# AMINO ACID BIOSYNTHESIS IN ISOLATED PEA CHLOROPLASTS: METABOLISM OF LABELED ASPARTATE AND SULFATE

W. Ronald MILLS* and Kenneth G. WILSON
*Department of Botany, Miami University, Oxford, OH 45056, USA*

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## 1. Introduction

Accumulating evidence points to chloroplasts as the site of nitrite and sulfate reduction and incorporation into carbon skeletons [1]. Furthermore, several enzymes of aspartate-family amino acid biosynthesis recently have been localized in plastids. These are: aspartate kinase [2], homoserine dehydrogenase [3], diaminopimelate decarboxylase [4], acetolactate synthetase [5] and homocysteine-dependent 5-methyltetrahydropteroyl glutamate transmethylase [6]. In whole plants Lys and/or Thr have been shown to regulate the production of the aspartate-derived amino acids [7]. Biosynthesis of this family of amino acids and its regulation in isolated chloroplasts has, however, received little attention. In this report we describe the biosynthesis of amino acids in pea chloroplasts from labeled aspartate and sulfate.

## 2. Materials and methods

### 2.1. *Growth of seedlings*

Plants of *Pisum sativum*, cv. Alaska, were grown as described [8]. Prior to harvest seedlings were placed in darkness 16–18 h to reduce chloroplastic starch.

*Abbreviations:* Asp, aspartic acid; Cys, cystine; Hse, homoserine; Ile, isoleucine; Lys, lysine; Met, methionine; Met(O), methionine sulfoxide; Ser, serine; Thr, threonine

* A portion of this work was included in the Doctoral Dissertation of W.R.M. Present address: Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts. AL5 2JQ, England

This was followed by 30 min in light to enhance photophosphorylation in vitro [9].

### 2.2. *Isolation, incubation and characterization of chloroplasts*

Chloroplasts were isolated from young pea leaves and incubated under conditions designed to allow light-driven protein synthesis [8]. Chloroplast intactness was estimated by phase contrast microscopy [10] and chlorophyll determined by the method of Arnon [11].

### 2.3. *Analysis of labeled amino acids and proteins*

Following incubation of chloroplasts with labeled precursors samples were made to 7% TCA and stored at 4°C for 1 h. They were centrifuged at 20 000 × *g* for 15 min and washed twice with 3% TCA. Supernatants were pooled and comprised the free amino acid fractions. After dilution with water to approximately pH 2, salts and TCA were removed by cation-exchange chromatography [12]. Amino acids were redissolved in citrate buffer (0.2 M sodium citrate (pH 2.2), 0.1% caprylic acid, 5% thiodiglycol and 120 nmol/ml norleucine). After addition of amino acid standards, samples were analyzed with a JEOL-JLC-6AH Amino Acid Analyzer [13]. Half of the column eluate was collected by an automated fraction collector at 1-min intervals. The recorder was equipped with an event marker pen which allowed comparison of radioactive and mass peaks. One-ml aliquots were added to 5 ml of Aquasol 2 cocktail (New England Nuclear, Boston, MA, USA) and analyzed by scintillation spectrometry with 84% counting efficiency.

The TCA insoluble fraction was resuspended in 2 ml of 5.7 M HCl containing 2 mg bovine serum

albumin. This was added to a Pierce 5-ml hydrolysis tube, flushed with nitrogen and sealed. Samples were hydrolyzed at 145 ± 2°C for 6 h [14], the humin being removed by filtration. Cation-exchange chromatography and amino acid analysis were performed as described.

Samples from protein synthesis experiments were prepared as described by Mans and Novelli [15] and analyzed by scintillation spectrometry at ca. 70% counting efficiency.

### 2.4. *Chemicals*

The following radioactively labeled chemicals were acquired from New England Nuclear, Boston, MA, USA: [U-$^{14}$C]L-aspartic acid (203 mCi/mmol) and [$^{35}$S]$H_2SO_4$ (43 Ci/mg). Other chemicals were obtained from commercial sources.

## 3. Results and discussion

Isolated pea chloroplasts incubated in light incorporated label from [$^{14}$C]ASP into compounds coeluting with all the aspartate-derived amino acids (table 1). In the hydrolyzed protein fraction label emerging with the Asp, Thr, Met and Met(O) standards was found. These compounds as well as Lys, Ile, Ser-Hse and several others were labeled in the soluble amino acid fraction (table 1).

