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Transient Breakdown in the Selective Permeability of the Plasma Membrane of *Chlorella emersonii* in Response to Hyperosmotic Shock: Implications for Cell Water Relations and Osmotic Adjustment

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Summary. In osmotic experiments involving cells of the euryhaline unicellular green alga *Chlorella emersonii* exposed to hyperosmotic stress by immersion in a range of low molecular weight organic and inorganic solutes, a temporary breakdown in the selective permeability of the plasma membrane was observed during the initial phase of transfer to media of high osmotic strength (up to 2000 mosmol kg^{-1}). Thus, although the cells appeared to obey the Boyle-van't Hoff relationship in all cases, showing approximately linear changes in volume (at high salinity) as a function of the reciprocal of the external osmotic pressure, the extent of change was least for the triitols, propylene glycol and glycerol, intermediate for glucose, sorbitol, NaCl and KCl, with greatest changes in media containing the disaccharides sucrose and maltose. In NaCl-treated cells, uptake of external solute and loss of internal ions was observed in response to hyperosmotic treatment while sucrose-treated cells showed no significant uptake of external solute, although loss of intracellular K^+ was observed. These observations suggest that the widely used technique of estimating cellular turgor, and osmotic/nonosmotic volume by means of the changes in volume that occur upon transfer to media containing increasing amounts of either a low molecular weight organic solute or an inorganic salt may be subject to error. The assumption that all algal cells behave as "ideal osmometers," with outer membranes that are permeable to water but not to solutes, during the course of such experiments is therefore incorrect, and the data need to be adjusted to take account of hyperosmotically induced external solute penetration and/or loss of intracellular osmotica before meaningful estimates of cell turgor and osmotic volume can be obtained.

Key Words *Chlorella emersonii* · unicellular alga · osmotic responses · cell volume · membrane permeability · hyperosmotic shock · osmometric behavior

Introduction

Studies concerned with the changes in cellular volume that accompany alterations in external osmotic pressure have played a key role in shaping present-day concepts of the water and ionic relations of plant cells. Following the pioneering studies of De Vries and Pfeffer in the latter half of the nineteenth century, many plant physiologists have attempted to quantify the basic parameters of plant cell water relations. Several recent studies, initiated by the research of P.S. Nobel and co-workers (Nobel, 1969; Nobel & Wang, 1970), have utilized the volume changes that occur upon transfer of individual cells and isolated organelles to media of high osmotic strength to facilitate such measurements. Subsequent research in other laboratories has extended the range of cell types used in osmotic pressure/volume studies, to include several diverse algal genera, e.g. *Poterioochromonas* (Kauss, 1974), *Dunaliella* (Ben Amotz, 1974; Rabinowitch, Grover & Ginzburg, 1975; Gimmler, Schirling & Tobler, 1977), *Tetraselmis* (*Platymonas*) (Kirst, 1977*a*), *Porphyra* (Reed, Collins & Russell, 1980) and *Chlorella* (Munns et al., 1983*a*). These studies have measured changes in cell volume over a range of external solute concentration, using several different solutes, including NaCl (Kauss, 1974; Gimmler et al., 1977), sea salt (Reed et al., 1980) and mannitol (Munns et al., 1983*a*) with the unanimous finding that, above a critical solute level (required to eliminate turgor), the cell protoplasts appeared to behave as "ideal osmometers" showing a linear relationship between volume and the reciprocal of the external osmolality. This behavior is in accordance with the physical relationships first described by van't Hoff and Boyle (*see* Nobel, 1974) and several important cellular parameters can be calculated from osmotic pressure/volume curves of this type. The intercept on the ordinate axis (i.e. the volume at infinite osmotic pressure) gives the so-called "nonosmotic volume" of the cell. This is taken to represent the volume of hydrophobic components within the cell interior (e.g. carbohydrate storage inclusions, membrane systems, etc. (Nobel &

Wang, 1970)). By subtraction of this value from the total cell volume, the osmotic volume of the cell can be calculated. The magnitude of the cell turgor (hydrostatic) pressure (due to the presence of a surrounding cell wall) can also be determined, by comparing the osmotic volume of cells in media of high osmotic strength (where turgor is dissipated and the internal osmotic pressure is identical to that of the surrounding medium) with that of cells in their original medium (Dainty, 1976).

