays was 0.083 ± 0.007 nmol min^{-1}(mg protein)$^{-1}$.

thus screened for its sensitivity to inhibition by protein-modifying reagents, including those reacting with residues of functional importance in anion-recognition centres [30].

(a) Sulphydryl reagents. The high nucleophilicity of the thiol group means that cysteine residues tend to be the most reactive amino-acid residues in proteins. Both *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzene sulphonate (PCMBS), two of the most widely used sulphydryl reagents, strongly inhibited the uptake of succinate into tonoplast vesicles from *K. daigremontiana*, and this inhibition could be prevented by 5 mM DTT (Table III and Fig. 3a).

(b) Lysyl-specific reagent. The ϵ-amino group of lysine in its unprotonated form is a very reactive nucleophile in proteins. Pyridoxal phosphate is a specific modifier of lysyl groups, and when stabilized by sodium borohydride is an irreversible inhibitor [27,31]. Pyridoxal phosphate greatly reduced succinate uptake into tonoplast vesicles of *K. daigremontiana* under such conditions (Fig. 3b), suggesting that one or more lysine residues are important in the dicarboxylate uptake system.

(c) Histidyl-specific reagent. Diethylpyrocarbonate (DEPC) modifies the imidazole side chain of histidine to form an *N*-carbethoxyhistidyl derivative [32]. Succinate uptake into tonoplast vesicles was strongly inhibited by incubation with DEPC (Fig. 3c). The reversal of DEPC inhibition by hydroxylamine, which is considered a test for the specificity of DEPC for histidine residues [32], could not be demonstrated in the present experiments because hydroxylamine itself caused considerable inhibition of succinate transport (data not shown).

(d) Arginyl-specific reagent. Arginine possesses a highly basic guanido group which plays an essential role in the active site of many enzymes that bind anionic substrates [30]. Phenylglyoxal is an irreversible arginyl-specific reagent and succinate uptake was strongly inhibited by this modifier (Table III), again suggesting that an arginine residue is important in the transport process.

(e) DIDS. 4,4'-Diisothiocyanatostilbene-2,2'-disulphonate (DIDS) is a general anion-transport inhibitor that is believed to modify lysine and cysteine residues [27,33,34]. DIDS was also found to be an effective inhibitor of the tonoplast dicarboxylate transport system (Table III).

Substrate-protection experiments

To test whether the amino-acid residues modified by the group-selective reagents are located at the substrate-recognition site, substrate-protection experiments were performed by preincubating tonoplast vesicles with or without 150 mM dicarboxylate (malate) in the presence of inhibitor. To avoid a reduced concentration gradient because of malate uptake, and an isotope-dilution effect because of residual malate, 150 mM malate was added to the minus-malate controls at the end of the preincubation period, and all vials incubated for a further 10 min. The membranes were then pelleted, resuspended in resuspension medium, and used for transport assays. Preincubation with malate completely protected the dicarboxylate transport system from inhibition by pyridoxal phosphate, and partially protected it from inhibition by phenylglyoxal (Fig. 4). However, preincubation with malate did not protect the dicarboxylate-uptake system significantly from inhibition by DEPC, NEM, or DIDS. Thus it appears that one or more lysine residues and possibly one or more arginine residues are located at the substrate-recognition site of the dicarboxylate transport system.

