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Involvement of Protons as a Substrate for the Sucrose Carrier during Phloem Loading in *Vicia faba* Leaves[1]

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ABSTRACT

The effect of pH on uptake of exogenous sucrose by broadbean (*Vicia faba* L.) leaf discs without the lower epidermis has been investigated at various sucrose concentrations. The concentration dependence of sucrose uptake showed a biphasic saturation response. At high sucrose concentrations (>20 millimolar), sucrose uptake showed no pH dependence. At low sugar concentrations (<5 millimolar), plots of $1/V$ against $1/H^+$ give straight lines which all intercept at the same point at the left of the ordinal axis. Calculations show that these data agree well with two-substrate kinetics for the carrier, the substrates being the protons and the sucrose molecules. Our results provide further evidence that protonation/deprotonation processes of the carrier are involved in phloem loading, especially for low sucrose concentrations of the apoplast.

In the past few years, the mechanism of sugar uptake has been extensively investigated with various plant materials: *Ricinus* cotyledons (15–17) or petioles (20), sugar beet leaf (9–14), and broadbean leaf (5, 6, 8). Komor (17) has provided evidence that a proton-sugar co-transport process is involved for sucrose uptake in castor bean cotyledons, although the tissue responsible for the observed phenomena cannot be precisely defined. Concerning the leaf tissues, where phloem loading normally has to operate in the plants, we have some indications that proton-sugar symport is involved. Giaquinta (13) showed that exogenous [^{14}C]sucrose uptake by sugar beet leaf tissue was stronger at an apoplast pH of 5.0 than at a pH of 8.0. Alkaline pH values increased the K_m of the carrier for sucrose, without affecting the V_m.[2] A proton-extruding activity has been described in leaf tissues and, on the basis of its sensitivity to effectors such as fusicoccin, K^+, Na^+, it has been attributed to the existence of a plasmalemma proton-pump (1, 6, 14). Autoradiographs of leaf discs incubated on ^{86}RbCl suggested this proton-pump to be more concentrated in the veins than in the surrounding mesophyll cells (6). However, although a proton-pumping ATPase is a necessary prerequisite for proton-substrate symport, its existence is not sufficient to prove such coupled transport. The carrier kinetic data reported here show that at low external sugar concentrations (1 to 5 mM), the carrier responsible for exogenous sucrose accumulation in *Vicia faba* leaf follows two-substrate kinetics, with the proton as one of the substrates. For high sucrose concentrations, another carrier is involved, which exhibits no pH dependence.

Our material was chosen because the lower epidermis can be easily stripped off. The floating of discs prepared in this way ensures a good contact between the tissue and the incubation solution. Moreover, this allows easy manipulations of the apoplast pH and suppresses the effects which would be due to a differential stomatal aperture. Geiger *et al.* (11, 23) and Giaquinta (13) have shown that leaf discs can be a valuable tool for studies on sucrose phloem loading when incubation times are kept short.

MATERIALS AND METHODS

Plant Material. Growth of seedlings and leaf disc preparation were performed as described (8).

Determination of Kinetic Parameters. After removal of the lower epidermis, the discs (12 mm diameter) were floated for 30 min on a buffered solution of 20 mM Mes (pH 5.0) containing 250 mM mannitol. To determine the kinetics of uptake, the discs then were transferred to incubation media containing sucrose concentrations from 1 to 150 mM. To avoid a loss in counting accuracy through too great a decrease of specific radioactivity, increasing amounts of [U-^{14}C]sucrose (3 to 12 μCi/10 ml) were added to the media. Mannitol (as the osmoticum) was added in varying amounts so that the total sugar (mannitol + sucrose) molarity was 250 mM. After 30-min incubation, the discs were rinsed in three changes of unlabeled solution for 2 min each, dry-ice frozen, lyophilized, and combusted to $^{14}CO_2$ with an Oxymat Intertechnique IN 4101 oxidiser. A 100-μl aliquot of the incubation medium was combusted under the same conditions in order to convert the value for the recovered radioactivity to 1 representing the amount of sucrose absorbed by the tissues. Similar rates of uptake were measured using either [U-^{14}C]- or [6,6′-(n)-^{3}H]sucrose. In the latter case, the label was recovered as ^3H_2O after combustion in the Oxymat apparatus. The [U-^{14}C]sucrose (403 mCi/mmol) and [6,6′-(n)-^{3}H]sucrose (2.1 Ci/mmol) were supplied by Amersham France (Versailles).

