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CONVERSION OF *o*-(β-D-GLUCOSYLOXY)-CINNAMIC ACID TO *o*-(β-D-GLUCOSYLOXY)-HYDROCINNAMIC ACID IN *MELILOTUS ALBA**

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Abstract—In sweet clover (*Melitolotus alba* Desr.) coumarin and related compounds occur primarily as β-D-glucosides. Synthetic *trans* *o*-(β-D-6-^{3}H-glucosyloxy) cinnamic acid α-^{14}C was administered to sweet clover shoots. Analysis of subsequently isolated *trans* and *cis* *o*-(β-D-glucosyloxy)-cinnamic acid and *o*-(β-D-glucosyloxy)-hydrocinnamic acid showed that in the *in vivo* conversion of the former glucosides to the latter glucoside, significant retention of glucose had occurred. These results indicate that in the *in vivo* conversion free coumarin is not an important intermediate. Identical results obtained from genotypes containing β-glucosidase and genotypes lacking β-glucosidase confirm the latter statement. Results from comparisons of the dilutions of specific activity of the isolated glucosides indicated that the *trans* isomer rather than the *cis* isomer of *o*-(β-D-glucosyloxy)-cinnamic acid is reduced to the corresponding hydrocinnamic acid derivative. Methods involving ion-exchange column chromatography and TLC separation of the three mentioned glucosides also are described.

INTRODUCTION

IN SWEET clover (*Melilotus alba* Desr.) the *cis* and *trans* isomers of *o*-hydroxycinnamic acid (coumarinic acid and *o*-coumaric acid respectively) and *o*-hydroxyhydrocinnamic acid (melilotic acid) occur primarily as β-D-glucosides.[1–3] Results from isotope labeling experiments[4–6] and studies on β-glucosidase,[5] coumarin reductase[7] and dihydrocoumarin hydrolase[8] indicate that the glucosides are formed by the pathway shown in Fig. 1.

Gorz and Haskins,[9] working on the genetics of coumarin production in sweet clover, developed genotypes which lack β-glucosidase activity. Genotypes heterozygous or homozygous dominant (genotype BB) for gene B are high in β-glucosidase and those homozygous recessive for the gene b (genotype bb) lack the enzyme.[10] In the proposed pathway (Fig. 1), β-glucosidase performs the function of freeing coumarinic acid from its bound (glucoside) form (II). In a neutral or acidic environment, coumarinic acid spontaneously lactonizes to yield coumarin (III). The latter is converted to melilotic acid (V) by the action of coumarin reductase and dihydrocoumarin hydrolase. Thus, in the proposed scheme, the β-glucosidase is required for the conversion of the *o*-hydroxycinnamic acid glucosides to melilotic acid.

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Plants lacking the enzyme should, therefore, accumulate very little melilotic acid and its β-glucoside. Such is not the case, however, since plants of the genotype bb were found to contain significant quantities of melilotic acid β-glucoside (VI).[3] This suggests that the *o*-hydroxycinnamic acids can be converted to melilotic acid by a pathway not involving hydrolysis by β-glucosidase. The most direct route for this conversion is a direct reduction of *o*-coumaric acid β-glucoside (I) or coumarinic acid β-glucoside (II) to VI (Fig. 1). The work reported here investigates this possibility.

By procedures previously published II and VI cannot be separated. Usually the glucosides are hydrolyzed and analyzed for aglycone content as the latter are easily separated by chromatography.[6] However, since the experimental approach used in our studies required isolation

