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The Effect of Sugars on the Binding of [^{203}Hg]-*p*-Chloromercuribenzenesulfonic Acid to Leaf Tissues[1]

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ABSTRACT

Replacement of mannitol with sucrose decreases the binding of [^{203}Hg]-*p*-chloromercuribenzenesulphonic acid (PCMBS) to *Vicia faba* leaf discs without epidermis. This decrease is optimal for 20 minutes on incubation, is concentration-dependent, and is also found with maltose and raffinose. In parallel experiments, the addition of sucrose, maltose, and raffinose during PCMBS pretreatment was shown to increase subsequent uptake of [U-^{14}C]sucrose. In contrast, D- or L-glucose, 3-*O*-methylglucose, galactose, fructose, palatinose, turanose, or melibiose had no effect either on PCMBS binding or on [^{14}C]sucrose uptake. The sucrose-induced decrease of PCMBS binding is retained after a cold and ionic shock. Measurements of specific activities of membrane fractions prepared from tissues incubated in labeled PCMBS show that the decrease concerns the 120,000 gravity pellet, but that very mild procedures must be chosen to prevent redistribution of label in the supernatant. Altogether, the data provide new support to the hypothesis that the active site of the sucrose carrier contains a group sensitive to PCMBS.

Sucrose uptake by plant tissues occurs with proton-symport, at least for low sucrose concentrations (3, 4, 12, 13, 17). However, beyond this relatively macroscopic evidence, the molecular properties of the sucrose carrier, and the changes induced by protonation are very little known. Only recently has some information on the recognition of sucrose by the carrier and on the active site of the enzyme become available. Synthesizing several analogs of sucrose, Hitz *et al.* (15) suggested that in protoplasts of soybean cotyledons the interaction between sucrose and its transporter is mainly hydrophobic and that both glucose and fructose moieties are involved in this interaction. Schmitt *et al.* (22) found that in the same material, maltose and inulobiose (50 mM) decreased uptake of 0.5 mM [^{14}C]sucrose while cellobiose, palatinose, raffinose (100 mM), and melezitose (50 mM) caused no inhibition. However, no direct evidence was given that the inhibiting sugars were acting at the carrier level.

We have proposed that, in broadbean leaf, the active site of the sucrose carrier contains a site sensitive to PCMBS[3] (5, 19) and that this property might be used for differential affinity labeling of the transporter. This assumption was grounded on the evidence summarized below: (a) kinetics of sucrose uptake after preincubation or incubation of leaf tissues with the nonpermeant inhibitor PCMBS were of 'competitive' type; (b) the presence of unlabeled sucrose during PCMBS pretreatment resulted in an enhanced subsequent uptake of [^{14}C]sucrose (compared to a PCMBS-treated control); (c) this protection by sucrose from PCMBS inhibition was specific (not brought about by glucose, fructose, or the sucrose analogs palatinose and turanose), was not due to exchange diffusion, and was also of 'competitive' type. The simplest explanation of these data was that the presence of sucrose in the active site of the carrier prevented the access of PCMBS to a functional group, presumably a thiol.

In the present paper, we have studied the effect of different sugars on the binding of labeled PCMBS to plant tissues, and compared this effect with the protection brought about by the same sugar against PCMBS inhibition of [^{14}C]sucrose uptake.

MATERIALS AND METHODS

Plant Material. Broad bean plants (*Vicia faba* L. cv Aguadulce; Clause, Brétigny-sur-Orge, France) were grown in a growth cabinet (16 h of light provided by Sylvania tubes F 65 w Gro-lux, at 20 ± 1°C, and 8 h of dark at 16°C). Light energy measured at the leaf level was 14 w·m^{-2}. RH was 60 ± 5% throughout. The plants were watered daily with Hoagland solution and used when 3 weeks old. Discs (12-mm diameter) were punched with a cork borer from mature leaves, after stripping off the lower epidermis. After excision, the discs were floated for 15 min on a medium (referred to as 'N' medium) containing 250 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$, and 20 mM K-phosphate (pH 6.0), and then processed as described below. The experiments were run in the light at 20°C.