Many of the studies described above for algal cells have provided rather higher values for the nonosmotic volume than would be predicted from measurements of cellular water content (Gimmler et al., 1977; Kirst, 1977*a*). Similar phenomena have also been observed in animal cells where the discrepancy between the (apparent) osmotic volume and the water content of any given cell type has been described by Ponder (1948) as the ratio of the two values (Ponder's R). Values for Ponder's R in animal cells are invariably less than 1.0 and have been interpreted in several ways (Dick, 1966; House, 1974). However, the *linearity* of response of both animal and plant cells at high osmotic strength has been regarded as sufficient evidence of ideal osmometric behavior, even though the assumptions of solute impermeability, upon which such an interpretation depends, have not always been tested rigorously.

A recent report using red blood cell ghosts gives us cause to question such assumptions. The osmotic responses of bovine red blood cell ghosts to a series of low molecular weight carbohydrates have been studied by light scattering, with the observation that changes in ghost volume varied as a function of the molecular weight of the solute used to generate the osmotic gradient (Chang et al., 1983). Thus the ghosts shrank to a greater extent in solutions containing raffinose and sucrose than in media containing isosmotic amounts of glyceraldehyde and erythritol. Osmotic pressure/volume curves obtained using different organic solutes gave different values for the nonosmotic volume (the intercept on the ordinate axis) in each case (Chang et al., 1983). Such observations are in contrast to a recent report for mouse fibroblast cells where, although osmotic pressure/volume responses were found to be dependent upon the nature of the external solute (in this case the ionic salts KCl, NaCl, LiCl and $CaCl_2$ were used), the curves all produced the same nonosmotic volume (Raaphorst & Kruuv, 1979). Similar observations regarding the effects of external solutes on the slope of the Boyle-van't Hoff plot using media containing amino acids and suspensions of isolated chloroplasts have been interpreted in terms of changes in the reflection coefficients (a term that, in part at least, takes account of the permeability of the membrane to solutes) of the amino acids, due to active chloroplast uptake systems for these solutes (Nobel & Wang, 1970).

The present study was carried out to establish the osmotic responses of cells of the euryhaline green alga *Chlorella emersonii*, a unicellular organism that is often regarded as a model system for the responses of higher plant cells, in media of varying external solute composition. Cell volume and solute uptake have been monitored to assess the behavior of this alga in terms of the Boyle-van't Hoff relationship.

Materials and Methods

C. emersonii (strain 211/8b: Culture Centre of Algae and Protozoa, Cambridge, U.K.) was grown in batch culture at 25°C in 1 dm^3 flasks containing 0.5 dm^3 of BG11 medium (Rippka et al., 1979) under continuous illumination at a photon fluence rate of 35 μmol m^{-2} sec^{-1}. Cells were harvested for experimental purposes in late exponential phase of growth.

Cell volume analysis was carried out using a particle size analyzer (Coulter Electronics Ltd., Luton, U.K., Model ZB, fitted with a C1000 Channelyzer) linked to an Acorn microcomputer to facilitate calculation of mean cell volume (*see* Rabinowitch et al., 1975; Owen, Thurston & Bazin, 1977). The aperture size was 50 μm, aperture length 60 μm. For each value of mean cell volume, six replicate samples were used, with 15,000 to 18,000 cells per sample.

Media of varying solute composition were prepared by adding the required amount of each solute (i.e. glucose, glycerol, KCl, maltose, NaCl, propylene glycol, sorbitol or sucrose) to a solution of BG11 medium plus added NaCl (315 mosmol kg^{-1}, as measured using a Wescor 5100C vapor pressure osmometer) to give a range of total osmolalities from 415 to 2000 mosmol kg^{-1}. NaCl at 315 mosmol kg^{-1} was found to be the minimum electrolyte concentration required for accurate size analysis using the Coulter ZB system (Parsons, 1973).

