

# Correlation between the Suppression of Glucose and Phosphate Uptake and the Release of Protein from Viable Carrot Root Cells Treated with Monovalent Cations

Received for publication January 8, 1971

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## ABSTRACT

Treating carrot (*Daucus carota* L.) discs with ice-cold NaCl solutions for 30 minutes caused three effects that appear to be functionally related: the exchange of tissue  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  for  $\text{Na}^+$ , the release of protein, and the suppression of active uptake of glucose and orthophosphate. Cyclosis continued apparently unabated after treatment with NaCl at concentrations of up to 0.25 M, so the cells remained viable and energetically competent. The correlation between the release of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  and release of protein, and between these effects and the suppression of glucose and orthophosphate uptake, supports the hypothesis that divalent cations maintain, and monovalent cations disrupt, linkages between the outer cell surface and proteins required for active solute uptake. Calcium preserved uptake activity only when it was added in time to prevent the release of protein. Cells gradually recovered some glucose uptake activity after it had been completely inactivated by treatment with 0.25 M NaCl. This recovery occurred in the absence of added  $\text{Ca}^{2+}$ . It was inhibited by puromycin and so appears to require some protein synthesis. Beet (*Beta vulgaris* L.) discs were more resistant than carrot discs to treatment with NaCl solutions, thus reflecting the difference in tolerance of the two species to sodicity.

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Biological membranes have fairly specific ion requirements and their properties can be markedly altered by changes in the ionic composition of the bathing milieu. Hence, some membrane alteration might be expected in plants growing on a  $\text{Na}^+$ -saline root medium and accumulating excessive amounts of salts. The electron micrographs of the halophyte *Atriplex halimus* L., published by Blumenthal-Goldschmidt and Poljakoff-Mayber (2), indicate considerable damage to membranes of plants grown on media containing high levels of  $\text{Na}^+$ :NaCl to -23 bars osmotic potential and  $\text{Na}_2\text{SO}_4$  to -17 bars OP. We are not aware of any published electron microscope studies of glycophytes grown at the more moderate levels of  $\text{Na}^+$ -salinity (-2 to -5 bars OP<sup>1</sup>) more commonly encountered, but membrane alteration might be inferred from some of the plant responses. A few examples are: (a) increased apparent root resistance to water flow (16, 20); (b) movement of  $\text{Na}^+$  into leaves of plants that normally exclude this ion from the shoot (17,

22); (c) suppressed uptake of <sup>14</sup>C-labeled glucose and L-leucine by roots (15, 23); (d) increased loss of polypeptide material from roots (authors, unpublished data).

The essential role of  $\text{Ca}^{2+}$  in preserving structural and functional integrity of plant membranes and the antagonistic effects of monovalent cations are well documented (5, 14). In their studies with soybean roots, Hanson and coworkers (7, 11, 26) found that both EDTA and monovalent cations removed  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , apparently from sites near the cell surface, along with UV-absorbing material that they suggested might be nucleotides. They also noted that removing divalent cations from roots suppressed their uptake of <sup>32</sup>P and <sup>36</sup>Cl, and that adding  $\text{Ca}^{2+}$  rapidly overcame the suppression, thus corroborating Tanada's earlier observations (27) with mung bean roots. Cations may influence uptake activity and specificity through their effects on the conformation of membrane macromolecules (19, 24). Also, divalent cations could aid in binding components of the transport system to the cell surface, whereas monovalent cations might promote their release. Heppel (13), Pardee (21), and others have obtained what appear to be membrane transport proteins from the outer surface of bacterial cells by relatively gentle EDTA-osmotic shock treatment. Uptake of certain solutes was impaired by the treatment and subsequently restored, in some cases, by adding back the released protein.

The objective of the experiments with carrot discs described below was to test the hypothesis that divalent cations maintain and monovalent cations disrupt linkages between the outer cell surface and proteins required for active solute uptake. Testing consisted of treating the discs with salt solution in the cold so as to minimize intracellular accumulation of salt, measuring the release of cations and of protein, and subsequently measuring the capacity of the treated tissue to take up glucose and  $\text{P}_i$ . Some expected consequences of the hypothesis are: (a) a release of protein following the exchange-replacement of tissue  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  by  $\text{Na}^+$ ; (b) correlation between the release of protein and the reduction of uptake capacity; (c) correlation between the preservation of uptake capacity by  $\text{Ca}^{2+}$  and preservation of protein attachment. These expectations were verified experimentally.

An experiment with beet discs was included to compare the effects of NaCl treatment on tissue of a plant that is much more tolerant than carrot to sodicity (1, 17). Sodidity commonly denotes a soil condition wherein 15% or more of the cation exchange sites on the soil exchange complex are occupied by  $\text{Na}^+$  instead of the usual  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . However, the important ionic conditions that a plant encounters on a sodic root medium, whether in the soil water or in nutrient culture solution, are: fairly high levels of  $\text{Na}^+$  (>20 meq/liter) and low levels of

<sup>1</sup> Abbreviation: OP: osmotic potential.

