Indole Compounds Synthesized by Diplodia natalensis 1, 2

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Since the isolation of indoleacetic acid (IAA) from *Rhizopus suinus* (15) numerous investigations have been conducted on the production of auxins by fungi (7). Some fungi produced auxin in the absence of an added precursor in the medium (16), although such synthesis usually required the addition of tryptophane (1, 15, 16, 18). This amino acid has generally been accepted as the primary precursor to IAA in both chlorophyllous and non-chlorophyllous plants. In most earlier investigations the production of IAA by fungi was determined by the Avena curvature test or some related bioassay (7). Recently employment of chromatographic methods and chemical reagents for the identification of specific compounds has supplied evidence for a variety of indole derivatives as the final products of tryptophane metabolism and added to our knowledge of biosynthetic pathways in lower organisms (8, 16, 18, 19).

Gentile and Klein (2), as part of a study to determine the possible importance of IAA in fungal growth, concluded that *Diplodia natalensis* Pole-Evans synthesized IAA with or without tryptophane added to the medium. IAA was determined by the procedure of Gordon and Weber (4). This method, however, gives similar color reactions with a number of indole derivatives and is not specific for IAA. In fact, preliminary chromatograms of extracts of culture fluids run in this laboratory suggested that other indoles as well as IAA are products of Diplodia metabolism.

The purpose of this study has been to separate and identify indole compounds synthesized by Diplodia when grown in synthetic media with and without added precursors and to determine whether these compounds possess growth-promoting properties. From the results obtained the possible pathways of biosynthesis of the indole compounds produced by this organism are considered.

**Materials & Methods**

> Culture of Organism. *Diplodia natalensis* Pole-Evans was obtained from Dr. R. M. Klein of the New York Botanical Garden and cultures were maintained on potato-sucrose agar and stored at 6 to 8 C. Two hundred grams fresh potato were autoclaved in 800 ml distilled water and strained through cheesecloth. To this broth were added 20 g sucrose, 15 g

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agar, 5 g yeast extract, and water to 1 liter, pH 6.8. For experimental purposes the liquid medium of Gentile and Klein was used (2). Where added, tryptophane or tryptamine were in a final concentration of 5 × 10^{-3} M. The medium was distributed in 50 ml quantities in twelve, 250 ml Erlenmeyer flasks, autoclaved, and inoculated with uniform plugs of mycelium grown on the potato-sucrose agar. Incubation was at 30°C for standing cultures and room temperature for shake cultures.

**Extraction.** After 7 or 10 days incubation, replicate cultures were combined (12 × 50 ml) and the culture fluid separated from the mycelium by suction filtration. The filtrate was adjusted to pH 2.9 as suggested by Gordon and Nieva (3), and extracted three times with peroxide-free ether. The ether extracts were pooled and reduced to 100 ml under vacuum and this volume was then extracted three times with a 2% aqueous solution of NaHCO₃ to remove the acid fraction, leaving the neutral fraction in the ether. The aqueous portion was readjusted to pH 2.9 and the acid fraction taken back into ether. The acid and neutral fractions were placed in a freezer for 3 hours to remove water, transferred to dry containers, and evaporated to dryness on a steam bath. The residues obtained were redissolved in 0.25 ml redistilled methanol and refrigerated.

The mycelium from the above cultures was washed thoroughly to remove all residual medium and the excess water removed by suction filtration. The tissue was ground in a mortar and extracted three times with ether at 3°C. Extracts were combined and acid and neutral fractions separated as with the culture fluids.

**Chromatography.** Separation of indole compounds was accomplished by paper chromatography using Whatman No. 1 filter paper. The solvent used was isopropanol, ammonia (28%), and water (10:1:1) (13). When two-dimensional chromatograms were necessary, the second solvent was isopropanol, glacial acetic acid, and water (4:1:1) (8). Both ascending and descending methods were employed, but best results were obtained using the ascending technique. Sheets of filter paper were spotted 2.5 cm from the bottom with 0.1 ml aliquots of the methanol solutions which were applied by means of drawn glass capillaries and dried with a small hair dryer. The sheets were equilibrated in a solvent atmosphere for 8 hours and then lowered into solvent troughs. Chromatograms were allowed to develop for approximately 12 hours, at which time the solvent front had moved 25 to 30 cm from the point of origin. Temperature was 22 ± 1°C.

