Potassium Transport in Suspension Culture Cells and Protoplasts of Carrot

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ABSTRACT

The properties of potassium transport in carrot (Daucus carota L.) suspension culture cells and their isolated protoplasts were examined. Cells cultured in Murashige and Skoog (MS) medium (Plant Physiol. 15: 473–497) were potassium saturated and, consequently, they exhibited little net potassium accumulation. Cells that transport and accumulate potassium were derived from the MS-grown cells by culturing them in a potassium-free modified medium. The transport properties of the modified medium cells included: (a) smooth nonsaturating kinetics with 80% of the maximum rates occurring at 0.1 millimolar KCl, (b) linear transport for at least 75 min, (c) alkaline pH optimum, and (d) a little accompanying anion uptake with increased malate concentrations balancing net increases in positive charge, and (e) a little effect on transport by plasmolysis. Potassium transport activity appeared to be 50% lower in protoplasts isolated from the modified medium cells. Nevertheless, the protoplasts exhibited essentially the same kinetics, time course, pH response, and malate adjustment as the intact cells. We concluded from these results that the low potassium cells and their isolated protoplasts are ideally suited to investigating potassium transport at the cell level without the complications associated with multilayered and highly differentiated tissues.

Potassium transport is a fundamental process in both the individual plant cell and the functioning plant as a whole. The transport system is known to be highly specific for potassium and dependent on aerobic respiration (2). This has led to the general conclusion that a plasma membrane protein is involved in potassium transport. Yet, direct evidence for the existence of possible carriers, pores, or channels has not been presented (14, 28). This deficiency may be, in part, the consequence of experimental complications associated with the tissue systems generally used in transport experiments. Our interest has been in developing and characterizing a system that eliminates many experimental problems encountered in the traditional transport systems.

The majority of our knowledge concerning potassium transport has been derived from studies utilizing aged storage tissue or excised roots. These intact tissue systems exhibit a number of characteristics that complicate investigations of ion transport into plant cells. In excised roots, loss of accumulated ions through vascular tissue (12), variable transport along the root (30), and possible diffusion barriers at the epidermis (11) are all important considerations in evaluating ion transport. Furthermore, heterogeneous cell types can contribute differentially to potassium uptake. Although storage tissue minimizes the effects of heterogeneous cell type and longitudinal ion transport encountered in roots, problems still arise concerning the effects of wounding on the isolated discs of storage tissue (2).

Investigating potassium transport at the cellular level in intact tissue is further complicated by the presence of the plant cell wall. The cell wall has a significant cation exchange capacity that influences any cation entering the cell wall solution. The effect of complex exchange chemistry on potassium transport has been difficult to assess. Furthermore, the cell wall is a significant barrier to electrophysiological measurements, membrane protein labeling, and plasma membrane isolation (3, 18).

To avoid the problems associated with intact tissue systems and the plant cell wall, we developed a carrot suspension culture system that produces homogeneous populations of undifferentiated cells that grow singly or in small clumps. These characteristics eliminate many experimental complications associated with multilayered and highly differentiated tissues. Other complications, attributable to the plant cell wall, have also been avoided by investigating potassium transport in protoplasts isolated from these cultured cells. Polley and Hopkins (24) also chose to work with suspension cultures in an effort to minimize the inherent difficulties encountered in intact tissues. However, they noted that the characteristics of their cells were similar to high salt barley roots in which potassium transport activity is very limited (4, 5, 8). Polley and Hopkins (24) tried to decrease the internal potassium levels of their cells, but they were unsuccessful because the cells would not grow in low potassium solutions.

In this paper we report on the first successful development and characterization of a low potassium carrot suspension culture system. This system produces low potassium suspension culture cells which vigorously transport and accumulate potassium. We also report on the transport properties of protoplasts isolated from these low potassium cells. The cultured cells and their isolated protoplasts are an excellent model system for investigating potassium transport into the cell.

