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Utilization of the Amide Groups of Asparagine and 2-Hydroxysuccinamic Acid by Young Pea Leaves[1]

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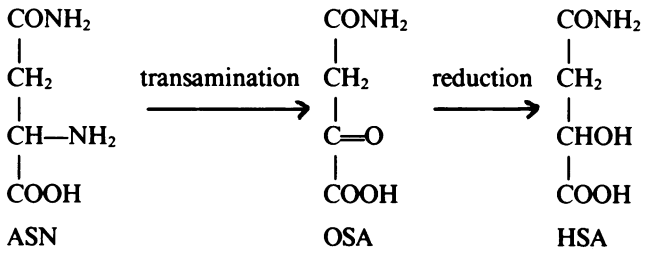
ABSTRACT

The fate of nitrogen originating from the amide group of asparagine in young pea leaves (*Pisum sativum*) has been studied by supplying [^{15}N-amide]asparagine and its metabolic product, 2-hydroxysuccinamate (HSA) via the transpiration stream. Amide nitrogen from asparagine accumulated predominantly in the amide group of glutamine and HSA, and to a lesser extent in glutamate and a range of other amino acids. Treatment with 5-diazo,4-oxo-L-norvaline (DONV) a deamidase inhibitor, caused a decrease in transfer of label to glutamine-amide. Virtually no ^{15}N was detected in HSA of leaves supplied with asparagine and the transaminase inhibitor aminooxyacetate. When [^{15}N]HSA was supplied to pea leaves, most of the label was also found in the amide group of glutamine and this transfer was blocked by the addition of methionine sulfoximine, which caused a large increase in NH_3 accumulation. DONV was not specific for asparaginase, and inhibited the deamidation of HSA, causing a decrease in transfer of ^{15}N into glutamine-amide, NH_3, and other amino acids. It is concluded from these results that use of the amide group of asparagine as a nitrogen source for young pea leaves involves deamidation of both asparagine and its transamination product HSA (possibly also oxosuccinamate). The amide group, released as ammonia, is then reassimilated via the glutamine synthetase/glutamate synthase system.

In many leguminous plants most of the N required for vegetative growth and seed formation is supplied in the form of asparagine (8, 12). Both ^{15}N labeling (14) and *in vitro* enzyme work (5, 13) suggest that asparagine is metabolized by two distinct routes, deamidation and transamination.

Deamidation of asparagine by asparaginase yields ammonia, which may be subsequently re-assimilated by the GS[2]/GOGAT system (1, 14), and aspartate. The amino N of asparagine can be utilized by transamination, producing alanine, homoserine, or glycine (5–7, 14) and the oxo-analog of asparagine, OSA (13). Further metabolism of the amide N remaining in OSA, which does not accumulate, is not yet clear, but there is evidence that two pathways are involved. The activity of an enzyme which catalyzes the deamidation of OSA has been reported (13) and the product of OSA reduction, HSA, has been detected in pea leaves (9).



Experiments with [^{14}C]OSA and [^{14}C]HSA showed that OSA was converted to HSA and the carbon skeleton of HSA was metabolized primarily to amino acids (9). There is no information on the metabolism of HSA amide N.

This report describes the fate of ^{15}N originating from the amide groups of asparagine and HSA when these compounds are fed to young pea shoots in the presence and absence of different metabolic inhibitors, and also shows that DONV, reported to be a specific inhibitor for asparaginase (4), has a broader effect on deamidation.

MATERIALS AND METHODS

Pea seedlings (*Pisum sativum* L. cv Little Marvel) were grown hydroponically and shoots removed as described previously (14). Detached shoots were fed through their cut ends via the transpiration stream. When inhibitors were supplied, these were prefed for 1 h prior to the supply of the ^{15}N compounds. Samples were taken, extracted and analyzed as described previously (14).

Enzyme Assays. Asparaginase and asparagine transaminase were extracted and assayed as described earlier (5, 6). Glutamine synthetase was extracted and assayed by the method of O'Neal and Joy (11).

Chemicals. [^{15}N-Amide]asparagine (99% atom excess) and [^{15}N-amide]HSA (99% atom excess) were obtained from Isotope Labelling Corp. (Whippany, NJ). DONV was synthesized by Dr. Ho H. Thoi, Department of Chemistry, Columbia University, NY, using the method modified by Handschumacher *et al.* (4). Other reagents and chemicals were obtained from Sigma.

RESULTS AND DISCUSSION

Effect of Inhibitors on Asparaginase and Asparagine Aminotransferase Activities. To determine the specific effects of DONV, an analog of asparagine, on the deamidation and transamination of asparagine, a series of experiments were done, both *in vivo* and *in vitro*. Asparaginase in young pea leaves was strongly inhibited by 20 mM DONV, both *in vivo* and *in vitro* (Table I). However, later results (see below) indicated that DONV had a broader inhibitory effect on deamidation, and was not specific for asparagine deamidation. DONV had no effect on asparagine transaminase or GS (Table I).