$\text{Ca}^{2+}$  (<1 meq/liter) (1, 17). Plant growth under these conditions seems to depend not so much on tolerance to  $\text{Na}^+$  as on the ability of the plant to make do with small amounts of  $\text{Ca}^{2+}$  in the presence of considerably greater amounts of  $\text{Na}^+$ . Beets grow quite well under these conditions (1) but carrots do not (17). One possible explanation for the difference is that beet may require less  $\text{Ca}^{2+}$  for membrane maintenance but binds the required  $\text{Ca}^{2+}$  more firmly than carrot does, making it more difficult to replace with  $\text{Na}^+$ . The resistance of the membranes of beet discs to treatment with  $\text{NaCl}$  and EDTA supports this point of view.

## MATERIALS AND METHODS

**Preparation and Treatment of Carrot Discs.** Carrots (*Daucus carota* L.) were purchased at a local market. A cylinder 9.5 mm in diameter was punched from the central xylem core with a No. 5 cork borer and sliced into 1-mm thick discs with a razor blade jig. The discs were washed and aged in several changes of aerated distilled water at room temperature (24–27 C; 25 C, in most cases) for a total of 18 hr. They were blotted dry with cleansing tissue and transferred in 20-g samples to 400-ml beakers. These were packed in ice on a rotary shaker. The discs were treated by shaking them (120 oscillations/min) for 30 min in 100 ml of ice-cold salt solution, or ice-cold distilled water in the case of the controls. The treatment solutions were decanted and saved. The discs were washed by shaking them for 30 min in 100 ml of ice-cold distilled water followed by a second wash of 30 min in distilled water at room temperature. The two washes were combined and saved. Treated and control discs were blotted dry, and then incubated at room temperature with shaking in 100 ml of 1 mM potassium phosphate buffer, pH 6.0, containing 1 mM glucose. The net uptake of glucose and  $\text{P}_i$  by the tissue was determined by analyzing aliquots of the glucose- $\text{P}_i$  solution. A 5-ml aliquot was withdrawn after 15 min of incubation and at hourly intervals thereafter.

The large, thin walled parenchyma cells making up the bulk of the discs showed very active cyclosis. This property was used to judge cell viability.

**Analyses.** The treatment solutions and the combined washes were each concentrated to about 5 ml by lyophilization. Salt-treatment solutions were dialyzed against cold distilled water for 1 hr; the water washes were not dialyzed. The concentrates were brought to 80% ethanol and allowed to stand at –20 C overnight. The precipitate was collected by centrifugation (35,000g, 0 C, 30 min), washed with absolute ethanol-acetone (1:1 v/v), dried in air and in a vacuum desiccator over  $\text{CaCl}_2$ , and then taken up in a small volume of 1 N NaOH. The protein content was determined by the procedure of Lowry *et al.* (18) and by the biuret reaction (9). Representative samples were also checked by Kjeldahl digestion and direct nesslerization. The proteinaceous nature of the material was also verified by hydrolyzing it with 3 N HCl in sealed tubes at 15 lbs., 120 C, for 5 hr and chromatographing the hydrolyzate on Whatman<sup>2</sup> No. 1 paper with phenol-water (100:39 w/v) and 1-butanolic-acetic acid-water (100:22:50 v/v). Spots were located with ninhydrin and isatin sprays. The total protein content of the carrot discs was estimated from the Kjeldahl nitrogen content of the residue remaining after discs had been triturated with 95% ethanol and extracted with 80% ethanol and with cold 5% perchloric acid.

The polysaccharide content of the alcohol-insoluble material obtained from the salt-treatment solutions and the water

washes was estimated by the  $\alpha$ -naphthol reaction (4), using galactose as a standard. This material was also hydrolyzed with 2 N HCl in sealed tubes at 15 lbs., 120 C for 45 min. The hydrolyzate was chromatographed on Whatman No. 1 paper with ethyl acetate-pyridine-water (8:2:1, v/v). Spots were located by the silver nitrate procedure of Trevelyan *et al.* (28).

Glucose was determined by the glucose oxidase procedure (Worthington Glucostat).  $\text{P}_i$  was determined by the Fiske-SubbaRow procedure (6). The cation content of the discs was determined after nitric-perchloric acid digestion. Sodium and potassium were determined by flame photometry, calcium and magnesium by EDTA titration (3).

## RESULTS

**Control Experiments.** The carrot xylem tissue consisted primarily of large, thin walled parenchyma cells with an average diameter of  $63 \pm 11 \mu$ . The discs were generally 15 to 16 cells thick. The diffusion paths from the surface to the center of the discs via the cell walls appeared to be sufficiently short for rapid equilibration of solution in the tissue free space with the external solution. Cyclosis was observed in cells at all depths including cells just below the cut surface. About 15% of the cells of the disc were located at the surface.

Assays on carrot discs that had been washed and aged for 18 hr indicated that they contained from 8 to 10 mmoles of glucose and from 0.3 to 0.9 mmole of  $\text{P}_i$  per 100 g fresh weight. The values fluctuated with the season of the year, tending to be higher in the winter and spring than in the summer. The minimum concentrations in the tissue, based on an average water content of 91%, would be of the order of 90 mM glucose and 3 mM  $\text{P}_i$ . Uptake of either from 1 mM solutions would be against an appreciable concentration gradient and, hence, is considered active uptake. Grant and Beevers (10) concluded from their studies with carrot discs that glucose uptake is an active process. The discs in the present case took up both glucose and  $\text{P}_i$  at constant rates for at least 3 hr (Fig. 1). The later decline in rate of glucose uptake is due to depletion of the solution, not to declining activity of the discs. Uptake returned to the higher rate when the solution was renewed. Measurements of uptake during the first 15 min in the glucose- $\text{P}_i$  solution were often erratic, presumably because of irregularities in the volume of solution carried over on the surface and in the free space of the discs. These irregularities were not entirely eliminated by blotting discs to constant weight. Consequently, the discs were allowed to equilibrate with the solution for 15 min before uptake measurements were begun. Zero on the time axis of the figures corresponds to the beginning of uptake measurements, 15 min after the beginning of incubation in the glucose- $\text{P}_i$  solution.