MATERIALS AND METHODS

Plant Material. Suspension cultures of Daucus carota L., obtained from Z. R. Sung at the University of California, were maintained in Murashige and Skoog (21) medium that was supplemented with 5 mM succinate and 1 mg/L dichlorophenoxy acetic acid. The cells were grown in 200 ml MS2 per 2 L flask. The succinate was included to buffer pH changes. The cells were subcultured with low density inoculations (4 mg/ml) at 10 d intervals. All cultures were maintained on an orbital shaker (95 rpm) in a constant temperature room at 28°C with continuous illumination provided by two banks of fluorescent lights suspended 0.914 m above the shaker platform. Growth was meas-

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2 Abbreviations: MS, Murashige and Skoog medium; MM, modified medium; Fw, fresh weight.
ured by following changes in turbidity (26) or through direct measurement of fresh weight. For these cells, Sung (26) has shown that increases in turbidity are directly proportional to increases in cell number and dry weight.

**Modified Medium Cells.** After 6 d in MS medium, near the end of the exponential phase of growth, cells destined for transport experiments were aseptically washed in a solution consisting of 5 mM CaCl₂, 1 mM NH₄NO₃, and 10 g/L sucrose (pH 5.7). The cells were then transferred into an equal volume of an experimentally developed medium. The MM composition was based on the MS medium formulation and had the following composition: (a) macronutrients = 10 mM CaCl₂, 3.0 mM CaCl₂, 1.0 mM NH₄NO₃, 0.6 mM MgSO₄, and 0.5 mM NaH₂PO₄; (b) micronutrients = the same as MS medium, except KI was eliminated; and (c) organic additions = the same as MS, except sucrose was lowered to 10 g/L. After 4 d in the modified medium, the cells were harvested for transport experiments. These cells are referred to as MM cells.

**Protoplast Isolation.** Protoplasts were isolated from 24 g of MM cells for each experiment. The isolation procedure was a modification of the methods of Uchimiya and Murashige (29) and Gronwald and Leonard (6). The MM cells were suspended in a digestion solution (5 ml/g tissue) containing 0.6 mM mannitol, 0.5 mM DTT, 0.2 mM CaCl₂, 0.05% BSA, 2.0% Cellulysin, and 1.0% Macerase (pH 5.8). The mixture was equally distributed among six Petri dishes and placed in the light on a reciprocal shaker (50 cycles/min) for 5 h at 29°C.

Protoplasts used in transport experiments are frequently separated from wall debris and undigested tissue by centrifugation through a Ficoll step gradient (6.11, 13). Even with significant modification, however, this technique was not successful with MM cell protoplasts. Consequently, a new procedure was developed. At the end of the digestion period, each Petri dish wasgently decanted into a 250 ml beaker containing 130 ml of protoplast suspension solution (0.6 mM mannitol, 0.2 mM CaCl₂, 0.25 mM DTT, and 5.0 mM Mes [pH 6.1]). Partially digested clumps of cells were allowed to settle out of solution for 15 min. The supernatants, containing suspended protoplasts, were gently decanted into 250 ml centrifuge tubes and centrifuged at 150g for 5 min. The supernatant was aspirated off the delicate pellet and the protoplasts were resuspended in 10 ml of the protoplast resuspension solution. After 10 min settling at 1 g, the supernatants were collected and centrifuged at 150g for 5 min. The final protoplast pellets were consolidated in 30 ml of the protoplast resuspension solution. This preparation was estimated to be 85% isolated protoplasts with a small population of partially digested cell clumps. These protoplasts were routinely used in potassium transport experiments. A highly purified preparation of protoplasts was obtained by filtering the supernatant of the first settling period through a 20 μm mesh Nytex cloth. This technique eliminated partially digested clumps. It was not routinely used, however, because the protoplast yield from the filtration step was very low. The transport properties of the highly purified protoplasts were the same as those of protoplasts isolated with the standard procedure. Thus, the small number of partially digested cells found in the routinely used protoplast preparation did not have a significant effect on the potassium transport results.

