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Changes in Enzyme Regulation during Growth of Maize

III. INTRACELLULAR LOCALIZATION OF HOMOSERINE DEHYDROGENASE IN CHLOROPLASTS[1]

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ABSTRACT

Extracts of leaf tissue of *Zea mays* L. seedlings were fractionated on nonlinear sucrose gradients to separate subcellular organelles. Homoserine dehydrogenase (EC 1.1.1.3) was identified in those fractions containing intact chloroplasts, as judged by the presence of chlorophyll and triosephosphate isomerase activity. Neither enzyme activity was detected in fractions containing ruptured chloroplasts, mitochondria, or microbodies. Quantitative measurements of enzyme activity and chlorophyll, and electron microscopic analysis of plastid preparations support the conclusion that maize mesophyll chloroplasts contain a significant fraction of the total cellular content of homoserine dehydrogenase.

A survey of representative kinetic, regulatory, and physical properties did not reveal any significant differences between enzyme released from isolated, undamaged chloroplasts and that obtained from soluble cellular fractions.

Examination of enzyme prepared from chloroplasts of different age seedlings indicated that the sensitivity of homoserine dehydrogenase to inhibition by the feedback modifier L-threonine was progressively diminished during growth of the plants. This systematic change in regulatory properties of the enzyme occurred to the same extent for the enzymes obtained from chloroplasts and soluble fractions.

Chloroplasts have long been known for their role in carbon assimilation but the extent to which they participate in nitrogen metabolism is less well established (12). The demonstration of light-dependent conversion of nitrite to amino nitrogen (18), and the recent identification of enzyme systems associated with ammonia metabolism (24) in isolated chloroplasts provide evidence of the importance of these organelles in nitrogen assimilation. Glutamate is the major assimilatory product and could contribute to the biosynthesis of other amino acids in chloroplasts as a substrate of aminotransferases. These and other observations collectively suggest that chloroplasts may be a major site of the synthesis of a wide range of amino acids in multicellular plants.

Homoserine dehydrogenase catalyzes the conversion of aspartic semialdehyde to homoserine and is associated with a multibranched pathway leading from aspartate to the synthesis of several amino acids. Homoserine is required for the biosynthesis of methionine, threonine, and isoleucine. Aspartic semialdehyde can also be metabolized to lysine in a separate branch of the pathway. During earlier investigations, evidence was obtained which suggested that the regulatory properties of homoserine dehydrogenase are progressively altered during maturation of a variety of maize tissues (7, 19). The present results, obtained with leaves of plants grown for different periods, indicate that the enzyme is present in chloroplasts and that the regulatory properties of the enzyme isolated from plastids of young plants differ from those of enzyme prepared from plastids of older plants.

MATERIALS AND METHODS

Plant Material. Seeds of *Zea mays* L. var. earliking, obtained from Joseph Harris Co., Rochester, N.Y., were washed in running tap water for 60 to 120 min and planted in vermiculite which had been moistened with a solution of Hyponex (1 g/l). Seedlings were grown at 26 C with 18 hr of light (600–800 ft-c) provided by fluorescent and incandescent lamps and 6 hr of darkness. Shoot material was harvested after 4 to 30 days growth by excision at the scutellar node, and shoot length was taken as an indication of plant age. The average length of samples was determined by direct measurement of each shoot or by values obtained from a random sample of 50 shoots. Fresh weight was determined for the total sample of plant material used in each experiment.

Chemicals. Ultrapure, enzyme grade sucrose was purchased from Schwarz/Mann and materials for electrophoresis were P.A.G.E. quality from Isolab Inc. Hyponex was obtained from Hydroponic Chemical Co. All reagents, substrates, and other chemicals were the highest purity available from commercial sources.

Enzyme Extraction. For complete extraction of homoserine dehydrogenase, plant material was cut into small segments and homogenized in a chilled Waring Blendor with 1 to 3 ml/g fresh weight of buffer (0.2 M tris-HCl, 30% glycerol [v/v], 1 mM EDTA, 1.4 mM 2-mercaptoethanol, and 5 mM L-threonine [pH 8.5]). The enzyme was concentrated with $(NH_4)_2SO_4$ as described previously (19). Concentrated preparations were dialyzed overnight against a large excess of 50 mM K-phosphate buffer containing 20% glycerol (v/v), 1 mM EDTA, 1.4 mM 2-mercaptoethanol, and 5 mM L-threonine (pH 7.5) prior to assay.