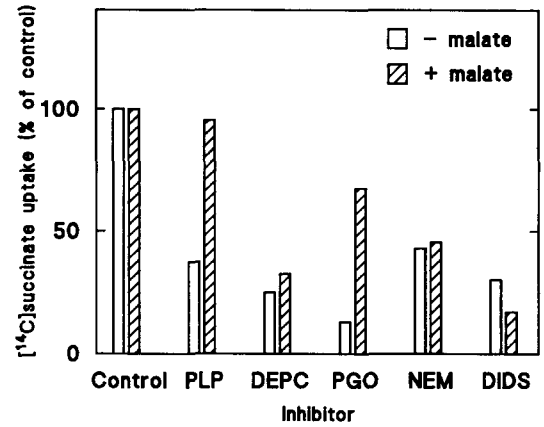

Fig. 4. Substrate protection of [^{14}C]succinate uptake by tonoplast vesicles of *Kalanchoë daigremontiana* from inhibition by group-specific reagents. Vesicles were preincubated with or without 150 mM malate for 5 min prior to incubation with an inhibitor on ice for 30 min. The concentrations of inhibitors used were 2 mM pyridoxal phosphate (PLP), 1 mM diethyl pyrocarbonate (DEPC), 10 mM phenylglyoxal (PGO), 5 mM *N*-ethylmaleimide (NEM), 3 mM 4,4-diisothiocyanatostilbene-2,2-disulphonate (DIDS). Inhibition was stopped where appropriate by adding 5 mM sodium borohydride to the PLP-treated vesicles after 5 min, or 30 mM imidazole to the DEPC-treated vesicles after 30 min. After 30 min, malate was added to the minus-malate samples and all samples incubated for a further 10 min. Vesicles were then pelleted at $200000 \times g_{av}$ (20 min, 4°C), resuspended in resuspension buffer, and used in standard uptake assays. The results shown are from several experiments and are expressed as a percentage of the appropriate control. Control uptake rates in the absence of inhibitor averaged 0.24 nmol min^{-1}(mg protein)$^{-1}$ for the minus-malate vesicles, and 0.36 nmol min^{-1}(mg protein)$^{-1}$ for vesicles preincubated with malate. Standard deviations averaged 20.1% of the means.

Kinetics of pyridoxal phosphate inhibition and protection by malate

Because one or more lysine residues were clearly implicated by the inhibitor experiments as critical for substrate recognition and transport, the kinetics of inhibition of dicarboxylate transport by pyridoxal phos-

phate were further investigated. A semi-log plot of percentage dicarboxylate transport activity remaining with time of incubation with pyridoxal phosphate did not show simple linear kinetics (Fig. 5a), suggesting that more than one class of lysine residue was modified by pyridoxal phosphate. However, a more detailed time-course of the initial phase of inhibition (0 to 30 s) did reveal pseudo-first-order kinetics (Fig. 5b). The second-order rate constant of inactivation (k) and the order of the reaction (n) with respect to the concentration of inactivator were obtained from the equation

$$v = V_0 \cdot e^x, \text{ where } x = -k[I]^n t$$

in which v is the dicarboxylate transport activity remaining after incubation with inactivator at a concentration I for time t, and V_0 is the initial dicarboxylate transport activity [35]. The best-fit estimates ($\pm$S.E.) for the constants calculated by non-linear regression analysis were $k = 10.8 \pm 3.5\ M^{-1}\,min^{-1}$, and $n = 1.03 \pm 0.13$. This value of n is consistent with the modification of a single lysine residue being sufficient for complete inactivation of the dicarboxylate transport system.

The kinetics of the protective effect of substrate on inhibition of dicarboxylate transport by pyridoxal phosphate were investigated by incubating tonoplast vesicles with 5 mM pyridoxal phosphate for a range of incubation times, in the presence of different concentrations of malate (Fig. 5c). Protection increased with increasing malate concentration. The dissociation constant (K_d) for the transporter-protector complex was obtained from the equation

