appeared in the blood after 5–6 hr., and the high concentration persisted.

3. The curves for excretion of the $^{15}N$-labelled total, nitrate, urea and ammonium nitrogen fractions in the urine lagged behind their corresponding curves for appearance and disappearance of these components in the blood by about an hour.

4. The formation of methaemoglobin in the blood followed the time course of nitrite formation in the rumen rather closely, suggesting that nitrite was passed rapidly and directly from the rumen to the blood. There was no direct evidence for nitrite formation from nitrate in the blood.

5. Nitrite was bound tightly to the haem of methaemoglobin, for it could be recovered in haem that had been subjected to treatment with acetone containing 1·2 ml. of 12N-hydrochloric acid/100 ml.

This paper is published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The work was supported in part by research grant E-848 from the Division of Research Grants, National Institutes of Health. We are indebted to R. E. Nichols and T. Kowalczyk for providing the fistulated cattle used in these experiments.

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*Biochem. J.* (1961) **81**, 242

# Plant Polyphenols

## 4. HYDROXYCINNAMIC ACID–SUGAR DERIVATIVES*

BY J. B. HARBORNE AND J. JANET CORNER
*John Innes Institute, Hertford, Herts.*

(*Received* 16 *February* 1961)

Hydroxycinnamic acids, which occur in most higher plants, are almost always present in bound form and are liberated from plant extracts by hydrolysis with acid or alkali. Of the organic substances known to occur in combination with hydroxycinnamic acids, quinic acid is the one most frequently mentioned in the literature: thus chlorogenic acid (a quinic ester of caffeic acid) appears to be almost ubiquitous and at least four other caffeoyl quinic acids are known. *p*-Coumaroylquinic acid is also reported to occur with some frequency (Cartwright, Roberts, Flood & Williams, 1955; Griffiths, 1959). In contrast, few simple sugar derivatives have been described (Karrer, 1958) and, with the exception of *o*-coumaroylglucoside (melilotoside) (Charaux, 1925), none has been fully characterized.

* Part 3: Harborne & Sherratt (1961).

In view of current interest in the biosynthesis (McCalla & Neish, 1959), natural distribution (Bate-Smith, 1954; Herrmann, 1958) and biological function (Lee & Le Tourneau, 1958; Rabin & Klein, 1957) of the hydroxycinnamic acids, a study was made of their derivatives occurring in the potato and a number of other higher plants. Besides the *p*-coumaroylglucose and caffeic acid 3-$\beta$-glucoside found in potato berries (Corner & Harborne, 1960), seven other naturally occurring cinnamic acid–glucose derivatives have been found. Further, simple glucose esters have been identified in nearly a hundred plant species.

The finding that glucose esters are of wide occurrence suggests that they may be formed during the metabolism of free hydroxycinnamic acids in plant tissues. Previous work has only indicated that phenolic glucosides (Kosuge & Conn, 1959) and quinic esters (Levy & Zucker, 1960) are

formed *in vivo* from the free acids. A number of these acids therefore were fed to a variety of plants and the metabolites isolated and identified. The results, which have already been reported briefly (Harborne & Corner, 1960), are now presented in detail.

## MATERIALS AND METHODS

*Hydroxycinnamic acids and derivatives.* The free acids were obtained from L. Light and Co. Ltd., or from Fluka A.G., Buchs, Switzerland, or were synthesized by standard procedures from malonic acid and the appropriate aldehyde (Johnson, 1942); they were recrystallized twice from water or aqueous ethanol. The methyl esters were prepared by heating the corresponding free acid in methanol containing 5% (v/v) of sulphuric acid for 2 hr. on a steam bath. Chlorogenic and *iso*chlorogenic acid were obtained by purchase; *p*-coumaroylquinic acid was supplied by A. H. Williams, Long Ashton Research Station. Melilotoside was synthesized from helicin and malonic acid (Helferich & Lutzmann, 1938).

*Sugars and enzymes.* Rutinose was prepared from rutin by hydrolysis with 10% (v/v) acetic acid soln. for 4 hr. at 100°. Gentiobiose and cellobiose were obtained commercially. The purified β-glucosidase, as supplied by L. Light and Co. Ltd., did not contain any free sugars or other contaminants of low mol. wt. and at a concn. of 0·5 mg./ml. hydrolysed aesculin (at a concn. of 5 mg./ml.) to aesculetin at pH 4·6 and 37°. A sample of anthocyanase, prepared from *Aspergillus niger* and supplied by Rohm and Haas Co., Philadelphia, U.S.A., was used as a source of esterase. The enzyme mixture (at a concn. of 7 mg./ml.) completely hydrolysed chlorogenic acid (at a concn. of 3 mg./ml.) to caffeic acid and quinic acid in 30 min. at 37° and pH 3·9.

*Paper chromatography and spectroscopy.* Whatman no. 1 and no. 3 papers (chromatography grade) were used. The solvent mixtures employed were: *A*, butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase); *B*, water; *C*, butan-1-ol–2N-ammonia (1:1, v/v; upper phase); *D*, butan-1-ol–ethanol–water (4:1:2·2, by vol.). Chromatograms were examined in u.v. light of 253 mμ and > 300 mμ sources, in the presence or absence of ammonia vapour.

