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Solute Accumulation in Soybean (*Glycine max* L.) Cells Adapted to NaCl Salinity

By

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With 3 Figures

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Summary

EL SAYED H. & KIRKWOOD R. C. 1992. Solute accumulation in soybean (*Glycine max* L.) cells adapted to NaCl salinity. – Phyton (Horn, Austria) 31 (2): 233–249, with 3 figures. – English with German summary.

Cells of soybean (*Glycine max* L. "Maple Arrow") adapted to NaCl salinity (up to 680 mM) and which have undergone extensive osmotic adjustment, accumulated Na^+ and Cl^- as principal solutes for this adjustment. With the exception of 85 mM NaCl, salinity decreased fresh weight (F. W.), dry weight (D. W.) and succulence but increased the dry matter content (D. M. C.); the water content however, increased with NaCl concentration. Although the concentrations of Na^+ and Cl^- correlated well with the level of adaptation, these ions apparently did not contribute to the osmotic adjustment which occurred during a culture growth cycle, because the concentrations of Na^+ and Cl^- did not increase during the period of most active adjustment. While the average concentrations of soluble sugars and total free amino acids increased as a function of the level of adaptation, the levels of these solutes did not approach those observed for Na^+ and Cl^-. The concentration of proline was positively correlated with cell osmotic potential, accumulating to an average concentration of 130.7 mM in cells adapted to 680 mM NaCl and representing about 75% of the total amino acid pool as compared to an average of 1.65 mM and about 17% of the pool in unadapted cells. These results indicate that although Na^+ and Cl^- are the principal components of osmotic adjustment, organic solutes also may make significant contributions.

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Zusammenfassung

El Sayed H. & Kirkwood R. C. 1992. Akkumulation von gelösten Stoffen in an NaCl-Salinität adaptierten Zellen von Sojabohne (*Glycine max* L.). – Phyton (Horn, Austria) 31 (2): 233–249, mit 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Zellen der Sojabohne (*Glycine max* L. „Maple Arrow"), welche an NaCl-Salinität angepaßt wurden (bis 680 mM) zeigen eine ausgeprägte osmotische Anpassung. Sie akkumulieren in Folge dessen hauptsächlich Na^+ und Cl^- als gelöste Stoffe. Die Salinität führte mit Ausnahme von 85 mM NaCl zu einer Verminderung des Frischgewichts (F. W.), des Trockengewichts (D. W.) und der Sukkulenz, aber zu einer Zunahme der Trockensubstanz (D. M. C.); der Wassergehalt stieg ebenfalls mit der NaCl-Konzentration an. Obwohl die Konzentrationen von Na^+ und Cl^- mit dem Grad der Anpassung gut übereinstimmen, dürften diese Ionen offensichtlich nichts zur osmotischen Anpassung beitragen, welche während einer Kulturperiode auftrat. Der Gehalt an Na^+ und Cl^- stieg nämlich nicht während der Phase der größten osmotischen Anpassung an. Während die mittlere Konzentration an löslichem Zucker und gesamten freien Aminosäuren funktionell mit dem Grad der Anpassung anstieg, erreichte das Ausmaß an diesen gelösten Stoffen nie jene für Na^+ und Cl^-. Die Konzentration von Prolin war positiv mit dem osmotischen Potential korreliert, indem diese Aminosäure bis zu einer mittleren Konzentration von 130,7 mM in den Zellen akkumulierten, welche an 680 mM NaCl adaptiert wurden. So repräsentiert der Prolingehalt etwa 75% des gesamten Aminosäurepools im Vergleich zu einem durchschnittlichen Wert von 1,65 mM und rund 17% unangepaßter Zellen. Diese Ergebnisse zeigen, daß auch gelöste organische Stoffe einen signifikanten Beitrag an der osmotischen Anpassung leisten können, obwohl Na^+ und Cl^- die Hauptkomponenten darstellen.

Introduction

Osmotic adjustment is a fundamental adaptive response of plant cells which are exposed to salinity (Flowers, Troke & Yeo 1977, Greenway & Munns 1980, Wyn Jones & Gorham 1983) and is necessary for survival and growth under saline conditions. Osmotic adjustment in response to salinity results from solute accumulation which occurs through the uptake of solutes, the synthesis of organic compounds, or both. The identification of solutes which accumulate in response to salinity is an initial step toward the elucidation of the biochemical and physiological mechanisms which are responsible for and regulate osmotic adjustment. Halophytes typically utilize Na^+ and Cl^- as principal osmotica, while organic solutes may serve an important role in balancing the osmotic presure of the cytoplasm with that of the vacuole; much of the Na^+ and Cl^- is thought to be compartmentalized in the vacuole (Flowers, Troke & Yeo 1977; Wyn Jones & Gorham 1983, Yeo 1983). In response to moderate levels of salinity, many glycophytic plants appear to exclude Na^+ and Cl^- as a mechnism of tolerance (Sacher, Staples & Robinson 1982), instead using the synthesis and accumulation of organic compounds for osmotic adjustment (Greenway &

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MUNNS 1980, YEO 1983). The ability of glycophytic plants to accumulate Na^+ and Cl^- and survive high levels of salinity has not been investigated thoroughly.

