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Elucidation of the Plants Accommodations to Water and Saline Stress at Cell Level

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a. COVER PAGE

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c. ABSTRACT

The first section of our work reports the development of salinity-resistant lines of tobacco cells in suspension culture adapted to grow in NaCl concentrations up to 500 mM. The character of salt resistance, and the structural characteristics of the adapted line, were stable even in the absence of selection pressure. Since the immediate source of energy for the uptake of many metabolites (or for their extrusion from the cell) is today believed to be the proton motive force established by a proton pump, we have studied proton fluxes as a response to salinity in wild type and adapted lines. Net proton extrusion by the adapted cells was greater, especially in the presence of KCl and fusicoccin; membrane potential was higher; and the degree of coupling between H^+ efflux and K^+ influx appeared to be higher in adapted as compared with wild type cells.

The second section of our work concerned the identification and characterization of proton pumps (i.e. H^+ -translocating ATPases which generate the protonmotive force and may play a key role in osmotic adaptation) in membrane preparations from the roots of plants of varying resistance to salinity. The U.S. partner has critically compared techniques for the isolation and purification of such membrane fractions. In Jerusalem we have studied the pump in the roots of the halophyte Atriplex. Interestingly, growing the plant in saline media doubled the proton-translocating activity per mg membrane protein and otherwise modified it a number of ways. By contrast, saline conditions during growth had no detectable effect in the case of cotton, a crop plant which can withstand mild saline stress. An important feature in cotton was the large stimulation of proton pumping produced by supplying K^+ .

d. OBJECTIVES OF THE RESEARCH

Our principal objective was to elucidate the mechanism of the plant's adaptation to saline stress. This we proposed to do -

(a) at the level of cells in suspension culture. We aimed to develop halophytic lines able to grow in high concentrations of NaCl (up to 500 mM). Having achieved this, our plan was to examine their physiological responses to external salinity and to compare them with those of the wild type;

(b) at the level of membrane preparations derived from whole plants. Proton-translocating membrane ATPases (proton pumps) are today believed to play a central role in energizing the flux of solutes into and out cells. They thus play an important part, not only in nutrient uptake, but also in osmotic adaptation; and further in controlling the level of Na^+ , and perhaps also of Cl^- in the plant. One of our objectives was to characterize and compare the proton pump in root membrane preparation from a halophyte (Atriplex) and a glycophytic crop plant (cotton), and in particular to examine whether saline conditions during growth modified the characteristics of the pump in each case.

e. BODY OF REPORT - DESCRIPTION OF THE RESEARCH RESULTS

I. STUDIES OF OSMOTIC ACCOMMODATION TO SALINE STRESS AT CELL LEVEL

A. Development of cell lines in suspension culture resistant to salinity stress

Attention has recently been strongly focused on the possibility of developing lines of cells in suspension culture possessing characters of potential value in agriculture. We have earlier reported the development of tobacco cell lines resistant to saline stress, and studied their K^+ , Na^+ and proline pools as function of external salinity. We have continued these studies in the context of the present project, and have produced a line capable of growing in 500 mM NaCl, a level of resistance higher than any previously reported in the literature. Below we report on the stability of the salt-resistance character.

C. NaCl-induced morphological and ultrastructural changes in the cell lines

Hasegawa, Bressan and their coworkers (Binzel et al., 1985) have reported that adaptation of Nicotiana cells to high NaCl brought about a reduction in cell volume. We have observed the same phenomenon in our laboratory. In the present investigation we have studied the morphological and ultrastructural characteristics of the various cell lines in greater detail, and have further examined the time course of the structural changes that occur when cells are transferred from high to low salt media or vice versa.

MATERIALS AND METHODS

Plant material

Wild type and NaCl adapted cell lines of Nicotiana tabacum/gossii were obtained and grown as described in Watad et al., 1983; 1985. After various treatments cell samples were taken for light and electron microscopical observation.

Light microscopy

1. Samples of cells were stained with Neutral Red and were observed and photographed with a light microscope. From these pictures cell diameter was determined.
2. Sections, 5 μ m thick, of cell samples which were fixed and embedded for electron microscopical observations (as described below) were stained with 1% Toluidine Blue O for 1-2 minutes, washed with distilled water and viewed with a light microscope after applying immersion oil and covering with a cover slip.
3. Samples of cells were stained with Sudan IV and 2% FeCl_3 for detection of lipids and phenols respectively.

Electron microscopy

Samples of cells were fixed in one of the two following ways:

- A) 2.5% glutaraldehyde in phosphate buffer (50 mM; pH 7) for 1 h.
- B) 1.5% glutaraldehyde in MOPS buffer (25 mM, pH 7) for 1 h.

The osmolarity of each sample of fixation fluid was adjusted to that of the growth medium by adding NaCl to the buffer. The cell samples were washed for 1 h. with the appropriately-adjusted buffer; encapsulated in 2% agar; post-fixed in 1% OsO_4 in water; dehydrated in a graded series of acetone; and embedded in epoxy resin (Spurr, 1969). Ultrathin sections were cut on a LKB Ultratome III Ultramicrotome, stained with a saturated solution of uranyl acetate in 50% ethanol for 30-60 minutes and in lead citrate (Reynolds, 1963) for 2-3 minutes, and viewed with a Philips 300 electron microscope.

RESULTS

Shape and size of cells grown in non-saline and saline media

The wild type cells growing in standard medium showed shape polymorphism: spherical, rod, irregular (Fig. 1). Both isolated cells, and cells grouped in clusters of between a few to 50 cells were observed. In contrast cells adapted to grow at 350 mM NaCl and grown continuously at this salt concentration were all spherical and formed larger clusters (up to 100 cells per group) (Fig. 2). When these cells were transferred gradually (during the course of one week) back to non-saline medium and had been growing in standard medium for over 20 generations (adapted cells) they maintained their spherical shape but were larger and formed smaller clusters than when grown in saline media (Fig. 3); isolated cells were also common. Cells growing at higher salt concentrations (up to 500 mM), looked similar to those growing in 350 mM NaCl (Fig. 4).

The volume (as calculated from measured diameters) of 350 mM NaCl adapted cells growing in this salt concentration was about 40% of the volume of the wild type cells growing in standard medium (Table I). When the adapted cells were gradually transferred back to standard medium and grown in absence of salt for 20 generations their volume was about 20% larger than that of wild type cells. Cells adapted to 450 and 500 mM NaCl and grown in these media had a volume about 20% of that of wild type cells in standard medium. The ratio of the fresh weight of the adapted cells in saline media to that of wild type cells growing in standard medium is similar to that obtained for their respective volumes (Table I).

Changes observed under the light microscope after transfer of adapted cells from standard medium to medium containing 300 mM NaCl

Cells adapted to 350 mM NaCl were gradually returned to standard medium and grown in absence of salt for 6 generations (Fig. 5), then transferred directly to medium containing 300 mM NaCl. They were observed at various

Table 1. Fresh weight and dry weight yields, and geometric parameters of wild type and adapted cells at stationary phase. The cells were growing in the medium to which they had been adapted. Growth assays were performed as described previously by Watad *et al.*, 1983, 1985. Cell diameter was that of the spherical cells within the cell population. Cell volume was calculated from the diameter assuming that the cells were spherical.

