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Genetic Control of the Metabolism of \( o \)-Hydroxycinnamic Acid Precursors in *Melilotus alba*

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GENETIC CONTROL OF THE METABOLISM OF
O-HYDROXYCINNAMIC ACID PRECURSORS IN MELILOTUS ALBA

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In sweetclover (Melilotus alba Desr.) the cis and trans isomers of o-hydroxy-
cinnamic acid occur primarily as the respective β-D-glucosides (RUDORF and
SCHWARZE 1958; KOSUGE 1961; HASKINS and GORZ 1961a; STOKER and BELLIS
1962). The review of BROWN (1963) summarizes evidence that these glucosides
are formed via the following pathway: phenylalanine (formed from shikimic
acid) → trans-cinnamic acid → trans-o-hydroxycinnamic acid (o-coumaric acid)
trans-β-D-glucosyl-o-hydroxycinnamic acid (o-coumaric acid glucoside) → cis-
β-D-glucosyl-o-hydroxycinnamic acid (coumarinic acid glucoside). Sweetclover
plants of the Cu Cu genotype contain substantial amounts of both o-coumaric
acid glucoside and coumarinic acid glucoside, and cu cu plants are low in content
of both glucosides (HASKINS and GORZ 1961a,b). The suggestion has been made
that the cu gene influences content of the glucosides by controlling the o-hydroxy-
lation of cinnamic acid (BROWN 1963). Another possibility is that the cu allele
somehow effects the destruction of o-coumaric acid as rapidly as this compound
is formed, thus preventing the synthesis of o-coumaric acid glucoside. Still other
possibilities could be suggested. In the present study, the role of the Cu/cu gene
pair in the formation of o-coumaric acid glucoside and coumarinic acid glucoside
was investigated by comparing plants of the cu cu and Cu Cu genotypes with
respect to the ability to incorporate 14C from 14C-labeled phenylalanine, trans-
cinnamic acid, and o-coumaric acid into the two glucosides.

MATERIALS AND METHODS

Plant material: Four closely related, highly inbred lines of biennial white-blossomed sweet-
clover (Melilotus alba) were used. With respect to the cu and b alleles, genotypes of the four
lines were Cu Cu BB, Cu Cu bb, cu cu BB, and cu cu bb. Derivation of the lines has been described
previously (HASKINS and GORZ 1965). The effect of the cu locus on content of the glucosides of
cis- and trans-o-hydroxycinnamic acid is indicated in the preceding paragraph. The b locus affects
β-glucosidase activity—homogenates of the leaves from BB plants are high in activity of this
enzyme, and homogenates of bb leaves appear to be devoid of such activity (HASKINS and GORZ
1965).

Seedlings were started in the greenhouse and were transplanted to the field at Davis, Cali-
ifornia, in mid-June, 1964. Portions of branches were removed for feeding experiments in mid-
July, at which time the plants were at a stage of rapid vegetative growth. Immediately following

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removal of the branches, cut ends were immersed in water and the branches were carried to the laboratory.

Chemicals: L-Phenylalanine-1-14C was purchased from the New England Nuclear Corporation, and trans-cinnamic acid-2-14C from Tracerlab, Inc. o-Coumaric acid-2-14C was obtained by hydrolyzing the corresponding β-glucoside with emulsin and extracting the free o-coumaric acid with ether. The labeled glucoside was synthesized by the procedure of Kosuge and Conn (1959).

Nonlabeled o-coumaric acid and coumarin were purchased from the K and K Laboratories and the Matheson Chemical Co., respectively. Almond emulsin was purchased from the California Corporation for Biochemical Research and from Worthington Biochemicals, Inc. Sweetclover β-glucosidase was prepared by a procedure similar to that described by Kosuge and Conn (1961). In assays of the sweetclover preparation using p-nitrophenyl-β-D-glucoside (Schaeffer, Haskins and Gorz 1960), 1.0 ml of the preparation hydrolyzed 3.7 μmoles of this substrate per min at room temperature (approximately 24°C).