Chloroplasts were isolated by extremely brief and gentle homogenization of leaf material which had been trimmed of obviously senescent tissue and cut into 1- to 3-cm segments. In most instances, this entailed one or two 1-sec bursts in a Waring Blendor regulated to 80% power with a rheostat. Four to 8 ml of 50 mM K-phosphate buffer containing 5% dextran (average mol wt. of 40,000; Sigma) (w/v), 0.2% BSA (w/v), 1.4 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.3 mM $MnCl_2$, 0.33 M D-sorbitol, 3 mM EDTA, and 5 mM L-threonine (pH 7.5) per g fresh weight of leaf material were utilized for extraction of plastids. The resultant extract was filtered through four layers of

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nylon mesh and layered directly on sucrose gradients or subjected to differential centrifugation. In our experience, a comparatively small volume of extraction buffer (*e.g.* 1 ml/g fresh weight) or a brief initial centrifugation designed to concentrate chloroplasts results in a reduced yield of intact plastids when such preparations are subjected to sucrose density gradient centrifugation.

In some experiments, chloroplast-enriched fractions were obtained by centrifugation of extracts for 1 min at 500*g* (maximum) to remove whole cells and debris and concentrating the plastids from the supernatant by centrifugation for 5 min at 1,500*g* (maximum). Enzymes were released by hypotonic shock from plastids isolated by differential centrifugation or obtained from sucrose gradients. In most cases, the phosphate buffer containing glycerol described above was used to dilute chloroplast preparations. Additional enzyme activity was not detected after freezing and thawing nor when a preparation was homogenized in a TenBroeck glass homogenizer.

During some experiments, plant material remaining after a gentle extraction of chloroplasts was reextracted under more vigorous conditions in 1 to 2 volumes of extraction buffer. When this procedure was utilized, homoserine dehydrogenase was concentrated from the second extract by precipitation with $(NH_4)_2SO_4$ and dialyzed against the phosphate buffer containing glycerol before assay.

Sucrose Density Gradient Centrifugation. The discontinuous sucrose gradients described by Miflin and Beevers (23) were modified for the present experiments to optimize recovery of intact chloroplasts from maize tissues and to permit use of larger samples of plant extract. All sucrose solutions were prepared with 50 mM Tricine (pH 7.5) containing 1.4 mM 2-mercaptoethanol. Sucrose concentration was determined by refractometry and corrected to 25 C. All experiments were carried out at 4 C, 2 to 18 hr after preparation of the gradients. Each gradient consisted of 2 ml of 55% sucrose followed by 8 ml of a linear 55% to 40% gradient, 8 ml of 40% sucrose, and a final 7-ml linear region from 40% to 24.5% sucrose. After addition of 28 to 30 ml of filtered leaf extract, gradients were centrifuged for 3 min at 4,000 rpm (2,520*g*, maximum) followed by 6 min at 10,000 rpm (16,300*g*, maximum) in a Sorvall, HB-4 swinging bucket rotor. When the centrifuge brake was used, the rotor was allowed to decelerate to 5,000 rpm before application. Fractions of 0.5 to 2 ml were collected from the bottom of the gradients.

Enzyme Assays. All enzymes were measured spectrophotometrically at 25 C using a Gilford model 240 spectrophotometer equipped with a constant temperature circulator and attached to a Sargent model SLR recorder. For most experiments, a full scale of 0.1 *A* was utilized. Catalase (EC 1.11.1.6) was measured under the conditions described by Lück (17). Triosephosphate iosmerase (EC 5.3.1.1) was determined by the coupled procedure of Norton *et al.* (25) and Cyt *c* oxidase (EC 1.9.3.1) by the method of Yonetani and Elliott (27). Fumarase (EC 4.2.1.2) was measured by the increase in A_{240} with 50 mM L-malate, buffered at pH 7.6 with potassium phosphate, as substrate. Homoserine dehydrogenase (EC 1.1.1.3) was measured as described previously (3) with 20 mM L-homoserine as substrate and 0.48 mM $NADP^+$ or 6.7 mM NAD^+ as coenzyme. When necessary, measurements were corrected for inhibition resulting from the small amounts of threonine present in the sample buffer. All enzyme activities were corrected for nonspecific reactions and measured under conditions where the initial linear rates were proportional to the amount of enzyme added. For each enzyme, one unit (U) of activity is defined as the amount of enzyme required to convert 1 μmol of substrate to product per min. The mM extinction coefficients used were: catalase, 0.04; Cyt *c* oxidase, 18.5; fumarase, 2.44; triosephosphate isomerase and homoserine dehydrogenase, 6.22. Chl was measured as described by Arnon (2), and protein was determined spectrophotometrically after concentration with $(NH_4)_2SO_4$ and dialysis (19).

