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Interactive Effects of Salinity and Phosphorus Nutrition on the Concentrations of Phosphate and Phosphate Esters in Mature Photosynthesizing Corn Leaves[1]

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ABSTRACT

The effects of salinity on corn plants (*Zea mays* L.) are influenced by the concentration of nutrient orthophosphate. Salinity (−2 bars each of NaCl and $CaCl_2$) was more injurious in combination with a high concentration of orthophosphate (2 mM) (that gave optimum yields in the absence of salinity) than it was with a lower concentration (0.1 mM). With 2 mM orthophosphate, salinity seemed to damage the plant mechanisms that normally regulate the internal concentration of orthophosphate resulting in excessive accumulation and P toxicity. On the other hand, with 0.1 mM orthophosphate, salinity decreased orthophosphate concentration in mature leaves. This effect was paralleled by decreases in the concentration of adenosine 5'-triphosphate and in the energy charge of the adenylate system, indicating an orthophosphate deficit. Even so, plants survived salinity better under these conditions than in the presence of 2 mM orthophosphate. The data indicated that salinity affected the phosphorylated state of the adenine nucleotides only indirectly through its effect on the concentration of orthophosphate in the cells.

Salinity, especially in the presence of 2 mM orthophosphate, resulted in an increase in the concentrations of sugar phosphates in mature photosynthesizing leaves, suggesting that translocation rather than photosynthesis was a limiting process. Decreased translocation could be a secondary effect of decreased growth. However, a decreased translocation rate could cause decreased growth by limiting the supply of essential metabolites reaching growing tissues.

The recent salinity-fertility studies of Bernstein *et al.* (7) showed a marked interactive effect of salinity and nutrient Pi on corn plants. Salinity was more deleterious in combination with a high concentration of Pi (2 mM) than with a low concentration (0.05 mM). There occurred a greater reduction in yield and a characteristic leaf injury. The injury seemed to be a result of excessive accumulation of P; other ions did not accumulate sufficiently to account for it. Also, the injury signs were similar to those caused by P toxicity in wheat (8) and barley (15). These observations suggested that salinity affected the uptake and utilization of Pi by the plants. Considering the central role of P in cell metabolism, evidence of salt-induced changes in P metabolism might also provide insight regarding the mechanism of salt injury to plants. The work reported here was concerned with the effects of salinity in combination with low and high concentrations of nutrient Pi on the concentrations of Pi and of P-esters[2] in mature photosynthesizing corn leaves. Preliminary work showed that salinity, like extremes in P nutrition (9), affected the concentrations of these compounds much more than it did the concentrations of lipid P and nucleic acid P.

MATERIALS AND METHODS

Plant Culture. Corn plants (*Zea mays* L. T-strain Golden Cross Bantam) were grown from seed in nutrient-irrigated sand cultures in a heated greenhouse from December to February. The minimum night temperature was 16 C, the maximum day temperature was 32 C. The relative humidity was not controlled and generally ranged from 40% during the day to 80% at night. There were eight cultures consisting of 19-liter glazed crocks filled with coarse sand. Each crock had its own 40-liter reservoir of nutrient solution. The solution was pumped from the reservoir to the top of the sand and allowed to drain back into the reservoir three times daily, at 0900, 1700, and 2400 hr. The sand, after draining, retained approximately 4 liters of solution. The nutrient solution was made with deionized H_2O and the following salts in mM concentration: $Ca(NO_3)_2$, 2.5; KNO_3, 2; $MgSO_4$, 1.5; KH_2PO_4, 0.1 (low P) or 2 (high P); K_2SO_4, 0.95 or 0 to equalize the K^+ concentration of low and high P solutions, respectively. Trace elements were supplied as follows with the amounts in mg/l: Fe, 0.5 as ferric citrate; Mn, 0.25 as $MnCl_2$; B, 0.25 as H_3BO_3; Zn, 0.05 as $ZnSO_4$; Cu, 0.02 as $CuSO_4$; Mo, 0.01 as H_2MoO_4. The saline solutions also contained 32 mM $CaCl_2$ and 48 mM NaCl. The osmotic potential of the base nutrient solution was −0.4 bar and that of the saline solution was −4.4 bars. The four treatments were determined by the irrigation solutions used; control (nonsaline) low P, control high P, saline low P, and saline high P. Two crocks (treatment replicates) were irrigated with each solution.

