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PROTON ELECTROCHEMICAL GRADIENTS AND SUCROSE ACCUMULATION IN THE MAIZE SCUTELLUM*

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Key Word Index—*Zea mays*; Gramineae; maize scutellum; protonmotive force; sucrose accumulation.

Abstract—Estimates of proton and sucrose gradients across the plasmalemma of maize scutellum cells were made in order to test previous conclusions that sucrose uptake occurs by a proton–sucrose symport with a 1 : 1 stoichiometry. Membrane potential and cytoplasmic pH were estimated from the distribution between cells and bathing solution of [^{14}C]tetraphenylphosphonium ions and 2,4-dinitrophenol (DNP), respectively. External pH in the range 3–8 had no effect on cytoplasmic pH which was 8.0, but external pH strongly influenced membrane potential which was 40 mV (inside negative) at pH 3 and 140 mV at pH 7–8. Additions of KCl and NaCl caused some depolarization of the membrane potential, as did the energy poison, DNP, but mannose (an energy poison in scutellum cells) had no effect. The electrochemical proton gradient (protonmotive force) varied from 340 mV at pH 3 to 163 mV at pH 7.6. During 5 hr incubations at pH 4 or 7, bathing solution sucrose $[S]_o$ appeared not to reach a steady-state with tissue sucrose $[S]_i$. The non-steady-state sucrose gradients were too small at pH 4 and too large at pH 7 to be accounted for only by the operation of a proton–sucrose symport. Sucrose gradients also were calculated from the measured protonmotive forces on the assumption that cytosol [S], in contrast to $[S]_i$, had reached a steady-state with $[S]_o$ at both pHs. It was concluded from these calculations that leak and/or 'slip' (sucrose exit via the porter without a proton) pathways for sucrose as well as a proton–sucrose cotransporter are present in the plasmalemma and that sucrose gradients exist across the tonoplast.

INTRODUCTION

Sugar-proton cotransport (symport) systems have been reported in a number of higher plants, e.g. [1–3] Of particular interest are the reports that phloem loading from the apoplast of the leaf [4, 5], cotyledon [6] and bark [7] is accomplished by sucrose–proton cotransport. In these cotransport systems, sugar accumulation is driven by the proton gradient, and at equilibrium the two gradients are related according to the equation of Mitchell [8]:

$$Z \log [S]_i/[S]_o = n(\psi_o - \psi_i) - n\,Z(pH_o - pH_i) = n\,\Delta\rho \quad (1)$$

where [S] is the sugar concentration, n is the proton : sugar stoichiometry, ψ is the electrical potential, Z is the factor that converts concentration ratios into mV ($Z = 60$, at 30°), the subscripts o and i refer to outside and inside the plasmalemma, and $\Delta\rho$ is the protonmotive force. In higher plants, this equation has been applied only to hexose proton cotransport in sugarcane suspension cells [1]. Sugarcane cells develop a protonmotive force sufficient to support a 100-fold hexose accumulation, but only a 15-fold accumulation was obtained using the glucose analog 3-*O*-methylglucose [1]. However, recently it was reported that hexose accumulation in sugarcane cells took place across the tonoplast and there was no hexose accumulation across the plasmalemma [9]. The mechanism for this active hexose transport across the tonoplast is not known but certainly equation (1) would not apply.

In this paper, equation (1) is applied to the sucrose–proton cotransport system of the maize scutellum [2, 10]. Recently it was shown that $n = 1$ [2] and $pH_i = 8$ [11] for the scutellum system. The stoichiometry was determined for sucrose–proton exit from the cell at a pH_o of 7.5–8, but it is assumed that the same stoichiometry holds for entry. The entry of hexose into *Chlorella* [12] and sugarcane cells [1] and of lactose into *Escherichia coli* [13] occurs with a sugar : proton stoichiometry of one. The pH_i determined for the scutellum was that of the cytoplasm, and it was independent of pH_o in the range 3–8. The present study was concerned with measurement of the other parameters of equation (1), ψ and $[S]_i/[S]_o$, and with the effect of pH_o on the proton and sucrose gradients.

