STUDIES ON THE PHENOLIC COMPOUNDS OF APPLE LEAF TISSUE

(*Malus pumila* Mill.)

A thesis presented in partial fulfilment of the requirements
for the degree of Master of Horticultural Science
in Plant Science

by

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Chapter 1

INTRODUCTION -
THE PHENOLIC COMPOUNDS IN HIGHER PLANTS

1.1 The Natural Phenolic Compounds in Higher Plants

A number of books dealing wholly or partly with the natural phenolic compounds have been published in recent years. A comprehensive treatment of phenolic compounds is presented in one of them (1). Specific discussion of the flavonoid group of phenolic compounds is also found elsewhere (2,3). In these references much of the literature on phenolic compounds is cited and reviewed.

1.1.1 The nature of phenolic compounds

Substances which possess at least one aromatic ring bearing an hydroxyl substituent constitute the phenolic compounds. Several hundred such compounds are known in plants. They represent a number of groups of compounds including the simple monocyclic phenols, the flavonoids and their relatives, the xanthones, phenolic quinones, alkaloids and sterols, besides polymeric materials such as the tannins and lignins (4).

Further discussion of the natural phenolic compounds in this thesis will be largely confined to the two groups of monocyclic phenols (benzoic and cinnamic acid derivatives) and the flavonoid group of compounds. This recognises the relative importance of these three groups of phenolic compounds in plants in general and in the genus Malus in particular. While the flavonoid compounds constitute the largest group of natural phenols (4), the phenolic acids of the benzoic and cinnamic acid groups are present in variety in many plants (5). The following outline of each of these three groups will illustrate their nature.

1.1.2 Benzoic acid derivatives (C6 - C1 compounds)

Most of the simple phenolic acids (see Table 1) are apparently widely distributed in Angiosperms. For example, an examination of the leaves of 122 plants representing 86 families showed that p-hydroxybenzoic and gentisic acids occurred in 97% of the plants (6).

Three acids, p-hydroxybenzoic, vanillic and syringic acids, occur as ester groups in lignin and, as a rule, plants which do not contain lignin lack these acids (6). Other benzoic acid derivatives occur in plants (5), some of which are rich sources of derivatives with unusual hydroxylation patterns (6).

The interrelationships of all these hydroxybenzoic acids are not clear at present since the origin of the carbon skeleton of some is unknown (7).

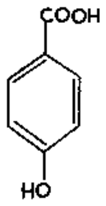
1.1.3 Cinnamic acid derivatives and related compounds (C6 - C3 compounds)

The common cinnamic acid derivatives are shown in Table 1 and are all derivatives of trans-cinnamic acid. These acids are represented in combined form in practically every higher plant. In a sample of the leaves of Angiosperms the frequencies of occurrence of

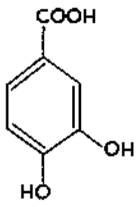
Table 1

STRUCTURE OF SOME BENZOIC AND CINNAMIC ACID DERIVATIVES

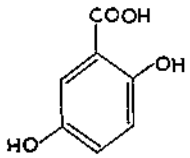
Benzoic Acid Derivatives



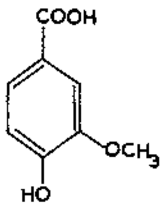
p-hydroxybenzoic acid



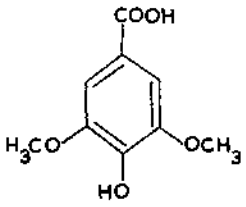
protocatechuic acid



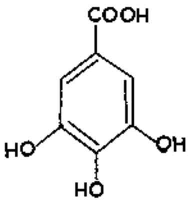
gentisic acid



vanillic acid

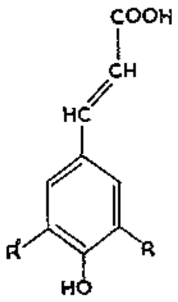


syringic acid



gallic acid

Cinnamic Acid Derivatives



General structure

R = R' = H	p-coumaric acid
R = OH; R' = H	caffeic acid
R = OCH_3; R' = H	ferulic acid
R = R' = OCH_3	sinapic acid

p-coumaric, caffeic, ferulic and sinapic acids were 49, 63, 48 and 32% respectively (6).

