Intracellular Localization of Enzymes in Leaves and Chloroplast Membrane Permeability to Compounds Involved in Amino Acid Syntheses

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1. Enzyme distribution between chloroplasts and the nonchloroplast parts of green leaf cells of *Spinacia oleracea*, *Nicotiana rustica*, *Vicia faba*, and *Phaseolus vulgaris* have been investigated by use of the nonaqueous chloroplast isolation technique. Whereas pyruvate kinase and peroxidase were located only or mainly outside of the chloroplasts, the other enzymes studied, isocitric dehydrogenase, glutathione reductase, NAD- and NADP-dependent pyridine nucleotide quinone reductase, malic dehydrogenase, NAD- and NADP-dependent glyoxylate reductase, glutamate-oxaloacetate transaminase, NAD-dependent glutamic dehydrogenase, and NADP-dependent aspartic dehydrogenase were both inside and outside of the plastids. In contrast, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase is located only within the chloroplasts.

2. Intact isolated spinach chloroplasts incorporated only a very small amount of labeled carbon from $^{14}\text{CO}_2$ into amino acids in the light. The addition of NH_4Cl did not increase the amount of labeled amino acids and had no effect on the total amount of ^{14}C fixed during short time photosynthesis. However, NH_4^+ caused changes in the pathway of carbon during photosynthesis. In the presence of NH_4^+ , more ^{14}C was incorporated into sugar monophosphates and phosphoglyceric acid than in the absence of NH_4^+ .

3. ^{14}C -labeled glycine and serine fed to intact isolated spinach chloroplasts were neither accumulated nor transformed into other compounds, but ^{14}C -labeled glutamic acid was converted into glutamine. This transformation took place only in the light in chloroplasts containing an intact outer envelope. The addition of NH_4^+ and certain substrates and cofactors did not increase the rate of transformation.

4. The penetration of some amino acids and substrates through the outer envelope of the chloroplasts was investigated on aqueously isolated spinach plastids. It was found that α -ketoglutarate, oxaloacetate, pyruvate, aspartate, and alanine are able to penetrate the envelope although at least for some of these compounds the outer membrane of the chloroplasts acts as a partial barrier.

5. From the experiments reported here and in connection with the results published by other investigators it can be concluded that the most common amino acids such as glutamic acid, aspartic acid, alanine, glycine, and serine are able to penetrate through the outer envelope of the chloroplasts and the synthesis of these amino acids can occur in the leaf cells inside as well as outside of the chloroplasts.

Although it is well known that protein synthesis occurs within the leaf cells of higher plants not only in the cytoplasm but also in the chloroplasts¹⁻⁴, little information is available on the role of chloroplasts in amino acid synthesis. ROSENBERG et al.⁵ found that aspartic acid was synthesized by isolated chloroplasts but LEECH and ELLIS⁶ presented evidence that this could be due to the contamination of the isolated chloroplasts by mitochondria. According

to HEBER⁷ part of the leaf aspartic acid and alanine seem to be synthesized in vivo in chloroplasts of higher plants, while SMITH et al.⁸ found evidence of amino acid synthesis by chloroplasts of *Chlorella*.

Considerable evidence is available for the existence of amino acids in at least two pools within the living cell. This was found in animal cells (literature by MOSES⁹), in yeast cells¹⁰, in *Chlorella*^{8, 11}, and in cells of higher plants¹²⁻¹⁴. The question then arises

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¹ U. HEBER, Z. Naturforschg. 15 b, 100 [1960].

² B. PARTHIER and R. WOLLGIEHN, in: P. SITTE, Probleme der biologischen Reduplikation, pg. 244, Springer-Verlag, Berlin/Heidelberg/New York 1966.

³ J. W. DAVIES and E. C. COCKING, Planta 76, 285 [1967].

⁴ M. M. MARGULIES and F. PARENTI, Plant Physiol. 43, 504 [1968].

⁵ L. L. ROSENBERG, J. B. CAPINDALE, and F. R. WHATLEY, Nature [London] 181, 632 [1958].

⁶ R. M. LEECH and R. J. ELLIS, Nature [London] 190, 790 [1961].

⁷ U. HEBER, Habilitationsschrift, Bonn 1963.

⁸ D. C. SMITH, J. A. BASSHAM, and M. KIRK, Biochim. biophysica Acta [Amsterdam] 48, 299 [1961].

⁹ V. MOSES, in: H. METZNER, Die Zelle, pg. 248, Stuttgart 1966.

¹⁰ D. B. COWIE and F. T. McLURE, Biochim. biophysica Acta [Amsterdam] 31, 236 [1959].

¹¹ J. A. BASSHAM, B. MORAWIECKA, and M. KIRK, Biochim. biophysica Acta [Amsterdam] 90, 542 [1964].

¹² A. ONGUN and C. R. STOCKING, Plant Physiol. 40, 819 [1965].

¹³ F. C. STEWARD and R. G. S. BIDWELL, J. exp. Bot. 17, 726 [1966].

¹⁴ H. G. AACH and U. HEBER, Z. Pflanzenphysiol. 57, 317 [1967].

concerning the permeability of the various cell membranes to different amino acids. How readily do amino acids exchange between the two or more amino acid pools in the cell?

The present paper presents results of a study (1) of the intracellular distribution of some of the enzymes that are necessary for amino acid synthesis and (2) of the permeability of the chloroplast envelope to various compounds involved in amino acid synthesis.

