PHENOLIC ACIDS AND LIGNIN IN WHEAT.
TRACER STUDIES OF THEIR FORMATION FROM PHENYLPROPANOID COMPOUNDS AND IN PHOTOSYNTHESIS

By

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ABSTRACT

Quantitative determinations were made, by a newly developed method, of p-coumaric, ferulic, sinapic, p-hydroxybenzoic, vanillic and syringic acids which occur as alkali-hydrolyzable derivatives in ethanolic extracts and in ethanol-insoluble residues of wheat tissues. Changes in the ethanol-soluble and ethanol-insoluble derivatives of these acids during growth and development were determined. A new phenolic acid, orthoferulic acid, was isolated from wheat. C^{14}-labeled compounds were administered to wheat shoots and their incorporation into lignin and into hydroxycinnamic acids was measured. Labeled CO_2, phenylalanine or tyrosine were incorporated more readily into the insoluble derivatives of hydroxycinnamic acids than into ethanol-soluble derivatives whereas the reverse was true for labeled precursors such as cinnamic and the hydroxycinnamic acids. Time-course studies with $^{14}CO_2$ suggested the sequence: CO_2 → aromatic amino acids → ethanol-insoluble derivatives of hydroxycinnamic acids → lignin. A scheme is suggested to explain these complex relationships.

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DISCUSSION

INTRODUCTION

Hydroxycinnamic acids, such as p-coumaric, caffeic, ferulic and sinapic acids, are widely distributed in vascular plants in the form of esters (Bate-Smith, 1962a). Together with cinnamic acid itself, they have been implicated as intermediates in the biosynthesis of lignin as well as of other phenolic plant products. This is the result of a number of tracer and enzyme studies (Neish, 1964a), many of which have been carried out with the wheat plant.

Apart from the work of Griffiths (1958a) and of Urban (1959) on the levels of hydroxycinnamic acids in certain species there is very little information available concerning the quantities of these acids in plant tissues, particularly at various stages of growth and development in any one species.

Reliable methods for the estimation of hydroxycinnamic acids did not appear to be available and the present work was concerned initially with the development of a quantitative procedure. A suitable method was developed and applied to the analysis for phenolic acids in wheat tissues.

In the course of this work several unidentified esters of hydroxycinnamic acids were detected in ethanolic extracts of wheat and attempts were made to identify these. In addition, it was found that relatively large amounts of phenolic acids, particularly p-coumaric and ferulic acids, are released on alkaline hydrolysis of the insoluble residue remaining after ethanolic extraction of wheat shoots. Quantitative determinations of these ethanol-insoluble esters, at various stages of growth and development of the plant, indicated that they were not end-products of metabolism but might, in fact, be concerned with lignification. Studies were made, therefore, of the formation of both soluble and insoluble ester forms of certain hydroxycinnamic acids and of their relationships to lignin formation employing the tracer technique.

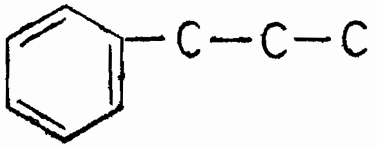
In common with all other organic plant products, the hydroxycinnamic acids and lignin must be ultimately derived from carbon dioxide in photosynthesis. Although a few studies on lignin formation in wheat, from $^{14}CO_2$ in photosynthesis, had been carried out more than a decade ago (Stone, 1953; Brown et al., 1953), the question of the relationships of the hydroxycinnamic acids to lignin formation was not examined in these experiments. Evidence for the participation of the acids in lignin biosynthesis stemmed from tracer experiments in which either these acids or closely related compounds were administered to shoot or stem cuttings of plants and their incorporation into lignin was determined. A number of questions arose out of these experiments; are the hydroxycinnamic acids natural precursors of lignin in wheat? i.e. can the relationships of these acids to lignin formation be demonstrated from $^{14}CO_2$ in intact plants? What is the relationship between the ethanol-soluble and the ethanol-insoluble esters of hydroxycinnamic acids?

A major part of the present study consisted, therefore, of a series of attempts to resolve some of these problems.

REVIEW OF LITERATURE

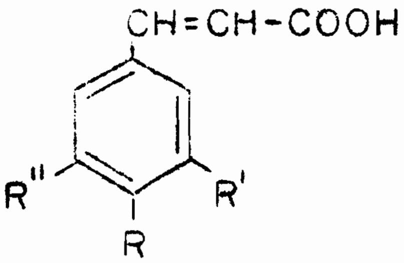
PART 1. THE PLANT PHENOLIC ACIDS

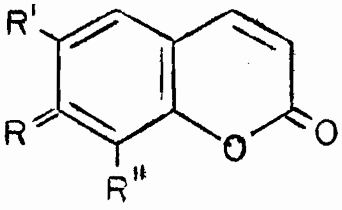
The most prominent structural unit among the phenolic substances in higher plants is the nine carbon moiety represented by the phenylpropanoid unit:



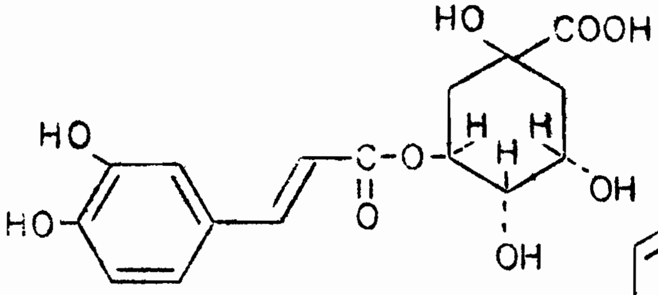
This structural unit forms the carbon skeleton of the aromatic amino acids, of the cinnamic acids and their derivatives, coumarins, lignans, flavonoid compounds and lignins. Representative examples of some of the naturally occurring phenylpropanoid compounds and their derivatives are shown in Figure I.

The more common naturally occurring phenolic acids in living tissues of higher plants are members of the cinnamic (C_6-C_3) acid series and of the benzoic (C_6-C_1) acid series. Phenylacetic (C_6-C_2) acids are much less frequently encountered. Coumarins, the lactones of cinnamic acids, and some cinnamyl and benzyl aldehydes and alcohols are probably as widely distributed in higher plants as their corresponding acids. So far there has been no single flowering plant examined which does not contain one or more of the aforementioned compounds. Evidence for these conclusions has come from the results of extensive screening of a vast number of plant species for their polyphenolic constituents (Bate-Smith, 1954, 1956, 1962a, 1962b; Kremers, 1957; Griffiths, 1958b; Ibrahim et al., 1961).



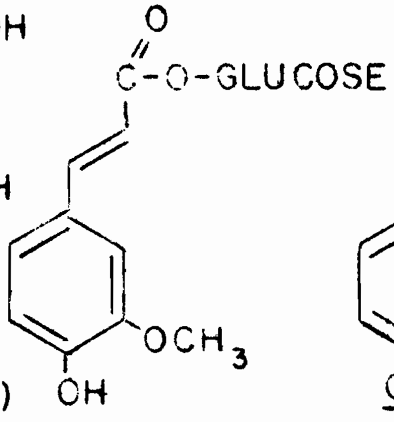


R, R', R" = H	CINNAMIC ACID	R, R', R" = H	COUMARIN
R', R" = H, R = OH	p-COUMARIC ACID	R', R" = H, R = OH	UMBELLIFERONE
R" = H, R, R' = OH	CAFFEIC ACID	R" = H, R, R' = OH	ESCULETIN
R" = H, R' = OCH_3, R = OH	FERULIC ACID	R' = H, R, R" = OH	DAPHNETIN
R = OH, R', R" = OCH_3	SINAPIC ACID	R" = H, R = OH, R' = OCH_3	SCOPOLETIN

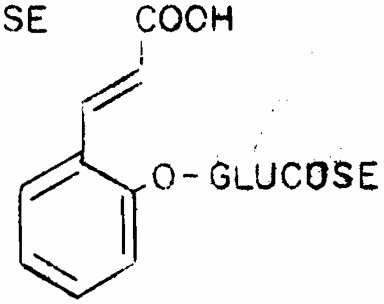


CHLOROGENIC ACID

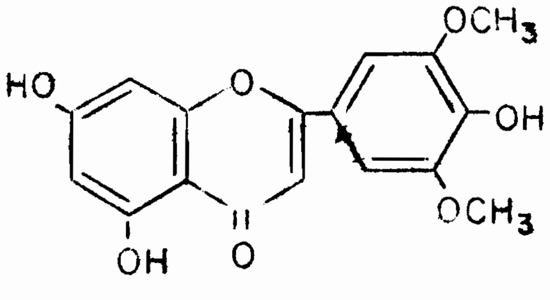
(3-O-CAFFEOYL-D-QUINIC ACID)



FERULOYL GLUCOSE ESTER

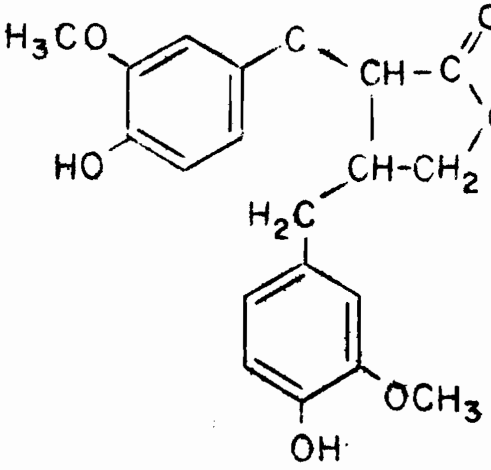


O-COUMARIC ACID GLUCOSIDE



TRICIN

(FLAVONOID)



MATAIRESINOL

(LIGNAN)

Figure 1. Some Naturally-occurring Phenylpropanoid Compounds in Higher Plants.

I. CHEMISTRY AND BIOCHEMISTRY

A. Naturally Occurring Forms

It is well known that the phenolic acids are almost always present in tissues of higher plants as conjugates of a variety of organic compounds, being linked together in glycosidic or ester linkages (Bate-Smith, 1962a). Some of the known naturally occurring conjugates are listed in Table I.

Compounds of the following classes have been found to occur as conjugates of phenolic acids: 1. aliphatic alcohols and acids; 2. phenols, phenolic alcohols and acids; 3. amino acids and alkaloids; 4. flavonoid compounds including anthocyanins; 5. various mono-, di- and tri-saccharides; 6. terpenoid compounds; 7. glycoproteins; and 8. cell wall material including lignin. Of these compounds, the most frequently reported is chlorogenic acid, a depside of quinic and caffeic acids. This compound was first discovered by Robiquet and Boutron in 1837. It has since been shown to be almost ubiquitous in higher plants (Politis, 1949; Sondheimer, 1962). At least three other isomers of chlorogenic acid have been detected and isolated. Isochlorogenic acid was isolated from unroasted coffee beans by Barnes _et al_. (1950); neochlorogenic acid was obtained as a crystalline material from peaches by Corse (1953) and the third isomer, pseudochlorogenic acid, was isolated by Uritani and Miyano (1955) from sweet potatoes infected with blackrot.

Table I

Some Naturally-occurring Esters of Phenolic Acids in Plants

Compound	Structure	Species	References
Gaultherin	Primveroside of methylsalicylate	*Gaultheria procumbens*, leaves	Procter, 1843
Linocinnamarin	Glucoside of methyl *p*-coumarate	*Linum usitatissimum*, seed	Klostermen *et al.*, 1955
Sinapine	Ester of sinapic acid and choline	Widely distributed in crucifers	Schultz and Gmelin, 1952
Sinalbin	Glucoside of choline sinapate	*Sinapis alba* and other crucifers	Karrer, 1958
Cinnamoylcocaine	Cinnamoyl ester of cocaine	*Erythroxylon* spp.	Manske and Holmes, 1950
Rescinnamine	3,4,5-trimethoxycinnamic acid ester of methyl reserpate	*Rauwolfia serpentina*	Klohs *et al.*, 1954
Theogallin	Ester of gallic and quinic acids	Thea leaves	Cartwright and Roberts, 1955; Roberts and Mayers, 1958
1-*p*-Hydroxybenzoyl-β-glucose	Ester of *p*-hydroxybenzoic acid and glucose	*Catalpa bignonioides* flowers	Birkofer *et al.*, 1961

The closely related compound p-coumaroylquinic acid was reported by Cartwright et al. (1955) in tea, apple and pear and by Williams (1958) in apple. This compound was critically identified by Haslam et al. (1961a) as 3-O-p-coumaroylquinic acid. Three different isomers of feruloylquinic acid in addition to chlorogenic, isochlorogenic and neochlorogenic acids were detected in green coffee, by paper and cellulose-column chromatography, by Pictet and Brandenberger (1960). Crystalline 3-O-feruloyl-D-quinic acid was subsequently isolated and identified by Corse et al. (1962) from unroasted coffee beans. It is of interest in this respect that no evidence has yet been obtained for the presence of the cognate compound, p-coumaroylquinic acid, in coffee beans. Similarly the natural occurrence of cinnamoylquinic acid has not yet been described although the identification of this compound in potato tubers administered L-phenylalanine or cinnamic acid is suggestive (Levy and Zucker, 1960; Hanson and Zucker, 1963). The 1,4-dicaffeoyl depside of quinic acid, cynarine, has been isolated and identified from artichokes (Panizzi and Scarpati, 1954; Panizzi et al., 1954). Another compound believed to be 1,4-di-p-coumaroylquinic acid has been reported to be present in stem tissues of the pineapple plant by Sutherland and Gortner (1959).

It was only recently that the natural occurrence of shikimic acid esters of hydroxycinnamic acids has been reported. Goldschmid and Hergert (1961) identified p-coumaroyl-, caffeoyl- and feruloyl-shikimic acids among the many phenolic constituents of the cambial sap of the western hemlock.