Administration of labeled compounds: Solutions of phenylalanine and trans-cinnamic acid for feeding contained 1.0 pmole/ml and the compounds had a specific activity of 1.61 μc/μmole. o-Coumaric acid was fed at a concentration of 0.77 pmole/ml and a specific activity of 0.38 μc/μmole. All solutions were neutralized before administration.

The terminal 4- to 6-cm shoot, consisting of several leaves in addition to stem tissue, was cut from each of the branches harvested from the field-grown plants. Each of the three labeled compounds was administered to shoots of all four genotypes, making a total of 12 samples. Each sample included five shoots with a total fresh weight of approximately 5 g. Three-ml portions of the radioactive solutions were fed to the plant samples in 10-ml beakers. In each case, uptake of the solution was followed by administration of at least two water rinses as previously described (Kosuge and Conn 1959). Shoots were then transferred to 50-ml beakers of water for a period of further metabolism. Solutions remaining in the 10-ml beakers were diluted to known volumes for measurements of residual activity. A total period of 21 hours was allowed from the initiation of feeding until extraction of the tissue. During this time the shoots were exposed to light from two 24-inch Sylvania Grolux lamps at a distance of approximately 12 inches.

Extraction of plant tissue: At the end of the period of metabolism, each 5 g sample of shoots was immersed in 100 ml of boiling water to provide for rapid inactivation of enzymes present in the tissue. Plant material and water were immediately autoclaved at 15 psi for 20 min. The resulting extract was filtered while still warm, and the tissue residue was immersed in 50 ml of water and reautoclaved for 10 min. This second extract was removed by filtration, and the tissue residue was subjected to a third extraction like the second. The three extracts were pooled, a 20 ml aliquot was saved, and the remainder (approximately 150 ml) was subjected to fractionation. In preliminary tests of the extraction procedure, first, second, and third extracts accounted for 91%, 7%, and 2%, respectively, of the total o-hydroxycinnamic acid glucosides extracted. Both trans and cis glucosides appeared to be extracted with equal efficiency. All extracts were held in a frozen condition except during processing, and in so far as possible all processing was done in weak light to minimize possible trans-cis interconversion.

Fractionation and purification: Extracts were fractionated according to the scheme presented in Figure 1. The first ether extraction, resulting in the fraction designated Ether I, was designed to remove free coumarin, cinnamic acid, and phenolic acids from the original extract. By virtue of the specificity of sweetclover β-glucosidase (Kosuge and Conn 1961), treatment of Aqueous I with this enzyme selectively hydrolyzed the glucosides of coumarinic acid and melilotic acid (o-hydroxyhydrocinnamic acid) while leaving o-coumaric acid glucoside intact. Extraction of this hydrolysate at neutrality was expected to remove free coumarin (the lactone of coumarinic acid) in Ether II while leaving behind melilotic acid. Melilotic acid was then extracted at pH 2 (Ether III). o-Coumaric acid glucoside, present in Aqueous III, was hydrolyzed with emulsin and the free o-coumaric acid was extracted at pH 2 (Ether IV).

Ether fractions I, II, III, and IV were concentrated to near dryness in a stream of air at room temperature. Each of these fractions was then diluted to 10 ml with 95% ethanol. Aliquots of these ethanolic solutions were used in subsequent procedures.

Aliquots of each original extract, each Aqueous IV fraction, each ether fraction, and appro-
HYDROXYCINNAMIC ACID SYNTHESIS

Original extract (approx. 150 ml)
  pH to 2.0
  extract, 3 50-ml portions ether

Aqueous I
  pH to 5.0
  hydrolyze, sweetclover β-glucosidase (0.5 ml)
  pH to 7.0
  extract, 3 50-ml portions ether

Aqueous II
  pH to 2.0
  extract, 3 50-ml portions ether

Aqueous III
  pH to 5.0
  hydrolyze, almond emulsin (25 mg)
  pH to 2.0
  extract, 3 50-ml portions ether

Aqueous IV
  pH to 2.0
  extract, 3 50-ml portions ether

Ether I

Ether II
  (coumarin)

Ether III
  (melilotic acid)

Ether IV
  (g-coumaric acid)

