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The Location of Nitrite Reductase and Other Enzymes Related to Amino Acid Biosynthesis in the Plastids of Root and Leaves[1]

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ABSTRACT

Density gradient separation of plastids from leaf and root tissue was carried out. The distribution in the gradients of the activity of the following enzymes was determined: nitrite reductase, glutamine synthetase, acetolactate synthetase, aspartate aminotransferase, catalase, cytochrome oxidase, and triosephosphate isomerase. The distribution of chlorophyll was followed in gradients from leaf tissue. The presence of plastids that have retained their stroma enzymes was denoted by a peak of triosephosphate isomerase activity. Coincidental with this peak were bands of nitrite reductase, acetolactate synthetase, glutamine synthetase, and aspartate aminotransferase activity. The results suggest that most, if not all, the nitrite reductase and acetolactate synthetase activity of the cell is in the plastids. The plastids were found to contain only part of the total glutamine synthetase, aspartate aminotransferase, and triosephosphate dehydrogenase activity in the cell. Some evidence was obtained for low levels of glutamate dehydrogenase activity in chloroplasts.

A proportion of the nitrite reductase extracted from plant cells is associated with a particulate fraction in both leaves and roots (10, 21). While most work has suggested that nitrite reductase is localized in the chloroplast, Lips and Avissar (17) have suggested that the enzyme is present in the peroxisome. Miflin (21) found that the enzyme from barley roots banded in sucrose density gradients below the mitochondria and microbodies but he did not assay for any plastid markers and was unable to identify the organelle in question. Subsequently, Dalling *et al.* (7) found that nitrite reductase from wheat roots, under isopycnic density gradient centrifugation, was distributed coincidently with only one of two peaks of plastid marker enzymes. Because of the unusual distribution of the plastid markers in the gradient and the lack of complete coincidence between them and nitrite reductase, it is not possible from these results to state unequivocally that nitrite reductase is located in root plastids.

If it is accepted that nitrite reductase is present in the plastids, then the question arises as to how many more of the enzymes involved in the synthesis of amino acids are also present in the plastid. Studies with isolated chloroplasts have shown that they are capable of a light-dependent reduction of nitrite and the incorporation of the product into α-amino nitrogen (19, 20, 23). Leech and co-workers (9, 16) and Lea and Thurman (15) have shown that chloroplasts have a light-stimulated glutamate dehydrogenase, which appears to be NADPH-dependent and bound tightly to the chloroplast lamellae. There is also evidence that chloroplasts can incorporate ammonia into glutamine (12, 25, 27). Further, chloroplasts contain a wide range of transaminases (13). In root tissues, glutamate dehydrogenase seems to be solely in the mitochondria (7, 21), but, apart from these observations, little seems to have been done on the location of these enzymes in roots.

Studies on $^{14}CO_2$ fixation in chloroplasts indicate that ^{14}C does not readily appear in amino acids, which suggests that the carbon skeletons for the amino acids are derived from outside the chloroplasts. In agreement with this α-ketoglutarate, pyruvate, and P-enolpyruvate stimulate the formation of α-amino nitrogen from nitrite by isolated chloroplasts (23); these carbon compounds are sufficient for the formation of amino acids regarded as the heads of the amino acid families (*e.g.*, aspartate and glutamate). Little is known of the location of enzymes responsible for the conversion of these carbon precursors to the α-keto acids required for the formation of amino acids at the end of their family tree (*e.g.*, leucine, isoleucine, valine, phenylalanine, and tyrosine). One enzyme in this category is acetolactate synthetase, the first enzyme unique to isoleucine, leucine, and valine biosynthesis. This enzyme has been ascribed to the mitochondria of *Neurospora* (31) but no work on its subcellular location has been done with green plants.

Recent studies by Miflin and Beevers (24) have produced a technique for the isolation of intact plastids in a relatively pure state. This technique has been used with leaf and root tissues and various enzymes of nitrogen metabolism assayed in order to answer some of the questions raised above. This paper presents the results of these experiments.

MATERIALS AND METHODS

Plant Material. Plants were grown as described previously (21, 24).