RESULTS AND DISCUSSION

Uptake of tetraphenylphosphonium ions into maize scutellum slices

Buffered solutions of the lipid-soluble cation tetra[U-^{14}C]phenylphosphonium ion ([^{14}C]TPP^+) were added to the slices at zero time and portions of the bathing solution were removed for ^{14}C assay at intervals over a 200 min period (Fig. 1). TPP^+ equilibrated between slices and bathing solution in *ca* 100 min (pH 6.8) or 140 min (pH 4). At equilibrium the slices contained *ca* 86% (pH 6.8) or 27% (pH 4) of the total TPP^+, and this distribution was maintained for at least 160–200 min (data not shown). The TPP^+ distributions indicate (from the Nernst equation, equation (2): see Experimental) membrane potentials

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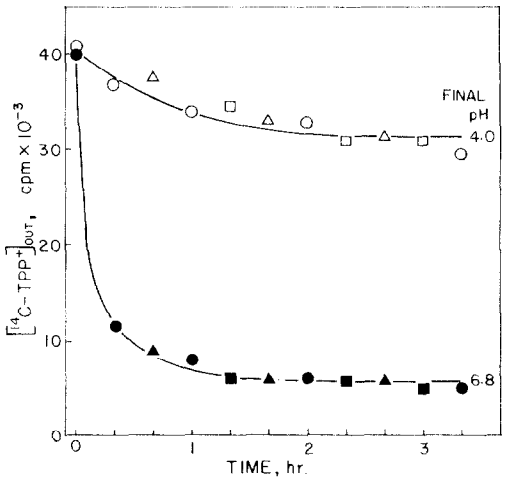

Fig. 1. Time course of [^{14}C]TPP$^+$ uptake into scutellum slices. Data from a single representative experiment (see Fig. 3) in which three groups of slices were run at each pH.

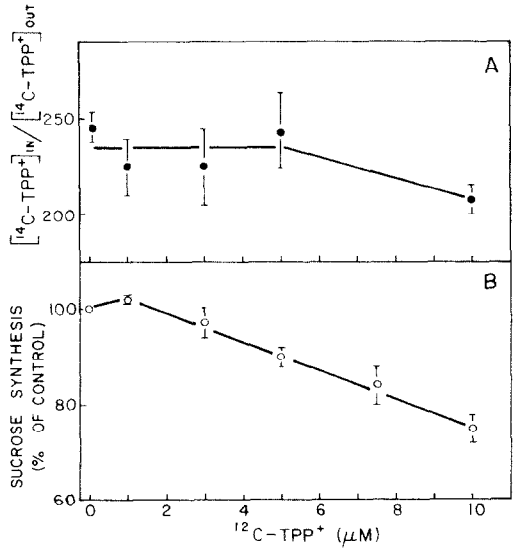

Fig. 2. Effect of TPP$^+$ concentration on the distribution of [^{14}C]TPP$^+$ between bathing solution and scutellum slices (A) and on sucrose synthesis (B). In (A), slices were incubated for 200 min at pH 6.8 (cf. Fig. 1). In (B), slices were incubated for 180 min in 0.1 M fructose at pH 6.8. Data are averages from four experiments.

(negative inside) of 144 mV (pH 6.8) and 67 mV (pH 4). Results similar to those of Fig. 1 were obtained with triphenyl[^{14}C]methylphosphonium ions (TPMP$^+$).

Unspecific binding of TPP$^+$ and TPMP$^+$ internally or externally, as well as the concentration of these ions at the surfaces of cell wall and plasmalemma in response to surface potentials, would introduce errors in the estimated membrane potential. Another source of error in using lipid-soluble cations results from their ability to diffuse freely across membranes and thereby influence the membrane potential being measured. At high concentrations they act as energy poisons [14]. With *Escherichia coli* [15], TPP$^+$ and ^{86}Rb$^+$ plus valinomycin gave nearly identical membrane potentials, whereas TPMP$^+$ gave higher absolute potentials which were considered to reflect unspecific binding of TPMP$^+$. With yeast [16], absolute membrane potentials obtained with TPP$^+$ were higher than those obtained with a microelectrode, but it is not clear if this was due to binding of TPP$^+$ or to ion leakage at the microelectrode puncture. Corrections for binding of TPP$^+$ in sugarcane cells were made by using heat-killed cells as controls; the corrections were considerable at membrane potentials smaller than 30 mV [1].

