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Transient Accumulation of Asparagine in Sycamore Cells after a Long Period of Sucrose Starvation

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ABSTRACT

The mobilization of stored carbohydrates (sucrose and starch) and proteins during sucrose starvation was studied with sycamore (*Acer pseudoplatanus* L.) cells. When almost all the intracellular carbohydrate pools had disappeared, the cell protein content declined progressively whereas asparagine determined by either ^{13}C nuclear magnetic resonance or reversed phase high performance liquid chromatography increased steadily. After a long period of sucrose starvation, the most intense resonances in the ^{13}C nuclear magnetic resonance spectra were from citrate and asparagine. The total amounts of asparagine (expressed as nitrogen) and free amino acids that appeared after a long period of sucrose deprivation corresponded roughly to the total amount of protein (expressed as nitrogen), that disappeared within the same period of time. Addition of sucrose in the culture medium after a long period of sucrose starvation led to a disappearance of asparagine. These results suggest therefore that the presence of asparagine in plant cells in large excess should be considered as a good marker of protein utilization after a long period of sucrose starvation and is very likely related to stress.

In previous publications Journet *et al.* (7) and Roby *et al.* (12) have shown that transfer of sycamore cells into flasks containing sucrose-free culture medium triggered the following cascade of reactions: (a) initially cellular carbohydrate reserves (vacuolar sucrose and starch present in plastids) were consumed; (b) when almost all the intracellular carbohydrate pools had disappeared the cell fatty acids deriving from membrane polar lipids such as phosphatidylcholine were utilized as oxidizable substrates for ATP production, whereas cytoplasmic phosphorylcholine increased symmetrically and was not further metabolized. These authors concluded, therefore, that the presence of a large excess of phosphorylcholine in plant cells should be considered as a good marker of membrane utilization after a long period of sucrose starvation and is likely to be related to stress.

In the present studies we have used ^{13}C NMR to investigate the changes in major carbon compounds including citrate, asparagine, and other amino acids of sycamore cells during a prolonged period of sucrose deprivation followed by sucrose replenishment. Our data shed new light on protein breakdown and on the origin of asparagine in higher plant cells.

MATERIALS AND METHODS

Plant Material

Sycamore (*Acer pseudoplatanus* L.) cells were grown at 21°C as a suspension in a nutrient medium according to Roby *et al.* (12). The cell suspensions were maintained in exponential growth by frequent subculture. Cells were harvested from the culture medium, rinsed three times by successive resuspension in fresh culture medium devoid of sucrose, and placed at zero time into flasks containing sucrose-free culture medium. At each time point, cells were harvested for the measurement of protein, sucrose, starch, and free amino acids including asparagine.

Perchloric Extract

For perchloric acid extraction, cells (9 g fresh weight) were quickly frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle with 1 mL of 70% (v/v) perchloric acid. The frozen powder was then placed at −10°C and thawed. The thick suspension thus obtained was centrifuged at 10,000 *g* for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M $KHCO_3$ to about pH 6.5. The supernatant was then centrifuged at 10,000 *g* for 10 min to remove $KClO_4$, the resulting supernatant was lyophilized and stored in liquid nitrogen. For the NMR measurements, this freeze-dried material was redissolved in 2.5 mL water containing 10% D_2O (perchloric acid extract).

The ^{13}C NMR spectra of neutralized perchloric acid extracts were measured on a Bruker NMR spectrometer (WM 250) equipped with a 10-mm multinuclear probe tuned at 62.9 MHz. Acquisition used 18 μs pulses (60°) at 4 s intervals (spectra of carboxyl groups were unchanged when a pulse interval of 10 s was employed). Two levels of decoupling were used: 2.5 W during the data acquisition (0.54 s) and 0.4 W during the delay period (3.46 s). Spectra were acquired over a period of 2 h. Free induction decays were accumulated using 8K data points and zero-filled to 32K prior Fourier transformation. A 1 Hz line broadening was applied. Chemical shifts were obtained by reference to the hexamethyldisiloxane resonance at 2.6 ppm. Spectra of standard solutions of known carbon compounds at pH 6.5 were compared with that of a perchloric acid extract of sycamore cells. The definitive assignments were made after running a series of spectra obtained by addition of the authentic compounds to the perchloric acid extracts (for the introduction to high-resolution NMR spectroscopy and its application to *in vivo* and *in vitro* studies, see ref. 11).

