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Release of Sucrose from *Vicia faba* L. Leaf Discs[1]

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ABSTRACT

The release of sucrose from leaf discs of *Vicia faba* L. to a bathing medium was studied for evidence of a relationship between this release and mesophyll export of photosynthate *in vivo*. Sucrose was released specifically over hexoses and represented over 85% of total photosynthate released. The sucrose appeared to be derived from the mesophyll tissue directly and release did not require concurrent photosynthesis. The data indicated two separate channels for sucrose release. The first was sensitive to inhibition by 1 millimolar *p*-chloromercuribenzenesulfonic acid and the second was promoted by lowering the Ca^{2+} concentration below 0.1 millimolar. Flow through both channels was about equal when tissue that had been actively photosynthesizing for several hours was used. The rate of release was not dependent on the extracellular pH, but was inhibited by 10 micromolar carbonylcyanide *p*-trifluromethoxyphenylhydrazone. Lowering the Ca^{2+} concentration below 0.1 millimolar or raising the K^+ concentration above 100 millimolar stimulated sucrose release. The stimulation by high K^+ was not reversed by adding Ca^{2+}. The data supported the postulate that Ca^{2+} removal or K^+ addition changed the permeability of the mesophyll plasma membrane to sucrose.

Central to an understanding of how plants utilize photosynthate is an understanding of the mechanism by which photosynthate is transferred from the mesophyll cell to the companion cell of the phloem. Two distinct pathways have been proposed for this transfer, symplastic and apoplastic transport. In symplastic transport, photosynthate remains in the symplasm and passes through the plasmodesmata into the phloem (2). Although this type of transport is important in several tissues, it is not the commonly accepted route for photosynthate transfer between mesophyll and phloem (9, 10). Instead, transfer of photosynthate probably involves the apoplastic pathway, in which release of photosynthate occurs across the mesophyll plasma membrane with subsequent uptake by the phloem companion cells.

Interest in the apoplastic route has promoted a considerable effort to characterize the process by which sucrose is loaded into the phloem (3, 7, 9–13, 29). Phloem loading of sucrose has been shown to be an energy dependent process that probably involves the cotransport of protons and sucrose into the phloem. Hence, the driving force for sucrose uptake was proposed to be a proton gradient established across the plasmalemma by a vectorial ATPase (12). The process of unloading sucrose from the mesophyll into the apoplast has received less attention than phloem loading.

Sucrose release from leaf discs or slices has been utilized to study sucrose unloading. Edelman and Schoolar (6) showed that iodoacetate promoted sucrose release from leaf slices of C_4 plants such as sugar cane. Sucrose release was dependent on continued photosynthesis. Hawker *et al.* (14) also demonstrated a sucrose exit from spinach, bean, and sugar beet leaves. The exit was specific for sucrose over reducing sugars and was inhibited by 10 mM $CaCl_2$. Photosynthate exit from sugar beets was also characterized by Doman and Geiger (5). In their study, increases in photosynthate release were potentiated by low concentrations of K^+ (15 mM) and correlated with an increased translocation of sucrose out of the leaf.

Sugar release from isolated leaf cells and protoplasts has also been studied (16, 17, 28). In the first study, Ca^{2+} was shown to inhibit flow of metabolites, including sucrose, out of isolated cotton leaf cells (28). In the second study, Huber and Moreland (16, 17) demonstrated that sugar release from tobacco and wheat protoplasts was an energy-dependent process where sucrose release was coupled to K^+ exit. However, data taken from isolated cells or protoplasts might not be representative of the *in vivo* state of the mesophyll. The palisade-mesophyll tissue may act as a single unit in transferring photosynthate to the phloem (26). The destruction of symplastic connections within the tissue during cell or protoplast isolation would dilute the ability of specialized transport cells within the mesophyll to release sugar.

Calcium has been implicated in controlling the release of metabolites from plant cells (22, 23, 26, 27). In fact, the removal of Ca^{2+} from the medium surrounding sections from various tissues has been shown to cause a rapid loss of K^+ from the tissue (13, 18, 20, 24, 30). Also, a release of glucose from germinating pollen, caused by the removal of Ca^{2+} from the bathing medium, has been noted (4), and Ca^{2+}, as well as Mn^{2+}, has been implicated in lowering sugar release from corn scutellum slices (8). Generally, the conclusion derived from experiments such as those listed above has been that Ca^{2+} is required for maintenance of the integrity of the plasma membrane. In a similar manner, Nieman and Willis (24) have shown that concentrations of monovalent ions above 0.1 M caused a loss in polysaccharides and protein from carrot root cells. Therefore, high concentrations of monovalent ions may also increase permeability of the plasma membrane.

In an effort to gain an understanding of how sucrose is exported from intact mesophyll, the release of sucrose from leaf discs to an artificial sink (the bathing medium) was investigated. Broad beans, (*Vicia faba* L.) were chosen as a tissue source because of the ease with which both leaf epidermises could be removed and because it has been used previously for both phloem uptake (3) and phloem unloading (31) experiments. The objectives of this investigation were to characterize the sucrose release from leaf discs, to determine the nature of the ionic regulation of this release, and to determine whether the sucrose release was directly from the mesophyll tissue or passed through the phloem first.

MATERIALS AND METHODS

Plant Material. *Vicia faba* plants were grown in the greenhouse until the fifth leaf was fully expanded (10–15 d). Leaves were used