Excess corn seed was planted in each crock. Initially, four crocks were irrigated with control low P solution, and four were irrigated with control high P solution. The salt treatment was initiated when seedlings were established by adding the $CaCl_2$ and NaCl to the nutrient solution in two equal increments 1 week apart. The plants were thinned to one per crock. Nutrient solution volumes were maintained throughout the experiment by adding deionized H_2O to the solution reservoirs. The pH value of the solutions was kept between 5 and 6 by adding nitric acid. Ferric citrate was added every few days to provide Fe at 0.5 mg/l. All solutions were replaced every 3 weeks. The minimum Pi concentrations determined in the 3-week-old low and high P solutions were 0.02 mM and 1.4 mM, respectively.

[1] This paper is dedicated to a fellow plant physiologist and friend, Leon Bernstein.

[2] Abbreviations: F6P: fructose 6-phosphate; FDP: fructose 1,6-diphosphate; G1P: glucose 1-phosphate; G6P: glucose 6-phosphate; P-esters: acid-soluble phosphate esters; ester-P: esterified phosphate; PGA: 3-phosphoglyceric acid; R5P: ribose 5-phosphate; UDPG: UDP-glucose.

Leaves 8, 9, 10, and 11 on the main axis (the 14th was the flag leaf) were harvested at 1300 hr on the 76th day, after 5 hr of exposure to bright sunlight. The salt treatment, which had been in full effect for 52 days, did not affect the number of leaves on the main axis. The leaves were cut off 1 cm above the sheath joint, weighed, rinsed with distilled H_2O, blotted dry, and frozen in liquid N_2. Only the saline high P treatment caused necrotic areas in the leaves. All of the necrotic tissue was cut away and discarded so that only green, healthy appearing tissue was used for analysis.

Extraction of Pi and P-esters. The eight leaves sampled from a given treatment were pulverized together in the presence of liquid N_2 and extracted with cold aqueous trichloroacetic acid according to the procedure recommended by Isherwood and Barrett (21). This procedure was shown (21) to give quantitative recovery of phosphate esters added to plant tissue and to remove all of the naturally occurring low mol wt P compounds. The extract was partitioned against ethyl ether (6×) to remove most of the trichloroacetic acid concentrated in a rotary evaporator at 37 C with reduced pressure, adjusted to pH 7 with NH_4OH, and centrifuged at 0 C. The dark brown precipitate was discarded. The tan supernatant was washed through a column (2.4 × 95 cm) of Sephadex G-15[3] with degassed distilled-deionized water, pH 5.5, at room temperature (20 to 23 C) and at a rate of 45 ml/hr. All P compounds, including Pi, eluted between 150 and 360 ml (1–2.4 void volumes). At least 10 different pigment bands remained adsorbed on the column after all P compounds had been eluted. The electrical conductivity of the eluate indicated that a considerable amount of salt also was held back and separated from the P compounds. Treating the Sephadex eluate with water-insoluble PVP at pH 3 did not seem to achieve additional purification.

