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Short Communication

Synthesis of Indoleacetic Acid via Tryptamine by a Cell-free System from Tobacco Terminal Buds[1, 2]

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Plants infected with the vascular pathogen, *Pseudomonas solanacearum*, exhibit symptoms associated with auxin imbalance. The content of 3-indoleacetic acid (IAA) has been shown to increase 100-fold in inoculated tobacco plants (7). The origin of IAA in diseased tissues has remained relatively obscure, although preliminary data suggest that the host contributes most of the auxin during early stages of pathogenesis (6). Progress in this area has been impeded by the similarity in pathways of biosynthesis of IAA from tryptophan (TTP) in higher plants and microorganisms.

A possible approach to the problem of IAA synthesis in the host-parasite complex is the determination of differences in synthetic pathways between both members, thus allowing the use of radioactive precursors which only one member can convert to IAA. Alternate pathways in the conversion of TTP to IAA have been reported for *P. solanacearum* (8) and one of its hosts, tobacco (6). The recovery of radioactive IAA from tobacco plants fed ^{14}C-labeled tryptamine (TNH_2) suggested that this compound was a possible precursor of IAA synthesis in tobacco. Confirmation of these results was sought by studying the synthesis of auxin in a cell-free system from tobacco terminal buds.

Apical stem sections, 3 cm in length, were removed from 4-week-old tobacco plants (*Nicotiana tabacum* var. Bottom Special) grown in sand culture at 28°, under 1500 ft-c from a combination of General Electric Cool White, Sylvania Gro Lux and incandescent bulbs on a 12-hour photoperiod. The apices were homogenized in a Sorvall Omnimixer for 4 minutes in cold 20 mM potassium phosphate (pH 6.0) at 2 ml per g fresh weight of tissue. The homogenate was centrifuged at 5000 g for 10 minutes at 0° and the supernatant fraction used without further treatment. If not homogenized immediately, apices were frozen in dry ice and stored at −20° for future use.

Reaction mixtures containing tobacco cell-free extracts in 20 mM phosphate buffer at pH 6.0 and 2.5 μmole/ml L-TTP were incubated for 1 hour at 30°. The total volume was 10 ml. Reactions were terminated by heating in a boiling water bath for 5 minutes or by adding an equal volume of 10 % trichloroacetic acid. The protein precipitated by heat or trichloroacetic acid treatment was removed by centrifugation at 10,000 g for 10 minutes and the supernatant was retained for extraction of indoles. Control mixtures containing heat-inactivated enzyme or no enzyme were treated similarly. In experiments utilizing labeled precursors, DL-TTP-1′-^{14}C (8.95 mc/mmole) or TNH_2-1′-^{14}C bisuccinate (2.73 mc/mmole) were added to reaction mixtures at 0.10 μc/ml.

Indole compounds were separated into: A) ether-soluble acidic, B) ether-soluble basic plus neutral, and C) ether-insoluble components. For this purpose, the reaction mixture was adjusted to pH 8.2 with solid $NaHCO_3$ and basic and neutral substances were removed by extraction with diethyl ether. The aqueous layer was adjusted to pH 3.0 with 1 N HCl and acidic substances were extracted with ether. The aqueous layer contained the ether-insoluble components. The 3 fractions were evaporated to 1 ml under reduced pressure at 25°.

Three-week-old tobacco plants, grown in the manner already described, were removed from the pots and the sand was washed off the roots. Each plant was placed in a 250 ml beaker containing either 9.5 mg TNH_2-HCl or 10.0 mg DL-TTP to which had been added 2 μc of the appropriate radioactive precursor. Each treatment involved 4 plants. Each plant absorbed the initial 50 ml of solution in about 3 hours. Distilled water was added as required over an incubation period of 44 hours, after which the plants were immediately frozen in dry ice.

The frozen plants were finely ground with a mortar and pestle at −78° and the powder was added slowly to 400 ml of hot ethyl acetate. The mixture was boiled for 15 minutes, filtered through cheesecloth and the plant material was ground in 300 ml distilled water in a Waring Blendor. The suspension was filtered through cheesecloth and the solids were discarded. The supernatant was adjusted to pH 3.8 and extracted 3 times with 100 ml ethyl acetate. The acetate fraction was washed once with distilled water and combined with the original acetate extract. The aqueous fractions were bulked, adjusted to pH 3.0 and extracted 3 times with 100 ml diethyl ether. This fraction was labeled 1. The aqueous layer was adjusted to pH 8.2 with 100 ml 5 % $NaHCO_3$ and was extracted

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3 times with 100 ml diethyl ether. The organic and aqueous fractions were designated 2 and 3, respectively.