The papers were dried and then sprayed with modified Salkowski reagent to detect the resolved indole compounds (4). Salkowski reagent was chosen because of its high sensitivity (0.1 μg IAA) (13) and near specificity for indole. It is recognized that this level of sensitivity is for the pure compound and cannot necessarily be expected for crude extracts or other indole compounds. Further there are reports of color occurring in the presence of phenolic compounds (14) as well as the interference of phenolic compounds in this reaction (12). Since we are concerned solely with the identification of indole compounds, and in recognition of the limitations of the Salkowski method, we have used this reagent in conjunction with other techniques for the identification of indole compounds. It is possible that indole compounds present in concentrations below the level of sensitivity of the Salkowski reagent were not detected.

**Spectrophotometry.** Absorption maxima in the visible region were determined for reaction products of the separated compounds with Salkowski reagent. Ultra violet (uv) spectra of the separated compounds in methanol were determined on a B. & L. Spectronic 505 recording spectrophotometer. One portion of a chromatogram on which a number of the same aliquots had been separated was sprayed with Salkowski reagent to determine the location of the compounds. Corresponding areas on the un sprayed portion were eluted with 3 ml of methanol and the uv spectra recorded from 220 to 350 nm. For the absorption maximum in the visible range, 2 ml of Salkowski reagent were added to 1 ml of methanol containing the eluted compound (4) and the absorption peak determined between 400 and 600 nm. Rates of color development of the eluates varied, but, once developed, the color proved to be stable for more than 24 hours.

**Biological Assay.** The wheat coleoptile straight growth test as developed by Nitsch and Nitsch (11) was employed using Dural variety wheat to assay the growth-promoting properties of the compounds from the acid fractions after separation by chromatography. 4 mm sections cut 3 mm from the tip of 3 day old coleoptiles, which were approximately 2.5 cm long, were added to culture tubes which contained 2 ml of 2% sucrose, pH 5.1, and discs of filter paper cut from Salkowski-sensitive areas on developed chromatograms. Controls were run with discs of blank filter paper and known amounts of IAA with every bioassay. The variety of wheat used in these assays was found to be as sensitive to IAA as the variety used by Nitsch and Nitsch (11). All sections were presoaked for sensitization. After 20 hours the length of test sections was compared with that of the controls to determine auxin activity. Throughout the experiment coleoptiles were kept in the dark or handled under a red photographic safety lamp.

**Experiments With C¹⁴-Labeled Tryptophane.** For cultures incorporating tryptophane 2° C¹⁴ (α-carbon of side chain) 5 μc of the labeled compound were appropriately diluted with unlabeled tryptophane to obtain the 5 × 10⁻⁸ M final concentration in the medium. Chromatograms of extracts of this medium were sprayed with Salkowski reagent to locate the spots. Equal sized discs were cut from the center of each Salkowski reactive spot and their radioactivity counted. To determine approximately the degree which each radioactive compound accumulated, the net CPM were multiplied by the total area of the colored spot.
Results

Maximum growth of the mycelium appeared to have taken place after 6 to 7 days in standing cultures and after 5 to 6 days with shaking. The chromatographic results were the same with both methods of incubation and metabolic end products were not altered by the longer growth period of 10 days. In Table I are listed the various chromatographic, spectrophotometric, and growth-promoting properties of the extracts with those of standard compounds. Biological activity was tested with the compounds from the acid fractions only because generally it is considered that an acid group on the side chain of the indole ring is necessary for auxin action (9).

Spot No. 1. This spot appeared only on chromatograms of the neutral fraction of the medium which had been supplemented with tryptamine. However, from the uv spectrum (fig 1) it is questionable whether this compound contains the indole ring (8). Comparison with indolealdehyde (fig 2), often found in the neutral fraction of plant extracts (20) at a similar Rf (13), leaves little doubt that they are not identical.

Spot No. 2. This spot occurred at Rf 0.77 of the tryptamine medium neutral fraction and also on chromatograms of the neutral fraction of the tryptophane supplemented medium. Eluates of this spot taken from chromatograms of both media exhibited broad undefined peaks in the visible spectrum after reaction with Salkowski reagent (table 1). However, the overall character of the curves of each resemble one another in both the visible and uv ranges (fig 3, 4), and both gave a yellow fluorescence in uv light. It was concluded that they were the same substance. The uv spectrum displayed indolic characteristics and comparison of the chromatographic and spectrophotometric qualities of this compound with the findings of Stowe and Thimann (13) suggested that it was possibly the indolic alcohol, tryptophol. Kaper and Veldstra (8) reported tryptophol to be present in cultures of Agrobacterium tumefaciens, presumably a product of indoleacetaldehyde reduction. Synthetic tryptophol was not available during this study and until comparison by co-chromatography is accomplished, identification of this spot (No. 2) must remain tentative.

