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Proton-motive-force-driven sucrose uptake in sugar beet plasma membrane vesicles

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Highly purified plasma membranes were prepared by aqueous two-phase partioning from sugar beet (*Beta vulgaris* L.) leaf microsomes. Uptake of sucrose was studied with non-energized vesicles and with vesicles artificially energized by a pH gradient (ΔpH) or a pH gradient plus an electrical gradient ($\Delta pH + \Delta\psi$). In non-energized vesicles, sucrose was taken up via a diffusion-like system until attainment of concentration equilibrium. Energized vesicles rapidly accumulated sucrose with accumulation ratios of 6 and 12 (vs the external concentration) for vesicles energized by ΔpH and $\Delta pH + \Delta\psi$, respectively. Energized uptake exhibited saturation kinetics, and was sensitive to carbonyl cyanide *m*-chlorophenylhydrazone and *N*-ethylmaleimide.

Sugar transport; Sucrose; Protonmotive force; Plasma membrane vesicle; (*Beta vulgaris* L.)

1. INTRODUCTION

Long-distance transport of sugars in plants is an important process for crop productivity [1]. This transport involves several carrier-mediated steps through the plasma membrane of various cells, and in most plants, sucrose is the major form of transported carbohydrate [1,2]. In spite of current attempts to identify the sucrose carrier [3–5], our knowledge of sucrose transport at the membrane level remains macroscopic. Studies with whole plant tissues [6–8] or protoplasts [9] have provided evidence that the sucrose carrier of the plasma membrane is powered by the proton-motive force. However, this kind of study may be limited by diffusional problems or cellular metabolism. Considerable insight could be gained by studying sucrose transport in purified plasma membrane vesicles of higher plants [10]. Because of the inner localization of the active site of the proton-translocating ATPase in right-side-out-oriented vesicles, generation of a proton-motive force via ATP hydrolysis must be excluded. Artificial generation of a transmembrane potential ($\Delta\psi$) and/or of a transmembrane pH gradient (ΔpH) has been developed successfully with bacterial membrane vesicles. These methods have allowed detailed studies to be performed on the transport of sugars [11,12] and amino acids [13]. The present work reports on the possible use of similar methods to investigate the transport of sucrose (and of other solutes taken up with proton symport) in plasma membranes purified from higher plants.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, dithiothreitol; NEM, *N*-ethylmaleimide

2. MATERIALS AND METHODS

2.1. *Plant material*

Sugar beet plants (*Beta vulgaris* L. var. Aramis) were grown as described in [4]. Mature exporting leaves (4–5 weeks old) were used for preparation of plasma membrane vesicles.

2.2. *Plasma membrane vesicle preparation*

Plasma membrane vesicles were prepared according to [4].

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Characterization of these preparations by using marker enzymes has shown that they are highly enriched in plasma membranes [4]. The final upper phases resulting from aqueous two-phase partitioning [14] were diluted with the following medium (medium K): 0.3 M sorbitol, 50 mM potassium phosphate (pH 6.0 or 7.5, see sections 2.3 and 2.4), 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 0.5 mM DTT. Vesicles were pelleted for 1 h at 100000 × *g*, diluted in the same medium K (30 ml) and incubated for 30 min at room temperature to ensure equilibration of medium within vesicles. After 45 min centrifugation, vesicles were resuspended in a small volume of medium K in order to give a concentrated vesicle suspension (about 15 mg protein/ml). Aliquots of this solution were frozen in liquid N_2 and stored at −80°C.

