Plant Physiol. (1982) 69, 553–556
0032-0889/82/69/0553/04/$00.50/0

Niacin Biosynthesis in Seedlings of *Zea mays*[1]

Received for publication February 20, 1981 and in revised form October 14, 1981

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ABSTRACT

Evidence obtained from incubation of corn (*Zea mays* cv. Golden Bantam) seedlings in DL-[benzene ring-U-^{14}C]tryptophan, L-[5-^{3}H]tryptophan, L-[U-^{14}C]aspartate and [U-^{14}C]glycerol indicates that niacin is synthesized in these plants via oxidative degradation of tryptophan. Aspartate and glycerol do not appear to be precursors of niacin in corn seedlings.

Niacin is synthesized in chicks, certain bacteria, some fungi, and mammals by the catabolism of tryptophan via kynurenine, 3-hydroxyanthranilic acid, and quinolinic acid. In *Escherichia coli* and *Mycobacterium tuberculosis*, this vitamin is synthesized from glyceraldehyde-3-P and aspartate. The pathway(s) of niacin biosynthesis in angiosperms are uncertain (for a review see 1). Findings with unlabeled substrates, suggesting that niacin is synthesized from tryptophan, are inconclusive. Several investigations with labeled tryptophan or intermediates of its catabolism to niacin have also produced uncertain results due to the experimental procedures used. Convincing evidence for the existence of the glyceraldehyde-aspartate pathway in flowering plants also is lacking (1). The studies reported here were undertaken for the purpose of obtaining firmer evidence regarding the pathway(s) of niacin biosynthesis in corn.

MATERIALS AND METHODS

Plant Material. Seeds of *Zea mays* cv. Golden Bantam (Burpee Seed Co.) were surface-sterilized by immersion in 5% (v/v) Clorox for 30 min. They were germinated under dim light and 18-h photoperiods on sterile washed vermiculite and irrigated with the mineral components of Knudson C medium (8). Four-d-old epicotyls were removed under aseptic conditions, cut into 1-cm sections, and incubated in precursor solutions.

Incubation. A total of eight sections (four epicotyl tips and four bases, combined weight 587–687 mg) were incubated in stoppered flasks containing 0.5 μmol filter sterilized precursor in 2-ml sterile 0.067 M K-phosphate (pH 7.0). Precursors were: DL-[benzene ring-U-^{14}C]tryptophan, 5 μCi; L-[5-^{3}H]tryptophan, 100 μCi; L-[U-^{14}C] aspartate, 25 μCi; or [U-^{14}C]glycerol, 10 μCi. All were Amersham products. Experiments with all compounds except L-[5-^{3}H]tryptophan, were replicated three times.

The sections were incubated for 24 and 48 h on a reciprocal shaker (60 oscillations/min) under 18-h photoperiods provided by two 40 w Sylvania Gro-Lux lamps (F40-GRO) at a temperature of 22°C (±2°C). Respiratory CO_2 was trapped in 2 N KOH contained in vials suspended above the incubation mixture.

Extraction. Following incubation, the tissues were washed with distilled H_2O, homogenized, and extracted four times with methanol:chloroform:water (12:5:3, v/v/v) to extract free niacin into the alcohol-aqueous phase (2). To release bound niacin, an additional 100 to 200 mg tissue were homogenized and autoclaved 30 min in 1.8 ml of 1 N H_2SO_4 (4). The autoclaved mixture was neutralized with 0.41 N $Ba(OH)_2$, centrifuged, and the pellet was extracted four times with methanol:chloroform:water, using the procedure described above. Niacin content of the acid extract is reported as total niacin. Aqueous extracts were evaporated in a flash evaporator, and the residue was dissolved in the appropriate chromatographic eluant.

Analysis. The extracts were analyzed by reverse-phase HPLC, using a Waters Associates system consisting of a μBondapak C_{18} chromatography column, 0.39 × 30 cm, a Model 6000A pumping system, a Model 440 UV absorbance detector set at 254 nm, a Model R401 differential refractometer, and Model U6K injector. Monitor outputs were recorded on a Houston Omni-Scribe dual pen chart recorder (11).

