Phosphate Absorption, Fluxes, and Symplasmic Transport in Osmotically-Shocked *Zea mays* Roots

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**ABSTRACT**

Osmotic shock with sequential 30 min treatments in ice-cold saline solutions and distilled water inhibited both the subsequent uptake of orthophosphate (Pi) and its transport into the xylem of excised corn (*Zea mays* L.) roots. Measurements of Pi fluxes with $^{32}$P indicated that the decrease in net Pi uptake over a 24 h period caused by osmotic shock was due primarily to delayed recovery of Pi influx rather than to increasing efflux. Despite complete recovery of Pi absorption within 2–6 h after shocking with 150–200 mM NaCl, transport to the xylem during the subsequent 24 h only partially recovered. Leucine uptake and incorporation into protein was also markedly inhibited by osmotic shock but both almost completely resumed control rates within 24 h after shocking with up to 150 mM NaCl. Tetracycline inhibited recovery of Pi uptake after NaCl treatment whereas puromycin did not. These results with corn roots are consistent with the hypothesis that recovery of Pi uptake activity after moderate osmotic shock requires *de novo* synthesis of membrane proteins. Incomplete recovery of Pi transport to the xylem suggests that osmotic shock may damage plasmodesmata.

Key words: Corn; Ion uptake; Leucine uptake; NaCl; Puromycin; Tetracycline.

**INTRODUCTION**

The osmotic shock technique, first used successfully on bacteria to remove membrane transport proteins (Anraku, 1967; Heppel, 1969; Pardee, 1968), has since been tested on higher plants (Amar and Reinhold, 1973; Attia and Jeanjean, 1983; Grunwaldt, Ehwald, and Goring, 1978; Maas and Finkel, 1979; Maas, Ogata, and Finkel, 1979; Migliaccio, 1980; Nieman and Willis, 1971; Rubinstein, Mahar, and Tattar, 1977; Rubinstein, 1982a, b). This technique involves treating plant tissues in cold hyper-osmotic solution followed immediately by transfer to a cold hypo-osmotic solution. As with bacteria, it removes proteins that seem to be implicated in solute transport, but so far no proteins with transport properties have been identified in higher plants. Evidence that some proteins released by osmotic shock are integral components of ion transport is circumstantial (Maas *et al.*, 1979; Migliaccio, 1980; Nieman and Willis, 1971; Rubinstein *et al.*, 1977; Rubinstein, 1982a, b). First, a close relationship exists between loss of ion transport activity and the release of proteins. Second, recovery of transport activity seems to require protein synthesis.
One of the major problems in interpreting the effects of osmotic shock on roots arises from their complicated heterogeneous multi-tissue structure. Roots, unlike bacteria, consist of several different tissues that are interconnected by plasmodesmata in a single functional unit known as the symplasm. Transport in roots not only involves transport across the plasmalemma into the cytoplasm, but also transport into the vacuole and radially into the xylem. Since little of the endogenous solutes of the tissue are lost during moderate osmotic shock treatments (Maas et al., 1979; Nieman and Willis, 1971), it seems unlikely that the tonoplast is affected. On the other hand, there is evidence that the plasmodesmata are markedly affected by osmotic shock (Greenway, 1974; Grunwaldt, Ehwald, Pietzsch, and Goring, 1979; Rubinstein, 1982a, b; Van Iren and Boers-van der Slujs, 1980). It was of interest, therefore, to determine the relative effect of osmotic shock on ion absorption and symplasmic ion transport to the xylem. If the symplast is the principal pathway for ion transport at low external ionic concentrations (below 1-0 mM) as generally believed (Anderson, 1976; Bange, 1973; Grunwaldt et al., 1978; Pitman, 1977), damage to the plasmodesmata should reduce symplasmic transport significantly.

Another question arising about the inhibition of ion absorption by osmotic shock is its relative effects on ion influx and efflux. If the plasmalemma is made sufficiently leaky that no net uptake can occur, this alone would explain the inhibitory effects of osmotic shock on ion absorption. Furthermore, the proteins released may not be directly involved in transport but serve only to maintain membrane semipermeability.

The goal of this study was to determine the effects of osmotic shock not only on net Pi uptake by corn roots, but also on Pi fluxes occurring in roots, i.e. principally influx and efflux at the plasmalemma, and translocation through the root into the xylem. Effects on leucine uptake and incorporation were also investigated.