Parameters	Wild type cells in stand. med.	A350 cells in stand. med.	A350 cells in 350 mM NaCl	A450 cells in 450 mM NaCl	A500 cells in 500 mM NaCl
F.W. Yield (mg·ml ⁻¹ suspension)	420 ± 20	520 ± 30	240 ± 20	150 ± 10	120 ± 10
D.W. Yield (mg·ml ⁻¹ suspension)	9 ± 0.8	11 ± 1	10 ± 1	10 ± 1	10 ± 1
Diam- eter (µm)	96 ± 13	102 ± 20	71 ± 13	58 ± 8	55 ± 7
Volume (mm ³)	4.6·10 ⁻⁴	5.55·10 ⁻⁴	1.8·10 ⁻⁴	10 ⁻⁴	8.5·10 ⁻⁵
Volume ratio <u>Adapted</u> Wild type		1.2	0.4	0.22	0.19
F.W. ratio <u>Adapted</u> Wild type		1.2	0.31	0.25	0.17

time intervals after transfer. Half an hour after transfer about 50% of the cells showed plasmolysis (Fig. 6). One day after transfer most of the cells had deplasmolysed, some cells still showed mild plasmolysis and about 5% of the cells had died (shrunken cytoplasm in mostly empty cells) (Fig. 7). Three days after transfer all living cells had deplasmolysed; their size was similar to that before transfer (Fig. 8). At this stage the cells dry weight had not yet started to divide. Eight days after transfer cell dry weight had doubled and cells about half the size of the cells originally transferred had appeared. At this point cells were transferred to fresh medium containing 300 mM NaCl and a week later (15 days after transfer to salt) all cells were observed to be about half the volume of the cells originally transferred from standard medium (Fig. 9).

Structure of cells as observed under the light and electron microscope

Wild type cells had only a thin layer of peripheral cytoplasm in which few organelles were observed (Fig. 10). The plastids were not well developed; only a few inner membranes were present. Rarely small osmiophilic droplets were observed attached to the inner side of the tonoplast.

Adapted cells grown in standard medium for a long period (50 generations), had a small cytoplasmic volume and undeveloped plastids like the wild type cells (Figs. 11, 12) but did not contain osmiophilic droplets. When these cells were grown in media containing increasing NaCl concentrations the cell size decreased while the ratio cytoplasmic volume to cell volume increased (Fig. 13). More mitochondria and plastids were observed in cells growing in saline media. The plastids were more differentiated (i.e. contained a larger amount of inner membrane and resembled chloroplasts) (Fig. 14).

One of the most striking features in adapted cells growing in NaCl was the presence of large osmiophilic droplets in the vacuole (Fig. 15). Their amount and size rose with increasing external NaCl concentration. At low NaCl concentrations the osmiophilic droplets were usually seen attached to the inner side of the tonoplast, while at high NaCl concentrations there were also large droplets free in the vacuole. Staining with Sudan IV gave negative results under the light microscope while staining with FeCl_3 indicated that cells growing in saline media contained phenols whereas cells grown in standard medium did not. Many particles staining positively with Toluidene Blue were observed in cells growing in the presence of NaCl while cells growing in standard medium did not (data not shown).

Ultrastructural changes during and after gradual transfer of cells from 350 mM NaCl to standard medium

Cells adapted to and growing in 350 mM NaCl were transferred every 6 h. to media containing 50 mM less NaCl until they reached standard medium. During the two days that the transfers lasted the cells retained their characteristic features, i.e., developed pastids, numerous mitochondria and osmiophilic droplets in the vacuole (Fig. 16). Figs 17 and 18 show that the cells retained the Toluidine Blue-positive particles even after reaching 0 NaCl. After growing in standard medium for three weeks the cells had lost these characteristics and had regained the characteristics of cells grown in standard medium (Fig. 19).

Ultrastructural changes in cells transferred directly from standard medium to media containing various NaCl concentrations

Cells adapted to 350 mM NaCl but growing in standard medium were inoculated directly into media containing 100 or 200 mM NaCl. One week after transfer the cells had developed the same characteristics as those which had been growing continuously in high NaCl concentrations (Fig. 22). However, one week after transfer of wild type cells to saline media such development was not observed, and a marked disruption of internal mitochondrial membranes had occurred (compare Figs. 20, 23 to Figs. 21, 24).

DISCUSSION

There are multiple structural differences between the wild type cells and cells adapted to high NaCl concentration. The wild type cells are polymorphic while the adapted cells when grown in saline media are all similar in shape, spherical, and are smaller. Adapted cells transferred back to non-saline medium were larger than wild type cells in standard medium and retained their spherical shape even after growing for many generations in absence of salt.

The small volume of adapted cells grown in NaCl-containing medium was earlier reported by previous authors (Dix and Street, 1975; Binzel et al., 1985); however, Dix and Street (1975) stated that adapted cells transferred back to non-saline medium regained the wild type shape.

The fact that in our study adapted cells brought back to standard medium kept their spherical shape indefinitely and did not revert to the polymorphic forms of the wild type, and further, that these cells were some 20% larger than the spherical cells present in the wild type population show that a permanent change occurred during adaptation.

Physiological (Watah et al., 1983; 1985; 1986) as well as morphological differences have been observed between the adapted and wild type cells including differences in size of proline pool as a function of external salinity, and growth rate in standard medium. Saline stress does not merely select the spherical wild type cells but somehow modifies them probably through modification of gene expression. Walbot and Cullis (1985) have reviewed the literature on rapid genomic changes (which are transferred to the following generation) as a response to environmental stress.

Adapted cells growing in saline media developed a slight green color due to chlorophyll synthesis (data not shown). With increasing salinity, plastids were more developed, progressively displaying inner membranes typical of chloroplasts, though not to the same extent as the latter. The increased number of mitochondria, RER cisternae, and ribosomes observed may indicate a higher rate of metabolism.

The intense staining by FeCl_3 of adapted cells grown in NaCl together with the presence of osmiophillic droplets in the vacuole suggests that these droplets may be phenols (Hayat, 1981). Walker et al. (1984) suggested that granular electron dense deposits in the vacuole of salt stressed citrus root cells were due to the presence of phenols. However, while in the latter case phenol accumulation was thought to be due to damage it is not evident in our case, as vigorously growing cell cultures could be maintained indefinitely (at least 3 years) in salt media.

Cells in our investigation are brought down from 350 mM to standard medium stepwise to prevent osmotic shock, a process taking about 2 days. Even though the cells are in non-saline medium they maintain the typical appearance of cells grown continuously in high salt medium. Only when cell divisions occur do the daughter cells take on the appearance of cells growing continuously in non-saline medium.

When adapted cells growing in non-saline media are transferred in one step to high salt, they plasmolyze. After three days they have deplasmolyzed (i.e. they are capable of osmotic adaptation) while maintaining the large size typical of adapted cells growing in standard medium. Only after cell division are the daughter cells smaller, and have the typical appearance of cells growing continuously in high salt medium.

