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Sucrose Transport and Phloem Unloading in Stem of *Vicia faba*: Possible Involvement of a Sucrose Carrier and Osmotic Regulation[1]

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ABSTRACT

Stems of *Vicia faba* plants were used to study phloem unloading because they are hollow and have a simple anatomical structure that facilitates access to the unloading site. After pulse labeling of a source leaf with $^{14}CO_2$, stem sections were cut and the efflux characteristics of ^{14}C-labeled sugars into various buffered solutions were determined. Radiolabeled sucrose was shown to remain localized in the phloem and adjacent phloem parenchyma tissues after a 2-hour chase. Therefore, sucrose leakage from stem segments prepared following a 75-minute chase period was assumed to be characteristic of phloem unloading. The efflux of ^{14}C assimilates from the phloem was enhanced by 1 millimolar *p*-chloromercuribenzene sulfonic acid (PCMBS) and by 5 micromolar carbonyl cyanide *m*-chlorophenly hydrazone (CCCP). However, PCMBS inhibited and CCCP enhanced general leakage of nonradioactive sugars from the stem segments. Sucrose at concentrations of 50 millimolar in the free space increased efflux of [^{14}C]sucrose, presumably through an exchange mechanism. This exchange was inhibited by PCMBS and abolished by 0.2 molar mannitol. Increasing the osmotic concentration of the efflux medium with mannitol reduced [^{14}C]sucrose efflux. However, this inhibition seems not to be specific to sucrose unloading since leakage of total sugars, nonlabeled sucrose, glucose, and amino acids from the bulk of the tissue was reduced in a similar manner. The data suggest that phloem unloading in cut stem segments is consistent with passive efflux of sucrose from the phloem to the apoplast and that sucrose exchange via a membrane carrier may be involved. This is consistent with the known conductive function of the stem tissues, and contrasts with the apparent nature and function of unloading in developing seeds.

Unlike the processes of sucrose loading into phloem of exporting leaves and sucrose uptake by ground parenchyma tissues, the process of sucrose unloading from the phloem is poorly understood. This lack of knowledge partially reflects the absence of an experimental system in which processes occurring in the phloem can be dissociated from those occurring in neighboring cells. Recently, several works have been published on assimilate unloading into seed coats (8, 12, 14). In this system, unloading is carried out by phloem sieve tubes, companion cells, and phloem parenchyma cells which may have specialized morphological and biochemical features for secretion. These studies suggest a possible involvement of energy dependence and carrier mediation. In another recent study (6), intact bean plants were utilized to study unloading into the stem. Assimilates in stems of *Vicia faba* parasitized by *Cuscuta* are unloaded into the apoplast prior to uptake by the ground parenchyma cells (13). Furthermore, that study also suggested that phloem unloading was under metabolic control, since it was inhibited by DNP[5] and low temperature. In contrast, it has been proposed that phloem unloading of sugars into the apoplast in bean stems is passive and that the sugar concentration in the free space is merely a balance between passive unloading and active uptake by sink parenchyma cells (6).

V. faba stems seem to be an appropriate system for phloem unloading studies. They are hollow and only a few layers of parenchyma cells separate the central cavity from the phloem sieve tubes. This anatomy minimizes the possibility that an unloaded sugar molecule will be retrieved before it 'escapes' to the bathing medium. We used *V. faba* stems to study the phloem unloading process. We assumed that (a) following a short pulse, [^{14}C]sucrose was restricted to the phloem tissue, and (b) specificity of the unloading process prevailed if the efflux of ^{14}C-labeled sucrose from the phloem sieve elements was altered differently than the efflux of nonphloem solutes, such as reducing sugars, by various experimental treatments.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Plants of *Vicia faba* L., cv Broadwinsor, were greenhouse-grown in 3-L pots with 7:3 sand:peat mixture. After germination, the plants were irrigated daily and fertilized weekly with complete nutrient solution. A 16-h photoperiod was provided using mixed incandescent and fluorescent lamps with a photon flux density of 500 μmol m^{-2} s^{-1} at canopy height. The average day and night temperatures were 25 and 18°C, respectively. All plants chosen for experimental use were uniform in size, 30 d old, and preanthesis.