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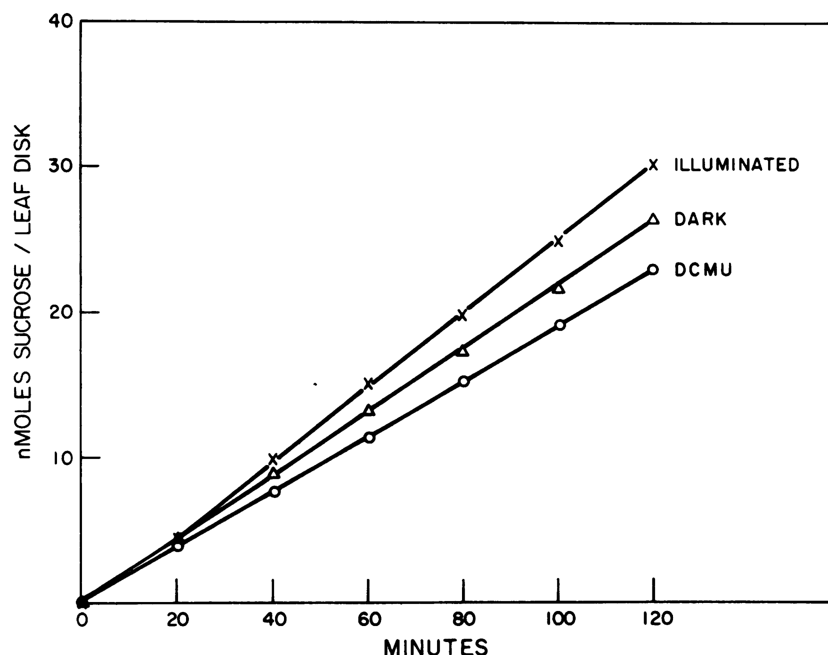

FIG. 1. Effect of photosynthesis on sucrose release from *V. faba* leaf discs. Sucrose was determined enzymically and leaf discs were incubated in 0.1 mM $CaCl_2$ in standard incubation medium (×——×), Incubated in the light; (△——△), incubated in the dark; (○——○), incubated in the light with 10 μM DCMU.

after plants had been in the light for 3 to 4 h. Following removal of the lower epidermis, leaf discs (5 mm in diameter) were cut from the fifth leaf. The leaf discs were placed in incubation medium (50 mM Hepes, pH 7.0; 1 mM NO_3^-; 50 mM K^+; and approximately 38 mM Cl^-) containing 100 μM $CaCl_2$ at 4°C. Leaf discs were vacuum infiltrated with standard bathing medium for approximately 3 min.

Assay of Sucrose Release. Five leaf discs (0.18 cm^2 each) were placed in 1.2 ml of incubation medium and incubated at 25°C. Vials containing samples were either incubated in the dark or in saturating illumination of about 200 $\mu E/cm^2 \cdot s$ provided by 75-w flood lamps. To illuminated samples, $KHCO_3$ was added at 7.5 mM. Aliquots of 0.08 ml were removed from the incubation mixture at various times and boiled for 1.0 min. Following cooling of the samples, each was assayed for sucrose content by a modification of the methods of Jones *et al.* (19) where one-half of the amounts of P-glucoisomerase and glucose-6-P dehydrogenase were used in the assay. All data were corrected for changes in the volume of the incubation medium caused by removal of samples. Data presented in figures were taken from actual experiments where treatments were run in duplicate and all experiments were reproduced at least three times.

$^{14}CO_2$ Fixation. Five leaf discs were incubated in 1.2 ml of incubation medium at 25°C. Radiolabel was added as 5.0 mM $NaH^{14}CO_3$ (0.3 μCi/μmol). Aliquots of 0.08 ml were removed from the incubation mixture, and the acid-stable radioactivity was determined in each. Representative samples were deionized as previously described (1). Also samples were treated with 1 unit/ml hexokinase, 2 mM MgATP, and 10 mM DTT for 15 min at 25°C prior to deionizing. The hexokinase treatment allowed quantitative removal of glucose and fructose from the fractions by the deionizing step. TLC of deionized fractions was done using cellulose plates and an elution solution consisting of butanol:benzene:pyridine:H_2O (5:1:3:3).

Sucrose Uptake. Sucrose uptake by leaf discs was determined by a modification of the methods of Delrot *et al.* (3). Five leaf discs were incubated in 1.2 ml of incubation medium that contained 20 mM radiolabeled sucrose (10 μCi/mmol [U-^{14}C]). After incubation in the dark at 25°C for 1 h, the tissue was rinsed in three changes of unlabeled incubation medium containing 20 mM sucrose for 3 min each. The leaf discs were then dried at 50°C and placed in 10 ml of scintillation fluid (7 ml toluene, 3 ml ethanol, 6 mg PPO, 2 mg POPOP) for 8 h to allow the Chl to dissolve. Samples were then counted and corrected to dpm by using a predetermined Chl quench curve. Similar results were obtained if the tissue was homogenized in and counted in Insta-Gel[2](Packard Instruments).

RESULTS

Release of Sucrose from Leaf Discs. Discs, prepared from mature leaves of *V. faba* L., released sugars to the bathing medium at rates that remained constant for at least 4 h (Fig. 1). The sugar released from the leaf discs was found to be primarily sucrose and not glucose or fructose. If aliquots of the bathing medium were assayed without the addition of invertase, the amount of sugar assayed was reduced by 95%.

To test the effect of peeling the leaves on sucrose release, leaves were peeled on the upper surface, lower surface, or not peeled at all. Leaf discs from all leaves still produced linear rates of sucrose release, but peeling produced about a 2-fold increase in rate of release. Peeling the leaf discs did not change the linearity of the exit rate, but did increase the reproducibility of the results obtained from different experiments. Leaves were peeled and situated so that no cut surfaces were in contact with the bathing medium. Again, sucrose release occurred with similar characteristics as when leaf discs were used. Finally, the rate of sucrose release from peeled leaf discs was dependent upon the total leaf area used in the assay, but independent of the actual size of the individual leaf discs over a range of 0.18 to 1.2 cm^2 (data not shown). Vacuum infiltrating leaf discs with the bathing medium increased the reproducibility of the results in a manner similar to peeling. These

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methods were used in the preparation of tissue for all experiments reported in this paper.

Illumination stimulated the rate of sucrose release by about 15% (Fig. 1) and the addition of 10 μM DCMU inhibited release in the light by 20 to 30% (Fig. 1) with no effect on release in the dark. Sucrose release apparently was not dependent upon concurrent photosynthesis. Therefore, the sucrose release must have come from a preexisting sucrose pool or from starch breakdown. If the plants were kept in the dark for several hours prior to use, the proportion of sucrose released that was light dependent increased (*i.e.* from 15% for control plants to 50% for plants maintained for 3 h in dark), indicating that photosynthesis was required to refill an export pool. In fact, if the plants were kept in the dark for 24 h prior to use, the rate of sucrose release in the dark was essentially zero. Pool sizes for representative light and dark periods are shown in Table I.