FIGURE 1.—Fractionation procedure for tissue extracts. Each ether fraction was washed with a small volume of water and washings were combined with the appropriate aqueous fraction prior to the next step. Enzymatic hydrolyses were done at room temperature. Incubation times were approximately 1½ and 5 hours for the treatments with sweetclover β-glucosidase and almond emulsin, respectively. Ether was driven from aqueous fractions I and III by aeration prior to the enzymatic treatment of these fractions.

appropriate control compounds were chromatographed on Whatman No. 1 filter paper with a descending solvent consisting of n-propyl alcohol and concentrated NH₃OH (70:30, v/v) (Kosuge 1961). The resulting chromatograms were examined for fluorescent and ultraviolet-absorbing areas and were scanned for radioactivity. For certain of the Ether IV fractions the other three solvents described by Kosuge (1961) also were used. These solvents were (1) the upper phase of a mixture of n-butyl alcohol, glacial acetic acid, and water (80:20:10, v/v/v); (2) 95% ethanol, concentrated NH₃OH, and water (80:5:15, v/v/v); and (3) 2% acetic acid.

For genotypes cu cu bb and Cu Cu bb, coumarin and o-coumaric acid were isolated from Ether I1 and Ether IV, respectively, by sublimation in vacuo. In each case the dried material representing 3 ml of ethanolic solution was sublimed. For the cu cu bb genotype, a solution containing 5 mg of nonlabeled coumarin was added to the aliquot of Ether II prior to drying of the sample in the sublimation apparatus. Collection of coumarin on the cold finger of the apparatus was begun when the bath temperature reached 45°C, and was terminated when the temperature reached 64°C. For both genotypes, 3 ml portions of Ether IV were diluted with 5 mg of nonlabeled o-coumaric acid, and sublimates were collected over a temperature range of 140°C to 175°C. A Fisher-Johns melting point block was used in determining melting points of the sublimed compounds, and a Cary model 14 recording spectrophotometer was used for determining ultraviolet absorption spectra.

Assay methods: Original extracts and the four ether fractions were assayed fluorometrically for coumarin and o-coumaric acid (Haskins and Gorz 1961b), using an Aminco-Bowman Spectrophotofluorometer with the excitation wavelength set at 385 mp and the emission wavelength at 505 mp (Kosuge 1961).

An attempt was made to assay the Ether III fractions for melilotic acid using diazotized p-nitroaniline as described by Snell and Snell (1953). However, results were disappointing because of the presence of interfering compounds. Appreciable readings were obtained for Ether III fractions from the cu cu bb and cu cu BB genotypes, but the color of the solutions was yellow rather than the red which characterizes melilotic acid. Ether III fractions representing the Cu Cu bb and Cu Cu BB genotypes appeared to produce the proper color for melilotic acid, but readings were not considered reliable because of the probable presence of impurities.

Determinations of radioactivity of original extracts and aqueous fractions were made with
Nuclear-Chicago gas-flow counting equipment. A Packard Tri-carb liquid scintillation spectrometer was employed for counting of all ether fractions. The counting fluid described by Waller and Henderson (1961) was used. Nuclear-Chicago equipment was used for scanning paper chromatograms for radioactivity.

RESULTS AND DISCUSSION

As shown in Table 1, uptake of the radioactive compounds by shoots of the cu cu bb and cu cu BB genotypes was somewhat greater than that by Cu Cu bb and Cu Cu BB shoots. The reason for this apparent difference is not known with certainty, but lack of uniformity of air movement and light during feeding may be involved. The Cu Cu genotypes incorporated 14C from phenylalanine and trans-cinnamic acid into the constituents of the aqueous extracts more extensively than did the cu cu genotypes. Incorporation values for trans-cinnamic acid were similar to those for phenylalanine, and were only about half as great as those for o-coumaric acid. Obviously, o-coumaric acid was converted to volatile and/or water insoluble compounds less extensively than were phenylalanine and cinnamic acid.

