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The Mechanism of Osmotic Accommodation to Water and Saline Stress in Plant Cells; and the Role of Hormones in Modulating this Response

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The Mechanism of Osmotic Accommodation to Water and Saline Stress in
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c. ABSTRACT

Plants able to resist water and saline stress do so by modulating the osmotic potential in the various intracellular compartments. In this Project we have investigated a number of aspects of this process.

We have developed a technique (involving poly-L-lysine treatment) for inducing selective leakage of the plasmalemma. This technique should allow elucidation of the osmoregulation of the cytoplasmic compartment (seat of major metabolic processes) as distinct from that of other compartments.

A stable NaCl-resistant line has been developed from suspension-cultured tobacco cells. The NaCl-resistant cells are seen to differ from wild type in growth characteristics, proline content, and control of internal K^+ and Na^+ concentration as a function of external salinity.

Na^+ and K^+ fluxes at the various membranes have been estimated and compared in root systems from *Atriplex*, a halophyte, and oat, a glycophyte. The transmembrane Na^+ flux was 5 to 10 times higher in the case of the halophyte. Studies on isolated membrane ATPase, today believed to play a central role in solute uptake, have not so far revealed the basis of this effect. However, they indicate that the enzyme from *Atriplex* roots tolerates NaCl concentrations ten times higher than those which inhibit pea enzyme. The *Atriplex* membrane preparations are seen to be capable of generating an ATP-dependent transmembrane electric potential difference, which probably reflects the operation of an electrogenic pump; the characteristics of this pump are being investigated.

Studies on isolated pea mesophyll protoplasts indicated that the mechanism of K^+ transport probably involves an ATP-fueled K^+ porter. Membrane sugar transport and proton extrusion by leaf cells were observed to be sensitive to turgor. We propose a model for osmotic regulation on the basis of these results.

RESEARCH RESULTS

Plant growth depends on the capacity of the cells to maintain turgor above a critical value. Plants capable of surviving under conditions of water and saline stress can accommodate to the stress by modulating the levels of solute pools in the various cellular compartments. The changes in solute pool levels are achieved by transporting solutes across membranes and by biochemical transformations. The present Project has been directed towards the clarification of a number of aspects of osmotic accommodation.

I. COMPARTMENTATION OF SOLUTE POOLS IN PLANT CELLS

I. A. *Identification and estimation of cytosol and vacuolar pools* (Jerusalem laboratory)*

The ability to achieve osmotic balance between intracellular compartments without inhibiting essential biochemical events in the cytosol may well be one of the major factors on which resistance to water and salinity stresses depends.

The urgent need is increasingly felt for a technique which allows determination of solute pool concentrations in the cytosol, as distinct from overall concentrations which include the large vacuolar content (see for example 1, 2). Knowledge of the nature and size of the pools in the cytosol (which represents only a few per cent of the total cellular volume) is essential for an understanding of the mechanism of osmotic adaptation of the cytosol.

Our approach to this problem is the induction of selective pore for-

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mation in the plasmalemma which would allow the direct measurement of cytosol solutes after they have leaked out of the cells. This method has been successfully applied to yeast by Wiemken and coworkers (2). We have shown that treatment of thin slices of beet root storage tissue with appropriate concentrations of poly-L-lysine, M.W. 30,000, induced selective pore formation in the plasmalemma (3). However Na^+ and K^+ compartmentation in beet root showed great variability. Since poly-L-lysine does not diffuse through multi-cellular layers of plant cells the study was continued using cells in suspension.

Results and Discussion

Addition of an appropriate amount of poly-L-lysine to a *Nicotiana tabacum* CV. Xanthi suspension initiated a rapid phase of leakage of low molecular weight solutes which lasted about 30 min; then the rate of leakage declined (Fig. 1). The amount of poly-L-lysine which induced selective pore formation in the plasmalemma depended both on the poly-L-lysine lot which was used and on the cells which were being treated. In our conditions 550 μg poly-L-lysine per ml packed cells was appropriate. Higher concentrations of poly-L-lysine induced leakage which did not reach clear plateaux and leaked both the cytosolic and vacuolar low molecular weight solute pools. Lower concentrations of poly-L-lysine induced leakages showing plateaux, however smaller amounts of each solute was leaked. Incubation of cells with 550 μg poly-L-lysine per ml packed cells, at pH 5.5, did not cause the leakage of neutral red preloaded in the vacuoles; and the cells still plasmolyzed when 750 mM KCl was added

and deplasmolyzed when the KCl was diluted to 250 mM indicating that the treatment did not affect adversely the tonoplast.

We considered that the rapid phase of leakage initiated by the addition of poly-L-lysine represented the leakage of the solutes present in the cytosol. The concentration of the solutes present in the cytosol were calculated on the approximate evaluation that the volume of the cytosol was 5% of the total cell volume and that the cell volume was 50% of the packed cell volume after centrifugation at 2000 g for 5 min as determined by mannitol free space using ^{14}C -labeled mannitol and tritiated water. In order to calculate the vacuolar solute concentrations it was assumed that once the cytosol solutes had been leaked the remaining solutes were mainly located in the vacuole and that the vacuolar volume represented 80% of the total cell volume.

The poly-L-lysine treatment leaked about 2% of the P_i , 4% of the ninhydrin positive compounds and 10% of the K^+ present in the cells. It is possible that the poly-L-lysine treatment was not leaking 10% of the K^+ from each cell, but rather 100% of the K^+ from a certain fraction of the cell population which for some reason was particularly vulnerable and happened to contain 4% of the ninhydrin positive compounds and 2% of the P_i present in the cells. In order to demonstrate that all the cells had in fact been affected by the treatment an attempt was made to estimate the change in potential caused by the treatment by measuring the distribution ratio of the tetraphenylphosphonium cation (TPP^+). If the treatment induced pore formation in the plasmalemma of all the cells it should cause the disappearance of the electrogenic component of the potential, and we should expect a substantial change in ratio $\text{TPP}_{\text{in}}^+/\text{TPP}_{\text{out}}^+$ at pH 5.5. On

the other hand if only 5% of the cells had been affected, the change in ratio $\text{TPP}_{\text{in}}^+/\text{TPP}_{\text{out}}^+$ should be scarcely if at all detectable. Table 1 shows that poly-L-lysine (PLL) treatment brought about 50 per cent drop in the ratio, i.e. an effect similar to that of FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone) (see table). The latter is known to short-circuit the proton pump. Azide, which interferes with the ATP supply to the pump via its effect on mitochondria, also reduced the ratio by about the same amount.

Table 1. Distribution of labeled tetraphenylphosphonium cation (TPP^+) in *Nicotiana* cells under various treatments

Treatment	$\frac{\text{TPP}_{\text{in}}^+}{\text{TPP}_{\text{out}}^+}$
Control	8.2 ± 0.3
Poly-L-lysine 800 $\mu\text{g/ml}$ pc [*]	4.2 ± 0.9
FCCP 50 μM	4.3 ± 0.5
NaN_3 2 mM	5.3 ± 0.3

Incubation with TPP^+ lasted for 3 h in absence or presence of poly-L-lysine or metabolic inhibitors.

^{*} ml pc = ml packed cells

A. Leakage of K⁺.

B. Leakage of ninhydrin positive compounds.

C. Leakage of P_i.

Fig. 1. Poly-L-lysine induced leakage of solutes from *Nicotiana* cells. The vertical arrow indicates addition of 550 μg poly-L-lysine (PLL) per ml packed cells. The horizontal arrow indicates the per cent of the solute present in the cytosol. The line in the upper part of the graph indicates the total amount of the solute present in the cells. The indicated solute concentrations in the cytosol and the vacuole were calculated as described in the text.

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I. B. *Quantitative assessment of cytosol and vacuolar pool sizes, and of trans-membrane fluxes, in a glycophyte and a halophyte (Purdue Laboratory).*

The Na^+ and K^+ flux rates at various root cell membranes (plasma membrane, tonoplast, and at the cytoplasm/xylem interface) and the pool sizes of Na^+ and K^+ in *Atriplex nummularia* (a halophyte) have been compared to the flux rates and pool sizes of these ions in oats (a glycophyte). In order to do this we have used the compartmental analysis as described by Pitman (1963) and others.

The compartmental analysis involves loading the tissue (roots in this case) with a radioactive tracer during an absorption period and then washing the radioisotope from the tissue during a subsequent period. The compartmental analysis assumes the system behaves as a 3-compartment system which in plant cells is equated to the cell surface — free space, cytoplasm, and vacuole. It also requires that the system be in a steady-state with respect to the ions being measured. This steady-state condition is approached by growing the plants in a nutrient medium for a substantial period of time and then carrying out the experiment *per se* in the same

medium but with radioisotope added.

For our studies, oats and *Atriplex* were germinated in vermiculite. After one week the seedlings were transplanted to one-half strength Hoaglands nutrient solution supplemented with 3 mM NaCl; the final K^+ concentration was also 3 mM. Oats were grown in an environmental chamber for an additional 14 days at 22 C, 14 hr photoperiod, light intensity of 215 Einsteins $cm^{-2} sec^{-1}$, and a day and night relative humidity of 60 and 85%, respectively. *Atriplex* plants were grown in an environmental chamber also for 14 days at a day temperature of 28 C and a night temperature of 22 C, 14 hr. photoperiod, light intensity of 500 Einsteins $cm^{-2} sec^{-1}$, and a day and night relative humidity of 60 and 85%, respectively.

Experiments were initiated by transferring single plants to 200 ml of nutrient solution containing either ^{86}Rb (10,000 cpm/ μ mole) or ^{22}Na (20,000 cpm/ μ mole for *Atriplex* and 40,000 cpm/ μ mole for oats). The radioisotope loading period was either 23 or 24 hours. Plants were detopped after the loading period and tygon tubing was attached to the stem for the collection of exudate. Immediately after excision the roots were transferred to an identical medium with the radioisotopes omitted. External medium, exudate, and tissue were then monitored for both radioactive and non-radioactive ion contents during the subsequent 9-12 hour period.

Figure 1 shows the K^+ and Na^+ contents of the roots during the loading and unloading periods. There is some variation in K^+ and Na^+ contents during the course of the experiment, however, the ion levels were sufficiently constant to allow the analysis. Figure 2 shows the concentration of K^+ and Na^+ in the exudate during the loading and unloading periods.

Fig. 1 Sodium and Potassium content in roots of oats.

Plants were grown on half-strength Hoagland solution plus 3 mM NaCl (final concentration) for 11 days before beginning the experiment and these salts were present during the experiment. Detopping of plants took place after 3 days. Each value is the mean $\pm$ SE of 6 root systems.

Fig. 2 Sodium and Potassium concentration in exudate of oat plants.

Growth conditions and experiment condition were the same as in Fig. 1. From 72 to 81 hrs. exudate was collected from the same plants. Prior to 72 hrs. the exudate data was obtained from separate plants.

Exudate ion concentrations prior to detopping for the unloading portion of the experiment were obtained by detopping plants and collecting exudate for 45 minutes. These plants were also used for tissue analysis of K^+ and Na^+ . K^+ content of the exudate decreased 4 hours after detopping whereas the Na^+ content of the exudate remained constant. The same experiments for *Atriplex* have been performed and the results are similar (data not shown), with the exception that both the Na^+ and K^+ content of the xylem declined at about 15 minutes after excision.

A comparison of the effect of detopping on the washout of the accumulated ^{86}Rb in oats is shown in Figures 3 and 4. The cytoplasmic and vacuolar half-times for intact plants was 84 minutes and 147 hours, respectively. For detopped plants (Fig. 4) the vacuolar half-times were 130 hours which is quite close to the 147 hours obtained for intact plants. Half-times for cytoplasm, when just considering loss from the surface of roots, was 111 minutes, and when considering both the surface and the exudate was 92 minutes. Again, these values are similar to the 84 minutes found for the half-time of ^{86}Rb exchange of cytoplasm in intact roots. Again, the same experiments have been conducted for *Atriplex* and similar results were obtained.

Based upon the compartmental analysis, the various fluxes of K^+ and Na^+ at the various membranes, and the ion contents of various compartments for both oat and *Atriplex* roots are summarized in Figure 5. Although these results illustrate some interesting features, only the major ones will be pointed out here. The plasma membrane of oat roots is clearly more discriminating between K^+ and Na^+ than the plasma membrane of *Atriplex*

Fig. 3 Time course of loss of ^{86}Rb from oat roots.

Intact plants were loaded for 24 hrs. in half-strength Hoagland solution (+3 mM NaCl) and then washed in aliquots of non-radioactive solution of the same composition. The radioactivity lost in successive washing periods was added to the final tissue radioactivity to give the total radioactivity in the tissue at the beginning of each washing period and finally at the beginning of the wash-out period. The slowest component is extrapolated to $t=0$ and its content subtracted from the total to give the content of the faster exchanging components (shown in inset).

Fig. 4 Time course of loss of ^{86}Rb from oat roots of detopped plants. Intact plants were loaded for 24 hrs. in half-strength Hoagland solution (+ 3 mM NaCl) and then detopped. A tube was attached for collecting exudate during the washout period. The radioactivity lost in the washing and in the exudate was added to give the total at the beginning of each period. Other details as in fig. 3.