The influence of some of the standard treatment conditions on the rates of glucose and  $\text{P}_i$  uptake were examined in preliminary experiments. The pH of the distilled water used for washing and aging the discs was in the range of 5.0 to 5.5 because of dissolved  $\text{CO}_2$ . The pH of the salt solutions was in the same range. Adjusting all to pH 6.0 before use by adding KOH to the water and appropriate base to the salt solution did not affect the results. Aging the discs in 0.10 mM  $\text{CaCl}_2$  instead of in distilled water did not affect the results, provided the discs were washed with water before incubation in the glucose- $\text{P}_i$  solution. Traces of  $\text{Ca}^{2+}$  in this solution stimulated the uptake of  $\text{P}_i$  but not the uptake of glucose. Varying the ratio of glucose to  $\text{P}_i$  in the absorbing solution had little or no effect on uptake rates. The uptake of one seemed to be unaffected by the presence or absence of the other.

The washed discs appeared to be substantially free from microbial contamination. Microorganisms were not observed

<sup>2</sup> 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 U. S. Department of Agriculture.

when material scraped from the surface of the discs or collected by centrifuging the treatment solutions, washes, and glucose- $P_1$  incubation solutions was examined under a phase contrast microscope ( $\times 1500$ ). The presence of puromycin did not affect the rate of disappearance of glucose and  $P_1$  from solution containing control discs, indicating the absence of microbial contribution to the disappearance.

**Suppression by NaCl.** Treatment with ice-cold NaCl solutions for 30 min suppressed the uptake of both glucose and  $P_1$  (Fig. 1). The 30-min treatment was selected on the basis of preliminary experiments indicating a leveling off of suppression by that time. The degree of suppression was nearly independent of NaCl concentration in the range of 0.05 M to 0.20 M. Uptake curves for 0.10 M and 0.15 M NaCl treatments (not shown) were essentially congruent with those for the 0.20 M treatment. Suppression increased abruptly when the concentration of NaCl was raised to 0.25 M. Net uptake of glucose was completely prevented for several hours;  $P_1$  uptake persisted, but only at about 40% of the control rate. Treatment with 0.30 M NaCl caused the discs to release approximately 15% of their glucose and 25% of their  $P_1$  and completely prevented net uptake of these solutes for at least 5 hr. Cyclosis was observed in the treated cells for at least 24 hr after treatment, indicating the absence of severe intracellular damage. Damage was observed readily enough when discs were treated with 0.35 M and 0.40 M NaCl: cyclosis stopped; the cytoplasm of some cells separated from the cell walls; the tissue became flaccid; cells became leaky to glucose and other sugars and to  $P_1$ , amino acids, and protein.

**Osmotic Potential.** All of the osmotic potential values given refer to solutions at 0 C. The OP of the cells of washed and aged carrot discs was estimated by determining the lowest concentration of mannitol, within 0.05 M, causing definite plasmolysis of cells. Changes in cell volume were estimated by weighing the discs before and after incubation in mannitol solutions. Plasmolysis was not definite until the mannitol concentration reached 0.45 M ( $-10.8$  bars OP). Correction for the amount of water lost before plasmolysis, which amounted to about 10% of the fresh weight of the discs, indicated an OP

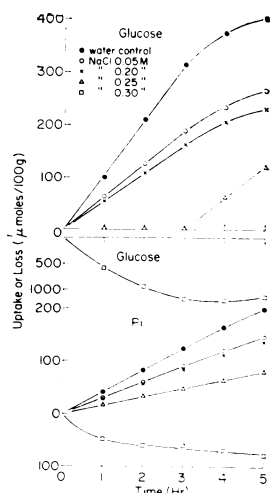


FIG. 1. Kinetics of uptake or release of glucose and  $P_1$  by control and NaCl-treated carrot discs. The standard procedure was to treat washed and aged (18 hr) discs with ice-cold solutions of NaCl or distilled water (control) for 30 min, wash them with ice-cold water for 30 min, followed by water at room temperature for 30 min. Control and NaCl-treated discs were incubated at room temperature in solution containing glucose and potassium phosphate, pH 6.0, each at a concentration of 1 mM. Uptake or loss was determined by analyzing aliquots of the solution.

TABLE I. Effect of NaCl Treatment on Cation Contents of Carrot Discs

Washed and aged (18 hr) carrot discs were analyzed before and after treatment with NaCl. They were treated with ice-cold NaCl solutions or distilled water for 30 min and washed with ice-cold water for 30 min and with water at room temperature for 30 min.

