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Effect of Cutting on Solute Uptake by Plasma Membrane Vesicles from Sugar Beet (*Beta vulgaris* L.) Leaves[1]

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The uptake of sucrose, 3-*O*-methylglucose (3-*O*-MeG), and valine were studied in discs and in purified plasma membrane vesicles (PMV) prepared from sugar beet (*Beta vulgaris* L.) exporting leaves. The uptake capacities of freshly excised leaf discs were compared with the uptake in discs that had been floated for 12 h on a simple medium (aging) and with discs excised from leaves that had been cut from the plant 12 h before the experiments (cutting). After cutting, sucrose uptake amounted to twice the uptake measured in fresh discs, whereas the uptake of 3-*O*-MeG and valine remained unaffected. In aged leaf discs, there was a general stimulation of uptake, which represented 400, 300, and 400% of the uptake measured in fresh discs for sucrose, 3-*O*-MeG, and valine, respectively. Sucrose uptake in fresh discs was sensitive to *N*-ethylmaleimide (NEM), to *p*-chloromercuribenzenesulfonic acid (PCMBS), and to mersalyl acid (MA). Although cutting induced the appearance of a sucrose uptake system that is poorly sensitive to NEM but sensitive to PCMBS and MA, aging induced the development of an uptake system that is sensitive to NEM but poorly sensitive to PCMBS and MA. Autoradiographs of discs fed with [^{14}C]sucrose show that cutting resulted in an increase of vein labeling with little effect in the mesophyll, whereas aging induced an increase of labeling located mainly in the mesophyll. The data show that cutting is sufficient to induce dramatic and selective changes in the uptake properties of leaf tissues and that the effects of cutting and aging on the uptake of organic solutes are clearly different. Parallel experiments were run with purified PMV prepared from fresh and cut leaves. The uptake of sugars and amino acids was studied after imposition of an artificial proton motive force (pmf). Comparison of the uptake properties of PMV and of leaf tissues indicate that the recovery of the sucrose uptake system in PMV is better than the recovery of the hexose and of the valine uptake systems. As observed with the leaf discs, cutting induced a 2-fold increase of the initial rate of sucrose uptake in PMV but did not affect the uptake of valine and 3-*O*-MeG. Cutting induced an increase of both V_{max} and K_m of the sucrose transport system in PMV. Measurements of the pmf imposed on the vesicles indicated that the increase of sucrose uptake induced by cutting was not due to a better integrity of the vesicles. Hexoses did not compete with sucrose for uptake in PMV from fresh and cut leaves, and maltose was a stronger inhibitor of sucrose uptake in PMV from cut leaves than in PMV from fresh leaves. The sensitivity of sucrose uptake to NEM, PCMBS, and MA in PMV from fresh and cut leaves paralleled that described above for the corresponding leaf discs. These data show that (a) the changes induced by cutting on sucrose uptake by leaf discs are due to membrane phenomena and not to the metabolism of sucrose; (b) the study of sucrose uptake with PMV gives a good account of the physiological situation; and (c) the specific effects induced by cutting on the sucrose uptake system are not lost during the preparation of the PMV.

Upon excision and flotation for a few hours on simple media, various plant tissues undergo the aging phenomenon, which results in dramatic physiological changes (Van Steveninck, 1975). The increase of solute uptake resulting from aging of leaf tissues has been documented in various species, including *Centranthus ruber* (Abraham and Reinhold, 1980), *Pelargonium zonale* (Carlier, 1980), *Beta vulgaris* (Giaquinta, 1980), *Triticum aestivum* (Léger et al., 1982), and *Vicia faba* (Lemoine et al., 1984). These data show that increased uptake of sugars induced by aging concerns Suc (Giaquinta, 1980; Léger et al., 1982; Lemoine et al., 1984) as well as metabolizable and nonmetabolizable hexoses (Carlier, 1980; Léger et al., 1982). Changes in sugar uptake induced by aging are sensitive to ions such as Li^+ (Carlier and Thellier, 1979), to inhibitors of protein synthesis, to transcription inhibitors, to polyamines, and to chemical reagents affecting ethylene biosynthesis (Lemoine et al., 1984). Autoradiographs of leaf tissues show that in wheat (Léger et al., 1982), in broad bean (Lemoine et al., 1984), and in sugar beet (Sakr et al., 1992), the increase of Suc uptake induced by aging is localized in the mesophyll tissue rather than in the veins. Aging does not promote the exit of Suc induced by counterflux experiments, whereas the counterflux of hexoses is stimulated 7-fold in aged tissues compared with fresh tissues (M'Batchi and Delrot, 1988). Sugar uptake is much more sensitive to pH in aged tissues than in fresh tissues, a phenomenon that has been termed "pH sensitization" (Léger et al., 1982; Lemoine et al., 1984).

Although the data summarized above suggest that aging may induce dramatic changes in the transport properties of the tissue, their interpretation in terms of membrane transport is limited. Indeed, these data may be complicated by the metabolism of Suc, or diffusion problems across the cell wall, which might be affected by aging. In an attempt to understand more clearly the changes in Suc transport induced by

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Abbreviations: ΔpH, transmembrane pH gradient; Δψ, transmembrane electrical gradient; DMO, dimethyloxazolidine dione; MA, mersalyl acid; NEM, *N*-ethylmaleimide; PCMBS, *p*-chloromercuribenzenesulfonic acid; pmf, proton motive force; PMV, plasma membrane vesicles; 3-*O*-MeG, 3-*O*-methylglucose; TPP, tetraphenylphosphonium bromide.

aging, we (Sakr et al., 1992) recently used the techniques allowing the study of pmf-driven Suc influx into PMV (Buckhout, 1989; Bush, 1989; Lemoine and Delrot, 1989). It was shown that the effects of aging on Suc uptake by peeled sugar beet leaf discs seem to be simply reproduced just by cutting leaves and dipping their petioles for 12 h into a simple medium (Sakr et al., 1992). Furthermore, pmf-driven uptake of Suc in PMV from leaves cut in this way was almost doubled compared with PMV prepared from fresh sugar beet leaves. This stimulation of Suc uptake was of the same magnitude as the one observed with the leaf discs (Sakr et al., 1992). Because it was impossible to peel the large amounts of leaves that are needed to prepare PMV by the phase-partition technique, the PMV used by Sakr et al. (1992) were prepared from cut leaves and not from leaves aged after peeling off the lower epidermis. Although both cutting and aging may lead to an increase in the uptake of organic solutes by leaf tissues, cutting represents only a small part of the treatments usually undergone by aged tissues (excision, peeling, and flotation onto a large volume of aqueous medium). Therefore, the present paper compares the uptake properties of fresh, cut, and aged leaf discs, as well as the pmf-driven uptake into PMV derived from fresh and cut tissues, with special reference to Suc.