Reports on the natural occurrence of sugar esters of phenolic acids indicate the wide distribution enjoyed by these compounds in living tissues of higher plants (Harborne and Corner, 1961) although they occur only in small amounts. So far, very few of them have been isolated and fully characterized. Glucose and gentiobiose esters of p-coumaric, caffeic and ferulic acids, as well as a caffeoyl-rutinose ester, have been isolated and identified by Birkofer et al. (1960, 1961). Similar sugar esters were isolated by Corner and Harborne (1960) and by Harborne and Corner (1961). These authors added to the list the glucose ester of sinapic acid and the rutinose ester of p-coumaric acid.

Corner et al. (1962) presented evidence for the presence in the roots of Polygala senega of a number of sugar esters of hydroxylated cinnamic acids. The analytical data suggested that these sugar esters are not all simple mono esters but that two of them are polycinnamoyl derivatives of D-glucose containing sinapoyl and 3,4,5-trimethoxycinnamoyl residues. However, the exact nature or molecular size of these compounds has not been determined because of the small amounts isolated. There were also some indications that two other compounds present may be depsides of ferulic acid and of ferulic and p-coumaric acid respectively.

Apparently, the glycosides of hydroxycinnamic acids are less frequently encountered in nature than are the sugar esters. The glucosides of o-coumaric acid and its dihydro form, melilotic acid, are well known in higher plants (Kosuge and Conn, 1959, 1961; Brown et al., 1960). Recently the monoglucosides of caffeic acid (Harborne and Corner, 1961) and of 2-hydroxy-4-methoxy-cis-cinnamic acid (Brown, 1963) have been reported to occur naturally in plants.

The occurrence of cinnamic acids in amide linkages has also been indicated. Karimoto et al. (1962) have reported the isolation of annuloline, which contains a 3,4-dimethoxycinnamic acid residue, from the grass Lolium multiflorum. An unstable complex ester, which breaks down in solution to give chlorogenic and caffeic acids in addition to at least six amino acids, has been isolated by Clark et al. (1959) from potato peel.

Ferulic acid together with two unidentified phenolic compounds was obtained upon alkaline hydrolysis of a glycoprotein fraction isolated from wheat flower pentosans (Fausch et al., 1963). However, treatment of the pentosans with pure hydrolytic enzymes released no phenolic compounds. It was tentatively assumed that ferulic acid is linked to the xylan chain of the glycoprotein via ester linkages. Similarly, the esterification of ferulic acid with terpenoid compounds has been reported. Tamura et al., (1959) have isolated crystalline dihydro-γ-sitosteryl ferulate in 0.01% yield from wheat germ oil. Ohta (1960) has identified oryzanol C, in oil of rice bran, as 24-methylene-cycloartenyl-ferulate.

p-Coumaric acid was reported as acylating glucosides of some flavonoid compounds. p-Coumaryl-3-glucosides of pelargonidin, paeonidin, cyanidin and petunidin were shown to be the main pigments present in cultivated diploid species of tuberous Solanum (Harborne, 1956, 1957; Harborne and Sherratt, 1957). Genetical studies have revealed the presence of a gene for acylation in these species (Dodds and Long, 1955, 1956). Other hydroxycinnamic acids have also been found as acylating the 7-hydroxyl group of some flavonoid compounds, e.g., p-coumaric acid in tiliroside (Bate-Smith, 1962a) and sinapic acid in rubrobrassicin (Hörhammer et al., 1959). Occasionally ferulic acid is condensed with coniferyl alcohol to form a lignan molecule such as matairesinol which has been isolated from the heartwood of Picea abies by Freudenberg and Knof (1957).

It is most interesting that the phenolic acids may be esterified with lignins. This was first reported by Smith (1955a) who showed that alkaline hydrolysis of native lignin (the methanol soluble fraction of lignin) from aspen (Populus tremula) releases p-hydroxybenzoic, vanillic, syringic and ferulic acids. In this particular case p-hydroxybenzoic acid was released in substantially large amount accounting for 6.9% of the native lignin. Subsequent studies by the same author (1955b) revealed the presence of similar ester groups in native lignins of other plants. Ferulic, p-coumaric, vanillic and sometimes syringic acids were released in varying proportions from native lignins of sugar cane, wheat, Douglas fir and chestnut. Smith (1955a) concluded that these phenolic acid molecules are linked individually by ester linkages to aliphatic hydroxyl groups of lignin.

Pearl and his associates in a series of papers (see Pearl et al., 1957, 1958, 1959, 1960 and 1961a) confirmed and extended Smith's results. Alkaline hydrolysis of sawdust of the heartwood, or of the bark, of more than forty species of woody dicotyledons and palms released p-hydroxybenzoic, vanillic and syringic acids as well as the corresponding aldehydes and p-coumaric and ferulic acids in varying proportions according to the species. Although syringic acid and syringaldehyde were consistently released on alkaline hydrolysis of all examined species, the corresponding sinapic acid has not been recorded as being present. Neither were the dihydroxy acids, protocatechuic and caffeic acids obtained.

More recently Stafford (1962) demonstrated the presence of p-coumaric and ferulic acids among other phenolic constituents in alkaline hydrolyzates of timothy grass, Phleum pratense. She emphasized that caffeic and sinapic acids were not detectable in these alkaline hydrolyzates.

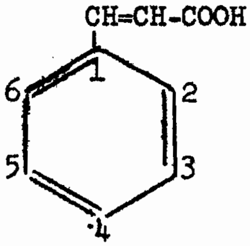
Apart from the more common phenolic acids mentioned previously, there are others which are much less frequently encountered in extracts of plant tissues. A list of these acids is presented in Table II. It should be borne in mind, however, that these acids also occur in a bound form, the exact nature of which remains to be investigated.

B. Properties and Isolation

The phenolic acids are much stronger acids than phenols. Their acidity increases with substitution especially in the ortho position. They are, in general, colorless or faintly colored compounds, very slightly soluble in cold water, soluble in hot water, ethanol and diethyl ether. The alkali and the alkali earth metal salts of these acids are soluble while the heavy metal salts are very sparingly or not at all soluble in water. Besides the typical properties of acids, these compounds show reactions characteristic of the aromatic nucleus.

Table II

Some Naturally, but Less Frequently Occurring Phenolic Cinnamic Acids in Higher Plants

Phenolic acid	Plant species	References
Cinnamic acid  	Free and esterified in many resins and essential oils e.g. *Cinnamomum casia*	Geissman and Henriener, 1952
p-Methoxycinnamic acid	As the ethyl ester in various species of *Aloë*	Geissman and Henriener, 1952
Isoferulic acid, (4-Hydroxy-3-methoxy-cinnamic acid)	*Catalpa ovata*	Hiramoto and Watanabe, 1940
3,4-Dimethoxycinnamic acid	Rhizomes of *Veronica verginiana*	Karrer, 1958
3,4-Methylenedioxy-cinnamic acid	*Piper sp.*	Simmonds and Stevens, 1956

The phenolic acids occur in plants largely as esters and glycosides (Bate-Smith, 1962a). The general procedure for their extraction from plant tissues involves the use of diethyl ether or, better still, hot aqueous ethanol, methanol or isopropanol. Boiling N NaOH has also been used (Pearl et al., 1957).

The method described by McCalla and Neish (1959b) has been found to be very satisfactory by other workers (Ibrahim and Towers, 1960). In this method, the plant material is extracted with aqueous ethanol and the ethanolic extract is evaporated to dryness. The residue thus obtained is re-extracted in a small volume of boiling water and filtered through Celite. The filtrate is extracted with ether, to remove the free acids, and then subjected to either alkaline or acid hydrolysis to release the phenolic acids bound in either ester or glycosidic linkages. The acids released on hydrolysis are subsequently extracted with ether under acidic conditions.

Phenolic acids may be separated as their insoluble silver, lead or barium salts with subsequent decomposition of the salts as silver or lead sulfides and barium sulfates. Better separation may be achieved by multiple extraction with buffers of different pH using the counter current distribution method (Freudenberg and Harkin, 1963). Other possible ways for separation of the individual phenolic acids or their derivatives are fractional crystallization, fractional distillation or fractional sublimation (Clarke and Nord, 1955). Generally speaking, all these methods are laborious and lack the sensitivity necessary for detection of the low amounts of phenolic acids present in plant extracts.

The application of the chromatographic technique to the analysis of phenolic compounds in higher plants has opened a new era in the study of these substances and their distribution in plant tissues. By means of chromatographic methods a larger number of plants has been screened for phenolic constituents and many phenolic substances infrequently detected in the past, such as chlorogenic acid, have shown to be almost ubiquitous in higher plants.

Paper chromatography was first used for separation of phenols by Bate-Smith (1948); Bate-Smith and Westall (1950). Since then it has been the most frequently used technique for the analysis of plant phenolics. Many methods have been developed and data for the separation of various phenolic substances have been summarized by Block _et al._ (1958) and more recently by Harborne (1961). Of particular importance in this respect are the papers published by Bate-Smith (1954, 1956); Pearl _et al._ (1957, 1958); Van Sumere _et al._ (1957); Griffiths (1957) and Ibrahim and Towers (1960) on the separation of phenolic acids and polyphenolic constituents in hydrolyzed plant extracts; and by Swain (1953); Reppel (1954); and Harborne (1960) for separation of the coumarins; and finally by Pearl _et al._ (1961b) and Harborne and Corner (1961) for the separation of simple glycosides and esters of phenolic acids. Comprehensive lists of R_f values for a large number of simple phenolic substances in six solvent systems were published by Reio (1960).

The isolation of various phenolic substances from plant extracts has also been achieved through chromatography on columns of cellulose (McCalla and Neish, 1959b); charcoal (Towers _et al._, 1963); a mixture of equal volumes of Darco G. charcoal and Celite (Neish, 1957);

silicic acid (Hanson and Zucker, 1963) and ion exchange resins (Seki et al., 1959; Towers, 1964b). Column chromatography is a particularly useful method for the isolation of single components present in appreciable amounts in plant extracts as well as for the partial purification of crude extracts. However, the very high resolution of complex mixtures possible on paper sheets is not achieved very often and the separations are not always reproducible.

Despite the fact that gas-liquid partition chromatography and adsorption chromatography on thin layer plates have proven ideal methods for the quick, sensitive resolution of primary plant metabolites in extremely complex mixtures, their application to the study of the plant phenolic substances has been limited. Esters of ferulic acid have been detected by gas chromatography in acetone extracts of tamarac heartwood (Nair and Rudloff, 1959); β-phenyllactic acid was determined in urine (Williams et al., 1963) and vanillic and ferulic acids (methyl esters) were detected in a yeast fermentation by-product (Robbins et al., 1963). The biochemical application of gas phase chromatography to the analysis of phenolic compounds has been recently discussed by Burg (1962) and by Burchfield and Storrs (1962).

Thin layer chromatography has also been used for separation of a variety of phenols and phenolic substances (Pastuska, 1961; Stundt and Saccardi, 1962). Stahl and Schorn (1961) have studied the application of this method for separation of coumarin, flavone derivatives, hydroxy acids, tannins, anthracene derivatives and lichen substances. The aromatic aldehydes produced on alkaline nitrobenzene oxidation of lignin have been separated and studied by this method (Kratzl, 1961).

Other references on the use of thin layer chromatography for separation of phenolic compounds can be found in the recent book by Bobbit (1963).

C. Detection and Quantitative Determination

There are various well-known chemical tests for the detection of the aromatic acids the details of which are given by Bray and Thorpe (1954) and by Clarke and Nord (1955). The hydroxamic acid test can be used for detection of the carboxyl group, whereas the nitration test is specific for the aromatic nucleus. The tests most frequently mentioned in the literature, however, are the phenolic color reactions with various chromogenic reagents. The reaction with ferric chloride (Svendsen, 1951) although useful is not specific for phenols but is given also by hydroxypyridines, quinolines, oximes, enols and some carboxylic acids. Blue, green, violet or red colors constitute a positive reaction. Stronger colors are produced with phenols containing two hydroxyl groups ortho "to each other".

Derivatives of phenols, formed through their reaction with nitrous acid, are condensed with excess phenol in the presence of concentrated sulfuric acid to form indophenols. p-Substituted phenols do not react. There are various modifications of this method (Gibbs, 1927b). Phenols also couple with diazonium salts in the presence of a base to form highly colored azophenol dyes. Substitution occurs predominantly in the para-position if it is free, otherwise in the ortho position or by expulsion of the group in the para-position if this position is substituted. Diazotized p-nitroaniline (Bray et al., 1950) and sulfanilic acid (Ames and Mitchell, 1952) have been commonly used. They have been adapted for use as spray reagents for detection of phenolic acids on paper chromatograms as well as a basis for colorimetric methods for quantitative analysis of phenols (see Block et al., 1958).

Recently Pearl and McCoy (1960a) have shown that aqueous solutions of thirty commercially produced stable diazo salts of a number of aromatic amines provide readily available spray reagents for locating and identifying phenolic compounds and aromatic amines on paper chromatograms. Comparison of the stabilized diazo salt of p-nitroaniline, known commercially as Fast Red Salt GG., and freshly prepared diazotized p-nitroaniline (Bray and Thorpe, 1954), as spray reagents for twenty phenolic compounds usually encountered in wood chemistry revealed that the colors produced were identical and further that the dark colored background obtained with the latter reagent was absent (Pearl and McCoy, 1960b).