Other cinnamic acid derivatives are rare, for example, 3,4,5-trimethoxycinnamic acid. The o-hydroxycinnamic acids are readily cyclized to coumarins during isolation and tend to be recorded as such. So far the only o-hydroxycinnamic acid isolated is o-coumaric acid (6).

The alcohols corresponding to p-coumaric, ferulic and sinapic acids (p-coumaryl, coniferyl and sinapyl alcohol respectively) are thought to be precursors of lignin, a polymer containing such C6 - C3 units (6).

Other C6 - C3 compounds include the coumarins, the <u>iso</u>coumarins and the chromones, while the lignans which occur in heartwoods are dimers of C6 - C3 units (6).

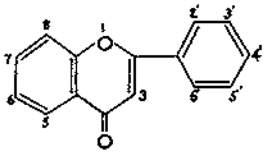
1.1.4 The flavonoid compounds (C6 - C3 - C6 compounds)

The flavonoid nucleus is represented by the structure of flavone, 2-phenylchromone, in Table 2 (8). The molecule consists of two benzene rings (A and B) joined by a three-carbon bridge which is formed into a γ-pyrone ring. In nature, various classes of flavonoid compounds occur and they are distinguished only by the state of oxidation of the three-carbon bridge (8). Details of these classes are presented in Table 2 and a few examples of specific compounds are given. The numbering of the flavone nucleus applies to all other classes except the dihydrochalkones and chalkones, where the C5 and C7 (flavone) become C2' and C4' and the aurones, where C5 and C7

Table 2

CLASSES OF THE FLAVONOID COMPOUNDS

The Flavonoid Nucleus



flavone

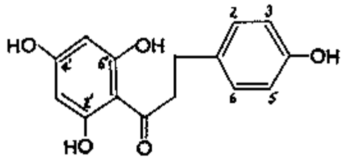
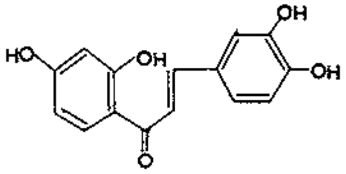
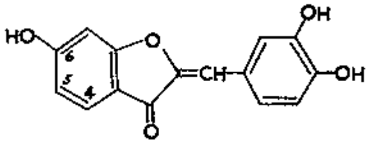
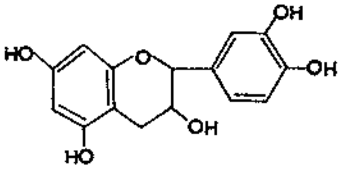
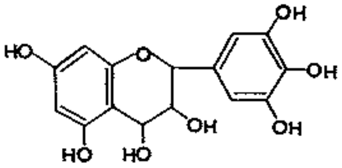
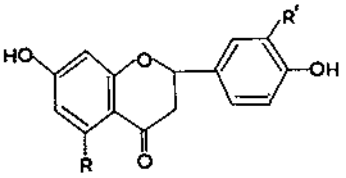
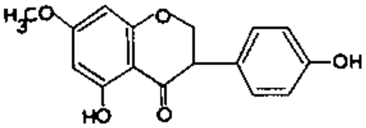
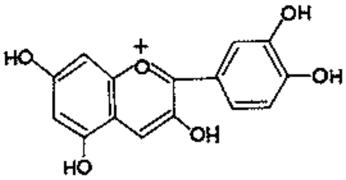
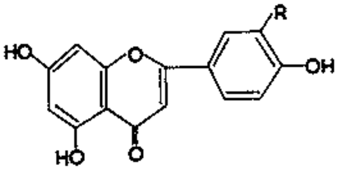
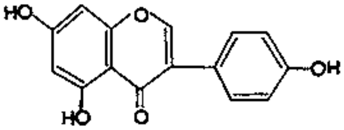
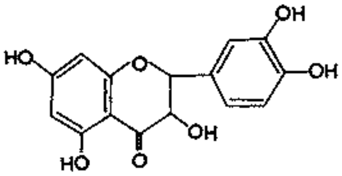
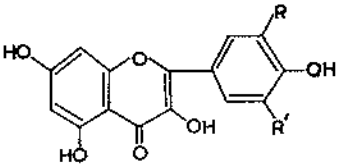
Class	Structure	Name of Compound
Dihydrochalkones		Phloretin
Chalkones		Butein
Aurones		Sulphuretin
Flavan-3-ols		Catechin
Flavan-3,4-diols		Leucodelphinidin