Amino Acids and Asparagine Measurements

Cell samples (20 g fresh weight) were removed, collected on a glass fiber filter (15 s, suction pressure 60 kPa), and

rapidly rinsed three times with 30-mL portions of sucrose-free culture medium. Cells (2 g fresh weight) were rapidly dropped into 10 mL of water/ethanol (1:1, v/v) containing 2.5 μmol amino-2-adipic acid (internal standard) and sonicated for 6 min at 20 kHz, 160 W, with a Vibra-cell (Sonics and Material, Inc, Danbury, CT). Cell walls, various debris and precipitated proteins were eliminated by a 3000*g* centrifugation (15 min).

Aliquots (100 μL) of the supernatant thus obtained were evaporated under vacuum. Samples were neutralized after adding 20 μL of ethanol/water/triethylamine (1:2:2, v/v) and again evaporated to dryness. The samples thus obtained were ready for derivatization. The derivatization reagent was freshly made daily and consisted of ethanol/triethylamine/water/phenylisothiocyanate (7:1:1:1, v/v). Phenylthiocarbamyl derivatives of the amino acids were allowed to form for 20 min at room temperature after 50 μL of reagent had been added to the dry samples. The derivatization reagents were then removed under vacuum and the amino acid derivatives were dissolved in 1 mL of medium containing 4 mM-KH_2PO_4 (pH 7.5) and 6% (v/v) acetonitrile (1).

Separation of the phenylthiocarbamyl derivatives was carried out using a Millipore liquid chromatograph which consisted of two Waters model-510 HPLC pumps and a M440 fixed-wavelength detector (254 nm). The temperature (39°C) was controlled within ±1°C with a column heater (Waters). The reversed phase Pico Tag column (15 cm × 3.9 mm) equipped with a Guard-PAK (Waters) precolumn insert, was equilibrated with eluent A (0.4 M-sodium acetate/4 mM-triethylamine, adjusted to pH 6.35 with acetic acid). Samples were injected in a volume of 20 μL (0.05–0.5 nmol of asparagine) using a M710 B WISP auto injector (Waters). The gradient run for the separation consisted of 90% eluent A + 10% eluent B (60% ⟨v/v⟩ acetonitrile in water) traversing to 49% eluent A + 51% eluent B in 13 min (flow rate 1 ml·min^{-1}) using a convex curve (no. 5) programmed with an M680 automated gradient controller (Waters). The gradient and flow programming used are very simple and produce a quiet baseline, thus allowing high sensitivity. Detection of 1 to 2 pmol phenylthiocarbamyl derivatives of amino acids was possible. Peak integration was carried out with a single-channel recorder/integrator (Waters 740 data module). To determine accurately the amount of asparagine in the samples a calibration of the asparagine peak with known amounts of external derivatized asparagine was first performed. In addition, all the data have been normalized to the internal standard amino-2-adipic acid added at the beginning of the amino acids extraction.

Measurement of Various Compounds

Sucrose, starch, protein, and phosphatidylcholine (lecithin) were determined according to previous publications (7). The ammonium content of the cells was determined by microdiffusion followed with nesslerization according to Shelp *et al.* (14).

RESULTS

Effect of Sucrose Starvation on the Intracellular Carbohydrate Pool, Protein, and Lecithin