Tissue Extraction. Spinach (*Spinacia oleracea*) leaves were homogenized in 2 volumes/g of tissue of an isolation medium, consisting of 10 mM KCl, 1 mM $MgCl_2$, 1% w/v Dextran T40, 1% w/v Ficoll, 0.1% w/v bovine serum albumin, made to volume with 30% (w/v) sucrose containing 79 mM Tricine buffer, pH 7.5, for 5 × 1 sec bursts in an Atomix blender. Pea and barley roots were ground in a chilled pestle and mortar in

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2 volumes of the same medium except that it also contained 1 mM glutathione and the sucrose concentration was 25% w/w. All homogenates were filtered through eight layers of fine nylon gauze and the filtrate layered on the density gradient.

Density Gradients were of w/w sucrose solutions made up to 100% with 0.1 M Tricine buffer pH 7.5. Gradients used for spinach leaves were 4 ml of a 60% sucrose cushion followed by 6 ml of a linear gradient from 60 to 42%, followed by 5 ml of 42%, followed by 10 ml of a linear gradient from 42 to 30% with a final 3 ml of 30% sucrose. Eight milliliters of filtered homogenate were placed on top of the gradient. The gradient used for short time centrifugation of the pea root tissue consisted of a 4-ml cushion of 60% sucrose, a linear gradient of 18 ml of 50 to 25%, followed by a final 3 ml of 25% sucrose with 10 ml of homogenate layered on top. The gradients were usually prepared about 2 hr before use.

Immediately after layering on the filtered homogenate the tubes were balanced and placed in a SW27 rotor in a Beckman Spinco L.2-65 centrifuge and, as soon as vacuum permitted, centrifuged to 4,000 rpm for 5 min and then at 10,000 rpm for a further 10 min. The rotor was allowed to decelerate to 5,000 rpm before turning on the brake. The gradients and centrifugation techniques for the longer spins are given in legends to the figures. After centrifugation all gradients were fractionated into samples of 1.2 ml on an ISCO density gradient fractionator, run at 2.0 ml/min. The sucrose concentrations of fractions were determined by refractometry.

Enzyme Assays. Catalase was determined by the method of Lück (18), triosephosphate isomerase by the method of Gibbs and Turner (8). Chlorophyll was determined by the method of Arnon (2), nitrite reductase by the method of Bourne and Miflin (4), acetolactate synthetase by the method of Miflin (22), glutamine synthetase by the method of Shapiro and Stadtman (29). Glutamate dehydrogenase was measured by incubating 100 μl of gradient fraction in 0.2 mM NADPH, 1 mM $CaCl_2$, 0.1% w/v Triton X-100, 10 mM $(NH_4)_2SO_4$, 10 mM Tricine buffer pH 7.8 in a final volume of 1 ml; the reaction was started by the addition of 100 μl of 0.1 M α-ketoglutarate, pH 7.5, in phosphate buffer and the absorption of NADPH at 340 nm followed on a recording spectrophotometer. Cytochrome oxidase was measured by the method of Hackett (11) after incubating aliquots of the fraction in 0.1% digitonin for 5 min. Aspartate aminotransferase was measured by the method of Sizer and Jenkins (30).

RESULTS

Leaf Tissue. The results of a rapid gradient separation of a spinach leaf homogenate are shown in Figure 1. The intact chloroplasts are identified by the marker enzyme triose-P isomerase (14). Coincident with this band of triose-P isomerase are bands of glutamine synthetase, acetolactase synthetase, and nitrite reductase. The intact chloroplast band was uncontaminated by microbody marker enzymes in that less than 2% of the total catalase activity was found under the plastid peak. Mitochondrial contamination, as measured by the Cyt oxidase recovered in the peak, was also less than 2%. Using this technique no evidence was obtained for the coincidental banding of nitrite reductase and catalase.