Binding of [^{203}Hg]PCMBS to Leaf Discs and the Effect of Sugars. Discs were incubated for 15 min with 0.5 mM [^{203}Hg] PCMBS (37 kBq·ml^{-1}) on 'N' medium (control) or on a medium where mannitol was replaced by another sugar (see "Results") at the same molarity (250 mM). After incubation, the discs were rinsed (3 × 3 min) on N medium or on a medium containing 250 mM of the sugar tested.

Protection by Sugars against PCMBS Inhibition of Sucrose Uptake. The experimental procedure was the same as that described by M'Batchi and Delrot (19). Briefly, control discs were preincubated for 20 min with 250 mM unlabeled sugars (see "Results") and without PCMBS, then rinsed on N medium (3 × 3 min) and incubated 30 min in N medium with 1 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.

Counting of Discs and Expression of Data. After the last rinse, discs were rapidly frozen with Dry-Ice, lyophilized, and their radioactivity was measured with a gas-flow counter (Manu 16, Numelec). Results were corrected for decay (^{203}Hg) and for counting efficiency.

The experiments were conducted on sets of randomized discs (usually 20 to 30 discs) and each disc was counted separately.

Preparation of a Crude 'Microsomal' Fraction. Fifty discs were

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

incubated for 15 min with 0.5 mM [^{203}Hg]PCMBS (2.3 to 1 mBq · ml^{-1}) on N medium (control) or on a medium containing 250 mM sucrose instead of 250 mM mannitol, and rinsed as above on N medium (control) or on a medium containing 250 mM sucrose. They were rapidly frozen with Dry-Ice and stored at −20°C until later steps. A crude 'microsomal' fraction was prepared from each set of discs according to the method of Yoshida *et al.* (25) with minor modifications. The frozen discs were broken into small pieces and ground for 60 s at 4°C in 3 ml of grinding medium with a 'Tri-R' homogenizer (Jamaica, NY). The grinding medium consisted of 500 mM sorbitol, 50 mM Mes, 10 mM EGTA neutralized with KOH, 5% soluble PVP (M_r = 24,500), 0.5% defatted BSA, 2 mM salycylhydroxamic acid, and 1 mM phenylmethylsulfonyl fluoride (pH 7.6). The homogenate was passed through an Amberlite XAD-4 column (1.5 × 15 cm) and subjected to differential centrifugations (1,500*g* for 7 min; 14,000*g* for 15 min, 120,000*g* for 5 h). The pellets were washed extensively with distilled H_2O and their specific activity was measured by combination of liquid scintillation counting and protein assay according to the method of Bradford (2). Since the use of frozen tissues for cell fractionation makes it impossible to give precise names to the fractions, the terms 'mitochondrial' and 'microsomal' are only used for convenience to design the 14,000*g* and 120,000*g* pellets, respectively.

Chemicals and Radiochemicals. Chemicals were purchased from Sigma Chemical Co., except PVP and Amberlite (Fluka, Buchs, Switzerland) and sucrose (Prolabo, Paris, France.). [U-^{14}C]sucrose (19.5 TBq · mmol^{-1}) and [^{203}Hg] *p*-chloromercuribenzensulfonic acid (500 MBq · mmol^{-1}) were purchased from Amersham France (Les Ulis). Purity of labeled PCMBS was checked by paper electrophoresis at pH 6.0.

RESULTS

[^{203}Hg]PCMBS Binding and PCMBS Inhibition of Sucrose Uptake. A close examination of the time course of [^{203}Hg] PCMBS binding shows to phases, from 0 to 20 min and from 20 to 120 min (Fig. 1A). Indeed, before 20 min, the rate of [^{203}Hg] PCMBS binding constantly decreased and should be expected to give the dotted lines drawn in Figure 1A for longer incubation times. Yet, the rate of binding increased again between 20 and 60 min. While several hypotheses may explain the appearance of the second phase (see "Discussion"), the first one can be considered as representative mainly of PCMBS binding to the external side of the cell.

