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Nitrite Assimilation and Amino Nitrogen Synthesis in Isolated Spinach Chloroplasts[1]

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ABSTRACT

The assimilation of nitrite leading to *de novo* synthesis of amino nitrogen in a chloroplast-enriched fraction isolated from freshly harvested young spinach (*Spinacia oleracea* L.) leaves was demonstrated. The preparations showed approximately 55% intact chloroplasts as determined by light scattering properties and fixed CO_2 at rates of approximately 100 μmoles hr^{-1} mg $chlorophyll^{-1}$.

The chloroplast-enriched fraction contained the enzymes, nitrite reductase and NADPH-glutamate dehydrogenase, needed for the reduction of nitrite and incorporation of ammonia into glutamate. Kinetic studies showed that the reduction of nitrite by the chloroplast-enriched fraction is light-dependent, and the process proceeds at rates of 6 to 12 μmoles hr^{-1} mg $chlorophyll^{-1}$. The addition of nitrite to the chloroplast preparation caused a 3-fold increase in the production of α-amino nitrogen when compared with the control without nitrite. There was a stoichiometric relation between amino-nitrogen synthesis and nitrite disappearance from the medium. The ratio of amino-nitrogen:NO_2^- ranged from 0.6 to 0.9. The initial rate of amino-nitrogen production was faster when α-ketoglutarate was added to the nitrite reducing chloroplast medium than when it was omitted. However, these high rates were not sustained and the total amino-nitrogen production at the end of a 30-minute period was only slightly higher. These data show that chloroplasts are functionally able and contain the enzyme complement necessary to utilize light energy for the reduction of nitrite to amino nitrogen. Thus, chloroplasts should be considered as a major site for *in vivo* amino-nitrogen synthesis in green plants.

The *in situ* location of the enzymes involved in the assimilation of nitrate to amino acids, especially nitrate and nitrite reductases, has been a subject of controversy. The lack of agreement as to their precise intracellular location arises, in part, from inadequate cell fractionation techniques. This is especially true for aqueous separation procedures, which permit, at best, only a relative approximation of the location of enzymes within the cell.

Although several investigators have reported that nitrate reductase is located in the cytoplasm (10, 13, 29, 30), other workers have suggested that this enzyme is located in the chloroplast (9, 11). The location of nitrite reductase in the chloroplast, indicated by several workers (10, 11, 29), seems most logical since its proposed electron donor, reduced ferredoxin, is found exclusively in the chloroplast. However, Grant *et al.* (13) have reported that this enzyme is located in the cytoplasm.

A number of investigations support the hypothesis that chloroplasts play a major role in amino acid synthesis. In 1964, Bassham and co-workers (4, 5) found from kinetic studies of $^{14}CO_2$ incorporation by intact *Chlorella* cells that glutamate is the primary amination product and suggested that chloroplasts could be an important site of amino acid synthesis. Subsequently, Kirk and Leech (21) and Tsukamoto (32) established that isolated chloroplasts have the capability of amino acid biosynthesis. They also found, in agreement with others (12, 30), that the addition of ammonia to isolated chloroplasts failed to enhance the incorporation of $^{14}CO_2$ into amino acids. Further, the NADPH-dependent glutamate dehydrogenase, considered to be involved with the anabolic production of glutamate, is located in the chloroplasts (23, 25, 32).

The objectives of this work were: (*a*) to determine the relative intracellular distribution of the enzymes involved with the assimilation of nitrite to amino acids; (*b*) to investigate the capability of chloroplast-enriched fractions to convert nitrite to amino nitrogen.

MATERIAL AND METHODS

Plant Material. Freshly harvested, young (not fully expanded) leaves of greenhouse-grown spinach (*Spinacia oleracea* L. var. Early Hybrid 7, Asgrow Seeds, Ill.) were used in all experiments.

