The Metabolism of $^{14}$C-Labeled Isatin and Anthranilate in Pisum Stem Sections$^{1,2}$

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Abstract. Isatin, previously shown to promote growth in green and etiolated pea stems, is converted mainly to anthranilate in those tissues; small amounts of isate are also formed. Fed anthranilate is converted mainly to its $\beta$-D-glucoside; smaller amounts are metabolized to anthranilamide, tryptophan and kynurenine. These data provide some basis for understanding the growth promoting activity of isatin.

The auxin activity of isatin (4) is surprising since its structure lacks many features, generally considered essential to an auxin molecule (22). The demonstrated in vitro conversion of isatin to isate (1) yields a molecule structurally closer to the usual conception of an auxin, but still does not explain its activity. Since high concentrations (ca. 0.1 mM) of isatin and isate are required to elicit growth responses, the possibility remains that both compounds are converted to some other molecule, which is a more active auxin. We have therefore studied the detailed metabolism of $^{14}$C-labeled isatin in pea plants, and have found that the predominant metabolite is anthranilic acid. Since information about the metabolism of anthranilic acid in plants is only fragmentary (7, 15, 16, 20, 21, 23) we undertook investigation of this compound as well.

Materials and Methods

Plant Material. Green or etiolated pea plants (Pisum sativum L. cv. Alaska) grown as previously described (5), were used for incubation experiments and for bioassays. Subapical stem sections 5 mm in length were cut from 7 day-old etiolated and 12 to 14 day-old green plants. Etiolated stem sections were cut under a dim green photomorphogenically inactive light (5); green sections were cut in the laboratory.

Chemicals. All chemicals used were of analytical purity. Beta-glucosidase from almonds (Schwarz) had a specific activity of 3.5 U/mg. Potassium isate was synthesized according to Stefanovic et al. (19). Benzene ring labeled isatin-$^{14}$C was synthesized as described by Marvel and Hiers (12). Aniline-$^{14}$C hydrochloride, U.L., 5 $\mu$C/m mole (New England Nuclear Corp.) was diluted with unlabeled aniline and reacted with chloralhydrate and hydroxylamine to form isonitrosoacetanilide. This was isolated and heated with H$_2$SO$_4$ to give isatin, which was recrystallized from water. The yield of isatin prepared from 100 or 200 mg of aniline was ca. 42% of the theoretical; e.g. 200 mg of aniline with total activity 250 $\mu$C yielded 132 mg of isatin-$^{14}$C with a specific radioactivity 65 $\mu$C/m mole. Ring labeled anthranilic acid-$^{14}$C was synthesized from isatin-$^{14}$C by oxidation with H$_2$O$_2$ in an alkaline solution at 10 to 15° and was recrystallized from water (17). By this procedure anthranilic acid-$^{14}$C of specific radioactivity 129 $\mu$C/m mole was prepared from isatin-$^{14}$C of specific radioactivity 97 $\mu$C/m mole. The practical yield of crude anthranilic acid was 70 to 89%, even from 100 mg isatin.

The identity and purity of the synthesized isatin-$^{14}$C and anthranilic acid-$^{14}$C were established by radiochromatography, color reactions on the chromatograms, comparison of ultraviolet spectra, and melting points.

Tissue Incubation. Five grams of subapical stem sections of green or etiolated peas were incubated in petri dishes with 10 ml of isatin and anthranilic acid solutions at concentrations of 1 mM or 5 mM. Experiments were performed at room temperature either in complete darkness (etiolated sections) or in light from cool white fluorescent and incandescent lamps, ca. 3000 ft-c (green sections). The incubation period was 3 or 6 hr, unless otherwise stated.

All experiments were first performed with non-radioactive substrates.

Extraction and Preliminary Partition of Metabolites. At the end of the incubation period, the sections were removed from the substrate solution, thoroughly washed with redistilled water, dried between filter papers and boiled 3 times for 2 min.
each in 20 to 30 ml of methanol. After cooling, the samples were stored overnight at 4°. The combined methanolic extract was filtered, taken to dryness in a rotary evaporator and the residue dissolved in 25 ml of warm water.