Solute uptake during the initial phase of hyperosmotic treatment was studied for cells in NaCl and sucrose. Cells of *C. emersonii* were transferred from basal growth medium to a hyperosmotic medium containing either NaCl or sucrose, together with the appropriate radioisotopic tracer ($^{24}Na^+$ or ^{14}C-sucrose, respectively, at specific activities up to 400 MBq mol^{-1}), to give final osmolalities within the range 315 to 2000 mosmol kg^{-1}. After a 5-min incubation period in each medium, 1 cm^3 aliquots of cell suspension were then transferred onto cellulose acetate membrane filters of pore size 0.45 μm and diameter 25 mm (Oxoid Ltd., Basingstoke, U.K.). The bathing medium was then removed rapidly by vacuum filtration and the cells were then rinsed quickly with 10 cm^3 of medium of identical ionic strength and composition but without added radioisotope, to remove tracer from all extracellular regions. This rinsing procedure took no longer than 20 sec in each case. The small amount of residual carry-over was estimated using cells treated for 30 min in 50% dimethyl sulfoxide (DMSO): this value (typically 2 to 5% of the radioactivity associated with viable cells in the case of $^{24}NaCl$ at high external concentration) was then subtracted from all experimental data.

Samples were counted in a Packard 300CD liquid scintillation spectrometer with fully automatic quench correction. A minimum of 5×10^7 cells was counted in each case (≥ 8 mm^3 total cell volume). Aliquots (10 to 50 mm^3) of bathing medium were also counted using the same system, in Packard 229 liquid scintillation cocktail (Packard Instruments, Illinois). Samples containing $^{24}Na^+$ were also corrected for decay during counting ($t_{1/2} = 15$ hr).

Experiments were carried out at 20°C under continuous illumination (35 μmol m^{-2} sec^{-1}). The osmolality of all experimental solutions was verified using a Wescor 5100C vapor pressure osmometer. Radioisotopic ^{14}C-sucrose was obtained from Amersham International, Buckinghamshire, U.K.; $^{24}Na^+$ was kindly provided by the Scottish Universities Research and Reactor Centre, E. Kilbride, U.K.

Results and Discussion

The cell volumes of *C. emersonii* following a 5-min incubation period in each test solution are shown in Fig. 1. Since the measured osmolality of cell sap from *C. emersonii* grown in nonsaline medium is less than 300 mosmol kg^{-1} (Greenway & Watkin, 1983), cell turgor should in theory be abolished under such conditions and osmotic pressure/volume curves should therefore be linear over this range, providing the reflection coefficients of the solutes involved are near to 1.0 (Nobel, 1974). However, it is clear that the extent of change in cell volume was dependent upon the solute used to establish the osmotic gradient. In the case of organic solutes, those carbohydrates with the lowest molecular weights (i.e. propylene glycol and glycerol) were found to be least effective in producing a decrease in cell volume at high osmotic strengths while the greatest reductions in cell volume were observed in concentrated solutions of the disaccharides maltose and sucrose. Any calculations of nonosmotic volume and hence osmotic volume would give very different answers, depending upon the solute. To illustrate this, the apparent nonosmotic volume of cells incubated in propylene glycol is 151 μm^3 while cells incubated in sucrose solutions show an apparent nonosmotic volume of 116 μm^3 (Fig. 1). Likewise, the monovalent salts NaCl and KCl produced different osmotic pressure/volume curves, with KCl-treated cells (apparent nonosmotic volume 136 μm^3) showing the greater change in volume at any given osmolality. In the case of KCl, the volume estimates at high external KCl concentration may be affected by any increase in membrane conductance, although this effect has not been noted by previous workers (Rabinowitch et al., 1975).

Time courses for the changes in cellular volume of *C. emersonii* transferred from basal medium to media containing either glycerol, glucose, NaCl or