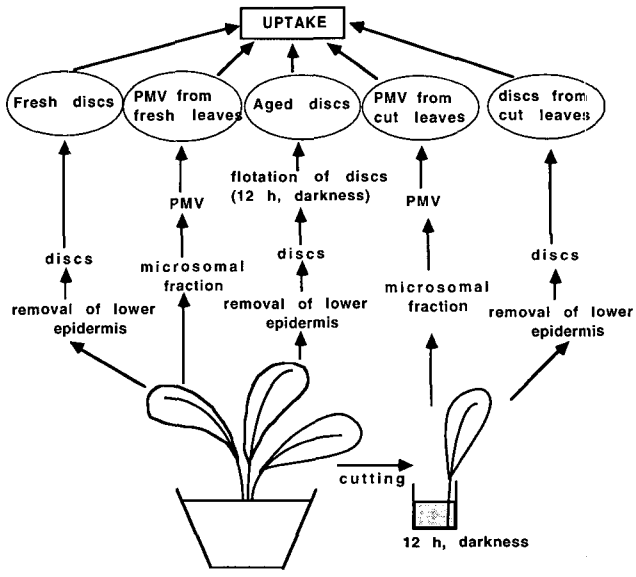

Figure 1. Experimental procedures used to study the effect of cutting and aging on uptake. Uptake into leaf discs without lower epidermis was studied by floating the discs onto solutions containing labeled sugars or amino acids. Uptake of the same labeled compounds into PMV was studied by imposing an artificial pmf across the plasma membrane. Other details are given in "Materials and Methods."

MATERIALS AND METHODS

Preparation of PMV

Conditions for growing the plants were described in Gallet et al. (1989). PMV were prepared by phase partitioning of microsomal fractions from fully expanded sugar beet (*Beta vulgaris* L.) leaves, in a dextran T 500/PEG 3350 mixture (Lemoine et al., 1991). Two millimolar DTT and 5 mM EDTA were included in the grinding buffer (Lemoine et al., 1991). Except when stated otherwise (see experiments with thiol reagents), the PMV were stored as a concentrated protein suspension (10–15 mg protein mL^{-1}) in buffer K (280 mM sorbitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ buffered at pH 7.5 with 50 mM K-phosphate) in the presence of 1 mM EDTA and 0.5 mM DTT.

Highly purified PMV were prepared by phase partitioning either from leaf blades taken directly from the plant (i.e. fresh leaves) or from leaf blades of cut leaves (Fig. 1). In the latter case, the leaves were excised from the plant, the extremity (about 2 cm) of their petiole was immersed into distilled water (100 mL for two or three leaves), and the leaves were maintained for 12 h in the dark and in saturating humidity to avoid excessive transpiration. After this treatment, the leaf blades were processed in the usual way to prepare a microsomal fraction, and the PMV were purified by phase partitioning (see above).

The purity and the sidedness of the PMV prepared either from fresh or from cut tissues were assessed using vanadate-sensitive, nitrate-sensitive, azide-sensitive ATPases and Chl, as described by Gallet et al. (1989), NADH Cyt *c* reductase, and IDPase (Hodges and Leonard, 1974). No difference was found in the purity (more than 90%) and in the sidedness (85–90% right side out) of the PMV from either fresh or cut leaves (data not shown). The plasma membrane fractions contained 0.7% and 0.4%, respectively, of the initial Cyt *c* reductase and IDPase present in the microsomal fractions.

Solute Uptake in PMV

The procedures we used for the energization of the PMV and for their filtration were as detailed previously (Lemoine and Delrot, 1989; Gallet et al., 1992). The uptake solutions contained various labeled compounds: 1 mM Suc plus 64.8 kBq mL^{-1} [6,6′(n)-^{3}H]Suc (or 27.8 kBq mL^{-1} [U-^{14}C]Suc), 1 mM 3-*O*-MeG plus 64.8 kBq mL^{-1} 3-*O*-methyl-D-[1-^{3}H]Glc, 1 mM Val plus 46.3 kBq mL^{-1} L-[2,3,4-^{3}H]Val. Although most experiments were run with a single labeled substrate, some experiments (as explained in "Results") were run with both [^{14}C]Suc and [^{3}H]Val added together. For uptake in nonenergized conditions (no gradient), the incubation medium was buffer K (pH 7.5) in which vesicles had been equilibrated before storage. For uptake in energized conditions, the concentrated vesicle suspension (2 μL), preincubated for 30 min with 5 μM valinomycin, was mixed with a vortex with 400 μL of buffer Na at pH 5.5, in the presence of 5 μM valinomycin. The combination of a 50-mM K-phosphate buffer (pH 7.5) inside the vesicles with a 50-mM Na-phosphate buffer (pH 5.5) in the incubation medium generates a ΔpH (outside acidic) and a K^+ diffusion potential resulting in a $\Delta\psi$ (inside negative). Active transport refers to the difference between total uptake in energized conditions (ΔpH + $\Delta\psi$) and the uptake in nonenergized (passive) conditions (ΔpH = $\Delta\psi$ = 0).

Unless otherwise stated, the uptake data presented for PMV are means of 12 replicates (from three independent experiments) ± SE.

Determination of the Internal Volume of the Vesicles

The internal volume of the vesicles was determined as detailed in Lemoine et al. (1991) except that the vesicles (1 mg protein mL^{-1}) were incubated in 50 mM K-buffer, pH 7.5, containing various concentrations (0–1000 mM) of sorbitol and a mixture of 69 kBq mL^{-1} ^3H_2O (measurement of water space) and 42 kBq mL^{-1} [^{14}C-carboxyl]dextran (mol wt 50,000–70,000) (measurement of extravesicular space) instead of [^{14}C]sorbitol. In the present work, [^{14}C-carboxyl]-dextran was preferred to [^{14}C]sorbitol because the latter compound has been reported to permeate the PMV in some instances (Lemoine et al., 1992; Maurousset et al., 1992). The total nonosmotic space was determined by adding the value found for nonosmotically active water at infinite osmolality to the space accessible to dextran. The osmotic space was determined from the difference between total and nonosmotically active water at different osmolalities of the medium (Lemoine et al., 1991).

