Sultes Contributing to Osmotic Adjustment in Cultured Plant Cells Adapted to Water Stress

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ABSTRACT

Osmotic adjustment was studied in cultured cells of tomato (Lycopersicon esculentum Mill cv VFNT-Cherry) adapted to different levels of external water potential ranging from -4 bar to -28 bar. The intracellular concentrations of reducing sugars, total free amino acids, proline, malate, citrate, quaternary ammonium compounds, K+, NO3-, Na+, and Cl- increased with decreasing external water potential. At any given level of adaptation, the maximum contribution to osmotic potential was from reducing sugars followed by potassium ions. The sucrose levels in the cells were 3- to 6-fold lower than reducing sugar levels and did not increase beyond those observed in cells adapted to -16 bar water potential. Concentrations of total free amino acids were 4- to 5-fold higher in adapted cells. Soluble protein levels declined in the adapted cell lines, but the total reduced nitrogen was not significantly different after adaptation. Uptake of nitrogen (as NH4+ or NO3-) from the media was similar for adapted and unadapted cells. Although the level of quaternary ammonium compounds was higher in the nonadapted cells than that of free proline, free proline increased as much as 500-fold compared to only a 2- to 3-fold increase observed for quaternary ammonium compounds. Although osmotic adjustment after adaptation was substantial (up to -36 bar), fresh weight (volume increase) was restricted by as much as 50% in the adapted cells. Altered metabolite partitioning was evidenced by an increase in the soluble sugars and soluble nitrogen in adapted cells which occurred at the expense of incorporation of sugar into cell walls and nitrogen into protein. Data indicate that the relative importance of a given solute to osmotic adjustment may change depending on the level of adaptation.

Osmotic adjustment in plants has been observed and studied for many years (38). Apart from the recognition of osmotic adjustment as an integral part of higher plant cell growth, much interest in the role of osmotic adjustment in water and salt stress tolerance has developed in recent years (12). It is generally known that, in the absence of various avoidance mechanisms, plant cells which actually experience desiccation to the point of turgor loss must regain turgor through osmotic adjustment to resume growth. In spite of the obvious importance of osmotic adjustment to the maintenance of growth under many circumstances of either osmotic or desiccation stress there is relatively little information on the physiological and biochemical nature of osmotic

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equilibrium with the medium to which they are adapted, the ψ of the cells was taken to be equal to that of the medium. The ψ of the latter was measured by using a Wescor model HR 33T hygrometer with model C52 thermocouple psychrometer chambers. Osmotic potential of the cells was measured by the method of incipient plasmolysis as described previously (12). Incipient plasmolysis is defined as occurring when 50% of the cells were visibly plasmolyzed.

Relative Partitioning of Glucose into Osmotic and Cell Wall Pools. Unadapted cells and cells adapted to 25% PEG were grown for approximately 10 generations in medium containing 30 g/l glucose as the carbon source instead of sucrose. No differences in growth in medium with glucose were observed. These cells were then subcultured into 50-ml Erlenmeyer flasks containing 10 ml of appropriate medium containing 30 g/l glucose. Cells were incubated until mid-log phase, and then 100 μl of filter-sterilized [U-14C]glucose (260 mCi/mmol) in the appropriate medium was added to a final specific activity of 4.2 μCi/μmol. Cells were incubated for up to 3 d, then filtered through GF/F glass fiber filter paper. Samples of the culture medium were assayed for glucose uptake. The unadapted cells and cells adapted to 25% PEG were rinsed with 5 ml of deionized H2O or with 0.9 m mannitol, respectively. After weighing, the cells were homogenized and incubated in 10 ml of 80% ethanol (v/v) at 70°C for 20 min. Cell wall material and cell debris was collected by centrifugation at 5,200 g for 10 min and the supernatant was decanted. The debris was re-extracted with 80% ethanol as before, and the supernatants were combined and taken to dryness under reduced pressure. The dry residue was dissolved in 5.0 ml water. Cell walls were purified according to Carpita et al. (5), and noncellulosic polymers in lyophilized cell walls were hydrolyzed in acetic-nitric acid according to Udepack (39). Unhydrolyzed cellulose was washed twice in deionized H2O, and both cellulose and aqueous samples were suspended in ACS aqueous counting solution (Amersham) and radioactivity was determined by liquid scintillation spectrometry.

Preparation of Samples for Solute Analysis. Cells were harvested by filtering on a Büchner funnel and washed with isotonic solutions of mannitol. After fresh weight determination, 100 to 200 mg of cells were quickly placed in 2 ml of ice-cold MCW2 (12:5:3, v/v/v) and stored at −20°C prior to proline and QAC analyses. Cells used for other solute analyses were frozen, lyophilized, and stored at −20°C.

