Proline Accumulation and the Adaptation of Cultured Plant Cells to Water Stress

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ABSTRACT

The transfer of cultured tomato cells (Lycopersicon esculentum cv VFNT-Cherry) to a low water potential environment resulted in an increased dry weight to fresh weight ratio accompanied by a rapid accumulation of proline. Proline content continued to increase as osmotic adjustment and growth occurred. The initial increase in proline concentration was accompanied by a drop in turgor. However, proline levels continued to increase with a gain in turgor during osmotic adjustment. Thus, the accumulation of proline depended not only on cell water potential, or on the initial loss of turgor but more closely on cell osmotic potential. The ultimate level of proline depended on the level of adaptation. Proline levels remained high after more than 100 cell generations in low water potential media, but declined rapidly after transfer to media with a less negative water potential. Addition of exogenous proline to the medium during water stress and during osmotic downshock alleviated the normally resulting inhibition of growth. The results suggest a positive role for proline accumulation in adaptation of cells to changing external water potentials.

It has been suggested for some time that the often observed accumulation of proline by plant tissues during water stress is an adaptive response even though investigators have obtained varying results relating proline accumulation to enhanced survival or tolerance under stress (4, 6, 7, 13, 16, 27, 29). Although the relationship between proline levels and osmotic adjustment has rarely been determined, the most common interpretation of proline accumulation is that proline at high levels acts as a cytoplasmic osmotic solute.

The relationship between proline accumulation and osmotic adjustment of plant cells during a prolonged period of exposure to a constant low water potential (ψw) has not been determined before. Also, the water status parameter which is primarily associated with the initiation of the accumulation of proline in response to water stress has not been determined, i.e. whether it is the turgor (ψt), osmotic potential (ψw), or ψ of the cell.

In plants, correlations between water stress tolerance and any single physiological parameter, such as cell ψw, are difficult to make inasmuch as tolerance may be affected by a number of factors, such as stomatal physiology and morphology, plant and leaf size, root characteristics, etc. It is possible to circumvent such problems by using cultured plant cells where tolerance to water or salt stress must involve osmotic adjustment to prevent permanent loss of turgor and subsequent cell dehydration. Since cultured cells are in ψw equilibrium with the medium, it is possible to keep the cells at a constant ψw over an extended period of time and eliminate the possibility that changes in growth which occur under imposed low external ψ are the result of an altered balance of water availability and loss.

The objective of the present investigation was to determine the relationship between proline accumulation and the adaptation of cultured plant cells to water stress through osmotic adjustment. An attempt has been made also to assess which aspect of cell water relations is involved in the initiation of the accumulation of proline.

MATERIALS AND METHODS

Growth of Cell Cultures. Cell suspensions of tomato (Lycopersicon esculentum, Mill. cv VFNT-Cherry) were adapted to increasing concentrations of PEG by sequential transfer to medium with 0, 15, 20, 25, and 30 g L⁻¹ PEG. These cell lines, designated P-0, P-15, P-20, P-25, and P-30 were grown in their respective concentrations of PEG as described earlier (2, 3) for at least 100 cell generations before use in experiments described here. Growth of cells was determined by measuring fresh weight and dry weight gain, also as described earlier (3).

Measurement of Osmotic Potential and Water Potential. Osmotic potential of the cells was measured by the method of incipient plasmolysis, as described earlier (9). The ψw of the cells was taken to be the same as that of the medium since the cells are in ψw equilibrium with the medium to which they are adapted. The ψ of the latter was measured by using a Wescor model HR 33T hygrometer with model CS2 thermocouple psychrometer chambers. All estimations of ψ were made by calculations of ψ - ψw. Values of ψ are sometimes negative because cells which are inoculated into medium with a ψ value which is more negative than their ψw are assumed to come to ψw equilibrium with the medium. Under such circumstances it is presumed that the cells are under tension or have cytorrhized to allow the cell ψ to equal ψ of the medium.

Measurement of Tolerance to Water Stress. Fresh and dry wt gain of the cells in media with various concentrations of PEG were determined using cells of different developmental stages as described in an earlier report (2).