Various concentrations of [^{12}C]TPP$^+$ were used with one concentration of [^{14}C]TPP$^+$ in order to detect binding of TPP$^+$ in scutellum slices. If binding occurs, [^{12}C]TPP$^+$ would displace [^{14}C]TPP$^+$ from the binding sites and the apparent $[[^{14}C]TPP^+]_i/[[^{14}C]TPP^+]_o$ ratio would decrease. Figure 2 shows the effect of [^{12}C]TPP$^+$ concentration on the $[[^{14}C]TPP^+]_i/[[^{14}C]TPP^+]_o$ ratio and on sucrose synthesis from exogenous fructose. Sucrose synthesis from fructose occurs at a rate that requires nearly 50% of the ATP produced in scutellum slices [17], and it should serve as a sensitive monitor for energy-poisoning effects of TPP$^+$. In the range of 0.32–5.0 μM, [^{12}C]TPP$^+$ had no effect on the [^{14}C]TPP$^+$ distribution, but there was a small decrease in the [^{14}C]TPP$^+$ ratio when the concentration of [^{12}C]TPP$^+$ was increased to 10 μM (Fig. 2A). Concentrations of TPP$^+$ greater than μM inhibited sucrose synthesis. At 10 μM, TPP$^+$ inhibited sucrose synthesis 25% (Fig. 2B). Therefore, the decrease in the [^{14}C]TPP$^+$ ratio when 10 μM [^{12}C]TPP$^+$ was used probably should be ascribed to TPP$^+$ acting as an energy poison and not to displacement of bound [^{14}C]TPP$^+$. This conclusion is supported by previous work [2] in which it was shown that at higher concentrations of lipid-soluble cation (TPMP$^+$, 10 mM, pH 7) sucrose synthesis is inhibited 65% and the inside/outside TPMP$^+$ ratio is reduced to 1.0. The buffers used in these experiments contained Na$^+$ at concentrations at least 2000-times greater than TPP$^+$, which probably prevented unspecific binding of TPP$^+$ and concentration of TPP$^+$ at the surfaces of negatively charged cell wall and plasmalemma. However, non-ionic binding of TPP$^+$ would not be influenced by Na$^+$. In the experiments that follow, 5 μM TPP$^+$ was used because it had no effect on [^{14}C]TPP$^+$ distribution and it inhibited sucrose synthesis only 10%.

Effect of pH_o *on membrane electrical potential*

The effect of pH_o on membrane potential is shown in Fig. 3. Between pH 3 and 6.8 absolute values of membrane potential increased *ca* 27 mV for each unit increase in pH_o, but there was no change between pH 6.8 and 7.6. Similar results have been reported for *Chlorella* [18] and sugarcane cells [1] using TPP$^+$ and for *Anabaena* [19] using TPMP$^+$. In leaf cells of *Kalanchoe*, membrane potential (measured with a microelectrode in the vacuole) was independent of pH_o between 9 and 5, but below pH_o 5 it depolarized by 54 mV per unit decrease in pH_o, i.e. the cell potential responded to pH_o in the same way as that of an H$^+$-electrode [20]. Many investigators using ion distribution (TPP$^+$, TPMP$^+$, Tl$^+$ or K$^+$) or microelectrodes have obtained membrane potentials in bacteria, algae and higher plants, e.g. [1, 13, 15, 18, 20–22] similar in magni-