Separation of Cellular Organelles. Leaves were washed, deribbed, and rapidly minced, immediately prior to homogenization. The material was ground with a semimicro water-jacketed Waring Blendor for 10 sec at 60 v, in a breaking medium A containing: 0.33 M sorbitol, 2 mM isoascorbate, 2 mM disodium EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 10 mM NaCl, 0.5 mM K_2HPO_4, 0.05 M MES buffer, pH 6.2, 5 mM dithiothreitol, 0.1% (w/v) bovine serum albumin, and 1% (w/v) insoluble Polyclar AT. The ratio of medium to leaves was 3:1 by weight. The homogenate was filtered through two layers of Miracloth, and the filtrate was centrifuged at 500*g* for 1 min. The pellet (P) was carefully resuspended with 5 ml of medium B. Medium B was identical to medium A, except that 0.05 M HEPES buffer, pH 7.2 was used instead of MES, and isoascorbate was omitted. The resuspended pellet (P) was centrifuged at

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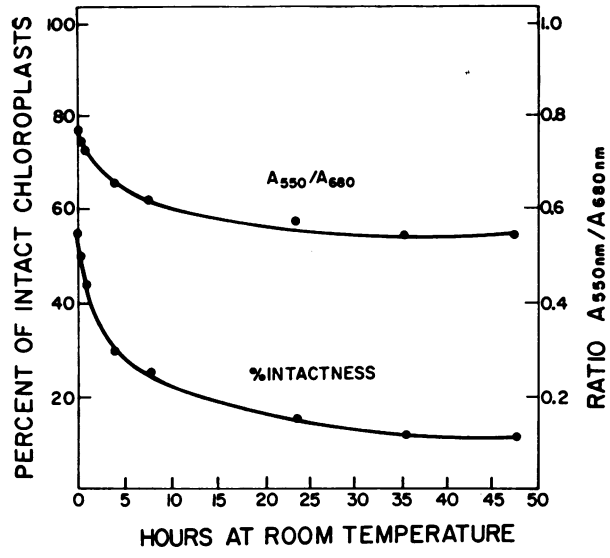

FIG. 1. Light absorption patterns and estimated degree of intactness of the chloroplast-enriched (P_2) fraction from spinach leaves.

500g for 1 min to give pellet (P_1). The supernatants S (from P) and S_1 (from P_1) were recombined. The combined supernatants were then subjected to successive differential centrifugation steps as follows: 1,500g for 5 min, 3,000g for 10 min and 20,000g for 20 min to give pellets P_2, P_3, and P_4 respectively. Each successive pellet was treated as described for P_1. The washing (resuspension) medium was always combined with the original supernatant.

For the enzyme assays, each pellet (P_1 through P_4) was hand-ground in a TenBroeck glass homogenizer with 3 ml of medium appropriate for each enzyme. In each case the homogenate was centrifuged at 30,000g for 15 min, and the resultant supernatant was used for enzyme assay and protein determination.

For measurement of chloroplast intactness (P_1, P_2, and P_3), CO_2-fixation (P_2) and NO_2^- assimilation (P_2), the washed pellets were carefully resuspended in 4 ml of medium B.

Assays. Nitrate reductase was extracted and assayed as described by Hageman and Hucklesby (16), except that 5 mM dithiothreitol was substituted for cysteine. Nitrite reductase was extracted and assayed by a modification of the method described by Beevers *et al.* (6), using a dithionite-methyl viologen system. NADH- and NADPH-dependent glutamate dehydrogenases were assayed according to the procedure described by Joy (18) by recording the decrease in absorbance at 340 nm for 3 min. EDTA was omitted from the extraction medium since it inhibits the enzyme (19). Fumarase was assayed in a total volume of 3 ml, and the reaction was recorded at 240 nm for 3 min at room temperature (8). Catalase activity was determined by the rate of H_2O_2 disappearance as described by Luck (27). Protein content of the extracts was measured by the method of Lowry *et al.* (26) using bovine serum albumin as standard. Chl concentrations were determined, as described by Arnon (1), on aliquots taken from the uncentrifuged homogenates used for the enzyme assays or the resuspended pellets.

Measurement of Chloroplast Intactness. The estimation of the relative amount of intact (class I) chloroplast present in P_1, P_2, and P_3 fractions was carried out by the spectrophotometric method of Karlstam and Albertsson (20) in the dark at 25 C. Chl concentration was 150 μg ml^{-1}.

