Phytochemistry, 1974, Vol 13, pp 1407 to 1412 Pergamon Press Printed in England

ASPARAGINE BIOSYNTHESIS BY *ORYZA SATIVA* SEEDLINGS

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(*Received* 24 *September* 1973)

Key Word Index—*Oryza sativa*, Gramineae, rice, ammonium nutrition, asparagine, biosynthesis, L-aspartate

Abstract—L-Aspartate-[U-^{14}C] was quickly metabolized in rice seedlings into amino acids, organic acids and sugars On feeding simultaneously with ammonium for 2 hr, about 1% of the total soluble radioactivity was recovered as asparagine Major amino acids labelled were aspartate, glutamate, glutamine and alanine in both shoots and roots On the other hand, on feeding L-aspartate-[U-^{14}C] to rice seedlings precultured in an ammonium medium, asparagine accounted for 35% of the total soluble radioactivity in the roots Different labelling patterns in amino acids from those of non-precultured tissues were observed, and the main amino acids labelled in this case were asparagine and γ-aminobutyrate in the roots; glutamate, asparagine and glutamine in the shoots It was observed in the roots that this increase of asparagine labelling was associated with a decrease of label in glutamate

INTRODUCTION

DESPITE the interest in asparagine synthesis, neither the mechanism by which asparagine is synthesized in plants, nor its role in the biochemistry of the plant is understood.

Asparagine synthetases have now been described in bacteria,[1–4] and in animal tissues:[5–9] these differ from glutamine synthetase (E.C. 6.3.1.2) in that AMP and pyrophosphate are reaction products whereas ADP and orthophosphate are the products of the reaction catalysed by glutamine synthetase. The animal systems also differ from the bacterial systems in that glutamine is the preferred amide nitrogen donor, although the ammonium ion acts as a substrate *in vitro*.

No similar enzyme has been described in plants, although plants are known to synthesize large quantities of asparagine; etiolated lupin seedlings for example have been reported to accumulate as much as 25% of their dry weight in the form of asparagine,[10] and asparagine may account for 43% of the total free amino acids and amides in dormant seeds of wheat.[11]

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[9] HOROWITZ, B and MEISTER, A (1972) *J Biol Chem* **247,** 6708
[10] SCHULZE, E (1898) *Z Physiol Chem* **24,** 18
[11] WANG, D (1968) *Contrib Boyce Thompson Inst* **24,** 109

Although glutamine synthesis from glutamic acid, ammonia and ATP has been demonstrated in plant tissues,[12-14] there is no convincing evidence that asparagine synthesis proceeds by an analogous route. Some authors[15-17] have claimed that asparagine synthesis in plant tissues takes place by a mechanism analogous to the glutamine synthetase reaction, but even recent attempts to carry out such a reaction, or demonstrate conversion of aspartate to asparagine *in vitro*, have so far been unsuccessful [18,19] Recently, Streeter[20] and Rognes[21] have demonstrated an active asparagine synthetase which uses glutamine rather than ammonia as its nitrogen source

In 1963, Blumenthal Goldschmidt, Butler and Conn reported that cyanide was incorporated into asparagine in certain cyanogenic plants [22] Since then, numerous investigators have demonstrated this conversion in many plant species,[18 19 23-26] fungi and bacteria [27] Lupins also synthesize asparagine from exogenous $K^{14}CN$, but Level and Butler reported that aspartate is normally the more important precursor [28] Mitchell and Bidwell[29] suggested that [^{14}C]aspartate was not converted directly to asparagine in pea roots, but had to first enter the Krebs cycle, and that the carbon left the Krebs cycle for asparagine synthesis via a symmetrical 4-carbon acid, such as succinate

Our previous papers revealed an activation of glutamate dehydrogenase by ammonia treatment,[30] and some regulatory properties of glutamine synthetase in rice roots supplied with ammonia were studied [14] The present experiments were undertaken to examine the synthesis of asparagine from [^{14}C]aspartate associated with ammonia assimilation in rice plants

RESULTS AND DISCUSSION

L-Aspartate-[U-^{14}C] is extensively metabolized by rice seedlings (Table 1) Label from [^{14}C]aspartate was converted within 2 hr into organic acids (anionic fraction, 39%) and sugars (neutral fraction, 34%) in detached shoots, whereas in roots no significant label was observed in the sugar fraction (5·4%) and most of the radioactivity was incorporated into the amino (cationic fraction, 55%) and organic acid fraction (40%) When [^{14}C]aspartate was fed to rice seedlings together with ammonia, radioactivity was increased in the amino acid fraction, both in shoots and in roots, whilst activity in the organic acid and the sugar fractions decreased

