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2-HYDROXYSUCCINAMIC ACID: A PRODUCT OF ASPARAGINE METABOLISM IN PLANTS

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SUMMARY: When [^{14}C]-asparagine was supplied to growing pea leaves aspartate and other compounds were formed, but after 4 hours more than half of the metabolised carbon skeleton was present as one compound, identified as 2-hydroxysuccinamate. This compound was also formed, with a high rate of conversion, when 2-ketosuccinamate (the product of transamination of asparagine) was supplied to the leaves. There was some synthesis of amino acids from 2-hydroxysuccinamate, and in the dark it was metabolised to release some carbon dioxide. Accumulation and metabolism of 2-hydroxysuccinamate has also been observed in soybean leaves. It is suggested that 2-hydroxysuccinamate is a major intermediate in the metabolism of the carbon skeleton of asparagine following transamination, an important route for asparagine utilisation.

Asparagine is a major tissue component in a number of plants, and appears to be a common storage and transport form of nitrogen. The relative importance of pathways of utilisation of this compound has not been clearly resolved (1). Asparaginase has been considered to be an important system, and appears to operate in some tissues, such as developing lupin seeds (2). However, transamination provides an alternate route for utilisation, and Streeter (3) was able to detect asparagine transaminase activity in soybean extracts. In vivo ^{15}N labelling studies (4) suggest that transamination plays an important role in the distribution of nitrogen from asparagine in growing pea leaves.

The fate of the carbon skeleton of asparagine is not clear. ^{14}C from labelled asparagine has been found in a "non amino" fraction (2) and in malate (3). Transamination of asparagine yields 2-ketosuccinamate (KSA), but Streeter (3) was not able to detect labelled KSA, or its deamidation product oxalacetate, in soybean leaves which had been supplied with [^{14}C]-asparagine. In this paper we show that in leaves of peas and soybeans carbon from asparagine or KSA

Abbreviations: HSA = 2-hydroxysuccinamate
KSA = 2-ketosuccinamate
TMS = trimethylsilyl

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accumulates as 2-hydroxysuccinamate, and this appears to be the first report of the presence of this compound in plant tissues.

METHODS

Pea plants (Pisum sativum L. cv Little Marvel) were grown as described previously (5). Shoot tips consisted of the apex and half expanded fifth leaf and labelled materials were supplied through the cut end of the stem, in a growth chamber. Soybeans (Glycine max, cv Neepewa) were grown in soil, and shoot tips consisted of apex and expanding first trifoliate leaf. Carbon dioxide, collected during dark incubation, was absorbed in monoethanolamine solution. At the end of each incubation period, a sample of three shoot tips was ground in liquid nitrogen and extracted with 80% ethanol. The ethanol insoluble fraction was re-extracted several times. Pigments were removed from extracts by partition against chloroform, or by absorption onto Celite.

Dowex 50 (H^+) and Dowex 1 (formate) columns were used to separate extracts into amino acid, acid and neutral fractions. Amino acids were eluted from Dowex 50 with 2N ammonium hydroxide to avoid acid hydrolysis of amides. Organic acids were eluted from Dowex 1 with 2N and 4N formic acid. In some cases the Dowex 1 step was omitted. Fractions were further separated by ascending paper chromatography (Whatman 3MM paper) in 1-butanol:acetic acid:water (12:3:5) or isopropanol:ammonia:water (8:1:1), or by electrophoresis on Whatman 3MM paper (1800v for 1 hour, in 0.5M acetate buffer, pH 4.1). It was necessary to adjust the column effluents to pH 9-9.5 before chromatography with butanol-acetic acid-water to prevent the variable hydrolysis of HSA to malate. TMS derivatives of some organic acids were separated by gas chromatography on an OV17 column.

Preparation of KSA. KSA was prepared from asparagine (labelled with L-[U-^{14}C] asparagine) using L-amino acid oxidase (Type I, Sigma Chemical Co.) in a scaled down modification of the method of Meister (6). KSA was used directly after Dowex 50 treatment, and was made alkaline to above pH 11, to convert the KSA to the monomer form (7), then readjusted to pH 7.5.

