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# Salt-induced Inhibition of Phosphate Transport and Release of Membrane Proteins from Barley Roots

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## ABSTRACT

**Osmotic shock with sequential 30-minute treatments in ice-cold saline solutions and $H_2O$ released proteins from excised barley roots and inhibited the subsequent uptake of orthophosphate (Pi). The amount of protein released increased sharply at NaCl concentrations above 0.05 molar, approximately the threshold concentration above which Pi uptake was increasingly suppressed. About 60% of the nearly 100 micrograms of protein per gram fresh weight of roots that was eluted in 0.16 molar NaCl treatments apparently had no function in Pi transport, since it was eluted at NaCl concentrations (≤0.05 molar) that did not affect Pi uptake. Although 0.16 molar NaCl completely inhibited Pi uptake, active uptake resumed at about 60% of control rates within 1 to 2 hours. The presence of either puromycin or cycloheximide greatly reduced the recovery of Pi uptake activity after the NaCl treatment. Mannitol and various monovalent and divalent salts at concentrations isosmotic with NaCl also inhibited Pi uptake, but $CaCl_2$ was consistently the least inhibitory. The correlation between the concentration of the osmotic treatments and the simultaneous loss of protein and Pi uptake activity, together with the evidence that uptake recovery requires protein synthesis, support the hypothesis that the proteins eluted are required for active Pi transport.**

Substantial evidence now indicates that periplasmic binding proteins are important components of transport mechanisms in bacteria (2, 5, 7, 14–16). According to the Singer-Nicolson (21) model of membrane structure, these binding proteins are peripheral proteins attached on the outer surface of the plasma membrane to certain integral proteins imbedded in a phospholipid matrix. Singer (20) postulated that this association between the peripheral and integral proteins creates a mechanism for transport caused by conformational changes and that the peripheral proteins serve as specific binding sites. A variety of binding proteins have recently been isolated through the use of an "osmotic shock" method, *i.e.* the rapid transfer of cells from isotonic to hypotonic medium (5, 15). Pardee (16) isolated and crystallized a sulfate-binding protein from the osmotic shock fluid of *Salmonella typhimurium*. Medveczky and Rosenberg (10, 11) purified a phosphate-binding protein released by osmotically shocking *Escherichia coli*. Generally, binding proteins are relatively easily dissociated from membranes, are lipid-free and soluble, and have mol wt between 22,000 and 42,000 (2, 15, 20).

Although most data have been obtained with bacteria, some evidence suggests that similar transport proteins are released by osmotically shocking fungi (23) and higher plant cells. Nieman and Willis (13) found that treating carrot discs in cold NaCl solutions released protein and suppressed the subsequent active uptake of glucose and Pi. Although the proteins were not identified as components of the transport mechanism, the close correlation between the amount of protein released and the degree of uptake suppression, and the inhibition by puromycin of the recovery of uptake activity strongly suggested that proteins were involved. Amar and Reinhold (1) described a similar study in which osmotic shock caused the loss of protein from bean leaf strips and at the same time sharply reduced the cells' ability to absorb α-aminoisobutyric acid, an amino acid analog. The leaf tissue also recovered partial uptake activity but not in the presence of cycloheximide or actinomycin D. Recently, Rubinstein *et al.* (18) found that osmotic shock reduced the uptake of α-aminoisobutyric acid, 3-*O*-methyl glucose, leucine, and Cl in oat coleoptiles. Chloride uptake was inhibited least but, as with the organic solutes, uptake activity partially recovered after 1 to 3 h.

The objective of this study was to determine if proteins released from barley roots by osmotic shock are essential components of ion transport mechanisms. In this paper, we report the effects of salt shock treatments on the reversible inhibition of Pi uptake and the related loss and synthesis of proteins.