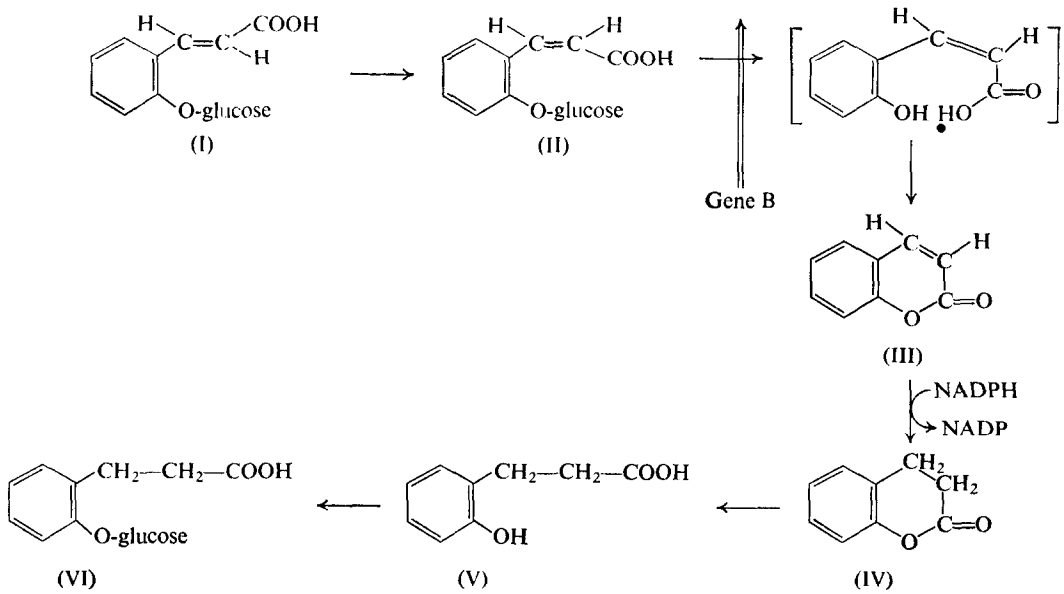

FIG. 1. POSTULATED PATHWAY FOR THE CONVERSION OF THE β-GLUCOSIDE OF *o*-COUMARIC ACID TO THE β-GLUCOSIDE OF MELILOTIC ACID.

I, *o*-Coumaric acid β-D-glucoside; II, coumarinic acidβ-D-glucoside; III, coumarin; IV, dihydrocoumarin; V, melilotic acid; and VI, melilotic acid β-D-glucoside. Gene B controls production of the β-glucosidase which is proposed to function in the pathway by freeing coumarin (III) from its glucosidic form (II).

of the intact β-glucosides, it was necessary to develop procedures for their separation and isolation. The first part of this paper describes the procedures which were developed to isolate and purify the glucosides from extracts of *M. alba*. The remainder of the paper describes results of experiments with *o*-(β-D-6-^{3}H-glucosyloxy)-cinnamic acid α-^{14}C which was used to detect direct reduction of I or II to VI.

RESULTS

Purification Procedures

A gradient elution with formate from a polyamine resin in the hydroxide form gave two well-separated fractions, one which contained II and VI and a second which contained I. The first fraction was then separated by stepwise elution with acetate from the same resin in

the acetate form. Attempts to reduce the column chromatography of the three glucosides to a single step using gradient elution from resin in the acetate form were unsuccessful. By such procedures the VI fraction contained considerably more phenolic components than was the case with the two-step procedure. Also, complete separation of I and II was not achieved.

TLC on DEAEc-ellulose separated the three glucosides. Resolution was markedly improved if the plates were irrigated twice with the ammonium formate buffer prior to application of sample. Both pH and ionic strength of the developing buffer affect the mobilities of compounds in this system (Table 1). This procedure also separated the glucosides from coumarin and *o*-coumaric acid. However, prior to TLC on a preparative scale, crude plant extracts of the three glucosides must be partially purified by the two-step column chromatography procedures described above. The steps prior to the separation of the I and II were done under low-light intensities in order to minimize *trans–cis* isomerization. In combination with the two-step ion-exchange chromatography, the TLC step yields three glucoside preparations that appear free of contaminants as determined by chromatography and by the appearance of their u.v. absorption spectra. A repeat of the TLC step is used to verify purity of each glucoside sample.

TABLE 1. DEAE-CELLULOSE CHROMATOGRAPHY OF COUMARIN AND COUMARIN DERIVATIVES

	R_f*	R_f†
Coumarin	0·70	0·69
o-Coumaric acid	0·19	0·31
Melilotic acid	0·41	0·61
Melilotic acid glucoside	0·58	0·80
Coumarinic acid glucoside	0·36	0·71
o-Coumaric acid glucoside	0·28	0·56

Mobilities on DEAE-cellulose:

* Plate was preirrigated two times with 0·05 M ammonium formate, pH 3·3, and developed in 0·05 M ammonium formate, pH 3·3.