Determination of ΔpH and $\Delta\psi$

The two components of the pmf were determined simultaneously by incubating the vesicles with 4 μM [^{3}H]TPP (4.8 TBq mL^{-1}) to evaluate $\Delta\psi$ and 7 μM [^{14}C]DMO (2.0 TBq mL^{-1}) to evaluate ΔpH. Procedures for uptake and conversion of the uptake values of the probes to the corresponding gradients were as detailed in Lemoine et al. (1991). DMO uptake was converted into ΔpH according to the equation $\Delta pH = \log([DMO]_i/[DMO]_{out})$. Passive adsorption of DMO was subtracted from total DMO uptake to calculate $[DMO]_i$. This passive adsorption was measured either by imposing the normal pH gradient ($pH_i = 7.5$, $pH_{out} = 5.5$) in the presence of 10 μM carbonyl-*m*-chlorophenylhydrazone or by imposing no pH gradient (pH 7.5 inside and outside). Both methods yielded the same results.

Leaf Disc Experiments

Three kinds of leaf discs, referred to as fresh, cut, and aged discs, were prepared (Fig. 1) and used for uptake experiments as described below. After cutting the petiole and removing the lower epidermis, leaf discs (6 mm diameter) were punched with a cork borer and floated for 30 min on solution P containing 20 mM Mes/NaOH, pH 5.0, 300 mM mannitol, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$. Fresh discs were then directly transferred to the incubation medium described below, and aged discs were transferred to fresh P solution and kept for 12 h in the dark before being transferred to incubation medium. Incubation was run for 30 min on P medium to which 1 mM labeled sugar or 1 mM labeled amino acid (3.6 KBq mL^{-1}) had been added. At the end of the incubation, the discs were rinsed (3 × 3 min) on medium P and blotted dry. In contrast to experiments with PMV, none of the media used for leaf discs experiments contained DTT. Depending on the experiments, leaf discs were either lyophilized for autoradiography or digested in a mixture containing 25 μL of 65% $HClO_4$, 50 μL of 33% H_2O_2, and 50 μL of 0.1% Triton X-100 before liquid scintillation counting. "Cut" discs were processed as fresh discs, except that they were prepared from "cut" leaves (leaves cut from the plant with the tip of the petiole immersed in distilled water, in the dark, and under saturating humidity, for 12 h before the removal of the lower epidermis and the preparation of the discs). Before the uptake *sensu stricto*, aged discs undergo the typical flotation period involved in aging (Fig. 1). In contrast, "cut" discs are prepared from leaves that have been separated from the plant by cutting the petiole 12 h before the uptake but otherwise remain intact until the beginning of incubation. Because it is very difficult to peel off the lower epidermis when the leaf blade is still attached to the plant, "fresh" discs were prepared and used immediately after cutting of the petiole. The uptake data presented for leaf discs are means of separate measurements of 30 discs from three independent experiments ± SE.

Suc Metabolism

Metabolism of [^{14}C]Suc was measured with PMV from either fresh or cut leaves after incubation of the vesicles for 10 min with 1 mM [^{14}C]Suc. Sugars were extracted and separated by paper chromatography as described by Lemoine et al. (1989).

[^{14}C]Suc metabolism was also measured in leaf discs from fresh and cut leaves and in aged discs that had been incubated on 1 mM [^{14}C]Suc for 30 min. Sugars were extracted as described by Dickson (1979) and separated by paper chromatography (Lemoine et al., 1989).

RESULTS

Stimulation of Solute Uptake in Leaf Discs by Cutting and Aging

Preliminary experiments showed that the uptake of Suc, 3-*O*-MeG, and Val was linear for at least 1 h in fresh and aged discs as well as in discs from cut leaves (data not shown). In fresh discs (Fig. 2, open columns), the uptake of 3-*O*-MeG and of Val was about twice the uptake of Suc. Aging of excised sugar beet leaf discs for 12 h resulted in a strong increase in the uptake of Suc, 3-*O*-MeG, and Val (Fig. 2, hatched columns). This increase was approximately the same for all three substrates tested, i.e. 300 to 400% of the control value measured in fresh discs. In discs from cut leaves (Fig. 2, dotted columns), the uptake of Suc was about twice the uptake measured in fresh discs, whereas the uptake of 3-*O*-MeG and of Val was not significantly affected compared with fresh discs. It may be concluded that although cutting of the leaf is sufficient to induce part of the increased Suc uptake observed after aging of leaf discs, this treatment is not sufficient to mimic the effects observed on the uptake of 3-*O*-MeG and of Val after flotation of the leaf discs.

Suc uptake in fresh leaf tissues is well known to be sensitive to various thiol reagents including NEM and PCMBS (Giaquinta, 1976; Delrot et al., 1980; Maynard and Lucas, 1982b). To test whether this sensitivity changed during aging, we studied the effect of various permeant (NEM) or relatively impermeant (PCMBS, MA) thiol reagents on Suc uptake by fresh and aged leaf discs and in discs from cut leaves. Uptake of Suc into fresh discs was inhibited about 35% by NEM and 45% by PCMBS (Fig. 3, left columns). In contrast, in discs prepared from cut leaves (Fig. 3, middle columns), Suc uptake was inhibited about 70% by PCMBS but only 30% by NEM.