Root Tissue. The result of an isopycnic gradient centrifugation of barley roots (Fig. 2) shows that the nitrite reductase activity peaks coincidentally with triose-P isomerase and that the microbodies and mitochondrial marker enzymes band separately at different densities, although there is a degree of overlap in the activities. Similar results (Fig. 3) are obtained with pea roots except that catalase and Cyt oxidase peak coincidentally. This has also been noted by Zschoche and Ting (32)

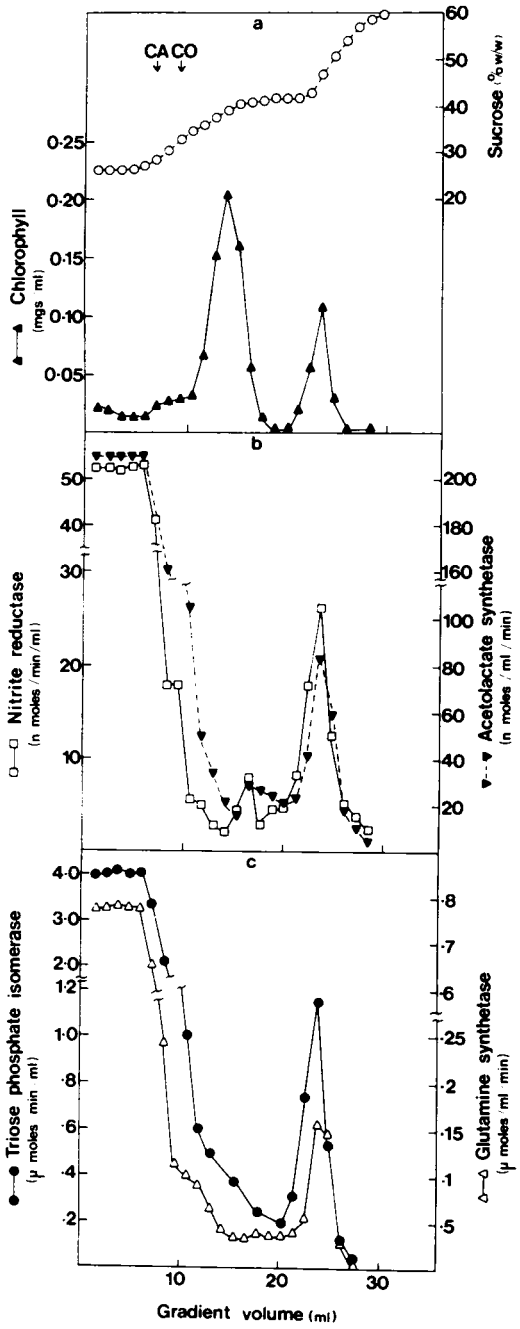

FIG. 1. Distribution of various enzyme activities in a sucrose density gradient centrifugation of a homogenate of spinach leaves. The arrows denote the region of the peak of activity for catalase (CA) and Cyt oxidase (CO).

but, as discussed elsewhere (24), it is probably due to the trapping of microbodies by the mitochondria. The results with barley (Fig. 2) and with other root tissues indicate that roots contain microbodies.

In further studies with root tissues the brief centrifugation technique was used since this gave a greater recovery of enzyme activity and less contamination of the plastids with other organelles. The results obtained with pea root homogenates are given in Figure 4. As in the leaf tissue, glutamine synthetase, acetolactate synthetase, nitrite reductase, and triosephosphate isomerase band together at a density of 1.21 g/cm^3. Aspartate aminotransferase gave two peaks of activity, one at