When wild type cells were observed under the electron microscope one week after direct transfer to 100 or 200 mM NaCl, the main change apparent was the disintegration of the internal mitochondrial membranes (Figs. 20, 23). In adapted cells treated in the same fashion the mitochondria looked normal (Figs. 21, 24). Similar destruction of internal mitochondrial membranes was observed by Smith et al. (1982) in roots of the inland ecotype of Agrostis stolonifera when grown for 2 weeks in 100 mM NaCl. The salt marsh ecotype of this plant showed normal mitochondria when subjected to the same conditions. Flowers et al. (1985) also reported that under saline conditions there was disorganization of leaf cellular structure including disintegration of the internal membranes of the mitochondria in the Amber variety of rice. However, the elite breeding line IR2153.26.3.5.2 showed normal mitochondria under similar conditions. It would seem that in the Nicotiana wild type cell-line we have been studying and in the roots of the inland ecotype of A. stolonifera as well as in leaf cells of the Amber rice variety the internal membranes of the mitochondria are sensitive to saline stress.

Interestingly Pomeroy (1977) showed that mitochondria isolated from cold stressed wheat seedlings swelled upon addition of KCl but did not contract upon further addition of succinate; mitochondria from non-cold stressed wheat seedlings contracted upon addition of succinate after that of KCl. Observations under the electron microscope showed that addition of KCl (swelling), caused a disorganization of the internal membranes of the mitochondria; however, only in the case of the non-cold stressed wheat mitochondria did addition of succinate cause reorganization of the internal membranes (contraction). These data may be relevant to the effect of NaCl on Nicotiana wild type cell mitochondria since they show, first, that disorganization of the internal mitochondrial membranes is reversible, and second, that reversibility is dependent on the mitochondrion's capacity to cope with a saline environment.

Compounds such as proline and betaine have been reported to accumulate in plants growing under saline conditions (Wyn-Jones and Gorham, 1983). While these substances probably play a role in osmoregulation and protection of

proteins and membranes, it has been shown for higher plant enzymes that amino acids, sugars or polyols could probably also fulfill this role (Lerner et al., 1980; Paleg et al., 1981; Nash et al., 1982; Lerner, 1985).

Interestingly some bacteria specifically use proline, betaine and their derivatives in order to osmoregulate when grown under saline conditions (Csonka, 1981; Le Rudulier et al., 1984). In view of the analogy between mitochondria and bacteria, it seems possible that the specific role of proline accumulation in adapted cells of Nicotiana might be to provide an osmoticum for the mitochondria.

Mitochondria require a compatible cytoplasm in order to survive. One may speculate that in the cell line we have been studying salinity modifies the cytoplasmic environment in such a way as to make it incompatible with the normal operation of the mitochondria. However, when the Nicotiana wild type cell-line is exposed to progressively increasing concentration of NaCl a rapid change in gene expression is induced which gradually modifies either the cytoplasmic environment or perhaps the mitochondria and restores compatibility between the cytoplasm and the mitochondria.

It is today accepted that cytoplasmic male sterility, cms, is due to a cytoplasm-mitochondrial incompatibility in the anther during pollen formation leading to abortion. In Tcms corn Warnike and Lee (1977) showed that disorganization of internal membranes of the mitochondria of the tapetum cells in sterile anthers is the first indication of abnormality and may initiate the events leading to pollen abortion. One might suggest that in principle the same kind of phenomenon is occurring during abortion in cms and in Nicotiana wild type cells exposed to salinity: a change in cytoplasmic physiology leads to incompatibility between the cytoplasm^{m)} and the mitochondria.

LITERATURE CITED

- Binzel, M.L., Hasegawa, P.M., Handa, A.K. and Bressan, R.A. 1985
Adaptation of tobacco cells to NaCl. *Plant Physiol.* 79: 118-125.
- Csonka, L.N. 1981 Proline over-production results in enhanced osmo-
tolerance in Salmonella typhimurium. *Molec. Gen. Genet.* 182: 82-86.
- Dix, P.J., Street, H.E. 1975 Sodium chloride-resistant cultured cell
lines from Nicotiana sylvestris and Capsicum annuum. *Plant Sci.*
Lett. 5: 231-237.
- Flowers, T.J., Duque, E., Hajibagheri, M.A., McGonigle, T.P. and Yeo, A.R.
1985 The effect of salinity on leaf ultrastructure and net photo-
synthesis of two varieties of rice: Further evidence for a cellular
component of salt-resistance. *New Phytol.* 100: 37-43.
- Hayat, M.A. 1981 Principles and Techniques of Electron Microscopy:
Biological Applications, 522 pp. 2nd ed. London, Edward Arnold.
- Lerner, H.R., 1985 Adaptation to salinity at the plant cell level.
Plant Soil. 89: 3-14.
- Lerner, H.R., Sussman, I. and Avron, M. 1980 Characterization and
partial purification of dihydroxyacetone kinase in *Dunaliella*
salina. *Biochim. Biophys. Acta.* 615: 1-9.
- Le Rudulier, D., Strom, A.R., Dandekar, A.M., Smith, L.T. and Valentine, R.C.
1984 Molecular biology of osmoregulation. *Science.* 224: 1064-1068.
- Nash, D., Paleg, L.G. and Wiskich, J.T. 1982 Effect of proline, betaine and
some other solutes on the heat stability of mitochondrial enzymes.
Aust. J. Plant Physiol. 9:47-57.

- Paleg, L.G., Douglas, T.J., van Dall, A. and Keech, D.B. 1981 Proline, betaine and other organic solutes protect enzymes against heat inactivation. Aust. J. Physiol. 8: 107-114.
- Pomeroy, M.K. 1977 Ultrastructural changes during swelling and contraction of mitochondria from cold-hardened and nonhardened winter wheat. Plant physiol. 59: 250-255.
- Reynolds, E.S. 1963 The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.
- Smith, M.M., Hodson, M.J., Opik, H. and Wainwright, S.J. 1982 Salt-induced ultrastructural damage to mitochondria in root tips of a salt-sensitive ecotype of Agrostis stolonifera. J. Exp. Bot. 33: 886-895.
- Spurr, A.R. 1969 A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.
- Walbot, V. and Cullis, C.A. 1985 Rapid genomic changes in higher plants. Ann. Rev. Plant Physiol. 36: 367-396.
- Walker, R.R., Sedgley, M., Blesing, M.A. and Douglas, T.J. 1984 Anatomy, ultrastructure and assimilate concentrations of roots of citrus genotypes differing in ability for salt exclusion. J. Exp. Bot. 35: 1481-1494.
- Warmke, H.E. and Lee, S.-L.J. 1977 Mitochondrial degeneration in Texas cytoplasmic male-sterile corn anthers. J. Hend. 68: 213-222.
- Watad, A.A., Reinhold, L. and Lerner, H.R. 1983 Comparison between a stable NaCl-selected Nicotiana cell line and the wild type. Plant Physiol. 73: 624-629.

