Changes in Pool Sizes of Free Amino Acids and Amides in Leaves and Plastids of *Zea mays* during Leaf Development[1]

Received for publication August 15, 1978 and in revised form October 16, 1978

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ABSTRACT

The concentrations of free amino acids and amides within isolated maize (*Zea mays* L.) plastids were determined and compared with concentrations in the leaf tissue. The concentrations were different for each individual amino acid and varied between 1 and 10 millimolar. At five different developmental stages concentrations in the plastids were greater than those in the intact leaf tissue. During development, from the proplastid stage to the mature chloroplast, the amount of each amino acid per plastid remained relatively constant, but there were decreases in concentrations of plastid amino acids resulting from the developmental increase in plastid volume. In proplastids, the free amino acids were present in greater concentrations than those previously found to inhibit partially amino acid-synthesizing enzymes located in chloroplasts. In the chloroplasts, the molarities of the free amino acids were within the range known to inhibit amino acid-synthesizing enzymes.

In studies of the control of chloroplast and leaf amino acid metabolism an important parameter is the concentration of the individual amino acids within the organelles. While several measurements of amino acid concentrations in leaf tissue have been made, their concentrations in intact plastids have not been established. Determinations which use nonaqueous techniques (1) to isolate the chloroplasts must be regarded with some caution because of the problems of cytoplasmic contamination in this type of procedure (4, 9, 27). Previous experiments in our laboratory (12) showed that chloroplasts isolated in aqueous media retain free amino acids but no attempt was made to estimate the amino acid concentrations.

The estimation of *in vivo* free amino acid and amide molar concentrations in plastids requires the quantitative analysis of free amino acids and amides, the estimation of amino acid losses from plastids during isolation (types C and E according to the nomenclature for plastid types by Hall [7]), and the measurement of the *in vivo* chloroplast volume. Reliable techniques for chloroplast isolation, assessment of losses due to rupture of envelopes, estimation of chloroplast volume, and quantitative amino acid analysis are now available. If the analysis is made on isolated plastids, some knowledge of the permeability of the chloroplast envelope to efflux of free amino acids and amides is required. Many amino acids are among the metabolites to which the chloroplast inner envelope membrane is not freely permeable (6, 10). The rapid transfer of aspartate and glutamate observed by Heldt's group (11) would not be expected to occur during aqueous isolation because the necessary exchange metabolites would not be available in effective concentrations as the cytosol is greatly diluted with the isolation medium.

Previously we have described the ultrastructural characteristics of plastids from different parts of the maize seedling shoot (15) which show a distinctive developmental sequence and can be used to study within a single leaf the changes that occur during *in vivo* plastid development. This paper reports the quantitative analysis of the free amino acid pool in isolated fully differentiated plastids and compares the *in vitro* concentration of each amino acid in isolated chloroplasts with its *in vivo* concentration in the leaf tissue. The changes in concentrations of free amino acids in plastids and in leaf tissue in five different developmental stages are also compared.

MATERIALS AND METHODS

Plant Materials. Seedlings of *Zea mays* L. (var. Kelvedon Glory, Thompson and Morgan, Ipswich, England) were grown for 7 days in constant environment cabinets as described previously (15). Shoots of seedlings were harvested by cutting through the meristematic tissue just above the mesocotyl. The coleoptile and first leaf were removed and the remaining leaf tissues cut transversely into five 2-cm-long sections (15, 16). As it has been shown that the free amino acid pools of photosynthetic tissue change dramatically during the light period of the diurnal cycle (3, 20, 25), leaves were always harvested at the same time of day, 4 to 5 h after the beginning of the light period.