I. Material and Methods

Leaves of spinach (*Spinacia oleracea* L.) grown in pots in the open or bought from the local market were used. Tobacco (*Nicotiana rustica* L.), broad bean (*Vicia faba* L.), and bush bean (*Phaseolus vulgaris* L.) were grown in the greenhouse.

Nonaqueous isolation of chloroplasts and enzyme assays. The intracellular distribution of enzymes was determined by isolating chloroplasts nonaqueously from tobacco and bean leaves by a modification of the method of HEBER et al.¹⁵ Enzyme activities were determined on samples resuspended in ice cold 0.1 M TRA* (pH 7.5). In some instances, 10^{-3} M EDTA was included in the resuspending medium. Isocitric dehydrogenase, malic dehydrogenase, glyoxylate reductase, glutathione reductase, glyceraldehyde-3-phosphate dehydrogenase, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, and glutamic dehydrogenase were determined under the conditions described by BERGMAYER¹⁶. The other enzyme reactions were carried out by using the following methods: pyruvate kinase¹⁷, pyridine nucleotide quinone reductase¹⁸, peroxidase¹⁹, and aspartic dehydrogenase²⁰. Absorption measurements were made with a Zeiss spectrophotometer. The distribution of the enzymes between the chloroplasts and the nonchloroplast part of the cell was calculated by the method of HEBER and WILLENBRINK²¹.

Aqueous isolation of chloroplasts. The isolation of chloroplasts from spinach leaves was performed as described by JENSEN and BASSHAM²², except that the chloroplasts were transferred, after sedimentation in buffer A, directly into buffer C, pH 7.6 ("intact" chloroplasts) or into distilled water ("broken" chloroplasts). The integrity of the intact chloroplasts was checked either microscopically or by using the amount of oxygen evolution as a criterion²³. Under the micro-

scope, the control showed between 40 and 60% of the chloroplasts in the preparation with intact outer envelopes. Oxygen evolution was determined with an oxygenograph (Gilson Medical Electronics) on samples of chloroplasts in buffer C immediately after isolation and at the end of the main experiment. If it is taken into consideration that only 40 to 60% of the chloroplasts are intact, the activities of the intact chloroplasts were about twice that calculated on a total chlorophyll basis.

¹⁴CO₂ feeding. Incorporation of ¹⁴CO₂ into isolated chloroplasts was carried out in small centrifuge tubes in buffer C (pH 7.6) at 26 °C under the conditions outlined in Table 4. The total volume was 90 to 95 μ l. After preillumination at approximately 2500 ft-c. (General Electric Reflector Flood 150 W) for 1 to 2 min, NaH¹⁴CO₃ (specific activity 5 mC/mmol) was added. After incubation in the light for 15 to 20 min, the chloroplasts were killed with hot 96% ethanol (final concentration 70%).

The compounds were separated by two dimensional paper chromatography on Whatman 4 paper which was previously washed 3 hrs in 0.5% oxalic acid and rinsed 15 times in distilled water. Chromatography was carried out in the first direction with n-butanol/propionic acid/water (47/23/30, v/v/v) and in the second direction with water saturated phenol. The location of the labeled compounds was determined by autoradiography and the radioactivity of the individual compounds was determined by counting the cut out paper in a liquid scintillation counter (Packard Model 500E).

Amino acid feeding. Feeding of isolated chloroplasts (89 to 135 μ g chlorophyll in the glycine and serine experiments, 27 to 72 μ g chlorophyll in the glutamic acid experiments) was performed in the light and the dark under conditions similar to those described for ¹⁴CO₂ feeding. In some cases (see below), different amounts of cofactors or substrates were added. At zero time the labeled amino acids were added to give final concentrations as follows: glycine 1.7 to $3.2 \cdot 10^{-5}$ M (specific activity 50.3 mC/mmol), serine 1.0 to $1.9 \cdot 10^{-5}$ M (specific activity 84 mC/mmol), and glutamic acid 1.7 to $2.8 \cdot 10^{-4}$ M (specific activity 10 mC/mmol DL-glutamic-3,4-¹⁴C acid). The total volume of the reaction medium was 70 to 140 μ l. At intervals, the chloroplasts were killed with hot 96% ethanol (final concentration 70%). In some experiments with glycine and serine, the suspensions were centrifuged for a short time in order to sediment the chloroplasts. The supernatant was transferred to another tube and hot

¹⁵ U. HEBER, N. G. PON, and M. HEBER, *Plant Physiol.* **38**, 355 [1963].

¹⁶ H. U. BERGMAYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim 1962.

¹⁷ T. BÜCHER and G. PFLEIDERER, in: *Methods in Enzymology*, vol. I, pg. 435, Academic Press, New York 1955.

¹⁸ W. D. WOSILAIT and A. NASON, *ibid.*, vol. II, pg. 725.

¹⁹ B. CHANCE and A. C. MAEHLY, *ibid.*, vol. II, pg. 764.

²⁰ G. JACOBI, *Planta* **49**, 561 [1957].

²¹ U. HEBER and J. WILLENBRINK, *Biochim. biophysica Acta* [Amsterdam] **82**, 313 [1964].

²² R. G. JENSEN and J. A. BASSHAM, *Proc. nat. Acad. Sci.* **56**, 1059 [1966].