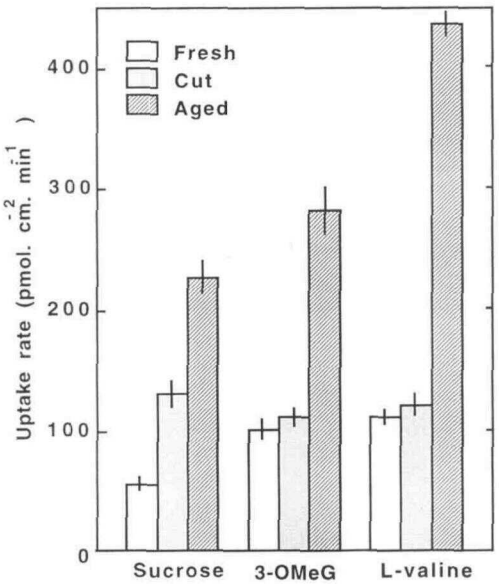

Figure 2. Aging increases the uptake rate of Suc, hexoses, and amino acids into leaf discs, whereas cutting only stimulates the rate of Suc uptake. Leaf discs from fresh or cut leaves and aged leaf discs were incubated for 30 min on 1 mM radiolabeled substrate. Data are means of 30 replicates (±SE) from three independent experiments.

MA was a strong inhibitor of Suc uptake both in fresh discs and in discs from cut leaves. Therefore, the increase of Suc uptake observed after cutting (difference between cut and fresh discs) was completely blocked by PCMBS and MA, but was only poorly sensitive to NEM. In aged discs (Fig. 3, right columns), Suc was inhibited only 30% by PCMBS and 60% by NEM. MA was also a poor inhibitor of Suc uptake after aging. In contrast to cutting, aging of leaf discs induced the appearance of a Suc uptake system that is poorly sensitive to PCMBS and to MA and is strongly sensitive to NEM.

Previous experiments (Sakr et al. 1992) had shown that after incubation with exogenous [^{14}C]Suc, the increase in uptake observed in aged discs, compared with fresh discs, concerned both the mesophyll and the veins. In the present paper, we have studied the effects of cutting on the localization of the [^{14}C]Suc absorbed. Autoradiographs showed an accumulation of exogenous [^{14}C]Suc into the veins of fresh discs (Fig. 4A). Compared with fresh tissues, cutting induced an increase of labeling that was located mainly in the veins (Fig. 4B). Confirming our earlier data in aged tissues (Sakr et al., 1992), the increase of labeling appears not only in the veins but is also evident in the mesophyll (Fig. 4C).

Chromatographic analysis showed that 9.3, 16.0, and 8.4% of the [^{14}C]Suc absorbed had been hydrolyzed to hexoses, respectively, in fresh discs, in aged discs, and in discs from cut leaves.

Effect of Cutting on Solute Uptake by PMV

The uptake of Suc, 3-*O*-MeG, and Val was measured after 1 min of incubation, which was considered a good estimate of the initial velocity of uptake (see Fig. 6, and Lemoine and Delrot, 1989, for Suc; Tubbe and Buckhout, 1992, and C. Gaillard and S. Delrot, unpublished data, for hexose; Gaillard et al., 1990, Lemoine et al., 1992, and C. Gaillard and S. Delrot, unpublished data, for Val). Figure 5 compares the initial velocity of active uptake of substrates into fresh and cut PMV. In fresh PMV (Fig. 5, open columns), the initial velocities of Suc uptake and Val uptake were equal and about twice the initial velocity of 3-*O*-MeG uptake. This situation strongly differs from that described above for leaf discs (Fig. 2), where 3-*O*-MeG and Val uptakes represented about twice the amount of Suc uptake. This shows that the different uptake capacities of the discs were not recovered with the same efficiency in PMV, suggesting that the Suc transport system was either less sensitive to inactivation or preferentially recovered during the purification of the PMV.

In PMV from cut leaves (Fig. 5, dotted columns), the initial velocity of active uptake of Suc was stimulated about 60% compared with PMV from fresh leaves, whereas the initial velocity of 3-*O*-MeG uptake and of Val uptake was not significantly different in the two types of PMV. Although somewhat lower than the stimulation noted in our preliminary study (200%, Sakr et al., 1992), the data show a clear

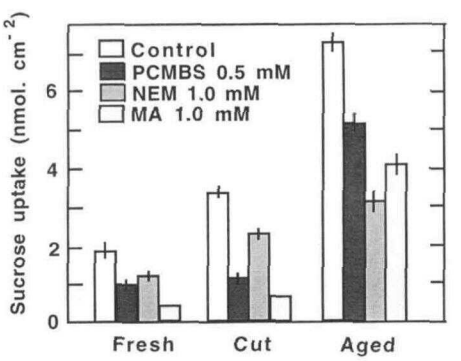

Figure 3. Effect of thiol reagents on Suc uptake into fresh, cut, and aged leaf discs. The reagents were present only during the incubation with [^{14}C]Suc. Data are means of 30 replicates (±SE) from three independent experiments.

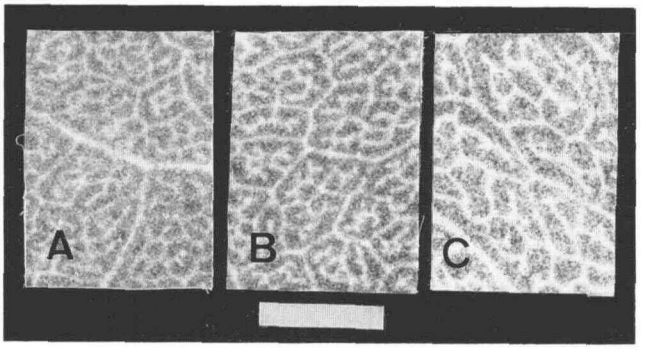

Figure 4. Localization of Suc uptake in fresh (A), cut (B), and aged (C) leaf tissues. The tissues were incubated for 30 min with 1 mM [^{14}C]Suc, rinsed, freeze-dried, and autoradiographed. The label appears in white. The scale bar = 0.2 mm. The dark area around the pictures shows the background of the film.

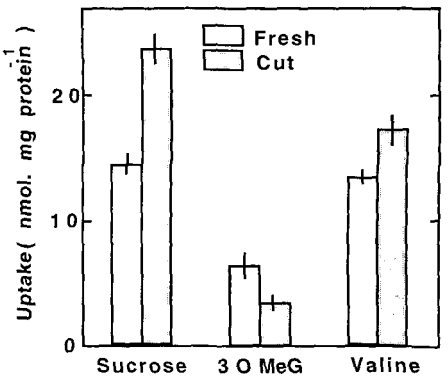

Figure 5. pmf-driven uptake of Suc, 3-*O*-MeG, and Val into PMV prepared from fresh and cut leaves. Data are means ± SE of 12 measurements from three independent experiments. The data for Suc and Val come from double-labeling experiments ([^{14}C]Suc/[^{3}H]Val). Similar results were obtained when these labeled compounds were provided separately.

increase in Suc uptake by PMV after cutting. The parallel between the data of Figure 5 and Figure 2 shows that the effects of cutting observed on the uptake of solutes by leaf discs may also be observed on the active uptake of solutes by PMV prepared from leaf material. In particular, cutting resulted in a strong increase in Suc uptake both in leaf discs and in PMV, whereas it did not affect the uptake of 3-*O*-MeG or of Val in either kind of material. Figures 2 through 5 show that the Suc uptake system behaves in a particular way after cutting of the leaves. Further experiments were aimed at a better characterization of this system in PMV prepared from cut leaves.

