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Evidence for Phloem Loading from the Apoplast

CHEMICAL MODIFICATION OF MEMBRANE SULFHYDRYL GROUPS[1]

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ABSTRACT

The water-soluble, sulfhydryl-specific, chemical modifier *p*-chloromercuribenzenesulfonic acid reversibly inhibited the accumulation of exogenously supplied ^{14}C-sucrose into leaf discs of *Beta vulgaris*. *P*-Chloromercuribenzenesulfonic acid treatment did not inhibit photosynthesis or respiration or induce membrane leakage to sucrose, indicating that the site of inhibition was the plasmalemma. The active loading of sucrose and $^{14}CO_2$-derived assimilates into the phloem and their translocation from the source leaf were inhibited by the nonpermeant modifier. Several nonpermeant sulfhydryl group modifiers also inhibited sucrose accumulation into leaf discs while two amino-reactive reagents had no effect. The results indicate that sugars are actively accumulated into the phloem from the apoplast and that membrane sulfhydryl groups may be involved.

The mechanism of photosynthate translocation is generally accepted as a mass flow of solutes along a gradient of hydrostatic pressure established between regions of photosynthetic sugar production and sink utilization. One aspect of translocation that has been studied only recently and warrants further investigation concerns the mechanism and pathway of sugar movement between the sites of production in the mesophyll cells and the sites of entry into the sieve elements (phloem loading). Recent studies by Geiger and co-workers (5) indicated that sugars are not transported solely along a diffusion gradient between the mesophyll cells and minor veins of the phloem. Instead, plasmolysis studies, using freeze substitution electron microscopy, indicated the existence of a substantial concentration gradient of solutes between the mesophyll cell cytoplasm and the transfer (companion) cell-sieve element complex. It was proposed that this osmotic potential gradient in the minor veins, with its resulting water influx, is the driving force for translocation.

Currently, two distinct pathways have been proposed for the movement of photosynthate from the mesophyll cells to the phloem. In the symplastic route (1, 14, 25), assimilates produced in the mesophyll are transferred entirely within the cytoplasm, via plasmodesmata, to the phloem. The increased concentration of solutes in the phloem could result from an active accumulation mechanism associated with specialized plasmodesmata at the mesophyll-phloem junction (6) or by the existence of an active intracellular transport compartment, possibly the endoplasmic reticulum, extending from the mesophyll cells to the sieve elements (1).

Proponents of the apoplastic route of transfer (6, 19, 20, 23) postulate that sugars at some point exit from the mesophyll cells into the free space and are then accumulated into the phloem by an energy-dependent process. Implicit in the apoplastic pathway of solute transfer is a membrane-mediated transport of sugars from the free space into the phloem.

The use of nonpermeant, water-soluble chemical modifers has been well documented in membrane transport studies finding application as either a covalently bound marker for specific membrane groups or as a means of perturbing surface-located membrane functions (3, 13, 15, 22). Modification of membrane surface groups as a tool to study solute transport has been widely documented in animal (13), fungal, and bacterial (21) cells but has been used only recently on plants, primarily with isolated chloroplast membranes (8, 9). In particular, the nonpenetrating, sulfhydryl-specific reagent, PCMBS,[2] has been extensively employed to investigate surface-located carrier proteins involved in the transport of amino acids, sugars, and ions in a variety of organisms (13, 15, 21, 22).

Nonpenetrating chemical modifiers can be used to distinguish between the apoplastic and symplastic pathway of sugar movement to the phloem and to provide information on the mechanism of phloem loading. The rationale is that if the sucrose derived from photosynthesis is actively transported across a membrane into the phloem from the free space, then a reagent that inhibits uptake of exogenously supplied sucrose into the phloem (and does not penetrate the cell membrane) would inhibit sucrose entry into the phloem and, thus, its subsequent translocation from the leaf. In addition, the chemical group selectivity of the modifier may indicate which functional groups in the membrane are involved in the uptake process. Alternatively, if the nonpermeant reagent effectively inhibits membrane-mediated uptake of exogenously supplied sucrose but does not inhibit translocation of assimilates derived from CO_2, then the symplastic route is indicated.

In this study, the nonpenetrating chemical modifier, PCMBS, is used to study phloem loading and translocation. The results indicate that the sugars are actively transported into the phloem from the apoplast and that a sulfhydryl-containing carrier protein may be involved.