At the beginning of incubation, the rate of binding was smaller in the 'sucrose' sets than in the control sets of discs. The difference between the two sets increased constantly up to 15 to 20 min (maximum relative difference = 18% at 20 min) and was statistically significant (Student's *t* test) between 5 and 20 min, but then disappeared between 20 and 30 min (Fig. 1A). Expressed as radioactivity, the difference between the control (800 cpm) and the sucrose discs (650 cpm) was about 150 cpm/disc after 15-min incubation. The experiment was repeated three other times with an incubation period of 15 min, and the same results were obtained. When the data of the four experiments are pooled, the following values are found for PCMBS binding: 1.94 ± 0.04 nmol cm^{-2} for control discs (mean ± SE; n = 130 discs) and 1.65 ± 0.03 nmol cm^{-2} for sucrose-protected discs (n = 130). The relative difference (15%) between both kinds of discs was significant at the 0.001 level. Time course inhibition of [^{14}C]sucrose uptake by 0.5 mM PCMBS (Fig. 1B) shows that maximal inhibition is also reached at 20 min of treatment. As will be detailed in the "Discussion," both the decrease in PCMBS binding in the presence of sucrose and its comparison with PCMBS inhibition of sucrose uptake are in good agreement with the hypothesis of PCMBS binding to the active site of the carrier. In further experiments designed for a better characterization of the decrease of PCMBS binding induced by sucrose, a 15-min incubation time in labeled PCMBS was chosen.

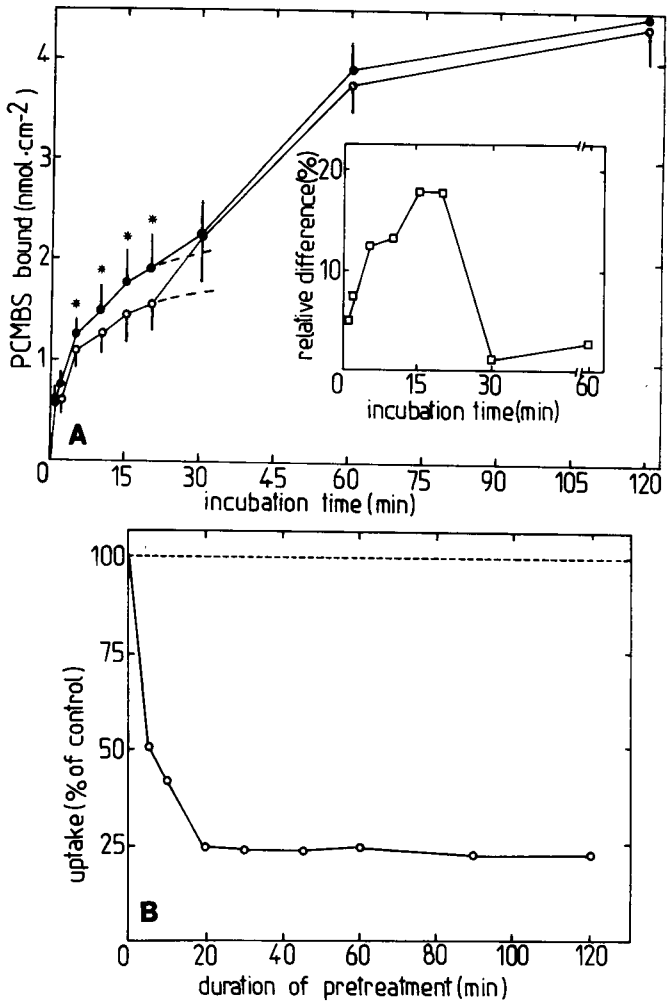

FIG. 1. Effect of sucrose on the binding of [^{203}Hg]PCMBS (A) and effect of PCMBS on the uptake of sucrose (B). A, Time course of PCMBS binding to leaf tissues incubated in 0.5 mM [^{203}Hg]PCMBS in the presence of 250 mM mannitol (●) or 250 mM sucrose (○). Each value is the mean of 50 discs ± SD (five experiments). (*), For a given time, the difference between the control and the sucrose sets is significant at the 0.05 level (*t* test). (----), Extrapolations from the first phase of uptake. Inset, relative difference of PCMBS binding, expressed as

$$\frac{\text{Control} - \text{"sucrose"}}{\text{Control}} \times 100$$

B, Inhibition of 1 mM [^{14}C]sucrose uptake as a function of pretreatment with PCMBS (0.5 mM). Discs were preincubated for various time with PCMBS, rinsed (3 × 3 min) and allowed to take up [^{14}C]sucrose for 30 min without inhibitor. Each value is the mean of five discs counted separately.

Concentration Dependence of the Decrease of PCMBS Binding. Figure 2 shows that the decrease in PCMBS binding was dependent on the concentration of sucrose, with a rapid increase between 0 and 25 mM sucrose, and a linear increase from 25 up to 250 mM sucrose. In comparison, D-glucose only induced a smaller decrease of binding, not statistically significant, and at concentrations greater than 100 mM.

