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Energetics of Sucrose Transport into Protoplasts from Developing Soybean Cotyledons[1]

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ABSTRACT

The accumulation of tetraphenylphosphonium (TPP^+), 5,5′-dimethyloxazolidine-2,4-dione (DMO), and a micro pH electrode were used to measure membrane potential, intracellular and extracellular pH, respectively, upon the addition of exogenous sucrose to soybean cotyledon protoplasts. Addition of sucrose caused a specific and transient (a) depolarization of the membrane potential (measured by TPP^+ accumulation), (b) acidification of the intracellular pH (measured by DMO accumulation), and (c) alkalization of the external medium (measured by a micro pH electrode). The time course for all these changes was similar (*i.e.* 5 to 10 minutes). Based on the rate of sucrose uptake and alkalization of the external medium, a stoichiometry of 1.02 to 1.10 for proton to sucrose was estimated. These data strongly support a proton/sucrose cotransporting mechanism in soybean cotyledon cells.

Kinetic analyses of sugar uptake into protoplasts isolated from a rapidly growing soybean cotyledon revealed that at low external sugar concentrations, sucrose is preferentially transported over glucose (14, 22). This difference in sugar uptake is mainly due to the presence of a saturable uptake component for sucrose but not for glucose (14, 22). The responses of sucrose uptake into protoplasts (14, 22) and excised intact soybean cotyledons (9, 26) to the external pH, temperature, and several metabolic inhibitors suggested a proton cotransport mechanism in sucrose uptake, but the evidence was circumstantial at best. Electrogenic sucrose/proton transport predicts that the addition of sucrose could cause (a) a transient depolarization of the membrane potential because of the initial influx of the charged proton, (b) a transient acidification of the internal cytoplasm resulting from proton entry, and (c) alkalinization of the external medium due to proton disappearance from the medium. These changes should both be transient (because of the re-establishing of the proton gradient by an active proton pump) and specific for sucrose.

In this study, we used TPP^+[2] (2, 6, 7, 12, 18–21, 27), DMO (3, 6, 24, 25), and external pH monitor (6–8, 17, 18, 31) to show that these predictions are met for sucrose transport into protoplasts isolated from developing soybean cotyledons. The stoichiometry of sucrose and proton in the system was also estimated. The results strongly support a sucrose/proton cotransport in soybean cotyledons.

MATERIALS AND METHODS

Protoplasts were isolated from developing soybean (*Glycine max* L. Merr cv Wye) cotyledons as described previously (14) and suspended for all experiments in a basic medium of 0.5 M sorbitol, 10 mM $CaCl_2$, and 25 mM Mes-KOH (pH 6.0). Sucrose uptake into the protoplasts was also according to Lin *et al.* (14) except as noted.

The electric potential difference between the protoplasts and the surrounding medium (membrane potential of the protoplast) was calculated using the Nernst equation (15) from the distribution of TPP^+ between protoplasts and medium. After incubation with labeled [^{3}H]TPP^+ (diluted with 8 μM unlabeled TPP^+, final specific radioactivity 125 μCi/mmol) for various times, aliquots of protoplast suspension were placed onto a silicone oil microfuge tube (11, 14) and protoplasts were separated from the medium by a 30-s centrifugation. Radioactivity and protein content of protoplast pellets were then measured. To estimate the internal volume of the protoplasts in each aliquot, 'water space' and 'mannitol space' were measured after simultaneous incubation with [^{14}C]mannitol and ^3H_2O (both from New England Nuclear) as previously described (28).