The combined ethyl acetate fraction was extracted 3 times with 100 ml 5 % $NaHCO_3$ and the acetate layer was retained as fraction 4. The bicarbonate layer was extracted 3 times with 100 ml diethyl ether, and this organic fraction was labeled 5. The aqueous layer was adjusted to pH 3.0 with 1 N HCl and then was extracted 3 times with 100 ml diethyl ether. The organic layer was labeled fraction 6. All fractions were evaporated to 1 ml at 25°, under reduced pressure. Compounds in the extracts were separated by paper chromatography and radioactivity was detected with a Baird Atomic 4 pi, open window gas flow strip scanner, model Scanogram II.

A radioactive, Ehrlich-positive substance at the R_F of IAA in isopropanol:ammonium hydroxide: water (10:1:1) solvent was recovered from cell-free systems containing TTP- or TNH_2-1′-^{14}C (fig 1). Scannings of chromatograms of ether-soluble acidic fractions from plants supplied with either precursor showed similar patterns of radioactivity. Other radioactive metabolites were Ehrlich-positive, except for one at R_F .52. This compound did not react with any of the color reagents used. The labeled compound, II, at R_F 0.18 gave an orange-pink color with Ehrlich's reagent similar to that of 3-indolecarboxylic acid (ICA), but its R_F and light purple reaction with *p*-dimethylaminocinnamaldehyde reagent (10) were different from those of ICA. Compound III (R_F .28) produced a light blue reaction with Ehrlich's reagent, typical of an unknown previously found in diseased and healthy tobacco plants supplied with labeled TNH_2 (6). Indolepyruvic acid (IPyA) was not detected in our

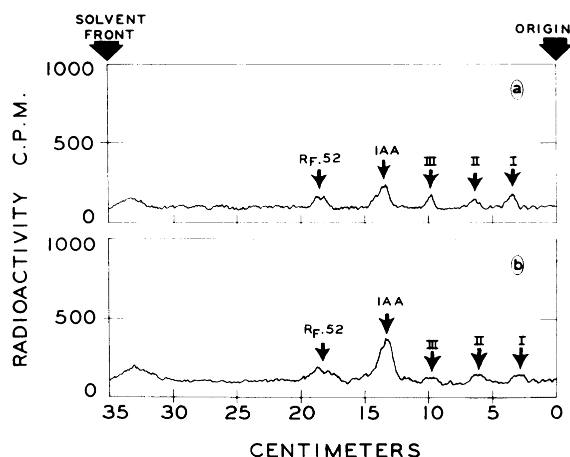

FIG. 1. Radioactivity (cpm) of 200 µl samples of ether-soluble acid fractions from tobacco terminal bud tissue homogenates incubated for 1 hr with (a) DL-tryptophan-1′-^{14}C and (b) tryptamine-1′-^{14}C. Tracings are of scanner records of chromatograms on Whatman No. 1 paper, using isopropanol:ammonium hydroxide:water (10:1:1) solvent.

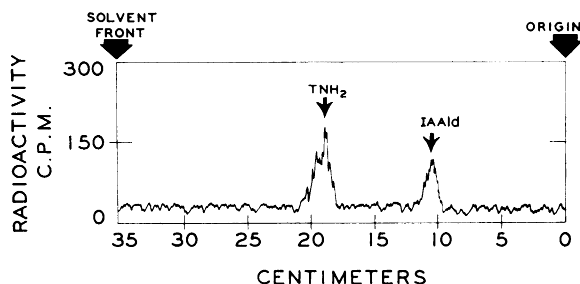

FIG. 2. Radioactivity (cpm) of a 100 µl sample of the ether-soluble basic plus neutral fraction from tobacco terminal bud tissue homogenate incubated for 1 hour with DL-tryptophan-1′-^{14}C and $NaHSO_3$. Tracing of a scanner record from a chromatogram on Whatman No. 1 paper, using 8 % NaCl:acetic acid (100:1) solvent.

preparations. One consistent feature of these experiments was the higher radioactivity of IAA produced by plant homogenates from ^{14}C-TNH_2 as compared with that produced from ^{14}C-TTP (table I). In both instances, product yields were low, indicating an unfavorable equilibrium in the system used.

When chromatograms of ether-soluble neutral and basic fractions from plant homogenates incubated with TTP-1′-^{14}C were examined, radioactivity was found only at the R_F of TNH_2. This compound gave a violet color with Ehrlich's reagent, typical of TNH_2. The amounts of TNH_2 increased with time of incubation, and this was correlated with increasing radioactivity peaks at the R_F of TNH_2. No other radioactive metabolic products were detected in these fractions. The aqueous fractions showed radioactivity only at the R_F of TTP.