Table I. *Size of Carbohydrate Pools of V. faba Leaf Discs*

Treatment	Starch[a]	Soluble	Total
	nmol sucrose equivalents/leaf disc		
Light[b], 3 h	16.5	40.2	56.7
Light, 7 h	45.1	120.4	165.5
Dark[c], 4 h	11.3	24.9	36.1
Dark, 24 h	4	12.3	16.3

[a] Starch was assayed by method of Huber and Israel (15), and the soluble fraction was assayed by same method as used for sucrose (see "Materials and Methods").

[b] Time in light after 13-h dark period.

[c] Time in dark after a 3-h photoperiod.

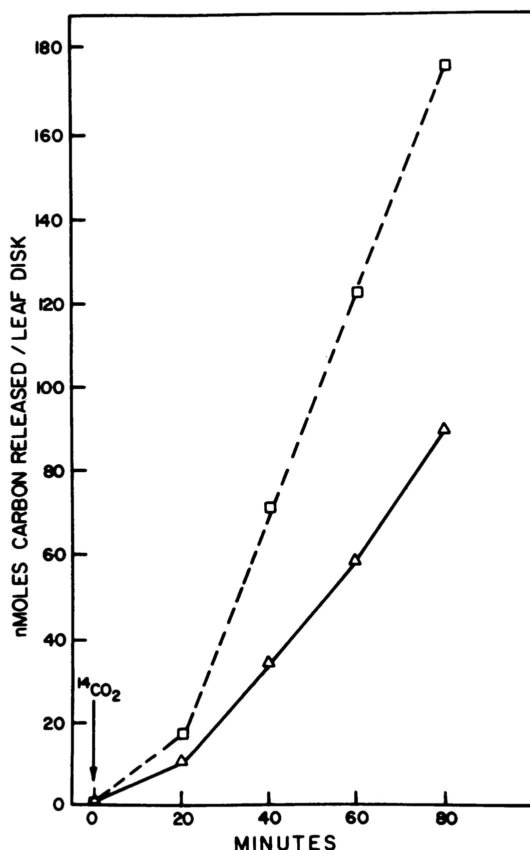

FIG. 2. Release of radiolabeled metabolites from *V. faba* leaf discs incubated in the light. The $KH^{14}CO_2$ was added at time zero. The Ca^{2+} and EGTA concentrations were adjusted to 0.1 mM at time 0, and aliquots were deionized by the method of Atkins and Canvin (1) prior to counting. (□- - -□), 0.1 mM EGTA; (△——△), 0.1 mM Ca^{2+}.

Sucrose release also was followed by monitoring the exit of acid-stable radiolabel into the bathing medium from leaf discs supplied with $^{14}CO_2$ (Fig. 2). Less than 15% of the radiolabel exiting from the leaf discs was charged (see "Materials and Methods"), and the neutral fraction was shown to be primarily sucrose and hexoses by TLC. Also, if the radiolabeled fractions were treated with a hexokinase mixture prior to deionizing, only an additional 3 to 4% of the radiolabel was removed. Therefore, greater than 80% of the radiolabel exiting the leaf discs was probably sucrose.

The release of radiolabel from leaf discs showed an increase in rate with time (Fig. 2). After 2 h of incorporation of $^{14}CO_2$, the proportion of released sucrose, measured enzymically, that was radiolabeled was as high as 35%. Release of radiolabel was similar to the release of sucrose measured enzymically including Ca^{2+} inhibition and sensitivity to inhibitors (see latter section).

Regulation of Sucrose Release by Ca^{2+}. The rate of sucrose release was increased about 2-fold by the chelation of Ca^{2+} ions with EGTA[3] (Figs. 2 and 3). The stimulation or inhibition of the rate of sucrose release, brought about by the addition of EGTA or Ca^{2+}, respectively, was fully reversible (Fig. 3). Therefore, the removal of Ca^{2+} probably did not cause irreversible damage to the tissue. Also, the removal of calcium ions did not alter the rate of photosynthesis in the leaf discs as monitored by the incorporation of $^{14}CO_2$ (data not shown). The proportion of radiolabel released that was sucrose did not change when the tissue was incubated in the presence or absence of Ca^{2+}. Also, removal of Ca^{2+} from the bathing medium did not cause an increase in the release of hexoses from leaf discs.

The concentration of Ca^{2+} required to reestablish the control rate of sucrose release, as opposed to the Ca^{2+}-free or -stimulated rate, was less than 0.1 mM with the transition from one rate to the other occurring in less than 30 μM Ca^{2+} (Fig. 4). Increasing the Ca^{2+} buffering capacity of the bathing medium with 100 μM EGTA instead of 10 μM had no appreciable effect on the results indicating that Ca^{2+} released from the plasma membrane or cell wall did not alter the free Ca^{2+} concentration. In all experiments reported in this paper, 0.1 mM $CaCl_2$ or 0.1 mM EGTA were used to designate plus and minus Ca^{2+}, respectively.

Inhibition of sucrose release did not specifically require Ca^{2+} because both Zn^{2+} and Mn^{2+} also inhibited release at similar concentrations to those required with Ca^{2+} (Table II). It was, however, impossible to exclude the possibility that both cell wall-bound and EGTA-bound Ca^{2+} were being released by the addition of Zn^{2+} and Mn^{2+}. In contrast to Zn^{2+} and Mn^{2+}, Mg^{2+} had no effect on sucrose release even at concentrations as high as 5 mM (Table II) and Ca^{2+} continued to return the rate to control levels even in the presence of 5 mM Mg^{2+}. Therefore, although Zn^{2+} and Mn^{2+} may be able to substitute for Ca^{2+}, the regulation of sucrose release was not simply a divalent ion effect.

Inhibition of the Release of Sucrose. The release of sucrose was inhibited by 1 mM PCMBS either in the dark (Fig. 5) or in the light. The inhibition was observed both when PCMBS was added during the assay (Fig. 5) or when leaf discs were pretreated with PCMBS for 10 min followed by washing with PCMBS-free medium. After a 40-min treatment with PCMBS, the rate of sucrose release was inhibited 95% in the presence of Ca^{2+} and only 57% in the absence of Ca^{2+} (Fig. 5).