Time Course of Suc Uptake in PMV from Fresh and Cut Leaves

Figure 6 shows total and passive uptake of Suc into PMV prepared from fresh and cut leaves. Cutting increased both the initial rate of total uptake (about 70% between 0 and 1 min) and the accumulation plateau (about 30% between 1 and 10 min), confirming earlier preliminary experiments (Sakr et al., 1992). However, it did not significantly affect passive uptake of Suc; Sakr et al. (1992) observed a slight stimulation of uptake under passive conditions in PMV from cut leaves.

Chromatographic analysis showed no evidence of [^{14}C]Suc metabolism into PMV from fresh and cut leaves (data not shown).

Concentration Dependence of pmf-Driven Suc Uptake in PMV from Fresh and Cut Leaves

Initial rates of active uptake of Suc were measured as a function of Suc concentration in PMV prepared from fresh leaves (Fig. 7, lower curve) and from cut leaves (Fig. 7, upper curve). Maximal velocities were about 40 and 80 nmol Suc mg^{-1} protein min^{-1}, respectively, in PMV from fresh and cut leaves, whereas Suc concentrations corresponding to half saturation were 0.5 and 0.8 mM, respectively. Therefore, the events occurring after cutting modify the Suc uptake system of the plasma membrane in such a way that the affinity for Suc is slightly lowered and the maximal velocity is strongly enhanced. To determine whether these changes were due to the energization of the PMV or to a direct change of the activity of the carriers, the pmf actually imposed on the PMV was measured. A prerequisite for this study is a precise determination of the internal volume of the vesicles.

Internal Volumes of PMV

The water-permeable, dextran-impermeable space of the PMV was measured at various osmolalities of the medium, allowing a calculation of the internal volume of the vesicles (Lemoine et al., 1991). Figure 8 gives the water space (open squares), the extravesicular space (closed squares), and the total nonosmotic space of the PMV (closed circles, calculated as the sum of nonosmotically active water at infinite osmolality and of the space accessible to dextran). Nonosmotically active water is the pool of water that cannot move freely into and out of the vesicles in response to osmotic changes. The internal volume of PMV is the difference between total water

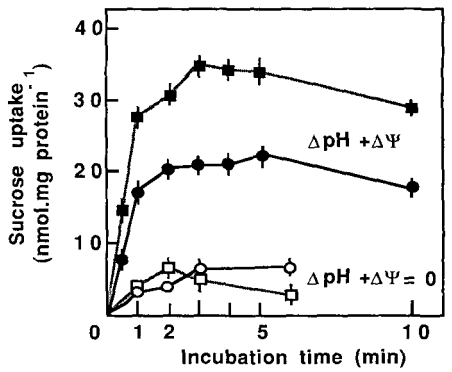

Figure 6. Effect of cutting on uptake of Suc into PMV from leaf tissues. Suc uptake was studied with PMV from fresh (○, ●) or cut (□, ■) leaves, under passive conditions (ΔpH = Δψ = 0; ○, □) or under energized conditions (●, ■).

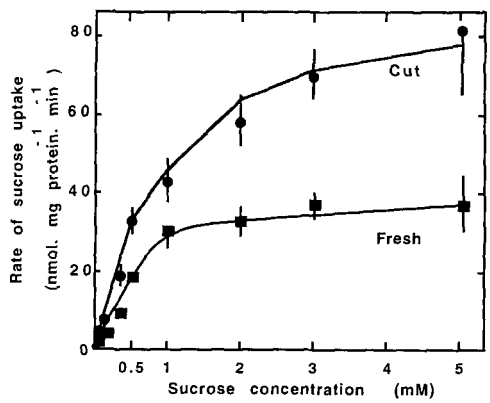

Figure 7. Concentration dependence of pmf-driven Suc uptake into PMV from fresh (■) or from cut (●) leaves. Uptake was measured after 30 s of incubation.

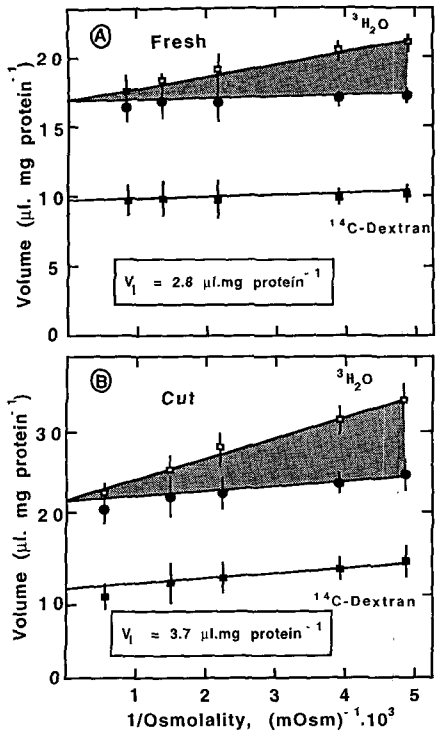

Figure 8. Measurement of the internal volume of the PMV at different osmolalities. ■, Volume accessible to [^{14}C]dextran; □, volume accessible to tritiated water; ●, volume accessible to nonosmotically active water. The shaded areas represent the variations of the osmotic space. The values given for the internal volume (V_i) are those corresponding to the osmolality of the experiments on sugar and amino acid uptake (330 mosmol).

and nonosmotically active water (shaded areas). At the osmolality used in the uptake experiments (330 mosmol), the internal volume of cut PMV was about 33% higher than the internal volume of fresh PMV (3.7 μL mg^{-1} protein versus 2.8 μL mg^{-1} protein, respectively).