Thus the deamidase inhibitor DONV (20 mM) and 4 mM AOA

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[2] Abbreviations: GS, glutamine synthetase; HSA, 2-hydroxysuccinamate; OSA, 2-oxo-succinamate; AOA, aminooxyacetate; MSO, methionine sulfoximine; DONV, 5-diazo,4-oxo-L-norvaline; GOGAT, glutamate synthase.

Table I. *Effect of DONV on Asparaginase, Asparagine Aminotransferase, and Glutamine Synthetase Activities (Expressed as Per Cent Inhibition Relative to Water Control)*

	Inhibition at Following DONV Concentration (mM)					Control Activity
	1	5	10	15	20	
			%			
Asparaginase						
In vitro[a]	23	70	71	86	99	34.5[d]
In vivo[b]	ND[c]	ND	58	70	81	33.1[d]
Asparagine aminotransferase						
In vitro	ND	ND	4	5	8	35.2[d]
In vivo	ND	ND	2	2	3	34.4[d]
Glutamine synthetase						
In vitro	ND	ND	4	4	5	0.83[e]
In vivo	ND	ND	2	2	3	0.82[e]

[a] The enzyme was first extracted from leaves and the inhibitor (or water) was added to the assay solutions.
[b] The leaves were supplied with the inhibitor (or water) for 1 h via the transpiration stream, then the enzyme was extracted and assayed.
[c] ND, not determined.
[d] nmoles ASN consumed·min^{-1}·g^{-1} fresh wt.
[e] μmol glutamyl hydroxamate produced·min^{-1}·g^{-1} fresh wt.

(an effective inhibitor of asparagine transamination [6]) were used in feeding experiments to investigate the utilization of asparagine.

Utilization of Asparagine Amide Nitrogen. ^{15}N[Amide]asparagine was supplied to detached pea shoots, with and without inhibitors. During the experimental period, amino acid levels were relatively constant, and for feeding of asparagine only, or asparagine plus AOA, the values were similar to those reported earlier (14). For asparagine plus DONV, after 60 min there was a decrease in levels of glutamine (by about 50%) and aspartate (by about 30%), while there was a small (about 20%) increase in glutamate. The recovery of total ^{15}N indicated that the uptake of asparagine was decreased by approximately 20% in the presence of DONV, and by less than 5% with AOA (Table II).

During feeding there was a linear increase in accumulation of ^{15}N from the amide group of asparagine in a range of metabolic products (Table II). The results show that most of the ^{15}N was incorporated into the amide group of glutamine. This transfer to glutamine was substantially inhibited by DONV, as was the transfer to the other amino compounds and ammonia. These results support our previous conclusion (14) that some amide N of asparagine is liberated as ammonia by direct deamidation, followed by reassimilation through GS.

The addition of AOA caused a less pronounced inhibition of ^{15}N flow to glutamine (and some other amino compounds) than DONV, but unlike DONV it was very effective in preventing transfer to HSA (Table II). The fact that AOA caused a 30% decrease in transfer of label to glutamine and a 40% decrease in transfer to ammonia suggests that at least 30 to 40% of the asparagine is transaminated prior to deamidation. The increase in HSA labeling seen when DONV was fed with asparagine could be due to inhibition of HSA deamidation, causing label to accumulate in this compound (see below).

Utilization of HSA Amide Nitrogen. When [^{15}N-amide]HSA was supplied to detached pea leaves there was again a linear increase in total label in the leaf, and in the flow of ^{15}N into a range of metabolites (Table III). MSO had little effect on HSA uptake, but DONV again caused about 20% decrease in uptake.

Table II. *Flow of ^{15}N from [^{15}N-amide]Asparagine (99% atom excess) Supplied to Expanding Pea Leaves, in the Presence and Absence of 20 mM DONV or 4 mM AOA*

Inhibitors were supplied for 1 h before addition of labeled asparagine.

	^{15}N Content								
	ASN (min)			ASN + DONV (min)			ASN + AOA (min)		
	15	30	60	15	30	60	15	30	60
					ng $^{15}N·g^{-1}$ fresh wt				
Asn-Amide	2462	5582	11199	2203	4741	10083	2530	5676	11981
Gln-Amide	221	476	999 (100)[a]	33	72	145 (18)	165	304	688 (68)
Gln-Amino	23	46	105 (100)	2	5	13 (16)	18	34	68 (65)
Glu	102	168	427 (100)	24	54	119 (35)	55	122	293 (68)
Asp	31	76	158 (100)	9	21	41 (33)	14	30	62 (39)
NH_3	14	33	75 (100)	4	9	16 (27)	10	21	44 (58)
HSA	141	328	647 (100)	129	265	604 (118)	17	36	66 (10)
Other amino nitrogen	117	267	514 (100)	55	109	202 (49)	64	135	262 (53)
Total	3111	6976	14124	2459	5276	11223	2873	6358	13464

[a] Values in parentheses are percentage of uninhibited control values at 60 min, corrected for differences in total ^{15}N content.