MATERIALS AND METHODS

Beta vulgaris L. (monohybrid A-1) plants were grown in a controlled environment under the following conditions: 18-hr photoperiod at 3800 ft-c, 24 C-19 C day-night temperature re-

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[2] Abbreviations: MOPS: morpholinopropane sulfonic acid; CCCP: carbonylcyanide-*m*-chlorophenylhydrazone; DNP: 2,4-dinitrophenol; TNBS: 2,4,6-trinitrobenzene sulfonic acid; SITS: 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid; DNFA: 2,4-dintro-5-fluoroaniline; DTNB: 5,5'-dithiobis-2-nitrobenzoic acid; PCMB: *p*-chloromercuribenzoic acid; Mersalyl-(α[3-hydroxymercuri-2-methoxypropyl)-carbamyl]phenoxyacetic acid); CPDS: 6,6'-dithionicotinic acid; PCMBS: *p*-chloromercuribenzene sulfonic acid; DTE: dithioerythritol; NEM: N-ethylmaleimide.

gime and 75% relative humidity. Accumulation of ^{14}C-sucrose was measured in 1-cm diameter discs obtained from expanded leaves of 5- to 6-week-old plants. Previous studies have shown that such leaves constitute an exporting source of assimilates (2, 7). Prior to collecting discs, the leaves were gently abraded with carborundum 300 (6, 23) to enhance solute entry through the cuticle. Experiments conducted on nonabraded tissue showed identical results except for lower rates of penetration of ^{14}C-sucrose.

Treatment of leaf discs with PCMBS (and other chemical modifiers) consisted of incubating the tissue in 20 mM K phosphate buffer (pH 7) containing varying concentrations of PCMBS for varying periods of time (see Fig. 1 for details). After PCMBS treatment, the tissue was washed in several changes of phosphate buffer to remove the unreacted modifier. For reversal of the inhibition, PCMBS-treated discs were transferred to 20 mM DTE or cysteine in 20 mM phosphate buffer (pH 7) for 5 to 10 min followed by several rinses in phosphate buffer. Discs (in triplicate) were transferred to 50 mM [^{14}C-U]sucrose (1000 μg C/μCi) containing 5 mM phosphate buffer for 60 min. The discs were exodiffused in three changes of H_2O for 10 min each which removed 95% of the free space label. Experiments revealed that ^{14}C-sucrose uptake into the discs was linear for at least 2 hr and was completely inhibited by the uncouplers DNP and CCCP and heat treatment, indicating its metabolic dependency. The tissues were frozen in solid CO_2, combusted to $^{14}CO_2$ by an Intertechnique Oxymat and the $^{14}CO_2$ recovered in a phenethylamine-toluene scintillation cocktail. The radioactivity (dpm) was determined by liquid scintillation spectroscopy.

Photosynthesis in leaf discs was measured in a photosynthetic Warburg using either ^{14}C-bicarbonate or $^{14}CO_2$ as the substrate. Control and PCMBS-treated discs were preilluminated (3700 ft-c, 25 C) for 15 min in open 50-ml Erlenmeyer flasks containing 1 ml of 10 mM MOPS buffer (pH 7). After the preillumination period, the flasks were stoppered and 10 mM ^{14}C-bicarbonate (1 μmol/μCi) injected and illumination was continued for 5 min. The reaction was terminated by ceasing illumination and adding 2 ml of 6 N acetic acid. The contents of the flasks were extracted with boiling 80% (v/v) ethanol and the ^{14}C-incorporated determined by liquid scintillation spectroscopy. Photosynthesis was also determined by injecting $^{14}CO_2$ (generated by phosphoric acid addition to [^{14}C]barium carbonate) with similar results. Dark respiration of leaf discs suspended in the phosphate buffer was determined polarographically with a YSI oxygen electrode (7).

Steady state translocation and whole leaf photosynthesis were determined as described previously (6). For autoradiography, leaf discs exposed to ^{14}C-sucrose were frozen in powdered solid CO_2 and lyophilized (2). The tissue was then flattened using a hand press and exposed on Kodak Industrex x-ray film (type M) until 6 to 8 $\times$ 10^6 cpm accumulated. The radiograms were processed in Kodak x-ray developer and fixer.

