H^+-sugar antiport as the mechanism of sugar uptake by sugarcane vacuoles

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The uptake of 3-*O*-methylglucose (3-OMG) by vacuoles isolated from sugarcane cells caused a depolarization of the internally positive membrane potential and a decrease of the pH gradient. The uptake of sugar was accompanied by a corresponding efflux of protons out of vacuoles with a stoichiometry of 1:1. The data support a model whereby sugar is transported via an H^+-antiport system.

Sugarcane vacuole *Sugar uptake* *Proton antiport*

1. INTRODUCTION

The vacuoles of higher plants are storage compartments for sugars, anthocyanin, and organic acids. The hydrophilic nature of these compounds precludes passive permeation across the membrane as a mechanism of uptake. Since sugar is accumulated in the vacuoles of sugarcane, an active uptake mechanism has been postulated [1]. Evidence in several plant species shows that a tonoplast-bound ATPase acidifies the intravacuolar space and polarizes the tonoplast to a positive value [2,3]. This proton gradient may be used for energization of sugar transport into vacuoles.

Sugarcane cells grown in suspension cultures were used for this study. These cells accumulate both hexose and sucrose in the vacuoles [4]. Vacuoles which have retained both proton-translocating ATPase [5] and glucose uptake [4] activities can be isolated from these cells. These vacuoles are, therefore, a useful material for the study of the coupling of sugar flow to proton flow.

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2. EXPERIMENTAL

Sugarcane cell suspensions (a subclone of var. H50-7209) were grown in White's basal salt medium supplemented with yeast extract, arginine, sucrose, vitamins, and 2,4-D [6]. Cultures were incubated on a rotary shaker at 27°C and were harvested after 9 days of growth.

Vacuoles were isolated from protoplasts by centrifugation on a Ficoll cushion [1]. Tonoplast vesicles were prepared by homogenization of vacuoles with a glass homogenizer in a large volume of buffer containing 25 mM Tricine, 10 mM $MgSO_4$, 5 mM K_2SO_4 and 250 mM mannitol (pH 6.5). The homogenate, in turn, was layered on a cushion of 10% dextran T70 in 25 mM Tricine (pH 6.5) containing 250 mM mannitol and was centrifuged for 60 min at 100000 × *g*. Membranes were collected at the interface and were used for all measurements. The volume of vacuoles and tonoplast vesicles was measured as the H_2O-permeable, but dextran-impermeable space [4].

Membrane potential ($\Delta\Psi$) was measured either by distribution of [^{14}C]thiocyanate (SCN^-) or by absorption spectroscopy of bis(3-propyl-5-oxo-isoxazol-4-yl)pentamethine oxonol (oxonol-VI).

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For the uptake of [^{14}C]SCN^-, vacuoles were incubated at 25°C in a medium containing 50 mM Tris–Mes (pH 6.5), 500 mM mannitol, 3 mM $MgSO_4$ and 5 μM SCN^- (spec. act. 20 μCi/μmol) and allowed to equilibrate for 15 min. ATP or other chemicals to be tested were added and samples were withdrawn at intervals, centrifuged through silicon oil, and the radioactivities of the pellet and supernatant determined. The distribution ratio of SCN^- across the membrane was used to calculate $\Delta\Psi$ according to the Nernst equation. For the optical measurement of $\Delta\Psi$, vacuoles or tonoplast vesicles were suspended in 25 mM Tricine, 10 mM $MgSO_4$, 250 mM mannitol, 4 μM oxonol-VI and 3 mM Mg-ATP (pH 6.5). Absorption changes of oxonol-VI were measured using either an Aminco-Chance or a Perkin-Elmer 356 dual wavelength spectrophotometer with the monochromator set at 603 and 580 nm. Calibration was performed with an imposed K^+ gradient in the presence of valinomycin (10 μg/ml).

The pH gradient (ΔpH) was measured as quenching of fluorescence of the permeant amine dyes, quinacrine or acridine orange, using an Aminco-Bowman spectrophotofluorometer. Changes in emission were measured at 425–505 nm (excitation ⟶ emission) for quinacrine and 493 ⟶ 530 nm for acridine orange. The ΔpH was calculated as in [7].