Carbon Dioxide Fixation. Aliquots (0.5 ml) of the P_2 fraction, which contained maximum amounts of intact chloroplasts, were immediately incubated with 0.3 ml of 50 mM sodium bicarbonate-^{14}C (1.5 $\times$ 10^6 dpm) and made to a final volume of 1.0 ml with resuspension medium with or without appropriate amounts of KNO_2. Light from incandescent bulbs, after passage through a layer of water, provided 2000 ft-c incident light at the surface of the 25-ml reaction flask. Temperature was 20 C. The reaction was stopped after 5 min by adding 1 N HCl, and the determination of the radioactivity incorporated into acid-stable products was carried out in a modified Bray's scintillation fluid, as described by Bjorkman and Gauhl (7). The ability of the chloroplasts to fix CO_2 was found to be a function of the time of preparation or standing prior to assay.

Nitrite Assimilation. An aliquot (2.0 ml) of the P_2 fraction was combined with KNO_2, KNO_3 and neutralized (pH 7) α-ketoglutarate, or water (control) to give 3 ml of final volume in a 25-ml Erlenmeyer flask. The reaction mixtures were incubated at 30 C in a water bath with continuous shaking. Light was provided by a 500-w flood lamp which gave an incident energy of approximately 1.5 $\times$ 10^5 ergs cm^{-2} sec^{-1}. Nitrite loss was followed by removing 0.1-ml aliquots of the reaction mixture at zero and subsequent time intervals. Nitrite was assayed as described by Hewitt and Nicholas (17), except that the solutions were clarified by centrifugation (1000g for 10 min) before reading at 540 nm.

Amino Nitrogen Determination. The measurements were made with the same reaction mixture used to follow NO_2^- assimilation, including zero time. The residual reaction mixture, after removal of the aliquot(s) for determination of NO_2^-, was immediately frozen with liquid nitrogen and stored at −20 C. The solutions were thawed at room temperature and centrifuged at 1000g for 10 min. Aliquots (0.05 ml) of the supernatant were used directly for amino-N determinations as described by Yemm and Cocking (33) with L-isoleucine as standard. Alternatively, 10 ml of 70% ethanol were added to the thawed solution, and the mixture was agitated vigorously for 5 min. The suspension was centrifuged (20,000g for 10 min), and the supernatant was washed with petroleum ether, concentrated by evaporation and made up to to 2.0 ml with water. The sample was then passed through a Dowex 50 W (200-400 mesh, H^+ form) column (6 $\times$ 0.5 cm), and the amino acids were eluted with 20 ml of 4 N NH_4OH. The eluate was evaporated to dryness and suspended in 1.0 ml of 10% isopropanol. Aliquots (0.2 ml) were used for amino-N determinations as previously described.

RESULTS AND DISCUSSION

Chloroplast Intactness. Although 55% of the chloroplasts in the P_2 fraction were judged intact when measured immediately after resuspension, the number of intact chloroplasts decreased rapidly (44% intact after 1 hr) upon standing at 25 C (Fig. 1). Smaller amounts of intact chloroplasts were found in the P_3 fraction than in the P_2 fraction. These estimates of intactness were also verified by observations made with a phase contrast microscope. This degree of intactness was also supported by the ability of the P_2 fraction to fix CO_2. Fixation rates of approximately 100 μmoles hr^{-1} mg Chl^{-1} were observed, when measurements were made within 5 min after isolation of the chloroplasts.

Enzyme Distribution. The distribution of nitrate and nitrite reductases, and NADPH- and NADH-dependent glutamate dehydrogenases among the several cellular fractions separated by differential centrifugation is presented in Table I. Although the highest level of nitrate reductase on a per fraction or per mg protein basis was in the P_4 (mitochondrial) fraction, this is

possibly due to indiscriminate binding to membranous material (10) rather than *in vivo* association with the mitochondria. Since approximately 90% of the activity was in the supernatant, this datam is in agreement with the contention that nitrate reductase is a cytoplasmic enzyme.