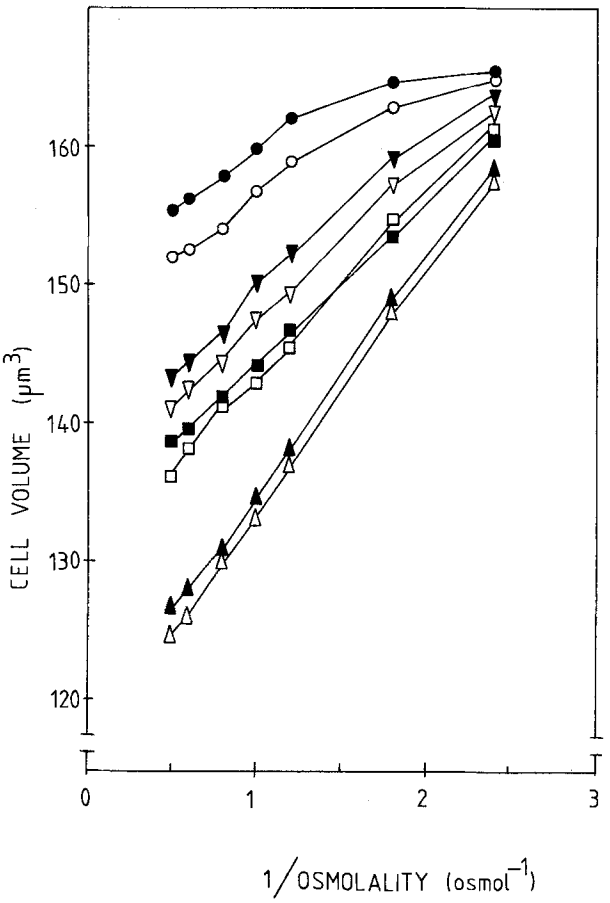

Fig. 1. Osmotic pressure/volume responses of *C. emersonii* in media of varying solute composition. Cells were transferred to test solutions containing NaCl at 315 mosmol kg^{-1} (the minimum electrolyte concentration for the particle size analyzer), together with either propylene glycol (●), glycerol (○), NaCl (▼), KCl (▽), glucose (■), sorbitol (□), maltose (▲) or sucrose (△) to give total osmolalities from 415 to 2000 mosmol kg^{-1}. Cell volume was measured after 5 min (6 replicates: standard deviation ≤ 1.8 μm^3 in all cases). The results are shown in the form of a Boyle-van't Hoff plot, i.e. cell volume versus the reciprocal of the external osmolality. The volume of *C. emersonii* in electrolyte solution free of added solutes (315 mosmol kg^{-1}) was 167.2 μm^3

sucrose showed an initial, rapid decrease, complete in 30 to 60 sec, followed by a smaller, slower rise over the next 2 to 5 min, with little evidence of any subsequent major change in cellular volume over the remainder of the experiment (Fig. 2).

Since the changes in cellular volume in media of high osmotic strength were found to vary as a function of the external solute (Fig. 1), a possible interpretation of these effects is that the changes in volume due to water flux from the cell interior in response to an increase in external osmolality may be mitigated by uptake of the external solute, due to transient changes in the permeability properties of the plasma membrane. Such an interpretation is

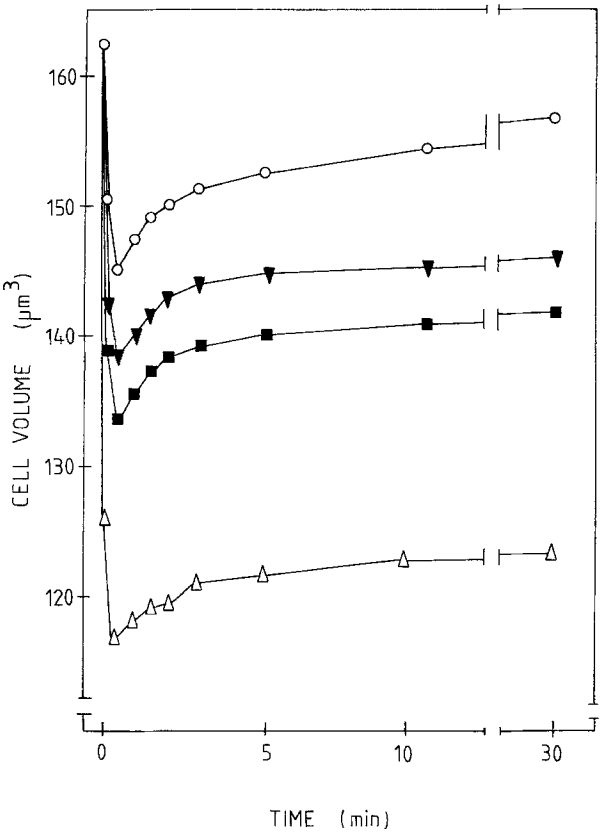

Fig. 2. Time courses for changes in cellular volume of *C. emersonii* in response to either glycerol (○), glucose (■), NaCl (▼) or sucrose (△) at high osmotic strength (total osmolality 2000 mosmol kg^{-1}). Representative data from individual experiments are shown