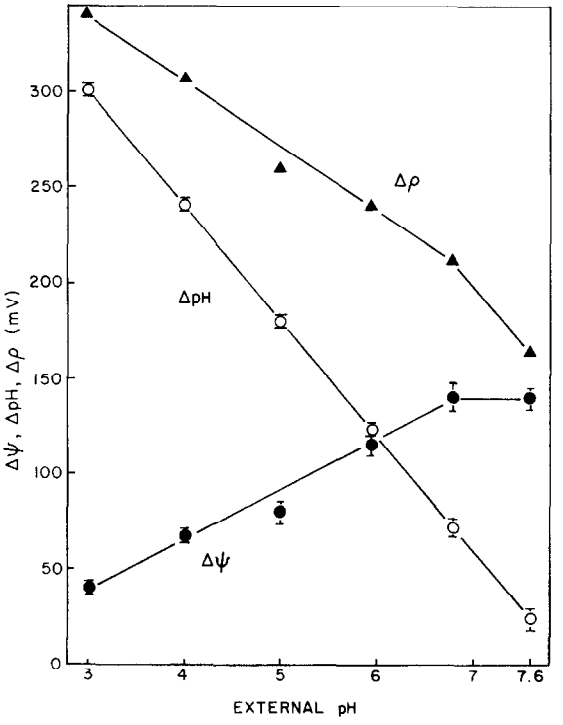

Fig. 3. Effect of external pH on membrane potential ($\Delta\psi$), ΔpH and protonmotive force ($\Delta\rho$). Data are averages from four to six experiments.

tude to those of Fig. 3. Using microelectrodes, absolute membrane potential values were obtained which were higher than those of Fig. 3 in fronds of *Lemna* [23] and lower than those of Fig. 3 in pea internodes and squash cotyledons [24].

The [^{14}C]TPP$^+$ distribution ratio was decreased by the addition of K^+ or Na^+ (Fig. 4). When 50 mM KCl was added 60 min after adding TPP$^+$, an exit of TPP$^+$

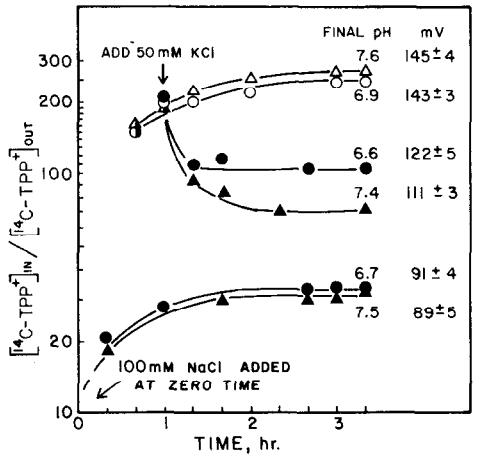

Fig. 4. Effect of K^+ and Na^+ on distribution of [^{14}C]TPP$^+$ between bathing solution and scutellum slices. Data are averages from three experiments.

occurred until a new equilibrium distribution was attained. When the initial pH_o was 7.0, the final pH_o was 6.6 and it took 20 min to attain an equilibrium corresponding to a potential of 122 mV, only *ca* 10 mV less than expected for that pH_o (cf. Fig. 3). When the initial pH_o was 8.0, the final pH_o was 7.4 and it took 80 min to attain an equilibrium corresponding to a potential of 111 mV, *ca* 29 mV less than expected for that pH_o (cf. Fig. 3). When the bathing solution initially contained 100 mM NaCl, the membrane potential was depolarized by *ca* 55 mV at both pH_os. Sucrose efflux from scutellum slices is increased by 50 mM KCl, especially when the initial pH_o is above 7.5 [14]. This probably results from depolarization of the membrane potential (Fig. 4) since sucrose exit is electrogenic and proceeds against the membrane potential [2].

Membrane potential was relatively insensitive to 2,4-dinitrophenol (DNP), which caused small depolarizations at pH_o 5.0 and 6.0 but had no effect at pH_o 6.9 (Table 1). Pretreatment of the slices with mannose, which sharply reduces the ATP content of scutellum slices while only slightly increasing the ADP content [25], had no effect on membrane potential (Table 1). In *Chlorella*, membrane potential (measured with TPP$^+$) also was relatively insensitive to energy poisons [18], and in *Lemna*, energy poisons had no effect on membrane potential (measured with a microelectrode) although they greatly reduced ATP levels [23]. Cells of maize scutellum [2], *Chlorella* [18] and *Lemna* [13] contain electrogenic, sugar–proton cotransport systems, and perhaps could maintain membrane potentials via sugar gradients in the absence of ATP. Nevertheless, energy poisons that act as protonophores would be expected to cause considerable depolarization.