Anion Exchange Chromatography. The P compounds were chromatographed on a column (2.4 × 70 cm) of Dowex 1-×8 formate, 200 to 400 mesh, with a void volume of 130 ml. The column was eluted with a formic acid-ammonium formate gradient at room temperature. The gradient system consisted of a Marriotte bottle reservoir connected in series with three straight walled, 1-liter, magnetically stirred mixing bottles. The third mixing bottle was connected to the column. The volume of solution in each mixing bottle remained constant at 1 liter. Solution was forced through the system by a hydrostatic head maintained by the Marriotte bottle. The latter contained 1 M ammonium formate in 4 N formic acid. The three mixing bottles initially contained: (*a*) 4 N formic acid; (*b*) 1 N formic acid; (*c*) degassed distilled-deionized water. The pH of the solution going on the column, initially 5.5 to 6, dropped to 3 with the first 10 ml and then slowly to a minimum of 2 by 400 ml; it increased to 2.5 by 1 liter and remained essentially constant thereafter. The concentration of formate increased sigmoidally with a nearly linear rate of about 1.27 eq/l between 0.4 and 4 liters.

The column and the gradient system were calibrated by chromatographing mixtures of known compounds (glucose, G1P, G6P, F6P, FDP, R5P, AMP, ADP, ATP, UMP, UDP, UTP, UDPG, Pi, NAD, NADP, PGA). Elution was monitored by measuring UV absorbance, sugars, Pi, and total P after digestion. Good recoveries were achieved with esters of known purity (G1P, 96%; G6P, 100%; F6P, 100%; AMP, 96–100%; ADP, 100%; ATP, 95–98%). Bartlett (5) also reported quantitative recovery of P-esters chromatographed on Dowex 1-×8 formate at room temperature. Eluting the column at room temperature gave a better separation of P-esters than was achieved at 2 to 4 C.

An aliquot of the Sephadex-treated extract equivalent to 25 g of fresh leaf was adjusted to pH 7 with NH_4OH and placed on the Dowex 1 column. The eluate was collected in 10-ml fractions. Cations and ampholites were washed off the column with H_2O and then gradient elution was commenced at a rate of 30 ml/hr at room temperature. The eluate was monitored by measuring pH, electrical conductivity, UV absorbance at 260 nm, total P after digestion, and sugars by the α-naphthol reaction.

Fractions from the center of each P peak, where the chance of cross contamination was minimal, were combined. Aliquots were removed to determine Pi, acid-labile P, and total P. Another aliquot was treated batch-wise with Dowex 50, H^+ form, to remove ammonium ion, with ether to remove formic acid (5), and then concentrated in a rotary evaporator at 37 C with reduced pressure. This solution was used for measuring UV absorption spectra and for determining sugars.

Analytical Methods. UV absorbance of solutions was measured in 1-cm path length cuvettes with a Beckman DU spectrophotometer. Nucleotide concentration was calculated by means of the molar absorbancy values in the Pabst Circular OR-10 (29). Pi was determined by the Fiske-SubbaRow procedure (13), acid-labile P by the same procedure after heating with 1 N H_2SO_4 at 100 C for 7 min. Total P was determined by the procedure of Bartlett (6) after digestion with H_2SO_4 and H_2O_2. Total sugar was determined by the α-naphthol reaction (12); hexose by the anthrone reaction (31); fructose by the resorcinol (2) and cysteine-carbazole (2) reactions; pentose by the orcinol reaction (2).

RESULTS

Growth. The control high P treatment produced the highest yield of green shoots (Table I), it was twice that of the low P treatment. This difference was primarily due to the absence of tillers on the low P treatment, which is a characteristic of P-deficient plants. No other signs of a P deficiency were evident. Plants on the two P treatments were similar with respect to the size and appearance of the main axis. Plants on the saline treatment produced no tillers with either low or high P. The combination of salinity and high P caused the greatest suppression of growth and also caused a severe leaf injury that did not occur on any of the other treatments. The injury appeared first as a water-soaked region at the leaf tip that later became necrotic and progressed as far as 25 cm back along the margins and between the veins. None of the salt-affected plants showed distinct signs of water stress like wilting or rolling of leaves.