Preparation of Cell Samples for Proline Measurement. The cells were harvested by filtering on a Büchner funnel, and adapted cells were washed with mannitol solution isosmotic with the medium to which they were adapted. After fresh weight determination, some cells were quickly weighed and placed in 2 ml of methanol:chloroform:water mixture (12:5:3) and kept at -20°C prior to proline measurements. The remaining cells were...
lyophilized and kept at −20°C.

Extraction and Assay of Proline and Total Free Amino Acids. Proline was extracted and assayed according to Singh et al. (27) except for endogenous proline measurements of amino acid treated cells, where proline was determined by capillary gas chromatography, flame ionization detection as described by Rhodes et al. (21). Total free amino acids were extracted from the lyophilized cells with 80% ethanol at 70°C, at least three times. The pooled extracts were evaporated under reduced pressure and the residue was redissolved in water. α-Amino nitrogen was determined according to Rosen (22). All μM concentrations of endogenous proline are from μmol proline/g fresh weight − dry weight) calculations.

RESULTS

Proline Content of Cells Is Associated with Osmotic Adjustment and Tolerance to Low External Water Potential. The proline level of the cells is highly correlated with the cell ψo (Fig. 1). However, as we had considered previously that this correlation was only strong at very negative cell ψo values (2), it seems that upon close inspection this appeared so only because the association was exponential. The log of proline concentration does appear correlated with cell ψo values over a very large range of osmotic adjustment (Fig. 1A). Thus, the log of the average proline concentration of each cell line is highly correlated with the tolerance of the cell line (Fig. 1B).

Upon initial exposure of nonadapted cells to low ψ (−21 bar) medium there was a rapid increase in dry weight/fresh weight ratio of the cells (data not shown) indicating loss of ψo, and subsequently a loss of cell volume. This was evidenced by the observance of many cells with partially collapsed walls (cytorrhized). By d 6, the cells had recovered from the cytorrhisism, and the ψc (−33 bar) of the cells had become more negative than the ψ (−21 bar), allowing restoration of ψo, and initiation of growth (Fig. 2B), indicating osmotic adjustment had occurred. In fact, osmotic adjustment in response to the stress results in the establishment of ψo levels which are above those found during the growth of cells in medium without PEG (2). The average ψo of the cells during the first growth cycle in 25% PEG was 9 bar, while the average ψo during growth of the same inoculum cells in medium without PEG was about 6 bar. The difference in ψo between cells growing in medium with and without PEG eventually becomes quite pronounced (2). Although we could not estimate cell ψo, and thus calculate the ψo of stressed cells before approximately d 6 because plasmolysis of the cytorrhized cells is too difficult to observe, it is possible that the osmotic adjustment may begin very soon after exposure to stress. Also it is possible that the mechanism which allows osmotic overadjustment and thus increased ψo may be initiated very quickly after exposure to low ψ.

Proline accumulation preceded growth and occurred, as far as we could determine, about the same time as osmotic adjustment (Fig. 2B). The proline level remained substantially increased after growth proceeded, and did not return to the normal level as long as the cells remained in low ψ medium. The level of total free amino acids increased rapidly after transfer to low ψ medium also, but only increased about 2-fold compared to a nearly 100-fold increase in the level of proline (Fig. 2, B and C).

When nonadapted cells were transferred to medium without PEG, instead of medium containing 25% PEG, the cells began to grow sooner and the proline levels were always much lower, although slight changes during growth were observed (Fig. 2A).

Water Potential and Proline Accumulation. Nonadapted cells were transferred to media with lower ψo, either directly (Fig. 3), or in a stepwise manner (Fig. 4A) to be able to expose the cells to a low ψ with minimum loss of turgor. The levels of proline which accumulated in nonadapted cells in media of similar ψs were 2- to 3-fold lower when the transfer was done stepwise (Fig. 4A) rather than directly (Fig. 3B). These results suggest that the levels of proline in cells exposed to osmotic stress are not strictly dependent on cell ψ.

Turgor and Proline Accumulation. After each stepwise transfer of cells to medium of lower ψo, a decrease in initial ψo occurred, and for the first two transfers the final ψo also decreased (Fig. 4A). However, proline accumulation (measured just prior to each inoculation and at the time of final ψo) began only as osmotic adjustment began, and this was when the final ψo had begun to increase. Although final ψo decreased to about +3 bar at the onset of proline increase, such low ψo is commonly observed in cells with low proline growing in nonstress medium.
FIG. 2. Time course of proline accumulation (●), osmotic adjustment (〇), and fresh weight growth (△) of nonadapted cells in medium containing 0% PEG (−4 bar initial ψ) (A) and 25% PEG (−21 bar initial ψ) (B); ψ of medium becomes slightly less negative as nutrients are absorbed. Nonadapted cells were transferred into both media at an inoculum density of 8 g L⁻¹. (C) Concentration of total free amino acids in cells growing in 25% PEG (▲) and in 0% PEG (●).