- Watad, A.A., Lerner, H.R. and Reinhold, L. 1985 Stability of the salt-resistance character in Nicotiana cell lines adapted to grow in high NaCl concentrations. *Physiol. Veg.* 23: 887-894.
- Watad, A.A., Pesci, P.A. Reinhold, L. and Lerner, H.R. 1986 Proton fluxes as a response to external salinity in wild type and NaCl-adapted Nicotiana cell lines. *Plant Physiol.* 81: 454-459.
- Wyn Jones, R.G. and Gorham, J. 1983 Osmoregulation. In *Encyclopedia of Plant Physiology, New Series, Vol. 12C*. Eds. O.L. Lange, P.S. Nobel, C.B. Osmond and H. Ziegler, pp. 35-58, Springer-Verlag, Berlin.

FIGURE LEGENDS

Abbreviations for Figures 1-24:

Cy	- cytoplasm	E.M.	- electron micrograph(s)
L.M.	- light micrograph(s)	M	- mitochondrion
OD	- osmiophilic droplet	P	- plastid
v	- vacuole	w	- cell wall
W.T.	- Wild type		

Fig. 1 L.M. of W.T. cells of Nicotiana tabacum/gossii growing in standard medium X 110.

Fig. 2 L.M. of cells adapted to 350 mM NaCl growing at this concentration. X 110.

Fig. 3 L.M. of cells adapted to 350 mM NaCl growing for 20 generations in standard medium. X 110.

Fig. 4 L.M. of cells adapted to 450 mM NaCl growing at this concentration. X 110.

Figs. 5-9 L.M. showing the changes with time of 350 mM-adapted cells which had been growing in standard medium for 6 generations and were then transferred in one step to 300 mM NaCl. X 110.

Fig. 5. Cells in standard medium before transfer.

Fig. 6. Cells 30 min after transfer to 300 mM NaCl.
Arrowheads indicate plasmolyzed cells.

Fig. 7. Cells 1 day after transfer. Most cells are deplasmolyzed; some cells are apparently dead (arrowheads).

Fig. 8. Cells 3 days after transfer. Cells are the same size as before transfer (compare with 5).

Fig. 9. Cells 15 days after transfer. After cell division occurs, cell size is smaller and cells appear in large clusters.

Figs. 10-15 E.M. of wild type and adapted cells.

Fig. 10. Part of a wild type cell growing in standard medium. Note typically sparse cytoplasmic content. X 10,000.

Fig. 11. Part of a cell adapted to 350 mM NaCl and growing in standard medium for 50 generations. X 1000. Large vacuole and peripheral cytoplasm are seen.

Fig. 12. The same cell as in Fig. 11, showing typical undeveloped plastids. X 30,000.

Fig. 13. Cells adapted to 500 mM NaCl and growing in this salt concentration have dense cytoplasm which occupies a larger portion of the cell volume as compared with cells growing in standard medium. X 3,000.

Fig. 14. Plastid of a 500 mM adapted cell growing at this salt concentration. The plastid is much more differentiated than that seen in Fig. 12. X 38,000.

Fig. 15. Part of a 400 mM NaCl adapted cell growing at this salt concentration. Typical large osmophilic droplets are seen in the vacuole. X 2500.

Figs. 16-19 L.M. and E.M. micrographs showing 350 mM-adapted cells at various stages during transfer from 350 mM NaCl to standard medium. (Cells were transferred to media successively lower in NaCl concentration by 50 mM every 6 h.).

Fig. 16. E.M. of cells growing for 6 h. in 50 mM NaCl.
X 6500.

Fig. 17. L.M. of sections through cells growing for 6 h. in 100 mM NaCl stained with Toluidine Blue O. X 100.

Fig. 18. L.M. of sections through cells growing for 6 h. in 0 mM NaCl stained with Toluidine Blue O. X 180. Cells had not yet divided and still had the same appearance as adapted cells growing in high-salt medium.

Fig. 19. E.M. of part of a cell growing in standard medium for 9 generations. X 26,000.

Figs. 20-24 E.M. micrographs showing cells which had been transferred in one step from standard medium to either 100 or 200 mM NaCl.

Fig. 20. Damaged mitochondria of wild type cells 7 days after transfer to 100 mM NaCl. X 31,500.

Fig. 21. Intact mitochondria in 350 mM-adapted cells which had grown in standard medium for 3 generations, 7 days after transfer back to 100 mM NaCl. X 31,500.

Fig. 22. Part of an adapted cell 7 days after transfer from standard medium to medium containing 200 mM NaCl. X 4500.

Fig. 23. As in Fig. 20 but after transfer to 200 mM NaCl. X 31,500.

Fig. 24. As in Fig. 21 but after transfer to 200 mM NaCl. X 31,500.

II. STUDY OF H^+ -TRANSLOCATING ATPASES IN ROOT MEMBRANE VESICLES

Improvement of our understanding of the mechanism of osmoregulation depends upon our understanding of the mechanism, energisation and control of solute transport into and out of the cell. The metabolic energy for solute transport must be supplied, directly or indirectly, via ATP or a similar energy-rich molecule. According to widely accepted views today a primary membrane pump of central importance in the cell is an H^+ -translocating ATPase ("proton pump") which moves protons against their electrochemical potential gradient and thus generates a protonmotive force which serves as an immediate source of energy for the transmembrane flux of a variety of other solutes. The latter flux may be mediated by proton symport or antiport mechanisms, or by uniport driven by the electric component of the electro-chemical proton gradient.

The proton pump is likely to play an important role in regulating the ion fluxes into and out of the cell which accompany adaptation to saline or osmotic stress, and we have been investigating H^+ -translocating ATPases in plants of varying resistance to salinity. Our studies are being carried out on isolated membrane vesicles. The major advantages of carrying out membrane transport studies at the level of membrane vesicles include the fact that transport can be studied in isolation from metabolism; and that the serious complications arising out of the continuous intracellular transfer of solutes between cytoplasmic and vacuolar compartments are avoided.

A. A critical comparison of techniques for isolating and purifying plant membrane vesicles (Purdue Laboratory)

The U.S. partner pioneered the isolation and characterization of plant membrane ATPases. He has now carried out a careful comparison of his original (and still widely used) technique based on separation of membrane fractions on a sucrose gradient, and a more recent technique based on two-phase separation.

[2] Isolation of the Plasma Membrane

By THOMAS K. HODGES and DAVID MILLS

Plasma membranes of plants have been purified primarily by using either sucrose gradients¹⁻⁴ or an aqueous two-phase system consisting of Dextran T 500 and polyethyleneglycol 3350.⁵⁻⁸ The sucrose gradient separation takes advantage of the different sizes and densities of the various membranes in the cell, whereas, the two-phase polymer system takes advantage of the different surface charges of membranes. Use of sucrose gradients for isolating plant plasma membranes has been in use since 1972,¹ whereas use of the two-phase system for isolating the plant plasma membrane was first reported in 1981.⁵ A thorough comparison of the two procedures in the same study has not been made, thus it is difficult to assess which is superior. However, the sucrose gradient procedure appears to purify plasma membrane vesicles that are a mixture of right-side-out and inside-out vesicles that are mostly permeable to ions and Mg-ATP^{9,10} whereas, the two-phase system appears to provide primarily a purification of right-side-out plasma membrane vesicles that are impermeable to Mg-ATP¹¹. Since the purification procedure of choice may depend upon the planned use of the membranes, both procedures will be described.