Spectra were determined with a Unicam SP. 500 spectrophotometer. In measuring the spectra of compounds, eluted from paper chromatograms, allowance for filter-paper impurities was made by using eluates of an appropriate paper blank.

*Feeding experiments.* Leaves were cut from the stems of young plants and the petioles were placed immediately in saturated aqueous solutions of the appropriate cinnamic acids, previously adjusted to pH 7 by the addition of 2N-sodium hydroxide. The leaves were placed, suitably supported, in a controlled environment room at 23° under light-intensity of 800 ft.-candles, applied for 12 hr./day for 1–7 days. Equal numbers of leaves were placed at the same time in distilled water and then treated similarly. Potato disks, 2 mm. thick and 12 mm. in diameter, were cut from scrubbed, freshly harvested tubers of *Solanum tuberosum* cv. 'King Edward', and then washed for 2 hr. with tap water. After drying on filter paper, the disks were placed in Petri dishes containing filter papers soaked in either the aqueous cinnamic acid solutions or in distilled water. The dishes were covered and left at room temp. for 3 days.

After the treatments the plant material was washed with water, dried, cut into small pieces and then plunged into boiling ethanol. After 30 min., the ethanolic extract was decanted and the residue was re-extracted for 30 min. with an equal volume of ethanol. The combined extracts were concentrated *in vacuo* at about 50° and stored overnight at 0° to allow the chlorophyll of green tissue or the starch of tuber tissue to separate out. The clear supernatant liquid was placed as streaks on no. 3 filter paper, which was developed with solvents *A* or *C*. Metabolic products of the cinnamic acids were detected by comparing chromatograms prepared from leaves fed with cinnamic acids with those from leaves fed with water. All new bands on the former chromatograms were cut out, eluted and purified by successive chromatography in solvents *A*–*D*. The substances present in these bands were identified by methods described below.

When doubt existed about the novelty of a particular band on the chromatogram in a feeding experiment, a band of the same $R_F$ was cut from the control chromatogram. The two bands were put through the same purification procedure; direct spectral and chromatographic comparison then indicated clearly whether the compound in question was new or not.

*Isolation of cinnamic acid–sugar derivatives.* These were isolated from fresh leaves, flowers or berries of plants known to contain these substances (see Results) by the procedures described above. Usually, the derivatives present in the concentrated plant extracts were separated in solvent *A*, and the individual bands were purified by chromatography in solvent *B* (to separate from flavonoids), in solvent *C* (to separate from quinic esters of cinnamic acids) and then again in solvent *A*.

*Identification of cinnamic acid–sugar derivatives and related compounds.* Known polyphenols and their hydrolysis products were identified by direct comparison with authentic samples (Harborne, 1960*b*). The cinnamic acid–sugar derivatives were recognized as new compounds by comparing their colour reactions, adsorption spectra and $R_F$ values (Table 1) with those of known, naturally occurring cinnamic acid quinic and methyl esters (e.g. chlorogenic acid and methyl *p*-coumarate). They were identified as follows. Pure solutions were treated with: (*a*) 2N-sodium hydroxide at room temp. for 2–3 hr. (under nitrogen, when *o*-dihydroxylic groups were known to be present in the cinnamic acid); (*b*) N-hydrochloric acid at 100° for 0·5 hr.; (*c*) β-glucosidase [0·2 ml. of aqueous 10% (w/v) solution] in citrate–phosphate buffer, pH 4·5, at 37° for 0·5 hr.; (*d*) esterase [1 ml. of aqueous 10% (w/v) solution] in citrate–phosphate buffer, pH 3·9, at 37° for 6 hr. If hydrolysis took place, the solutions were acidified with Amberlite IRC-120 (H) resin, the free cinnamic acids being extracted into ether and identified by standard procedures (Harborne, 1960*b*). The sugars present in the aqueous residues were identified by comparison with authentic compounds by chromatography in three solvent systems, by spectral measurements on the colour complex formed with resorcinol in sulphuric acid and by their behaviour towards glucose oxidase. The diphenylamine spray reagent (Schwimmer & Bevenue, 1956) and paper electrophoresis were used to distinguish between 1:4- and 1:6-linked disaccharides. The aqueous residues were shown to be free

(except for PC3 and CA5) of non-reducing sugars (benzidine spray), amino acids (ninhydrin spray) and quinic acid and other organic acids (bromophenol blue, silver nitrate and periodate–benzidine sprays).

The ratios of cinnamic acid to sugar were determined in the glycosides by the method used for anthocyanins (Harborne, 1960*a*). The positions of the sugar residues in the glycosides were confirmed by methylation with methyl sulphate and potassium carbonate in acetone for 1 hr. at the b.p., and subsequent hydrolysis with 2N-hydrochloric acid for 0·5 hr. at 100°. The methylated cinnamic acids were extracted into ether and identified by standard procedures (see e.g. Dunlap & Wender, 1960).