Chloroplast Isolation. Chloroplast samples were prepared from about 35 g fresh weight of leaf material by a modification of the method developed by Leese and Leech (16). Leaf pieces about 5 mm wide were cut and homogenized in about 400 ml of an ice-cold medium containing 0.5 M sucrose and 1 mM $MgCl_2$ in 67 mM phosphate buffer at pH 7.4 using a M.S.E. Atomix at full speed for successive periods of 3 s, 5 s, and 5 s for the two most mature leaf sections from 6 to 8, and 8 to 10 cm from the shoot base. A pestle and plastic spatula were used to homogenize the three shoot sections from the region between the base and 6 cm from the base of the shoot. The preparation of the pelleted chloroplasts was completed within 3 min. Samples of the chloroplast preparation were examined by phase contrast light microscopy, representative photomicrographs were taken (about 800 plastids per photograph), and the proportion of intact chloroplasts counted in 10 photographs of each batch of plastids. Only the plastids with a distinct bright, shiny appearance, defined as type A or B by Hall (7), were counted as intact and this number was expressed as a percentage of the total number of plastids. Chl concentration was determined in 80% acetone according to Arnon (2). Bundle sheath chloroplasts were recognizable in the plastid suspensions from sections between 4 and 10 cm from the leaf base but these suspensions always contained fewer than 5% intact bundle sheath plastids.

Composition of Isolation Medium. The isolation of intact plas-

[1] This work was supported by a grant to D. J. C. from the Science Research Council.

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Table I. Characteristics describing developmental differences of shoot sections.

All the characteristics given were determined using maize seedlings which had been grown for seven days in the same constant conditions and had been selected to be between 14 and 18 cm tall. Values in lines 1, 2 and 4 were measured by H.M. Nice in our laboratory; values in lines 5, 6 and 7 were determined as described in the methods section of this paper; values in line 3 were calculated by dividing values in line 1 by those in line 2. All values are given to two significant figures.

		Position of section in leaf centimetres from base of shoot				
	Units	0-2	2-4	4-6	6-8	8-10
Chlorophyll/ section	μg	1.6	4.1	11	22	33
Number of plastids/ section	millions	36	66	64	44	38
Chlorophyll per plastid	$ngx10^{-6}$	44	62	170	500	870
Average plastid volume	μm^3	5.0	5.6	9.9	13.0	28.0
% intact isolated chloroplasts	$\frac{intact}{total}$	36	46	45	37	35
Fresh weight of section	mg	40	46	49	53	53
Aqueous tissue volume	mm^3	37	43	46	49	49

tids with intact envelope membranes from maize seedlings requires careful choice of isolation media. In our laboratory and elsewhere, isolation media containing BSA (8, 16, 26) or PVP (21) have been used. These compounds were not included in the isolation medium used in the experiments reported here so the proportion of intact plastids was less than usually obtained in their presence. Free amino acids and amides were extracted more easily from these plastids since the intact envelope membranes were broken readily in the absence of BSA and PVP and the losses due to adsorption of amino acids onto the large BSA or PVP molecules eliminated. The sucrose molarity of the isolation medium was chosen to be slightly hypertonic (0.5 M) to maintain plastid integrity.

Preparation of Amino Acid Samples from Isolated Plastids. Isolated plastids were broken by resuspension in ice-cold water, followed by 10 rapid strokes of a TenBroeck homogenizer and then stirred by a mechanical rotamixer until more than 95% of the plastids had lost their envelopes. The suspension of broken plastids was centrifuged at 3,000*g* for 20 min at 1 C. The supernatant was added to ethanol to a final concentration of 80% (v/v) ethanol and recentrifuged at 3,000*g* for 45 min at −5 C. After reducing the volume of the supernatant to 10 ml by rotary evaporation below 40 C, it was mixed with 4 g of Dowex 50W-X8 cation exchange resin (200–400 mesh; hydrogen form) (12). The resin was washed three times with 20 ml glass-distilled H_2O and the amino acid fraction removed by three washes with 4 mM NH_4OH. When the volume of the combined washes had been reduced by rotary evaporation, samples were stored at below −10 C for subsequent amino acid analysis.

