11-1965

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

Francis A. Haskins
United State Department of Agriculture, fhaskins@neb.rr.com

T. Kosuge
University of California - Davis

GENETIC CONTROL OF THE METABOLISM OF *O*-HYDROXYCINNAMIC ACID PRECURSORS IN *MELILOTUS ALBA*[1]

F. A. HASKINS AND T. KOSUGE

Department of Agronomy, University of Nebraska, Lincoln, and Department of Plant Pathology, University of California, Davis

Received July 9, 1965

IN sweetclover (*Melilotus alba* Desr.) the *cis* and *trans* isomers of *o*-hydroxycinnamic 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*-hydroxylation 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 ^{14}C from ^{14}C-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 sweetclover (*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, California, 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

[1] Research was supported in part by the National Science Foundation (Grant No. GB-1148). Published with the approval of the Director as Paper No. 1710, Journal Series, Nebraska Agricultural Experiment Station.

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

Chemicals: L-Phenylalanine-1-^{14}C was purchased from the New England Nuclear Corporation, and *trans*-cinnamic acid-2-^{14}C from Tracerlab, Inc. *o*-Coumaric acid-2-^{14}C 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 μmole/ml and the compounds had a specific activity of 1.61 μc/μmole. *o*-Coumaric acid was fed at a concentration of 0.77 μmole/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-

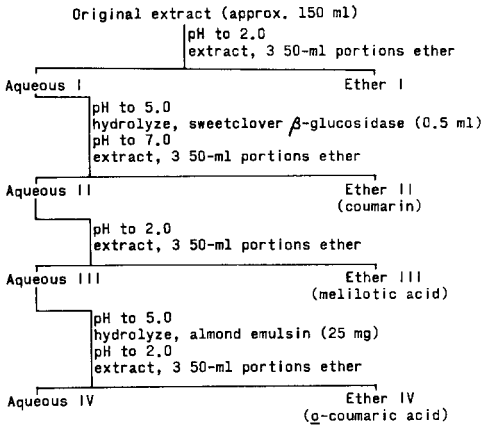

FIGURE 1.—Fractionation procedure for tissue extracts. Each ether fraction was washed with a small volume of water and washings were combined with the appropriate aqueous fraction prior to the next step. Enzymatic hydrolyses were done at room temperature. Incubation times were approximately 1¼ and 5 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.

priate control compounds were chromatographed on Whatman No. 1 filter paper with a descending solvent consisting of *n*-propyl alcohol and concentrated NH_4OH (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 (80.20:100, v/v/v); (2) 95% ethanol, concentrated NH_4OH, 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 II 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°. 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° to 175°. 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 mμ and the emission wavelength at 505 mμ (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 ^{14}C 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

Uptake of administered radioactivity and incorporation of radioactivity into constituents of aqueous plant extracts

Compound fed	Genotype	Percentage uptake*	Percentage incorporation into aqueous extract†
L-Phenylalanine-1-^{14}C(4.83 μc)	*cu cu bb*	93	42
	cu cu BB	94	47
	Cu Cu bb	83	55
	Cu Cu BB	79	55
trans-Cinnamic acid-2-^{14}C(4.83 μc)	*cu cu bb*	94	49
	cu cu BB	92	52
	Cu Cu bb	83	58
	Cu Cu BB	85	59
o-Coumaric acid-2-^{14}C(0.89 μc)	*cu cu bb*	96	101
	cu cu BB	95	98
	Cu Cu bb	77	101
	Cu Cu BB	83	106

* $\dfrac{\text{activity administered} - \text{residual activity}}{\text{activity administered}} \times 100$

† $\dfrac{\text{activity in aqueous extract}}{\text{activity administered} - \text{residual activity}} \times 100$

TABLE 2

Distribution of cis- *and* trans-o-*hydroxycinnamic acids following fractionation of plant extracts*

Compound fed	Fraction	o-HCA isomer*	Quantity (μmoles) from genotype			
			cu cu bb	*cu cu BB*	*Cu Cu bb*	*Cu Cu BB*
L-Phenylalanine-1-^{14}C	Original extract	*cis*	>2.1, <4.1	>3.3, <5.3	170	173
		trans	<2.0	<2.0	31.4	32.4
	Ether II	*cis*	1.9	2.1	154	150
	Ether IV	*trans*	0.30	0.35	18.6	18.6
trans-Cinnamic acid-2-^{14}C	Original extract	*cis*	>1.4, <4.9	>1.7, <5.1	177	170
		trans	<3.5	<3.4	35.9	34.6
	Ether II	*cis*	2.0	1.8	171	158
	Ether IV	*trans*	0.55	0.59	20.2	20.2
o-Coumaric acid-2-^{14}C	Original extract	*cis*	>1.1, <4.5	>1.9, <6.2	170	160
		trans	<3.4	<4.3	32.1	32.4
	Ether II	*cis*	1.4	2.4	158	145
	Ether IV	*trans*	1.4	1.4	22.7	23.1

* In the original extracts both isomers of o-hydroxycinnamic acid (o-HCA) were present primarily as the respective β-D-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 mμ respectively, and lacked the 505 mμ peak characteristic of the fluorescence of o-coumaric acid. Thus, the 505 mμ 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*-hydroxycinnamic acid content because the attainment 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 phenylalanine 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