Effect of pH on Sucrose Uptake. Leaf discs without lower epidermis were floated for 30 min on a 250 mM mannitol solution containing various buffers: 10 mM citrate -20 mM Na-phosphate (pH 3.0, 3.5, 4.0, and 4.5), 20 mM Mes (pH 5.0, 5.5, 6.0, and 6.5), or 20 mM Tricine (pH 7.0, 7.5, 8.0, and 8.5). After preincubation, the discs were incubated for 30 min in the same buffered solution with 1 (set I) or 20 mM (set II) [^{14}C]sucrose. For set II, the mannitol concentration was 230 mM. After incubation, the tissues were rinsed and collected as described above.

Effect of pH on Kinetic Parameters of Sucrose Uptake. The results of the experiments described above led us to select five pH values for studying the effect of proton concentration on the kinetics of sucrose uptake at low and high sucrose concentrations. Thus, exogenous sugar uptake was studied at pH 4.0, 5.5, 7.0, 7.5, and 8.0 with 1, 1.5, 2, 3, and 5 mM sucrose (set I), respectively, or 20, 40, 60, 80, 100 mM sucrose (set II), respectively. In experiment I, besides the buffer, all media contained 250 mM mannitol. In experiment II, mannitol was added to keep the over-all sugar concentration at 250 mM. These experiments were performed in

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[2] Abbreviations: V_m, maximal velocity; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

the same way as described above, except that the label in the incubation medium was 5 μCi [^{3}H]sucrose.

Computation of Data. The data concerning system 1 have been computed with a program (21) which carries out a weighted linear and nonlinear least-squares regression analysis using the Lineweaver-Burk and Michaelis-Menten equations, respectively.

RESULTS AND DISCUSSION

Evidence for Two Exogenous Sucrose Uptake Systems. Preliminary experiments showed that the uptake of sucrose was linear with time during the first 30 min incubation. This duration of uptake was chosen for all subsequent experiments because it gave a good account of the initial uptake velocity. Figure 1 shows the concentration dependence of sucrose uptake and the effect of the proton-permeating compound CCCP (50 μM) which was the most powerful inhibitor among many others tested (2,4-dinitrophenol, *N*-ethylmaleimide, *p*-chloromercuribenzenesulfonic acid; diethylstilbestrol, and dicyclohexylcarbodiimide). The data have been reported in Figure 2 using Lineweaver-Burk and Eadie-Hofstee plots. Our results are best explained by the involvement of two saturable carriers operating in addition to a simple diffusive pathway. Indeed, phase 1 (well apparent from 1 to 3 mM sucrose, Fig. 2) and phase 2 (well apparent from 5 to 50 mM, Fig. 2) are both sensitive to CCCP (Fig. 1). However, the inhibition exerted by CCCP (50 μM) varied from 80% with 1 mM sucrose to 60% with 100 mM sucrose (Fig. 1). This inhibition was not greater when CCCP concentration was increased to 0.1 mM. Efflux experiments have been performed with discs loaded for 30 min on a 1 or 100 mM [^{3}H]sucrose solution and exodiffused for 30 min after the usual rinsing process (2 min, three times). During this 30-min efflux, the discs loaded with 1 mM sucrose lost 10 to 15% of the label still present after the three 2-min washes, whereas the discs loaded with 100 mM sucrose lost 35% of this label. This result suggests that the CCCP-insensitive component of uptake can be considered as a diffusive (effluxible) one. Above 75 mM sucrose, the second carrier (system 2) is nearly saturated, whereas the passive component of uptake increases linearly with increasing sucrose concentrations (Fig. 1). Therefore, the third phase, which has the characteristics of a mainly diffusive component, clearly appears above 75 mM, using either double-reciprocal or Eadie-Hofstee plots (Fig. 2). Particularly, this phase parallels the vertical axis, using Eadie-Hofstee plots. Such a diffusive component of sucrose uptake has already been reported for castor bean cotyledons (19).