Preparation of Amino Acid Samples from Leaf Tissue. The 2-cm sections of leaf lamina were frozen in liquid N_2, crushed and extracted four times with ethanol and once with water in a TenBroeck homogenizer. The insoluble material was removed after each extraction by centrifuging at 1,000*g* for 5 min. The combined extracts (10 ml for 300 mg fresh weight tissue) were centrifuged at 1,000*g* for 10 min and the supernatant used for further analysis. Pigments were removed by chromatographing the extract on TLC plates of 0.25-mm-thick Silica Gel H in chloroform-methanol-water (65:25:4, v/v). Plate material below the 0.5 R_F position was removed and washed with water (8.0 ml per TLC plate) three times and centrifuged at 1,000*g* for 5 min to precipitate plate material. The combined washes were reduced by 20 ml by freeze-drying or rotary evaporation below 40 C. Subsequent preparation of the amino acid fractions was as described for chloroplast extracts. Recovery of radioactive amino acid standards from TLC plates was 95 to 100%.

In order to check the complete procedure, a standard quantity

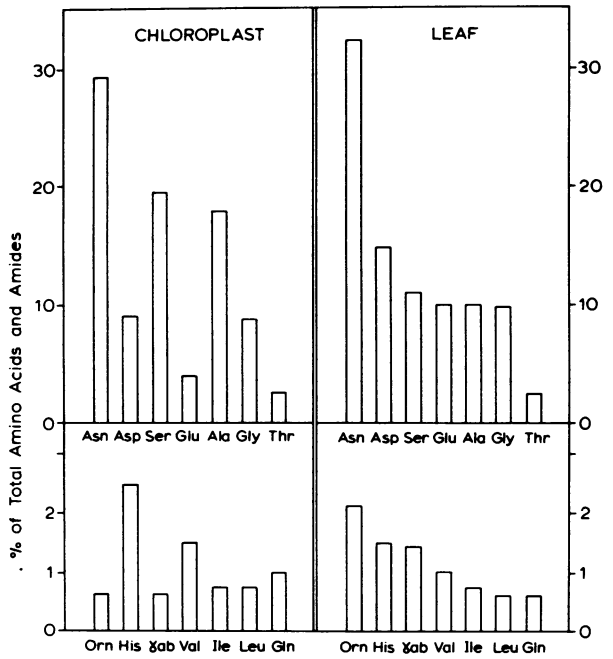

FIG. 1. Percentage composition of pools of free amino acids and amides in chloroplasts and leaf tissue. The abundance of each amino acid and amide is expressed as a percentage of the total free amino acid and amide pool. The amino acids and amides are arranged in order of their abundance in the leaf tissue pool. (The amino acid and amide percentages from ornithine to glutamine [lower two histograms] are represented on a scale that is twice the scale used to represent the percentages of the more abundant amino acids and amides.)

of D-allo-isoleucine was added to each leaf homogenate and extracted along with plant amino acids. The amount of this amino acid in the final analysis of the amino acid samples measured the recovery through all of the extraction and analysis procedures. D-allo-Isoleucine is not found normally in maize seedling leaf tissues and was clearly separated in the amino acid analysis. The average value for recoveries was 90.0 ± 6.6%. Amino acid levels in each extract were corrected according to the recovery of this standard.

Amino Acid Analysis. Samples for analysis were prepared in lithium citrate buffer adjusted to pH 2.7 and separated on a column of sulfonated-polystyrene (0.8 × 46 cm) using a JEOL fully automatic amino acid analyzer, model JLC-6AH. Two standard amino acid mixtures and four samples were loaded in the refrigerated sample store at one time and analyzed successively. Amino acids and amides were identified by their retention times and their concentrations determined by reference to the ninhydrin absorbance of the standards analyzed in the same batch of samples. The system used gave rapid analysis of samples and clear separation and identification of all of the abundant amino acids, including asparagine and glutamine.

