Plant Physiol. (1994) 106: 991-998

Kinetics Analysis of the Plasma Membrane Sucrose-H^+ Symporter from Sugar Beet (*Beta vulgaris* L.) Leaves[1]

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The kinetics behavior of the H^+-sucrose (Suc) symporter was investigated in plasma membrane vesicles from sugar beet (*Beta vulgaris* L.) leaves by analyzing the effect of external and internal pH (pH_o and pH_i, respectively) on Suc uptake. The apparent K_m for Suc uptake increased 18-fold as the pH_o increased from 5.5 to 7.5. Over this same pH_o range, the apparent V_{max} for Suc uptake remained constant. The effects of pH_i in the presence or absence of internal Suc were exclusively restricted to changes in V_{max}. Thus, proton concentration on the inside of the membrane vesicles ($[H^+]_i$) behaved as a noncompetitive inhibitor of Suc uptake. The K_m for the proton concentration on the outside of the membrane vesicles was estimated to be pH 6.3, which would indicate that at physiological apoplastic pH Suc transport might be sensitive to changes in pH_o. On the other hand, the $[H^+]_i$ for half-maximal inhibition of Suc uptake was approximately pH 5.4, making regulation of Suc transport through changes in $[H^+]_i$ unlikely. These results were interpreted in the framework of the kinetics models for co-transport systems developed by D. Sanders, U.-P. Hansen, D. Gradmann, and C. L. Slayman (J Membr Biol [1984] 77: 123–152). Based on their analysis, the behavior of the Suc symporter with respect to the $[H^+]_i$ is interpreted as an ordered binding mechanism by which the binding of Suc on the apoplastic side of the membrane and its release on the symplastic side precedes that of H^+ (i.e. a first-on, first-off model).

Suc transport across the PM is catalyzed by an H^+-Suc symporter (Buckhout, 1989; Bush, 1989; Lemoine and Delrot, 1989; Williams et al., 1990). Transport is driven by the proton motive force established across the PM by the H^+-ATPase in vivo and is electrogenic (Bush, 1990; Slone and Buckhout, 1991). The movement of H^+ and Suc is tightly coupled with a stoichiometry of 1:1 (Slone and Buckhout, 1991), and the carrier is specific for the Suc molecule, although phenylglucosides are also recognized by the carrier (Hecht et al., 1992). Finally, a gene encoding the H^+-Suc symport protein was recently identified in spinach leaf cells (Riesmeier et al., 1992). In the plant, this transporter is responsible for phloem loading and, thus, photoassimilate export from leaves in many plant species (Giaquinta, 1983; Bush, 1993; Riesmeier et al., 1993).

The membrane vesicle system offers the advantage for the kinetics analysis of transport processes that the driver ion and solute concentration on both sides of the vesicle membrane can be varied, and it has the further advantage that effects of tissue thickness and substrate access are eliminated (Ehwald et al., 1979). Determination of the kinetics parameters of a transport process can provide predictive insight into the behavior of the transporter in vivo. For example, if the apparent K_m for $[H^+]_o$ of the H^+-Suc symporter is approximately equal to pH_o, then changes in pH_o could significantly affect the rate of Suc uptake. The same would hold true for $K_{0.5}$ for inhibition of uptake by $[H^+]_i$. Thus, a decrease in pH_o might significantly inhibit Suc uptake, as is the case for H^+-dependent Cl^- uptake in *Chara* (Sanders and Hansen, 1981).

On the other hand, a single carrier might be involved in influx and efflux depending on the local concentrations of the ligands and the relative sensitivity of the carrier to changes in ligand concentration. Such macroscopic reversibility is found, for example, in the Na^+-HCO_3^- symporter in glial cells. This symporter catalyzes the movement of Na^+ and HCO_3^- with inward transport being stimulated by increasing concentration of HCO_3^- outside of the membrane vesicles or by membrane depolarization, but transport being reversed by acidification of the extracellular space. Changes in pH_o of less than 0.5 pH unit in the range of 7.2 are large enough to influence the direction of bicarbonate transport. Because of its sensitivity to changes in pH, the Na^+-HCO_3^- symporter may be involved in the regulation of extracellular pH (Munsch and Deitmer, 1994).

A prerequisite for evaluating the potential affects of pH and Suc concentration changes on the rate of Suc transport is a knowledge of their concentration in vivo and knowledge of the kinetics parameters associated with transport. Determination of the kinetics parameters for Suc transport is the subject of this report. The concentrations of Suc and H^+ in the symplast and apoplast have been determined in several cases. The cytoplasmic pH in plant cells ranges between 7.0 and 7.5 (reviewed by Kurkdjian and Guern, 1989), and changes in pH of approximately 0.5 pH unit have been observed under anaerobic conditions (Roberts et al., 1984), with light to dark transitions (Kurkdjian and Guern, 1989), following extracellular acidification (Felle, 1988) and with changes in temperature (Aducci et al., 1982). Changes of 1.5

[1] This project was supported by a grant from the Deutsche Forschungsgemeinschaft.