NaCl Treatment		Cation per 100 g Discs				Increase in Na <sup>+</sup> /Loss of Ca <sup>2+</sup> , Mg <sup>2+</sup> , K <sup>+</sup>
Concentration	Osmotic potential	Ca <sup>2+</sup>	Mg <sup>2+</sup>	K <sup>+</sup>	Na <sup>+</sup>	
M	bar	meq				meq/meq
		Before treatment				
		1.42	1.74	5.41	1.76	
		After treatment				
0	0	1.43	1.73	5.42	1.74	
0.10	-4.2	0.95	1.48	4.98	2.70	0.82
0.15	-6.3	0.86	1.43	4.98	2.82	0.82
0.20	-8.3	0.83	1.37	5.01	3.01	0.93
0.25	-10.4	0.80	1.33	5.00	3.08	0.92
0.30	-12.5	0.76	1.29	5.04	3.46	1.15

of about  $-9.7$  bars for turgid cells. In order to gain some insight regarding the osmotic effects of 0.25 M NaCl ( $-10.4$  bars OP) on the carrot cells, sections were examined with a microscope as they were subjected at room temperature to the following sequence of treatments: (a) changing the bathing medium from distilled water to 0.25 M NaCl; (b) incubation in 0.25 M NaCl for 30 min; (c) replacing the salt solution with distilled water. None of these treatments changed the appearance of the cells under the microscope. There was no definite indication of plasmolysis on adding the salt, and cyclosis continued throughout the entire sequence of treatments.

**Ion Exchange.** During the treatment with ice-cold NaCl solutions, the discs took up Na<sup>+</sup> and released Ca<sup>2+</sup>, Mg<sup>2+</sup>, and K<sup>+</sup> (Table I). Virtually all of the cations released appeared in the NaCl solutions, only traces appearing in subsequent water washes. Control discs, treated with distilled water only, released none of these cations. With NaCl concentrations up to 0.25 M, the meq of Na<sup>+</sup> taken up was slightly less than the total meq of cations released, and so could all be accounted for by ion exchange. Increasing the concentration of NaCl to 0.30 M increased the amount of Na<sup>+</sup> taken up apparently by mechanisms other than cation exchange for it caused very little increase in the amounts of Ca<sup>2+</sup> and Mg<sup>2+</sup> released and no increase in the amount of K<sup>+</sup> released. Only a small fraction of the total cell K<sup>+</sup> (about 8%) was accessible for exchange with Na<sup>+</sup>, and this fraction was completely removed by 0.10 M NaCl. The retention of most of the K<sup>+</sup>, even after treatment with 0.30 M NaCl, supports the assumption that the salt effects studied here were restricted to the outer surface of the cells.

**Protein Release.** The carrot discs released or secreted small amounts of protein to the bathing medium, and the treatment with NaCl solutions enhanced this release. Representative data are shown in Table II. The amounts of protein released varied somewhat with different batches of carrots, but the response to salt treatment followed a consistent pattern. Most of the protein released by salt came off into the water washes following the salt treatment rather than into the salt solutions. At concentrations of 0.05 M and 0.10 M, NaCl actually suppressed the release of protein in its presence. The amount of protein released to the wash went up with the first increment of NaCl and then remained essentially independent of NaCl concentration up to 0.20 M. Increasing the concentration of NaCl to

Table II. *Effect of NaCl Concentration on the Release of Protein by Carrot Discs to the Salt Solutions and Water Washes*

Washed and aged (18 hr) carrot discs were treated with ice-cold solutions of NaCl or distilled water for 30 min and washed with ice-cold water for 30 min and with water at room temperature for 30 min. The treatment solutions and combined washes were concentrated and analyzed.

NaCl Concn	Protein Released per 100 g Discs		
	Treatment solution	Combined washes	Total
<i>M</i>		<i>μg</i>	
0	135	210	345
0.05	86	337	423
0.10	80	355	435
0.15	133	337	470
0.20	147	343	490
0.25	190	543	733
0.30	313	624	937

Table III. *Effect of NaCl Concentration on the Release of Polysaccharide by Carrot Discs to the Salt Solutions and Water Washes*

Treatment conditions were as described in Table II.

NaCl Concn	Polysaccharide Released per 100 g Discs		
	Treatment solution	Combined washes	Total
<i>M</i>		<i>μg</i>	
0	38	51	89
0.05	59	190	249
0.10	126	453	579
0.15	162	376	538
0.20	199	685	884
0.25	506	825	1331
0.30	557	930	1487

0.25 M caused an abrupt increase in protein release. This concentration evidently exceeded some threshold for protein release. The threshold may be osmotic or ionic, perhaps related to the extent of Na<sup>+</sup> substitution for divalent cations at the cell surface. The actual amount of protein released by 0.25 M NaCl was small, only about 0.2% of the total protein of the discs. The average total protein content of this tissue ± SD for 12 batches of discs was 0.363 ± 0.022 g of protein per 100 g of fresh weight. Paper chromatography of protein hydrolysates indicated that the amino acid composition of the protein released by NaCl was qualitatively similar to that of the total water-soluble protein of the cells. Hydroxyproline, characteristic of some cell wall proteins, was not detected.

**Polysaccharide.** The ethanol-insoluble material recovered from the salt-treatment solutions and from the subsequent water washes contained, in addition to protein, polysaccharide that was soluble in 1 N NaOH (Table III). The amount tended to increase linearly with the concentration of NaCl from 0 to 0.30 M and to be independent of the amount of protein released. Paper chromatography of the hydrolysate of polysaccharide released by control and NaCl-treated discs indicated a single spot for galactose. The NaCl-treatment appeared to enhance the release of the same polysaccharide fraction that was released to water.