The uncoupler FCCP (10 μM) also inhibited sucrose release either in the dark (Fig. 5) or in the light. Similar results were found with 3 mM DNP (Table III). In contrast to treatment with PCMBS, maximum inhibition was delayed by at least 20 min

[3] Abbreviations: EGTA, (ethylene bis[oxyethylenenitrilo])tetraacetic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol.

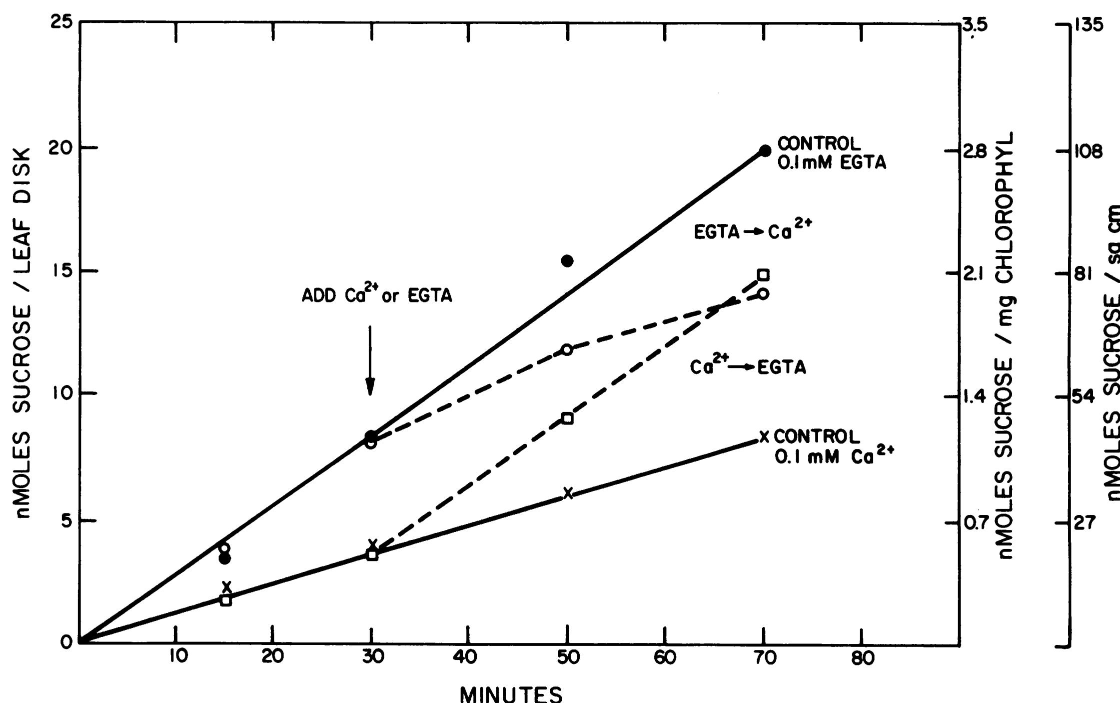

FIG. 3. Effects of EGTA and Ca^{2+} on the release of sucrose from illuminated *V. faba* leaf discs. (●——●), 0.1 mM EGTA; (×——×), 0.1 mM Ca^{2+}; (○- - -○), 0.2 mM $CaCl_2$ added at 30 min; (□- - -□), 0.2 mM EGTA added at 30 min.

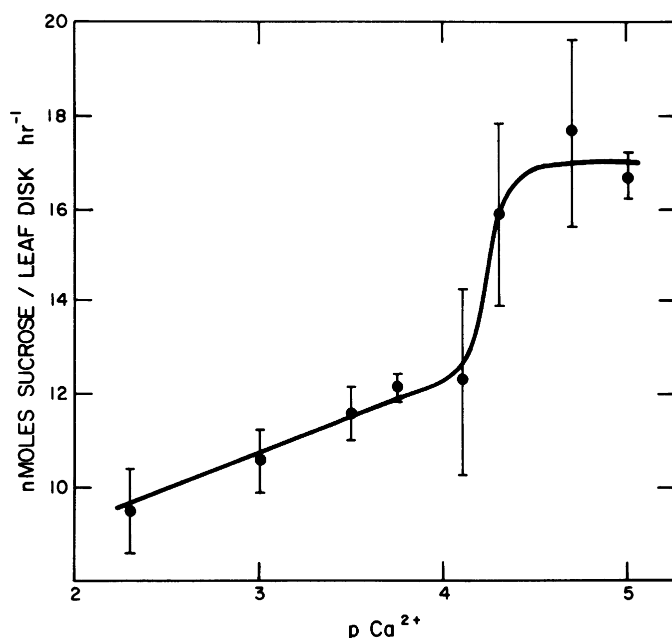

FIG. 4. Effect of exogenous $CaCl_2$ on release of sucrose from illuminated *V. faba* leaf discs. All treatments contained 10 μM EGTA. Free Ca^{2+} concentrations were calculated from the dissociation constant for EGTA and Ca^{2+} at pH 7.0. Standard deviation for three replications at each point represented by bars, and the line drawn represents an average fit of three separate experiments.

following addition of the uncoupler (FCCP shown in Fig. 5). The data suggested that uptake of the inhibitor might be delayed. However, O_2 evolution from leaf discs was inhibited in the light in less than 10 min and, therefore, the uptake of FCCP by the leaf discs was probably only partially responsible for the delayed inhibition. The sensitivity of sucrose release to inhibition by FCCP and DNP indicated that energy was required for sucrose transport out of the mesophyll or for refilling of the sucrose pool. However,

Table II. *Effect of Divalent Cations on the Rate of Sucrose Release from Illuminated V. faba Leaf Discs*

Addition	Free Ion Concn.[a]	Inhibition of Rate in 0.1 mM EGTA[b]
	mM	%
None		0
Ca^{2+}	0.1	45
Mg^{2+}	0.1	0
Mg^{2+}	5.0	0
Mg^{2+} + Ca^{2+}	5.0 + 0.1	44
Mn^{2+}	0.1	43
Zn^{2+}	0.1	35
Fe^{2+}	0.1	5

[a] All solutions except the first listed contained 0.1 mM EGTA.
[b] Rate in 0.1 mM EGTA was 40 nmol sucrose released/leaf disc·h.

the inability of valinomycin or gramicidin (1 μM) to inhibit sucrose release (data not shown) was difficult to reconcile with the data given above.