Table 2 (continued)

Flavanones		R = H;R' = OH Butin R = OH;R' = H Naringenin R = R' = OH Eriodictyol
Isoflavanones		Padmakastein
Anthocyanidins		Cyanidin
Flavones		R = H Apigenin R = OH Luteolin
Isoflavones		Genistein
Dihydroflavonols		Taxifolin
Flavonols		R = R' = H Kaempferol R = OH;R' = H Quercetin R = R' = OH Myricetin R=OCH$_3$;R'=H Isorhamnetin

become C4 and C6. While the dihydrochalkones, chalkones, aurones, isoflavanones and isoflavones lack the basic 2-phenylchromone skeleton they are nevertheless classed as flavonoids on the grounds of their close chemical and biosynthetic relationship with the group (8).

A number of compounds are known within each class of the flavonoids as a consequence of the substitution of largely hydroxyl and methoxyl groups in both the A and B rings. All these compounds may be referred to as flavonoid aglycones. There are certain common patterns of substitution in the A and B rings which may be explained on the basis of their biosynthetic origin. Usually hydroxyl groups in the A ring are at both the C5 and C7 positions or only at C7 and they normally remain unmethylated. From one to three hydroxyl or methoxyl groups may be present in the B ring. It is usual to find a free hydroxyl group in the C4' position with further orthohydroxyl or methoxyl groups at the C3' and C5' positions. These substitution patterns in the A and B rings are consistent with the derivation of the former ring from acetate units and the latter from cinnamic acid type precursors (8).

Compounds with less usual substitution patterns are the result of the introduction of hydroxyl or methoxyl groups into other positions in the rings or the removal of such groups from normal positions, or both (8). The extent of this variation in substitution differs with the particular class of flavonoid and where it is most marked and varied the greater is the variety of aglycones in the class.

With almost 100 flavonol and flavone aglycones known, the former class accounting for nearly two-thirds of the total, variation in the substitution pattern is wide - from flavone itself to digicitrin, 3',5-dihydroxy-3,4',5',6,7,8-hexamethoxyflavone (8). An outline of the substitution pattern in flavones and flavonols is presented in Table 3.

Table 3

SUBSTITUTION IN FLAVONES AND FLAVONOLS (8)

Location of hydroxyl or methoxyl substituents	Frequency of occurrence
C5 and C7	over 90%
C4'	under 80%
C3'	about 50%
C6	over 33%
C8	about 25%
C2'	about 10%
C5'	about 10%

The hydroxyl substituent at C6 and C8 is methylated in 70-80% of cases but the C5 substituent is methylated in about only 15% of cases (8). A 4'-methoxyl group is rather common in flavones but rare in other flavonoids (6).

In the flavones only apigenin and luteolin are considered of frequent occurrence in Angiosperms (6). Kaempferol, quercetin and myricetin are the three most commonly occurring flavonols and in some groups of plants they can almost be regarded as common metabolites (8).