Spot No. 3. From the uv spectrum of spot No. 3 (fig 5), found only in the acid fraction of the tryptophane medium it seemed questionable whether the compound was an indole derivative. Studies with tryptophane 2' C14 demonstrated that at least the α-carbon of the side chain was not present. The complete disappearance of the spot from chromatograms of the cultures without tryptophan suggested that this compound was derived from tryptophan or a derivative of tryptophan.

Spot No. 4. This compound was obtained from the culture fluid and mycelium from tryptophane and tryptamine supplemented media. It has been identified as indoleacetic acid, which gave an identical uv spectrum.

Fig. 1. Spot No. 1; Rf 0.83. This compound was obtained from the neutral fraction of tryptamine supplemented medium. It has an Rf value very close to that of indolealdehyde, but is not indolealdehyde (see fig 2).

Fig. 2. Ultra violet absorption spectrum of indolealdehyde.

Fig. 3-4. Spot No. 2; Rf 0.77. This compound was obtained from the neutral fractions of tryptamine (fig 3) and tryptophane (fig 4) supplemented media. It has been identified tentatively as tryptophol.

Fig. 5. Spot No. 3; Rf 0.43. This compound was obtained from the acid fraction of tryptophane supplemented medium only. Its uv spectrum suggests that it is not an indole derivative.

Fig. 6. Spot No. 4; Rf 0.25. This compound was obtained from the culture fluid and mycelium from tryptophane and tryptamine supplemented media. It has been identified as indoleacetic acid, which gave an identical uv spectrum.
Fig. 7. Two-dimensional chromatogram of the acid fraction from the tryptophane supplemented medium in which Diplodia was grown. Indoleacetic acid and indolelactic acid samples were chromatographed in the second dimension only for comparison with unknowns. This figure indicates the resolution of spots Nos. 4 and 5, which were superimposed following one-dimension chromatography of the fraction. These spots are identified as indoleacetic acid and indolelactic acid, respectively.
grams using the acetic acid solvent suggests that the substance is acid labile. Since the compound appears only in this fraction it seems certain that it is produced from tryptophane.

- **Spot No. 4.** Spot No. 4 appeared on chromatograms of the acid fraction of each of the different media used as well as on those of the acid fraction of the mycelium grown in the tryptophane medium. The color reaction, R<sub>t</sub>, and fluorescence of this lavender spot suggested that the compound might be IAA, the presence of which had been assumed by Gentile and Klein (2). Eluates of this compound were taken by the method of Gordon and Paleg (5) from a number of chromatograms of each different fraction. When reacted with Salkowski reagent, each eluate displayed an absorption peak at 535 nm, that of IAA. Assay by the wheat coleoptile test showed positive auxin activity and comparative chromatography with synthetic IAA confirmed that in each case this compound was indeed IAA.

It was noted that spot No. 4 was far more intense on chromatograms from the tryptophane supplemented medium. Evaporation of the ether from the acid fraction following extraction from the medium originally containing 5 x 10<sup>-9</sup> mg tryptophane left a residue of crystals. These, when redissolved in methanol and chromatographed, revealed an intense IAA spot. Preliminary experiments showed that, although tryptophane is relatively insoluble in ether, if it is present in aqueous solution detectable amounts will be carried over by the extraction technique employed. Evidence of unmetabolized tryptamine was not found on chromatograms of either fraction. Although quantitative determinations were not undertaken, it would appear that tryptamine is readily converted to IAA by Diplodia. The IAA spot was extremely weak on chromatograms of the basic medium and some difficulty was encountered in detecting IAA in the tryptophane medium.

- **Spot No. 5.** This compound, present only in the acid fraction of the medium with added tryptophane, appeared as a bright yellow spot at the R<sub>t</sub> attended by IAA in extracts from the basic and tryptophane media. Chromatographic and spectrophotometric analysis showed the compound to be identical in all respects to indolelactic acid, except that this spot was definitely active whereas indolelactic acid shows no growth-promoting properties (10). Kaper and Veldstra (8) stated that IAA and indolelactic acid coincided on chromatograms using the isopropanol, ammonia, water solvent, but were separated on two-dimensional chromatograms using isopropanol, acetic acid, and water for the second solvent. Application of this technique in initial experiments with Diplodia failed to produce a separate spot corresponding to IAA. In later experiments, in which C<sup>14</sup>-labelled tryptophane was used, chromatograms were spotted with larger aliquots of extract to insure sufficient radioactivity on developed papers. The second dimension of these chromatograms (fig 7) revealed, in advance of the yellow spot (No. 5) a faint lavender spot, which, although extremely weak was concluded