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Abbreviations: Btp, bis-Tris propane; $\Delta\psi$, membrane potential difference; $[H^+]_{i/o}$, proton concentration on the inside/outside of the membrane vesicles; $K_{0.5}$, concentration for half-maximal effect; $pH_{i/o}$, pH on the inside/outside of the membrane vesicle; PM, plasma membrane; $[S]_{i/o}$, substrate concentration on the inside/outside of the membrane; TPP^+, tetraphenylphosphonium; zero-trans, $[Suc]_i = 0$.

pH units or more have been observed following exposure to acidic gases such as SO_2 or NO_2 (Pfanz et al., 1987), although acidification of this magnitude is likely responsible for the tissue damage associated with exposure to low concentrations of these gases. Values for the apoplastic pH vary widely between 5.0 and 6.5 with extreme values of 4.0 and 7.0 having been reported (reviewed by Grignon and Sentenac, 1991).

The concentration of Suc in the leaf symplast and apoplast is strongly dependent on the metabolic state and the cell type. Fondy and Geiger (1977) estimated the concentration of Suc in the sugar beet (*Beta vulgaris* L.) leaf apoplast to be approximately 70 μM; the concentration in the metabolic space was approximately 100 times this value. These values were averages based on the surface area of the leaf and did not reflect cellular differences. Thus, the cytoplasmic Suc concentration in the mesophyll cells is in the low millimolar range and in the companion cell-sieve element complex it is between 0.4 and 0.8 M (Giaquinta, 1983). Factors regulating Suc uptake are largely unknown, although possible regulation of the Suc carrier activity by the sink-to-source transition has been suggested (e.g. Lemoine et al., 1992).

In this report, we describe a series of investigations in which we determined the effects of a systematic variation of $[H^+]_i$, $[H^+]_o$, $[Suc]_i$, and $[Suc]_o$ on apparent K_m and V_{max} for Suc uptake under the restricted conditions of saturating $\Delta\psi$ and in some cases saturating $[H^+]_o$ and zero-trans Suc ($[Suc]_i = 0$). The results of this investigation provide insight into the predicted behavior of the carrier with respect to these parameters in vivo as well as providing insight into the reaction mechanism of the symporter.

MATERIALS AND METHODS

Plant Material and Isolation of PM

Sugar beets (*Beta vulgaris* L. cv Tina EE; Kleinwanzlebener Saatzucht, Göttingen, Germany) were grown in hydroponic culture as described by Bush (1989). Fully expanded, basal leaves from 56- to 168-d-old plants were harvested 1 to 3 h after the beginning of the light period, and PM vesicles were isolated by aqueous two-phase partitioning (Buckhout, 1989) from 160 g fresh weight of sugar beet leaves with the midrib removed. Seventy-four-gram phase systems were used. Following phase separation, the PM fraction was diluted 5-fold in buffer containing 0.1 mM Hepes-Btp (pH 7.5), 200 mM sorbitol, 50 mM K_2SO_4, 1 mM Suc where indicated, and 1 mM DTT (dilution buffer) and pelleted (50,000*g* for 30 min, DuPont Sorvall SS34 rotor). The vesicles were resuspended in dilution buffer containing 10 mM Hepes-Btp at a pH and Suc concentration as required. This suspension was incubated at 10°C for 30 min and then concentrated by centrifugation as above. Next, the pellets were resuspended in the appropriate buffer at an approximate protein concentration of 10 mg mL^{-1} and frozen at −70°C until needed. Protein concentration was determined by the method of Bradford (1976).

ΔpH- and Δψ-Dependent Suc Uptake

Suc uptake was determined by the filtration method as described by Buckhout (1989). ΔpH- and Δψ-dependent Suc uptake was initiated by a 200-fold dilution of the PM fraction into 500 μL or, in some cases, 1 mL of transport buffer (10 mM Hepes-Btp, pH 5.5, or as indicated, 200 mM sorbitol, 50 mM Na_2SO_4, and 1 mM DTT) in the presence or absence of 1 μM valinomycin. Energy-dependent Suc uptake was defined as the difference in uptake between vesicles diluted into transport buffer at pH 5.5 in the presence of valinomycin and vesicles diluted into transport buffer at pH 7.5 or as indicated with 50 mM K_2SO_4 in place of Na_2SO_4 and without valinomycin. The Suc incorporation was determined at 0.5, 1, 2, and 4 min, and the uptake rate was determined from these data by linear regression analysis. Each series of experiments reported here was conducted on a minimum of three different PM preparations (two preparations in the case of the data shown in Table II and Figs. 1 and 7), and, unless otherwise stated, the data are reported as means of those experiments. Uptake of acetate and TPP^+ into membrane vesicles was conducted as described by Tubbe and Buckhout (1992). The internal volume used for calculating internal concentrations was taken to be 4 μL mg^{-1} protein as was previously determined (Tubbe and Buckhout, 1992).

Materials

The sources for chemicals were as previously stated (Buckhout, 1989; Slone and Buckhout, 1991; Hecht et al., 1992).

RESULTS

Conditions for Saturating Δψ

Investigation of the behavior of Suc uptake in response to variations in internal and external driver ion (H^+) and ligand (Suc) concentration can be simplified if certain experimental conditions are imposed in the test system. Two such conditions are zero-trans ligand concentration and saturating membrane potential. In the membrane vesicle system, the condition of zero-trans ligand concentration can readily be achieved for Suc and approached for H^+ by simply equilibrating vesicles in solutions of the desired composition (see below). Conditions for saturating Δψ were empirically determined. Δψ was generated as a K^+ diffusion potential by equilibrating PM vesicles in 100 mM K^+ and diluting them into K^+-free medium in the presence of valinomycin. The ability of the procedure to generate a negative Δψ relative to the external medium and the ability of Δψ to drive sugar uptake have been demonstrated (Fig. 1A; Bush, 1990; Tubbe and Buckhout, 1992).