In the analysis of tryptophan metabolites, the initial eluant was 10 mM Na-acetate (pH 4.84), followed by 3% (w/v) methanol in 10 mM Na-acetate (pH 4.84). The change in eluants was initiated 35 min after sample injection and the second eluant reached the detectors 9 min later. Flow rate was 1.0 ml/min, maintained by a pressure of 6,200 kPa (900 p.s.i.). This system resolved all major intermediates of the tryptophan-niacin pathway except quinolinic acid.

Niacin was determined in tissues incubated with [^{14}C]aspartate or [^{14}C]glycerol by reverse-phase ion-pair HPLC, using 50 mM sodium tetrabutylammonium phosphate (pH 7.0; Eastman), in 10% (v/v) methanol as eluant (6). Flow rate was 1.5 ml/min, maintained by a pressure of 9,700 kPa (1,400 p.s.i.). Quinolinic acid was resolved in standard solutions by this system but was not measurable in extracts due to interference from other radioactive products.

The chromatographic system was maintained at 20 (±2)°C during operation. To complete each analysis, the column was washed briefly with 75% methanol. Compounds were quantified by measurement of areas of their UV absorption peaks.

Eluates were collected in 0.5-min fractions, and radioactivity in these and all other samples was determined in a Beckman LS7000 liquid scintillation counter. Scintillation fluids consisted of 0.4% PPO and 0.05% POPOP in toluene:Triton X-100 (2:1, v/v) for extracts and chromatographic fractions, and 0.5% PPO and 0.01% POPOP in toluene:methyl cellosolve:ethanolamine (13:11:1, v/v/v) for CO_2 solutions. To allow for comparisons, the amounts of niacin and pathways intermediates are expressed as nmol tryptophan, aspartate, or glycerol equivalents (Tables I and II).

RESULTS

All identifiable intermediates of the tryptophan-niacin pathway as well as the vitamin itself were detected in the tissues or incubation mixtures as radioactivity labeled compounds following exposure to ^{14}C or ^{3}H precursor (Table I). The amounts of *N'*-

[1] Supported by National Science Foundation Grant PCM 76-21483.

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formylkynurenine and kynurenine formed during incubation with [^{14}C]tryptophan were always higher than those of 3-hydroxykynurenine, 3-hydroxyanthranilic acid, or total niacin (Table I). Levels of *N'*-formylkynurenine and kynurenine detected after 48 h were lower than those measured following 24-h incubation periods. The reverse is true for 3-hydroxyanthranilic acid, 3-hydroxykynurenine, and total niacin (Fig. 1). Acid treatments following incubation with [^{14}C]tryptophan resulted in the detection of niacin other than in the free form (Table I). However, this was not the case after incubation in [^{3}H]tryptophan (Table I). The proportion of [^{3}H]tryptophan converted into niacin was lower than that of ^{14}C-labeled precursor (Table I).

Both [^{3}H] and [^{14}C]tryptophan served as precursors of IAA. The molar amounts of both converted were similar following 24 h incubation. However, a higher percentage of the ^{3}H precursor (10.36 *versus* 7% for [^{14}C]tryptophan) is converted into the hormone (Table I). After 48 h incubation, the amounts and percentage of label from [^{14}C]tryptophan in IAA increased, whereas the reverse was true for the ^{3}H precursor (Table I). An unknown metabolite of [^{14}C]tryptophan was not detected in ^{3}H extracts (Table I). Radioactivity from both [^{3}H] and [^{14}C]tryptophan was detected in large amounts in the pellet and the organic fraction of the extract, but only a very small proportion of ^{14}C was recovered as CO_2 (Table I).

The amount of ^{14}C from aspartic acid detected in niacin was inconsequential (Table II). Most of the ^{14}C from aspartic acid taken up by the tissues was given off as respiratory CO_2. Only relatively small amounts were present in the pellet and the organic and chromatographic fractions (Table II).