The *invitro* isolation of glycophytic cells with enhanced salt tolerance has helped facilitate the study of cellular responses to salinity (BEN-HAYYIM & KOCHBA 1983, BEN-HAYYIM & KOCHBA 1983, BEN HAYYIM, SPIEGEL-ROY & NEUMANN 1985, BINZEL & al. 1985, HEYSER & NABORS 1981, WATAD, REINHOLD & LERNER 1983). These salt tolerant cells are especially usefull in studies to identify specific adaptive responses of glycophytes to salinity. They provide a system with which to separate those processes which are involved in salinity adaptation as opposed to those which are a response to the imposition of stress. The use of isolated cells does restrict studies of salt tolerance mechanisms to those which are characteristic of individual cells. It does not lend itself to examination of more complex multicellular processes such as root ion exclusion, xylem / phloem ion exchange and redeposition (JESCHKE 1984), or mechanisms involving differentiated highly specialized cells, i. e. salt glands and bladders or transfer cells. It is the intrinsically cellular mechanisms however, which under conditions of extreme salinity ultimately may be the most important to cell survival; they are most readily modified genetically in glycophytic crop species.

The purpose of the present work was to study the responses of cell suspensions of soybean (*Glycine max* L. "Maple Arrow") to NaCl salinity stress using the cell suspension cultures of the glycophyte soybean as a comparative system.

Materials and Methods

Abbreviations – 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; GB5, Gamborg B5; ψ_π Osmotic Potential; ψ_w Walter Potential; ψ_p Turgor; HFBI, N(O,S)-heptafluorobutyryl isobutyl; QAC, Quaternary Ammonium Compound; ACC, 1-Amino Cyclopropane-1-Carboxylate.

Growth and Preparation of Cell Suspension. A cell suspension of soybean (*Glycine max* L. "Maple Arrow") was developed from the callus culture (stem). Cells were grown in 500 ml conical flasks each containing 200 ml of Gamborg's B5 (GB5) salt medium (GAMBORG, MILLER & OJIMA 1968) with addition of GB5 3.87 g l^{-1}, sucrose 30 g l^{-1}, IAA 0.75 mg l^{-1}, Kinetin 1 mg l^{-1}, and 2,4-D 5-50 μM. The cells were maintained with continuous shaking (100 rotations/min) at 25° C under fluorescent light with a 14 h photoperiod (3,000 lux).

Osmotic stress: The resulting cells were transfered to GB5 basal medium with different concentrations of added salinity; control, 85, 170, 340, 510 and 680 mM NaCl. Cells were harvested for analysis each week from the first week after inoculation (beginning of stationary phase). Illumination was provided for 14 h in 24 h using cool white fluorescent lamps, the maximum light intensity being 3,000 lux.

Analysis of Cells for Growth and Solutes:

Cell Growth: Fresh weight was determined by weighing the cells left on Whatman Glass Microfibre (GF/C, 2.5 cm) filters after filtering under vacuum; the

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cells had been washed three times in distilled water at a ratio of 3 to 4 g/50 ml of distilled water. The dry weight of cells was determined after incubating the cells at 85° C for 24 h. Succulence was determined by taking the ratio of FW/DW, whereas, the dry matter content (DMC) content was determined by taking the ratio of DW/FW x 100.

Sampling of Cells for Solute Analysis:

The changes in ψ_π and tolerance to salinity of cells during a culture growth cycle were indicative that solute content also fluctuated cell as a function of growth cycle stage, therefore cell samples for solute analysis were collected throughout the growth cycle for each cell line at approximately equivalent stages of growth (Fig. 1). Cells in the later linear phase of growth were inoculated at a fresh weight density of 0.02 g/ml into 2 l of nutrient medium contained in a 4 l Erlenmeyer flask. The medium contained the same concentration of NaCl as that of the stock culture medium. Cells used for solute analyses were harvested on Buchner funnel, rinsed with isotonic solutions of mannitol, frozen, lyophilized, and then stored at −20° C.

Osmotic potential were determined by plasmometry (BRESSAN & al. 1982, HANDA & al. 1982) and were based on the concentration of NaCl causing incipient plasmolysis in 50% of the viable cells. Water potential of the culture media were measured by determination of the freezing point with a Precision Systems, Inc. (Springfield, MA) automatic osmometer or by the dew point method with a Wescor (Logan, UT) thermocouple psychrometer-hygrometer model HR33T, with model C52 sample chambers (BRESSAN & al. 1981). Calibration was accomplished using NaCl solutions. For the purpose of estimating turgor, cells were assumed to be in water potential equilibrium with the culture medium and cell turgor values were calculated as the difference between the water potentials and osmotic potentials.

Solutes Analysis: Amino acids, organic acids, and QACs were analyzed from a common extract obtained by homogenizing 50 mg of lyophilized cells in 2.5 ml of methanol : chloroform : water (12 : 5 : 3). After centrifugation, the supernatant was removed and the procedure repeated three times. Three ml of H_2O and 2 ml of chloroform were added to the pooled extracts, the aqueous phase was removed and the organic phase reextracted with an additional 5 ml of H_2O. The aqueous extracts were pooled and evaporated to dryness at 40° C under a stream of compressed air. Amino acids and organic acids were separated by ion exchange chromatography after the residue was resuspended in 2 ml of H_2O (RHODES, MYERS & JAMIESON 1981). Half the extract was loaded on to a 1 × 2 cm column of Dowex 50-H^+ (200–400 mesh) while the remaining 1 ml (to be used for QAC analysis) was stored at −20° C. The void and water washes (2 × 2.5 ml) were collected and stored at – 20° C for analysis of organic acids. The amino acids were eluted from the column with three 2 ml volumes of 6 M NH_4OH and the eluate brought to dryness under a stream of compressed air at 40° C. The residue was then resuspended in 2 ml H_2O and passed over a 1 × 2 cm column of Dowex 1-CH_3COO^-; the void and H_2O washed (3 × 2 ml) contained the neutral and basic amino acids while the acidic amino acids were eluted with 2 M CH_3COOH (3 × 2 ml). Both fractions were brought to dryness under a stream of compressed air at 40° C.