To establish the conditions under which Δψ was saturating, potential driven uptake was measured as a function of protein concentration. Since the final protein concentration was achieved by varying the volume of added membranes and the K^+ concentration difference, and concomitantly the theoretical Δψ decreased with increasing volume of PMs added, the expected result was confirmed by TPP^+ uptake. The observed accumulation of TPP^+/mg protein was greatest at the highest dilution of the PM suspension in the assay buffer (Fig. 1A). As the dilution factor was decreased, so was the accumulation of TPP^+. Δψ-dependent Suc uptake increased linearly with added membrane protein, and with Δψ, up to an added volume of 5 μL in a total volume of 500 μL (Fig.

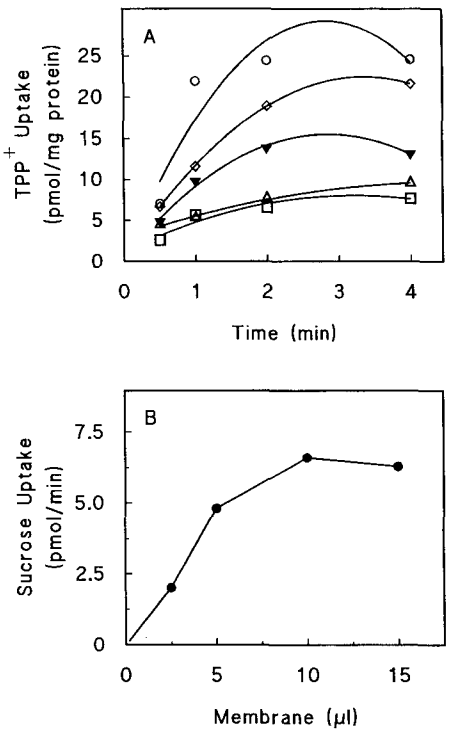

Figure 1. The $\Delta\psi$-dependent Suc and TTP^+ uptake in PM vesicles. $\Delta\psi$ was generated as a K^+ diffusion potential by equilibrating PM vesicles in 100 mM K^+ at pH 7.0 and diluting them into K^+-free medium (pH 7.0) in the presence of valinomycin. The $[K^+]_o$ and the theoretical $\Delta\psi$ were, thus, dependent on the volume of PM fraction added. A, TPP^+ uptake was determined by diluting PM vesicles into 1 mL of assay buffer. The dilutions shown are: 1000- (○), 400- (◇), 200- (▼), 133- (△), and 100-fold (□). B, $\Delta\psi$-dependent Suc uptake experiments were conducted by diluting the given volume of PM vesicles in 500 μL. For further details, see "Materials and Methods" and Tubbe and Buckhout (1992).

1B), thus indicating that uptake activity at a dilution of 100-fold was independent of $\Delta\psi$. In vivo transport was also found to be independent of the membrane potential, indicating that co-transport is likely saturated with respect to $\Delta\psi$ (Hansen and Slayman, 1978; Felle, 1981). The results reported here are also supported by the finding of Schwab and Komor (1978) that $\Delta\psi$ of less than −30 mV was sufficient to saturate H^+-dependent 6-deoxyglucose uptake in *Chlorella*. To ensure conditions of saturating membrane potential, all subsequent $\Delta\psi$-dependent Suc uptake experiments were conducted at a dilution of 200-fold or greater.

Effect of $[H^+]_o$ on Suc Uptake

The influence of $[H^+]_o$ on the rate of Suc uptake was investigated under conditions of saturating $\Delta\psi$ to determine the conditions necessary for saturating $[H^+]_o$ and to determine the effects of $[H^+]_o$ on the behavior of the H^+-Suc transporter. As has been previously shown (Buckhout, 1989), the rate of Suc uptake increased in a simple hyperbolic manner with respect to increasing Suc concentrations (Fig. 2A). Increasing the pH_o from 5.5 to 7.5 resulted in a decrease in Suc uptake by approximately 80% over the range of Suc concentrations tested (Fig. 2A; Bush, 1990). Transforming these data by the method of Hanes-Woolf resulted in a series of parallel lines (Fig. 2B). Therefore, the effect of increasing pH_o on Suc uptake was an increase in the apparent K_m for Suc with little or no effect on V_{max}. The values for the apparent K_m and V_{max} at various pH_o are summarized in Table I.

The effect of pH_o was analyzed further by comparing the rate of Suc uptake as a function of $[H^+]_o$ at fixed external Suc concentrations ranging from 0.05 to 1 mM (Fig. 3A). The analysis resulted again in a series of hyperbolic curves; however, the Hanes-Woolf transformation of these data resulted in intersecting lines, which indicated that an increase in the external Suc concentration decreased the apparent K_m for $[H^+]_o$ and increased the V_{max} (Fig. 3B). Again, the apparent values for K_m and V_{max} for the various $[Suc]_o$ concentrations are given in Table I. The differential effect of $[Suc]_o$ and