*Chromatographic survey of cinnamic acid derivatives.* Fresh leaf, berry, petal or root samples were extracted with boiling ethanol for 0·5 hr., and a portion of the extract, after concentration, was placed as a spot on chromatograms, subsequently developed in solvents *A–D*. A cinnamic acid derivative was recorded as present in a particular plant if its colour reactions and $R_F$ values in at least two solvent systems were the same as the authentic compounds, run on the same chromatograms.

## RESULTS

### *Chemical identification*

Fifteen glucose-containing hydroxycinnamic acid derivatives isolated from natural sources or from plants fed on cinnamic acids (for details, see below) have been characterized. Their properties are shown in Table 1. Compounds OC1, MC1, PC1, CA1, FE1, SN1 and T1 all yield on hydrolysis glucose and the relevant cinnamic acid in equimolecular amounts. The following evidence shows that in these seven compounds D-glucose is attached from its 1-position by a $\beta$-linkage to the carboxyl and not to a phenolic group of the parent cinnamic acid: (1) They are all easily hydrolysed by alkali, esterase and $\beta$-glucosidase, the sugar produced being rapidly destroyed by glucose oxidase. (2) Their colour reactions and spectral characteristics closely resemble those of authentic methyl and quinic esters of the corresponding cinnamic acid. For example, their spectra show the large bathochromic shift of 60–65 m$\mu$ typical of cinnamic acid esters on passing from acid to alkaline solution; by contrast, the alkaline spectrum of melilotoside, (*o*-coumaroylglucoside) shows a hypsochromic shift of 7 m$\mu$. (3) Their chromatographic properties when compared (see Table 1) with quinic esters [i.e. lower $R_F$ values in butanol–acetic acid–water (solvent *A*) but higher $R_F$ values in butanol–2N-ammonia (solvent *C*)] and with the free acids ($\Delta R_F$ values are shown in Table 1) are consistent with their proposed structures. (4) Methylation and subsequent hydrolysis of PC1, CA1 and FE1 give the expected fully methylated cinnamic acid (*p*-methoxy- from PC1 and 3:4-dimethoxy- from CA1 and FE1). By contrast, CA2, 3-glucoside of caffeic acid (see below), gives a partially methylated cinnamic acid, isoferulic acid, on similar treatment. Thus OC1, MC1, PC1, CA1, FE1, SN1 and T1 are the simple D-glucose esters of *o*-, *m*- and *p*-coumaric, caffeic, ferulic, sinapic and 3:4:5-trimethoxycinnamic acid respectively. Compound C1 could not be obtained in sufficient quantity for its characterization to be completed. However, on the basis of its recorded properties and its mode of formation (Table 3), it is provisionally identified as 1-cinnamoylglucose.

The substances OC2, PC2 and CA4 are also simple esters of *o*- and *p*-coumaric and caffeic acid respectively, but they all contain two sugar residues (Table 1). On alkaline hydrolysis, PC2 yields a rhamnosylglucose, chromatographically and electrophoretically identical with rutinose; OC2 and CA4 yield a glucosylglucose, identical with gentiobiose. Thus, although the nature of the disaccharide moieties in these compounds has not been rigorously proved, it is almost certain that OC2 is 1-*o*-coumaroylgentiobiose, PC2 is 1-*p*-coumaroylrutinose and CA2 is 1-caffeoylgentiobiose.

CA2 and CA3, like CA1, give equal amounts of caffeic acid and glucose on hydrolysis with acid or $\beta$-glucosidase. Both compounds have different spectral and colour properties from caffeic acid or its esters and, unlike caffeoylglucose, neither is hydrolysed by alkali or esterase. When methylated, and the product hydrolysed, CA2 yields *iso*ferulic acid. CA2 is therefore the 3-$\beta$-glucoside of caffeic acid; and, by elimination, CA3 must be the 4-$\beta$-glucoside. Thus, all the three possible monoglucose derivatives of caffeic acid (CA1, 2 and 3) have been identified.

Two other compounds have been found, each of which contains a cinnamic acid, glucose and an unidentified residue. The first of these is a *p*-coumaric acid derivative (PC3) which on acid hydrolysis gives glucose and an organic acid, which may be quinic acid. PC3 is not a simple ester, however, and must have an unusual structure, since it is not hydrolysed by $\beta$-glucosidase, by esterase or even by alkali. The other compound, CA5, is hydrolysed by acid or alkali to give caffeic acid and glucose. It is not hydrolysed by $\beta$-glucosidase and is clearly different in its chromatographic properties from the simple glucose derivatives (CA1, 2 and 3). Further work on the characterization of these compounds is in progress.