Measurement of the pmf in Fresh and Aged PMV

Using the internal volumes calculated above and the time course of DMO uptake (to estimate ΔpH) and TPP uptake (to estimate $\Delta\psi$), the pmf actually imposed upon the membrane under our experimental conditions was compared for PMV from fresh and cut leaves. Cutting resulted in only a small increase of the pmf imposed on the vesicles at the beginning of incubation, but the pmf decreased more slowly in PMV from aged leaves than in PMV from fresh leaves (Fig. 9). From the Nernst equation, and assuming a 1:1 stoichiometry between protons and the cotransported solute, it may be calculated that, even if significant, the slight difference observed for the pmf after 1 min of incubation (201 versus 208 mV in PMV from fresh and cut tissues, respectively) would account only for a 3.5% increase in the concentration of intravesicular Suc after 1 min of incubation. Given the internal volume calculated above (Fig. 8) and the time course of Suc uptake (Fig. 6), the concentration of Suc after 1 min of incubation is 6.4 and 7.6 mM in PMV from fresh

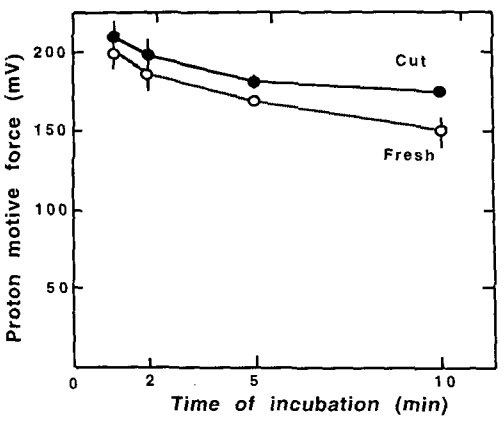

Figure 9. Time course of the pmf imposed on the vesicles for the energization of solute uptake. The pmf (ΔpH − ZΔpH) was calculated after determining ΔpH from the uptake of [^{14}C]DMO and $\Delta\psi$ from the uptake of [^{3}H]TPP. PMV from fresh (○) or cut (●) leaves.

and cut tissue, respectively, which corresponds to an 18% increase. The slight increase in the pmf observed in PMV from cut leaves cannot account for the increase in the accumulation ratio of Suc. Furthermore, if the increased Suc uptake depended only on the pmf, a more general effect on the uptake of 3-*O*-MeG and of Val would be expected. This effect is not observed (Fig. 5).

Sensitivity of Uptake to Thiol Reagents

The sensitivity of Suc uptake to thiol reagents was studied with PMV from fresh leaves and from cut leaves (Fig. 10). It

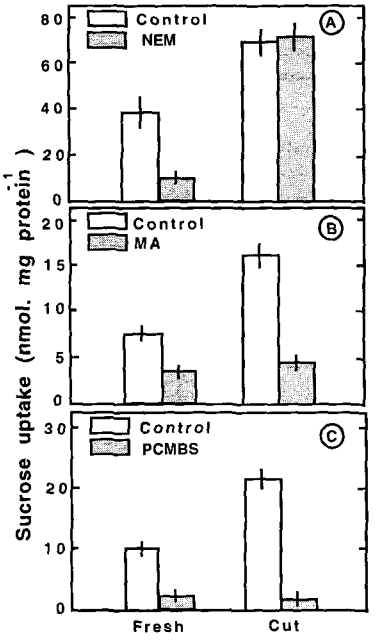

Figure 10. Effect of cutting on the sensitivity of pmf-driven Suc uptake to thiol reagents. PMV were incubated for 2 min with 1 mM [^{14}C]Suc under energized conditions in the presence of 1 mM NEM (A), 1 mM MA (B), or 1 mM PCMBS (C).

was interesting to compare this sensitivity with the effects of the same inhibitors on Suc uptake by leaf discs (Fig. 3). Suc uptake in fresh PMV from sugar beet leaves has been reported to be NEM sensitive (Lemoine and Delrot, 1989). However, Bush (1989) and Williams et al. (1990), using PMV from sugar beet leaves and castor bean cotyledons, respectively, reported that NEM did not inhibit proton-driven Suc uptake. This discrepancy may be explained simply by use of different experimental conditions (S. Sakr and S. Delrot, unpublished data). The presence of DTT in the storage medium of the PMV and in the incubation medium is particularly important.

The conditions used in the present paper to study the effect of thiol reagents were selected because they allowed the strongest inhibition of uptake in fresh tissues. Therefore, the inhibiting effect of NEM was studied with PMV stored at −70°C in the presence of DTT and with DTT in the incubation medium containing the label. The effect of MA was studied with PMV stored in the presence of 0.5 mM DTT but without DTT in the incubation medium. To study the effect of PCMBS, DTT was omitted from both the storage medium and the incubation medium. Due to these different experimental conditions and to some variations between different batches of PMV (see also Figs. 5, 6, 7, and 11), the control values (in the absence of inhibitor) cannot be directly compared across the three panels of Figure 10. Indeed, the presence of DTT during storage (Fig. 10A) resulted in a high uptake compared with vesicles stored in the absence of DTT.

However, for a given inhibitor, the same conditions were used for PMV from fresh and cut leaves. This allowed us to study the change in inhibitor sensitivity induced by cutting. The data in Figure 10A confirm that, under our experimental conditions, pmf-driven uptake of Suc is sensitive to NEM in PMV from fresh leaf. Data from leaf discs led us to conclude that the Suc uptake induced by cutting was only slightly sensitive to NEM (Fig. 3). This conclusion is at least partially confirmed by the NEM insensitivity of Suc uptake in PMV from cut leaves. The different sensitivity of uptake to NEM observed in PMV (totally insensitive) versus discs (poorly inhibited) may be explained by side effects of NEM on metabolism in leaf discs and/or by the use of DTT at different steps of membrane preparation and utilization. Both mersalyl (Fig. 10B) and PCMBS (Fig. 10C) are strong inhibitors of pmf-driven influx of Suc in PMV from fresh leaves and from cut leaves. These patterns of sensitivity strongly resemble those previously observed with leaf discs (Fig. 3).