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[5] Abbreviations: DNP, 2,4 dinitrophenol; PCMBS, *p*-chloromercuribenzene sulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; BTP, 1,3-bis-tris(hydroxymethyl)-methylamino-propane.

Pulse-Chase Treatment. Each selected plant (50 cm high) was trimmed of all but one uppermost fully expanded source leaf. This leaf was wrapped with a 13 × 33 cm plastic bag and pulsed for 30 min with 100 μCi $^{14}CO_2$, generated by mixing 100 μCi [^{14}C]$NaHCO_3$ (51.5 mCi/mmol) with 4 M HCl. Chase periods of various duration began after the bag had been removed.

Efflux Treatment. The ^{14}C preloaded stems were excised at the soil level and 2 cm segments averaging 0.25 g fresh weight were cut from the four uppermost internodes (below the source leaf). The segments were washed for 2 min in distilled H_2O and transferred to 4 ml of efflux media in plastic weighing boats. (The shallow weighing boats were selected to avoid anerobiosis which might have occurred in tubes.) These solutions were replaced periodically as indicated in each experiment. All solutions were buffered with 10 mM Mes adjusted to pH 5.5 with BTP. All solutions were adjusted to the same osmolality by varying the mannitol content. Each treatment consisted of four replicates and was repeated at least three times. Unloading was calculated as the percentage of the total radioactivity in the tissue that leaked into the bathing medium. In some experiments, first order rate constants describing ^{14}C efflux were calculated. Radioactivity of the incubation solution was determined after 11 ml of scintillation cocktail (Aquasol II)[6] was added to each sample. The remaining stem tissue was digested in 4 ml of H_2O_2-$HClO_4$ (60:40 v/v) digestion solution and incubated at 50°C until complete discoloration occurred. After cooling, 11 ml of scintillation liquid was added and radioactivity was determined in a scintillation counter (Packard, Tri-Carb 460 CD).

Determination of Labeled Sugar. To identify radioactive sugars present in the efflux medium, the medium was freeze-dried and redissolved in 400 μl of distilled H_2O. To identify sugars in the stem, segments were extracted three times with 80% ethanol at 80°C. The ethanolic fractions were evaporated to dryness and resuspended in 400 μl of distilled H_2O. Aliquots (20 μl) of the resuspended samples were injected into an HPLC (Bio-Rad, carbohydrate HPX-42, 300 × 7.8 mm) and eluted with water and detected with a differential refractometer. The peaks of interest were collected and radioactivity was determined.

Other Analytical Procedures. Total amino acids were determined with ninhydrin (9). Determination of soluble sugar was done with anthrone reagent (5). Reducing sugars were determined by Nelson's method (7).

RESULTS

The transport pathway of the ^{14}C-labeled assimilates from the source leaf downwards appears to be via the phloem sieve elements, as indicated by the following: (a) a transport velocity of 60 to 80 cm/h was measured, a value characteristic of phloem flow rates (4); (b) sucrose was the only labeled sugar transported throughout the pulse chase period, as shown in Figure 1A. Autoradiography of stem cross-sections indicated that the transported radioactive material was still confined to the phloem tissue at the termination of a 2-h pulse-chase period (not shown). After that 2-h period of time, [^{14}C]sucrose was distributed along the entire stem. For the efflux experiments, we used only the upper four internodes to eliminate variability within replications which might have occurred due to large differences in internode age.

The kinetics of ^{14}C-labeled sucrose efflux from the stem segments is shown in Figure 2. After a chase period of 45 min, two phases could be distinguished: a rapid and a slow phase, with rate constants of 1.8×10^{-2} and 5×10^{-3} min^{-1} and $t_{1/2}$ of 38.5 and 138 min, respectively, which corresponded to efflux from the free space and cytoplasm. When the efflux assay was carried out on plants chased for 5 h, we observed a similar kinetic pattern indicating that ^{14}C assimilates were still being unloaded from the free space and cytoplasmic compartments. Following a 20-h chase, the rate and percentage of ^{14}C-efflux was much slower ($k = 5 \times 10^{-4}$ min^{-1}, $t_{1/2}$ 48 h), presumably reflecting leakage from a different compartment. HPLC chromatography of the efflux media revealed that following the 45-min chase, and during the 120 min exposure to the efflux medium, ^{14}C was exclusively in sucrose (Fig. 1, A–C). Following a chase of 5 h, we observed equal proportions of [^{14}C]sucrose and [^{14}C]glucose; however, no fructose was labeled (Fig. 1D). We concluded, therefore, that in the shorter chase period (45 min), [^{14}C]sucrose is unloaded exclusively from the phloem, whereas following the 5-h chase, sucrose had been cleaved by invertases probably in the cytoplasm of the parenchyma cells. For these reasons, experiments were not extended beyond the 45-min chase and 120-min efflux.

