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Asparagine metabolism and nitrogen distribution during protein degradation in sugar-starved maize root tips

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Abstract: Excised maize (*Zea mays* L.) root tips were used to monitor the effects of prolonged glucose starvation on nitrogen metabolism. Following root-tip excision, sugar content was rapidly exhausted, and protein content declined to 40 and 8% of its initial value after 96 and 192 h, respectively. During starvation the contents of free amino acids changed. Amino acids that belonged to the same "synthetic family" showed a similar pattern of changes, indicating that their content, during starvation, is controlled mainly at the level of their common biosynthetic steps. Asparagine, which is a good marker of protein and amino-acid degradation under stress conditions, accumulated considerably until 45 h of starvation and accounted for 50% of the nitrogen released by protein degradation at that time. After 45 h of starvation, nitrogen ceased to be stored in asparagine and was excreted from the cell, first as ammonia until 90–100 h and then, when starvation had become irreversible, as amino acids and aminated compounds. The study of asparagine metabolism and nitrogen-assimilation pathways throughout starvation showed that: (i) asparagine synthesis occurred via asparagine synthetase (EC 6.3.1.1) rather than asparagine aminotransferase (EC 2.6.1.14) or the β-cyanoalanine pathway, and asparagine degradation occurred via asparaginase (EC 3.5.1.1); and (ii) the enzymic activities related to nitrogen reduction and assimilation and amino-acid synthesis decreased continuously, whereas glutamate dehydrogenase (EC 1.4.1.2–4) activities increased during the reversible period of starvation. Considered together, metabolite analysis and enzymic-activity measurements showed that starvation may be divided into three phases: (i) the acclimation phase (0 to 30–35 h) in which the root tips adapt to transient sugar deprivation and partly store the nitrogen released by protein degradation, (ii) the survival phase (30–35 to 90–100 h) in which the root tips expel the nitrogen released by protein degradation and starvation may be reversed by sugar addition and (iii) the cell-disorganization phase (beyond 100 h) in which all metabolites and enzymic activities decrease and the root tips die.

Key words: Asparagine – Nitrogen metabolism – Protein degradation – Root meristem – Sugar starvation – *Zea* (nitrogen metabolism)

Abbreviations: AlaAT = alanine aminotransferase; AspAT = aspartate aminotransferase; AS = asparagine synthetase; Asnase = asparaginase; AsnAT = asparagine aminotransferase; β-CS = β-cyanoalanine synthase; GDH = glutamate dehydrogenase; Glnase = glutaminase; GOGAT = glutamate synthase; GS = glutamine synthetase; NiR = nitrite reductase; NR = nitrate reductase

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Introduction

Carbohydrate deprivation commonly occurs in higher plants during senescence (Thomas 1978; Peoples and Dalling 1988), in darkness (Postius et al. 1976; Baysdorfer et al. 1988) and under post-harvest (King et al. 1990) conditions. In order to survive carbohydrate deprivation, plant cells have to adapt to the lack of carbohydrates and either partially or totally substitute protein and lipid metabolism for sugar metabolism through autophagic processes (Thomas 1978; Journet et al. 1986; Brouquisse et al. 1991).

Protein degradation, via amino-acid catabolism, leads to a supply of carbon skeletons which are oxidized in the tricarboxylic-acid cycle and to the release of nitrogen, mainly as ammonium (Mazelis 1980). The role of asparagine as a detoxification product, acting as a nitrogen storage compound synthesized under high concentrations of ammonium, is now well established (Givan 1979; Sieciechowicz et al. 1988a). Asparagine accumulation, in response to high levels of protein degradation, was first reported in carbohydrate-starved barley leaves (Yemm 1937). In addition, asparagine appears to play a crucial role in plant growth, development and senescence since, as with glutamine, it is a primary nitrogen-transport compound within the plant (Urquhart and Joy 1981).

In green tissues, asparagine and glutamine synthesis are regulated by light and consequently by carbon status (MacGrath and Coruzzi 1991). Thus, while in light-grown plants glutamine is preferentially synthesized, in dark-grown plants (i.e., in which photosynthetic carbon is limiting) asparagine is synthesized preferentially to transport nitrogen (Urquhart and Joy 1981). In root tissues, although some studies report that the enzymes of nitrogen reduction and assimilation are regulated by sugars and energy level (Sahulka and Lisa 1978; Oaks et al. 1980), there is little information available on the changes in activity of the majority of these enzymes during senescence or starvation (Peoples and Dalling 1988). In addition, apart from asparagine and glutamine, little is known concerning the changes in amino acids and the role of other amino acids in senescent or starved root tissues.

In the present work we investigated the effects of long-term sugar starvation on excised maize root tips. The changes in sugars, proteins, free amino acids and ammonium were analyzed in parallel with the changes in the activities of the main enzymes involved in nitrogen reduction and assimilation, amino-acid transamination and asparagine metabolism. Our results show that, in root meristems, starvation may be divided into three phases i.e., acclimation, survival and cell-disorganization phases. These phases correspond to different cellular carbon states and are characterized by specific changes in free-amino-acid content and enzymic activities. Nitrogen appears first to be preferentially accumulated in asparagine during the acclimation phase, and then strongly expelled into the incubation medium, essentially as ammonia, during the survival phase and, finally when starvation becomes irreversible, freely released as protein, aminated compounds and ammonium during the cell-disorganization phase.

Materials and methods

Plant material. Maize (*Zea mays* L. cv. DEA, 1990) seeds were purchased from Pionneer France Maïs (Toulouse, France). The germination and incubation conditions, and the starvation and control treatments used, have been described in detail elsewhere (Brouquisse et al. 1991).

Amino-acid analysis. Amino acids were extracted from the root tips according to the method of Stitt and ap Rees (1978) as described by Brouquisse et al. (1991) and analysed by reversed-phase high-performance liquid chromatography (HPLC) using the *o*-phthaldialdehyde-thiol (OPA-thiol) method described by Joseph and Marsden (1986). Separation of the OPA-amino acids was carried out using a Vista 5500 liquid chromatograph (Varian Associates, Palo Alto, Cal., USA) and a reversed-phase Hypersil H5 MOS C_8-column (22 cm long, 4 mm i.d.).

The gradient mixture used for HPLC was as follows: buffer A, 50 mM potassium acetate (pH 7.23) : methanol : tetrahydrofuran (THF) (90 : 7.5 : 2.5, by vol.); buffer B, 50 mM potassium acetate (pH 7.23) : methanol (20 : 80, v/v); and buffer C, pure methanol. Amino-acid elution was carried out on a linear gradient of 100% buffer A to 30% buffer A and 70% buffer B in 25 min and then to 100% buffer C in 10 min (flow-rate 1 ml · min^{-1}). To separate histidine and glutamine, elution was performed with a different gradient mixture: buffer A, 50 mM potassium phosphate (pH 7.5) : methanol (80 : 20, v/v); buffer B, same solutions as A but a ratio of 20 : 80 (v/v); buffer C, pure methanol. Conditions of elution were as described by Joseph and Marsden (1986).

Detection of the OPA-amino acids was performed with a Varian 9060 polychrome variable-wavelength detector preset at 340 nm. Peak integration was carried out with a Varian D654 Data system. Calibration was made with a solution of authentic amino-acid standards (AA–S18; Sigma Deisenhofen, FRG).

Analysis of intra- and extracellular ammonium and total reduced nitrogen. Total reduced nitrogen was measured after mineralization of the root tips or the incubation medium. Intracellular ammonium was measured after extraction of the root tips with 0.1 N HCL whereas extracellular ammonium was measured directly in the incubation medium. In all cases, measurements were made by either Nesslerization or the phenol-hypochlorite method (King et al. 1990) with the modification that the alkaline hypochlorite solution contained 0.5% NaOCl.