Polyacrylamide Gel Electrophoresis. Disc gel electrophoresis was performed under the conditions described by Davis (6) except that 1 mM L-threonine was included in both large and small pore gels and the upper reservoir buffer. Bromphenol blue was used as the tracking dye and detection of the enzyme after electrophoresis was based on substrate-dependent dye precipitation (19). After visualization of the enzyme distribution, gels were scanned at 600 nm using a linear transport device attached to the Gilford spectrophotometer.

Electron Microscopy. Sucrose gradient fractions containing intact or fragmented chloroplasts were collected and concentrated by centrifugation at 10,000 rpm (12,100*g*, maximum) for 30 min. The resultant pellets were fixed in 2% glutaraldehyde (pH 7.4) followed by a treatment with 1% OsO_4, at the same pH. The fixed pellets were dehydrated and embedded in Epon 812. Thin sections were doubly stained with uranyl acetate and lead citrate and examined with a Siemens 101 electron microscope.

RESULTS

Localization of Homoserine Dehydrogenase. Subcellular components can be identified by measurements of enzymes which are considered to be soluble constituents of specific organelles. The distribution of several such enzymes among fractions of a sucrose density gradient is illustrated in Figure 1A. Undam-

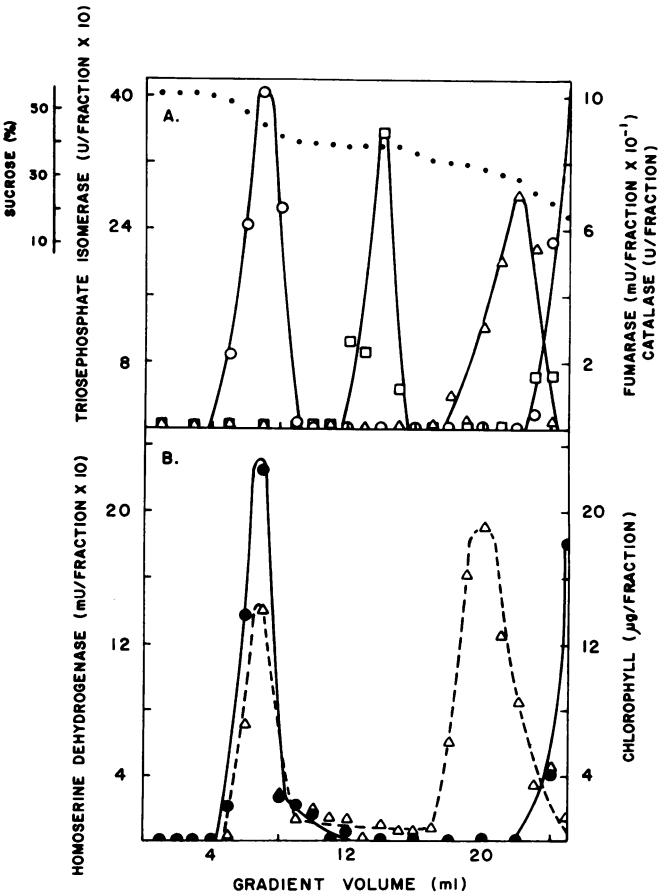

FIG. 1. Distribution of various cellular constituents in fractions of a sucrose density gradient. Young maize shoots (length, 4.9 cm) were harvested and extracted and the filtered homogenate subjected to centrifugation as described under "Materials and Methods." One-ml fractions were collected from the bottom of the tube. A: Distribution of triosephosphate isomerase (○), fumarase (□), and catalase (△) activities; sucrose concentration: (· · · ·). B: distribution of homoserine dehydrogenase activity (●) and Chl (△---△).

aged chloroplasts were characterized by the release of triosephosphate isomerase when exposed to hypotonic conditions. Catalase activity reflected the presence of microbodies in gradient fractions and fumarase was used to identify mitochondria. The latter enzyme consistently migrated into fractions of slightly higher density than the bulk of Cyt *c* oxidase activity. This difference probably reflects the distribution of damaged and intact mitochondria, since Cyt *c* oxidase is associated with the inner mitochondrial membrane, whereas fumarase is a soluble matrix enzyme (11).

A portion of the activity of homoserine dehydrogenase coincided with that of triosephosphate isomerase and the band of Chl representing intact chloroplasts (Fig. 1). The remainder of homoserine dehydrogenase was present in the soluble extract and the top few fractions where mixing between the extract and the gradient occurred. Measurements of the extract are not illustrated, but the reduced sucrose concentrations and the presence of several enzyme activities characteristic of mixed fractions can be observed in Figure 1. Occasionally, small amounts of homoserine dehydrogenase activity were detected in the dense fractions at the bottom of the gradients. This enzyme is assumed to be associated with whole cells, rapidly sedimenting membranous material, or starch granules, as suggested from the results of other studies (22, 23). This enzyme, when present at the bottom of the gradient, was quite labile and was never detected after the fractions had been frozen and thawed. In contrast, homoserine dehydrogenase associated with chloroplasts and that present in the soluble extract was stable for several days at −20 C.