Light-driven protein synthesis in isolated pea chloroplasts is inhibited by Lys and/or Thr and this is reversed by Met [8]. One interpretation of the data is that Lys, Thr, Ile and particularly Met are biosynthesized in isolated chloroplasts and their synthesis regulated by Lys and Thr. Hence, one or more of these amino acids becomes limiting to protein synthesis. When Lys and Thr (0.5 mM each) were present during incubation with [$^{14}$C]Asp the distribution of label was quite different (table 1). Of the aspartate-family amino acids, only Asp, Thr, Met and Met(O) contained radioactivity and this was reduced. Moreover, Asp was the only labeled amino acid in the protein fraction.

Table 1
Incorporation of [$^{14}$C]aspartic acid into amino acids by isolated pea chloroplasts[a]

| | Control | | | | Lys + Thr | | | |
|---|---|---|---|---|---|---|---|---|
| | Protein amino acids | | Free amino acids | | Protein amino acids | | Free amino acids | |
| | cpm sample ($\times 10^{-3}$) | % of protein amino acids | cpm sample ($\times 10^{-3}$) | % of free amino acids | cpm sample ($\times 10^{-3}$) | % of protein amino acids | cpm sample ($\times 10^{-3}$) | % of free amino acids |
| Aspartic acid | 140.8 | 97 | 75.5 | 49 | 35.0 | 100 | 397.3 | 98 |
| Methionine sulfoxide | 1.4 | 1 | 4.1 | 3 | 0.0 | 0 | 1.2 | <1 |
| Methionine | 0.3 | <1 | 14.6 | 10 | 0.0 | 0 | 0.4 | <1 |
| Lysine | 0.0 | 0 | 6.4 | 4 | 0.0 | 0 | 0.0 | 0 |
| Threonine | 2.3 | 2 | 4.2 | 3 | 0.0 | 0 | 1.4 | <1 |
| Isoleucine | 0.0 | 0 | 4.6 | 3 | 0.0 | 0 | 0.0 | 0 |
| Serine-homoserine | 0.0 | 0 | 2.1 | 1 | 0.0 | 0 | 0.0 | 0 |
| Other amino acids | 0.0 | 0 | 41.5[b] | 27 | 0.0 | 0 | 6.6[c] | 2 |
| Totals | 144.8 | 100 | 153.0 | 100 | 35.0 | 100 | 406.9 | 100 |

[a] A final volume of 2.0 ml contained 55.1 μg chlorophyll and 0.5 μCi [$^{14}$C]Asp. Incubation was for 15 min. Lys + Thr were at 0.5 mM each

[b] Several unidentified peaks of radioactivity with retention times less than the Met(O) standard contained ca. 21 000 cpm. Other peaks of radioactivity eluting with the following standard amino acids were observed: alanine, β-alanine, arginine, glutamic acid, phenylalanine, tryptophan and tyrosine

[c] Two unidentified peaks of radioactivity with retention times less than the Met(O) standard contained ca. 5000 cpm. The remaining radioactivity eluted from the column with glutamic acid

Table 2
Characteristics of light-driven incorporation of $[^{35}S]SO_4^{2-}$ into protein in isolated pea chloroplasts[a]

| Treatment | Incorporation (% light control) |
|---|---|
| Dark | 18 |
| Lysed | 17 |
| Chloramphenicol (100 μg/ml) | 66 |
| Cycloheximide (100 μg/ml) | 102 |

[a] A final volume of 0.2 ml contained 11.6 μg chlorophyll and 2 μCi of $[^{35}S]SO_4^{2-}$. Incubation was for 15 min. Mean cpm in light control was 3147

To further study the biosynthesis of Met in isolated pea chloroplasts they were incubated with $[^{35}S]SO_4^{2-}$ under conditions designed for light-driven protein synthesis [8]. Isolated pea chloroplasts incorporated labeled sulfate into protein (table 2). Both light and intact chloroplasts (70–90%) were required. Synthesis was inhibited by chloramphenicol but unaffected by cycloheximide. This agrees with the hypothesis that $[^{35}S]SO_4^{2-}$ is assimilated into amino acids in isolated intact chloroplasts and subsequently is incorporated into chloroplastic protein.