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[17] NAIR, P M (1969) *Arch Biochem Biophys* **133**, 208
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[19] TING I P and ZSCHOCHE, W C (1970) *Plant Physiol* **45**, 429
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[29] MITCHELL, D J and BIDWELL, R G S (1970) *Can J Botany* **48**, 2001
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Subsequently, the radioactive compounds in the eluate from the Amberlite IR-120 (H^+ form) were separated by 2-D PC by the systems described in the Experimental, and were located by radioautography. Radioactive areas were cut out from the paper chromatograms, and the radioactivities were determined. The results are presented in Table 1 (lower part). Chromatographic analysis of the amino acid fraction revealed that aspartate, glutamate, glutamine and alanine were major amino acids labelled with ^{14}C. Only a small proportion of the radioactivity from aspartate was recovered as asparagine (0·5% in shoots and 2·5% in roots), consistent with the results obtained by Naylor *et al.*[31]

TABLE 1 EFFECT OF AMMONIA ON THE METABOLISM OF ASPARTATE-[U-^{14}C] IN *Oryza sativa* SEEDLINGS

Fraction	Detached shoots		Roots	
	Control (cpm)	Ammonia (cpm)	Control (cpm)	Ammonia (cpm)
Ethanol soluble	307700	329400	173700	286900
Neutral	105800	96200	9500	6000
	(34 4)*	(29 2)	(5 4)	(2 1)
Anionic	121200	88300	69400	106200
	(39 4)	(26 8)	(40 0)	(37 0)
Cationic	80600	144900	94800	174700
	(26 2)	(44 0)	(54 6)	(60 9)
Compounds analysed		% Of total metabolized ^{14}C		
Aspartic acid	27 5 (7 2)*	10 5 (4 6)	20 5 (11 2)	8 2 (5 0)
Glutamic acid†	44 1 (11 6)	43 3 (19·1)	37 3 (20 4)	25 6 (15 6)
Serine, Glycine	3 4 (0 9)	2 2 (1 0)	0 8 (0 4)	0 3 (0 2)
Asparagine	0 5 (0 1)	1 0 (0 4)	2 5 (1 4)	2 0 (1 2)
Glutamine	5 0 (1 3)	32 2 (14 2)	33·0 (18 0)	59 6 (36 3)
Alanine	17 4 (4 6)	7 2 (3 2)	3 4 1 9)	2 1 (1·3)
γ-Aminobutyrate	1 4 (0 4)	0 9 (0 4)	1 9 (1 0)	1 7 (1 0)
Others	0 7 (0 2)	2 8 (1 2)	0 7 (0 4)	0 5 (0 3)

L-Aspartate-[U-^{14}C] (5 μCi, 227 mCi/mmol) was supplied to rice seedlings via roots or stems (in the case of detached shoots) together with or without ammonium (7 2 mM NH_4Cl) for 2 hr The tissues were then rinsed, killed with 80% EtOH and then processed as described in the Experimental

* Numbers in parentheses represent % of the total EtOH-soluble ^{14}C

† Includes pyrrolidone carboxylic acid

In both shoots and roots, the ammonia treatment (only for 2 hr) accelerated the disappearance of label from the supplied aspartate and increased the labelling of glutamine. This glutamine accumulation after ammonia treatment suggested the operation of the enzyme glutamine synthetase, which has been reported from rice plant seedlings.[14] There was, however, only a minor effect of ammonia treatment on asparagine labelling. In these experiments in which it was expected that asparagine synthesis would be favoured, more label from aspartate appeared in glutamine than in asparagine, and asparagine labelling was negligible in spite of ammonia supply, suggesting that asparagine is not a primary product in ammonia assimilation as indicated by Oji *et al.*[32]

Maxwell *et al.*[33] reported that wheat shoots supplied with $^{14}CO_2$ during a light period, then placed in darkness, accumulated label in asparagine and the label in sugars was reduced. They suggested that sugars produced in the dark period were preferentially converted to asparagine, mainly as a storage compound in the dark. Our preliminary experiments in which rice seedlings grown on an ammonium medium or nitrogen(*N*)-deficient

[31] NAYLOR, A W, RABSON, R and TOLBERT, N E (1958) *Physiol Plant* **11**, 537

[32] OJI, Y and IZAWA, G (1973) *Plant Cell Physiol* **14**, 139

[33] MAXWELL, M A B and BIDWELL, R G S (1970) *Can J Botany* **48**, 923

medium were supplied with $^{14}CO_2$ for 2 hr in light showed that little radioactivity was present in asparagine in the ammonia-cultured shoots, and none in the roots (unpublished data)