Intracellular pH of the protoplasts following DMO incubation was calculated according to the following equations (16, 20)

$$pH_i = pK_i^{DMO} + \log\left[\frac{C_i}{C_o}\left(10^{pH_o - pK_o^{DMO}} + 1\right) - 1\right] \quad (1)$$

and

$$\frac{C_i}{C_o} = \frac{^{14}\text{C taken up}}{^{14}\text{C administered}} \times \frac{V_o}{V_i} \quad (2)$$

Where '*i*' and '*o*' denote the inner and outer phases, respectively, '*C*' is the concentration of H-DMO + DMO^-, and '*V*' is the volume. Also pK_i^{DMO} is assumed to be equal to pK_o^{DMO}. DMO concentration was calculated from the amount of DMO accumulated after protoplasts were incubated with labeled [^{14}C] DMO (diluted with 20 μM unlabeled DMO, final specific radioactivity 50 μCi/mmol) for various times. Aliquots of protoplasts suspension were placed on a silicone oil microfuge tube and centrifuged as described above. Internal volume was also determined as described above.

Exchange of TPP^+ or DMO accumulated within the protoplasts with external solution was measured by incubating protoplasts in [^{3}H]TPP^+ or [^{14}C]DMO solution as described above for 40 and 10 min, respectively, and transferring protoplasts into 25 ml of 8 μM unlabeled TPP^+ or 20 μM unlabeled DMO solution.

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[2] Abbreviations: TPP^+, tetrophenylphosphonium ion; DMO, 5,5′-dimethyl-oxazolidine-2,4-dione; DNP, 2,4-dinitrophenol; FCCP, *p*-trifluoromethoxy-carbonylcyanide phenylhydrazone; 3-O-MG, 3-*O*-methyl-D-glucose; PCMBS, *p*-chloromecuribenzenesulfonic acid; 1799, bis-(hexafluoroacetonyl)acetone.

Suspensions were taken at each interval to measure the radioactivity and protein content of the protoplasts. The radioactivity released to the external medium was also measured.

External pH changes were monitored by a MI-710 micro combination pH probe (Microelectrodes, Inc., NH), an Orion model 901 Microprocessor Ionalyzer, and a strip chart recorder. The protoplast sample chamber was described by Zamski and Wyse (manuscript in preparation). Stoichiometry of H^+/sucrose was calculated by measuring ^{14}C sucrose influx and the rates of alkalization of the external pH changes.

Protoplast respiration was measured as previously described (11). TPP^+ was purchased from Aldrich, DMO from Sigma, and other chemicals were ACS reagent grades. All radiochemicals were from New England Nuclear.

RESULTS AND DISCUSSION

Measurement of Membrane Potential by TPP^+. Although lipophilic cations have been used successfully to evaluate membrane potentials of animal cells (10) and isolated membrane vesicles from bacteria (23), their applicability in estimating membrane potentials in plants have been questioned recently (4) due to large binding of lipophilic cations with cell walls and other components. Isolated protoplasts eliminate a large portion of the bound lipophilic cation and improve the applicability of these cations to estimate membrane potentials of the protoplasts (12, 19, 21).

Gimmler and Greenway (4) and Harold and Papineau (5) suggested there are four essential prerequisites for the applicability of using TPP^+ to estimate the membrane potential in plant and bacterial cells, respectively. They are: (a) cellular TPP^+ concentration should reach equilibrium with time; (b) TPP^+ molecules should be freely moving across the membrane, thus a complete exchange of cellular and external TPP^+ molecules should occur; (c) TPP^+ should not affect metabolic reactions which are related to the membrane potential maintenance in the cell; and (d) there should be a substantial difference in the amount of TPP^+ accumulated into living and dead (or energetic and nonenergetic) cells. Figure 1 shows that TPP^+ accumulation in protoplasts isolated from developing soybean cotyledons reached a maximal value within 50 min of incubation. When all metabolism is inhibited by treating protoplasts with FCCP plus O°C, TPP^+ accumulation is markedly decreased (~90% inhibition). The TPP^+ accumulated in these inhibited cells probably represents the binding of TPP^+ molecules to membrane and other cell components. At 30°C, the uncoupler FCCP and several other metabolic inhibitors partially depolarized the membrane potential of the cell (Fig. 1). FCCP and other metabolic inhibitors have been shown to decrease membrane potential to the diffusion potential level (13 and references therein) which is 40 to 60% of the total membrane potential. Figure 1 also shown at concentrations tested, DNP, Na arsenate, and NaN_3 are not as effective as FCCP or Na cyanide in depolarizing membrane potentials of the protoplasts. By subtracting the 'binding component' of TPP^+ on the membranes from the total TPP^+ accumulated in the cell, it was estimated the membrane potential of isolated soybean cotyledon protoplasts was −50 to −55 mv. The electrogenic component which is sensitive to FCCP and other metabolic inhibitors is 20 to 25 mv.

