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Parachloromercuribenzenesulfonic Acid[1]

A POTENTIAL TOOL FOR DIFFERENTIAL LABELING OF THE SUCROSE TRANSPORTER

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ABSTRACT

Vicia faba **leaf discs without epidermis were pretreated with parachloromercuribenzenesulfonic acid (PCMBS), rinsed and incubated on [^{14}C]sucrose (1 or 40 millimolar). Those sucrose concentrations were chosen as representative of the apparent uptake system 1 (1 millimolar) and system 2 (40 millimolar) previously characterized. Pretreatment with 0.5 millimolar PCMBS for 20 minutes inhibited system 1 and system 2 by about 70%.**

Addition of unlabeled sucrose during PCMBS-pretreatment protected the carrier(s) from the inhibition, whereas glucose, fructose, and sucrose analogs were unable to afford protection. At 1 millimolar [^{14}C]sucrose, the protection resulted in a small but consistent reduction of normal inhibition (from 63 to 45%) for sucrose concentrations of 50 millimolar and more during pretreatment. Contrarily, at 40 millimolar [^{14}C]sucrose, the protection increased linearly with the sucrose concentration in the pretreatment medium, and complete prevention of inhibition was reached for 250 millimolar sucrose.

The protection was not due to exchange diffusion and was located in the veins. Michaelian kinetics indicated that PCMBS and sucrose compete with each other at the active site of the carrier.

Among 14 compounds tested (sugars, amino-acids, hormones, ^{32}P), sucrose uptake was by far the most sensitive to PCMBS. Sucrose preferentially protected its carrier(s) from inhibition. Treatment with 20 millimolar cysteine or 20 millimolar dithioerythreitol reversed inhibition by PCMBS pretreatment.

The purification and the study of carriers mediating the Na^+-coupled uptake of solutes in animal cells is now underway (3, 19, 20, 21, 23, 30, 32). A more detailed investigation has been performed with the lactose and the glutamine carriers of *Escherichia coli* (10, 17, 18, 26), where the mechanisms of the ion-carrier interaction are investigated. Although a great deal of evidence now supports the occurrence of proton-coupled transport in various plant cells (5, 9, 13, 16, 22, 28), our knowledge of the general properties of the carriers (6, 13, 22) and of the details of the H^+-carrier interaction (6, 9, 13) is scarce and we treat it at a relatively macroscopic level due to the lack of available methods for purification of these proteins.

Previous work from this laboratory (6) has shown that sucrose uptake by broadbean leaf is apparently mediated by two carriers (K_{m1} = 3 mM, K_{m2} = 35 mM); the high affinity carrier exhibits two-substrate (proton and sucrose) kinetics (6) and is coupled to proton influx (5) while the low affinity carrier is less dependent on protons (5, 6). To gain further information on the H^+-transporter interaction and on the nature of these carriers (*e.g.* does system 2 derive from system 1 by allosteric change?), we have started a research program aimed at the quantification and the purification of the sucrose carrier. In addition to the above-mentioned problems, such a method would be helpful in many other questions where the quantification of sucrose carriers/mg protein would provide interesting information: import/export transition of the leaf, changes in transport properties associated with aging, screening of varieties according to their density in sucrose carriers.

Among the methods already used with success in animal or bacterial cells, we have chosen differential affinity labeling by thiol-reacting compounds. This choice was guided by the following reasons: (a) the method yields good results without requiring the painstaking synthesis of a substrate-derivative; (b) the rationale can be extended to carriers other than the sucrose carrier with minor modifications; (c) some information on the sensitivity of sugar (5, 7, 12, 24) and amino-acid (9) uptake by leaf tissue was already available.

Differential labeling was designed by Fox and Kennedy (11) who used *N*-ethylmaleimide to label a protein involved in the transport of β-galactoside by *Escherichia coli*. The rationale is based upon the protection afforded by the substrate towards inhibitors which bind to the active site of the carrier. When present, the substrate in the binding site impairs the access of inhibitor. The labeling involves two steps. Step 1: incubation of tissues with nonradioactive inhibitor in the presence of the substrate. Reactive groups other than those of the active site of the carrier are blocked. Step 2: removal of substrate and cold inhibitor and incubation with radioactive inhibitor. The radioactivity preferentially binds to the active site of the carrier which was protected by the substrate during step 1.

Rather than *N*-ethylmaleimide which can also mark intracellular proteins, we decided to test PCMBS[2] which does not cross the plasmalemma (1, 7, 12) and which inhibits the sucrose carrier without affecting the proton pump in our material (5, 7). The present work shows that PCMBS fulfills most of the requirements needed to mark the sucrose carrier in *Vicia faba* leaves.