Preparation of HSA. Sodium borohydride solution was added in equimolar proportion to KSA in the monomer form, and the mixture was incubated for 30 min at room temperature. The solution was adjusted to pH 2 with hydrochloric acid to decompose residual borohydride. After neutralisation, HSA was absorbed on Dowex 1 (formate) and eluted in 2N formic acid.

Radioactivity was estimated by liquid scintillation counting, and was detected on paper by use of a gas flow radiochromatogram scanner.

All asparagine solutions (labelled and unlabelled) were passed through Dowex 1 (chloride) before use to remove any contaminating aspartic acid.

IDENTIFICATION OF 2-HYDROXYSUCCINAMATE IN LEAVES

When [^{14}C]-asparagine was supplied to pea shoots to follow the fate of the asparagine carbon skeleton, a substantial amount of an unknown compound was formed. Accumulated samples of the unknown did not react with ninhydrin, but showed the presence of an amide group with Rydon-Smith procedure (7). Initially it was thought that the compound was KSA, formed by transamination of asparagine. However, no dinitrophenylhydrazone derivative could be produced, and the compound could be separated chromatographically from authentic KSA.

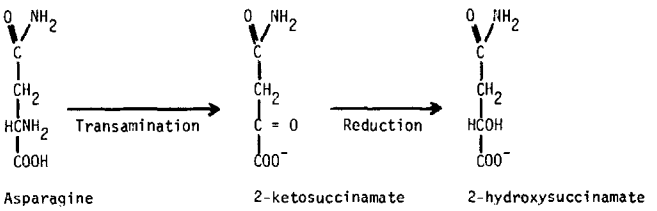

Figure 1. Production of HSA from asparagine

Table 1. SEPARATION OF 2-HYDROXYSUCCINAMATE AND SOME RELATED SUBSTANCES. Results are given for paper chromatography (Butanol-acetic acid-water) and high voltage paper electrophoresis. See Methods section for details.

	R_F(B.A.W.)	$*R_p$(Electrophoresis)
HSA	32	110
KSA(monomer)	25	140
KSA(dimer)	8	160
Malate	50	122
Citrate	39	120
Asp	20	87
Glu	26	52
Asn	16	Small displacement from origin towards cathode

$*R_p = \frac{\text{Movement of compound x100}}{\text{Movement of picric acid marker}}$

2-Hydroxysuccinamate (structure shown in Fig. 1) was synthesised by borohydride reduction of KSA which should specifically reduce the keto group to a hydroxyl group. The identity was confirmed by acid hydrolysis of the HSA when the amide group was lost and malate was produced (identified by co-chromatography in two systems, and coincidence of the peaks of the TMS-derivatives on gas chromatography). The unknown compound from pea leaves also produced malate on acid hydrolysis, and ran identically on chromatography in two solvent systems (Butanol-acetic acid-water and isopropanol-ammonia-water) and also on high voltage paper electrophoresis. Properties of HSA and some related compounds in two useful separation systems are shown in Table 1.

Table 2. METABOLISM OF [^{14}C]ASPARAGINE BY GROWING PEA LEAVES.
Zero time samples were taken after young pea shoots had been supplied with labelled solution (1 mM Asn) for 30 min in light. Incubation continued in light or dark, with a supply of unlabelled asparagine.

		Incubation conditions: Light		Dark
*Recovery in:	0 min	90 min	210 min	210 min
Asn	79.7%	60.0	25.2	55.5
CO_2	ND	ND	ND	8.7
Other compounds	20.3	40.0	74.8	35.8
**Distribution of activity in other compounds:				
HSA	41.8%	47.1	57.1	48.6
Amino acids	48.6	29.4	13.0	30.7
Neutral cpds	1.5	3.6	2.2	2.0
Insolubles	8.1	19.8	27.6	18.6

* Expressed as percentage of total radioactivity recovered
** Expressed as percentage of radioactivity in ethanol insoluble fraction plus soluble material other than Asn
ND - Not determined

No suitably sensitive reagent for detection of HSA has been found, and no accurate estimates of natural concentrations of HSA have been determined. Identification has largely relied on detection of radioactive peaks using ^{14}C-labelled unknown and authentic compounds. Sufficient hydroxysuccinamate was obtained from untreated soybean leaves to form a TMS-derivative, which produced a peak on gas chromatography that coincided with the derivative of authentic HSA. Calculations from this data suggest, very approximately, that the natural concentration in soybean leaves could be in the region of 1 mg/g fresh material. The instability of the TMS-derivative has not yet permitted use of gas chromatography as an accurate quantitative method.