Results of the fluorometric assay of various fractions for the cis and trans isomers of o-hydroxycinnamic acid are shown in Table 2. Almost all of the o-hydroxycinnamic acid present in the original extracts was in a bound form as shown by the facts that fluorescence readings on unhydrolyzed samples were negligible and that very little (less than 1%) of the compound appeared in the

TABLE 1

<table>
<thead>
<tr>
<th>Compound fed</th>
<th>Genotype</th>
<th>Percentage uptake*</th>
<th>Percentage incorporation into aqueous extract†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Phenylalanine-1-14C(4.83 μC)</td>
<td>cu cu bb</td>
<td>93</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>cu cu BB</td>
<td>94</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>83</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Cu Cu BB</td>
<td>79</td>
<td>55</td>
</tr>
<tr>
<td>trans-Cinnamic acid-2-14C(4.83 μC)</td>
<td>cu cu bb</td>
<td>94</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>cu cu BB</td>
<td>92</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>83</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>Cu Cu BB</td>
<td>85</td>
<td>59</td>
</tr>
<tr>
<td>o-Coumaric acid-2-14C(0.89 μC)</td>
<td>cu cu bb</td>
<td>96</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>cu cu BB</td>
<td>95</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>77</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Cu Cu BB</td>
<td>83</td>
<td>106</td>
</tr>
</tbody>
</table>

* activity administered—residual activity

† activity administered

activity in aqueous extract × 100
Distriubtion of cis- and trans-o-hydroxycinnamic acids following fractionation of plant extracts

<table>
<thead>
<tr>
<th>Compound fed</th>
<th>Fraction</th>
<th>o-HCA isomer*</th>
<th>Quantity (μmoles) from genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cis</td>
<td>Cu Cu bb</td>
</tr>
<tr>
<td>1-Phenylalanine-1-14C</td>
<td>Original extract</td>
<td>cis</td>
<td>&gt;2.1, &lt;4.1</td>
</tr>
<tr>
<td></td>
<td>Ether II</td>
<td>cis</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td></td>
<td>Ether IV</td>
<td>cis</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trans</td>
<td>0.30</td>
</tr>
<tr>
<td>trans-Cinnamic acid-2-14C</td>
<td>Original extract</td>
<td>cis</td>
<td>&gt;1.4, &lt;4.9</td>
</tr>
<tr>
<td></td>
<td>Ether II</td>
<td>cis</td>
<td>&lt;3.5</td>
</tr>
<tr>
<td></td>
<td>Ether IV</td>
<td>cis</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trans</td>
<td>0.55</td>
</tr>
<tr>
<td>o-Coumaric acid-2-14C</td>
<td>Original extract</td>
<td>cis</td>
<td>&gt;1.1, &lt;4.5</td>
</tr>
<tr>
<td></td>
<td>Ether II</td>
<td>cis</td>
<td>&lt;3.4</td>
</tr>
<tr>
<td></td>
<td>Ether IV</td>
<td>cis</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>trans</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* In the original extracts both isomers of o-hydroxycinnamic acid (o-HCA) were present primarily as the respective β-glucosides; following hydrolysis the cis isomer was in the form of the lactone, coumarin, and the trans isomer was in the form of free o-coumaric acid.

Ether I fraction. Comparison of the fluorescence spectrum of known o-coumaric acid with spectra of base-hydrolyzed original extracts from Cu Cu plants indicated excellent agreement. However, for cu cu plants, fluorescence spectra of the solutions used in assaying for trans and total o-hydroxycinnamic acids had peaks at 480 and 495 μm respectively, and lacked the 505 μm peak characteristic of the fluorescence of o-coumaric acid. Thus, the 505 μm readings on original extracts of cu cu plants overestimated the content of trans-o-hydroxycinnamic acid and, probably to a lesser extent, of total o-hydroxycinnamic acid, and the usual calculation of cis-o-hydroxycinnamic acid by difference provided an estimate that was too low. These difficulties in assay contributed to the lack of preciseness evident in certain of the quantities given in Table 2.