Specificity of Protection against PCMBS. To test the relationship between the decrease of [^{203}Hg]PCMBS binding induced by sugars and the protection of sucrose carrier, we have assayed various mono-, di-, and trisaccharides for their ability: (a) to

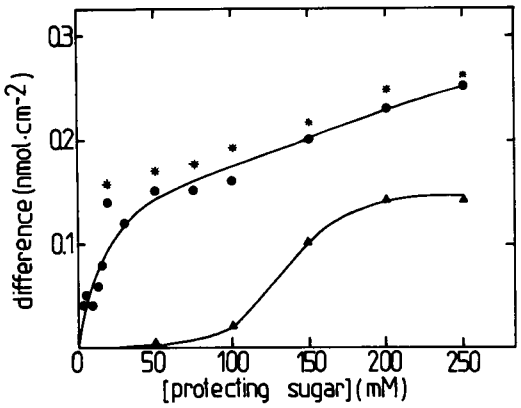

FIG. 2. Effect of various concentrations of sucrose and glucose on the binding of [^{203}Hg]PCMBS. The values reported are the differences between control discs incubated for 15 min in 0.5 mM labeled PCMBS plus 250 mM mannitol and discs incubated in labeled PCMBS with the specified concentration of sucrose (●) or glucose (▲). In all cases, mannitol was added to keep the overall sugar concentration of the medium at 250 mM. Each point was obtained from 50 control discs and 50 treated discs counted separately. (*), The difference is significant at the 0.01 level.

Table 1. *Effect of Various Sugars on the Binding of [^{203}Hg]PCMBS and on PCMBS Inhibition of [^{14}C]Sucrose Uptake*

The difference of PCMBS binding is calculated as: 'mannitol' set (50 discs) minus 'sugar-treated' set (50 discs). The difference of [^{14}C]sucrose uptake is calculated as: 'uptake by tissues pretreated with PCMBS in the presence of sugar' (50 discs) minus 'uptake by tissues pretreated with PCMBS alone' (50 discs). In both tables, sugars were present at 250 mM, except the mixture glucose plus fructose (125 mM each).

Sugar Added	Difference in PCMBS Binding	Difference in [^{14}C]Sucrose Uptake
	nmol·cm^{-2}·15 min	*nmol·cm^{-2}·30 min*
3-*O*-MeG	−0.19	−0.45[a]
L-Glucose	−0.08	0.01
D-Glucose	0.11	−0.09
D-Fructose	0.05	0.08
D-Galactose	0.04	−0.32[b]
D-Glucose + D-fructose	0.17	−0.04
Palatinose	0.07	0.04
Turanose	0.06	−0.02
Melibiose	0.06	0.01
Maltose	0.20[c]	0.14[a]
Sucrose	0.25[d]	0.48[c]
Raffinose	0.24[d]	0.13[b]

[a,b,c] Mean that the difference in [^{14}C]sucrose uptake between 'inhibited' (pretreated with PCMBS alone) and 'sugar-treated' (pretreated with PCMBS + sugar) discs is significant at the 0.05, 0.01, and 0.001 levels, respectively. Uptake by inhibited discs was 1.20 nmol sucrose·cm^{-2}·30 min, and uptake by control (no PCMBS 250 mM mannitol) was 4.28 nmol·cm^{-2}·30 min. [c,d] Mean that the difference in PCMBS binding between the mannitol and the sugar-treated discs is significant at the 0.01 and 0.001 levels, respectively. PCMBS binding in mannitol discs was 1.75 nmol·cm^{-2}·15 min.

inhibit PCMBS binding; (b) to protect sucrose uptake from PCMBS inhibition. The results (Table I) show that L-glucose, D-glucose, 3-*O*-MeG, galactose, and fructose neither decreased significantly the binding of labeled PCMBS nor increased sucrose uptake from PCMBS-treated discs. In contrast, 3-*O*-MeG has a tendency to enhance the binding of PCMBS and its inhibition of sucrose uptake. Among disaccharides, palatinose (6-*O*-α-D-glucopyranosyl-D-fructofuranose), turanose (3-*O*-α-D-glucopyranosyl-D-fructose), and melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) were ineffective, while maltose (4-*O*-α-D-glycopyranosyl-D-glucose) and sucrose (1-*O*-α-D-glucopyranosyl-β-D-fructofuranoside) significantly reduced PCMBS binding and PCMBS inhibition of sucrose uptake. The trisaccharide raffinose (*O*-α-D-galactopyranosyl-[1 → 6]-α-D-glucopyranosyl-β-fructofuranoside) was also effective (Table I).