Estimates of the extent to which the integrity of various organelles was preserved and the degree of separation achieved can be made from the results presented in Table I. In this experiment, 18.7% of the total Chl was recovered in gradient fractions containing intact chloroplasts. Since chloroplasts were not concentrated prior to fractionation of the extracts, the observed recovery represents a true yield and is comparable to that obtained from other plant species (22, 23). Apparent yields based only on the distribution of Chl among gradient fractions would be significantly higher. No detectable contamination of undamaged chloroplasts with mitochondria or microbodies was observed during this or most other experiments. The recovery of mitochondria was 21.7% as judged by the distribution of fumarase activity. Similar results were obtained in other experiments and cross-contamination of marker enzymes was consistently less than 5%. Gradient fractions containing mitochondria and microbodies were contaminated with variable amounts of chloroplast fragments.

Morphological Characterization of Isolated Chloroplasts. To characterize further the two well separated regions of Chl present in discontinuous sucrose gradients, a separate experiment was carried out in which samples were examined by electron microscopy. Representative fields of the rapidly and slowly sedimenting bands of Chl are illustrated in Figure 2, *A* and *B*, respectively. Although some damage to both preparations undoubtedly occurred during concentration of the plant material, a larger fraction of the "enzymically intact" chloroplasts appears to be associated with outer membranes (Fig. 2A) than those devoid of soluble enzyme (Fig. 2B). Plastids in the fragmented preparation which appear to have maintained their structural integrity may have ruputred and resealed during extraction (16). The preparation representing intact chloroplasts was virtually free of contaminating material, and the lack of a significant number of mitochondria in the fragmented preparation is consistent with the conclusion that undamaged mitochondria sediment to regions of higher density during this procedure. Thus, based on both enzymic and morphological criteria, the uncontaminated and undamaged chloroplasts isolated from maize contain homoserine dehydrogenase.

Distribution of Homoserine Dehydrogenase in Cells of Older Plants. Shoots of seedlings grown for different periods of time were harvested and the enzyme content of their subcellular organelles examined after separation on sucrose gradients. Homoserine dehydrogenase activity was always associated with intact, but never with damaged chloroplasts. It was noted that Chl derived from extracts of older plants exhibited a more

Table I. Enzyme and Chlorophyll Distribution Among Sucrose Density Gradient Fractions Containing Subcellular Components.

Component[1]	Intact Chloroplasts	Disrupted Chloroplasts	Microbodies	Mitochondria	Soluble Fraction
Chlorophyll					
µg	25.6	73.4	-	-	-
% of total	18.7	53.6	(∿50)[2]	(<3)	37.9
Triosephosphate isomerase					
U	10.5	-	-	0	170
% of total	5.8	(<4)	(<2)	0	94.2
Fumarase					
mU	0	-	-	147	526
% of total	0	(<5)	(<5)	21.8	78.2
Catalase					
U	0	-	22.3	0	77.1
% of total	0	(∿20)	22.4	0	77.6
Homoserine dehydrogenase					
mU	4.3	-	-	-	35.8
% of total	10.6	(<1)	(<1)	(<0.3)	89.4

1. Young maize shoots were extracted and the subcellular components isolated as described in Materials and Methods and illustrated in Fig. 1.

2. Bracketed values indicate the extent to which cross contamination occurred due to overlap in the distribution of components or to mixing of the soluble fraction with the upper region of the gradient. Measurements of enzymes or chlorophyll in such fractions were included with those representing cellular components or the soluble material for purposes of the calculations.