Table III. *Flow of ^{15}N from [^{15}N-amide]hydroxysuccinamic Acid (99% atom excess) Supplied to Expanding Pea Leaves, in the Presence and Absence of 1 mM MSO or 20 mM DONV*

Inhibitors supplied 1 h before addition of labeled HSA.

	^{15}N Content								
	HSA only (min)			HSA + MSO (min)			HSA + DONV (min)		
	15	30	60	15	30	60	15	30	60
	ng $^{15}N \cdot g^{-1}$ fresh wt								
HSA	4403	8671	16562	4396	8836	16534	3692	7516	14453
Gln-amide	295	632	1308 (100)[a]	30	63	132 (10)	41	90	202 (20)
Gln-amino	21	44	96 (100)	2	5	12 (13)	2	5	9 (13)
Glu	98	181	397 (100)	16	51	115 (30)	12	29	45 (15)
Asp	51	121	253 (100)	7	20	44 (18)	6	16	24 (18)
NH_3	32	71	148 (100)	214	594	1712 (1156)	6	16	28 (24)
Other amino nitrogen	56	204	412 (100)	19	48	101 (25)	23	55	113 (35)

[a] Values in parentheses are percentages of uninhibited control values at 60 min, corrected for differences in total ^{15}N content.

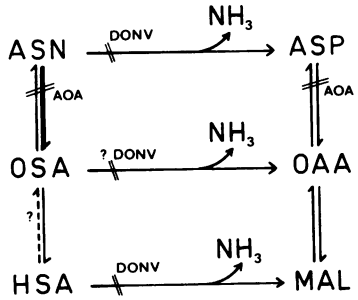

FIG. 1. Pathways of asparagine metabolism in developing *Pisum sativum* shoots. The ammonia is reassimilated, predominantly by glutamine synthesis (unless inhibited by methionine sulfoximine). OSA, 2-oxo-succinamate; HSA, 2-hydroxysuccinamate; OAA, oxalacetate; MAL, malate.

The major transfer of label by metabolism was to the amide N of glutamine. Lesser amounts were found in glutamate, aspartate, ammonia, and the amino N of glutamine. The addition of MSO to this system caused a drastic reduction of the label transferred to glutamine and the other amino compounds, and a massive accumulation in ammonia, consistent with its inhibitory effect on GS (Table III). This accumulation of ammonia supports our hypothesis that most of the HSA derived from asparagine is further metabolized by deamidation. DONV reduced the transfer of ^{15}N to all compounds analyzed, including ammonia, by about 80%. This appears to be due to an inhibition of HSA deamidation, indicating that DONV has a more general inhibitory effect on deamidation rather than the specific effect on asparaginase previously reported (4). Transfer of ^{15}N from HSA to glutamate and aspartate is probably via glutamine and the actions of GOGAT and transaminases: the inhibition of this transfer by MSO (Table III) confirms the role of glutamine as an intermediate in this flow. Transfer to glutamate was least affected by MSO, and this could be due to some assimilation of ammonia by glutamate dehydrogenase, which is not affected by MSO (3).

Transfer of ^{15}N to the total pool of other soluble amino compounds (excluding those reported individually in Table III) was also detected, although the labeling in each was very low when these amino acids were analyzed (data not shown). The amino nitrogen of these compounds is probably not derived directly from HSA, but via transamination of glutamate or aspartate.

CONCLUSIONS

By combining these and our earlier ^{15}N feeding studies (2, 14) with our *in vitro* enzyme work (5–7) we are able to propose the scheme shown in Figure 1. Asparagine arriving in the transpiration stream can be directly deamidated by asparaginase in the cytosol or transaminated in the peroxisome (7). The OSA does not accumulate but is immediately reduced to HSA (9), and may also be deamidated to oxalacetate (13); we have not yet isolated the enzyme system responsible for OSA reduction. HSA accumulates, and is metabolized by release of the amide group as ammonia. Direct deamidation of HSA would presumably give malate; however, preliminary estimates of HSA deamidation in crude extracts from pea leaves give low activities. The results do not rule out the possibility that metabolism of HSA could involve reconversion to OSA and deamidation of the latter, although ^{14}C labeling experiments suggest a strong equilibrium toward HSA formation (9).

Transamination of asparagine occurs with glyoxylate or pyruvate (6), transferring the amino N to glycine or alanine. Thus, taking into account the action of both asparaginase and asparagine transaminase, the amino group of asparagine can be transferred to alanine, glycine, or aspartate, three central amino acids which can be further metabolized to a range of amino and other compounds. The amide N, on the other hand, is transferred to the amide position of glutamine via deamidation of asparagine itself, OSA, or HSA, and the subsequent action of GS. The activity of GOGAT transfers this N to glutamate, which is also in a central position in N metabolism (10). These pathways provide an adaptable system for the utilization of both nitrogenous groups of asparagine, a major source of nitrogen for the developing pea leaves.

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