† Plate was preirrigated two times with 0·5 M ammonium formate, pH 3·3, and developed in 0·5 M ammonium formate, pH 3·3.

Radioisotope Feeding Experiments

To determine whether direct reduction of *o*-coumaric or coumarinic acid glucosides (I and II) to the dihydro acid occurs in sweet clover, *trans o*-(β-D-glucosyloxy)-cinnamic acid α-^{14}C was administered to *Melilotus alba* cuttings. If direct reduction of the administered compound occurred, the $^{3}H/^{14}C$ ratio would remain constant during *in vivo* conversion of I or II to VI. However, if the conversion involves glucoside hydrolysis as an intermediate step, a substantial loss of ^{3}H relative to ^{14}C would be expected to occur. The latter expectation assumes a dilution and/or metabolism of the freed tritiated glucose in the plant.

The labeled *trans* acid (I) was used in the feeding experiments to minimize the possibility of hydrolysis of the glucoside by the β-glucosidase liberated from the cut end of shoots. The β-glucosidase of sweet clover is highly active on the *cis* isomer (II) but inert towards the *trans* compound (I).[5, 11]

[11] F. A. HASKINS and H. J. GORZ, *Biochem. Biophys. Res. Commun.* **6**, 298 (1961).

When the labeled I was fed to cuttings, about 95 per cent of the administered counts were recovered in the water extract of the shoots at the end of 18 hr. About 92 per cent of the isolated ^{14}C counts were accounted for by the three mentioned glucosides.

Results of several typical experiments shown in Table 2 suggest direct reduction of *o*-coumaric acid glucoside or its *cis* isomer occurs in sweet clover. In some experiments, the $^{3}H/^{14}C$ ratio of the melilotic acid glucoside (VI) was essentially the same as that of *cis* compound (II), indicating complete retention of glucose during the reduction.

In all experiments with plants of BB and bb genotypes (Table 2), the specific activity of VI was higher than that of II with one exception and represented only a two-fold dilution or less from that of I.

TABLE 2. METABOLISM OF β[D-6-^{3}H-GLUCOSYL]-*o*-HYDROCINNAMIC-α-^{14}C ACID IN SWEET CLOVER

Genotype	Isolated compound	% of total* CPM recovered	% of ^{3}H retained†			Specific activity of isolated compound‡			Dilution§		
			Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
BB	Coumaric acid glucoside	70	61	54	56	44	48	46	4·8	4·4	4·6
	Coumarinic acid glucoside	20	63	58	56	22	6·6	8·3	9·5	32	25
	Melilotic acid	2 to 4	20	55	13	23	8·1	36	9·1	26	5·8
bb	Coumaric acid glucoside	70	84	91	81	23	53	44	9·2	4·0	4·7
	Coumarinic acid glucoside	20	73	50	71	10	32	9·5	20	6·7	21
	Melilotic acid glucoside	2 to 4	26	55	20	13	—¶	21	16	—¶	10

* Based on CPM of both ^{14}C and ^{3}H.

† Measured relative to ^{14}C (administered ^{14}C-^{3}H coumaric acid glucoside = 100%).

‡ Specific activity is based upon ^{14}C and is expressed as mμc/mmole.

§ Dilution is expressed as $\frac{\text{specific act. cpd. administered.}}{\text{specific. act. cpd isolated}}$

¶ The sample was lost before spectral analysis for melilotic acid glucoside content was made. Therefore, the values for specific activity and dilution were not obtained. Amount administered was 1·5 μc of ^{14}C-^{3}H-coumaric acid glucoside per 2 g fr. wt. Specific activity was 0·45 μc/mmole for ^{3}H and 0·21 for ^{14}C.

DISCUSSION

o-Coumaric acid glucoside (I) and its *cis* isomer (II) are readily interconvertible by light.[12] Therefore, unless protected from light, the various manipulation of the samples allow ample opportunity for conversion to occur. However, the initial purification step separates the two isomers and thus minimizes the inconvenience of such interconversions. Subsequent appearance of the corresponding isomer in either the *cis* or *trans* sample indicates interconversion during the purification procedure and appropriate corrections for such interconversions can be made.

