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Preparation of Membrane Vesicles Enriched in ATP-Dependent Proton Transport from Suspension Cultures of Tomato Cells

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FRANCES M. DUPONT*[1] AND MARIA DE GRACIA ZABALA[2]
ARCO Plant Cell Research Institute, 6560 Trinity Court, Dublin, California 94568

ABSTRACT

Membranes enriched in ATP-dependent proton transport were prepared from suspension cultures of tomato cells (*Lycopersicon esculentum* Mill cv VF36). Suspension cultures were a source of large quantities of membranes from rapidly growing, undifferentiated cells. Proton transport activity was assayed as quench of acridine orange fluorescence. The activity of the proton translocating ATPase and of several other membrane enzymes was measured as a function of the cell culture cycle. The relative distribution of the enzymes between the 3,000, 10,000, and 100,000*g* pellets remained the same throughout the cell culture cycle, but yield of total activity and activity per gram fresh weight with time had a unique profile for each enzyme tested. Maximal yield of the proton translocating ATPase activity was obtained from cells in the middle logarithmic phase of growth, and from 50 to 90% of the activity was found in the 10,000*g* pellet. The proton translocating ATPase activity was separable from NADPH cytochrome *c* reductase and cytochrome *c* oxidase on a sucrose gradient. Proton transport activity had a broad pH optimum (7.0–8.0), was stimulated by KCl with a K_m of 5 to 10 millimolar, stimulation being due to the anion, Cl^-, and not the cation, K^+, and was not inhibited by vanadate, but was inhibited by NO_3^-. The activity is tentatively identified as the tonoplast ATPase.

Since methods are available to obtain sealed vesicles from tonoplast or plasma membrane of plant tissues and to measure ATP-dependent proton transport *in vitro* (1–5, 10, 13, 16), it is reasonable to contemplate using the isolated vesicles to investigate the response of the plasma membrane or tonoplast to environmental stress. We investigated the effect of chilling temperature on suspension cultures of tomato cells (6) and planned to examine the effects of chilling temperatures on proton transport in membrane vesicles isolated from the suspension cultures (7). Prior to doing such studies, it was necessary to optimize the conditions for obtaining the proton translocating vesicles from tomato cells and to characterize the vesicles and determine their origin. Transport rates and enzyme activities of cells in culture are reported to vary greatly during a passage cycle. For example, enzymes of lipid metabolism (15), nitrate metabolism (8, 9), and carbohydrate metabolism (17), and rate of uptake and incorporation of thymidine and leucine (12) vary with time in passage. We therefore examined the effect of time in passage upon the activity and distribution of the proton translocating ATPase activity and that of other membrane-bound enzymes, in addition to characterizing the effect of assay conditions on enzyme activity.

MATERIALS AND METHODS

Cell Culture. Methods were as previously described (6). Callus was initiated from roots (growth curve experiment) or hypocotyls (all other experiments) of aseptic tomato seedlings (*Lycopersicon esculentum* Mill cv VF36) germinated on agar. Suspension cultures were initiated 2 months to 1 year after initiation of callus, and maintained for up to 1 year prior to the experiments shown. The cultures were maintained in 200 ml of medium in 500 ml flasks on a reciprocating shaker at 28°C. The medium (6) contained ammonium as its sole nitrogen source.

Membrane Preparation. Cells were harvested by vacuum filtration on Whatman No. 4 filter paper in a Büchner funnel, rinsed with an equal volume of distilled H_2O, and weighed. Cells were homogenized using either a mortar and pestle, or by blending with glass beads for 40 s in a Bead Beater Cell Homogenizer (Biospec Products, Bartlesville, OK). Similar results were obtained with either method, but the Bead Beater gave a much greater efficiency of cell breakage. The homogenization buffer consisted of 4 mM EDTA and 25 mM Tris, which together give a pH of 8.0, and 2 mM DTT in 0.25 M sucrose. The homogenate was filtered through two layers of Miracloth (Calbiochem), centrifuged at 3,000*g* for 5 min, 10,000*g* for 20 min, and 100,000*g* for 30 min. Membrane pellets were resuspended in a buffer consisting of 0.25 M sucrose and 5 mM Tris-HCl (pH 7.5). All operations were performed at 4°C in a cold room.