Fig. 5 Estimated value of individual fluxes (umol/g fresh wt/hr) and content (umol/g fresh wt) of Na^+ and K^+ in Atriplex and oat root cells. The "cell" represent a series of cells between the root epidermis and the xylem. Values are the mean of 6 determinations. C and C_o refer to internal concentrations and external concentrations, respectively. The numbers at the bottom of the diagram are aligned to correspond to the appropriate cellular compartment.

roots. This is primarily due to a low flux rate for Na^+ in oats, or stated differently, a high flux rate for Na^+ in *Atriplex*. Similarly the flux of Na^+ into the oat root xylem is much lower than the transport of Na^+ into the xylem of *Atriplex*. Thus, *Atriplex* appears to have evolved a system for transporting Na^+ more rapidly than the oat plant.

These results support the view that, in general, halophytes accumulate Na^+ more readily than glycophytes (Jeschke and Stelter, 1976; Jeffries, 1973). The present study provides a quantitative assessment of the fluxes across the various membranes and the pool sizes for both a glycophyte and a halophyte.

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II. STUDIES OF OSMOTIC ACCOMMODATION TO SALINE STRESS AT CELL AND PROTOPLAST LEVEL

Although encouraging progress has recently been made towards elucidation of osmotic regulation in Algae, embracing both ion transport systems and the synthesis of organic osmotica (see p.49, Bisson and Gutknecht 1980) accommodation to water and saline stress is considerably less well understood in the case of higher plant cells. Three systems have been used for study in the present investigation, cells in suspension culture, isolated

protoplasts, and leaf segments with the epidermis removed.

II. A. *Cells in suspension culture (Jerusalem laboratory)*

Strong interest has recently been shown in using techniques of cell suspension culture for developing lines of cells possessing characters of potential value in agriculture. Thus cell lines derived from crop plants have been obtained which show resistance to salinity (9, 10, 16, 23), water stress (5), disease (4, 21), herbicides (7) and chilling injury (11). Such cell lines might obviously prove useful tools for the elucidation of the mechanism of resistance at cell level.

We have developed a stable NaCl-resistant tobacco cell line, and are comparing the responses of this line and the wild type to rising external salinity. One of the parameters we report on in the present communication is proline accumulation, known to be a striking consequence of exposure to water deficit or to salinity stress, both in whole plants (2) and in callus cultures (18). Its role if any, in adaptation to stress is not yet understood and is the subject of controversy. Stewart and Lee (28) for instance regard it as playing a potentially important role in the osmotic adaptation of the cytosol; while Hanson *et al.* (14) have suggested that its accumulation may result from stress damage in particular to the mitochondrial membranes. We bring evidence here that proline accumulated in the selected cells in NaCl concentrations where they could grow and develop indefinitely; that NaCl-selected cells differ from the wild type in growth characteristics, proline content and the control of internal K^+ and Na^+ concentration as a function of external salinity; and that these traits are stable in the selected cells through many generations in the absence of imposed stress.

MATERIALS AND METHODS

Cell Cultures. Tobacco callus (*Nicotiana*, genome tabacum, with cytoplasm substituted from *gosii*, kindly donated by the Department of Genetics, the Volcani Agricultural Research Organization) was grown on medium which contained Murashige and Skoog salts (22) and the following in mg./litre: thiamine HCl 0.1; myo-inositol 100; 2,4-dichlorophenoxyacetic acid 1; sucrose 20,000; glycine 2; nicotinic acid 0.5; pyridoxine HCl 0.5; Bactoagar 10,000. The pH was adjusted to 5.8 - 6.0.

Suspension cultures were obtained from the callus by shaking portions in 250 ml Erlenmeyer flasks containing 70 ml of a liquid medium of the same composition as that given above, without the agar. The cell suspensions obtained were transferred to fresh medium every 7 to 10 days. They were maintained on a gyratory shaker (120 cycles min^{-1}) kept at 25 C, in continuous light at 2000 lux.

Selection of NaCl-resistant Cells. Callus was transferred to standard agar medium to which 85 mM NaCl had been added. Such portions of the callus as had persisted and developed after 2 weeks were removed and shaken in liquid medium containing the same NaCl concentration. CaCl_2 was added to the medium and to all experimental and washing solutions to a final concentration of 10 mM. This was done firstly in order to maintain the integrity and ion-selective properties of the cell membrane (15, 20); but also in order to minimize binding of monovalent cations in the Donnan free space of the cell walls (6, 31) as will be discussed in the Results section.

After 4 weekly subcultures the suspension cultures were transferred

to 110 mM NaCl, and the process was repeated with gradual rises in NaCl concentration until the cells were growing in 200 mM NaCl. This was the resistant cell line used in the experiments reported here, but further culturing through graded NaCl media has resulted in a cell line capable of growing in 400 mM NaCl.

Experimental Procedure. Experiments were carried out in duplicate. Aliquots of cell suspensions corresponding to 5 ml of packed cells were inoculated into 250 ml flasks containing 70 ml of the appropriate experimental solution. Samples were withdrawn from the flask at intervals for the assays as detailed below.

Growth Assay. Growth of the cells was assayed by measuring packed cell volume after centrifuging the suspension in a Sorval GLC centrifuge at 1000 *g* for 4 min. In certain cases fresh weight and dry weight were determined after filtration of the cells.

Analysis of Intracellular Solutes. Cells were filtered out of the suspension medium through Miracloth, and washed with a solution containing 10 mM CaCl₂ and mannitol of the same osmolarity as the suspension medium, in order to avoid osmotic shock (1). They were then resuspended twice in the washing medium after which they were centrifuged. Extracellular space in the pellet was estimated by determining the volume accessible to Dextran Blue, molecular weight about 2,000,000. The pellet was made up to 10 ml with distilled H₂O and boiled for 10 min, then centrifuged for 5 min at 1000 *g*.

Proline was determined in the supernatant by the acid-ninhydrin method (3). Amino acids excluding proline were determined by the method

of (27), but using acetate buffer.

Na⁺ and K⁺ were determined by means of an atomic absorption spectrophotometer (Varian Techtron Type AA-5).

Reproducibility of Experiments. Each experiment was carried out in duplicate and was repeated at least twice. Standard errors are indicated on the Figures.

Chemicals. All chemicals were of the purest grade commercially available. Thiamine HCl and nicotinic acid were obtained from Nutritional Biochemicals Co., U.S.A.; pyridoxine HCl from BDH Biochemicals, U.K.; 2,4-dichlorophenoxyacetic acid from Sigma, U.S.A.; Bacto-agar from Difco Laboratories, U.S.A. and myo-inositol from Colbiochem, U.S.A.

RESULTS

Development of the cell lines obtained in this investigation was achieved by raising the external NaCl concentration by 50-100 mM at a time. An increase of 150-200 mM in one step was lethal. This observation showed that the capacity to withstand salinity was acquired by degrees.

Figure 1 gives growth curves for selected and wild type cells, when inoculated into standard medium or into media containing 150 mM or 200 mM NaCl respectively. In standard medium the growth of the selected cell line closely resembled that of the wild type cells (curves b and c). This was true also for selected cells which had undergone 6 successive transfers through standard medium after their removal from high salt solution (curve a). The wild type cells failed to grow in

Figure 1 Growth curves for NaCl-selected and wild type *Nicotiana* cells in standard and saline media. Curves b and g: wild type cells grown in standard medium and then inoculated respectively into standard medium or medium with addition of 150 mM NaCl. Curves c, d and e: Selected cells grown in high-NaCl medium, then transferred gradually over 7 days to standard medium and subsequently inoculated into standard medium (c), 150 mM NaCl (d) or 200 mM NaCl (e). Curves a and f: Selected cells grown in high NaCl media, then transferred gradually to standard medium and subsequently transferred 6 times through standard medium before inoculation into standard medium (a) or 160 mM NaCl (f).

either of the high salt media (curve g). Selected cells, on the other hand, developed, though after a somewhat longer time lag than in standard medium (curves d and e). The total volume of the selected cells at stationary phase in high salt medium (curves d and e) was approximately 50% of those in standard medium (curve c). The percentage dry weight of the selected cells (curve e) at stationary phase however was 2.2% as compared with 1.5% in the case of both wild type and selected cells in standard medium (curves b and c).

Resistance to high salt medium showed stability through 6 successive transfers through standard medium. It is striking that the performance of selected cells which had been transferred in this manner (curve f) almost equalled that of the cells continuously maintained in high salt media (curve e). A lag of some days however occurred before rapid growth started in their case.

Proline Accumulation as a Response to External Salt Concentration in Selected and Wild Type Cells. Selected cells were transferred in gradual stages from 150 mM NaCl to standard solution during the course of one week, and then inoculated into media of various NaCl concentration (up to 100 mM). After a further week their proline content was compared with that of wild type cells exposed to the same NaCl concentrations. Figure 2 shows that, above a threshold concentration of 35 mM NaCl, proline concentration in the selected cells rose almost linearly with external NaCl. By contrast, proline content increased only slightly in the wild type cells. The level reached in 100 mM NaCl was approximately 8 times higher than that in standard solution in the case of

Figure 2. Proline accumulation in wild type and NaCl-selected *Nicotiana* cells respectively as a function of NaCl concentration in the medium. Proline measured 7 days after inoculation. The selected cells had been grown in 150 mM NaCl medium, then transferred gradually over 7 days to standard medium before inoculation into the experimental media.

Figure 3. Progress curves for proline accumulation in wild type and NaCl-selected *Nicotiana* cells. The NaCl concentration in the experimental media is indicated on the curves. Selected cells growing in 180 mM NaCl had been transferred gradually to standard medium, then transferred twice (curves a, c, and f) or 6 times (curve b) through standard medium before inoculation into experimental media.

the selected cells, but only 1.5 times higher in the case of wild type cells.

No detectable change in the general amino acid level accompanied the rise in proline content in these experiments. Determination of total amino acids excluding proline showed no change in selected cells with increasing external NaCl concentration (not shown) though it is possible that the level of individual amino acids changed, a fall in one compensating for a rise in another.

Stability of Proline Response. Figure 3 gives the time course of proline accumulation in selected and wild type cells in standard medium, in 150 mM NaCl solution, and, in the case of selected, in 180 mM NaCl (the wild type cells did not survive in 180 mM NaCl). The selected cells had been transferred in gradual stages to standard medium, and had then passed through two further weekly transfers in standard solution before the start of the experiment. The proline content of selected and wild type cells remained approximately equal in standard medium (curves e and f). By contrast in 150 mM NaCl proline reached a level approximately 8 times the initial in selected cells, as compared with only twice the initial in wild type cells (curves c and d respectively). In 180 mM NaCl proline concentration was 27 times the initial in selected cells after 8 days (curve a). Consideration of Figure 3 in conjunction with Figure 2 indicates that the curve for proline accumulation rises with increasing slope as external NaCl concentration is increased. When selected cells were transferred straight from high salt medium (250 mM) to a fresh high salt medium without the intervening

period in standard solution experienced by the selected cells in this experiment, their proline content remained unchanged at approximately 12 $\mu\text{mol/ml}$ packed cell volume (not shown).

The stability of the proline response is shown by curve b in Fig. 3. Here the selected cells had passed through standard medium 6 times before inoculation into 160 mM NaCl. Proline accumulation was 17 times as high as initial in these transferred cells. The wild type cells in 150 mM NaCl accumulated a limited amount of proline for as long as they survived. The curves for both cell lines were similar in standard medium (curves e and f).

The Reversibility of Proline Accumulation. When NaCl-selected cells were transferred from high salt medium to media of lower NaCl concentration their proline content fell rapidly (within a few hours) as is seen in Figure 4. Care must be taken in such an experiment not to damage the cells by osmotic shock (1). It was therefore arranged that the drop in external NaCl concentration experienced by the cells in this experiment was only 50 mM, from 200 to 150 mM NaCl. An earlier experiment (see inset in Fig. 4) where cells were transferred more slowly (at 24 h intervals) through a series of falling NaCl concentrations indicated that the drop from 200 to 150 mM NaCl would be sufficient to bring about the disappearance of the greater part of the proline accumulated. This observation accords with that pointed out above, that changes in proline concentration are steeper in the higher region of the NaCl concentration range. The fall in proline concentration could not be accounted for on the basis of a change in volume accompanying decrease in external osmotic potential, since no volume change was detectable by light microscopic measurements under the conditions of this experiment (measurements 24 h after transfer to less concentrated NaCl medium). Figure 4 shows that the half time for decreasing the proline

Figure 4 Kinetics of the reversibility of proline accumulation in NaCl-selected *Nicotiana* cells. The cells had been growing in 200 mM NaCl, were transferred for 5 min to 150 mM NaCl to free them from 200 mM medium, and then resuspended in fresh 150 mM NaCl medium for the periods indicated. Inset: Proline content of cells grown in 180 mM NaCl and then transferred at daily intervals through a series of NaCl concentrations as indicated on the graph.