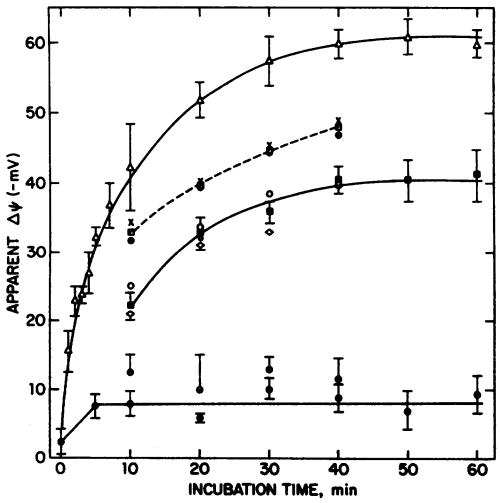

FIG. 1. Time course of $[^3H]TPP^+$ accumulation and the apparent membrane potentials of isolated soybean cotyledon protoplasts, (Δ), control; (●——●), 0°C + 15 μM FCCP; (⊖), °C; (●), 50 μM DNP; (▨), 1 mM $Na\text{-}N_3$; (×), 1 mM Na-arsenate; (■), 15 μM FCCP; (◇), 15 μM 1799; (○), 1 mM Na-cyanide.

After protoplasts were allowed to accumulate $[^3H]TPP^+$ for 40 min, most of free $[^3H]TPP^+$ in the cell can be exchanged with external unlabeled TPP^+ (Fig. 2). TPP^+ concentration up to 20 μM caused no detectable change of protoplast respiration for at least 40 min. Also, addition of less then 20 μM of TPP^+ in the uptake solution did not affect the amino acid influx into the protoplasts (Table I). However, pretreatment of protoplasts with TPP^+ for 40 min before the uptake of sugar and amino acid caused a 20 to 50% increase of the influx of solutes (Table II).

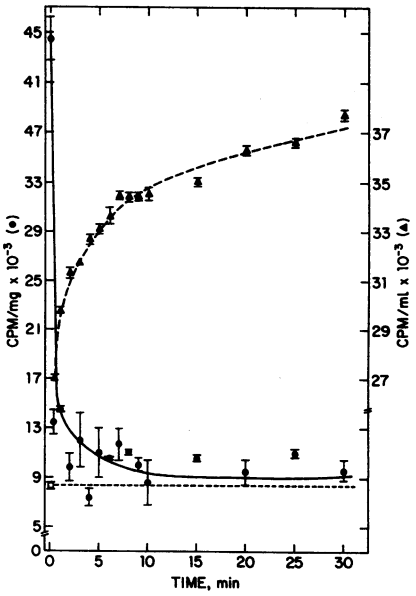

FIG. 2. Time course of the exchange of TPP^+ molecules between protoplasts and external solution. Protoplasts were preincubated with $[^3H]TPP^+$ for 40 min and transferred at time 0 to 25 ml of 8 μM unlabeled TPP^+ solution. (○), Radioactivity remaining in protoplasts; (Δ), radioactivity released into the external solution. The basal dashed line indicates the radioactivity level in the cold- and FCCP-treated protoplasts.

Table I. *Effect of TPP^+ on Glutamine Uptake into Protoplasts Isolated from Developing Soybean Cotyledons*

TPP^+ was added in the uptake solution. Glutamine uptake was measured with 1 mM glutamine (+ $[^{14}C]$glutamine) at pH 6.0 and 30°C for 20 min.