Effect of Competing Sugars on Suc Uptake

In PMV from fresh leaves, hexoses did not compete with Suc for uptake, whereas maltose inhibited the uptake by about 50% (Fig. 11, left columns). These data are in good agreement with earlier results obtained from leaf discs (Geiger and Fondy, 1980; Giaquinta, 1980; Maynard and Lucas, 1982b) and PMV (Buckhout, 1989; Bush, 1989) from sugar beet. α-Phenylglucoside strongly competed for Suc transport, as previously shown in soybean cotyledon protoplasts (Hitz et al., 1986) and in broad bean leaf tissues (M'Batchi and Delrot, 1988).

In PMV from cut leaves (Fig. 11, right columns), the hexoses did not compete for Suc uptake either, but the maltose sensitivity was much weaker than in fresh discs. Again, α-phenylglucoside was a strong inhibitor of Suc uptake. Raffinose significantly stimulated the uptake in PMV from cut leaves, whereas no effect was observed in fresh PMV.

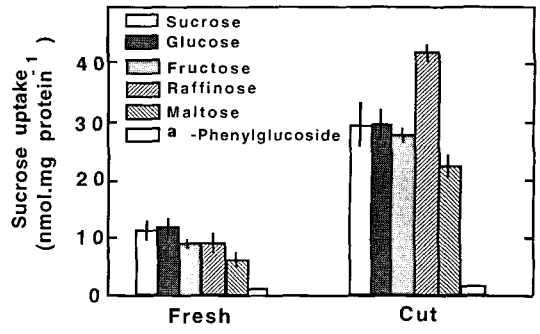

Figure 11. Effect of various competing sugars on pmf-driven Suc uptake into PMV from fresh and cut leaves. The competing sugars were present at 10 mM during the incubation (1 min) with 1 mM [^{14}C]Suc.

DISCUSSION

The aim of the present paper was to characterize the effects of aging and of cutting on the uptake of organic compounds by leaf tissues and to provide a deeper insight into the biochemical mechanisms underlying this phenomenon by studying the uptake into purified PMV. The discussion focuses on three major points: (a) the different effects of cutting and aging at the physiological level; (b) the comparisons of the effect of cutting on solute uptake by leaf tissues and by purified PMV; and (c) the mechanisms underlying the increase of Suc uptake observed with PMV from cut tissues.

The physiological effects of aging on solute uptake have long been known. The effects of cutting on metabolism have been well characterized (see, for example, Sugiharto et al., 1992), but its effects on the transport of organic solutes have not yet been studied in detail. One may wonder whether the effects of aging just depend on the isolation of the tissue from the mother plant or if they also involve the continuous contact of the tissues with large volumes of solution.

Figures 2 through 4 indicate that the effects induced by cutting on solute uptake by leaf discs are clearly different from that induced by aging. Indeed, in aged discs, there is a general stimulation of the uptake of Suc, of hexoses, and of amino acids, whereas the stimulation of uptake concerns Suc only after cutting (Fig. 2). Moreover, the stimulation of Suc uptake is much stronger after aging than after cutting. The sensitivity of Suc uptake to thiol reagents in discs from cut tissue is not the same as in aged discs and it is also different from that observed in fresh tissues (Fig. 3). On one hand, aging induces an uptake system sensitive to NEM and poorly sensitive to PCMBS and MA. This system appears preferentially in the mesophyll. On the other hand, cutting induces, preferentially in the veins, an uptake system poorly sensitive to NEM and sensitive to PCMBS and MA. [^{14}C]Suc hydroly-

sis, although limited, is 2-fold higher in aged discs than in fresh discs and in discs from cut leaves. These physiological data do not allow any clear explanation of the effects induced by cutting and by aging, yet they do show that, upon mechanical treatment such as cutting and aging, the plant is able to regulate the uptake capacity of the leaf tissue, and more particularly the uptake and phloem loading of exogenous Suc.

The particular response of Suc after cutting may be related to the basic importance of this sugar for the carbon balance in the plant. The different responses observed after cutting and aging, in terms of quality and quantity, indicate that these treatments probably involve different mechanisms of transduction. The effects of cutting on sugar transport may be mediated indirectly by numerous events such as a wounding signal, blocking of sugar export, or a change in water or hormonal content. Whether the responses observed involve only signal transduction *sensu stricto* (via calcium, electric signals), particularly in the case of cutting, or also depend on a hormonal and nutritional imbalance, will be investigated in the future. It will be interesting to study the effect of cutting with $^{14}CO_2$-labeled leaves, to know whether the change in the relative location (mesophyll versus veins) observed with exogenous [^{14}C]Suc is also apparent for endogenous assimilates. In this regard, it may be stressed that, following heat girdling or cooling of broad bean petiole, which strongly inhibit Suc export, an apparent decrease in the efficiency of phloem loading is observed (Ntsika and Delrot, 1986), apparently due to a diversion of the assimilated carbon from Suc to starch (Grusak et al., 1990).

Aging of tissues is accompanied by a stimulation of the plasma membrane ATPase, as measured by the proton-extruding activity (Delrot, 1981) or the transmembrane potential (Mertz and Higinbotham, 1976; Lemoine, 1984). This explains, at least in part, the general stimulation of transport observed in aged leaf discs. It is not possible to discard completely the hypothesis that part of the increased Suc uptake in aged discs (400%) is due to the lower stimulation (200%) induced by cutting on Suc uptake. However, fresh discs, aged discs, and discs from cut leaves exhibit a different sensitivity of Suc uptake toward thiol reagents (Fig. 3). Whereas aging leads to a stimulation of Suc uptake both in the mesophyll and in the veins of various species (wheat, Léger et al., 1982; broad bean, Lemoine et al., 1984; sugar beet, Sakr et al., 1992), cutting is characterized by an increased uptake of exogenous Suc localized mainly in the veins (Fig. 4). This argues for completely separated mechanisms leading to stimulation of Suc uptake in aged tissues and in cut tissues.