The ultraviolet absorption spectra of a variety of phenolic compounds including coumarins (Goodwin and Pollock, 1954), aldehydes and free acids (Lemon, 1947a; Sutherland, 1958) and the methyl (Geissman and Harborne, 1955; Jurd, 1957), quinyl (Sutherland, 1958) and sugar esters of phenolic acids (Harborne and Corner, 1961) have been determined. In neutral solutions the spectra of the phenolic acids are almost identical with those of their esters (Geissman and Harborne, 1955) and often they cannot be readily distinguished from phenols lacking a carboxyl group (Jurd, 1957). Nevertheless, a distinction can be made by the addition of sodium acetate when a hypsochromic shift of about

10 mμ is obtained with the free acids (Jurd, 1957). The esters and simple phenols either remain unaffected or show slight bathochromic shifts.

The ultraviolet absorption spectra of o-dihydroxyphenolic compounds are altered characteristically in the presence of a mixture of boric acid and sodium acetate showing considerable bathochromic shifts of their absorption maxima. According to Jurd (1956) this method is particularly useful for characterizing the various phenolic pigments in plant extracts. The individual spots may be cut out and the spectrum determined directly on the paper strip by the method of Bradfield and Flood (1952). The strip is then dipped into ethanol saturated with boric acid and sodium acetate, allowed to dry, and the spectrum redetermined.

Quantitative determination of phenolic compounds has been achieved by means of a variety of methods which are, in the main, colorimetric. A well known method of this sort is that published by Folin and Denis (1915). To determine free (non-conjugated) phenols, the urine sample is treated to remove uric acid and traces of proteins which may be present and filtered. Twenty ml of this filtrate are placed in a 50 ml flask to which are added 5 ml of phosphotungstic-phosphomolybdic acid reagent and 15 ml of saturated sodium carbonate solution. After diluting to volume with lukewarm water (30 - 35° C) and allowing to stand for 20 minutes the deep blue solution is read in a colorimeter against a standard solution of phenol. The phosphotungstic-phosphomolybdic acid reagent is prepared by boiling together for two hours 100 g of sodium tungstate, 20 g phosphomolybdic acid, 50 ml of

85 per cent phosphoric acid and 75 ml of water. The mixture is then cooled, made up with water to a volume of 1 litre and filtered if necessary. This method has been recommended by Bray and Thorpe (1954) as being very sensitive and widely applicable provided that non-phenolic reducing substances are absent from the extract. Roberts and Link (1937) reported another method, for the determination of coumarin, melilotic acid and coumaric acid in plant tissues, based on the colors produced by reaction of these compounds with diazotized p-nitroaniline. Similar methods were published by Pridham (1957) for determination of phenolic glycosides and aglycones on paper chromatograms and by Booth and Boyland (1957) for determination of aromatic amines using diazotized sulfanilic acid.

The fact that all simple phenols absorb ultraviolet light strongly, with principal absorption maxima being in the 270 - 280 mμ wavelength region, has been utilized as a basis for the spectrophotometric determination of phenolic compounds separated by paper chromatography. Stone and Blundell (1951) reported such a method for the determination of the aromatic aldehydes obtained on alkaline nitrobenzene oxidation of lignin. The oxidation mixture was chromatographed on paper and the appropriate spots were cut out and extracted in a small Soxhlet apparatus for two hours with about 25 ml of ethanol. Following the procedure of Lemon (1947b), 4 ml of 0.2% alcoholic potassium hydroxide solution were added to the extract which was made up to 50 ml with ethanol. The optical densities were measured at 352 mμ for vanillin, 368 mμ for syringaldehyde and 335 mμ for p-hydroxybenzaldehyde.

This method was adopted by Pearl et al. (1957, 1960) for the determination of vanillin, syringaldehyde and p-hydroxybenzoic, vanillic, syringic, p-coumaric and ferulic acids, separated by paper chromatography, from alkaline hydrolyzates of thirty-four representative hardwoods.

Ruckenbrod (1955) estimated chlorogenic and caffeic acids in plant extracts by means of paper chromatography. The spots, detected by their fluorescence in ultraviolet light, were extracted with 10 ml water, filtered and the optical densities were determined at 324 mμ for chlorogenic acid and 290 mμ for caffeic acid. Similar methods were also applied to the determination of cinnamic (Koukol and Conn, 1961) and p-coumaric acids (Neish, 1961) dissolved in 0.05 N NaOH solution and read at 268 and 333 mμ respectively. Recently Seligson et al. (1963) described an easy method for the isolation and identification of phenolic acids using paper chromatography and infrared spectroscopy. In this method, the phenolic acids were eluted from paper chromatograms, separated from paper impurities and applied to powdered potassium bromide which was then made into a micropellet for infrared spectrophotometry. Twenty-five micrograms of compounds such as salicylic acid and other phenolic acids have been reasonably purified and identified by this method.

II. BIOSYNTHESIS AND METABOLISM

The available evidence indicates that phenylpropanoid compounds, in higher plants, are formed via the shikimic acid pathway and it is fairly certain that plant phenolic acids owe their existence to the aromatic amino acids, phenylalanine and tyrosine, or to intermediates involved in their synthesis (Neish, 1960, 1964b). Therefore a description of the biosynthetic pathway leading to the formation of the aromatic amino acids is necessary.

A. The Aromatic Amino Acids

Plants and some microorganisms share the ability to synthesize the aromatic amino acids from carbohydrates and the elucidation of this biosynthetic pathway, in microorganisms, is due to Davis and his collaborators. The subject has been extensively reviewed (Davis, 1955, 1958; Sprinson, 1960). This outstanding work was carried out, over several years, with nutritional mutants, obtained by irradiation of a wild strain of *Escherichia coli*. Shikimic acid was found to satisfy uniquely the growth requirements for a quintuple auxotrouph incapable of synthesizing five aromatic compounds including phenylalanine and tyrosine. It was obviously a key compound in aromatic biosynthesis in this organism.

The individual reactions, in the sequence, were worked out by means of mutants blocked at different stages, which were found to accumulate one or more of the intermediates in the culture medium. Subsequent studies involved the use of the tracer technique as well as of enzyme systems (Sprinson, 1960; Srinivasan et al., 1963; Higuchi and Kawamura, 1964). The sequence has been fully developed and the presently accepted shikimic acid pathway is outlined in Figure 2.

In higher plants, proof for the shikimic acid pathway as a functioning scheme in aromatic biosynthesis is not as conclusive as it is for microorganisms. However, there are several definite reasons, advanced by workers in this field (Neish, 1961, 1964b) for believing that the shikimic acid pathway does function in higher plants. Shikimic acid is of widespread occurrence in meristematic and cambial tissues as well as in the leaves of higher plants (Hasegawa, 1962). Tracer studies have shown that shikimic acid is readily converted in higher plants to the aromatic amino acids, phenylalanine and tyrosine, as well as to a vast variety of other aromatic compounds including lignins (Neish, 1960).

The conversion of uniformly labeled shikimic acid to phenylalanine and tyrosine, labeled only in the ring, was demonstrated by McCalla and Neish (1959a) in *Salvia splendens* and by Gamborg and Neish (1959) in *Triticum vulgare* and *Fagopyrum tataricum*. This establishes the direct conversion of shikimic acid, without previous rearrangement, to the aromatic nucleus in members of three different families belonging to both mono- and dicotyledonous plants. This direct conversion of shikimic acid into the aromatic nucleus was also demonstrated with

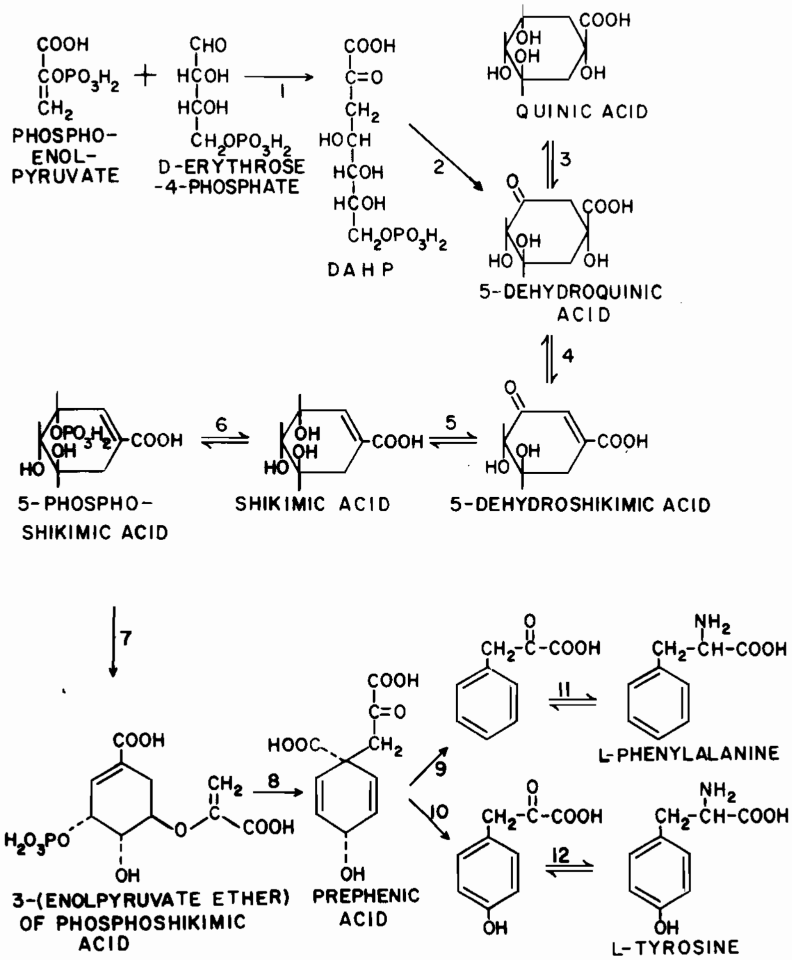


Figure 2. The shikimic acid pathway.

DAHP = 3-Deoxy-D-arabino-heptulosonic acid 7-phosphate.

microorganisms (Davis, 1955; Gross, 1958). Similarly the incorporation of phenylpyruvic and phenyllactic acids into the bound phenylalanine of young wheat and buckwheat plants was shown by Gamborg and Neish (1959) to be as good as the incorporation of phenylalanine itself. The corresponding p-hydroxyphenyl compounds were readily incorporated into the tyrosine of both species.

Nevertheless, the burden of proving the functioning of the shikimic acid pathway in higher plants lies largely in tracer studies on incorporation of shikimic acid into the aromatic nuclei of lignin and other phenolic compounds in a variety of plant species. Brown and Neish (1955a) demonstrated this incorporation into lignins of both wheat and maple. Eberhardt and Schubert (1956) demonstrated it in sugar cane and further provided evidence for the direct conversion, without rearrangement, of the carbon skeleton of specifically labeled shikimic acid into vanillin isolated from lignin. Appreciable incorporation of radioactivity from glucose-^{14}C into both shikimic acid and lignin of a species of *Eucalyptus* was reported by Hasegawa and Higuchi (1960). Finally Acerbo *et al.* (1960) found that distribution of carbon-14 in the aromatic nuclei of lignin, after incorporation of specifically labeled glucose-^{14}C, was the same as that found in phenylalanine and tyrosine formed by microorganisms via the shikimic acid pathway.

The suggestion that the closely related compound quinic acid is an intermediate between sugars and aromatic compounds was made as early as 1930 by Kiesel who followed the changes in the acid contents of young spruce shoots during their growth. The distribution of quinic acid in plants is also wide, and in many cases, the coexistence of this acid with shikimic acid has been demonstrated (Hasegawa, 1962). Furthermore, the ready conversion of quinic acid to shikimic acid, phenylalanine and tyrosine was demonstrated in many plants by Weinstein et al. (1959a, 1959b, 1961, 1962). In their experiments with young rose plants (1959b) there was some indication that quinic acid is preferred to shikimic acid as an intermediate in aromatic biosynthesis.

Although enzymes responsible for all individual reactions of the shikimic acid pathway, shown in Figure 2, have been detected and/or purified from microorganisms (Higuchi and Kawamura, 1964), few studies have so far been carried out in higher plants. The enzyme, 5-dehydroquinase (reaction 4 of Figure 2) was detected by Mitsuhashi and Davis (1954) in peas and spinach and purified from cauliflower buds by Balinsky and Davis (1961c). Dehydroshikimic acid reductase (reaction 5) was purified and studied by Nandy and Ganguli (1961a) from mung bean seedlings and from pea seedlings by Balinsky and Davis (1961a, b). Furthermore, Nandy and Ganguli (1961b) have shown that a cell free extract of mung beans was capable of converting glucose-6-phosphate to dehydroshikimic acid. However, so far, there has been no demonstration of all the enzymes in any one species.

In conclusion, there seems to be a close resemblance between the biosynthetic pathway of aromatic compounds in microorganisms and that in plants, although more work at the enzyme level is required in order to establish this.

B. The Phenolic Cinnamic Acids

In addition to the fact that the hydroxycinnamic acids are the most common of all occurring plant phenolic acids, they apparently occupy a central position in the synthesis of other plant phenolic products. As already mentioned they are widely distributed as conjugates of various compounds or as coumarins. They possibly yield the C_6-C_2 and the C_6-C_1 acids as products of their oxidative degradation. Furthermore they have been postulated as the key intermediates in formation of lignans (Swain, 1962) and lignins (Neish, 1960), the flavonoids and related compounds (Watkin et al., 1957; Swain and Bate-Smith, 1962; Geissman, 1963).

The basic synthesis of the parent phenylpropane skeleton has already been discussed (Figure 2). The first aromatic compounds formed are the two keto acids, phenyl- and p-hydroxyphenyl-pyruvic acids which, until recently, were considered to be the aromatic precursors of a great many plant products including the phenolic cinnamic acids (Neish, 1960, 1964b). The first evidence from tracer investigations was advanced by Geissman and Swain (1957), who showed that while phenylalanine-^{14}C was converted to caffeic acid without rearrangement of the carbon skeleton, by tobacco plants, there was no incorporation of activity from acetate-^{14}C. These results were corroborated by Reid (1958) who further showed that phenylacetic acid could not act as a precursor of caffeic acid in tobacco. Brown et al. (1959) in their studies on lignin biosynthesis in wheat, demonstrated the conversion of labeled phenylalanine and tyrosine to both p-coumaric and ferulic acids.