N Determination. Total reduced N in cells, and NH4+ and NO3− present in media, were measured by the Kjeldahl method (1). For total reduced N in cells, 50 to 100 mg of lyophilized cells from each sample were digested in H2SO4 and H2O2. Five ml samples were taken directly from the media for NH4+ and NO3− estimation.

Protein Estimation. Ninety to 100 mg fresh weight of cells from each sample were homogenized in 50 mm K-phosphate (pH 7.5). Homogenates were centrifuged at 30,000g for 10 min. The soluble proteins were precipitated with 10% TCA. The protein precipitates were redissolved in 0.1 m NaOH and the amount of protein was measured by the method of Lowry et al. (23).

Solute Assays. For sugars and amino acids, 50 mg lyophilized cells were extracted three times for 30 min at 70°C with 2.5 ml of 80% ethanol. The pooled extracts were evaporated under reduced pressure and the residue was redissolved in 100 mM Na acetate buffer (pH 5.0) for total amino acids or in Beckman Physiological buffer for individual amino acids. Reducing sugars were analyzed by the modified version of Nelson (29) as described by Somogyi (34). Sucrose was measured by assaying for reducing sugars after hydrolysis with invertase.

Alpha-amino nitrogen was determined according to Rosen (31) using glycine standards. Levels of other individual amino acids were measured with a Beckmann amino acid analyzer.

Proline was extracted and assayed according to Singh et al. (33) except the permittus step was omitted as it was found unnecessary. Cells in MCW were homogenized twice in 2 ml of ice-cold MCW, and the pooled supernatants were extracted with 2.5 ml chloroform (CHCl3) and water (2:3, v/v) at 4°C overnight. The aqueous layer was evaporated to dryness and redissolved in water before measurement of proline and QAC.

Organic acids were extracted by homogenization in 3 ml of MCW (1:1:1, v/v/v) per 100 mg of lyophilized cells. If necessary, methanol was added until a single phase was obtained and the samples were then incubated at 45°C for 30 min. One ml each of chloroform and water was then added and the aqueous phase was removed. The organic phase was extracted with another 2 ml of water. The pooled aqueous phases were partitioned with equal volumes of chloroform and the aqueous phase was purified and assayed for organic acids with GC by the method of Stumpf and Burris (37) except that the samples were derivatized after drying over Na2O2 for at least 24 h. [6-14C]Succinate was added prior to extraction to measure recovery and maleate was used as the internal standard for GC. Trimethyl silylated acids were separated on a 12.5-m OV-1 vitreous silica capillary column (Hewlett-Packard) with a Hewlett-Packard 5790 gas chromatograph. Oven temperature was programmed for 120°C for the first 7 min, then increased (45°C/min) to 200°C. Column N2 carrier gas flow was 1 ml min−1 with a 1:100 split ratio. Some samples of organic acids were subjected to electron impact MS after similar chromatography with a Finnigan/MAT 9610 gas chromatograph coupled to a Finnigan-MAT 4021 quadrupole mass spectrometer interfaced to a Finnigan/MAT 2100 C INCOS data systems at 70 ev and a source temperature of 200°C. Mass spectra of peak fractions of samples were identified by comparison to published spectra.

Nitrate and chloride ions were extracted by incubating 100 mg of the lyophilized cells in 10 ml water at 40°C for 30 min. The cell material was removed by centrifugation and extracted as before, and the supernatants were pooled. Nitrate was measured in 100-μl fractions of this supernatant after conversion into nitrite by using freshly extracted nitrate reductase from frozen soybean nodules according to Lowe and Hamilton (22).

Chloride was estimated from the same supernatant according to the method of Schales and Schales (32) with reagent kits from Sigma Chemical Co.

Fifty to 100 mg of the lyophilized cells were solubilized in concentrated H2SO4, prior to measurement of Na+ and K+ ions by atomic emission spectrophotometry with a Micro Tek UniCam SP90 Atomic absorption/emission spectrophotometer, using NaCl and KCl as standards.

QAC was measured by the method of Storey and Wyn Jones (35), but the individual QACs were not resolved. KI + I2 reagent (0.2 ml) was added to the sample (0.5 ml of the MCW extract) and the mixture was agitated in an ice water bath for 90 min. Two ml of ice-cold H2O and 10 ml of ice-cold ethylene dichloride were added, and the two layers were mixed under a stream of N2 for 5 min. The absorbance of the lower layer was read at 365 nm within 10 min (with dilution if necessary). Glycine betaine was used as the standard.