²³ J. M. ROBINSON and C. R. STOCKING, *Plant Physiol.* **43**, 1597 [1968].

* Abbreviations: ATP = adenosine triphosphate, EDTA = ethylene diamine tetraacetate, NAD = diphosphopyridine nucleotide, NADP(H) = (reduced) triphosphopyridine nucleotide, PGA = 3-phosphoglyceric acid, TRA = triethanolamine.

ethanol was added to each tube. The weights of chloroplasts and supernatant were taken before the addition of ethanol.

Aliquots were plated in ultrathin layers on planchets and the total radioactivity determined (Nuclear Chicago Automatic Planchet Sample Changer combined with a Series Decade Scaler). The ^{14}C -labeled compounds were separated by one dimensional paper chromatography on washed Whatman 4 paper. The following solvents were used: n-butanol/propionic acid/water (47/23/30, v/v/v), isobutyric acid/ NH_3 /EDTA²⁴, and in a few cases phenol/water (80/20, v/v), and pyridine/water (80/20, v/v). The radioactive spots were identified by running the chromatograms through a strip counter (Vanguard Automatic Chromatogram Scanner 800) and the radioactivity was determined as described previously.

In some instances the distribution of radioactivity among the different compounds was checked by two dimensional paper chromatography as described above. In these cases the solvents were (1) n-butanol/propionic acid/water (47/23/30, v/v/v) and phenol/water (80/20, v/v) or (2) isobutyric acid/ NH_3 /EDTA²⁴ and n-butanol/propionic acid/water (as above).

Substrate translocation. In experiments on the penetration of substrates through the chloroplast envelope *in vitro*, the activities of glyceraldehyde-3-phosphate dehydrogenase, glutamate-oxaloacetate transaminase, and glutamate-pyruvate transaminase were measured as described by BERGMAYER¹⁶. Substrate penetration was calculated according to the method of HEBER et al.²⁵ in which three different preparations from each sample were used: A. broken chloroplasts which show the total activity of the enzyme in the plastids; B. "intact" chloroplasts which always contained a certain amount of broken plastids; C. the supernatant of the "intact" chloroplasts after short centrifugation of the chloroplast suspension. The stimulation rate (B-C/A-C) is a measure of the penetration of the substrates. The higher the stimulation due to plastid rupture, the more impermeable the chloroplast envelope for at least one of the involved compounds under the conditions of the experiment.

Breaking of the chloroplasts was achieved by osmotic shock or by ultrasonic vibration (5 sec at full intensity; Biosonic II, Bronwill Scientific, Rochester, N.Y.).

II. Results and Discussion

1. Intracellular distribution of enzymes

a) Purity of the nonaqueous chloroplasts

The determination of the distribution of a series of enzymes between the chloroplasts and nonchloroplast part of the cell should give some information

on the sites in which synthesis of different substrates can take place. The technique of isolation of chloroplasts in a nonaqueous medium from freeze-dried material makes possible the quantitative determination of enzyme distribution between chloroplasts and the nonchloroplast part of the cell. In some cases, it might be possible that the enzyme activity which is calculated for the nonchloroplast part of the cell is too low because of the inhibition effects which could occur in this fraction. This fraction contains all of the material which is normally located in the vacuole and, therefore, separated from the cytoplasm and the different cell organelles in the intact cell. Vacuolar compounds when mixed with the cytoplasm could inhibit at least a part of the enzyme reactions. However, even if this should be the case, the results should still show at least whether an enzyme is located only outside of the chloroplasts or in both compartments, inside and outside of the plastids.

The prerequisite for obtaining reliable results is the isolation of a chloroplast fraction which is relatively free from contamination by cytoplasm, mitochondria, or other cell material. The purity of the isolated chloroplasts may be checked by testing for enzymes absent *in vivo* from the chloroplasts, e. g. pyruvate kinase¹. Table 1 shows the distribution of pyruvate kinase in the separated material which was used for the further enzyme studies. Since in all cases less than 10% of the pyruvate kinase was found in the chloroplast fractions, it may be concluded that the contamination of the isolated plastids by cytoplasmic material was less than 10 per cent.

Since the pyruvate kinase is sometimes inhibited in the total leaf extracts, efforts were made to find other enzymes which are located only outside of the chloroplasts and thus can be used as a measure of cytoplasmic contamination of the isolated plastids. SMILLIE²⁶ found, for instance, in *Euglena* that isocitric dehydrogenase and glutathione reductase both appear to be located only outside the chloroplasts. Our results (Table 1) indicate that these enzymes and in addition pyridine nucleotide quinone reductase were present both in the chloroplasts and the nonchloroplast samples obtained from the leaves of the higher plants tested.

²⁴ E. TYSZKIEWICZ, *Analyt. Biochem.* [New York] **3**, 164 [1962].

²⁵ U. HEBER, U. W. HALLIER, and M. A. HUDSON, *Z. Naturforsch.* **22b**, 1200 [1967].

²⁶ R. M. SMILLIE, *Canad. J. Bot.* **41**, 123 [1963].