Preparations enriched in sealed vesicles were prepared by centrifugation onto a cushion of 10% 70 kD Dextran (4, 18).

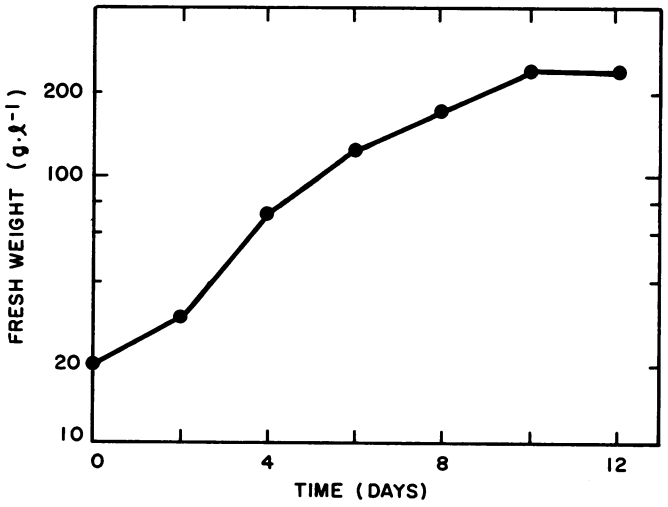

FIG. 1. Fresh weight of tomato cell cultures as a function of time in passage.

[1] Present address: USDA Western Regional Laboratory, 800 Buchannan Street, Albany, CA 94710.

[2] Present address: Department of Biological Sciences, Stanford University, Stanford, CA 94305.

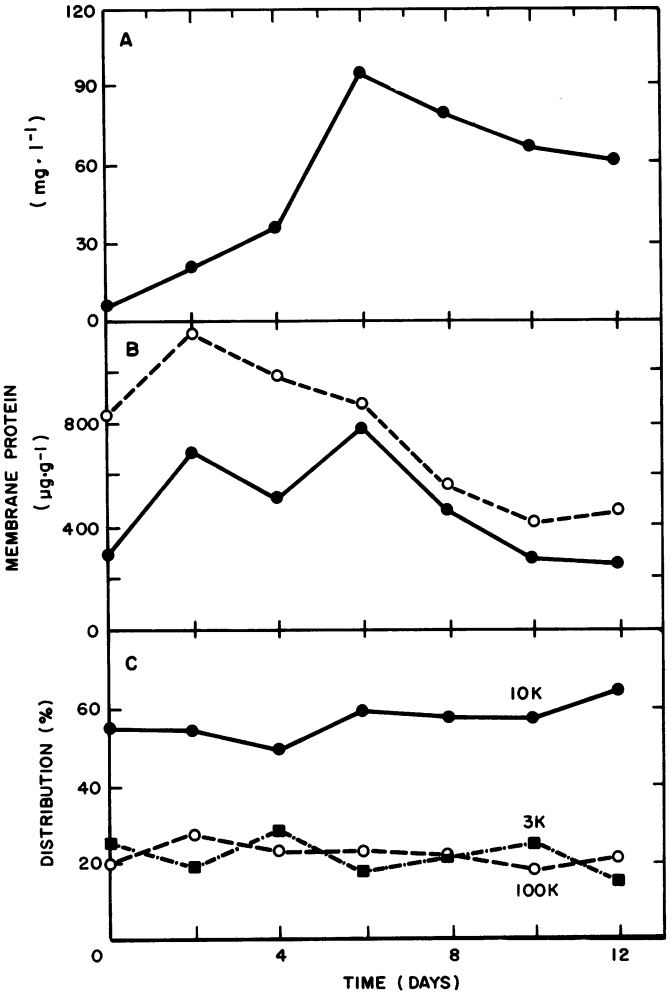

FIG. 2. Membrane protein as a function of time in passage. A, Total protein per liter of cell suspension, calculated as the sum of the protein in the three membrane fractions. B, Protein per g fresh weight (●); additional experiment (○). C, Distribution of protein between the 3,000 (■), 10,000 (●), and 100,000*g* (○) pellets.