The good correlation observed between the effects of sugars on PCMBS binding and PCMBS inhibition of sucrose transport suggests that the decrease in binding is due to the impairment by some sugars of access of the active site of the sucrose carrier to PCMBS. However, the possibility that these results are due to proteins other than the sucrose carrier must be examined. We have paid special attention to the possibility that the results described above might be due to a protecting effect of sucrose on the active site of a cell wall invertase. Indeed, as with the sucrose carrier, the invertase must fit with sucrose, and the active sites of both enzymes might be spatially and/or structurally related. A slight invertase activity can be recovered from the washing medium of peeled broadbean leaves (7).

The Effect of Osmotic Shock on PCMBS Retained by the Tissues. Part of the cell-wall invertase may be released by a combination of a cold, osmotic and ionic shock (10). In contrast, carriers catalyzing coupled translocations embedded in the membranes, cannot be easily released by such treatments (9, 21). We have investigated the effect of a cold osmotic shock subsequent to the binding of labeled PCMBS on the radioactivity remaining in the discs. This procedure was preferred to the reverse one (shock, then binding of PCMBS) to avoid artefacts due to a possible general alteration of the membrane permeability by the osmotic shock. Table II shows that incubation of tissues in 600 mM NaCl for 1 h at 20° or 4°C released some label from the tissues but hardly affected the difference between the control and the sucrose discs.

Specific Activity of Membrane Proteins. Differential centrifugations were initially made according to the procedures of Yoshida *et al.* (25). However, it rapidly turned out that several

Table II. *Effect of Ionic and Cold Shock on the Binding of [^{203}Hg] PCMBS*

Tissues were incubated for 15 min in 0.5 mM labeled PCMBS in the presence of 250 mM mannitol or 250 mM sucrose, rinsed and harvested after incubation for 1 h on 'N' medium (control) or on 600 mM NaCl + 250 mM mannitol, at 20° or at 4°C. Each value is the mean of 15 discs ± SE. Differences between (a,c), (a,e), (b,d), (b,f) were significant at the 0.05 level; differences between (a,b), (c,d), and (e,f) were significant at the 0.001 level. Relative difference was calculated as

$$\frac{\text{Control} - \text{'sucrose'}}{\text{Control}} \times 100$$

Condition	PCMBS Binding	Difference
	nmol·cm^{-2}·15 min	*relative*
No shock, control	1.98 ± 0.21[a]	20.20
No shock, sucrose	1.58 ± 0.17[b]	
NaCl, 20°C, control	1.54 ± 0.26[c]	21.43
NaCl, 20°C, sucrose	1.21 ± 0.22[d]	
NaCl, 4°C, control	1.49 ± 0.31[e]	23.49
NaCl, 4°C, sucrose	1.14 ± 0.28[f]	

improvements were needed to increase the yield in pelletable proteins and the specific activity of proteins recovered. After grinding, filtration on cheese-cloth and elution through the Amberlite column, 85 to 90% of initial label of the discs was recovered. No attempt was made to minimize the loss due to these steps. Specific activity of proteins solubilized after grinding in the medium used by Yoshida *et al.*(25) showed the expected decrease of PCMBS binding in the 120,000*g* pellet when sucrose had been added to the incubation medium (Table IIIA). However, although no difference was noticed between the control and 'sucrose' 14,000*g* pellets, their specific activities were surprisingly higher than those of the 120,000*g* pellet (Table IIIA). Also, a high proportion (about 40% of the initial label) remained in the final supernatant. Efflux experiments to be described elsewhere (S. Delrot, D. R. Geiger, unpublished data) showed that the sodium metabisulfite and the EGTA of the grinding medium strongly promoted PCMBS release from discs and thus could be responsible for redistribution of protein and/or of label during the extraction process. Consequently, measurements of specific activity were repeated with omission of both compounds from the extraction medium (Table IIIB). Again, the expected decrease in PCMBS binding induced by sucrose was found in the microsomal pellet. No difference between the specific activity of control and sucrose discs could be detected in the other pellets, and more importantly, the highest specific activities were recovered in the 120,000*g* pellet. The increase in the specific activity of the 120,000*g* pellet resulting from omission of metabisulfite and EGTA seems to result more from a change in the distribution of proteins than that of radioactivity (Table III). This omission had little effect on the radioactivity of the different fractions, while it decreased their protein content and particularly that of the 120,000*g* pellet. The radioactivity recovered in this pellet only accounted for 7% of the initial radioactivity of the discs. This low yield may be explained by the usual losses due to grinding, column chromatography, incomplete centrifugation, incomplete recovery of the pellets, and possible redistribution of label as discussed below.