(Fig. 4B). Thus, even though ψᵢ loss can be associated with the initiation of proline accumulation, ψᵢ loss does not always result in proline accumulation (Fig. 3). Once proline accumulation is initiated, osmotic adjustment and ψᵢ increase almost simultaneously with a continued increase in intracellular proline levels (Fig. 4A). During deadaptation (Fig. 4B) ψᵢ decreases substantially while proline levels decline dramatically along with ψᵢ.

Osmotic Potential and Proline Accumulation. It follows from the preceding data that although accumulation of proline under water stress can be associated with changes in ψᵢ and ψ of the cells, it is not strictly correlated with either of these parameters. When cells from different stages of growth with different ψᵢ were transferred to medium of differing ψᵢ, high proline accumulation was not observed in cells which had a relatively positive ψᵢ, i.e., −6.0 bar and −5.2 bar even though ψᵢ was completely lost (Fig. 3). In contrast, cells with a relatively more negative ψᵢ, (−10.6, −12.2, and −15.0 bar) were able to accumulate high levels of proline to high (Fig. 3B) over similar intervals of time. Thus, the magnitude of cell ψ at which the increase in proline occurs may change depending on the initial ψᵢ (Fig. 3), and although proline accumulation can appear associated with loss of ψᵢ in some instances cells which have lost ψᵢ completely fail to increase proline to high levels (Fig. 3). Although we cannot rule out that the former response also may be related to the phase of growth of the cells, once proline accumulation is initiated, increases and decreases in proline content are proportional only to the cell ψᵢ (Figs. 1A and 4). It seems that ψᵢ is the water relations component with which proline accumulation is most closely associated.

The Effect of Deadaptation on Proline Levels. A rapid decline in proline level occurred when cells were transferred back into PEG-free medium after either direct adjustment (Fig. 5) or after stepwise adjustment (Fig. 4B) to low external ψ. This decline in proline concentration persists as long as cells are kept in medium without PEG, and although some decline in proline concentration is observed in cells growing in 25% PEG at the end of their growth cycle (Fig. 5B) (10), high proline persists as long as the cells are continually cultured in the PEG medium. Apparently,
the accumulation of high intracellular levels of proline in cells adapted to water stress is not an injury response during stress, since the cells were able to revert back to a normal pattern of growth upon removal of the stress (Fig. 5A). A subpopulation of uninjured cells which did not accumulate proline during stress would most likely exhibit an extended lag before beginning to grow when subcultured on medium without PEG.

Effect of Exogenous Proline on Tolerance to Water Stress. In the absence of stress, the growth of cells could be enhanced somewhat (20–80% relative to untreated control) by exogenously supplied proline (Fig. 6). Although in some cases, probably related to the age and handling of cells, exogenous proline slightly inhibited the growth of nonstressed cells (Table I). However, the effect of proline was much greater and always stimulatory when the cells were subjected to water stress. The increase in growth was as much as 28-fold in cells transferred to medium with 30% PEG (Table I). Even very high concentrations of exogenous proline (50–100 mM) were not inhibitory to growth in the presence of stress (Fig. 6) in contrast to the effect of other amino acids (Table I).

To some extent, the stimulatory effect of proline depends on the growth phase of the cells (Fig. 7). Growth enhancement of cells in the absence of stress (up to 160%) by proline was mostly observed when the inoculum cells were in the rapid growth phase (Fig. 7A). The enhancement of growth of nonadapted cells under water stress (30% PEG) was always much greater, but was also maximum at the rapid growth phase of the inoculum (Fig. 7B).

The stimulatory effect of exogenous proline on growth was due more to a decrease in the lag period than to a stimulation of the growth rate.