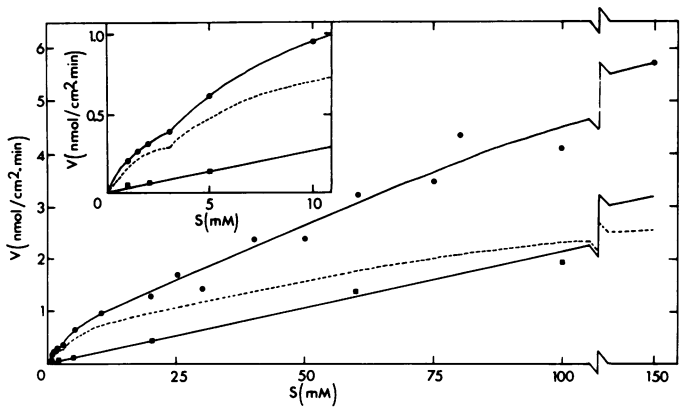

FIG. 1. Concentration dependence of sucrose uptake by broadbean leaf discs at pH 5.0. This figure summarizes the results of five separate experiments. (●), control; (■), 50 μM CCCP. CCCP was present in the preincubation and incubation media. (- - -),: "active" uptake = total uptake minus CCCP-insensitive uptake. For 1 mM sucrose, the data are the mean of 30 triplicates; for 2 and 5 mM mean of 25 triplicates; for 1.5, 3, 10, 20, 50, 75, and 100 mM, mean of 10 triplicates; for 25, 30, 40, 60, 80, and 150 mM, mean of five triplicates.

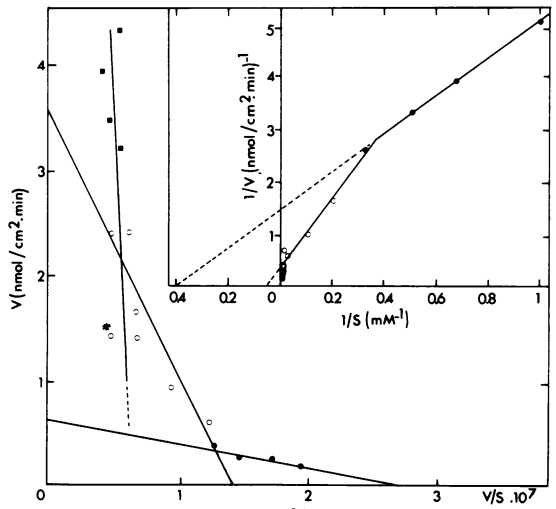

FIG. 2. Eadie-Hofstee (left) and Lineweaver-Burk (inset) plots of sucrose uptake. The different symbols identify the concentration ranges used for the regression analysis (data from Fig. 1). (*), not considered.

The apparent kinetic parameters deduced from Figure 2 are K_{m1} = 2.70 mM, V_{m1} = 0.71 nmol cm^{-2} min^{-1}, and K_{m2} = 25.8 mM, Vm_2 = 3.6 nmol cm^{-2} min^{-1}. After subtracting the passive component of uptake (CCCP-insensitive, Fig. 1) from the total amount of sugar absorbed, an inflexion point still appears for 3 mM sucrose (Fig. 3). This indicates that phase 2 does not simply result from the combination of phases 1 and 3. After this subtraction, the values for K_{m1} and K_{m2} are 2.66 and 35.3 mM, respectively, whereas V_{m1} and V_{m2} are lowered to 0.56 and 3.12 nmol cm^{-2} min^{-1}, respectively. The occurrence of two carriers has been reported for sucrose uptake in a number of materials (4, 13, 23, 24) and also for sucrose export from soybean leaves (22). In terms of our data, phases 1 and 2 could result from multiphasic kinetics, with a single system which changes its properties, both K_m and V_m, in response to substrate concentration. Another possibility is that a high affinity and a low-affinity system function simultaneously. In this case, the values given above for K_{m1} and V_{m1} would be slightly overestimated, whereas the values given for K_{m2} and V_{m2} would be underestimated. Our data cannot distinguish between these two possibilities. For all following results, the passive component shown in Figure 1 has been subtracted from the total uptake measured.

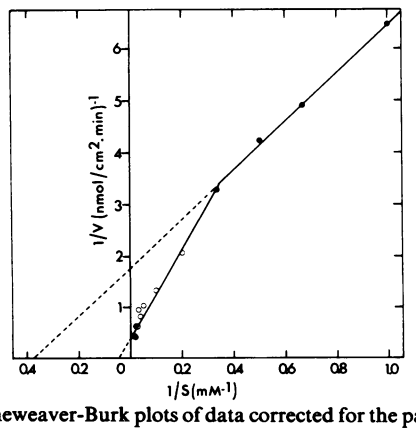

FIG. 3. Lineweaver-Burk plots of data corrected for the passive (CCCP-insensitive) component of uptake shown in Figure 1. An inflexion point still appears at 3 mM sucrose, showing that two saturable carriers are involved in the active uptake of sucrose.