Effect of pH_o *on cytoplasmic* pH *and protonmotive force*

Previously it was shown that the cytoplasmic pH of scutellum cells is 8.0 and is independent of pH_o in the range 3–8 [11]. In the present experiments, scutellum slices were incubated for 200 min in buffer plus 5 μM TPP$^+$ and then cytoplasmic pH was estimated from the distribution of DNP between slices and bathing solution [11]. The long incubation in buffer and the presence of TPP$^+$ had no effect on cytoplasmic pH, which remained at 8.0. The calculated pH gradients and protonmotive forces ($\Delta\rho$) are plotted in Fig. 3 as functions of pH_o. Values of $\Delta\rho$ range from 340 mV at pH_o 3 to 163 mV at pH_o 7.6.

The estimates of electrical potential and cytoplasmic pH are averages of the values in the various cell compartments, whereas values of the bulk cytosol are needed for equation (1). Because of the low pH of the scutellum vacuoles, they have a negligible influence on DNP distribution and are ignored in calculating pH_i [11]. Therefore, the estimated pH_i of 8.0 is an average value that includes cytosol and organelles. When scutellum cells are treated with mannose, the pH_i falls to 7.5, but it is not influenced by pH_o. It was suggested previously [11] that pH_i after mannose treatment (7.5) is that of the cytosol, mannose having abolished the pH gradient between mitochondrion and cytosol. If this is so, the ΔpH and $\Delta\rho$ values in Fig. 3 should be decreased by 30 mV. The electrical potential across the inner membrane of the mitochondrion of higher plants has been reported to be *ca* 120 mV, inside negative [26], whereas only small potential differences appear to exist across the tonoplast (e.g. 25 mV, inside positive [20]). To some extent, errors in estimating the

Table 1. Effects of DNP (10^{-4}M) and mannose on membrane potential*

Final pH_o	Membrane potential (mV) Control	+ DNP	+ Mannose	% of control + DNP	+ Mannose
5.0	76.8	57.0	75.3	74	98
6.0	120.2	105.0	115.2	87	96
6.9	140.1	138.6	141.0	99	101

*Data are averages from two experiments. DNP (0.1 mM) was added to the [^{14}C]TPP$^+$ solution. Slices were pretreated with 50 mM mannose for 60 min (second preliminary incubation, cf. Experimental) and then placed in a mannose-free [^{14}C]TPP$^+$ solution.

potential across the plasmalemma introduced by the membrane potentials of mitochondrion and vacuole would compensate for one another, but because of the proportionately large volume of the vacuoles, the plasmalemma potentials of Fig. 3 are probably slightly underestimated. More reliable estimates of plasmalemma potentials would require a microelectrode positioned in the cytosol of these multivacuolate cells.

Sucrose gradients and protonmotive force

Scutellum slices were placed in buffer at pH 4 or 7 and incubated for 5 hr. Initially, there was a net exit of sucrose followed after 1–1.5 hr by a net uptake (Fig. 5). When slices were removed after 1–2 hr, washed and replaced in fresh buffer, a similar 'overshoot' of sucrose exit occurred and this could be repeated at least once again, although the heights of the peaks declined each time (data not shown). The 'overshoot' suggests that a small sucrose reservoir with a leak exists in the slices. At pH 4, a constant sucrose concentration in the bathing solution $[S]_o$ was attained even though the tissue concentration $[S]_i$ continued to decline (Fig. 5). At pH 7 (final pH 6.8), $[S]_o$ did not reach a constant level and became too low to measure (glucose oxidase–peroxidase–dye method) when the incubation time exceeded 5 hr. Therefore, at pH 6.8, $[S]_o$ and $[S]_i$ values at 5 hr were used to calculate sucrose gradients (Table 2) which are on the small side since $[S]_o$ was declining at a proportionally greater rate than $[S]_i$. Sucrose gradients at pH 4 were calculated from the $[S]_i$ at the time $[S]_o$ became constant (Fig. 5). Clearly, the sucrose gradients of Table 2 are not equilibrium gradients (see below).

In the sucrose gradient experiments, pH_i and membrane potential were measured on separate groups of slices. Distributions of [^{14}C]TPP$^+$ similar to those of Fig. 1 were obtained, and constant equilibrium distributions were maintained to the end of the 5 hr period. After 5 hr at pH_o 4 or 6.8, pH_i remained at 8.0.