MATERIALS AND METHODS

Plant Material. Growth of broadbean (*Vicia faba* L. cv Aguadulce) and preparation of leaf discs (12 mm diameter) have been described elsewhere (7). After excision, the discs were floated for 15 min on a medium (referred as 'N' medium, containing 250 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 20 mM phosphate buffer (pH 6.0), and then processed as described

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[2] Abbreviations: PCMBS, parachloromercuribenzenesulfonic acid; 3-*O*-MeG, 3-*O*-methylglucose; α-AIB, α-aminoisobutyric acid.

below. All experiments were performed in the light at 20°C.

Optimization of PCMBS Treatment. All membrane proteins possessing free external SH groups are expected to be sensitive to PCMBS. Since we were interested in blocking the protein(s) involved in sucrose transport, we attempted to optimize the PCMBS treatment for these protein(s), and as a test we measured the amount of [^{14}C]sucrose taken up after a pretreatment with the inhibitor. The discs were pretreated for various times (5, 10, 20, 30, 45, 60, 90, 120 min) with 0.05, 0.10, 0.50, and 1 mM PCMBS in N medium. The tissues were then incubated for 30 min on a solution containing 1 or 40 mM [U-^{14}C]sucrose without the inhibitor. These sucrose concentrations were chosen as representative for system 1 and system 2 which had previously been characterized in broadbean leaf (5, 6). Subsequent procedure for rinsing of discs and analysis of results have been described elsewhere (6). In these experiments as in the following ones, the osmolarity of all media was kept at 250 mM by varying appropriately the mannitol concentration.

Protective Effect of Sugars against PCMBS Treatment. Control discs were preincubated for 20 min without inhibitor in the presence of unlabeled sugar (sucrose, glucose, fructose, palatinose, turanose) at the concentration specified in the results, rinsed (3 × 3 min) and incubated (30 min) with 1 or 40 mM [U-^{14}C]sucrose, and rinsed again (3 × 2 min). In the same time, inhibited discs were processed exactly as control discs, except that 0.5 mM PCMBS was present in the preincubation medium. For each treatment, the experiment was run on a set of 10 discs, and was repeated two or three times.

Protective Effects of Sucrose on Other Carriers. Leaf discs were pretreated for 20 min with 0.5 mM PCMBS in the presence of 100 mM sucrose, rinsed (3 × 3 min) on N medium and incubated for 30 min with various labeled compounds: sugars (1 mM sucrose, glucose, 3-*O*-methylglucose or fructose), amino-acids (1 mM α-AIB, glycine, valine, serine, threonine, asparagine, glutamic acid), hormones (0.01 mM ABA or IAA), ions (1 mM PO_4H_2Na). The radioactivity of all incubation media was 1 to 3 μCi/10 ml, except for IAA (0.8 μCi/10 ml) and ABA (0.4 μCi/10 ml). Specific activities of the compounds are listed below. For each compound, a control set and an inhibited set preincubated only with sucrose or PCMBS, respectively, were run.

Sucrose Protection and Exchange Diffusion. Discs (15/set) preloaded for 20 min in 100 mM labeled sucrose (20 μCi/10 ml) with or without 0.5 mM PCMBS were rinsed as in protection experiments (3 × 3 min). This duration of rinsing is sufficient to remove all free space label and inhibitor because, in peeled discs, the apoplastic compartment is washed within 3 min (8). The discs were then effluxed in a medium containing various amounts of sucrose, in the absence of the inhibitor. Efflux was monitored every 6 min during 35 min by measuring the radioactivity of a 50-μl sample of the medium. Rates of efflux were calculated from the slopes between 5 and 35 min.

Reversion of PCMBS Treatment. Discs preincubated for 20 min with 0.5 mM PCMBS were rinsed on N medium (3 × 3 min) and incubated for various times on DTE or cysteine. Afterwards they were rinsed again (3 × 3 min) and incubated with 1 mM radioactive sucrose, following the usual process.

Radiochemicals. The following compounds were purchased from Amersham France (Les Ulis) or from the Commissariat à l'Energie Atomique (Saclay): [U-^{14}C]sucrose (555 mCi/mmol), [U-^{14}C]glucose (240 mCi/mmol), [1-^{3}H]-3-*O*-methylglucose (5 mCi/mmol), [U-^{14}C]fructose (260 mCi/mmol), [1-^{14}C]-α-aminoisobutyric acid (57 mCi/mmol), [U-^{14}C]glycine (100 mCi/mmol), [U-^{14}C]valine (285 mCi/mmol), [U-^{14}C]asparagine (140 mCi/mmol), [3-^{3}H]serine (28 mCi/mmol), [U-^{14}C]threonine (226 mCi/mmol), [U-^{14}C]glutamic acid (285 mCi/mmol), [G-^{3}H]ABA (2 Ci/mmol), [5-^{3}H (n)]-β-IAA (29 Ci/mmol), ^{32}Pi (0.5 mCi/mmol).