Proton flux was measured in a chamber containing 30 μl vacuole suspended in 5 mM KCl, 10 mM $MgSO_4$, 3 mM Mg-ATP, and 500 mM mannitol in a total volume of 300 μl. The suspension was gently stirred and pH changes were measured with a flat-bottom combination glass electrode. The proton flux was calibrated by addition of known quantities of HCl.

Protein was measured as in [8] using the commercially available dye from Bio-Rad Laboratories.

Oxonol-VI was a gift from G. Hauska, Regensburg. The fluorescent probes for ΔpH were from Sigma and [^{14}C]thiocyanate was from Amersham.

Table 1

Effect of hexose uptake on membrane potential, pH gradient, and H^+ flux

	Intact vacuoles	Tonoplast vesicle
Decrease of membrane potential		
measured with oxonol-VI	8.3 ± 5.5 mV	2.9 ± 1.7 mV
measured with SCN^-	9.8 ± 3.2	ND
Decrease of pH gradient measured with quinacrine or acridine orange	0.06 ± 0.04	0.12 ± 0.00
H^+ efflux	0.45 ± 0.29[a]	ND

[a] nmol·h^{-1}·μl vacuole^{-1}

The incubation mixture for the optical measurement of $\Delta\Psi$ contained 20 μl vacuoles (200 μg protein) in 3 ml buffer. The SCN^- accumulation was measured in 52 μl vacuoles in 1.0 ml buffer. The ΔpH change was determined using 10 μM quinacrine or 4 μM acridine orange. Incubation mixture contained 13 μl vacuoles (127 μg protein) in 1.0 ml buffer. The proton flux was determined using 30 μl vacuoles in 0.3 ml medium. All values indicate the change caused by the addition of 1 mM 3-OMG and corrected for the change by a second addition of 3-OMG. Values were the means from 3 different vacuole or tonoplast preparations

3. RESULTS

3.1. *Effects of hexose uptake on membrane potential*

The addition of 3-OMG caused a depolarization of the vacuoles of about 8 mV when measured as oxonol-VI absorption changes (table 1). This depolarization was correlated with 3-OMG uptake since a second addition after the first saturating pulse gave no further response of the optical probe (fig.1). The time course and the value of oxonol-VI absorption changes varied with different vacuole preparations: sometimes there was a continuous, gradual depolarization; more often, there was a small but rapid response, within 10 s. Therefore, results were variable. The membrane potential depolarization of tonoplast vesicles was small compared to that of intact vacuoles. Vesicle preparation procedures have been reported to yield tightly sealed vesicles [9], but a large proportion of the vesicles may still be leaky or vesicles may be inside out.

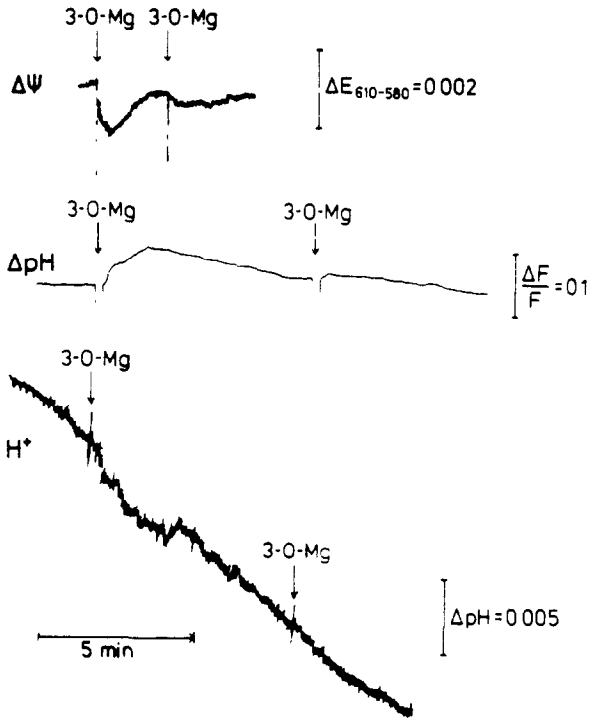

Fig.1. Effect of 3-OMG on membrane potential, pH gradient, and proton flux in intact vacuoles. Membrane potential was measured as the change in absorption of oxonol-VI during hexose uptake; ΔpH change was measured as change in acridine orange fluorescence; proton flux was measured as an acidification of the external medium using a combination glass electrode. Experimental conditions were the same as in table 1.