MATERIALS AND METHODS

Seeds of Zea mays L., cv. Northrop King PX 20, were germinated on cheesecloth-covered stainless steel nets suspended over continuously aerated 0-5 mM CaSO$_4$. After 4 d growth in the dark at 26 °C, roots were excised and used in the experiments.

Phosphate uptake

Four-day-old corn roots were excised from the plants, cut into segments 1-2 cm long and treated with various NaCl concentrations for 30 min at about 1 °C. The roots were then rinsed for 30 min with distilled water at 1 °C. During this period of osmotic shock the roots regain turgor but release significant amounts of protein (Maas and Finkel, 1979; Maas et al., 1979).

Subsequently, net Pi uptake was measured while incubating the roots for up to 24 h in a solution of 0-5 mM CaSO$_4$ and 0-2 mM KH$_2$PO$_4$. The solutions were continuously shaken and aerated. Ion uptake or loss was estimated by analysing 1-0 ml aliquots periodically removed from the absorption solutions. Phosphate was determined by the phosphomolybdate method of Taussky and Shorr (1953). The phosphate content of the roots was about 15 μmol g$^{-1}$ fr. wt.

Phosphate fluxes

Phosphate fluxes were determined with $^{32}$P and the apparatus described by Pitman (1971) and Weigl (1969). Briefly, it consisted of a plexiglass box divided into two chambers by a rubber membrane. Ten excised roots, 6-0 cm long, were inserted through holes in the membrane, with about 5-5 cm of the apical end protruding into the main chamber and the cut ends into the small chamber. After inserting unlabelled roots through the membranes, the main chamber was filled with 70 ml of solution consisting of 0-2 mM KH$_2$PO$_4$, carrier-free $^{32}$P (specific activity between 0-60 and 1-35 Ci mol$^{-1}$) and 0-5 mM CaSO$_4$. The small chamber contained 20 ml of CaSO$_4$ solution iso-osmotic with that in the main chamber. Ion translocation into the xylem of both control and osmotically-shocked roots was estimated by measuring periodically the concentration of labelled Pi in the small compartment. Calculations were based on the specific activity of the outer solution. The volume of solution in the
small chamber was maintained at 20 ml and the radioactivity removed during sampling was accounted for in the calculations.

Ion efflux was determined by inserting prelabelled roots in the boxes and measuring the release of $^{32}$P from the cortex (main chamber) and from the xylem (small chamber) to the outer solutions. The roots initially contained about 15 μmol g$^{-1}$ of Pi labelled with $^{32}$P at specific activities varying between 0.54 and 4.13 Ci mol$^{-1}$ at the beginning of different experiments. The $^{32}$P retained in roots after the efflux experiments was determined by solubilizing the roots in hot nitric acid and counting the activity. Ion efflux was calculated from the specific activity of whole roots.

**Leucine uptake and Incorporation**

Leucine uptake and incorporation into excised roots were measured on control and salt-shocked roots immediately after shocking and 24 h after recovery in a solution of 0.2 mM KH$_2$PO$_4$ and 0.5 mM CaSO$_4$. Tetracycline (6.0 mg l$^{-1}$) was added to the recovery solutions to control bacterial contamination. Leucine uptake and incorporation were determined by floating 5.0 mm long root sections on $^{14}$C leucine solution (final concentration 1.0 mM with specific activity between 1.17 and 2.41) for 1 h. The sections were then washed in 10 mM unlabelled leucine and 1.0 g samples were ground in 1.0 ml of 0.1 N NaOH with a glass homogenizer. The apparatus was then washed with 1.0 ml of 0.1 N NaOH and 200 μl aliquots of the homogenate were placed on each of two filter paper discs. After drying, one of the discs was counted immediately as a measure of leucine uptake. The other was treated as follows to remove leucine that was not incorporated into protein: washed for 15 min in 10% (w/v) TCA; 1 h in 5% TCA at 90 °C; 15 min in 95% (v/v) ethanol–anhydrous ether (1:1, by vol.); 15 min in ethanol–anhydrous ether (1:3, by vol.); and finally 15 min in ether. The residue was then dried and $^{14}$C was counted to obtain a measure of leucine incorporation in the proteins.

Radioactive Pi was counted on a Nuclear-Chicago$^2$ scintillation detector and $^{14}$C-leucine on a Beckman liquid scintillation analyser.