Effects of pH on Sucrose Accumulation. The maximal accumulation rate from a 1 mM [U-^{14}C]sucrose solution was observed for acidic pH values, whereas sugar accumulation from a 20 mM sucrose solution exhibits no pH dependence (Fig. 4). No difference could be noticed between the rates of uptake from a 1-mM sucrose solution in citrate/phosphate or Mes buffers (pH 5.5), or between the rates of uptake in morpholinopropanesulfonic acid, Hepes, or Tricine buffers (pH 7.5). Discs which are preloaded for 30 min on 1 mM [^{3}H]sucrose and effluxed for 30 min at pH 5.0 or 8.0 lose the same amount of label during this efflux. Thus, the decrease in radioactivity which appears at alkaline pH values for discs floating on 1 mM labeled sucrose is not due to a buffer effect nor to the effect of pH on sucrose efflux. As the over-all time for preincubation and incubation was relatively short (about 1 h), we assume that the variations of uptake velocity can be attributed to the effect of pH on the uptake process itself rather than on the metabolic activity of the tissues. Also, similar results have been reported for the uptake of sucrose in sugar-beet leaves (13) and of the nonmetabolized sugar 3-*O*-methylglucopyranoside by *Pelargonium zonale* leaf discs (3). The accumulation of 3-*O*-methylglucopyranoside was inhibited by increasing pH values when the external concentration was low (10^{-4}–10^{-6} M), whereas no effect of pH could be detected with high external concentration (10^{-1}–10^{-2} M). Figure 5 shows that sucrose uptake is mainly confined to the minor veins network. Decreasing the pH of the incubation medium from pH 8.0 to pH 5.5 increases both phloem loading and sugar uptake by mesophyll cells. Under our experimental conditions, the clearest pictures of loading were observed at pH 3.5 or 4.0.

Effects of pH on Kinetic Parameters of Sucrose Uptake. Plots of $1/V$ against 1/S (sucrose) for low sucrose concentrations in the external medium show that the K_m of system 1 is increased at alkaline pH values, whereas the V_m is not affected (Fig. 6). This result is in good agreement with those previously published (13, 18). In Figure 6, the values of uptake at 5 mM sucrose have not been considered for the regression analysis because system 2 is clearly involved at this concentration (see Figs. 1–3 and inset of Fig. 6). For high sucrose concentrations (20 to 100 mM), no consistent variations of K_m and V_m could be detected.

Assuming a proton-sucrose symport mechanism for phloem loading (12), the proton can be considered as a substrate for the carrier to the same extent as sucrose. Thus, plots of $1/V$ against $1/H^+$ should give straight lines, as already shown (13, 18). Figure 7A shows this to be the case for sucrose concentrations between 1 and 5 mM. Moreover, all the lines intercept at the same point. The K_m for protons at 1 mM sucrose is approximately 10 mM, a value similar to that found by Giaquinta for sugar beet leaves (13). With high sucrose concentrations, no correlation between pH and uptake velocity could be detected (Fig. 7B). Figure 7A is typical for two-substrate kinetics with the proton and the sucrose molecules as the substrates. Alternatively, the data of Figure 7A could be considered as linear competitive inhibition by OH^- because a plot of $1/H^+$ is equivalent to a plot of $OH^-/10^{-14}$. Two-substrate

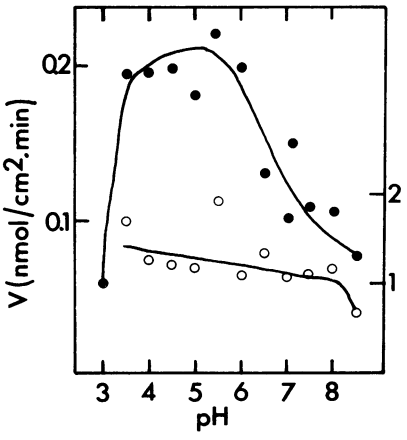

FIG. 4. Effects of pH on the rate of sucrose uptake from a 1 mM (●, left ordinal axis) or a 20 mM (○, right ordinal axis) sucrose solution. Each point is the mean of five triplicates.