Expression of Data. All experiments reported here have been repeated two or three times on sets of 10 discs for each point, with similar results. Unless otherwise stated, radioactivity measurements were made on the entire set (10 discs).

RESULTS

Optimization of PCMBS Pretreatment. To achieve successfully differential affinity labeling, suitable conditions of treatment (duration, concentration of inhibitor, and of protective agent) must be carefully tested (27). Time course and concentration dependence studies of PCMBS inhibition showed no marked difference when the subsequent uptake was studied either at 1 mM or at 40 mM [^{14}C]sucrose. The inhibition plateaued after 45, 30, 20, and 10 min of pretreatment for PCMBS concentrations ranging from 0.05, 0.10, 0.50, and 1 mM, respectively; for those durations of pretreatment, there was about 45, 60, 67, and 72% inhibition with 0.05, 0.10, 0.50, and 1 mM PCMBS, respectively. Because there was only a small difference between the plateaus obtained with 0.5 and 1 mM PCMBS, and in order to minimize the side effects such as increase in cation permeability, reaction with internal thiols or with other groups than thiols which may appear at high PCMBS concentration (29), for all subsequent experiments, we chose to block the sucrose carrier by a 20-min pretreatment with 0.5 mM inhibitor.

Protecting Effects of Sugars against PCMBS Inhibition of the Sucrose Carrier. The effect of adding various concentrations of unlabeled sugars (sucrose, glucose, fructose) during the PCMBS pretreatment on the subsequent uptake of [^{14}C]sucrose (1 or 40 mM) has been studied. The results of pretreatment with sucrose are reported in Table I. In control sets without inhibitor (columns 2 and 5), pretreatment with increasing concentrations of unlabeled sucrose inhibited subsequent uptake of label, either at 1 or 40 mM [^{14}C]sucrose. In contrast, in discs pretreated with the inhibitor (column 3 and 6, Table I), the addition of increasing sucrose concentration in the pretreatment medium enhanced subsequent uptake of label; the increase in uptake was more marked for tissues taking up 40 mM [^{14}C]sucrose than for discs taking up 1 mM [^{14}C]sucrose. Residual uptake, given for each sucrose concentration in the pretreatment medium by the ratio 'uptake by PCMBS pretreated discs/control uptake', is reported in column 4 (Table I) for 1 mM [^{14}C]sucrose, and in column 7 for 40 mM [^{14}C]sucrose. Clearly, the addition of sucrose in the pretreatment medium decreases the inhibition exerted by PCMBS since residual uptake increased from 36.6% (no sucrose) to 53.1% (250 mM sucrose) and from 35.5 to 96.7%, at 1 and 40 mM [^{14}C]sucrose, respectively. The degree of protection afforded by sucrose on system 1 (1 mM [^{14}C]sucrose) was very different from that obtained on system 2 (40 mM [^{14}C]sucrose). For system 1, residual uptake plateaued at about 55% for sucrose concentrations equal or greater than 50 mM, whereas for system 2 it still increased up to 96.7% for 250 mM sucrose.

Similar experiments with glucose as a protecting agent in the preincubation medium have been performed. When studying either system 1 or system 2, in contrast to sucrose, preincubation with glucose did not decrease subsequent uptake of sucrose in discs not treated by PCMBS (column 2 and 5, Table II). Except for 250 mM glucose on system 1, there was no evident protection by glucose (columns 4 and 7, Table II).

Like glucose but unlike sucrose, fructose in the preincubation medium did not decrease uptake of 1 or 40 mM [^{14}C]sucrose by tissues not treated with PCMBS (Table III). Despite some variability in the rate of sucrose uptake, fructose induced no evident increase in residual absorption in the presence of PCMBS on system 2 (column 7, Table III) and only a slight increase on system 1 (column 4).

Results reported in Tables I, II, and III are the mean of two or three separate experiments where measurements have been made

Table I. *Effect of Sucrose on the PCMBS Inhibition of the Sucrose Carrier*

Discs were pretreated with (+ PCMBS) or without (control) 0.5 mM PCMBS for 20 min in the presence of various unlabeled sucrose concentrations. After rinsing, they took up sucrose from a 1 or 40 mM [^{14}C]sucrose solution without inhibitor. Residual uptake is the ratio PCMBS/control. Mean of three sets of 10 discs for each value.

Sucrose in Pretreatment	1 mM [^{14}C]Sucrose			40 mM [^{14}C]Sucrose		
	Uptake (control)	Uptake (+PCMBS)	Residual uptake	Uptake (control)	Uptake (+PCMBS)	Residual uptake
mM	*nmol cm^{-2}/30 min*		%	*nmol cm^{-2}/30 min*		%
0	3.91	1.43	36.7	87.07	30.90	35.5
5	3.74	1.60	42.8	75.59	29.83	39.5
10	3.48	1.60	46.0	69.19	28.61	41.4
30	2.95	1.43	48.5	71.44	30.96	43.3
50	3.01	1.66	55.1	62.27	33.74	54.2
100	3.03	1.68	55.4	69.01	38.73	56.1
150	3.20	1.74	54.4	62.24	45.07	72.4
200	3.13	1.82	58.2	47.01	37.90	80.6
250	2.93	1.55	52.9	51.92	50.25	96.8

Table II. *Effect of Glucose on the PCMBS Inhibition of the Sucrose Carrier*

Details as in Table I, except that glucose replaced sucrose during pretreatment. Mean of two sets of 10 discs.