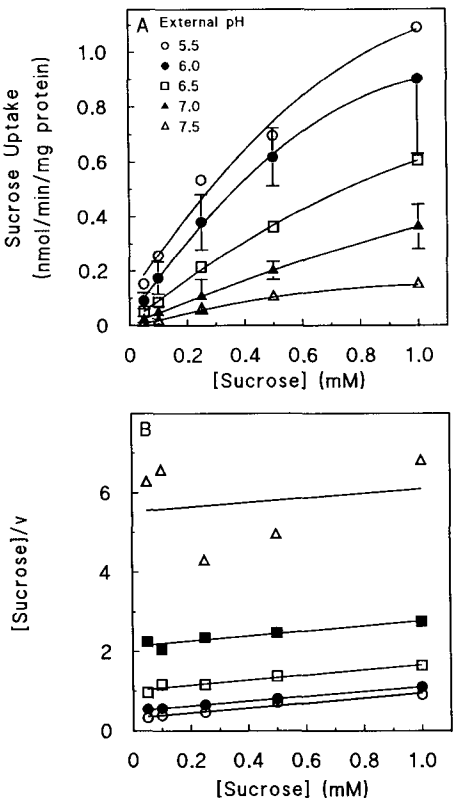

Figure 2. Effect of pH_o on the rate of Suc uptake at varying $[Suc]_o$. A, Suc uptake was determined in PM vesicles equilibrated at pH 7.5 in the presence of 100 mM K^+ and diluted in buffer at the pH indicated and containing 100 mM Na^+ and 1 μM valinomycin. The rate of uptake was determined as a function of pH_o and $[Suc]_o$. Bars represent SE and are shown only for pH_o of 6.0 and 7.0 for reasons of clarity of illustration. The SEs illustrated in this figure are indicative of the average SE in all experiments in this report. B, The data in A were replotted by the Hanes-Woolf method. With this method, the slope is equal to V_{max}^{-1}, and the x intercept is equal to $-K_m$. Values shown are averages of four determinations. The apparent K_m and V_{max} values were determined by linear regression analysis and are reported in Table I.

Table I. *Summary of the kinetics constants obtained from the analyses in Figures 2 and 3.*

Values were determined by linear regression analysis of the uptake data transformed by the Hanes-Woolf method. The inherently low activity at pH 7.5 (Fig. 2A) resulted in a significant error within single experiments and produced variability greater than 50% among the replicate experiments. This error was particularly evident when these data were transformed (Fig. 2B, Δ). Therefore, the constants derived from these data have been enclosed in parentheses to draw attention to this fact.

pH_o	Sucrose	$K_{m[H^+]}$	$K_{m[sucrose]}$	V_{max}
	mM	μM	mM	nmol min^{-1} mg^{-1} protein
5.5			0.52	1.60
6.0			0.87	1.69
6.5			1.57	1.54
7.0			3.41	1.59
7.5			(9.61)	(1.74)
	0.05	0.86		0.19
	0.10	0.86		0.32
	0.25	0.48		0.61
	0.50	0.35		0.76
	1.00	0.23		1.18

$[H^+]_o$ on the Suc transport process indicated that Suc and H^+ interacted with the symporter on the external surface of the membrane in an ordered manner. The relative insensitivity of V_{max} to Suc uptake with respect to pH_o has been previously shown for Suc uptake into leaf segments of *Vicia faba* L. (Delrot and Bonnemain, 1981) and *B. vulgaris* L. (Giaquinta, 1977). The apparent K_m for H^+ of 0.01 μM at 1 mM Suc calculated in those reports is significantly lower than the value of 0.23 μM reported here (Table I). It seems possible, however, that in intact tissues the equilibration of the bulk pH with the cell wall pH may not be complete. In fact, the cell wall pH has been shown to be significantly lower than the bulk pH (Grignon and Sentenac, 1991). Thus, a more acid cell wall pH with respect to the bulk pH would result in an underestimation of the half-maximal H^+ concentration for Suc uptake.

The condition of saturating $[H^+]_o$ was taken to mean that an increase in $[H^+]_o$ resulted in no further change in the Suc uptake rate. Saturation was confirmed by analysis of the data shown in Table I. A comparison of the apparent K_m for Suc with the inverse of the $[H^+]_o$ results in a linear function ($r >$ 0.99; Fig. 4). The limiting K_m for Suc at relatively large $[H^+]_o$ was equal to 0.55 mM, a value that was approximately reached at pH 5.5 (Table I). Thus, at pH_o of 5.5 and below, both V_{max} and K_m for Suc uptake were insensitive to changes in pH_o.

Effect of $[H^+]_i$ on Suc Uptake at Saturating $[H^+]_o$ and $\Delta\psi$

Of interest to the general kinetics behavior of the H^+-Suc symporter is the effect of pH_i on the rate of Suc uptake. If, for example, the rate of uptake is sufficiently sensitive to changes in pH_i at or about the cytoplasmic pH of approximately 7.2, then changes in pH_i would affect the rate of Suc uptake. An analysis of the effects of $[H^+]_i$ on Suc transport

