BIOGENESIS OF ASPARAGINE FROM VARIOUS POSSIBLE PRECURSORS BY LENTIL SEEDLINGS[1]

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There are a number of possible pathways for the biogenesis of asparagine. Some of these are: (a) direct amidation of aspartate in the presence of ATP, in analogy of glutamine synthesis; (b) transamidation between aspartate and some amide; (c) amination of α-ketosuccinamate; (d) carboxylation of β-alanylamide, and (e) carbamylation of α-alanine. There is some evidence for or against the occurrence of several of these reactions. Thus, Webster and Varner (1955) found that extracts of wheat germ and of lupine catalyze the conversion of C^{14}-aspartate into asparagine in the presence of ATP and both ammonium and magnesium ions. Mardashev and Lestrovaya (1951) reported that liver extracts can form asparagine by transamidation between glutamine and aspartate. Similarly, Levintow (1957a, b) has observed that HeLa cells will incorporate the amide group of N^{15}-glutamine into asparagine, but will not incorporate the nitrogen of N^{15}-ammonia. In contrast, Webster and Varner (1955) were not able to find any evidence of a transamidation between glutamine and C^{14}-aspartate in extracts of wheat germ or lupine. The possibility of asparagine formation by amination of α-ketosuccinamate is raised by the demonstration of this reaction by Meister and Fraser (1954) but its biological significance is uncertain, because α-ketosuccinamate is not known to arise from sources other than asparagine itself. Finally, Webster and Varner (1955) could not demonstrate asparagine formation in wheat germ extracts by the carboxylation of β-alanylamide.

It is of considerable interest, therefore, to examine the formation of asparagine from various possible precursors by intact plant cells to determine whether evidence for or against any of the above pathways might be obtained. This paper reports on asparagine formation by lentil seedlings in the presence of various possible precursors.

METHODS AND MATERIALS

Lentil seeds were washed for 5 minutes in a 1 percent ethanol solution (by volume). They were then soaked for 30 minutes in water and planted in water-saturated vermiculite. Seedlings were grown for 5 days in the dark at 25° C. The seedling tissue was cut into 0.5-cm sections. These were shaken in the incubation medium at 38° C for the time periods indicated. After incubation, the tissue sections were washed thoroughly with distilled water and ground in a mortar with 1 ml of hot water. The extract was centrifuged at 5000 x *g* for 15 min. Amino acids and amides of the supernatant solution were separated by paper chromatography with Whatman No. 3MM filter paper and 90 percent aqueous phenol as the solvent. Duplicate chromatograms were prepared for each experiment. After a 24-hr development period at room temperature, the phenol was evaporated in a strong current of air. One chromatogram was sprayed with a 0.01 percent ninhydrin solution in acetone, and dried in an oven for 5 min at 65° C. Amino acids and amides were identified by R_F values and color. This chromatogram was used to help locate and identify radioactive areas on the second chromatogram. The distribution of radioactivity was determined initially with a Forro windowless chromatogram scanner and recording ratemeter; later with a Geiger-Muller tube and standard scaling circuit adapted for chromatogram

[1]Supported in part by a grant from the Graduate School of The Ohio State University.
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scanning. Location of radioactive amino acids and amides was confirmed by autoradiography with Eastman No-Screen X-ray film.

DL-aspartate-4-C^{14} and DL-alanine-1-C^{14} were obtained from the New England Nuclear Corporation. DL-glutamate-2-C^{14} was obtained from the Volk Radiochemical Company, and $C^{14}O_2$ was prepared from $BaC^{14}O_3$ obtained from the Oak Ridge National Laboratory.

RESULTS

Five-day-old lentil stems, roots, and cotyledons are able to incorporate aspartate-C^{14} into asparagine (table 1). Not only do stems absorb the greatest

TABLE 1

*Incorporation of asparate-C^{14} into the asparagine of lentil stems, roots and cotyledons**

System	Total radioactivity taken up by the cells (counts/min)	Total radioactivity incorporated into asparagine (counts/min)	Percent of absorbed activity incorporated into asparagine
Stems	6100.	1451.	23.8
Roots	5300.	975.	18.4
Cotyledons	3300.	363.	11.9

*One gm of tissue was incubated for 60 min at 38° C in 5 ml of a solution containing 0.075 M tris-(hydroxymethyl)-aminomethane-HCl (pH 7.0), 0.05 M L-aspartate, 0.04 M NH_4Cl, 0.04 M glucose and DL-aspartate-4-C^{14} containing a total activity of 228,500 counts per minute.

TABLE 2

*Incorporation of the carbons of various possible precursors into asparagine**

Substance	Percentage of absorbed radioactivity incorporated into asparagine	
	1-hour incubation	4-hour incubation
Aspartate-4-C^{14}	24.	19.
$C^{14}O_2$	10.	18.
Glutamate-2-C^{14}	11.	11.
Alanine-1-C^{14}	10.	8.

*Experimental conditions were the same as described with table 1.

quantities of aspartate into their cells, but they are also the most effective at the incorporation of aspartate into asparagine. Incorporation proceeds steadily for approximately 60 min, but further incubation (up to 240 min) results in little or no additional incorporation of aspartate into asparagine.

Aspartate is incorporated into asparagine more readily than any of the several possible precursors examined (table 2). The carbons of glutamate and alanine are incorporated only about half as effectively as aspartate; the carbon of CO_2 is incorporated more slowly, but more extensively, than the carbons of either glutamate or alanine. The fact that the carbon of CO_2 is incorporated into

asparagine over a 4-hr period indicates that the failure of aspartate to be incorporated into asparagine for longer than 60 min is not due to an inactivation of the tissue during the incubation period. Instead, some other factor must be either inactivated or consumed completely during the measured incorporation of aspartate into asparagine.