For example, these compounds are represented in the leaves of 90% of a sample of woody dicotyledons (8). In a sample of 1000 Angiosperms a leaf survey showed the frequency of occurrence of kaempferol, quercetin and myricetin to be 48%, 56% and 10% respectively. Probably every higher plant contains a flavone or flavonol (9). However, many of the reported flavones and flavonols (6) have been found so far only in a single species (8). The dihydroflavonols often co-occur with the related flavonols and those corresponding to kaempferol, quercetin and myricetin occur widely in heartwoods (6).

Of the twelve known anthocyanidins, six are common. They are flavylium cations structurally related to the flavonols and are unstable in the free state; salt formation or glycosylation stabilises them. The common ones are widely distributed in higher plants, in leaves, flowers and fruits, where they may make a considerable contribution to plant colour (6).

Flavanones are of fairly general distribution and naringenin and eriodictyol are relatively common (6). The *iso*flavanones are rare (8). *Iso*flavones are largely confined to one sub-family on the Leguminosae, the Papilionatae. Twenty-six *iso*flavones are known and 20 of them have been isolated from the Leguminosae (6).

The 20 naturally occurring chalkones are probably very limited in their distribution. Chalkones undergo partial or complete ring closure to yield the corresponding flavanones when treated with dilute alkali, or usually dilute acid. This conversion is a ready one in the case of 2',4',6'-trihydroxychalkones and is mediated enzymatically by flavanone synthetase in several plants (8). Only six aurones are known (8). With the chalkones they constitute the anthochlor pigments which are abundant only in the Compositae, despite their occurrence in several other families (9).

Four dihydrochalkone compounds are known to occur naturally. Phloretin, the most common one, is present in Malus and in the Ericaceae, where asebogenin, the 4'-methylether of phloretin, is also present. Smilax glycyphylla (Liliaceae) contains phloretin too. Four species of Malus contain a 3-hydroxyphloretin compound. The fourth dihydrochalkone occurs in the essential oil of Populus balsamifera (6).

The flavan-3-ols may be collectively called catechins (8,10) and several natural ones are recorded (10). In substitution patterns they correspond to known aglycones in other classes of flavonoid compounds. The name catechin is given to a specific flavan-3-ol. In nature it occurs as (+)-catechin, often called d-catechin, and its laevorotatory diastereoisomer (-)-epicatechin, or l-epicatechin, is also a natural product. Both isomers are of wide distribution and frequently occur together (10).

Leucoanthocyanins, or leucoanthocyanidins, are compounds which yield anthocyanidins when boiled with aqueous or alcoholic hydrochloric acid. All leucoanthocyanidins for which structures are definitely established are flavan-3,4-diol derivatives, but their widespread occurrence in nature may mean that other structures await discovery (11). Leucoanthocyanidins are especially widespread in the woody dicotyledons, 60% having these compounds in their leaves (8). They undergo oxidation and polymerisation readily in vitro (11). The main mode of occurrence is in the polymeric form, as flavolans, which are especially abundant in heartwoods where they are usually responsible for the dark colour (8). Structural determinations have been restricted to the flavan-3,4-diol derivatives (10). With three asymmetric centres in the molecule, a variety of optically active forms is possible (11).

The flavan-3-ols and the flavan-3,4-diols are oxidised in plants to brown pigments. Flavolans, themselves coloured, are also oxidised (8). The condensed tannins are polymeric forms of the flavan-3-ols and flavan-3,4-diols (8,11). The hydrolysable tannins, based on gallic acid (3,4,5-trihydroxybenzoic acid) is the other group of commercial tanning agents (6).

1.1.5 Biosynthetic interrelationships

A brief outline of the current understanding of the biosynthesis of the benzoic and cinnamic acid derivatives and the flavonoid compounds is presented here to underline the interrelationships of these groups of natural phenols in higher plants. (See Figure 1 (12,13).)