Protein analysis. Protein nitrogen was measured using the phenol-hypochlorite method after extraction with 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes; pH 7.3) and 0.2% (w/v) sodium deoxycholate, precipitation with 10% trichloroacetic acid and mineralization of the root-tip extract. The amount of protein was calculated from the measured nitrogen data based on the assumption that the nitrogen content of protein is 16.7%.

Enzyme assays. For each enzyme assay 50–100 maize root tips were crushed in a mortar at 4° C with sufficient extraction medium (i.e., 0.2 g fresh weight · ml^{-1}). Addition of chymostatin to the extraction medium was necessary to minimize the effects of proteases on the measured enzymic activities (Dieuaide et al. 1992). Crude extracts were then centrifuged (12000 · *g*, 15 min) and the supernatants were either used directly or treated with ammonium sulfate (see below) and desalted through an Econo-Pac 10 DG column (Biorad, Paris, France). Spectrophotometric determinations were made with an Uvikon 930 spectrophotometer (Kontron, Zürich, Switzerland). Enzymic activity measurements by amino-acid determination were an adaptation of the OPA-thiol method described above.

Asparagine synthetase (EC 6.3.1.1, EC 6.3.5.4) (AS) extraction was adapted from the method of Oaks and Ross (1983). The extraction medium contained 0.1 M phosphate buffer (pH 8.0), 25 mM β-mercaptoethanol, 10% glycerol, 10 mM $MgCl_2$, 10 μM chymostatin, 1 mM ATP and 1 mM aspartate. Ammonium sulfate was added to the crude-extract supernatant to a final concentration of 55%. The pellet obtained after centrifugation (20000 · *g*, 15 min) was resuspended in the extraction buffer, without chymostatin, and desalted using the extraction buffer. Each assay contained 2.5 mM ATP, 10 mM glutamine or NH_4Cl, 11.5 mM $MgCl_2$, 1 mM aminooxyacetate (a transaminase inhibitor), 10 mM aspartate and 0.4 ml enzyme extract in a total volume of 0.65 ml. All substrates were dissolved in 50 mM phosphate buffer (pH 8.0) containing 5 mM β-mercaptoethanol. The reaction was run at 35° C for 15 min. Aliquots of 100 μl were sampled at 5-min intervals and the reaction terminated by addition of 100 μl of 95% ethanol. The precipitated proteins were removed after centrifugation (20000 · *g*, 15 min), the supernatant reduced to dryness in a rotary evaporator, and the residue resuspended in 0.1 ml of water. The asparagine in the aqueous extract was quantified by HPLC using the OPA-thiol derivatization method (see above). The derivatives were eluted within 15 min by isocratic elution at a flow-rate of 1 ml · min^{-1}. The elution buffer was: 50 mM potassium acetate (pH 7.23) : methanol : THF (69 : 30 : 1, by vol.). Assay blanks consisted of (i) boiled enzymic extract plus reagent and (ii) enzymic extract without glutamine or NH_4Cl.

Asparaginase (EC 3.5.1.1) (Asnase) extraction and assay were adapted from the method of Joy and Ireland (1990). The extraction medium contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol, 10 μM chymostatin, and 1 mM $CaCl_2$ which is an inhibitor of asparagine synthetase. The enzymic extract was pre-

cipitated with ammonium sulfate (60% saturation), then pelleted and desalted using the extraction medium without $CaCl_2$. Assay of Asnase was performed according to the method of Joy and Ireland (1990) and the aspartate formed was analysed by HPLC using the same elution buffer as for AS.

Asparagine:α-ketoacid aminotransferase (EC 2.6.1.14) (AsnAT) was extracted in the following medium: 50 mM 3-(N-morpholino)ethanesulfonic acid (Mops; pH 7.5), 1 mM EDTA, 5 mM dithiothreitol, 0.2% (w/v) sodium deoxycholate, 10 μM chymostatin and 1% (w/v) soluble polyvinylpyrrolidone (PVP). The enzymic extract was precipitated with ammonium sulfate (60% saturation), then pelleted and desalted using 20 mM N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine; pH 8.2). The activity of AsnAT was measured with pyruvate or glyoxylate as co-substrate. Each assay contained 50 mM Tricine (pH 8.2), 1 mM asparagine, 1 mM pyruvate or glyoxylate and 0.4 ml enzyme extract in a final volume of 0.65 ml. The measurement of AsnAT activity was performed as for AS. The alanine or glycine formed were analyzed by HPLC using an isocratic elution gradient containing 50 mM potassium acetate (pH 7.5) : methanol : THF (43 : 56 : 1, by vol.).

β-Cyanoalanine synthase (EC 4.4.1.9) (β-CS) was extracted in a medium containing 50 mM (Bicine; pH 8.5) and 10 μM chymostatin. The enzymic extract was precipitated with ammonium sulfate (80% saturation), then pelleted and desalted using 50 mM Bicine (pH 8.5). The β-CS assay was performed according to the method of Hendrickson and Conn (1969).

Alanine:α-ketoglutarate aminotransferase (EC 2.6.1.2) (AlaAT) and aspartate:α-ketoglutarate aminotransferase (EC 2.6.1.1) (AspAT) were co-extracted in a medium containing 50 mM 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid (Tes; pH 7.5), 1 mM cysteine, 1 mM EDTA, 10 μM chymostatin and 1% (w/v) soluble PVP. The supernatant of the crude extract was used for enzymic assay. The activities of AspAT and AlaAT were measured at 25° C according to Rej and Horder (1983a and b, respectively).

Glutamate dehydrogenase (EC 1.4.1.2–4) (GDH) was extracted, after freezing and thawing of the root tips, with a medium containing 50 mM Tricine (pH 8.0), 1 mM cysteine, 1 mM EDTA, 10 μM chymostatin and 1% (w/v) soluble PVP. The enzymic extract was precipitated with ammonium sulfate (65% saturation), then pelleted and desalted using 50 mM Tricine (pH 8.5). The assay mixture for NAD(P)H-GDH activity contained 0.1 M Tricine (pH 8.0), 1 mM ADP, 1 mM $CaCl_2$, 0.2 mM NAD(P)H, 12 mM α-ketoglutarate and 0.1 M NH_4Cl. The GDH-NAD and GDH-NADP activities were measured at room temperature, the former in 0.1 M 2-(N-cyclohexylamino)ethanesulfonic acid (Ches; pH 9.2), 50 mM glutamate and 0.5 mM NAD, and the latter in 0.1 M Ches (pH 9.6), 50 mM glutamate and 0.5 mM NADP.

Glutaminase (EC 3.5.1.2) (Glnase) was co-extracted with AS. The reaction mixture contained 10 mM glutamine, 1 mM amino-oxyacetate, 5 mM NH_4Cl and 0.4 ml of desalted extract in a final volume of 0.65 ml. The assay was performed at 35° C for 15–30 min. The method of activity measurement was the same as for AS. The glutamate formed was measured by HPLC using the same elution buffer as for AS.

Glutamine synthetase (EC 6.3.1.2) (GS) was extracted in a medium containing 0.1 M Tricine (pH 8.0), 20 mM $MgSO_4$, 10 μM chymostatin and 1 mM EDTA. The supernatant of the crude extract was used for enzymic measurement. Glutamine synthetase was determined according to a modified version of the method of O'Neal and Joy (1973). The assay mixture consisted of 0.1 M Tricine (pH 7.8), 4 mM $MgSO_4$, 0.2 mM EDTA, 80 mM glutamate, 6 mM NH_2OH, 8 mM ATP, and enzyme extract. The total reaction volume was 1 ml. The reaction was started by addition of NH_2OH and terminated, after 10 min at 37° C, with 1 ml $FeCl_3$ reagent. Extracts were then centrifuged and the absorbance of the δ-glutamyl hydroxamate formed was measured at 540 nm. Assay blanks contained all the reagents except ATP.