The metabolites of isatin present in the extract were divided into 3 groups, according to their solubility in ethyl acetate (Fig 1). The extraction with 25 ml of ethyl acetate was repeated 5 times. As isatate is converted to isatin in strongly acidic media, such conversion was minimized by the acidification of the aqueous phase by acetic acid; the ethyl acetate extract was then made alkaline with ammonia and re-extracted with water before evaporation. Conversion of isatate was monitored by including unlabeled isatate during the extraction. In the case of radioactive substances the partial solubility of ethyl acetate in water contributed to a slight overlapping of the groups, which was only slightly improved by further extraction.

**Paper Chromatography of the Extracts.** The metabolites in the extracts were separated by paper chromatography on 4 cm wide strips of Whatman No. 1 paper. The chromatographic systems used were:

1) Acidic: benzene — acetic acid — water 2:2:1 v/v/v. 2) Neutral: a) n-butanol saturated with water, b) n-butanol — benzene — water 1:1:1 v/v/v. 3) Alkaline: isopropanol-ammonia (28 %) — water 8:1:1 v/v/v.

The alkaline system gave the best separation, but the stability of the β-glucoside of anthranilic acid in it was poor. Acidic systems, on the other hand, partially decomposed isatate. System 2c served to separate tryptophan from kynurenine. The spots of metabolites were usually located on the chromatograms in UV light and detected by p-dimethylaminocinnamaldehyde reagent (PDACA; 1 % solution in a mixture of equal parts of 37 % HCl and alcohol. w/v), Prochaska's reagent (40 % formaldehyde-37 % HCl-water 1:1:2 v/v/v), Ehrlich's or ferric chloride reagents (6). For the specific detection of isatin and isatate as blue spots, the proline reagent (0.1 % solution of proline in 4 % acetic acid w/v) was suitable. For their visualization as pink spots, the indican reaction (0.1 % solution of potassium indoxyl sulphate in 1 N HCl w/v) was effective (3). Anthranilic acid could be specifically detected as a

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**Table I. Chromatographic Data for Some Substances Related to Isatin and Anthranilate**

<table>
<thead>
<tr>
<th>Substance</th>
<th>System 1</th>
<th>System 2a</th>
<th>System 2b</th>
<th>System 3</th>
<th>UV</th>
<th>Detection PDACA</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-L-Kynurene</td>
<td>0.00</td>
<td>0.00</td>
<td>0.10</td>
<td>0.17</td>
<td>0.07</td>
<td>0.09</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>0.03</td>
<td>0.04</td>
<td>0.10</td>
<td>0.17</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Xanthurenic acid</td>
<td>0.03</td>
<td>0.04</td>
<td>0.10</td>
<td>0.17</td>
<td>0.10</td>
<td>0.13</td>
</tr>
<tr>
<td>3-Hydroxyanthranilic acid</td>
<td>0.06</td>
<td>0.08</td>
<td>0.53</td>
<td>0.91</td>
<td>0.69</td>
<td>0.83</td>
</tr>
</tbody>
</table>
| Isatate              | Decomp   | Decomp    | 0.10      | 0.17      | 0.08| 0.17            | 0.64 1.16 | ... ...

... Weak blue Violet...

| Isatin               | 0.43     | 0.55      | 0.76      | 1.31      | 0.74| 0.98            | 0.82 1.49 | Weak blue Violet |
| p-Aminobenzoic acid  | 0.44     | 0.56      | 0.54      | 0.93      | 0.64| 0.85            | 0.37 0.67 | Dark blue Blue   |
| Anthranilamide       | 0.49     | 0.63      | 0.65      | 1.12      | 0.64| 0.85            | 0.78 1.42 | Yellow-blue Blue |
| Anthranilic acid     | 0.78     | 1.00      | 0.58      | 1.00      | 0.75| 1.00            | 0.55 1.00 | Blue Blue Blue   |
| Anthranilonitrile    | 0.83     | 1.06      | 0.84      | 1.44      | 0.79| 1.06            | 0.88 1.60 | Blue Blue Blue   |

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violet-red spot by the Ekman dipping reagent (Solution I: 2 N HCl-acetone-5% NaNO₂ 5:45:1, v/v/v; solution II: 5% solution of ethyl-α-naph-thylamine in ethanol, v/v) (18).