Determination of the Contribution of Extracellular Water to Fresh Weights and of Mannitol to Dry Weights. The solute concentrations and dry weights obtained as described above were corrected for the presence of extracellular water in fresh weights using inulin-[6-14C]carboxylic acid (19), and for differential amounts of mannitol remaining on the lyophilized cells, using [14C]mannitol since the cells were washed with different concen-
trations of mannitol. To determine the amounts of extracellular water, 0.5 μCi of insulin-[14C]carboxylic acid was added to 50 ml of cell suspension of each individual cell line (P0, P20, P25, and P30). After 5 h of incubation on a reciprocal shaker, different volumes (containing 0.4-2.0 g cells) of cell suspensions were filtered through Whatman No. 4 filter paper. The filtered cells were washed twice with mannitol solutions which were isotonic with the medium to which the cells were adapted. The mannitol solution used for washing contained insulin-[14C]carboxylic acid at the same specific radioactivity (0.01 μCi/ml) used during incubation of cell suspensions with insulin-[14C]carboxylic acid. Washed cells were lyophilized and weighed to determine the apparent dry weight to fresh weight ratio. A known amount of the lyophilized cells (5-15 mg) from each sample was transferred to scintillation vials and 0.5 ml water was added to each vial before adding 4.5 ml dioxane based scintillation fluid. The addition of water allowed a better distribution of the sample within the scintillation fluid and resulted in improved efficiency of radioactivity measurement. The cpm of all samples were quench corrected to determine dpm. Using the dpm of insulin-[14C]carboxylic acid per mg dry weight and the specific radioactivity of insulin-[14C]carboxylic acid (0.5 μCi/50 ml), the average extracellular water contents per mg dry weight of cells (±SD) were calculated from three experiments and found to be 8.41% ± 0.73, 15.70% ± 1.31, 27.64% ± 3.13, and 22.65% ± 2.61 of the total cell water for the P0, P20, P25, and P30 lines, respectively.

To determine the contribution of mannitol to the dry weight of cells, the isotonic solutions of mannitol used for washing contained [3H]mannitol (2 μCi/100 ml). To reproduce conditions that were used for the preparation of samples for solute analyses, the cells were not preincubated with [3H]mannitol before filtration and washing. The cpm of all samples were quench corrected to determine dpm. Using the specific radioactivity of [3H]mannitol used for washing and the dpm of [3H]mannitol per dry weight, the average contributions of mannitol to the dry weights (±SD) were calculated from three experiments and found to be 9.29% ± 0.14; 17.27% ± 5.78, 28.30% ± 2.76, and 21.78% ± 2.93 for P0, P20, P25, and P30 cell lines, respectively.

RESULTS

Growth and Water Relations. Nonadapted cells exhibited a typical growth curve upon transfer to fresh medium, i.e., a lag phase of 3 d, followed by a rapid growth phase until about 16 d when the cells entered stationary phase (Fig. 1, A and B). The cell lines which are adapted and grow in medium with low water potentials, i.e., −16, −22, and −28 bar, show similar patterns of growth, but tend to gain fresh weight at reduced rates compared to unadapted cells (Fig. 1A). The final fresh weight gain in the adapted cell lines was reduced in comparison with that in the unadapted cell line and the decrease in growth was proportional to the level of adaptation (Fig. 2A). However, the final dry weight gain at stationary phase (10.26 ± 0.45 g l−1) was similar for all cell lines (Fig. 1B). This indicates that the cell lines were adapted to the stress, i.e., the cells were not actually in a state of stress with respect to dry weight accumulation.

The ψw, ψs, and ψf of the nonadapted and the adapted cell lines changed during the growth cycles (Ref. 4; Fig. 3). The ψs were most negative during the exponential phase of growth, becoming as low as −14 bar for the P0 cells and −60 bar for the P30 cells. At any point during the growth cycle, the ψs of the cells was more negative than the water potential of the medium, and so positive turgor always was maintained in all of the cell lines (Fig. 3A) also indicating the lack of significant stress to the adapted cells. Thus, in the cells adapted to media with ψw values of −4 bar (0% PEG), −16 bar (20% PEG), −22 bar (25% PEG), and −28 bar (30% PEG), the average ψw values were −11, −22, −31, and −47 bar, respectively. The average ψw values of the cells increased