To purify the glucosides from plant extracts, they should be isolated by the two ion-exchange chromatography steps before final purification by DEAE–cellulose TLC. Isolation

[12] A. KLEINHOFS, F. A. HASKINS and H. J. GORZ, *J. Chromatog.* **22**, 184 (1966).

from enzyme reaction mixtures usually can be accomplished satisfactorily by the TLC procedure.

The retention of ^3H in melilotic acid glucoside suggests that direct reduction of either *trans* or *cis* *o*-glucosyloxycinnamate occurs in the sweet clover plant. In all three experiments (Table 2) with plants of both the BB and bb genotypes, the specific activity of VI was greater than that of the *cis* precursor. This observation suggests that *o*-coumaric and not coumarinic acid was reduced. In all experiments, however, the $^3H/^{14}C$ ratio of VI was equal to or significantly lower than that of II or I. Essentially similar ^3H retention values were obtained with glucosides from both β-glucosidase-rich and β-glucosidase-deficient genotypes. Thus, it appears that the same pathway of conversion occurs in both genotypes and that the β-glucosidase appears to play a minor role in the conversion of the *o*-hydroxycinnamic acids to melilotic acid in BB genotypes. Reduced glucose retention which occurred in the bb genotype in some experiments may be due to the enhanced activity of the reversible reaction: UDP + melilotic acid-β-D-glucoside ⇌ UDPG + melilotic acid. This would allow reaction of melilotic acid with unlabeled UDPG and synthesis of untritiated melilotic acid glucoside. This assumes that the pool size of UDPG is larger than that of melilotic acid. In addition, Kleinhofs *et al.*[13] obtained no reaction between coumarin and UDPG with the sweet clover glucosylating system. Thus, if the reaction, coumarinic acid glucoside + UDP → coumarin + UDPG occurs in the plant, it would occur only in the direction of free coumarin formation. Dilution of ^3H in the *cis* compound by transglucosylation therefore would not occur and ^3H retention values for *cis* and *trans* glycosyloxy cinnamic acids should be identical if no other glucose-exchange reactions occurred. Such appears to be the case because in all experiments the tritium retention values for the *cis* compound were essentially the same as those for *trans* (Table 2). Any free ^{14}C-coumarin generated by a reaction between UDP and II would be converted to ^{14}C-melilotic acid by the reductase–hydrolase system.[8,9] The melilotic acid could then be glucosylated and such a reaction sequence would reduce the $^3H/^{14}C$ ratio in VI.

Although coumaric acid derivative from the bb genotypes exhibited almost complete retention of glucose, that from the BB genotypes consistently showed a 50% loss. These results could be explained if some hydrolysis of the administered compound had occurred during the feeding period due to release of some β-glucosidase from the cut end of the shoot. However, this idea is contrary to the demonstrated lack of activity of the β-glucosidase toward the *trans* compound (I). Perhaps the reaction, UDP + *o*-coumaric acid-β-P-glucoside ⇌ UDPG + *o*-coumaric acid, occurred in the plant.[13] This would involve equilibration of UDPG-^3H with and dilution by the unlabeled UDPG pool. Subsequent resynthesis of the glucoside with unlabeled UDPG would account for the reduced retention of ^3H in the isolated compound. However, the difference between the two genotypes cannot be explained solely on this basis unless the exchange reaction occurred more rapidly in plants of the BB genotype.

In addition to the B gene, another gene has been implicated in coumarin metabolism in sweet clover.[10] This gene (Cu) appears to act at a site in the pathway prior to the three glucosides discussed in this paper. Plants of the cucu genotype show markedly decreased levels of all *o*-hydroxycinnamic acid derivatives relative to the wild or CuCu genotype. The experiments discussed so far involved plants with the CuCu allele. Other experiments with plants have the cucu allele gave results identical to those already described for the CuCu genotypes.