The membrane potential, measured as the accumulation of SCN^-, had been shown previously to have a positive value on the internal surface of the tonoplast [5]. The addition of 3-OMG to a vacuole suspension decreased the membrane potential by 10 mV (table 1).

The depolarization of tonoplast by 3-OMG indicates that either a positive charge leaves the vacuole during hexose uptake or a negative charge enters together with hexose.

3.2. *Change of intravacuolar pH during hexose uptake*

The addition of 3-OMG to intact vacuoles caused a small decrease of the ΔpH so that the vacuole interior became slightly less acidic (table 1). The internal pH of tonoplast vesicles was more significantly changed after the addition of 3-OMG (table 1, fig.1). This effect was observed with quinacrine or acridine orange as probes. The internal pH of tonoplast vesicles changed by about 0.1 unit. This is evidence for proton efflux during hexose uptake.

The flux of protons in the suspension medium can also be measured with a pH electrode. The addition of 3-OMG to intact vacuoles caused a slight transient acidification of the medium. A second addition did not result in a similar response (fig.1). The rate of hexose uptake by isolated vacuoles has been previously determined to be 0.5 $nmol \cdot h^{-1} \cdot \mu l$ $vacuole^{-1}$ [4]; therefore, a stoichiometry of 1 H^+ per 3-OMG was calculated. The uptake of hexose, therefore, is a monovalent H^+ antiport.

4. DISCUSSION

The active transport of hexose into vacuoles of sugarcane occurs either via an electrogenic H^+ antiport with a stoichiometry of 1:1 or via an hydroxyl ion symport. It was previously shown that isolated sugarcane vacuoles contained a proton-translocating ATPase which generated an inside acid pH and a positive membrane potential ([5], unpublished). Under these conditions, the downhill electrochemical gradient for proton is

outward from the vacuole and, therefore, a proton antiport system for hexose uptake is probably the simplest way to make use of this protonmotive force.

The different response of intact vacuoles and of tonoplast vesicles to the addition of 3-OMG is expected if two facts are considered. First, the internal buffering capacity of intact vacuoles is significantly higher than that of tonoplast vesicles since intravacuolar compounds are washed out during tonoplast vesicle preparation. Hence, change in ΔpH should be and was more readily detectable in vesicles than in vacuoles. Second, during vesicle preparation, the membranes may become inverted and/or they may seal incompletely. Therefore, a less pronounced effect on the membrane potential may be seen.

The effects of hexose on membrane potential and pH gradient are small, probably because the H^+-translocating ATPase counteracts the hexose-caused proton exit.

The best known examples of antiport systems are the various exchanger systems of chloroplast envelopes and mitochondria. For instance, the ATP/ADP exchanger works in an electrogenic manner where in the presence of Mg^{2+} the same binding site is able to bind either ATP^{4-} or ADP^{3-} [10]. The mechanism of H^+-hexose antiport can be visualized to function in a similar manner. If the binding site for H^+ and hexose is located on the same site of the protein, then the binding of H^+ will prevent the binding of hexose and vice versa. If the transporter protein can change its contact to the two sides of the membrane only in the loaded state, then inevitably the ATPase-caused acidification of the vacuolar space and polarization of the membrane will favor proton binding at the vacuolar side and proton release at the cytoplasmic side with greater possibility of hexose binding at the cytoplasmic side and release into the vacuole. Evidence for a very close proximity of H^+ and sugar binding sites has been found recently for the sugar-H^+ symport system of *Chlorella* [11]. It is possible that during evolution, a shift of the H^+ binding site would transfer a symport system to an antiport system.

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