Glutamate synthase (EC 2.6.1.53) (GOGAT) extracts were prepared according to the method of Oaks et al. (1980), 10 μM chymostatin being added to the extraction medium, and the assay carried out at 25° C (Oaks et al. 1979).

Nitrate reductase (EC 1.6.6.1) (NR) was extracted and assayed as described by Long and Oaks (1990). Assay blanks contained enzymic extracts and all reagents except NADH.

Nitrite reductase (EC 1.7.7.1) (NiR) was prepared and assayed using a modified version of the method described by Suzuki et al. (1985). The root tips were crushed with 25 mM potassium phosphate pH 7.5 containing 1 mM EDTA, 10 μM chymostatin and 0.5% (w/v) insoluble PVP. The enzymic extract was precipitated with ammonium sulfate (75% saturation), then pelleted and desalted using 25 mM potassium phosphate (pH 7.5). Nitrite reductase was assayed, at 25° C, in 25 mM potassium phosphate (pH 7.3) containing 1 mM $NaNO_2$ and 50 μM methylviologen. The total reaction volume was 1 ml. The reaction was started by addition of 10 μl of 125 mM sodium dithionite in 250 mM sodium bicarbonate. Samples were maintained under a nitrogen-flow during the incubation and 20-μl aliquot fractions were sampled, diluted in 1.68 ml H_2O, and shaken energetically for 30 s. A 2-ml aliquot of the nitrite reagent (Long and Oaks 1990) was then added and the absorbance measured at 540 nm after 30 min. Assay blanks contained (i) enzymic extract plus reagents except dithionite and (ii) boiled extract plus reagents.

Results

Removal of the supply of carbohydrates to root meristems immediately triggered a sharp decline in sugar content (Fig. 1). Sugar content then remained constant at 15% of its initial value, followed by a total disappearence by 120 h. During the same period, protein content declined continuously to reach 40 and 8% of its initial value at 96 and 192 h, respectively (Fig. 1). In the presence of 0.1 mM glucose in the incubation medium (control), the protein content did not vary significantly during 96 h of incubation (data not shown), indicating that the changes in sugar and protein contents in starved root tips were not caused by excision stress. In previous work it has been shown that 4 h after excision the respiratory quotient of maize root tips was close to 0.75 (Saglio and Pradet 1980) and that after 15–20 h proteins and lipids were the only substrates for respiration (Brouquisse et al. 1991). The following results focus on the changes in content and metabolism of the amino acids released during protein degradation.

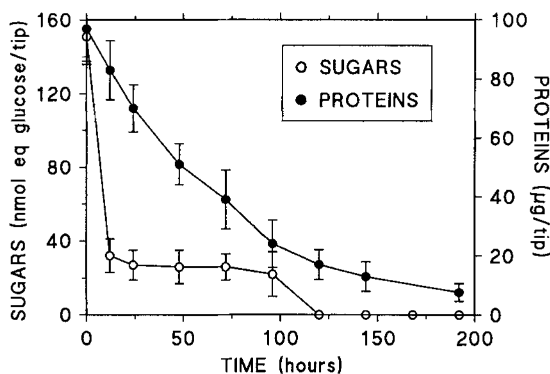

Fig. 1. Changes in total protein (●) and total sugar (○) contents of excised maize root tips during glucose starvation. These data represent the means ± SD of three independent analyses

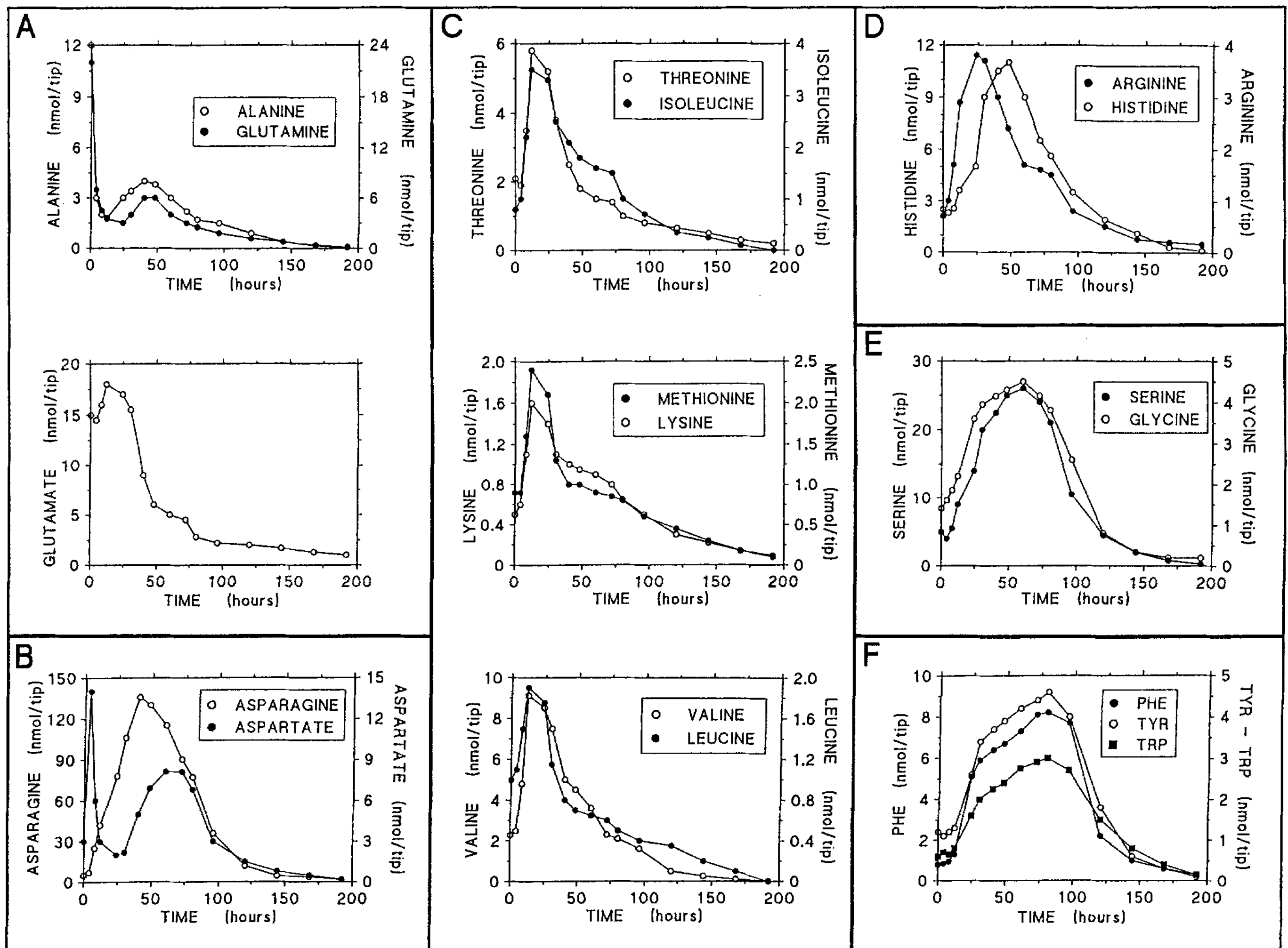

Fig. 2A–F. Changes in free-amino-acid contents of excised maize root tips during sugar deprivation. Amino acids are pooled according to common patterns of change in content throughout starvation (**A, C, E, F**) or their metabolic relations (**B**). These data represent the means of five independent experiments

Intracellular changes of free-amino-acid content during starvation. Within few hours of carbohydrate starvation there were significant changes in the levels of amino acids (Fig. 2). Alanine, glutamine and glutamate (Fig. 2A) were the predominant free amino acids in non-starved root tips (12, 22 and 15 nmol per tip, respectively). Alanine and glutamine declined to a quarter of their initial concentration within 12 h, and showed a transient peak between 40 and 50 h and then decreased continuously until tissue death. In contrast, the glutamate level showed a slight increase (i.e., 20%) after 12 h and did not start to decline till 30 h (Fig. 2A). Glutamate is both the synthetic precursor and degradation product of glutamine and arginine. However, there appears to be no correlation between either glutamate and glutamine content (Fig. 2A) or between these amino acids and arginine contents (Fig. 2D).