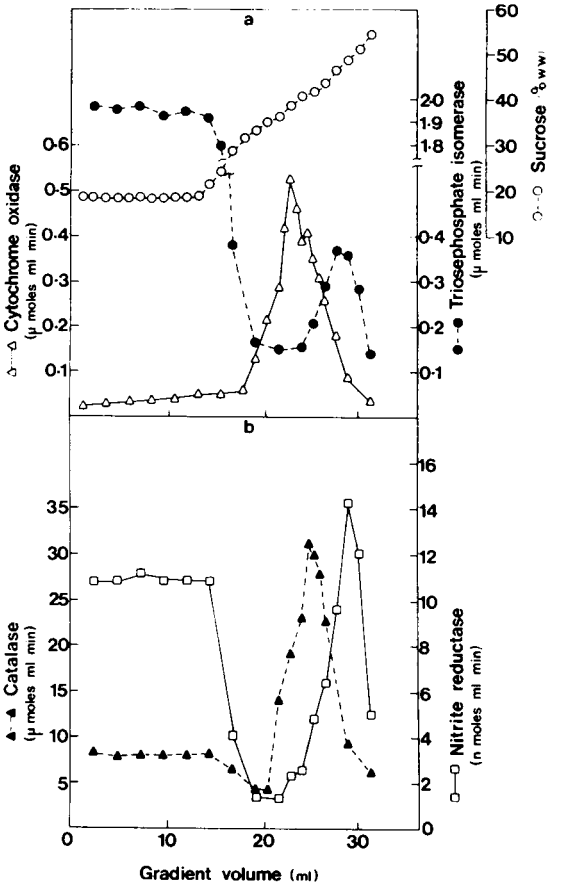

FIG. 2. Distribution of various enzyme activities in a sucrose density gradient of a filtered homogenate of barley roots. Fifteen ml of homogenate were layered on a linear gradient of 25 to 55% sucrose over a 5-ml cushion of 60% sucrose and centrifuged for 3.5 hr at 20,000 rpm in a SW 27 rotor.

1.21 g/cm^3 and one that coincided with Cyt oxidase activity. The intact plastid region contained less than 1% of either the total catalase or the total Cyt oxidase, showing that contamination with mitochondria and microbodies was negligible. In neither type of separation is there any peak of triose-P isomerase at the same density as the mitochondria. This is in contrast to the findings of Dalling *et al.* (7) with wheat roots.

Enzyme Recoveries. Table I gives the percentage of the various enzyme activities present in the plastid peak as calculated from the results presented in Figures 1 to 4. Although the results are not clear cut, there is an indication in leaves that there is a greater proportion of the nitrite reductase and acetolactate synthetase activities associated with the intact plastids than there is of the triose-P isomerase and glutamine synthetase activities. This may indicate, but in no way proves, that, whereas the latter two enzymes are located in both the cytosol and the plastids, nitrite reductase and acetolactate synthetase are predominantly, if not solely, in the plastids. Positive evidence for the dual location of the triose-P isomerase has been found by Anderson and Advani (1). In root tissue the difference in the amount of these three enzymes associated with the plastids is much more marked, reinforcing the above suggestion. The recovery of the pea root plastids, as based on nitrite reductase and acetolactate synthetase levels, was over 30% compared with around 18% for leaf chloroplasts, as based on Chl measurements.

Although not presented here, similar results were obtained with soya bean tissue culture cells. However, the recoveries were very low, particularly with long term density gradients, and were negatively correlated with the level of starch in the tissue. This correlation may also explain some of the difficulties encountered with barley preparations in that electron micrographs show that barley root plastids are rich in starch grains. It is probable that the starch grains are centrifuged out of the plastids during separation on the gradient and pellet at the bottom of the tube. This has been shown to happen in leaf tissue (26) and would explain why a proportion of the nitrite reductase is always found in the pellet at the bottom of barley root gradients (21).

Nitrite Reduction in Plastids. Previous studies have shown the presence of glucose-6-P and gluconate-6-P dehydrogenase in root plastids (7, 24). Since nitrite reductase is also present in the plastid, experiments were carried out to see if either of these potential electron donors could serve as a source of reductant for nitrite reductase in whole plastids. The plastids were isolated by a rapid technique and assayed immediately for their nitrite-reducing capabilities. Results (Table II) showed that glucose-6-P but not gluconate-6-P was capable of supporting nitrite reduction. The rates were not increased by the addition of NADP. Reduced pyridine nucleotides and ATP were without effect. Ammonia was produced in approximately