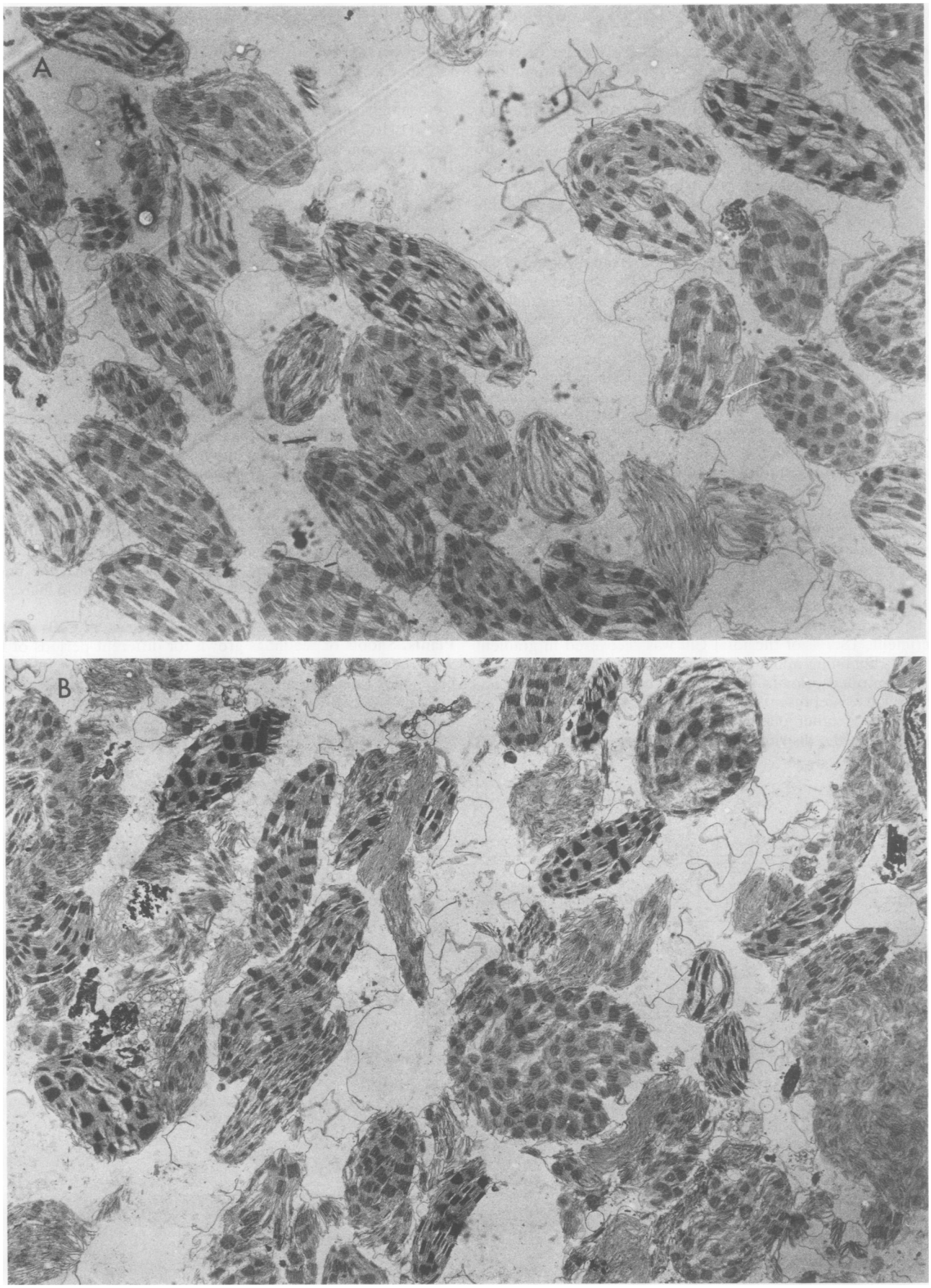

FIG. 2. Electron micrographs of sucrose density gradient fractions containing Chl. Leaf material harvested from shoots 14 cm long was extracted and the resultant homogenate was fractionated on sucrose gradients. Fractions considered to contain intact or disrupted chloroplasts were separated and their particulate contents concentrated into pellets by centrifugation. These pellets were then treated and thin sections examined as described under "Materials and Methods." A: Preparation of rapidly sedimenting intact chloroplasts obtained from fractions with a mean density of approximately 1.2 g/cm^3. (× 6,000); B: preparation of material present in fractions with a density of about 1.15 g/cm^3. (× 6,000).

diffuse distribution among sucrose gradient fractions. Undamaged chloroplasts of older plants appeared to be more heterogeneous than those prepared from young plants, as evidenced by a concomitant increase in the asymmetrical distribution of homoserine dehydrogenase, triosephosphate isomerase, and Chl. Although several factors may contribute to this phenomenon, it does not appear to be due to the well known chloroplast dimorphism of maize. The gentle extraction procedure employed should yield very few bundle sheath chloroplasts (14), and only mesophyll chloroplasts were observed in electron micrographs of comparatively old preparations.