Asparagine increased considerably from 5 to 135 nmol per tip after 45 h of starvation and accounted for two-thirds of the total free-amino-acid content at that time (Fig. 2B). Such an accumulation of asparagine has already been found to be a good marker of protein and amino-acid degradation under different stress conditions (Sieciechowicz et al. 1988a) and particularly during starvation (Genix et al. 1990; King et al. 1990). On the basis of 16.7 g nitrogen per 100 g protein, it could be calculated that the asparagine accumulation, between 0 and 45 h of starvation, accounted for 50% of the nitrogen released from protein degradation (Fig. 1, Table 1). The most common precursor for asparagine synthesis is aspartate: it may be noted that there existed a balance between the patterns of aspartate and asparagine content in the course of the starvation (Fig. 2B). The asparagine increase was preceded by a sharp peak of aspartate during the first 4 h after excision. Similarly, beyond 40 h, the slowing down of asparagine accumulation was followed by a slower accumulation of aspartate whose maximum occurred at approx. 70 h. Such changes indicated that during starvation aspartate first acted as the asparagine precursor and then as its primary degradation product. The involvement of the other possible routes for asparagine synthesis (i.e., β-cyanoalanine pathway and

Table 1. Distribution of total nitrogen between proteins, amino acids, primary amines and ammonium in the maize root tips and in the incubation medium during sugar starvation. Results are expressed as nanomoles of nitrogen contained or released per root tip after 0, 45, 96 and 192 h of starvation

		Nitrogen/tip (nmol)			
		0 h	45 h	96 h	192 h
Total nitrogen	root tips	2050	1600	1150	400
	incubation medium	0	390	850	1560
	Σ total nitrogen	2050	1990	2000	1960
Root tips	proteins	1160	610	285	92
	asparagine	10	270	72	4
	other amino acids	102	128	59	3
	NH_4^+	20	62	75	5
Incubation medium	proteins	0	17	46	128
	primary amines[a]	0	75	195	334
	NH_4^+	0	130	420	540
	Σ nitrogen	1292	1290	1152	1106

[a] Under the heading "primary amines" in incubation medium, we pooled all the compounds which reacted with OPA reagent; 30 to 40% of them were classical amino acids. On the four major peaks detected on HPLC chromatograms, one of them was glycine, another was putatively attributed to putrescine and the two others remained unidentified. The nitrogen amount, at 96 and 120 h, could thus be underestimated because unidentified compounds may be polyamines

asparagine : α-ketoacid transamination) will be discussed below.

The other amino acids, after a 4-h lag, increased, reached a maximum occurring between 12 and 90 h and then decreased continuously to nearly zero up to 190 h.

Threonine, isoleucine, lysine, methionine, leucine and valine contents (Fig. 2C) showed a similar pattern: an initial rapid increase (two- to fourfold) with a maximum at 12 h and then a decrease until 200 h. Interestingly threonine, lysine, methionine and isoleucine belong to the same "biosynthetic family", i.e. the aspartate family (for review, see Bryan 1990 and Ireland 1990), and share common synthetic steps, i.e. aspartate kinase and aspartate semialdehyde dehydrogenase. Similarly, leucine and valine are both synthesized from pyruvate and share with isoleucine, the third branched-chain amino acid, several common enzymes in their synthesis (Bryan 1990; Ireland 1990).

Arginine content (Fig. 2D) increased threefold, reached a maximum at 24 h starvation and then decreased after 30 h. Its pattern did not follow that of glutamate although they both decreased in the same period. It should be noted that arginine, which is a good nitrogen carrier in the Gramineae and coniferous species (C/N = 1.5), did not function as a nitrogen-storage compound during starvation.

Histidine (Fig. 2D) which does not share any common synthetic step with other amino acids (Bryan 1990), and whose degradation pathway in plants remains unknown (Mazelis 1980), showed a peak with a maximum at 50 h.

Serine and glycine levels showed the same pattern (Fig. 2E), reaching their maximum at approx. 60 h of starvation at which time asparagine had already begun to decrease. These two amino acids, which belong to the same "synthesis family", seemed to be of particular importance since serine was, after asparagine, the most-accumulated amino acid during starvation, reaching 26 nmol per root tip, and glycine was the only amino acid that continued to be released into the incubation medium during the last period of starvation (data not shown).

Finally, the changes in the levels of tyrosine, phenylalanine and tryptophan during starvation were similar (Fig. 2F) and characterized by a shoulder at around 30–40 h and a maximum at 80–90 h when the starvation became irreversible. These amino acids, which are synthesized along the shikimate pathway, appeared to be only partially recycled or degraded since they accumulated continuously up to the assumed disorganisation of the limiting membranes of the cell (Brouquisse et al. 1991).

After 30–35 h of starvation, some non-amino-acid nitrogen compounds were detected in the tissues but their levels remained low compared with the free amino acids. One of these compounds co-eluted with putrescine in the HPLC analysis of the OPA derivatives (data not shown).

Nitrogen status of the root tips. During starvation, the oxidation of the amino acids produced by protein degradation to CO_2 resulted in the release of nitrogen which could either be stored within the cell or excreted. From the beginning of the starvation the total nitrogen content of the root tips declined continuously to reach 20% of its initial value after 200 h starvation (Fig. 3A). This decrease in tissue nitrogen content corresponded to the release of nitrogen into the incubation medium (Fig. 3B). During the same period, ammonium concentration increased within the tissue up to 60 h, levelled off at about 75 nmol per root tip and then decreased beyond 100 h (Fig. 3A). This increase in intracellular ammonium content was accompanied after 24 h by a prominent and continuous ammonium efflux out of the tissue (Fig. 3B). Thus it appears from the data concerning changes in proteins (Fig. 1), amino acids (Fig. 2), ammonium and total nitrogen (Fig. 3) that, during starvation, protein

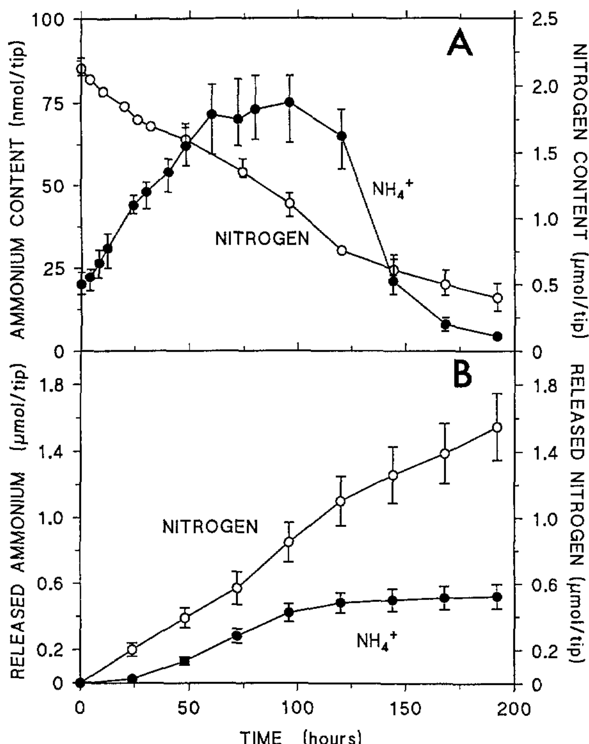

Fig. 3A, B. Changes in the contents of ammonium and total nitrogen during sugar starvation (**A**) within maize root tips, and (**B**) excreted by the root tips. These data represent the means ± SD of three independent experiments

nitrogen (i) was not stored entirely as asparagine and other free amino acid but was partially released as NH_4^+ inside and outside the cell and (ii) was redistributed according to the progress of the starvation.