**Intact Cell Uptake.** Intact cell uptake experiments were conducted in 3.5 L treatment solutions at 25°C. The solutions were vigorously aerated with CO₂-free air to maintain constant O₂ levels and homogeneously mix the cells during the course of the experiment. Cultured cells were rinsed with deionized H₂O and then added to the treatment solutions (2 g/treatment). After the desired uptake period, the cells were collected on a Whatman No. 1 filter paper inside a Büchner funnel mounted on a 20 L carboy. Treatment solutions were aspirated into the carboy with the house vacuum. The collected cells were rinsed with 80 ml 2.5 mM CaCl₂ followed by three rinses with deionized H₂O. The calcium rinse eliminated exchangeable potassium associated with cell wall exchange sites (data not shown). After determining the fresh weight of the collected cells, they were transferred to 50 ml beakers and extracted in 15.0 ml 0.33 n HNO₃. Transport data were computed by subtracting the initial concentration of nonexchangeable potassium from the final. This was a reliable measure of potassium influx because potassium efflux was not observed in the MM cells.

**Protoplast Uptake.** Protoplast uptake experiments were initiated by adding 1 ml of the final protoplast suspension (10⁶ cells) to a 250 ml flask containing 40 ml 0.6 mM mannitol, 2.0 mM Na or Tris Mes (pH 6.1), 0.2 mM CaCl₂, 0.25 mM DTT, and KCl at the desired final concentration. The uptake solutions were placed on an orbital shaker (60 rpm) and maintained at 25°C. To terminate the experiment, the solutions were transferred into 50 ml conical centrifuge tubes, pelleted, resuspended in potassium-free uptake solution, and pelleted again. Resuspension in the potassium-free solution corresponded with the desired end of the uptake period. The final pellet was extracted in 5.00 ml 0.01 N HNO₃. This collection procedure minimized potassium carryover from the uptake solution. Control, e.g. treatment in potassium-free solution, and time zero samples were collected using the same procedure so that potassium efflux and carryover could be quantitatively measured.

**Analytic Techniques.** Potassium, sodium, calcium, and magnesium concentrations were determined from acid extracts using a Perkin-Elmer Atomic Absorption Spectrometer (model 372). Chloride was measured with an Amino-Cotlove Chloride Titrator. Malate concentrations were determined with the enzymic analysis of Gutmann and Wahlefeld (7). Respiration rates were determined by measuring O₂ consumption with an O₂ polarographic electrode. Protein concentrations were measured by the Lowry method (16). All experiments were repeated at least twice with replicate samples run in each experiment. The data are expressed on a gram fresh weight, mg protein, or μmol Mg²⁺ basis.

**RESULTS AND DISCUSSION**

**Intact Cell Uptake.** Preliminary experiments with cells harvested from the Murashige and Skoog medium, MS cells, showed that their capacity to transport and accumulate potassium was low, and in uptake solutions containing less than 1 mM KCl, the cells actually lost potassium. These results were not too surprising considering the high concentration of potassium (21 mM K⁺) in the growth medium from which the cells were harvested. Under these conditions, the MS cells are saturated with potassium and, like high salt roots, their potassium transport capacity was significantly diminished (5, 8). To minimize the complications associated with high internal potassium levels, we developed a procedure for producing low potassium cells.

Plant tissue can accumulate required nutrients in excess of metabolic needs (2). If this occurred with potassium in the MS cells, we reasoned that transferring a vigorously growing culture of cells into a potassium-free medium may not have a deleterious effect until available potassium pools drop below metabolic needs. If such cells are harvested before affecting metabolic requirements, they may prove useful in studying potassium transport. To test this hypothesis, a 6 d old culture of MS cells was transferred into a modified growth medium containing all the required nutrients except potassium. When these cells were harvested after 4 d in the modified medium, internal potassium concentrations had dropped by 50%, and they rapidly accumulated potassium in short-term transport experiments.

