Light-Induced Trans to Cis Conversion of \( \beta \)-D-Glucosyl \( \alpha \)-Hydroxycinnamic Acid in Melilotus alba Leaves

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Isotope studies indicate that \( \alpha \)-coumaric acid glucoside (\( \beta \)-D-glucosyl \textit{trans-\textit{o}}\)-hydroxycinnamic acid) is the immediate precursor of coumarinic acid glucoside (\( \beta \)-D-glucosyl \textit{cis-\textit{o}}\)-hydroxycinnamic acid) in sweetclover (5,7). Structures of these compounds are as follows: Preliminary reports (3,4)

\[
\begin{align*}
\text{\( \alpha \)-Coumaric acid glucoside} & \quad \text{Coumarinic acid glucoside} \\
\end{align*}
\]

suggest that this \textit{trans} to \textit{cis} conversion is a nonenzymatic photochemical reaction. However, the published data do not preclude the possibility that the reaction is effected by a light-sensitive isomerase system. The present paper deals with an investigation of the conversion of \( \alpha \)-coumaric acid glucoside to coumarinic acid glucoside in intact sweetclover leaflets and in leaflet extracts. Evidence to be presented supports the conclusion that in sweetclover leaves this \textit{trans} to \textit{cis} conversion is nonenzymatic and is induced by ultraviolet irradiation.

Materials and Methods

\textit{Plant Material.} Sweetclover (\textit{Melilotus alba} Desr.) of the \textit{CuCuBB} genotype was used in these experiments. The derivation of this genotype has been described elsewhere (2). Hot water extracts of \textit{CuCuBB} sweetclover leaves are high in content of glucosidically bound \( \alpha \)-hydroxycinnamic acid, and homogenates of such leaves contain a highly active \( \beta \)-glucosidase.

Except where otherwise indicated, plants were grown in plant growth chambers (Instrumentation Specialties Company)\(^3\), in 1-pint plastic-coated milk cartons containing a mixture of soil, sand, and vermiculite. Some of the plants were subjected to continuous light and others to a photoperiod of 19 hours. Chambers were equipped with cool white fluorescent lamps (General Electric Power Groove tubes) which provided a light intensity of approximately 1,500 ft-c at the level of the plants. The chambers were maintained at a temperature of 27\(^\circ\) and at 50\% relative humidity.

Plants from the growth chambers ranged in age from 27 to 44 days when sampled. Only the youngest fully expanded leaf was taken from each plant used.

\textit{Steam Treatment.} The aim of this treatment was the inactivation of any \textit{trans-cis} isomerase that might be present. Leaflets to be treated were held individually in a stream of steam for 10 to 15 seconds. Tests for \( \beta \)-glucosidase in the steamed leaflets indicated that this enzyme was effectively inactivated by the treatment.

\textit{Light Treatment.} Ultraviolet light: A Gates MR4 lamp equipped with the TF8 tube was used as a source of ultraviolet light (peak near 360 ma). In the experiment employing steamed leaflets, the leaflets were placed on glass microscope slides surrounded by moist filter paper during irradiation. The slides prevented extensive leaching which occurred when steamed leaflets were placed directly on moist filter paper. In all other experiments using detached leaflets, the leaflets were placed on moist filter paper or on ice cubes during irradiation. The distance from the light source to the leaflets was approximately 3.5 cm. Leaflets were turned over every 30 minutes during treatment. Leaf extract and solutions of authentic \( \alpha \)-coumaric acid and coumarinic acid glucosides (approximately 30-ml volumes) were irradiated in 50-ml beakers placed on magnetic stirrers. The distance from the light source to the surface of the solutions was approximately 3.5 cm.

Fluorescent light: Solutions were treated with cool white fluorescent light from General Electric Power Groove lamps in the growth chambers where, as previously indicated, the light intensity was approximately 1,500 ft-c. Solutions were stirred by means of magnetic stirrers during irradiation.

Sunlight: Experiments utilizing the sun as a light source were conducted on clear summer days.
with light intensities ranging from about 7,100 to over 10,000 ft-c. In the experiment on the influence of light quality on \textit{trans} to \textit{cis} conversion, detached leaflets were placed on ice cubes in metal cans that were left open to the sun or were covered by various filters. A partial characterization of the filters is given in Table I. The ice was necessary to prevent excessive wilting and shriveling of leaflets, especially in the open can and under the colorless and yellow filters. Leaflets were inverted every 30 minutes during the treatments. In another experiment the influence of unfiltered sunlight on young leaves of intact chamber-grown plants was investigated.