Table 1 summarizes the calculated nitrogen balance of the major nitrogen compounds, related to protein degradation, after different periods of starvation. The sum of intra- and extracellular nitrogen amounts was approximately constant, indicating that the recovery of the different nitrogen compounds was close to 100%. In non-starved maize root tips, proteins and amino acids accounted for 62% of the total nitrogen (Table 1), adenine nucleotides and nucleic acids accounted for 0.7% and 2.4%, respectively (Brouquisse et al. 1991; C. Chevalier, (INRA, Bordeaux, France)). On the basis of a maize-root-tip fresh weight of 2.15 mg (Brouquisse et al. 1991) the initial concentration of intracellular ammonium was found to be approx. 10 mM, assuming an even distribution in cell water (see *Discussion*), which was in the range of other reported NH_4^+ concentrations in maize root tips (Oaks et al. 1980; Lee and Ratcliffe 1991).

After 45 h of starvation, which corresponds to the maximum of asparagine accumulation (Fig. 2B), 47% of the proteins had been degraded. Only 50% of the nitrogen lost from protein (550 nmol per root tip) ended up in asparagine whereas the remaining 50% was distributed between other amino acids (3.6%), ammonium (31.3%, most of which was found external to the cells) and extracellular amino acids and proteins (13.6% and 3.1%, respectively) (Table 1).

From 45 to 96 h, asparagine accumulation ceased (Fig. 2B), the intracellular content of free amino acids declined continuously and the level of intracellular ammonium stabilized (Fig. 3A). After 96 h, more than 75% of the nitrogen issued from protein degradation had been expelled outside the cells (Table 1). At that time, the starvation process was irreversible as indicated by free leakage of metabolites out of the cells (Brouquisse et al. 1991). Thus, large quantities of ammonium, amino acids and proteins were found in the incubation medium (Table 1).

It is important to emphasize that until 100 h starvation asparagine levels in the incubation medium were

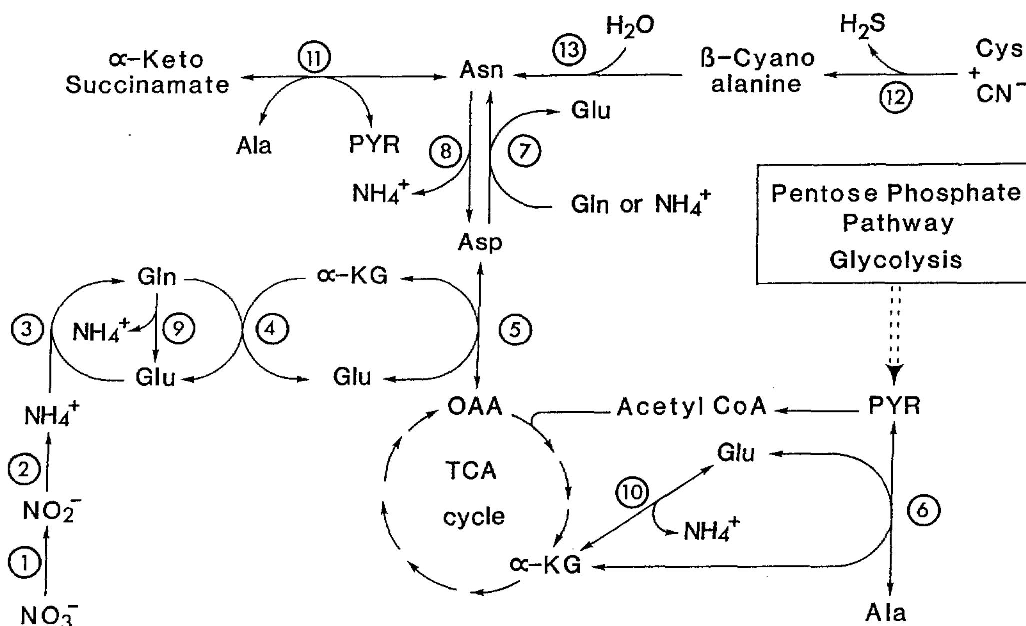

Fig. 4. Diagram of the main pathways of asparagine metabolism and nitrogen assimilation in plant cells. *1*, nitrate reductase; *2*, nitrite reductase; *3*, glutamine synthetase; *4*, glutamate synthase; *5*, aspartate aminotransferase; *6*, alanine aminotransferase; *7*, asparagine synthetase; *8*, asparaginase; *9*, glutaminase; *10*, glutamate dehydrogenase; *11*, asparagine aminotransferase; *12*, β-cyanoalanine synthase; *13*, β-cyanoalanine hydratase. *Ala*, alanine; *Asn*, asparagine; *Cys*, cysteine; *Gln*, glutamine; *Glu*, glutamic acid; *αKG*, α-ketoglutarate; *OAA*, oxaloacetate; *PYR*, pyruvate; *TCA*, tricarboxylic acid

negligible. Therefore, in the root meristem under starvation, asparagine could not be considered as a nitrogen-transport compound as in senescing green tissues (Peoples and Dalling 1988).

Changes in the activity of enzymes involved in nitrogen metabolism. The accumulation of asparagine during the first 45 h of starvation (Fig. 2B), showed that asparagine could primarily serve as a transient storage compound for the nitrogen issued from the degradation of other amino acids. Figure 4 summarizes the main pathways related to asparagine metabolism, nitrogen assimilation and the main transamination reactions.

Although little is known concerning amino-acid catabolism in plants, it is now accepted that acetyl-CoA is the end product of the degradation of about twelve amino acids (Mazelis 1980; Gerbling and Gerhardt 1989). Deamination of these amino acids often occurs via a transamination mechanism which involves α-ketoglutarate (Mazelis 1980). The glutamate thus formed may (i) react with oxaloacetate to form α-ketoglutarate plus aspartate through AspAT, (ii) react with NH_4^+ to form glutamine through GS or (iii) be deaminated to α-ketoglutarate and NH_4^+ through GDH (Fig. 4). Three routes are known to account for asparagine synthesis in plants (for review, see Sieciechowicz et al. 1988a). Glutamine and ammonium may react with aspartate to form asparagine, this route being mediated by AS, and is generally considered as the main pathway for asparagine synthesis in plants. The two other routes of asparagine synthesis involve AsnAT and the β-cyanoalanine pathway (Fig. 4), and are considered as secondary routes.

Figure 5 reports the changes in the enzyme activities, shown in Fig. 4 (with the exception of β-cyanoalanine hydratase), during the course of starvation.

Initial glutamine (Gln) and NH_4^+-dependent AS activities per root tip were 26 and 10 pmol · min^{-1}, respectively (equivalent to 12 and 4.6 nmol · min^{-1} · g FW^{-1}, respectively). Their activities greatly increased during the first 24 h of starvation (by factors of 13–17) and then decreased continuously up to 200 h (Fig. 5A). The ratio of Gln-AS/NH_4^+-AS activity remained constant at a value of 2.5. Glutamine is generally considered as the main substrate of AS activity in vivo. The transient increase and decrease of AS agrees with the opposite changes observed between intracellular aspartate and asparagine contents during the first 100 h of starvation (Fig. 2B). Moreover, the increase of AS activity (up to 350 pmol · min^{-1} per root tip with glutamine as co-substrate) is sufficient to account for the apparent flux of nitrogen transferred in asparagine (on average 50 pmol · min^{-1} per root tip) between 0 and 45 h.