The fraction from the Dowex 50-H^+ column containing the organic acids (void and water washes) was brought to dryness and resuspended in 1 ml of H_2O. One half ml was passed over a 1 × 2 cm column of Dowex 1-$HCOO^-$. After washing the column

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with 3 × 2 ml volumes of H_2O the organic acids were eluted with 6N HCOOH (4 × 2 ml) and brought to dryness under a stream of compressed air at 40° C.

HFBl ester of the amino acids and O-heptafluorobutyryl isobutyl and/or isobutyl ester of organic acids were prepared according to the methods of RHODES, MYERS & JAMIESON (1981). ∞- Amino-n-butyric acid (0.25 μmol) was added to the samples as an internal standard prior to derivatization. The derivatives in ethyl acetate : acetic anhydride (1 : 1) were subjected to GLC (Hewlett Packard Model 5794 A) using a fused silica capillary column (DB5-30 N, 30 m × 0.2 mm ID, J&W Scientific, Rancho Cordova, CA). The split ratio at the injector port was 20 : 1; column pressure 19 psi N_2 carrier gas, column flow rate 1 ml min^{-1}; 22 ml N_2 sweep gas at the flame ionization detector, 222 ml min^{-1} air and 35 ml min^{-1} H_2 at the detector; 20 ml min^{-1} total N_2 flow at the split vent. The injector temperature was set at 250° C; the detector at 280° C and the column subjected to a variable temperature programme (100° C for 4 min, then increased from 100 to 260° C at a rate of 6° C min^{-1} and held at 260° C for 4 min). Peak areas were determined by a Hewlett-Packard 3390 A reporting integrator and were related to the area of the internal standard, ∞-amino-n-butyric acid. The response factors for individual amino acids and organic acids were determined by GLC of HFBl esters of an amino acid standard mixture (Sigma No. AA-S-18) and HFBl esters of a 1 mM mixture of malonic, maleic, malic, citric, isocitric, tartric, and succinic acids. The only amino acids without unique retention times, as determined by GC-MS (RHODES, MYERS & JAMIESON 1981), were valine, which co-chromatographed with β-alanine, and methionine, which co-chromatographed with tyramine. The presence of ACC in the free amino acid pool was verfied by electron impact GC MS (RHODES, MYERS & JAMIESON 1981).

In order to separate QACs, the extract was first passed over a 1 × 2 cm column of Dowex 1-OH^-. The void and water washes (2 × 4 ml) were collected and passed over a 1 × 2 cm column of Dowex 50-H^+. After washing the column with 2 × 4 ml H_2O, the QACs were eluted with 2.5 N HCL (3 × 2 ml) and brought to dryness under a stream of compressed air. QACs were measured according to the procedure of LADYMAN & al. (1983) except that absorbance of the samples and standards was determined at 500 nm.

For sugar analyses, 25 mg of lyophilized cells were extracted in 2.5 ml of 80% ethanol for 30 min at 70° C, centrifuged, and the supernatant collected. This procedure was repeated twice. The pooled extracts were evaporated under vacuum at 40° C and the residues redissolved in 2.5 ml of water. Reducing sugars were analyzed according to SOMOGYI (1952) and sucrose by measuring reducing sugars after hydrolysis with invertase (Sigma, No. I-4753), and protein contents were analyzed according to BRADFORD (1976).

Na^+ and K^+ were measured by atomic emission spectrophotometry using a Micro-Tek Unicam SP-90 Atomic Absorption/Emission Spectrophotometer and NaCl and KCl standards. K^+ measurements were verified by atomic absorption to ensure that the emission values had not been influenced significantly by changes in the atomic emissivity of K^+ due to the variation in the Na^+ content of the samples. Lyophilized cells were wet ashed using a combination of HNO_3 and $HCLO_4$. Two ml of concentrated HNO_3 were added to 25 mg of cells in a Folin-Wu tube, 2 ml of 70% $HCLO_4$ were added 10 min later, and the mixture heated on a digestion block at 180° C until a colourless solution remained. The final volume was diluted to 25 ml with H_2O prior to analysis.

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Cl^- and NO_3^- analyses were performed on water extracts of lyophilized cells. A 25 mg sample was incubated in 5 ml of water for 30 min at 60° C and following centrifugation the supernatant was collected. This procedure was repeated twice. The supernatants were pooled, evaporated to dryness (in a 40° C water bath under a stream of compressed air), and the residues resuspended in 2 ml of water. CL^- was estimated according to the titrametric method of SCHALES & SCHALES (1941) using a reagent Kit from the Sigma Chemical Co. (No. 830). NO_3^- was converted to NO_2^- by nitrate reductase freshly isolated from frozen soybean nodules, and the NO_2^- colorimetrically determined as described by LOWE & HAMILTON (1976).