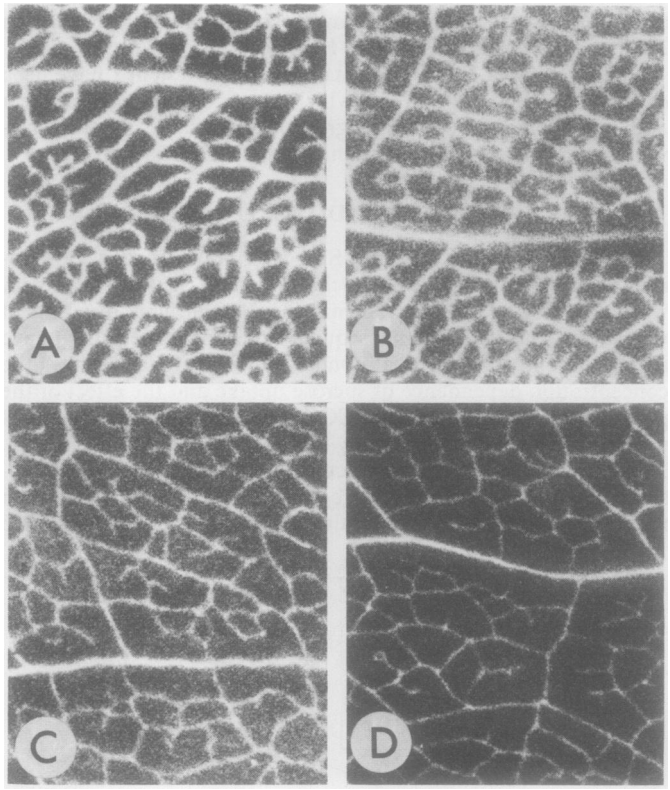

FIG. 5. Autoradiographs from leaf discs incubated for 30 min on a 1 mM [U-^{14}C]sucrose solution at pH 3.5 (A), 5.5 (B), 6.0 (C), and (8.0) (D). The lyophilized tissues have been exposed to Kodirex films for 7 days.

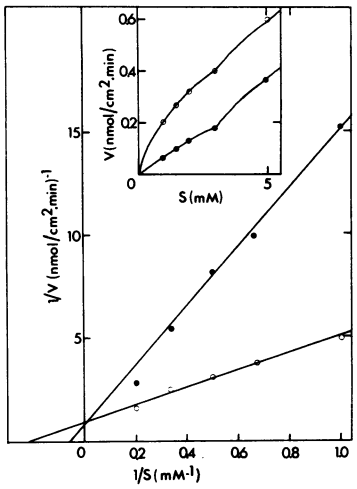

FIG. 6. Reaction kinetics of sucrose uptake at different pH values. Reciprocal initial velocity *versus* reciprocal sucrose concentration at pH 5.5 (○) or pH 8.0 (●). At pH 5.5, K_m = 4.65 mM, V_m = 1.23 nmol cm^{-2} min^{-1}. At pH 8.0, K_m = 18.2 mM, V_m = 1.31 nmol cm^{-2} min^{-1}. The points at 5 mM have not been considered for the regression analysis because the system 2 is involved in the uptake (see inset and Figs. 1–3).

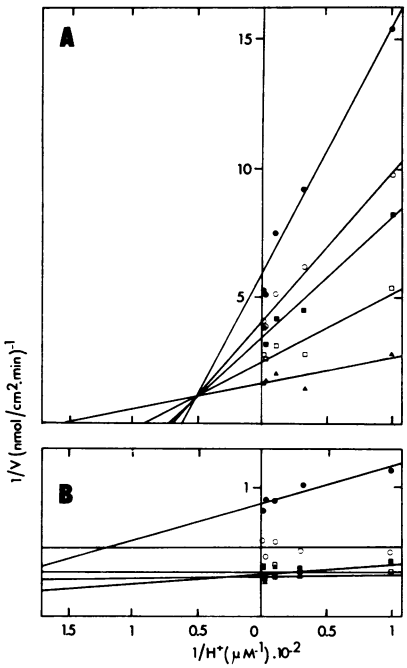

FIG. 7. Reaction kinetics of sucrose uptake at different pH values. Reciprocal initial velocity *versus* reciprocal proton concentrations. A, low sucrose concentrations: (●), 1 mM; (○), 1.5 mM; (■), 2 mM; (□), 3 mM; (▲), 5 mM. B, high sucrose concentrations: (●), 20 mM; (○), 40 mM; (■), 60 mM; (□), 80 mM; (▲), 100 mM. In A, the correlation between $1/V$ and $1/H^+$ was statistically significative for the five sucrose concentrations (t test). In B, the correlation was poorly significative for 20 mM sucrose, and there was no correlation for the other concentrations. Each point is the mean of five triplicates. This experiment was repeated another time with similar results.

kinetics are expressed as:

$$V = \frac{V_m(A)(B)}{K_A K_B' + K_B'(A) + K_A'(B) + (A)(B)} \quad [1]$$

with $K_A' = (EB)(A)/(EAB)$, $K_A = (E)(A)/(EA)$, $K_B' = (EA)(B)/(EAB)$ where A and B can be either a proton or a sucrose molecule (21). In the following discussion, the constants K will be termed K_H or K_S, depending on the substrate considered.