Quantitative Estimates of the Enzyme Content of Chloroplasts. Although homoserine dehydrogenase was consistently associated with intact chloroplasts, it is more difficult to establish the amount of enzyme activity characteristic of these organelles *in vivo*. Comparison of the enzyme and Chl content of isolated plastids with the total enzyme and Chl present in leaf extracts led to an estimate that 60% of the enzyme was localized in chloroplasts (average of data presented in Table II). However, the results of the independent experiments were quite variable; in some experiments, the vast majority of the enzyme was associated with chloroplasts (Table IIA) while in others the estimates were significantly lower (Table IIB). There was little obvious correlation between the estimated homoserine dehydrogenase content of chloroplasts and the characteristically reduced yield of apparently undamaged plastids from older maize plants (26). If some of these chloroplasts were damaged during isolation, then homoserine dehydrogenase would have to be more readily lost from the stroma than triosephosphate iosmerase. The distribution of the latter enzyme was reasonably constant and consistent with the presence of both plastid and soluble forms (Table II). An alternative explanation of the variability of the results observed for homoserine dehydrogenase relates to a differential distribution of unidentified enzyme inhibitors or activators, although no evidence of such factors was obtained when preparations were dialyzed.

If enzyme and Chl are uniformly distributed among chloroplasts, then the measured enzyme specific activity (SA), expressed as mU/mg Chl, would be similar in all sucrose density gradient fractions of reasonably pure, undamaged chloroplasts. Based on eight independent experiments, the average ratios (SA in adjacent fraction/SA in peak fraction) ±SE were 1.12 ± 0.11 and 0.72 ± 0.08 for the enzyme in fractions which preceded and followed the peak, respectively. A similar analysis of triosephosphate isomerase resulted in values of 0.89 ± 0.14 and 0.74 ± 0.16 for the leading and trailing fractions, respectively ($N = 6$). Although the slightly reduced specific activities in trailing fractions may reflect a degree of contamination with damaged chloroplasts, the possibility that some intact chloroplasts contain low levels of enzyme relative to their Chl content should not be eliminated. The general similarity of enzyme-specific activities among gradient fractions does, however, provide convincing quantitative evidence of a true association of homoserine dehydrogenase with maize chloroplasts.

Properties of Homoserine Dehydrogenase. If maize cells contain a soluble form of the enzyme in addition to the form associated with chloroplasts, such forms might be characterized by differences in kinetic, regulatory, or physical properties. Although we have not yet isolated and purified sufficient quantities of the chloroplast enzyme for detailed characterization, several representative properties have been examined. For example, maize homoserine dehydrogenase, isolated and partially purified by conventional techniques, exhibits dual coenzyme specificity (3). Every sample of the chloroplast enzyme that was tested catalyzed the conversion of homoserine to aspartic semialdehyde with either NAD^+ or $NADP^+$ as the coenzyme. In addition, the ratio of NAD^+- to $NADP^+$-dependent activity, measured under standard assay conditions, averaged (±SE) 1.6 ± 0.1 for enzyme derived from chloroplasts and compares favorably with an average of 1.4 ± 0.1 obtained with enzyme present in extracts from which intact plastids had been removed. These results are based on analysis of 12 different preparations.

Table II. Estimates of the Intracellular Distribution of Enzymes *in vivo*.

Shoot Length (cm)	Enzyme Distribution (%)1. Homoserine dehydrogenase		Triosephosphate isomerase		Yield of Intact Chloroplasts (%)2.
	Plastids	Cytoplasm	Plastids	Cytoplasm	
A.3.					
5.3	96	4	..	..	20.2
7.1	98	2	..	..	13.6
12.6	74	26	53	47	10.2
45.6	83	17	45	55	3.6
B.					
4.9	63	37	33	67	18.7
6.9	48	52	..	..	22.8
9.0	28	72	..	..	14.5
10.6	21	79	44	56	17.3
25.5	17	83	..	..	7.5

1. Estimates of the *in vivo* enzyme content of chloroplasts were obtained by the following calculation: [enzyme activity/mg chlorophyll in isolated intact chloroplasts) X total chlorophyll recovered]/(total enzyme activity recovered) X 100. Intact plastids were isolated by sucrose density gradient centrifugation as described in Materials and Methods. Enzyme activity which was apparently not associated with chloroplasts was assumed to be cytoplasmic, based on results such as those illustrated in Fig. 1.

2. Chloroplast yield was calculated from the fraction of total chlorophyll recovered in intact plastids as judged by the presence of triosephosphate isomerase and/or homoserine dehydrogenase activity.

3. The results of independent experiments are arbitrarily divided into two sets. Even though the experimental procedures were equivalent for all experiments, the results presented in Set A differed from those presented in Set B. This unexplained variability is discussed in the text.