RESULTS AND DISCUSSION

Since any agent that inhibits cellular metabolism indirectly inhibits metabolism-dependent transport, it is crucial that the action of PCMBS be limited solely to interactions with sulfhydryl groups on the membrane surface rather than with intracellular sulfhydryl groups. Preliminary experiments demonstrated that PCMBS markedly inhibited ^{14}C-sucrose accumulation into leaf tissue. To determine whether the inhibition was due to modification of the membrane surface or due to impairment of intracellular metabolism, the following experiments were conducted. The time and concentration dependence of PCMBS inhibition of ^{14}C-sucrose uptake are shown in Figure 1, a and b, respectively. Complete reversal of the inhibition was achieved when the inhibited tissue was subsequently treated with the sulfhydryl-containing reagents DTE or cysteine (data not shown). Since the

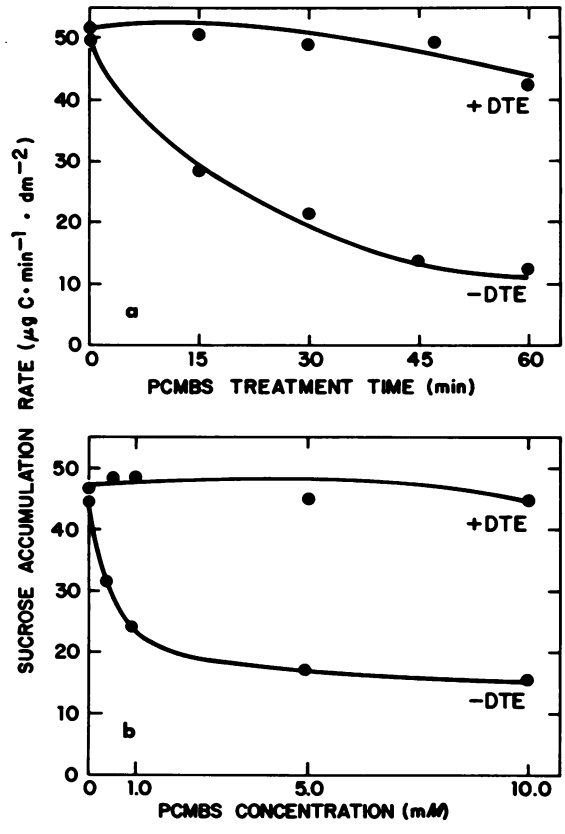

FIG. 1. Time and concentration dependence of PCMBS treatment on the inhibition of sucrose accumulation. (a): PCMBS concentration was 2 mM. (b): Treatment time was 15 min. DTE (20 mM) treatment was subsequent to PCMBS treatment.

inhibition could be substantially reversed even after a 60-min PCMBS treatment, intracellular metabolism was not permanently altered by PCMBS. That the site of inhibition is limited to the plasmalemma and not to intracellular metabolism is further shown in Table I. Treatment with 2 mM PCMBS inhibited ^{14}C-sucrose accumulation by 60% without affecting photosynthesis or dark respiration. In contrast, the membrane-penetrating chemical modifier, NEM, which is also specific for sulfhydryl groups, completely inhibited photosynthesis and respiration, indicating the sensitivity of these processes to sulfhydryl-reactive compounds. Similar results were observed with iodoacetamide, a membrane-penetrating, sulfhydryl-specific reagent. Thus, the lack of sensitivity of photosynthesis and respiration must be due to lack of penetration of PCMBS across the cell membrane.

The data presented in Table II indicate that PCMBS is not simply increasing the efflux rate of accumulated sucrose, thereby giving an apparent inhibition of uptake. Several leaf discs which had accumulated ^{14}C-sucrose (and washed of free space label) were incubated in 20 mM phosphate buffer, ±3 mM PCMBS for

Table I. *Effect of p-Chloromercuribenzenesulfonate and N-ethylmaleimide on Sucrose Accumulation, Photosynthesis, and Dark Respiration in Beta Leaf Discs*

Discs were treated with 2 mM PCMBS or 1 mM NEM for 15 min.