Nr.	plant	fraction	pyruvate kinase		isocitric dehydrogenase		glutathione reductase		pyridine nucleotide quinone reductase			
			activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts	NAD-dependent		NADP-dependent	
									activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts
1	<i>Vicia</i>	chloroplasts	0.008	2.2	0.34	19	1.52	56	1.37	12	3.47	67
		nonchl. parts	0.214		0.76		0.76		6.13		1.08	
2	<i>Vicia</i>	chloroplasts	0.009	2.6	0.42	29	1.44	70	1.39	17	3.92	68
		nonchl. parts	0.280		0.85		0.52		5.53		0.75	
3	<i>Vicia</i>	chloroplasts	—	—	0.50	31	—	—	1.82	20	3.51	65
		nonchl. parts	—	—	0.71		—		4.57		1.18	
4	<i>Nicotiana</i>	chloroplasts	0.009	3.5	0.40	38	0.57	97	2.98	34	5.46	56
		nonchl. parts	0.135		0.36		0.025		3.28		2.41	
5	<i>Nicotiana</i>	chloroplasts	0.016	5.7	0.44	52	0.77	109	2.95	27	6.11	47
		nonchl. parts	0.132		0.20		0.00		3.83		3.40	
6	<i>Phaseolus</i>	chloroplasts	0.026	8.1	0.27	29	0.67	57	3.45	40	4.72	53
		nonchl. parts	0.268		0.60		0.46		4.65		3.79	

Table 1. The distribution of pyruvate kinase, isocitric dehydrogenase, glutathione reductase, NAD-dependent and NADP-dependent pyridine nucleotide quinone reductase between the chloroplasts and the nonchloroplast parts of green leaf cells.

Nr.	plant	fraction	peroxidase		glyceraldehyde-3-phosphate dehydrogenase		malic dehydrogenase		glyoxylate reductase			
			activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts	NAD-dependent		NADP-dependent	
									activity in μ moles/mg dry weight/h	% in the chloroplasts	activity in μ moles/mg dry weight/h	% in the chloroplasts
1	<i>Vicia</i>	chloroplasts	9.3	7.0	4.40	99	16.0	22	0.61	24	0.216	40
		nonchloro. parts	78.6		0.04		30.9		1.20		0.208	
2	<i>Vicia</i>	chloroplasts	12.2	12.6	4.12	102	19.8	28	0.53	26	0.173	46
		nonchloro. parts	70.0		0.00		41.1		1.26		0.226	
3	<i>Vicia</i>	chloroplasts	6.7	5.7	8.49	102	20.7	23	—	—	—	—
		nonchloro. parts	69.0		0.00		43.8		—		—	
4	<i>Nicotiana</i>	chloroplasts	inhib.	—	—	—	25.6	33	1.25	28	0.193	—
		nonchloro. parts	inhib.		inhib.		29.4		1.80		inhib.	
5	<i>Nicotiana</i>	chloroplasts	inhib.	—	—	—	30.1	45	1.70	27	0.282	—
		nonchloro. parts	inhib.		inhib.		18.2		2.21		inhib.	
6	<i>Phaseolus</i>	chloroplasts	4.7	2.9	4.50	128	23.2	31	1.33	57	0.213	78
		nonchloro. parts	142.0		0.00		45.8		0.89		0.059	

Table 2. The distribution of peroxidase, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, malic dehydrogenase, NAD-dependent and NADP-dependent glyoxylate reductase between the chloroplasts and the nonchloroplast parts of green leaf cells.

In contrast, peroxidase (Table 2) was located in the nonchloroplast fraction of the cells and the small amount of activity associated with the plastids could have been due to the cytoplasmic contamination (compare the pyruvate kinase activity of the same samples). The peroxidase activity was relatively high, but decreased rapidly during the first minutes after the H_2O_2 was added, especially in the freeze-dried tobacco leaves. On the basis of these results, peroxidase activity could be used for measuring the

contamination of the isolated chloroplasts by non-plastid cytoplasmic material in preparations from *Vicia* and *Phaseolus* leaves.

In contrast to the peroxidase activity, NADP-dependent glyceraldehyde-3-phosphate dehydrogenase was found only in the chloroplasts (Table 2). This is in agreement with the results of other laboratories^{15, 26}. Besides constituting evidence of an intracellular compartmentation of this enzyme, the results also show that no secondary translocation

across the chloroplast membrane has taken place during the nonaqueous isolation procedure.

On the basis of the results of the distribution of pyruvate kinase, peroxidase, and glyceraldehyde-3-phosphate dehydrogenase (Tables 1, 2) it can be concluded that the chloroplasts which were isolated from the freeze-dried leaf material contained definitely less than 15% and probably less than 10% contamination by nonplastid cytoplasmic material.

b) Enzymes involved in amino acid synthesis

In the following, the intracellular distributions of some enzymes which are involved in the synthesis of amino acids have been investigated. The results are given in Tables 2 and 3.

Malic dehydrogenase and glyoxylate reductase are enzymes which catalyze the synthesis of amino acid precursors, e.g. oxaloacetic acid and glyoxylic acid. According to SMILLIE²⁶, in *Euglena* these enzymes appear to be located only outside the chloroplasts. As is evident from Table 2, our results indicate that malic dehydrogenase and both glyoxylate reductases are present in the chloroplasts and the nonchloroplast part of the cell. In *Vicia*, tobacco, and *Phaseolus*, about 20 to 45% of the malic dehydrogenase were found in the chloroplasts. Data of HEBER¹ also show that a considerable amount of

the total malic dehydrogenase of leaf cells resides in the plastids. In contrast, YAMAZAKI and TOLBERT²⁷ concludes that malic dehydrogenase is not located in the plastids but in mitochondria and in the peroxisomes. Table 2 also shows that peroxidase activity associated with the chloroplast fraction was within the same range as the pyruvate kinase activity and on this basis it could be concluded that in these plants peroxidase was absent from the plastids.