The distribution of nitrite reductase shows that this enzyme is associated with the chloroplast-enriched fractions. Approximately 10% of the total activity was associated with the P_2 fraction which had the highest percentage of whole chloroplasts. The P_3 fraction (mostly broken chloroplast) had the highest specific nitrite reductase activity and retained 7% of the total activity. Thus, it would appear that nitrite reductase binds, to some degree, to cellular components. However, it does not appear to be bound tenaciously, since 70% of the activity appeared in the supernatant. Data obtained with tobacco and wheat suggested that nitrite reductase is located in the stroma of the chloroplasts (10). No detectable catalase activity was found in the P_2 fraction, showing that this fraction was essentially free of peroxisomes.

Approximately 38% of the total NADPH-glutamate dehydrogenase was associated with the P_2 and P_3 fractions, but unlike nitrite reductase very little activity was found in the P_1 fraction. Although no direct evidence was obtained, it is conceivable that the enzyme was inhibited by some component of the P_1 fraction. The high specific activity and retention of 20% of the NADPH-glutamate dehydrogenase by the P_3 fraction suggest that this enzyme is more tightly bound to the broken chlorolplasts than nitrite reductase. From this and other data (25), it was concluded that NADPH-glutamate dehydrogenase is located in the chloroplasts.

The highest specific activity of NADH-glutamate dehydrogenase was found with the P_4 (mitochondrial) fraction, although among the pellets, the P_2 fraction retained the highest percentage of total activity. The amount (8% of total) of NADH-glutamate dehydrogenase in the P_2 fraction is considered to be due to mitochondrial contamination as 10% of the total fumarase activity was also recovered in the same fraction. As observed previously with the NADPH-glutamate dehydrogenase, little NADH activity was found in the P_1 fraction. The presence of 80% of the total NADH activity in the supernatant may be attributed to the rupture of the mitochondria during isolation (25).

The distribution pattern of NADH-glutamate dehydrogenase among the various fractions illustrates again the problems encountered in attempting to deduce *in situ* localization of enzymes by differential centrifugation with aqueous media. In spite of the fact that NADH-glutamate dehydrogenase is considered to be located in or with the mitochondria, 80% of the total activity was found in the supernatant.

The data (Table I and Fig. 1) show that the P_2 fraction contains approximately 50% intact chloroplasts and adequate levels of the two enzymes, nitrite reductase and NADPH-glutamate dehydrogenase needed in the assimilation of nitrite to amino nitrogen.

Nitrite Assimilation. The effect of increasing NO_2^- concentration in the reaction mixture from 0.1 to 0.6 mM on NO_2^- assimilation and CO_2 fixation by the P_2 fraction (chloroplast-enriched) is shown in Figure 2. Concentrations of NO_2^- above 0.25 mM in the reaction medium did not produce linear increases in the rates of NO_2^- assimilation. On the other hand, the same order of increase in NO_2^- concentrations caused a gradual decrease in the rates of CO_2 fixation. Whether the inhibition of CO_2 fixation is due to a direct toxic effect of NO_2^- on the chloroplasts or results from a competition for light energy cannot be determined from these data.

The kinetics of NO_2^- assimilation is shown in Figure 3.

Table I. *Distribution of Enzyme Activities among the Cellular Fractions Separated from Spinach Leaf Homogenate by Differential Centrifugation*