One of the long-term problems in isolating plasma membranes from plants is that it still appears to be impossible to achieve extremely pure preparations; claims vary from 75 to 95% purity. In efforts to improve the purification, other techniques have been tried in association with the procedures mentioned above such as surface labeling of the plasma mem-

¹ T. K. Hodges, R. T. Leonard, C. E. Bracker, and T. W. Kennan, *Proc. Natl. Acad. Sci. U.S.A.* 69, 3307 (1972).

² R. L. Anderson and P. M. Ray, *Plant Physiol.* 61, 723 (1978).

³ G. Nagahashi, R. T. Leonard, and W. W. Thomson, *Plant Physiol.* 6, 993 (1978).

⁴ W. S. Pierce and D. L. Hendrix, *Planta* 146, 161 (1979).

⁵ S. Widell and C. Larsson, *Physiol. Plant.* 51, 368 (1981).

⁶ T. Lundborg, S. Widell, and C. Larsson, *Physiol. Plant.* 52, 89 (1981).

⁷ S. Yoshida, M. Uemura, T. Niki, A. Sakai, and L. V. Gusta, *Plant Physiol.* 72, 105 (1983).

⁸ M. Uemura and S. Yoshida, *Plant Physiol.* 73, 586 (1983).

⁹ H. Sze and T. K. Hodges, *Plant Physiol.* 58, 304 (1976).

¹⁰ J. Cambrina and T. K. Hodges, in "Plant Membrane Transport: Current Conceptual Issues" (R. M. Spanswick and W. J. Lucas, eds.), p. 211. Elsevier/North-Holland Biomedical Press, Amsterdam, 1980.

¹¹ C. Larsson, P. Kjellbom, S. Widell, and T. Lundborg, *FEBS Lett.* 171, 271 (1984).

brane with radioactive iodine,¹² lectins,^{13,14} or silica particles in combination with polyanions.¹⁵ Although still promising, these additions have not substantially improved the purification. Other techniques such as isoelectric focusing of plant membranes,¹⁶ or free-flow electrophoresis, which has been useful in isolating various animal membranes,^{17,18} have not been evaluated thoroughly for separating plant membranes.

Purification Procedures

Sucrose Gradient. Figure 1 is a diagrammatic sketch of the steps involved in purifying the plant plasma membrane using either the sucrose gradient procedure or the phase system. For both procedures the initial steps are the same.¹⁹ Plant tissue is homogenized in an ice cold grinding medium consisting of 0.25 M sucrose, 3 mM EDTA, 2.5 mM dithiothreitol (DTT), 25 mM Tris-MES, pH 7.2. Commonly 4 ml/g of tissue is used. All steps of the isolation procedures are carried out at 2–4°. The tissue is homogenized using either a mortar and pestle, by chopping with a razor blade, or by some mechanical chopping device. The mortar should be smooth surfaced and no abrasives should be added. The amount of tissue frequently used varies from 5 to 50 g fresh weight. The homogenate is strained through 4 layers of cheesecloth or through miracloth and then centrifuged at 13,000 g for 15 min. The supernatant is then centrifuged at 80,000 g for 60 min. The pelleted membranes (microsomes) are suspended in 3–6 ml of 0.25 M sucrose and 5 mM KPi buffer, pH 7.8 initially with a smooth glass rod and finally by repeated pipetting through a small bore 1-ml pipette. The composition of the suspending medium can vary depending on the intended use of the membranes. The microsomal membranes are carefully layered onto a previously prepared gradient of sucrose solutions. We use solutions consisting of 20, 30, 34, and 45% (w/w) sucrose containing 1 mM MgSO₄, 2.5 mM DTT, and 1 mM Tris-MES, pH 7.2. The gradient containing the membranes is centrifuged at 95,000 g for 2 hr. In our laboratory, we use a Beckman Type 27 or 28 rotor for this centrifugation step. Plasma membranes are concentrated at the 34/45% interface. Sucrose and membranes above this interface are removed by aspiration,

¹² P. H. Quail and A. Browning, *Plant Physiol.* **59**, 759 (1977).

¹³ W. F. Boss and A. W. Ruesink, *Plant Physiol.* **64**, 1005 (1979).

¹⁴ R. L. Travis and R. L. Berkowitz, *Plant Physiol.* **65**, 871 (1980).

¹⁵ L. K. Chaney and B. S. Jacobson, *J. Biol. Chem.* **258**, 10062 (1983).

¹⁶ L. R. Griffing and R. S. Quatrano, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4804 (1984).

¹⁷ K. Hannig and H. G. Heidrich, this series, Vol. 31, p. 746.

¹⁸ S. Menashi, H. Weintroub, and N. Crawford, *J. Biol. Chem.* **256**, 4095 (1981).

¹⁹ T. K. Hodges and R. T. Leonard, this series, Vol. 32, p. 392.

FIG. 1. Flow chart depicting the steps involved in the purification of plasma membranes using either a sucrose gradient or an aqueous two-phase separation. See text for details.

and, using a pasteur pipette that is bent 90° at the tip, the plasma membranes are removed and kept on ice until ready to be used.

Phase System. The phase-separation procedure involves mixing the microsomal membranes with a mixture of Dextran T 500 and polyethyleneglycol 3350 (PEG) containing NaCl. This procedure was first developed by Albertsson,²⁰ and it takes advantage of the different surface

²⁰ P. A. Albertsson, *Biochim. Biophys. Acta* 27, 328 (1958).

TABLE I
STOCK SOLUTIONS AND PROCEDURE FOR PREPARING TWO-PHASE SEPARATION SYSTEM

Stock solutions	Desired final concentrations (% w/w) of dextran and PEG (g)					
	6.0	6.1	6.2	6.3	6.4	6.5
1. 20% (w/w) Dextran T 500	1.20	1.22	1.24	1.26	1.28	1.30
2. 40% (w/w) PEG 3350	0.60	0.61	0.62	0.63	0.64	0.65
3. 1 M sucrose in 20 mM K ₂ P ₄ buffer, pH 7.8 ^a			Add 0.75 ml			
4. 0.464 g NaCl/10 ml			Add 0.10 ml (4.64 mg of NaCl) ^b			
5. Membrane fraction ^c			Add 1.0 ml and weigh			
6. Water			Bring to 4.0 g			

^a Final concentrations of sucrose and K₂P₄ are to be 0.25 M and 5 mM, respectively.

^b Other NaCl concentrations to test are shown in Fig. 4b and d.

^c Membranes are suspended in 0.25 M sucrose and 5 mM K₂P₄, pH 7.8.

charges of membranes. Since plasma membranes of different species, and probably different tissues, possess different surface charges, it is first necessary to evaluate a series of polymer and salt solutions for each species or tissue studied.⁵⁻⁸ For an excellent discussion of the theoretical aspects of phase partitioning of membranes, consult the review of Albertsson *et al.*²¹ The stock solutions required for evaluating the phase separation procedure are shown in Table I. It is important to prepare these solutions very carefully, and since the polymers vary from batch to batch they need to be tested for their effectiveness each time they are purchased.²² Larsson²² even suggests that the water content of the dextran should be determined, however, in our use of the phase system, satisfactory results were obtained by assuming the water content of Dextran T 500 to be 7.5%. The range of polymer concentrations to test will normally be from about 5.5 to 7.0% (w/w) for each polymer, and the NaCl concentrations will range from 0 to 40 mM. Four stock solutions plus water and membranes are required (Table I). It is important to prepare the final experimental solutions by weighing them. Thus, to prepare an experimental solution of 6.3% dextran and 6.3% PEG, one would add 1.26 g of a 20% solution of Dextran T 500 (Pharmacia), 0.63 g of a 40% solution of PEG 3350 (Sigma, average MW 3350), 0.75 ml of 1 M sucrose in 20 mM

²¹ P. A. Albertsson, B. Anderson, C. Larsson, and H. E. Akerlund, *Methods Biochem. Anal.* **28**, 115 (1982).