Ion Exchange Chromatography. The chromatogram of the leaf extract from the saline high P treatment (Fig. 1) showed 19 major P peaks and several minor ones. Baseline separation was not always achieved, but the major peaks were reasonably distinct. Similar chromatograms, but with generally smaller peaks, were obtained for the three other treatments. The chromatogram for the control low P treatment lacked peaks 2, 6, and 8; that for the control high P treatment lacked peaks 2 and 9. The amounts of P present in the major peaks are indicated in Table II. The assay was reliable at least down to 0.1 μmole Pi/100 g fresh tissue. The main P compound in each peak is indicated in those cases where identification is reasonably certain. The criteria used for identification were: the position of the peak relative to peaks of known compounds: UV absorption spectra at pH 2 and pH 7; the presence of Pi, acid-labile P,

Table I. *Effects of Salinity and Nutrient Pi on Yield of Corn Shoots*

Treatment	Fresh Wt/Two Plants
	g
Control high P	2259
Control low P	1101
Saline low P	549
Saline high P	252

[3] Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the United States Department of Agriculture.

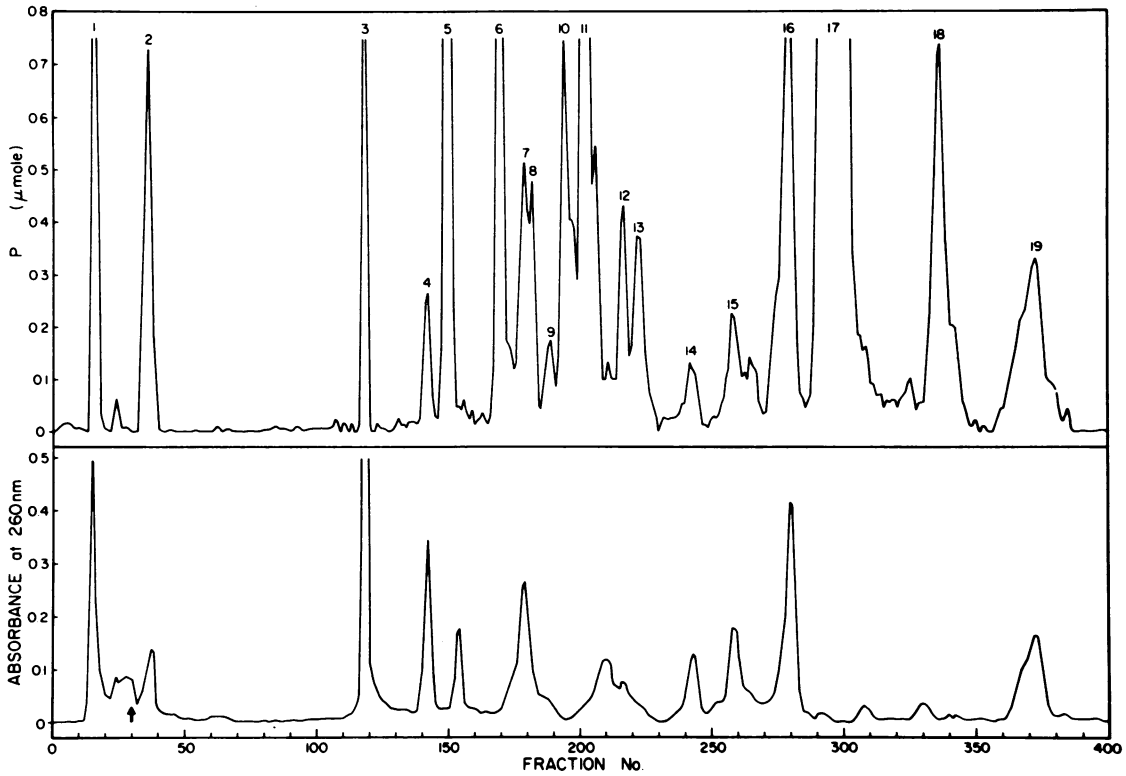

FIG. 1. Chromatogram of phosphorus compounds in the trichloroacetic acid extract of mature photosynthesizing leaves of corn plants grown on saline high P cultures. The compounds were separated on a 2.4 × 70 cm column of Dowex 1-formate with a formic acid-ammonium formate gradient (pH 6 to 2.5, formate 0 to 4.2 M). The gradient was started at the arrow after eluting with water. The phosphorus concentration (ordinate) is in μmoles/10 ml fraction. The UV absorbance at 260 nm is corrected for the absorbance of the eluant.