The activity of β-CS exhibited the same trend as that for AS, with a maximum at 24 h and then a decrease until 120 h at which time activity was no longer detectable (Fig. 5A). Initial β-CS activity (473 pmol · min^{-1} per root tip or 220 nmol · min^{-1} · g FW^{-1}) was 20-fold higher than initial AS activity. Such observations have already been reported in maize root tips by Stulen et al. (1979), although we found 12- and 24-fold higher AS and β-CS activities, respectively, than these authors. An ex-

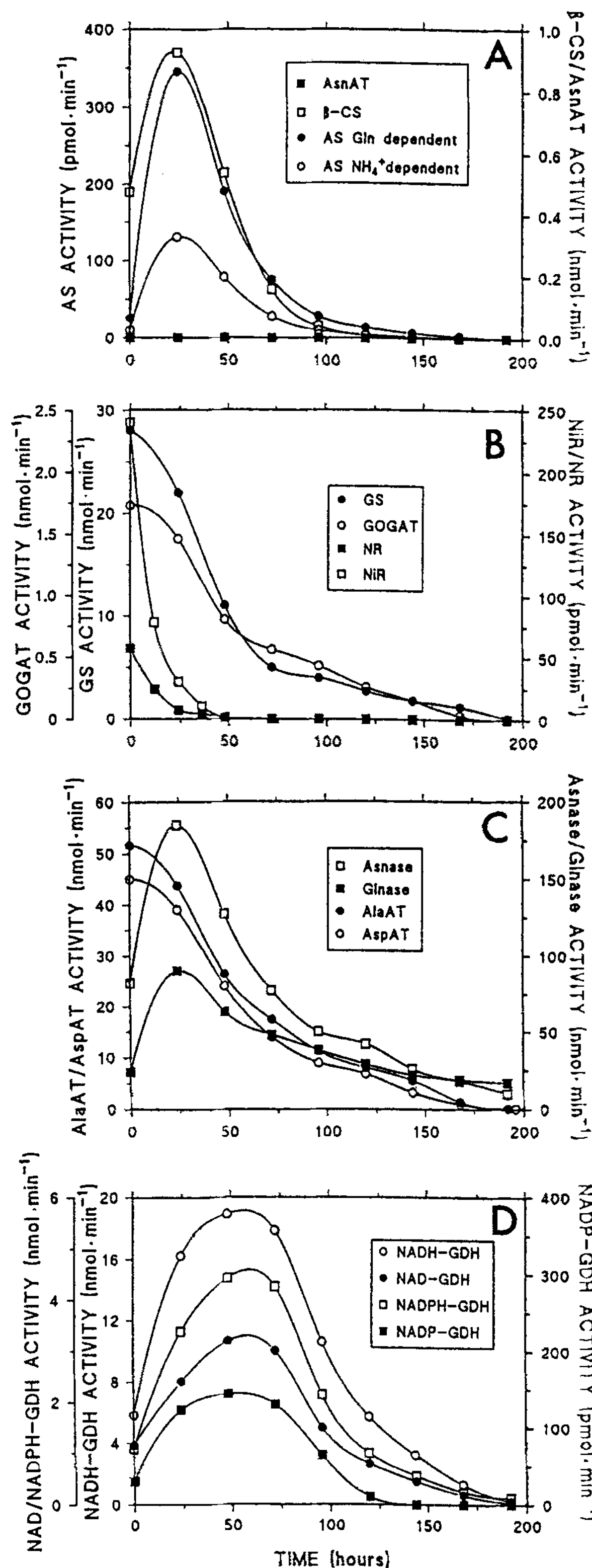

Fig. 5A–D. Changes in some enzymic activities of excised maize root tips during glucose deprivation. **A** asparagine metabolism: AsnAT, β-CS, Gln- or NH_4^+-dependent, AS. **B** Nitrate reduction and ammonia assimilation: NR, NiR, GS and GOGAT. **C** Amino-acid deamidation and transamination: Asnase, Glnase, AlaAT and AspAT. **D** NAD- and NADP-dependent GDH activities in aminating and deaminating reactions. The data are expressed on a per-root-tip basis and represent the means of two to five independent experiments

planation for the presence of such high β-CS activity in maize is, to date, unclear since this species is non-cyanogenic. In contrast to the other enzymes, whose activities were significantly different when they were measured in starved or on-starved root tips, the activity of β-CS showed the same relative increase in starved and non-starved root tips (data not shown). This indicates that the increase of β-CS activity (Fig. 5A) was caused by excision stress rather than starvation. No AsnAT activity was found throughout starvation (Fig. 5A) making this pathway ineffective for asparagine synthesis or degradation during starvation. Such an absence of AsnAT has already been reported in non-green tissues (Lea et al. 1990). These data provide convincing support for the hypothesis that AS is the route for asparagine synthesis during starvation.

From the onset of sugar deprivation, the activities of enzymes involved in nitrogen assimilation decreased (Fig. 5B). Nitrate reductase and NiR decreased very rapidly and were undetectable after only 48 h following excision. Such a rapid decrease in activity has already been observed in darkened maize seedlings (Bowsher et al. 1991) and more generally under stress conditions (Kleinhofs and Warner 1990). Glutamate synthase and GS decreased more slowly and reached 15 and 25% of their initial activity, respectively, after 96 h (Fig. 5B). It is noteworthy that during starvation GS activity always remained higher than that of AS (two orders of magnitude) indicating that, despite its decreasee, GS remained potentially a route for NH_4^+ transfer into asparagine. However the continuous accumulation of NH_4^+ (Fig. 3A), the maintenance of the free-glutamate level (Fig. 2A) and the drop in free-glutamine content (Fig. 2A) were not in favour of a very active GS, in vivo, during the first stage of starvation.

Alanine aminotransferase and aspartate AspAT are of particular importance in meristematic cells because they generate the synthesis of numerous amino acids (Givan 1980). Moreover, AspAT is the principal route for aspartate synthesis from oxaloacetate and is thus critical for asparagine synthesis during starvation. Initial AlaAT and AspAT activities were very high (52 and 46 nmol · min^{-1} · per root tip, respectively) (Fig. 5C). They decreased continuously throughout starvation (Fig. 5C) in a way similar to those of GS, GOGAT (Fig. 5B) and the glycolytic enzymes (Brouquisse et al. 1991). However, AspAT always remained high enough (more than 500-fold) to account for the carbon flux from oxaloacetate to aspartate during asparagine accumulation.

The transient increases of glutaminase and Asnase activities during the first 24 h of starvation (4- and 2.3-fold, respectively) (Fig. 5C) are more difficult to explain. Indeed, both activities would decrease asparagine accumulation through (i) removal of glutamine which is the preferential substrate for AS (Glnase) and (ii) deamination of newly formed asparagine (Asnase). However, Rognes (1980) showed that AS and Glnase activities are associated with the same enzyme in the cotyledon of lupin seedlings; to our knowledge, no other published work exists concerning Glnase in plants. The increase of Glnase we observed could thus be a consequence of the increase of AS activity. The initial level of Asnase activity (82 pmol · min^{-1} per root tip) was 3.2-fold higher than that of AS. However, after 24 h, which corresponds to the period of most active asparagine accumulation (Fig. 2B), and up to 48 h, the AS/Asnase activity ratio reversed in favour of AS activity (AS/Asnase = 1.9 and 1.5 after 24 and 48 h, respectively). Beyond 48 h, this ratio favoured Asnase when asparagine was degraded and ammonium expelled from the cell (AS/Asnase = 0.92 and 0.56 after 72 and 96 h).