## MATERIALS AND METHODS

Barley roots (*Hordeum vulgare* L. cv. CM 67) were excised from 5-day-old seedlings grown in aerated 0.2 mM $CaSO_4$ solutions in the dark at 25 C. The roots were cut into approximately 1-cm lengths, rinsed, mixed thoroughly in fresh 0.2 mM $CaSO_4$, and centrifuged 5 min in wire mesh baskets at 65*g* to obtain a uniform moisture content. Ten-g root samples were pretreated for 30 min at 2 C in 200 ml of deionized water (controls) or salt solutions, washed 30 min in deionized water at 2 C, and then rinsed briefly at room temperature. The roots were transferred to absorption solutions containing 0.5 mM $CaSO_4$ and 1 mM $KH_2PO_4$ at pH 5 and 25 C. The solutions were continuously shaken and aerated and pH was maintained between 4 and 5. Ion uptake or loss was measured by analyzing 1-ml aliquots of the absorption solution. At the end of the absorption period, the roots were removed, rinsed twice in deionized water, oven-dried at 65 C, and analyzed. Phosphate was determined by the phosphomolybdate method of Taussky and Shorr (22) and cations by atomic absorption spectrophotometry.

Protein eluted in the pretreatment and wash solutions was dialyzed against cold deionized $H_2O$ for 2 days and then precipitated in cold 0.4 M $HClO_4$. The precipitate was collected on Celite over a Millipore filter and dissolved in 0.1 M NaOH at room temperature overnight. The soluble protein was removed from the Celite by filtering the suspension through fritted glass and was assayed by the Lowry procedure (8).

## RESULTS

**Phosphate Absorption.** The first experiment was conducted to

determine the salt concentration that would reversibly inhibit Pi uptake without rupturing the plasmalemma and causing loss of internal cell constituents.

The effects of 30-min pretreatments in various ice-cold NaCl solutions on the subsequent rate of Pi uptake from 1 mM $KH_2PO_4$ ranged from essentially none at 0.05 M NaCl to a net loss at 0.25 M as compared with either the 2 or 25 C water-treated control roots (Fig. 1). The results of several experiments verified that rates of Pi uptake by control roots pretreated at 2 and 25 C were similar; therefore, all subsequent control roots were pretreated in water at 2 C. The NaCl pretreatments did not significantly affect Pi uptake until the concentration exceeded 0.10 M, and then the rate of uptake decreased abruptly with increasing salt concentrations. As the pretreatment concentrations were increased, a distinct lag period developed before uptake resumed. At the higher concentrations a net loss of endogenous Pi was observed, but despite the initial loss and the 3-h lag after the 0.20 M pretreatment, Pi uptake activity still recovered some. At 0.25 M, however, inhibition appeared irreversible, at least within the 7-h absorption period. These data indicate that a concentration between 0.16 and 0.18 M reversibly inhibited net Pi uptake without causing irreparable membrane damage.

Root K levels at the end of the 7-h absorption period showed that K uptake was also reduced by the salt pretreatments and that the nature of the salt effect resembled that observed for Pi uptake. Although steady-state rates could not be determined from final root contents, total K uptake in the 7-h period ranged from about 34 μeq/g fresh roots for the controls to 29 at 0.10 M NaCl, to 3.7 at 0.18 M. A net loss of 7.9 μeq K/g occurred from the 0.25 M NaCl-pretreated roots.

To test whether Pi uptake after the 0.16 M NaCl pretreatment was metabolically mediated and not merely exchange diffusion across damaged membranes, we added 0.05 mM DNP[1] to the absorption solution. The gradual net loss of Pi uptake from the roots indicated that a metabolic rather than a diffusive mechanism was involved (Fig. 2). The data suggest that the uncoupler not only suppressed Pi uptake, but increased membrane permeability. DNP is known to increase membrane permeability to protons and K (6).