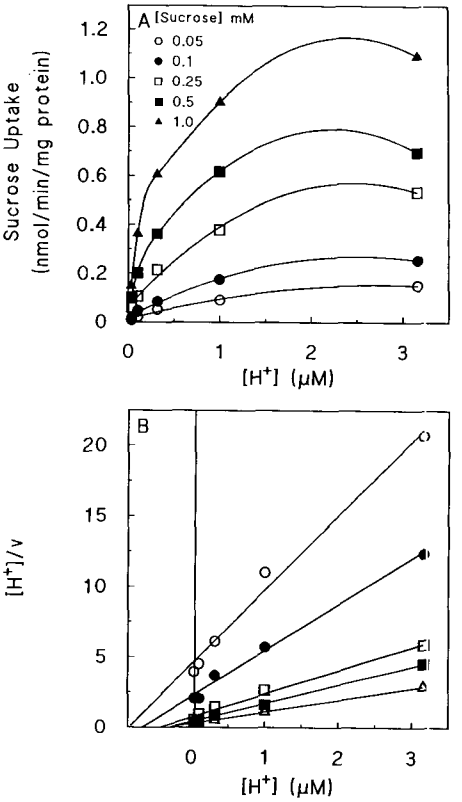

Figure 3. Analysis of Suc uptake under increasing $[H^+]_o$ at varying $[Suc]_o$. A, Suc uptake was determined in PM vesicles equilibrated at pH 7.5 in the presence of 100 mM K^+ and diluted in buffer containing 10 mM Na^+ and 1 μM valinomycin at the pH indicated. The rate of uptake was determined as a function of pH_o and $[Suc]_o$. B, The data in A were replotted by the Hanes-Woolf method. Values shown are averages of four determinations. The apparent K_m and V_{max} values are reported in Table I.

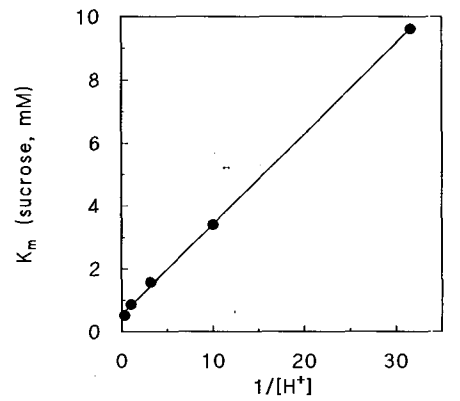

Figure 4. The effect of $[H^+]_o$ on the apparent K_m for Suc uptake. The data were taken from Table I, and the inverse of the $[H^+]_o$ was plotted as a function of the apparent K_m for Suc determined at the corresponding $[H^+]_o$. The relationship of K_m to $[H^+]_o$ was highly linear (r = 0.99) with the limiting K_m at infinitely large $[H^+]_o$ equal to 0.55 mM.

was conducted either with or without 1 mM internal Suc. In the first experiments, PMs from the two-phase system were equilibrated in buffer adjusted to pH 7.5, 6.5, or 5.5 in the presence of 1 mM Suc as described in "Materials and Methods." To test whether the pH_i had reached equilibrium, pH_i was determined by acetate uptake, assuming an internal vesicle volume of 4 μL mg^{-1} (Tubbe and Buckhout, 1992). The results of this analysis showed that the measured pH_i was in agreement with the theoretically expected value (Table II). For simplicity, it was subsequently assumed that pH_i was equal to that in the buffer used for equilibration.

Suc uptake experiments were conducted in vesicles with a pH_i of 7.5, 6.5, and 5.5 as described above. Decreasing the pH_i from 7.5 to 5.5 resulted in an approximately 75% decrease in the rate of Suc uptake (Fig. 5A). The apparent V_{max} for Suc uptake decreased from 976 pmol min^{-1} mg^{-1} protein at pH_i 7.5 to 243 pmol min^{-1} mg^{-1} protein at pH_i 5.5, and the apparent K_m for Suc was largely independent of the $[H^+]_i$; the values varied less than 10% over a 100-fold decrease in $[H^+]_i$ (Fig. 5B; Table III). Therefore, $[H^+]_i$ behaves as a noncompetitive inhibitor of Suc uptake when $\Delta\psi$ and $[H^+]_o$ are saturating and when $[Suc]_i$ is 1 mM. The $[H^+]_i$ for half-maximal inhibition of Suc uptake was estimated between 0.5 and 1.8 μM. Because of the variability of the data (cf. Fig. 5B), a more accurate determination was not possible.

Kinetics analyses similar to those reported above were conducted in vesicles that were equilibrated in dilution buffer (10 mM Hepes-Btp, 200 mM sorbitol, 50 mM K_2SO_4, and 1 mM DTT) adjusted to pH 7.5, 7.0, 6.5, 6.0, or 5.5 but in the absence of internal Suc. Also with this protocol, Suc uptake was affected by pH_i through a decrease in V_{max} with little or no change in K_m (Fig. 6; Table III). Thus, V_{max} decreased from 2.98 nmol min^{-1} mg^{-1} protein at pH_i 7.5 to 1.69 nmol min^{-1} mg^{-1} protein at pH_i 5.5, and the apparent K_m for Suc remained constant at approximately 0.5 mM. As above, $[H^+]_i$ in the absence of internal Suc behaved as a noncompetitive inhibitor of Suc uptake with a half-maximal inhibition of Suc uptake of 4.3 μM.