The apparent involvement of TNH_2 as an intermediate in IAA synthesis from TTP by tobacco tissue homogenates suggested that 3-indoleacetaldehyde (IAAld) should be an additional intermediate in the reaction. To test this possibility, 2.5 µmoles/ml $NaHSO_3$ were added to standard reaction mixtures containing labeled TTP, and the preparations were incubated for 1 hour. Radioactivity measurements of chromatograms of neutral and basic fractions developed with 8 % NaCl:acetic acid (100:1) solvent revealed a labeled compound giving an immediate yellow color with 2,4-dinitrophenylhydrazine reagent (10) at the R_F of IAAld (fig 2). Also, when homogenates were supplied with IAAld, a compound with the R_F and color reactions characteristic of IAA was detected in acid fractions. Extracts of acidic substances from heat-inactivated homogenates incubated with labeled TNH_2 and labeled TTP contained small amounts of non-radioactive IAA. No radioactive metabolites were observed in any of the extracts from the control preparations.

The results of experiments in which radioactive intermediates were supplied to growing tobacco plants corroborated the conclusions from the in vitro studies. A compound chromatographically

Table I. *Amounts and Specific Activities of IAA from Reactions in Tobacco Tissue Homogenates and in Intact Tissue of Plants Each Supplied with* DL-*Tryptophan-1′-^{14}C or Tryptamine-1′-^{14}C*

System	Precursor	Total amt of precursor	Specific activity of precursor	IAA recovered*	Specific activity of IAA****
		μmoles	cpm × 10^3/μmole	μmoles	cpm × 10^3/μmole
Homogenate	TTP-1′-^{14}C	25.0	80.0	0.16**	49.6
	TNH_2-1′-^{14}C	25.0	80.0	0.19	70.0
Intact plant	TTP-1′-^{14}C	49.0	81.6	0.05***	33.9
	TNH_2-1′-^{14}C	48.6	82.3	0.08	72.1

* Determined by fluorimetry of 0.1 M phosphate buffer (pH 7.4) extracts from ether eluates of silica gel chromatograms.
** Total from 10 ml of homogenate.
*** Total from 4 plants.
**** Calculated from radioactivity measurements of chromatograms at the R_F of IAA, after correction for background activity.

similar to IAA was synthesized by tobacco plants from TTP and TNH_2. Also, radioactive TNH_2 could be recovered from plants fed labeled TTP. As was found in the in vitro experiments, the IAA from plants supplied with TNH_2-1′-^{14}C appeared to be more radioactive than that produced from DL-TTP-1′-^{14}C (table I). This could have been due to channeling of TTP into other metabolic pathways and to lack of conversion into IAA of the D isomer of TTP. The other Ehrlich-positive metabolites found in acidic fractions (fraction 6) from plant extracts corresponded in R_F and color reactions to those metabolites produced in vitro. Also, the patterns of radioactivity in acidic fractions from both TNH_2-1′-^{14}C and TTP-1′-^{14}C fed plants were similar to those obtained in extracts from in vitro experiments. Most of the TNH_2 extracted from plants supplied with this precursor was found in fraction 2, but some remained in the aqueous fraction (fraction 3). Chromatogram scannings of fraction 2 from plants fed labeled TTP showed only 1 highly radioactive metabolite which, on the basis of its R_F values in several solvents and its color reactions, was tentatively identified as TNH_2.

The fluorescence and excitation maxima of IAA and TNH_2 recovered from tobacco cell-free systems or intact plants were identical to those of authentic IAA (288 and 378 mμ), and TNH_2 (288 and 360 mμ). The product identified as IAA showed growth-promoting activity on wheat coleoptiles similar to that of authentic IAA.

Studies with tomato (14), oat coleoptile (9), watermelon (2), pineapple (3), *Coleus* (11) and tobacco (6) have implicated TNH_2 in the biosynthesis of IAA from TTP. The presence of TNH_2 has been demonstrated in *Acacia* (13), tomato (12) and maize (4), and high yields of IAAld from TNH_2 were obtained with a purified amine oxidase from pea seedlings (1). Peroxidase-catalyzed oxidations of TNH_2 and IAAld also occur in crude enzyme preparations from pea seedlings. Thus, the machinery for the conversion of TNH_2 to IAA appears to be present in a number of plant species. The results of this study strongly suggest that IAA synthesis from TTP in Bottom Special tobacco proceeds via TNH_2 deamination.

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