Before routinely using cells harvested from the modified medium for transport experiments, they were compared to MS-grown cells for signs of physiological stress. During their growth

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in the modified medium, the carrot cells maintain the same color and densities as parallel groups of cells growing in MS medium. The growth rate, as measured by changes in fresh weight, of the modified medium cells, MM cells, was the same as the MS cells, and the average MM cell size, 17 ± 4 μm, remained constant during the 4 d growth period. Thus, the increase in fresh weight was the result of continued cell division. Also, when harvested the respiration rates of the MS and MM cells were 39.0 and 38.6 μmol O₂/g fresh weight-h, respectively. Taken together, these results suggest that the MM cells were physiologically unstrressed carrot cells.

After 4 d growth in the modified medium, cation concentrations changed in response to the decrease in internal potassium levels (Table I). The loss of positive charge on a cellular basis, due to the dilution of potassium through continued growth, was partially compensated for by increases in sodium and magnesium concentrations. These changes were relatively small when compared to cation differences between low and high salt barley roots (23) and, overall, the cation concentrations of the MM cells did not differ substantially from other plant materials (23, 25).

The time course of potassium uptake into the MM cells was linear for 75 min before beginning to level off. After 75 min of accumulation, internal potassium concentrations were nearing those found in MS cells.

Potassium uptake by the MM cells exhibited a broad pH response with optimum transport occurring around pH 8.0 (Fig. 1). The alkaline pH optimum is also characteristic of excised oat roots (28) and beet storage tissue (25).

Potassium transport kinetics produce a smooth nonsaturating curve which approaches linearity above 1 mM KCl (Fig. 2). These kinetics are similar to those reported for corn root potassium uptake (10). Although the MM cells did not display simple Michaelis-Menten kinetics, an approximate Km for potassium was estimated to be 35 μM.

After standard transport experiments (treatment solution: 0.5 mM KCl, 0.05 mM CaCl₂, 1 mM Na-Mes [pH 6.1] 1 h), MM cell

Table I. Cation Concentrations of Murashige and Skoog and Modified Medium Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>K⁺</th>
<th>Na⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
<th>Total Cations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>74.0</td>
<td>1.3</td>
<td>14.7</td>
<td>10.0</td>
<td>100</td>
</tr>
<tr>
<td>MM</td>
<td>39.0</td>
<td>4.3</td>
<td>14.7</td>
<td>18.0</td>
<td>76</td>
</tr>
</tbody>
</table>

potassium concentrations increased by approximately 20 μmol/g fresh weight. This resulted in a 30% increase in the total cation concentration of the cells. During the same period, the other major cation concentrations did not change. To compensate for this charge increase, there was a net increase in cellular malate and chloride concentrations (Table II). Adjustment of organic acid concentrations is a well-known response to differential ion accumulation (9, 17). When K₂SO₄ was used as the potassium salt, potassium uptake kinetics were unaffected (data not shown). Since malate is the primary anion balancing accumulated potassium, the absence of a rapidly transported anion in the transport solution did not affect potassium uptake.

Protoplast Uptake. It is necessary to isolate plant cell protoplasts in solutions whose water potential closely matches the osmotic potential of the experimental tissue. Under such conditions, the pressure potential of the cell is close to zero and the cell is flaccid. There are conflicting reports in the literature concerning the effect of low water potentials on ion transport (1, 27). To avoid possible misinterpretation of protoplast potassium transport due to secondary effects of low water potentials, we investigated potassium uptake in intact MM cells as a function of water potential (Fig. 3). When CaCl₂ or mannitol was used as osmoticum to lower treatment solution water potentials, there were no apparent effects on potassium accumulation. We concluded that negative water potentials did not significantly affect potassium uptake in the MM cells.