The relatively efficient incorporation of aspartate into asparagine may occur by the amidation of aspartate. It might be expected that $C^{14}O_2$ is incorporated by a carboxylation of either α-alanine or β-alanine, followed by the conversion of the aspartate thus produced into asparagine. The data of table 3 are not in

TABLE 3

Effect of D-*aspartate on the incorporation of aspartate-*C^{14} *and* $C^{14}O_2$ *into asparagine**

Precursor	Incubation time	
	1-hour	4-hours
Aspartate-4-C^{14}		
plus L-aspartate	23.8	19.2
plus D-aspartate	12.4	9.0
$C^{14}O_2$		
plus L-aspartate	11.7	18.5
plus D-aspartate	10.6	17.6

*Experimental conditions were the same as described with table 1, except that, where indicated, $NaHC^{14}O_3$ (total activity; 220,000 counts/min) was substituted for aspartate-C^{14}, and D-aspartate was substituted for L-aspartate.

TABLE 4

*Incorporation of the carbons of various possible precursors into glutamine**

Substance	Percentage of absorbed radioactivity incorporated into glutamine	
	1-hour incubation	4-hour incubation
Glutamate-2-C^{14}	40.	46.
$C^{14}O_2$	15.	18.
Aspartate-4-C^{14}	12.	10.
Alanine-1-C^{14}	6.	5.

*Experimental conditions were the same as described with table 1.

agreement with this thesis, however. While the presence of D-aspartate strongly inhibits the incorporation of aspartate-C^{14} into asparagine, D-aspartate hardly affects the incorporation of $C^{14}O_2$. If $C^{14}O_2$ were merely being incorporated into aspartate prior to its conversion to asparagine, one would expect the incorporation of $C^{14}O_2$ into asparagine to be inhibited also by D-aspartate.

The fact that glutamate is a less effective precursor of asparagine than aspartate is apparently not due to a block in the assimilation of glutamate. In contrast to its relatively poor utilization for asparagine synthesis, glutamate is utilized more effectively for glutamine synthesis than any other precursor examined (table 4).

Further evidence that aspartate is converted directly into asparagine is obtained from the degradation of asparagine with ninhydrin. Little radioactivity is found in the alpha carboxyl group of asparagine after the incorporation of aspartate-4-C^{14} (table 5). This suggests that aspartate is converted fairly directly into asparagine without extensive metabolism of the aspartate molecule (through the Krebs cycle, for example). In contrast, asparagine which has been formed in the presence of glutamate-2-C^{14}, alanine-1-C^{14}, or $C^{14}O_2$ contains about half of its radioactivity in the alpha carboxyl.

Likewise, glutamine which has been formed from glutamate-2-C^{14} contains little radioactivity in its alpha carboxyl, but contains much radioactivity if formed from aspartate-4-C^{14}, or from $C^{14}O_2$. These experiments agree with the data of tables 2 and 4 which show the preferential utilization of aspartate and glutamate for asparagine and glutamine syntheses respectively.

TABLE 5

Effect of ninhydrin treatment on the radioactivity of asparagine formed from various precursors

Precursor	Percentage of radioactivity lost by ninhydrin treatment	
	Asparagine	Glutamine
Aspartate-4-C^{14}	7.	47.
Glutamate-2-C^{14}	51.	5.
Alanine-1-C^{14}	54.	—
$C^{14}O_2$	44.	45.

*The reaction system was the same as described with table 1. Each precursor contained 1,000,000 counts per minute.

DISCUSSION

The data reported here are in agreement with the view that asparagine is formed from aspartate, and glutamine is formed from glutamate. The relatively poor incorporation of alanine, CO_2, and glutamate into asparagine suggests that the formation of asparagine by the carbamylation of alanine, the carboxylation of β-alanylamide, or the Krebs cycle conversion of glutamine (via α-ketoglutaramate, succinamate, etc.) to asparagine are either non-existent or quantitatively much less important than a direct conversion of aspartate to asparagine. These findings agree with those of Nelson and Krotkov (1956) that the pattern of labeling in broad bean leaves exposed to $C^{14}O_2$ is the same both in glutamate and glutamine and in aspartate and asparagine.

Increasing evidence that aspartate itself is a precursor of asparagine (in at least some organisms) raises a question as to the mechanism of the aspartate-asparagine conversion. The most likely mechanism for the conversion of aspartate to asparagine would seem to be either the direct amidation of aspartate with ammonia or a transamidation to aspartate from some amide. Evidence has been presented (Webster and Varner, 1955) for the occurrence in wheat germ and lupine of an enzyme system catalyzing a direct amidation of aspartate with ammonia in the presence of ATP. In contrast, no evidence has been found for asparagine formation by transamidation from glutamine to aspartate in lupine or wheat germ (ibid.), although evidence exists (Levintow, 1957a, b) for the occurrence of these reactions in animal cells. At present, the conversion of aspartate to asparagine measured in this investigation seems most probably to proceed by the direct reaction of aspartate and ammonia.

SUMMARY

Incorporation of the carbons of various possible precursors into asparagine and into glutamine in lentil seedlings has been measured. Glutamate carbon is most readily incorporated into glutamine and aspartate carbon is most readily incorporated into asparagine. Partial degradation of the formed glutamine and asparagine shows that glutamate and aspartate undergo little metabolism prior to their incorporation into glutamine and asparagine respectively. The incorporation of aspartate into asparagine is inhibited by D-aspartate, but the incorporation of CO_2 into asparagine is unaffected.

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