The above data plus other evidence [16] suggest that Cys and possibly Met are biosynthesized in chloroplasts. When $[^{35}S]SO_4^{2-}$ was fed to pea chloroplasts a small amount of radioactivity (0.1–0.2%) was recovered in the free amino acid fraction (table 3), confirming the findings of Trebst and Schmidt [16]. Five percent co-chromatographed with Cys. A small amount eluted with Met, 62% with the Met(O) standard and 30% with several unidentified peaks before it. The large amount of radioactivity migrating with Met(O) was unexpected. Glutathione has been found to elute near Met(O) under similar chromatographic conditions [17] and may have contained a portion of the radiolabel.

Trials incubated with $[^{35}S]SO_4^{2-}$ and Lys + Thr gave different results (table 3). No radioactivity was found in the Met peak and there was large reduction in label migrating with Met(O). However, a notable increase in label emerging before Met(O) was observed (table 3).

We were surprised by a number of our findings. First, the relatively large amount of tracer derived from both precursors eluting before Met(O) was unexpected, but this may represent labeled peptides. Second, since homoserine is known to accumulate in young pea seedlings we expected to find it strongly labeled in the soluble fraction. Yet only a relatively small amount of radioactivity co-eluting with Ser-Hse was observed. Third, a large number of amino

Table 3
Incorporation of $[^{35}S]SO_4^{2-}$ into free pool amino acids by isolated pea chloroplasts[a]

| | Control | | Lys + Thr | |
|---|---|---|---|---|
| Amino acids | cpm sample ($\times 10^{-3}$) | % of free amino acids | cpm sample ($\times 10^{-3}$) | % of free amino acids |
| Methionine | 0.2 | <1 | 0 | 0 |
| Methionine sulfoxide | 20.1 | 64 | 2.4 | 5 |
| Cystine | 1.5 | 5 | 0.8 | 2 |
| Unidentified[b] | 9.7 | 31 | 48.5 | 93 |
| Total | 31.5 | 100 | 51.7 | 100 |

[a] A final volume of 2.0 ml contained 43.0 μg chlorophyll and 20 μCi of $[^{35}S]SO_4^{2-}$. Incubation was for 15 min. Concentrations of Lys + Thr were 0.5 mM each

[b] The unidentified radiolabeled compounds eluted from the column in several peaks with the retention times less than the Met(O) standard

acids outside the aspartate family were apparently biosynthesized from [$^{14}$C]Asp in the isolated chloroplasts.

The production of labeled Lys, Thr and Ile from [$^{14}$C]Asp confirms that isolated pea chloroplasts can synthesize these compounds, as enzyme localization studies suggested (see Introduction). Furthermore, labeled compounds co-chromatographing with Met and Met(O) were produced from both radiolabeled sulfate and Asp, suggesting that Met is also biosynthesized in chloroplasts. However, confirmation using various separation techniques will be required to unequivocally establish that these amino acids are synthesized in isolated chlorplasts. Such studies are underway. As the level of labeled Met and Met(O) produced from both tracers and the amount of Lys, Thr and Ile produced from [$^{14}$C]Asp was reduced by Lys + Thr, this combination appears to regulate chloroplastic biosynthesis of aspartate-family amino acids.

Chloroplasts incubated with [$^{14}$C]Asp produced a number of compounds co-eluting with amino acids outside the aspartate family. Amino acids derived from pyruvate, phosphoenolpyruvate, glutamate and others were labeled. Thus, extensive metabolism of [$^{14}$C]Asp must have occurred. Indeed, only 20–36% of the added label was recovered as Asp. It seems unlikely that such vast metabolism of [$^{14}$C]Asp could have occurred in 15 min. The extensive washing of the cation-exchange columns with water may have selectively removed much of the [$^{14}$C]Asp [18]. The production of aromatic amino acids in isolated plastids might be expected since enzymes of their synthesis have been localized there [2,19–22] and they have been shown to be produced from labeled precursors in isolated chloroplasts [23]. However, further study will be necessary to establish if arginine is produced in chloroplasts.

The biosynthesis of both aspartate-derived and other amino acids was reduced by exogenous Lys + Thr (table 1). Since Lys + Thr inhibit protein synthesis in isolated pea chloroplasts [8], chloroplastic amino acid biosynthesis may be enhanced by concomitant protein synthesis. Possibly protein synthesis reduces the concentrations of endogenous amino acids which act as feedback inhibitors thus stimulating carbon flux through amino acid biosynthetic pathways.

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