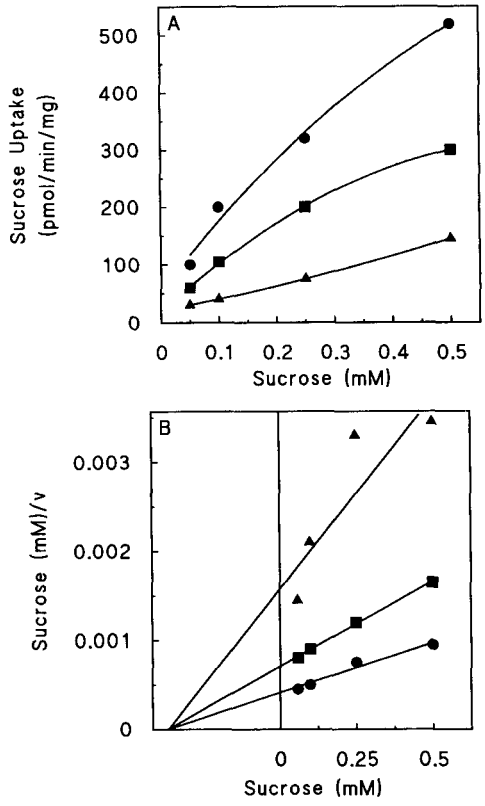

Figure 5. Analysis of the effect of 1 mM internal Suc and $[H^+]_i$ on the rate of Suc uptake. A, Suc uptake was determined in PM vesicles equilibrated at the indicated pH in the presence of 1 mM Suc. Uptake was initiated by dilution of vesicles in pH 5.5 buffer containing 100 mM Na^+ and 1 μM valinomycin (see "Materials and Methods" for details). The rate of uptake was determined as a function of pH_i and $[Suc]_o$. B, The data in A were replotted by the Hanes-Woolf method. Values shown are averages of four determinations. The apparent K_m and V_{max} values determined from this analysis are presented in Table III.

Table II. *Determination of vesicle pH_i*

PMs from the two-phase system were diluted in dilution buffer (10 mM Hepes-Btp, 200 mM sorbitol, 50 mM K_2SO_4, and 1 mM DTT) adjusted to pH 7.5, 7.0, 6.5, 6.0, or 5.5 and concentrated by centrifugation (50,000*g* for 30 min). The resulting pellets were resuspended in the dilution buffer at the appropriate pH and incubated at 10°C for 30 min. PMs were again concentrated by centrifugation, and the resulting pellets were resuspended in dilution buffer at a protein concentration of approximately 10 mg ml^{-1} and were frozen at −70°C until needed. Acetate uptake experiments were conducted by the method of Tubbe and Buckhout (1992), assuming a vesicle internal volume of 4 μl mg^{-1}. Results are averages of two experiments.

Theoretical pH_i	Measured pH_i
5.5	5.3
6.0	6.1
6.5	6.7
7.0	7.2
7.5	7.4

Table III. *Summary of kinetics constants calculated from Figures 5 and 6*

The apparent K_m values were determined from the Hanes-Woolf plots by linear regression analysis. The units for K_m and V_{max} are mM and nmol min^{-1} mg^{-1} protein, respectively. N.D., Not determined.

Parameter	pH_i				
	5.5	6.0	6.5	7.0	7.5
K_m	0.50	0.48	0.54	0.47	0.51
K_m + 1 mM Suc	0.41	N.D.	0.42	N.D.	0.43
V_{max}	1.69	1.85	2.80	2.92	2.98
V_{max} + 1 mM Suc	0.24	N.D.	0.56	N.D.	0.98

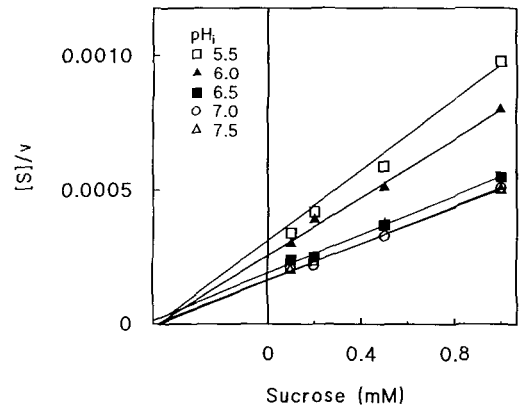

Figure 6. Analysis of the effect of $[H^+]_i$ on the rate of Suc uptake in the absence of internal Suc. A, Suc uptake was determined in PM vesicles equilibrated at the indicated pH in the absence of internal Suc. Uptake was initiated by dilution of vesicles in pH 5.5 buffer containing 100 mM Na^+ and 1 μM valinomycin (see "Materials and Methods" for details). The rate of uptake was determined as a function of pH_i and $[Suc]_o$. The data were plotted by the Hanes-Woolf method, and the values shown are averages of six determinations. The apparent K_m and V_{max} values determined from this analysis are presented in Table III.

Effect of $[Suc]_i$ on Suc Uptake at Saturation $[H^+]_o$ and $\Delta\psi$

In experiments analogous to those reported above, PMs were resuspended in buffer (10 mM Hepes-Btp, pH 7.5, 200 mM sorbitol, 50 mM K_2SO_4, and 1 mM DTT) containing 0, 0.1, 0.25, or 0.5 mM Suc. Vesicles were equilibrated, concentrated, and stored as described above. Suc uptake was determined as a function of $[Suc]_o$ with the three $[Suc]_i$ concentrations listed. The actual $[Suc]_i$ was assumed to equal that in the buffer used for equilibration and resuspension. Suc uptake was not significantly affected by any of the $[Suc]_i$ tested (Fig. 7). Thus, Suc uptake under the conditions of saturating $[H^+]_o$ and $\Delta\psi$ was not sensitive to $[Suc]_i$. For technical reasons, higher concentrations of internal Suc were not investigated, although one would expect that, at $[Suc]_i$ significantly greater than those tested here, an inhibition of Suc uptake would result (Komor, 1977; see below).