Fraction	Protein Content	Chl Content	Nitrate Reductase[1]		Nitrite Reductase	
			Total activity	Specific activity	Total activity[1]	Specific activity
	mg/ml		*μmoles hr^{-1} fraction^{-1}*	*μmoles hr^{-1} mg protein^{-1}*	*μmoles hr^{-1} fraction^{-1}*	*μmoles hr^{-1} mg protein^{-1}*
P_1	2.88 (5)	0.229 (20)	0.14 (0.2)	0.02	11.88 (8)	1.38
P_2	2.30 (4)	0.556 (47)	0.98 (1)	0.14	13.86 (10)	2.01
P_3	1.42 (3)	0.146 (12)	1.94 (3)	0.45	10.91 (7)	2.56
P_4	1.16 (2)	0.097 (8)	4.39 (6)	1.26	7.08 (5)	2.03
S	1.84 (86)	0.006 (12)	65.87 (90)	0.49	101.47 (70)	0.76
			NADPH-glutamate dehydrogenase		NADH-glutamate dehydrogenase	
P_1	2.56 (5)	0.243 (25)	0.72 (2)	0.09	0.57 (1)	0.07
P_2	2.18 (4)	0.487 (50)	6.54 (18)	1.01	3.06 (8)	0.47
P_3	1.30 (3)	0.106 (11)	7.68 (20)	1.97	1.89 (5)	0.48
P_4	1.04 (3)	0.064 (7)	0.93 (2)	0.30	2.19 (6)	0.70
S	1.68 (85)	0.003 (8)	22.63 (58)	0.18	32.12 (81)	0.26

[1] Total volume of the fractions; P_1, P_2, P_3, P_4: 3 ml; S: 73 ml.

[2] Number in parenthesis indicate percentage of total component or activity for each fraction.

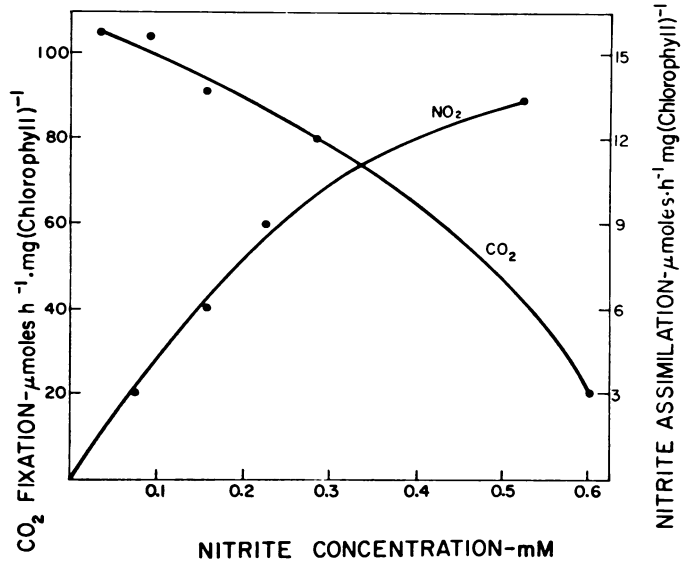

FIG. 2. Effect of nitrite concentration on nitrite assimilation and CO_2 fixation in a chloroplast-enriched (P_2) fraction from spinach leaves. These were separate experiments conducted with the same preparation. Chl concentration in the reaction flasks was 125 to 150 μg ml^{-1}.

There was no loss of NO_2^- as long as the flasks were kept in the dark, but upon illumination the rate of disappearance of NO_2^- ranged from 6 to 8 μmoles hr^{-1} mg Chl^{-1}. The observed transients indicate that NO_2^- reduction is completely dependent on light.

Nitrite Assimilation and Concurrent Amino-N Production. The production of amino-N coupled with the assimilation of NO_2^- by the chloroplast-enriched fraction (P_2) with and without the addition of 0.4 mM α-ketoglutarate in the light is shown in Figure 4. Although the control (minus NO_2^-) exhibited a steady increase in amino-N throughout, the addition of NO_2^- to the system caused a 3-fold increase in amino-N production over the control during the 30-min period. The addition of α-ketoglutarate plus NO_2^- further enhanced the amino-N production, especially during the first 10 min. This enhanced

production of amino-N, in response to added α-ketoglutarate, did not appear to affect the rate of NO_2^- reduction. That the α-ketoglutarate was being aminated from an endogenous source, would have been a logical explanation for the observed results, except that the two controls (minus NO_2^- with or without α-ketoglutarate) exhibited identical increases in amino-N. Net losses of NO_2^- were 1.26 and 1.59 μmoles/30 min^{-1} and net increases in amino-N were 1.16 and 1.38 μmoles/30 min^{-1} for the $+NO_2^-$ and $+NO_2^-$ + α-ketoglutarate treatments, respectively. The stoichiometric ratios between amino-N:NO_2^- were 0.92 and 0.86, respectively.