**Ion Specificity.** Several different salts were tested to determine their effectiveness in releasing protein and in suppressing

the uptake of glucose and P<sub>i</sub>. All of the solutions contained 250 meq of salts per liter. Table IVA shows the amounts of protein and polysaccharide released to the combined washes following salt treatment, and Figure 2 shows the effect of treatments on the uptake of glucose and P<sub>i</sub>. Both Na<sup>+</sup> and K<sup>+</sup> promoted the release of protein and polysaccharide and both suppressed the uptake of glucose and P<sub>i</sub>. Both were more effective as chloride salts than as sulfate salts, possibly because of the lower OP of the chloride solutions (−10.4 bars for NaCl and KCl compared to −6.9 bars for Na<sub>2</sub>SO<sub>4</sub> and K<sub>2</sub>SO<sub>4</sub>). However, part of the difference may be due to the tendency of sulfate to form ion pairs and to stabilize macromolecules. Treatment with CaCl<sub>2</sub> released polysaccharide more effectively than treatment with Na<sub>2</sub>SO<sub>4</sub>, but it did not promote the release of protein nor did it suppress the uptake of glucose. It had a slight stimulating effect on the uptake of P<sub>i</sub>. When Ca<sup>2+</sup> and Na<sup>+</sup> were supplied together, they were mutually antagonistic; Ca<sup>2+</sup> prevented the Na<sup>+</sup>-induced release of protein and suppression of glucose uptake, and Na<sup>+</sup> reduced the Ca<sup>2+</sup> stimulation of P<sub>i</sub> uptake. The combination was also less effective than either NaCl or CaCl<sub>2</sub> alone in releasing polysaccharide.

**Calcium Reversal.** Since most of the protein released by the NaCl treatment came off into the water wash following the treatment rather than into the salt solution (Table II), it was of interest to determine whether the effect of NaCl on protein release and on the uptake of glucose and P<sub>i</sub> could be reversed by the presence of Ca<sup>2+</sup> in the wash. Discs were treated with NaCl and then washed with 0.5 mM CaCl<sub>2</sub> (−0.03 bar OP) instead of with water. A low concentration of Ca<sup>2+</sup> was used to avoid appreciable reduction of the OP of the wash. The results (Table IVB and Fig. 3; compare with Fig. 1) show that the Ca<sup>2+</sup> wash did tend to reverse the effects of the earlier NaCl

Table IV. *Effect of Treatment on the Release of Protein and Polysaccharide to Water or CaCl<sub>2</sub> Washes*

Washed and aged (18 hr) carrot discs were treated with ice-cold solutions of the indicated salts or distilled water for 30 min and washed with ice-cold water or 0.5 mM CaCl<sub>2</sub> for 30 min and with water or 0.5 mM CaCl<sub>2</sub> at room temperature for 30 min. The two washes in each case were combined and concentrated for analysis.

Treatment	Wash	Protein	Poly-saccharide
		<i>μg/100 g</i>	<i>μg/100 g</i>
A. Water	Water	183	94
CaCl <sub>2</sub> 0.25 N	Water	196	651
CaCl <sub>2</sub> 0.125 N + NaCl 0.125 N	Water	196	372
Na <sub>2</sub> SO <sub>4</sub> 0.25 N	Water	562	206
NaCl 0.25 N	Water	612	1141
K <sub>2</sub> SO <sub>4</sub> 0.25 N	Water	500	723
KCl 0.25 N	Water	610	978
B. Water	Water	210	61
Water	CaCl <sub>2</sub> 0.5 mM	238	147
NaCl 0.20 M	Water	343	495
NaCl 0.20 M	CaCl <sub>2</sub> 0.5 mM	220	310
NaCl 0.25 M	Water	543	768
NaCl 0.25 M	CaCl <sub>2</sub> 0.5 mM	413	349
NaCl 0.30 M	Water	624	930
NaCl 0.30 M	CaCl <sub>2</sub> 0.5 mM	568	348
C. Water	Water	306	170
Na-EDTA 1 mM	Water	324	311
NaCl 0.20 M	Water	618	757
NaCl 0.20 M + Na-EDTA 1 mM	Water	832	856

treatment. Some reversal also was obtained with 0.5 mM  $MgCl_2$ . After treatment with 0.20 M NaCl, the  $Ca^{2+}$  wash kept the protein release down to the control level and completely prevented the suppression of glucose uptake. It reduced, but did not prevent, the suppression of  $P_i$  uptake. Increasing the concentration of the NaCl treatment solution to 0.25 M and 0.30 M increased the amount of protein released to the salt solution (Table II) and diminished the protection of uptake activity afforded by the  $Ca^{2+}$  wash. In order for  $Ca^{2+}$  to preserve uptake activity, it evidently must be added before protein has been released. As another test of correlation between prior release of protein and diminished protection by  $Ca^{2+}$ , discs were treated with 0.20 M NaCl, washed with water to remove protein, and then with 0.5 mM  $CaCl_2$ . The subsequent rates of glucose and  $P_i$  uptake (Fig. 3), show that removing the protein diminished the protective effect of  $Ca^{2+}$ .