Figure 5 Proline accumulation in wild type and NaCl-selected *Nicotiana* cells as a response to the addition of mannitol to the medium. Continuous curves: standard medium; dashed curves: 300 mM mannitol. Selected cells had been grown in 180 mM NaCl, then transferred gradually to standard medium and transferred twice through standard medium before inoculation into the experimental media.

concentration from the level characteristic of cells in 200 mM NaCl to that characteristic of 150 mM NaCl (cf. Fig. 3) was about 6 h.

The Response of Salt-Selected and Wild Type Cells to High External Mannitol Concentration. Proline accumulation as a response to decreased (i.e. a change to more negative) external osmotic potential was examined in experiments where mannitol was added to the media. The course of proline accumulation is seen in Figure 5. Appreciable accumulation was not observed in either wild type or selected cells at 100 mM mannitol (not shown). At 300 mM both cell lines accumulated proline, but the salt-selected cells achieved a considerably higher concentration (3.8 $\mu\text{mol ml}^{-1}$ packed cell volume as compared with 1.2 in the case of wild type cells). Since the selected cells had experienced 2 weekly transfers through standard medium before the start of the experiments, the results also indicate stability of the potential to respond to negative water potential by accumulation of proline.

K⁺ and Na⁺ Content as Affected by External NaCl Concentration in Wild and Selected Cell Lines. Part of the cation content of cells that have been growing in salt-containing media will be located in the Donnan free space of the cell walls bound by electrostatic forces, and will thus not be removable by washing in water. This is a problem that has frequently been neglected in previous investigations reported in the literature. Monovalent cations in the Donnan free space of the cell walls can, however, be displaced by divalent cations (see

6, 31).

In preliminary experiments we observed that when cells were grown in 100 mM NaCl media containing 0.2 mM CaSO_4 and then washed in water, half their apparent Na^+ and K^+ content could be removed by subsequently washing 3 times in 0.2 CaSO_4 for 5 min. When the Ca^{++} concentration in the growing medium was raised to 10 mM, one subsequent wash in 10 mM CaCl_2 (+ mannitol isotonic with the growing medium to avoid osmotic shock) removed the Na^+ and K^+ from the cell walls. This was evidenced by the failure of 4 further washes in 10 mM CaCl_2 to lower the measured monovalent cation content of the cells. In the experiments reported below the cells were therefore grown and later washed in solutions containing 10 mM CaCl_2 . Experiments showed that the addition of CaCl_2 at even twice this concentration had no adverse effect on cell growth.

When the two cell lines were grown in standard medium, where Na concentration is only 0.36 mM, no appreciable difference in K^+ and Na^+ content was noted as between cell lines. In both types of cell internal Na^+ concentration was very low as compared with K^+ .

When, however, the cells were grown in media of increasing NaCl concentration, differences between the 2 cell types were noted (Fig. 6). These differences in ion content developed even though the NaCl-selected cells had previously been transferred gradually from their high-salt medium through media of decreasing salt concentration during the course of 7 days and had subsequently spent 4 days in standard medium before the start of the experiment.

At 20 mM external NaCl (i.e. where external K^+ concentration

Figure 6 K^+ and Na^+ content of wild type and NaCl-selected *Nicotiana* cells as a function of NaCl concentration in the medium. Figs. 6a and b, 2 days after inoculation; c and d, 7 days after inoculation. The selected cells had been growing in 180 mM NaCl, had then been transferred gradually over 7 days to standard medium, and had been 4 days in standard medium before inoculation into the experimental media.

equalled external Na^+) internal Na^+ concentration was higher in the selected than in the wild type cells after 2 days (Fig. 6a and b). Internal K^+ concentration was somewhat lower than in the wild type. Over the whole concentration range the Na:K ratio was higher in the selected than in the wild type cell after 2 days.

After 7 days the K^+ content of the wild type cells had fallen considerably in the presence of NaCl. The decline in K^+ content increased with increase in external NaCl concentration. No fall with time in K^+ content had occurred in the absence of external NaCl. In the selected cells, on the other hand, K^+ content had not fallen in NaCl media since the 2 day reading, but on the contrary had risen. At 80 mM external NaCl, K^+ content was $35 \mu\text{mol ml}^{-1}$ packed cell volume, while it was 15 in the wild type. (Even at 120 and 175 mM external NaCl the K^+ content of selected cells was 25 and $20 \mu\text{mol ml}^{-1}$ packed cell volume respectively, in other experiments, data not shown.)

Figure 6 also shows that between the 2-day and the 7-day readings, Na^+ content of the selected cells fell slightly, whereas it was unchanged in the wild type cells. As a result the Na:K ratio in the wild type cells, which had been lower than that in selected cells after 2 days, rose above the ratio for the selected after 7 days.

DISCUSSION

The NaCl-resistant tobacco cell line developed in this investigation resembles those described by Dix and Street (10) and Nabors *et al.* (24) in that the resistance showed stability through many generations

when grown in the absence of selection pressure, i.e. in NaCl-free medium. In this it differed from the line developed by Hasegawa *et al.* (16) which quickly reverted back to the sensitivity of the original population. On the other hand it resembled the line developed by these last authors in that at stationary phase the fresh weight of the selected cell cultures was about 50% of that for the wild type.

Striking differences in proline content were observed as between wild type and NaCl-selected cells when exposed to increasing concentrations of NaCl (Fig. 2). Moreover, the ability to accumulate proline as a response to high external salt media, like the ability to grow in these media, was preserved through many transfers through salt-free media (Fig. 3). Both abilities were stable through about 24 generations in the absence of selection pressure (i.e. for as many generation times as were tested). As pointed out in the Introduction, there is a continuing controversy in the literature as to whether proline accumulation is related to resistance to salinity and drought (28), or whether it is not so related (29) and may even be the result of damage (14). In our investigation cell lines which had the ability to grow and develop indefinitely in high NaCl media accumulated proline when grown in these media. When the two cell lines were grown at the highest external NaCl level at which the sensitive wild type could survive, far less proline accumulated in the sensitive cells than in the resistant ones (Fig. 2). Since the sensitive cells were clearly suffering from saline stress, while the resistant cells could withstand much higher salt concentrations, this observation suggests that proline accumulation in our cells is not

the visible result of damage, e.g. to mitochondrial membranes (2) but accompanies survival and growth in a saline environment.

This conclusion is reinforced by the fact that when cell lines are developed by gradually increasing, stepwise, the NaCl concentration in the media, internal proline concentration rises at each step. There is thus a correlation between the ability of the cells to survive in a higher NaCl environment and proline content.

Proline accumulation was fully reversible. On transfer sequentially to media of lower and lower NaCl concentration, the proline content of the selected cells fell progressively to the lower and lower steady state levels corresponding to the external NaCl concentrations (Inset in Fig. 4). The new level was reached within 24 h after transfer to a lower external NaCl concentration, and the half-time for this decline in proline content was 6 h (Fig. 4). This half-time is far less than the generation time for this cell line, which is estimated as 4 days. Disappearance of proline was thus not due to dilution resulting from cell replication. Handa *et al.* (13) have recently briefly reported reversibility of proline accumulation in a tomato cell line in the case of water stress. In whole plants proline accumulation has also been observed to be reversible, after a lag of 10-24 h (8, 12).

It has often been shown that proline accumulates in plants both in the case of salinity stress and in that of water stress (2). Our present paper shows that a cell line selected for resistance to salinity stress responds to water stress (external mannitol) by accumulating markedly more proline than do wild type cells (Fig. 5). The capacity

for this response was stable through at least 8 generation in standard medium. Heyser and Nabors (17) have recently reported that a NaCl-adapted tobacco cell line survived better on dextran medium (i.e. under water stress) than did a non-adapted line.

Analysis of internal K^+ and Na^+ concentrations showed that Na^+ content rose steadily in both cell lines as external NaCl concentration was increased. Since Ca^{++} was present in excess, it is unlikely that the Na^+ was in bound form in the Donnan free space. Na^+ therefore appeared to be involved in the osmotic adaptation of these cells; alternatively their power of excluding Na^+ was limited. At low external NaCl concentrations the interesting fact emerged that in the lag phase 2 days after inoculation from standard into NaCl medium the resistant cells were less discriminating against Na^+ than the wild type — their internal Na:K ratio was markedly higher than that of the wild type cells, even though they had passed through salt-free media for several generations (see Figure 6). The comparative lack of discrimination against Na^+ when external NaCl concentrations were relatively low was thus inherited in the absence of selection pressure. However with increased length of exposure to NaCl in the external medium wild type cells lost part of their K^+ content, while selected cells did not, and in fact gained K^+ (compare Figs. 6a and b with 6c and d). The K^+ loss by the wild cells increased in severity with rise in external NaCl concentration, and may well be a reflection of the stress imposed on these non-selected cells. The retention of K^+ content in the face of high external Na^+ concentration may well be central to the selected cells' resistance to salt stress. The

curves for K^+ content for the two cell types after 7 days in media of increasing NaCl concentration (Figs. 6c and d) resemble those presented by Croughan *et al.* (9) for a NaCl tolerant and a wild type alfalfa callus respectively.

It is of interest to consider the nature of the change which has conveyed NaCl resistance on the selected cell lines in our investigation. The fact that resistance developed in a stepwise manner strongly points to the possibility of gene amplification, since the latter is also a stepwise phenomenon (see 19, 30). Stepwise development would not be expected in the case of mechanisms such as mutation or selection of a small number of resistant cells present from the start in the cell population. Gene amplification involves not only one gene, but a segment of a chromosome. The fact that in our investigation resistance was stable through many generations in the absence of imposed stress suggests that the segment of the chromosome undergoing amplification in this case was not extrachromosomal (19, 30). Gene amplification has been proposed as the explanation of adaptive changes in urease levels in tobacco cells (26) and aminopterin resistance in maize cell lines (25). Accumulation of the urease in the former case, as of proline in our own case, was dependent on the presence of the stress and did not occur in its absence; re-imposition of the stress in both cases elicited accumulation as if by a "remembered" mechanism. In the animal examples of gene amplification, on the other hand, accumulation of the proteins occurred in the selected cells even in the absence of stress. The possible role of gene amplification as a mechanism underlying the development of salt resistance in plant cells remains to be demonstrated.

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II. B. K^+ uptake by isolated protoplasts (Jerusalem laboratory)

In the alga *Valonia* it has been convincingly demonstrated that the effector system bringing about the adjustment of internal osmotic potential is the K^+ pump and leak system (see Bisson and Gutknecht 1980). A prerequisite for understanding the osmoregulatory responses in higher plant cells is an understanding of the driving forces governing solute flux. In the case of K^+ there is as yet no consensus as to whether uptake is the result of passive flux into the cell along the electrochemical potential gradient or whether it involves the operation of a K^+ pump.

The absence of an extracellular compartment (cell walls and intercellular spaces) in isolated protoplast preparations offers considerable advantages for membrane transport studies. No allowance need be made for solutes present in the cell wall; flux may be observed over very brief time intervals; and consequently the effect of treatments, or of alterations in experimental conditions can be rapidly monitored. We have used mesophyll protoplasts for recent investigations into plasmalemma and

tonoplast sugar transport mechanisms (Guy et al. 1979, 1980, 1981). Ion transport studies on protoplasts have also been reported (e.g. Mettler and Leonard 1979, Lin 1980) using protoplasts derived from cultured *Nicotiana* cells and oat roots, respectively. Mesophyll protoplasts might be a particularly promising subject for experiments on the energization of ion transport in view of the widely-observed effect of light on such transport in green cells (see Jeschke 1976). Previous workers investigating algae and leaf tissue from a variety of sources have examined the spectral requirements, O_2 dependence and inhibitor sensitivity of light-stimulated cation transport, and have concluded that ATP generated in cyclic photophosphorylation is the basis of the light effect (Jeschke 1976). It is not yet decided, however, whether ATP directly energizes a K^+ transporter (or K^+-H^+ antiporter), or whether its action is indirect — for example it may fuel an electrogenic H^+ pump, thereby increasing the driving force for passive K^+ flux into the cell along its electrochemical potential gradient (see Bellando et al. 1979, Poole 1978). We report here an investigation into the light effect on Rb^+ transport in pea mesophyll protoplasts, in which flux has been measured over considerably shorter intervals than has been possible for higher plant cells in the past.

Abbreviations — CCCP, carbonyl cyanide m-chlorophenylhydrazone; DES, diethylstilbestrol; MeG, methyl-3-glucose; TCA, trichloroacetate; TPP^+ , tetraphenyl phosphonium.