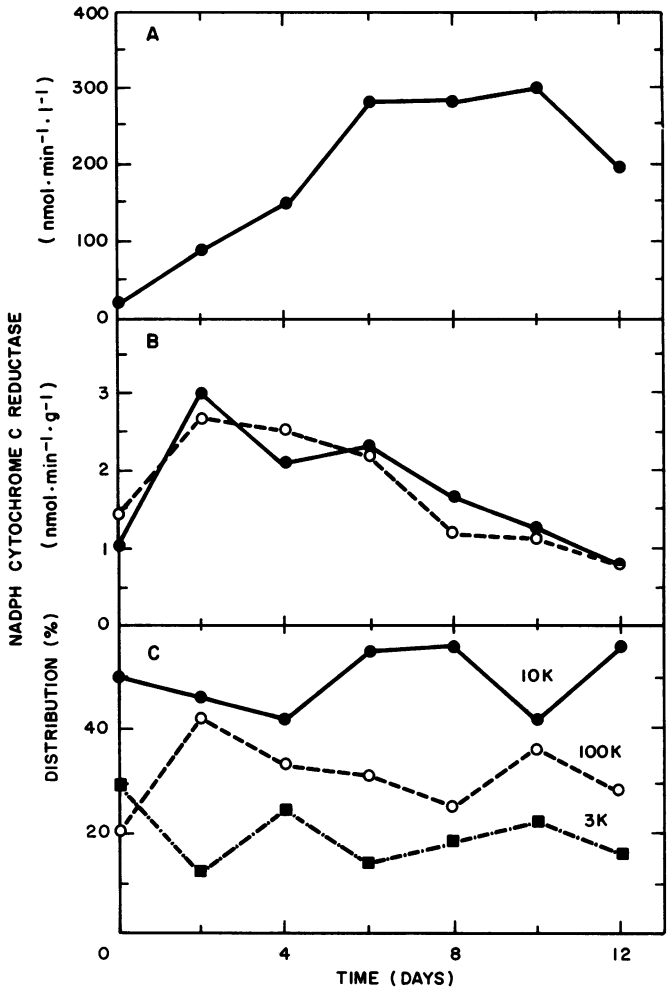

FIG. 3. NADPH-Cyt *c* reductase activity of cell membranes as a function of time in passage. A, Total activity per liter of cell suspension, calculated as the sum of the activity in the three membrane fractions. B, Activity per g fresh weight (●); additional experiment (○). C, Distribution of activity between the 3,000 (■), 10,000 (●), and 100,000*g* (○) pellets.

Growth Curve Experiments. Cells were inoculated into 600 ml of medium in a single 1 L flask, grown for 10 d to obtain the primary inoculum, then subcultured into a series of 500 ml Erlenmeyer flasks by a 1 to 10 dilution to a total of 250 ml of medium, and maintained at 28°C on a reciprocating shaker. Every other day, two flasks were harvested, fresh weight determined, and 10 g taken for the membrane preparations. The cells were ground with mortar and pestle, the homogenate filtered through Miracloth, and 3,000, 10,000, and 100,000*g* pellets prepared by differential centrifugation. A portion of each pellet was immediately assayed for proton transport, and the remainder was divided into aliquots and frozen at −70°C.

Assays. ATP-dependent proton transport was assayed by measuring the decrease in fluorescence of acridine orange (19) upon addition of ATP to a mixture of membranes, 0.25 M sucrose, 1 μM acridine orange, 1 mM $MgSO_4$, 50 mM KCl, and 10 mM Tris-HCl (pH 7.5) in 3.0 ml. The final concentration of ATP was 1 mM unless otherwise indicated. Fluorescence was assayed using a Perkin Elmer L5 spectrofluorometer, with excitation at 493 nm, emission at 528 nm, and slit widths at 5 nm. Cyt *c* oxidase, NADPH Cyt *c* reductase, uridine diphosphatase, and protein were assayed as previously described (4). All activities were assayed at room temperature, approximately 23°C. All biochemicals were purchased from Sigma.