Release of $^{14}CO_2$-labeled photosynthate was inhibited by sucrose added to the bathing medium (Table IV). Sucrose concentrations 25 mM or above caused inhibition of release, but sucrose added at concentrations as high as 200 mM only inhibited release 64% (Table IV).

Sucrose release from *V. faba* leaf discs was insensitive to the addition of a wide range of compounds, whether they were added in the presence or absence of Ca^{2+}. Included in these compounds were plant hormones (naphthaleneacetic acid, BA, GA_3, at 10 μM and ABA at 0.1 mM), nucleotides (AMP, GMP, cAMP, cGMP, cCMP at 10 μM), amino acids (Asp, Asn, Glu, Gln, Arg, Ser, Pro, Lys, Ala at 1 mM), and other miscellaneous compounds (coumarin, 0.1 mM; acetylcholine, 10 μM; thio-D-glucose, 0.1 mM; and invertase, 30 units/ml). The data do not, however, preclude that any of the compounds tested might have long-term effects on sucrose release.

Comparison of Sucrose Release to Uptake. Sucrose uptake has previously been characterized for *V. faba* leaf discs (3). In agree-

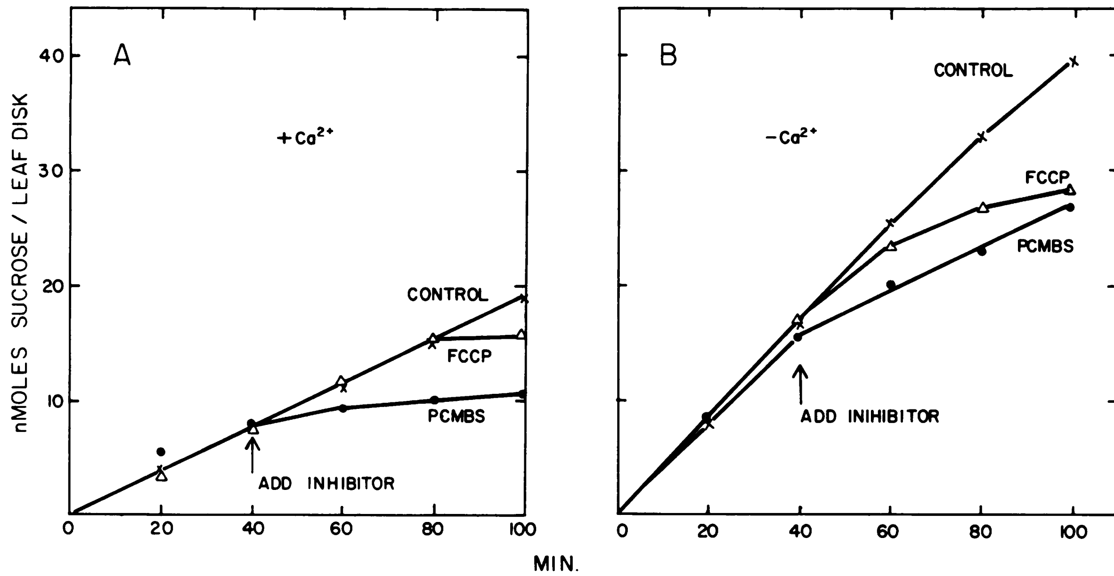

FIG. 5. Effect of inhibitors and Ca^{2+} on release of sucrose from *V. faba* leaf discs maintained in the dark. A, 0.1 mM Ca^{2+}; B, 0.1 mM EGTA; (×——×), control; (△——△), 10 μM FCCP; (●——●), 1 mM PCMBS. The inhibitors were added at 40 min.

Table III. *Effect of PCMBS and DNP on Sucrose Release and Uptake in V. faba Leaf Discs Maintained in the Dark*

Treatment[a]	Uptake[a]		Release	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
	% inhibition of control rate[b]			
None (control)	0	19	0	0
PCMBS, 1 mM	85	82	85	44
DNP, 3 mM	82	74	50	60

[a] Both the $CaCl_2$ and EGTA rate were compared to control in $CaCl_2$.
[b] Control rates were 34 nmol sucrose uptake/leaf disc·h and ($+Ca^{2+}$) 20 nmol sucrose released/leaf disc·h; ($-Ca^{2+}$) 38 nmol sucrose released/leaf disc·h.

Table IV. *Effect of Sucrose on Release of $^{14}CO_2$-Labeled Photosynthate from V. faba Leaf Discs*

Treatment	$+Ca^{2+}$	$-Ca^{2+}$
	% inhibition of control rate[a]	
None (control)	0	0
Sucrose, 1, 5, 10 mM	0	
Sucrose, 25 mM	31	
Sucrose, 50 mM	43	55
Sucrose, 100 mM	52	60
Sucrose, 200 mM	64	

[a] Rate was averaged for 120 min. Control rate was 40.8 nmol, or 122.4 nmol, of fixed carbon released/leaf disc·h into medium containing 0.1 mM $CaCl_2$ or 0.1 mM EGTA.