**Effects of Other Salts and Mannitol.** Mannitol and several salts of monovalent and divalent ions were compared with NaCl at isosmotic concentrations of −5 and −8 bars OP to determine if the effect of the NaCl pretreatment was ion-specific or osmotic (because of an inadvertent error in temperature corrections, the actual OP of mannitol was about 0.5 bar lower than that of salts). The two OP values were chosen to provide comparisons of the osmotic effects at the threshold salt concentration as well as the concentration at which reversible inhibition of Pi uptake could be expected. The effects of mannitol and the various Cl and $SO_4$ salts at −5 bars OP were similar to that of NaCl, *i.e.* they slightly reduced Pi uptake (Fig. 3). With the −8 bars OP pretreatments, Pi uptake was reversibly inhibited by all osmotica. The experiments were repeated several times, and although the recovery times and subsequent rates of Pi uptake varied somewhat among experiments and treatments, the results were basically alike. Uptake was completely inhibited for 1 or more h and then activity resumed at between one-third and two-thirds the control rate. The effect of mannitol, a nonionic osmoticum, was essentially the same as that of NaCl. Recovery was always slowest with Mg salts. The $CaCl_2$ pretreatment consistently had the least effect on Pi uptake, but it appears from the effects of increasing concentrations of $CaCl_2$ (Fig. 4) that Ca acts similarly to Na (*cf.* Fig. 1). Pi uptake was suppressed about the same by −9.6 bars (0.15 M) $CaCl_2$ and −8 bars (0.17 M) NaCl.

Unlike its effect on carrot discs (13), Ca (0.5 mM) did not preserve uptake activity when added to the wash solutions in our experiments.

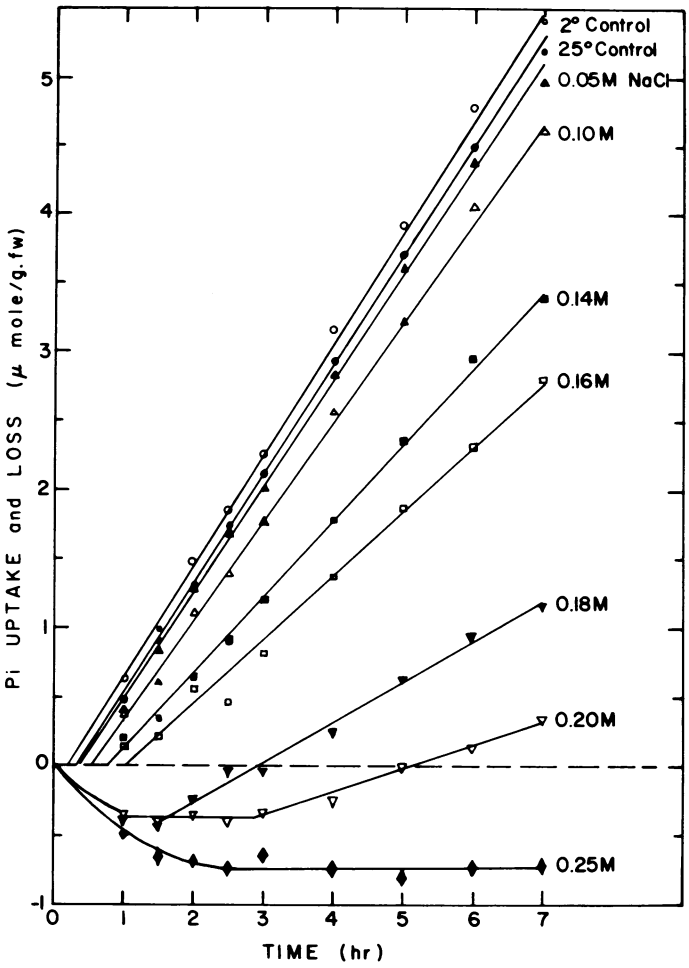


FIG. 1. Time course of Pi uptake or loss by control and NaCl-pretreated barley roots. Absorption solutions contained 1 mM $KH_2PO_4$ and 0.5 mM $CaSO_4$ at pH 5 and 25 C.

**Protein Release.** The loss of protein from barley roots to the pretreatment and combined wash solutions is shown in Figure 5. A significant but variable amount of protein was always lost to the control, cold-water pretreatments. Since Pi uptake activity was not reduced by water pretreatments, the proteins lost presumably served no role in Pi transport. More protein was consistently released in the hypotonic rinse solutions than in the salt solutions. The amount released in the rinse solutions was related to the degree of inhibition of Pi uptake after pretreatments at various salt concentrations. The loss of protein to the rinse solutions increased very little until NaCl concentrations exceeded 0.05 M. It was at concentrations above this threshold at which NaCl began to interfere with Pi uptake (Fig. 1).