$$v = V_0 \cdot e^z, \text{ where } z = -\{k' \cdot K_d / (K_d + [P])\}t$$

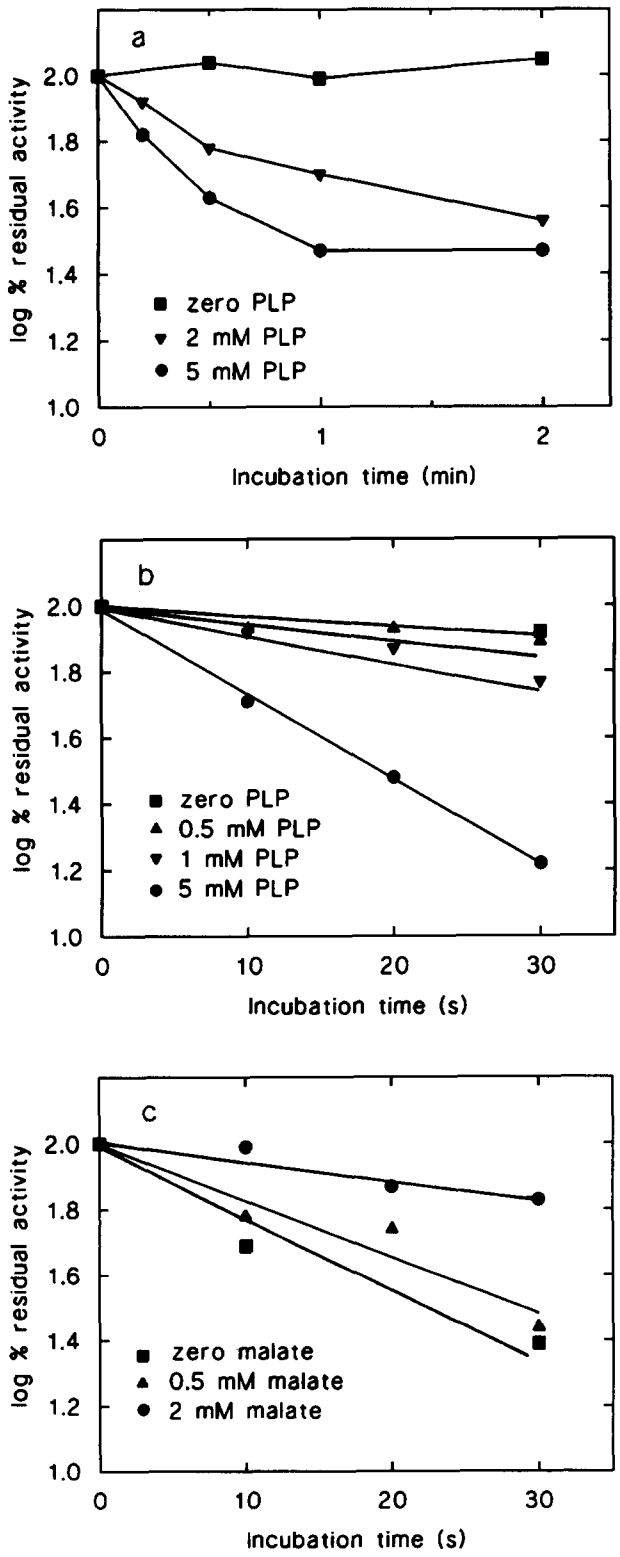

Fig. 5. Time-course of pyridoxal phosphate inhibition for succinate transport in *Kalanchoë daigremontiana* tonoplast vesicles. Tonoplast vesicles were incubated with the indicated concentration of pyridoxal phosphate at room temperature. At various times, aliquots were removed and the inhibition stopped by adding 5 mM sodium borohydride to stabilize the reversible bond and reduce the Schiff base formed between pyridoxal phosphate and the ϵ-amino group of the lysine residue. Vesicles were then assayed for [^{14}C]succinate uptake activity. (a) Inhibition over a 2-min time-course. Data are expressed as the log of the percentage of mean initial transport activity remaining. For the untransformed data standard deviations averaged 10.5% of the means ($n = 3$). Initial succinate uptake activity was 1.19 ± 0.06 nmol min^{-1}(mg protein)$^{-1}$. (b) 30-s time-course. Data are expressed as above. For the untransformed data standard deviations averaged 14.0% of the means ($n = 3$). Initial succinate uptake activity was 0.79 ± 0.14 nmol min^{-1}(mg protein)$^{-1}$. (c) Protection by malate against pyridoxal phosphate inactivation of the succinate uptake activity. Tonoplast vesicles were preincubated with various concentrations of malate for 5 min before adding 5 mM pyridoxal phosphate to all assays. At various times, aliquots were removed and the inhibition stopped by adding 5 mM sodium borohydride. Samples were incubated for 10 min, and the vesicles then pelleted to remove excess external malate, resuspended in resuspension medium, and assayed for remaining succinate uptake activity. Data are expressed as above. For the untransformed data standard deviations averaged 18.7% of the means ($n = 3$). The initial succinate uptake activity was 0.54 ± 0.06 nmol min^{-1}(mg protein)$^{-1}$.

in which v, V_o and t are defined as above, k' is the apparent pseudo-first-order rate constant, and [P] is the concentration of protector P [36]. The best-fit estimates (±S.E.) calculated by non-linear regression analysis in two experiments were 0.72 ± 0.29 mM (Fig. 5c) and 2.9 ± 1.5 mM (data not shown).