Glucose in Pretreatment	1 mM [^{14}C]Sucrose			40 mM [^{14}C]Sucrose		
	Uptake (control)	Uptake (+PCMBS)	Residual uptake	Uptake (control)	Uptake (+PCMBS)	Residual uptake
mM	*nmol cm^{-2}/30 min*		%	*nmol cm^{-2}/30 min*		%
0	3.46	1.29	37.3	73.16	25.79	35.3
5	3.24	1.27	39.2	69.07	26.39	38.2
10	4.03	1.59	39.5	73.16	32.70	44.7
30				84.18	30.84	36.6
50	3.85	1.60	41.6	74.39	28.59	38.4
100	3.46	1.32	38.2	74.51	22.42	30.1
150	4.08	1.62	39.7	61.33	25.64	41.8
200	3.55	1.38	38.9			
250	3.55	1.80	50.7	73.70	27.63	37.5

Table III. *Effect of Fructose on the PCMBS Inhibition of the Sucrose Carrier*

Details as in Table I, except that fructose replaced sucrose during pretreatment. Mean of two sets of 10 discs for each point.

Fructose in Pretreatment	1 mM [^{14}C]Sucrose			40 mM [^{14}C]Sucrose		
	Uptake (control)	Uptake (+PCMBS)	Residual uptake	Uptake (control)	Uptake (+PCMBS)	Residual uptake
mM	*nmol cm^{-2}/30 min*		%	*nmol cm^{-2}/30 min*		%
0	3.46	1.29	37.3	68.87	26.60	38.6
5	3.20	1.25	39.1			
10	4.33	1.72	39.7	72.05	33.13	46.0
30				65.22	29.69	45.0
50	3.65	1.45	39.7	67.10	27.45	40.9
100	3.44	1.32	38.4	66.41	27.31	41.1
150	5.26	2.22	42.2	70.01	32.22	46.0
200	4.22	1.96	46.4	64.76	25.18	38.9
250	3.99	1.85	46.4	58.64	24.45	41.7

on sets of 10 discs per experiment. Because of some variability in the rates of uptake, and to ascertain the effect of sucrose, we performed other experiments where the radioactivity of each disc was counted separately so that a *t* test could be made. The results (Table IV) indicate that, in the absence of PCMBS, addition of 100 or 250 mM sucrose during pretreatment significantly decreased subsequent uptake of 1 mM [^{14}C]sucrose, whereas in the presence of PCMBS, this resulted in a significant increase, when compared to discs not treated by sucrose. In contrast, hexoses did not affect the uptake of sucrose by PCMBS-pretreated discs. In the absence of PCMBS, fructose (100 or 250 mM) and glucose (100 mM) did not affect subsequent sucrose uptake. For unknown reasons, in this experiment, pretreatment of tissues with 250 mM glucose promoted sucrose uptake, but this effect was not consistently observed (see Table II).

Two hypotheses might account for the increase in residual uptake which occurs after sucrose pretreatment (columns 4 and 6, Table I; column 3, Table IV): (a) this increase is due to sucrose protection from PCMBS inhibition; and (b) since pretreatment with sucrose inhibits subsequent uptake of this sugar, the increase

Table IV. *PCMBS Inhibition and Sugar Protection of 1 mM [^{14}C] Sucrose Uptake*

Details as in Tables I, II, III. Data are expressed as mean ± SD, with the number of counted discs given in parentheses.

Sugar in Pretreatment	Rate of Uptake		Residual Uptake
	Not PCMBS pretreated	PCMBS pretreated	
mM	*nmol cm^{-2}/30 min*		%
None	4.28 ± 1.50 (40)[a]	1.20 ± 0.25 (37)[e]	28.0
Sucrose, 100	3.65 ± 1.16 (36)[b]	1.89 ± 0.35 (40)[f]	51.8
Sucrose, 250	3.44 ± 1.17 (38)[c]	1.68 ± 0.27 (38)[g]	48.8
Glucose, 100	3.96 ± 0.72 (19)	1.30 ± 0.29 (19)	32.8
Glucose, 250	5.19 ± 1.65 (18)[d]	1.11 ± 0.22 (20)	21.4
Fructose, 100	4.51 ± 0.78 (17)	1.26 ± 0.24 (19)	27.9
Fructose, 250	3.79 ± 0.98 (20)	1.28 ± 0.23 (21)	33.8

[a,b,c,d] Differences between a and b, a and d significant at the 0.05 level; difference between a and c significant at the 0.01 level.