PRODUCTS OF ASPARAGINE UTILISATION

Table 2 shows the metabolism of asparagine by young pea leaves in light and dark, following a 30 minute feed with [^{14}C]-asparagine in light. There was a progressive utilisation of asparagine during incubation in the light,

Table 3. METABOLISM OF 2-KETOSUCCINAMATE BY GROWING PEA LEAVES. Distribution of radioactivity after young pea shoots had been supplied continuously with [^{14}C]-KSA (0.5 mM) for 2 hours in the light. Distribution expressed as percentage of total radioactivity recovered.

KSA	13.9%
HSA	57.4
Amino acids	20.8
Insolubles	7.9

and an increasing proportion of the carbon from asparagine accumulated as HSA. Amino acids were highly labelled at first, particularly aspartic acid, and with lesser amounts of label in glutamic acid, homoserine and alanine; there was a continuing incorporation of label into the ethanol insoluble fraction. No attempt was made to collect CO_2 during light incubation, as other experiments had shown an insignificant release of CO_2 from asparagine in the light, and the small amount of label in sugars and sugar phosphates confirmed that there was little production and refixation of labelled CO_2. During dark incubation however there was a substantial production of labelled CO_2, about one-fifth of the metabolised carbon. Less asparagine was metabolised in the dark.

Similar results were obtained using soybean leaves in the light.

METABOLISM OF 2-KETOSUCCINAMATE

During a continuous 2 hour feed in light, 80% of the [^{14}C]-KSA entering young pea leaves was converted to other compounds, as shown in Table 3. Amino acids (mainly aspartic acid, glutamic acid and homoserine) became labelled, but two-thirds of the metabolised carbon was recovered as HSA, indicating an efficient conversion process.

METABOLISM OF 2-HYDROXYSUCCINAMATE

In contrast to KSA, HSA fed to pea leaves accumulated and was less readily metabolised (Table 4). There was some conversion to amino acids (again mainly aspartic acid) and a progressive incorporation into the insoluble fraction.

Table 4. METABOLISM OF 2-HYDROXYSUCCINAMATE BY GROWING PEA LEAVES. Zero time samples were taken after young pea shoots had been supplied with [^{14}C]-HSA (0.1 mM) for 30 min in light. Incubation then continued without HSA, in light or dark. Distribution expressed as percentage of total radioactivity recovered.

		Incubation conditions: Light		Dark
Recovered in:	0 min	90 min	210 min	210 min
HSA	86.2%	89.0	82.4	76.7
CO_2	ND	ND	ND	9.7
Amino acids	11.6	4.9	4.9	7.8
Insolubles	2.2	6.1	12.6	5.8

ND - Not determined

In the dark there was a slightly greater utilisation of HSA, and a considerable conversion to CO_2. It is possible that oxidation could follow deamidation of HSA to form malate. However, no labelled malate was found in extracts when precautions were taken to ensure that there was no breakdown of HSA during processing of ion-exchange effluents (see methods).

Soybean leaves also metabolised HSA, somewhat more actively than peas.

CONCLUSIONS

It is clear that metabolism of asparagine in leaves produces substantial amounts of HSA, a substance which until now does not seem to have been detected in plant tissue. This is not surprising, in view of the ready breakdown to malate during processing of organic acid fractions, and since it does not react (or the concentration is too low for detection) with common chromogenic reagents. It is reasonable to assume that HSA accumulates after transamination of asparagine (Fig. 1). KSA can be detected in cell free preparations as a product of transamination (3) but could not be detected in vivo; our feeding experiments show that the conversion of KSA to HSA is rapid. Deamidation (to

produce oxalacetate) could provide an alternate route for KSA metabolism. A KSA deamidase has been detected (3, 6) and in fact its presence in pea leaf preparations has complicated initial attempts to detect an enzyme capable of reducing KSA. However it is clear that in the leaf tissue, reduction to HSA provides the major pathway for KSA conversion, possibly in a compartment separate from the deamidase. Although HSA accumulates, it is also metabolised, particularly in the dark. Further experiments to investigate the production and utilisation of HSA are in progress.

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