Incorporation of [^{14}C]glycerol into niacin was as low as that of [^{14}C]aspartate (Table II). Glycerol was respired to a lesser extent than aspartic acid, and more of the label appeared in the pellet and the organic fraction.

DISCUSSION

Our results indicate that in corn seedlings, niacin is synthesized from tryptophan by the oxidative pathway which is known to exist in animals and some microorganisms (1). Total niacin production by a whole shoot under our incubation conditions was 25 to 50 pmol/d. This biosynthetic rate is sufficient to account for the niacin content of 4-d shoots, which is less than 200 pmol (unpublished results). The rate of production of niacin from tryptophan (about 0.1% of uptake/day) is low in comparison to humans who convert 2.8% dietary tryptophan to the vitamin (5, 7).

The presence of intermediates in the incubation medium is indicative of leakage from the tissues, either through the cut surfaces or across cell membranes. An indirect relationship exists between the levels of formylkynurenine and kynurenine in the tissues and amount of leakage, but the reverse is true for 3-hydroxykynurenine and 3-hydroxyanthranilic acid (Table I).

It is not clear from our observations whether some of the [^{14}C]tryptophan taken up by the tissues may be lost by leakage. However, inasmuch as the endogenous levels of [^{12}C]tryptophan

Table I. *Incorporation of ^{14}C and ^{3}H from Tryptophan in Tryptophan-Niacin Pathway Intermediates and Products by 10 mg Corn Seedling Tissue*

Substance	Incubation Time											
	24 h						48 h					
	DL-[benzene ring-U-^{14}C]tryptophan				L-[5-^{3}H]tryptophan		DL-[benzene ring-U-^{14}C]tryptophan				L-[5-^{3}H]tryptophan	
	Amount, tryptophan equivalent (nmol)			Percent of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percent of total tryptophan taken up	Amount, tryptophan equivalent (nmol)			Percent of total tryptophan taken up	Amount, tryptophan equivalent (nmol)	Percent of total tryptophan taken up
	Incubation medium	Tissue extract	Total				Incubation mixture	Tissue extract	Total			
Tryptophan[a]	52.30	14.00	66.30	37.57	15.00	74.00	33.00	11.00	44.00	32.68	19.00	83.19
Formylkynurenine	0.63	0.05	0.68	1.83	0.03	0.15	0.26	0.11	0.37	1.10	0.02	0.09
Kynurenine	1.40	0.10	1.50	4.03	0.07	0.35	0.87	0.13	1.00	2.97	0.12	0.53
3-Hydroxykynurenine	0.26	0.04	0.30	0.81	0.01	0.05	0.49	0.18	0.67	1.99		
3-Hydroxyantranilic acid		0.01	0.01	0.03	0.03	0.15	0.004	0.12	0.12	0.36	0.03	0.13
Free niacin		0.01	0.01	0.03	0.003	0.015		0.01	0.01	0.03		
Total niacin		0.04	0.04	0.11	0.001	0.005		0.08	0.08	0.24	0.01	0.04
Trigonelline		0.03	0.03	0.08			0.001	0.01	0.01	0.03		
Niacin plus its intermediates and metabolites	2.29	0.28	2.57	6.92	0.14[b]	0.72[b]	1.63	0.64	2.26	6.72	0.18[b]	0.79[b]
IAA	0.81	1.80	2.61	7.00	2.10	10.36	1.20	3.60	4.80	14.26	0.10	0.44
Unknown	5.80	0.80	6.60	17.71			5.10	1.20	6.30	18.72		
Unidentified fractions	5.60	1.10	6.70	17.98	1.70	8.39	1.80	1.80	3.60	10.70	1.40	6.13
CO_2		0.01	0.01	0.03				0.01	0.01	0.03		
Pellet		2.00	2.00	5.37	0.98	4.83		2.90	2.90	8.62	1.80	7.89
Organic fraction	2.30	0.47	2.77	7.43	0.35	1.73	2.20	0.59	2.79	8.29	0.36	1.58
Total	69.10	20.46	89.56	100.01	20.27	100.03	44.93	21.74	66.66	100.02	22.84	100.02

[a] The value for tryptophan was derived by dividing tissue tryptophan by tissue tryptophan plus the total of all other metabolites and intermediates; values for all others are derived by dividing amount in tissue plus medium by tissue tryptophan plus all metabolites and intermediates.