\textit{Assay Procedure.} Previously published procedures (3) were slightly modified for use in extraction and assay of \textit{o}-coumaric acid and coumarinic acid glucosides. The fluorometric procedure designated Method I in the cited publication was employed. Fluorescence readings were made with a Turner model 110 fluorometer equipped with primary filter 7-60 and secondary filter 8.

\textbf{Results and Discussion}

In Experiment I (table II) the effect of ultraviolet irradiation on steamed and unsteamed leaflets from greenhouse-grown plants was studied. Twelve leaflets (4 leaves) were used. One leaflet from each leaf was held in the dark as a control, one was steamed and then exposed to ultraviolet irradiation, and one was exposed to ultraviolet irradiation without prior steaming. The irradiation treatment caused decreases in the concentration of the \textit{trans} isomer and corresponding increases in the \textit{cis} isomer in both steamed and unsteamed leaflets. If the \textit{trans} to \textit{cis} conversion were dependent upon the activity of a heat-labile isomerase, great differences in conversion between the steamed and unsteamed leaflets would be anticipated. The observed difference between the treatments probably did not result from the presence of a heat-stable isomerase, but rather from the shriveling of the steamed leaflets during irradiation. Such deformation of the leaflets would be expected to decrease the effective exposure to ultraviolet irradiation, and hence to decrease \textit{trans} to \textit{cis} conversion.

In Experiment II (table II), measurements were made of the ultraviolet-induced \textit{trans} to \textit{cis} conversion occurring at room temperature and at a temperature slightly above 0°. Eighteen leaflets (6 leaves) were used. Six leaflets served as the dark controls, 6 were treated with ultraviolet light at room temperature, and 6 were placed on ice cubes during the irradiation treatment. Results indicate that conversion of \textit{o}-coumaric acid glucoside to coumarinic acid glucoside in irradiated leaflets was at least as extensive in the cold as at room temperature. This lack

<table>
<thead>
<tr>
<th>Filter*</th>
<th>Color specification</th>
<th>Approximate range µm</th>
<th>Wavelength of peak µm</th>
<th>Transmittance at peak %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorless</td>
<td>Pyrex brand glass</td>
<td>290 to &gt; 750</td>
<td>uniform, 350 to 750</td>
<td>90</td>
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<tr>
<td>Ultraviolet transmitting</td>
<td>7-60</td>
<td>310 to 390</td>
<td>355</td>
<td>70</td>
</tr>
<tr>
<td>Blue</td>
<td>5-60</td>
<td>360 to 490</td>
<td>415</td>
<td>65</td>
</tr>
<tr>
<td>Green</td>
<td>4-65</td>
<td>470 to &gt; 750</td>
<td>545</td>
<td>63</td>
</tr>
<tr>
<td>Yellow, sharp cut-off</td>
<td>3-69</td>
<td>520 to &gt; 750</td>
<td>uniform, 560 to 750</td>
<td>88</td>
</tr>
</tbody>
</table>

* Summarized from \textit{Glass Color Filters}, a bulletin of the Corning Glass Works, copyright 1948.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of plant material</th>
<th>Treatment</th>
<th>No. of leaflets</th>
<th>Total \textit{o}-hydroxy-cinnamic acid (% of fr wt)</th>
<th>Percentage trans</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Greenhouse</td>
<td>Dark</td>
<td>4</td>
<td>mean ± SE 1.30 ± 0.15</td>
<td>mean ± SE 59 ± 1</td>
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<tr>
<td></td>
<td></td>
<td>Ultraviolet, 4½ hr Steamed</td>
<td>4</td>
<td>1.29 ± 0.14</td>
<td>35 ± 2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Unsteamed</td>
<td>4</td>
<td>1.27 ± 0.16</td>
</tr>
<tr>
<td>II</td>
<td>Growth Chamber</td>
<td>Dark</td>
<td>6</td>
<td>1.50 ± 0.06</td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ultraviolet, 2 hr 29°</td>
<td>6</td>
<td>1.45 ± 0.05</td>
<td>57 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>About 0°</td>
<td>6</td>
<td>1.50 ± 0.05</td>
<td>51 ± 2</td>
</tr>
</tbody>
</table>
of temperature sensitivity would not be expected of an enzymic reaction. In other experiments amounts of the 2 isomers were not affected by exposure of the leaflets to cold or to steam without subsequent irradiation.