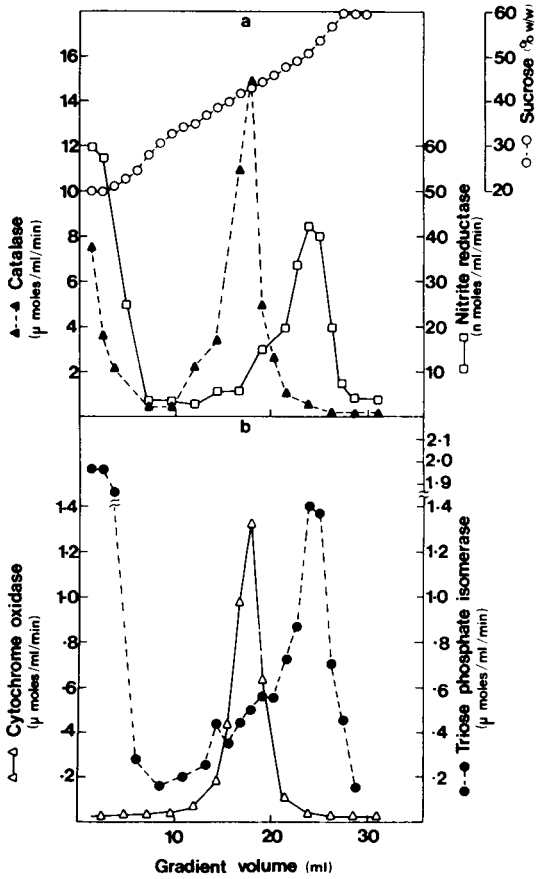

FIG. 3. Distribution of various enzyme activities in a sucrose density gradient of a 10,000*g* pellet from pea root tissue. Root tissue was homogenized, centrifuged at 10,000*g* for 15 min, resuspended in 2 ml of 20% sucrose, and layered on a gradient of 5 ml of 22% sucrose, 20 ml of a linear gradient of 25 to 55% sucrose with a 5-ml cushion of 60% sucrose. All solutions were w/w sucrose made up with 0.1 M Tricine buffer, pH 7.5. The gradient was centrifuged at 20,000 rpm in a SW 27 rotor for 3.5 hr.

stoichiometric amounts and the activity was present in extracts from aseptically grown peas. Enzyme activity declined rapidly with time over a period of about 2 hr and it was necessary to carry out the experiments rapidly.

DISCUSSION

In the experiments reported here the nitrite reductase activity in the gradient bands coincidentally with the plastid marker enzyme triose-P isomerase at a density commensurate with the known density of intact plastids. In no case is there any coincidence of nitrite reductase with catalase, the microbody marker enzyme. These and other studies (6, 7) refute the idea propounded by Lips and Avissar (17) that nitrite reductase is in the microbodies. The results obtained from the short term centrifugation techniques used in this study are particularly convincing because of the very low contamination of the plastid band with catalase, in contrast to the much higher levels of cross contamination between microbodies and plastids that can occur in isopycnic density gradient separations (6, 7, 28). It is probably the closeness in equilibrium banding densities of peroxisomes and intact chloroplasts that has caused Lips and Avissar to attribute nitrite reductase activity to the peroxisomes, especially since they did not assay for plastid marker enzymes in their gradients. Location of nitrite reductase in the intact chloroplast is consistent with the recent findings of Miflin (23) and Magalhaes *et al.* (19, 20) that intact chloroplasts are capable of a high and continuous rate of light-dependent nitrite reduction in the absence of any added enzyme or cofactors.