	Glutamine Uptake	
	nmol/10^6 protoplasts·h	%
Control	2.30 ± 0.16	100
8 μM TPP^+	2.30 ± 0.31	100
20 μM TPP^+	2.02 ± 0.25	88
50 μM TPP^+	1.72 ± 0.10	75

Table II. *Effect of TPP⁺ Pretreatment on Sugar and Amino Acid Uptake into Protoplasts Isolated from Developing Soybean Cotyledons*

Sugar and amino acid uptake were measured with 1 mM sugar or amino acid (+ corresponding ^{14}C labeled substrate) at pH 6.0 and 30°C for 20 min.

	Control	TPP-Treated[a]	Δ
	nmol/10⁶ protoplasts·h		%
Sucrose	8.34 ± 2.88	13.04 ± 3.72	156
3-O-MG	4.71 ± 1.21	6.76 ± 1.38	144
Glutamine	6.33 ± 1.97	7.78 ± 3.16	123
AIB	2.35 ± 0.75	3.19 ± 0.76	136

[a] Protoplasts were preincubated in 8 μM TPP⁺ sorbitol buffer medium for 40 min before being transferred to TPP⁺ containing uptake solution.

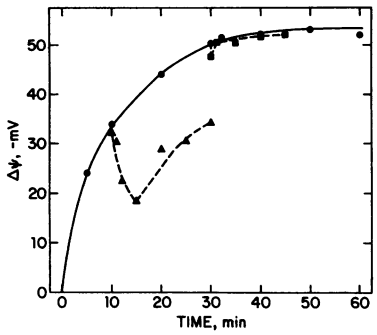

FIG. 3. Effect of exogenous sucrose on the apparent membrane potential of isolated protoplasts. The apparent membrane potentials were calculated from: the accumulation of TPP with time in protoplasts in the absence of sucrose (●); 10 min TPP accumulation in the presence of 1 mM sucrose plus 0 to 20 min 1 mM sucrose pretreatment of protoplasts (▲); or 30 min TPP accumulation in the presence of 1 mM sucrose plus 0 to 15 min 1 mM sucrose pretreatment of protoplasts (■). Time in *x* axis is the duration of TPP accumulation or TPP accumulation plus sucrose pretreatment.

These data showed that the four essential prerequisites for using TPP⁺ to estimate membrane potential (4, 5) have been met for protoplasts isolated from developing soybean cotyledons. By subtracting a relatively small amount of bound TPP⁺, TPP⁺ distribution was used to estimate membrane potentials of protoplasts. Although the calculated membrane potentials are lower than those reported for excised cotyledon tissue (9) using microelectrodes, the values reported here are similar to most membrane potential values reported in several isolated protoplasts measured either with TPP⁺ or microelectrodes.

Sucrose-Dependent Depolarization. After protoplasts were pretreated with 1 mM sucrose for various time periods in a 0- to 30-min range, a 10- or 30-min TPP⁺ accumulation period was applied to calculate the concentration of cytoplasmic TPP⁺ concentration and thus the membrane potentials. Figure 3 shows a time course of the membrane potential change upon the addition of exogenous sucrose. A 20-mv depolarization of protoplast membrane potential lasting 5 to 10 min was observed upon the addition of 1 mM exogenous sucrose. Treating protoplasts with exogenous sucrose for longer than 30 min did not cause any change of membrane potential, thus demonstrating that the change of membrane potential with sucrose influx is transient. Results are consistent with those reported for the excised whole soybean cotyledons (9).

When protoplasts were pretreated with different concentrations of sucrose for 5 min and then presented with TPP⁺ for 10 min, a biphasic concentration curve of membrane potential change *versus* sucrose concentration was shown (Fig. 4). This biphasic curve consisted of saturable and linear components similar to those observed in the sucrose influx in these same