Table II. *Effects of Salinity and Nutrient Pi on Concentrations of Pi and P Esters in Mature Photosynthesizing Corn Leaves*

Peak	Main P Compound	Treatment			
		Control		Saline	
		Low P	High P	Low P	High P
No.		*μmoles P/100 g fresh wt*			
1	Pi	4.7	13.6	28.4	37.5
2	Unknown	0	0	1.4	10.5
3	NAD	3.6	6.1	4.8	9.3
4	AMP	3.4	1.4	4.9	2.7
5	Unknown	0.7	7.3	5.7	24.8
6	Unknown	0	9.3	6.2	21.2
7	NADP	5.6	5.1	5.4	8.6
8	Unknown	0	2	2.1	6
9	Unknown	2	0	0.9	2.9
10	G1P	5.3	7.3	9.6	11.1
11	G6P	9	15.2	18.3	29.6
12	F6P	1.5	2.8	5.5	6
13	R5P	0.9	2.2	3.6	7.1
14	UMP	0.9	4.9	3.2	2.2
15	ADP	5	4	3.2	4.9
16	UDPG	5.8	15.3	6.1	15.3
17	Pi	127.3	173.1	49.4	683.6
18	PGA	9.3	5.8	9	15.3
19	ATP	7.7	11.1	5.3	13.8
Total Pi (1 + 17)		132	186.7	77.8	721.1
Total acid-soluble P		192.7	286.5	173	912.4

and/or acid-stable P; the kind of sugar indicated by the reaction with the sugar reagents; ratio of sugar-P; ratio of P-pentose-nucleotide, determined by UV absorbance, and hexose-P-pentose-nucleotide for UDPG.

Peak 1 contained cations and neutral compounds that eluted in the void volume during the water wash. All of the P present in this fraction reacted as Pi. It appeared to be complexed with Ca^{2+} and Mg^{2+} and so was not retained by the resin. The UV absorbance was caused by a tan phenol-like pigment with an absorption peak at 335 nm, pH 7.

The unidentified peaks, 2, 5, 6, 8, and 9 all gave a positive reaction with α-naphthol indicating a sugar moiety. The position of these peaks tended to rule out sugars with fewer than six carbons. A decrease in the chain length of sugar phosphates tends to increase retention on the resin (5). Peak 2 gave no reaction with the other sugar reagents. Peaks 5 and 6 reacted with anthrone but with low color yield/mole of P, suggesting mannose or galactose. Neither reacted with orcinol or resorcinol, thus, ketoses were ruled out. Peak 9 gave a weak positive reaction with orcinol and resorcinol; these reactions, together with the position of the peak, suggest that it may contain sedoheptulose 7-phosphate. None of the five peaks indicated specific UV absorbance, thus, nucleotides were ruled out. P peaks 2 and 5 were closely followed by UV absorbing peaks, but distinct from them.

At least 500 fractions were collected for each extract, but there were no discrete P peaks after 400. Known UTP eluted as a distinct peak well after ATP. No UTP was detected in any of the extracts, so its concentration evidently was below 0.1 μmoles P/100 g fresh weight. UDP was not detected either. Known UDP eluted between PGA and ATP. Possibly a small UDP peak might have been obscured by the leading edge of a broad ATP peak.