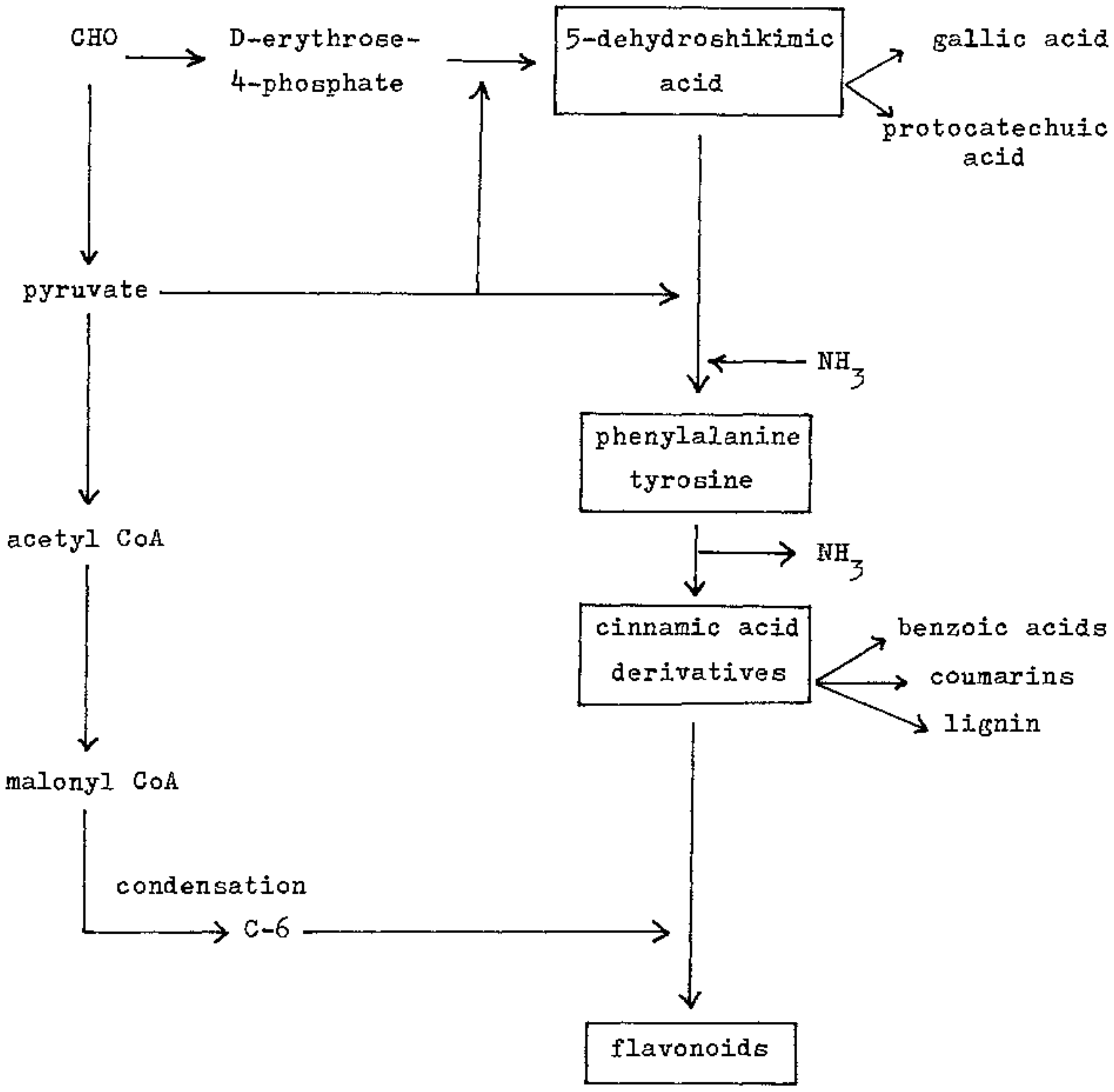
Cinnamic acid derivatives are thought to be derived from the amino acids phenylalanine and tyrosine, themselves synthesised in plants via the shikimic acid pathway.

