Incorporation of ^{15}N-Ammonia into Serotonin in Cotyledons of Maturing Walnuts

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Cotyledons isolated from maturing seeds of *Juglans regia* incorporate supplied [^{15}N]-ammonia into serotonin (5-HT). A ^{15}N incorporation of about 9% into the indolic nitrogen of 5-HT is detectable by gas chromatography-mass spectrometry following isolation of 5-HT by preparative HPLC. This ^{15}N incorporation supports the hypothesis that biosynthesis and accumulation of 5-HT play a role in ammonia detoxification in maturing walnut seeds.

Introduction

Serotonin (5-HT), the well known central neurotransmitter in mammalian brain, is also detectable in mature seeds of walnuts (*Juglans regia* L.) [1]. The biosynthesis of 5-HT occurs from tryptophan during maturation of these seeds [2] and is regulated by *de novo* synthesis of an aromatic amino acid decarboxylase [3, 4], together with a hydroxylase needed for the transformation of tryptophan to 5-HT.

As with amides, ureids, alkaloids, and other nitrogen-containing secondary plant products [5, 6] the accumulation of 5-HT is considered to be involved in ammonia detoxification [7]. Such a role for 5-HT is supported by a decrease of ammonia content during maturation accompanied by 5-HT accumulation following tryptophan biosynthesis [8] in these seeds.

In order to verify the hypothesis of ammonia detoxification by 5-HT biosynthesis, [^{15}N]NH_4^+ incorporation experiments were carried out and are described below.

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Materials and Methods

Plant material

Seeds of *Juglans regia* L. were a generous gift from E. Moll, Botanical Garden of the City of Cologne. 15 weeks after anthesis the seeds were harvested, freed from pericarp and integuments, and placed aseptically on agar plates made from 1% agar in water.

Incubation experiments

8 µmol [^{15}N]NH_4Cl (CEA-France, Gif-sur-Yvette), dissolved in double-distilled water and sterilized by membrane filtration (Sartorius, Göttingen, West Germany, 0.2 µm pore size), were applied to the surface of each cotyledon, which was then incubated aseptically at 25 °C.

Chromatography

Analytical HPLC was carried out as described elsewhere [8] and preparative HPLC as indicated in the figure. The prepacked preparative Whatman column was a generous gift from Dr. D. Strack, Department of Botany, University of Cologne. The samples were prepared by extraction of the seeds with 0.05 N HCl [8] and injections were made using a 100 µl loop. For determination of ^{15}N incorporation into 5-HT a combined gas chromatography-mass spectrometry (in the mass fragmentographic mode) was performed as described elsewhere [9], except for the use of GC capillary columns.

Results and Discussions

Young seeds of walnuts contain about 8 µmol NH_4^+ g^{-1} fresh weight [8]. To prevent toxic concentrations of ammonia accumulating due to the loss of moisture in the subsequent period of ripening, this NH_4^+ must be assimilated through the synthesis of N-containing compounds. The accumulating 5-HT is believed to be such an ammonia detoxification compound, synthesized via glutamine and tryptophan [7].

In order to confirm ammonia consumption by 5-HT biosynthesis, 8 µmol [^{15}N]NH_4Cl were applied to the surface of each of a number of cotyledons isolated aseptically from walnut seeds. After an incubation period of 9 days at 25 °C, 5-HT was extracted from the cotyledons with 0.05 N HCl and

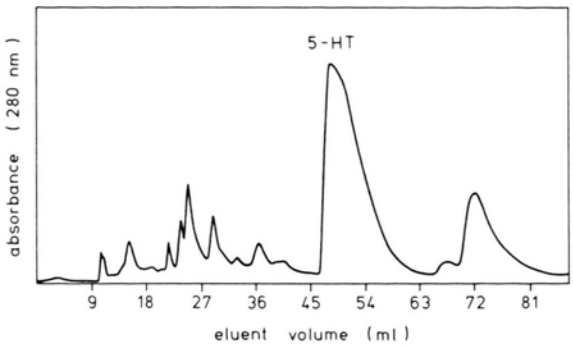

Fig. 1. Purification of serotonin (5-HT) by preparative reversed-phase HPLC on a prepacked Whatman Partisil M9-column 10/29 ODS (RP-18). Flow rate: 3 ml min^{-1}. Mobile phase: isocratic 1% aqueous CH_3COOH.

purified by preparative HPLC as indicated in the figure. 5-HT samples – identified by analytical and preparative HPLC – were pooled from successive preparative runs, evaporated to dryness and stored under N_2.

To determine ^{15}N incorporation into 5-HT, the dried sample was derivatized by perfluoroacylation to yield the pentafluoropropionic derivative of 5-HT (= 5-HT-3PFP) and analysed by gas chromatography-mass spectrometry in the mass fragmentographic mode according to previously described procedures [9]. Since the PFP derivative of the 5-HT synthesized from $[^{15}N]NH_4Cl$ would contain ^{15}N atoms, the fragment corresponding to the β-cleavage in the side chain (*m/e* 451 in the standard 5-HT-3PFP) would have a *m/e* of 452. Thus, 5-HT isolated from walnuts should have a $\frac{m/e\ 452}{m/e\ 451}$ ratio higher than that of authentic 5-HT-3PFP. To test this hypothesis, three ion fragments (*m/e* 451, 452, and 453) were focused, corresponding to a major fragment (451) and its isotopic peaks *m/e* + 1 (452) and *m/e* + 2 (453), all of them containing the indolic nitrogen. The ratios between the response at 452/451 and 453/451 respectively were as follows:

Response ratio	5-HT-3PFP standard [%]	5-HT-3PFP from walnuts [%]
$\frac{m/e\ 452}{m/e\ 451}$	20.1 ± 0.2	28.9 ± 0.7
$\frac{m/e\ 453}{m/e\ 451}$	2.4 ± 0.2	4.1 ± 0.1

The results ($\bar{x} \pm$ S.D.) were found to be statistically different ($P < 0.001$, Student's *t*-test, $n = 3$) for each response ratio. Taking into account these mean values, the precentage of ^{15}N incorporation into the 5-HT sample from walnut seeds can be calculated as 8.8% $\left(\text{ratio } \frac{452}{451}\right)$ and 8.6% $\left(\text{ratio } \frac{453}{451}\right)$. These findings demonstrate very well that cotyledons of maturing seeds are able to use ammonia for 5-HT biosynthesis. Beside ^{15}N-labeled 5-HT these cotyledons will also have synthesized unlabeled 5-HT from their own unlabeled ammonia and tryptophan. Therefore the detectable ^{15}N incorporation into 5-HT of about 9% is high enough to verify the hypothesis of ammonia detoxification by 5-HT biosynthesis in maturing walnut seeds.

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