Materials and methods

The plant material (*Pisum sativum* L. var. Dan) was grown and sampled as described earlier (Guy et al. 1979, 1980). Protoplasts were isolated from the leaf strips by incubation for 20 h in a 0.6 M sorbitol solution containing 1% cellulysin (Calbiochem) and titrated to pH 5.8 with Trizma base. Pretreatments and incubation took place in a medium which contained 10 mM Mes, 10 mM Hepes, 10 mM CaCl_2 , 0.2 mM H_3PO_4 , 1 mM MgSO_4 and 0.5 M sorbitol, brought to the desired pH with Trizma base. ^{86}Rb was used as a tracer for following K^+ uptake. $^{86}\text{RbCl}_2$ (about 50 kBq/tube), with the addition of KCl at the desired concentration, was added to the protoplast suspension at the start of the uptake period. At the conclusion of this period protoplasts were separated from the medium by centrifugation through a 6 cm KCl-CaCl_2 layer into a mixture of dibutyl and dioctyl phthalates (Guy et al. 1979). The pellet was resuspended in water, bringing about disintegration of the protoplasts, and aliquots were taken for protein determination by the Lowry (1951) method. Other aliquots were diluted with distilled H_2O and counted by Cerenkov radiation in a Packard Tri-Carb 3385 liquid scintillation spectrophotometer.

The light source for treatments was a water-cooled General Electric Quartzline lamp, the light intensity at the level of the incubation tubes being $50 \text{ nE cm}^{-2} \text{ s}^{-1}$ in the range of 400-700 nm. Protoplasts to be incubated in the light were also pretreated for at least 1 h in the light; "dark" samples spent this period in darkness. In experiments with inhibitors, the inhibitor was added to the protoplast suspension 3 min before the addition of the labelled solution.

Electric potential difference ($\Delta\psi$). The $\Delta\psi$ between the protoplasts and the surrounding medium was calculated from the distribution between cells and medium of TPP^+ , phenyl- ^3H . After incubation with labelled TPP^+ (diluted with 8 μM unlabelled TPP^+ , final specific activity 10,250 MBq/mmol) for various periods, aliquots of protoplast suspension were placed on a KCl-CaCl_2 layer in a plastic microfuge tube and centrifuged (Microfuge B, Beckman Instruments) through a layer of silicone oil (60 μl of 4:1 F-50 and RTV-910, Silicone Oil Division, General Electric, Waterford, NY) into a layer containing 2 M sorbitol and 12% TCA, 3:1. The microfuge tubes were frozen in liquid N_2 , and the tip containing the pellet was excised and transferred to 0.5 ml distilled H_2O , which disrupted the protoplasts. Samples were then withdrawn for counting in toluene-triton scintillation liquid. To estimate the internal volume of the cells in each aliquot, "water space" and "mannitol space" were measured after simultaneous incubation with ^{14}C -mannitol and tritiated H_2O as described by Volokita et al. (1981).

Reproducibility. Experiments were carried out in triplicate or quadruplicate, and were repeated at least 2 to 3 times. Error bars in the figures indicate the SD.

Labelled chemicals were obtained from the Radiochemical Centre, Amersham, England.

Results

The course of Rb^+ uptake in the light and the dark. The curve for Rb^+ uptake in the light did not decrease in slope with time — on the contrary, an increase in slope was detectable after about 7 min. This change in the slope of the curve was more pronounced at pH 7 than at pH 5 (Fig. 1a; see also Fig. 3, control curves). On the other hand uptake in the dark was relatively fast for approximately 7 min, after which the slope of the uptake curve decreased. This flattening of the curve was more marked at pH 5.5 than at pH 7.0 (Fig. 1b).

The dependence of uptake on pH. In the dark, uptake was relatively independent of pH between pH 4.5 and pH 6.5 (Fig. 2). Above this pH there was a steep rise with increasing pH (Fig. 2, Curve c). The curve for uptake in the light, on the other hand, rose between pH 4.5, and pH 5.5, decreased in slope between pH 5.5 and 6.3, and rose again with further increase in pH (Fig. 2, Curve a). In many experiments the curve did not merely tend to flatten between pH 5.5 and 6.3, but actually fell, creating a peak in the profile at pH 5.5, as exemplified by Curve b in Fig. 2. Both in the light and the dark the dependence on pH became more marked with increasing length of incubation period — after only 7 min of uptake the characteristic pH profiles described above had not yet developed (Fig. 2, Curves d and e). The development of the pH profile with time is related to the fact that the curve for uptake against time (Fig. 1) rose more steeply at high than at low pH. This was true both in the light and in the dark.

Fig. 1. Progress curves for $(K^+ + Rb^+)$ uptake by isolated pea mesophyll protoplasts in the light and in the dark. Circles, pH 5.5; triangles, pH 7.4. $(K^+ + Rb^+)$ concentration 0.1 mM. Error bars indicate $\pm$ SD from plotted means of triplicates.

Fig. 2. $(K^+ + Rb^+)$ uptake by isolated pea mesophyll protoplasts as a function of pH. Open symbols, in the light; solid symbols, in the dark. —, 7 min of uptake; —, 25 min of uptake; . . ., 25 min of uptake in a different experiment. $(K^+ + Rb^+)$ concentration 0.1 mM. Error bars indicate $\pm$ SD from plotted means of triplicates.

Fig. 3a and b. The effect of 5 μ M CCCP on the course of $(K^+ + Rb^+)$ uptake by isolated pea mesophyll protoplasts in the light and in the dark at pH 5.5 and 7.4, respectively. Open symbols, light; solid symbols, dark; circles, without CCCP; triangles, + 5 μ M CCCP. $(K^+ + Rb^+)$ concentration 5 mM. Error bars indicate $\pm$ SD from plotted means of triplicates.

Fig. 4. The effect of increasing CCCP concentration on $(K^+ + Rb^+)$ uptake by isolated pea mesophyll protoplasts. Open symbols, in the light; solid symbols, in the dark. $(K^+ + Rb^+)$ concentration 5 mM; pH 7.4; uptake period 27 min. Error bars indicate $\pm$ SD from plotted means of triplicates.

Studies of the relationship between uptake rate and K^+ concentration have revealed complex kinetics suggestive of multiphasic uptake (M. Rahat and L. Reinhold, unpublished results). Similar pH profiles were obtained, however, irrespective of whether the ($Rb^+ + K^+$) concentration was in the range of the first phase (up to approx. 0.2 M) or above it.

The effect of CCCP on Rb^+ uptake. The effect of the proton uncoupler CCCP was studied both in the K^+ concentration range of the first uptake phase and in that of the second. Very similar pictures were obtained for both phases. The results shown here (Fig. 3) are for 5 mM KCl (second phase). In the dark, little or no inhibitory effect of 5 μ M CCCP was detectable, either at pH 5.5 or at pH 7.4 (Fig. 3a, b). In the light, on the other hand, CCCP had a strong inhibitory effect. The latter was more marked at pH 7.4 than at pH 5.5, the curve for uptake in the light in the presence of CCCP being depressed below that for uptake in the dark (Fig. 3). The percentage inhibition by CCCP rose with time, particularly after the first 10 min at pH 7.4, when the slope of the curve for the control samples increased (Fig. 3; cf. Fig. 1), whereas that for the CCCP-treated samples did not.

In view of the lack of effect of 5 μ M CCCP on uptake in the dark, a range of CCCP concentrations was investigated (Fig. 4). In the light, sensitivity was shown at the lowest CCCP concentration tested (0.5 μ M CCCP), and increasing concentrations produced progressively more and more severe inhibition until the maximum effect was achieved at 10 μ M.

In the dark, however, inhibition was only observed at 1 and 5 μM CCCP, and even in this range the inhibition was very slight. Higher concentrations produced an increase in uptake to a level somewhat above that of the control. The shapes of the curves shown in Fig. 4 bear a striking resemblance to those reported by Prins (H. B. Prins, 1974. Thesis, Univ. of Groningen, The Netherlands) for *Vallisneria* leaves.

The effect of ATPase inhibitors and arsenate. Figure 5 shows the effect of quercetin (known as an inhibitor of mitochondrial ATPase — Lang and Racker 1974); rutin, a related inhibitor; DES, believed to be a specific inhibitor of plasma membrane ATPase (Balke and Hodges 1977); and arsenate, which interferes with ATP synthesis. All these inhibitors depressed uptake in the light. Inhibition was more marked at pH 7.4 than at pH 5.5. In the dark, however, sensitivity was displayed only at pH 5.5. At the higher pH, uptake in the dark appeared to be somewhat stimulated in the presence of arsenate and DES.

The effects of the inhibitors in the KCl concentration range of the first uptake phase were similar to those in that of the second, both in the light and in the dark (not shown).

The effect of transition from light to dark on Rb^+ uptake. If protoplasts are switched from light to dark, the light driving force for MeG uptake appears to persist for some minutes (Guy et al. 1980). The protoplasts take up MeG at the accelerated rate characteristic of light conditions for the first 7 min in the dark, after which the uptake rate re-

Fig. 5. The effect of various inhibitors on $(K^+ + Rb^+)$ uptake by isolated pea mesophyll protoplasts in the light and in the dark. ○, control, light; ●, control, dark; ◆, 100 μM DES; □, 20 μM quercetin; △, 50 μM rutin; ▼, 5 mM arsenate. $(K^+ + Rb^+)$ concentration 5 mM. Error bars indicate $\pm$ SD from plotted means of triplicates.

verts to the dark rate. Figure 6a shows this effect for MeG; in the same experiment, however, no "residual light driving force" could be detected in the case of Rb^+ flux (Fig. 6b). On the contrary, the switch from light to dark resulted in an uptake rate actually below that of protoplasts held continuously in the dark. This result was obtained in the case of Rb^+ flux both at pH 7.7 and at pH 5.5, and over a wide ($\text{Rb}^+ + \text{K}^+$) concentration range (0.1 mM to 5.0 mM).

Measurement of the cell "potential" by TPP^+ distribution. Figure 7 shows the course of entry of TPP^+ into protoplasts, in the light and in the dark. Discontinuities are detectable in both curves, and these discontinuities may not be solely due to inaccuracies in the measurements but may reflect the successive entry of TPP^+ into the various intracellular compartments. It is, therefore, not easy to judge which level of the curve reflects the "plasmalemma potential". However, the results clearly indicate a higher plasmalemma potential in the light than in the dark, since the whole of the curve for protoplasts in the light lies above that for protoplasts in the dark. Furthermore, the initial slope of the curve is higher in light than in darkness.

Treatment with CCCP produced no large effect on the apparent potential, either in the light or in the dark (Fig. 8). At the first reading the values for CCCP-treated protoplasts lay above the control values, possibly owing to increased permeability to TPP^+ or to slight hyperpolarisation (cf. Keifer and Spanswick 1978, Kishimoto 1981). No appreciable depolarisation as compared with the control occurred within 20 min.

Fig. 6. Effect of light-to-dark transition on the course of methyl-3-glucose (MeG) uptake (a) or (K^+ + Rb^+) uptake (b) by isolated pea mesophyll protoplasts. Light-to-dark: protoplasts transferred to dark at 0 min after 1 h in light. MeG concentration 1 mM; (Rb^+ + K^+) concentration 0.5 mM; pH 5.5. Error bars indicate $\pm$ SD from plotted means of triplicates.

Fig. 7. Effect of light on the apparent membrane potential of isolated pea mesophyll protoplasts. Open symbols, light; closed symbols, dark. pH 5.5. Membrane potential calculated from TPP⁺ distribution between protoplasts and medium. Error bars indicate $\pm$ SD from plotted means of quadruplicates.

Fig. 8. Effect of CCCP on the apparent membrane potential of isolated pea mesophyll protoplasts. Open symbols, light; closed symbols, dark; circles, without CCCP; triangles, + 5 μ M CCCP. Membrane potential calculated from TPP⁺ distribution between protoplasts and medium. Error bars indicate $\pm$ SD from plotted means of quadruplicates.

The rate of TPP^+ uptake was higher at pH 7.7 than at pH 5.5 both in the light and the dark (not shown) in accordance with a number of reports that the cell potential rises with pH (e.g. Kitasato 1968, Spanwick 1974b).

Discussion

A recent review (Jeschke 1976) has stressed the need for identifying the membrane or membranes involved in light-stimulated ion fluxes in higher plant cells. Previous studies, which were of necessity of relatively long duration, have provided indirect evidence in this respect. Our results strongly support the earlier deduction (Jeschke 1976) that the plasmalemma is involved on several grounds. Firstly, the light effect was detectable within 1 min of supply of Rb^+ to isolated protoplasts (e.g. Fig. 6; cf. Guy et al. 1980). Second, on switching to darkness, the light effect on uptake was abolished with no detectable delay (within 1 min, Fig. 6). Third, uptake in the light was sensitive to DES, regarded as a specific inhibitor of plasma membrane ATPase (Balke and Hodges 1977) (Fig. 5).

The progress curve for uptake in the light showed no tendency to flatten, in contrast to that for dark uptake (Fig. 1); rather it increased in slope after about 10 to 15 min of uptake. This suggests some autocatalytic step. Another possible explanation is that uptake was now occurring into an additional intracellular compartment not accessible in the dark. The chloroplasts are obvious candidates. However, light-stimulated uptake of K^+ into chloroplasts is not a recognised phenomenon

(Jeschke 1976). Further, the rate of uptake after 15 min exceeded initial flux. Thus, if the shape of the light curve in fact reflects uptake into an inner compartment after 15 min, coordination of the regulatory mechanisms for fluxes at the boundary membrane of this compartment and at the plasmalemma must occur to explain the accelerated uptake rate.