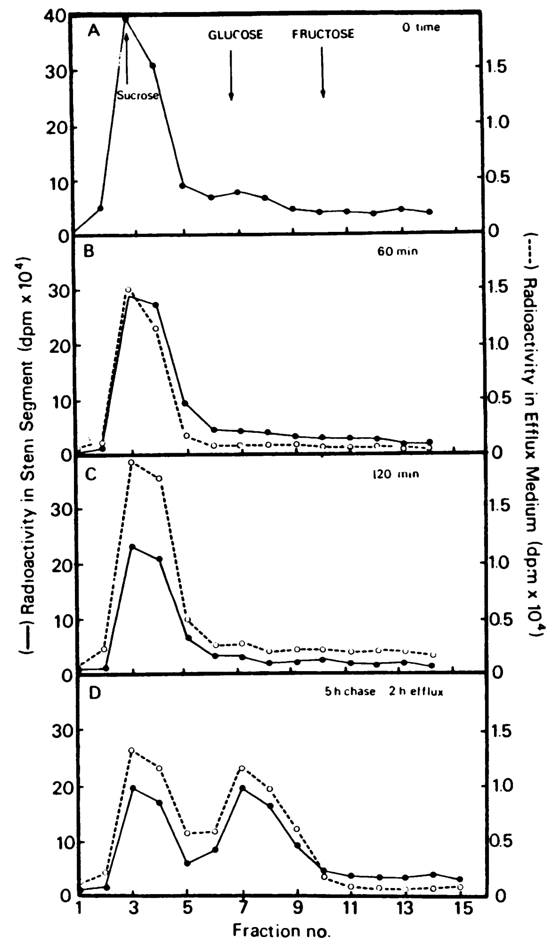

FIG. 1. HPLC analysis of ^{14}C labeled soluble sugars in the efflux media and in the stem segments. (A–C), Sugar analysis after 30 min pulse with $^{14}CO_2$ and 45-min chase followed by 0 (A), 60 (B), and 120 min (C) of efflux. (D), Sugar analysis after 30 min pulse, 5 h chase, and efflux for 2 h.

Carrier Involvement in Sucrose Unloading. To determine whether phloem unloading was mediated by a plasma membrane carrier, ^{14}C-labeled stem segments were subjected to treatment with 1 mM PCMBS. At this concentration, PCMBS has been shown to react as a nonpermeant sulfhydral reagent on the surface of the plasma membrane and to block sucrose uptake by leaf discs (2).

The efflux of ^{14}C-assimilates was slightly enhanced by 1 mM

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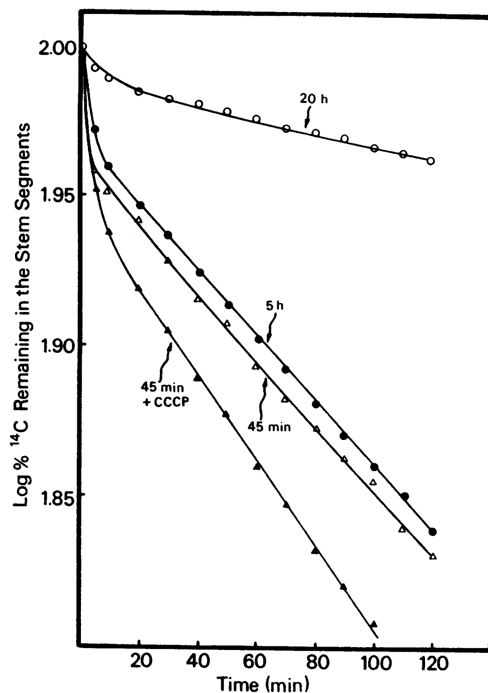

FIG. 2. Efflux of ^{14}C solutes from prelabeled *V. faba* stem segments. Efflux was measured following 30 min of pulse with $^{14}CO_2$ and chase periods for 45 min (△), 5 h (●), and 20 h (○). (▲), Efflux in the presence of 5 μM CCCP after $^{14}CO_2$ for 30 min and chase for 45 min.