That phenylalanine and trans-cinnamic acid play a key role in the biosynthesis of plant phenolic acids was further indicated by the work of McCalla and Neish (1959b) on the biosynthesis of phenolic cinnamic acids in stem cuttings of Salvia and substantiated by Levy and Zucker (1960) using tissue slices of potato tubers. Recently this role was clearly demonstrated by the isolation of two distinct aspartase-like enzymes, phenylalanine ammonia lyase (Koukol and Conn, 1961) and tyrosine ammonia lyase (Neish, 1961). These two enzymes act on L-phenylalanine and L-tyrosine respectively producing the corresponding cinnamic and p-coumaric acids. More recently, a third enzyme, 3,4-dihydroxyphenylalanine (DOPA) ammonia lyase, was reported to occur in barley shoots. This enzyme acts in the same way on DOPA to produce caffeic acid (MacLeod and Pridham, 1963).

C. Interconversion of Members of the Cinnamic Acid Series

L-Phenylalanine, cinnamic, dihydrocinnamic (phenylpropionic) and (-)-phenyllactic acids were all found to be good precursors of the phenolic cinnamic acids in stem cuttings of Salvia splendens by McCalla and Neish (1959b). These authors carried out a kinetic study on the formation of cinnamic acids from phenylalanine in Salvia. Their results were consistent with the view that these compounds are related in the following sequence:

$$\begin{tikzcd}
\text{Dihydrocinnamic acid} \arrow[d] & & & & \\
\text{cinnamic acid} \arrow[r] & \text{\textit{p}-coumaric acid} \arrow[r] & \text{caffeic acid} \arrow[r] & \text{ferulic acid} \arrow[r] & \text{sinapic acid} \\
\text{(-)-phenyllactic acid} \arrow[u] & & & & \\
\text{phenylpyruvic acid} \arrow[u] \arrow[d, shift left] & & & & \\
\text{L-phenylalanine} \arrow[u, shift left] & & & &
\end{tikzcd}$$

Each member of the cinnamic acid series, when fed to *Salvia*, was readily converted to the more complex members but not so readily to a simpler member. The phenolic acids, in *Salvia* stem cuttings, were largely present in a combined form of unknown constitution. These authors suggested that although the cinnamic acids could be detected only as esters, nevertheless the series of interconversions occur at the free acid level before esterification in this manner:

$$\begin{tikzcd}
\text{Precursor} \arrow[r] & \text{\textit{p}-coumaric acid} \arrow[r] \arrow[d] & \text{caffeic acid} \arrow[r] \arrow[d] & \text{ferulic acid} \arrow[r] \arrow[d] & \text{sinapic acid} \arrow[d] \\
& \text{ester} & \text{ester} & \text{ester} & \text{ester}
\end{tikzcd}$$

This suggestion, together with differences noted in the pool sizes of the acids, was offered by the authors as a justification for their finding, in some feeding experiments, that later members of the series acquired higher specific activities than earlier members.

In their studies of the biosynthesis of chlorogenic acid, Levy and Zucker (1960) found that L-phenylalanine and *trans*-cinnamic acid, but not *p*-coumaric acid, gave a pronounced stimulation of net synthesis of chlorogenic acid in 48 hours by tissue slices of potato tubers. Neither phenylpyruvate, phenyllactate nor *p*-coumarate gave any detectable stimulation and, in the absence of quinic acid, phenylalanine was twice as effective as cinnamic acid. These findings suggested to the authors that the synthesis of some intermediate between cinnamic acid and the caffeoyl moiety of chlorogenic acid, other than free p-coumaric acid, is rate limiting. Two such intermediates, cinnamoylquinic ester and p-coumaroylquinic ester, were subsequently identified in tissues cultured on trans-cinnamic acid and sodium quinate.

L-Phenylalanine-U-^{14}C was converted to both p-coumaroylquinic and chlorogenic esters having essentially the same specific activity which was about equal to that of the administered phenylalanine. Addition of non-labeled cinnamic acid with labeled phenylalanine resulted in dilution of the activity in both esters. These results led Levy and Zucker (1960) to propose the following sequence for the synthesis of chlorogenic acid: phenylalanine → cinnamic acid → cinnamoylquinic acid → p-coumaroylquinic acid → chlorogenic acid. The hydroxylation in the last step was successfully demonstrated in cell free extracts from potato tubers.

This work was further supported by Hanson and Zucker (1963) whose polyphenol oxidase preparation oxidized 3-O-p-coumaroylquinic acid to chlorogenic acid, and the corresponding shikimic acid depside to caffeoylshikimic acid. However, Haslam et al. (1961b) were unable to detect the conversion of synthetic 3-O-p-coumaroylquinic acid to chlorogenic acid when incubated with a mushroom tyrosinase preparation (Mallette et al., 1948).

In contrast to the results reported by Levy and Zucker (1960) Runeckles (1963a) found that p-coumaric acid is a better precursor than cinnamic acid for chlorogenic acid synthesis by tobacco leaf disks while caffeic acid was a poor precursor. On the other hand, cinnamic acid displayed a better conversion to p-coumaroylquinic ester and p-coumaroyl-glucose ester than did p-coumaric acid itself. Runeckles stressed this observation as being a constant finding in a number of experiments. He offered one of the following explanations: if p-coumaroylquinic acid is the immediate precursor of chlorogenic acid, and is produced from both cinnamic and p-coumaric acids, the levels of p-coumaroylquinic acid detected represent equilibrium concentrations. The observed differences could then be accounted for by an effect of cinnamic acid in partially inhibiting the turnover of p-coumaroylquinic acid. A second explanation is that the conversion of p-coumaroylquinic acid to chlorogenic acid is a process involving one or more intermediates.

In a continuation of this study, Runeckles (1963a) further observed that the glucose esters of carbon-14 labeled cinnamic and p-coumaric acids, administered to tobacco leaf disks, were more readily converted to both p-coumaroylquinic and chlorogenic acids than were the free acids. Although these results were obtained from a single experiment carried out on small scale, nevertheless, they suggest the possibility that interconversions between the various forms of esters of cinnamic acids may occur *in vivo*. It is interesting in this respect that the fungus, *Polystictus versicolor*, can effect the hydroxylation of cinnamic acid to p-coumaric acid but intermediates such as quinyl esters have not been reported (Farmer *et al.*, 1959). Similarly, O-methylation of caffeic acid to ferulic acid was demonstrated by the use of S-adenosyl-methionine and a meta-O-methyltransferase from higher plants (Finkle and

Nelson, 1963).

Thus far, the question concerning the true form in which cinnamic acids may undergo hydroxylation and methylation or methoxylation has not been fully answered. It may be true that direct interconversions can be effected between individual cinnamic acid conjugates such as quinyl esters (Levy and Zucker, 1960; Hanson and Zucker, 1963), shikimyl esters (Hanson and Zucker, 1963), glucose esters (Harborne and Corner, 1961), or glucose and quinyl esters (Runeckles, 1963a, b). Nevertheless, none of these esters is known to be universally distributed and they often display characteristics to be expected of end products rather than intermediates (Neish, 1964b). It is more likely that the true intermediates are more active compounds such as CoA esters (Neish, 1960, 1964b; Sondheimer, 1962; Towers, 1964a). These could be visualized as giving rise to esters of quinic acid, shikimic acid, glucose, etc., if not used for some other purposes such as reduction to coniferyl alcohol in lignin biosynthesis or condensation with malonyl CoA to form flavonoids and related compounds (Neish, 1964b).

D. The Detoxication Process; Metabolism of Administered Phenolic Acids

The phenomenon of esterification and/or glycosylation of aromatic compounds when introduced into animals or plants was described a long time ago as a detoxication process. In the recent review by Towers (1964a) a good account has been given of this phenomenon.

The sugar esters of cinnamic acids have been reported by Harborne and Corner (1961) not only to occur in higher plants with the same order of frequency as the well known quinic acid esters, but also to be the main products of feeding leaves, of a variety of plant species, with the free acids. The formation of particular esters, however, depends on the particular organ or tissue being used as well as on its physiological state. Thus, while cinnamoylglucose, p-coumaroylglucose, p-coumaroylquinic and chlorogenic acid were formed, from administered L-phenylalanine-^{14}C or cinnamic acid-^{14}C, by leaf disks of Malus (Avadhani and Towers, 1961) and Pyrus (Lawson, 1961), only the two glucose esters were synthesized by leaves of Grevillea robusta (Grisdale and Towers, 1960; Lawson, 1961), a species which does not normally contain chlorogenic acid (Bate-Smith, 1962a). Similar results were reported by Harborne and Corner (1961) whose repeated attempts to detect the quinyl esters of p-coumaric and caffeic acids, after administration of the two acids to plants not normally containing these quinyl esters, were unsuccessful.

In wheat shoots, Higuchi and Brown (1963a) failed to detect synthesis of glucose esters of cinnamic acids after administration of phenylalanine-U-^{14}C, although these glucose esters were readily detectable after administration of hydroxycinnamic acids. The qualitative and/or the quantitative variability in the sugar esters formed from any one precursor was well illustrated in the work of Harborne and Corner (1961). They found that the main products of feeding leaves of a variety of plant species for periods of one to three days with any of phenylalanine, cinnamic, p-, m-, o-coumaric, caffeic, ferulic, sinapic and 3,4,5-trimethoxycinnamic acids were glucose esters. The esters formed from p-coumaric, caffeic, ferulic and sinapic acids were found to be identical, in every respect, with material isolated from natural sources.

In contrast to the well established observation that administration of simple phenols or phenolic alcohols gives rise to glycosides in many plant species (Hutchinson et al., 1958; Nystrom et al., 1959; Pridham and Saltmarch, 1960; Pridham, 1960), the preferential synthesis of esters rather than glycosides from phenolic acids was clearly demonstrated in this study. The only plant found to produce glycosides of hydroxycinnamic acids was the tomato plant in which small amounts of the 3- and 4-β-glucosides of caffeic acid were formed in addition to much larger amounts of caffeoyl glucose. The formation of o-coumarylglucoside from o-coumaric acid was not detected although previous work with ^{14}C-labeled o-coumaric acid indicated that appreciable quantities of radioactive o-coumarylglucoside, in which the aglycone was labeled, were formed in sweet clover (Kosuge and Conn, 1959) and in sweet grass (Brown et al., 1960).

The possibility that these anomalous results are due to the methods used by Harborne and Corner, for detection of the glucosides was emphasized by Runeckles and Woolrich (1963). These authors pointed out the fact that whereas the glucose esters of ferulic and sinapic acids could be easily detected through their fluorescence under ultraviolet light and their reactivity towards phenolic reagents, the glycosides of these two acids lack these characteristics. It is of interest, in this respect, that the two glucosides of caffeic acid detected by Harborne and

Corner (1961) are fluorescent. Radioactive o-coumarylglucoside was detected by means of autoradiography or else by its color reaction with phenolic reagents after being hydrolyzed with emulsin on chromatograms (Kosuge and Conn, 1959). Runeckles and Woolrich (1963), however, demonstrated that incorporation of radioactivity, from administered ^{14}C-labeled p-coumaric and ferulic acids to tobacco leaf disks for 40 hours, was substantially higher in their respective glucosides than in the glucose esters.

E. The Benzoic Acids

Although esters and/or glycosides of a wide variety of substituted benzoic acids are known as common constituents of higher plants (Tomaszewski, 1960), relatively little is known about their mode of synthesis and their interrelationships. It has been assumed, in general, that they may arise by the breakdown of hydroxycinnamic acids or phenylpropanoid compounds or from non-aromatic precursors of the shikimic acid pathway (Figure 2) and of the acetate pathway (Figure 4) to be described below.

1. By breakdown of larger molecules: That compounds of the C_6-C_1 (benzoic acid) class can be formed by removal of a two-carbon fragment from the three-carbon side-chain of phenylpropanoid compounds was suggested by Geissman and Henreiner (1952). It was only recently that this possibility was experimentally examined and verified. Thus

Gross and Schütte (1963) showed that the benzoic acid moiety of cocaine was radioactive after administration of phenylalanine-3-^{14}C to Erythroxylon novogranatense. Furthermore, Grisebach and Vollmer (1963) have demonstrated that the radioactivity of salicylic acid, isolated from Gaultheria procumbens supplied with cinnamic acid-3-^{14}C, was confined to the carboxyl carbon.

Good evidence for the formation of the ubiquitously occurring p-hydroxylated benzoic acids from phenylpropanoid compounds has come from the work of Chen (1963). He fed the common natural members of the cinnamic acid series labeled with carbon-14 in the β-position to a variety of plant species and examined the distribution of radioactivity in the phenolic acid fraction extracted from the plant material. His results indicated that while cinnamic acid-^{14}C was converted to salicylic, o-pyrocatechuic, gentisic, protocatechuic and vanillic acids by leaf tissue of Gaultheria procumbens, only gentisic and protocatechuic acids were formed by Hydrangea macrophylla, and vanillic acid was the sole C_6-C_1 acid labeled in Teucrium lusitanicum. In subsequent experiments, he showed the conversion of p-coumaric acid to p-hydroxybenzoic acid by three different species and to both this acid and vanillic acid by yet another species. Protocatechuic and vanillic acids were formed from caffeic acid in only one species while from ferulic and sinapic acids, the corresponding vanillic and syringic acids, respectively, were formed by all species examined.