Fig. 1. Dry weight and fresh weight growth characteristics of adapted and nonadapted cell lines as a function of growth cycle stage. Cells adapted to growth in the presence of 0, 20, 25, and 30% PEG were inoculated into 2 l of the respective medium to which they were adapted. The cells were harvested on the days indicated and samples were used for all of the analyses reported. (O), 0% PEG; (△), 20% PEG; (□), 25% PEG; (●), 30% PEG.
fraction (Fig. 4A) to that incorporated into the cell wall fraction (Fig. 4B) was 2 to 3 times higher in the adapted cells compared to unadapted cells. These results represent only logarithmically growing cells and the partitioning of carbon may change at different growth stages. Uptake of both NO$_3^-$ and NH$_4^+$ was similar in adapted and unadapted cells (Fig. 5, D and F) but endogenous levels of NO$_3^-$ (Fig. 6, E and F) and NH$_4^+$ (data not shown) are higher in adapted cells while soluble protein levels in adapted cells are lower (Fig. 5A). This altered partitioning of carbon and nitrogen also reflects the increase in the concentration of cell solutes in general which is associated with osmotic adjustment.

**Inorganic Solute Levels.** Unlike halophytes (19, 20) or cultured cells adapted to NaCl (14) which accumulate large concentrations of Na$^+$ and Cl$^-$ ions in order to adjust the cellular $\psi_s$, cell lines adapted to PEG-induced water stress would not be expected to do so simply because the amount of inorganic solutes available in the nutrient medium is not sufficient to allow high levels of adjustment. However, it appears that some significant accumulation of ions does occur in these cell lines (Fig. 6). The unadapted cells and P30 cells accumulated 8 to 10 mM Na$^+$ against the low concentration (less than 1 mM) in the medium, but these concentrations did not vary much over the growth cycle (Fig. 6, A and B). The concentration of Na$^+$ increased by 3- to 5-fold in P25 and P30 cells, respectively (Fig. 6, A and B). Cells accumulated substantially more K$^+$ (40–200 mM) than Na$^+$ at similar levels of adaptation (Fig. 6, C and D). In contrast to Na$^+$, the K$^+$ concentrations in the cells increased proportionally as the cells were adapted to higher levels of stress (Fig. 6C) and the changes in the concentrations through the growth cycle were similar to changes in the $\psi_s$ of the cells (Figs. 3, C and 6D), thus indicating a relatively more important role for K$^+$ ions in osmotic adjustment of these cells. The amounts of Cl$^-$ which accumulated were similar to the amounts of Na$^+$ which accumulated at the respective level of adaptation (Fig. 6, G and H) and like Na$^+$, did not vary much over the growth cycle except in P30 cells. The intracellular concentration of NO$_3^-$ also increases with adaptation to higher levels of stress (Fig. 6E). Accumulation of NO$_3^-$ in the adapted cells (up to 60 mM in the P30 cells) appears to reflect a change in metabolism, e.g. decreased NO$_3^-$ reduction, since uptake of NO$_3^-$ from the medium appeared similar in all the cell lines (Fig. 5D).

**Organic Solute Levels.** The levels of several low mol wt organic compounds in each cell line were examined (Figs. 7 and 8). In most cases, the changes in the solute concentrations were similar to the changes in $\psi_s$ of cells during the growth cycle. In each cell line, the concentrations of solutes reached a maximum level during the exponential phase of growth. The concentration of reducing sugars in the cells increased with the degree of adapta-
adapted cells and total cells. B, but the levels of cells. The concentration reached as high as 600 mM in the P20 cells. The cells also accumulated some sucrose (Fig. 7, C and D) but the levels of sucrose were 3- to 8-fold lower than those of reducing sugars (Fig. 7, A and B). The sucrose concentration increased in moderately adapted P20 cells but did not increase with further adaptation to water stress.

The total free amino acid concentrations increased 2-fold in P20 cells (Fig. 7, E and F). The free amino acid content had increased by 6-fold in P25 cells although no additional increase was observed in P30 cells (Fig. 7E). Since the total reduced N in the adapted cells did not decrease significantly at the levels of adaptations examined here, it follows that the increased levels of free amino acids (Fig. 7E) must occur at the expense of proteins (Fig. 5A), either by reduced incorporation into protein (15) or by degradation of protein (8).

Increases in proline content were more marked (Fig. 7, G and H) than of any other solute examined. Below a certain threshold (corresponding to \( \psi \) of \(-11 \) bar), proline levels increased rapidly with increasing levels of adaptation (Fig. 7G). Thus, the proline level in the nonadapted cell line did not exceed 0.65 mM at any time during the cell growth cycle whereas the maximum concentration reached as high as 10, 29, and 97 mM in P20, P25, and P30 cells, respectively (Fig. 7H). The per cent increases in free proline were higher than the per cent increases observed in total free ninhydrin positive amino acids. The average per cent of free proline in total free amino acids increased from 0.7% in the P0 cell line to 44.7% in the P30 cell line (Table I). This suggests that the mechanism by which free proline is generated in the adapted cell line may be independent of that which regulates the level of other free amino acids.