Discussion

Kinetics of dicarboxylate transport

Tonoplast membrane vesicles prepared from leaf mesophyll tissue of the CAM plant *Kalanchoë daigremontiana* were transport-competent and showed a time-course of dicarboxylate uptake (Fig. 1) similar to that of malate uptake by tonoplast vesicles of *Catharanthus roseus* and chlorate uptake by tonoplast vesicles of red beet [37,38]. Comparison of dicarboxylate uptake in different membrane fractions with the observed specific activity of the tonoplast H^+-PP_iase showed that dicarboxylate transport was almost exclusively associated with the tonoplast membrane (Table I). Dicarboxylate uptake was also stimulated by the activity of both the tonoplast H^+-ATPase and H^+-PP_iase, presumably in response to the enhanced inside-positive membrane potential generated by these H^+ pumps. The substrate kinetics experiments (Fig. 2) revealed K_m values for malate (4.0 mM) and succinate (1.8 mM) lower than those estimated by White and Smith [21] (14 mM) for the same transport system. However, their calculations were based on the effect of dicarboxylates on proton transport and were therefore only an indirect measure of dicarboxylate transport kinetics. The present calculation is close to the K_m values for malate determined for the barley tonoplast malate transporter (2.5 mM) and the *Catharanthus roseus* transporter (4.5 mM) [39,40]. We also found that ATP increased the V_{max} but did not alter the K_m for dicarboxylate uptake (data not shown), as noted in other studies [39,40].

In substrate-competition experiments, succinate and malate appeared to be transported across the tonoplast membrane by the same transport system (Table II, [21]). Either dicarboxylate can therefore be used to study the transport system in *K. daigremontiana*, and most experiments described here used [^{14}C]succinate as a tracer for malate transport in view of the cost of radiolabelled malate.

Inhibitor sensitivity

The use of amino-acid specific inhibitors is a powerful technique that can yield important information about residues of functional importance in proteins, although care must be taken over experimental conditions to maintain the specificity of the inhibitor [26]. The inhibitors used here are directed against basic amino-acid residues, which, being positively charged, are likely to be important as potential substrate-recognition sites in anion-transport proteins.

Sulphydryl reagents were potent inhibitors of dicarboxylate uptake by tonoplast vesicles of *K. daigremontiana* (Table III, Fig. 3a), as found for the tonoplast malate transporter of barley [41]. However, the tonoplast malate transporter of *Catharanthus roseus* is apparently not inhibited by sulphydryl reagents [15].

Of the basic amino acids, lysine residues are important in the function of a number of anion-transport proteins, as for example the chloroplast phosphate translocator [42] and the aspartate/glutamate carrier from heart mitochondria [33]. A lysine residue was also found to be important in the function of the dicarboxylate transport system of both *K. daigremontiana* (Fig. 3b) and barley mesophyll vacuoles [43]. Histidine residues are also important in malate transport across the tonoplast of barley and *Catharanthus roseus* [14,44], and the histidyl-specific reagent DEPC inhibited dicarboxylate uptake into tonoplast vesicles of *K. daigremontiana* (Fig. 3c). Reversal of DEPC inhibition by hydroxylamine (a test for DEPC specificity) could not be directly demonstrated in the present experiments because transport itself was inhibited by hydroxylamine, as has been found for other transport systems [45,46]. However, the DEPC dosage used for covalent modification was within the range in which reaction with histidine residues is highly selective [32]. An arginine residue also appears to be important in the malate transport processes of *K. daigremontiana* (Table III) and barley [43], as well as in other anion transport mechanisms [47].