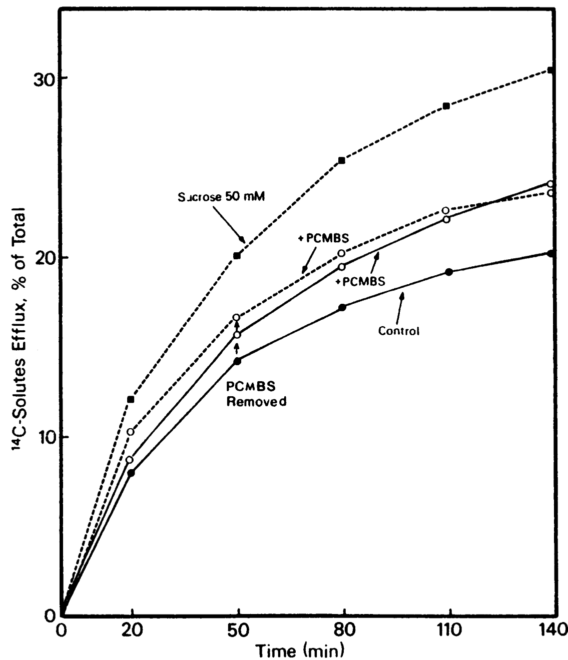

FIG. 3. The effect of PCMBS (1 mM) and sucrose (50 mM) on efflux of ^{14}C solutes from *V. faba* stem segments. (●——●), Efflux into buffer; (○——○), control + PCMBS; (■---■) + sucrose; (○---○), sucrose + PCMBS. Free PCMBS was removed from both the control and the sucrose treatments after 50 min of incubation (indicated by arrows).

PCMBS in the absence of sucrose. PCMBS inhibited sucrose enhanced efflux (Fig. 3). These effects of PCMBS were maintained even after PCMBS had been removed from the incubation media (after 50 min), as indicated by the similarity of the efflux curves. Incubation of the stem segments with the protonophore CCCP at a concentration of 5 μM substantially enhanced sucrose efflux (Fig. 2). DNP at 0.1 mM and NaCN at 1.0 mM had similar effects (not shown). These data suggest that sucrose efflux is through a sucrose carrier but is not active. CCCP at a concentration of 5 μM enhanced leakage of nonlabeled reducing sugars, total sugar, and amino acids from the bulk of the tissue (Fig. 4). Thus, these compounds enhanced the general permeability of the membrane. Conversely, PCMBS at a concentration of 1 mM inhibited leakage of nonlabeled reducing and total sugars from the stem sections but enhanced leakage of nonradioactive amino acids (Fig. 5).

Effect of Osmotic Concentration and Apoplastic Sucrose. Since assimilate flow from source to sink tissue is primarily a function of the turgor pressure gradient along the conductive elements, changes in the osmotic potential of the apoplast in the sink would affect this gradient. Therefore, changes in apoplastic solute concentrations may influence unloading and translocation rates. To examine this possibility, mannitol was used to manipulate apoplastic osmotic concentrations during the unloading assay. Efflux of [^{14}C]sucrose was reduced by increasing mannitol concentrations in the bathing media (Fig. 6). At 0.6 M mannitol, efflux was slightly enhanced over the rate observed at 0.4 M. However,

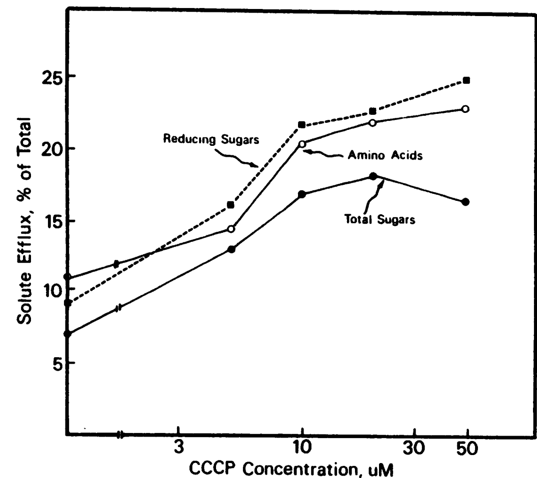

FIG. 4. Effect of CCCP on leakage of solutes from stem segments of *V. faba*. Total efflux time was 120 min. The tissue contained 73.2, 58.4, and 15 mol g^{-1} of total sugars, reducing sugars, and amino acids, respectively.