RESULTS AND DISCUSSION

Published procedures for obtaining membrane vesicles enriched in ATP-dependent proton transport from corn seedlings and tobacco callus (5, 18) were tested on cells from tomato suspension cultures. When sealed vesicles were obtained from a 10,000 to 100,000*g* microsomal pellet by flotation on 10% Dextran and assayed for ATP-dependent fluorescence quench using 5 mM ATP:Mg (5) proton transport activity was barely detectable. Additional experiments indicated that the majority of the activity was not in the microsomal, but in the mitochondrial fraction, and also that concentrations of ATP:Mg above 1 mM inhibited transport. Good activities were obtained when the mitochondrial fraction was assayed using 1 mM ATP:Mg. We decided to examine the activity of the proton-translocating ATPase throughout a single passage cycle to determine if the sedimentation behavior of the activity was peculiar to one phase of the growth cycle, as well as to determine the optimum time to harvest the cells to obtain maximum yield of the proton transport activity. The activity of several other membrane marker enzymes was also followed during the cell cycle, to test whether marker enzymes for different membranes would have different patterns of activity during the cell cycle and to determine whether the pattern of proton transport activity was correlated with that of any other enzyme activity.

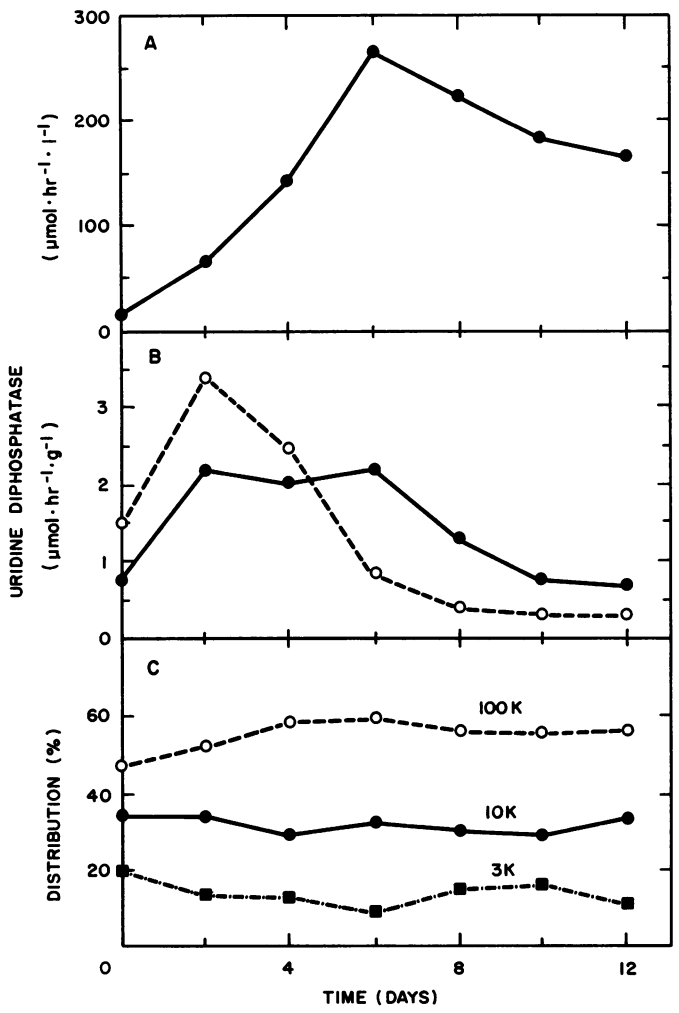

FIG. 4. Uridine diphosphatase activity of cell membranes as a function of time in passage. A, Total activity per liter of cell suspension, calculated as the sum of the activity in the three membrane fractions. B, Activity per g fresh weight (●); additional experiment (○). C, Distribution of activity between the 3,000 (■), 10,000 (●), and 100,000*g* (○) pellets.