As pointed out in the Introduction, it has remained an open question as to whether or not ATP formed in photophosphorylation (believed to be responsible for the light effect on cation uptake) directly energizes a K^+ transporting system. An alternative view is that it energizes an electrogenic proton extrusion pump which contributes substantially to the driving force for passive K^+ influx (see Bellando et al. 1979). Our results with CCCP provide a strong indication that the passive uniport model is not applicable to uptake in the light by pea mesophyll protoplasts. CCCP was an immediate and effective inhibitor of Rb^+ transport in the light, and the reduction in Rb^+ uptake cannot be ascribed to a reduction in membrane potential since no appreciable depolarization was detectable during the first 20 min (Fig. 8). This result is unlikely to be an artifact dependent on the use of TPP^+ to estimate membrane potential, since the failure of CCCP to bring about depolarization for many minutes after its application has been observed by a number of other workers using penetrating microelectrodes (e.g. H. B. Prins, 1974: Thesis, Univ. of Groningen, The Netherlands, Keifer and Spanswick 1978, Kishimoto 1981). These workers have in fact observed slight initial hyperpolarization, and depolarization below control value was only observed after more than 1 h (Keifer and Spanswick 1978). Our results

in general resemble those of Prins (see above) for *Vallisneria* leaves. We conclude with him that Rb^+ uptake in the light cannot be accounted for by passive flux along the electrochemical potential gradient, since uptake was severely curtailed even though the potential gradient was maintained. Mercier and Poole (1980) have also recently concluded that uptake is correlated with the ATP level rather than the membrane potential.

A possibility to be considered is that the ATP requirement nevertheless relates to the fuelling of the proton extrusion pump, and that a proton- K^+ cotransport mechanism operates to drive K^+ inwards. Slayman (1974) has postulated a carrier which cotransports K^+ and H^+ inwards in exchange for Na^+ outwards in the case of *Neurospora*. Evidence in favour of similar cotransport models for K^+ uptake has recently been accumulating for bacteria (Bakker and Harold 1980, Rhoads and Epstein 1977). The results summarized in Fig. 7, however, argue against such a model in our case. We have recently concluded (Guy et al. 1980) that the light-dependent driving force which, in the case of MeG uptake, persists for about 7 min after the protoplasts are switched from light to darkness, is most reasonably explained as an electrochemical potential gradient for protons. Our grounds for this conclusion include consideration of the inhibitor sensitivity — MeG uptake is far more sensitive to CCCP in protoplasts switched from light to dark than in those continuously in the dark, but on the other hand shows stability to arsenate. We have suggested that in the switched protoplasts the proton conductor is dissipating a trans-membrane ΔpH generated by the light-sensitive proton pump. The implication of the absence of a residual light driving force in the

case of Rb^+ uptake (Fig. 6), therefore, is that ΔpH does not play a dominant role here. The contrast between the responses of sugar and Rb^+ flux, respectively, to a switch from light to dark suggests that a co-transport model is not applicable to mesophyll protoplasts. We conclude that an ATP-fueled K^+ porter, or K^+-H^+ antiporter, is the most likely explanation of our results.

Uptake in the dark contrasted strikingly with uptake in the light in a number of respects, notably the response to CCCP (Figs. 3 and 4). At pH 7.4 dark uptake was completely stable to 5 μM CCCP, a concentration which produced severe inhibition of light uptake within 5 min. The result was that light uptake in the presence of CCCP was depressed to a level actually below that of uptake in the dark. Light and dark uptake of K^+ appear to be different processes, and this conclusion recalls findings (Rhoads and Epstein 1977) that there are several different systems, differently energized, for transport of K^+ in *Escherichia coli*. Higher concentrations of CCCP actually produced slight stimulation of uptake in the dark (Fig. 4), as reported also for *Vallisneria* leaves by Prins (H. B. Prins, 1974. Thesis, Univ. of Groningen, The Netherlands). The author attributed this finding to increased permeability of the cell membranes at high CCCP concentrations.

There are reports in the literature (see Jeschke 1976) of CCCP inhibition of K^+ flux in leaf tissue in the dark. In these studies, however, the duration of CCCP treatment was hours rather than minutes as in our own experiments.

Uptake in the dark at pH 7.4 was stable not only to CCCP, but to all

the inhibitors tested (Fig. 5). Passive flux along the electrochemical potential gradient may be an appropriate model for dark uptake. The rising profile for uptake as a function of pH (Fig. 2) could be understood on the basis of the increase in electric potential with increase in pH which has been reported for a number of systems (Kitasato 1968, Bakker and Harold 1980), and which was also observed in the present investigation (p. 11). At pH 5 a certain degree of sensitivity to the ATPase inhibitors was noted even in the dark. A greater dependence on metabolic energy at pH 5 as compared with pH 7.4 would be expected if K^+ influx depends to any extent on exchange with H^+ , since more energy is required at the lower pH to move H^+ outwards against the higher external concentration of H^+ ions. That the cell potential itself appears to have been stable to the inhibitors tested may reflect the fact that the contribution of electrogenic pumps to the potential under these conditions was relatively slight — electrogenic pumps function to only a very limited extent in green cells in the dark (Spanswick 1974a, Rubinstein and Tattar 1980).

While the curve for dark uptake rose smoothly with pH (cf. Lin 1980 for root tissue) a peak at pH 5.5 could frequently be distinguished in the light (Fig. 2). The latter pH is optimal for sugar transport by these protoplasts (Guy et al. 1981). The peak might result from a requirement for protonation of some component of the K^+ transport system (see Reinhold et al. 1980). The generally rising trend of the pH profile is likely to be related to the increase in outward H^+ flux with rising pH (Poole 1974, Spanswick 1974). With increasing pH the gap between

the dark and the light curves lessened. However, although the dark curve approached the light curve, uptake was different in kind, as evidenced by the differing inhibitor sensitivity at pH 7.7 (Fig. 5).

Spanswick (1974b) has suggested that the observed increase in membrane potential with rise in pH is due to an effect on the electromotive force of the electrogenic pump. An alternative explanation offered by Kitasato (1968) invokes passive H^+ fluxes. A third possibility that merits consideration is homeostatic control of the electrogenic H^+ pump. This suggestion is based on the observation that the transmembrane electrochemical potential gradient for protons in microorganisms is held constant with a rise in external pH, the decline in ΔpH across the membrane being compensated for by an increase in cell potential (Zilberstein et al. 1979). In the present study there were indications that the increase in cell potential between pH 5.5 and pH 7.4 was at least as great in the presence of CCCP as in its absence, since the increase in level of dark uptake was at least as great. The maintenance of the pH profile in spite of the presence of a CCCP concentration which promptly and drastically inhibited uptake in the light suggests that the profile does not depend on operation of the proton pump. This finding favours the second explanation set out above, i.e. that passive H^+ fluxes are involved. The point deserves further investigation.

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II. C. *The sensitivity of membrane sugar transport to turgor — a possible model for osmotic regulation (Jerusalem laboratory)*

In a series of earlier investigations we have studied the energization and control of sugar transport across the plasmalemma and tonoplast of isolated mesophyll protoplasts and vacuoles (e.g. 6, 7). In the framework of the present project we have extended our studies to the effects of external water potential on transport. Because experiments involving manipulations of external osmotic pressure in the hypotonic range are impractical with isolated protoplasts, these studies were carried out with leaf tissue of *Senecio mikenoides*, a species where the entire epidermis is very readily removable, allowing unhindered exchange of solutes between inner cell layers and medium.

We have observed that the sugar transport system (as assessed by measuring uptake of the non-metabolized analogue 3-o-methyl glucose) shows sensitivity to turgor. We propose that this effect of turgor on sugar transport is in fact mediated via the proton extrusion pump, since we have also observed that a change towards more negative external water potential brings about an increase in the rate of proton extrusion (also noted by (4) and (5)). The increased proton motive force which is generated would, according to our suggestion, drive sugars into the cell by proton-symport.

The possibility suggested itself that this response to external water potential may reflect osmotic adaptation. Before coming to a conclusion, however, we have thought it necessary to investigate a number of alternative explanations which likewise suggested themselves:-

(a) The effect might relate to the "aging" phenomenon rather than to osmotic adaptation. The development of uptake capacity associated with aging (1) might for some reason be promoted or facilitated when the cell is at low turgor. However we have reported (1) that aging can be suppressed by treatment with the antibiotic cerulenin which inhibits the biosynthesis of membrane lipids. Figure 1 shows, firstly, that the course of aging in the presence of mannitol runs parallel to that in its absence, i.e. that mannitol does not appear to accelerate the aging process; and, secondly, that in the presence of a cerulenin concentration which has substantially slowed aging, mannitol still stimulates MeG uptake by approximately the same percentage as observed in the absence of the antibiotic. The mannitol effect is thus not attributable to an effect on aging.

(b) More negative external osmotic pressure might be exerting its effect on net inward sugar flux, not via a stimulating effect on unidirectional influx, but by reducing unidirectional efflux (possibly by diminishing the passive permeability of the membrane and hence reducing outwards "leak" — cf. 3). We have investigated this possibility in 2 types of experiments:- (1) Compartmental analysis (wash-out experiments) gave no indication that the rate constant for efflux, either from the "cytoplasmic" compartment or from the "vacuolar" compartment, was lower in mannitol-treated tissue. (2) The unidirectional fluxes were "visualized" in experiments of the following design: The cells were first loaded with ^{14}C -methylglucose. They were then very briefly rinsed and transferred to a methylglucose solution of the same concentration but now

Figure 1. The effect of 0.3 M mannitol on MeG uptake by leaf segments of *Senecio mikenoides*; and on development of MeG uptake capacity during aging in absence and presence of 35 mg/l cerulenin. After aging for various periods as indicated, samples were incubated in 1 mM MeG, with or without mannitol, for 1 h to determine uptake capacity. All media contained 2 mM KCl, 0.2 mM CaSO_4 and 2.5 mM fusaric acid.

labelled with tritium instead of ^{14}C . Loss of ^{14}C by the tissue was followed with time and gave a measure of efflux while uptake of T with time provided a measure of influx. Figure 2 shows that an effect of mannitol on unidirectional influx is observable; on the other hand there is no indication that mannitol treatment decreased unidirectional efflux. The observed effect of more negative external osmotic pressure on net inward sugar flux was thus due to enhanced influx, rather than decreased efflux.

(c) We have obtained the effect of more negative external water potential on sugar uptake not only by adding mannitol to the medium but also sorbitol or polyethyleneglycol. (In the case of the polymer a higher osmolarity — about 0.5 osmomolar — was required for maximum effect as compared with about 0.3 osmomolar in the case of mannitol and sorbitol.) In order to investigate whether the effect might relate not to osmoregulation but to some protective effect of poly-ols on the cell membranes, we tested the effect of two substances which do not contain such groups, but which penetrate the membrane only slowly and are thus suitable for use as osmotica — choline and betaine. Both these substances when added to the medium at 300 milliosmomolar, produced a stimulation of sugar uptake of the order of magnitude of 50%. The effect was thus independent of the presence of poly-ol groups.

(d) We have also considered the possibility that the tissue which is sensitive to turgor and thus responsible for the effect might be the phloem companion cell-sieve tube complex. However, it is generally accepted that the phloem loading system is highly specific for sucrose and that methylglucose does not penetrate into it.

Figure 2. Progress curves for net MeG flux inwards (square symbols), influx (triangles) and efflux (circles) into and out of leaf segments of *Senecio mikenoides*. The segments (from which the lower epidermis had been removed) were first incubated in ^{14}C -labelled MeG (1 mM) for 90 min, then transferred to ^3H -labelled MeG at the same concentration. Efflux was followed as loss of ^{14}C from the segments, influx as gain in ^3H . All media contained 10 mM KCl, 0.2 mM CaSO_4 , 2.5 mM fusicoccin and 2.5 mM MES buffer pH 5.8. Solid symbols, + 0.3 M mannitol; open symbols, without mannitol.

Having eliminated the possibilities listed above, we propose that the observed effects of external water potential may indeed reflect osmotic adaptation. In a discussion of the problem Bisson and Gutknecht (2) have pointed out that a specific regulatory mechanism must have 3 components: A detector to measure turgor or some quantity associated with it; an effector; and a means of passing information between the detector and the effector. On the basis of our observations on leaf cells we should like to point to the possibility that a key role in the mechanism of osmotic adaptation is played by the proton extrusion pump. If, as is widely believed, this pump is plasmalemma-sited, it could function at one and the same time both as detector and as effector. Changes in the plasmalemma associated with changes in the hydrostatic pressure gradient across the plasmalemma-cell wall complex (see 2) could have direct effects on the operation of the proton pump. Alterations in the rate of proton pumping, and hence in the pmf generated, would, in turn (according to the chemiosmotic viewpoint), have immediate repercussions on the transmembrane fluxes of a variety of solutes. The response of one membrane-sited system to a change in the pressure gradient could thus effect changes in the internal concentration of a considerable number of the solutes which make up the cell's osmotic potential, without the requirement for information transfer between detector and effector.