The presence of  $Ca^{2+}$  in the wash also reduced the amount of polysaccharide released after treatment with NaCl. In this case, the  $Ca^{2+}$  wash appeared to be equally effective after treatment with 0.20 M, 0.25 M, and 0.30 M NaCl.

**EDTA Treatment.** In their studies with soybean roots, Foote and Hanson (7) observed that treatment with 1 mM EDTA released  $Ca^{2+}$  and  $Mg^{2+}$  from the roots, along with UV-absorbing material, and suppressed the subsequent uptake of  $^{32}P$  and  $^{36}Cl$  by the roots. EDTA has also been used to release enzymes and membrane transport proteins from the outer surface of bacterial cells (13). Hence, it was of interest to compare the effects of EDTA and NaCl on carrot cells. Discs were treated for 30 min with ice-cold 1 mM Na-EDTA, pH 6.0, without and with 0.20 M NaCl, washed twice with water as in the salt treatments, and then incubated in glucose- $P_i$  solution. The EDTA alone had little or no effect on protein release (Table IVC) or on glucose uptake (Fig. 4). It did, however, suppress  $P_i$  uptake by about 30%. Together, EDTA and 0.20 M NaCl were about additive in suppressing  $P_i$  uptake but more than additive in promoting the release of protein and in suppressing the uptake of glucose, indicating a synergistic effect. The EDTA presumably sequestered divalent cations as they were released by exchange for  $Na^+$  and thus increased the exchange.

**Puromycin Treatment.** The delayed appearance of glucose uptake after treatment with 0.25 M NaCl (Figs. 1 and 2), with 0.125 M  $K_2SO_4$  (Fig. 2), or with 0.30 M NaCl followed by a  $Ca^{2+}$  wash (Fig. 3) indicates that the cells were able to repair salt damage to the glucose uptake system, but this repair took

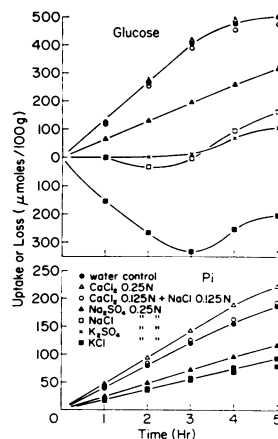


FIG. 2. Kinetics of uptake or release of glucose and  $P_i$  by carrot discs after treatment with the indicated 0.25 N salt solutions. The procedure for treatment and incubation was as described for Figure 1.

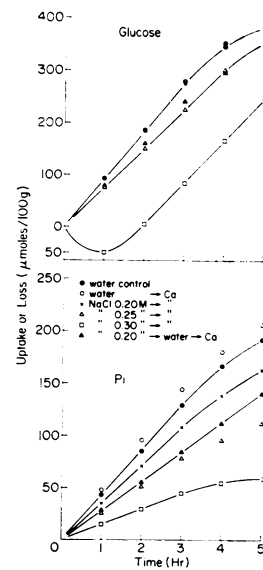


FIG. 3. Reversal by calcium of the NaCl-induced suppression of glucose and  $P_i$  uptake. Carrot discs were treated with ice-cold NaCl solutions as in Figure 1, but washed with 0.5 mM  $CaCl_2$  instead of with water. The filled triangles represent discs that were treated with 0.20 M NaCl, washed twice with water as usual, and then with 0.5 mM  $CaCl_2$  at room temperature. The incubation conditions were as described in Figure 1. In the glucose plot, the open circles, filled circles, and crosses coincide.

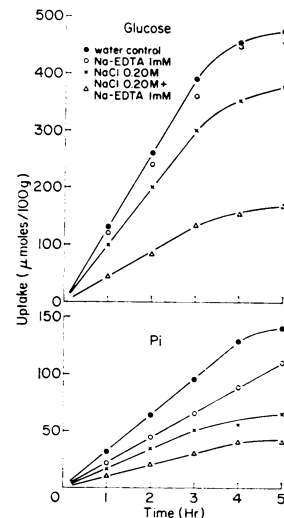


FIG. 4. Effect of treatment with Na-EDTA on the uptake of glucose and  $P_i$ . Carrot discs were treated with ice-cold 1 mM Na-EDTA, without and with 0.20 M NaCl. The procedure for treatment and incubation was as described in Figure 1.

several hours at room temperature. Membrane repair, which seems to be indicated, would very likely require protein synthesis, perhaps to replace the protein removed by salt treatment. Hence, it was of interest to determine whether salt-treated discs would recover glucose uptake activity in the presence of puromycin, an inhibitor of protein synthesis. Discs were treated with 0.25 M NaCl, washed with water to remove protein and completely inactivate glucose uptake, and then incubated at room temperature in glucose- $P_i$  solution without and with 25  $\mu g/ml$  puromycin. The uptake of glucose and  $P_i$  from this solution was measured over a 5-hr period. The discs subsequently were washed for 90 min in several changes of aerated distilled water, and then returned to fresh glucose- $P_i$ ,

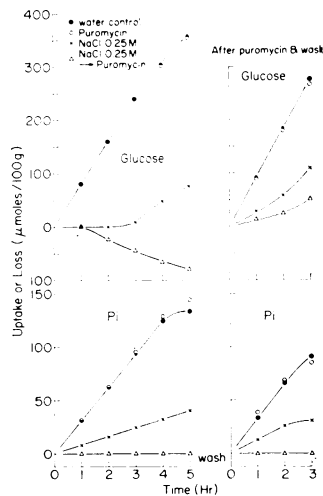


FIG. 5. Effect of puromycin on the uptake of glucose and  $P_i$  by control and NaCl-treated carrot discs. Discs were treated with ice-cold 0.25 M NaCl or water and washed with water (standard procedure, see Fig. 1). Control and treated discs were incubated at room temperature in glucose- $P_i$  solution without and with puromycin (25  $\mu\text{g}/\text{ml}$ ) for 5 hr. At the end of this time, they were washed for 90 min in several changes of aerated distilled water at room temperature and returned to fresh glucose- $P_i$  solution without puromycin. The open and filled circles coincide in most cases.