A subsequent experiment, identical to those described in Figure 4, was conducted except that the amino-N was extracted with ethanol and column separated prior to assay. The results again show that the addition of α-ketoglutarate without NO_2^- did not increase the production of amino-N over that of the minus α-ketoglutarate control. The net increases in amino-N in the control ($-NO_2^-$), α-ketoglutarate control ($-NO_2^-$ + α-ketoglutarate) and complete ($+NO_2^-$ + α-ketoglutarate) were 0.71, 0.72 and 1.92 μmoles mg Chl^{-1}/30 min^{-1} respectively. The increase in amino-N in the controls can be attributed to endogenous levels of α-ketoglutarate in the chloroplasts (12) that could be aminated, or to the hydrolysis of protein under the experimental conditions. The net increase in amino-N was 1.20 μmoles compared with a net loss of NO_2^- of 2.02 μmoles, giving a stoichiometric relationship between amino-N:NO_2^- of 0.6.

These results show that a chloroplast-enriched preparation, having a capability of fixing CO_2 at high rates (100 μmoles hr^{-1} mg Chl^{-1}), is capable of reducing NO_2^- and synthesizing amino-nitrogen *de novo*, when supplied with NO_2^- and light. The rates of NO_2^- reduction (6–12 μmoles hr^{-1} mg Chl^{-1}) observed with the isolated chloroplasts are sufficiently high to accommodate the rates of nitrate assimilation *in situ*. This assumes that nitrate reductase is the rate-limiting step.

Grant *et al.* (13) ruled out the involvement of chloroplasts in NO_3^- assimilation and amino acid synthesis and proposed that the enzymatic reduction of NO_3^- and NO_2^- occurs in the cytosol. In subsequent studies, Grant *et al.* (15) and Winkenback *et al.* (34) concluded that with spinach chloroplasts, nitrite inhibits photosynthesis because the chloroplasts cannot carry out nitrite detoxification by reducing it to ammonia and amino acids. However, Winkenback *et al.* (34) have suggested

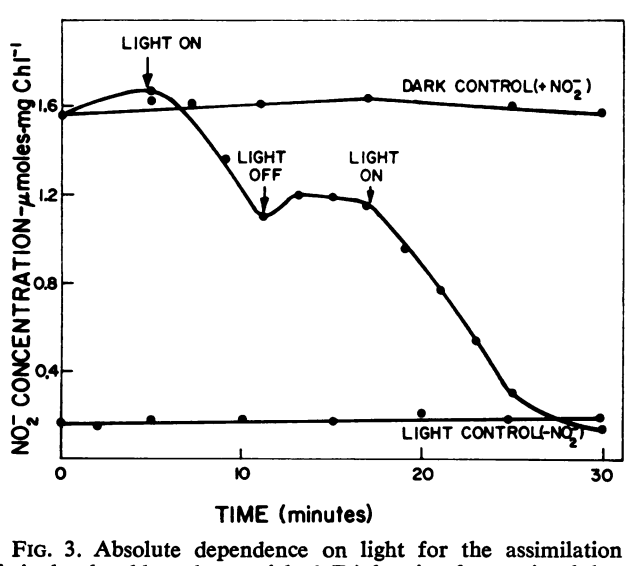

FIG. 3. Absolute dependence on light for the assimilation of nitrite by the chloroplast-enriched (P_2) fraction from spinach leaves.