### *Natural occurrence*

Nine cinnamic acid derivatives containing glucose have been found to occur naturally. The plant sources of the seven that have been completely characterized are shown in Table 2. Three of these, 1-*p*-coumaroyl-, 1-caffeoyl- and 1-feruloylglucose, occur frequently. This is apparent from the

Table 1. *Properties of cinnamic acid–glucose derivatives*

For details see text.

| Designation | $R_F$ values* in solvent | | | | $\Delta R_F$ in solvent *A*: sugar ester minus free acid | Colour | | Spectral maxima | | Hydrolysis products and ratios |
|---|---|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | | Ultraviolet | Ultraviolet/ ammonia | 95% Ethanol | Sodium ethoxide | |
| PC1 | 0·66 | 0·73, 0·81 | 0·30 | 0·75 | 0·28 | Colourless | Blue | 235, —, 313 | 240, 365 | *p*-Coumaric acid (1·00), glucose (1·10) |
| PC2 | 0·49 | 0·84 | 0·16 | 0·64 | 0·43 | Colourless | Blue | 232, —, 314 | 240, 364 | *p*-Coumaric (0·75), glucose (1·00), rhamnose (1·00) |
| PC3 | 0·63 | 0·90 | 0·00 | 0·27 | — | Colourless | Blue | 218, —, 312 | 235, 365 | *p*-Coumaric, glucose |
| CA1 | 0·51 | 0·61, 0·72 | 0·08 | 0·63 | 0·28 | Blue | Green | 250, 305, 334 | 265, 400 | Caffeic (1·00), glucose (1·10) |
| CA2 | 0·46 | 0·46 | 0·02 | 0·55 | — | Blue | Intense purple | 228, 295, 311 | 235, 335 | Caffeic (1·00), glucose (1·20) |
| CA3 | 0·29 | 0·77, 0·83 | 0·00 | 0·46 | — | Blue | Turquoise | —, 300, 322 | 247, 365 | Caffeic (1·00), glucose (1·10) |
| CA4 | 0·29 | 0·77, 0·83 | 0·03 | 0·46 | — | Blue | Green | 245, 305, 335 | 265, 400 | Caffeic (1·00), glucose (1·88) |
| CA5 | 0·29 | 0·81, 0·88 | 0·00 | 0·18 | — | Blue | Green | 240, 302, 328 | 265, 405 | Caffeic, glucose |
| FE1 | 0·57 | 0·64, 0·73 | 0·19 | 0·68 | 0·31 | Blue | Green | 240, 300, 330 | 250, 382 | Ferulic (1·00), glucose (1·13) |
| SN1 | 0·47 | 0·47, 0·57 | 0·16 | 0·66 | 0·37 | Blue | Green | 238, —, 330 | 255, 397 | Sinapic (1·00), glucose (1·20) |
| T1 | 0·62 | 0·74, 0·83 | 0·45 | 0·83 | 0·25 | Mauve | Mauve | 230, —, 307 | 230, 307 | 3:4:5-Trimethoxycinnamic (1·00), glucose (0·96) |
| OC1 | 0·75 | 0·79 | 0·40 | 0·76 | 0·18 | Yellow | Orange | 228, 277, 325 | 287, 387 | *o*-Coumaric (0·51)†, glucose (1·00) |
| OC2 | 0·45 | 0·87 | 0·12 | 0·53 | 0·48 | Yellow | Orange | 230, 277, 327 | 285, 389 | *o*-Coumaric (0·61)†, glucose (2·00) |
| MC1 | 0·70 | 0·85 | 0·46 | 0·74 | 0·21 | Colourless‡ | Mauve | 233, 277, 312 | 287, 344 | *m*-Coumaric (1·00), glucose (0·98) |
| C1 | 0·79 | 0·89 | 0·65 | 0·79 | 0·14 | Colourless‡ | Colourless‡ | — — — | —, — | Cinnamic, glucose |

* By comparison, *p*-coumaroylquinic acid has $R_F$ values of 0·71, 0·92, 0·09, 0·54, and caffeoylquinic acid (chlorogenic acid) has $R_F$ values 0·59, 0·67 and 0·84, 0·01, 0·50, in solvents *A*–*D* respectively.

† Figures are low, because material is lost by cyclization to coumarin (detected by its odour).

‡ Visible as absorbing spots on a fluorescent background in ultraviolet light of 253 mμ wavelength.

Table 2. *Occurrence of cinnamic acid–sugar derivatives in Nature*

Key to methods of comparison, where applicable, with authentic material prepared in feeding experiments: 1, co-chromatography; 2, identification of glucose and the corresponding cinnamic acid on β-glucosidase hydrolysis; 3, spectral measurements; 4, measurement of glucose–cinnamic acid ratio.