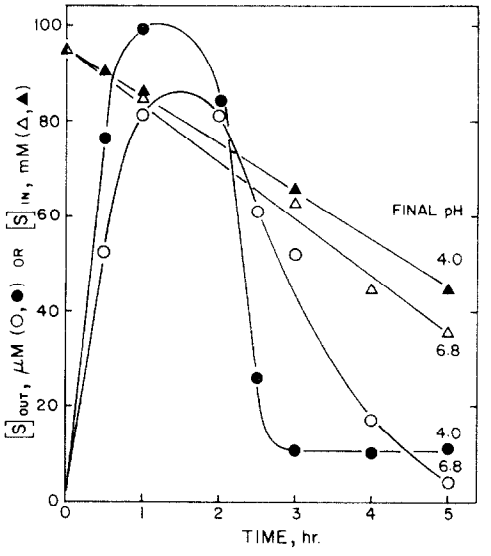

Fig. 5. Sucrose concentration of bathing solution $[S]_o$ and scutellum slices $[S]_i$ as a function of time of incubation at pH 4 and 6.8. Data from a single experiment (see Table 2).

The sucrose gradients at pH_o 4 and 6.8 are given in Table 2 as simple ratios or converted into mV (i.e. 60 log $[S]_i/[S]_o$). If sucrose–proton cotransport is the only sucrose transport system in the scutellum, then according

Table 2. Sucrose and proton gradients across the plasmalemma*

pH_o	$[S]_i$ (mM)	$[S]_o$ (μM)	$[S]_i/[S]_o$	60 log $[S]_i/[S]_o$ (mV)	$\Delta\rho$ (mV)
4.0	63.6	11.0	5782	226	305
4.0	53.6	15.0	3573	213	310
6.8	35.9	4.7	7638	233	210
6.8	36.1	4.0	9025	238	212

*Gradients were calculated on the assumption that sucrose and TPP$^+$ were uniformally distributed in the cell water, and therefore are said to be across the plasmalemma. Data are from two experiments. Time courses of $[S]_i$ and $[S]_o$ from one experiment are shown in Fig. 5. pH_i was 8.00 ± 0.05 after 5 hr at both pH_os. Membrane potentials were *ca* 65 mV at pH 4 and 140 mV at pH 6.8 (cf. Fig. 3).

to equation (1), the chemical potential gradients of sucrose and protons should be equal but opposite in sign. However, equation (1) holds only for equilibrium conditions, and the sucrose gradients were not in equlibrium. Nevertheless, if it is assumed that the cytosolic sucrose concentration was constant in these experiments (the vacuoles serving as a sucrose reservoir) and that the sucrose fluxes across the plasmalemma were at or near the steady-state, some interesting conclusions can be drawn by applying equation (1).

At pH 4, the constant $[S]_o$ attained after 3 hr (Fig. 5) may indicate that the cytosolic sucrose concentration also was constant and that steady-state conditions prevailed across the plasmalemma. From equation (1), a cytosolic sucrose concentration of 1.1 M would be required to balance the measured $\Delta\rho$. If pH_i is taken as 7.5 instead of 8 (see above), the required cytosolic sucrose concentration would be 0.42 M. These values are unrealistic because the slices have a cytosol volume of 40 μl/0.5 g [11] and do not contain enough sucrose for such high concentrations. Apparently, 'leak' pathways in the plasmalemma prevent build-up of sucrose gradients to match $\Delta\rho$. Alternatively, sucrose may exit on the cotransport porter without a proton, a 'slip' reaction [27].