2. From non-aromatic precursors of the shikimic acid pathway: In addition to their wide distribution in higher plants the mono-, di- and trihydroxybenzoic acids (p-hydroxybenzoic, protocatechuic and gallic

acids respectively) are also produced by many fungi. Since these acids have the same number of carbon atoms and distribution of oxygen function as shikimic acid and related compounds, it was assumed that they were formed by oxidative dehydration of the latter. Thus protocatechuic acid (Gross et al., 1956; Gross, 1958) and gallic acid (Haslam et al., 1961b) were shown to be directly synthesized from 5-dehydroshikimic acid in Neurospora and in Phycomyces respectively. The formation of gallic acid probably occurs by an independent reaction from that of protocatechuic acid, since incubation of the latter compound with Phycomyces did not give rise to the former.

That gallic acid is derived from a non-aromatic precursor in higher plants is indicated by the fact that glucose-^{14}C was found to be a far better precursor than phenylalanine-^{14}C in Geranium pyrenaicum (Conn and Swain, 1961).

The possibility of direct hydroxylation and O-methylation of C_6-C_1 precursors, in higher plants, was indicated from the work of Ibrahim and Towers (1959). They showed that salicylic acid-^{14}C was hydroxylated in the 3- or 5-position when introduced into plants giving rise to o-pyrocatechuic and gentisic acids respectively. Carboxyl-labeled benzoic acid was oxidized to salicylic, o-pyrocatechuic, gentisic and p-hydroxybenzoic acids in leaf disks of both Gaultheria and Primula (Ibrahim, 1961). p-Hydroxybenzoic acid was hydroxylated to protocatechuic acid, and the latter when administered was either hydroxylated to gallic acid or O-methylated to vanillic acid depending on the plant species (Chen, 1963). On the other hand, Chen (1963) was not able to detect syringic acid on radioautographs of extracts of leaf segments of four species administered vanillic acid-^{14}C. Swain (1962) suggested that syringic acid may be formed by O-methylation of gallic acid in higher plants. A scheme for the interrelationships of the C_6-C_3 and C_6-C_1 acids, as suggested by Chen (1963), is shown in Figure 3.

3. The acetate pathway: That the o-hydroxylated benzoic acids, salicylic and gentisic acids have strikingly similar distributions in higher plants and that their synthetic pathways are probably similar was pointed out by Griffiths (1959). In fungi, however, these o-hydroxylated acids were shown to be synthesized by a totally different route involving the de novo synthesis of the aromatic nucleus from acetate units (Gatenbeck and Lönroth, 1963).

The concept of the acetate pathway for aromatic biosynthesis originated nearly 70 years ago when Collie (1907) suggested and proved chemically that polyacetic acids, formed by head-to-tail condensation of acetate units, may cyclize to form the aromatic nucleus. This suggestion was later emphasized by Birch and Donovan (1953, see also Birch, 1957) who drew attention to the fact that a large number of natural products of fungal or plant origin contain aromatic rings with hydroxyl functions on alternate carbon atoms and which consequently may have arisen in this way (Figure 4).

The hypothesis has now been confirmed by isotopic tracer experiments, the first evidence being provided by Birch's group (Birch et al., 1955). They showed that 6-methylsalicylate was formed from acetate-1-^{14}C by Penicillium griseofolvum. The labeling pattern was in accordance with the hypothesis (see Figure 4). Since then many compounds of fungal origin have been shown to arise from acetate. In higher plants

Figure 3. Scheme illustrating the relationships of the hydroxycinnamic and hydroxybenzoic acids in plants.

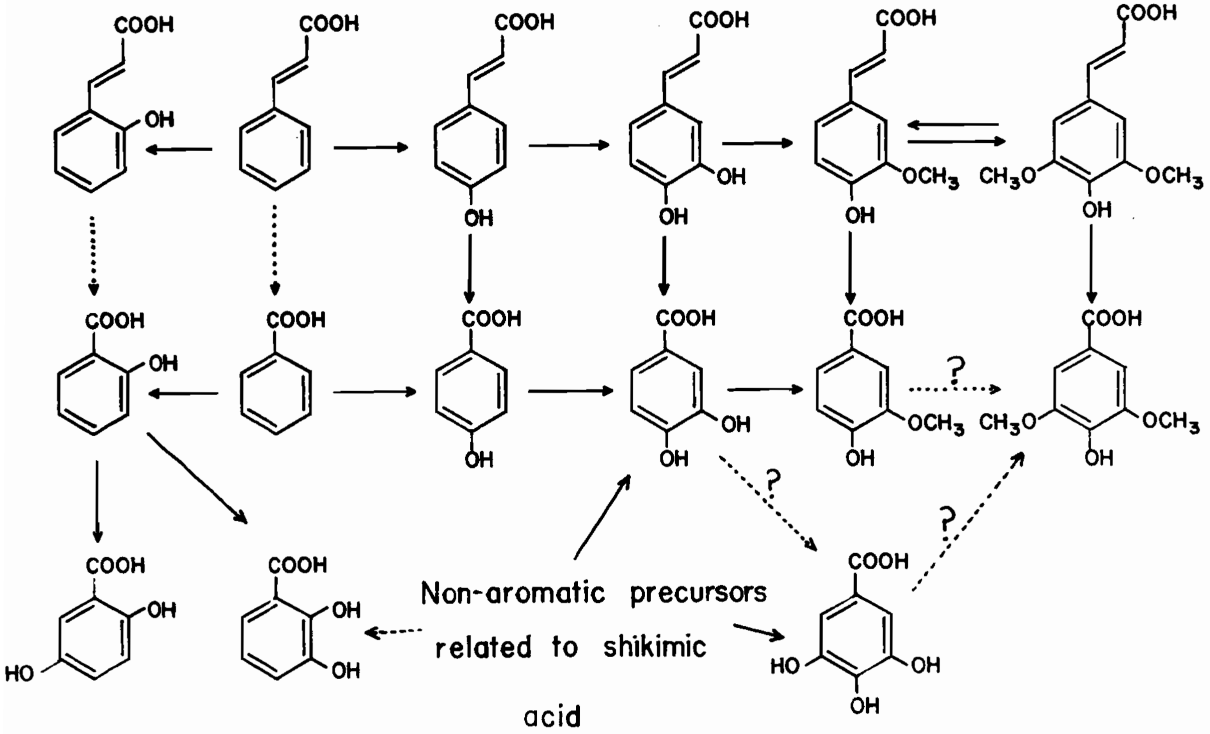
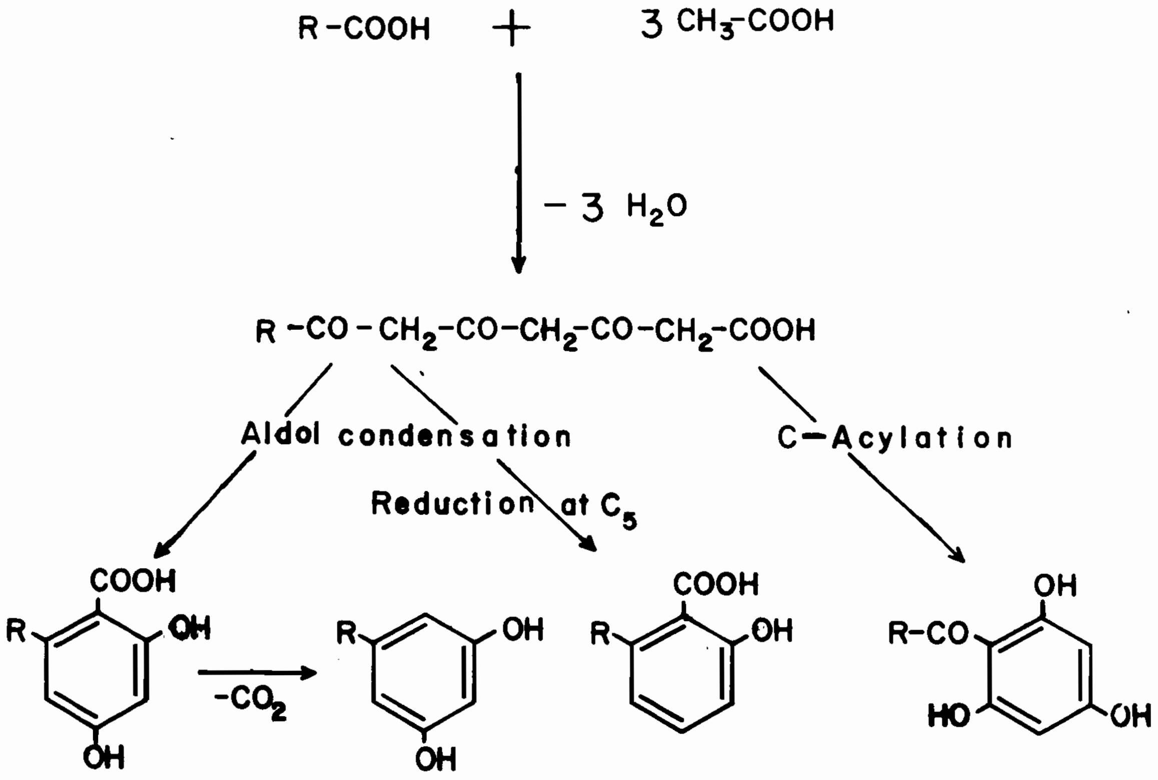


Figure 4. The acetate pathway according to Birch and Donovan (1953).



so far only the A ring of the flavonoid compounds (Bogorod, 1958; Neish, 1960, 1964a, b) has been shown to be derived from acetate.

The recent work on the mechanism for the biosynthesis of aromatic rings from acetate has been reviewed by Geissman (1963) and by Neish (1964b). It is considered to be essentially analogous to fatty acid synthesis in that acetyl CoA is required as a starter and the rest of the acetate-derived carbons are incorporated via malonyl CoA. Experimental support for this mechanism has come from studies on the biosynthesis of 6-methylsalicylate by species of _Penicillium_ both _in vivo_ (Birch _et al._, 1961; Bu'Lock and Smalley, 1961) and by cell free extracts (Lynen and Tada, 1961).

F. The Phenolic Acids as Intermediate Metabolites in Higher Plants

Although the phenolic substances of plants have usually been regarded as metabolic end-products there is some indirect evidence suggesting their possible utilization as respiratory substrates. Almost all this evidence is based on the observed reduction or disappearance of certain compounds in certain organs during some stages of growth and development (Towers, 1964a). One example of such evidence is represented by the work of Urban (1958, 1959) on the diurnal changes in the amounts of flavonoid and hydroxycinnamic acid derivatives extractable with methanol from leaves of sunflower, corn and wheat. She reported fluctuations of considerable amplitude with the flavonoid constituents which increased during the day to a maximum in the evening. The cinnamic acids increased during the night returning to almost their original values at midday. Such changes imply a cycle of synthesis, from non-phenolic precursors, and catabolism or else translocation, of a proportion of the phenolic product.

Studies with microorganisms have shown that many species of soil bacteria and fungi are capable of growing on phenolic compounds as sole carbon sources. Ring cleavage and complete oxidation of the aliphatic intermediates to CO_2 has been established as a familiar feature of microbial metabolism of aromatic compounds and various pathways have been mapped out (Evans, 1958, 1963; Towers, 1964a).

In higher plants, however, the situation is still vague since only very little attention has been paid to this problem. Zaprometov (1959) reported that cut tea shoots were capable of oxidizing an externally supplied mixture of randomly labeled catechins. The cut shoots were allowed to absorb the radioactive solution in the light and were subsequently transferred to the dark. Complete oxidation of catechins to $^{14}CO_2$ was found to occur in two stages with 73 - 82% of the radiocarbon appearing as $^{14}CO_2$ in the second stage, 45 - 70 hours after administration. In view of the apparent oxidation of considerable amounts of catechins and of the long metabolic period involved in these experiments, Towers (1964a) drew attention to the possibility that microbial activities may have been the actual factor involved in this oxidation.

As already mentioned, the C_6-C_2 and the C_6-C_1 acids may be formed as a result of oxidative degradation of the C_6-C_3 acids. Labeled hydroxybenzoic acids were obtained by administration of the corresponding labeled hydroxycinnamic acids to a variety of plant species (see pp. 34 - 35). The formation of homogentisic (2,5-dihydroxyphenylacetic) acid by oxidation of L-tyrosine has been achieved with a homogenate of nutsedge, Cyperus rotundus L. (Palmer, 1961). The oxidation of L-phenylalanine, caffeic and other C_6-C_3 acids to pungenin aglycone (3,4-dihydroxyacetophenone) by Picea pungens (Neish, 1959) and of L-phenylalanine, L-tyrosine and a variety of C_6-C_3 acids to dhurrin aglycone (p-hydroxymandelonitrile) by Sorghum vulgare (Koukol and Conn, 1962) has been demonstrated.

Decarboxylation and possible ring cleavage of carboxyl-labeled hydroxy- and hydroxymethoxybenzoic acids were demonstrated both *in vivo*, by a variety of plant species, and *in vitro*, using acetone powders of higher plants by Chen (1963). Similar results were obtained by the same author for hydroxy- and hydroxymethoxy-cinnamic acids labeled in the β-position indicating the complete removal of the 3-carbon side-chain. However, no evidence was obtained for ring cleavage although the fact that, in experiments with carboxyl-labeled vanillic acid all attempts to detect guaiacol were unsuccessful, is suggestive. Evidence for the cleavage of the ring of tyrosine was obtained by Ibrahim *et al.* (1961). In three different experiments 28%, 36% and 46% of the total activity of uniformly labeled tyrosine taken up by leaf disks of Pyrus communis could be accounted for in aliphatic compounds including sugars and organic acids. Incorporation of more than 33-1/3% of the activity from uniformly labeled tyrosine in non-aromatic compounds was taken as an indication of ring cleavage. Sugar formation from phenylalanine and cinnamic, p-coumaric, caffeic and ferulic acids by tobacco leaf disks has been reported by Runeckles (1963c).