Apart from proline, increases were observed in the average levels of serine, glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine, \( \gamma \)-amino butyric acid, lysine, histidine (Table I), and also in free ammonia (not shown). The increases ranged from 2- to 30-fold, but apart from proline, only the concentration of \( \gamma \)-amino butyric acid rose to comparatively high levels (up to 33 mM). In addition to the increases observed in the individual amino acids, decreases were observed in the concentrations of glutamate and glutamine (2- to 3-fold, respectively) in the adapted cell lines (Table I). Our results are consistent with data that indicate that glutamate is the precursor to proline. It has been demonstrated that \([{^4}\text{C}]\) glutamate is converted into proline in detached leaves of tobacco which have been subjected to water stress (2). The observed levels of glutamate or glutamine in unadapted cells were not high enough to account for the levels of proline observed in adapted cells. Pool sizes of amino acids do not reflect the fluxes through the pools however. Even though total levels of glutamine decreased our data suggest that increased synthesis of proline from glutamine or through other biosynthetic pathways, i.e. via arginine and ornithine, occurs at higher levels of adaptation. GABA is generally formed by the decarboxylation of glutamate (25) or from glutamine (e.g. in rice leaves) and it is possible that the high levels of GABA observed in adapted cells result from accelerated decarboxylation of glutamate. Streeter and Thompson (36) have speculated that the accumulation of GABA under anaerobic conditions is due to the acceleration of glutamate decarboxylation as well as to the arrest of GABA transamination.

Malate and citrate were the only organic acids found in significant levels. In a given cell line, the concentrations of these two organic anions were similar and increased as the cells became adapted to increasing levels of water stress (Fig. 8, A and B). Some other compounds (mainly sugars) besides malate and citrate were identified by mass spectrometric analyses but none occurred in significant concentrations.

A small but significant increase was observed in levels of QAC (Fig. 8C) with increasing levels of adaptation. Although in the nonadapted cell line, the average level of QAC was almost 10-fold higher than that of proline, the process of adaptation to low external \( \psi \) did not bring about the large increases in QAC as observed for proline accumulation (2- to 3-fold for QACs as compared to 500-fold for proline; Figs. 7, G and H, and 8C; Table I).

**DISCUSSION**

The present results as well as earlier results in this laboratory (4) show that tomato cells in suspension culture have large capacities for osmotic adjustment when subjected to water stress. Osmotic potential changes can result from both active and passive accumulation of solutes (38). It appears that the cultured cells of tomato undergo active osmotic adjustment because of the observed maintenance and increase in turgor during the adjustment (Figs. 2 and 3). Moreover, the fact that upon transfer to fresh medium, the cells increase their fresh weight or grow at the same time that \( \psi \), is decreasing also indicates that active solute accumulation must occur. Cell solute levels increase as the cells are adapted to increasing levels of stress (Fig. 9A), and since these accumulations are most pronounced during the exponential phase of the cell growth cycle (Fig. 9B), it follows that osmotic adjustment is more pronounced during the time the cells are growing.

It has already been shown in an earlier study (11) that PEG does not contribute to the osmotic adjustment of the cells. In
general, the concentration of all of the low mol wt solutes examined increased with the degree of adaptation, although in some cases a linear relationship between specific solute concentration and \( \psi_r \) was observed and in others it was not. The solutes which were examined in the present investigation could be divided into three categories: (a) those solutes for which the concentration did not change with a lower level of osmotic adaptation but was proportional to \( \psi_r \) at higher levels of adaptation, \textit{i.e.} Na\(^+\), proline, and QAC; (b) those solutes for which the concentration was proportional to \( \psi_r \) only at lower levels of adaptation, \textit{i.e.} sucrose and total amino acids; and (c) the majority of solutes which increased in concentration proportionally or nearly proportionally to \( \psi_r \), at all the levels of adaptation studied, \textit{i.e.} K\(^+\), NO\(_3\)\(^-\), Cl\(^-\), reducing sugars, malate, and citrate.

The maximum contribution to \( \psi_r \) was made by reducing sugars and by K\(^+\) ions. However, the relative contribution of all the inorganic ions to \( \psi_r \) did not change significantly with the increase in level of adaptation while the relative contribution to \( \psi_r \) of organic solutes changed only slightly with the exception of proline. The relative osmotic contribution of proline changed greatly (30-fold) during adaptation even though its maximum contribution to osmotic adjustment was small compared to sugars or potassium. This suggests that the levels of proline are more specifically regulated than the other solutes during adjustment to stress.