Experiments were conducted to determine if the culture of rice seedlings with ammonium affects the accumulation of asparagine Rice seedlings were grown for 5 days in the ammonium medium or N-deficient medium (as a control) as previously reported [30] L-Aspartate-[U-^{14}C] was administered to plants from both treatments via the roots for 2 hr The results of this experiment are shown in Table 2 The L-Aspartate-[U-^{14}C] was

TABLE 2. EFFECT OF AMMONIA ON THE METABOLISM OF ASPARTATE-[U-^{14}C] IN RICE SEEDLINGS PRECULTURED WITH OR WITHOUT AMMONIUM MEDIUM FOR 5 DAYS

Fraction	Shoots		Roots	
	Control (cpm)	Ammonia (cpm)	Control (cpm)	Ammonia (cpm)
Ethanol soluble	101700	203100	96800	97800
Neutral	36700	5900	18600	6800
	(36 1)*	(2 9)	(19 2)	(7 0)
Anionic	39800	25200	47800	8800
	(39 1)	(12 4)	(49 4)	(9 1)
Cationic	25200	172000	30400	82100
	(24 8)	(84 7)	(31 4)	(83 9)
Compounds analysed	% Of total metabolized ^{14}C			
Aspartic acid	19 3 (4 8)*	6 9 (5 8)	25 6 (8 0)	5 9 (5 0)
Glutamic acid†	45 4 (11 3)	38 4 (32 5)	48 7 (15 3)	8 8 (7 4)
Serine, Glycine	7 4 (1 8)	3 2 (2 7)	1 3 (0 4)	0 9 (0 8)
Asparagine	1 4 (0 3)	21 9 (18 5)	4 5 (1 4)	42 0 (35 2)
Glutamine	2 0 (0 5)	17 8 (15 1)	3 8 (1 2)	1 3 (1 1)
Alanine	8 4 (2 1)	3 4 (2 9)	1 7 (0 5)	0 9 (0 8)
γ-Aminobutyrate	11 2 (2 8)	5 1 (4 3)	11 2 (3 5)	38 1 (32 0)
Others	4 9 (1 2)	3 4 (2 9)	3 1 (1 0)	2 1 (1 8)

Rice seedlings, precultured for 5 days with ammonium medium (14 3 mM NH_4Cl) or N_2-deficient medium (as a control) were treated as in Table 1, * and † are identical to Table 1

again rapidly metabolized. By comparison with the controls, in the ammonium-cultured seedlings, a considerable proportion of the radioactivity of the ethanol soluble fraction remained in amino acid fractions. Moreover, label from [^{14}C]aspartate was converted to a lesser extent into the organic acids (anionic fraction), and no significant label was observed in the sugar (neutral fractions) in the ammonium-cultured seedlings.

Subsequent chromatographic analysis of the amino acid fraction revealed that major products labelled with ^{14}C were glutamate, asparagine and glutamine in ammonium-cultured shoots, asparagine and γ-aminobutyrate in ammonium-cultured roots. The preculture with ammonium medium significantly accelerated the accumulation of labelled asparagine from the supplied [^{14}C]aspartate in both shoots and roots (Table 2, lower part) Glutamate labelling was strongly reduced in the roots. Label converted to asparagine in roots accounted for 35% of the total soluble radioactivity. This value is higher than the result obtained in corn roots using K^{14}CN as a precursor.[25] Oaks *et al.*[25] concluded that the cyanide pathway was potentially present, but was probably not normally active in corn root tips, indicating that cyanide was probably not readily available endogenously

The marked increase in asparagine labelling and substantial decreases in glutamate and glutamine labelling in rice plant roots following pre-incubation with ammonium were

suggestive of the operation of glutamine-dependent asparagine synthetase demonstrated in soybean[20] and yellow lupin seedlings.[21] The decreases in glutamate and glutamine labelling, however, might be also attributable to the lesser incorporation of [^{14}C]aspartate into them, caused by the dilution of label with cold aspartic acid Analysis of free pool amino acids indeed showed that rice plant seedlings were evidently capable of making substantial amounts of aspartate, glutamate, asparagine and glutamine when supplied with ammonia. The enhanced accumulation of asparagine associated with ammonia assimilation might suggest that the important role of asparagine synthesis is in the detoxification of the ammonia excessively present; consistent with the suggestion made by Lees *et al.*[18]

The conversion of [^{14}C]aspartate to [^{14}C]glutamate and [^{14}C]glutamine has been interpreted by Oji *et al.*[32] Aspartate-[4-^{14}C] seems to be deaminated first to C-4 labelled oxalacetate by transaminase, and transformed into glutamate-[1-^{14}C] via the tricarboxylic acid cycle, with glutamate being converted to glutamine-[1-^{14}C], in barley plants. A similar metabolic conversion might be involved in rice seedlings.