[b] Does not include trigonelline.

Table II. *Fate of ^{14}C from Aspartate and Glycerol in 100 mg Corn Seedling Tissue*

Fraction	L-[U-^{14}C]Aspartate				[U-^{14}C]Glycerol			
	24 h		48 h		24 h		48 h	
	Amt., aspartate equivalent	Total taken up	Amt., aspartate equivalent	Total taken up	Amt., glycerol equivalent	Total taken up	Amt., glycerol equivalent	Total taken up
	nmol/100 mg	%	*nmol/100 mg*	%	*nmol/100 mg*	%	*nmol/100 mg*	%
Free niacin	0.01	0.03	0.01	0.03				
Total niacin	0.002	0.006	0.002	0.005	0.003	0.01	0.002	0.008
Other	9.70	30.50	4.90	12.73	11.20	48.07	12.00	45.80
CO_2	21.00	66.04	32.00	83.12	1.20	5.15	1.40	5.34
Pellet	0.88	2.77	1.30	3.38	2.10	9.01	3.40	12.98
Organic fraction	0.21	0.66	0.29	0.75	8.80	7.77	9.40	35.88
Total taken up	31.80	100.00	38.50	100.02	23.30	100.00	26.20	100.00

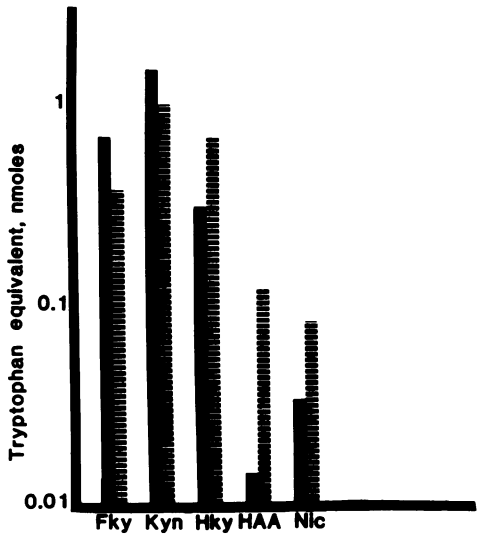

FIG. 1. Levels (nmol/100 mg tissue) of *N'*-formylkynurenine (Fky), kynurenine (Kyn), 3-hydroxykynurenine (Hky), hydroxyanthranilic acid (HAA), and niacin (NIC) in corn seedlings following 24- (solid bars) and 48- (dashes) h incubation periods.

are very low and the occurrence of leakage is not central to the question of niacin biosynthesis our calculations are based on the assumption that this amino acid did not leak (Table I). If it did, incorporation percentages would probably be affected minimally.

Niacin was not detected in the medium. This is probably due to the fact that most of it is bound in acid-labile products which cannot diffuse across cell membranes. The small amounts of free niacin in the tissues (if any exist) may not leak or are effectively reabsorbed if they do. In any case, losses due to leakage (if they did occur) were too small to be detected by our experimental methods or have a major effect on the results. Altogether, niacin production by experimental plants is low and this may be responsible, at least in part, for the inability of previous investigators to detect it in their systems without the sensitivity of HPLC.