Effect of Different Amino Acids on Tolerance to Stress. From Table I, it can be seen that apart from proline, exogenous arginine, asparagine, aspartate, valine, glutamine, and glutamate also enhanced the tolerance of nonadapted cells to water stress.
In these experiments, nonadapted cells were used from late log phase of growth. Large variation was observed in some cases likely because of unavoidable differences in the phase of growth of inoculum cells in different replicate experiments. In the absence of stress many of the amino acids tested had an inhibitory effect on growth. Inhibition of growth by various amino acids has been observed by others (30). Also, proline and glutamine were the only amino acids which stimulated rather than inhibited growth under stress at concentrations higher than 1 mM, and this stimulation was much higher than that of other amino acids (Table I).

Effect of Exogenous Proline on Cell Growth during Osmotic Downshock. It appears that exogenous proline not only enhanced the water stress tolerance of the cells, but also increased the tolerance to osmotic downshock (Table II). In the absence of proline, growth of P₀₀ cells transferred to medium with lower concentrations of PEG was severely inhibited compared to growth in the presence of exogenous proline (Table II). This growth enhancement by proline appeared maximal when osmotic downshock was sufficient but not too severe. Thus, the transfer of cells grown in 30% PEG to 20% PEG resulted in a 24-fold increase in growth when proline was added to the 20% PEG medium (Table II), and no growth at all was observed when P₀₀ cells were transferred to 0% PEG medium with or without proline.

**DISCUSSION**

The initiation of proline accumulation in plant tissues in response to a decrease in tissue \( \psi \) could conceivably be a response to changes in either the osmotic or pressure component of the water potential. Our results indicate that when plant cells in culture are transferred to a medium of a lower \( \psi \) the resulting drop in \( \psi_p \) is followed by osmotic adjustment. It seems that osmotic adjustment may commence slightly before proline levels begin to increase (Fig. 4A), and although an initial decrease in \( \psi_p \) accompanies proline accumulation, the \( \psi_s \) of the cells prior to transfer appears critical in determining whether or not cells will accumulate high levels of proline (Figs. 3 and 4). Furthermore, adapted cells in culture can be maintained in low \( \psi \) media for over 100 cell generations and the proline level remains high even though osmotic adjustment has fully restored \( \psi_p \). It is clear that if there is any involvement of \( \psi_p \) loss in the initiation of the accumulation of proline, it is not necessary to maintain elevated proline levels. However, elevated osmotic adjustment appears to be necessary to maintain a high proline level since raising the \( \psi \) of the external medium diminishes osmotic adjustment, \( \psi_p \), and the proline level of adapted cells back to prestress levels (Figs. 4B and 5B). Our results suggest that the signal for altering the cellular level of proline is not simply a change of turgor. As Chu et al. (5) have shown for barley leaves exposed to NaCl, and as Hanson and Hitz (12) have suggested, proline accumulation is associated with some process related to cell \( \psi_s \) change, perhaps
Table I. Effects of Different Amino Acids on Tolerance to Water Stress

Nonadapted cells from late exponential phase of the growth cycle were transferred (8 g fresh wt L⁻¹) into media containing 0 or 30% PEG in the presence of different amino acids at the concentration indicated. Cells were harvested after 15 d and 25 d for cultures growing in the absence or presence of PEG, respectively. Values shown for growth in 0 or 30% PEG as percentage of control without amino acid ± SE. Average fresh weight gain of cells growing in 0% PEG and 30% PEG without addition of amino acid were 320 ± 1.76 g L⁻¹ and 1.6 ± 1.40 g L⁻¹, respectively. Values are averages of at least three separate experiments each with two replicates except where noted. Endogenous proline is given as nmol/mg dry weight.

<table>
<thead>
<tr>
<th>1.0 mm Amino Acid</th>
<th>10.0 mm Amino Acid</th>
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<tbody>
<tr>
<td>Growth in 0% PEG</td>
<td>Growth in 0% PEG</td>
</tr>
<tr>
<td>Endogenous proline</td>
<td>Endogenous proline</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
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<tr>
<td>Asparagine</td>
<td>98.8 ± 1.6</td>
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<tr>
<td>Aspartate</td>
<td>102.3 ± 6.8</td>
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<tr>
<td>Glutamate</td>
<td>97.3 ± 3.9</td>
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<tr>
<td>Glutamine</td>
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<tr>
<td>Proline</td>
<td>86.0 ± 6.5</td>
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<tr>
<td>Valine</td>
<td>89.3 ± 4.8</td>
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<tr>
<td>Arginine</td>
<td>87.6 ± 7.4</td>
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<tr>
<td>Ornithine</td>
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<tr>
<td>Glycine</td>
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<tr>
<td>Leucine</td>
<td>39.0 ± 7.7</td>
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<tr>
<td>Serine</td>
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<tr>
<td>γ-Aminobutyrate</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Alanine</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Methionine</td>
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<tr>
<td>Phenylalanine</td>
<td>ND</td>
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<td>Tryptophan</td>
<td>ND</td>
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</tbody>
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*ND, nondetectable amount of growth.  aOne experiment, 2 replicates.