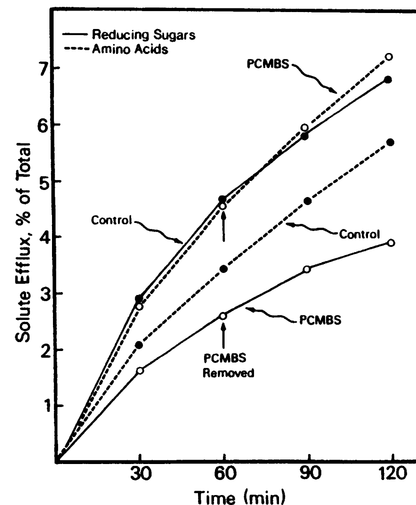

FIG. 5. Effect of PCMBS (1mM) on leakage of reducing sugars and amino acids from *V. faba* stem segments. The free PCMBS was removed after 60 min.

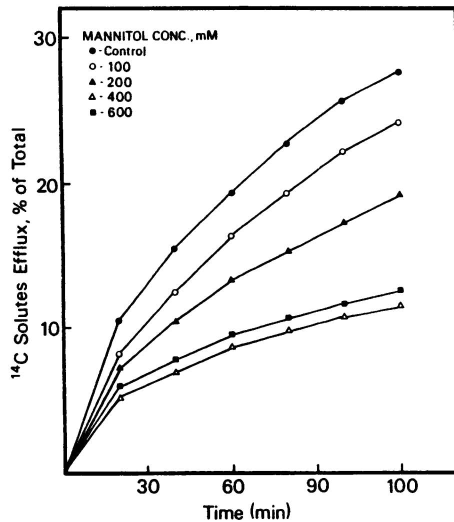

FIG. 6. Effect of various mannitol concentrations on efflux of ^{14}C solutes from *V. faba* stem segments.

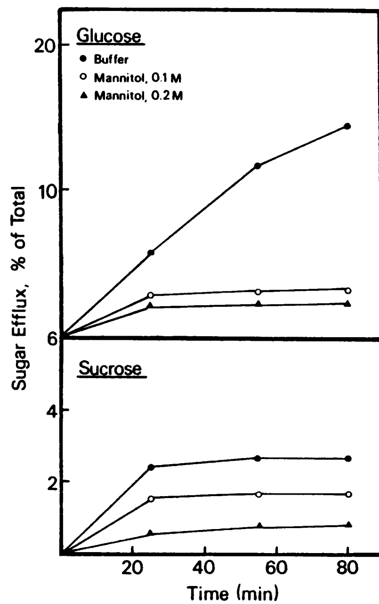

FIG. 7. Effect of mannitol on leakage of glucose and sucrose from stem segments of *V. faba*. The sugars were determined by HPLC chromatography. The tissue contained 42.3 and 17.5 mol g^{-1} of glucose and sucrose.

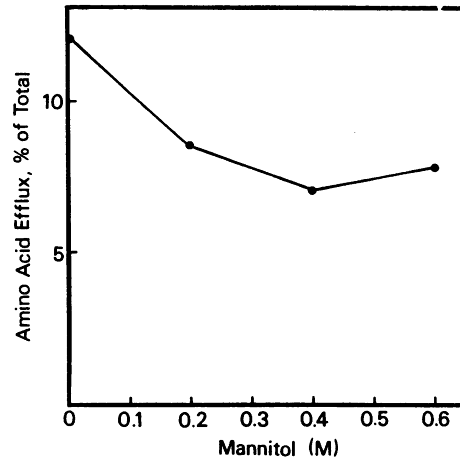

FIG. 8. Effect of mannitol on amino acid leakage from stem segments of *V. faba* plants. Total efflux time was 120 min. The initial amino acid content was 13.8 mol g^{-1}.