**Protein Synthesis.** Assuming that the salt pretreatments and subsequent rinses released Pi-binding proteins and that recovery of Pi uptake activity required *de novo* protein synthesis, we tested the effects of two protein synthesis inhibitors on the recovery of salt-pretreated roots. Pi uptake by control (2 C water) and 0.18 M NaCl-pretreated roots was measured in the absence and presence of 1 mg/l cycloheximide and 200 mg/l puromycin (Fig. 6). Puromycin had no effect on Pi uptake by the control roots but strongly suppressed uptake by the salt-pretreated roots. Cycloheximide also inhibited recovery by pretreated roots, but seemed to reduce uptake by the control roots as well. This same inhibitory effect of cycloheximide on Pi uptake has been reported for bean roots (12).

[1] Abbreviations: DNP: 2,4-dinitrophenol; OP: osmotic potential.

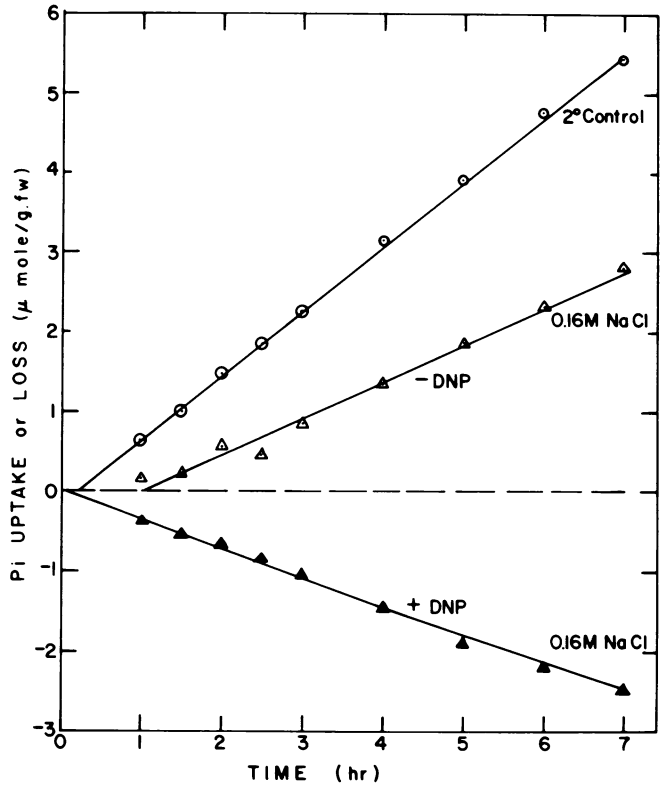


FIG. 2. Time course of Pi uptake by control and NaCl-pretreated barley roots in absence and presence of 0.05 mM DNP. Absorption conditions as given in Figure 1.