Dermination of Intracellular Solute Concentrations

The solute concentrations reported here in have been corrected for the contribution of extracellular water to the total water volume and solute contents expressed on the basis of dry weight have been corrected for the contribution of residual mannitol to the apparent dry weight of the cells. The extracellular volume and residual mannitol were determined by adding (^3H) mannitol solutions used to rinse samples of late linear phase cells from each line. Mannitol was not taken up by the cells in the time elapsed during the rinsing (data not shown). After the cells were frozen and lyophilized, the ^3H dpm mg/dry weight as well as the specific radioactivity of the mannitol solution ^3H dpm μl^{-1} were determined using a Beckman Scintillation Spectrometer (Model LS – 6800).

Determinations of extracellular water using (^{14}C) inulin carboxylic acid in place of (^3H) mannitol yielded similar results. Utilizing these values, the solute concentrations were based upon the intracellular water content of the cells where intracellular water equaled (total fresh weight – extracellular water – dry weight). These intracellular water values were used to calculate the solute concentrations of the cells from throughout the growth cycle; therefore if fluctions in the extracellular volume of the cells occurred during the growth cycle, the actual solute concentrations would vary accordingly from those reported.

Results and Discussion

Growth: The effect of NaCl treatments on growth (FW, DW & DMC) and succulence of cell suspensions of soybean (*Glycine max* L. "Maple Arrow") are shown in Fig. 1 and Table 2. With the exception of 85 mM NaCl, the rate of growth decreased with NaCl concentration. Succulence decreased while DMC content increased with increased NaCl concentration in the external medium.

In these studies F. W., D. W. and succulence of soybean cell suspensions generally decreased with increase in NaCl concentration, while the D. M. C. increased. The contribution of Na^+ and Cl^- to cell D. W. did not increase in proportion to the level of NaCl to which the cells were adapted. BINZEL & al. (1985) and HASEGAWA, BRESSAN & HANDA (1987) reported that, generally, the water content of cells declined during adaptation to salinity, resulting in as much as a 5-fold reduction in cell volume; in glycophytes, however, increase in water content or succulence generally increase in response to salt exposure.

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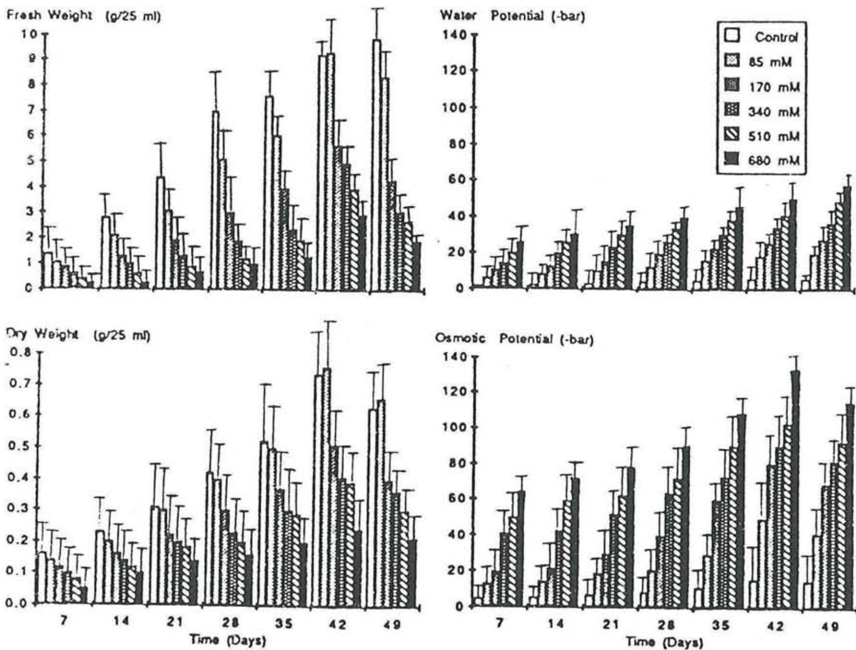

Fig. 1. Fresh and dry weights, cell osmotic and water potential of unadapted and cells adapted to 85, 170, 340, 510 and 680 mM NaCl which were used for solute analyses. Each value is the mean of five observations; Vertical bars, Standard Error means.

Organic Solute Accumulation: The intracellular concentrations of soluble sugars increased as a function of the level of adaptation (Fig. 2). The magnitude of these changes, however, was not comparable to that observed for Na^+ and Cl^-. While sucrose levels increased more than 10-fold between unadapted cells and those adapted to 680 mM NaCl, the patterns of carbohydrate accumulation observed in halophytes (BRIENS & LARHER 1982) also indicate that the accumulation of sucrose is favoured over that of glucose in response to salinity.

The concentrations of both sucrose and reducing sugars increased after the cells were inoculated into fresh media and then returned to near original levels as the cultures approached the stationary phase (Fig. 2). This trend in sugar concentration closely follows the pattern of osmotic adjustment by the cells in a culture cycle (Fig. 2) and may be related to the availability of carbohydrate in the medium at these stages of growth. During the course of the growth cycle, ionic solutes may replace organic solutes as sugars are depleted from the medium and metabolized as sources of energy (MOTT & STEWARD 1972).