A variable amount of glyoxylate reductase was found associated with the plastids in the different samples of leaves tested. The NAD-dependent glyoxylate reductase activity was over 70% outside of the chloroplast samples (except in *Phaseolus*) while more than 40% of the NADP-dependent glyoxylate reductase was located in the chloroplast samples. TOLBERT et al.²⁸ found up to 55% of the NAD-dependent but no NADP-dependent glyoxylate reductase in spinach leaf peroxisomes.

The two transaminases tested, glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase, showed activity both inside and outside of the chloroplasts although only a low percentage of the total glutamate-pyruvate transaminase activity was associated with the chloroplasts. These results are in agreement with the results published by

Nr.	plant	fraction	glutamate-oxaloacetate transaminase		glutamate-pyruvate transaminase		NAD-dependent glutamic dehydrogenase		NADP-dependent aspartic dehydrogenase	
			activity in $\mu\text{moles/mg dry weight/h}$	% in the chloroplasts	activity in $\mu\text{moles/mg dry weight/h}$	% in the chloroplasts	activity in $\mu\text{moles/mg dry weight/h}$	% in the chloroplasts	activity in $\mu\text{moles/mg dry weight/h}$	% in the chloroplasts
1	<i>Vicia</i>	chloroplasts	1.36	38	1.27	20	0.010	16	0.026	22
		nonchloroplast parts	1.42		3.26		0.031		0.059	
2	<i>Vicia</i>	chloroplasts	1.28	41	1.40	26	0.014	27	0.019	20
		nonchloroplast parts	1.53		3.35		0.032		0.060	
3	<i>Vicia</i>	chloroplasts	1.39	38	0.95	22	—	—	—	—
		nonchloroplast parts	1.50		2.10		—		—	
4	<i>Nicotiana</i>	chloroplasts	1.66	42	1.47	—	0.023	25	0.000	—
		nonchloroplast parts	1.32		inhib.		0.039		0.000	
5	<i>Nicotiana</i>	chloroplasts	1.64	34	1.75	—	0.010	8	0.057	—
		nonchloroplast parts	1.59		inhib.		0.052		0.000	
6	<i>Phaseolus</i>	chloroplasts	1.36	57	0.65	58	0.000	—	0.053	63
		nonchloroplast parts	0.91		0.42		0.000		0.030	

Table 3. The distribution of glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, NAD-dependent glutamic dehydrogenase and NADP-dependent aspartic dehydrogenase between the chloroplasts and the nonchloroplast parts of green leaf cells.

²⁷ R. K. YAMAZAKI and N. E. TOLBERT, Plant Physiol. **43**, Suppl. 9 [1968].

²⁸ N. E. TOLBERT, A. OESER, T. KISAKI, R. H. HAGEMAN, and R. K. YAMAZAKI, J. biol. Chemistry **243**, 5179 [1968].

WILSON et al.²⁹ who found that transaminase activity was present in medium particles and in the corresponding supernatant. In contrast, HEBER¹ reported for *Vicia* leaves that glutamate-pyruvate transaminase was located only outside the plastids, while glutamate-oxaloacetate transaminase was found in both fractions. KASPAREK³⁰ has reported that barley contain transaminases capable of transferring amino groups from 16 amino acids to pyruvate. Other transaminases which were tested by the technique of JACOBI²⁰ showed no activity in the freeze-dried material.

Although the total activity of both NAD-dependent glutamic dehydrogenase and NADP-dependent aspartic dehydrogenase was low, a small but significant percentage (16 to 27%) of the total activity of these enzymes was associated with the chloroplast fraction in *Vicia*. The values are too high to be explained by the low cytoplasmic contamination of the chloroplast fraction indicated by the pyruvate kinase and peroxidase data and show in contrast to reports of other laboratories^{1,31} that low levels of these enzymes occur in the chloroplasts. LEECH and KIRK³² have recently reported a NADP-dependent L-glutamic dehydrogenase to be located exclusively in *Vicia* chloroplast lamellae and suggest that the NAD-dependent dehydrogenase is localized in mitochondria.

In summary none of the enzymes involved in amino acid metabolism that showed significant activity in the employed test system was absent from the plastids. On this basis alone the possibility of amino acid synthesis both inside and outside of the chloroplasts must be considered.

2. ¹⁴CO₂ metabolism by isolated chloroplasts

Fixation of ¹⁴C-labeled carbon dioxide by isolated chloroplasts has been reported by many investigators^{22,33,34}. In general, most of the reduced ¹⁴C in isolated chloroplasts is incorporated into inter-

mediates of the carbon reduction cycle, particularly PGA, dihydroxyacetone phosphate, sugar phosphates, and glycolic acid. The labeling of secondary products of low molecular weight, for instance of amino acids, is rather small. ROSENBERG et al.⁵ found that at least aspartic acid can be synthesized in isolated chloroplasts, but LEECH and ELLIS⁶ suggested that this result may have been due to a contamination of the chloroplast preparation with mitochondria. HEBER⁷ and EVERSON et al.³⁵ observed that aspartic acid and alanine are synthesized during photosynthesis by isolated chloroplasts and TOLBERT et al.^{36,37} obtained ¹⁴C-labeling of glycine and serine in isolated plastids. Recently it was found that *Acetabularia* chloroplasts fed with ¹⁴CO₂ synthesize aspartic acid, glutamic acid, alanine, serine and glycine³⁸.