Compound fed	Genotype	Activity taken up by shoots (μc)	Compound Isolated: Coumarin — Incorporation* (percent)	Coumarin — Dilution†	*o*-Coumaric acid — Incorporation* (percent)	*o*-Coumaric acid — Dilution†
L-Phenylalanine-1-^{14}C	*cu cu bb*	4.49	0.014	4,970	0.29	38
	Cu Cu bb	4.01	0.024	268,000	9.2	82
trans-Cinnamic acid-2-^{14}C	*cu cu bb*	4.54	0.16	460	2.22	9.1
	Cu Cu bb	4.01	0.14	47,400	15.4	53
o-Coumaric acid-2-^{14}C	*cu cu bb*	0.85	0.48	127	48.4	1.2
	Cu Cu bb	0.69	0.21	42,200	60.9	21

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.

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-NH_4OH 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 4

Recovery of fluorescence and radioactivity following chromatography of Ether IV fractions. Compound fed: trans-*cinnamic acid-2-*^{14}C

Genotype	Chromatogram band	Recovery*	
		Fluorescence	Radioactivity
cu cu bb	*o*-coumaric acid	86	5
	Rf 0.30	19	36
Cu Cu bb	*o*-coumaric acid	82	74
	Rf 0.30	3	10

* 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.

Most of this study was done during the summer of 1964 when the senior author was a Research Associate in the laboratory of DR. ERIC E. CONN, Department of Biochemistry and Biophysics, University of California, Davis. Grateful acknowledgment is made to DR. CONN and the members of his group for their hospitality, their helpful suggestions, and the use of their equipment and supplies.

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 β-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

BROWN, S. A., 1963 Recent studies on the formation of natural coumarins. Lloydia **26**: 211–222.

BROWN, S. A., G. H. N. TOWERS, and D. WRIGHT, 1960 Biosynthesis of the coumarins. Tracer studies on coumarin formation in *Hierochloë odorata* and *Melilotus officinalis*. Can. J. Biochem. Physiol. **38**: 143–156.

HARBORNE, J. B., and J. J. CORNER, 1961 Plant polyphenols. 4. Hydroxycinnamic acid-sugar derivatives. Biochem. J. **81**: 242–250.

HASKINS, F. A., and H. J. GORZ, 1961a A reappraisal of the relationship between free and bound coumarin in Melilotus. Crop Sci. **1**: 320–323. —— 1961b Assay of *cis*- and *trans*-*o*-hydroxycinnamic acids in sweetclover extracts. Biochem. Biophys. Res. Comm. **6**: 298–303. —— 1965 Absence of dominance of the *B* gene in influencing β-glucosidase activity in *Melilotus alba*. Genetics **51**: 733–738.

HASKINS, F. A., L. G. WILLIAMS, and H. J. GORZ, 1964 Light-induced *trans* to *cis* conversion of β-D-glucosyl *o*-hydroxycinnamic acid in *Melilotus alba* leaves. Plant Physiol. **39**: 777–781.

KLEINHOFS, A., 1964 Studies concerning the effect of the *Cu/cu* alleles on coumarin biosynthesis in *Melilotus alba*. M.S. thesis. University of Nebraska, Lincoln.

KOSUGE, T., 1961 Studies on the identity of bound coumarin in sweet clover. Arch. Biochem. Biophys. **95:** 211–218.

KOSUGE, T., and E. E. CONN, 1959 The metabolism of aromatic compounds in higher plants. I. Coumarin and *o*-coumaric acid. J. Biol. Chem. **234:** 2133–2137. —— 1961 The metabolism of aromatic compounds in higher plants. III. The β-glucosides of *o*-coumaric, coumarinic, and melilotic acids. J. Biol. Chem. **236:** 1617–1621.

RUDORF, W., and P. SCHWARZE, 1958 Beiträge zur Züchtung eines cumarinfreien Steinklees und Untersuchungen über Cumarin und verwandte Verbindungen. Z. Pflanzenzücht. **39:** 245–274.

RUNECKLES, V. C., and K. WOOLRICH, 1963 Tobacco polyphenols. I. The biosynthesis of *o*-glucosides and *o*-glucose esters of hydroxycinnamic acids. Phytochem. **2:** 1–6.

SCHAEFFER, G. W., F. A. HASKINS, and H. J. GORZ, 1960 Genetic control of coumarin biosynthesis and β-glucosidase activity in *Melilotus alba*. Biochem. Biophys. Res. Comm. **3:** 268–271.

SNELL, F. D., and C. T. SNELL, 1953 *Colorimetric Methods of Analysis*. Vol. III. Edition 3 (p. 150). Van Nostrand, New York.

STOKER, J. R., 1964 The biosynthesis of coumarin in *Melilotus alba*. Biochem. Biophys. Res. Comm. **14:** 17–20.

STOKER, J. R., and D. M. BELLIS, 1962 The isolation and identification of bound coumarin from *Melilotus alba*. Can. J. Biochem. Physiol. **40:** 1763–1768.

WALLER, G. R., and L. M. HENDERSON, 1961 Biosynthesis of the pyridine ring of ricinine. J. Biol. Chem. **236:** 1186–1191.