PART II. LIGNIN BIOSYNTHESIS

The term lignin was introduced by Anselme Payen (1838-1842) to designate the "matiéres incrustantes" or "matiéres ligneuses" of cell wall components, differing from cellulose, which he removed from wood with nitric acid and alkali (caustic soda). Over one hundred years later, lignin was defined by Brauns (1952, page 15) as "the incrusting material of the plant which is built up mainly, if not entirely, of phenylpropane building stones; it carries the major part of the methoxyl content of the wood; it is unhydrolyzable by acids, readily oxidizable, soluble in hot alkali and bisulfite and readily condenses with phenols and thio compounds". It is rather discouraging to note that the differences between these two definitions reflect the results of a tremendous amount of research in lignin chemistry during the intervening years. Although the accumulated data within this "century of research" have shed light on most of the chemical and physical properties of the lignin complex, its structure is still far from being completely understood.

Brauns (1952, p. 2) noted that one of the major stumbling blocks to an understanding of lignin structure is the great confusion which prevails in the lignin literature and seems to become greater, instead of less, each year. A confusion that was best described by Pepper (1958) as recalling to mind the Hindu fable of the Blind Men and the Elephant as written by J. G. Saxe:

It was six men of Indostan to learning much inclined,
Who went to see the Elephant (though all of them were blind)
That each by observation might satisfy his mind.

The First approached the Elephant, and happening to fall
Against his broad and sturdy side, at once began to bawl:
"God bless me! but the Elephant is very like a wall!"

The Second, feeling of the tusk, cried: "Ho! what have we here
So very round and smooth and sharp? To me 'tis very clear
This wonder of an Elephant is very like a spear!"

The Third approached the animal, and happening to take
The squirming trunk within his hands, thus boldly up and spake:
"I see," quoth he, "the Elephant is very like a snake!"

The Fourth reached out an eager hand, and felt about the knee.
"What most this mighty beast is like is mighty plain", quoth he;
"'Tis very clear the Elephant is very like a tree!"

The Fifth, who chanced to touch the ear, said: "E'en the blindest man
Can tell what this resembles most; deny the fact who can,
This marvel of an Elephant is very like a fan!"

The Sixth no sooner had begun about the beast to grope,
Than, seizing on the swinging tail that fell within his scope,
"I see," quoth he, "the Elephant is very like a rope!"

And so these men of Indostan disputed loud and long,
Each in his own opinion exceeding stiff and strong,
Though each was partly in the right, and all were in the wrong!

The principal factors contributing to this confusion lie firstly in the fact that, so far, no method is known by which lignin can be isolated from the coexisting carbohydrates in the cell wall in an unaltered form, and secondly in the apparent inhomogeneity of the lignin polymer. Different types of "lignin" are obtainable from the same plant material by means of different isolation procedures. Consequently, quantitative and/or qualitative variations in the lignin degradation products recovered by any given procedure are inevitable. The term lignin can no longer be considered to designate a chemically defined compound but is rather a collective term for a group of high molecular weight amorphous compounds which are related chemically (Brauns, 1952, page 14) and hence the use of the plural "lignins" was rightly recommended by Kremers (1959).

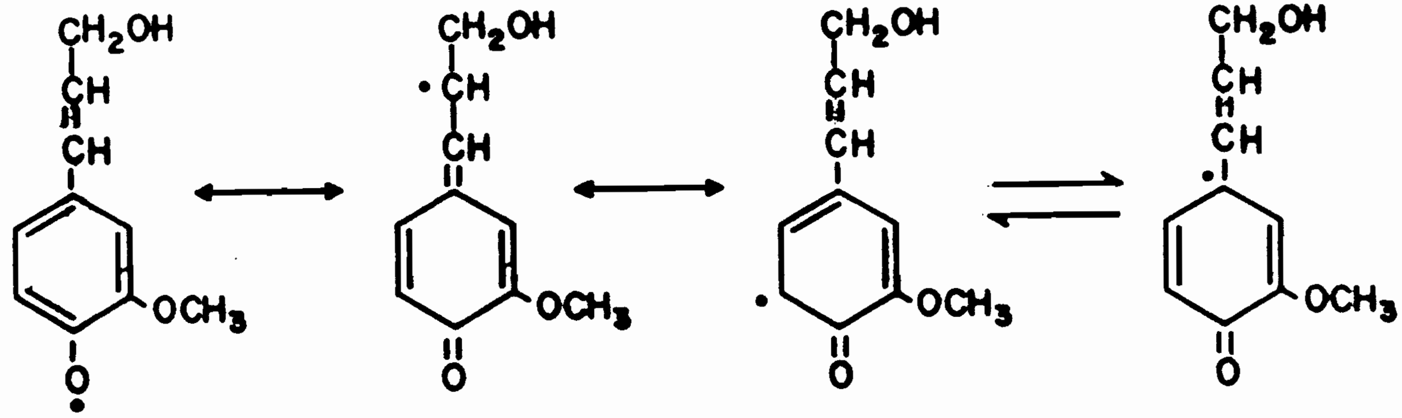
Recent work has provided increasing support for the proposal of the Swedish chemist, Klason, in 1897 that lignin is a complex polymer of coniferyl alcohol or sinapyl alcohol (see Figure 5). Because of the difficulties, involved in the isolation and purification of lignins, workers in the field of lignin biosynthesis have had recourse to chemical degradation for the recovery of certain monomeric products which possess known structures and which can be purified by crystallization. The most frequently used degradation procedure is oxidative degradation with nitrobenzene or metallic oxides in an alkaline medium. This type of reaction yields products which still contain the benzene ring.

Alkaline nitrobenzene oxidation of lignin yields one or more of the three substituted benzaldehydes: p-hydroxybenzaldehyde, vanillin and syringaldehyde (Figure 6). So far, coniferous lignins have been found to yield only vanillin whereas lignins of woody dicotyledonous plants yield both vanillin and syringaldehyde; monocotyledonous lignins give rise to all three substituted benzaldehydes. The work of Gibbs and his associates (example, Towers, 1951; Towers and Gibbs, 1953; Gibbs 1958) has been of major significance in this respect. Two other, equally important but not as frequently used, degradation procedures are: ethanolysis, which is a prolonged heating in ethanol containing a small amount of mineral acid; and hydrogenolysis over Raney nickel catalyst. These two methods are sometimes preferred to oxidation in that they yield products retaining all the carbon atoms of the phenylpropanoid

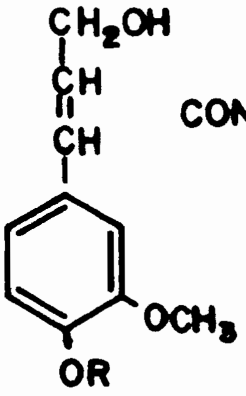
<u>Figure 5.</u> A. Free radical mechanism of lignin synthesis proposed by Freudenberg.

B. Primary lignin building stones according to Klason.

A



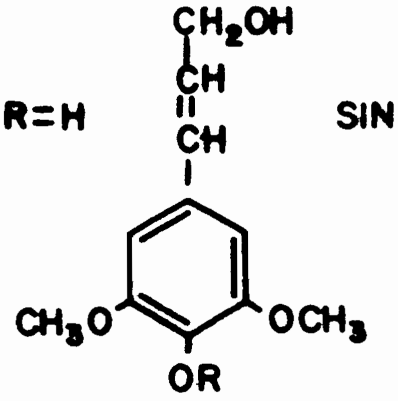
B



CONIFERYL ALCOHOL R=H

CONIFERIN

R = GLUCOSE



SINAPYL ALCOHOL R=H

SYRINGIN

R= GLUCOSE

skeleton (Figure 6) although in a much lower yield (Brown, 1961b).

That the lignin building stone has a phenylpropane carbon skeleton may be regarded as proven (Brauns, 1960, page 5), but how the stones are linked together in lignin is still a mystery, since not even one dimeric lignin degradation product has been isolated from lignin preparations.

Many suggestions have been made in the past in an effort to explain the nature of the precursors of lignin and the mechanisms of their synthesis in plants (Brauns, 1952). These suggestions, however, have been described as being either purely speculative, or else as being based on evidence of an indirect or fragmentary character (Nord and Schubert, 1963a, b). It is only during the last two decades or so, as a result of the application of the tracer technique as well as of enzymatic studies, that progress has been gained in the knowledge of lignin biosynthesis.

Although the total integrated series of biochemical reactions leading from carbon dioxide ultimately to lignin is still largely obscure, nevertheless the information available for some isolated stages of lignin biosynthesis will be briefly reviewed in the following sections.

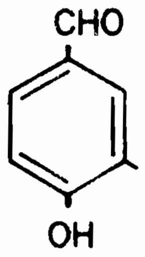
A. Lignin Formation from Carbon Dioxide:

For many years it has been the consensus of lignin chemists that lignin, like other plant products, is formed in photosynthesis

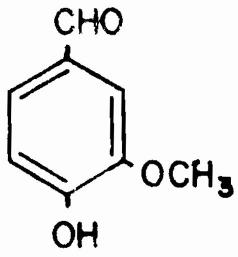
Fig. 6

Figure 6. Monomeric products of chemical degradation of lignin.

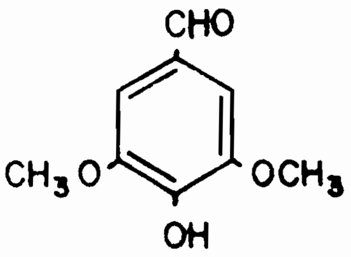
I. OXIDATIVE DEGRATION PRODUCTS



p-HYDROXY-BENZALDEHYDE

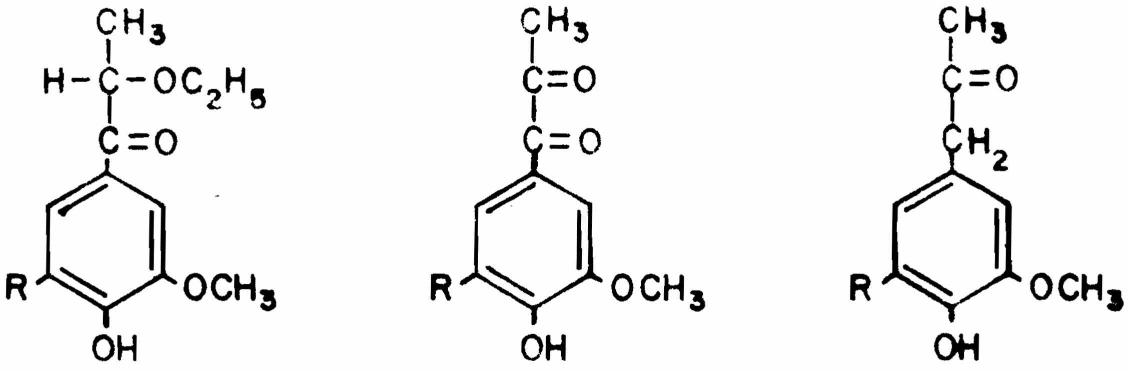


VANILLIN



SYRINGALDEHYDE

2. ETHANOLYSIS PRODUCTS



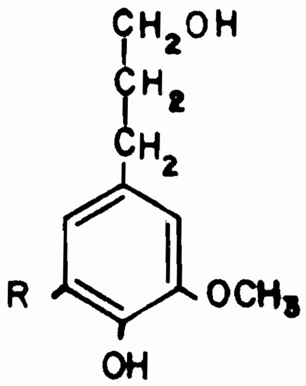
HIBBERT'S KETONES

R = OCH_3 OR R = H

3. HYDROGENOLYSIS PRODUCTS

R=H DIHYDROCONIFERYL ALCOHOL

R=OCH_3 DIHYDROSINAPYL ALCOHOL



from carbon dioxide via carbohydrates. Stone (1953) provided the first evidence for this, in an experiment in which he subjected wheat plants, at the stage of growth corresponding to their most rapid rate of lignification, (Stone et al., 1951), to ^{14}C-labeled carbon dioxide. Samples of treated plants were harvested every few days until the stage of maturity was reached. The plants were dried in a current of air at 35° C and oxidized with nitrobenzene in alkali. The specific and total radioactivity of the plants and of the isolated oxidation products, vanillin, syringaldehyde and p-hydroxybenzaldehyde were determined. The results showed that $^{14}CO_2$ was still being respired from the plants at maturity and that all the labeled carbon which entered the lignin did so within 24 hours of its administration. The total activity of p-hydroxybenzaldehyde dropped continuously throughout the life of the plant.

This was interpreted by Stone as a further support for his earlier contention (Stone et al., 1951) that this aldehyde is derived chemically from tyrosine present in the plant protein, and not from the lignin fraction. While the activity acquired originally by the vanillin portion of the lignin suffered an initial drop for about 2 weeks after activation, it then became constant. The activity acquired by the syringaldehyde portion remained constant throughout the growth of the plant, indicating that lignin, as represented by syringaldehyde, is an end product of metabolism.

In short-term experiments (Brown et al., 1953) the changes taking place in the incorporation of activity from $^{14}CO_2$ into the lignin of wheat plants were examined. Plants undergoing a stage of rapid lignification were fed with $^{14}CO_2$ for 20 minutes and then allowed to grow for 1 - 24 hours in a normal atmosphere before being harvested for analysis. The results showed that lignin was synthesized most rapidly from 4 to 6 hours after activation with the syringyl units being formed more slowly than the guaiacyl. A slower build-up in radioactivity of lignin continued for the rest of the 25-hour experimental period.