Table I summarizes the effect of sucrose starvation of sycamore cells on intracellular starch, sucrose, protein, and lecithin. After 24 to 30 h of sucrose starvation, when almost all the intracellular carbohydrate pool (starch + sucrose) had disappeared, the cell lecithin content declined progressively, although sucrose starvation for up to 96 h had no significant effect on fresh weight. This table also indicates that the total protein of the cells decreased progressively during the starvation period; for example, within 96 h, the total protein had decreased to half of its normal level. We calculate from Table I that the rate of net breakdown of protein at 21°C, during the period of most rapid disappearance of protein, was approximately 0.1 mg h^{-1} g^{-1} fresh weight (16 μg N h^{-1} g^{-1} fresh weight). The lag phase observed for protein breakdown was comparable with that observed for lecithin breakdown. In other words, the protein content began to decline when the intracellular carbohydrate pool had almost disappeared. These observations confirm that a large proportion of the endomembrane system disappears in starving cells, while tonoplast and plasmalemma, enriched in sterol compounds, escape the autophagic process (4). Since the levels of ammonium within sycamore cells monitored over a 96 h sucrose starvation remained negligible (not shown), we wished to determine whether protein breakdown resulted in a marked accumulation of amino acids including asparagine, a well known scavenger of cellular ammonium (15).

Effect of Sucrose Starvation on Amino Acid and Asparagine Content

The amino acids present in sycamore cells were derivatized using the procedure described under "Materials and Methods." The separation of the amino acids from normal and 72-h sucrose starved cells by reversed-phase HPLC is shown in Figure 1. The gradient shape was chosen to optimize the spacing of the separated peaks in the minimum analysis time. In normal (nonstarved) cells alanine (A) was the most predominant amino acid followed by glutamic acid (E). The other amino acids were present but in very low amounts. Under these conditions asparagine was almost undetectable. After 72 h of sucrose starvation, when the total cell protein content had considerably declined (the total amount of pro-

Table I. *Effect of Sucrose Starvation on the Concentration of Carbohydrate (Sucrose + Starch), Protein, and Lecithin in Sycamore Cells*

These data are from a representative experiment and have been reproduced at least three times. Cells were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated for various lengths of time in flasks containing sucrose-free culture medium.

Period without External Sucrose Supply	Fresh Weight	Sucrose + Starch	Protein	Lecithin
h	*mg/mL*	*μmol glucose/g fresh wt*	*mg/g fresh wt*	*μg/g fresh wt*
0	47	180	13	750
12	49	98	13	740
24	50	20	12.5	720
48	50	5	10	560
96	52	Trace	6	310