Organic acids accumulated appreciably only in unadapted cells and cells adapted to < 170 meq l^{-1} NaCl salinity. Of the seven organic acids

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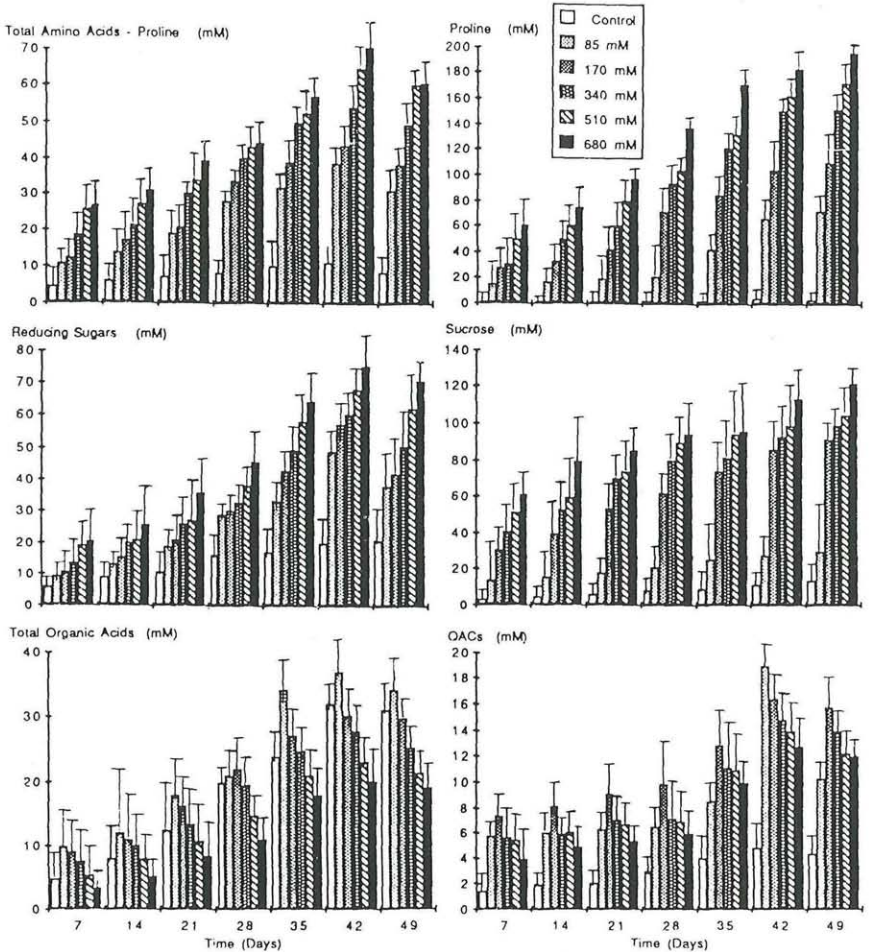

Fig. 2. Total free amino acids, free proline, total reducing sugar, sucrose, total organic acids and quaternary ammonium compound concentrations of unadapted cells and cells adapted to 85, 170, 340, 510 and 680 mM NaCl.
Solute concentrations measured through a culture growth cycle are plotted as a function of days after Inoculation.
Each value is the mean of five observations; Vertical bars, Standard Error means.

identified (Table 1) citrate and malate were the only ones to accumulate in excess of 1 mM and represented the major components of the free organic acid pool.

Total free amino acid levels increased with cell osmotic adjustment both as a function of the level of adaptation and the stage of the culture growth cycle (Fig. 2), with alanine, glutamine, α-amino butyrate, and particularly proline (data not shown) being predominant. The growth cycle

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Table 1.

Organic acid concentrations (mM) are the averages of data collected through a culture growth cycle for unadapted cells and cells adapted to 85, 170, 340, 510 and 680 mM NaCl

Organic acid (mM)	NaCl (mM)					
	0	85	170	340	510	680
Citrate	3.74	5.13	3.40	2.40	2.00	1.75
Isocitrate	0.03	0.07	0.0	0.05	0.04	0.02
Malate	13.10	15.87	14.90	13.81	10.96	8.96
Maleate	0.77	0.87	0.8	0.79	0.70	0.60
Malonate	0:31	0.58	0.50	0.43	0.40	0.32
Succinate	0.46	0.44	0.40	0.32	0.30	0.14
Tartrate	0.33	0.62	0.59	0.41	0.40	0.24
Total	18.74	23.56	20.63	18.21	14.80	12.03

related accumulation of free amino acid could be partly attributed to the availability of amino acids from 1% (w/v) casein hydrolysate which was routinely included in the medium. Further experiments with cells grown in the absence of external casein hydrolysate have established that salt adapted cells also accumulate high levels of proline as a function of ψ_π (data not shown), indicating that proline accumulation in these cells is not solely a function of altered transport but must result from alterations in proline metabolism.

On average, proline represented about 17% of the total free amino acid pool in the unadapted cells and about 75% of the pool in cells adapted to 680 mM NaCl (Table 2). Intracellular proline concentration was correlated positively with ψ_π; the relationship is logarithmic for unadapted cells and linear for cells adapted to salt (Fig. 2). The relationship between proline concentration and ψ_π for unadapted cells is indicative that proline accumulation is a component of osmotic adjustment and that proline does not accumulate solely in response to osmotic stress. This does not preclude a significant role for proline in osmotic stress adaptation. It appears that proline accumulation does not commence only at a threshold level of adaptation (WEIMBERG & al. 1984). In the absence of a way to obtain cells with zero ψ_π however, such a conclusion is by no means definitive.

Although there was a small increase in the levels of Quaternary Ammonium Compounds (QACs) in cells adapted to NaCl (Fig. 2 and Table 2), the concentrations of these compounds were considerably lower than the measured concentrations of sugars and proline. Thus the contribution of QACs to osmotic adjustment in salt tolerant cells must be small if they are uniformly distributed within the cell. The identity of the QACs in soybean

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Table 2.