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III. STUDIES ON MEMBRANE ATPase

Plasma membrane ATPases are today believed to play a central role in solute uptake in all organisms. Nutrient uptake, it is believed, is energized by these membrane-sited proteins either directly, or through the electrochemical potential gradients for ions which they establish. The membrane ATPases of halophytes should be a particularly rewarding subject for study in view of the successful osmoregulation effected by these plants in saline habitats. The present investigation has included studies in both the Purdue and Jerusalem laboratories of membrane ATPase preparations obtained from roots of the halophyte *Atriplex nummularia*.

III. A. Comparison of plasma membrane ATPase activity in preparations from oat and from *Atriplex* roots (Purdue laboratory).

From the flux studies reported above (IB) it is apparent that Na^+ transport in *Atriplex* roots is different (either quantitatively or qualitatively) from the Na^+ transport in oats. Based upon the premise that the plasma membrane ATPase is involved in ion transport (Hodges, 1976), we have evaluated whether this enzyme in *Atriplex* is similar to or different from the enzyme in oats.

Membrane fractions enriched in plasma membranes of both oat roots and *Atriplex* roots were isolated as previously described (Leonard and Hodges, 1973) using discontinuous sucrose gradients. The oat and *Atriplex* plants were grown as described in IB. ATPase assays were carried out as described (Leonard and Hodges, 1973).

In our previous work, oat plants were grown aeroponically for one

week over a solution of 1 mM CaSO_4 . The plasma membrane ATPase of oats grown in the CaSO_4 solution and in the Hoagland solution used in the present studies are shown in Table 1. Interestingly, the ATPase activity of plants grown in Hoagland solution was about one-half the activity of those grown in CaSO_4 . This may be due to the preferential synthesis of other plasma membrane proteins in the more complete nutrient medium.

With *Atriplex*, the plants were also grown in a half-strength Hoagland solution for 3 weeks and the entire root system was used for analysis. Since this contrasts with other studies in which young roots are harvested periodically from the same plant, we have compared the ATPase activities of the plasma membrane from these 2 types of roots (Table 2) and no significant differences were obtained. This experiment does demonstrate that the plasma membrane from *Atriplex* roots contains an active ATPase that requires Mg^{2+} and is further stimulated by the addition of KCl.

The pH optimum for the *Atriplex* ATPase was between 6.0 and 6.5 for the K^+ -stimulated component. The optimum for the Mg^{2+} component was between 7.0 and 7.5. These results are similar for the oat plasma membrane ATPase (Hodges and Leonard, 1973).

The kinetic constants, K_m and $V_{\max}$, for the ATPase of both oats and *Atriplex* were determined at varying Mg-ATP concentrations in the absence and presence of 50 mM KCl (Table 3). In the absence of KCl, the K_m and $V_{\max}$ for the oat and *Atriplex* enzymes were nearly identical. In the presence of KCl, the $V_{\max}$'s of the ATPase from both plants were similar, but the K_m in *Atriplex* was somewhat lower. Based upon these kinetic constants, it appears that the plasma membrane ATPase of oat and *Atriplex*

Table 1. ATPases activity of plasma membrane fraction of oat roots. Plants were grown aeroponically over 1.0 mM CaSO_4 or in half-strength Hoagland solution.

Addition	ATPase	
	(μmoles/mg protein/hr)	
	CaSO_4	Hoagland
MgSO_4	34.0 ± 4.1	16.2 ± 1.2
$\text{MgSO}_4 + \text{KCl}(50 \text{ mM})$	56.2 ± 7.8	28.0 ± 1.9
ΔKCl	22.2 ± 9.0	11.8 ± 0.8

Table 2. ATPase activity of plasma membrane fraction of Atriplex roots. Entire roots of 3-week old plants or the young actively growing region of roots of several month old plants were evaluated.

Addition	ATPase	
	μmoles/mg protein/hr	
	Young roots from young plants	Young roots from old plants
MgSO_4 (3 mM)	18.8 ± 4.9	21.7 ± 12.0
$\text{MgSO}_4 + \text{KCl}$ (50 mM)	38.7 ± 10.3	39.8 ± 15.8
ΔKCl	19.9 ± 7.6	18.8 ± 8.2

Table 3. Kinetic constants for plasma membrane ATPase of oats and Atriplex.

<u>Experimental Conditions</u>	<u>Kinetic Constants</u>	
	<u>Km (mM)</u>	<u>Vmax (μmoles/mg/hr)</u>
Oats		
Mg - ATP (- KCl)	0.96	20.2
Mg - ATP (+ KCl)	0.52	33.8
Atriplex		
Mg - ATP (- KCl)	0.92	21.8
Mg - ATP (+ KCl)	0.37	33.3

is qualitatively similar.

In previous work we have shown that the plasma membrane ATPase of oats is sensitive to cations with the order of effectiveness in stimulations being $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$ (Sze and Hodges, 1977). The *Atriplex* plasma membrane ATPase, at pH 6.5, shows a similar sensitivity to cations (Table 4). However, at pH 8.5, although the ATPase activity is lower (see Legend of Table 4), the relative sensitivity toward the cations is different, i.e. $Rb^+ > Cs^+ > K^+ > Na^+ > Li^+$. Ammonium is not included in the alkali cation series, but its effect on the enzyme at pH 6.5 and 8.5 is very different — being quite effective as a stimulator at the high pH. Anions also appear to have some effect on the *Atriplex* ATPase at both pH 6.5 and 8.5 (Table 4). At pH 6.5 fluoride was very toxic to the enzyme and the other anions, with the exception of acetate, depressed the ATPase by 20-40% in comparison to Cl^- . At pH 8.5 the anions depressed the ATPase markedly as compared to Cl^- . Of particular interest was the inhibitory effect of NO_3^- at pH 8.5. The basis for this is unknown, but NO_3^- also inhibits an ATPase of a membrane fraction of oats that has a lower density (30/34% sucrose interface) than the main plasma membrane fraction (34/45% sucrose interface) (Cambraia and Hodges, 1980). It is also interesting that the two anions frequently toxic to enzymes, i.e. I^- and Fl^- , affect the ATPase in an opposite manner at pH 6.5 and at pH 8.5. Thus, in general, the *Atriplex* plasma membrane ATPase, like the oat plasma membrane ATPase, is sensitive to cations, but the *Atriplex* ATPase appears also to be substantially affected by anions.

Because the *Atriplex* ATPase was somewhat sensitive to anions, and our

Table 4. Effect of cations and anions on the plasma membrane ATPase of Atriplex. ATPase activities were assayed at pH 6.5 and 8.5.

Salt	ATPase (% of control)	
	pH 6.5	pH 8.5
KCl	100	100
NH ₄ Cl	84	202
RbCl	78	142
CsCl	65	118
NaCl	66	69
Choline Cl	44	17
LiCl	18	35

KCl	100	100
K Acetate	104	34
K Fumarate	67	70
K ₂ SO ₄	84	27
KNO ₂	67	22
KBr	73	37
KI	57	-6
KF	-72	24
KNO ₃	61	-36
KHCO ₃	—	14

In the chloride series of salts, the ATPase activity at pH 6.5 for MgSO₄ and MgSO₄ + KCl was 17.2 and 41.7, respectively, and at pH 8.5 the corresponding values were 6.8 and 13.0, respectively. In the potassium series of salts, the ATPase activity at pH 6.5 for MgSO₄ and MgSO₄ + KCl was 20.0 and 41.4, respectively and at pH 8.5 the corresponding values were 9.9 and 20, respectively.

earlier studies had shown the 30/34% sucrose interface membranes of oats to be uniquely sensitive to anions, we have evaluated this same membrane fraction from *Atriplex* (i.e. 30/34% sucrose interface) for ion sensitivity (Table 5). At pH 6.5 this membrane ATPase also exhibits sensitivity toward the cations, however, it is not quite as sensitive as the plasma membrane ATPase. We have not yet evaluated the cation sensitivity of this membrane fraction at pH 8.5. Anions affect this membrane ATPase at pH 6.5 similar to their effect on the plasma membrane ATPase. However, at pH 8.5 anions stimulate the ATPase of the 30/34% fraction considerably more than they stimulate the plasma membrane ATPase. These results are similar to that found for the membrane ATPases of oats, i.e. the less dense membrane ATPase appears to be more sensitive to anions than the plasma membrane ATPase, especially at the higher pH.

We have also found that the plasma membrane ATPase of *Atriplex* is not stimulated synergistically by K^+ and Na^+ even when the *Atriplex* was grown at 3, 50, and 100 mM NaCl in the nutrient medium. This was also true for the oat plasma membrane ATPase.

Tables 6 and 7 show the sensitivity of the plasma membrane and 30/34% membrane fraction ATPase activities to various inhibitors. The plasma membrane ATPase of both oats and *Atriplex* (Table 6) respond similarly to the inhibitors with the exception of oligomycin. The KCl-stimulated component of the *Atriplex* ATPase is less affected by oligomycin than in oats. This suggests that the oat plasma membrane fraction is more heavily contaminated by mitochondrial ATPase than the *Atriplex* plasma membrane fraction. The slightly greater sensitivity of the oat preparation to azide

Table 5. Effect of cations and anions on the ATPase activity of the membrane fraction collected at the 30/34% sucrose interface. ATPase activities were assayed at pH 6.5 and 8.5.

Salt	ATPase (% of control)	
	pH 6.5	pH 8.5
KCl	100	
NH ₄ Cl	99	
RbCl	94	
CsCl	79	
NaCl	87	
Choline Cl	43	
LiCl	25	

KCl	100	100
K Acetate	105	107
K Fumarate	65	112
K ₂ SO ₄	93	69
KNO ₂	83	81
KBr	109	69
KI	108	7
KF	-106	90
KNO ₃	48	-3
KHCO ₃	—	55

In the chloride series of salts, the ATPase activity at pH 6.5 for MgSO₄ and MgSO₄ + KCl was 12.5 and 27.9, respectively. In the potassium series of salts, the ATPase activity at pH 6.5 for MgSO₄ and MgSO₄ + KCl was 16.2 and 31.2, respectively and at pH 8.5 the corresponding values were 5.0 and 10.8, respectively.

Table 6. Effect of various inhibitors on plasma membrane ATPase of oat and Atriplex roots.

Treatment	Salts ¹	Oats		Atriplex	
		Specific activity ²	%	Specific activity ²	%
Control(with 0.1% Ethanol)	MgSO ₄	16.4 ± 0.6	100	18.6 ± 1.8	100
	MgSO ₄ +KCl	30.7 ± 1.0	100	34.6 ± 3.6	100
	Δ KCl	14.3 ± 0.7	100	16.0 ± 2.0	100
Oligomycin (5 µg/ml)	MgSO ₄	14.2 ± 1.8	86	14.5 ± 2.4	78
	MgSO ₄ +KCl	23.1 ± 2.3	75	30.5 ± 2.0	88
	Δ KCl	8.9 ± 1.7	62	16.0 ± 0.8	106
DES (0.1 mM)	MgSO ₄	5.3 ± 2.1	32	1.8 ± 1.8	10
	MgSO ₄ +KCl	6.1 ± 1.7	20	3.9 ± 1.6	11
	Δ KCl	0.8 ± 1.4	6	2.1 ± 1.1	13
Control (H ₂ O)	MgSO ₄	17.1 ± 2.1	100	16.1 ± 1.8	100
	MgSO ₄ +KCl	28.8 ± 3.1	100	33.9 ± 4.7	100
	Δ KCl	11.7 ± 1.4	100	17.8 ± 3.6	100
Azide (0.5 mM)	MgSO ₄	16.7 ± 2.9	98	13.7 ± 2.3	85
	MgSO ₄ +KCl	25.5 ± 3.7	88	29.2 ± 4.3	86
	Δ KCl	8.8 ± 0.8	75	15.5 ± 2.7	87
Vanadate (0.1 mM)	MgSO ₄	13.2 ± 1.3	77	11.6 ± 1.8	72
	MgSO ₄ +KCl	15.0 ± 2.1	52	15.2 ± 1.3	45
	Δ KCl	1.8 ± 1.8	15	3.6 ± 0.5	20

1) MgSO₄ and KCl was 3 and 50 mM, respectively

2) Specific activity equals µmoles/mg protein/hr.

supports this notion. DES and vanadate are both potent inhibitors of the plasma membrane ATPase. DES inhibits both MgSO_4 and $\text{MgSO}_4 + \text{KCl}$ activity, whereas vanadate primarily inhibits the KCl-stimulated component of the ATPase. It is generally believed that vanadate is a specific inhibitor of the plasma membrane ATPase; thus, the sensitivity of the ATPase in both oats and *Atriplex* supports the conclusion that this membrane fraction is highly enriched in plasma membranes.

The 30/34% membrane fraction of *Atriplex* (Table 7) is much less sensitive to vanadate than the plasma membrane fraction. However, the 59% inhibition of the K^+ -stimulated component of the ATPase indicates that this fraction contains substantial amounts of plasma membranes. Mitochondrial ATPase, however, must be essentially absent from this fraction based upon the lack of azide inhibition.