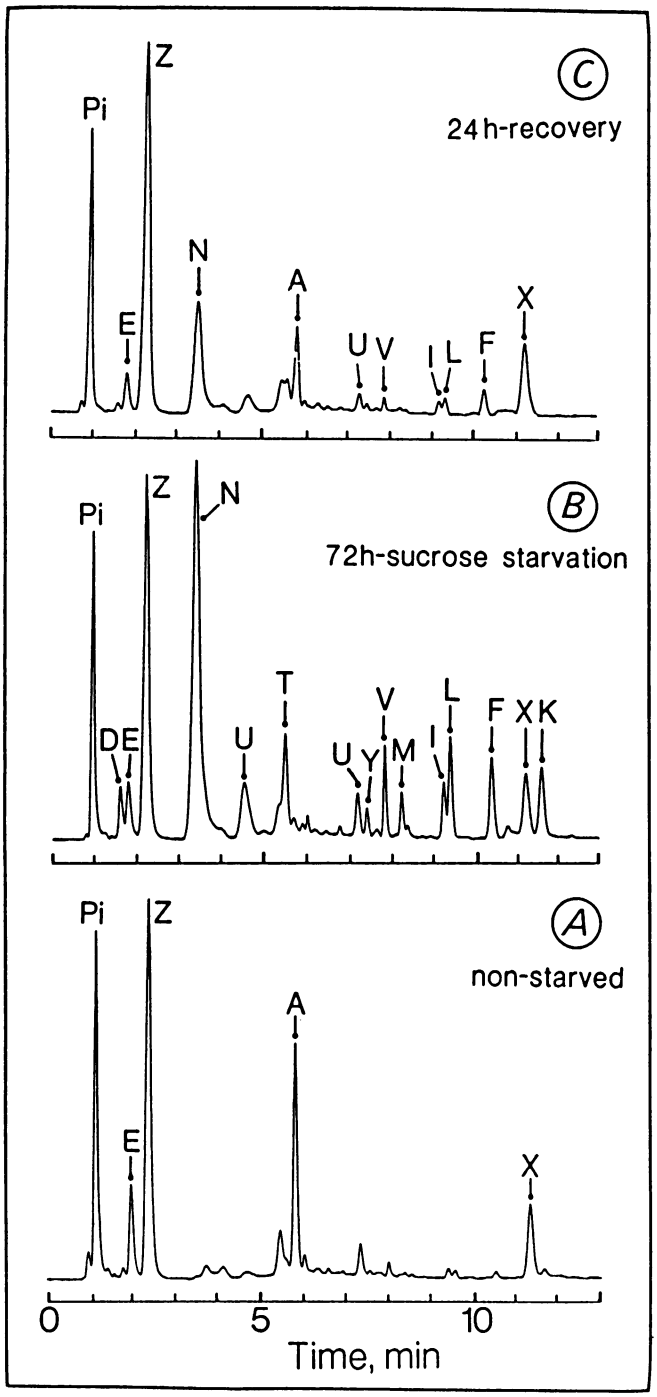


Figure 1. Separation of the major phenylthiocarbamyl derivatives of the amino acids isolated from sycamore cells via reversed-phase HPLC on a Pico-Tag column. Absorbance was monitored at 254 nm. The protocol used is given under "Materials and Methods." Amino acids were extracted from (A) normal aerobic cells, (B) 72 h-sucrose starved cells, and (C) 72 h-sucrose starved cells followed by 24 h-recovery after addition of 50 mM sucrose to the culture medium. At each time, cells (20 g fresh weight) were harvested and their amino acids were isolated and derivatized as described under "Materials and Methods." Z, Amino 2 adipic acid (internal standard); Pi, orthophosphate; X, contamination peak resulting from the derivatization reagent; U, unknown. Single letter amino acid designations.

tein was reduced to 8.5 mg protein g^{-1} fresh weight after growth for 72 h without external sucrose, compared with 13 mg g^{-1} fresh weight in controls supplied with sucrose), free amino acid levels in sycamore cells increased markedly. In contrast, the 72-h sucrose-starved cells exhibited very low amounts of alanine indicating that this amino acid was rapidly metabolized when the external supply of sucrose was withdrawn. Interestingly, during the course of sucrose starvation a peak with the same retention time as asparagine (N, Fig. 1) increased considerably. The phenylthiocarbamyl derivative of asparagine was characterized by its absorption spectrum in the UV with a maximum at 247 nm and a broad shoulder around 270 nm (not shown). Asparagine was further identified by the position of its resonance peaks in the ^{13}C NMR spectra obtained from perchloric acid extracts of sycamore cells (see below). Asparagine accumulation only occurred after 24 to 30 h of sucrose starvation and it is noteworthy that the lag phase observed for protein breakdown was comparable with that observed for the increase in asparagine. In fact, the asparagine content of cells deprived of sucrose for more than 4 d began to increase when the intracellular sucrose had been consumed (Fig. 2). After 72 h of sucrose starvation the total amount of asparagine present in intact sycamore cells was considerable, approximately 10 μmol per g fresh weight (Fig. 2) and the total amount of protein that had been broken down was approximately 4 to 5 mg per g fresh weight. From these results we calculated that the total amounts of asparagine