[e,f,g] Differences between e and f, and e and g significant at the 0.001 level.

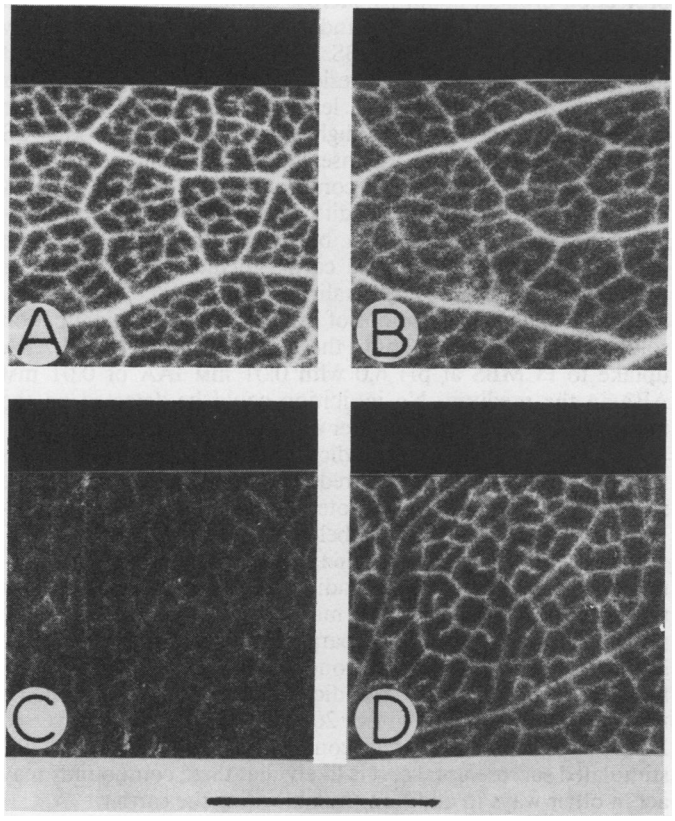

FIG. 1. Autoradiographs from PCMBS-treated sucrose-protected discs. Before incubation on 1 mM [^{14}C]sucrose, the tissues were pretreated under the following conditions: A (control 1), O mM PCMBS, O mM sucrose; B (control 2), O mM PCMBS + 100 mM sucrose; C (inhibited), 0.5 mM PCMBS + O mM sucrose; D (inhibited-protected), 0.5 mM PCMBS + 100 mM sucrose. The scale bar stands for 5 mm; the radioactivity appears in white and the background of the film is shown at the top.

might be due to PCMBS protection from sucrose inhibition. The second posibility could result in a smaller decrease in sucrose uptake with increasing sucrose concentrations in the pretreatment medium of PCMBS-treated discs (column 2, Table IV). However, this hypothesis cannot explain the stimulation of uptake which is observed in the presence of 100 or 250 mM sucrose

Table V. *Effect of PCMBS on Exchange Diffusion*

Discs loaded for 20 min with 100 mM [^{14}C]sucrose, with or without 0.5 mM PCMBS, were rinsed (3 × 3 min), and effluxed for 35 min on a medium (without inhibitor) containing no sucrose, or 100 mM unlabeled sucrose. Initial content of the discs was 106,020 dpm cm^{-2} leaf disc (control) and 45,160 dpm (PCMBS pretreated). The experiment was repeated another time with the same results.

Sucrose in Efflux Medium	Rate of Efflux	
	Not PCMBS pretreated	PCMBS pretreated
mM	*dpm/min·cm^2 leaf disc*	
0	192	162
100	431	181

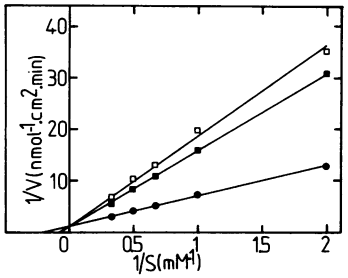

FIG. 2. The effects of PCMBS on the kinetics of sucrose uptake. PCMBS was present only during the uptake (30 min) of [^{14}C]sucrose (0.5–3 mM). (●), Control; (■), 0.25 mM PCMBS; (□), 0.5 mM PCMBS. Each point is the mean of 10 discs. The experiment was repeated another time with similar results.