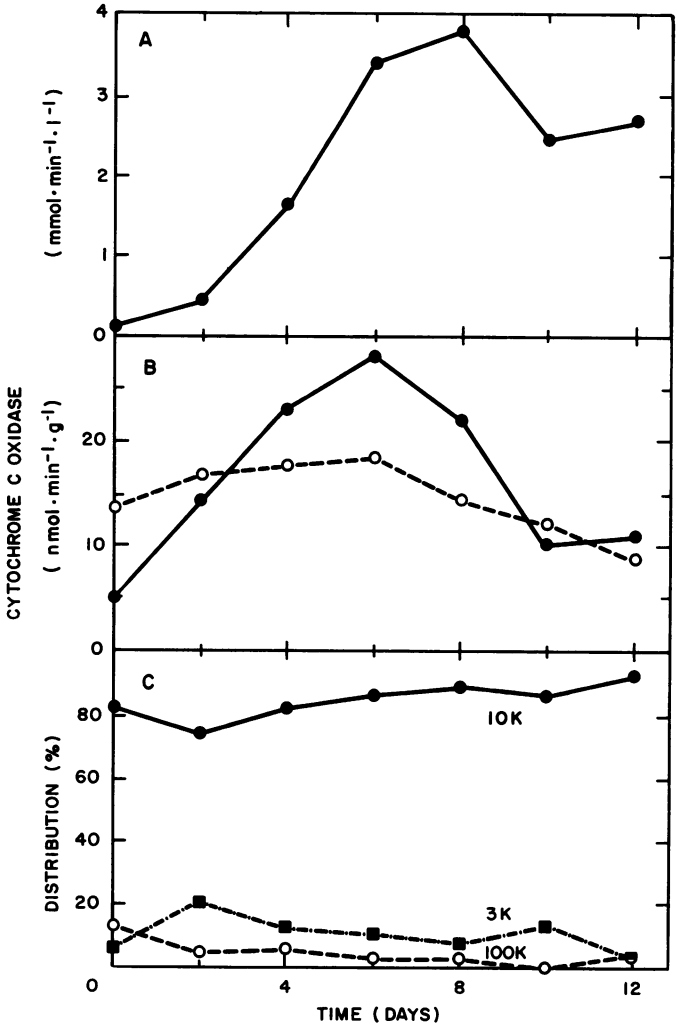

FIG. 5. Cyt *c* oxidase activity of cell membranes as a function of time in passage. A, Total activity per liter of cell suspension, calculated as the sum of the activity in the three membrane fractions. B, Activity per g fresh weight (●); additional experiment (○). C, Distribution of activity between the 3,000 (■), 10,000 (●), and 100,000*g* (○) pellets.

Growth Curve. Cells were grown and harvested as described in "Materials and Methods." Figure 1 shows the growth curve for the cells used for the experiment. The cells were homogenized with a mortar and pestle and the membranes were separated by a differential centrifugation into 3,000, 10,000, and 100,000*g* pellets. The pellets were assayed for protein, NADPH Cyt *c* reductase, UDPase, Cyt *c* oxidase, and ATP-dependent quench of acridine orange fluorescence. The results of the enzyme assays are shown in Figures 2 to 6. The top panel of each figure shows the total activity per liter of cell suspension, the middle panel shows activity per g fresh weight, and the bottom panel shows the relative distribution of activity between the three membrane pellets. The middle panel also shows the data for a separate experiment to indicate the reproducibility of the activities on a per g fresh weight basis. A similar distribution of activities was found for both experiments. Determinations of each activity were made separately, in triplicate, on the three pellets. Since the distribution of activities between the pellets did not change during the growth cycle, only the sum of the activities of the three pellets is shown.

Total membrane protein reached a maximum at day 6, and then declined (Fig. 2). The maximum yield of protein per g fresh weight was obtained between day 2 and day 6, during the logarithmic phase of cell growth (Fig. 2). NADPH-Cyt *c* reductase activity, a marker for ER, was highest during the early logarithmic phase of growth (Fig. 3), as was UDPase, a marker for Golgi membranes (Fig. 4). The activity of the mitochondrial marker, Cyt *c* oxidase peaked in the mid- to late logarithmic phase of growth (Fig. 5). Proton transport showed the most variability between the two experiments (Fig. 6). The different results for the first and the second experiments, with a dip in transport activity on day 2 in the first experiment, and a peak on day 2 in the second, was found whether fresh or frozen vesicles were assayed. Our main interest was to determine the time of maximum yield of transport activity, which was between days 6 and 8 for both experiments, so we did not attempt further analysis of the phenomenon that occurred immediately after transfer of the cells.