### Table 1

<table>
<thead>
<tr>
<th>Reference compounds</th>
<th>R&lt;sub&gt;t&lt;/sub&gt;</th>
<th>Salkowski characteristics</th>
<th>Auxin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indolealdehyde</td>
<td>0.80</td>
<td>Orange</td>
<td></td>
</tr>
<tr>
<td>Tryptophane*</td>
<td>0.70</td>
<td>Yellow-brown</td>
<td></td>
</tr>
<tr>
<td>Tryptamine</td>
<td>0.65</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Indolelactic acid</td>
<td>0.25 (0.77)**</td>
<td>Yellow</td>
<td></td>
</tr>
<tr>
<td>Indoleacetic acid</td>
<td>0.25 (0.87)**</td>
<td>Lavender</td>
<td></td>
</tr>
<tr>
<td>Tryptophane</td>
<td>0.18</td>
<td>Yellow</td>
<td></td>
</tr>
</tbody>
</table>

** Diplodia compounds **

| Spot No. 1          | 0.83        | Lavender                  | No            | Not tested |
| Spot No. 2          | 0.77        | Yellow-brown              | Yes           | Not tested |
| Spot No. 3          | 0.43        | Lavender                  | Yes           | Inactive   |
| Spot No. 4          | 0.25 (0.87)** | Lavender                 | Yes           | Active     |
| Spot No. 5          | 0.25 (0.77)** | Yellow                  | Yes           | Not tested |
| Spot No. 6          | 0.21        | Pink***                   | Yes           | Inactive   |

* Data of Stowe and Thimann (13)
** Isopropanol-acetic acid-water
*** Color develops after 6 to 12 hours.

Fig. 8. Ultraviolet absorption spectrum of spot No. 5. This compound is identified as indolelactic acid, which gave an identical uv spectrum.

Fig. 9. Ultraviolet absorption spectrum of spot No. 6, R<sub>t</sub> 0.21. This compound was present in all fractions from the culture fluid and mycelium, but remains unidentified. Its uv spectrum is, however, typical of those obtained from indolic compounds.
to be IAA by comparative chromatography. Thus it became clear that spot No. 5 was identical with indolelactic acid and indeed masked IAA on chromatograms using basic solvent. The uv absorption spectrum of the yellow spot was identical with that of pure indolelactic acid when tested.

Spot No. 6. This pink spot was present in all fractions from both the media and mycelium in each experiment. In all cases it was more strongly concentrated in the acid fractions of the media and its position on chromatograms indicated that the substance is slightly more acidic than IAA. The presence of spot No. 6 in neutral fractions is presumably due to incomplete separation into the acid fraction.

The pink color developed slowly, appearing first 6 to 12 hours after spraying and becoming more intense and fluorescent with time. Because of its atypical color characteristics, the possibility that this spot was caused by a phenolic substance was considered, but the uv absorption curve (fig 9) suggested strongly that the compound contained the indole nucleus. A spot of similar Rf and color reaction was tentatively identified as indoleglycolic acid by Kaper and Veldstra (8). However, indoleglycolic acid is reported to be active as an auxin (6) whereas this spot was found to be inactive in our bioassay. Attempts to identify it as any other indole derivative were unsuccessful.

Table II

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Compound</th>
<th>Tryptophol ?</th>
<th>Indoleacetic acid</th>
<th>Indolelactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Net cpm</td>
<td>$13 \times 10^2$</td>
<td>$11 \times 10^2$</td>
<td>$134 \times 10^2$</td>
<td></td>
</tr>
<tr>
<td>Spot area</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* As determined from cpm and spot size.

Cultures of Diplodia containing $^{2}C^{4}$-labelled tryptophane, when extracted and chromatographed, confirmed that spots Nos. 5 and 2, and probably spot No. 4 were derived from tryptophane. Table II shows the approximate relative degree to which each compound accumulated under the culture conditions.

The mycelium which developed in each of the three variations of media yielded only spot No. 6 with the exception of that grown in the tryptamine medium. This mycelium produced a strong IAA spot even after thorough washing to remove residual compounds present in the medium only.