Table III gives the calculated values for four adenine nucleotide ratios. The ratio [ATP]/[ADP] has a controlling effect on many enzymes (3, 30). It also indicates the availability of Pi for phosphorylation (24), a low ratio, as observed on the low P treatments, indicating a Pi deficit. This ratio was influenced by nutrient Pi, but not by salinity. The energy charge of the adenylate system, ([ATP] + ½ [ADP])/([ATP] + [ADP] + [AMP]), a metabolic regulatory parameter (4, 10, 30), decreased with the concentration of Pi in the tissue. It seemed to be only indirectly

Table III. *Effects of Salinity and Nutrient Pi on Phosphorylated State of Adenine Nucleotides in Mature Photosynthesizing Corn Leaves*

Nucleotide Concn	Treatment			
	Control		Saline	
	Low P	High P	Low P	High P
ratio				
$\frac{[ATP]}{[ADP]}$	1.0	1.9	1.1	1.8
$\frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$	0.45	0.66	0.31	0.60
$\frac{[ATP]}{[ADP] \times [Pi]}$ ($\times 10^{-3}$ M)	0.79	0.99	1.45	0.26
$\frac{[ATP] \times [AMP]}{[ADP]^2}$	1.42	1.29	3.45	1.98

affected by salinity, through changes in tissue Pi. The phosphate potential, [ATP]/([ADP] × [Pi]), has been shown to control mitochondrial respiration (22, 23, 28, 33). It influences the redox state of Cyt *c* (22) and a_3 (28). Whereas it was little affected by nutrient Pi in the absence of salinity, it was influenced by the extremes in tissue Pi caused by salinity, increasing on the saline low P treatment and decreasing on the saline high P treatment. The apparent equilibrium constant of the adenylate kinase reaction, K = ([ATP] × [AMP])/[ADP]², tended to remain constant in the absence of salinity and to increase in its presence, especially with low nutrient Pi.

DISCUSSION

Plants on the nonsaline high P treatment are regarded as "normal" controls for comparison with plants on the three other treatments. The concentrations of Pi and of P esters in the leaves (Table II) were in the range expected for plant tissue adequately supplied with Pi (9). The ratios of AMP-ADP-ATP, ATP-ADP, and the energy charge were similar to those of photosynthesizing wheat leaves (10) and indicative of an active adenylate kinase system (10). As others have noted (25), the energy charge of tissue of higher plants tends to be lower than that of microorganisms and animal tissue.

Several criteria indicate that the plants on the control low P treatment were deficient in Pi: the reduced shoot yield, the absence of tillers, the reduced concentration of Pi and of most P esters, the low concentration of ATP, the low ratio of ATP-ADP, and the low energy charge. The geometry and extent of the root system and the volume of substrate available to the roots are important factors in determining the ability of plants to take up Pi (9). In our experiments, the small size of the cultures (19-1) may have contributed to a Pi deficiency on the 0.1 mM treatment. When corn plants were grown on 1000-l cultures a Pi concentration of 0.005 mM has been reported to be sufficient (32).

Considering the 20-fold difference in the initial Pi concentration of the high and low P nutrient solutions, the difference in the Pi concentration in leaves on the two treatments was relatively small. The plants evidently maintained considerable control over the internal concentration of Pi.

The interactive effects of salinity and nutrient Pi on growth were similar to those reported earlier by Bernstein *et al.* (7). Salinity was more damaging to corn plants in the presence of a high concentration of nutrient Pi; it caused visible leaf injury only under these conditions.

There were two effects of salinity on P utilization that suggest damage to transport processes: a loss of control of internal Pi concentration and an accumulation of P-esters in source leaves. In the presence of 0.1 mM Pi, salinity decreased the concentration of Pi in leaves by nearly half. This effect was not attributable to decreased availability of Pi in the saline-nutrient solution, a possibility that was considered because of the higher concentration of Ca^{2+}. Sugar phosphates, on the other hand, increased so there was relatively little change in the concentration of total acid-soluble P.