Table III. *Specific Activities, PCMBS, and Protein Content of Cell Fractions Obtained by Differential Centrifugation*

A, Tissues ground in the medium of Yoshida *et al.* (25). B, Tissues ground in the same medium but where sodium metabisulfite and EGTA have been omitted. (A) and (B) are the mean of four and three different experiments, respectively, made on sets of 50 discs for each condition. Discs have been incubated in [^{203}Hg]PCMBS for 15 min with 250 mM mannitol (control) or 250 mM sucrose (sucrose).

Condition	Pellet Specific Activity		
	1,500*g*	14,000*g*	120,000*g*
	pmol PCMBS bound/μg protein		
A			
Control	3.15	5.33	4.11
Sucrose	2.52	5.12	3.74
B			
Control	1.59	6.64	12.70
Sucrose	1.70	6.70	9.51
	PCMBS content		
	nmol		
A			
Control	2.98	8.16	5.01
Sucrose	2.13	5.57	4.63
B			
Control	1.29	7.88	6.11[a]
Sucrose	1.15	5.92	4.57
	Protein content		
	μg		
A			
Control	945	1,530	1,218
Sucrose	845	1,088	1,237
B			
Control	810	1,187	481[a]
Sucrose	675	883	480

[a] In parallel experiments where control discs were ground as in B but without BSA, the protein and PCMBS contents of the final supernatant were 7,600 μg and 42 nmol, respectively.

DISCUSSION

This paper gives the first data on the binding of PCMBS, an inhibitor widely used in uptake experiments (6, 8, 11, 18–20, 23, 24), and on the effect of various sugars on this binding. The experiments were designed to test the hypothesis that PCMBS binds to the active site of the sucrose carrier (19).

The first phase of PCMBS binding (0 to 20 min) may correspond mainly to the filling of the apoplast with the radioactive solution (2 to 3 min [7]) and to the binding of the inhibitor to thiols of the cell wall proteins and to external thiols of plasma membrane proteins. The second phase may be due either to the binding of PCMBS to a secondary category of sites, less sensitive to PCMBS, or to the uptake of label within the cells. Although the latter hypothesis cannot be completely ruled out because of the lability of the mercaptide bond and of possible redistribution of label, several results could not be explained if PCMBS entered the cells. Photosynthesis (1, 11), respiration (6), proton extruding activity of leaf tissues (6), and the transmembrane potential difference of the cell (20) have been reported to be insensitive to PCMBS. Furthermore, results to be described elsewhere (S. Delrot, D. R. Geiger, unpublished data) show that the time course of binding of a permeant thiol inhibitor (^{3}H-*N*-ethylmaleimide) plateaus at a value 10 times higher than that of [^{203}Hg] PCMBS under similar conditions.

The smaller rate of [^{203}Hg]PCMBS binding to the tissues in the presence of sucrose (Fig. 1A; Table I) may be explained by the fact that the presence of sucrose on the active site of the sucrose carrier prevents PCMBS from reaching its target. Figure 1B shows that PCMBS reacts with the sucrose carrier during the first 20 min of treatment, since maximal inhibition of uptake was reached within this time. Consequently, if the decrease of PCMBS binding is actually due to binding of sucrose at the active site of the carrier, it should be optimal during the first 20 min of incubation, (Fig. 1A). The absence of difference of PCMBS binding between the control and the 'sucrose' sets during the very first min of incubation may be due to the fact that, at this time, PCMBS binds mainly to cell-wall proteins; the disappearance of the difference after 20 min of treatment can be explained by the reaction (in both sets) of proteins not directly involved in sucrose uptake, resulting in a background which masks the initial difference. Also, since the interaction of sucrose with its carrier is only transient and very rapid, PCMBS can hit the carrier in its 'unloaded' form, and this process may neutralize all sucrose carriers, even in the presence of sucrose, after long incubation times. A difference of 18% may appear small, but it is highly significant and specific (Table I). Given the background due to all other proteins, the actual difference at the level of the protein protected by sucrose must be much higher.