Although additional characterizations need to be made, our results to date indicate that the plasma membrane ATPase of *Atriplex* roots is similar to the plasma membrane ATPase of oat roots. We still need to examine whether Na^+ stimulation of the *Atriplex* enzyme is unique in some way. However, based upon the similarities observed to date between the oat and *Atriplex* ATPases, it does not look promising that this enzyme will account for the different flux rates of Na^+ at the plasma membrane of these 2 species.

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Table 7. Effect of various inhibitors on ATPase activity of membranes obtained from 30/34% sucrose interface of gradient using Atriplex roots.

Treatment	Salts	ATPase		% Δ K
		Δ moles/mg protein 1 hr		
Control (H ₂ O)	MgSO ₄	12.1	1.9	100
	MgSO ₄ + KCl	22.4	3.6	
	Δ KCl	15.9	0.8	
Vanadate (0.1 mM)	MgSO ₄	7.5	1.6	59
	MgSO ₄ + KCl	16.9	2.4	
	Δ KCl	9.4	2.1	
Azide (0.5 mM)	MgSO ₄	7.3	2.3	98
	MgSO ₄ + KCl	22.9	3.2	
	Δ KCl	15.6	1.4	
Control (1.0% Ethanol)	MgSO ₄	13.2	2.1	100
	MgSO ₄ + KCl	26.0	6.7	
	Δ KCl	12.8	4.7	
DCCD (50 mM)	MgSO ₄	-1.0	2.9	20
	MgSO ₄ + KCl	1.5	1.8	
	Δ KCl	2.5	0.8	
Control H ₂ O	MgSO ₄	13.7	1.1	100
	MgSO ₄ + KCl	28.8	1.8	
	Δ KCl	15.1	1.3	
EDAC 10 mM	MgSO ₄	13.0	1.7	85
	MgSO ₄ + KCl	25.9	3.7	
	Δ KCl	12.9	2.8	

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III. B. *Salt activation and inhibition of membrane ATPase from roots of the halophyte Atriplex nummularia (Jerusalem laboratory).*

In view of its ability to grow in highly saline environments, it was interesting to study the ATPase activity of membrane preparations obtained from roots of the halophyte *Atriplex nummularia* in an extended range of salinity similar to soil conditions encountered by *Atriplex* in nature. The ATPase activity of preparations from *Atriplex* roots was compared with that of *Pisum* which has a relatively low salt tolerance. Some data concerning the plasma membrane ATPase of the halophyte *Plantago maritima* have been provided by Kuiper and his coworkers (for references see Erdei & Kuiper, 1980). A more detailed report has also appeared on the membrane-bound ATPases of the desert halophyte *Salicornia pacifica* (Kim & Weber, 1980; Weber, 1982).

Materials and methods

Atriplex nummularia Lindl. and *Pisum sativum* L., cv Dan, were grown in the greenhouse in aerated half-strength Hoagland and Snyder solution (Hewitt, 1963). The apical 5 cm of white young roots were removed from *Atriplex* plants that were 3 to 6 months old. In the case of the pea, the apical 5 cm of the roots were excised from 2-week-old seedlings. The root samples were weighed, rinsed 3 times in ice-cold de-ionized water, and ground in an ice-cold mortar and pestle as described by Leonard *et al.* (1973). In the earlier experiments a 13,000-80,000 g pellet (Beckman centrifuge 5L 50, rotor SW 41 Ti) was used for the ATPase assay. In subsequent experiments the preparation was fractionated on a discontinuous sucrose gradient as described by Leonard *et al.* (1973).

ATPase activity was assayed by measuring Pi liberation. The total volume of the assay mixture was 500 mm³; ATP and MgSO₄ concentrations were 3 mol m⁻³. The buffer was MES-HEPES-3-3-dimethylglutaric acid, 11 mol m⁻³ each, adjusted to the desired pH with tris or HCl. The reaction was initiated by addition of the membrane preparation and terminated 30 to 90 min later, depending on activity, by addition of ice cold TCA to a final concentration of 5 per cent. The supernatant after centrifugation was used for Pi estimation (Taussky & Shorr, 1953). Optical density was measured at 660 nm in a Gilford spectrophotometer with rapid sampling attachment. Protein was determined according to Lowry *et al.* (1951).

Salt-stimulated ATPase activity was calculated (a) as the differ-

ence between the values observed in the presence of Mg^{+2} alone and those observed in the presence of Mg^{+2} plus the appropriate salt or (b) as the percent activation of MgATPase induced by salt.

All experiments were carried out in triplicate and each experiment was repeated at least 3 times.

Chemicals. ATP was obtained from Boehringer, Mannheim, West Germany.

Results

Monovalent salts have been reported to stimulate plant membrane ATPase preparations at pH values both below and above 7 (Cambara & Hodges, 1980). In preliminary experiments we examined the effects of KCl and NaCl at a number of concentrations both at pH 5 and at pH 8 on ATPase activity of membranes from roots of *Atriplex* and pea. In view of the difficulties involved in obtaining adequate amounts of the *Atriplex* material, the unfractionated membrane preparations were used for these preliminary experiments. They indicated that the effects of the salts were complex: both stimulation and inhibition of ATPase activity were observed (Figs. 1, 2 & 3).

Figs. 1 & 2 show the effect of increasing salt concentration on monovalent ion-stimulated ATPase activity at pH values 5 and 8. At pH 5 the pea ATPase (Fig. 1a) was stimulated by low salt concentration but inhibited by higher concentrations. The transition from stimulation to inhibition (that is, depression of ATPase activity to a level below that observed in the presence of Mg^{+2} alone) occurred at about 150 mol m^{-3} for both KCl and NaCl. By contrast the *Atriplex* ATPase (Fig. 1b) was stimulated maximally by salt concentrations of about 100 mol m^{-3} , and stimulation was maintained even in the presence of 450 mol m^{-3} salt.

The curves for pH 8 differed from those for pH 5 (Fig. 2). The curve for pea was shifted towards lower concentration — maximal stimulation was obtained at 5 to 10 mol m^{-3} , and threshold inhibition at about 15 mol m^{-3} (see inset in Fig. 2a). The inhibition observed at higher concentrations was severe. Transition from stimulation to inhi-

bition as salt concentration increased was also observable at pH 8 in the *Atriplex* curve. Maximal stimulation was obtained between 50 and 100 mol m⁻³ salt, and transition to inhibition occurred at about 150 mol m⁻³. The interaction of monovalent ion concentration and pH is thus especially noteworthy in the case of *Atriplex* ATPase — high salt concentrations stimulate activity at pH 5 (Fig. 1b) but are clearly inhibitory at pH 8 (Fig. 2b).

Although in the case of the pea, the size of the salt stimulation was small in proportion to the background MgATPase activity, this stimulation was reproducible in repeated experiments. The most striking feature of Figs. 1 and 2 is the widely differing response of the two species to increasing salt concentration, and this, too, was highly reproducible.

In view of the contrasting responses to increasing salt concentration at pH 5 and pH 8, we studied the pH profile at various monovalent salt concentrations. In the case of pea (Fig. 3a) stimulation was observed only at pH 5. At all other pH values the presence of salt inhibited ATPase activity below that for the presence of Mg⁺² alone. This finding is consistent with the earlier results shown in Figures 1a and 2a. The curves for 50 mol m⁻³ and higher NaCl concentrations showed a pronounced trough with a minimum at pH 7. The results for KCl (not shown) were very similar to those for NaCl, except that inhibition was somewhat less severe.

In the case of the *Atriplex* NaCl-MgATPase (Fig. 3b) salt stimulated the activity over almost the entire concentration range tested, but the

Figure 1. The effect of increasing KCl or NaCl concentration on monovalent ion-stimulated ATPase activity of crude membrane preparations from pea roots (a) and *Atriplex* roots (b) at pH 5. Salt-stimulated ATPase is calculated as the difference between the activities measured in the presence and absence of the indicated salt concentration.

Figure 2. The effect of increasing KCl or NaCl concentration on monovalent ion-stimulated ATPase activity of crude membrane preparations from pea roots (a) and *Atriplex* roots (b) at pH 8. Salt-stimulated activity calculated as in Fig. 1.

Figure 3. NaCl-stimulated ATPase activity of crude membrane preparations from pea (a) and *Atriplex* (b) roots as a function of pH, at various NaCl concentrations in mol m^{-3} as indicated on the curves. Salt-stimulated activity calculated as in Fig. 1.

Figure 4. Discontinuous sucrose gradient fractionation of *Atriplex* membrane preparation. KCl-stimulated ATPase activity (calculated as in Fig. 1) was measured in the presence of 100 mol m^{-3} KCl at pH 7.

pH optimum appeared to be progressively displaced towards lower pH values as salt concentration increased. The height of the peak also increased with rising NaCl concentration. The shift in pH optimum was less pronounced in the case of KCl-MgATPase (not shown), displacement only being observed above 100 mol m^{-3} .

These features of the *Atriplex* enzyme justified an attempt to purify the ATPase preparations by fractionation on a discontinuous sucrose gradient. Fig. 4 shows that the highest KCl-stimulated ATPase activity was obtained with fractions 6 and 7, as is the case for oat roots (Hodges, 1976) though not for pea epicotyl (Hendrix & Pierce, 1980).

The phosphate hydrolysis activity of fractions 6 plus 7 is given in Fig. 5 as a function of pH, for four different substrates. At pH values of 7 and 8 by far the highest hydrolysis rates were observed when the substrate was ATP. At lower pH values, however, ADP and pyrophosphate supported rates of phosphate liberation two fold greater than that observed for ATP; while the rate of phosphate hydrolysis from β -glycerophosphate was low at all pH values tested.

Walker and Leigh (1981) have reported KCl stimulation of an Mg-dependent pyrophosphatase in red beet tonoplasts. The effect of KCl on phosphate hydrolysis for the 4 different substrates shown in Fig. 5 is given in Table 1. There was no appreciable stimulation of phosphatase activity by KCl (Line 1). KCl-stimulated MgADPase activity and Mg-pyrophosphatase activity was about $1 \mu\text{mol Pi} \cdot \text{mg}^{-1} \text{ protein} \cdot \text{h}^{-1}$. Thus KCl-stimulated pyrophosphatase activity could only account for about 15 per cent of the observed KCl stimulation at 100 mol m^{-3} KCl when MgATP was

Figure 5. Liberation of P_i from ATP, ADP, β -glycerophosphate, and pyrophosphate by *Atriplex* membrane fractions 6 plus 7 as a function of pH. Substrate concentrations as in Table I. (○—○) ATP, (●—●) ADP, (□—□) pyrophosphate, (Δ····Δ) β -glycerophosphate. The bars indicate the standard error where this exceeded the size of the symbol.

Figure 6. KCl-stimulated ATPase activity of *Atriplex* membrane fractions 6 plus 7 in the presence of various concentrations of KCl as a function of pH: (a) calculated as the difference between the activity in the presence and absence of KCl; (b) calculated as per cent activation produced by KCl. Numbers on curves indicate KCl concentrations in mol m^{-3} . Standard error bars are shown for the points which demonstrate the shift in pH optimum with change in KCl concentration.

Figure 7. pH profile of MgATPase activity of crude pea root membrane preparation.

Table 1. Phosphatase and pyrophosphatase activity in purified root membrane preparations from *Atriplex nummularia*, in the presence and absence of KCl.

Substrate concentrations chosen such that the amount of phosphate was equal in all cases. Pyrophosphate supplied as sodium salt. KCl concentration 100 mol m^{-3} ; pH 6. Activities given as $\mu\text{mol Pi mg}^{-1} \text{ protein h}^{-1}$, $\pm \text{S.E.}$

	Substrate (and concentration mol m^{-3})	Activity		Calculated KCl-stimulated activity
		-KCl	+KCl	
1	Mg β -glycerophosphate (9)	1.0 ± 0.1	1.2 ± 1.1	0.2 ± 1.1
2	Pyrophosphate (4.5)	4.5 ± 0.5	5.5 ± 0.4	1.0 ± 0.7
3	Mg pyrophosphate (4.5)	4.4 ± 0.4	5.5 ± 0.9	1.0 ± 1.0
4	ADP (4.5)	3.5 ± 0.4	4.3 ± 0.4	0.8 ± 0.6
5	MgADP (4.5)	2.9 ± 0.3	4.0 ± 1.6	1.2 ± 1.6
6	ATP (4.5)	1.7 ± 0.1	3.7 ± 0.2	2.0 ± 0.3
7	MgATP (3)	1.2 ± 0.7	8.3 ± 0.5	7.1 ± 0.9

the substrate. (The stimulation of $2 \mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$ noted in line 6 where ATP was the substrate can probably be attributed to the presence of a low amount of Mg^{+2} introduced by the membrane preparation.)