The fractionation procedure effected a clear separation of the cis and trans isomers of o-hydroxycinnamic acid. o-Coumaric acid was not detected in Ether II fractions, nor was coumarin found in Ether IV. The Ether II fraction accounted for an average of 92% of the bound cis-o-hydroxycinnamic acid present in the original extracts of Cu Cu shoots, indicating satisfactory hydrolysis of coumarinic acid glucoside by the sweetclover β-glucosidase preparation. The yield of o-coumaric acid following emulsin treatment was somewhat lower, averaging 62% of the bound o-coumaric acid present in the original extracts of Cu Cu shoots.

A small amount of coumarin but no o-coumaric acid was detected in the Ether III fractions from Cu Cu genotypes. This contamination may be attributed primarily to the formation of emulsions which decreased the efficiency of ether extraction. Although the contamination represented only about 1% of the coumarin present in the Ether II fractions, it was sufficient to interfere significantly with the determination of melilotic acid in the Ether III fractions. Because of this interference, together with that mentioned in the section on assay methods,
melilotic acid values are considered unreliable and are not reported here. In
general, however, levels of this constituent were low, amounting to less than a
tenth of the levels of o-coumaric acid in extracts from the Cu Cu genotypes.

In chromatographic behavior fractions originating with the cu cu bb genotype
resembled closely those from cu cu BB, and fractions from Cu Cu bb were very
similar to those from Cu Cu BB. The aliquots chromatographed were not equal
in terms of radioactivity or o-hydroxy-cinnamic acid content because the attain-
ment of such equality was not feasible. Despite this obvious weakness in the
chromatographic investigations, several points are worthy of note. Thus, labeled
coumarin was not detected on chromatograms of any of the Ether II fractions.
Labeled melilotic acid was not detected in the Ether III fractions following phen-
ylalanine or cinnamic acid feeding, but apparently was formed by all genotypes
after administration of labeled o-coumaric acid. Labeled o-coumaric acid was
readily detected in all Ether IV fractions except those resulting from extracts of
cu cu shoots to which labeled phenylalanine and cinnamic acid had been fed.
Chromatograms of the Ether IV fraction from cu cu shoots fed labeled cinnamic
acid revealed a single radioactive peak at Rf 0.30 which was easily distinguished
from the o-coumaric acid peak (Rf 0.39).

Melting points of the coumarin and o-coumaric acid isolated by sublimation
agreed well with the melting points of authentic coumarin (68–69°C) and
o-coumaric acid (215–216°C). Similarly, ultraviolet absorption spectra indicated
a high degree of purity for the isolated compounds. As expected on the basis of
the radioactivities observed on chromatograms, coumarin was not extensively
labeled, and the activity of o-coumaric acid was highly dependent upon genotype
and compound fed.

Dilution values based on isolated coumarin and o-coumaric acid (Table 3)
were in every instance considerably lower for the cu cu genotypes than for Cu Cu.
The large quantities of non-labeled glucosides of o-coumaric and coumarinic

TABLE 3

Incorporation of labeled precursors into coumarin and o-coumaric acid isolated
by sublimation from Ether II and Ether IV fractions, respectively

<table>
<thead>
<tr>
<th>Compound fed</th>
<th>Genotype</th>
<th>Activity taken up by shoots (μc)</th>
<th>Incorporation* (percent)</th>
<th>Dilution† (percent)</th>
<th>Incorporation* (percent)</th>
<th>Dilution† (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Phenylalanine-1-14C</td>
<td>cu cu bb</td>
<td>4.49</td>
<td>0.014</td>
<td>4,970</td>
<td>0.29</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>4.01</td>
<td>0.024</td>
<td>268,000</td>
<td>9.2</td>
<td>82</td>
</tr>
<tr>
<td>trans-Cinnamic acid-2-14C</td>
<td>cu cu bb</td>
<td>4.54</td>
<td>0.16</td>
<td>460</td>
<td>2.22</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>4.01</td>
<td>0.14</td>
<td>47,400</td>
<td>15.4</td>
<td>53</td>
</tr>
<tr>
<td>o-Coumaric acid-2-14C</td>
<td>cu cu bb</td>
<td>0.85</td>
<td>0.48</td>
<td>127</td>
<td>48.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Cu Cu bb</td>
<td>0.69</td>
<td>0.21</td>
<td>42,200</td>
<td>60.9</td>
<td>21</td>
</tr>
</tbody>
</table>

Calculations are based on activities of the sublimed compounds and on the quantities of these compounds present in the
Ether II and IV fractions. Specific activities of the compounds fed were 1.61 μc/μmole for phenylalanine and cinnamic
acid, and 0.38 μc/μmole for o-coumaric acid.