Several properties of the major unidentified metabolite of tryptophan provide useful clues regarding its structure. The long retention time indicates that it contains an intact indole moiety. Ring cleavage introduces polar functional groups which would decrease retention time. Further, its retention time (longer than that of tryptophan but shorter than IAA) suggests either the loss of one of the charged moieties on the side chain of tryptophan or the introduction of a nonpolar functional group. Moreover, the absence of a corresponding radioactive peak after incubation with [5-^{3}H]tryptophan indicates that a substitution has occurred at the 5-position. Two metabolites of tryptophan with these properties, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, are well known in animals and have been detected in the West African legume *Griffonia simplicifolia* (3) and in barley and tomato seedlings (9, 10). Authentic 5-hydroxytryptamine, but not 5-hydroxyindoleacetic acid (both Sigma products) coeluted with the labeled unknown in HPLC.

The similarity in quantitative distributions of metabolites of DL-[^{14}C] and L-[^{3}H]tryptophan may suggest that the enzymes of the tryptophan-niacin pathway are equally active with D- and L-forms of the precursor and the asymmetric intermediates. If so, our findings are in agreement with previous reports (12) that both D- and L-tryptophan are substrates of tryptophan pyrolase, which catalyzes the initial ring cleavage in pea seedlings. However, it is also possible that the metabolism of a single ^{14}C isomer would produce the same relative amounts of intermediates.

The relatively low levels of niacin in the experiments with [5-^{3}H]tryptophan suggest caution in its use as a precursor in this system. One reason for this could be that some of the ^{3}H in niacin, derived from the 5-position of tryptophan, may have been removed during acid treatment of the tissues to release bound niacin. However, removal of ^{3}H from this position would merely reduce the number of counts without fundamentally affecting the results.

Levels of formylkynurenine and kynurenine decreased slightly during the second day, whereas those of 3-hydroxykynurenine acid and niacin increased (Fig. 1). This suggests that hydroxykynureninase (3-hydroxykynurenine → 3-hydroxyanthranilic acid) may be rate-limiting in the biosynthesis of niacin in corn seedlings, and increases in activity during incubation with tryptophan or early intermediates in the pathway. A similar increase in activity kynurenine hydroxylase (kynurenine → 3-hydroxykynurenine) may also occur.

Most of the niacin was detected in acid-labile bound forms, which probably include NAD, NADP, and other pyridine nucleotides. This suggests that niacin may initially be formed as part of a larger molecule, possibly nicotinic acid mononucleotide as in animals (1). Quinolinic acid mononucleotide(s) may be another logical intermediate. However, rapid turnover of niacin in the pyridine nucleotide cycle could also account for these results, since the initial product could be the free vitamin which may be quickly incorporated into pyridine nucleotides.

Quinolinic acid was not resolved among the metabolites of tryptophan in corn seedlings. Therefore, our numbers (Table I) are underestimations of the total production of intermediates. Quinolinic acid may be converted directly to the vitamin, or give rise to niacin nucleotides via quinolinate ribonucleotide (1). Identification of quinolinate ribonucleotide among the metabolites of labeled tryptophan would suggest that niacin first appears in the form of a ribonucleotide.

Incorporation of aspartate and glycerol into niacin was so low and so near the limits of the detection methods as to be inconsequential. However, the possibility that the glyceraldehyde-aspartate pathway is operative to a limited extent cannot be completely excluded, inasmuch as incorporation of the precursors was equimolar and the amounts of niacin detected were constant (Table II). Still, the evidence for a precursor role of aspartate and glycerol is very limited and questionable because incorporation of label into the vitamin could also be due to recycling of radioactivity through tryptophan. On the whole, our findings suggest very strongly that niacin may not be synthesized directly from glyceraldehyde-3-P and aspartate in corn seedlings. Consequently, it appears that biosynthesis of niacin from these precursors is at least not a universal in the plant kingdom, as previously suggested (see 1 for a review).

Acknowledgments—We thank Drs. K. K. Tewari, E. Rodriguez, G. C. Stephens and G. Nicolson for seeds and apparatus; G. Reynolds and W. Wisdom for assistance with high pressure liquid chromatography; and Jan L. Cooper for assistance with the manuscript.

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