Gradient Centrifugation. To test whether a good separation could be obtained between the proton transport activity and mitochondrial activity, the membranes of the 10,000*g* pellet were separated by centrifugation on a continuous sucrose gradient (Fig. 7). There was good separation between the proton transport activity, Cyt *c* oxidase, and NADPH-Cyt *c* reductase. The proton transport activity was found at a density (1.12 g/cm^3) similar to that found for the activity from corn (4, 10, 13), oats (3), and beet (1, 14). The fact that the vesicles are of a light density but

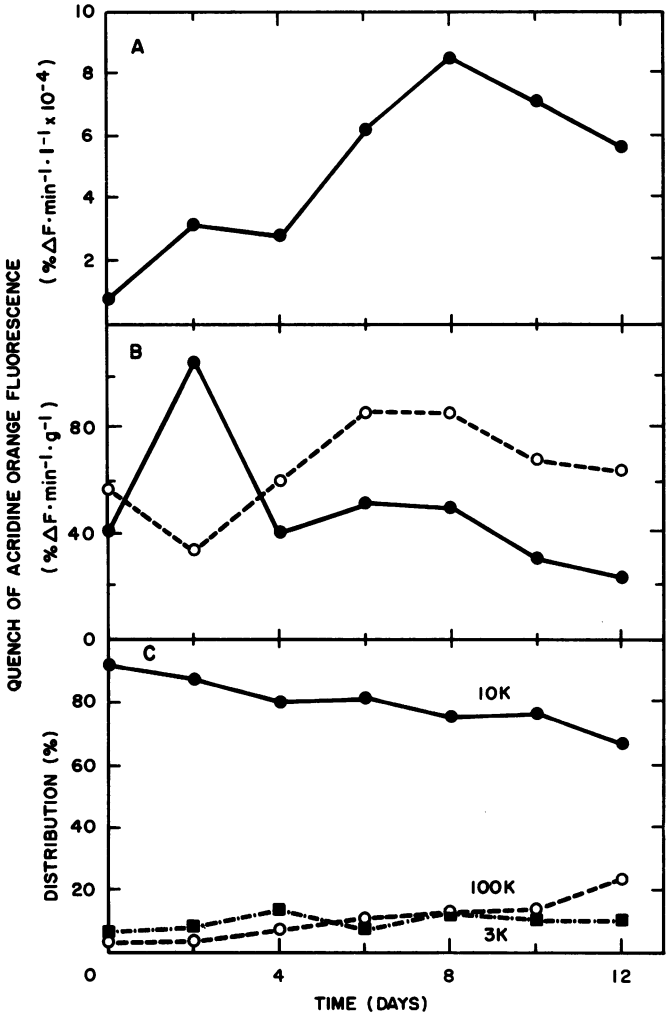

FIG. 6. Proton transport, measured as initial rate of quench of acridine orange fluorescence, of cell membranes as a function of time in culture. A, Total activity per liter of cell suspension, calculated as the sum of the activity in the three membrane fractions. B, Activity per g fresh weight (●); additional experiment (○). C, Distribution of activity between 3,000 (■), 10,000 (●) and 100,000*g* (○) pellets.