²² C. Larsson, in "Moderne Methoden der Pflanzenanalyse" (H. F. Linskens and J. F. Jackson, eds.), New Ser. Springer-Verlag, Berlin and New York, (in press).

KPi , pH 7.8, 0.10 ml of 0.464 g $\text{NaCl}/10$ ml, and 1.0 ml of membranes in 0.25 M sucrose and 5 mM KPi , pH 7.8. This mixture is then weighed and brought to 4.0 g with water.

The phase system containing the membranes is inverted and returned upright 20 times and then centrifuged at 1000 g for 5 min in a swinging bucket rotor (Fig. 1). The upper (U_1) and lower (L_1) can then be treated as follows: (1) the phases can be repartitioned or washed (Fig. 2) which yields further purification, or (2) the two phases can be removed separately as depicted in Fig. 1, diluted with grinding medium, and centrifuged at 150,000 g for 60 min. If the second option is followed the membrane pellets are resuspended in 1–3 ml grinding medium and used for enzyme assays or other characterizations.

In most situations step 1 would be followed and the U_1 and L_1 phases would be washed to purify the membranes further and these steps are shown in Fig. 2. Wash phases (Fig. 2) are prepared in centrifuge tubes in exactly the same manner as already described in Table I, but with 1.0 ml of 0.25 M sucrose and 5 mM KPi buffer, pH 7.8, in place of the 1.0 ml of membranes. About 90% of U_1 phase (primarily PEG) with plasma membranes is removed with a Pasteur pipette, taking care not to disturb the material at the interphase. This material is placed on a bottom wash phase that has had 90% of the top PEG phase removed. This top wash phase is added to everything remaining in the original tube, i.e., that part of U_1 phase not removed, plus the interface, and L_1 . It is very important that

FIG. 2. Steps involved in the washing or repartitioning of the initial upper (U_1) and lower (L_1) phases of the phase-separation system. Wash phases are in centrifuge tubes containing all the same solutions and treated in the same way as the experimental tubes, but with no membranes. Membranes can be pelleted from the phases by dilution and centrifugation as shown in Fig. 1, or the wash can be repeated.

the original 4 g weight be maintained in each of the subsequent washes. The washed U₂ and L₂ phases can be washed any number of times following the procedure just described. When the washings are completed both phases are diluted 10-fold with grinding medium, and membranes are sedimented as already described (Fig. 1).

Once the optimum concentrations of dextran, PEG, and NaCl have been determined for a particular tissue, large volumes of washing phases can be prepared that contain the proper concentrations of these components rather than preparing individual wash phases in centrifuge tubes as shown in Fig. 2. However, if this is done, it is important to follow the procedure in Table I for the first partitioning so that the desired dextran and PEG concentrations are achieved. After the first partitioning one can use the bulk washing solutions. These bulk wash solutions are prepared by weighing the desired amounts of the primary stock solutions (Table I) of PEG, dextran, and NaCl and adding them to a separatory funnel. Sucrose, KPi buffer, and water are added to give the final weight desired. After temperature equilibration (4°), the solutions are mixed and allowed to separate overnight. The two phases are separated with care being taken to avoid the interface. It is much more convenient to have these wash solutions available in bulk than to have to prepare them separately in centrifuge tubes for each experiment.

Membrane Markers

In order to assess the purity of a plasma membranes preparation one must assess the enrichment of plasma membrane markers as well as the contamination by other membranes. The following markers have been used to follow the various membranes of plant cells during purification attempts:

Plasma membranes, K⁺-stimulated ATPase,¹ vanadate inhibited^{23,24}
phosphotungstic acid/chromic acid stain^{1,25}
NPA binding²⁶
UDPG-sterol glucosyltransferase²⁷
glucan synthetase II^{1,28}

²³ B. J. Bowman and C. W. Slayman, *J. Biol. Chem.* 254, 2928 (1979).

²⁴ S. R. Gallagher and R. T. Leonard, *Plant Physiol.* 70, 1335 (1982).

²⁵ J.-C. Roland, in "Electron Microscopy and Cytochemistry of Plant Cells" (J. L. Hall, ed.), pp. 1-2. Elsevier/North Holland, Amsterdam, 1978.

²⁶ C. A. Lembi, D. J. Morré, K. St.-Thomson, and R. Hertel, *Planta* 99, 37 (1971).

²⁷ A. Chanson, E. McNaughton, and L. Taiz, *Plant Physiol.* 76, 498 (1984).

²⁸ P. M. Ray, in "Plant Organelles" (E. Reid, ed.), pp. 135-146. Ellis Horwood, Chichester, 1979.

Mitochondria, cytochrome *c* oxidase^{1,19}

Endoplasmic reticulum, NADH cytochrome *c* reductase, antimycin A insensitive^{19,29}

Golgi vesicles, IDPase^{1,30}

glucan synthetase I^{1,30}

Tonoplast, NO₃⁻-inhibited ATPase^{31,32} (still uncertain)

None of the plasma membrane markers, when used alone, is totally satisfactory. The one marker that is used most commonly is the K⁺-stimulated, vanadate-inhibited, ATPase. However, there are K⁺-stimulated ATPases associated with other plant cell membranes,³³ and the specificity of vanadate for the plasma membrane ATPase is not absolute.³⁴ The NO₃⁻-inhibited ATPase is now used almost routinely as a marker for the tonoplast,³⁵ however, the evidence that it is ubiquitous on and restricted to the tonoplast membrane is lacking. Thus, conclusions about the distribution of the tonoplast in a purification procedure based upon this enzyme activity are useful, but must still be considered as tentative. In practice, a test for the presence of several of the markers is recommended, especially if one is attempting to isolate plasma membranes from a tissue that has not been studied previously.

Critique of the Sucrose Gradient and Phase Separation Procedures

Most studies of the plant plasma membrane have been carried out using the discontinuous sucrose gradient originally described by Hodges *et al.*¹ or a slight modification (see Fig. 1). The modifications usually involved changing the sucrose concentrations because the density of cellular membranes varies slightly from tissue to tissue. An example of this is shown in Fig. 3 which illustrates the densities of Golgi vesicles and plasma membrane vesicles from corn and oat roots.³⁶ From these data it is obvious that it is much more difficult to obtain a pure preparation of plasma membranes from corn roots than from oat roots because of the overlapping densities of the Golgi and plasma membranes of corn roots.

²⁹ J. M. Lord, T. Kagawa, T. S. Moore, and H. Beevers, *J. Cell Biol.* 57, 659 (1973).