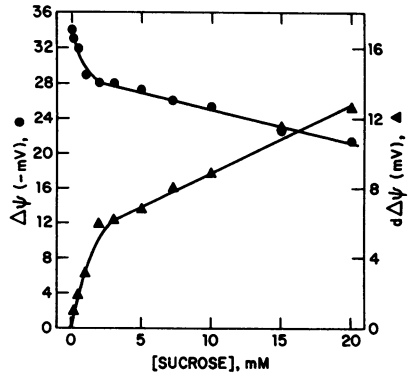

FIG. 4. Concentration (kinetic) curve of sucrose-induced relative transient depolarization of membrane potentials in isolated protoplasts. Protoplasts were pretreated with different concentrations of exogenous sucrose for 5 min which were followed by a 10-min accumulation of [³H]TPP⁺. Apparent membrane potentials were then calculated (●). Difference between the 0 sucrose and each different sucrose pretreatment (▲) is equal to relation values of the sucrose-depolarized membrane potentials.

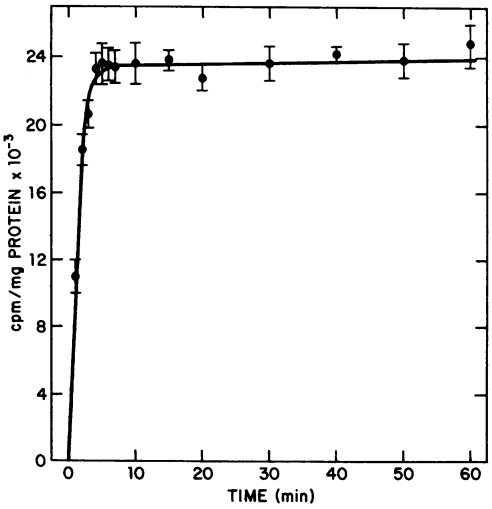

FIG. 5. Time course of [¹⁴C]DMO uptake by isolated protoplasts.

protoplasts (14, 22). Due to the uncertainty in the calculated membrane potentials, no attempts were made to define the rate of sucrose uptake as a function of membrane potential in the course of sucrose influx.

Measurement of Intracellular pH Using DMO. The weak acid, [¹⁴C]DMO, has been used as a chemical probe to estimate the intracellular pH in plant cells (3, 6, 24, 25). Again, controversial statements concerning the accuracy of the pH value obtained through this technique have been raised (16, 20). In isolated *Asparagus* mesophyll cells, a rapid equilibration of [¹⁴C]DMO between the cells and external medium was reached (3). Recently, DMO has further been used to monitor the change of intracellular pH in responding to the change of external pH in *Chara corallina* (24). The fast distribution of [¹⁴C]DMO between the external medium and plant cells may be useful in monitoring the change of intracellular pH during the course of sucrose influx in isolated protoplasts.

The use of [¹⁴C]DMO to determine intracellular pH is based on the assumption that DMO is an inert substance to the cell and the undissociated acid, H-DMO, is transported passively across biological membranes and that the membrane is impermeable to the DMO^- anion (1, 16, 20). This requires the establishment of passive equilibrium of DMO distribution during

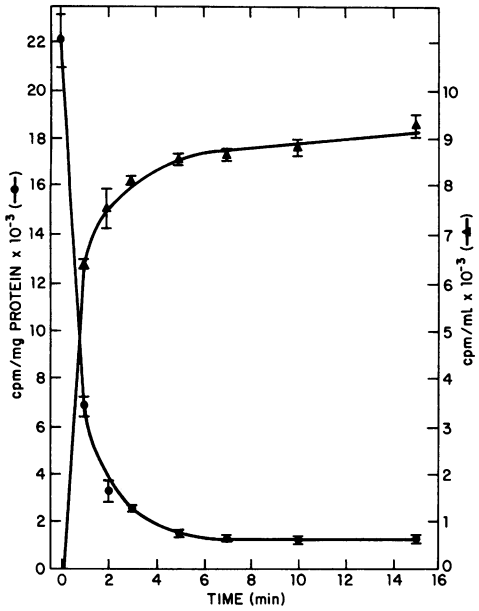

FIG. 6. Time course of the exchange of DMO molecules between the protoplast and external solution. Protoplasts were preincubated with [^{14}C]DMO for 10 min and transferred to 25 ml of 20 μM unlabeled DMO at time 0, (●). Radioactivity remaining in the protoplasts; (▲), radioactivity released to the external solution.