Assuming A to be the proton, Figure 7A would be a plot of $1/V$ against $1/A$ with the lines intersecting at a single point given by (21):

$$1/A = -1/K_H \quad \text{and} \quad 1/V = 1/V_m(1 - K_H'/K_H) \quad [2]$$

K_S' and V_m can be determined by a plot of the ordinal intercepts $1/V$ of Figure 7A against 1/sucrose. K_H' then can be determined from equation 2. The calculations give: K_H = 18.9 nM, K_H' = 1.1nM, K_S' = 4.55 mM, and V_m = 0.97 mol cm^{-2} min^{-1}. Good agreement was found between the experimental velocities and the velocities calculated with these values.

Supposing that A was sucrose in equation 1, Figure 7A would be a plot of $1/V$ against $1/B$ with all the lines intersecting at (21):

$$1/B = K_S'/K_H'K_S \quad \text{and} \quad 1/V = 1/V_m(1 - K_S'/K_S) \quad [3]$$

Following the same procedure as reported above gives: K_S' = 4.55 mM, K_S = 79.50 mM, K_H' = 1.1 nM, and V_m = 0.97 nmol cm^{-2} min^{-1}. The uptake activities computed with these parameters are very close to the ones calculated by the previous method and to the experimental points. The theoretical intercept in Figure 6 in the case of two substrate kinetics is $1/S = -0.02$ mM and $1/V$ = 1.05 $nmol^{-1}cm^2min$. This value is very close to the value derived from the data in Figure 6.

Taken together, our results confirm and extend previous findings from Giaquinta (13). Our data show that, at low external sucrose concentrations, phloem loading can be explained by two-substrate kinetics in which the binding of one substrate (proton or sucrose) favors the binding of the other one. On the external (mesophyll) side of the membrane, all carrier molecules are under the protonated form because, in our material, the pH of the apoplast is regulated in the 4.5 to 5.0 range (S. Delrot, unpublished data). In the case of two-substrate kinetics, the abscissal intercept of $1/V$ against $1/H^+$ cannot be strictly taken as $-1/K_H'$ but as $-K_S/K_H'K_S'$. Thus, the unloaded carrier is approximately half-protonated at pH 7.7, whereas the loaded carrier is approximately half-protonated at pH 9.0 and the pH 8.0 value given by Giaquinta (13) for half-protonation probably refers to the unloaded carrier. As previously mentioned (13), the value of pH 9.0 is close to the pK of thiol groups (about 10). In our material, the carrier possesses sulfhydryl groups on the external side of the membrane, whereas the ATPase does not possess such groups in this position (8). It is tempting to suggest that the conformational changes induced by the protonation/deprotonation of the carrier are mediated by sulfhydryl groups. This can be considered as only tentative because others groups have pK values near pH 9.0, for example the α-NH_3^+ of lysine and arginine (pK = 9.0). Because of the acidic pH of the apoplast, the limiting step of the loading process should be the release of the sucrose molecule on the internal side of the membrane. The rate of release will depend both on the pH of the phloem symplast and on the local sucrose concentration of the phloem. The ATPase activity detected in the plasmalemma of the transfer cells (2) could be responsible for the maintenance of the phloem symplast pH at values which are likely alkaline because the pH of the phloem sap is in the 7.5 to 8.5 range (25).

Therefore, the affinity of the carrier for sucrose is probably decreased at the internal side of the membrane, due to the alkaline pH. The question now arises as to what is the sucrose concentration of the apoplast *in vivo* and to test if the protons are really translocated with the carrier through the membrane. Preliminary work showing that this is indeed the case (7) will be developed in a subsequent paper. The conformational changes induced by protonation/deprotonation are merely dependent on the transmembrane pH gradient, but this does not exclude the possibility that part of the loading process is powered by the electrical component of the protonmotive force.

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