Table II. Effect of Exogenous Proline on Osmotic Downshift

Stationary phase cells which were adapted to growth in medium with 30% PEG were transferred into media containing different amounts of PEG, each with different levels of exogenous proline, as indicated. Cells were harvested for fresh weight determination after 17 d. The fresh weights of cells (g L⁻¹) in the control media (0 mm Proline) were: 2.8, 15% PEG; 2.4, 20% PEG; 44.0, 25% PEG; and 14.8, 30% PEG.

<table>
<thead>
<tr>
<th>Concentration of PEG in Medium</th>
<th>Exogenous Proline (mm)</th>
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<tbody>
<tr>
<td>%</td>
<td>0</td>
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<tr>
<td>0</td>
<td>ND*</td>
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<td>15</td>
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<td>20</td>
<td>100</td>
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*Growth not detected.

the concentration of a specific solute, or as suggested by Goring and Thien (8) and more recently indicated by Pesci and Belfaguna (20), perhaps cytoplasmic pH. Although proline accumulation was associated with loss of $\psi_w$ (38) and injury in intact barley plants (13), the results of Voetberg and Stewart (36) using excised barley leaves indicated that high proline levels were closely associated with accumulation of exogenous Na⁺ ions, and proline levels remained elevated even after $\psi_w$ was regained.

It seems clear from Figures 2 and 5 that once the cultured cells have begun to adapt by osmotic adjustment, the level of proline is representative of the degree of adaptation and not of injury, unlike what may occur in leaf tissues of intact plants (13, 28). Watad et al. (37) also found elevated proline levels to be associated with increased osmotic adaptation of cultured tobacco cells to NaCl. When studying whole plant tissues, it is difficult to establish that the cells which have accumulated high levels of proline are not a subpopulation of cells which are injured by the initial stress. Using cultured cells, such injured high proline-containing cells should not persist in subcultures in the presence of stress, and the proline level of the cells of subsequent subcultures therefore should diminish. Since it does not (Fig. 1), it is clear that it is the adapted (growing in the presence of the stress) cells and not necessarily cells injured by the initial stress which have high proline levels. Moreover, no proline was detected in the medium containing the stress-adapted cells, which would likely have released proline had they been injured (result not shown).

We have shown that high proline levels are compatible with and maintained in cells growing actively at low $\psi_w$. Such results have been reported in other plant species as well, e.g. Phaedachylyl tricornatum (24), Triglochin maritamina (29), Rupplia (4), and jojoba (32). A recent survey of various drought-resistant plants in the Namib desert showed a strong ability of such plants to respond to drought by accumulation of proline to high levels (35).

Although some workers, e.g. Singh et al. (26, 27), Hubac and...
Guerrier (16), Stewart and Lee (29) report positive correlations between proline accumulation and stress tolerance or adaptation to stress, the results of others such as Hanson et al. (13), Tal et al. (33), and Ferreira et al. (7) indicate a negative correlation between the two characteristics. One of the reasons for such a discrepancy could be that in some intact plants, the mechanism of tolerance may be other than or in addition to simple osmotic adjustment, e.g. in barley cultivars studied by Hanson et al. (13), where the mechanism of drought stress tolerance appears to be through drought avoidance. In other words, the different cultivars may not only reach different leaf water potentials in response to the same magnitude of stress, but the tissues which are examined of both sensitive and tolerant cultivars may never osmotically adjust or may not osmotically adjust sufficiently to evoke large changes in proline level (Fig. 1). Such tolerance differences could result from morphological adaptations, e.g. a better developed root system (7). In such cases, it is difficult to imagine any straightforward relationship between proline accumulation and drought tolerance.