Finally, we measured the changes in NAD- and NADP-dependent GDH activities in both the aminating and the deaminating direction (Fig. 5D). These activities increased by 2.8 to 4.8-fold during starvation (Fig. 5D). However, in contrast to β-CS, AS, Glnase and Asnase, the maximum activity was reached after 48 h and maintained until 72 h. Such a specific pattern indicates that GDH may have different roles during the course of starvation, particularly during and after the period of asparagine accumulation. It should also be noted that, contrary to previously reported observations (Singh and Srivastava 1983; Loyolas Vargas and Jimenez 1984; Cammaerts and Jacobs 1985; Srivastava and Singh 1987; Tirado et al. 1990), the NADH/NADPH, NADH/NAD and NADPH/NADP activity ratios did not vary significantly during starvation.

Discussion

It has been reported previously that, in root tips, sugar content is low (approx. 80 mM glucose equivalents) compared with mature roots and that respiration varies greatly depending on the sugar level (Saglio and Pradet 1980; Brouquisse et al. 1991). Moreover, in roots of plants submitted to dark/light cycles, sugar content and respiration decrease in the dark and increase in the light (Kerr et al. 1985; Frossard 1985). Therefore, in root meristems it appears that starvation of sugars occurs at the end of the night.

Removal of the carbohydrate supply to maize root tips induces a rapid decrease of the soluble sugar content (Fig. 1) and triggers an immediate degradation of proteins (Fig. 1) and lipids (Brouquisse et al. 1991; Dieuaide et al. 1992). In response to protein degradation, the level of asparagine increased within the tissue up to 45 h (Fig. 2B). Such an accumulation of asparagine has already been reported in numerous green and non-green plant tissues undergoing senescence, and submitted to light/dark transitions or starvation stresses (Yemm 1937; Thomas 1978; Genix et al. 1990; King et al. 1990). However, after 45 h, the level of asparagine decreased (Fig. 2B); the absence of asparagine in the medium indicates that it is degraded, presumably to aspartate and NH_4^+. The cellular level of ammonium increased upon the start of starvation (Fig. 3A) but the excretion of NH_4^+ into the medium is delayed by about 24 h (Fig. 3B). This raises the question of benefits and cost of asparagine formation during short-term and long-term sugar deprivation.

Considering the expression of biological costs in terms

of moles of glucose, according to Pate and Layzell (1990), it may be calculated that the synthesis of asparagine and its subsequent transformation to 2 glutamate (after glucose re-feeding) costs 1.56 glucose equivalents, whereas the assimilation of 2 NO_3^- into 2 glutamate would cost 2.26 glucose equivalents (the difference, 0.7 glucose equivalents, corresponds to the cost of nitrate uptake and reduction). Thus, the transient storage of 130 nmol of asparagine per root tip will save 90 nmol of glucose equivalent, i.e. 60% of the sugar content of non-starved root tips. The storage of nitrogen in glutamine would be less efficient in terms of carbohydrate economy (Pate and Layzell 1990). However, the storage of nitrogen in arginine which would be more efficient (C/N = 1.5, instead of two for asparagine) does not occur: the ease of mobilization of asparagine nitrogen during recovery after glucose re-feeding may have been another important factor in the selection of the form of nitrogen storage during the first 2 d of starvation.

Our results therefore show that root meristems are able to adapt to temporary carbon deprivation and have adopted, through the storage of asparagine, the less expensive mechanism (in terms of carbon consumption) for nitrogen preservation. The decrease of asparagine after 45 h may indicate that at this time the constraint of carbon supply becomes so strong that the asparagine carbon has to be used by the cells and, consequently, ammonium is lost into the medium (Fig. 3B). It is interesting to note that, in contrast to mature or senescent leaves or nodules where asparagine and glutamine are re-exported to regions of more active growth (Peoples and Dalling 1988), the starved root meristem behaves as a sink tissue and does not release asparagine from of the cell.

The nitrogen balance (Table 1) shows that asparagine is not the only protein-degradation product in maize root tips, as it is in sycamore cells (Genix et al. 1990), since, between 0 and 45 h, one-half of the protein nitrogen was released in the form of ammonium and aminated compounds. Ammonium represents 30% of the nitrogen lost by proteins after 45 h and more than 50% between 45 and 96 h (Fig. 3, Table 1). Although ammonium was essentially expelled from the tissue, intracellular content increased greatly up to 75 nmol per root tip after 70 h. Such a concentration (approx. 35 mM) raises the problem of in-vivo NH_4^+ toxicity if we consider that an in-vitro concentration of 5 mM is largely sufficient to uncouple biological membranes. Although NH_4^+ was found to be toxic (Givan 1979), there are very few reports examining toxic concentrations of ammonia in vivo since it is not easy to determine its subcellular concentration. Recently, however, Lee and Ratcliffe (1990) have used in-vivo ^{14}N-nuclear magnetic resonance to show that in maize root tips NH_4^+ is essentially located in the vacuole and absent from the cytoplasm. These observations agree with theoretical calculations which show that, on the basis of certain physiological parameters (i.e., NH_4^+/NH_3 pKa = 9.2, pH vacuole = 5.8, pH cytoplasm = 7.3) and assuming that the cytoplasm and the vacuole each occupy 45% of the total tissue volume (Patel et al. 1990), ammonium concentration varies from 20 to 70 mM in the vacuole and from 0.6 to 2 mM in the cytoplasm of the root tips during starvation. Such compartmentation explains the accumulation of high amounts of NH_4^+ in the root tips without damage.

Besides asparagine, the changes in free amino acids (Fig. 2) show that they are metabolized differently throughout starvation. In vivo, the intracellular amino-acid concentration is controlled by four factors: synthesis, degradation or transformation, utilisation for protein synthesis and release through protein degradation. While the expected trend would be for free-amino-acid levels to reflect the intense protein degradation, free amino acids may be pooled on the basis of a "biosynthesis pathway" criterion. For instance, threonine, lysine, methionine and isoleucine (Fig. 2C), whose biosynthesic precursor is aspartate, exhibit the same 4-h lag phase, 8-h increase and stepwise decrease. This behavior indicates that their content during starvation is mainly controlled at the level of common synthetic steps, i.e. aspartate kinase and-or aspartate semialdehyde dehydrogenase, as previously shown for aspartate kinase II in *Bacillus subtilis* submitted to nutrient limitation (Graves and Switzer 1990). The degradation and-or inactivation of aspartate kinase would consequently lead to a better availability of aspartate for asparagine synthesis. Similarly, valine and leucine (Fig. 2C), serine and glycine (Fig. 2E), and phenylalanine, tyrosine and tryptophan (Fig. 2F), which respectively share common synthesis enzymes, may be pooled together. The regulation of free-amino-acid content and their changes is of particular interest if we consider that, as in mammals, some of them are important regulators of protein degradation (Mortimor et al. 1988; Caro et al. 1989). For example, serine which has been found to promote protease activity in senescent oat leaves (Veierskov et al. 1985) was, after asparagine, the most abundant amino acid in starved root tips, and its change parallels the increase in proteolytic activities during starvation (James et al. 1992, submitted for publication). After 30 h of starvation the levels of glutamate and arginine, which are synthetic precursors of polyamines, decreased (Fig. 2A, D) and aminated compounds (including putrescine) accumulated in the root tips. Polyamine synthesis has been reported to occur in plant tissues undergoing senescence or submitted to various stresses (Flores 1990).

The changes in enzymic activity (Fig. 5) are in keeping with the modifications of the amino-acid and ammonium levels, and corroborate the conclusions drawn from these measurements. From the onset of starvation, enzymes of nitrogen assimilation and transaminases (NR, NiR, GS, GOGAT, AlaAT, AspAT) decrease continuously, as the enzymes involved in glucose metabolism (Brouquisse et al. 1991), whereas the enzymes involved in asparagine metabolism (AS, β-CS, Asnase), Glnase and GDHs increase transiently at different stages of starvation.