	Sucrose Accumulation	Photosynthesis	Respiration
	μgC/min·dm^2		
Control	31.0	41.0	2.0
PCMBS	13.0	39.0	1.9
NEM	3.2	2.0	0.0

30 min. There was no difference in the amount of ^{14}C label that exodiffused into the external medium in control and PCMBS-containing buffer, indicating that PCMBS did not increase sucrose leakage from the cells by some perturbation to the membrane. Similar results were obtained when $^{14}CO_2$ was supplied to discs while measuring the amount of ^{14}C label entering the external solution. These efflux experiments were also performed on tobacco leaf discs in which the lower epidermis was removed prior to ^{14}C-sucrose or $^{14}CO_2$ uptake (to eliminate the diffusion barrier imposed by the epidermis between the mesophyll and external solution) with similar results.

The above data show that PCMBS markedly inhibits sucrose uptake into leaf tissue and that the site of inhibition is limited to the plasma membrane, possibly by reacting with sulfhydryl groups of a carrier protein. That sulfhydryl groups may be involved in sucrose accumulation is indicated further in Table III. Gaudemer and Latruffe (3) have recently documented a variety of penetrant and nonpenetrant sulfhydryl reagents on their ability to oxidize membrane-enclosed glutathione. Table III shows that treatment of leaf discs with these nonpenetrating sulfhydryl reagents inhibited sucrose accumulation by approximately 50%. Perhaps the lack of inhibition by CPDS is due to steric factors between the membrane and modifier (21). Two nonpenetrating amino-reactive reagents, TNBS and SITS (13, 15), did not markedly interfere with sucrose accumulation, suggesting that the inhibition of sugar uptake is specific for chemical modification of sulfhydryl groups and not due to general membrane perturbation.

Autoradiography of leaf tissue supplied with ^{14}C-sucrose shows the label confined to the minor veins (Fig. 2). In autoradiographs of tissues treated with 2 mM PCMBS prior to sucrose accumulation, little label was observed in the tissue. That PCMBS treatment inhibits sucrose accumulation into the minor veins of the phloem as visualized by autoradiography indicates that the membranes of the phloem (*i.e.* transfer cells and sieve elements) also show sensitivity to PCMBS treatment. The PCMBS sensitivity of phloem and the calculations of Geiger and co-workers (4, 23) that there are 70 cm of minor veins/cm² leaf or 0.88 cm² of minor vein surface/cm² leaf (*i.e.* the minor veins constitute a substantial portion of the leaf discs) suggest that the inhibition of sucrose uptake by PCMBS into leaf disc tissue is also reflecting inhibition of sucrose entry into the minor veins.

Table II. *Effect of p-Chloromercuribenzenesulfonate on the Efflux of Accumulated ^{14}C-Sucrose from Beta Leaf Discs*

Details given in text.

	Amount of Sucrose Exodiffused	Amount of Sucrose Retained
	μgC/dm²	
Control	28.7	362
PCMBS-treated (3 mM)	27.8	353

Table III. *Effect of Nonpermeant Sulfhydryl Reagents on Sucrose Accumulation into Beta Leaf Discs*

Sulfhydryl reactive	Reagents at 2 mM for 15 min. Sucrose accumulation μgC/min·dm²
Control	29.8
PCMBS	12.3
PCMB	14.5
DTNB	14.1
DNFA	15.6
Mersalyl	10.1
CPDS	24
Amino reactive	
TNBS	26
SITS	27

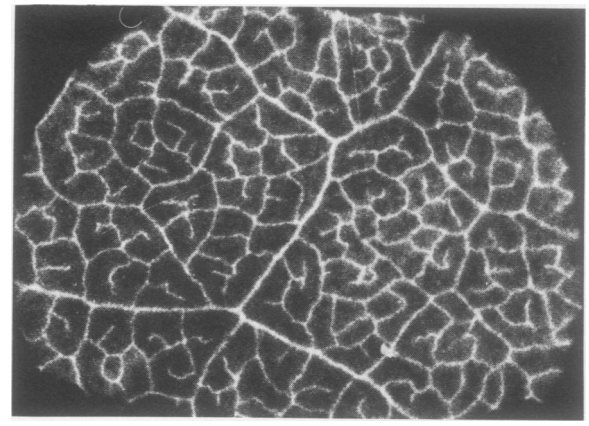

FIG. 2. Autoradiograph of leaf disc incubated on 50 mM ^{14}C-sucrose in 5 mM phosphate buffer; white area is ^{14}C localization.