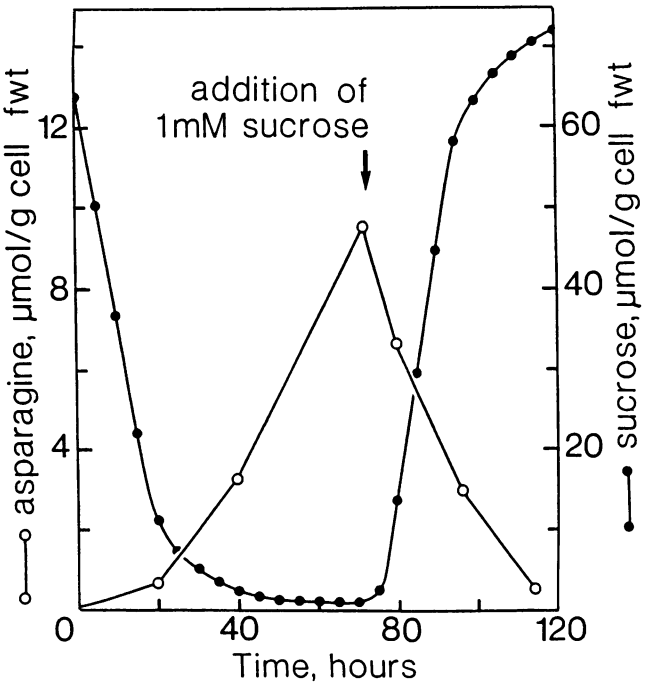
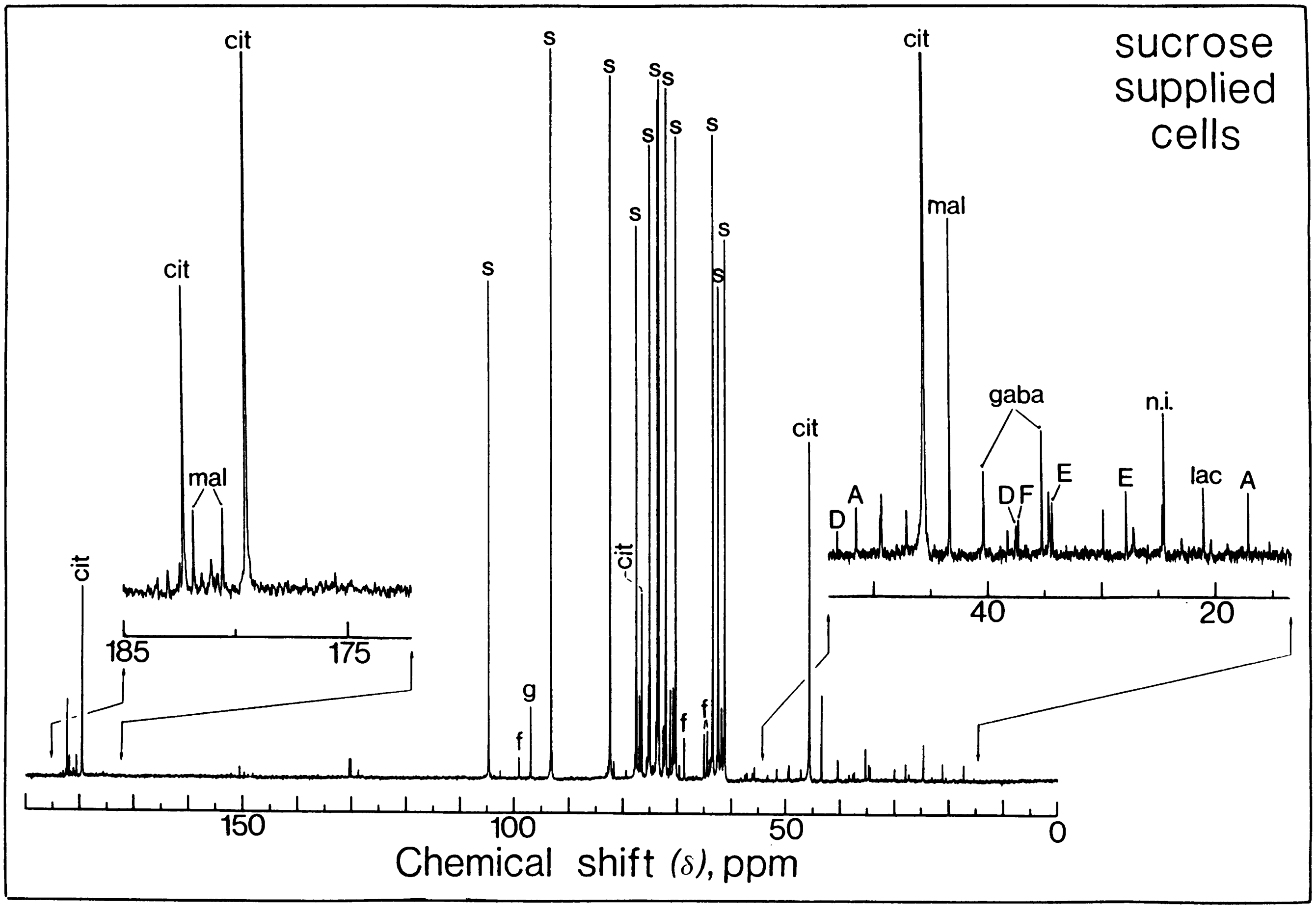