³⁰ P. M. Ray, T. L. Shining, and M. M. Ray, *Proc. Natl. Acad. Sci. U.S.A.* 64, 605 (1969).

³¹ R. R. Walker and R. A. Leigh, *Planta* 153, 140 (1981).

³² B. Marin, H. Crtin, and J. D'Auzac, *Physiol. Veg.* 20, 333 (1982).

³³ T. K. Hodges, in "Transport in Plants" (U. Lttge and M. G. Pitman, eds.), Vol. 2, Part A, p. 260, Springer-Verlag, Berlin and New York, 1976.

³⁴ D. R. McCarty, K. Keegstra, and B. R. Selman, *Plant Physiol.* 76, 584 (1984).

³⁵ S. D. O'Neill, A. B. Bennett, and R. M. Spanswick, *Plant Physiol.* 72, 837 (1983).

³⁶ R. T. Leonard, in "Advances in Plant Nutrition" (P. B. Tinker and A. Lauchli, eds.), Vol. 1, p. 209, Praeger, New York, 1984.

FIG. 3. Distribution of membrane associated ATPase and IDPase activities on continuous sucrose gradients. Membranes were obtained from corn and oat roots.¹⁶ Reprinted by permission of Praeger Publishers.

The other major contaminant that must be reckoned with in purifying plasma membranes, when using sucrose gradients, is mitochondria.¹ Since mitochondria possess densities only slightly higher than plasma membranes (about 1.18–1.22 as compared to 1.14–1.17 g/cm³, respectively), one must remove the mitochondria by differential centrifugation prior to sucrose gradient separation.¹⁹ However, even the combination of differential and gradient centrifugation is less than 100% effective in eliminating mitochondria from the plasma membrane fraction.

The phase-separation procedure appears to be quite effective in the preparation of plant plasma membranes,⁵⁻⁸ however, there are no reports where this procedure has been compared directly to the sucrose gradient procedure. For this reason we have compared both procedures in terms of certain marker enzyme activities of the purified plasma membrane fractions. First, however, it is necessary to illustrate the importance of evaluating the polymer and NaCl concentrations used in the phase-separation procedure. Figure 4a and b shows the effect of polymer concentrations (both dextran and PEG) and NaCl concentrations, respectively, on the percentages of protein and various marker enzymes in the upper (U₁) phase of the two-phase system using a microsomal preparation of oat root membranes. Figure 4c and d shows the results of a similar experiment using a microsomal preparation of Atriplex roots. With just the polymers and no salt (Fig. 4a and c) very little purification occurred with oat membranes, but substantial purification occurred with Atriplex membranes. At polymer concentrations of 6.3%, increasing salt concentrations resulted in a marked reduction in protein and enzyme activity in the U₁ phase for both the oat and Atriplex preparations, however, higher concentrations of salt were required for oats than for Atriplex. For membrane preparations of both species, mitochondrial membranes were nearly completely removed from the U₁ phase. With Atriplex, the endoplasmic reticulum was also nearly all transferred to the lower phase, but this was not so for oats. Golgi vesicles (IDPase) were substantial contaminants of the U₁ phase in membrane preparations of both species (Fig. 4b and d). Most of these contaminations can be reduced however by repeated washes.⁵⁻⁸ In oats, two washes removed most of the IDPase (see U₁ in Table II), but in Atriplex, about 6% of the activity initially in the microsome fraction remained after two washes (data not shown). The important point here is that when using the phase separation procedure, one must carefully determine the proper polymer and salt concentrations for each tissue studied.²²

Table II shows a comparison of the enzyme activities of microsomal membranes of oat roots purified using both the sucrose gradient and phase-separation procedures. Specific activity of K⁺-ATPase is routinely enriched 2- to 3-fold over the microsome fraction when using sucrose

FIG. 4. Separation of membrane marker enzymes from oat roots (a and b) and Atriplex roots (c and d) using the phase-separation procedure. (a, c) Effect of polymer (both PEG and dextran) concentrations on the percentage of enzymes in the upper phase after the first separation, i.e., U_1 . (b, d) Effect of NaCl concentrations, keeping the PEG and dextran concentrations constant at 6.3%, on the percentage of enzymes in the upper phase after the first separation, i.e., U_1 .

FIG. 4. (continued)

gradients. Curiously, a similar enrichment of ATPase was not observed using the phase separation that involved 3 washes (U_3). Total ATPase activity in U_3 was less than one-half that found in the sucrose gradient. A similar lack of enrichment of the ATPase was observed by Uemura and Yoshida.⁸ It should be noted that the sum of U_3 and L_3 should not add to

TABLE II
PROTEIN AND MARKER ENZYME ACTIVITIES OF MICROsome FRACTION, 34/45% SUCROSE INTERFACE FRACTION, AND LOWER (L_1) AND UPPER (U_1) PHASES OF PHASE PARTITION OF Os ROOT CELL MEMBRANES*

Isolation procedure	Fraction	Protein (mg)	K^+ -ATPase		IDPase		Cytochrome oxidase		NADH cytochrome c reductase, antimycin A resistant	
			SA	Total	SA	Total	SA	Total	SA	Total
Sucrose gradient Phase	Microsome	4.488 (100)	0.212	0.950 (100)	0.082	0.367 (100)	0.048	0.215 (100)	0.210	0.942 (100)
	34/45%	0.194 (4)	0.627	0.122 (13)	0.005	0.001 (0.3)	0.062	0.012 (6)	0.029	0.006 (1)
	U_1	0.241 (5)	0.190	0.045 (5)	0.005	0.002 (0.5)	0.006	0.001 (1)	0.009	0.002 (0.2)
	L_1	2.655 (59)	0.157	0.403 (42)	0.185	0.492 (100)	0.061	0.162 (75)	0.172	0.458 (49)

* Sucrose gradient was performed as described here and the phase partition system contained 6.3% PEG and dextran and 4.64 mg NaCl. Specific activities (SA) are in $\mu\text{mol mg}^{-1} \text{min}^{-1}$ and total activities are in $\mu\text{mol min}^{-1}$.

TABLE III
EFFECT OF 0.15% TRITON^a

Isolation procedure	Fraction	ATPase activity, $\mu\text{mol mg}^{-1} \text{min}^{-1}$			
		-Triton		+Triton	
		MgSO ₄	KCl	MgSO ₄	KCl
—	Microsome	0.313	0.175	0.523	0.253
Sucrose gradient	34/45%	0.507	0.322	1.283	0.495
Phase partition	U ₂	0.490	0.238	1.850	0.930
	L ₂	0.207	0.185	0.247	0.148

^a Effect of 0.15% Triton X-100 on oat root membrane ATPase of a microsomal fraction, a 34/45% sucrose interface fraction, and fractions (U₂ and L₂) obtained from phase partitioning. The phase system contained 6.3% PEG and dextran and 4.64 mg NaCl.

100% because the contents of other washes are not included in the table. Other enzyme activities reported in Table II are partitioned into the lower phases, and in particular cytochrome *c* oxidase (mitochondria) and NADH cytochrome *c* reductase (endoplasmic reticulum) are effectively moved from the upper to the lower phase. Thus, with the exception of the ATPase data, the phase-separation procedure appears to be somewhat more effective than the sucrose gradient for purifying the plant plasma membrane.