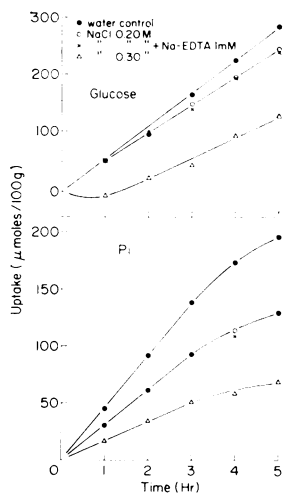


FIG. 6. Effect of treatment with NaCl and with NaCl plus 1 mM Na-EDTA on the uptake of glucose and  $P_i$  by beet discs. Beet discs were prepared, treated, and incubated according to standard procedures (legend, Fig. 1). The open circles and crosses coincide.

solution without puromycin. The presence of puromycin had no effect on the uptake of either glucose or  $P_i$  by control discs not treated with salt (Fig. 5). In the case of the salt-treated discs, puromycin completely inhibited the reduced  $P_i$ -uptake activity that had survived the salt treatment; it also prevented the recovery of glucose-uptake activity and caused the discs to become leaky to glucose. Washing the NaCl-puromycin-treated discs in distilled water permitted a gradual recovery of some glucose uptake activity but there was no indication of recovery of  $P_i$  uptake activity.

**Beet Experiment.** Garden beets (*Beta vulgaris* L.) were obtained at a local market. Discs were prepared, aged (18 hr), and treated in the same way as the carrot discs. The beet discs were not as active as the carrot discs in taking up glucose but they were comparable in taking up  $P_i$  (Fig. 6). Judging from the uptake and retention of glucose and  $P_i$ , beet was more re-

sistant than carrot to treatment with NaCl. Beet discs continued to take up glucose and  $P_i$ , although at reduced rates and after a lag in the case of glucose, following treatment with 0.30 M NaCl. This concentration completely prevented net uptake by carrot discs and caused the cells to become leaky (Fig. 1). Treatment with 1 mM Na-EDTA in the presence of 0.20 M NaCl had no effect at all on the uptake activity of beet. Either there were no divalent cations released by 0.20 M NaCl to be sequestered by EDTA or, if there were, it made no difference to the glucose and  $P_i$  uptake mechanisms whether they were sequestered or not.

Van Steveninck (30), also working with red beet discs, noted that 0.1 mM EDTA caused the beet membranes to become leaky to  $K^+$ . However, his experimental conditions were somewhat different from ours. He used freshly cut discs, which are more leaky to begin with, and he treated them with EDTA, apparently as the free acid, at 24 C for several hours.

**Reconstitution.** Attempts at restoring the glucose and  $P_i$  uptake activity of salt-treated carrot discs by adding back the moieties they release have so far been unsuccessful. One possible explanation is that one of the protein fractions released from the cells is precipitated by traces of  $Ca^{2+}$ . If this fraction is required for reconstitution, its precipitation enroute to the cell surface may thwart attempts at reconstitution.

## DISCUSSION

Treating carrot discs with ice-cold NaCl solutions for 30 min caused three effects that appear to be functionally related: the exchange of tissue  $Ca^{2+}$  and  $Mg^{2+}$  for  $Na^+$ ; the release of protein; and the suppression of active uptake of glucose and  $P_i$ . These appear to be nonspecific monovalent cation effects; they were caused by KCl as well as by NaCl. Chloride did not appear to be directly involved, but the nature of the anion did influence the effectiveness of the cation; both  $Na^+$  and  $K^+$  were more effective as chloride salts than as sulfate salts. The correlation between the release of  $Ca^{2+}$  and  $Mg^{2+}$  and the release of protein, and between these effects and the suppression of glucose and  $P_i$  uptake, supports the hypothesis that divalent cations maintain and monovalent cations disrupt linkages at the outer cell surface with proteins required for solute uptake, perhaps analogous to the membrane transport proteins of bacteria.

Divalent cations were released to NaCl treatment solutions apparently by ion exchange. They were not released to distilled water before NaCl treatment and only traces appeared in the water washes following NaCl treatment. Protein, on the other hand, was released mostly to water washes following NaCl treatment. The reasons for this are not clear. The amount of protein released to the wash was increased by adding EDTA to the NaCl treatment solution and decreased by adding  $Ca^{2+}$  to either the NaCl treatment solutions or to the wash, suggesting that ion-exchange equilibria are involved. Washing the tissue with water after NaCl treatment may affect protein release in part by reducing the concentration of free divalent cations at the cell surface. Preliminary experiments indicate that a reduction in the total salt concentration is also a factor. The data in Table II suggest that there is some ionic effect on the protein or other membrane constituent that suppresses the release of protein to NaCl solutions even after removal of a third or more of the tissue  $Ca^{2+}$ .