B. Lignin Formation from Carbohydrates

That carbohydrates are intermediates in the lignification process was shown by the conversion of D-glucose-C^{14} to lignin by a young Norway Spruce tree (Schubert and Acerbo, 1959); and to both shikimic acid and lignin in a species of Eucalyptus (Hasegawa and Higuchi, 1960) as well as in a tissue culture of Pinus strobus (Hasegawa et al., 1960).

An important step in metabolism of carbohydrates to lignin lies in their conversion to aromatic rings. The work of Davis and co-workers on aromatic biosynthesis from shikimic acid by microorganisms (Weiss et al., 1954) suggested the possibility of a similar biosynthetic pathway in higher plants. Brown and Neish (1955a) administered randomly ^{14}C-labeled shikimic acid, L-phenylalanine, and protocatechuic acid through the cut ends of growing wheat plants and maple twigs. The plants were allowed to metabolize for 24 hours after absorption of the radioactive solutions and the cell wall fraction was oxidized with nitrobenzene. The results showed that the efficiency with which shikimic acid was converted to both the vanillin and syringaldehyde-yielding residues of lignin was of the same order as that of L-phenylalanine, whereas protocatechuic acid was not measurably transformed into lignin. The sequence of reactions in lignin formation was suggested by these authors to be: (1) attachment of a three-carbon side-chain to a non-aromatic ring, (2) aromatization and (3) substitution of the ring.

Shikimic acid-2,6-^{14}C was further shown to be a precursor of the lignin vanillin in sugar cane plants without any rearrangement of its carbon skeleton (Eberhardt and Schubert, 1956). From these results, Nord and Schubert (1959) concluded that shikimic acid is an intermediate on the pathway from carbohydrates, formed from atmospheric CO_2 by photosynthesis, to the aromatic rings of the lignin building stones. The direct transformations of shikimic acid, phenylpyruvic and phenyllactic acids to phenylalanine and tyrosine (Gamborg and Neish, 1959) and of phenylalanine to the hydroxycinnamic acids (McCalla and Neish, 1959b) have been described previously.

C. Lignin Formation from Aromatic Compounds:

Studies were carried out by Neish and his associates on the transformation of some carbon-14 labeled aromatic monomers into the lignin residues which yield phenolic aldehydes on nitrobenzene oxidation. As a criterion for the efficiency of these compounds as lignin precursors they used the degree of dilution of the radioactive carbon during the transformations of the administered compound into aldehyde-yielding residues of lignin. Substances with a phenylmethane (C_6-C_1) skeleton were found, in general, to be inefficient precursors in the wheat plant, although the carbon-14 of vanillin and of p-hydroxybenzoic acid was incorporated to some extent into parts of the lignin (Brown and Neish, 1955b) indicating a possible condensation with a two carbon metabolite. This condensation would then form the C_6-C_3 lignin building stone (Brown and Neish, 1959) as was suggested by Higuchi (1957) and corroborated more recently by Kratzl (1961). Similarly, the C_6-C_2 compound, mandelic acid, was not converted to lignin by the same plant species (Wright *et al.*, 1958).

In contrast, several compounds with the phenylpropane structure (C_6-C_3) were found to be efficient precursors of lignin. Thus L-phenylalanine, L-tyrosine and cinnamic, phenylpyruvic, ferulic (Brown and Neish, 1955b), phenyllactic, phenylhydracrylic (dihydrocinnamic) and phenylglyceric acids (Wright *et al.*, 1958) were readily transformed into two or all three of the types of lignin residues in the wheat plant. The administration of L-phenylalanine, p-hydroxycinnamic, caffeic, ferulic and sinapic acids to wheat and maple and cinnamic acid to maple, showed a similar degree of efficiency, on the part of the two plant species, in transforming a given precursor into a specific lignin residue (Brown and Neish, 1956). More recently, Higuchi (1962) found that L-phenylalanine, p-coumaric and ferulic acids were quite good precursors of lignin in tissue cultures of white pine. Sinapic acid was converted not only to syringyl lignin but also to the guaiacyl part of lignin indicating that demethoxylation can occur in lignification. This had been reported by Kratzl (1961).

Degradation studies by Brown and Neish (1955b, 1959) showed that the benzene ring and the side-chain of the phenylpropane derivative were incorporated into lignin as a unit. Nord et al. (1957) administered carboxyl-labeled p-hydroxyphenylpyruvic acid to a growing sugar cane plant. A comparison of the radioactivity of the administered acid, of the isolated lignin, and of the barium carbonate obtained on combustion of the lignin revealed that most of the activity from the introduced acid was incorporated into lignin. Alkaline nitrobenzene oxidation of the isolated lignin (Nord and Schubert, 1957) yielded inactive vanillin, whereas alkaline fusion produced radioactive oxalic acid (Acerbo et al., 1958). It was apparent, in this work, that p-hydroxyphenylpyruvic acid was utilized intact as a unit, with the three carbon side-chain probably serving as a connecting link between the aromatic rings of the lignin molecule (Nord and Schubert, 1963a, b). Nord therefore concluded that p-hydroxyphenylpyruvic acid is an intermediate on the pathway between shikimic acid and lignin building stones in the sugar cane plant. He suggested the following tentative outline for the mechanism of the lignification process: carbon dioxide → carbohydrate → shikimic acid → p-hydroxyphenylpyruvic acid → primary lignin building stones → secondary lignin building stones → lignin.

Marked differences in the utilization of tyrosine as a lignin precursor by different plant species were obtained by Brown and Neish (1955b). Whereas L-tyrosine was as efficient as L-phenylalanine in the wheat plant, it was poorly utilized by maple, poplar and caragana plants. Studies of these apparent differences were extended to cover a total of twenty-one species representing twelve families of both mono- and dicotyledonous plants (Brown and Neish, 1956; Brown, 1961a). The results suggested that the utilization of tyrosine for the synthesis of lignin is apparently restricted to members of the Gramineae (grasses). These findings were corroborated by Siegel (1955) who was unable to obtain a lignin-like polymer from tyrosine using a dicotyledon, the Alaska pea, and by Dougall (1962) whose tobacco callus tissues failed to convert tyrosine to the vanillin and syringaldehyde-yielding residues of lignin.

The same situation as is reported for tyrosine is encountered in the utilization of the p-hydroxylated derivatives of phenylpyruvic and phenyllactic acids. Brown et al. (1959) showed these two compounds to be utilized efficiently for the formation of the guaiacyl and syringyl parts of lignin by the wheat plant but not by Salvia or buckwheat plants. Similarly, Kratzl and Billek (1957) found that spruce will not convert p-hydroxyphenylpyruvic acid to lignin while the sugar cane plant possesses this ability (Nord et al., 1957). These findings were interpreted by Brown et al. (1959) as an indication that neither p-hydroxyphenylpyruvic nor p-hydroxyphenyllactic acid is a general intermediate in lignification and differences between grasses and non-grasses probably result from the unique ability of grasses to convert p-hydroxyphenyllactic acid to p-hydroxycinnamic acid. This idea is supported by the demonstrated ability of wheat to form p-hydroxycinnamic and ferulic acids readily from both phenylalanine and tyrosine whereas only phenylalanine is a good precursor of these two acids in Salvia (Brown et al., 1959).

An alternative suggestion was offered by Neish (1960) who drew attention to the possibility that cinnamic and p-coumaric acids might arise directly by removal of elements of ammonia from the corresponding amino acids, phenylalanine and tyrosine. The latter suggestion was substantiated by the characterization of the enzyme phenylalanine ammonia lyase by Koukol and Conn (1961), which catalyzes the first reaction, in both grasses and non-grasses; and of the enzyme tyrosine ammonia lyase by Neish (1961) which catalyzes the second reaction and which is apparently restricted to grasses.

Brown and Neish (1955b, 1956) have pointed out that while caffeic acid was a good precursor for both guaiacyl and syringyl lignin building stones in maple and wheat, p-coumaric, ferulic and sinapic acid showed a preferential conversion into the corresponding p-hydroxyphenyl, guaiacyl or syringyl residues. These authors (1956) therefore suggested that each type of lignin polymer has a corresponding monomer which would mean that m-substitution of the ring precedes polymerization. This idea is supported by the findings that shoot tissues of bamboo and root tip tissues of kidney beans produced syringaldehyde upon incubation with syringylpropane derivatives only and not with guaiacylpropane derivatives (Higuchi et al., 1954).

In view of their widespread distribution in plants and their ready incorporation into lignin and because of the fact that they are highly ionizable under physiological conditions, the phenolic cinnamic acids have come to be regarded as important intermediates in lignin biosynthesis (Neish, 1960; Nord and Schubert, 1963a, b). A scheme covering the reactions from prearomatic compounds to lignin was proposed by Brown et al. (1959) in which phenylpyruvic acid is reduced to phenyllactic acid and the latter is dehydrated to cinnamic acid.

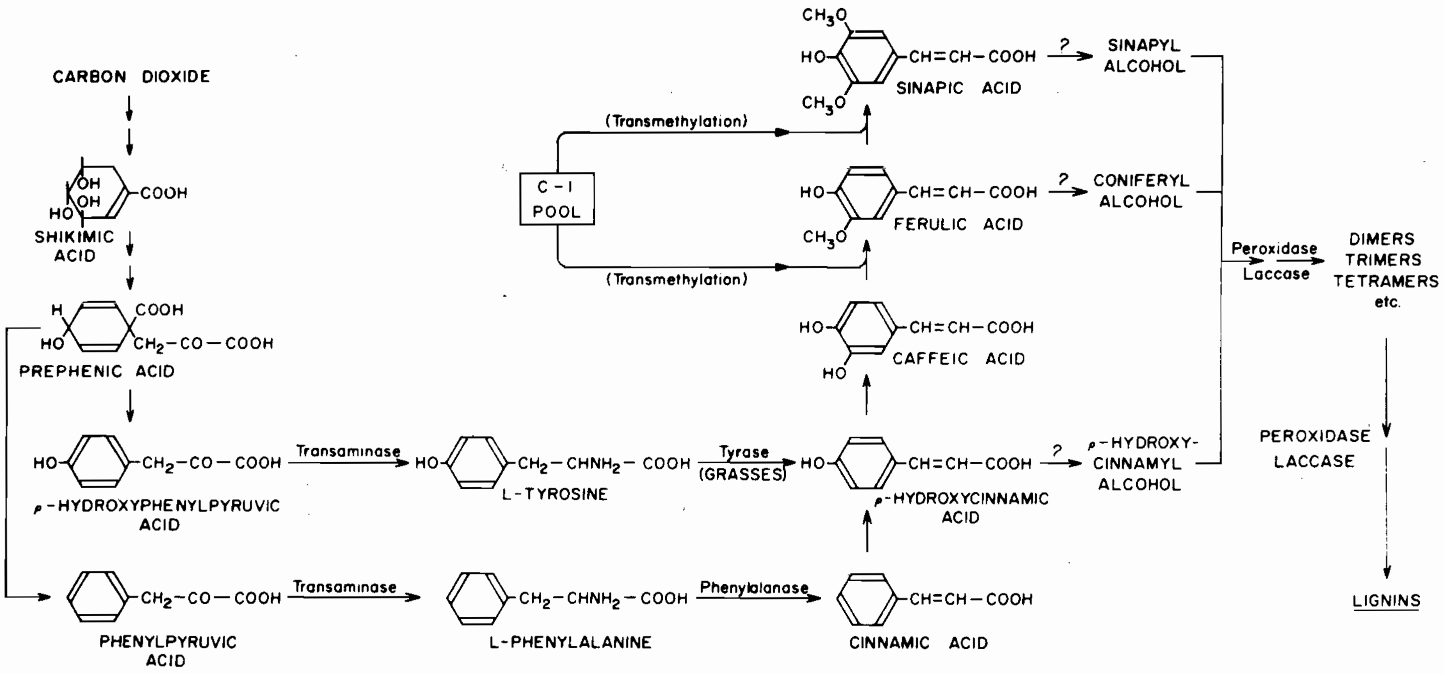
Phenylpyruvic acid arises via aromatization of prephenic acid as well as by transamination of phenylalanine. An alternative pathway, limited only to grasses, involves the corresponding p-hydroxylated acids formed from tyrosine or prephenic acid. In later reviews (Brown, 1961b, 1962) this scheme was modified to exclude the phenyllactic acids in view of the discovery of phenylalanine and tyrosine ammonia lyases (Figure 7). Recent studies by Higuchi and Brown (1963c), however, have indicated that formation of cinnamic and p-hydroxycinnamic acids by irreversible dehydration of the corresponding phenyllactic acids must still be considered as a possible alternative pathway.

D. Polymerization of Lignin Building Stones

Almost all recent speculations concerning lignification have centered on coniferyl alcohol as the primary unit in a process of oxidation and polymerization. This prevailing concept has its origin in Klason's conjecture in 1897 that coniferyl alcohol, or its glucoside coniferin, is the parent substance of lignin in the plant. Historically, this idea was based primarily on three facts: the frequent occurrence of coniferyl alcohol in the form of its glucoside coniferin in conifers, its similar elemental analysis to that of Klason lignin and its tendency to resinify (Kremers, 1959).

Basing their work on this concept, Freudenberg's group at Heidelberg has been working for nearly the last three decades on the _in vitro_ formation of lignin-like material by the action of enzymes on

Figure 7. A scheme showing the reactions from carbon dioxide to lignin as proposed by Brown (1961b).



coniferyl alcohol, and on the in vivo formation of lignin from carbon-14 labeled coniferin and related compounds. They conducted extensive investigations on the behaviour of coniferyl alcohol in dilute aqueous solutions exposed to air, to a mushroom or cambium oxidoreductase, a laccase, or a peroxidase and dilute H_2O_2 (for example, see Freudenberg, 1957, 1959, 1962a, b). This work has been reviewed in detail by Brauns and Brauns (1960, page 689).