Further support for the conclusion that this trans to cis conversion is photochemical and nonenzymic comes from the observation that ultraviolet light effected the change in aqueous solutions of authentic o-coumaric acid glucoside (fig 1). In fact, various workers (5,6) have employed ultraviolet irradiation of o-coumaric acid glucoside to prepare coumarinic acid glucoside. Coumarinic acid glucoside was affected only slightly by the ultraviolet treatment, as shown in the figure. According to the fluorometric assay, in the solution of coumarinic acid glucoside about 3% of the compound was in the trans form initially, and this level was increased to about 6% after 1½ hours of ultraviolet treatment. The equilibrium mixture resulting from exposure of aqueous solutions of either glucoside to ultraviolet light contained approximately 94% of the compound in the cis form and 6% in the trans form.

As indicated in figure 1, cool white fluorescent light was not effective in the conversion of o-coumaric acid glucoside to coumarinic acid glucoside. This result is in apparent disagreement with a preliminary observation from this laboratory that such light could cause this trans to cis conversion (3). In the earlier work Amplex cool white fluorescent lamps were used in contrast to the General Electric Power Groove cool white fluorescent lamps employed in the present experiments. Further tests have shown that Amplex lamps are indeed effective in the trans to cis conversion, and General Electric Power Groove lamps of like intensity are not. This difference suggests that the 2 kinds of lamp differ with respect to the spectral characteristics of the light emitted.

The response of an aqueous extract of chamber-grown sweetclover leaves to ultraviolet irradiation (curve A, fig 2) was very similar to that of a solution of synthetic o-coumaric acid glucoside (fig 1). Also, the ultraviolet-induced trans to cis conversion observed in detached leaflets (curves B and C, fig 2) was qualitatively similar to that noted in aqueous solutions. In the latter experiment 5 chamber-grown leaves and 5 greenhouse-grown leaves provided detached leaflets for irradiation. Mid-leaflets were irradiated for 2 hours (1½ hours for the greenhouse-grown leaves), one set of lateral leaflets for 4 hours, and the remaining lateral leaflets for 6 hours. Non-treated leaflets were obtained from a sixth leaf (3 leaflets) from each source of plant material. Content of o-hydroxycinnamic acid in the 18 chamber-grown leaflets was 0.76 ± 0.04% (mean ± SE, fr wt basis) and in the 18 greenhouse-grown leaflets, 1.41 ± 0.04%. As indicated in figure 2, the rate of trans to cis conversion was considerably slower in detached leaflets than in an aqueous leaf extract. Probable reasons for the decreased rate are A) the concentration of o-hydroxycinnamic acid glucoside was much higher in the leaflets than in the irradiated extract, and B) the anatomical and chemical composition of the leaflets doubtless interfered with exposure of the glucoside to the ultraviolet light. Nevertheless, extensive trans to cis conversion occurred in the irradiated leaflets.

As indicated in table II and figures 2, 3 and 4, 95 to 100% of the o-hydroxycinnamic acid glucoside present in chamber-grown leaflets was in the trans form. According to information supplied by the lamp manufacturer, sunlight is relatively much richer than cool white fluorescent light in radiant energy at both ends of the spectrum (below 420 mμ and above 640 mμ). The preceding experiments demonstrate that ultraviolet light is effective in converting o-coumaric acid glucoside to coumarinic acid glucoside, but do not rule out the possibility that light of wavelengths greater than 640 mμ might also be effective. This possibility is precluded, however, by an experiment employing filtered sunlight (fig 3). In this experiment detached leaflets from 7 leaves were used. The 3 leaflets from a single leaf were subjected to 3 different durations of one of the 7 kinds of light treatment indicated in the figure legend. Thus the mid-leaflets of leaves A through G received ½ hour of the respective light treatments listed, one set of lateral leaflets received light treatments for 1 hour, and the remaining lateral leaflets for 2 hours. Content of o-hydroxycinnamic acid in the 21 leaflets was 2.06 ± 0.06% (mean ± SE, fr wt basis). Only the unfiltered sunlight and the light transmitted by clear glass and ultraviolet-transmitting filters were effective in the trans to cis conversion. These results, considered in conjunction with the transmission characteristics of the filters (table I), indicate that wavelengths above approximately 360 mμ were virtually ineffective in the conversion.