* Activity in compound isolated / activity taken up) × 100.
† Specific activity of compound fed → specific activity of compound isolated.
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acids present in the Cu Cu plants prior to the administration of the labeled compounds greatly diluted the radioactivity of the compounds formed from the labeled precursors. For this reason, percentages of incorporation of the labeled precursors into the isolated compounds provide a better measure of the metabolic potentials of the different genotypes than do dilution values.

The incorporation data (Table 3) indicate that the Cu Cu bb and cu cu bb genotypes were similar in ability to convert o-coumaric acid to its glucoside; but Cu Cu bb shoots appeared to convert approximately 32 times as much phenylalanine and 7 times as much trans-cinnamic acid to o-coumaric acid glucoside as did cu cu bb shoots. Incorporation values further indicate that cu cu bb plants synthesized o-coumaric acid glucoside considerably more readily from cinnamic acid than from phenylalanine. The validity of the latter apparent difference was doubtful in view of the previously mentioned chromatographic separation of o-coumaric acid and the principal radioactive component in the Ether IV fraction resulting from feeding labeled cinnamic acid to cu cu bb shoots. Furthermore, such a difference, if valid, would suggest that cu cu plants possessed a diminished capacity to deaminate phenylalanine, and measurements of phenylalanase activity in preparations of cu cu and Cu Cu plants have not disclosed appreciable differences (KLEINHOFS 1964).

In further work the Ether IV fractions from cinnamic acid-fed shoots of the cu cu bb and Cu Cu bb genotypes were chromatographed on a larger scale with the n-propyl alcohol-NH₄OH solvent. The o-coumaric acid band (Rf approximately 0.39) was marked in ammonia vapor under ultraviolet light. This band and the adjacent 3cm band (Rf approximately 0.30) were cut out and were eluted with water. Eluates were assayed for fluorescence and radioactivity. The results shown in Table 4 clearly indicate that in the Ether IV fraction from the Cu Cu genotype o-coumaric acid was responsible for the bulk of the radioactivity, whereas in the corresponding fraction from the cu cu genotype o-coumaric acid accounted for no more than a small fraction of the radioactivity. Based on the activity of o-coumaric acid isolated by chromatography, values for incorporation (15.2%) and dilution (53) for the Cu Cu bb genotype agreed well with the values based on the sublimed compound (compare Cu Cu bb, cinnamic acid, in Table 3). However, for the cu cu bb genotype, activity of the chromatographically isolated

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chromatogram band</th>
<th>Recovery*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cu cu bb</td>
<td>o-coumaric acid</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>Rf 0.30</td>
<td>19</td>
</tr>
<tr>
<td>Cu Cu bb</td>
<td>o-coumaric acid</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Rf 0.30</td>
<td>3</td>
</tr>
</tbody>
</table>

* Recoveries are expressed as percentages of quantities applied to the chromatograms.
o-coumaric acid provided much lower incorporation (0.26%) and higher dilution (76) estimates than those suggested by sublimation results. Considered in total, the results support the conclusion that in the cu cu genotype, conversion of trans-cinnamic acid to o-coumaric acid glucoside was no more extensive than was the conversion of phenylalanine to this glucoside.

A portion of the Rf 0.30 eluate from the cu cu genotype was mixed with non-labeled o-coumaric acid and the mixture was subjected to conditions under which o-coumaric acid sublimes. Assays of the sublimate indicated that the radioactive material and o-coumaric acid sublimed to approximately the same extent. Thus, sublimation obviously is not a satisfactory means of purifying o-coumaric acid from the Ether IV fraction following cinnamic acid feeding.