Figure 2. Effect of sucrose starvation of sycamore cells on intracellular asparagine and sucrose contents. Cells harvested from the culture medium were rinsed three times by successive resuspension in fresh culture medium devoid of sucrose and incubated at zero time (45 mg fresh weight mL^{-1}) into flasks containing sucrose-free culture medium. At each time, cells were harvested and the sucrose and asparagine contents were measured as described under "Materials and Methods."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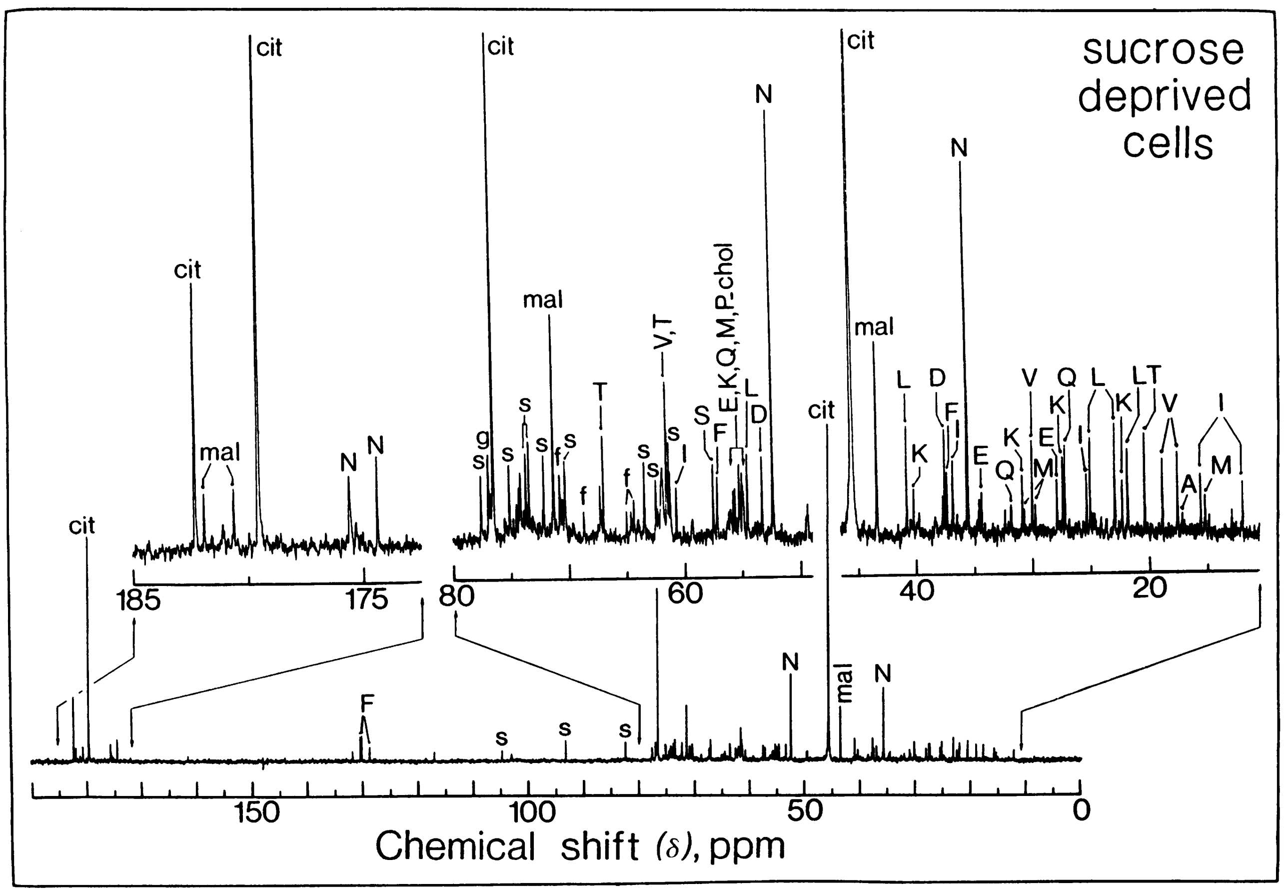
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(expressed as nitrogen) and free amino acids (see Fig. 1) that appeared after 72 h of sucrose deprivation correspond roughly to the total amount of protein (expressed as nitrogen), that disappeared within the same period of time. However, it is of interest to note that after a long period of sucrose starvation (80 h), asparagine previously accumulated disappeared progressively (not shown).