The sunlight-induced trans to cis conversion is not peculiar to detached leaves, but also occurs in intact plants (fig 4). In this experiment 5 chamber-grown plants were moved to open sunlight at zero time. The mid-leaflet was taken from the youngest fully expanded leaf on each plant after an exposure of ½ hour, one lateral leaflet was taken after 1 hour, and the remaining lateral leaflet after 2 hours. Three young leaflets from a sixth plant from the chamber served as the zero-exposure control. Content of o-hydroxycinnamic acid in the 18 leaflets was 1.76 ± 0.04% (mean ± SE, fr wt basis). The rate of conversion in this case was somewhat less than that observed in detached leaflets exposed to sunlight (curve A, fig 3), possibly because wind caused movement of the plants, thereby preventing maximal exposure of the leaves to the sun. Nevertheless, conversion in excess of 50% was effected in a 2-hour period. Other work (1) has shown that in young field-grown sweetclover leaves approximately 11% of the o-hydroxycinnamic acid glucoside is in the trans form and 89% is in the cis form. These levels resulted from extended exposure of the leaves to sunlight, and they may be considered as representing the
situation at equilibrium. Obviously, equilibrium had not been attained in the experiment referred to in figure 4.

Summary

The light-induced conversion of o-coumaric acid glucoside (β-D-glucosyl trans-o-hydroxycinnamic acid) to coumarinic acid glucoside (β-D-glucosyl cis-o-hydroxycinnamic acid) was studied in steamed and unsteamed leaflets of sweetclover (Melilotus alba Desr.) of the CucuBB genotype, in aqueous leaf extracts, and in aqueous solutions of the two glucosides. Ultraviolet light (peak near 360 m), cool white fluorescent lamps, and variously filtered sunlight were used as sources of illumination. Results of the experiments support the conclusion that in sweetclover leaves the
indicated trans to cis conversion is a nonenzymic photochemical reaction effected by light of wavelengths shorter than 360 μ.

Acknowledgment

The authors are grateful to Dr. T. Kosuge, Department of Plant Pathology, University of California at Davis, for supplying authentic samples of o-coumaric acid and coumarinic acid glucosides.

Literature Cited


Investigations on the Occurrence and Biosynthesis of Indolepyruvic Acid in Plant Tissues and Bacteria

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Introduction

The presence of IPyA3 in sweet corn kernels was reported by Stowe and Thimann (18) using methods of ammoniacal chromatography. Since then several workers have reported its occurrence (2, 12, 19, 20) in plant tissues as well as its formation from l-tryptophan (3, 6, 16) by the plant tissues. These identifications are based on the appearance of auxin activity at an Rf value lower than of IAA on isopropanol-ammonia chromatograms or on the production of pink to crimson colors with indole reagents in that zone. The work done by Bentley, Farrar, Housley, Smith and Taylor (1), Kaper and Veldstra (10) and Schwarz and Bitancourt (14) has shown, however, that IPyA breaks down on chromatography in ammoniacal solvents. The question of the occurrence of IPyA and of its formation from tryptophan by plant tissues has remained open due to the lack of its critical identification. In the present work, corn kernels and tomato seedlings have been examined for the natural occurrence of IPyA, and corn kernels, corn coleoptiles, tomato seedlings and crown gall bacteria have been tested for their capacity to synthesize this auxin from tryptophan.

Materials and Methods

Plant Material and Reagent. Sweet corn kernels (Zea mays L. var. Country Gentleman) of 1963 crop were obtained from W. Atlee Burpee Company, Clinton, Iowa, and stored at 4°. When corn coleoptile sections were required, the seeds were grown in moist vermiculite at 22° and 90% relative humidity in the dark until the coleoptiles had become 3 to 4 cm long. The coleoptile sections, 1 cm long were cut 3 mm behind the tip. Tomato seedlings (Lycopersicon esculentum L. var. Marglobe) were grown in the greenhouse for one month before being used for analysis. The culture of Agrobacterium tume-