Another general pathway which is well established for the synthesis of the aromatic ring in plants is the one involving the head to tail condensation and subsequent cyclisation of acetate units. This pathway may give rise to benzoic acid derivatives which may also arise by β-oxidation of appropriate cinnamic acid derivatives.

The carbon skeleton of the flavonoid compounds (C-15) comprises a C-6 unit (acetate derived) and a C6 - C3 unit (cinnamate derived);

Figure 1

SCHEME FOR BIOSYNTHESIS OF PHENOLIC COMPOUNDS IN HIGHER PLANTS



thus the biosynthesis involves the condensation of precursors derived from the two major pathways of aromatic ring biosynthesis in plants.

1.2 The Occurrence of Phenolic Compounds

In this section the main forms in which the phenolic compounds introduced above are found in higher plants will be discussed briefly.

1.2.1 Occurrence of the three groups of phenolics in the plant kingdom

Many organisms, from bacteria to higher plants, synthesise benzoic and cinnamic acid derivatives, but the combination of C-6 and C6 - C3 precursors in C-15 flavonoid compounds is restricted almost entirely to the flowering plants and ferns (7,14). Microorganisms can synthesise the C6 - C3 (phenylpropane) unit while fungi can synthesise both the C-6 and C6 - C3 precursors but cannot combine them into flavonoid compounds (14). All parts of higher plants contain flavonoid compounds but certain compounds tend to be restricted to particular tissues (8,14).

1.2.2 Natural forms of phenolic compounds

In the living cells of higher plants phenolic compounds normally occur in combined forms, as glycosides and esters (15,12,7). Storage tissues (seeds, berries) and dead or dying tissues (heartwood of trees) may contain free phenols (15). The main combined form of phenolic compounds is the glycosides and, with several hundred sugar-aglycone combinations recorded, the phenolic-O-glycosides is the most extensive known group of plant glycosides. In the glycosides an hydroxyl group of the phenol is linked in β-configuration (with a few exceptions) to the sugar molecule (15). Most plants contain more than one glycoside of any aglycone (8).

Some general discussion on the sugars of the phenolic glycosides is presented by Harborne (15). The five common plant monosaccharides found attached to phenols are, in order of decreasing frequency of occurrence, D-glucose, D-galactose, D-xylose, L-rhamnose and L-arabinose. D-glucuronic acid may also occur in these glycosides.

Many phenolic diglycosides occur in plants, but the structures of only ten of their disaccharide units have been elucidated (15). Trisaccharide, but not tetrasaccharide, units occur in phenolic glycosides, and while structures for six trisaccharides have been proposed, they have not been rigorously established. More trisaccharide units have still to be characterised (15). The oligosaccharides of phenolic glycosides contain only $1 \longrightarrow 2$ or $1 \longrightarrow 6$ linkages, with the sugar at the reducing end nearly always glucose. Many are specific to the phenolic glycosides, being absent elsewhere in plants in the free or combined state (15).

The phenolic compounds which occur as esters are the phenolic acids chiefly of the benzoic and cinnamic acid types (7,12).

1.2.3 Flavonoid glycosides

In plants, flavonoid compounds occur typically as glycosides, in which an hydroxyl group is joined in a semi-acetal link to the sugar molecule. In some cases more than one hydroxyl group in a molecule may be linked in this manner to an equal number of sugar molecules. The sugars found include mono-, di- and trisaccharides, combined through the C-1 of the sugar to form monosides, biosides and triosides. The frequency of occurrence of simple sugars in the glycosides, in decreasing order, is D-glucose, D-galactose, L-rhamnose, L-arabinose and D-xylose. D-glucuronic acid also occurs, at low frequency (8).

Flavonol glycosides constitute by far the largest and most varied class of phenolic glycosides and they have been isolated from many sources (15). Glycosylation is usually through the 3-position (about 70% of cases) while the 7-position is less commonly glycosylated (about 20% of cases) (8). C-4'-glycosides are known (8) as are 3,4'-diglycosides, but no 5-glycosides are recorded (15). In some more complex flavonol glycosides, p-coumaric, ferulic and caffeic acids are found as acyl groups attached to the sugar residues (15).