The inhibitor DIDS binds strongly to many anion transporters and it inhibits tonoplast malate transport in *K. daigremontiana* (Table III), *Catharanthus roseus* [48] and barley [41]. DIDS also inhibits the relatively non-selective 'SV (slow-activating vacuolar) channels' commonly observed in tonoplast membranes [49]. Such channels are activated at inside-negative vacuolar membrane potentials and will conduct malate and a number of other anions as well as cations. The transport system described here for *K. daigremontiana* is likely to be distinct from the SV-type channel, as it is specific for four-carbon dicarboxylates [21] and opens only at inside-positive membrane potentials (Refs. 4 and 12, Pennington, A.J., Pantoja, O. and Smith, J.A.C., unpublished data).

In substrate-protection experiments with these group-selective reagents, malate was able to reverse the inhibitory effects only of pyridoxal phosphate and phenylglyoxal, and the latter only partially (Fig. 4). These results suggest that one or more lysine residues, and possibly one or more arginine residues, are impor-

tant in the binding of malate to the 'malate-recognition site' of the transport protein. Similarly, the malate transport system of barley tonoplast is also proposed to have a lysine residue at the substrate-recognition site [43]. Positively charged amino-acid residues such as lysine and arginyl groups are known to play an important role in the recognition of negatively charged substrates by enzymes [30]. For the dicarboxylate transport system described here, such residues might form part of a 'selectivity filter' [50] at the surface of the membrane protein, allowing only selected ions to pass through. The tonoplast dicarboxylate uptake system of *K. daigremontiana* has a specificity for four-carbon dicarboxylates in the *trans*-carboxyl configuration [21]. This specificity could be caused by a precise spatial requirement for electrostatic interaction between the carboxyl groups and positively charged amino-acid residues at the substrate-recognition site of the transport protein.

The sites of action of DEPC, NEM and DIDS must be some distance removed from the substrate-recognition site, since malate binding did not prevent inhibition by these modifiers (Fig. 4). However, the tonoplast malate transport system of *Catharanthus roseus* is proposed to have a histidine residue at the active site, as DEPC inhibition of malate transport can be partially prevented by the presence of malate [44]. This suggests that the essential histidine residue may be located closer to the substrate-recognition site in *Catharanthus* than in *Kalanchoë*, perhaps representing a difference between the transport systems of C_3 and CAM plants.

The method of Levy et al. [35] for determining the apparent first-order rate constant (k') and the order of the reaction with respect to the concentration of an inhibitor (n) has been used by numerous workers to study the kinetics of inhibition of amino-acid-specific reagents, although there are potential problems with the experimental design and interpretation of some of these studies [51]. Recent examples from plant membranes include arginine residues in the nitrate uptake system in corn roots, and histidine residues in the plasma membrane H^+-ATPase of red beet [47,52]. Pyridoxal-phosphate inhibition of the vacuolar malate uptake system in *K. daigremontiana* appears to show complex kinetics (Fig. 5a), which might implicate more than one class of lysine residue with different reactivities to pyridoxal phosphate. This is similar to the pattern of inhibition of red beet plasma membrane H^+-ATPase by the tyrosyl-specific reagent *N*-acetylimidazole [53]. However, the initial phase of inhibition (0 to 30 s) of dicarboxylate uptake did show pseudo-first-order kinetics (Fig. 5b). The calculated K_d value was close to the calculated K_m for malate, which strongly supports the suggestion that malate exerts its protective effect by binding to the substrate-recognition site of the transport protein.