Since multiple forms of homoserine dehydrogenase had been isolated from several tissues of maize (7), enzyme preparations obtained from intact chloroplasts and soluble material which did not enter sucrose gradients were analyzed by polyacrylamide gel electrophoresis. Recorder traces of the substrate-dependent dye precipitation after electrophoresis of such preparations are illustrated in Figure 3. Not only were the same forms present in both the chloroplasts and soluble fractions as judged by their relative mobilities during electrophoresis, but the relative patterns of dye precipitation were essentially identical. The relationships among multiple forms of this enzyme are complex and will be discussed in a separate communication (DiCamelli and Bryan, in preparation). Based on the results of several experiments, no evidence of a unique chloroplast form has been obtained.

One of the unusual features of maize homoserine dehydrogenase is its variable sensitivity to inhibition by the feedback modifier, L-threonine (7, 19). Both the effectiveness of low concentrations of the inhibitor and the extent of inhibition by high inhibitor levels are progressively reduced during growth of the plants. The latter effect is illustrated in the inset of Figure 4 with preparations derived from light-grown shoots under conditions designed to recover nearly all of the enzyme but not to preserve the integrity of chloroplasts. To establish whether enzyme obtained from chloroplasts exhibited equivalent changes in regulatory properties, several types of preparations were examined. First, enzyme present in soluble extracts from which chloroplasts had been removed by centrifugation (○, Fig. 4) can be compared with that obtained from isolated chloroplasts (●, Fig. 4). Plastids were prepared by differential centrifugation for some of these experiments and, therefore, may be contaminated with other organelles. The absence of detectable homoserine dehydrogenase activity in any other particulate fraction renders this method suitable for comparative purposes. In addition, enzyme prepared from all of the soluble fractions and some of the chloroplast fractions was extensively dialyzed to remove any low mol wt inhibitors. Second, enzyme was initially obtained from chloroplast-enriched extracts and subsequently from cells which were not ruptured during the first extraction. The sensitivity of enzyme isolated after gentle and vigorous homogenation is depicted in Figure 4 by closed and open triangles, respectively. If the enzyme was desensitized upon release from the chloroplasts or if a different form of the enzyme was isolated by the harsh procedure, then consistent differences in enzyme sensitivity should have been observed among the preparations. Instead, a general decrease in enzyme sensitivity, correlating only with shoot length, can be observed with all of the different types of preparations.

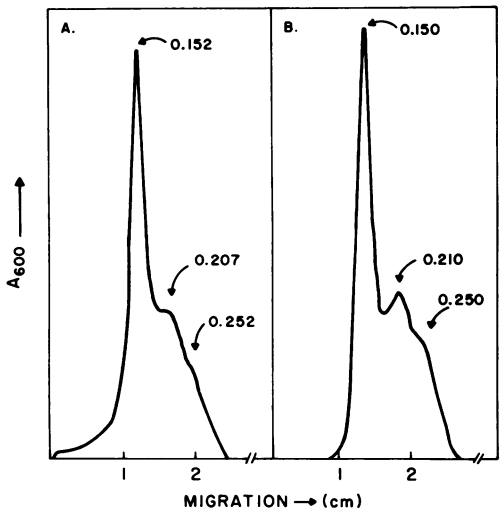

FIG. 3. Recorder scans of polyacrylamide gels stained for homoserine dehydrogenase activity after electrophoresis. Extracts of young shoots (length, 5.2 cm) were fractionated on sucrose gradients. Intact chloroplasts and the soluble material that failed to enter the gradients were collected and the enzyme isolated and concentrated as described under "Materials and Methods." After electrophoresis in 6.75% acrylamide, the gels were stained for enzyme activity and duplicate gels were incubated in the staining mixture in the absence of homoserine to correct for nonspecific reactions. Bromphenol blue was used as the tracking dye. Its migration is not indicated since only the first 3 cm of the gels are depicted. The relative mobility of each peak of activity is indicated on the figure and was calculated as: migration of the enzyme/migration of bromphenol blue. A: Enzyme prepared from intact chloroplasts; sample contained 116 μg protein and 2.9 mU NAD^+-dependent activity; B: enzyme prepared from soluble fractions; sample contained 179 μg protein and 3.6 mU NAD^+-dependent activity.