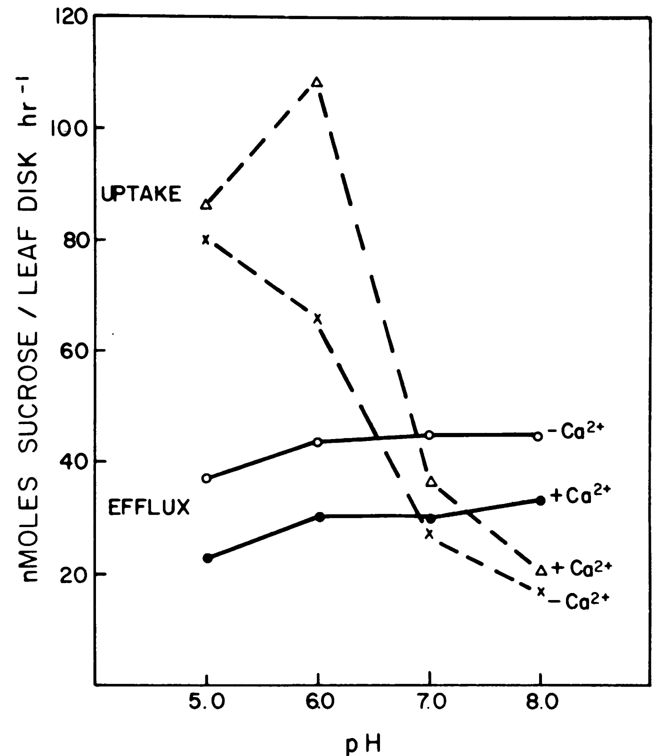

FIG. 6. Effect of exogenous pH on [U-^{14}C]sucrose uptake and on enzymically measured sucrose release by *V. faba* leaf discs incubated in the dark. The pH of the bathing medium was established with a mixture of 20 mM Mes and 20 mM Hepes. The K^+ concentration was held constant at 50 mM. (△- - -△), uptake in 0.1 mM Ca^{2+}; (×- - -×), uptake in 0.1 mM EGTA; (●——●), release in 0.1 mM Ca^{2+}; (○——○), release in 0.1 mM EGTA.

ment with these published results, 1 mM PCMBS or 3 mM DNP was found to inhibit sucrose uptake in *V. faba* leaf discs when the same incubation conditions were used as were used for release studies (Table III). However, DNP was not as effective on inhibition of release as it was on uptake (Table III). Also in agreement with published results (3, 10, 11), photosynthesis in leaf discs was not affected by PCMBS (data not shown). Sucrose uptake and release in the presence of Ca^{2+} were inhibited by PCMBS about equally (85%), but PCMBS did not inhibit release as strongly in the absence of Ca^{2+}.

The rate of sucrose uptake into the phloem has been shown to be strongly dependent on the pH of the surrounding medium (3, 10). A similar pH dependence for sucrose uptake was found using the incubation medium that was used for sucrose release (Fig. 6). In contrast to sucrose uptake, the rate of sucrose release was essentially independent of the pH of the bathing medium (Fig. 6). At pH 7.0, where most of the experiments on sucrose release were done, Ca^{2+} had less of an effect on sucrose uptake than on sucrose release (Fig. 6), and Ca^{2+} actually stimulated uptake while inhibiting release.

Effect of Monovalent Cations. The rate of sucrose release in the presence of Ca^{2+} was dependent upon the concentration of K^+ in the bathing medium (Fig. 7). In the absence of Ca^{2+} only 18 mM K^+ was required for the maximal rate of sucrose release, but in the presence of Ca^{2+}, 150 mM K^+ was required. A K^+ concentration of 50 mM was used in experiments reported in this paper. Increasing the Ca^{2+} concentration to 5 mM did not reverse the stimulation of sucrose release caused by 100 mM K^+ so that K^+ was probably not displacing Ca^{2+} from its binding sites. This stimulation of release by K^+ was probably not the result of changes in the osmolarity of the bathing medium, as concentrations of sorbitol at similar osmotic strengths to K^+ caused an inhibition rather than stimulation of sucrose release (Table V).

At a monovalent cation concentration of 50 mM, the rate of sucrose release from *V. faba* leaf discs was found to be slightly inhibited by substitution of Na^+ for K^+ in the medium (Fig. 8). This inhibition occurred either in the presence or absence of Ca^{2+} in the medium. At higher monovalent cation concentrations, Na^+ continued to cause about a 20% reduction in the rate of release over K^+, when the experiment was done in the presence of Ca^{2+} (data not shown).

Anions (SO_4^{2-}, Cl^-, NO_3^-, BO_4^{3-}, acetate, and PO_4^{3-}) added at 0.1 mM concentrations as the K^+ salts did not affect the rate of sucrose release either in the presence or absence of Ca^{2+}.

Sucrose Release from Species Other Than *V. faba*. Preliminary investigations have shown that sucrose release also occurs from leaf slices of plant species other than *V. faba*. Tissue slices from soybean (*Glycine max* [L.] Merr., *G. falcata* Benth.), peanut (*Arachis hypogea* L.), and asparagus (*Asparagus officinalis* L.) all show a linear rate of sucrose release (data not shown). However, the rates of sucrose release were always less than those observed with *V. faba* and the effect of Ca^{2+} was much less pronounced.

DISCUSSION

The data presented in this paper are supportive of data presented elsewhere (9–12) which suggest that photosynthetically derived sucrose is unloaded from the mesophyll into the apoplast prior to phloem loading. The sucrose release that occurred from leaf discs of *V. faba* possibly is a demonstration of this sucrose unloading, where the large volume of the bathing medium interrupts normal flow into the phloem. In support of this contention are results that show a high specificity for sucrose release over other sugars, linearity of release rate over long periods of time, PCMBS sensitivity of exit rate, and the release of photosynthetically derived sucrose.

There are, however, other possible sources for sucrose release into the bathing medium from leaf discs. Some of these are simple diffusion of sugars out of the intercellular spaces, leakage from cells ruptured or damaged by cutting or peeling, and leakage of sugars out of the phloem. The first of these sources, simple diffusion of sucrose from intercellular spaces, has been reported previously (21). However, the possibility that the sucrose release reported here resulted from simple diffusion is somewhat diminished by the linear rate of sucrose release and by the fact that photosynthetically radiolabeled sucrose can represent as high as 35% of the sucrose released from leaf discs.