In the presence of 2 mM Pi, salinity caused a 4-fold increase in the concentration of Pi in leaves, and leaf injury. The course of injury suggested a gradual breakdown of cell membranes with increasing concentrations of Pi in the tissue. A similar loss of control over internal Pi concentrations has been observed with P-deficient barley plants (15). Supplying the deficient plants with Pi resulted in excessive accumulation and leaf injury signs similar to those we observed on corn. The high concentrations of sugar phosphates in the source leaves of salt-stunted plants suggest that photosynthesis was not limiting for growth. These results are reminiscent of the earlier work of Gauch and Eaton (14) showing increased accumulation of sucrose and starch in the shoots of salt-stunted barley plants. This accumulation of metabolites in mature photosynthesizing leaves of salt-stunted plants might be attributed to a reduced number of active sinks using these metabolites for growth. It is also possible that salinity impaired translocation and thus retarded or prevented the development of sinks.

Drought clearly curtails translocation (11, 20). Information regarding specific effects of salinity on this process is scarce. Abrupt salination of sugarcane suppressed translocation (16), but this effect was probably due more to osmotic shock than specifically to the excess salt. There is some evidence that salinity suppressed the translocation of photosynthate from the leaves to the root of beet (19) and that high atmospheric humidity tended to overcome this effect. Here, translocation seemed to be responding to the combined effects of salinity and high transpiration on plant water potential. Both stresses would reduce the osmotic potential of tissues surrounding the phloem and this could increase the osmotic work required to maintain phloem loading and transport. Hayward and Long (18) observed that salinity suppressed the development of phloem relatively more than the development of xylem and parenchymatous tissue, so the transport system itself could become limiting in the salt-affected plant.

Salinity seemed to affect the phosphorylated state of the adenine nucleotides only indirectly, through its effect on the concentration of Pi in the tissue. When this was high, as it was on the saline-high P treatment, the concentration of ATP, the ratio of ATP-ADP, and the energy charge were also high. All of these were low when the concentration of Pi in the tissue was low. Hasson-Porath and Poljakoff-Mayber (17) have reported that saline concentrations of NaCl and Na_2SO_4 decreased the concentration of ATP in pea root tips. The concentration of Pi in the tissue was not given, but the ratio of ATP-ADP decreased with salinity, suggesting a Pi deficit. Both NaCl and Na_2SO_4 have been shown to damage the Pi uptake mechanism of carrot root cells (27).

Salinity increased the apparent K for the adenylate kinase reaction. This effect also was linked to the concentration of Pi in the tissue with the greatest increase occurring under conditions of a Pi deficit. Increased use of the β bond of ATP presumably would be advantageous under these conditions. Changes in the apparent K are not unusual; they may be caused by changes in pH, Mg^{2+} concentration, and nutritional status of the cells (10).

UDPG followed a pattern similar to that of ATP, responding to the concentration of Pi in the tissue rather than to salinity *per se*. With low Pi, ATP may have become limiting for the nucleosidediphosphate kinase reaction. G1P did not seem to be limiting.

The phosphate potentials for the corn leaves are not firm because of uncertainty regarding the concentration of Pi around the mitochondria. Presumably, it was proportional to the con-

centration in the tissue as a whole. The phosphate potential was little affected by changes in the nutrient Pi concentration in the absence of salinity. It was affected by the extremes in tissue Pi caused by salinity. The greatest change was the decrease, caused by increased Pi, on the saline high P treatment. Since a low phosphate potential would favor a high rate of respiration, it is interesting that salinity, with adequate nutrient Pi, does in fact, increase leaf respiration of corn (1) and a number of other species (26).

From an agronomic point of view, it is encouraging that salinity did not prevent the synthesis of P esters or directly affect the phosphorylated state of the adenine nucleotides in mature photosynthesizing leaves. These leaves, though stunted, seemed metabolically capable of supporting a greater rate of growth than realized with the salt-affected plants. The basis for the growth-suppressive effects of salinity is still moot. The results of these experiments and others (14, 19, 27) indicate a close link between disturbed transport of Pi and other metabolites, growth suppression, and leaf injury.

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