2.3. *Uptake experiments under 'non-energized' conditions*

For these experiments, the medium inside the vesicles and that used for incubation were the same, namely medium K (see section 2.3), buffered at pH 6.0. Vesicles were rapidly thawed in a water bath (23°C). A 2 μl aliquot (25–30 μg protein) of vesicle suspension was taken with a 10 μl Hamilton syringe and deposited on the wall of a 5 ml test tube containing 400 μl incubation medium (medium K plus sucrose at the indicated concentration and 55.5 KBq [6,6′(n)-^{3}H]sucrose; Amersham France). For uptake experiments at different sucrose concentrations, the sorbitol level was adjusted in order to maintain a constant sugar concentration of 300 mM. Uptake was initiated by rapidly mixing vesicles with the incubation medium on a vortex apparatus. During experiments, solutions and test tubes were kept in a water bath at 23°C. At the desired times, uptake was terminated by adding 1.75 ml chilled rinsing medium (medium K plus 5 mM $HgCl_2$ to minimize efflux) in the test tube, vortex-mixing and rapidly filtering the contents of the test tube on a Millipore HAWP filter (pore size 0.42 μm, diameter 25 mm) pre-wetted with 1.75 ml rinsing medium. The test tube was further rinsed with 1.75 ml of the same medium and this solution added onto the filter. When the filter surface was dry, the filter was withdrawn from the filtering unit and placed in a 6 ml scintillation vial. All of these operations took less than 30 s. After drying the filters in an oven at 55°C for 1 h, 5 ml liquid scintillation cocktail (PCSII, Amersham France) was added to the vial.

Filters became transparent within 5 min. The radioactivity on the filter was then counted and all radioactivity measurements were corrected via the external standard method. For determination of radioactivity not associated with vesicles and retained non-specifically on the filter, the following control experiment was run. Chilled rinsing medium (1.75 ml) was first added to the incubation medium in the test tube and the tube kept on ice. Vesicles (2 μl) were then added to this solution and immediately filtered and processed as described before. The radioactivity thus determined was subtracted from all other measurements and was essentially equal to that retained on the filter in the absence of vesicles. This demonstrates that the radioactive sucrose recovered at the end of the incubation period had been taken up in the vesicles.

2.4. *Uptake experiments under 'energized' conditions*

Plasma membrane vesicles were suspended in medium K buffered at pH 7.5 before freezing. Uptake experiments were basically the same as those described in section 2.3 except for the incubation medium. To study the effects of a pH gradient (ΔpH) and a transmembrane potential difference ($\Delta\psi$) on sucrose uptake, we used the procedure described in [15]. The vesicles were thawed rapidly at 23°C and then incubated with 5 μM valinomycin in order to make the plasma membrane specifically permeant to K^+. 2-μl aliquots of vesicle suspension were then incubated as described in section 2.3 in medium Na (0.3 M sorbitol, 50 mM sodium phosphate pH 5.5, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$ and 0.5 mM DTT) containing sucrose at the desired concentration plus 26 kBq [6,6′(n)-^{3}H]sucrose and 5 μM valinomycin. Due to the presence of valinomycin, when the vesicles are diluted in the incubation medium, K^+ leaves the vesicles down the concentration gradient. Because the plasma membrane is not as permeant to the other cations present in the medium (Na^+ or H^+), the K^+ flux creates a negative charge inside the vesicles compared to the outside. This potential difference adds to the pH gradient arising from the use of two buffers at different pH (7.5 inside, and 5.5 outside the vesicles).

To study the effect of a pH gradient (acidic outside the vesicles) alone, the same procedure was used except that the incubation medium was medium K buffered at pH 5.5, thus abolishing the K^+ gradient. Valinomycin was also added in these determinations to simplify comparison with the previous experiments. For both types of experiment, vesicles were processed at the end of the incubation period as described in section 2.3. This procedure allows one to control ΔpH and $\Delta\psi$ well because of the high concentrations of phosphate buffer used (either sodium or potassium phosphate), and also because a very small aliquot (2 μl) of the vesicle suspension is diluted into a large volume (400 μl) of incubation medium. Thus, the extravesicular buffer contained in the 2 μl of vesicle suspension will not cause any change in the pH of the incubation medium.

2.5. *Other methods*

Protein was determined according to [16] using 0.01% (w/v) Triton X-100 to solubilize membrane proteins, with bovine serum albumin as a standard.

To check for sucrose metabolism, vesicles retained on filters were extracted with 80% ethanol and the extract analyzed via paper chromatography [5].

Orientation of the vesicles was determined by measuring the latency of the plasmalemma ATPase as in [17].

