Indoleacetic Acid Synthesis in Soybean Cotyledon Callus Tissue

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ABSTRACT

Growth of an auxin-requiring soybean cotyledon callus tissue (Glycine max L., Merr. var. Acme) was promoted by tryptophan, tryptamine, indole, indoleaceticamide and, to a very slight degree, anthranilic acid. When tryptophan-3-14C was supplied in the growth medium, labeled indoleacetic acid (IAA) was found in both the tissue and the medium. An external source of tryptophan produced labeled IAA. Soybean callus contained 0.044 µmole/g free tryptophan, but this is apparently not available for conversion to IAA. These results suggest that while exogenously supplied tryptophan could elevate a specific internal pool where IAA synthesis occurs some of the growth on a tryptophan medium can be accounted for by external conversion.

Callus tissue provides an interesting system for the study of IAA biosynthesis in plants. Since the tissue requires an external auxin supply for growth, it presents a sensitive bioassay tissue for potential IAA precursors. Another advantage is that the tissue is free from bacterial contamination which has presented difficulties in the interpretation of some prior studies of IAA synthesis (4). Callus tissue from soybean cotyledons has been isolated and grows with good consistency (5). This investigation was undertaken to investigate IAA biosynthesis in this tissue.

MATERIALS AND METHODS

Plant Material. The callus tissue was originally isolated from soybean cotyledon (Glycine max L., Merr. var. Acme). Stock cultures were grown on an agar medium of Miller (5) with 3% sucrose, 0.5 mg/l kinetin, and 2 mg/l naphthaleneacetic acid.

Growth Studies. In the bioassay for possible IAA precursors, the growth medium referred to above was used with the exception of the NAA2 which was deleted for these experiments. The medium (49 ml) was poured into 125-ml Erlenmeyer flasks and autoclaved for 15 min at 121 C. An aqueous solution of the test compounds was prepared such that 1 ml of the solution added to 49 ml of the basal medium resulted in a concentration of the compound of 10-4 M. After the medium had been autoclaved, it was allowed to cool to about 60 C at which time the test solutions were aseptically added (Gelman 0.2-µm filter). After the medium had solidified, three pieces of callus (2-5 mg/piece) were planted in each flask. The flasks were incubated under continuous low intensity fluorescent light at 27 C for 28 days. Following incubation, the tissue was removed and weighed.

Labeling Studies. The callus tissue used in these experiments had been grown on the complete medium described above for 21 days. The tissue was aseptically removed from the flasks and cut into small pieces. Approximately 10 g of tissue was placed into a flask which contained 20 ml of autoclaved medium which had been modified as follows. The NAA and agar were deleted and the water was reduced by 20%. Five ml of an aqueous solution containing 2 µCi of tryptophan-3-14C (50.7 mCi/mmol, New England Nuclear) were added to the flask by means of a syringe equipped with a 0.2-µm filter (Gelman). The flask was placed on a shaker at low speed for 72 hr. The tissue was then separated from the medium using a Gelman filter. The tissue and medium were analyzed for IAA-14C by the method described below.

In order to test for external conversion of tryptophan to IAA, a modification of the above procedure was carried out. The tissue was incubated for 72 hr in a shake culture which contained an amount of unlabeled tryptophan at a molar concentration equal to 2 µCi of the labeled tryptophan (1.58 × 10-4 M). Growth studies showed that the concentration produced a 2- to 3-fold increase in growth over the control. After incubation, the tissue was separated from the medium by filtering through a Gelman filter into an autoclaved flask. Two µCi of tryptophan-14C were added to the filtered medium, which was then shaken at room temperature for 24 hr and analyzed for IAA-14C. A control was included in which the filtered medium was boiled for 15 min prior to the addition of the labeled tryptophan.

The IAA was isolated by previously reported methods (1). The tissue in 4.5 volumes of ice-cold absolute methanol was homogenized in a Waring Blender, and the filtered residue was washed with cold 80% methanol. Following evaporation of the methanol under vacuum at 40 C, the remaining aqueous material was adjusted to pH 3.5 and extracted three times with equal volumes of diethyl ether. In experiments where the medium was analyzed for IAA, the filtered medium was adjusted to pH 3.5 and extracted with ether.