Calculation of Concentrations of Amino Acids in Plastids. Amino acid analysis gave values for the nmol quantities of free amino acids and amides extracted from isolated plastids. From these values the molarities per intact plastid *in vivo* were calculated. For this calculation the Chl content of the plastid suspension, a figure relating the Chl level and number of plastids *in vivo*, the proportion of the plastids with intact envelopes, and the *in vivo* volume of plastids have to be known. The amino acid concentrations as nmol/mg Chl were first determined and converted to concentration per plastid using the values for Chl per plastid previously determined (16). The concentrations of amino acids as mol per ml were then calculated using the average plastid volume characteristic of each shoot section, measured by electron microscopy and light microscopy of chloroplasts *in vivo* (unpublished results). Average plastid volume was calculated from 20 plastid profiles at a magnification of × 10,000 assuming that a chloroplast approximates to an oblate spheroid, *i.e.* volume = $4/3\ \pi b^2 a$ where a and b are the short and long radii, respectively. Plastid volume was corrected for the volume of included starch. Plastid volumes and numbers of plastids per mg leaf Chl are given in Table I. The calculations assume all of the plastids to be intact. To correct for the percentage without envelope membranes (which would have lost all of their free amino acids), the proportion of intact plastids was determined by phase contrast microscopy. Any error due to counting as intact any "intermediate" (24), or "resealed" (13) plastids that have only partially lost stromal compounds will underestimate the amino acid concentration. Correction was not made for any diffusive loss from intact plastids during the isolation procedure. The calculation is as follows:

$$A = B\left(\frac{x}{w}\right)\left(\frac{100}{y}\right)\left(\frac{1}{z}\right)$$

where A is the mM concentration of an amino acid or amide in plastids *in vivo*; B is the nmol of an amino acid or amide in the total extract; w is the mg Chl in the plastid suspension; x is the mg Chl per plastid *in vivo*; y is the percentage of isolated plastids with intact envelope membranes; z is the average volume (μl) of plastids *in vivo*.

Calculation of Concentrations of Amino Acids in Leaf Tissue. To calculate the concentrations of free amino acids and amides in leaf tissue, the total aqueous volume of the leaf section was estimated. Accurate average fresh weights of the specific leaf sections were determined from a large number of plants (3,000) grown in 14 batches in the same controlled conditions as plants for amino acid extractions. We assumed that leaf section fresh weight minus dry weight was the aqueous tissue weight and represented the aqueous tissue volume in μl (1 ml H_2O assumed to equal 1 g). Quantities of amino acids extracted from leaf tissue were determined as nmol per leaf section and divided by the aqueous tissue volume expressed in mm^3 to give estimates of mM concentrations. Aqueous tissue volumes are given in Table I.

RESULTS

Comparison of Free Amino Acid and Amide Pools of Chloroplasts and Leaf Tissue. The analysis of the free amino acids and amides extracted from isolated chloroplasts from the most mature leaf tissue showed that the major component of the samples was the amide asparagine which accounted for 29 mol % of the total amino acids and amides (Fig. 1). Serine and alanine accounted for 19 mol % and 18 mol % respectively, and with the asparagine, glycine, and aspartic acid, comprised 85 mol % of the total free amino acid and amide pool extracted from chloroplasts. Glutamate and glutamine, 3.9 and 0.9 mol %, respectively, were relatively minor components in the chloroplasts.

For leaf tissue assays, maize seedlings were grown under the same strictly controlled conditions as plants for chloroplast isolation and the same areas from the same positions of the shoot were harvested. Extraction and analysis of the free amino acids and amides from the most mature tissue studied showed that the amide asparagine was the most abundant component of the pool, 32% of total. Aspartic acid, serine, glutamic acid, alanine, and glycine were the most abundant free amino acids with respectively 15, 11, 10, 10, and 10% of the total free amino acid and amide pool. Together with asparagine these five amino acids constituted 88% of the total free amino acid and amide pool in the leaf tissue.