The ammonium ion stimulated the incorporation of ¹⁴CO₂ into amino acids in *Chlorella in vivo*³⁹. We have studied whether an excess of NH₄⁺ (final concentration 0.01 M NH₄Cl) caused a change in the pattern of ¹⁴C-labeled compounds formed during photosynthesis in isolated spinach chloroplasts. Fig. 1 shows the total fixation of ¹⁴C into organic compounds during photosynthesis of isolated chloroplasts. During the first 5 to 10 min, incorporation occurred more or less linearly. Although rates of only 10 to 16 μ moles CO₂/mg chlorophyll·hr were obtained for the first 8 min of this experiment, the actual rates must have been at least double this amount since only intact chloroplasts are able to fix CO₂ under the conditions of the experiments^{23,40,41}. Only about 40 to 60% of the total chlorophyll in these preparations is in the intact plastids. No difference was observed in the amount of ¹⁴C incorporation in the presence or in the absence of 0.01 M NH₄Cl (Table 4).

However the presence of 0.01 M NH₄⁺ does cause a change in the pathway of carbon during photosynthesis (Table 4). In the presence of a high concentration of NH₄⁺, much more ¹⁴C was incorporated into sugar monophosphates and especially into PGA.

²⁹ D. G. WILSON, K. W. KING, and R. H. BURRIS, *J. biol. Chemistry* **208**, 863 [1954].

³⁰ M. KASPAREK, *Physiol. Vég.* **6**, 19 [1968].

³¹ G. L. RITENOUR, K. W. JOY, J. BUNNING, and R. H. HAGEMAN, *Plant Physiol.* **42**, 233 [1967].

³² R. M. LEECH and P. R. KIRK, *Biochem. biophys. Res. Commun.* **32**, 685 [1968].

³³ M. GIBBS and N. CALO, *Plant Physiol.* **34**, 318 [1959].

³⁴ M. LOSADA, A. V. TREBST, and D. I. ARNON, *J. biol. Chemistry* **235**, 832 [1960].

³⁵ R. G. EVERSON, W. COCKBURN, and M. GIBBS, *Plant Physiol.* **42**, 840 [1967].

³⁶ W. H. CHANG and N. E. TOLBERT, *Plant Physiol.* **40**, 1048 [1965].

³⁷ P. C. KEARNEY and N. E. TOLBERT, *Arch. Biochem. Biophysics* **98**, 164 [1962].

³⁸ D. C. SHEPHARD, W. B. LEVIN, and R. G. S. BIDWELL, *Biochem. biophys. Res. Commun.* **32**, 413 [1968].

³⁹ R. G. HILLER and C. P. WHITTINGHAM, *J. exp. Bot.* **15**, 21 [1964].

experiment Nr.		R 7		R 9		R 11		R 12	
experimental conditions	NaH ¹⁴ CO ₃ (10 ⁻³ M)	4.2		5.5		5.9		6.2	
	pyridoxal phosphate (10 ⁻³ M)	0		1.3		1.3		0	
	NH ₄ Cl (10 ⁻² M)	0	1.0	0	1.0	0	1.0	0	1.0
	chlorophyll (mg/ml)	0.56		0.64		0.425		0.40	
	photosynthesis (min)	20		20		15		16	
% of the radio-activity in the compounds	rate of photosynthesis (μmoles/mg chlorophyll · h)	4.1	3.9	2.8	3.5	10.8	11.6	9.3	9.0
	sugar diphosphates	31.8	23.1	13.7	9.1	26.8	12.4	14.0	5.0
	sugar monophosphates	5.9	9.2	5.3	7.5	4.4	10.4	8.9	22.8
	dihydroxyacetone phosphate	29.5	22.4	45.6	33.9	7.7	9.5	24.4	11.7
	phosphoglyceric acid	3.3	17.4	4.6	20.7	18.9	40.6	19.5	37.1
	aspartic acid	0.9	1.3	1.8	1.8	1.2	0.9	0.4	0.4
	glycine	14.5	9.1	6.0	5.5	8.4	4.1	8.1	2.9
	alanine	1.7	1.6	1.8	1.6	2.0	1.5	1.0	0.8

Table 4. Incorporation of ¹⁴C into various compounds during photosynthesis of intact spinach chloroplasts with ¹⁴CO₂ in the presence and absence of NH₄Cl.

Fig. 1. The total amount of ¹⁴C-labeled products after photosynthesis of intact isolated spinach chloroplasts with ¹⁴CO₂ in the presence and absence of NH₄Cl. Abscissa: time in min. Ordinate: total amount of ¹⁴C-labeled compounds in counts per min per μg chlorophyll.

In contrast, a much smaller amount of ¹⁴C was found in the sugar diphosphates (including uridine diphosphoglucose) while the synthesis of dihydroxyacetone phosphate decreased only slightly under the conditions of the experiment.

After 15 to 20 min of photosynthesis in isolated chloroplasts, significant labelling was found in aspartic acid, alanine, and glycine. In the presence of 0.01 M NH₄⁺, glycine and to a lesser extent alanine labelling was reduced.