supported by the data shown in Fig. 3 for Na^+ uptake in cells of *C. emersonii* incubated for 5 min in a range of media from 315 to 2000 mosmol kg^{-1} NaCl. Within this range, cells of *C. emersonii* showed a significant initial uptake of external Na^+ in response to hyperosmotic treatment, with the greatest Na^+ uptake being observed in 2000 mosmol kg^{-1} NaCl. This Na^+ influx will reduce the cell volume change upon transfer to 2000 mosmol kg^{-1} NaCl by at least 8 μm^3 (calculated on a total cell volume basis), with a doubling of this value if Cl^- also enters the cell to the same extent. Similar experiments using ^{14}C-sucrose failed to show any substantial uptake during this period (*data not shown*), with no evidence of sucrose penetration during hyperosmotic treatment. Thus the values for ^{14}C-sucrose in viable and DMSO-treated cells were not significantly different from each other. It thus seems reasonable to suggest that the changes in volume that occur upon transfer to media containing NaCl are lower than those for cells in sucrose-containing media, due in part at least to uptake of external solute. However, this explanation is insufficient to explain all of the shortfall in cell volume change in NaCl-containing media. The possibility of loss of intracellular solutes during hyperosmotic shock was investigated using cells of *C. emersonii* preloaded with ^{42}KCl (at 5 mmol dm^{-3}, specific activity 1 GBq mol^{-1}, in BG11 medium) for 24 hr to allow exchange and equilibration of intracellular and extracellular K^+. Cells preincubated in this manner were then assayed for their internal K^+($^{42}K^+$) content using the rinsing procedure described above for $^{24}Na^+$. Intracellular K^+ was measured at 32.24 fmol · cell^{-1} (192.8 mmol dm^{-3} cell volume) under these conditions (4 replicates). Cells transferred to a medium of the same K^+ concentration and specific activity, but with added NaCl or sucrose (to give a final osmolality of 2000 mosmol kg^{-1}) showed a net loss of K^+ during the first 5 min following transfer. The intracellular K^+ level in 2000 mosmol kg^{-1} sucrose was found to be lower than that in 2000 mosmol kg^{-1} NaCl (at 24.73 fmol · cell^{-1} and 26.43 fmol · cell^{-1}, respectively), showing that loss of internal ions may occur as a result of hyperosmotic treatment. If other internal osmotica are also lost during hyperosmotic treatment, then the osmotic pressure/volume curves will be steeper than predicted for an "ideal osmometer," with a lower value for the nonosmotic volume due to net solute loss from the cell interior.

The results outlined above show that linearity of change in cell protoplast volume in response to the reciprocal of the external osmolality is an inadequate criterion to use in support of "ideal osmometric" behavior, prior to the calculation of nonosmotic and osmotic volume using the Boyle-van't Hoff relationship. Future studies must therefore combine measurements of cell volume change with estimates of the extent of solute uptake and/or loss of intracellular osmotica during incubation in media of high osmotic strength. Given the not insubstantial problems associated with such multiple measurements, it may prove difficult to conduct suitable experiments, since the osmotic pressure/volume relationships of cells showing hyperosmotically-induced changes in membrane permeability will be a function of (1) water flux in response to a gradient of water potential across the membrane (the "classical" osmotic response; Dainty, 1976; Nobel, 1974), (2) penetration of external solute, and (3) loss of intracellular solutes.

The present study also illustrates the potential errors involved in measuring changes in cell volume using ^{14}C-labeled carbohydrates (Munns et al., 1983*a*), since these may penetrate the cell plasma membrane during the initial period following transfer to a hyperosmotic medium and thus lead to in-

correct estimation of the extracellular volume (and, by subtraction, the intracellular volume). Further problems may arise if the volume is measured using ^{14}C-labeled carbohydrates under conditions where the external osmolality is varied using NaCl or an alternative solute (Munns et al., 1983*a*).

The data shown in Fig. 1 also provide a further possible explanation for the discrepancy between cell osmotic volume and water content (i.e. low values for Ponder's R (osmotic volume/water content); House, 1974). Thus in plant cells showing similar responses to those of *C. emersonii,* NaCl, KCl and low molecular weight carbohydrates will produce osmotic pressure/volume curves that may underestimate the osmotic volume due to hyperosmotically induced external solute uptake or, conversely, may lead to overestimation, due to loss of intracellular osmotica.