Growth and solute concentrations are the averages of data collected through a culture growth cycle for unadapted cells and cells adapted to 85, 170, 340, 510 and 680 mM NaCl.

Growth and solute conc.	NaCl (mM)					
	0	85	170	340	510	680
F. W.	6.04	5.02	3.02	2.18	1.69	1.21
D. W.	0.43	0.42	0.30	0.25	0.22	0.16
Succulence	14.10	12.00	10.10	8.72	7.68	7.56
DMC	7.12	8.37	9.93	11.47	13.02	13.22
K^+	94.54	78.79	76.34	50.17	37.30	27.46
Na^+	28.12	210.57	337.31	372.14	410.40	444.72
K^+/Na^+	3.36	0.37	0.23	0.14	0.09	0.06
Cl^-	5.87	90.06	238.00	303.63	343.96	392.00
NO_3^-	16.86	26.40	21.13	17.49	14.39	12.00
TAA-P	7.80	24.61	29.16	37.47	43.74	46.83
P	1.65	35.72	66.94	93.79	108.02	130.70
TOA	18.74	23.56	20.63	18.21	14.81	12.03
QAC	3.04	8.87	11.29	9.34	8.84	7.79
R. S.	14.07	27.07	31.20	35.97	41.74	48.05
S	7.68	21.34	62.30	73.76	81.53	92.60
$\psi\pi$	9.22	26.70	45.63	63.39	85.67	126.20
ψw	3.60	13.74	19.23	26.59	34.14	40.90

F. W. = fresh weight (g/25 ml); D. W. = dry weight (g/25 ml); Succulence = F. W./D. W.; DMC = (D. W./F. W.) × 100; K^+, Na^+, Cl^-, NO_3^- = mM; K^+/Na^+ = Ratio; TAA-P = total amino acid-Proline; P = Proline; TOA = total oganic acid; QAC = quaternary ammonium compound; R. S. = reducing sugar; S = Sucrose; $\psi\pi$, ψw = −bars.

cells is currently being investigated; preliminary results suggest that these compounds are not betaines (e. g. glycine betaine or proline betaine), but rather choline and/or phosphoryl choline (data not shown). Many plant species, particularly halophytes and genotypes in the *Chenopodiaceae* and *Gramineae* accumulate large quantities of QACs, particularly glycine betaine in response to osmotic stress such as salt and water deficits (HANSON & al. 1985; WYN JONES & GORHAM 1983). These QACs are thought to play an important role in osmotic stress adaptation. This could be due to either the inherent lack of choline oxidizing activity in tobacco or the plastid localization of this activity and light dependence of glycine betaine synthesis (HANSON & al. 1985).

Measurements of polyamines in salt adapted cells indicate that some small changes in the levels of these compounds may have occurred in response to salinity. However, polyamines did not accumulate in sufficient

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quantities to make a significant contribution to the osmotic adjustment of the cells (data not presented).

Ion Accumulation: Based on the measurements of solute concentrations, salinity adaptation involves the accumulation of Na^+ and Cl^- as the major contributors to osmotic adjustment (Table 2). Collectively the other solutes contributed less than half that of Na^+ and Cl^-. The contribution of K^+ to the ψ_π declined 5-fold and may be indicative of a shift from osmotic and metabolic roles in unadapted cells to solely a metabolic function in adapted cells. Conversely whereas the concentration of Na^+ increased substantially with the level of adaptation the concentration of this ion to ψ_π remained over a relatively wide range of salinity. While the concentrations of individual organic solutes increased significantly, their relative contribution to whole cell osmotic adjustment failed to increase substantially, largely due to the magnitude of the changes in the ψ_π values. Their relative contribution to adaptation however, cannot be assessed without knowledge of their function in stress responses.

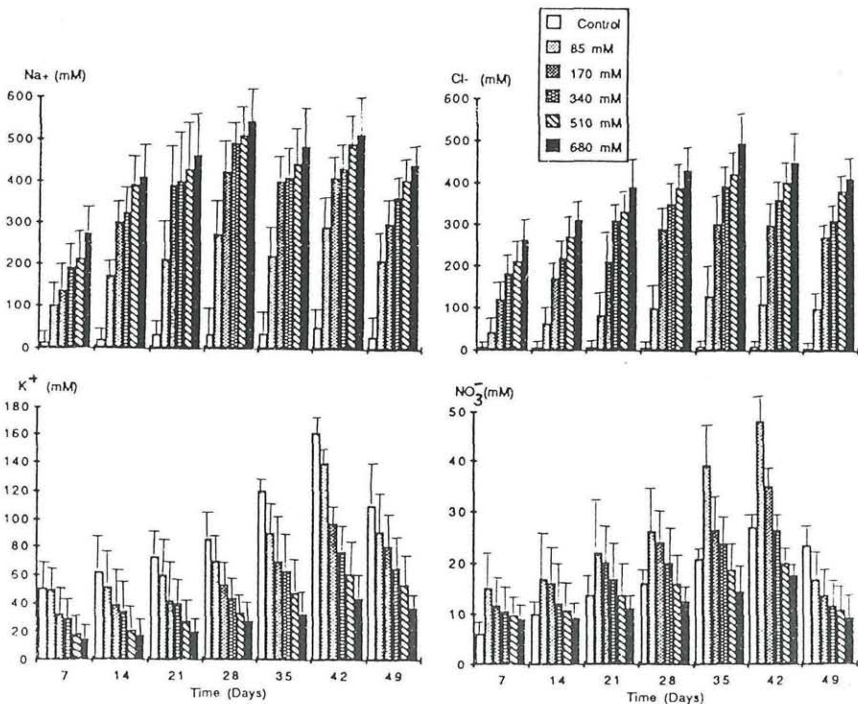

Fig. 3. The concentrations of Na^+, Cl^-, K^+ and NO_3^- of unadapted cells and cells adapted to 85, 170, 340, 510 and 680 mM NaCl.
Solute concentrations measured through a culture growth cycle are plotted as a function of days after inoculation.
Each value is the mean of five observations; Vertical bars, Standrd Error means.