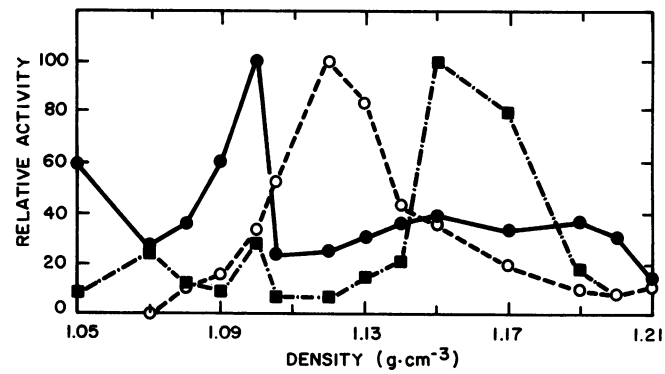

FIG. 7. Distribution of proton transport (○), NADPH-Cyt *c* reductase (●), and Cyt *c* oxidase activity (■) on a continuous sucrose gradient. The 10,000*g* pellet was layered on a 15 to 45% sucrose gradient containing 2 mm EDTA and 1 mm DTT and centrifuged for 2 h at 80,000*g*. Proton transport was assayed as initial rate of quench of acridine orange fluorescence. The absolute activities equal to 100% of relative activity are: 25.0 nmol/min/fraction for NADPH-Cyt *c* reductase, 12.6%/min/fraction for quench, and 0.74 μmol/fraction/min for Cyt *c* oxidase.

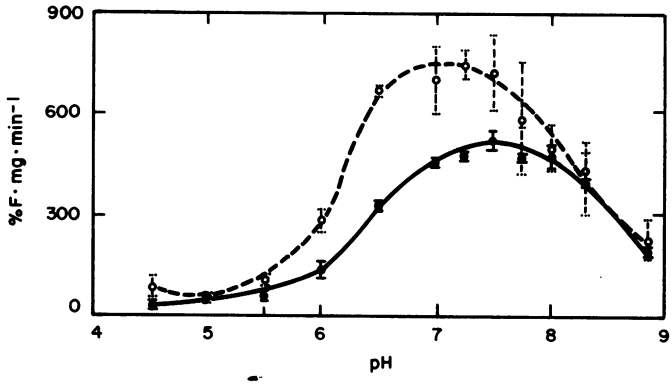

FIG. 8. Rate of proton transport, measured as initial rate of quench of acridine orange fluorescence, as a function of pH. Vesicles from the 10,000*g* pellet (○) or 100,000*g* pellet (●) were further purified by centrifugation onto a 10% Dextran step gradient. Transport was initiated by introducing the membrane vesicles into the assay buffer containing 1 mM Mg:ATP adjusted to the indicated pH with 10 mM Tris Mes.

sediment at the relatively low speed of 10,000*g* suggests that they are large vesicles. Simple preparative procedures were then tested for separating the transport activity from the mitochondrial activity. Sucrose step gradients and Dextran step gradients were compared, and the best results in terms of purification and yield were obtained using the standard Dextran cushion procedure developed by Sze (18).

Characterization. When the experiments to characterize the proton translocating ATPase were begun, the cells had been in continuous culture for several months. Although no significant changes were made in the procedures, there was an approximately 50:50 distribution of the proton transport activity between the 10,000 and 100,000*g* pellets, instead of the 90:10 distribution found previously. We were apparently now obtaining more small vesicles. At this time, we also began to use the Bead Beater Cell Homogenizer. Similar results were found with the Bead Beater and with mortar and pestle, except more efficient cell breakage was obtained with the Bead Beater. Similar results were also found whether the origin of the cultures was from root or hypocotyl. Sealed vesicles were obtained by flotation on a Dextran cushion, and the activity of membranes obtained from the 10,000*g* and the 100,000*g* pellet were compared. The effect of pH (Fig. 8) and of concentration of KCl (Fig. 9) on activity were determined. There was a broad pH optimum, between pH 7.0 and 8.0, with a tendency towards a lower pH optimum for the vesicles obtained from the 10,000*g* pellet (Fig. 8). Specific activities from the two pellets were similar (Figs. 8 and 9). The stimulation by KCl gave saturation kinetics, with a K_m for KCl of 5 to 10 mM (Fig. 9), in agreement with the value reported by Bennett and Spanswick for corn (2). No linear component of KCl stimulation was found, unlike results for the vesicles from corn roots (2). When activity was measured as a function of Mg:ATP concentration, maximal activity was found at 1 mM Mg:ATP, with a half maximal velocity between 30 and 80 μM ATP and Mg. Similar results were found for vesicles from the mitochondrial or microsomal pellets. The activity from both pellets was inhibited by nitrate (7), and the initial rate of quench was not affected by vanadate (not shown).