At pH 6.8, a constant $[S]_o$ was not attained. Either the cytosolic sucrose concentration was falling or $[S]_o$ had not yet come to the steady-state with a constant cytosolic sucrose concentration. Assuming that the $[S]_o$ values at pH 6.8 (Table 2) are correct, a cytosolic sucrose concentration of 12.6 mM (4 mM if the vaue of pH_i is taken as 7.5) would be required to match the measured $\Delta\rho$, according to equation (1). This concentration would be less than one-third of the vacuolar concentration. These calculations suggest that sucrose gradients exist across the tonoplast. If the 'leak' or 'slip' pathways postulated from the results obtained at pH 4 also were present at pH 6.8, the cytosolic sucrose concentration would be smaller and the sucrose gradient across the tonoplast would be greater. Previously, it was shown that energy poisons cause release of stored sucrose (presumably, vacuolar) into the cytosol of scutellum cells [14, 28]. This is consistent with the suggestion that sucrose can be accumulated in scutellum vacuoles against a concentration gradient. A direct demonstration of such accumulation has been made using isolated vacuoles from storage tissue of red beet which accumulate sucrose in the presence of ATP [29, 30].

A simple working hypothesis to explain these results (Fig. 5, Table 2) includes the following: (a) cytosolic sucrose concentration is maintained constant by transport of sucrose across the tonoplast; (b) cytosolic sucrose concentration is independent of pH_o; (c) 'leak' and/or 'slip' pathways for sucrose as well as a sucrose–proton cotransporter are present in the plasmalemma; (d) sucrose fluxes through the three pathways are influenced by pH_o which thereby influences $[S]_o$.

EXPERIMENTAL

Preparation of scutellum slices. Maize grains (*Zea mays* L. cv Funks 4949A) were soaked in running tap H_2O for 24 hr, then placed on moist paper towels and grown in the dark at 24–25° for 72 hr. Scutella were excised and cut transversely with a razor blade into slices of 0.5 mm or less in thickness (3–7 cells in thickness [31]). Slices were washed in H_2O until washings were clear, blotted on filter paper, and weighed in groups of 0.5 g.

Incubation conditions. Each group of slices was placed in a 50 ml beaker to which 10 ml H_2O or experimental soln was added as described below. Solns and wash H_2O were removed from the beakers with a Pasteur pipette connected to a H_2O aspirator pump. Incubations were carried out on a water-bath at 30°. Slices were incubated in H_2O for 30 min, rinsed once with H_2O and then incubated for 60 min in buffer. The buffers (20 mM) were the Na^+ salts of citric acid (pH 3, 4, 5) MES (pH 6), MOPS (pH 7) and EPPS (pH 8). At the end of these preliminary incubations, the slices were rinsed with 10 ml buffer, the beakers were wiped dry, and a filter paper wick was inserted under the pile of slices to remove the surface soln.

Measurement of membrane potential. Membrane potential was estimated from the distribution of tetra[U-^{14}C]phenyl phosphonium ions (TPP^+) between scutellum slices and bathing soln using the Nernst equation:

$$\Delta\psi = 60 \log [[^{14}C]TPP^+]_i/[[^{14}C]TPP^+]_o. \quad (2)$$

Groups of washed slices from the preliminary incubations were incubated in 10 ml buffer (20 mM) containing $[^{14}C]TPP^+$ (0.1 μCi) and various concns of $[^{12}C]TPP^+$. Portions (0.1 ml) of the bathing soln were removed and placed on planchets immediately after adding the ^{14}C soln and at intervals thereafter over a 3–5 hr period. The ^{14}C content of the planchets was measured using a low-background, Geiger counting system.

Measurement of cytoplasmic pH. Cytoplasmic pH was estimated from the distribution of 2,4-DNP between bathing soln and scutellum slices by a previously described procedure [11].

Measurement of sucrose. Each expt used 14 groups of slices, 7 at pH 4 and 7 at pH 7. One group from each pH was killed in boiling 80% EtOH after the preliminary incubations, and the other groups were incubated in 10 ml 20 mM buffer. At intervals over a 5 hr period, two 1.0 ml portions of the bathing soln were removed from one beaker at each pH, and the slices in that beaker were removed and killed in boiling 80% EtOH. Sucrose contents of bathing soln and extract were determined as previously described [32].

Calculation of internal concentrations of TPP^+ and sucrose. The slices contained 70% H_2O (350 μl/0.5 g). In calculating internal concs it was assumed that 80% (280 μl) of the tissue H_2O was inside the plasmalemma in which TPP^+ and sucrose were uniformly distributed. From measurement of the glucose space of the slices [11], it was estimated that the vol. of the cytosol was 40 μl/0.5 g or 14.3% of the vol. inside the plasmalemma.

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