## RESULTS

### Phosphate uptake

Net Pi uptake by excised corn roots was unaffected by 50 mM NaCl pretreatments but was inhibited at concentrations higher than 50 mM during the first hours of the experiment (Fig. 1). In fact, a significant Pi loss occurred initially at the highest concentrations. However, within 2–6 h depending on salt concentration, the roots recovered and Pi uptake resumed at rates comparable to the controls in all treatments except for roots stressed with 250 mM NaCl. Nevertheless, some recovery was evident even at this high concentration. The lag time between net loss of Pi and net Pi uptake increased with the intensity of the shock. Net Pi uptake in roots shocked with 250 mM NaCl did not occur until 20 h after the beginning of the experiment.

### Phosphate translocation

Phosphate translocation measured in exudate from cut ends of excised roots was small compared with net Pi uptake. After 48 h of incubation, only 0.36 μmol Pi g$^{-1}$ fr. wt. were released from the xylem of control roots (Fig. 2). Measureable differences in translocation among the stressed roots were not apparent until 24 h after shocking. Translocation was stimulated in roots pretreated with 50 mM NaCl, but was clearly reduced in roots shocked with NaCl at concentrations above 100 mM. Nearly complete inhibition occurred at 250 mM NaCl. In fact, the inhibition was greater than that caused by 3.0 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP), an inhibitor of active ion transport and oxidative phosphorylation. Unlike absorption which recovered after moderate osmotic shock, translocation in shocked roots never resumed the rates for control roots.

$^2$ Citation of trade names is for the benefit of the reader and does not imply any endorsement, guarantee, or preferential treatment by the U.S.D.A. or its agents.
**Phosphate fluxes**

The effect of osmotic shock on Pi efflux was determined with roots prelabelled with $^{32}$P and then shocked with NaCl at various concentrations (Fig. 3). Phosphate efflux from the cortex was little affected by NaCl concentrations up to 150 mM but it was increased by higher concentrations. The time course shows that like the control, most of the efflux occurred within a few hours after treatment at the lower concentrations but it continued for up to 24 h after the 250 mM and 300 mM treatments. The time course of efflux from the xylem was similar to that of the cortex but the amount was much less and it generally increased with increasing NaCl concentrations. Apparently, a breakthrough occurred at 300 mM NaCl because the amount of efflux from the xylem was nearly double that at 150 mM. In any case, the amount of Pi efflux from both the cortex and xylem caused by moderate osmotic shock up to 200 mM NaCl was very small (cf. Fig. 1).

**Protein synthesis**

To test the hypothesis that recovery of Pi uptake activity after osmotic shock requires protein synthesis, uptake of $^{14}$C-leucine and its incorporation into proteins of stressed and non-stressed roots was determined. The results show that both the uptake of leucine and its
incorporation into proteins immediately after shock were partly reduced by 50 mM and 100 mM NaCl but were strongly inhibited by concentrations above 100 mM (Fig. 4). Within 24 h after shock with up to 150 mM NaCl, the roots significantly recovered their ability to take up leucine and incorporate it into proteins. Some recovery was observed after osmotic shock at 200 mM but very little at 250 mM. Since leucine incorporation into proteins is dependent upon leucine uptake, the inhibition of leucine incorporation by osmotic shock does not necessarily indicate that protein synthesis was inhibited. Consequently, the recovery of Pi uptake was tested in the presence of two inhibitors of protein synthesis. Pi uptake by control and NaCl-pretreated roots (200 mM) was measured in the absence and presence of 200 mg l\(^{-1}\) puromycin or tetracycline. Figure 5 shows that tetracycline suppressed the recovery of Pi uptake by osmotically-shocked roots for approximately 6 h. Puromycin, while it caused greater loss of Pi from the roots, had little or no effect on recovery. Unfortunately, both inhibitors partially reduced Pi uptake by control roots.
DISCUSSION

The effects of osmotic shock on corn roots during the first 10 h after treatment were similar to those found by Maas et al. (1979) with barley roots. Net Pi uptake was increasingly inhibited as NaCl concentrations were increased above 50 mM. Concentrations above 100 mM caused the net loss of Pi during the first few hours. However, within 2–6 h after shocking with up to 200 mM NaCl, the roots appeared to recover almost completely and uptake rates approached that of the control. Even after a very strong osmotic shock treatment, e.g. with 250 mM NaCl, the roots partially recovered, although net Pi uptake did not occur until 20 h later. This recovery was not apparent in the shorter experiments with barley (Maas et al., 1979). The experiments with both barley and corn showed that NaCl treatments of 200 mM and higher caused significantly greater Pi loss and inhibition of Pi uptake than 150 mM NaCl. It is likely that plasmolysis and additional membrane damage occurred at NaCl concentrations between 150 and 200 mM (−0.7 and −0.95 MPa, osmotic potential).