Studies on the distribution of glutamate dehydrogenase did

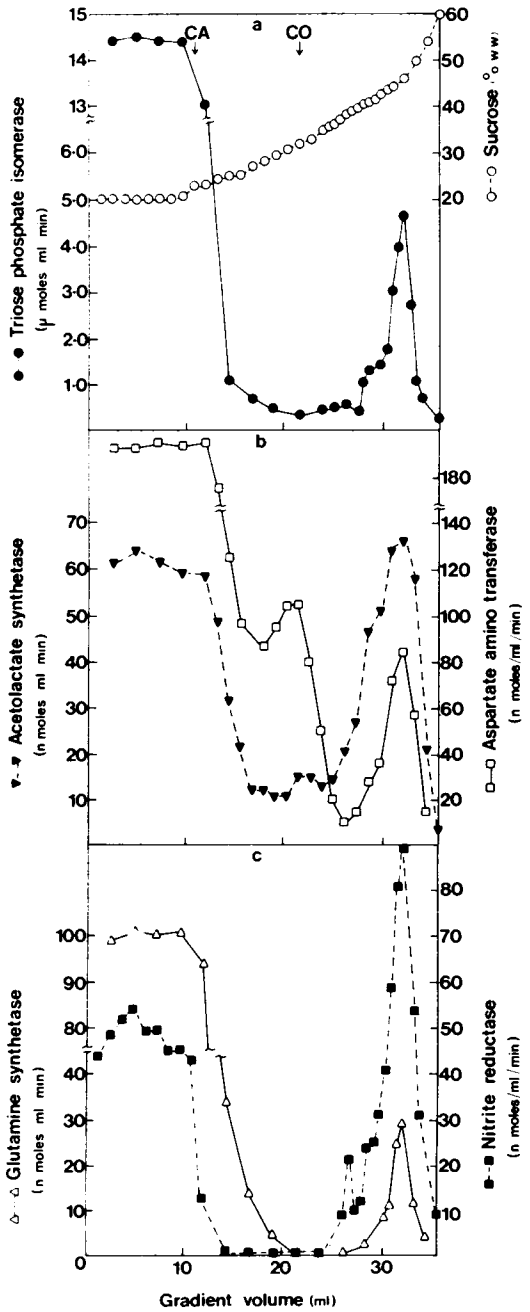

FIG. 4. Distribution of various enzyme activities in a sucrose density gradient of a filtered homogenate of pea roots after a brief centrifugation. The arrows denote the region of the peak of activity for catalase (CA) and Cyt oxidase (CO).

Table I. *Percentage Recovery of Various Enzymes in Intact Plastids*

The results were derived from the separation shown in Figures 1, 3, and 4 and are given as percentages of the total activity that were recovered under the intact plastid peaks.

Enzyme	Tissue		
	Spinach leaves	Pea roots	Barley roots
	%		
Triosephosphate isomerase	8	8	5
Nitrite reductase	13	33	25
Acetolactate synthetase	13	32	
Glutamine synthetase	7	7	
Chlorophyll	18		

Table II. *Effect of Various Compounds on Reduction of Nitrite by Isolated Pea Root Plastids*

Pea root plastids were prepared by grinding root tissue in a pestle and mortar using the standard isolation medium. The homogenate was filtered into centrifuge tubes and spun at 3000*g* for 2 min. The pellet was resuspended in a small volume of 0.4 M sucrose, 5 mM $CaCl_2$, and 50 mM HEPES buffer pH 7.5. Aliquots (100 μl) were incubated with 100 nmoles of nitrite plus the above additions in a final volume of 250 μl for 20 min at 24 C. At the end of this time the reactions were stopped with $ZnSO_4$ and the nitrite determined in the standard way (4).

Additions		Experiment				
		1		2		3[1]
		NO_2^- lost	NH_4^+ produced	NO_2^- lost	NH_4^+ produced	NO_2^- lost
	μmoles	nmoles/assay/hr		nmoles/g original fresh wt·hr		
None		0	11	0	0	0
Glucose-6-P	0.25	52	87	94	87	165
6-Phosphogluconate	0.25	4	9			
NADPH	1.2					0
NADH	1.2					0
Glucose-6-P + NADP	0.25 + 0.14	49	65			
Glucose-6-P + ATP	0.25 + 2.5			74	86	90
ATP	2.5			2	2	

[1] Peas for this experiment were grown under aseptic conditions.

Table III. *Activities in Chloroplasts of Certain Enzymes Involved in Amino Acid Biosynthesis*

Enzyme	Activity	Reference
	nmoles/mg Chl·min	
Nitrite reductase (methyl viologen assay)	270	Figure 1
	155–300	20
Nitrite reduction (light-dependent by intact chloroplasts,	150	23
	200	20
Light-dependent α-amino N production	145	23
	180	20
Glutamate dehydrogenase	40	See text
	80–100	16
	20–75	20
Light-dependent α-ketoglutarate consumption	10	9
Glutamine synthetase	1,500	Figure 1
Acetolactate synthetase	725	Figure 1
Amino acid incorporation	0.3[1]	3

[1] Based on the value of 1 nmole leucine incorporated/mg Chl·hr assuming that the leucine content of the protein synthesized was at least 5%.

not show the presence of this enzyme in any significant amount under the intact plastid peaks in gradients from either leaves or roots. This may be due to the difficulty in solubilizing the enzymes from the chloroplast lamellae (9, 15, 16). In two experiments with leaf tissue the chloroplasts were concentrated by a preliminary differential centrifugation and then layered on the gradient; in this case some activity of glutamate dehydrogenase was found under both the Chl peaks with a rate of approximately 50 nmole NADPH oxidized/mg Chl·min. This is of the same order of magnitude as reported by other workers but, in view of the Chl concentrations normally present in the gradients, it would be at the limit of detection in the assays used. In contrast, peaks of glutamine synthetase activity were easily discernable and follow the activity of triose-P isomerase closely. This confirms the suggested presence of the enzyme in chloroplasts and shows that it is also present in root plastids.