These findings confirm the previous reports that chloroplasts can synthesize at least several of the most common amino acids.

3. Metabolism of ¹⁴C-labeled amino acids fed to isolated chloroplasts

Many experiments have been made in which ¹⁴C-labeled amino acids have been fed to leaf cells *in vivo*. For example JIMENEZ et al.⁴² found that in wheat leaves ¹⁴C-labeled serine was converted into sucrose in the light. In addition ONGUN and STOCKING¹² observed that ¹⁴C-labeled serine moves rapidly into chloroplasts when fed to detached tobacco leaves; however, rapid metabolism of the serine took place only in the light. NAYLOR and TOLBERT⁴³ fed ¹⁴C-labeled glutamic acid to barley leaves and found that it was converted into glutamine, aspartate, malate, citrate, and γ-aminobutyric acid. Also these reactions were associated with the metabolism in the light.

In contrast, little work has been done in which intact isolated chloroplasts have been fed amino acids to determine the permeability properties of the plastid envelope to amino acids. SISSAKIAN⁴⁴ found that labeled glycine was incorporated into protein in a chloroplast preparation although the extent of whole chloroplasts in the preparation was not determined. CHAN and BASSHAM⁴⁵ fed ¹⁴C-glycolic acid to chloroplasts and obtained glycine.

⁴⁰ D. A. WALKER and R. HILL, *Biochim. biophysica Acta* [Amsterdam] **131**, 330 [1967].

⁴¹ J. A. BASSHAM, M. KIRK, and R. G. JENSEN, *Biochim. biophysica Acta* [Amsterdam] **153**, 211 [1968].

⁴² E. JIMENEZ, R. L. BALDIN, N. E. TOLBERT, and W. A. WOOD, *Arch. biochem. Biophysics* **98**, 172 [1962].

⁴³ A. W. NAYLOR and N. E. TOLBERT, *Physiol. Plant.* **9**, 220 [1956].

⁴⁴ N. M. SISSAKIAN, *Proc. Intern. Congr. Biochem.*, 3rd Congr., Brussels 1955, pg. 13, Acad. Press, New York 1956.

⁴⁵ H. W. S. CHAN and J. A. BASSHAM, *Biochim. biophysica Acta* [Amsterdam] **141**, 426 [1967].

In an attempt to determine whether certain amino acids were able to penetrate into intact chloroplasts and either be accumulated there or transformed within the plastids, ^{14}C -glycine, ^{14}C -serine, and ^{14}C -glutamic acid were fed to isolated chloroplasts which were provided with or without cofactors (e.g. pyridine phosphate, pyridine nucleotides, ATP), substrates (e.g. pyruvate, oxaloacetate), NH_4^+ , and NO_3^- . In no case was either glycine or serine accumulated or transformed either in the light or in the dark after 15 to 20 min in these experiments. These results are surprising in view of the finding that in intact leaves these amino acids are rapidly metabolized in the light^{12, 42}.

On the other hand, exogenously fed glutamic acid is rapidly converted to glutamine by intact spinach chloroplasts in the light but not in the dark (Fig. 2).

Fig. 2. Feeding of ^{14}C -glutamic acid to intact isolated spinach chloroplasts in the light and in the dark. Reaction mixture: buffer C contains $1.9 \cdot 10^{-4} \text{ M}$ glutamic acid and 0.47 mg chlorophyll/ml. Abscissa: time in min. Ordinate: % of the total amount of glutamic acid changed to glutamine.

Fig. 3. Feeding of ^{14}C -glutamic acid to intact and broken chloroplasts in the light. Reaction mixture: buffer C contains $2.8 \cdot 10^{-4} \text{ M}$ glutamic acid and 0.45 mg chlorophyll/ml. Abscissa: time in min. Ordinate: % of the total amount of glutamic acid changed to glutamine.

The ^{14}C from the added glutamic acid only appeared as glutamine. When the chloroplasts were swollen in dilute buffer to break the outer envelope, they lost their ability to transform glutamic acid into glutamine (Fig. 3). These results indicate that exogenous glutamic acid can penetrate rapidly into intact chloroplasts and that the enzyme system necessary for the transformation of glutamic acid to glutamine in the light is present in the chloroplasts.

4. Penetration of substrates through the chloroplast envelope

Little is known about the *in vivo* permeability of the chloroplast envelope to amino acids and precursors that are necessary for amino acid synthesis. ONGUN and STOCKING¹², who fed labeled serine to tobacco leaves, found that at least serine moved freely through the chloroplast envelope. AACH and HEBER¹⁴ observed that there is a compartmentation at least for certain amino acids within the cell, but ^{14}C -aspartic acid, ^{14}C -serine, and ^{14}C -alanine showed, after short time labeling with ^{14}C , about the same distribution between chloroplasts and the nonchloroplast part of the cell as the nonlabeled forms. From this they concluded that at least these amino acids can penetrate easily through the chloroplast envelope.

In the present study, the penetration of some amino acids and substrates was investigated on the assumption that in intact isolated chloroplasts, an enzyme reaction proceeds only as long as all the substrates and cofactors which are necessary for the reaction are present in the plastids, and that in order for exogenously added substrates effectively to stimulate the reaction they must pass through the chloroplast envelope. Therefore, the rate of an enzyme reaction *in vitro* in intact chloroplasts compared to the rate in broken plastids, where there would be no permeability barrier, should give some information about the permeability of the outer envelope of the plastids to the substrates and cofactors that are originally absent from or in limiting amounts in the intact isolated plastids.