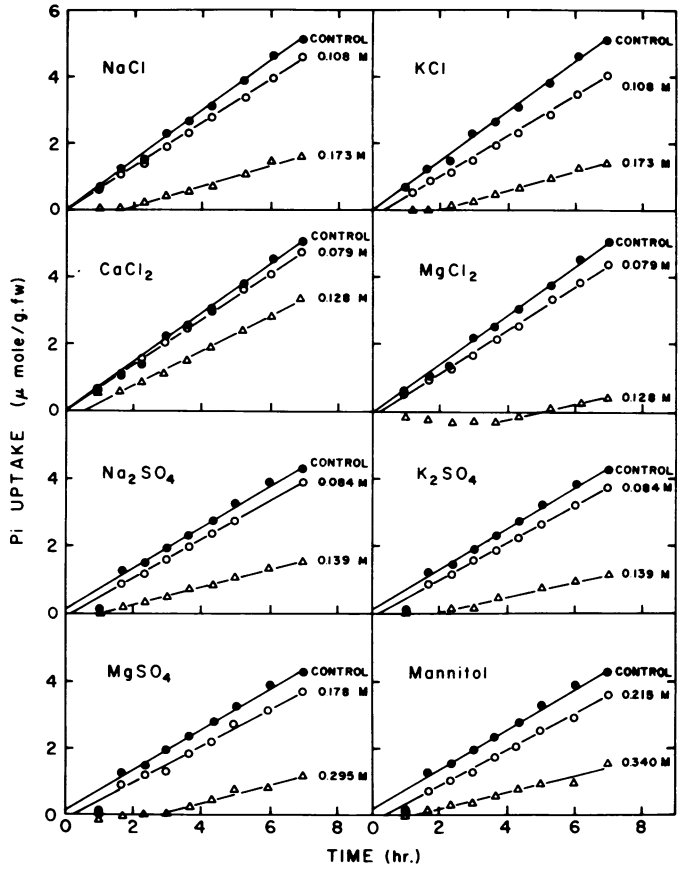


FIG. 3. Time course of Pi uptake by excised barley roots pretreated with various salts and mannitol at 0, −5, and −8 bars OP.

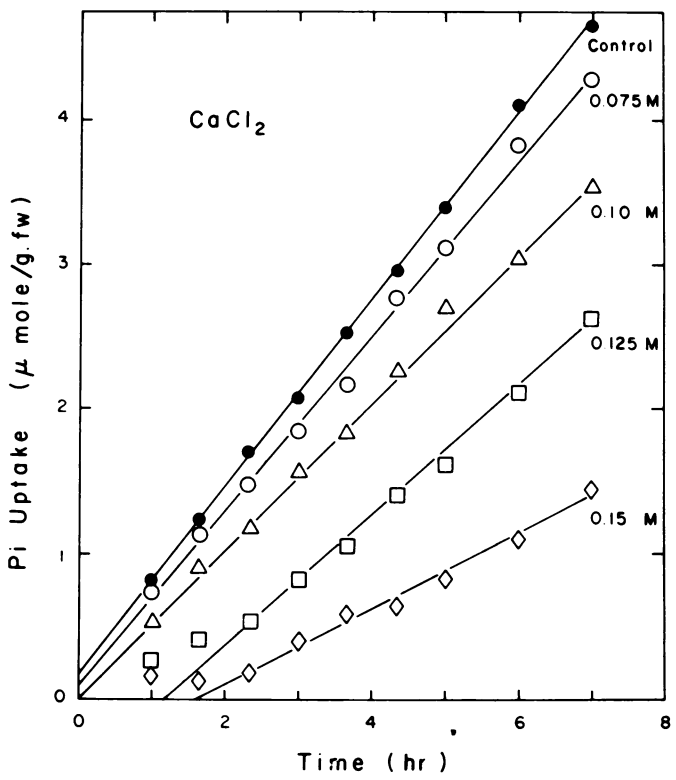


FIG. 4. Time course of Pi uptake by control and $CaCl_2$-pretreated barley roots. Absorption conditions as given in Figure 1.

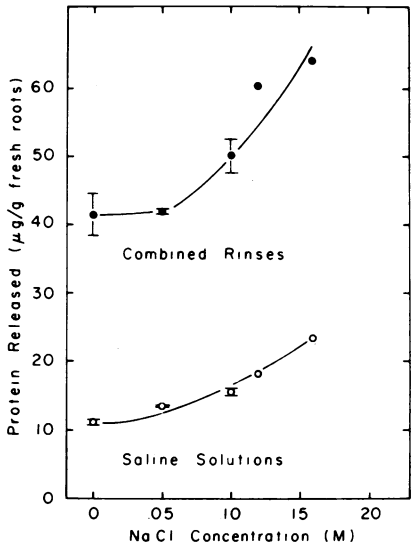


FIG. 5. Protein released by barley roots to NaCl pretreatment solutions and subsequent water washes as a function of NaCl concentration. Bars denote variation of duplicate samples.