The pH profiles for fractions 6 plus 7 at different KCl concentrations (Fig. 6a) resembles those earlier obtained for the crude preparation (Fig. 3b). The optimum pH for the lower salt concentrations was 7 while that for the higher salt concentrations was shifted to pH 6. Except in the case of the higher concentrations at pH 8, the addition of salt stimulated the activity over the entire concentration range tested. It is of special interest, however, that the shift in the optimal pH was only observed when salt-stimulated ATPase activity was calculated as the difference between the values observed in the presence and absence of salt respectively (Fig. 6a). When salt stimulation was expressed as percentage activation by KCl (Fig. 6b) all concentrations tested showed a pH optimum at pH 6. Very similar results were obtained with NaCl (not shown), where the concentration range tested extended to 400 mol m^{-3} . Even the latter concentration was stimulatory at pH values of 7 or lower.

Discussion

MgATPase activity in the crude preparation from both plants is liable to inhibition by added monovalent ions. This is indicated for both *Atriplex* and pea by the fact that for crude preparations large portions of the curves shown in Figs. 1a, 2a & b and 3a lie below the zero line. Inhibition is also evident for the purified enzyme at pH 8 (Fig. 6). A striking difference between the pea and *Atriplex* lies in their differing degree of sensitivity to salt inhibition. At pH 8 the threshold salt concentration causing inhibition was an order of magnitude higher in the case of *Atriplex* than in that of pea (Fig. 2). At pH 5 no inhibition at all was superficially apparent for *Atriplex* at the salt concentrations investigated (up to 450 mol m^{-3}), whereas 150 mol m^{-3} produced threshold inhibition in pea (Fig. 1).

The effect of salt (anion and cation) on plasmalemma ATPases is complex, as has been pointed out by Cambraia and Hodges (1980). Our results show up the complications arising from the fact that inhibition of MgATPase activity by monovalent salt is occurring concomitantly with salt activation of the salt-stimulated ATPase activity. That the inhibitory effects on MgATPase can produce substantial distortion of the curve for calculated monovalent ion-stimulated ATPase emerges from consideration of the pH profiles shown in Figs. 3 & 6a. Figs. 3b & 6a do not present the true pH profile for the *Atriplex* salt-stimulated ATPase, since the value used for the MgATPase baseline was that observed at zero NaCl or KCl, and did not take into account salt inhibition of the MgATPase. The latter inhibition will have been greatest in absolute

amount at pH values 7 and 8, the pH range where MgATPase activity is greatest (Fig. 5). At pH 5 and 6 the inhibition will have been very small in absolute amount, since MgATPase activity at these pH values were very low. Low monovalent salt concentrations will therefore only have produced sufficient inhibition to affect the results perceptibly at pH 7 and 8, not at lower pH values. Higher salt concentrations, however, will progressively bring about some inhibition at pH 6 as well, thus producing displacement of the apparent pH optimum towards lower pH values (Fig. 3b).

This interpretation of the results is supported by Fig. 6b. Plotting the effect of salt as a percentage of the MgATPase activity, as in this Figure, would be expected to minimize the effects which result from the varying level of this activity at the different pH values. Figure 6b shows that this plot indeed eliminates the shift in pH optimum with rise in salt concentration. Here the pH optimum is 6 for all salt concentrations.

A second possible explanation for the shift in pH optimum — and one that does not exclude the first — is that more than one enzyme hydrolyzes the MgATP supplied. The experimental results would be accounted for if 2 enzymes participated, of differing sensitivity to inhibition by high salt concentration. The first would have low activity in the absence of salt and would be susceptible of salt stimulation over a wide range of salt concentrations. The pH optimum of this enzyme would be near pH 6, thus accounting for the peaks in Fig. 6b. The second enzyme would have high activity in the absence of the salt and would have a

lower threshold for inhibition by salt as compared with the first enzyme. The pH optimum would be near 7 (see ATP hydrolysis curve in Fig. 5). Calculated as a percentage, the salt stimulation shown by the second enzyme would be small as indicated by the lack of peaks at pH 7 in Fig. 6b; when calculated as the difference between control and salt-treated (Fig. 6a), however, the salt stimulation would be large at pH 7 since activity is very high at this pH (Fig. 5) thus explaining why peaks are observed at pH 7 at low salt concentrations.

In the case of pea (Fig. 3a) the pH profiles lie for the most part below the zero line, and thus show that MgATPase activity was substantially depressed. This depression will have been greatest in absolute amount at approximately pH 7, where MgATPase activity is highest (Fig. 7). Since, in calculating salt-stimulated ATPase activity, no correction was made for the accompanying inhibition of MgATPase activity, salt stimulation will have been underestimated; and maximum underestimation of the salt stimulated activity will occur at approximately pH 7. The troughs in the pH profile (Fig. 3a) can thus be best interpreted as reflecting the maximum depression of MgATPase activity.

Our work has pointed to the difficulties inherent in interpreting values for salt-stimulation when working with mixed membrane ATPase activities, where one is stimulated by salt while another is simultaneously depressed. As a consequence the position of the baseline for MgATPase is liable to error, and the meaning of differences in level above the baseline is difficult to interpret. However, the salt concentrations at which the curves in Figures 1 and 2 start to decline is

easier to interpret; and this concentration is 10-fold higher in the case of *Atriplex* as compared with pea both at pH 5 and at pH 8. The enzyme from *Atriplex* thus appears to tolerate salt concentrations well in excess of those causing decline in activity of the enzyme from *Pisum*.

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III. C. ATP-dependent generation of a trans-membrane electric potential in vesicles prepared from the roots of the halophyte Atriplex nummularia.

One of the aims of the study of the ATPase activity in root membrane preparations was to prepare the ground for studies of trans-membrane solute transport and its energization.

The *Atriplex nummularia* preparation, fraction 6+7 from the sucrose gradient, (see III B for details) was assayed for electrogenic ion transport properties by examining its capacity to establish a trans-membrane potential ($\Delta\psi$). The method used was as described by Rasi-Caldogno *et al.* (1981). The vesicle preparation is incubated with (^{14}C) SCN^- (thiocyanate) in the absence and the presence of 5 mM ATP. After the appropriate incubation period the diluted reaction mixture is filtered on a millipore filter which is then assayed for radioactivity.

Figure 1 shows that the presence of ATP greatly enhances SCN^- accumulation suggesting the presence of an ATP-fueled electrogenic pump. It is also evident from Figure 1 that about 10 min is required for a plateau of SCN^- accumulation to be attained. From Table 1 it is clear that the proton ionophore FCCP (10 μM) prevents generation of the ATP-dependent $\Delta\psi$, indicating that a proton gradient is involved. Since the FCCP was dissolved in ethanol, the effect of ethanol was also examined but found to be very small (Table 1). It is likely that the vesicles contain a proton pump as has been demonstrated recently in work with vesicles derived from glycophyte tissue (1, 2, 3, 4).

The addition of KCl or K_2SO_4 dissipates potential, more markedly in

Fig. 1. ATP-Dependent SCN⁻ accumulation in *Atriplex* membrane vesicles.

Accumulation of SCN⁻ in the vesicles was measured as a function of time at pH 6.5.

● in the presence of ATP;

▲ in absence of ATP.

Vertical bars indicate S.E.

Table 1. Effect of the uncoupler FCCP on ATP-dependent SCN^- accumulation in *Atriplex* root membrane vesicles.

Treatment	ATP-Dependent	
	SCN^- uptake (dpm)	% of control
Control	$225 \pm 16^*$	100
+ ethanol**	202 ± 23	90
+ 10 μM FCCP**	4 ± 7	2

Measurements were made at pH 7.

* S.E.

** FCCP was added as an ethanol solution; the effect of the same ethanol concentration was therefore also tested.

the former case. This is probably an effect due to the anions penetrating the vesicles.

The effect of oligomycin on ATP-dependent SCN^- accumulation was examined to check that the membranes responsible for $\Delta\psi$ generation did not originate from the mitochondria (which should be eliminated when the 13,000 g precipitate is discarded — see Chapter 4). Table 2 shows that the ATP-dependent SCN^- uptake is not inhibited by oligomycin, indicating that the preparation is not contaminated by the F_0F_1 mitochondrial enzyme. Table 3 shows that oligomycin has no effect on the ATPase activity, reinforcing this conclusion. The investigation of this electrogenic pump is continuing.

Table 2. Effect of oligomycin on ATP-dependent SCN^- accumulation in *Atriplex* root membrane vesicles.

Treatment	ATP-Dependent	
	SCN^- uptake (dpm)	% of control
Control	$110 \pm 25^*$	100
+ ethanol	81 ± 15	73
+ 30 μg oligomycin/mg protein	131 ± 23	119

Measurements were made at pH 7.

*S.E.

Table 3. Effect of oligomycin on MgATPase activity in *Atriplex* root membrane vesicles.

μg oligomycin mg protein	$\mu\text{mole P}_i$ mg protein $\cdot$ h	% of control
0	$9.9 \pm 0.6^*$	100
25	9.1 ± 0.2	92
50	9.0 ± 0.8	91
100	9.3 ± 1.5	94

Measurements were made at pH 6.

*S.E.

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Evaluation of Research Achievement with respect to the Original Proposal

A major aim in our Proposal was to develop a technique to leak cytosol solutes independently of vacuolar solutes for the purpose of identifying and estimating the size of cytosol pools. A large measure of success has been achieved by use of the polycation poly-L-lysine (see 1A). This method has enabled us to distinguish cytosol and vacuolar pools of ions and metabolites of cells. The limitation of the method resides in the fact that poly-L-lysine does not diffuse through multilayers of plant cells, and its use is thus confined to monolayers or cell suspension. A modification of the principle, possibly using a molecule which raises the permeability of the plasmalemma, may extend its application to multicellular plant organs.

Flux analysis has been used as another approach towards estimating Na^+ and K^+ levels in cytosol as distinct from the vacuole (see 1B). This method also provides estimates of trans-membrane fluxes. Our experiments have brought to light the fact that Na fluxes are 5 to 10 times higher in root systems of the halophyte *Atriplex* as compared with the glycophyte oat.

It was also a major aim of this investigation to work towards understanding osmotic accommodation at cell level. Our proposed work on tonoplast transport met with serious technical hindrances owing to the difficulties encountered in isolating stable vacuoles from halophytes. However, we are carrying out a productive investigation at cell level into ion and metabolite pool sizes as a function of salinity in a stable NaCl-resistant tobacco cell line which we have developed; and which is capable

of growing at 450 mM NaCl (see IIA). We see this as a promising line of research and we shall continue to pursue it. At protoplast level our experiments have yielded significant information regarding the mode of passage of K^+ through the plasmalemma (see IIB). We have also carried out work on the response of membrane transport mechanisms in a leaf preparation to changes in external water potential, work which has led us to suggest a possible model for osmotic regulation (see IIC).

As set out in the Proposal we have investigated the monovalent salt stimulated ATPase activity in membrane fractions derived from the halophyte *Atriplex nummularia* and compared it with that of similar preparations derived from two glycophytes. Our investigations have shown up two points of special interest. First, that the *Atriplex* enzyme tolerates salt concentrations more than 10-fold higher than those causing decline in activity of that from the glycophyte *Pisum*. Second, that inhibition of MgATPase activity by monovalent salt occurs concomitantly with activation of the salt-stimulated ATPase activity. No clear evidence has been obtained that this enzyme can account for the different Na^+ flux rates in the halophyte as compared with the glycophyte which we have observed. We have proceeded to study ATPase-generated trans-membrane potentials in membrane vesicles. These potentials are the consequence of transfer of positive ions (probably protons) into vesicles. We shall investigate the effect of degree of salinity during the growth of the halophyte on the ATP-dependent membrane proton pump.

Cooperation

Principal investigators from the Israeli and U.S. Laboratories had the opportunity of face-to-face discussions both in Jerusalem and in Purdue, and as a result much of the research programme was jointly planned and assessed. The U.S. laboratory had been the pioneers in work on membrane ATPase in cereal roots and their know-how greatly assisted the Israeli laboratory. The Israeli laboratory for its part, had been conducting studies on the halophyte *Atriplex nummularia* for some years and supplied Dr. Hodges with plant material and information for the comparative studies in Purdue.

Benefits to Agriculture

The expected benefits to agriculture from this work are long-term. Lack of sufficient understanding of the physiology of the plant's response to saline and water stress at present impedes progress towards practical solutions to the problem of growing commercial crops on marginal or saline land. We are collecting information with regard to the mechanisms required for survival under conditions of these stresses. This basic knowledge will pave the way towards the practical goals, and must also be on hand before efficient use can be made of techniques such as genetic engineering for improving the resistance of commercial crop plants.

List of Published Papers

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- Reinhold, L., M. Rahat, M. Volokita, A. Seiden and M. Guy. 1982. Plasmalemma transport of sugar and ions in isolated pea mesophyll protoplasts, with a suggestion as to the possible mechanism of osmoregulation. *In: "Plasmalemma and Tonoplast: their Functions in the Plant Cell"*, eds., D. Marmé, E. Marrè and R. Hertel, pp. 271-276. Elsevier Biomedical Press: Amsterdam.
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- Watad, A. A., L. Reinhold and H. R. Lerner. 1983. Comparison between a stable NaCl-selected *Nicotiana* cell line and the wild type. *Plant Physiol.* 73 (In Press).
- Several other MSS. are at present being prepared.