Concentration dependence studies of sucrose uptake in broadbean leaf have shown the existence of two apparent saturable systems (K_{m1} = 3 mM; K_{m2} = 35 mM) superimposed by a linear, nonsaturable system (4). The concentration dependence of the decrease of PCMBS binding induced by sucrose (Fig. 2) exhibits

two different phases which cannot be interpreted with certainty at the present time. The first phase could be ascribed to a saturable system with a K_m of about 30 mM. Yet, since PCMBS titrates the SH groups at the active site of the carrier, sucrose is not truly competitive with PCMBS, and K_m value computed from Figure 2 can be only indicative. The second phase (above 75 mM) does not seem to be simply a nonspecific phenomenon (osmotic for example) since in this concentration range D-glucose may induce some decrease (although not significant) of PCMBS binding (Fig. 2) while other sugars such as D-galactose, 3-*O*-MeG or L-glucose do not, or may even cause an increase in PCMBS binding (Table I). Several pieces of evidence indicate that the decrease of PCMBS binding induced by sucrose is due neither to a nonspecific effect nor to an effect on a free space invertase, but actually due to the impairment of PCMBS access to the active site of the sucrose carrier.

For various sugars, there is a good qualitative relationship between the effect on PCMBS binding and the effect on sucrose uptake in the presence of PCMBS (Table I). L-Glucose, D-glucose, palatinose, turanose, and melibiose have no effect on either phenomenon. D-Fructose and the mixture D-glucose plus D-fructose only weakly and marginally ($t = 0.1$) decreased PCMBS binding and did not affect sucrose uptake. For reasons still unknown, 3-*O*-MeG and D-galactose seem to increase significantly the sensitivity of sucrose uptake towards PCMBS, although they exerted no clearcut effect on PCMBS binding. More importantly, three sugars (maltose, sucrose, raffinose) are highly efficient in both assays, with sucrose being the most efficient. It has been previously shown that the protecting effect of sucrose against PCMBS inhibition is not due to sucrose exchange (19). Our results can give some indirect information on the specificity of the sucrose carrier, and in this respect, they can be compared to recent data obtained on other materials with other methods. In broadbean leaf as in soybean cotyledon, maltose can be recognized by the sucrose carrier while palatinose and turanose cannot (22). However, raffinose is unable to inhibit sucrose uptake in soybean, whereas it decreases PCMBS binding and it protects the sucrose carrier in broadbean (Table I). Therefore, the conclusion that the sugar must be near a disaccharide in size to be recognized by the sucrose carrier (22) is not confirmed in our material. Similarly sucrose transport into vacuoles isolated from barley mesophyll photoplasts is inhibited by maltose, but also by raffinose (16). Whether these discrepancies are due to the plant material or to the different rationale of the experiments is not known.

The comparison of the differences in PCMBS binding and in sucrose uptake in Table I can be only qualitative, and not quantitative, because: (a) we do not know if PCMBS binds to other cysteines of the sucrose carrier, and the number of thiols in the active site (dithiol ?) is also unknown; (b) the uptake of [^{14}C]sucrose was measured at 1 mM [^{14}C]sucrose, which is not the optimal concentration for the measurement of the protection of uptake (see Table I; Ref. 19); (c) during the 30-min uptake of [^{14}C]sucrose, subsequent to PCMBS pretreatment, the lability of the mercaptide bond may allow redistribution of PCMBS from (and to) the sucrose carrier.

The hypothesis that the phenomena observed can be ascribed to a cell wall invertase and not to the sucrose carrier does not fit with the lack of effect of the cold osmotic shock. Indeed, the difference between the control and the sucrose discs remains after the shock, although this treatment releases some proteins (decrease in labeling), including invertase (10). Moreover, this hypothesis seems to be ruled out by the difference of specific activity induced by sucrose in the 'microsomal' fraction (Table III).