Chromatographic data for some of the compounds are collected in table 1. Unknown metabolites are designated by their RF in system 3.

Bioassays. For evaluation of the biological activity of pure substances or metabolites, straight growth test on 5 mm subapical pea stem sections (5) and straight growth test on wheat coleoptile sections (14) were used.

Determination of Radioactivity. Zones of radioactivity on the chromatographic strips were located by the Vanguard paper strip counter. The radioactivity of zones eluted with methanol (or methanol-water 2:1, v/v) was determined in an Anistron liquid scintillation spectrometer.

UV Spectra. UV spectra were determined by a Perkin-Elmer recording spectrophotometer, model 350.

Enzymatic Degradation of the β-glucoside of Anthranilic Acid. An equal volume of almond β-glucosidase solution (1 mg/1 ml) was mixed with an aqueous solution of the extracted spot of the unknown substance RF 0.67. The control treatment was similar, but lacked enzyme. The mixtures were incubated at 37° and at definite time intervals (5–120 min) samples of the incubated solutions were withdrawn and mixed with the same volume of ethanol. After centrifugation the supernatant liquid was analyzed chromatographically.

Results

Biological Activity. Anthranilic acid was ineffective in promoting growth of pea stem sections, but isatin and isatate promoted elongation at concentrations of 0.1 mM and higher (fig 2). Isatate was slightly more effective than isatin, and both were less active than IAA. Fresh weight increase gave the same picture. These results corroborate earlier reports (1,4).

Metabolism of Isatin-14C in Green and Etiolated Tissues. Both etiolated and green pea stem sections were incubated for 45 min to 24 hr in 1 mM or 5 mM solutions of benzene ring-labeled isatin-14C. After 6 hr. the uptake of isatin-14C by etiolated sections was greater than by green sections (40.7 % vs 26.5 %). The radioactive substances in the extract were fractionated into 3 groups, as outlined in figure 1. The activity was ca. 95 % in extract I, 4 % in extract II, and 1 % in extract III, in both green and etiolated tissue.

Most of the label in extract I was located in a spot identified by the RF values and color reactions as unmetabolized isatin (fig 3). The second most prominent radioactive zone fluoresced blue in UV light and had RF values corresponding to anthranilic acid. Its identity was confirmed: (1) by the specific Ekman reagent; (2) by co-chromatography with cold and labeled anthranilic acid in systems 1, 2a, and 3; (3) by its UV spectrum (max. 320 μ and minimum 273 μ standard substance, max. 320 μ and minimum 279 μ extracted substance). Other labeled spots included substances with RF values of 0.0, 0.67, and 0.85 in system 3. The first, with RF 0.0 in system 3, giving a blue-violet spot when reacted with p-dimethylaminocinnamaldehyde, also had an RF 0.0 in system 1. It is probably a degradation product of isatin, since it also appears after chromatography of old isatin stock solutions. The substance with an RF of 0.67 was later identified as the β-glucoside of anthranilic acid, while the spot with an RF 0.85 is probably anthranilamide.

Extract II contained much less radioactivity. The chromatogram in system 3 showed a small peak of isatin and a peak of isate which separated in system 2a into a minor isate peak and a major anthranilate peak. Extract III contained only a small amount of activity, corresponding to substances overlapping from previous extracts. The weak activity coincided with zones for tryptophan and kynurenine.