To assess the degree to which the levels of each of the solutes are regulated during the growth cycle before and after adaptation to water stress, we examined the correlations between solute levels and \( \psi_r \) values of the cells (as these values change during a culture growth cycle) before adaptation (\( P_0 \)) and after adaptation to 30% PEG (\( P_{30} \)). We found that the levels of two solutes, reducing sugars and sucrose, were nearly equally correlated with \( \psi_r \) before \( (r = 0.65 \text{ and } 0.73, \text{ respectively}) \) and after \( (r = 0.71 \text{ and } 0.85, \text{ respectively}) \) adaptation. The correlation coefficients for most of the other solutes were low before adaptation (proline, 0.51; citrate, 0.24; total amino acids, 0.50; Cl\(^-\), 0.55; K\(^+\), 0.51;
and nitrate, 0.51) but increased significantly after adaptation (proline, 0.86; citrate, 0.60; total amino acids, 0.71; Cl\(^{-}\), 0.86; K\(^{+}\), 0.85; and nitrate, 0.90). Malate and Na\(^{+}\) were exceptions where the correlation coefficient for malate decreased from 0.72 to 0.14 and Na\(^{+}\) remained low, 0.40 to 0.31. Sugars appear to be general osmotic agents, the concentrations of which are generally correlated with \(\psi_s\) adjustment and growth regardless of the levels of stress adaptation. Sugars are known to be involved in normal osmotic adjustment, i.e., those not associated with stress (16).

Many of the other solutes seem to become more correlated to the \(\psi_s\) changes associated with the cell growth cycle after substantial adaptation to stress, suggesting that increased regulation over their intracellular levels has occurred during adaptation.

Since only proline levels appear to change dramatically in terms of relative importance to overall osmotic adjustment, synthesis of proline may represent the most highly regulated metabolic event during adaptation and as such would appear to be suitable as a parameter to evaluate the degree of adaptation. However, this relationship may be applicable only to cultured cells or whole plant systems (e.g., 27), where osmotic adjustment is the only or major means of adaptation. Proline is very unlikely, as Hanson has shown (see 12) to be well correlated with the level of adaptation or tolerance in systems where tolerance or adaptation is brought about by other mechanisms, e.g., avoidance.

In earlier studies, it was shown that osmotic adjustment by tissues of whole plants was almost completely accounted for by the solutes measured. For example, in wheat (28), 60% to 100% of the decrease in \(\psi_s\) in response to decreased \(\psi\) was accounted for by the accumulation of free amino acids and sugars. Similarly, in fully expanded leaves of sorghum (18), 84 to 100% of the osmotic adjustment (7–11 bar) could be accounted for by increases in concentration of potassium, sugars, free amino acids, chloride, and carboxylic acids. Meyer and Boyer (26) showed that the growing region of the hypocotyl of soybean seedlings growing under water stress conditions underwent osmotic adjustment (5 bar), 70% of which could be explained by increases in sugars and free amino acids.

Recently, the accumulation of solutes was studied in salt-tolerant cells of *Nicotiana tabacum* (13, 14) and alfalfa (7). However, the osmotic adjustment observed in these cells was small (about 5 bar) and could be explained almost entirely (80–89%) by uptake of Na\(^{+}\) and Cl\(^{-}\) alone. It is clear from such studies that the contribution of typical solutes could almost totally account for moderate amounts of osmotic adjustment (5–11 bar).

In the present study, adaptation involving significantly greater levels of osmotic adjustment has been observed. Even after corrections were made to account for the presence of extracellular fluid, the different solutes which we measured accounted for 46% of the \(\psi_s\) in the nonadapted cells and in cells adapted to −16 bar stress, and for 65% and 56% of the \(\psi_s\) in cells adapted to −22 and −28 bar of stress, respectively. In spite of the high solute levels observed, the inability to account completely for the \(\psi_s\) of the cells could be due to (a) presence of unidentified solutes, (b) over-estimation of the \(\psi_s\) of cultured cells by the plasmolytic method used, (c) under-estimation of the nonosmotic volume, and (d) non-ideal behavior of solutes contributing to \(\psi_s\). It is clear that there are numerous solutes present in the cells for which we have not accounted. The contribution of these solutes to osmotic potential is unknown but it seems unlikely that there are other individual solutes which would make a major osmotic contribution.