incubation of a tissue with [^{14}C]DMO outside (C_i/C_o). As shown in Figure 5, [^{14}C]DMO inside reached a plateau within 5 min. A similar fast equilibrium of [^{14}C]DMO distribution was also observed in *Asparagus* mesophyll cells (3) and mitochondria (1). Also, a fast and near complete exchange of DMO in the cell with the external DMO molecules was observed in isolated soybean cotyledon protoplasts (Fig. 6). This is consistent with the notion that DMO is freely transported across the biological membranes (1, 23, 25, 29, 30). Furthermore, addition of up to 40 μM DMO in the protoplast suspension did not affect protoplast respiration.

To measure the effect of sucrose influx on intracellular pH, a 5-min accumulation of [^{14}C]DMO was measured in protoplasts which had been pretreated with 1 mM sucrose for different lengths of time (0–15 min). Intracellular pH was then calculated according to equation 1. The time course of this change is shown in Figure 7. A 5- to 10-min transient decrease of intracellular pH of about 0.2 pH unit was observed in soybean cotyledon protoplast upon the addition of 1 mM exogenous sucrose. The same concentration of 3-O-MG did not induce any intracellular pH change, even though 3-O-MG was accumulated by the protoplasts (14, 22). PCMBS, which inhibits sucrose influx (10, 22, 26), abolished this transient acidification of the intracellular pH.

DMO uptake decreased with increasing external pH (from pH 4.0 to pH 8.0) (Fig. 8A). Using equation 1, the effect of external pH on the intracellular pH was then estimated (Fig. 8B). A biphasic intracellular pH change upon changing of external pH was observed. Increasing external pH from 4.0 to 6.0 resulted in an increase of intracellular pH from 6.67 to 6.69. The intracellular pH jumped to pH 7.55 after the external pH was further increased to pH 8.0. This biphasic intracellular pH change upon the change of external pH was also observed in *Chara corallina* (24).

Figure 8B also shows that 1 mM exogenous sucrose induced a greater transient acidification of the intracellular pH at lower external pH than that at higher external pH. A greater than 0.3 pH unit change was calculated at pH 4.0 in response to the addition of 1 mM exogenous sucrose. This pH change decreased linearly to ≤0.05 pH unit at 8.0 external pH. This decrease of intracellular pH change correlated well with the decrease of

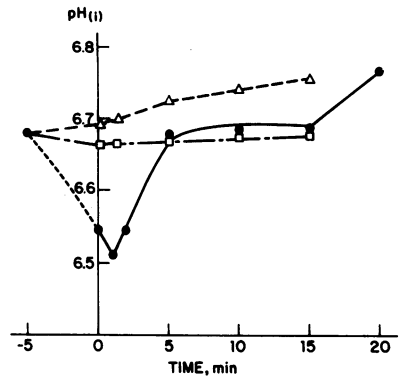

FIG. 7. Effect of exogenous sucrose on the intracellular pH of isolated protoplasts. pH_i at −5 min is the intracellular pH calculated from the accumulation of DMO in protoplasts in the absence of sucrose. Other values were calculated after protoplasts were preincubated in 1 mM sucrose (●), 1 mM 3-O-MG (□), or 0.2 mM PCMBS + 1 mM sucrose (△) at pH 6.0 for 0 to 15 min, in which [^{14}C]DMO was then added. Five min later, protoplasts were separated from the medium and DMO accumulation was measured. Time in x axis is the duration of sucrose pretreatment plus 5 min DMO accumulation.