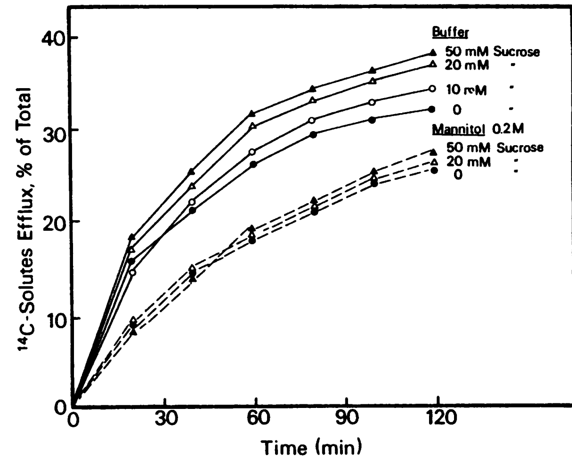

FIG. 9. Effects of increasing apoplastic sucrose concentration on efflux of ^{14}C solutes from stem segments of *V. faba* and its elimination by 0.2 M mannitol.

the effect of mannitol was not unique to [^{14}C]sucrose efflux. Increasing mannitol concentrations reduced the leakage rate of nonlabeled glucose, sucrose (Fig. 7), and amino acids (Fig. 8) similar to its inhibitory effect on efflux of [^{14}C]sucrose. These data suggest that changes in the osmotic concentration within the apoplast affects solute leakage from the bulk of the stem cells and does not specifically affect phloem unloading.

Increasing the sucrose concentration in the efflux medium to 50 mM enhanced the efflux of [^{14}C]sucrose (Fig. 9). The enhanced [^{14}C]sucrose efflux in 50 mM sucrose was reduced by 1 mM PCMBS (Fig. 3) and abolished in isotonic mannitol (Fig. 9). This is evidence for a turgor sensitive sucrose-sucrose exchange mechanism.

DISCUSSION

Stems of *Vicia faba* plants appear to be an appropriate system for studying phloem unloading, for the following reasons: (a) Anatomical simplicity—only 10 layers of parenchyma cells separate the phloem sieve elements from the central cavity into which the exported sugars are unloaded in our system. (b) Sucrose is the major sugar, if not the only one, which is transported and unloaded into the free space for 2 h following a $^{14}CO_2$ pulse (Fig. 1, A–C). (c) Cleavage of the unloaded sucrose, either by cell wall acid invertase or intracellular invertase, is not evident until several hours after unloading.

Wolswinkel and Ammerlaan (15) argued for high acid invertase activity in stems of *V. faba* with pH optima of 4.5 to 5.0. The pH of 5.5 maintained in our experiment may have minimized the 'risk' of sucrose cleavage during efflux. The origin of labeled glucose that appeared in the efflux medium after 5 h of chase is not known. If it resulted from sucrose cleavage by cell wall acid invertase, [^{14}C]glucose would probably appear in efflux experiments carried out after a 45-min chase. It is more likely that the free space [^{14}C]sucrose is taken up by the parenchyma cells and cleaved by cytosolic invertase. In that case, a lag period would be expected before a substantial amount of radioactivity could be detected in glucose, as was observed (Fig. 1D). The kinetic pattern of efflux of [^{14}C]labeled material (Fig. 2) indicates that 9% of the labeled assimilates leaked from the free space after 45 min and 5 h chase periods, and the rest was unloaded from the cytoplasmic compartment. After a 5-h chase, radioactive material is apparently unloaded from the cytoplasm of both

phloem and parenchyma cells. Following 20 h of chase, the cytoplasm unloads only a small fraction (approximately 3.7%); the rest is associated with ethanol insoluble material, presumably starch.