Mechanism of dicarboxylate uptake

Previous studies on tonoplast malate transport have described this transport system as a 'carrier' or 'permease' [10,11,37,43,44]. However, recent results from patch-clamp studies show that the tonoplast of CAM plants possesses a voltage-dependent, malate-selective ion channel (Refs. 4 and 12, Pennington, A.J., Pantoja, O. and Smith, J.A.C., unpublished data). But patch-clamp measurements will not detect electroneutral exchange, so it is conceivable that the tonoplast might also contain exchange carriers capable of transporting dicarboxylates. If malate uptake is via a carrier, the stimulation of labelled dicarboxylate uptake by preincubation of vesicles with unlabelled malate (legend to Fig. 4) could be attributable to *trans* stimulation, with internal malate exchanging for external labelled succinate [54]. Alternatively, if uptake is via an ion channel, preincubation with substrate could generate an inside-positive malate diffusion potential, which would drive subsequent dicarboxylate uptake (cf. Refs. 55 and 56). Consistent with this latter interpretation, 10 mM $TPMP^+$ (a permeant lipophilic cation which dissipates transmembrane electrical potentials) abolishes the stimulation of [^{14}C]succinate uptake caused by preincubation of tonoplast vesicles with malate (data not shown). These findings, combined with the patch-clamp studies, suggest that malate uptake by tonoplast vesicles of *K. daigremontiana* is mediated at least in part via an ion channel, but there may in addition be a carrier-mediated component to malate uptake under the conditions of these experiments. Further work to determine the relative contribution of channel- and carrier-mediated dicarbxylate transport might help resolve some of the apparent differences between the malate transport systems described for C_3 and CAM plants.

Acknowledgements

This work was supported by a grant from the Science and Engineering Research Council. We would like to thank Dr. N.J. Kruger for helpful discussions concerning the derivation of kinetic constants.

References

1 Boller, T. and Wiemken, A. (1986) Annu. Rev. Plant Physiol. 37, 137–164.
2 Wink, M. (1993) J. Exp. Bot. 44 (Suppl.), 231–246.
3 Leegood, R.C. and Osmond, C.B. (1990) in Plant Physiology, Biochemistry and Molecular Biology (Dennis, D.T. and Turpin, D.H., eds), pp. 274–298, Longman Scientific and Technical, Harlow, Essex.
4 Smith, J.A.C. and Bryce, J.H. (1992) in Plant Organelles, Society for Experimental Biology Seminar Series 50 (Tobin, A.K., ed.), pp. 141–167, Cambridge University Press, Cambridge.
5 Ni, M. and Beevers, L. (1991) Plant Physiol. 97, 264–272.
6 Betz, M. and Dietz, K.-J. (1991) Plant Physiol. 97, 1294–1301.