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EXPERIMENTAL

Preparation of Synthetic ^3H Glycosides

To prepare acetobromoglucose,[14] anhydrous glucose (360 mg) containing 1 mc of D-glucose-6-^3H (New England Nuclear), and acetic anhydride (0·4 ml) were used. The mixture was warmed with stirring for 10 min at 90°. The solution was cooled, freshly distilled acetyl bromide (0·8 ml) was added, the temperature of the reaction mixture kept below 30°. After 1 hr at room temperature, the solution was poured slowly with stirring into 10 ml of ice H_2O. The resultant crystalline residue was collected by filtration, and crystallized from ether-light petroleum[15] yielding colorless crystals, 444 mg, m.p., 84–86° (lit. m.p., 88–89°).[14,15]

o(6-^3H-tetraacetylglucosyloxy)-benzaldehyde was prepared according to Robertson and Waters.[16] The quantities of constituents used were: acetobromoglucose-^{6-3}H (444 mg); salicylaldehyde (0·23 ml), quinoline (1·5 ml); $CaSO_4$ (0·5 g); and Ag_2O (460 mg). The product, crystallized from a hot EtOH–H_2O mixture, although light-brown in color, was used in the next step without further purification. Yield, 469 mg; m.p. 137–138° (lit. m.p., 142°).[16]

trans o-(β-D-6-^3H-tetraacetylglucosyloxy)-cinnamic acid α-^{14}C was prepared by a method adapted after Helferich and Lutzman.[17] The quantities used were: (6-^3H-tetraacetylglucosyloxy)-benzaldehyde (275 mg); malonic acid 2-^{14}C (68 mg, 0·2 mc); pyridine (1 ml); and aniline (0·01 ml). The mixture was kept at 60° for 10 hr. Additional malonic acid (169 mg) was added and the solution was held 12 hr at 60°. Then 10–15 ml of N HCl were added and the precipitated material collected and washed with 1 N HCl. The product was decolorized (charcoal) and crystallized from acetone–water. Yield, 272 mg, m.p. 188–190° (lit. m.p. 187–188°).[17]

trans o-(β-D-6-^3H-glycosyloxy)-cinnamic acid α-^{14}C was prepared by a method described by Tsou and Seligman[18] for other acetylglycosides. The above tetraacetyl derivative (272 mg) was suspended in absolute methanol (1·5 ml, dried over Mg). Dry NH_3 was passed through the solution until saturated. The flask was stored overnight at room temperature in the dark. The solution was then taken to dryness, the residue taken up in warm EtOH, acidified with 5 N HCl, and $CHCl_3$ added slowly to effect precipitation. The amorphous residue was collected, taken up in a minimum volume of hot H_2O, and decolorized (charcoal). Large white needles formed overnight at 2°, yield 98 mg; m.p. 242–243° (lit. m.p. 245°).[17] The product moved as one component in TLC and had the same R_f, absorption spectrum and color reaction characteristic of authentic *o*-coumaric acid β-glucoside. Its specific activity was 0·21 μc/μmole for ^{14}C and 0·45 μc/μmole for ^3H. The overall yield from glucose-6-^3H was 35 per cent.

Source of Plant Material

Seeds of the CuCuBB, CuCubb, cucuBB and cucubb sweet clover genotypes were obtained through the courtesy of Drs. F. A. Haskins and H. J. Gorz, University of Nebraska. The plants were grown in a greenhouse and were over a year old when used. A supply of vigorous young shoots was maintained by frequent pruning of the plants.

Specific activity of β-glucosidase in the genotypes used in these studies were CuCuBB, 0·35; CuCubb, 0·0014; cucuBB, 0·33; and cucubb, 0·0050. Values are expressed as μmoles of *p*-nitrophenyl-β-D-glucoside hydrolyzed per minute per mg protein under the assay conditions described by Schaeffer *et al.*[19]

Administration of the Synthetic Glucoside

Solutions containing 14·7 μmoles/ml of the labeled *o*-coumaric acid β-glucoside were used for the feeding experiments. Terminal 4–6 cm shoots were cut from plants and the stems trimmed under water. 0·5 ml (1·5 μc of ^{14}C) of the radioactive solution was fed to each sample of several shoots (2 g fresh weight) in a 1 ml beaker. After the solution was absorbed, it was washed with three water rinses (0·5 ml). Shoots were then transferred to 30-ml beakers with water; given a short exposure (30 min) to a 15-W, 24-in., long-wave u.v. lamp to promote isomerization of coumaric to coumarinic acid derivatives;[20] and maintained for a period of 18 hr under two 24-in. "cool white" fluorescent lamps at a distance of approximately 15 cm.