Effects of Inhibitors. Several recent studies, using seed coats of legumes, suggested that unloading into sink tissue is carrier mediated and active. This suggestion is based on the observation that PCMBS, CCCP, other metabolic inhibitors, and low temperature inhibited the release of transported ^{14}C-assimilates to the bathing medium (14, 17). In the present study, PCMBS as well as CCCP enhanced the release of [^{14}C]sucrose from the phloem at concentrations which have been reported to inhibit sucrose uptake by leaf discs (2) and sugar beet taproot (10). Both reagents could interfere with the intergrity of the phloem plasma membrane by modifying membrane proteins (PCMBS) or depleting the cells of energy needed to maintain intact membranes (CCCP). Alternatively, [^{14}C]sucrose efflux could be enhanced because [^{14}C]sucrose uptake by the parenchyma cells adjacent to the phloem sieve tubes is inhibited, allowing leakage of more radioactive solutes from the apoplast to the bathing medium. The former possibility is more likely since we did not find sucrose cleavage in short-term experiments, suggesting that there is negligible uptake by parenchyma cells. CCCP seems to affect phloem and parenchyma cell leakage in similar manner; namely, it enhances leakage of phloem-originated ^{14}C solutes and also increases leakage of sugars and amino acids from the parenchyma (Fig. 4). However, PCMBS exerted different effects on these tissues. PCMBS increased ^{14}C solute efflux from the phloem, but inhibited total sugar and reduced sugar efflux from the parenchyma cells and concomitantly enhanced amino acid efflux (Fig. 5). These data suggest that sugars unloaded from the phloem and leakage from parenchyma cells occur through different routes in the plasma membranes; unloading is enhanced and the leakage from parenchyma is blocked by PCMBS modification. In the seed coat of *Pisum sativum*, where sugars are unloaded by parenchymatous transfer cells, PCMBS inhibited unloading (17). In stem of bean plants, however, PCMBS enhanced release of sucrose to the bathing medium (15).

Effects of Osmotic Concentration. Sucrose uptake by the sink (17, 18) and source (1) tissue from the apoplast has been shown to be sensitive to the osmotic potential of the bathing medium. We showed that increasing the osmotic concentration of the outer medium by mannitol causes substantial reduction in ^{14}C-assimilate efflux. Such a reduction is not expected if water flow through the plasma membrane is limiting unloading but is consistent with the known increase in solute leakage at high turgor (3). We suggest that the plasma membrane is altered as mannitol concentration decreases from 0.5 M to 0, thus enhancing [^{14}C]sucrose unloading. This hypothesis is further supported by the fact that a decrease in mannitol concentration is associated with an increase in leakage of total amino acids and nonlabeled sugars (Figs. 7, 8), an indication that mannitol concentration affects membrane permeability. Our findings are not consistent with those of Wolswinkel and Ammerlaan (16) who showed that increasing mannitol concentrations from 0 to 350 mM in the empty seed coats of *P. sativum* enhanced ^{14}C efflux into the seed cavity. Further increasing of the mannitol concentration strongly reduced assimilate unloading. These dissimilar responses to osmotic changes indicate that there may be different pathways and mechanisms of unloading in stem and seed coat tissues. In seed coats of soybean, assimilates are unloaded through transfer parenchyma cells (11). These cells might be affected differently by metabolic inhibitors and the osmotic environment.

Effect of External Sucrose. Increasing the sucrose concentration in the apoplast to 50 mM in isoosmotic buffered solutions enhanced unloading of [^{14}C]sucrose (Fig. 9). This was undoubtedly due to enhancement of the exchange of radioactive sucrose in the phloem with apoplastic nonradioactive molecules by the putative sucrose carrier, presumably located on the phloem plasma membrane. The fact that PCMBS inhibits the exchange (Fig. 3) reinforces this proposition. In 0.2 M mannitol, the sucrose effect was abolished, suggesting that the exchange process is turgor dependent. An alternative explanation is that the nonlabeled sucrose competes with the unloaded radioactive sucrose at uptake sites on the adjacent parenchyma cells, therefore leaving more [^{14}C]sucrose to diffuse into the bathing medium as previously suggested (6). If so, the effect would not be abolished by mannitol. Due to the large 10 to 1 volume difference between the bathing media and that of the tissue the effect cannot be explained as isotope trapping.

In conclusion, we propose that phloem unloading in the *V. faba* stem operates via a passive leakage-type mechanism, one which is primarily dependent on plasma membrane permeability. The chemical nature of the unloading site on the plasma membrane of the sieve element is not known, but it appears to be sensitive to the osmotic concentration of the apoplast and may involve protein components sensitive to PCMBS.

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