We have examined sugars and sugar alcohols by GC and did not find any major compounds other than those shown which accumulated during adjustment to stress. We know that estimations of the \(\psi_s\) by the plasmolytic method yields...
values which are 10 to 20% more negative than those obtained by freezing point depression or by vapor pressure psychrometry. These other methods could be expected to yield more positive values since they allow the mixing of extracellular and intracellular fluids. However, possible errors in the plasmolytic method could result in more negative values. Since this method measures ψ₀ at zero turgor, shrinking of the cells could concentrate the cell solutes. We could not, however, measure by volume displacement any volume change in the cells during turgor loss indicating that the cell walls are quite inelastic. Separation of the plasmalemma from such rigid walls could result in a situation of considerable tension which might then delay the point of plasmolysis beyond the true ψ₀. The cells may take up salt during the incubation. However, we find that per cent plasmolysis does not decrease over the incubation period in a plasmolyzing NaCl solution. We have corrected the ψ₀ values which are calculated from solute concentrations for extracellular fluid volume only. Nonosmotic volume errors remain for the bound water and for solid structures within the cell. These would make our total solute concentration calculations low and may represent significant errors. Moreover, as shown by other workers, the nonosmotic volume may change during long term adaptation (see 38). It seems likely that the ψ₀ values obtained by psychometric and plasmolytic methods are nearly equal in reliability.

It has been proposed (20), that, as in most algae, inorganic ions may play their major role as osmotic agents in the vacuoles of higher plants whereas organic solutes may be restricted largely to the cytoplasm. It can be seen from Table II that the sums of the contributions of the inorganic ions Na⁺, K⁺, Cl⁻, and NO₃⁻ to the total measured solute concentration ranged from 34 to 45%. The contribution of organic solutes to the total measured solute concentration ranged from 55 to 66%. To be in equilibrium, the vacuolar ψ₀ (theoretically largely due to ions) should be balanced by the cytoplasmic ψ₀ (theoretically largely due to organic solutes). Assuming the vacuolar volume to be approximately 90% of the total volume, it is clear that the contribution of organic solutes to the total solute concentration is much too high to assume they are largely restricted to the smaller cytoplasmic compartment. It seems that many of the organic solutes which are present in high concentrations such as sugars may be sequestered in the vacuole. Most evidence suggests that much of the free sugar in plants is located in the vacuoles (21, 30). Potassium ions, on the other hand, are believed to be located in the cytoplasm of plant cells, e.g., marine alga Halicystis (9) in spite of high concentrations (up to 400 mM). Limited evidence suggests that proline is localized also in the cytoplasm (21). In fact, if proline was sequestered in the cytoplasm, and the cytoplasm represented 10% of the total cell volume, the concentration of proline would contribute 7 bar equivalent of osmoticum in the cytoplasm of P₂₅ cells and nearly 15 bar equivalent of osmoticum in the cytoplasm of P₀ cells which are significant contributions. Such speculations emphasize the need for further investigations on the exact distributions of the different solutes in the cell in order to assign specific roles to them.

Since there are numerous solutes which appear to accumulate significantly during adaptation of the cultured cells to water stress it will be important to know if all the solute changes observed in the adapted cells arise independently of each other or if some primary biochemical response induces changes in the level of several solutes. It is possible that such a response is transduced through changes in hormonal levels. Such changes in turn must affect N assimilation since many of the solutes accu-
Table 1. Average Intracellular Pools of Amino Acids in Tomato Cells Adapted to Medium with Different Water Potentials

Total amino acid levels were measured on a Beckman amino acid analyzer. Some amino acids, such as arginine, were present in quantities too low to detect. The values shown are the means of two measurements.

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<th>Amino Acid</th>
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<td>Threonine</td>
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</tr>
<tr>
<td>Glutamate</td>
<td>1.96</td>
</tr>
<tr>
<td>Glutamine</td>
<td>6.42</td>
</tr>
<tr>
<td>Proline</td>
<td>0.12</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.28</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.59</td>
</tr>
<tr>
<td>Valine</td>
<td>0.98</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.02</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.18</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.27</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.16</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>2.43</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.15</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.17</td>
</tr>
</tbody>
</table>

FIG. 8. Concentrations (average over the growth cycle) of citrate, malate, and quaternary ammonium compounds (QACS) in adapted and nonadapted cell line as a function of the ψᵣ, (average over the growth cycle) of each cell line. Horizontal bars represent maximum and minimum values observed during a single growth cycle. Symbols and other details are as described in Figure 1.