Because Suc (or its metabolic products) have recently been shown to induce the expression of a number of different genes related or unrelated to carbohydrate partitioning (for review, see Delrot, 1993), one may wonder if the increased uptake capacity of the plasmalemma after leaf aging or cutting is due to a depletion of Suc (due to the dark treatment) or to an accumulation of Suc in the leaf (due to the fact that the export is blocked). In some experiments, the petioles of the excised leaves were dipped either into a 20-mM Suc solution (to feed the leaf via the transpiration stream) or into an EDTA solution (to prevent callose synthesis and allow continuation of export). These treatments did not affect the uptake capacity of the leaf discs subsequently excised from leaves aged in these conditions, compared with leaves aged in the standard medium (data not shown). Besides, in broad bean leaf discs, although the Suc content dramatically decreases when the aging is performed in the dark, the stimulation of Suc uptake is the same for discs aged in the dark or in the light (Delrot, 1981). Therefore, these data suggests that Suc itself is not the trigger of the increase of the Suc uptake system.

Comparison of the uptake rates measured for Suc, 3-*O*-MeG, and Val in fresh discs (Fig. 2) and in PMV prepared from fresh discs (Fig. 5) shows that the Suc uptake system was either preferentially recovered or less affected during the preparation of the PMV. Although the uptake of Suc (Buckhout, 1989; Bush, 1989; Lemoine and Delrot, 1989; Lemoine et al., 1991), of amino acids (Gaillard et al., 1990; Li and Bush, 1990), and of hexoses (Tubbe and Buckhout, 1992) has already been characterized with PMV from sugar beet leaves, the recovery of the uptake capacities in this system had not yet been compared. The preferential recovery of the Suc uptake system may result either from the preferential recovery of some populations of vesicles (phloem vesicles), from a better preservation of the functional state of the Suc carrier during the preparation of the PMV, and/or from a better sealing ability of the PMV containing the Suc carrier. It may be argued that in leaf discs, uptake depends not only on the transport at the plasma membrane but also on the metabolism of the substrates taken up, which affects their concentration in the cytoplasm. This may limit the interpretation of the comparison between leaf discs and PMV in terms of recovery of transport capacity. However, at least in the case of Suc and 3-*O*-MeG, the interference of metabolism with transport in leaf discs cannot account for the apparently higher recovery of Suc transport than of hexose transport after the preparation of PMV. Indeed, in leaf discs, 3-*O*-MeG, which is usually considered as a nonmetabolite, is taken up 2-fold more than Suc, which is at least partially metabolized (Fig. 2). In contrast, in PMV, where metabolism is absent both for Suc and 3-*O*-MeG, Suc transport is 2-fold higher than 3-*O*-MeG transport (Fig. 5).

The data obtained on the uptake of Suc into PMV from fresh and cut leaves are in good agreement with the data obtained from leaf disc experiments. The PMV from cut leaves exhibit a specific increase in the Suc transport rate (Fig. 5), as observed in the discs (Fig. 2), and the sensitivity of Suc transport to various thiol reagents in PMV (Fig. 10) is in good agreement with that observed in the corresponding leaf tissue (Fig. 3). Indeed, Suc transport is sensitive to NEM, PCMBS, and MA in PMV from fresh leaves (and in fresh discs), but it is poorly inhibited by NEM and strongly sensitive to PCMBS and MA in PMV from cut leaves (and in discs from cut leaves). This parallel indicates that the changes induced by cutting on the uptake of Suc by leaf tissues are due to phenomena taking place in the plasma membrane and not to changes of Suc metabolism or intracellular compartmentation of sugars. That Suc metabolism is not involved is also shown by the fact that the hydrolyses of [^{14}C]Suc measured were small and similar in fresh discs and in discs from cut leaves, and there was no trace of invertase activity in the

PMV from fresh and cut leaves. The parallel between Suc uptake in PMV and Suc uptake in leaf tissues indicates that the membrane phenomena leading to an increased Suc uptake after cutting are not reversed during the preparation of the PMV. This also shows that studies on PMV provide a good picture of the changes in Suc fluxes observed at the physiological level.

Suc uptake into sugar beet leaf discs has been described as the superimposition of a saturable and of a linear component whose sensitivity to various treatments, such as NEM and maltose, differ (Maynard and Lucas, 1982a, 1982b). Our data with PMV confirm the complexity of the sensitivity of Suc uptake to NEM and to maltose and indicate that this sensitivity may be affected differentially by treatments such as cutting. It is not possible to ascribe a precise tissue localization for these components. Indeed, like the system operating in fresh tissues, the uptake system induced by aging is localized mainly in the veins. However, both systems differ by their sensitivity to thiol reagents and maltose.

Further experiments were aimed at achieving a better understanding of the membrane phenomena involved in the stimulation of Suc uptake observed with PMV from cut leaves. These phenomena lead to a doubling of the maximal velocity and to an increase in the K_m (Fig. 7). The increase of V_{max} may be due to the greater sealing ability of the vesicles, to an increase in the number of carriers/mg protein (the carrier being the same as in fresh tissues), to the synthesis and incorporation in the membrane of a new set of carrier proteins, to the covalent modification of preexisting carriers, and to a modification of the lipidic environment of the carrier. The fact that the stimulation of uptake into PMV after cutting concerns only Suc (Fig. 5), and not hexoses and amino acids, whose uptake is also driven by proton symport (Gaillard et al., 1990; Li and Bush, 1990; Tubbe and Buckhout, 1992), and the minor differences found in the pmf imposed on PMV from fresh and cut leaves (Fig. 9) indicate that the effect of cutting on V_{max} does not likely depend on proton permeability. Although the initial values of the pmf (after 1 min of incubation) are only slightly higher in aged PMV than in fresh PMV, the difference observed may account for only a 3% increase in the intravesicular concentration of Suc, whereas this increase actually reaches 18% or more depending on the experiments.

At the present time, none of the other explanations can be ruled out. Indeed, the change in sensitivity to thiol reagents may be due either to the incorporation of a new set of carriers (induction, *sensu stricto*) or to a conformational change of the preexisting carrier, itself dependent on a covalent modification of the protein or a modification of the lipidic environment. This also holds true for the modification of carrier specificity observed in PMV from cut leaves (Fig. 11). The increase in the internal volume of the vesicles recovered after cutting may also be due to the recovery of a different population of vesicles, to a synthesis or to a modification of the membrane lipids, or to a preferential degradation of proteins (compared with lipids) after cutting. Therefore, a number of different possibilities are still obviously open and will be tested in future work. It is clear that cutting induces remote changes in the plasma membrane properties of leaf cells that lead to a selective increase of Suc uptake and this provides a model worthy of further investigation to understand the regulation of sugar transport.

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