Acetolactate synthetase activity is located in intact root and leaf plastids but in contrast to the situation in *Neurospora* (31), there is little evidence for any activity associated with the mitochondrial peak. Whether or not the presence of acetolactate synthetase indicates the existence in plastids of other enzymes, either for branched chain amino acid biosynthesis or for the synthesis of carbon skeletons of other amino acids, awaits further study.

Table III shows the rates of activity for some enzymes involved in nitrogen metabolism, derived from results in this study and from the literature. These values show that the levels of glutamate dehydrogenase are scarcely sufficient to account for the rate of formation of α-amino nitrogen found in intact chloroplasts. The disparity is probably accentuated *in vivo* due to the high K*m* values for ammonia (5 mM) (15) and the necessity of high levels of ammonia to achieve the quoted rates. Such levels, if they occurred *in vivo*, would lead to uncoupling of photosynthetic phosphorylation. Glutamine synthetase activities are more than sufficient to cope with the rate of nitrite reduction and the enzyme has a much greater affinity for ammonia (K*m* = 0.02 mM) (K. W. Joy, personal communication). If ammonia enters into the amide group of glutamine then some mechanism to transfer it to the α-amino position of amino acids must exist if it is to lead to amino acid formation.[3] Superficially, these results suggest that it is unlikely that much ammonia enters into organic form via glutamate dehydrogenase in the chloroplasts. However, little is known of the spatial distribution of the nitrogen-metabolizing enzymes within the chloroplasts, and the activity and characteristics of glutamic dehydrogenase *in situ* in the chloroplast lamellae may be considerably different from those of the extracted enzyme; until further information is available no conclusions can be drawn. A further theoretical entry point for ammonia is via aspartate dehydrogenase which has been claimed by some workers (27), but not others (9), to be present in chloroplasts. The rates of all the enzymes leading to amino acid synthesis are well in excess of those required to support protein synthesis as measured in isolated chloroplasts.

These results suggest that intact plastids are capable of carrying out several steps in the synthesis of amino acids. Many studies have shown that the synthesis and metabolism of amino acids is compartmentalized within the cell. The presence of various amino acid-synthesizing enzymes suggests that, at least for some amino acids, plastids could be one of the compartments involved. This intracellular separation has obvious regulatory advantages, *e.g.*, in the case of pyruvate metabolism, pyruvate destined for leucine, isoleucine, and valine synthesis is utilized in a different compartment from that destined for respiration via tricarboxylic acid cycle. These conclusions are true of both leaf and root plastids. Root plastids have received little attention and are usually referred to as proplastids, implying that they are really misplaced and frustrated chloroplasts. The results here, and in the previous paper should dispel this idea since they show that the root plastids contain a wide range of enzymes and are probably performing an important role in the metabolism of nongreen tissues.

The finding that glucose-6-P is able to support a degree of reduction of nitrite by root plastids indicates that this compound might be the way in which reducing power, derived from translocated carbohydrate, is transported to the site of nitrite reduction. This is consistent with the observation of Butt and Beevers (5) that nitrite stimulates the pentose phosphate pathway in root tissue. It does not necessarily tell us a great deal more about the electron transport chain to nitrite in nongreen tissues since there may be any number of intervening steps. Although the rates are low in comparison with total activity/g fresh weight assayed by the methyl viologen assay, the very nature of the isolation procedure would only result in a very low extraction of the plastids from the tissue.

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[3] **Note Added in Proof.** Recent studies have shown that chloroplasts contain a ferredoxin-dependent glutamate synthase which is capable of carrying out this transfer (P. J. Lea and B. J. Miflin. 1974. Nature, in press).

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