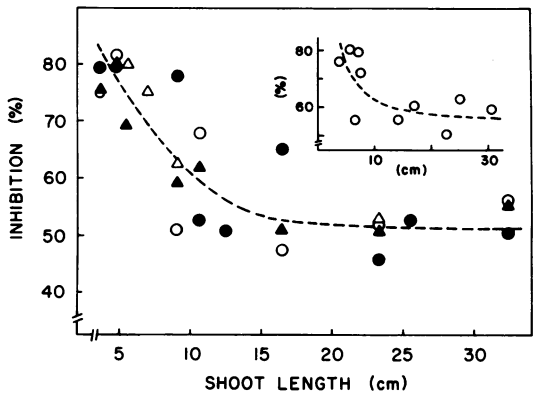

FIG. 4. Inhibition of maize homoserine dehydrogenase prepared from chloroplasts and soluble fractions of plants of different ages. Inhibition was calculated from the results of assays in the presence and absence of 10 mM L-threonine and saturating concentrations of homoserine and NAD^+. The results illustrated in the insert represent analysis of enzyme preparations which were extracted and concentrated by conventional techniques (19). The origins of remaining preparations were: (●), intact chloroplasts; (○), soluble extracts from which plastids had been removed; (▲), plastid-enriched extracts prepared by gentle homogenization of the plants; (△), extracts devoid of chloroplasts prepared by vigorous homogenzation of the plant material remaining after an initial gentle homogenization. Additional details relating to these preparations are given in the text.

DISCUSSION

The inclusion of homoserine dehydrogenase, which catalyzes the third reaction in a multibranched pathway of amino acid biosynthesis, within the stroma of mesophyll chloroplasts is strongly supported by the following observations: (*a*) enzyme activity was consistently associated with intact chloroplasts isolated by methods which result in little contamination with other subcellular organelles; (*b*) isolated chloroplasts were characterized by a typical mesophyll morphology; (*c*) neither homoserine dehydrogenase nor triosephosphate iosmerase activity was detected in fractions containing damaged chloroplasts; (*d*) similar activities per unit Chl were observed in several fractions of sucrose gradients which were judged to contain intact chloroplasts. No attempts were made to establish specifically whether the enzyme was also present in bundle sheath chloroplasts. Preliminary results, obtained during separate studies (Bryan and Miflin, unpublished results), do indicate that homoserine dehydrogenase is also present in chloroplasts of *Vicia faba*, a species which is characterized by typical C_3 photosynthetic metabolism. These results, combined with those of Mazelis *et al.* (20), Miflin (22), and Kirk and Leech (15) lead to the conclusion that lysine, threonine, and isoleucine can be synthesized in chloroplasts of higher plants. Among the end products of the aspartate pathway of amino acid biosynthesis, only the synthesis of methionine has

been associated with a cellular compartment other than chloroplasts. In this case, the activities of several enzymes considered to be involved in the synthesis of methionine were detected in mitochondrial preparations of *Pisum sativum* (5). Only a very small fraction of the total cellular content of most of the enzymes was recovered from mitochondria, and additional data are required before the extent to which methionine is uniquely synthesized in this organelle can be assessed.

It would be premature to conclude that chloroplasts are the exclusive subcellular site of homoserine dehydrogenase in maize leaves, since consistent estimates of its intracellular distribution were not obtained. Any enzyme not present in chloroplasts would be considered cytoplasmic, as no evidence of an association of homoserine dehydrogenase with mitochondria, microbodies, or any other particulate fraction was obtained. Nevertheless, the failure to detect kinetic, regulatory, or physical differences between the enzyme present in soluble fractions and that present in chloroplasts is consistent with a common subcellular origin of maize homoserine dehydrogenase. If distinct chloroplast and cytoplasmic enzymes exist, more sensitive techniques than those employed during the present investigation will be required to establish their unique characteristics. Even in the absence of such distinct enzymes, cellular changes which result in differential compartmentation are not precluded.

Homoserine dehydrogenase is one of several key regulatory enzymes in the aspartate pathway which are subject to inhibition by one or more end product amino acids (4, 21). However, very little definitive information concerning the intrachloroplast concentration of amino acids or factors which may influence their transport is available. A further complication is introduced by the progressive desensitization of maize homoserine dehydrogenase. The extent to which the activity of this enzyme is modulated *in vivo* would depend upon both the local concentration of threonine and the sensitivity of the enzyme. The possibility that homoserine dehydrogenase is also desensitized during the growth of peas is suggested by the results of two independent investigations. Aarnes and Rognes (1) isolated a sensitive enzyme from young seedlings, but DiMarco and Grego (9), using much older plants, concluded that the pea enzyme was not subject to inhibition by threonine.

The present results indicate that sensitive and insensitive enzymes are not differentially localized in unique subcellular compartments. Although the molecular basis of maize homoserine dehydrogenase desensitization has not been established, it is currently viewed as a form of post-translational modification (8). If the enzyme is altered within chloroplasts, then only endogenous factors, or those which are accessible to these organelles, could influence the process *in vivo*. In this context, reports of changes in the permeability of chloroplast membranes during development and maturation are particularly intriguing (10, 13). These and other observations reinforce the concept that metabolic regulation in multicellular plants need be neither quantitatively nor qualitatively constant.

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