Glycosylation in flavones is most commonly at the C-7 hydroxyl group (80%), followed by the C-5 and C-4' groups (8). Flavone 8-C-glycosides are formed in plants, and nearly all 8-C-glycosides isolated are of the flavone class (15). The glycosidic pattern in flavanones is akin to that of the flavones, for example, 7-glucosides are common and 5-glycosides are known (15). Ten isoflavone glycosides are known and five are 7-glucosides. In the heartwood of trees many isoflavones occur in the free state (15).

Glycosylated anthocyanidins are called anthocyanins, and here the pattern of glycosylation is similar to that in flavonols (15). However, if a second position in the anthocyanin molecule is glycosylated, it is normally at the 5- rather than at the 7-position as with flavonols. Glycosylation in the 3-position appears essential for the stability of the anthocyanin pigments as they are unknown without a sugar in the 3-position. Some anthocyanins with hydroxycinnamoyl residues attached to their sugars are known (15).

Catechins and leucoanthocyanidins occur as the aglycones in heartwood and even in leaf tissue (7). In the leaf of the tea plant, Camellia sinensis, the common catechins occur esterified in the 3-position with gallic acid (16).

Aurones are present only as glucosides (8), and mainly as 6-glucosides (15). Glucose is the only sugar found in chalkone glycosides, which include monosides and biosides, so far (8). The sugar is usually in the 4'-position. Sometimes chalkone-2'-glucosides co-occur with the corresponding flavanone-5-glucosides (15).

Six dihydrochalkone glycosides occur. In Malus, phloretin occurs as the 2'- and 4'-O-glucosides and also as the arabinosylglucoside and xylosylglucoside. The same genus yields 3-hydroxyphloretin-4'-O-glucoside. Phloretin-2'-O-rhamnoside occurs in Smilax glycyphylla (15).

1.2.4 Natural forms of cinnamic acid derivatives

The cinnamic acid derivatives occur in plants in more combined forms than any other group of plant phenols (15). They are usually found as esters of a variety of compounds, including cyclohexane carboxylic acids like quinic acid, sugars, organic acids such as tartaric, phenolic alcohols, flavonoids and alkaloids. Glucosides are also known (7,15).

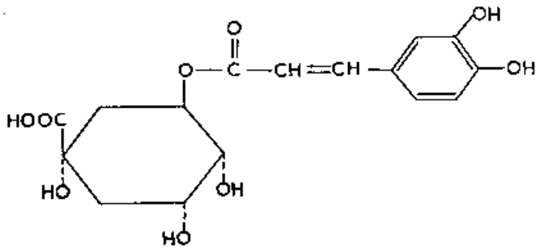
Conjugates of p-coumaric, caffeic and ferulic acids with quinic acid and with glucose occur widely in plant tissues. Many are found as trace components in very complex mixtures of phenolic compounds (17). Esters with quinic acid are common, especially in the case of caffeic acid; the 3-O-caffeoylquinic acid ester being known as chlorogenic acid (see Table 4). Other isomers are known (15,18). Many plants contain 3-O-p-coumaroylquinic acid, while the occurrence of 3-O-feruloylquinic acid is known (15). Glucose esters of p-coumaric, caffeic and ferulic acids are of common occurrence. These are mono-esters with the structure shown in Table 4 (12). p-Coumaroyl, caffeoyl and feruloyl esters of shikimic acid have also been isolated (15).

Feeding the leaves of a variety of plants with L-phenylalanine, cinnamic, o-coumaric, m-coumaric, p-coumaric, caffeic, ferulic, sinapic and 3,4,5-trimethoxycinnamic acids, for periods of 1-3 days, yields mainly the glucose esters of the phenolic acids. The structures of the esters with p-coumaric, caffeic, ferulic and sinapic acids are identical with the natural esters of the same composition (7) (see Table 4). Plants may form some glycosides as well as the predominant esters when fed cinnamic acid derivatives, for example, the 3- and 4-β-glucosides of caffeic acid are formed in addition to large amounts of caffeoyl-D-glucose, in tomato plants fed caffeic acid (7). (See Table 4 for the structure of the glucoside.)