## DISCUSSION

Several studies have shown that osmotic shock causes a loss of protein from plant tissues that appears to be correlated with the inhibition of active uptake of substrates, both organic and inorganic. The results of this study on excised barley roots are consistent with the hypothesis that osmotically released proteins are functionally involved in phosphate transport. The rapid transfer of roots from saline solutions (NaCl at −8 bars OP) to a hypotonic medium (distilled $H_2O$) released approximately 100 μg protein/g fresh weight. This treatment also reversibly inhibited the active

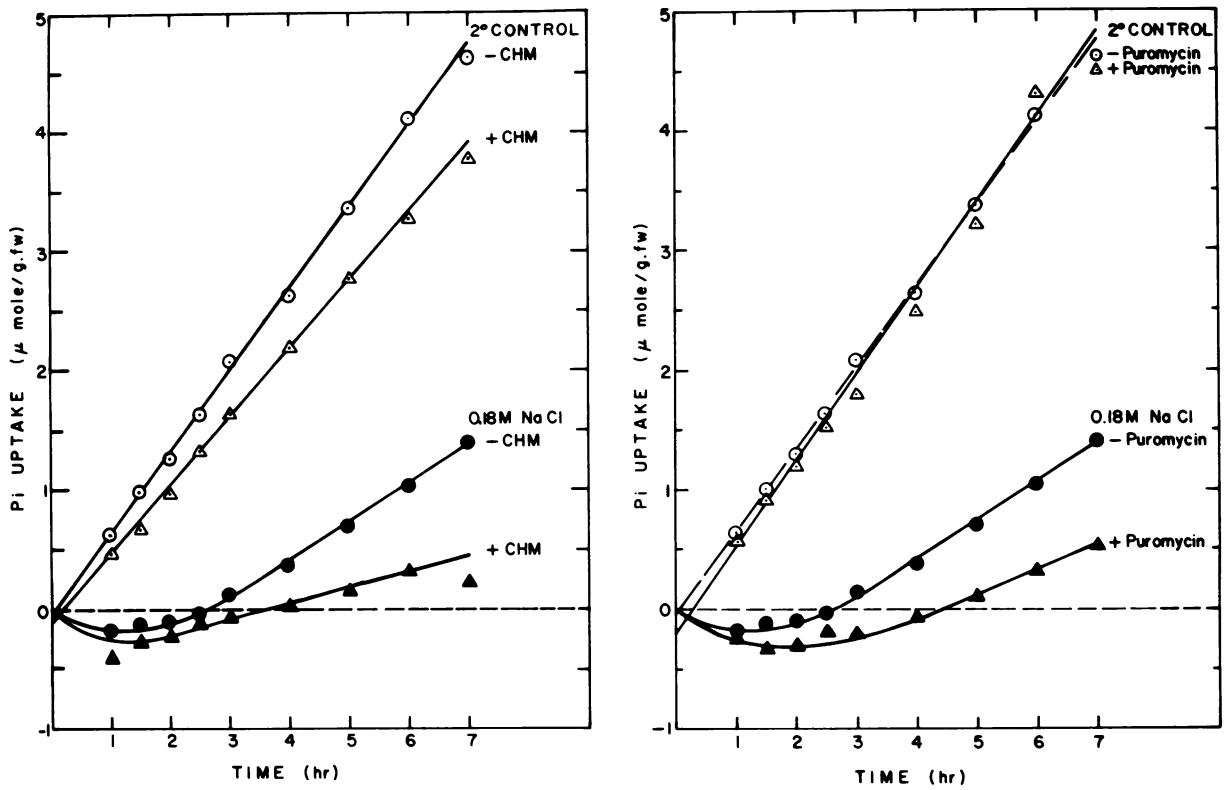


FIG. 6. Time course of Pi uptake by control and NaCl-pretreated barley roots in the absence and presence of 1 mg/l cycloheximide (CHM) or 200 mg/l puromycin. Absorption conditions as given in Figure 1.

uptake of Pi for 1 to 2 h. The amount of protein released was related to NaCl concentrations above 0.05 M as was the suppression of Pi uptake. If protein was involved in Pi transport, it apparently was eluted in treatments above 0.05 M, since only higher concentrations inhibited active Pi uptake. The root protein eluted in water control and low salt treatments obviously had no function in Pi transport. Subsequent experiments, to be published later, showed that bacteria did not contribute appreciably to the protein released from the roots.