Convincing evidence for the involvment of the AS-Asnase pathway for asparagine synthesis and degradation during starvation comes from different arguments. The first argument is the observation that AsnAT is undetectable and that the increase of β-CS (Fig. 5) is due to excision rather than starvation stress, and thus would

not occur in whole plants. The second argument is the transient peak of aspartate just before asparagine accumulation and the second larger peak of aspartate when asparagine accumulation is slower and then decreases (Fig. 2B). The third argument refers to the fact that AS activity increases during starvation (13- to 17-fold) and is sufficient to account for asparagine accumulation during all starvation time (Fig. 2B, 5A). The AS increase has already been reported under starvation and dark conditions (Stulen and Oaks 1977; Tsai and Coruzzi 1990) and has been clearly attributed to an increase in the level of both AS mRNA and protein (Tsai and Coruzzi 1990). Despite the calculated increase of cytoplasmic NH_4^+ (from 0.6 to 2 mM), the ammonium concentration always remains below that of glutamine (i.e., [glutamine] > 3 mM, Fig. 2A). These data combined with the steady ratio of Gln-AS/NH_4^+-AS activity indicates that glutamine remains the principal substrate for AS during asparagine accumulation.

The increase of Asnase during the same period that AS increased (Fig. 5C) was unexpected since, in green leaves, its activity increases in the light and decreases in the dark, in contrast to AS (Sieciechowicz et al. 1988a, b). However, their activities differ in sensitivity to Cl^-, K^+ and Ca^{2+} (Sieciechowicz et al. 1988a) and are subjected to substrate-product regulation. Moreover, although during starvation AS and Asnase compete for asparagine synthesis and degradation, it should be noted that there is an inversion of the AS/Asnase activity ratio in favour of AS during asparagine accumulation and in favour of Asnase after 45 h (Fig. 5A, C). The increase of Asnase is also an additional argument in support of asparagine degradation to aspartate after 45 h.

Little is known concerning the role of Glnase in plants. Rognes (1980) has shown that Glnase and AS activities are catalyzed by the same enzyme in lupin cotyledons. However, Glnase activity increased only by 2.3-fold, compared with 13- to 17-fold for AS, and remained above its initial activity for up to 100 h of starvation (Fig. 5C). In animal cells, it has been shown that Glnase is induced after starvation (Watford et al. 1984) and requires both phosphate and ammonia as activators. In such situation, Glnase is suspected to have a regulatory role in ureagenesis by providing glutamate, as a precursor of ornithine, and thus by increasing the rate of urea synthesis and NH_4^+ disposal (Szweda and Atkinson 1990). In root tips, a similar role for Glnase in polyamine synthesis may be hypothezised since aminated compounds (i.e., putrescine) accumulated in the root tips after 30–35 h of starvation.

The activities of NR and NiR decreased much more rapidly than those of GS, GOGAT or transaminase (Fig. 5B, C). Sahulka and Lisa (1978) reported previously that pea-root NR is more sensitive to variation in sucrose level than GS. Moreover, in darkened maize seedlings (Bowsher et al. 1990), the activities and mRNAs of root NR and NiR have been found to decrease over the same time scale as that reported in Fig. 5B. The rapid decrease of NR and NiR is consistent with the fact that, in roots, NO_3^- uptake and reduction depend on the carbon status (Clarkson 1986) and that NR is inactivated rapidly by a specific protease, i.e. the "corn inactivating protein", particularly abundant in maize roots (Wallace and Shannon 1981). The decrease of GS, GOGAT and transaminases during the first 24 h is gradual (Fig. 5B, C). It is of interest to note that, despite their degradation, GS and AspAT activities remain always high enough to account for aspartate and glutamine synthesis during asparagine accumulation. It is only when carbon starvation reaches a critical threshold, after 30 h, that the enzymes of nitrogen assimilation and amino-acid synthesis, like those of glucose metabolism (Brouquisse et al. 1991), are rapidly degraded and serve as survival substrates for roots. The increases in NAD- and NADP-GDH activities (Fig. 5D) have been observed previously in numerous tissues undergoing senescence and submitted to darkness, starvation or high NH_4^+ concentrations (for review, see Srivastava and Singh 1987). Our results which show that GDHs activities increased and remained high up to 72 h (Fig. 5D) do not allow us to ascribe a preferential direction to the GDH reaction during the first 24 h of starvation (see Lea et al. 1990, for a review); however, the GDH activities are obviously in the direction of glutamate catabolism after 30–35 h, when the other enzymes of nitrogen metabolism and the level of glutamate decrease continuously (Figs. 2A, 5A–C) and NH_4^+ is produced (Fig. 3).

Considered together, metabolite analysis and enzymic measurements show that starvation may be divided into three phases. (i) The acclimation phase (0 to 30–35 h) in which the root tips adapt to transient sugar deprivation. They partly store, in asparagine, the nitrogen released by protein degradation. The enzymes of nitrogen assimilation decrease slowly whereas those of asparagine metabolism increase. (ii) The survival phase (30–35 to 90–100 h) in which the constraint of carbon supply becomes so strong that asparagine progressively ceases to be accumulated and is then degraded to aspartate and ammonium. Ammonium is expelled from the tissue. Enzymic activities decrease except those of protein and amino-acid catabolism (GDH, proteases). During this phase, starvation may still be reversed by sugar addition (Brouquisse et al. 1991). (iii) The disorganization phase (beyond 90–100 h) in which all metabolites and enzymic activities decrease, the starvation becomes irreversible and the root tips die (Brouquisse et al. 1991).

Recently, MacGrath and Coruzzi (1991) reviewed the mechanisms of light regulation of glutamine and asparagine levels in green plant tissues. In the light, GSs, GOGATs and Asnase are induced and AS is repressed, thus favouring the reassimilation of the free NH_4^+ issued from photorespiration and the synthesis of glutamine. In darkness, the opposite is observed, favouring the synthesis of asparagine. However, although it has been shown that light may exert a direct effect on AS1 gene expression in roots (MacGrath and Coruzzi 1991), light regulation is unlikely to account for the excised root meristem adaptation to starvation. It is the rapid decline in sugar content, immediately after excision (Fig. 1), which triggers the sharp fall of respiration (Brouquisse et al. 1991), the decrease of the respiratory quotient from 1 to 0.75 within 4 h (Saglio and Pradet 1980), and the

rapid changes in free-amino-acid content (Fig. 2). Sugars, and possibly ester phosphates, thus appear to play a central regulatory role in the early stage of starvation, as suggested previously (Sahulka and Lisa 1978; Oaks et al. 1980). However, it is unlikely that adenylate energy charge or ATP/ADP ratio play a regulatory role during starvation, as hypothesized by different authors (Sahulka and Lisa 1978; Oaks et al. 1980; Cammaerts and Jacobs 1985; Lea et al. 1990), since these parameters are independent of the carbohydrate level in normoxic plant cells (Pradet and Raymond 1983). Indeed, previous studies show clearly that, in spite of the decrease of sugar and adenine-nucleotide pools, the energy charge and the ATP/ADP ratio remain high throughout starvation (Saglio and Pradet 1980; Roby et al. 1987; Brouquisse et al. 1991) and are therefore not able to serve as signals in the adaptation to starvation. Beyond 15 h, the sugar level remains steady (Fig. 1) and, unless through changes in compartmentation, is not able to control the sequence of further events. More likely is the hypothesis that other metabolites, such as amino acids, ammonium, nucleotides and inorganic phosphate, whose contents are modified throughout starvation, may in turn become regulatory signals during the periods of acclimation and survival.

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