The combined ether fraction, containing the IAA, was evaporated to near dryness and chromatographed on Whatman No. 3MM paper using isopropanol-ammonium hydroxide-water (8:1.1, v/v). One hundred µg of carrier IAA or tryptophan were added to the concentrated extracts prior to chromatography, and the bands containing the compounds were located by placing the papers under an UV light. The region of the paper containing the compound was eluted with 80% methanol which was then evaporated to a volume of 2 to 3 ml. Ten percent of the sample was counted in a liquid scintillation counter (Nuclear Chicago Unilux II). The scintillation fluid contained 5.5 g of PPO and 0.1 g of POPOP dissolved in 333 ml of Triton X-100 and 666 ml of toluene. Additional carrier (50 mg) was added to the remaining sample, and the compounds were recrystallized two or three times (to constant specific radioactivity) from 50% ethanol.

A colorimetric determination of the free tryptophan level in the tissue was made by the method of Udenfriend and Peterson (9) following ion exchange and paper chromatography (1).

1 Contribution No. 142 from Department of Biology, The Pennsylvania State University.
2 Abbreviation: NAA: naphthaleneacetic acid.
RESULTS

Growth Studies. The results obtained when soybean callus tissue was grown on media containing some possible IAA precursors are presented in Table I. Preliminary studies were carried out for all precursors in the concentration range of $10^{-3}$ to $10^{-5} \text{M}$ with $10^{-4} \text{M}$ producing the optimum response in each case. This concentration was then used for all subsequent experiments. Growth tests on each compound were repeated at least two times. While the relative response was the same the variation in absolute growth was such that the data were not combined. Table I shows the means and standard errors of one complete experiment. All the tested precursors of IAA stimulated growth at $10^{-4} \text{M}$ but anthranilic acid gave less stimulation than the others. The greatest growth stimulation was produced by indoleaceticamide followed by tryptophan and tryptamine. Growth induced by these compounds is not significantly different from IAA at $10^{-4} \text{M}$. Indole was less active than the other three derivatives while anthranilic acid was only slightly active. The control tissue (no IAA or possible IAA precursors) did not grow to any extent and had turned brown after the 28 day incubation period.

Labeling Studies. In order to determine if tryptophan is actually being converted to IAA as the growth studies suggest, labeling studies were carried out using tryptophan-$^{14}$C. The results of these experiments are presented in Table II. Radioactive IAA was recovered from tissue which was grown in medium containing the labeled tryptophan. About one-third of the IAA-$^{14}$C was also recovered from the medium itself. These results suggest that the conversion of tryptophan to IAA may take place in the medium. Therefore tryptophan-$^{14}$C was added to medium from which callus tissue had been removed by filtration. More labeled IAA was recovered from cell-free medium in 24 hr than when the tissue itself was incubated with the tryptophan-$^{14}$C for 72 hr. The low level of activity recovered from the boiled control indicates that the conversion is enzymatic.

DISCUSSION

Plant callus tissue is used frequently as a system in which to investigate auxins and other plant hormones and growth regulators. This research was carried out in an attempt to determine if IAA could be synthesized in callus tissue by a mechanism similar to that found in normal plant tissue. Recent reports (1, 3) have supported the hypothesis that tryptophan is the IAA precursor in many plants. The results of the growth studies (Table I) suggest that tryptophan is also a precursor of IAA in soybean callus tissue. Tryptophan was active in promoting growth, as was indole which is a precursor of tryptophan. An earlier report from this laboratory showed that *Avena* coleoptiles and excised bean shoots convert indole to IAA with tryptophan as the apparent intermediate (1). The growth-promoting activity of tryptamine would be expected if the route from tryptophan to IAA involves a decarboxylation of tryptophan to tryptamine as has been reported in tobacco (6). Anthranilic acid is also a more remote precursor but might have been expected to show more activity as we had previously observed with *Avena* stem tips (1). These findings, however, do agree with previous reports of low growth-promoting activity by anthranilic acid in other systems (2).

The labeling studies with tryptophan-$^{14}$C (Table II) give direct evidence that tryptophan is converted to IAA in soybean callus tissue. The amount of conversion, based upon the IAA-$^{14}$C recovered, compares well to that found for *Avena* and bean (1). From the growth studies, it also appeared that exogenously supplied tryptophan is converted to IAA with an efficiency sufficient to allow growth about equal to IAA at $10^{-4} \text{M}$.