The identity of the radioactive substance in the Rf 0.30 eluate was not conclusively established. However, in the four solvents of Kosuge (1961) the chromatographic behavior of this substance was identical to that of p-coumaric acid. Furthermore, under vacuum sublimation at approximately 150°C, p-coumaric and o-coumaric acids sublimed together; and the admixture of small amounts of p-coumaric acid with o-coumaric acid produced a surprisingly small effect on the melting point. Available evidence indicates, therefore, that the Rf 0.30 substance is p-coumaric acid. Other work, for example the studies of Brown, Towers and Wright (1960) on Heirochloë odorata, has demonstrated the conversion of trans-cinnamic acid to p-coumaric acid. In addition, Harborne and Corner (1961) demonstrated the formation of the glucose ester from administered p-coumaric acid in several plants, and Runeckles and Woolrich (1963) showed that both the glucose ester and the β-d-glucoside were formed when p-coumaric acid was fed to tobacco leaf disks. In the present case, the p-coumaric acid that appeared in the Ether IV fraction obviously existed in the original extract as an emulsin-labile compound. Both the glucose ester and the β-d-glucoside of p-coumaric acid are hydrolyzed by emulsin (Harborne and Corner 1961; Runeckles and Woolrich 1963); thus a conclusion as to the form in which bound p-coumaric acid existed in the original extracts is not possible at present. The occurrence of a radioactive substance with an Rf of approximately 0.30 in the Ether I fractions following cinnamic acid feeding suggests that some free p-coumaric acid also may have existed in the original extracts.

The extensive formation of o-coumaric acid glucoside in cu cu shoots supplied with o-coumaric acid provides direct evidence that cu cu plants are low in content of the glucosides of o-hydroxycinnamic acid because of an effective block in the biosynthetic pathway and not because the rate of destruction of o-coumaric acid or o-coumaric acid glucoside is abnormally high. Furthermore, the large difference between the cu cu and Cu Cu genotypes with respect to ability to convert phenylalanine or cinnamic acid to o-coumaric acid glucoside, and the similarity of these genotypes in ability to glucosylate o-coumaric acid are in agreement with the hypothesis that this cu-controlled block is concerned with the o-hydroxylation of trans-cinnamic acid, as suggested by Brown (1963). However, the possibilities that o-coumaric acid glucoside is formed by direct o-glucosyloxylation of
trans-cinnamic acid, or that a compound other than o-coumaric acid is normally the immediate precursor of o-coumaric acid glucoside, are not excluded.

The incorporation of $^{14}$C into coumarinic acid glucoside was very slight. This result was expected inasmuch as, in sweetclover leaves, the isomerization of o-coumaric acid glucoside to coumarinic acid glucoside has been reported to require ultraviolet light (Haskins, Williams and Gorz 1964). If it occurred, enzymatic isomerization of the type reported by Stoker (1964) was not extensive.

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**SUMMARY**

L-Phenylalanine-1-$^{14}$C, trans-cinnamic acid-2-$^{14}$C, and o-coumaric acid-2-$^{14}$C were fed to sweetclover shoots of the cu cu bb, cu cu BB, Cu Cu bb, and Cu Cu BB genotypes. Aqueous extracts of the shoots were fractionated by a procedure involving the selective enzymatic hydrolysis of the $\beta$-D-glucosides of coumarinic acid (cis-o-hydroxycinnamic acid) and o-coumaric acid (trans-o-hydroxycinnamic acid) and the separate extraction of the respective aglycones as coumarin and o-coumaric acid. Labelling results indicated that extensive incorporation of administered o-coumaric acid into o-coumaric acid glucoside occurred in both cu cu and Cu Cu shoots, but extensive conversion of administered phenylalanine and trans-cinnamic acid to o-coumaric acid glucoside occurred only in Cu Cu shoots. In no case was coumarin (from coumarinic acid glucoside) extensively labeled. Results support the hypothesis that the cu-controlled block in o-hydroxycinnamic acid biosynthesis is concerned with the o-hydroxylation of trans-cinnamic acid.

**LITERATURE CITED**