FIG. 9. Sum of organic and inorganic solutes (computed from Figs. 6–8) in adapted and nonadapted cell lines. A, Sum of average values of all measured solutes as a function of average ψᵣ over a single growth cycle of each cell line. Horizontal bars represent maximum and minimum values observed during the growth cycle. B, Sum of concentrations of all measured solutes as function of growth cycle stage of each cell line. Symbols and other details are as described in Figure 1.

mulated are nitrogenous solutes, e.g. NO₃⁻, proline, other amino acids like γ-amino butyric acid and QAC.

The quantitative and qualitative changes in free amino acids suggest that the cells may primarily respond to (and eventually adapt to) water stress by altering the rates of assimilation, synthesis, utilization, and interconversion of amino acids. For example, judging from the observed decrease in glutamate (Table 1), it appears that rates of reduction of glutamate to proline or of decarboxylation to γ-amino butyric acid may be affected. It is possible that the observed changes in organic acids could occur via amino acids also by pathways such as oxidative deamination. Similarly, changes in quaternary ammonium compounds can be mediated by amino acid metabolism (see 12).

In addition, there is an increase in free amino N and free sugars at the cost of a reduction in soluble protein and cell wall formation in adapted cells. Therefore, the process of conservation and/or generation of osmotic solutes such as sugars and free amino acids itself limits the synthesis of macromolecules which are likely required for growth processes. This is clearly evidenced by the fact that the adapted cells do not expand as much as the unadapted cells even though their turgor appears to be consid-
Table II. Relative Contribution of Solutes to Osmotic Potential

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Average ψ of Medium</th>
<th>Average ψ of Cells</th>
<th>Su-</th>
<th>Reducing sugars</th>
<th>Free amino acids</th>
<th>Proline</th>
<th>QAC</th>
<th>Malate</th>
<th>Citrate</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>NO₃⁻</th>
<th>CI⁻</th>
<th>Average ψ of Total Solutes</th>
<th>Average Contribution to Osmotic Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀</td>
<td>-4.0</td>
<td>-10.0</td>
<td>422</td>
<td>2.2</td>
<td>17.4</td>
<td>4.4</td>
<td>0.1</td>
<td>0.6</td>
<td>0.2</td>
<td>0.8</td>
<td>1.9</td>
<td>13.1</td>
<td>4.2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>P₀₀</td>
<td>-16.0</td>
<td>-22.0</td>
<td>901</td>
<td>4.9</td>
<td>14.4</td>
<td>3.9</td>
<td>0.4</td>
<td>0.4</td>
<td>0.9</td>
<td>1.0</td>
<td>0.9</td>
<td>13.8</td>
<td>3.2</td>
<td>1.7</td>
<td>-10.0 410</td>
</tr>
<tr>
<td>P₀₋₀₀</td>
<td>-22.0</td>
<td>-31.0</td>
<td>1270</td>
<td>2.1</td>
<td>26.3</td>
<td>8.1</td>
<td>2.1</td>
<td>1.4</td>
<td>1.3</td>
<td>1.5</td>
<td>2.4</td>
<td>12.5</td>
<td>4.5</td>
<td>2.7</td>
<td>-19.5 798</td>
</tr>
<tr>
<td>P₀₋₀₀₋₀₀</td>
<td>-28.0</td>
<td>-48.0</td>
<td>1954</td>
<td>2.0</td>
<td>20.9</td>
<td>5.4</td>
<td>3.0</td>
<td>1.4</td>
<td>1.3</td>
<td>1.3</td>
<td>2.4</td>
<td>12.2</td>
<td>3.7</td>
<td>2.2</td>
<td>-25.1 1032</td>
</tr>
</tbody>
</table>

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erably higher (Figs 2 and 3). Although many workers have shown that tissue elongation is directly related to turgor pressure (see 6, 10), our results are not necessarily contradictory to these observations. In recent years, such a lack of relationship between turgor and growth has been observed in other osmoregulating systems, e.g. the growing region of soybean hypocotyl (25) and the basal growing tissue of barley leaves (24). Changes in cell wall properties have been observed (17) in plants undergoing long term adaptation to water stress. In the present study, the adaptation of the tomato cells was also a long term process (3). It seems that insufficient turgor in adapted cells compared to unadapted cells (Fig. 3) is not the reason for the limited expansion of the adapted cells, but rather the amount of expansion/amount of turgor (elasticity and extensibility) has decreased during adaptation. A decrease in elasticity (and/or extensibility) could account for the higher turgor pressures observed here as well as the restricted growth. In view of observed differential growth response of stress-adapted and unadapted cell lines to auxins (unpublished results in this laboratory), these cell lines may also constitute an excellent system to examine the role(s) of hormones, particularly auxins, in regulating the cell wall properties which are important to stress adaptation.