The striking accumulation of γ-aminobutyrate in labelling from [^{14}C]aspartate was also observed in rice plant roots cultured in the ammonium medium (Table 2, lower part). Streeter *et al.*[34] reported the remarkable accumulation of γ-aminobutyrate in radish leaves exposed to anaerobic conditions, and ascribed this to the acceleration of glutamate decarboxylation and arrest of γ-aminobutyrate transamination. Glutamate decarboxylase may have contributed significantly to the increase of γ-aminobutyrate labelling followed by glutamate loss in the rice plant roots.

For asparagine biosynthesis, two major pathways may be found; the conventional aspartate pathway[15–17] and the cyanide pathway.[18,19,22–26] Oaks *et al.*[25] suggested that the aspartate pathway was the principal pathway in corn roots because the synthesis of cyanide and cysteine have been shown to be rate limiting. More recently, experiments using cycloheximide or azaserine, as potent inhibitors, showed that a glutamine-dependent asparagine synthetase might be active in maize root tips and altered in mature root sections in some way such that it no longer recognizes the inhibitor.[35]

Our results also suggest that the supplied aspartate is substantially converted to asparagine in rice seedlings cultured with the ammonium medium, but it is necessary at the moment to keep an open mind concerning the major precursor of asparagine in rice plants, until the asparagine forming system is demonstrated *in vitro*.

EXPERIMENTAL

Plant material Rice seedlings (*Oryza sativa* cv Kyoto Asahi) grown for about 3 weeks in tap H_2O were used as reported previously[30] Treatment with ammonia and cultural conditions were identical to the method used previously except as otherwise noted

Feeding experiments The feeding experiments were carried out at 30° under natural light in a greenhouse by immersing intact roots (50 seedlings) in 10 ml medium containing 5 μCi of L-aspartate-[U-^{14}C] (227 mCi/mmol) with or without ammonium (as NH_4Cl) In the case of detached shoots, cut ends of stems (40 seedlings) were placed in 2 ml medium containing the radioactive compound with or without ammonia After the 2-hr uptake period, the seedlings were quickly washed with running tap and dist H_2O, divided into shoots and roots, and extracted as follows, respectively

Extraction and fractionation of EtOH-soluble materials After sampling, the tissues were cut into small pieces, and ground with 80% EtOH in a mortar repeatedly The EtOH-soluble fraction was separated into three fractions, namely cationic (mainly amino acids), anionic (mainly organic acids) and neutral (mainly sugars), by use of ion-exchange resins. EtOH-soluble materials were first passed through a column of the sulphonic resin Amberlite IR-120 (H^+ form) The column was washed well with H_2O, then the effluent was passed through a column

[34] STREETER, J G. and THOMPSON, J F (1972) *Plant Physiol* **49**, 572
[35] OAKS, A and JOHNSON, F J (1973) *Can J Botany* **51**, 91

of the weakly basic resin Amberlite IR-45 (OH^- form) The effluent from this column contained the sugars Amino acids were eluted from the Amberlite IR-120 resin with 2 M NH_4OH The effluent was evaporated below 40°, dissolved in 6 ml of dist H_2O, and finally reduced to 0 2 ml in a desiccator Organic acids were eluted from the Amberlite IR-45 resin with 2 M $(NH_4)_2CO_3$ A portion of each fraction was used to measure radioactivity in a Kobe Kogyo Corp type PC-26 gas-flow counter Further fractionations of organic acids and sugar fractions were not done

PC and radioautography of ^{14}C-labelled amino acids The 2 M NH_4OH eluates from the Amberlite IR-120 (H^+ form) resin were subjected to 2-D radioautography to characterize the ^{14}C-labelled amino acids and to assay their radioactivities PC was carried out by ascending development on filter paper (Toyo No 51A), using the following solvent system PhOH H_2O (4 1) followed by *n*-BuOH–HOAc–H_2O (4 1 1) Appropriate amino acids were co-chromatographed on each sheet as authentic materials to identify the ^{14}C-labelled amino acids Radioactive areas on the paper chromatogram were located by radioautography by using Fuji X-ray film (Type KX) Amino acids were detected by their reaction with ninhydrin reagents For measurement of radioactivity of the ^{14}C-labelled compounds, radioactive areas were cut out from the paper chromatograms, and placed in vials containing 10 ml of a toluene-based scintillator solution This solution contained 4 g 2,5-diphenyloxazole (PPO) and 0 1 g *p*-bis-2-(5-phenyloxazolyl)-benzene (POPOP)/l toluene Measurements were made in a Beckman LS-100 liquid scintillation counter

Acknowledgement— The authors thank Professor E Takahashi for his encouragement during the course of this work