Extraction of Plant Tissue

Each sample of shoots was boiled in 100 ml H_2O for 15 min. The suspension was filtered through glass wool and the residue extracted 2× with boiling H_2O. The combined extracts were concentrated and used for ion exchange chromatography described below.

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Fractionation of the Extract

The initial separation step involved anion exchange chromatography on an epoxypolyamine resin (Bio-Rex No. 5). The plant extract was applied to a small column of the above resin in the OH^- form. The column was washed with distilled water and then eluted with a formate gradient (200 ml of distilled water in the mixing chamber and 200 ml of 1 *M* ammonium formate pH 3·4 in the reservoir). The elution of glucosides was followed by radioactivity and TLC analyses of aliquots of fractions.

The coumaric acid glucoside fraction was desalted by adsorption onto a column of partially deactivated charcoal.[21] After a water rinse (10–20 ml), the compound was eluted from the charcoal with water-saturated ethyl acetate made basic with concentrated NH_4OH.

The fraction containing coumarinic (II) and melilotic acid (VI) glucoside was taken to dryness and the residue taken up in distilled water. The fraction was applied to a column of epoxypolyamine resin (acetate form). After washing with 10–20 ml of distilled water, VI was removed from the column with 0·2 *M* acetic acid and II was recovered by elution with 1·0 *M* acetic acid. Each fraction was taken to dryness and the residue taken up in distilled water. An ether extraction at about pH 2 was performed to remove any aglycones present.

All fractions were freeze-dried and taken up in a small volume of absolute methanol. These solutions were then chromatographed twice by TLC procedures described below.

Thin-layer Chromatography

Prior to application of samples, TLC plates of DEAE–cellulose (Mackerey, Nagel, and Co. TLC grade) were irrigated twice in 0·5 M ammonium formate, pH 3·3, and dried in air at room temperature. Samples were then applied and the plate developed in 0·05 M ammonium formate, pH 3·3. The developed plates were examined briefly under u. v. light[12] to locate I and II. The glucosides (e.g. melilotic acid glucoside) were also located by spraying the plates with a 0·5 % emulsion solution followed 15 min later by a spray of diazotized *para*-nitroaniline.[22] The distribution of radioactive compounds was checked by combining scrapings from selected small areas of the plate directly with scintillation fluid for counting. To recover glucosides from the chromatogram, sections of DEAE–cellulose bearing the compounds were scraped from the plate into glass columns, and the columns eluted with 0·2 M acetic acid. The eluates were freeze-dried and the residue taken up in absolute methanol for a repeat of the TLC procedure or in distilled water for the analyses described below.

Determination of Radioactivity and Concentration of the Glucosides

The content of ^{14}C and ^{3}H of each glucoside was determined by differential channels counting in a liquid scintillation system. The concentration of the glucosides was determined spectrophotometrically, using VI, $A_m^{267} = 1{\cdot}04 \times 10^3$; I, $A_m^{267} = 1{\cdot}65 \times 10^4$; II, $A_m^{253} = 1{\cdot}02 \times 10^4$ (all extinction coefficients expressed as $M^{-1}\,cm^{-1}$). The concentration of VI was verified by performing an acid hydrolysis in 1 N HCl as described by Akeson *et al.*[3] and determination of melilotic acid concentration both spectrophotometrically and by a color assay using diazotized *p*-nitroaniline.[23] In the spectrophotometric method, following molar extinction coefficients in 0·05 N NaOH were used: melilotic acid, $A_m^{290} = 4{\cdot}16 \times 10^3$, $A_m^{330} = 0$; coumarin, $A_m^{330} = 6{\cdot}09 \times 10^3$; *o*-coumaric acid, $A_m^{360} = 8{\cdot}82 \times 10^3$ (all expressed as $M^{-1}\,cm^{-1}$).

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