DISCUSSION

The kinetics behavior of the H^+-Suc symporter was investigated. The concentrations for half-maximal activity have been determined with the goal of understanding possible effects of changes in cytoplasmic and cell wall pH on the Suc transport process in vivo. In summary, the rate of Suc uptake into PM vesicles showed saturating kinetics with respect to $[Suc]_o$ and $[H^+]_o$. The $K_{m[Suc]o}$ at saturating $[H^+]_o$ and $\Delta\psi$ and at very low $[H^+]_i$ and $[Suc]_i$ was determined to be 0.55 mM (y intercept of Fig. 4). The corresponding $K_{m[H^+]o}$ was 0.55 μM (pH 6.3, x intercept of Fig. 4). As mentioned above, this value is significantly larger than the $K_{m[H^+]o}$ determined in intact tissues (Giaquinta, 1977; Delrot and Bonnemain, 1981); however, it agrees well with 0.1 and 0.3 μM determined by Komor and Tanner (1974) and Bush (1990), respectively. Noteworthy is the possibility of a regulation of Suc uptake through changes in the apoplastic pH. Bush (1990) concluded, based on the $K_{m[H^+]o}$ of 0.3 μM and an assumed pH_o of 5.5, that Suc uptake would be unaffected by changes in the apoplastic pH. Yet, estimations of apoplastic pH vary widely, ranging typically between 5.0 and 6.5 (Grignon and Sentenac, 1991), and the data in Table I clearly show that changes in pH_o within this range result in a corresponding 2- to 3-fold change in the apparent K_m for Suc uptake. Thus, depending on the apoplast pH, the Suc uptake might be significantly affected by changes in the apoplastic pH in vivo.

The likelihood of the cytoplasmic pH regulating Suc uptake was also investigated. Both in the presence and absence of internal Suc, $[H^+]_i$ behaved as a noncompetitive inhibitor with an apparent $K_{0.5}$ for inhibition of 4.3 μM in the absence and 0.5 to 1.8 μM in the presence of 1 mM internal Suc. Although the $K_{0.5[H^+]}$ for inhibition of uptake was somewhat variable, even the lowest apparent $[H^+]_i$ for inhibition was significantly above the H^+ concentration in the cytoplasm. Thus, a decrease in cytoplasmic pH of 0.5 unit as a result of temperature change, anaerobia, or a light-dark transition (Kurkdjian and Guern, 1989) would not be expected to greatly affect the rate of Suc uptake in vivo.

A comparison of the K_m and V_{max} values determined in the presence or absence of internal Suc (Table III) might lead to the conclusion that the rate of Suc uptake was sensitive to changes in $[Suc]_i$ in the mM range. However, a detailed comparison of the rate of Suc uptake at various $[Suc]_i$ resulted in no statistically significant affect on uptake (Fig. 7). This result would indicate that the apparent differences in K_m and V_{max} values (Table III), determined with or without internal Suc, were only a result of variability in the data. In fact, the V_{max} for Suc uptake is somewhat variable depending on the actual preparation of PMs used, and this variability does not correlate with any known physical or physiological factor. Thus, it seems likely that variations in the membrane preparations might be responsible for the differences observed.

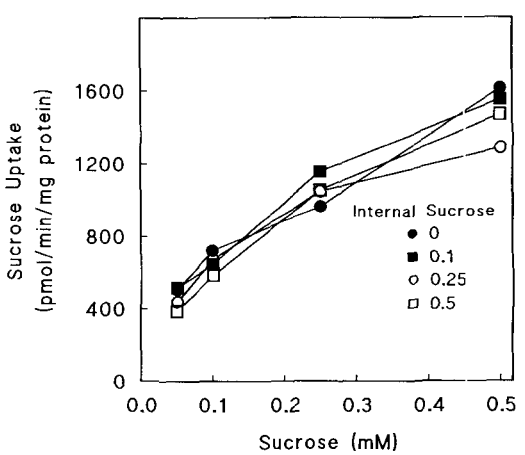

Figure 7. An analysis of the effect of $[Suc]_i$ on the rate of Suc uptake into PM vesicles. PM vesicles were equilibrated with $[Suc]_i$ as indicated at pH 7.5 and 50 mM K_2SO_4 (see "Materials and Methods"). The rate of Suc uptake was determined as a function of the internal and external Suc concentration. Transport was initiated by diluting the vesicles in pH 5.5 buffer containing 50 mM Na_2SO_4 and 1 μM valinomycin. Results are averages of two determinations.

Kinetics Model for the Symporter

In addition to providing insight into the behavior of a transport process with respect to the substrate and product concentrations, kinetics data can be useful in determining the reaction mechanism. Although several approaches for kinetics analysis of transport are available (Stein, 1989), we have chosen the approach described by Sanders et al. (1984). They mathematically analyzed the behavior of class 1 co-transporters based on the steady-state uptake of labeled ligand. Class I systems are co-transport models that have only one transport loop, where the carrier crosses the membrane as a charged species in one direction and returns uncharged. For a co-transporter with two ligands (H^+ and Suc in this case) and six discreet states, four different reaction sequences are obtained depending on the order of binding and release from the carrier (Fig. 8). Since a fully loaded positively charged carrier or an unloaded negatively charged carrier could cross the membrane, a total of eight binding sequences and charge transfer reactions results. The major assumptions in developing the theoretical rate equations for the eight models were: (a) only the charge translocation step is affected by the membrane potential, (b) the transport system is in steady state, and (c) the total concentration of the carrier on the membrane was constant. These represent minimal assumptions, avoiding a priori assignment of rate-limiting steps to the membrane transit reactions and providing the advantage that the resulting models are mathematically flexible. These advantages are accompanied by an inherent complexity in the mathematical descriptions, and as a result, the behavior of the model is difficult to predict. However, applying the simple physical restrictions on the transport system and theoretical rate equations of saturating $[H^+]_o$ and $\Delta\psi$ and in some cases zero-trans ligand (Suc), the mathematical complexity is greatly simplified, and the predicted behavior of each of the eight binding and transport sequences is in most cases unique. For a complete description of the model and a discussion of its merits and limitations, see Sanders et al. (1984).