The lack of enrichment of the ATPase, using the phase separation, could be due to the vesicles being tight and right-side-out.¹¹ This was tested by evaluating the effect of Triton X-100 on the ATPase activity and it was found that this detergent increased ATPase activity by 3- to 4-fold in the U₂ fraction but only about 2-fold in the 34/45% sucrose gradient fraction (Table III). These results support the earlier report that the phase-separation procedure maintains tightly sealed plasma membrane vesicles that are probably right-side-out.¹¹ In contrast, the microsomal vesicles must become leaky during their tenure on the sucrose gradient. This is consistent with the observations that microsomal vesicles purified on dextran gradients are tightly sealed as determined by ion flux studies.³⁷ When the substrate (probably ATP) accessibility problem is eliminated by Triton, it becomes clearer that the phase separation procedure, when using 3 washes, results in a purer plasma membrane fraction from oat roots than that obtained using the sucrose gradient procedure. The yields

¹¹ H. Sze and K. A. Churchill, *Proc. Natl. Acad. Sci. U.S.A.* 78, 5578 (1981).

are lower in the phase separation procedure, but this can be overcome by simply starting with larger quantities of plant material.

In other comparisons of the two procedures, they both require similar laboratory equipment, and the time required for each is about the same if one pellets the membranes from the polymers prior to further assay. The time might be slightly shorter for the phase separation procedure, but this is also dependent upon how many washes are involved. PEG tends to interfere with several assays, e.g., phosphate and protein, thus it is generally important to remove the membranes from the polymers before proceeding with further analyses. It is especially important to precipitate the proteins with trichloroacetic acid from the washed membranes obtained from the polymers before determining the protein. This is not as great a problem with the membranes in sucrose. The phase-separation procedure purifies one membrane at a time, i.e., plasma membranes are purified in the upper phase (in the procedure described here) while all other membranes are transferred to the lower phase. The sucrose gradient, on the other hand, can be prepared such that different membranes can be enriched at different sucrose interfaces at the same time.³³ The procedure of choice for purifying the plasma membrane, i.e., sucrose gradient or phase partitioning, will therefore depend upon the intended use of these membranes.

Acknowledgments

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B. Membrane H^+ -translocating ATPases in plants of varying resistance to salinity (Jerusalem Laboratory)

Studies of the proton pumps of halophytes might be specially rewarding, since ion fluxes across the cellular membranes are likely to be particularly high. In many cases plants adapted for growth in saline environments accumulate NaCl. Since accumulation in the vacuole is marked, tonoplast H^+ -pump activity might be exceptionally high. Plasmalemma H^+ -pump activity might also be high, because Na^+-H^+ antiport at the cell surface, driven by protonmotive force, might contribute substantially towards controlling Na^+ concentration in the cytoplasm. Below we report on H^+ -translocating ATPase activity in membrane vesicles derived from the halophyte Atriplex and the crop plant cotton, a glycophyte with moderate tolerance to salinity.

f. DESCRIPTION OF COOPERATION

The American partner has reappraised and compared two techniques, sucrose gradient and two-phase separation, for obtaining and purifying plasmalemma-enriched membrane fractions from the roots of higher plants. In the Israeli laboratory the sucrose gradient technique was applied to the study of membrane H^+ -translocating ATPases in plants of varying tolerance to salinity.

During the second year one of the Israeli principal investigators visited the U.S. laboratory where results and problems were discussed in depth. Further, the U.S. principal investigator organized visits for him to other prominent U.S. laboratories where membrane H^+ -translocating ATPases are under investigation (Professor Leonard at the University of California, Riverside and Professor Sze at the University of Maryland).

The Israeli laboratory supplied the U.S. investigators with plant material for their work on the halophyte *Atriplex*.

g. MAIN ACHIEVEMENTS OF THE RESEARCH

1) Saline-resistant cell lines of tobacco cells have been developed in suspension culture which can grow in NaCl concentrations up to 500 mM.

2) We have demonstrated that the character of salt resistance is stable in the absence of selection pressure.

3) We have further demonstrated that adaptation to growth in saline medium is accompanied by irreversible modification of the morphology of the cells, and a faster growth rate in the absence of salt.

4) Adapted cells in high-salt medium have ultrastructural features different from those of wild type cells or adapted cells in standard medium. We have shown that when adapted cells are transferred from high-salt to standard medium, or vice versa, the characteristic ultrastructural changes occur only in the daughter cells produced after transfer.

5) We have also been able to demonstrate physiological differences between the cell lines, particularly with regard to their rates of proton pumping in the presence of KCl and fusicoccin; and the apparent degree of coupling between H^+ efflux and K^+ influx.

6) Membrane-sited H^+ -translocating ATPases ("proton pumps") have a key role in energizing solute fluxes into and out of plant cells, and are thus of central importance in nutrient supply and osmotic adaptation. Up to now studies on such ATPases at membrane level in isolated vesicles have been limited to a small number of non-halophytic plant species. We have succeeded in demonstrating and characterizing H^+ -translocating ATPase activity in membrane vesicles which we have isolated from the roots of the halophyte Atriplex nummularia, and have observed the interesting fact that salinity during growth modulates this activity.

7) We have also succeeded in demonstrating H^+ -translocating ATPase activity in membrane vesicles derived from roots of cotton, a glycophytic crop plant which shows a certain degree of salinity tolerance. In this case the activity was not modulated by saline conditions during growth.

h. LIST OF PUBLICATIONS

- 1) Mills, D., Robinson, K. and Hodges, T.K. 1985 Sodium and Potassium Fluxes and Compartmentation in Roots of Atriplex and Oat. Plant Physiology, 78: 500-509.
- 2) Reinhold, L., Seiden, A., and Volokita, M. 1984 Is Modulation of the Rate of Proton Pumping a Key Event in Osmoregulation? Plant Physiology, 75: 846-849.
- 3) Watad, A.A., Lerner, H.R. and Reinhold, L. 1985 Stability of the Salt-Resistance Character in Nicotiana Cell Lines Adapted to Grow in High NaCl Concentrations. Physiol. Veg. 23: 887-894.
- 4) Watad, A.A., Pesci, P.A., Reinhold, L., and Lerner, H.R. 1986 Proton Fluxes as a Response to External Salinity in Wild Type and NaCl-Adapted Nicotiana Cell Lines. Plant Physiology 81: 454-459.
- 5) Hodges, T.K. and Mills, D. 1986 Isolation of the Plasma Membrane. Methods in Enzymology, 118: 41-54.
- 6) Braun, Y., Hassidim, M., Lerner, H.R., and Reinhold, L. 1986 Studies on H^+ -Translocating ATPases in Plants of Varying Resistance to Salinity: I. Salinity during growth modulates the proton pump in the halophyte Atriplex nummularia. Plant Physiology, 81:1050-1056.
- 7) Hassidim, M., Braun, Y., Lerner, H.R. and Reinhold, L. 1986 Studies on H^+ -Translocating ATPases in Plants of Varying Resistance to Salinity: II. K^+ strongly promotes development of membrane potential in vesicles from cotton roots. Plant Physiology, 81: 1057-1061.

Further publications are in preparation.