Table 5 shows that the activity of both glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase was 2 to 5 times higher on a total chlorophyll basis in the preparation where all of the chloroplasts were broken than when the preparation contained some intact as well as broken plastids. In contrast, the activity of glyceraldehyde-3-phosphate

experiment Nr.	glutamate-oxaloacetate transaminase		glutamate-pyruvate transaminase		NADP-dependent glycerol- dehydro-3-phosphate dehydrogenase	
	activity *	stimulation	activity *	stimulation	activity *	stimulation
1	30	2.5	10	4.9	149	∞
2	31	2.4	10	2.6	—	—
3	37	3.1	8	3.7	165	22
4	41	3.4	11	2.9	262	42

Table 5. Comparison between enzyme activities of intact and osmotically shocked spinach chloroplasts. The stimulation rate shows how much faster the enzyme reactions occur after the destruction of the outer envelope of the chloroplasts. * Enzyme activity of the shocked chloroplasts in $\mu\text{moles/mg chlorophyll} \cdot \text{h}$.

dehydrogenase was stimulated 22 to more than 100 times by the osmotic shock. In this latter case, the large stimulation on breaking the plastids results from the fact that the NADPH, that is necessary for the enzyme reaction, is unable to penetrate through the chloroplast envelope of the intact plastids^{23, 46}.

Further evidence of the resistance of the chloroplast envelope to some of the reactants in the two transaminase reactions is given in Figs. 4, 5 and 6. In these instances, the intact plastids were broken by ultrasonic vibration in the vessel after the reaction had been initiated. The change in slope represents the stimulation due to the breaking of the intact plastids. Treatment of the osmotically swollen chloroplasts with ultrasonic vibration gave little or no stimulation of the reactions while treatment of

Fig. 4. The activity of glutamate-pyruvate transaminase in suspensions of intact and broken spinach chloroplasts before and after treatment with ultrasonic vibration (US). Abscissa: time in min. Ordinate: optical density as measured with a Zeiss spectrophotometer. $25 \mu\text{g}$ chlorophyll per ml isotonic reaction medium in the cuvette.

Fig. 5. The activity of glutamate-oxaloacetate transaminase in suspensions of intact and broken spinach chloroplasts before and after treatment with ultrasonic vibration (US). Abscissa: time in min. Ordinate: optical density as measured with a Zeiss spectrophotometer. $10 \mu\text{g}$ chlorophyll per ml isotonic reaction medium in the cuvette.

the "intact" (a mixture of intact and broken) plastids did. These results indicate that the chloroplast envelope acted as a partial barrier to the passage of at least one or more of the reactants in each transaminase reaction *in vitro* (glutamate, pyruvate, α -ketoglutarate, alanine and glutamate, oxaloacetate, α -ketoglutarate, aspartate). However, all of these compounds must have been able to pass through the chloroplast envelope at significant rates since in no case was the stimulation on plastid rupture comparable to that observed when exogenous NADPH was one of the reactants. The possible influence of the isolation procedure and the suspending buffer medium on the permeability of the plastid envelope cannot be evaluated in these experiments.

⁴⁶ U. HEBER and K. A. SANTARIUS, *Biochim. biophysica Acta* [Amsterdam] **109**, 390 [1965].

Fig. 6. The activity of NADP-dependent glyceraldehyde-3-phosphate dehydrogenase in suspensions of intact and broken spinach chloroplasts before and after treatment with ultrasonic vibration (US). Abscissa: time in min. Ordinate: optical density as measured with a Zeiss spectrophotometer. $5 \mu\text{g}$ chlorophyll per ml isotonic reaction medium in the cuvette.

5. Conclusions concerning the synthesis of amino acids within the chloroplasts of leaf cells

The present experiments confirm and extend earlier reports that the chloroplasts contain the essential metabolic machinery for the synthesis of some of the most common amino acids.

According to BASSHAM and KIRK⁴⁷ the incorporation of inorganic nitrogen into organic compounds

occurs in *Chlorella* via glutamic acid. They postulated that the reductive amination to form glutamic acid is the primary route for the incorporation of inorganic nitrogen during photosynthesis and assumed that the amino groups in the other amino acids could all have arisen by transamination of their respective keto acids with glutamic acid acting as the amino donor. Our experiments have demonstrated the presence of aspartic dehydrogenase as well as glutamic dehydrogenase in chloroplasts. Consequently the possibility of the formation of aspartic acid from oxaloacetate by reductive amination cannot be ruled out in this tissue. Glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase were both present in relatively high concentrations in chloroplasts. However, we were unable to detect in plastids any transaminases capable of transferring the amino group from either glutamic acid or aspartic acid to either hydroxypyruvate or glyoxylate. Hence, at least in our *in vitro* experiments the glycolate pathway for the synthesis of serine and glycine as described for instance by RABSON et al.⁴⁸ was not detected. The pathways for serine and glycine formation in chloroplasts cannot be determined from the results of our experiments.

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⁴⁷ J. A. BASSHAM and M. KIRK, *Biochim. biophysica Acta* [Amsterdam] **90**, 553 [1964].

⁴⁸ R. RABSON, N. E. TOLBERT, and P. C. KEARNEY, *Arch. Biochem. Biophysics* **98**, 154 [1962].