When sucrose was used to lower solution water potentials, increasing concentrations of solute progressively inhibited potassium influx (Fig. 3). Since potassium uptake was unaffected by negative water potentials when using CaCl₂ and mannitol as osmotica, the inhibition encountered with sucrose appears to be unrelated to low potentials. Recent evidence suggests sucrose is transported into the cell by a proton-sucrose symport (15). Perhaps the high concentrations of sucrose used in these studies saturated the symport, depolarized the membrane potential, and
The efflux of potassium from protoplasts was shown to be a function of external water potential. The adjusting osmotica were CaCl_2 (●), sucrose (Δ), and mannitol (○). The potassium concentration was 5 mM for CaCl_2 and sucrose, and 0.5 mM in the mannitol experiments. The pH was buffered at 6.0. The rate of potassium uptake in mannitol is expressed as a function of Mg concentration.

Table III. Effects of Protoplast Potassium Uptake on Cellular Potassium and Magnesium Concentrations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cellular Cation Concentrations</th>
<th>μmol Mg(^{2+})/mg protein</th>
<th>μmol K(^{+})/mg protein</th>
<th>μmol K(^{+})/μmol Mg(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sample</td>
<td>0.248 ± 0.002</td>
<td>0.86 ± 0.01</td>
<td>3.48 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Minus K(^{+})</td>
<td>0.245 ± 0.002</td>
<td>0.87 ± 0.01</td>
<td>3.55 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Plus K(^{+})</td>
<td>0.250 ± 0.002</td>
<td>1.09 ± 0.01</td>
<td>4.33 ± 0.03</td>
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</table>

The results with mannitol (Fig. 3) were expressed as a function of MM cell magnesium concentration. While characterizing the transport properties of the intact MM cells, cellular magnesium concentrations were found to be very stable. Expressing the mannitol results as a function of magnesium made analysis much easier and eliminated complications associated with changes in fresh weight. In parallel analysis, the mannitol results were the same whether expressed as a function of fresh weight or magnesium (data not shown).

The stability of intracellular magnesium concentration was exploited in the analysis of protoplast potassium uptake. Most investigators have expressed protoplast ion uptake as a function of protein concentration or protoplast number (6, 13, 18). While protein concentration is usually a reliable reference for measuring potassium flux, protoplast number can be misleading because of differential cell lysis during the experiment. We chose magnesium to evaluate potassium uptake because analysis was fast, accurate, and unaffected by experimental conditions.

The stability of magnesium concentrations in the MM protoplasts was demonstrated in two ways. First, the magnesium concentration of the protoplasts, as a function of protein concentration, did not change during the course of standard transport experiments (Table III). Second, analysis of spent uptake solution showed no significant loss of magnesium. The protein and efflux results confirmed magnesium's reliability as a reference for measuring potassium fluxes in the MM protoplasts.

The cytological and biochemical integrity of the MM cells did not appear to be significantly affected by the loss of the cell wall. The protoplasts exhibited active cytoplasmic streaming and no microscopically visible cytological aberrations. When treated with Evans blue, the dye penetrated fewer than 1% of the cells. These results suggest that the plasma membrane and metabolic activity were relatively unaffected by the isolation procedure. Furthermore, the respiration rate of the protoplasts was the same as the intact cells, 0.98 versus 1.01 μmol O_2/μmol K\(^{+}\)-h, respectively. Respiration was expressed as a function of potassium content because this allowed for a direct comparison between the protoplasts and intact cells. We concluded that the carrot cells were not significantly damaged by the removal of the cell wall.

The time course of potassium uptake by MM protoplasts was linear for greater than 90 min (not shown). Similar periods of linear transport were reported for protoplasts isolated from root cortical tissue (6). However, shorter periods of linearity were reported for protoplasts isolated from cultured tobacco cells (18) and whole corn roots (13). The initial ion status of these tissues may have contributed to these differences. The cultured tobacco cells were high potassium cells grown in MS medium, and intact corn roots contain more potassium than separated cortex (6). Higher initial concentrations of potassium could account for shorter periods of linear transport.

Protoplast potassium uptake exhibited a broad pH response with optimum rates centered around pH 7.5 (Fig. 4). The effect of pH on transport was similar to that seen in the intact cells (Fig. 1) and other protoplast systems (6, 18).