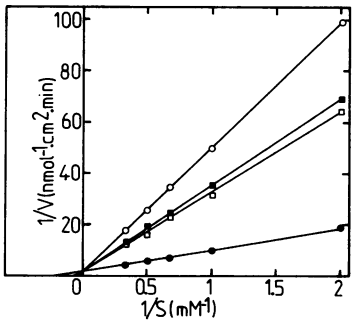

FIG. 3. The effects of PCMBS and sucrose on the kinetics of uptake. Discs were pretreated for 20 min with 0.25 mM PCMBS (■), 0.5 mM PCMBS (O), 0.5 mM PCMBS + 100 mM sucrose (□), rinsed, and allowed to take up [^{14}C]sucrose for 30 min without PCMBS. (●), No pretreatment. Each point is the mean of 40 (●, ■, O) or of 20 (□) discs.

(column 2, Table IV) and one has to conclude that sucrose may protect its carrier from PCMBS inhibition.

However, it cannot be concluded that this protection is due to a steric hindrance by the sugar of PCMBS access to the active site of the carrier. One could argue that the protection is afforded indirectly, via the metabolism, or via exchange diffusion of ^{14}C-sugar with unlabeled sugar loaded in the discs pretreated with high sucrose concentrations. Also, sucrose protection may not be located at the vein network, and thus would not be related to phloem loading. The following experiments were designed to provide information on these points.

Where Does the Protection Occur? Autoradiographs of inhibited and of inhibited-protected tissues show that the protection brought about by sucrose affects sucrose uptake by the veins, at 1 mM (Fig. 1) or 40 mM (not shown) [^{14}C]sucrose.

Protection or Exchange Diffusion? In the case of exchange

Table VI. *Effect of PCMBS and Sucrose (100 mM) on the Uptake of Sugars, Amino Acids, Phytohormones, and Ions*

The discs were preincubated for 20 min in the presence of 0.5 mM PCMBS with or without sucrose. The results are expressed as per cent of uptake by control discs. Each value is the mean of 20 or 30 discs (two or three experiments). All solutes presented at 1 mM except ABA and IAA which were 0.01 mM.

Substrate Taken Up	Sucrose in the Preincubation Medium	
	0[a]	100 mM[b]
Sucrose	26.3	55.8
Glucose	91.2	95.5
3-*O*-MeG	76.4	98.6
Fructose	88.0	90.1
α-AIB	91.4	69.8
Glycine	96.9	91.3
Valine	90.9	84.8
Serine	100	80.9
Threonine	77.0	89.3
Asparagine	87.7	79.6
Glutamic acid	87.5	85.1
IAA	93.9	99.9
ABA	97.5	100
PO_4H_3	89.4	91.3

[a] Control preincubated on N medium.
[b] Control preincubated in the presence of 100 mM sucrose.

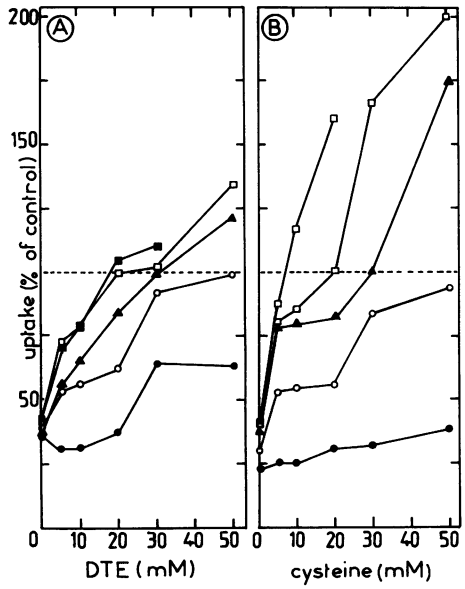

FIG. 4. Reversion of PCMBS inhibition by DTE and cysteine. Each point is the mean of seven discs. After pretreatment by PCMBS, the tissues were treated with DTE (A) or cysteine (B) for 5 s (●), 5 (○), 10 (▲), 15 (□), or 20 (■) min.

diffusion, the presence of sucrose in the efflux medium should increase the rate of efflux of the label. This did occur (Table V) since there was about a 2-fold increase of efflux, compared to a medium without sucrose, when 100 mM sugar was present during efflux. However, when the loading phase had been run in the presence of 0.5 mM PCMBS, there was only about a 10% increase between 0 and 100 mM sucrose. In other words, exchange diffusion is PCMBS sensitive. This, as well as many other considerations which will be detailed in the discussion section, makes it unlikely that the protection observed in the presence of sucrose results from exchange diffusion.

Kinetics of PCMBS Inhibition and of Sucrose Protection. The hypothesis according to which both PCMBS inhibition and sucrose protection concern the active site of the carrier is very clearly demonstrated in Figures 2 and 3. Figure 2 yields data from experiments where PCMBS (0.25 or 0.5 mM) was added during incubation with [^{14}C]sucrose. The kinetics obtained are of competitive type. This experiment was repeated two times with similar results. In other experiments, PCMBS was present with (or without) 100 mM sucrose during a 20-min pretreatment. The kinetics of sucrose uptake by a control, by inhibited and by inhibited-protected sets of discs are reported in Figure 3. This experiment shows that both inhibition by PCMBS and protection by sucrose are of competitive type. The data provide direct evidence for the existence of a SH group in the active site of the carrier (system 1); moreover, they strongly suggest that the protection afforded by sucrose against PCMBS inhibition also occurs at the level of this site.