| Designation | Sugar derivative | Source | Methods of comparison |
|---|---|---|---|
| PC1 | 1-*p*-Coumaroylglucose | *Lathyrus odoratus* (petals and leaves) | 1, 2, 3 |
| | | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | Tuberous *Solanum* spp. (berries, petals and leaves) | 1, 2, 3, 4 |
| | | *Primula obconica* (petals) | 1, 2, 3 |
| | | *Antirrhinum majus* (petals) | 1, 2, 3, 4 |
| | | *Brunfelsia calycina* (petals) | 1 |
| | | *Raphanus sativus* (young leaves) | 1, 2 |
| PC2 | 1-*p*-Coumaroylrutinose | *Lathyrus odoratus* (petals) | — |
| CA1 | 1-Caffeoylglucose | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | *Ranunculus bulbosus* (leaves, petals) | 1 |
| | | Tuberous *Solanum* spp. (leaves) | 1 |
| | | *Clematis lawsoniana* (petals, leaves) | 1, 2, 3, 4 |
| | | *Helleborus foetidus* (leaves) | 1 |
| | | *Datura knightii*, *D. meteloides* (leaves) | 1 |
| | | *Cestrum newellii* (leaves) | 1 |
| | | *Raphanus sativus* (leaves) | 1, 2, 3 |
| | | *Brunfelsia calycina* (petals, leaves) | 1 |
| | | *Anagallis arvensis* (leaves) | 1, 2 |
| | | *Antirrhinum majus* (petals) | 1, 2, 3 |
| CA2 | Caffeic acid 3-glucoside | Tuberous *Solanum* wild spp. (berries) | 1, 2, 3, 4 |
| CA4 | 1-Caffeoylgentiobiose | *Petunia hybrida* (petals) | — |
| FE1 | 1-Feruloylglucose | *Antirrhinum majus* (petals) | 1, 2, 3, 4 |
| | | *Petunia hybrida* (petals) | 1, 2, 3 |
| | | *Eulalia japonica* (leaves) | 1, 2 |
| | | *Raphanus sativus* (leaves) | 1, 2 |
| | | Tuberous *Solanum* spp. (leaves) | 1 |
| | | *Brunfelsia calycina* (petals) | 1 |
| SN1 | 1-Sinapoylglucose | *Brassica oleracea* (leaves) | 1, 2, 3 |

results presented in Table 2 and also from a chromatographic survey, which was carried out on the leaves and petals of various herbaceous plants grown at this Institute. Of 204 species chosen at random, 43% contained chlorogenic acid, 31% 1-caffeoylglucose, 13% *p*-coumaroylquinic acid and 5% 1-*p*-coumaroylglucose. Although the identification of these substances was based only on chromatographic comparison, the figures for the quinic esters agree with the results of earlier surveys (e.g. Herrmann, 1958) and, together, indicate that sugar esters occur in higher plants with the same order of frequency as the well-known quinic esters.

Of the plant families examined in more detail, two, Solanaceae and Ranunculaceae, appear regularly to contain glucose esters. Thus these substances have been found in the genera: *Brunfelsia*, *Cestrum*, *Lycopersicum*, *Nierembergia*, *Petunia* and *Solanum* of the Solanaceae; and in *Aconitum*, *Anemone*, *Aquilegia*, *Caltha*, *Clematis*, *Delphinium*, *Helleborus*, *Pulsatilla* and *Ranunculus* of the Ranunculaceae. Probably, close examination of the cinnamic esters of plants in other families would reveal a similar situation.

Glucose esters often occur in association with quinic and other esters. Thus leaves of the radish, *Raphanus sativus*, contain what may be choline esters of hydroxycinnamic acids [e.g. sinapin, the sinapic acid ester in the seeds of various Cruciferae (Karrer, 1958)], besides the glucose esters reported in Table 2. Also, chlorogenic acid and 1-caffeoylglucose have been found together in many plants, especially in members of the Solanaceae. As might be expected, the glucose ester content of a particular plant depends upon age and environment. Young radish leaves contain considerable amounts of glucose esters, but older leaves lack them completely; and leaves of tuberous *Solanum* species grown in the glasshouse and in the field show variation in the nature and amounts of their sugar esters.

Table 2 shows that a number of plants each contain more than one sugar ester. Thus petals of *Petunia hybrida* cv. 'Flaming velvet' contain 1-*p*-coumaroyl-, 1-feruloyl- and 1-caffeoyl-glucose as well as 1-caffeoylgentiobiose, a result which has been confirmed by the parallel studies of Birkhafer, Kaiser & Kosmol (1960). The three glucose esters also occur together in young radish leaves, in petals of *Brunfelsia calycina* and in petals of *Antirrhinum majus*. The earlier report (Schütte & Böhme, 1958) that quinic esters are present in the petals of the white genotype, *nivea*, of this latter

plant has been shown to be unfounded (Harborne & Corner, 1961).

Phenolic glucosides are much more restricted in their distribution in Nature than the glucose esters. Only one such compound, caffeic acid 3-β-glucoside, has been found in the present survey and this occurs exclusively in berries of wild tuber-bearing *Solanum* species (i.e. all 19 species examined). It is absent from the berries of the cultivated potato (12 clones of varying ploidy examined) and of non-tuberous *Solanum* plants (11 species examined). In contrast, berries of cultivated potatoes contain a considerable amount of 1-*p*-coumaroylglucose and free 7-hydroxycoumarin (umbelliferone), which are absent or very rare in the wild species. This finding is of considerable taxonomic interest, since it has a bearing on the relationship between wild and cultivated potatoes. Similar chemical differences in polyphenolic content of tubers (Dodds & Long, 1955; Harborne, 1960*a*) and of petal constituents (Harborne, 1960*b* and unpublished work) have been noted.

The natural sources of the incompletely identified glucose derivatives PC3 and CA5 are as follows: PC3 accompanied by some 1-*p*-coumaroylglucose is the major hydroxycinnamic acid derivative in both leaf and petal of the various colour forms of the garden *Geranium*. CA5 occurs with chlorogenic acid in the leaves of the tomato, cv. 'Potentate', of *Solanum neohawkesii* and of *S. canasense*. A number of other cinnamic acid derivatives, probably containing both sugar and other residues, have been noted during this work and it is hoped to examine those occurring in the leaves of wild potato species in due course.