3. RESULTS AND DISCUSSION

3.1. *Uptake under non-energized conditions*

In the absence of ΔpH or $\Delta\psi$, sucrose was taken up very slowly, maximum uptake being reached 120 min after the beginning of incubation (fig.1, circles). Similar results were obtained when the sucrose concentration was 50 mM (fig.1, squares). Assuming equilibration of internal sucrose concentration with that present externally to be reached after 120 min incubation, the method of Rottenberg [18] allowed us to estimate the internal volume of the vesicles to be 4.7 ± 0.4 μl/mg protein (mean of 4 experiments, ± SE). When uptake

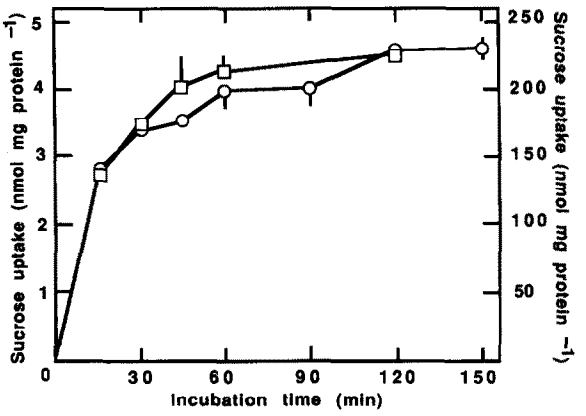

Fig.1. Time dependent uptake of 1 mM [(○), left-hand scale] and 50 mM sucrose [(□) scale on the right] under non-energized conditions. Results are averages of 4 [(○) 2 experiments] or 6 replicates [(□) 3 experiments] ± SE. For the sake of clarity only some of the standard errors are shown.

was studied as a function of sucrose concentration, no saturation was detected over a low concentration range (between 10 μM and 1 mM sucrose, not shown). The uptake rate was linear vs concentration of sucrose up to 50 mM, but some indications of saturation appeared at higher concentration (fig.2). The data obtained were consistent, within experimental error, with pure diffusion, or facilitated diffusion via a carrier having a very low affinity for sucrose.

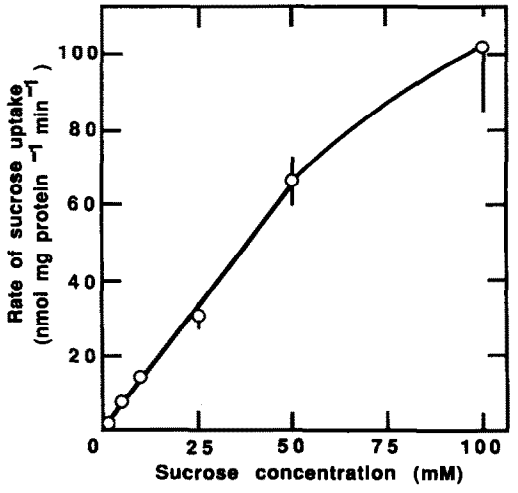

Fig.2. Concentration dependence of sucrose uptake under non-energized conditions after 15 min incubation. Results are means of 5 replicates (2 experiments) ± SE.

3.2. *Uptake of sucrose under energized conditions*

In the presence of a ΔpH, rapid accumulation of sucrose occurred during the first minute of incubation (fig.3). Intravesicular sucrose concentration then decreased over the next 2 min, reaching a value that was stable up to 10 min. Given the internal volume estimated previously (see section 3.1), which corresponds to a value for uptake of 4.66 nmol/mg protein, an accumulation ratio of 6 was determined, confirming that sucrose uptake occurred against a concentration gradient. In the presence of ΔpH + $\Delta\psi$, much greater accumulation of sucrose was observed, with an accumulation ratio of about 12 between 45 s and 2 min of incubation (about 55 nmol/mg protein). The maximum accumulation of sucrose in the presence of ΔpH + $\Delta\psi$ was approximately twice that noted in the presence of ΔpH alone. The internal sucrose concentration then decreased between 3 and 10 min. An important point to be noted is the shape of the curves obtained, demonstrating the rapid and transient accumulation of sucrose. These curves, particularly in the presence of ΔpH + $\Delta\psi$, are typical of the overshoot phenomenon which has often been reported in studies of cotransport