The Selectivity of Sucrose Protection. Differential affinity labeling of the sucrose carrier will be feasible only if the protection afforded by sucrose is selective and concerns no carrier other than the one mediating the uptake of this sugar. We have tested the inhibition exerted by PCMBS on the absorption of various substrates (sugars, amino-acids, ions, phytohormones) and the protection brought about by sucrose against PCMBS inhibition (Table VI). Among all compounds tested, sucrose uptake was by far the most sensitive to PCMBS. For all other substrates, except for threonine and 3-*O*-MeG, residual uptake after the inhibitor pretreatment still attained at least 85% of the control. The absorption of 3-*O*-MeG was slightly more sensitive to PCMBS than that of glucose and fructose, although the three sugars are generally thought to share a common hexose carrier (4). IAA and ABA have been shown to diffuse freely through plant membrane in their protonated form, but part of their uptake may be carrier-mediated (14, 2). The carrier-mediated component is more apparent at relatively alkaline pH values (6.0 and above), and with small concentrations of the hormone in the incubation solution. This led us to study the sensitivity of IAA and ABA uptake to PCMBS at pH 6.0 with 0.01 mM IAA or 0.01 mM ABA in the medium. No inhibition could be detected on the uptake of these hormones under our experimental conditions.

Statistical analysis (*t* test) indicated that the only compounds for which sucrose significantly reduced the effect of PCMBS were sucrose and 3-*O*-MeG. The protective effect of sucrose on 3-*O*-MeG uptake will be discussed below.

Reversal of PCMBS Inhibition. Obtaining a carrier in a functional state demands that the radioactive label that is located on the inhibitor which serves as a marker can be removed after the protein of interest has been separated from the other ones. This led us to search for suitable conditions of reversal of PCMBS binding. The results (Fig. 4) indicate that incubating the tissues for 15 min in 20 mM cysteine or 20 mM DTE completely reversed PCMBS inhibition. Since high concentrations of DTE or cysteine stimulated sucrose uptake, it is likely that these compounds may act in other ways in addition to unblocking the carrier.

DISCUSSION

The need of a method allowing the identification of plant plasmalemma transporters and their recovery in a functional state has been underlined in the introductory section of this paper. Two-dimensional electrophoresis of crude extracts of leaf cells proteins allow the visualization of about 250 spots, but their separation is not always easy (25). In an attempt to mark a plasmalemma transporter, a nonpermeant marker should prove to be very useful in decreasing the number of spots appearing on the electrophoretogram. This led us to focus our attention on PCMBS, which was previously shown to inhibit sucrose uptake but not the proton pump in broadbean leaf (unlike in sugar beet [13]), both by pH measurements (5, 6) and by electrophysiological data (Mounoury, Delrot, and Bonnemain, in preparation).

For an inhibitor to be useful as a differential labeling probe, the following requirements must be fulfilled: (a) the inhibitor treatment has to be optimized so as to mark preferentially the carrier under investigation; (b) the protection brought about by the substrate must be selective and preferentially concern the transporter studied; (c) the inhibitor must bind to the active site of the carrier, so that only its substrate can protect this carrier from inhibition; (d) for the recovery of a functional carrier, it should be possible to remove the inhibitor from the protein.

The results of Table VI extend to broadbean the previous observation in sugar beet (12, 24) and in soybean (31) that sucrose but not hexose uptake is PCMBS-sensitive (12, 24). The protective effect of sucrose on 3-*O*-MeG uptake does not seem to concern the hexose carrier, since the absorption of glucose and fructose was neither significantly inhibited by PCMBS nor protected by sucrose. A minor part of 3-*O*-MeG uptake might be mediated by the sucrose carrier, since competition experiments revealed that 3-*O*-MeG could reduce the uptake of sucrose, while the reverse was not true (Delrot, unpublished).

The small inhibition exerted by PCMBS on hexose uptake (as in sugar beet leaf [12, 13, 24]), the weak ability of hexoses to protect the sucrose carrier, and the preferential localization in the veins of the protection afforded by sucrose (our data) are in good agreement with previous results indicating the occurrence of two separate carriers for hexoses and sucrose (5, 12, 15, 24) and preferential loading of sucrose into the phloem.

Among 14 compounds tested including sugars, amino acids, ions, and hormones, sucrose absorption was by far the most sensitive to PCMBS. This may suggest that labeled PCMBS could preferentially mark this carrier, but does not exclude that the inhibitor may bind to other proteins in 'nonactive' sites, hence creating a background of labeled molecules. Although a single step labeling might be sufficient to identify the sucrose transporter, the selectivity of the method should be improved by differential labeling, provided that sucrose affords some protection and that the protection is selective.