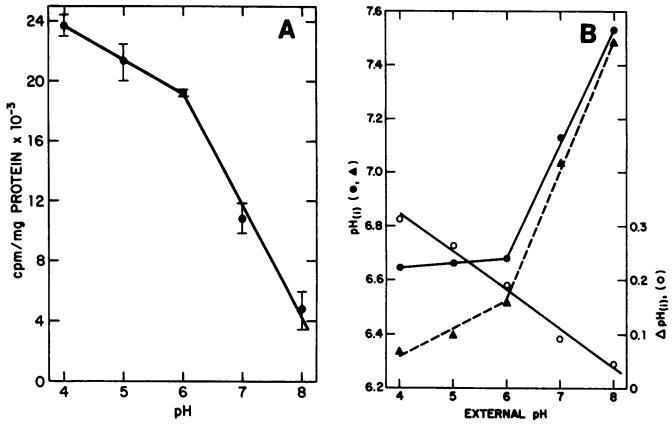

FIG. 8. A, Effect of external pH on the [^{14}C]DMO accumulation in isolated protoplasts. B, Effect of external pH on the intracellular pH of isolated protoplasts with (▲) and without (●) 5 min of 1 mM sucrose pretreatment; (○), sucrose-induced intracellular pH changes (equal to the difference of with and without sucrose pretreatments).

sucrose influx with increasing external pH values.

Sucrose-Dependent Alkalizations. One of the most commonly used criteria for establishing the coupling of H^+ to the substrate influx is the occurrence of a transient alkalization of external media upon substrate addition (6–8, 17, 18, 31). To further demonstrate H^+/sucrose cotransport in isolated soybean cotyledon protoplasts, external pH changes were monitored upon the addition of exogenous sucrose. Due to a relatively high buffering of the protoplasts in the medium and a relatively lower sucrose influx (compared to the other systems (6, 8, 31), 5 mM exogenous sucrose was required to produce a measurable transient alkalization of the medium (Fig. 9). The transient alkalization is specific to sucrose since the same amount of mannitol failed to induce any pH change of the medium. This alkalization of the external medium lasted for about 10 min and a second addition of sucrose did not induce any further pH changes. These changes are consistent with most substrate-induced transient alkalization H^+ cotransporting systems (6, 8, 18, 31). Comparison of the initial rate of alkalization and sucrose influx gave a 1.02 to 1.10 H^+/sucrose ratio.

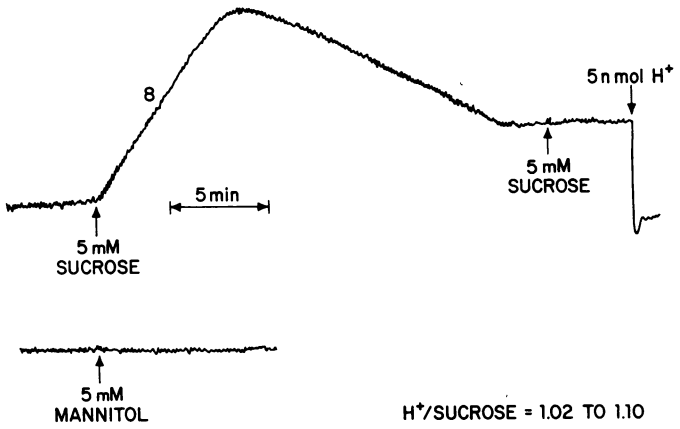

FIG. 9. Recorder traces of external pH changes of the protoplast suspension upon the addition of 5 mM sucrose or mannitol. Suspension media consisted of 0.5 M sorbitol, 10 mM $CaCl_2$, and about 5 million protoplasts at 20°C. Starting pH was 6.0. The numeral '8' above the pH trace was the initial net H^+ influx in nmol $H^+/10^6$ protoplasts·h. H^+/sucrose was the ratio of initial net H^+ influx (rate of alkalization) and sucrose influx when both rates were measured simultaneously.

CONCLUSION

Measurements of membrane potential, intracellular pH, and external pH in soybean cotyledon protoplasts following sucrose addition show that sucrose uptake into these protoplasts is coupled to the cotransport of protons. The removal of cell wall has greatly enhanced the applicability of TPP^+ and DMO as chemical probes to estimate membrane potentials and intracellular pH of plant cells, respectively. These chemical probes can further be used to monitor the changes of membrane potential and cytoplasmic pH upon the alteration of the cellular energetic status.

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