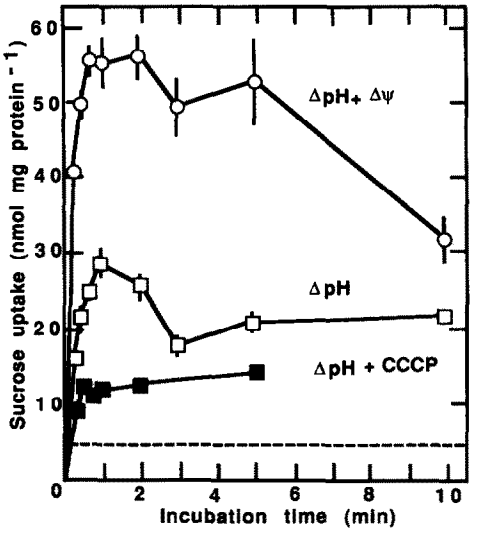

Fig.3. Time-dependent uptake of 1 mM sucrose under energized conditions. Experiments were run in the presence of ΔpH + $\Delta\psi$ (○), ΔpH (□) and ΔpH + 10 μM CCCP (■). The dashed line represents passive equilibration of sucrose. Results are means of 16 replicates (4 experiments) or 8 replicates for time points 3, 5 and 10 min (2 experiments). SE values are shown only when larger than the corresponding symbols.

systems involving artificially energized membrane vesicles. Overshoot has been noted for H^+/glucose 6-phosphate cotransport in *Escherichia coli* [15], Na^+/glucose cotransport [19], Na^+/amino acid cotransport [20] and H^+/glycine cotransport [21] in brush border membrane vesicles. The overshoot is due to the fact that, in the absence of metabolism inside vesicles, no energy is available to fuel proton pumps and therefore the electrochemical gradient of protons soon dissipates towards equilibrium, especially when protons are taken up with sucrose. Hence, in such experiments, the initial rate of sucrose uptake is increased, and sucrose accumulates transiently above equilibrium (see [22] for a theoretical analysis of overshoot). The overshoot observed here, as in above-cited works, is in contrast with the smooth time course recently reported for amino acid uptake into microsomes from zucchini energized by ΔpH [23]. Part of this discrepancy may be due to the fact that those authors performed experiments at a lower temperature (10–14°C), reported to stabilize the pH gradients [24].

Addition of CCCP (10 μM) to the incubation medium caused marked but incomplete inhibition of uptake in vesicles energized by ΔpH. Incomplete inhibition may be due to relatively slow dissipation of the $\Delta\mu H^+$ already reported [25]. Nevertheless, this significant degree of inhibition together with the very low uptake observed in the absence of ΔpH or ΔpH + $\Delta\psi$ (fig.1) strongly suggests that sucrose uptake depends on the proton-motive force.

Chromatographic analysis of the radioactivity recovered in vesicles energized by ΔpH + $\Delta\psi$ (2 min incubation) showed that no cleavage of sucrose occurred prior to uptake.

The present data were obtained using vesicles which had been frozen once. Measurements of ATPase latency showed that, whereas fresh vesicles obtained after phase partitioning were mainly right-side-out (latency 86%), samples used for uptake experiments contained equal proportions of inside-out and right-side-out vesicles (latency 49%). Although some experiments run with fresh vesicles gave results similar to those presented here in terms of uptake rates and response to $\Delta\psi$ and ΔpH (not shown), no attempt was made to ascertain the importance of vesicle orientation. However, the technique reported here, combined with methods allowing separation of right-side-out and inside-out vesicles [17,26], should enable a detailed study of the symmetry of transport to be performed.