^{13}C NMR of Neutralized Perchloric Acid Extracts

^{13}C NMR spectra obtained from perchloric acid extract of sycamore cells (9 g fresh weight) at pH 6.5 showed that the major resonances in the natural-abundance spectra corresponded to those of sucrose, citrate, and malate sequestered in the vacuole. The resonances of highest intensity corresponded to those of the glucosyl and fructosyl moieties of sucrose (17) and were estimated to correspond to an intracellular concentration of approximately 70 μmol g^{-1} fresh weight, in good agreement with biochemical determinations (7). Signals from citrate (intracellular concentration: 30 mM) centered at 182.3, 179.5, 76.4, and 45.5 ppm and malate (intracellular concentration: 5 mM) centered at 181.7, 180.6, 71.2, and 43.4 ppm were well characterized (Fig. 3). In addition, the spectra showed resonances arising from the natural abundance of ^{13}C in D-glucose and fructose. There were also signals from γ-amino butyrate, lactate, glutamate, alanine, and aspartate emerging from the background noise. All the carbon compounds present in the perchloric acid extract at concentrations lower than 500 μM were not distinguishable from the background noise in our experimental conditions (see "Materials and Methods."). This means that there are many compounds that are known to be present but are not seen in the NMR spectra of the extracts, such as the intermediates of the glycolytic and oxidative pentose phosphate pathways.

After 72 h of sucrose deprivation, several important changes occurred in the perchloric acid extract of the cells. As expected this extract showed small vestigial glucose, fructose, and sucrose resonances, but these peaks were absent or below the noise level in spectra of 96-h sucrose-starved cells (not shown). Of particular interest was the marked increase in the amount of asparagine. The definitive assignment of resonances to asparagine centered at 35.6 and 52.3 ppm was made after running a series of spectra obtained by addition of the authentic compound to the perchloric acid extracts at various pHs. The 72 h spectrum also exhibited a multitude of resonance peaks emerging from the background noise which were ascribed to amino acids including: isoleucine, methionine, valine, threonine, leucine, lysine, glutamine, glutamic acid, phenylalanine, aspartic acid, and serine. The other amino acids were present in significant amounts. It is interesting to draw a parallel between the results obtained using HPLC and those using ^{13}C NMR for the resolution of aminoacids: both techniques led to the same conclusion that asparagine and, to a lesser extent, other amino acids accumulated after a long period of sucrose starvation. Finally, throughout the starvation period, citrate which had been previously sequestered in the vacuole was not restored to the cytoplasm for metabolic processes. In other words, citrate did not effuse from the large vacuole reservoir to sustain mitochondrial respiration. It is clear therefore, that after a long period of sucrose starvation, the most intense resonances in the spectra were from citrate and asparagine.

Effect of Sucrose Replenishment

Addition of 1 mM sucrose to the medium after 72 h of sucrose starvation resulted in a marked decrease in the cell content of asparagine (Figs. 1 and 2), and 30 h after replenishment of sucrose asparagine was almost entirely metabolized. It is of interest to note that the decrease in asparagine level correlates with the increase in the amount of protein (not shown). This result strongly suggests that during the course of sucrose replenishment, although the medium contained NO_3 in large excess, nitrogen derived from asparagine is mobilized for protein synthesis.