After 6 hr of incubation, the balance of the conversion of isatin-14C was computed from the totals of all 3 extracts (table II). Isatin was transformed slowly in both etiolated and green peas, 75 and 63 %, respectively, remaining unconverted after 6 hr. The major product was anthranilic acid, which formed more readily in green sections than in etiolated ones. Minor products included the β-glucoside of anthranilic acid and a trace of isate. The incubation mixtures presented essentially the same picture as the segments.

Metabolism of Anthranilic Acid-14C. After 0 hr of incubation in 1 mM anthranilic acid-14C, etiolated sections (table II). The weak activity was the same as that in green sections 44.0 %
of the applied radioactivity in methanolic extracts. For chromatographic analysis, the methanol extracts were divided into 2 groups: those soluble in ethyl acetate (83% of the radioactivity) and those soluble in water (17%).

The ethyl acetate extract contained, in addition to the unmetabolized anthranilic acid, a conspicuous new substance appearing soon after the beginning of the incubation period and rapidly increasing in quantity with time (fig 4). This substance was

<table>
<thead>
<tr>
<th>Zone</th>
<th>Etiolated plants</th>
<th>Green plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>% Of Total</td>
</tr>
<tr>
<td>0.0</td>
<td>6,210</td>
<td>0.5</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>198,960</td>
<td>16.0</td>
</tr>
<tr>
<td>Isatate</td>
<td>9,692</td>
<td>0.8</td>
</tr>
<tr>
<td>β-Glucoside of anthranilic acid</td>
<td>70,332</td>
<td>5.6</td>
</tr>
<tr>
<td>Isatin</td>
<td>931,119</td>
<td>75.2</td>
</tr>
<tr>
<td>Anthranilamide</td>
<td>23,942</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Table II. Balance Sheet for Isatin-14C Absorbed and Metabolized by Pea Stem Sections

The boiling methanol extraction procedure was used.
tentatively designated as the \( \beta \)-glucoside of anthranilic acid (21). Another labeled substance, with an \( R_F \) value corresponding to that of anthranilamide, appeared in smaller amounts.

Identification of the \( \beta \)-glucoside of anthranilic acid was performed, as follows: 1) The substance showed a bluish fluorescence in UV light on chromatograms which was less intense than that of anthranilic acid. 2) It reacted with \( p \)-dimethylaminocinnamaldehyde, giving an intense red-violet spot. 3) After elution from chromatograms it was incubated with \( \beta \)-glucosidase, during which anthranilic acid was rapidly released (fig 5). Anthranilic acid and glucose also resulted from prolonged acidic hydrolysis with 2 N HCl. 4) After rechromatography in alkaline system 3, the original substance was partially degraded, giving traces of anthranilamide and anthranilic acid, but most of the glucoside was still intact (16). The less active, more hydrophobic spot of \( R_F \) 0.85 was identified as anthranilamide by its \( R_F \) values in 3 systems and by the specific pink color of the spot with \( p \)-dimethylaminocinnamaldehyde, which appears several hrs after spraying. Extract III contained some traces of anthranilic acid and its glucoside, but a new radioactive zone in system 3, distinctly separated from anthranilic acid, appeared near \( R_F \) 0.35 to 0.40. After rechromatography of this zone in system 2c, labeled tryptophan and kynurenine were clearly demonstrable (fig 6).

![Diagram](https://via.placeholder.com/150)

**Fig. 7.** Scheme of isatin and anthranilate metabolism in pea plants. Only the underlined compounds were identified in this investigation.

The substrates remaining after incubation again contained the same components as present in the extracts from plant material.

The balance of the conversion of anthranilic acid in etiolated and green segments is presented in table III. Anthranilic acid was more rapidly taken up and more actively metabolized than isatin, especially in green plants. The main transformation product was the \( \beta \)-d-glucoside; anthranilamide appeared to a much smaller extent. Another metabolic pathway leads to the formation of small quantities of kynurenine and tryptophan.