The difference in PCMBS binding induced by sucrose is not due to an osmotic effect of the sugar on the membrane, since it does not appear with other disaccharides (palatinose, turanose) and since the maximal difference appears at a different time with another thiol inhibitor (2 min for ^{3}H-*N*-ethylmaleimide; B. M' Batchi, D. Pichelin, S. Delrot, unpublished data).

Table III shows that the relative difference (25%) between the specific activities of the 120,000*g* fractions of control and sucrose discs is higher than that observed on the radioactivity of whole discs (15–18%). This result, which was repeated in two other series of three experiments each (21 and 26%) may be due to the removal of some 'background' proteins binding PCMBS. However, the label was also present in the 14,000*g* pellet and in the final supernatant. The same distribution was observed if fresh tissues were used instead of frozen discs (data not shown). The presence of label in the 14,000*g* pellet may be explained by cross-contamination of this fraction by the plasma membrane. Although this pellet contains more radioactivity in the control than in the sucrose discs, dilution by proteins from other membranes does not allow to observe clearcut differences in the specific activities of control and sucrose discs (see Table IIIB), although a difference of 20% appeared in some other experiments (data not shown).

The widespread distribution of label in the different pellets, and particularly in the final supernatant raises the possibility of redistribution of proteins and/or of label during the grinding and centrifugation procedures. Four explanations may be proposed to explain the high relative proportion of label recovered in the final supernatant (about 40% of total radioactivity of the discs): (a) [^{203}Hg]PCMBS can enter the cells during the incubation; (b) [^{203}Hg]PCMBS was contaminated with free ^{203}Hg (highly permeant); (c) the label is associated with cell-wall proteins released during the grinding process; (d) [^{203}Hg]PCMBS or proteins labeled with [^{203}Hg]PCMBS are released from the plasma membrane during grinding and high speed centrifugations. The first possibility does not fit with the long known properties of PCMBS nor with its lack of effect on respiration (1, 11), on photosynthesis (6), on proton extruding activity (6), and on the transmembrane potential difference (20). The second possibility can be discarded since the purity of [^{203}Hg]PCMBS was checked. Moreover, we checked that ^{203}Hg or [^{203}Hg]-PCMBS did not enter the cells in substantial amounts since the intracellular sap recovered by freeze-thawing only contained 5% of the initial radioactivity content of the discs. This figure is much smaller than the 40% found in the supernatant and rules out hypothesis (a) and (b).

We think that the label of the supernatant is released from the cell walls and the membranes during grinding and centrifugations. About 25% of the initial label of the discs may be assumed to be bound to the cell wall since it is released by a cold and ionic shock (Table II). On the other hand, we found recently that grinding in a mortar and a pestle (instead of electrical grinding) could decrease the proportion of label in the supernatant from 40 to 25 to 30% of the initial content of tissues. When the 1,500*g*, the 14,000*g* and the 120,000*g* pellets recovered from a first cycle of centrifugation are centrifuged again at 120,000*g* for 4 h, they release, respectively, 12, 10, and 7% of their label in the supernatant (data not shown). These figures, which are certainly higher during the first cycle of centrifugation, may give an estimate of losses due to incomplete pelleting (or solubilization) of membrane proteins.

In conclusion, the data presented in this paper give new support to the hypothesis (5, 19) that PCMBS binds to the active site of the sucrose carrier, presumably to a thiol. The occurrence of a functional thiol, selectively protected by sucrose, might be used for differential affinity labeling of this protein with PCMBS or with other thiol inhibitors.

One may argue that PCMBS is not a specific inhibitor for the sucrose carrier. However, the methods used for differential affinity may overcome this problem, since three different proton-

sugar systems have been characterized in bacteria with *N*-ethylmaleimide, which is even less specific than PCMBS (14). The difference of specific activity of crude microsomal fractions prepared from tissues incubated with [^{203}Hg]PCMBS in the presence or in the absence of sucrose and some improvements brought to the medium of Yoshida *et al.* (25) for our specific purpose provide the first steps towards the characterization of the protein by sucrose. However, much remains to be done to prevent fully the redistribution of PCMBS and to minimize the background due to other proteins. This can be achieved by using very mild procedures for the preparation of membranes and by appropriate detergent treatments (D. Pichelin, B. M'Batchi, S. Delrot, unpublished data).

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