Analysis of soybean callus tissue grown on the complete medium showed that the tissue contained free tryptophan at a level of $0.044 \mu\text{mole/g}$ fresh weight. This level compares quite closely to those previously reported for soybean callus (10) as well as for *Avena* and bean (1). This presents the paradox of a tissue which is apparently capable of synthesizing tryptophan in a quantity comparable to other plants but not capable of converting this tryptophan to IAA since an auxin or auxin precursor must be added to the medium. Evidence from carrot tissue culture (8) indicates the compartmentation of tryptophan in the cells such that internally synthesized tryptophan occurs in one or more pools which are different from the pool which exogenously added tryptophan enters. This suggests the possibility that the tryptophan synthesized by soybean callus tissue is not converted to IAA because it is sequestered away from the site of IAA synthesis. In fact, we previously postulated a similar compartmentation (1) based on rather contrary results to these. *Avena* coleoptile tips did not grow on external tryptophan but could grow on indole or tryptamine. Indole enhanced the internal free tryptophan levels as well as the labeling in both free tryptophan and IAA while tryptophan labeled proteins much more than indole.

Another alternative to internal compartmentation is suggested by the fact that the filtered medium in which the callus tissue had been grown can also carry out an enzymic conversion of tryptophan-$^{14}$C to IAA-$^{14}$C (Table II). It is well known that plant callus secretes enzymes into the growth medium, especially peroxidase. Peroxidase has been demonstrated to catalyze the conversion of tryptophan to indoleaceticamide with the subsequent appearance

Table I. Growth of Soybean Cotyledon Callus Tissue Induced by Possible Precursors of IAA

<table>
<thead>
<tr>
<th>Compound</th>
<th>Final Fresh Weight (mg/piece) (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>13.1 ± 3.9</td>
</tr>
<tr>
<td>IAA</td>
<td>625.4 ± 93.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>596.3 ± 128.9</td>
</tr>
<tr>
<td>Tryptamine</td>
<td>530.7 ± 126.9</td>
</tr>
<tr>
<td>Indole</td>
<td>356.6 ± 88.1</td>
</tr>
<tr>
<td>Anthranilic Acid</td>
<td>68.6 ± 18.7</td>
</tr>
<tr>
<td>Indoleacetamide</td>
<td>701.4 ± 155.5</td>
</tr>
</tbody>
</table>

The concentration of each test compound was $10^{-4} \text{M}$. Each mean weight was determined from 15 pieces of callus.

Table II. Conversion of Tryptophan-$^{14}$C to IAA-$^{14}$C by Soybean Cotyledon Callus Tissue

<table>
<thead>
<tr>
<th>Experimental Material</th>
<th>Incubation with 2 μCi Tryptophan-$^{14}$C (Hours)</th>
<th>IAA-$^{14}$C Recovered $^1$ cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture Tissue</td>
<td>72</td>
<td>2399</td>
</tr>
<tr>
<td>Medium</td>
<td>72</td>
<td>1208</td>
</tr>
<tr>
<td>Cell-free Medium $^2$</td>
<td>24</td>
<td>8045</td>
</tr>
<tr>
<td>Cell-free Medium Boiled $^3$</td>
<td>24</td>
<td>250</td>
</tr>
</tbody>
</table>

$^1$ Data corrected for losses during recrystallization.

$^2$ Medium preincubated 72 hours and filtered (0.2 $\mu$) prior to addition of 2 μCi tryptophan-$^{3}$-$^{14}$C to the cell-free medium.

$^3$ Medium preincubated 72 hours and filtered (0.2 $\mu$) prior to addition of 2 μCi tryptophan-$^{3}$-$^{14}$C to the cell-free medium.

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of small quantities of IAA (7). This may explain the activity of indoleacetamide in the growth studies where its growth-promoting effect exceeded the IAA treatment. Part of this effect was probably due to the fact that the indoleacetamide is more stable in the medium than is IAA. However, rapid growth began almost immediately and at least paralleled the IAA response through the incubation period.

At present we are unable to decide if the exogenously supplied tryptophan elevates a specific tryptophan pool in the tissue which is available for conversion to IAA; in any event considerable conversion can take place in the medium. A recent report (8) indicates that 5-methyl tryptophan-resistant mutants of carrot tissue which accumulate abnormally high levels of tryptophan are auxin-independent. This observation is consistent with the findings reported here. The excess tryptophan could either leak out of the cells and be converted to IAA in the medium or it might enter compartments in the cells where conversion could occur. Crown gall tumor or habituated tissue may also become auxin-independent due to tryptophan leakage between compartments or due to external tryptophan leakage.

LITERATURE CITES