*Formation from cinnamic acids*

The main products of feeding the leaves of a variety of plant species for periods of 1–3 days with the cinnamic acids listed in Table 3 are glucose esters. The esters formed from *p*-coumaric, caffeic, ferulic and sinapic acids are identical in every way with material isolated from natural sources. During metabolism, glucose is attached to the carboxyl in preference to a phenolic hydroxyl group of the cinnamic acids in all the plants studied. Even if the carboxyl group is protected and a compound such as methyl *p*-coumarate is fed, the product is not the expected phenolic glucoside but the glucose ester (in this instance, 1-*p*-coumaroylglucose). This must be formed via the free cinnamic acid which was also detected after feeding and is presumably produced on hydrolysis of the methyl ester by one of the well-known plant esterases. In this study, the only plant found to produce phenolic glucosides is the tomato; caffeic acid is metabolized in the leaves mainly to caffeoylglucose but small amounts of the 3- and 4-β-glucoside of caffeic acid are also formed.

Plants allowed to metabolize *o*-coumaric acid for longer periods (3–7 days) produce the glucose ester accompanied by lesser amounts of the glucosylglucose (probably gentiobiose) ester. No appreciable quantities of the related quinic esters appear to be formed during the course of these experiments. Thus a careful search for *p*-coumaroylquinic acid among the metabolites of *p*-coumaric acid, in plants not naturally containing this ester, was completely unsuccessful. A similar examination of plants for chlorogenic acid after feeding caffeic acid

Table 3. *Sugar derivatives formed in leaves fed with hydroxycinnamic acids and related compounds*

Relative amounts formed are indicated thus: + + +, over 50%; + +, about 20%; +, 10% or less. Plant species are: A, various cultivated and wild *Solanum* species; B, *Cestrum newellii*; C, *Raphanus sativus*; D, *Clematis lawsoniana*; E, *Lycopersicum esculentum*; F, *Datura knightii*.

| Compound fed | Sugar derivatives produced | Plant species |
|---|---|---|
| L-Phenylalanine | 1-*p*-Coumaroylglucose | B |
| | 1-Feruloylglucose (+) and 1-*p*-coumaroylglucose (+ + +) | A |
| Cinnamic acid | 1-Cinnamoylglucose (+) and 1-*p*-coumaroylglucose (+ + +) | A, C |
| | 1-*p*-Coumaroylglucose | B, D, E |
| *o*-Coumaric acid | 1-*o*-Coumaroylglucose | A, D |
| | 1-*o*-Coumaroylglucose (+ + +) and 1-*o*-coumaroylgentiobiose (+ +) | E |
| *m*-Coumaric acid | 1-*m*-Coumaroylglucose (+), 1-feruloylglucose (+ + +) and caffeic acid 4-β-glucoside (+) | A, E |
| *p*-Coumaric acid | 1-*p*-Coumaroylglucose | A, D, E |
| | 1-Caffeoylglucose (+ +) and 1-*p*-coumaroylglucose (+ + +) | C |
| Caffeic acid | 1-Caffeoylglucose | A, B, D |
| | 1-Caffeoylglucose (+ + +), caffeic acid 3-β- (+) and 4-β-glucoside (+) | E |
| | Scopolin | F |
| Ferulic acid | 1-Feruloylglucose | A, E |
| Sinapic acid | 1-Sinapoylglucose | A, E |
| 3:4:5-Trimethoxycinnamic acid | 1-(3:4:5-Trimethoxycinnamoyl)glucose | A, E |

was not so easy, since this ester occurs naturally in large amounts in many plant species. However, no chlorogenic acid was found in the leaves of radish or of *Clematis lawsoniana* plants either before or after feeding with caffeic acid.

Several other products, besides the simple glucose esters, have also been found (Table 3). The major product of feeding cinnamic acid to potato and radish is not cinnamoylglucose, but 1-*p*-coumaroylglucose, presumably formed by *p*-hydroxylation *in vivo*. The introduction of a *m*-hydroxyl group into *p*-coumaric acid occurs in radish leaves; 1-caffeoylglucose is a minor metabolite of *p*-coumaric acid in this plant. *o*-Hydroxylation is also carried out in radish leaves, since caffeic acid is converted into a mixture of 1-caffeoylglucose and the related coumarin, aesculin. Similarly, scopolin is formed from caffeic acid in the leaves of *Datura knightii*. Hydroxylation and methylation are both involved in the unexpected production in some solanaceous plants of 1-feruloylglucose from *m*-coumaric acid. A number of cinnamic acid precursors were also fed to potato leaves. These were DL- and L-phenylalanine, L-tyrosine, phenylpyruvic acid, β-phenylpropionic acid and DL-2-phenyl-lactic acid. Of these, L-phenylalanine is the only effective precursor, large quantities of 1-*p*-coumaroylglucose, accompanied by traces of 1-feruloylglucose, being formed after feeding. Other solanaceous plants also convert phenylalanine into 1-*p*-coumaroylglucose; in contrast, leaves of the radish and *Impatiens balsamina* were not able to utilize phenylalanine in this way.