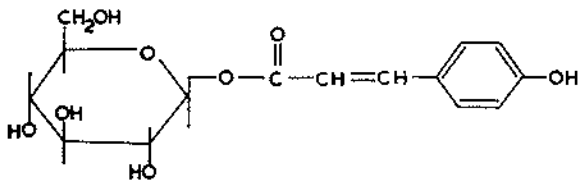
Shakuchirin (Table 4) is an example of a more complex compound containing a sugar and hydroxycinnamic acids. Many derivatives of this type exist but the complete structures of only a few have been

Table 4

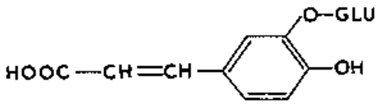
STRUCTURES OF SOME NATURAL HYDROXYCINNAMIC ACID DERIVATIVES



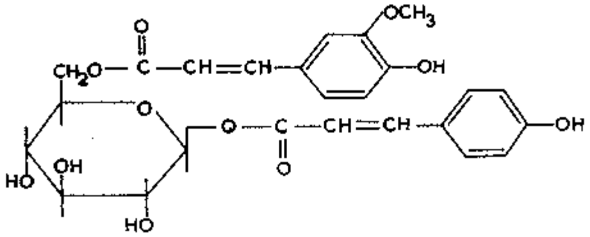
3-O-caffeoylquinic acid
(chlorogenic acid)



p-coumaroyl-D-glucose



caffeic acid-3-glucoside



1-p-coumaroyl-6-feruloylglucose
(chakuchirin)

determined (15).

1.2.5 Benzoic acid derivatives

Either esters or glycosides, or both, of a variety of benzoic acid derivatives are found in all higher plants (7). Apparently the composition and structures of many of these esters and glycosides are not known (5,7).

1.2.6 The significance of ester and glycoside formation

The reasons for the occurrence of phenolic compounds as esters and glycosides in actively metabolising plant tissues are not clearly understood.

Since plants convert administered phenolic compounds to glycosides and esters, these compounds may be looked upon as the products of detoxication reactions (7) which may prevent the interference of free phenols with some vital cellular mechanisms (15). The greater sap solubility and mobility in the cell vacuole of the glycosides over the parent phenols (8,15) may be significant. Glycosylation may protect the phenol from some enzymatic attack; potato phenolase does not oxidise the 3-glycosides of quercetin and myricetin, presumably because of steric reasons, for it will oxidise the corresponding aglycones (8).

1.2.7 The function of phenolic compounds in plants

Several functions have been suggested for phenolic compounds found in higher plants, but any vital function has yet to be uncovered.

The classification of phenolic compounds as 'secondary metabolites' (12) suggests functions which are more specific to particular plants than to plants in general. The function of some flavonoid compounds, particularly the anthocyanins, as plant pigments in leaves, flowers and fruits is forwarded (8,9). It is also recognised that phenolic compounds may play a part in determining the resistance of certain plants to attack by pathogenic organisms (19).

Utilisation of phenolic glycosides and esters as respiratory or reserve materials seems not to occur, the compounds remaining as inert metabolic by-products once synthesised. In the light of this knowledge it is difficult to understand the not uncommon occurrence of from 1-5% of the fresh weight of some plant tissues as a particular flavonoid compound (7).

For some phenolics, labelling studies have indicated the compounds to be in a state of metabolic flux in the living plant (20) and other studies have demonstrated the effect of light duration (21) and light quality (22) on the levels of them. Taken together with the well known effects of phenolics on the activity of the indolylacetic acid oxidase enzyme system from plants (23), these results provide some indication of a possible physiological function for certain phenolics.