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Salt adapted soybean cells accumulated both Na^+ and Cl^- as principal solutes for osmotic adjustment (Fig. 3). Although Cl^- accumulation increased proportionately with a decrease in ψ_π as did Na^+, the concentration of the latter always exceeded that of Cl^- (Fig. 3). NO_3^- did not appear to function as the counterion for the Na^+ in the salt adapted cells (Fig. 3). Similar imbalances of inorganic cation and anion accumulation have been observed for a number of halophytic species (FLOWERS & al. 1977). Since the concentrations of Na^+ and Cl^- accumulated by the salt adapted cells, particularly at high levels of NaCl, are considered to be inhibitory to cytoplasmic metabolic function, it is likely that vacuolar compartmentation of these ions is occurring (FLOWERS, TROKE & YEO 1977, GREENWAY & MUNNS 1980, WYNN JONES & GORHAM 1983, YEO 1983). The contribution of Na^+ and Cl^- in relation to the total D. W. was similar for cells adapted to varying levels of NaCl (Table 2), a phenomenon which is common to many halophytes over extreme ranges of salinity (GLENN & O'LEARY 1984).

The concentrations of Na^+ and Cl^- (Fig. 3) did not increase during the peroid of most active osmotic adjustment which occurs in the phase of the growth cycle just prior to and during the initial period of maximal fresh weight gain (Table 2). Thus, although accumulation of Na^+ and Cl^- was fundamental to adaptation of NaCl, these ions did not appear to function as the principal solutes for the osmotic adjustment which occurred during the growth cycle. If the Na^+ and Cl^- accumulated by the cells is compartmentalized in the vacuole, however, the observed changes in the whole cell salt concentration could be mediated in part by smaller (characteristic of rapidly dividing cells) vacuolar volumes (MOTT & STEWARD 1972).

The average concentrations of K^+ in the cells adapted to varying levels of NaCl were lower than that of the unadapted cells (Fig. 3). The reduced concentrations of K^+ in the salt adapted cells may be indicative of a reduced concentration of K^+ in the vacuole rather than in the cytoplasm. The accumulation of K^+ in glycophytes is often inhibited in response to salinity (GREENWAY & MUNNS 1980, JESCHKE 1984) due to a decreased K^+/Na^+ ratio. The resulting K^+ deficiencies have been implicated as a contributing factor in the deleterious effects of salinity on plant growth. WATAD & al. (1983) observed that salt adapted cells maintain higher concentrations of K^+ in the presence of high external Na^+ concentrations than do unadapted cells. The ability of salt adapted cells to maintain a constant K^+/Na^+ ratio (Table 2) under increasing salinity indicates that the cells were able to preferentially absorb K^+ under conditions of decreasing K^+/Na^+. The data presented here indicates that uptake of K^+ was competitively reduced due to salinity (NaCl), confirming the observations of DAS & MEHROTRA (1971), HASSON-PORATH, KAHONE & POLJAKOFF-MAYBER (1972), MATAR, DOESING & MARCHNER (1975), LESSANI & MARSCHNER (1978) and CHAVAN & KARADGE (1980). This contrasts with WATAD & al. (1983) who observed that salt adapted cells

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maintain higher concentrations of K^+ in the presence of high external Na^+ concentrations than do unadapted cells.

Ion exclusion is often considered to be a mechanism used by glycophytes to tolerate NaCl, particularly at moderate levels of salinity (BEN-HAYYIM, SPIEGEL-ROY & NEUMANN 1985, FLOWERS, TROKE & YEO 1977, GREENWAY & MUNNS 1980; SACHER, STAPLES & ROBINSON 1982). There appears to be genetic variability for the ability to exclude NaCl within glycophytes species with greater exclusion being correlated with greater tolerance (SACHER, STAPLES & ROBINSON 1982, WYN JONES, GORHAM & MCDONNELL 1984). In general, these observations have been interpreted to indicate that mechanisms for ion accumulation and presumably vacuolar compartmentation are not traits which are inherently efficient in glycophytes and may be one of the reasons for their relative susceptibility to saline environments. Although soybean is considered to be a glycophyte, when grown in culture the cells adapt to salinity in a manner similar to halophytes, utlizing Na^+ and Cl^- as primary osmotica and exhibiting a positive correlation between tolerance and intracellular concentrations of Na^+ and Cl^- (Fig. 2). The degree of accumulation of Na^+ and Cl^- by tissues of soybean after adaptation to high (> 200 mM NaCl) levels of salinity is undetermined, although tissues of tobacco plants do accumulate quite high levels of these ions after relatively brief periods of exposure to moderate (< 200 mM NaCl) salinity (FLOWERS, FLOWERS & GREENWAY 1986). In the presence of external NaCl, smaller accumulation of Na^+ and Cl^- occurred in the NaCl- unadapted compared to NaCl-adapted cell lines. While it is difficult to determine which of these ions is phytotoxic, it is known that the inhibitory effect of Na_2SO_4 is similar to that of NaCl (BEN-HAYYIM & KOCHA 1982), indicating toxicity of enhanced Na^+ rat her than Cl^-. BEN-HAYYIM & KOCHBA (1983) reported that lack of a clear correlation between growth and internal K^+ concentration did not permit identification of a growth inhibitory level of any specific ion.