The kinetics of potassium uptake into the protoplasts produce
the same smooth nonsaturating curve seen with the intact MM cells (Fig. 5). Concentrations of potassium greater than 0.5 mM produced a small linear increase in transport, and, as in the intact cell, an approximate \( K_a \) for potassium was 35 \( \mu \)M. These results suggest that the plant cell wall does not significantly influence potassium transport. Similar kinetics were reported for corn root protoplasts (11), although considerable variability was encountered in those experiments. Potassium transport kinetics in cultured tobacco cell protoplasts (19) were also similar except that system’s apparent affinity for potassium was much lower, \( K_a \) 360 \( \mu \)M. The lower affinity of the tobacco protoplasts may have been due to their high initial potassium concentrations.

Malic acid concentrations of the protoplasts increased by 0.34 \( \mu \)eq malate/\( \mu \)eq Mg\( ^{2+} \) when potassium content increased by 0.85 \( \mu \)eq K/\( \mu \)eq Mg\( ^{2+} \). As with the intact cells, this increase is associated with the need to maintain electrical neutrality. However, the protoplast malate increase balanced only 40% of the net cation increase, and the method by which the protoplasts balanced the remaining charge was not determined.

Protoplast transport activity was approximately 50% lower than in the intact cell (Table IV). This result was consistent whether the data were expressed as a function of magnesium or protein concentration. The initial potassium/magnesium ratio was higher in the protoplasts because some magnesium was lost with the cell wall during protoplast isolation. The initial potassium/protein ratio of the intact cells was higher than the protoplast ratio because a portion of the intact cell’s protein remained with the cell debris after filtering the acid extracts during analysis. The apparent decrease in protoplast transport activity was not an artifact of the methods of expression since the initial potassium ratios deviate in opposite directions. The decreased transport activity of the MM protoplast does not appear to be the result of an abnormally functioning transport apparatus. Had the protoplast isolation procedure significantly altered the transport system, one would expect a concomitant change in kinetics, time course, pH response, and overall protoplast viability. Instead, the transport properties of the MM protoplasts do not deviate substantially from the intact cells. Furthermore, the rate of potassium transport in 0.5 mM KCl compares favorably with other low-potassium protoplast systems (6, 11). The lowered activity may be the result of an energy diversion to other cellular activities, such as synthesis of a new cell wall. Similar decreases in uptake activity were reported for both glucose and amino acid transport in protoplasts isolated from leaf tissue (20).

In conclusion, the tissue culture system developed in this study produces suspension culture cells which actively transport and accumulate potassium. The cultured cells possess several experimental attributes which minimize transport problems encountered in highly differentiated tissues, and their isolated protoplasts eliminate the cell wall as a complicating factor. The transport properties of the intact cells were similar to other well-characterized systems and, importantly, the transport properties of the isolated protoplasts matched those of the intact cells. We concluded from these results that cells grown in the modified medium, and the protoplasts derived from them, represent an excellent model system for investigating potassium transport in plants.

LITERATURE CITED
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Table IV. Protoplast and Intact Cell Potassium Uptake

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Rate of K(^+) Uptake</th>
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<tr>
<td></td>
<td>( \mu )mol K(^+)/mg protein ( h )</td>
</tr>
<tr>
<td>Intact cell</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>3.19</td>
</tr>
<tr>
<td>Initial</td>
<td>2.05</td>
</tr>
<tr>
<td>Protoplasts</td>
<td></td>
</tr>
<tr>
<td>Final</td>
<td>1.09</td>
</tr>
<tr>
<td>Initial</td>
<td>0.86</td>
</tr>
</tbody>
</table>

The transport solution for the intact cells included 0.5 mM KCl, 0.05 mM CaCl\(_2\), and 1.0 mM Mes-Na (pH 6.1). The transport solution for the protoplasts included 0.5 mM KCl, 0.6 M mannitol, 2.0 mM Mes-Tris (pH 6.1), 0.25 mM DTT, and 0.2 mM CaCl\(_2\).