**Discussion**

Figure 7 summarizes what we think we understand of the metabolism of isatin in pea stem tissue. The main pathway of this metabolism leads to the formation of anthranilic acid. *via* opening of the bond between the \( \alpha \) and \( \beta \) carbons of the pyrrole ring. A similar degradation is known for tryptophan, in which splitting of the bond between the \( \alpha \) and \( \beta \) carbons of the pyrrole ring is catalyzed by the enzyme l-tryptophan: \( \text{H}_2\text{O}_2 \)-oxidoreductase (10, 13). The second pathway of degradation of isatin, initiated by fission of the bond between the \( \alpha \)-carbon and nitrogen of the pyrrole ring, leads to the formation of isatate (o-aminophenylglyoxylate). This process may well be non-enzymatic, for as previously described, its formation can occur *in vitro* in aqueous solutions of isatin (1). The *in vivo* yield of isatate was low, and this total amount might be due to non-enzymatic formation. When fed to pea tissue, isatate appears not to be converted to anthranilate.

The metabolism of externally supplied anthranilic acid in plants also proceeds in 2 directions: by the pathway leading to detoxification (*via* glucosylation and amide formation) and by the pathway leading to the formation of tryptophan. An analogous production of glycoside has been described earlier for IAA (8, 11, 25), benzoic acid and naphthylacetic acid (9). The pathway leading to tryptophan formation from anthranilic acid, well studied in microorganisms and animals, occurs also in plants, but at a lower efficiency (24). We do not understand why anthranilate promotes growth in *Avena* coleoptiles (24) but not in pea epicotyl tissue.

**Table III. Balance Sheet for Anthranilate-\(^{14}\)C Absorbed and Metabolized by Pea Stem Sections**

<table>
<thead>
<tr>
<th>Zone</th>
<th>Etiolated cpm</th>
<th>% Of total</th>
<th>Green plants cpm</th>
<th>% Of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptophan + kynurenine</td>
<td>25,534</td>
<td>5.2</td>
<td>11,297</td>
<td>4.6</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>244,998</td>
<td>49.2</td>
<td>36,798</td>
<td>15.1</td>
</tr>
<tr>
<td>( \beta )-Glucoside of anthranilic acid</td>
<td>203,803</td>
<td>40.8</td>
<td>181,376</td>
<td>74.8</td>
</tr>
<tr>
<td>Anthranilamide</td>
<td>23,862</td>
<td>4.8</td>
<td>13,325</td>
<td>5.5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>...</td>
<td>2.1</td>
<td>...</td>
<td>1.9</td>
</tr>
<tr>
<td>Kynurenine</td>
<td>...</td>
<td>3.1</td>
<td>...</td>
<td>2.7</td>
</tr>
</tbody>
</table>
Four possibilities must be considered in an attempt to explain the auxin activity of isatin: A) Isatin may have auxin activity per se. This concept is weakened by the observation that older isatin solutions are more active than fresh solutions (1). B) Isatin may act through its conversion to isatale. This hypothesis is weakened by the very slight differences in comparative auxin activity of these substances. C) Isatin may promote growth as a precursor of tryptophan and IAA. The main difficulty with this explanation lies in the fact that anthranilic acid, the main intermediary metabolite of isatin, does not promote the growth of pea stem sections when applied externally, although it has been reported to do so in Avena (24). D) Isatin may act through formation of traces of a highly active substance not revealed on our chromatograms. On balance, it would appear that (C) is the favored hypothesis.

Isatale is a relatively unstable compound, the metabolism of which has not yet been determined in detail. After its administration to plants, a part of this compound remains unaltered after 6 hr. but a major part might be metabolized in some yet unknown way. Neither the formation of anthranilic acid, nor the formation of appreciable amounts of other p-dimethyleniminocinnamaldehyde positive spots was observed.

Acknowledgments

We thank Dr. H. Schraudolf for useful suggestions, Dr. S. Banerjee for help with growth tests, and Dr. R. Satter and Dr. P. J. Davies for useful discussions.

Literature Cited