3.3. *Kinetics of sucrose uptake under energized conditions*

Experiments were run using vesicles energized by $\Delta\psi$ + ΔpH in order to ensure maximum uptake rates and with an incubation time of 15 s, assumed to allow measurement of the initial rate of uptake (fig.3). The concentration dependence of sucrose uptake exhibits saturation kinetics, consistent with carrier-mediated transport (fig.4). This is further confirmed by comparison with the linear kinetics observed under non-energized conditions (fig.2). Fig.4 allows one to estimate the values for K_m (300 μM) and V_{max} (90 nmol/mg protein per 15 s). However, careful examination of fig.4 shows that the curve displays a sigmoidal shape rather than that of typical Michaelis Menten plots. This is confirmed by the double-reciprocal plots (fig.5), showing strong curvature to occur at low sucrose concentrations. This behaviour may be due either to allosteric regulation, which has been reported for cotransport systems [27], or to the existence of a preferred kinetic pathway in the binding sequence of substrates leading to the ternary complex [28]. It should also be stressed that the determination of kinetic parameters with mem-

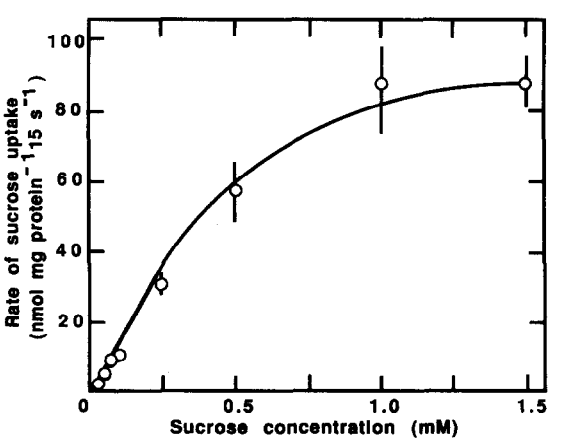

Fig.4. Concentration dependence of sucrose uptake under energized conditions ($\Delta\psi$ + ΔpH) after 15 s incubation. Results are means of 4 replicates (1 typical experiment). SE values shown only when larger than the symbols.

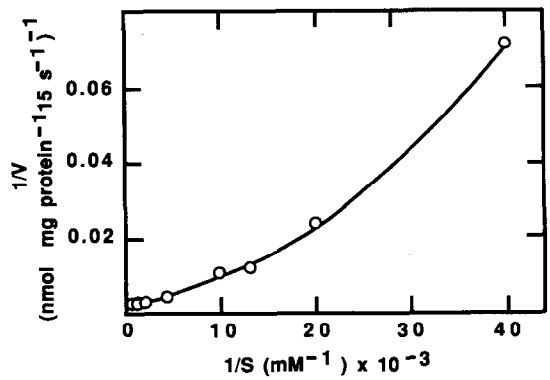

Fig.5. Plots of $1/V$ vs $1/S$ derived from fig.4.

brane vesicles depends strongly on the incubation time [29].

The involvement of a carrier, as indicated by the saturation kinetics, is also demonstrated by the inhibitory effect of thiol reagents such as *N*-ethylmaleimide on sucrose uptake (fig.6).

In conclusion, our data provide the first direct evidence of the energization of sucrose uptake by the proton-motive force in plasma membrane vesicles purified from plant material. Previously, the various proton/sugar and proton/amino acid transport systems of plants had been characterized with tissues or organs. The technique described here should prove useful in detailed studies of these systems at the membrane level.

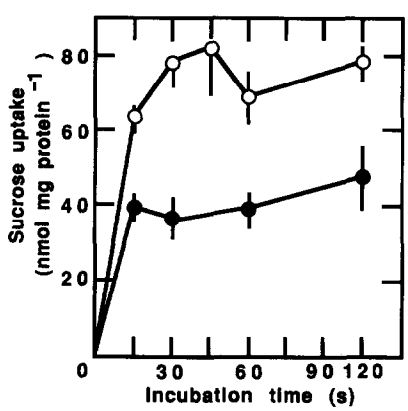

Fig.6. Time-dependent uptake of 1 mM sucrose under energized conditions ($\Delta\psi$ + ΔpH) in the absence (○) or presence (●) of 0.5 mM NEM. Vesicles were preincubated with NEM for 30 min before initiating uptake. Results are averages of 8 replicates (2 experiments) ± SE.

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