As it was impossible to observe sucrose release without some disruption of the cuticle and, therefore, damage to some cells, the second pathway for sucrose release through damaged cells could not be excluded. However, such a pathway was difficult to reconcile with the specificity for sucrose release over hexoses. Leakage of cellular contents from damaged cells should be nonspecific and Ca^{2+} effects on this leakage should be nonreversible. The inhibition of sucrose release by PCMBS and FCCP is also difficult to reconcile with a cellular leakage of metabolites. If sucrose was

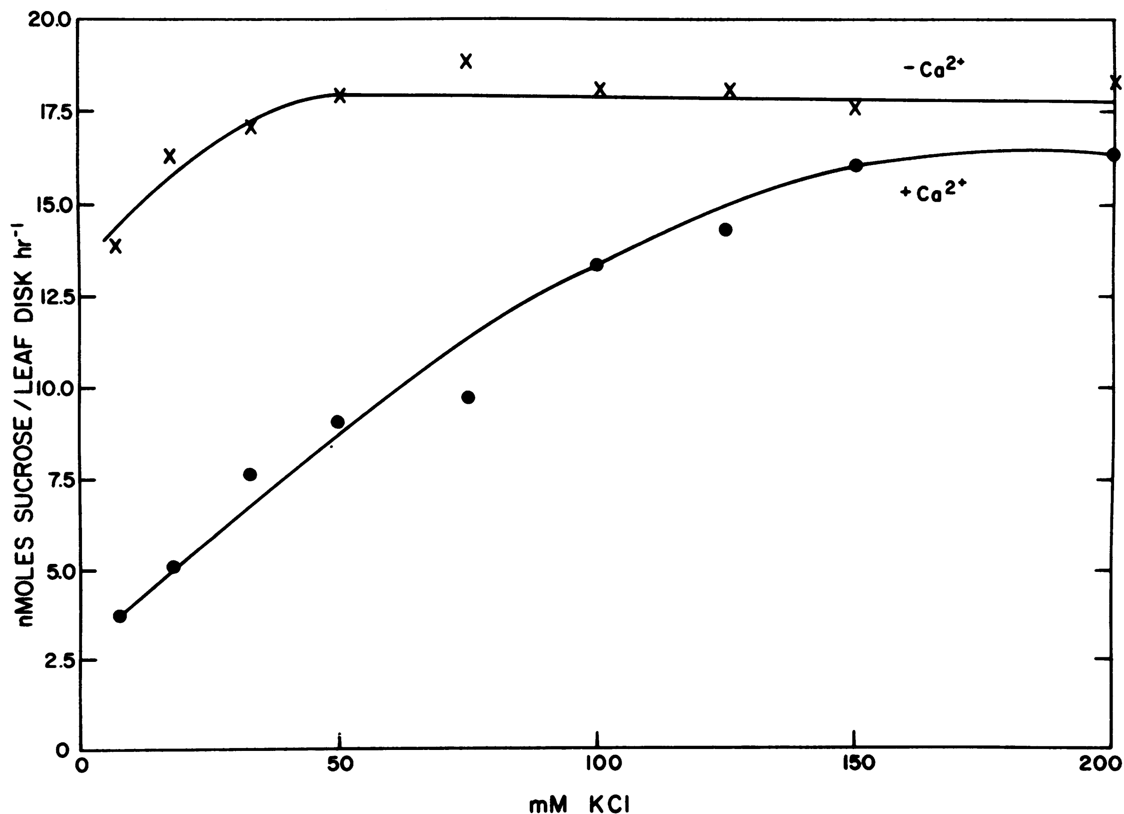

FIG. 7. Effect of exogenous KCl in the presence and absence of Ca^{2+} on the rate of sucrose release from illuminated *V. faba* leaf discs. (●——●), 0.1 mM Ca Cl_2; (×——×), 0.1 mM EGTA. The bathing medium consisted of 50 mM Hepes (Tris), pH 7.0; 7.5 mM $KHCO_3$; and the appropriate concentration of KCl. The minimum K^+ concentration was assumed to be the concentration of bicarbonate.

Table V. *Effect of Sorbitol on Sucrose Release in V. faba Leaf Discs Maintained in the Light*

Treatment	$+Ca^{2+}$	$-Ca^{2+}$
	nmol sucrose released/leaf disc·h	
None (control)	15.4	34.8
Sorbitol, 0.2 M	14.4	19.6
Sorbitol, 0.4 M	11.7	18.4

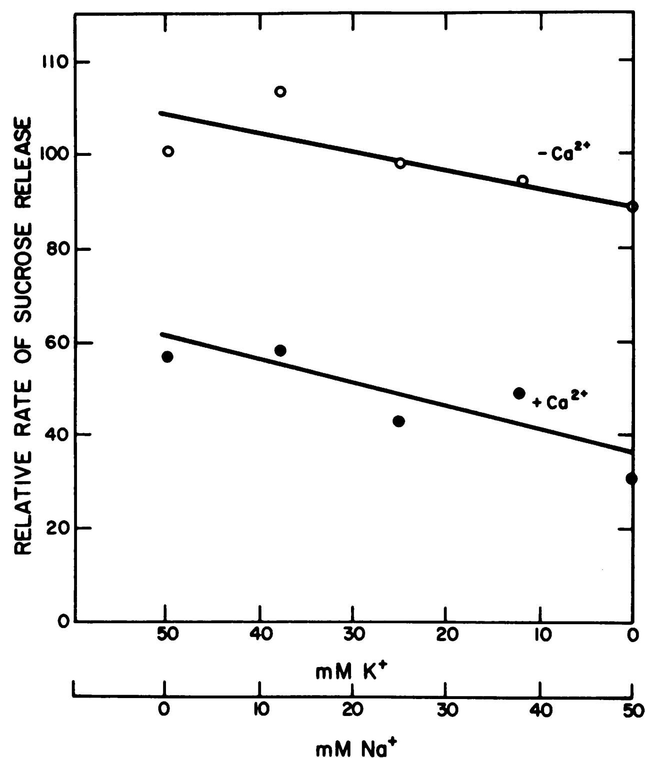

FIG. 8. Effect of the relative concentrations of Na^+ and K^+, in a 50 mM mixture of the ions, on the rate of sucrose release from illuminated *V. faba* leaf discs. (○——○), 0.1 mM EGTA; (●——●), 0.1 mM Ca^{2+}.

leaking out of damaged mesophyll cells, or cut phloem elements, then the number of such cells should change the rate of release. Sucrose release was, however, independent of how the tissue was prepared, *i.e.* either the method of peeling or cutting of leaf discs (or strips).

Finally, sucrose release could occur from the phloem by means of leakage across the phloem membrane. Such a route would provide an explanation for the stimulation of sucrose release by Ca^{2+} removal. However, photosynthetically formed sucrose would have to be transferred to the phloem concomitantly with release. Photosynthetically formed sucrose obviously does transfer to the phloem even in leaf discs suspended in a bathing medium (3, 5). Transfer to the phloem by an apoplastic route was minimized in this paper by use of a bathing medium buffered at pH 7.0 (Fig. 6). With *V. faba* leaf discs, 50% of the total soluble photosynthate, labeled with $^{14}CO_2$, is transferred to the bathing medium after 120 min. Therefore, passage of sucrose into the phloem by an apoplastic route prior to release is difficult to reconcile with the data presented above and with the pH independence of sucrose release from leaf discs. Sucrose uptake into the phloem shows a strong pH dependency (3, 11).