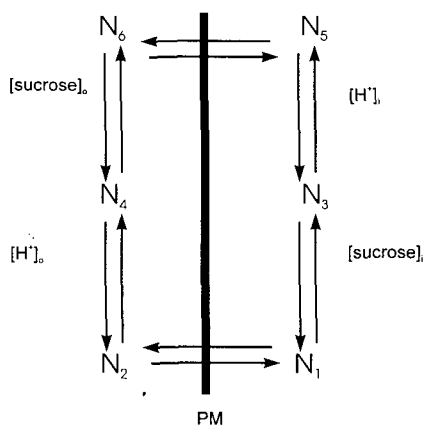

Figure 8. Generalized co-transport model for a six-state carrier. The model consists of six independent physical forms of the carrier (N_1 to N_6) with those with even-numbered subscript on the external side of the membrane and those with the odd-numbered subscripts on the inside. The unidirectional reaction constants between any two states (e.g. N_6, N_4) are given by k_{64} and, in the reverse direction, k_{46}. The effect of Suc or H^+ concentration is incorporated into the reaction constants, and the effect of membrane potential is incorporated into the models at the charge transit steps, either k_{21}/k_{12} or k_{56}/k_{65}. For a complete description of the models and a discussion of their merits and limitations, see Sanders et al. (1984). Shown is the first-on, first-off model.

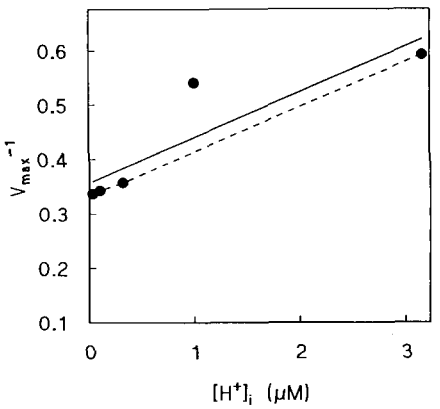

Figure 9. An analysis of increasing $[H^+]_i$ on the V_{max} for Suc uptake. $[H^+]_i$ was plotted against V_{max}^{-1}. The data were taken from Table III. The straight line was drawn by linear regression analysis (r = 0.88) with all data points included. The dashed line was drawn excluding the data at 1 μM H^+. The linear relationship indicates that at relatively large $[H^+]_i$ the V_{max} decreases to zero. This behavior would be expected if positive charges were translocated on the fully loaded carrier (see text).

The H^+-Suc symporter was inhibited by $[H^+]_i$ in a noncompetitive manner. This was true regardless of the concentration of internal Suc (Figs. 5 and 6; Table III). Of the eight possible reaction sequences that can be described by a six-state model (Fig. 8), only two reaction sequences would predict such a result; a last-on, last-off model, in which negative charge is carried on the fully unloaded carrier, and a first-on, first-off model, in which a positive charge is carried on the fully loaded carrier (table 3 in Sanders et al., 1984). The lack of inhibition of $[Suc]_i$ at saturating $\Delta\psi$ and $[H^+]_o$ might argue for a last-on, last-off model with negative charge being carried on the unloaded carrier. However, the cytoplasmic Suc concentration did affect Suc uptake in *Ricinus* cotyledons (Komor, 1977), and thus, it seems likely that the Suc concentrations used in this study were too low and that their effect on transport was too small to be detected.

Distinguishing between the two possible models presented above might be possible if the reaction sequence for the charge-transport step could be determined. As pointed out by Sanders (1986) for the conditions of $[Suc]_i = 0$ and $\Delta\psi$ very negative, Suc uptake by a carrier that transports macroscopic positive charge on the fully loaded carrier would decrease to zero at relatively large $[H^+]_i$. In contrast, a symport transporting macroscopic negative charge on the fully unloaded carrier would decrease in activity to a constant value at relatively large $[H^+]_i$. A comparison of $[H^+]_i$ against V_{max}^{-1} for the data in Table III resulted in a linear relationship (Fig. 9), suggesting that, in fact, V_{max} decreases to zero at relatively large $[H^+]_i$. The correlation coefficient was, however, relatively low (r = 0.89). Thus, for the H^+-Suc symport, we

tentatively conclude that macroscopic charge is carried by the fully loaded carrier. Furthermore, an ordered mechanism of transport is proposed in which the binding of Suc is on the external surface of the PM and release on the internal surface precedes that of H^+ (first-on, first-off ordered bireactant biproduct mechanism).

ACKNOWLEDGMENTS

The technical assistance and patience of Mrs. Sigrid Bittmann are gratefully acknowledged. This project is dedicated to the late Dr. Robert W. Warmbrodt.

Received February 28, 1994; accepted June 22, 1994.
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