In a field situation, the magnitude of water stress does not remain constant as progressive dehydration of unirrigated soil occurs, and the metabolic and adaptive responses of different tissues may vary greatly. Even though the use of cultured tissues may circumvent such problems, it is important to realize that the behavior of excised tissues or cell cultures may not always be consistent with the behavior of the whole plants from which they are derived (31).

Since the growth of cells subjected to sudden and large negative or positive water potentials was enhanced when proline was added to the medium, it seems that proline can have a beneficial role in adaptation of cultured plant cells to osmotic stress. The stimulatory effect of exogenous proline on cells adapting to low water potentials was not entirely specific, and it is uncertain how other amino acids might act to enhance adaptation. All treatments which allowed growth under stress including the control also resulted in elevated levels of endogenous proline (Table 1). However, only addition of proline to the medium caused the level of endogenous proline in unstressed cells to increase, indicating that the stimulatory effect of other amino acids on growth under stress may not be through directly enhancing endogenous proline levels. Also, as indicated earlier, the maximum stimulatory effects of all other amino acids were marginal compared to the maximum stimulation caused by proline and glutamine which still caused less stimulation than proline (Table 1) and may serve as a precursor to proline. In addition to proline, limited protection against high salt concentrations by amino acids, such as γ-aminobutyric acid, glycine, serine, β-alanine, sodium aspartate, and sodium glutamate, has been observed (15).

Recently, Itai and Paleg (17) have reported beneficial effects of exogenous proline and betaine during recovery of barley plants from stress, but not during stress, while others (I. Parvez; see [39]) have shown an effect during stress. The exact nature of the beneficial effect of proline is at present not clear, but has been attributed to proline acting as a compatible osmotic solute (29, 34), as a stabilizing or solubilizing factor for proteins under limiting cell water conditions (19, 25), or as a source of reduced nitrogen and carbon (1). It may be argued that in adapted cell lines of tomato, in spite of large increases in proline levels (100-fold on the average in cells adapted to 30% PEG), the levels of proline are still not large enough to constitute a significant contribution to the cell ψ', unless it were compartmentalized in the cytoplasm. Some evidence (11, 14) suggests that this may be true.

The magnitude of the growth stimulatory effect of exogenous proline is dependent on the age of the cells. Thus, the physiological status of the cells determines their response to exogenous proline, by perhaps differential efficiency of uptake of proline.

**FIG. 7. Effect of age of culture on the response to exogenous proline.** Nonadapted cells were grown as usual in medium with no PEG. Inset in (A) shows growth (■) curve of the inoculum cell culture. At the times indicated, cells from the inoculum culture were inoculated (8 g L⁻¹) into medium with no PEG (A) or with 30% PEG (B), in the presence of the amounts of proline indicated on the abscissa. Relative growth is expressed as percentage of fresh weight gain of the control (no proline added to medium) 20 d after inoculation at the given PEG level. Final fresh weight gains of the controls in 0% PEG and ages of the inocula in days were: 208 g L⁻¹, 3 d (Δ); 125 g L⁻¹, 7 d (○); 127 g L⁻¹, 10 d (□). Final fresh weight gains of the controls and ages of the inocula in days in 30% PEG were: 11.4 g L⁻¹, 3 d (Δ); 9.5 g L⁻¹, 5 d (□); 1.6 g L⁻¹, 7 d (○); 3.2 g L⁻¹, 10 d (□). Inset in (A) also indicates the maximum relative growth produced by treatment with exogenous proline of unstressed cells and inset in (B) indicates the maximum relative growth produced by treatment with exogenous proline in water-stressed cells, both as a function of inoculum age.
Since adapted cells undergoing osmotic downshock already have high levels of endogenous proline, the protective effect of exogenous proline may be by preventing the loss of endogenous proline during shock. In any case, the effect of exogenous proline on relieving osmotic downshock (Table II) is undoubtedly other than as an osmotic solute, and perhaps may be related to an interaction of proline with proteins, especially membrane proteins, by increasing their stability as suggested by Schobert (23, 24). In fact, Low (18) has recently summarized evidence which indicates that the major function of proline and other 'compatible' solutes which are accumulated during osmotic stress may be to protect the structure and function of proteins by being excluded from the protein hydration sphere.

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