DISCUSSION

These results indicate that after a long period of sucrose starvation, that is when almost all the intracellular carbohydrate pools have disappeared, the cell protein content declines progressively, probably via macroautophagy (9) with a parallel increase in asparagine. Such a result strongly suggests that amino acids released during the course of proteins breakdown, could be metabolized to provide the mitochondria with oxidizable substrates for ATP production (10). The peroxisomes from nonfatty plant tissues are able to oxidize via β-oxidation the branched-chain 2-oxo acids, which are formed by deamination of leucine, isoleucine, and valine, the major amino acids of membrane proteins (6). Furthermore, plant tissues such as pea root apices possess the capacity to use valine as a respiratory substrate (5). Preliminary results carried out in our Laboratory indicate that during the early step of sucrose deprivation there is an increase in the number of peroxisome/cell. Since ammonia was not released throughout the starvation period it might be expected that aspartate aminotransferase, glutamine synthetase, glutamate synthase, and asparagine synthetase play a key role in NH_3 reassimilation, and therefore in asparagine accumulation (13). We can conclude that the presence of a large excess of asparagine in plant cells should be considered as a good marker of protein breakdown after a long period of sucrose starvation and, like phosphorylcholine (3, 12), is also related to stress. In support of this

Figure 3. Proton-decoupled natural abundance ^{13}C NMR spectra of perchloric acid extracts of sycamore cells. Perchloric acid extracts were prepared from normal (upper panel) and 72 h sucrose starved cells (lower panel). Freeze-dried extracts corresponding to 9 g fresh weight were solubilized and supplemented with 2 mM EDTA as described under "Materials and Methods." The samples (2.5 mL) were put in a 10-mm diameter NMR tube and spectra recorded at 62.89 MHz on a Bruker NMR spectrometer (WM 250) as described under "Materials and Methods." Spectra were acquired over a period of 2 h. All the interesting regions are shown on an expanded scale. Peak assignments: cit, citrate; mal, malate; S, sucrose; g, glucose; f, fructose; gaba, γ-amino butyrate; lac, lactate; n.i., not identified; single letter amino acid designations.

conclusion, numerous interesting results (15, 16) have indicated that asparagine (along with other amides) is synthesized during exposure of plants to periods of environmental stress such as mineral deficiency, drought, or conditions of increased salinity. In addition, asparagine is actively synthesized in higher plants when proteins are hydrolyzed, for example during seed germination or leaf senescence.

The results presented in this article also demonstrate that in the presence of sucrose, asparagine was found to be metabolized in sycamore cells and does not behave, therefore, as a dead end metabolite. Of interest is the fact that neither NO_3^- nor sucrose present in the cell growth medium prevent asparagine catabolism. Many questions remain to be answered regarding the fate of asparagine. It might be expected that asparagine is metabolized either by transamination or deamidation by an asparaginase (15). However, in mature leaves that no longer require nitrogen for growth, asparagine molecules formed during senescence (*i.e.* the series of events subjected to direct genetic control and concerned with cellular disassembly) (18) escape from the cells and are exported toward the xylem where they accumulate. For example, high concentrations of this amino acid have been found in the xylem sap of peas, accounting for up to 70% of the xylem contents (19), and a variable proportion of this amino acid could be utilized in the developing plant at a later period. Since accumulated asparagine did not significantly leak out of sycamore cells during the course of sucrose starvation, it is obvious that these cells would be an appropriate model for studying the catabolism of asparagine.

The results presented in this article indicate that citrate exhibits a high metabolic inertness. Such an observation is very intriguing because after a long period of sucrose starvation this tricarboxylic acid cycle intermediate remains sequestered in the vacuole out of equilibrium with cytoplasmic enzymes. It is possible therefore that in these cells citrate plays a critical function in vacuolar homeostasis.

Finally, nothing is known about the mechanisms by which protein degradation is controlled during the course of sucrose starvation. It is possible that the increase in amino acid levels observed when almost all the intracellular carbohydrate pools had disappeared plays a critical role in the control of cell autophagy. In support of this suggestion, Leverve *et al.* (8) and Caro *et al.* (2) have shown that various amino acids and especially leucine at physiological concentrations strongly inhibit autophagic proteolysis in isolated rat hepatocytes.

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