Transfer into the phloem possibly could be by a symplastic route. Such a symplastic route, however, should be insensitive to inhibition by PCMBS, a nonpenetrating, sulfhydryl-specific reagent (10). Both sucrose uptake and release are strongly inhibited by PCMBS (Table II). Giaquinta (10) also showed that PCMBS inhibited the transport of $^{14}CO^2$-labeled metabolites in whole leaves of sugar beets (*Beta vulgaris* L.) and, therefore, symplastic transport does not appear to fit available data. In a complex structure such as the leaf disc, it is difficult to define completely the path of sucrose release. The data presented in this paper, however, do support a direct passage of sucrose from the mesophyll into the bathing medium.

The addition of PCMBS to leaf discs caused a reduction in the steady-state rate of sucrose release. This inhibition was stronger in the presence of Ca^{2+} than in its absence (85% *versus* 44% for data averaged from five separate experiments). Hence, two separate pathways for sucrose release might be indicated. Only one pathway would operate in the presence of Ca^{2+} and both in its absence. The removal of Ca^{2+} from the bathing medium caused a 2-fold stimulation of the rate of sucrose release, and the addition of PCMBS caused a 44% reduction of this EGTA-stimulated rate. Therefore, the addition of PCMBS slowed the rate of release by an amount equal to the rate seen in Ca^{2+} (0.85×0.50). The data, therefore, suggest that the removal of Ca^{2+} from the bathing medium opens a second pathway which is not inhibited by PCMBS. Data from experiments where sucrose release was measured either by enzymic assay or by radiolabeling support two routes. One route is promoted by removal of Ca^{2+} but is insensitive to PCMBS treatment. This Ca^{2+}-sensitive pathway is probably generated by removal of the naturally high Ca^{2+} concentrations found in the cell walls of the mesophyll. Consequently, this pathway may not exist *in vivo*. The possibility remained, however, that removal of Ca^{2+} from specific regions of the cell wall, possibly near the phloem, might be used to regulate sucrose release by the mesophyll. The presence of a Ca^{2+}-sensitive leakage pathway for sucrose release strengthened the possibility that the second route, the Ca^{2+}-insensitive, PCMBS-sensitive pathway, was representative of *in vivo* sucrose unloading from the mesophyll.

Both routes for sucrose release were found to have the same specificity for sucrose. A similar specificity for sucrose over reducing sugars was noted previously for bean and sugar beet leaf segments (14). Although release in this system was specific for sucrose in the absence as well as presence of Ca^{2+}, the concentration of Ca^{2+} required to lower the rate of release was 100 times that required for *V. faba* leaf discs (0.1 mM).

The transition from an EGTA-stimulated rate to control rate of sucrose release occurred over a rather narrow range of Ca^{2+} concentrations. Hence, a specific site for Ca^{2+} binding rather than nonspecific Ca^{2+} binding to cell wall carbohydrates or plasma membrane phospholipids was indicated. Ca^{2+} was not bound as tightly as would be expected for a cytoplasmic binding site where Ca^{2+} concentrations are probably kept below 1 µM. The data, therefore, indicated that Ca^{2+} was being removed from a specific binding site, possibly a protein on the plasma membrane surface. This removal caused an increase in the sucrose permeability of the membrane.

The stimulation of sucrose release by calcium removal suggests a permeability change in the plasma membrane of the mesophyll cells and, therefore, supports a passive flow of sucrose out of a sucrose pool within the mesophyll (Table I). In support of the existence of a sucrose pool is the fact that sucrose release occurs at a rate almost as rapid in the dark as in the light. Dark sucrose release, however, is dependent on sufficient build-up of photosynthate prior to the experiment. The size of the sucrose pool may actually be small if sucrose is continually synthesized from the starch pool. Sucrose in the export pool appears to be exchangeable with external sucrose since release is inhibited by external sucrose concentrations above 25 mM. Also, 36% of the exit rate remains at 200 mM sucrose.

The counter argument to a passive sucrose release from the tissue comes from the sensitivity of the release to the metabolic inhibitors FCCP (Fig. 5) and DNP. This inhibition was, however,

delayed from the point of application of the inhibitor and possibly represents an inability to refill the export pool rather than a direct inhibition of transport. Although the data presented in this paper are insufficient to support one mechanism of sugar transport over another, a process of facilitated transport is not inconsistent with any of the data presented.

Monovalent ions also have been implicated in regulating sucrose release from mesophyll cells (5, 16, 17). Doman and Geiger (5) showed that low concentrations of K^+ (15 mM- caused an increase in sucrose release from sugar beet leaves but higher concentrations gave variable effects. This increase in release rate was correlated with an increase in transport to sink (5). Leaf discs from *V. faba* showed a similar stimulation of sucrose release with increasing concentrations of K^+. Sodium ions could only partially substitute (80%) for K^+ in stimulating sucrose release. In a manner similar to removal of Ca^{2+}, high concentrations of K^+ ions are probably not physiologically relevant *in vivo*, but rather change the permeability properties of the plasma membrane (24).

Huber and Moreland (16, 17) proposed that sucrose release from mesophyll protoplasts from wheat and tobacco was by a K^+-sugar symport and this symport was an energy dependent process. In contrast to their data, the rate of sucrose release was lowered by substituting Na^+ for K^+ in the bathing medium in *V. faba* leaf discs and was stimulated by low concentrations of external K^+. Also, sugar release from leaf discs was highly specific for sucrose over hexoses which was not the case for wheat and tobacco protoplasts (17). The question of whether the differences in the data are related to species differences or the result of protoplast isolation remains unanswered. Sucrose release from the mesophyll may occur only from specialized cells located adjacent to the phloem tissue so that sucrose flow within the mesophyll is symplastic and between mesophyll and phloem is apoplastic. Such a system would keep sugar concentrations in the free space of the leaf to a minimum.

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