The results described above were obtained by feeding cinnamic acids through the petioles of leaves and also, for the potato and radish, by infiltration into tuber and root disks. Metabolic experiments were carried out mostly with leaves of the potato and other Solanaceae but a sufficient number of plants of other families was examined to show that glucose ester formation is a general property of most higher plants. It is also clear that plants vary in the ways they metabolize these cinnamic acids to other derivatives. In general, the nature of the cinnamic acid derivatives produced in the leaves of a particular plant appeared to be independent of the environment that plant was grown in, or the age of the leaves when sampled.

The possibility that some of the products of feeding experiments shown in Table 3 occur naturally in trace amounts cannot be ruled out. One such product, scopolin, was identified as a trace constituent of untreated *Datura knightii* leaves. However, spectral measurements on carefully purified samples from leaves fed with water or caffeic acid solution indicated that 8·2 times more scopolin was produced as a result of the feeding. There can thus be no doubt that caffeic acid is an efficient precursor of scopolin in this plant.

## DISCUSSION

Since the majority of natural phenols occur in plants as glycosides, it is not surprising to find that cinnamic acids also occur frequently in Nature in combination with sugars. The fact that simple glucose esters have not been described before may be attributed to the earlier emphasis on the quinic esters, especially on chlorogenic acid, which was isolated first by Payen (1846) and later characterized by Görter (1908). It is now apparent that, in a number of surveys, chlorogenic acid has not been satisfactorily distinguished from 1-caffeoyl- or 1-feruloyl-glucose. Indeed, 1-feruloylglucose and chlorogenic acid have identical $R_F$ values in a number of common solvent systems and can only be separated in butanol–ammonia or butanol–ethanol–water. The glucose esters of *Antirrhinum majus* were first thought to be quinic derivatives (Schütte & Böhme, 1958), partly because only quinic esters were known to occur widely at that time. Now glucose esters are known to occur widely, it seems probable in retrospect that the caffeic acid ester (designated as 'band 510'), isolated by Sondheimer (1957) from green coffee beans and several common fruits, was 1-caffeoylglucose.

Only three different sugars (glucose, gentiobiose and rutinose) have been found in cinnamic acid esters, but it is probable that others await recognition. Already, Kameswaramma & Seshadri (1947) have described a compound, pajaneelin, which, on hydrolysis, gives *p*-coumaric acid and fructose. Besides the simple glucose esters, there are a number of complex glucose derivatives of cinnamic acids, which are more difficult to characterize. A few such compounds have already been described and two substances found during the present investigation fall into this class. One of these (CA 5) is a caffeic acid derivative which differs from all the known caffeic acid–sugar compounds (see Table 4).

The discovery that glucose esters are formed from free hydroxycinnamic acids when fed to plants is unexpected, in view of two facts. First, that most $C_8$ phenols are rapidly converted into the corresponding β-glucosides when infiltrated into plant tissue (Hutchinson, Roy & Towers, 1958; Pridham & Saltmarsh, 1960); and, secondly, that Kosuge & Conn (1959) found that *o*-coumaric acid was converted into its phenolic glucoside (melilotoside) in sweet-clover leaves. A different enzyme from that required for synthesizing phenolic glucosides (Cardini & Yamaha, 1960) is presumably required for ester formation. Esterification

Table 4. *Naturally occurring caffeic acid glycosides and their $R_F$ values*

| Name | Sugars and other residues | $R_F$* in solvent *A* | $R_F$* in solvent *D* | Reference |
|---|---|---|---|---|
| 1-Caffeoylglucose | Glucose (1) | 0·51 | 0·63 | This paper |
| 1-Caffeoylgentiobiose | Glucose (2) | 0·29 | 0·47 | This paper |
| Caffeic acid 3-β-glucoside | Glucose (1) | 0·46 | 0·55 | This paper |
| Caffeic acid 4-β-glucoside† | Glucose (1) | 0·29 | 0·46 | Klosterman & Muggli (1959) |
| Echinacoside | Glucose (2), rhamnose (1), 3:4-dihydroxyphenylethanol (1) | 0·44 | 0·31 | Stoll, Renz & Brack (1950) |
| Compound CA5 | Glucose (1) | 0·29 | 0·18 | This paper |
| Orobanchin‡ | Glucose (1), rhamnose (1) and catechol deriv. (?) | 0·56 | 0·63 | Bridel & Charaux (1925) |

* Chlorogenic acid has $R_F$ 0·59 in solvent *A*, $R_F$ 0·50 in solvent *D*.
† Only known to occur naturally in combined form, in the seed of flax, *Linum usitatissimum*.
‡ This compound, occurring in *Orobanche* plants, is incorrectly claimed to be chlorogenic acid by Privat (1959). Buddleoside, a compound present in *Buddleia davidii*, is either identical with, or very similar in structure to, orobanchin (W. D. Ollis, personal communication).

probably occurs in preference to glycosylation because it both neutralizes the acid grouping and also improves the sap solubility of the cinnamic acids.