The contribution of Na^+ and Cl^- to cell D. W. did not increase proportionately to the level of NaCl to which the cells were adapted (Table 2). BINZEL & al. (1985) and HASEGAWA & al. (1987) reported that the water content of cells declines during adaptation to salinity, resulting in as much as a 5-fold reduction in cell volume (even though increased water content is a common response when glycophytes are exposed to salt). Thus the accumulation of high concentrations of Na^+ and Cl^- (Table 2) may be facililated by the limited expansion of salt adapted cells.

It is conceivable that smaller cells inherently would have an increased capacity to accumulate ions, perhaps mediated by enhanced transport capabilities conferred to them as a function of their plasma membrane and tonoplast surface area to volume ratios. If the transport capacity of a cell is determined very early in its ontogeny following cell division, then greater ion transport efficiency would be intrinsic to small cells. By remaining

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smaller the transport capacity per unit area of the plasma membrane or tonoplast of adapted cells would not be reduced as the cell or vacuole expanded. Perhaps more importantly the surface area of the tonoplast of adapted cells has apparently increased substantially as evidenced by the appearance of numerous trans-vacuolar strands (BINZEL & al. 1985) and these changes could significantly alter the rate at which the cells transport ions into the vacuole.

Apparently the osmotic adjustment which commences after inoculation into fresh medium (Fig. 1) is inherently different from that which occurs in response to decreasing ψ_w values of the culture media. The osmotic adjustment necessary to compensate for the saline environment is accomplished largely through the accumulation of Na^+ and Cl^-. Consequently, the concentrations of Na^+ and Cl^- correlated well with ψ_π values when cells from different levels of salinity are compared. At a given salinity level, however, changes in ψ_π which occur during the growth cycle are due mainly to changes in the concentrations of organic solutes, especially sugars and proline. This is not to say that ions do not play a role in cell expansion since their whole cell concentrations increase during the expansion phase of the growth cycle indicating that they must be accumulating in the cells.

Changes in the levels of osmotic solutes while occur during the culture growth cycle and their relative contribution to the ψ_π may be partly a consequence of the ontological state of the cells. During cell division and the initial stages of cell expansion, accumulation of solutes in the cytoplasm would cause its enlargement. The later stages of cell enlargement would involve expansion of the vacuole through accumulation of ions in this compartment. This pattern of accumulation of organic solutes in dividing cells and accumulation of ions in expanding cells is similar to the accumulation of organic solutes in the dividing cells of the shoot meristem and ions in the expanding cells of leaves of plants under saline conditions (RHODES & al. 1981).

Unlike certain algal species which utilize a single osmoticum, soybean cells accumulate several organic solutes along with Na^+ and Cl^- in response to NaCl stress. Apparently multiple physiological and metabolic changes are induced by external NaCl which result in the accumulation of various solutes, supporting the concept that adaptation of NaCl is genetically complex. The mechanism by which plant cells perceive a signal of an osmotic change in their environment and then transduce the signal into an apparently complex response such as the accumulation of several solutes is unknown. While a reduction in ψ_π might be an initial signal, it is unclear how a change in ψ_π could itself directly regulate solute accumulation, instead chances in ψ_π may initiate a chemical signal or series of signals which are involved in the regulation of solute accumulation (CRAM 1976). It is not inconceivable that a simple primary response to an altered osmotic

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environment induces several other responses. Therefore, the genetic basis of the primary response may not be very complex.

Additional experimentation is necessary to verify the assumption that Na^+ and Cl^- are compartmentalized in the vacuole and to ascertain and characterize the plasma membrane and tonoplast transport mechanisms which permit salt adopted cells to accumulate and compartmentalize Na^+ and Cl^-. Furthermore, it appears that reduced cell expansion is not a constraint caused by exposure to high levels of NaCl but rather is induced by NaCl and may in fact play a functional role in osmotic adaptation. Since the inhibition of growth by osmotic stress is of fundamental importance to crop productivity the precise role of growth reduction in the adaptation process merits further investigation.

These studies show that the response of soybean cell suspension culture to NaCl treatments involved the following effects: (1) The rate of growth (F. W. & D. W.) decreased except at the 85 mM NaCl concentration (2) Succulence decreased but D. M. C. content increased with increased salinity (3) Na^+ and Cl^- concentrations in soybean cell suspension increased, whereas K^+ and NO_3^- decreased. (4) Total amino acid and proline contents accumulated under NaCl concentrations. The reductions in F. W., D. W. and succulence but increased D. M. C. contents under saline stress (NaCl) may be due to the expenditure of energy on the synthesis of organic or inorganic solutes for osmotic adjustment rather than for growth. The increase in D. M. C. may occur because soybean cell suspension grown in saline medium tends to accumulate high levels of salt and an osmotic adjustment is required to maintain a cell water content higher than the external medium. The accumulation of the organic solutes and proline under saline stress may provide an alternative source of solutes in order to adjust osmotically to the conditions of the external environment.

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