The amino acids and amides are shown as proportions of the total extract from chloroplasts and leaf tissue in Figure 1. The distribution patterns are clearly different. The amide asparagine is the most abundant component of the total pools of free amino acids and amides in both the isolated chloroplasts and the leaf tissue. Serine and alanine are present in slightly higher proportions

Table II Concentrations of free amino acids and amides in chloroplast and leaf tissue pools

Concentrations in chloroplasts were calculated from measured and corrected free amino acid concentrations. The difference in the two values is the result of correcting for losses from 'broken' chloroplasts. The complete calculation is given in the Materials and Methods section and uses values for chlorophyll content of chloroplasts and the average *in vivo* chloroplast volume. Concentrations in leaf tissue pools were calculated from measured free amino acid and amide concentrations of leaf tissue and the aqueous tissue volume. The amino acids and amides are arranged in order of their abundance in the leaf tissue pool.

AMINO ACID OR AMIDE	PLASTID * A uncorrected	PLASTID * B corrected	LEAF
	millimolarity		
Asparagine	5.76	15.95	4.77
Aspartate	1.80	5.20	2.20
Serine	3.82	10.69	1.57
Glutamic acid	0.78	2.27	1.47
Alanine	3.51	9.90	1.46
Glycine	1.69	4.78	1.42
Threonine	0.48	1.37	0.35
Ornithine	0.13	0.38	0.32
Histidine	0.46	1.28	0.22
γ-aminobutyric acid	0.12	0.35	0.19
Valine	0.30	0.83	0.16
Lysine	0.10	0.29	0.12
Isoleucine	0.16	0.45	0.12
Tyrosine	0.13	0.36	0.11
Leucine	0.15	0.43	0.10
Glutamine	0.18	0.51	0.10
Phenylalanine	0.05	0.14	0.08
Total	19.62	55.18	14.76

in the chloroplast pool (19 and 18%) than in the leaf tissue pool (11 and 10%). In contrast, aspartic acid, glutamic acid, and ornithine are present at relatively lower proportions in the chloroplast pool (9, 4, and 0.7%) than in the leaf tissue pool (15, 10, and 2.2%). The amide glutamine was a minor component of both chloroplast (0.9%) and leaf (0.7%) pools.

The chloroplast amino acid and amide molar concentrations given in Table II were calculated from the nmol quantities and chloroplast volume measurements. The concentrations were calculated directly from the quantities of amino acids measured by the amino acid analyses (A) and also from figures which had been corrected to account for the presence of broken chloroplasts (B). The concentrations of amino acids and amides in leaf tissue were calculated from the measured nmol per leaf section and the aqueous tissue volume of the leaf section.

The molarity of the total pool of amino acids and amides was greater in the chloroplasts (20 mM uncorrected, 55 mM corrected) than in the complete leaf tissue (15 mM). It can be concluded that the molarity of the chloroplast pool must exceed the molarity of the total pool in the nonchloroplast part of the leaf tissue. This lower molarity in the nonchloroplast part could be due to lower concentrations in cytoplasmic, vacuolar, and noncellular compartments. Each individual amino acid and amide was estimated to have a greater molarity in the chloroplast pool than in the leaf tissue pool.

Concentrations at Different Stages of Development. The concentrations of free amino acids and amides in the plastids isolated from the five different shoot sections were calculated (Fig. 2). Asparagine, aspartate, serine, glutamate, alanine, and glycine are the six most abundant free amino acids and amides from plastids at all five developmental stages. The molarities of each of these and the other amino acids are greatest in the proplastids and molarities decline continuously during the development of proplastids to chloroplasts. The greatest difference between adjacent shoot sections was found in the plastids from the two most immature tissues sampled. Each of the molarities of glutamate,