Discussion

IAA was synthesized by *Diplodia natansis* in a synthetic medium using only asparagine as a nitrogen source. Neither indolepyruvic acid, tryptamine, nor indoleacetaldehyde, compounds which have been reported as intermediates in the synthesis of IAA, were found in quantities detectable by paper chromatography. Possibly, the unidentified spot No. 6 may be an intermediate compound in the pathway to IAA. The appearance of this spot with similar intensity on chromatograms of the basic medium as well as on those from media containing tryptophane or tryptamine suggests that it is not a product of tryptophane or tryptamine metabolism.

At the end of the culture period residual tryptamine could not be detected in cultures to which this compound initially had been added. Conversion of tryptamine to other substances, predominantly IAA and possibly tryptophol, would seem complete, which may explain why tryptamine is not usually found in culture extracts (8, 16). Wolf (18) found that *Ustilago zeae* could not synthesize IAA from tryptamine, whereas Gordon and Nieva (3) found that it was readily converted to IAA by spinach leaves. No evidence was obtained for a compound which could be identified as indoleacetaldehyde, although presumably tryptamine is first deaminated to the aldehyde. If the aldehyde is an intermediate in the synthesis of IAA from tryptamine by this organism, then it appears to be predominantly oxidized to the acid, although possibly some is reduced to the alcohol, tryptophol. Evidence for a dismutation reaction of this type in *Agrobacterium tumefaciens* was presented by Kaper and Veldstra (8).

The experiments with $^{2}C^{4}$-labelled tryptophane demonstrated that tryptophane proceeded to indolelactic acid far more extensively than to tryptophol or IAA. Probably indolepyruvic acid serves as the intermediate in the synthesis of these end products. Kaper and Veldstra (8) determined that synthetic indolepyruvic acid displayed a distinct septenary pattern of decomposition products when chromatographed in basic solvent. The appearance of this same pattern in culture extracts of *Agrobacterium tumefaciens* confirmed the presence of indolepyruvic acid in the medium. The chromatograms of *Agrobacterium tumefaciens* extracts grown under aerobic conditions with tryptophane showed indolelactic acid, tryptophol, and IAA. However, because IAA was also one of the non-enzymatic breakdown products of indolepyruvic acid it could not be determined whether IAA arose as a physiological end product or as a result of the spontaneous decomposition of indolepyruvic acid. No evidence for the presence of indolepyruvic acid was obtained from the experiments with Diplodia and it must be concluded that, if this compound is at all synthesized, it is rapidly converted to other end products. Although measures of radioactivity indicate that at least some of the IAA present was derived from tryptophane, the fact that IAA can arise from the spontaneous breakdown of indolepyruvic acid in solution (17) prohibits the conclusion that IAA is solely an end product of a series of enzymatic reactions. A complete separation of indolelactic acid and IAA on chromatograms was not
possible because the large quantities of extract necessary to show the IAA color caused overrunning of this spot by the strongly radioactive indoleacetic acid. Consequently, there was the possibility of contamination of the IAA spot by a small amount of radioactive indoleacetic acid. Since Diplodia is able to synthesize IAA in similarly small amounts in the absence of tryptophane, it is possible that the synthesis follows a pathway not including tryptophane. Although it is evident that the fungus readily converts tryptamine to IAA, proof that this compound serves as the natural precursor cannot be offered at this time.

Based on the evidence obtained the following reactions involving certain indole compounds and their precursors are suggested as a part of the metabolism of Diplodia natalensis.

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\begin{align*}
RCH_2CHHNH_2COOH & \xrightarrow{AH_2} RCH_2COOH
\end{align*}
\]

**Summary**

*Diplodia natalensis* Pole-Evans was grown aerobically in synthetic medium with or without added tryptophane or tryptamine. Chromatographic and spectrophotometric techniques were used to separate and identify the indole compounds produced by this organism.

Indoleacetic acid (IAA) was found to be a metabolic product in the absence of added precursor and was the only indole compound detected which possessed growth-promoting properties. When tryptamine was added to the medium it was readily converted to IAA. Chromatograms of the mycelium which had grown in the tryptamine supplemented medium revealed a strong IAA spot. Using TTP 2° C it was determined that indoleacetic acid is the predominant end product when tryptophane is added to the culture medium, and only small amounts of IAA are synthesized. Another indole compound, tentatively identified as tryptophol, was present in the extracts from both the tryptamine and tryptophane media.

An unidentified spot was found on chromatograms of extracts of both the culture fluids and mycelium of all the three medium variations. From its ultraviolet spectrum it seems to be indolic in nature, although it is questionable that this substance is a product of either tryptamine or tryptophane metabolism.

### Literature Cited