It should be noted that excision, cold shock, or even handling causes an initial reduction in ion uptake. However, Gronewald and Hanson (1980, 1982) have shown that corn roots recovered within 2–3 h. A similar 2 h lag phase was observed with our control roots before steady-state uptake occurred.

Fig. 3. Efflux of Pi from control and NaCl-pretreated corn roots. Roots were labelled with carrier-free $^{32}$P for 24 h, then excised and treated with 0–300 mM NaCl at 1 °C. Efflux was measured with roots immersed in an unlabelled solution of 0.2 mM KH$_2$PO$_4$ and 0.5 mM CaSO$_4$ at 25 °C.
The inhibition of net Pi uptake by moderate osmotic shock during the first few hours was caused primarily by decreasing Pi influx rather than by increasing efflux. Pi efflux from the cortex of roots that were prelabelled with $^{32}$P and then osmotically shocked with up to 150 mM NaCl was no higher than that from control roots. Higher salt concentrations caused continuous and appreciably more efflux confirming that further membrane damage had occurred which was neither readily nor completely repaired. Although Pi efflux from the xylem of shocked roots initially exceeded that of the control, the loss was very small compared to the decrease in net Pi uptake discussed above.

Recovery of Pi uptake activity by plant roots after moderate osmotic shock appears to depend upon de novo protein synthesis (Attia and Jeanjean, 1983; Maas et al., 1979). Our results show that leucine uptake and incorporation into proteins recovered within 24 h after osmotic shock with NaCl at concentrations up to 150 mM. Some recovery occurred at higher concentrations, but like Pi uptake activity, the recovery was incomplete. While these results do not necessarily indicate that protein synthesis was inhibited, they do show that after leucine uptake recovered, leucine incorporation and presumably protein synthesis were able to function normally. Results with protein synthesis inhibitors were inconclusive. While tetracycline inhibited recovery of Pi uptake in osmotically-shocked roots, it also affected uptake by control roots. The effect of puromycin on corn roots differed with that found with barley roots (Maas et al., 1979) where it had no effect on control roots, but strongly suppressed recovery of Pi uptake in salt-pretreated roots.
Several investigators working with bacteria and yeast have shown that these shock proteins are membrane binding proteins involved in ion transport (Ichihara and Mizushima, 1977; Jeanjean, 1981; Medveczky and Rosenberg, 1970; Pardee and Watanabe, 1968). A similar role has been suggested for proteins released from higher plants (Attia and Jeanjean, 1983; Maas et al., 1979; Nieman and Willis, 1971). Rubinstein (1982a) concluded from studies with oat leaf segments that cold hypo-osmotic shock inhibits proton excretion; plasmolysis alone was ineffective. It is worth noting that inhibition of Pi uptake with increased concentrations of salt correlates best with the amount of protein released in the hypo-osmotic solution (Maas et al., 1979).

Pi translocation to the xylem was also inhibited by osmotic shock but unlike Pi uptake activity, it did not completely recover within the 48 h after salt treatments. Apparently, some cellular damage that restricted translocation across the root persisted long after Pi uptake activity recovered. At high salt concentrations, symplasmic transport may have been inhibited because plasmodesmata were damaged during osmotic shock. The investigations of
Van Iren and Boers-van der Sluijs (1980) and Grunwaldt et al. (1978, 1979) showed that strong osmotic shock reduced ion transport to the internal part of corn and barley roots. Rubinstein (1982a, b) suggested that the plasmodesmata are damaged when the protoplasm swells during the hypo-osmotic treatment. He further suggests that some of the shock protein may come from the plasmodesmata. However, the nature and degree of injury to plasmodesmata from moderate osmotic shock is not known.

We conclude that moderate osmotic shock decreases net Pi uptake primarily by inhibiting active Pi influx and not by increasing membrane permeability and Pi efflux. This finding is consistent with the hypothesis that hypo-osmotic shock releases membrane proteins that are functionally involved in Pi transport (Maas et al., 1979; Nieman and Willis, 1971). It is possible, of course, that these proteins include ATPase proton pumps as Rubinstein (1982b) and Attia and Jeanjean (1983) suggest. The ability of the roots to resume Pi uptake and leucine uptake and incorporation into proteins after moderate osmotic shock shows that the membranes become functional again, presumably as a result of de novo protein synthesis; however, this conclusion requires further verification.

LITERATURE CITED