Some of the results obtained have a bearing on the biosynthesis of cinnamic acids and related compounds. Levy & Zucker (1960) have suggested that the hydroxylation of cinnamic to *p*-coumaric to caffeic acid *in vivo* occurs with the quinic esters, and not with the free acids. The present results show that hydroxylation can equally well take place with the glucose esters as enzyme substrates. The role of L-phenylalanine and cinnamic acid as precursors in the synthesis of the hydroxycinnamic acids in *Salvia splendens* (McCalla & Neish, 1959) and of chlorogenic acid in potato disks (Levy & Zucker, 1960) has now been extended to cover the hydroxycinnamic acids of the leaves of potato, tomato and other plants. It thus appears that the route:

phenylalanine → cinnamic acid → *p*-coumaric acid

is a fairly general one for hydroxycinnamic acid biosynthesis.

It has often been suggested (see, for example, Grisebach & Ollis, 1961) that cinnamic acids can be converted *in vivo* by *o*-hydroxylation followed by *trans–cis* isomerism and intramolecular cyclization into the related coumarins. The present discovery that aesculin and scopolin are formed from caffeic acid in the radish and *Datura knightii* respectively shows that such a pathway is followed in some plant tissues.

Finally, it is apparent that cinnamic acid glucose esters are as important as the well-known quinic esters in the economy of plant cells. The ease with which they are formed from the free acids, their turnover in young radish leaves and their presence in many plants in amounts much smaller than the quinic esters suggest that they may be more important biosynthetic intermediates than compounds such as chlorogenic acid.

## SUMMARY

1. Fifteen hydroxycinnamic acid–sugar derivatives have been isolated from plants or produced by feeding plants with cinnamic acids.

2. They are the glucose esters of cinnamic, *o*-, *m*- and *p*-coumaric, caffeic, ferulic, sinapic and 3:4:5-trimethoxycinnamic acid, caffeoyl and *o*-coumaroylgentiobiose, *p*-coumaroylrutinose and caffeic acid 3- and 4-β-glucoside. The remaining two cinnamic acid derivatives contain glucose and different unidentified residues.

3. 1-*p*-Coumaroyl-, 1-caffeoyl- and 1-feruloylglucose occur widely in the leaves and flowers of many plants. In contrast, caffeic acid 3-glucoside is known only in the berries of wild potato species.

4. Free cinnamic acids, when fed to plants, are generally converted into the corresponding glucose esters. Caffeic acid is also converted in some plants into aesculin or scopolin or into a mixture of its 3- and 4-β-glucoside. The hydroxylation and methylation of cinnamic acids has been observed *in vivo*.

5. It is suggested that the simple hydroxycinnamic acid glucose esters may be important biosynthetic intermediates in plants.

The authors wish to thank Dr G. Hussey for useful discussions and his helpful provision of plant material. They acknowledge with gratitude a gift of *p*-coumaroylquinic acid from Mr A. H. Williams.

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*Biochem. J.* (1961) **81**, 250

# The Synthesis of *meso*Inositol in Germ-free Rats and Mice

BY N. FREINKEL AND R. M. C. DAWSON*
*Thorndike Memorial Laboratory, Second and Fourth Medical Service (Harvard), Boston City Hospital, Mass., U.S.A.*

(*Received* 19 *May* 1961)

The precise role of dietary *meso*inositol in mammalian nutrition remains controversial despite more than two decades of inquiry. Early reports linking inositol to the promotion of growth and prevention of alopecia in mice (e.g. Woolley, 1940, 1941) and rats (e.g. Pavcek & Baum, 1941; Cunha, Kirkwood, Phillips & Bohstedt, 1943) have not been uniformly reduplicated (e.g. Martin, 1941; Fenton, Cowgill, Stone & Justice, 1950; Ershoff & McWilliams, 1943; McCormick, Harris & Anderson, 1954). In addition, no characteristic syndrome of inositol deficiency has yet been described in man. However, the recent findings that added inositol is required for the propagation of human cells in tissue culture (Eagle, Oyama, Levy & Freeman, 1957), and unbound inositol is concentrated in most mammalian tissues (Dawson & Freinkel, 1961) prompted a re-examination of the endogenous biosynthesis of inositol.

* Permanent address: Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge.

Excellent relevant data were available in this area before the inception of the present studies. Needham's (1924) observations of sustained inositoluria in rats maintained on low inositol diets had been re-enforced by the independent reports of Daughaday, Larner & Hartnett (1955) and Halliday & Anderson (1955) that [$^{14}$C]inositol could be recovered from rats given repeated injections of [$^{14}$C]glucose. However, as was first suggested by Woolley (1942), such 'endogenous' inositol could have originated from alimentary tract micro-organisms which are known to profoundly affect the picture in other fields of mammalian metabolism, e.g. the [$^{14}$C]urea catabolism of mammals is entirely due to associated micro-organisms (Kornberg, Davies & Wood, 1954). To minimize intestinal contributions, Daughaday *et al.* (1955) excised the entire gut immediately before analysis of the carcass for inositol. The possibility of microbial biosynthesis and absorption of labelled inositol from the intestine during the 3 days