Osmotic shock did not appear to be a major factor either in releasing protein or in suppressing the uptake of glucose and  $P_i$ . Neither of these effects occurred when discs were subjected to osmotic shock in the presence of  $Ca^{2+}$  as, for example, when they were treated with 0.125 M  $CaCl_2$  (-7.8 bars OP) and then transferred to water. On the other hand, both occurred when discs were treated with 0.05 M NaCl (-2.1 bars

OP) and then water. Also, both the NaCl-induced release of protein and suppression of uptake were reduced or prevented by an osmotically insignificant amount of  $\text{Ca}^{2+}$  in the wash.

The amount of protein released by salt is assumed to be the difference between the amounts released by salt-treated tissue and water-treated control tissue. The total amount released by treatment with NaCl at concentrations up to and including 0.20 M was roughly proportional to the amount of  $\text{Ca}^{2+}$  released (about 200  $\mu\text{g}$  protein/meq  $\text{Ca}^{2+}$ ). Treatment with 0.25 M NaCl released much more protein but only a little more  $\text{Ca}^{2+}$ . The ratio in this case was about 600  $\mu\text{g}$  protein/meq  $\text{Ca}^{2+}$ . This higher concentration of NaCl exceeded some threshold for protein release, perhaps by displacing  $\text{Ca}^{2+}$  at particularly critical sites.

There also appeared to be a correlation between the amount of protein released by salt, but appearing in the wash, and the degree of suppression of glucose and  $\text{P}_i$  uptake. On the average the rates of glucose and  $\text{P}_i$  uptake declined about 23% and 14%, respectively, per microgram of protein released per gram of tissue. Thus, the  $\text{P}_i$  uptake mechanism appeared to be the more resistant of the two.

The  $\text{P}_i$  uptake mechanism also differed in showing a stimulation by free or loosely held  $\text{Ca}^{2+}$ . This  $\text{Ca}^{2+}$  was accessible to EDTA and apparently was not involved in protein attachment. A  $\text{Ca}^{2+}$  stimulation of  $\text{P}_i$  uptake by root tissue has been observed earlier by several workers, for example, Tanada (27) and Foote and Hanson (7). More recently, Hanson and co-workers (12, 29, and references cited therein) have shown that the uptake of  $\text{P}_i$  by isolated corn mitochondria requires activation by  $\text{Ca}^{2+}$ . Apparently  $\text{P}_i$  and  $\text{Ca}^{2+}$  are transported into the mitochondrion together by an energy dependent process that substitutes for ATP production. It is not clear whether the  $\text{Ca}^{2+}$  stimulation of  $\text{P}_i$  uptake by tissue and the  $\text{Ca}^{2+}$  activation of  $\text{P}_i$  uptake by mitochondria are related effects.

Monovalent cation-induced damage to the cell membrane may help to explain the growth suppression caused by sodic root media. Such media provide conditions promoting this damage, that is, a fairly high concentration of  $\text{Na}^+$  coupled with a very low concentration of  $\text{Ca}^{2+}$ . In the case of the two plant species examined, one sensitive to sodicity (carrot) and one tolerant (beet), tolerance was associated with resistance of the cell membrane to damage by  $\text{Na}^+$ . The protective effect of  $\text{Ca}^{2+}$  against  $\text{Na}^+$  injury to the carrot cell membrane has a parallel in the observation that carrot plants were specifically sensitive to  $\text{Na}^+$  only on  $\text{Ca}^{2+}$  deficient media (17). The concept that a  $\text{Ca}^{2+}$  deficiency might contribute to reduced plant growth on sodic root media apparently was first suggested by Ratner (25).

Since the monovalent cation-induced injury described here was reduced or prevented by the presence of  $\text{Ca}^{2+}$ , it would not be expected to play a role in suppressing plant growth on saline media. By definition, a saline medium contains sufficient  $\text{Ca}^{2+}$  for plant growth. Its growth suppressive effect is related not to a low level of  $\text{Ca}^{2+}$  but to a high level of soluble salts;  $\text{Ca}^{2+}$ , in some cases, is the dominant cation. Salination of a complete nutrient solution with  $\text{CaCl}_2$  was just as effective in suppressing the growth of bean plants as salination with osmotically equivalent amounts of NaCl (8). Tissues of the  $\text{CaCl}_2$  stunted plants contained up to twice the normal amounts of  $\text{Ca}^{2+}$ , so a  $\text{Ca}^{2+}$  deficiency was clearly not at issue.

The amount of  $\text{Ca}^{2+}$  required at the cell surface to prevent monovalent cations from releasing protein and suppressing glucose and  $\text{P}_i$  uptake has not been established, but it appears to be low enough to be satisfied by most saline root media. However, it is quite possible that saline solutions, even containing high levels of  $\text{Ca}^{2+}$ , may cause some other type of membrane damage that was not detected in our short term

experiments but nevertheless might contribute to plant growth suppression.

From another point of view, salt treatment of plant tissue may be a useful tool for disassembling the cell membrane and for studying membrane transport processes.

*Acknowledgment*—We are grateful to Mr. Martin D. Derderian for expert assistance with the cation analyses.

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