The characteristics described fall within the range of values reported in the literature for similar vesicles from corn and beet. Although good activity of vesicles from corn roots was obtained at 5 mM Mg:ATP (5), Mandala and Taiz (10) reported inhibition of the proton-translocating ATPase of corn coleoptiles by high concentrations of substrate. The majority of the proton translocating ATPase activity from corn and tobacco was found in the

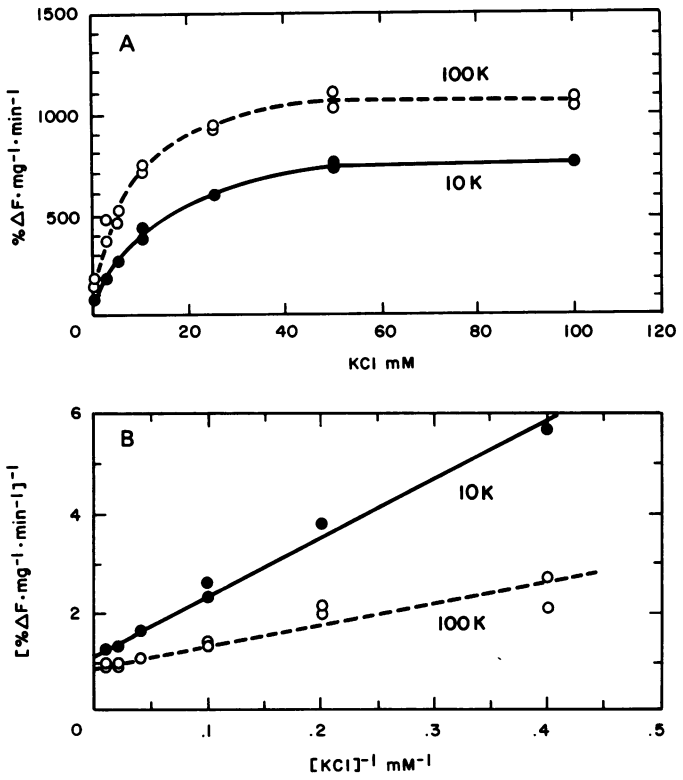

FIG. 9. A, Rate of proton transport, as a function of increasing KCl concentration. Vesicles from the 10,000*g* pellet (●) or 100,000*g* pellet (○) were collected on a cushion of 10% Dextran. Transport was initiated by adding 1 mM ATP into a mixture of vesicles, 1 mM Mg, and assay buffer. B, Lineweaver-Burk plot. Kinetic constants were calculated by linear regression analysis. K_m was 10.0 mM for the 10,000*g* pellet and 5.0 mM for the 100,000*g* pellet.

microsomal fraction (4, 18), but Poole *et al.* found about 50% of the activity from beet to be in the mitochondrial fraction (14). The majority of the activity appeared to be of the type of anion-sensitive proton translocating ATPase described for corn (2, 4, 5, 10, 13, 16), beet (1, 14), and oats (3) that has been suggested to be the tonoplast ATPase.

Enzyme activities and transport activities can vary greatly as a function of time during the growth cycle of cell cultures (8, 9, 11, 12, 15). The variations in activity are probably related to the changing behavior of the cells during a passage cycle, as they respond to fresh medium and new hormones, undergo increased cell division, gradually deplete the medium, and again reduce growth. A detailed description of changes in medium nutrients, pattern of cell division, and changes in uptake and incorporation of amino acids and nucleic acids for tomato cells in a culture medium similar to the one used in this paper is presented by Nover *et al.* (12). It is not surprising, then, that membrane-bound enzymes also vary with the cell cycle, and one might speculate that the early rise in activity of enzymes associated with ER (NADPH-Cyt *c* reductase) and with Golgi (UDPase) are related to an early burst of cell division and membrane and protein synthesis. The subsequent rise in activity of Cyt *c* oxidase and of proton transport activity by tonoplast vesicles suggests that the activities are more closely correlated with total increase in fresh weight of the cells.

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