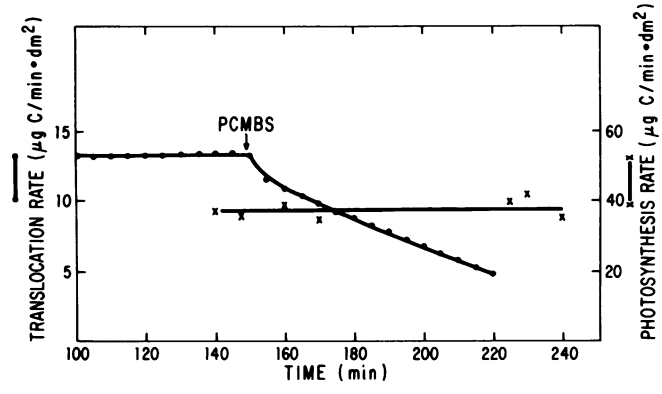

FIG. 3. Time course of translocation rate and photosynthesis rate after PCMBS treatment. Translocation and photosynthesis rates were measured simultaneously in an apparatus described recently by Geiger *et al.* (6). Phosphate buffer or buffer plus PCMBS was applied to the abraded upper leaf surface while supplying $^{14}CO_2$ to the lower surface. The phosphate buffer was replaced with buffered PCMBS (10 mM) for 25 min and then replaced with phosphate buffer alone. When ^{14}C-sucrose was applied to the upper leaf surface instead of $^{14}CO_2$, translocation was inhibited from 11 to 6 μg of C/min·dm² after 60 min.

Thus, if sucrose synthesized in the mesophyll cells entered the free space prior to phloem loading, its active accumulation into the phloem would be inhibited by PCMBS treatment and this would be manifested in a selective inhibition of translocation from the leaf. Figure 3 shows that the steady-state rate of translocation from intact leaves using $^{14}CO_2$ is inhibited by PCMBS under conditions where photosynthesis is not affected. A similar extent of inhibition was observed when ^{14}C-sucrose was applied to the source leaf instead of $^{14}CO_2$ (data given in figure legend).

The data presented in this study support the hypothesis that photosynthetically produced sucrose enters the apoplast prior to being actively transported into the phloem (6) and that membrane-bound sulfhydryl groups may be involved in the uptake process. The most likely sites of accumulation are the plasma membranes of the transfer cells at the phloem-mesophyll interface or the sieve element membranes. The osmotic potential generated by the active concentrating step located at the transfer cell membrane would generate the driving force for translocation.

The entry of solutes into the apoplast would have to occur so that the deposition is limited to regions close to the transfer cells

or to regions where the resistance imposed by transpirational flow (occurring in the opposite direction) would be minimal. Thus, sugars probably travel in the symplasm through the mesophyll and then enter the apoplast at the phloem region. There is some evidence that suggests that apoplastic water surrounding the phloem regions of the vascular bundle is not in direct communication with the transpirational stream (11) and, thus, may not represent an obstacle to solute uptake. Also, the entry of sugars into the free space may involve a transport mechanism itself (6) and, thus, it is conceivable that PCMBS may be inhibiting both the transfer of assimilates into the free space and their reaccumulation into the transfer cells of the phloem. In any event, the data indicate that sucrose loading into the phloem involves a membrane-mediated process and, thus, supports the apoplastic route of assimilate transfer between the mesophyll cells and phloem.

The energization of the proposed transport mechanism for sucrose is not known but may involve direct use of ATP (18, 23) or may possibly be coupled to the co-transport of ions in which the electrochemical potential generated by differences in ion concentrations drives sugar uptake. The latter hypothesis is intriguing for several reasons. Chemical analyses studies of phloem contents have demonstrated a relatively high concentration of K^+ ions and low proton concentration (pH 8–8.5) (12) in addition to a high sucrose content. This raises the possibility of whether sucrose uptake into transfer cells is coupled either to an active proton influx or K^+ efflux. Assuming the xylem or apoplast solution to be approximately pH 5 to 5.5, a substantial electrochemical potential of protons (or OH^-) exists which can be coupled to sugar translocation. The recent experiments by Komor and Tanner (16, 17) have shown that hexose transport is stoichiometrically coupled to the co-transport of protons in *Chlorella*. Whether this scheme approaches the mechanism involved in phloem loading is not known, but there are many precedents for the active transport of nonelectrolytes (*i.e.* sugars) being coupled to ion transport in a variety of organisms (10, 16, 17, 24) and, thus, this hypothesis warrants further consideration.

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