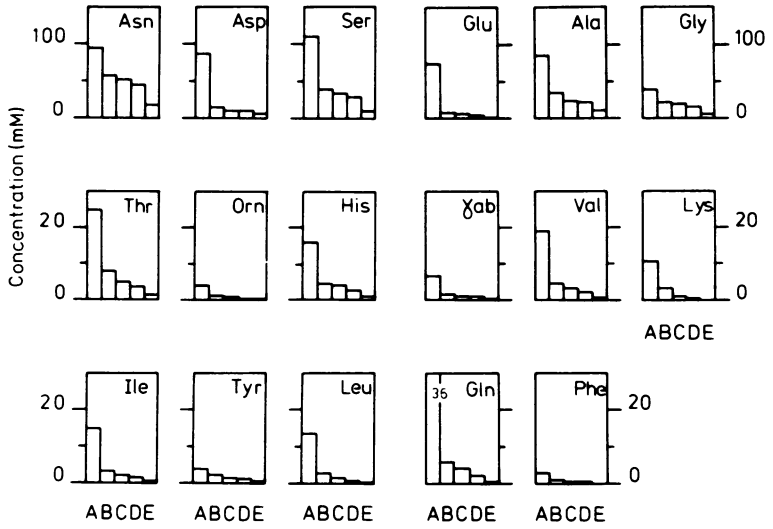

FIG. 2. Individual free amino acid and amide concentrations in plastids isolated from leaf tissue at five different stages of development. Concentration of each free amino acid and amides was determined as described under "Materials and Methods." On two separate occasions chloroplasts were isolated, extracted, and the content of free amino acids determined in several samples of each extract and the values given are averages for the determinations. This procedure was repeated for each of the five shoot sections. The 2-cm-long shoot sections are denoted by the letters A, B, C, D, E, with section A in the position between the shoot base and 2 cm from the base, section B between 2 cm and 4 cm from the base, and C, D, E, the subsequent 2-cm sections up to 10 cm from the base. The values for each amino acid are given in the order from left to right of the abundance of the amino acids in extracts from the most mature leaf tissue sample (section E). γab: γ-aminobutyric acid. (Concentrations of the six amino acids in the top row of the figure are given on a scale that is five times that used for the other amino acids.)

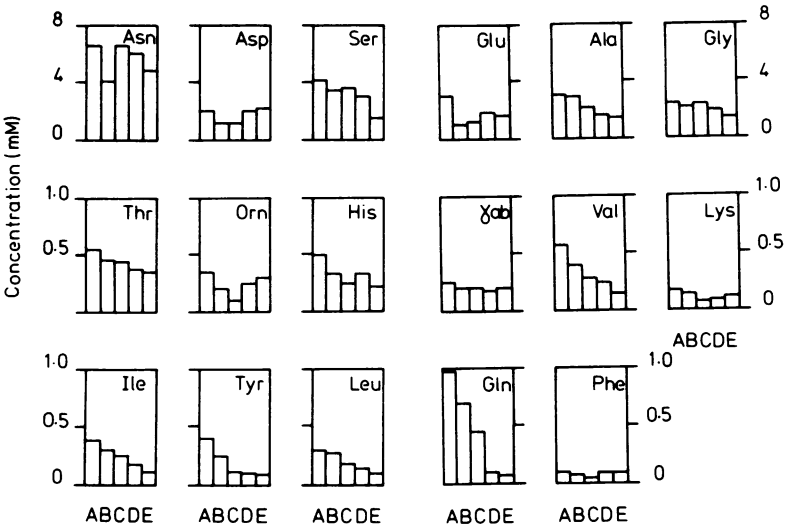

FIG. 3. Concentrations of individual free amino acids and amides extracted from leaf tissue samples. Concentration of each free amino acid and amide was determined as described under "Materials and Methods." On four separate occasions six plants were harvested and free amino acids and amides extracted from the five different sections of the shoot as described under "Materials and Methods." The 2-cm-long shoot sections are denoted by the letters A, B, C, D, E, with section A from the position between the shoot base and 2 cm from the base, section B between 2 cm and 4 cm from the base, and C, D, E the subsequent 2-cm sections up to 10 cm from the base. γab: γ-aminobutyric acid. (Concentrations of the six amino acids in the top row of the figure are given on the scale which is eight times that used for the other amino acids.)

glutamine, aspartate, isoleucine, leucine, and γ-aminobutyric acid in plastids from the most immature shoot section (0–2 cm from the base) is at least four times that of the same amino acid or amide in the plastids of the adjacent shoot section (2–4 cm from the base). The plastids always contain more asparagine than glutamine. Through the complete range of plastid development the concentrations of the two amides in the plastids are different. The proplastids have large pools of both amides asparagine and glutamine in a ratio of 3:1, but the mature chloroplasts have a large pool of free asparagine and a small pool of free glutamine (ratio 33:1).