There are also clear implications for osmotic studies using algal cells contained within the data shown in Figs. 1 and 3. NaCl results in a limited change in protoplast volume when compared to sucrose and the data indicate a significant salt influx during the initial stages of transfer to hyperosmotic conditions. The cell interior of *C. emersonii* will therefore be exposed to higher levels of NaCl upon upshock in saline media than have previously been considered, since most of the earlier studies have been concerned with the steady-state responses of salt-adapted cells (Munns et al., 1983*b*). Transient increases in intracellular NaCl levels following upshock have been observed in several algae, including *Tetraselmis* (Kirst, 1977*b*), *Ulva* (Dickson, Wyn Jones & Davenport, 1980) and *Synechococcus* (Blumwald, Mehlorn & Packer, 1983) and these increases have often been interpreted as part of a short-term mechanism of osmotic adjustment using ions (*see* Kirst, 1977*b*), in contrast to longer-term acclimation involving changes in intracellular organic osmotica (mannitol, Kirst, 1977*b*; dimethylsulfoniopropionate, Dickson et al., 1980; and sucrose, Blumwald et al., 1983, respectively). The present study suggests that such changes in ion levels may be due to transient (passive) changes in membrane permeability rather than to metabolic (active) processes involved in osmotic adjustment. Recent studies using *Dunaliella tertiolecta* (Ehrenfeld & Cousin, 1984) have also shown a rapid uptake of Na^+ upon transfer to hyperosmotic media containing NaCl, suggesting that the phenomenon of transient membrane disruption in media of high osmotic strength may be widespread among diverse algal types. Greenway and Setter (1979) have also reported transient increases in cell Na^+ content with concomitant loss of intracellular K^+ in *C. emersonii* exposed to hyperosmotic shock (616

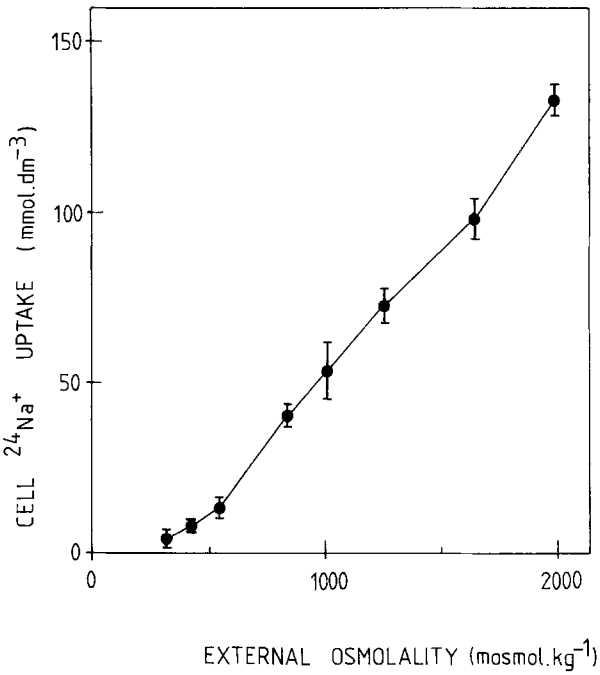

Fig. 3. Uptake of Na^+ (as $^{24}Na^+$) during hyperosmotic treatment in cells of *C. emersonii* immersed in a range of media containing NaCl at 315 to 2000 mosmol kg^{-1} for 5 min (4 replicates, mean ± standard deviation)

mosmol kg^{-1} NaCl), although their data were obtained using chemical analyses and rinsing procedures (isoosmotic mannitol): such techniques may be subject to error due to (i) contamination of samples with extracellular ions (Reed & Collins, 1980) and/or (ii) net ion loss during rinsing (Ehrenfeld & Cousin, 1984).

The present study illustrates the importance of cytoplasmic compatible solutes (Borowitzka & Brown, 1974), which are accumulated in response to osmotic stress at concentrations high enough to effect osmotic balance, in protecting against enzyme inactivation by NaCl (Brown, 1976, 1978). A number of possible cytoplasmic compatible solutes have been identified in plant cells, including glycerol (Borowitzka & Brown, 1974), sorbitol, proline and glycine betaine (*see* Wyn Jones & Gorham, 1983) and it may now prove necessary to assess their role in limiting the potentially damaging effects of NaCl influx into the cytoplasm in response to hypersaline stress (*see* Pollard & Wyn Jones, 1979) rather than simply considering their compatibility with enzyme function and metabolic activity.

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