7 Bremberger, C. and Lüttge, U. (1992) Planta 188, 575–580.
8 Hedrich, R., Kurkdjian, A., Guern, J. and Flügge, U.I. (1989) EMBO J. 8, 2835–2841.
9 Martinoia, E. (1992) Bot. Acta 105, 232–245.
10 Buser-Suter, C., Wiemken, A. and Matile, P. (1982) Plant Physiol. 69, 456–459.
11 Nishida, K. and Tominaga, O. (1987) J. Plant Physiol. 127, 385–393.
12 Iwasaki, I., Arata, H., Kijima, H. and Nishimura, M. (1992) Plant Physiol. 98, 1494–1497.
13 Oleski, N., Mahdavi, P. and Bennett, A.B. (1987) Plant Physiol. 84, 997–1000.
14 Rentsch, D. and Martinoia, E. (1991) Planta 184, 532–537.
15 Marigo, G. and Bouyssou, H. (1989) in Plant Membrane Transport: The Current Position (Dainty, J., DeMichelis, M.I., Marrè, E. and Rasi-Caldogno, F., eds.), pp. 155–160, Elsevier, Amsterdam.
16 Ou Yang, L.-J., Udvardi, M.K. and Day, D.A. (1990) Planta 182, 437–444.
17 Hoagland, D.R. and Arnon, D.I. (1950) California Agriculture Experiment Station Circular 347, 1–39.
18 Hewitt, E.J. and Smith, T.A. (1975) Plant Mineral Nutrition, The English Universities Press, London, UK.
19 Pope, A.J. and Leigh, R.A. (1988) Planta 176, 451–460.
20 Smith, J.A.C., Uribe, E.G., Ball, E., Heuer, S. and Lüttge, U.(1984) Eur. J. Biochem. 141, 415–420.
21 White, P.J. and Smith, J.A.C. (1989) Planta 179, 265–274.
22 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
23 Marquardt-Jarczyk, G. and Lüttge, U. (1990) J. Plant Physiol. 136, 129–136.
24 Bremberger, C., Haschke, H.-P. and Lüttge, U. (1988) Planta 175, 465–470.
25 Sze, H., Ward, J.M. and Lai, S. (1992) J. Bioenerg. Biomembr. 24, 371–381.
26 Eyzaguirre, J. (1987) Chemical Modification of Enzymes: Active Site Studies, Ellis Horwood, Chichester, Sussex, UK.
27 Cabantchik, Z.I., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302.
28 Flügge, U.-I. and Heldt, H.W. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliarello, E., Palmieri, F., Papa, S. and Klingenberg, E.M., eds.), pp. 373–382, Elsevier, Amsterdam.
29 Garcia, M.L., Patel, L., Padan, E. and Kaback, H.R. (1982) Biochemistry 21, 5800–5805.
30 Riordan, J.F., McElvany, K.D. and Borders, C.L. (1977) Science 195, 884–886.
31 Hartman, F.C. and Norton, I.L. (1977) Methods Enzymol. 47, 479–498.
32 Miles, E.W. (1977) Methods Enzymol. 47, 431–442.
33 Dierks, T., Stappen, R., Salentin, A. and Krämer, R. (1992) Biochim. Biophys. Acta 1103, 13–24.
34 Stappen, R., Dierks, T., Bröer, A. and Krämer, R. (1992) Eur. J. Biochem. 210, 269–277.
35 Levy, H.M., Leber, P.D. and Ryan, E.M. (1963) J. Biol. Chem. 238, 3654–3659.
36 Kiick, D.M., Allen, B.L., Rao, J.G.S., Harris, B.G. and Cook, P.F. (1984) Biochemistry 23, 5454–5459.
37 Bouyssou, H., Canut, H. and Marigo, G.(1990) FEBS Lett. 275, 73–76.
38 Chodera, A.J. and Briskin, D.P. (1990) Plant Sci. 67, 151–160.
39 Martinoia, E., Flügge, U.I., Kaiser, G., Heber, U. and Heldt, H.W. (1985) Biochim. Biophys. Acta 806, 311–319.
40 Marigo, G., Bouyssou, H. and Laborie, D. (1988) Bot. Acta 101, 187–191.
41 Martinoia, E., Vogt, E., Rentsch, D. and Amrhein, N. (1991) Biochim. Biophys. Acta 1062, 271–278.
42 Flügge, U.-I. and Heldt, H.W. (1977) FEBS Lett. 82, 29–33.
43 Martinoia, E., Vogt, E. and Amrhein, N. (1990) FEBS Lett. 261, 109–111.
44 Dietz, K.-J., Canut, H. and Marigo, G. (1992) J. Membr. Biol. 129, 137–143.
45 Morjana, N. and Scarborough, G.A. (1989) Biochim. Biophys. Acta 985, 19–25.
46 Beaumier, B. and Béliveau, R. (1991) Biochim. Biophys. Acta 1068, 142–148.
47 Ni, M. and Beevers, L. (1990) Plant Physiol. 94, 745–751.
48 Bouzayen, M., Latché, A. and Marigo, G. (1991) Plant Physiol. Biochem. 29, 379–383.
49 Hedrich, R. and Kurkdjian, A. (1988) EMBO J. 7, 3661–3666.
50 Hille, B. (1992) Ionic Channels of Excitable Membranes, 2nd Edn., Sinauer Associates, Sunderland, MA.
51 Carlson, G.M. (1984) Biochim. Biophys. Acta 789, 347–350.
52 Gildensoph, L.H. and Briskin, D.P. (1990) Plant Physiol. 94, 696–703.
53 Ho, I. and Briskin, D.P. (1992) Plant Physiol. Biochem. 30, 549–557.
54 Stein, W.D.(1990) Channels, Carriers and Pumps: An Introduction to Membrane Transport, Academic Press, San Diego.
55 Garty, H., Rudy, B. and Karlish, S.J.D. (1983) J. Biol. Chem. 258, 13094–13099.
56 Landry, D.W., Reitman, M., Cragoe, E.J. and Al-Awqati, Q. (1987) J. Gen. Physiol. 90, 779–798.