The fall in concentrations observed for free amino acids and amides of plastids during development reflects either a decrease in a number of moles in each plastid or an increase in the plastid volume. Figure 2 shows that from the second stage of development to the most mature stage studied, the number of mol of amino acid per plastid is constant but there is a marked change in chloroplast volume (Table I). The developmental change of amino acid concentrations in chloroplasts is largely the result of the maintenance of a relatively constant amount of each amino acid while the chloroplast volume increases as development proceeds.

The most abundant free amino acids and amides in the tissues of all five of the shoot sections were the same as the most abundant ones in the plastid pool, *i.e.* asparagine, aspartate, serine, glutamate, alanine, and glycine. The patterns of the changes in free amino acid and amide molarities in the five tissue samples from

the different parts of the leaf are shown in Figure 3. Glutamine molarity declined 10-fold with development of the tissue and this decline was much greater than found for the other amino acids and amides. There was a gradual (30%) decline in molarity of most free amino acids with age of tissue sampled but the molarities of asparagine, aspartate, ornithine, γ-aminobutyric acid, lysine, and phenyalanine did not decline significantly.

DISCUSSION

The concentrations of free amino acids and amides found in plastid preparations from maize seedling leaf tissue were higher than the concentrations in the leaf tissue. This relationship was found at the five stages of leaf tissue development and indicates that the free amino acid concentrations in plastids *in vivo* are greater than those in leaf tissue. The retention of free amino acids by plastids during the isolation procedure suggests that the envelope acts as an effective barrier to passive diffusion of amino acids. Experiments involving direct measurement techniques developed by Heldt (6, 10) have also demonstrated that the inner chloroplast envelope membrane does not allow free passive diffusion of some amino acids.

The estimated molarities of the plastid amino acids *in vivo* declined through the range of young to old tissues. The proplastids contain the highest concentrations of free amino acids and amides and they develop very rapidly in both volume and inner membrane structure. The patterns of relative abundance of individual free amino acids and amides are also different in proplastids and chloroplasts. The differing lipid (16) and protein (unpublished results) compositions of maize proplastids and chloroplasts have already been characterized.

Studies of isolated chloroplasts have shown that key enzymes involved in nitrogen reduction and synthesis of amino acids are found with chloroplasts of higher plants (for reviews see refs. 14, 18, 19). When the activities of some of these enzymes are assayed, the concentration of amino acids in the assay system is found to regulate activity (e.g. 5, 17–19, 23). Bryan *et al.* (5) recently reported that homoserine dehydrogenase of maize seedling plastids is controlled *in vitro* by 10 mM threonine (5). The 25 to 2 mM threonine concentration range of plastids found in our study of maize seedling plastids suggests that in the case of homoserine dehydrogenase the demonstrated *in vitro* control could indeed be physiologically significant. A developmental change in the enzyme regulation was also found (5) which parallels the change in threonine molarity reported here. The investigation of amino acid molarities and regulation of enzymes from plastids of plants grown under exactly the same conditions would be a further step toward demonstrating the physiological significance of *in vitro* controls.

The amino acid concentrations required to inhibit activities *in vitro* (usually above 1.0 mM) are often greater than those normally found in photosynthetic tissues, and also greater than the concentrations in maize seedling leaf tissue that are reported here.

Two examples of enzymes with important roles in amino acid synthesis which have well documented regulation of activity by amino acids are glutamine synthetase (22, 23) and acetolactate synthetase (17). These enzymes have also been shown to be present in chloroplasts (19). The concentrations of amino acids found to inhibit these enzymes *in vitro* are of the same order as the concentrations found in plastids from maize seedlings.

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