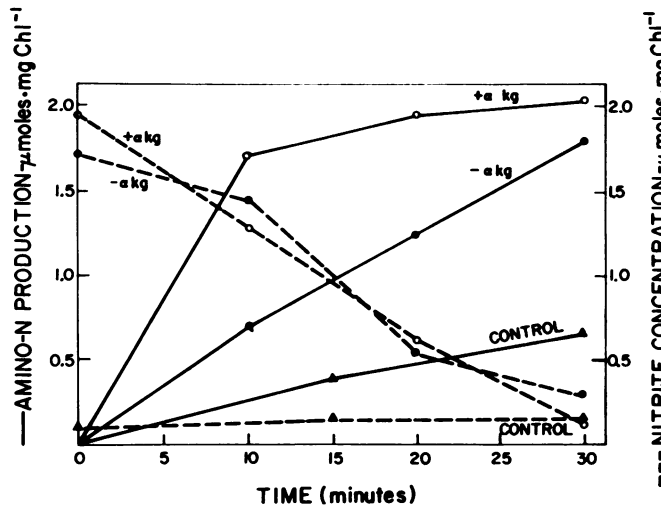

FIG. 4. Time course of amino-N synthesis and nitrite assimilation by the chloroplast-enriched (P_2) fraction from spinach leaves. The two control treatments (± α-ketoglutarate — NO_2^- gave identical results and are shown as one. Chl concentration in the reaction flasks was 150 μg ml^{-1}. Amino-N determinations were made directly on the cleared reaction mixture.

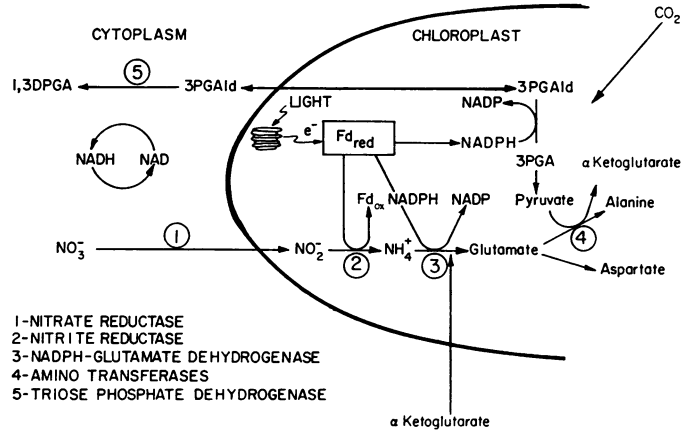

FIG. 5. Proposal for intracellular localization of the enzymes of nitrate assimilation. The scheme emphasizes that the chloroplast is a major site of nitrite reduction and amino nitrogen synthesis in leaf tissue.

that technical difficulties of chloroplast isolation might prevent the demonstration of their potential capability for amino acid synthesis. Support for this view might be deduced from previous work of Atkins and Canvin (2).

The finding that NO_2^- is converted into amino-N in the chloroplasts permits the development of the metabolic pathway of NO_3^- assimilation shown in Figure 5. The work of Klepper *et al.* (22) suggests that NO_3^- is reduced to NO_2^- in the cytoplasm via the 3-P-glyceraldehyde dehydrogenase-nitrate reductase coupled system. To minimize accumulation of free NO_2^- in the cytoplasm, it would seem logical for the nitrate reductase and nitrite reductase to be juxtaposed but separated by the chloroplast membranes and associated proteins. The NO_2^- would then have only to traverse a minimal distance to its site of reduction and incorporation into amino-N. The toxicity of free NH_3 to the normal functioning of the chloroplasts would suggest a very tight coupling between nitrite reductase and NADPH-glutamate dehydrogenase. The amino group from glutamate can be transferred to keto acids or amino acids

forming other amino acids or amides for transport throughout the cell.

The contention that ammonia should be converted to the amino form in the chloroplasts immediately after its production is substantiated by the following lines of evidence. The chloroplast membranes are relatively impermeable to ammonia (31), which would retard transference to other parts of the cell and as a consequence ammonia would accumulate inside the chloroplasts. Such accumulation would be toxic to the chloroplasts as ammonia has been shown to inhibit photosynthesis by uncoupling electron transport (3), to block ATP formation (24), and to induce structural changes (28).

Addendum. We have recently been informed by personal communication with Dr. B. J. Miflin, Department of Biochemistry, Rothamsted Experimental Station, Harpenden, Herts, England that he and this co-workers have independently and concurrently completed experiments that support the conclusions presented in this paper.

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