The hypothesis of PCMBS protection from sucrose (trans) inhibition does not account for the data of Table IV. In contrast, these data and those of Table I are in good agreement with the hypothesis of sucrose protection from PCMBS inhibition. This last hypothesis is also supported by the decrease in the rate of [^{203}Hg]PCMBS binding to the discs which is observed in the presence of sucrose, but not of glucose (M'Batchi and Delrot, in preparation). A crucial point is to know how is the protection brought about. The fact that easily metabolized hexoses do not confer protection (Tables II and III) and the fact that sucrose protection does not extend to other carriers exclude the possibility that sucrose would act as an energy source stimulating the general metabolism. Another possibility would be that exchange diffusion of ^{14}C with sugar loaded in the discs preincubated in sucrose would leak to an apparent protection. Several considerations enable us to discard this possibility. (a) In the absence of inhibitor, loading with unlabeled sucrose decreases subsequent uptake of label instead of increasing it as would be anticipated. (b) Even if exchange diffusion occurs at the same rate in sucrose-PCMBS-pretreated discs as in sucrose-pretreated discs, its effect is excluded by calculating the ratio of radioactivity 'sucrose-PCMBS-pretreated/sucrose-pretreated tissues' (residual uptake). These ratios are always higher in discs loaded with sucrose than in control discs (columns 4 and 7, Table I). Moreover, exchange diffusion is inhibited when discs are preloaded in the presence of PCMBS (Table V). This effect cannot simply be ascribed to a lower uptake of sucrose during the loading period in the presence of PCMBS; indeed, when taking the efflux in a medium without sucrose as a reference, there is about a 10% increase of efflux in the presence of 100 mM sucrose in the efflux medium for discs treated with PCMBS, instead of a 125% increase in untreated discs. The sensitivity of exchange diffusion to PCMBS does not favor higher values of residual uptake. (c) It would be unlikely that the effects of exchange diffusion on total uptake, which are rather indirect, would consistently result in competitive kinetics for sucrose-PCMBS-pretreated discs.

On the contrary, the hypothesis that sucrose exerts protection by partially impairing the access of PCMBS to the active site of the carrier gives a simple explanation of several observations. (a) PCMBS does inhibit the carrier at the level of the active site (Fig. 2 and 3). (b) The sucrose analogs (palatinose = 6-*O*-α-D-glucopyranosyl-D-fructofuranose and turanose = 3-*O*-α-D-glucopyranosyl-D-fructose), which did not competitively inhibit sucrose uptake, only poorly protected the sucrose carrier from PCMBS inhibition (data not shown). This suggest that stereochemical requirements must be fulfilled to afford protection. (c) Comparison of uptake in untreated discs, in sucrose-PCMBS- and in PCMBS-pretreated discs (Fig. 3) indicate that, like PCMBS inhibition, sucrose protection is of competitive type.

Of particular interest are the different degrees of protection afforded by sucrose when comparing 1 mM (system 1) or 40 mM (system 2) [^{14}C]sucrose uptake (Table I).The occurrence of two phases in Michaelian kinetics may be explained by several possibilities: one carrier undergoing allosteric changes, two carriers operating on the same membrane or on different membranes, and the inadequacy of Michaelian kinetics (initially proposed for enzyme in solutions) for enzymes embedded in membranes. The long term purpose of our work is the identification and the purification of the sucrose carrier(s) to study their functioning, and it should help to choose between these possibilities. However, already now, the results available from the comparison of the protective effect of sucrose on the uptake from a 1 mM (system 1) or a 40 mM (system 2) ^{14}C-sugar solution indicate that the distinction of two systems previously grounded on kinetic data (6) and measurements of proton fluxes associated with sugar uptake (5) also appears for protection studies. Therefore, the occurrence of these systems is not simply an artefact due to the use of Michaelian kinetics, but corresponds to true differences in the molecular functioning of the protein(s) mediating the uptake. In this respect, we are currently investigating the pattern of PCMBS inhibition at low or high sucrose concentration.

The data of Figure 4 show that, as in *Beta vulgaris* (12, 24), DTE or cysteine are able to reverse the effect of thiol blocking compounds, and this opens the possibility to recover an active carrier. However, the stimulation of uptake induced by high DTE or cysteine concentrations suggests that the effect of these compounds may not be restricted to the active site of the carrier. Hence, optimization of the reversion treatment must await the results of experiments where the release of radioactive PCMBS and the ability to take up sucrose will be compared.

The particular sensitivity of the sucrose carrier to PCMBS, the specific protection afforded by sucrose and the fact that PCMBS binds to the active site of the carrier underline the possibility to use this inhibitor as a differential affinity label. Further work is now underway in our laboratory using [^{203}Hg]PCMBS to mark the sucrose transporter.

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