Nieman and Willis (13) concluded that osmotic shock was not the major factor in releasing protein and suppressing uptake activity of carrot discs because these effects were diminished or prevented by the presence of Ca at low concentration in the wash solutions. They showed that monovalent cations displaced Ca and Mg ions and suggested that this loss disrupts linkages between proteins and cell membranes. Our results with barley roots differ from those obtained with carrot discs in that rapid changes in OP rather than monovalent cation concentration seem to be of major importance in releasing peripheral proteins. The effects of mannitol and several monovalent and divalent salts on Pi uptake were all essentially the same. Although $CaCl_2$ was not as effective as NaCl—perhaps because Ca may be involved in binding peripheral proteins to the membrane—it did act similarly to Na. Barley roots may behave more like beet root tissue (13), which either binds Ca more tenaciously or has a lower requirement than does carrot. We agree that Ca is essential in maintaining the structural and functional integrity of membranes, but the effects observed with barley roots seem to be primarily osmotic. Shapovalov (19) reported that the loss of fluorescent components from soybean roots was induced equally by plasmolysis with monovalent and divalent chloride salts and sucrose. However, a comparison on the basis of OP shows that $CaCl_2$ caused less leakage at −12.8 bars than NaCl at −9.2 bars and about the same leakage as sucrose at −10.4 bars.

Evidence that proteins released by osmotic shock are involved in Pi transport comes from the effects of protein synthesis inhibitors on the recovery of Pi uptake activity. Within 1 to 3 h after treatment in any of the −8 bar OP media, Pi uptake resumed at rates 30 to 65% of the control rate. This recovery apparently required *de novo* protein synthesis. Both puromycin and cycloheximide greatly reduced Pi uptake after treatment with NaCl. These results agree with the findings for carrots (13) and bean leaves (1).

Grunwaldt *et al.* (4) recently suggested that transport reductions and protein release from corn roots after osmotic shock are a result of membrane damage and are not analogous to the phenomena found with bacteria. They found that older vacuolated root cells were badly damaged or killed by osmotic shock while the youngest, less vacuolated cells in the root tip were little affected. Unfortunately, this conclusion was based on osmotic treatments that would ensure irreversible membrane damage and the loss of cell constituents from vacuolated tissue. A 0.6 M sorbitol solution would exert a −15 bar osmotic potential that few glycophytic plant tissues could withstand. Repp *et al.* (17) found that osmotic potentials between −14 and −18 bars (0.3 and 0.4 molal NaCl) killed 50% of the cells of many cultivated plants. On the other hand, the lower internal OP and greater resistance of nonvacuolated tissues to osmotic shock have been documented before. Greenway (3) found that a 20-bar decrease in OP caused a much greater leakage of metabolic constituents from vacuolated than from nonvacuolated corn root segments. Mauney (9) reported that young cotton embryos have a very low OP and require culture media of low OP to develop, but as the ovule matures, the OP of the medium must be increased.

These observations clearly emphasize the importance of selecting osmotic treatments that are compatible with the tissue. Obviously, one can damage cell membranes irreversibly with rapid transfers between osmotica with OP differentials greater than the internal cell OP.

In this study, we demonstrated that a −8 bar osmotic shock treatment can be used successfully to release proteins from barley roots without irreparable membrane damage as indicated by loss

of cell constituents. Although we were unable to demonstrate *in vitro* binding of Pi with any of the proteins separated electrophoretically on acrylamide gels, we believe that with continued efforts, a phosphate-binding protein will be found in higher plant membranes.

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