These enzyme systems result in the synthesis of a water-soluble dehydrogenation polymer (DHP) with properties remarkably similar to those of spruce milled-wood lignin (Freudenberg, 1957). If the polymerization reactions are interrupted at a certain stage, namely, when all the added coniferyl alcohol has been used up and only very little lignin has been formed, a large number of intermediates of low molecular weight are obtained (Freudenberg, 1962a, b). About forty substances have been separated so far by means of paper chromatographic techniques. These consist mainly of dimers (Freudenberg and Schlüter, 1955) and to a lesser extent of trimers and tetramers (Freudenberg and Sakakibara, 1959; Freudenberg and Friedmann, 1960; Freudenberg and Nimz, 1962). The structures of some of these compounds are shown in Figure 8. Quantitatively speaking, the most important of these are the phenylpropane dimers, dehydrodiconiferyl alcohol, DL-pinoresinol, and guaiacylglycerol-β-coniferyl ether. By the continued action of the enzymes, these substances are dehydrogenated further and condense with each other or with coniferyl alcohol to form a lignin-like polymer (Freudenberg, 1959, 1962a, b).

Coniferin itself cannot be transformed into a dehydrogenation

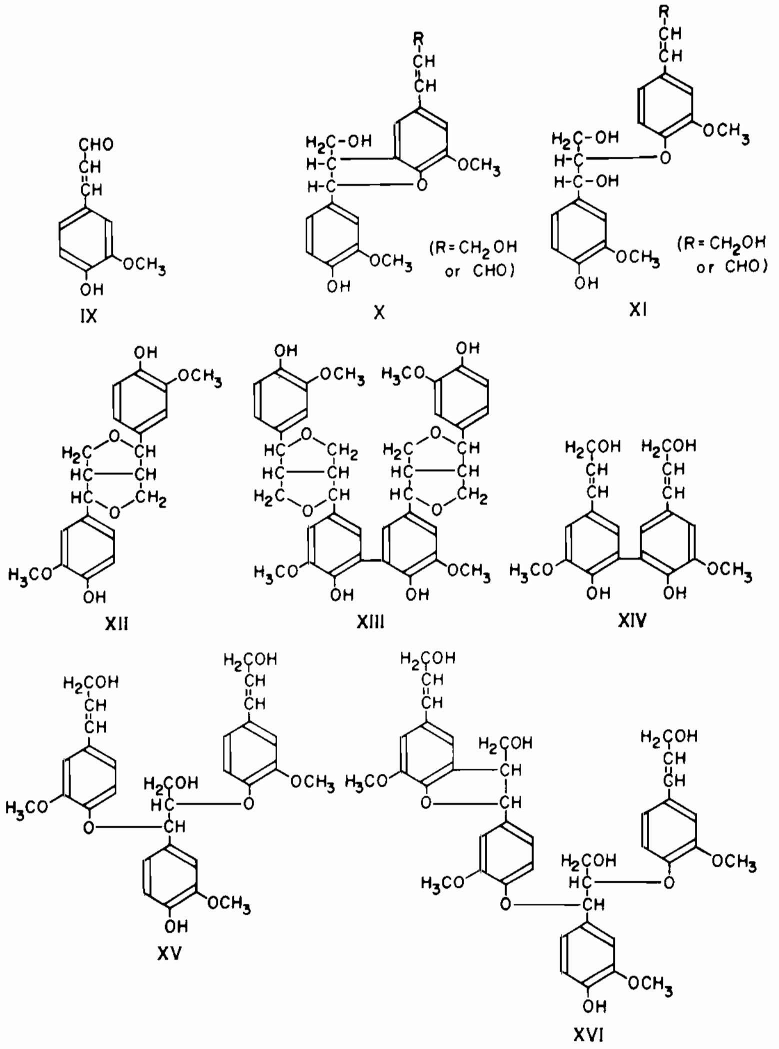


Figure 8. Intermediates in the formation of the dehydrogenation polymer of coniferyl alcohol.

IX = coniferylaldehyde; X = C_6-C_3 dimer; XI = guaiacylglycerol-β-coniferyl ether (R=CH_2OH); XII = D,L-pinoresinol; XIV = dehydrodiconiferyl alcohol. The others are trimers and tetramers of coniferyl alcohol.

polymer by these enzymes since it must first undergo hydrolysis by the action of β-glucosidase (Freudenberg, 1962b). The presence of β-glucosidase in the cambial layer of spruce was shown by Freudenberg (see Brauns and Brauns, 1960, p. 704).

The mechanism of the lignification process in living spruce trees was further studied by introducing into the plant specifically labeled D- and L-coniferin-^{14}C (Freudenberg, 1959, 1962b). The L-glucoside was not utilized by the plant since it is not cleaved by β-glucosidase although it was distributed through the plant to the same extent as was the D-glucoside. Administration of a solution of D-coniferin-^{14}C through the trimmed off needles of a young spruce branch caused almost 60% of the activity to appear in the wood and bark and about 40% in the needles. The radioactivity was confined almost entirely to the lignin fraction. Alcoholysis of this fraction gave radioactive Hibbert's ketones (Figure 6) which were considered by Freudenberg (1956, 1962b) to offer unequivocal proof that the radioactive substance had been converted to lignin. Since coniferin and coniferyl alcohol do not give these ketones, the coniferyl alcohol must have been converted during the process into a guaiacylglycerol-β-coniferyl ether type structure from which Hibbert's ketones are subsequently recovered on alcoholysis. Lignin formation from administered coniferin to plants was also reported by Kratzl (1961).

Freudenberg and Niedercorn (1958) administered DL-phenylalanine-2-^{14}C to young spruce trees and isolated radioactive coniferin from the plant 2.5 days later. These results indicated clearly that not only was phenylalanine converted by the plant to coniferyl alcohol but also that the latter compound was converted to its glucoside coniferin. Furthermore, the conversion of ferulic acid (Freudenberg, 1956) and of cinnamic acid (Neish, 1959) to lignin of spruce has been demonstrated. Higuchi and Brown (1963a) presented evidence for participation of coniferyl alcohol, formed through the mediation of coniferyl aldehyde, from administered ferulic acid in lignin synthesis by the wheat plant. Since coniferin formation could not be detected in these experiments, the authors adopted the view expressed previously by Neish (1960) that coniferin is a storage product formed only by a few species. Higuchi and Brown suggested the following scheme:

$$\begin{tikzcd} \text{ferulic acid} \arrow[r] & \text{coniferyl aldehyde} \arrow[r] & \text{coniferyl alcohol} \arrow[r] \arrow[d, shift left, "\text{(conifers)}"] & \text{lignin} \\ & & \text{coniferin} \arrow[u, shift left] & \end{tikzcd}$$

According to Freudenberg, coniferin which is found in the cambium, is hydrolyzed by a tissue-bound β-glucosidase into glucose and coniferyl alcohol (Freudenberg, 1962b). The glucose may be used by the plant for the formation of cellulose whereas the coniferyl alcohol, by the action of redoxases, is converted into lignin.

The conversion mechanism as explained by Freudenberg (1956) starts with enzymic dehydrogenation of coniferyl alcohol at the phenolic hydroxyl group forming a free radical, a quinone methide, which exists in several mesomeric forms as shown in Figure 5. Freudenberg considers the dimeric compounds, which could be formed by condensation of two such mesomeric forms (see Figure 8) to be the most important secondary building stones, and coniferyl alcohol to be the primary building stone of lignin. Since the radical formed by dehydrogenation of coniferyl alcohol is optically inactive and the secondary building stones are formed without the participation of enzymes, they are also optically inactive. This may explain the optical inactivity of the DHP and of protolignin (Freudenberg, 1959).

Freudenberg's theory also offers a basis for the formation of angiosperm lignins as being due to participation of sinapyl alcohol in lignin formation since its glucoside, syringin (Figure 5), has been found in the cambial sap of many species (Kremers, 1959). Model systems with laccase (Freudenberg, 1959) showed that sinapyl alcohol will not produce a DHP by itself since the reaction stops after dimerization to syringaresinol. However, in a mixture of sinapyl and coniferyl alcohols copolymerization occurs and a DHP is formed.

On the basis of his finding that the methoxyl content of lignin of leaves and stalks of barley does not remain constant during growth as would be anticipated if coniferyl alcohol is the primary lignin progenitor, Phillips (1935) stated: "it appears more probable that several intermediate substances are utilized by the plant in the synthesis of lignin. These substances do not contain the same percentage of methoxyl and some may be entirely free of methoxyl. In the process of lignin synthesis these building stones are combined in different proportions during the development of the plant." More recently the presence of small amounts of p-hydroxyphenyl and syringyl units in lignin of conifers has been established by several workers. Nord and DeStevens (1952) reported the recovery of appreciable quantities of p-hydroxybenzaldehyde on alkaline nitrobenzene oxidation of native and enzymatically liberated lignins of white pine. Since these two types of lignin are devoid of nitrogenous impurities, as demonstrated by analysis (Nord and DeStevens, 1958), the likelihood that p-hydroxybenzaldehyde is derived from protein tyrosine (Stone et al., 1951) is eliminated.

Anisic (p-methoxybenzoic) acid and trimethylgallic acid were also isolated in very small amounts from a mixture of the methoxylated aromatic acids obtained after oxidative degradation of spruce milled-wood lignin (Freudenberg and Chen, 1960; Freudenberg et al., 1962). These findings indicate that small quantities of p-hydroxycinnamyl (p-coumaryl) alcohol and sinapyl alcohol are copolymerized, as their dehydrogenated derivatives, along with coniferyl alcohol in spruce, with coniferyl alcohol being the major building stone (Freudenberg, 1962b).

Very recently the β-glucosides of the three substituted cinnamyl alcohols, p-coumaryl-4-0-glucoside, syringin and coniferin were detected in green shoots from young spruce saplings. Furthermore, all three glucosides were found to be radioactive when phenylalanine-β-^{14}C had been administered to the plants (see Freudenberg and Harkin, 1963). The identities of these glucosides were established by their isolation from the cambial sap of spruce trees by Freudenberg and Harkin (1963).

In summation it may be said that lignins are products of the shikimic acid pathway. p-Coumaric, ferulic and sinapic acids are formed from the aromatic amino acids, phenylalanine and tyrosine, by deamination followed by hydroxylation and methylation (or methoxylation). These acids are probably reduced to their corresponding alcohols, which may be stored temporarily as their glucosides in species like spruce. An oxidative polymerization, or copolymerization, in varying proportions according to the species, of the alcohols may then form lignins. There is also a possibility that syringyl groups may be formed by further methoxylation of the nuclei of the guaiacyl lignin (Neish, 1960).

MATERIALS AND METHODS

I. PLANT MATERIAL

Grains of Kharkov wheat (*Triticum aestivum* L. emend Thell. Spp. *vulgare* var. Kharkov 22 M. C.) were obtained from Dr. H. R. Klinck, Macdonald College of McGill University. Cut shoots of *Hydrangea macrophylla* Ser., *Gaultheria procumbens* L., *Pinus resinosa* Ait. and *Malus spp.* were obtained from plants grown in the greenhouse of McGill University, Montreal.

II. CULTIVATION OF PLANTS

The following two methods of germination were used:

A. Grains, sterilized by soaking in sodium hypochlorite solution (4 - 6% available chlorine) for 10 minutes, were washed thoroughly and aerated in tap water for 24 hours. They were germinated on cheesecloth spread over a perforated aluminum rack in a plastic pan (12 x 12 x 6") containing tap water up to the cheesecloth level (Neish, 1961). The cultures were placed under a bank of cool white fluorescent tubes in a temperature-controlled growth room where they received 18 hours of light daily. The light intensity, at the top level of the plastic pan, was 1500 f.c. The temperature of the light period was maintained at 25 ± 2° C and that of the dark interval at 17 ± 2° C. On the fourth day of germination, the tap water was replaced by Hoagland's nutrient solution which was changed twice weekly.

B. Grains were sown in soil in 8" clay pots at an average of 20 grains per pot. They were germinated and subsequently grown under the prevailing daylight conditions in the greenhouse of McGill University with no artificial light supplement. Plants were irrigated with tap water daily.

III. CHEMICALS

A. Non-radioactive Compounds

Most of the phenolic acids, aldehydes and coumarins used in this study were purchased from Light and Co., England. Isoferulic acid and coniferyl alcohol were purchased from Fluka, Switzerland. Ortho-ferulic acid was prepared by Dr. B. Bohm, University of Rhode Island. A small sample of tricin was obtained from Dr. S. Yoshida, University of Tokyo, Japan. p-Hydroxyphenyllactic acid was a preparation of Dr. A. C. Neish, Director of the Atlantic Regional Laboratory, National Research Council, Halifax. 2-Hydroxy-4-methoxy-cinnamic acid was a gift from Dr. S. A. Brown, Prairie Regional Laboratory, Saskatoon, to Dr. G. H. N. Towers.

B. Radioactive Compounds

Sodium carbonate-^{14}C, L-phenylalanine-U-^{14}C and L-tyrosine-1-^{14}C were obtained from Merck, Sharp and Dohme, Montreal. Cinnamic, p-coumaric, caffeic, ferulic and sinapic acids, all labeled in the 3-position, were preparations of Dr. A. C. Neish.

IV. ADMINISTRATION OF COMPOUNDS

A. $^{14}CO_2$

Two different types of photosynthetic chambers illustrated in Figures 9 and 10 were used. Whole rooted seedlings, when used, were placed in chamber X with their roots completely buried in wet, washed and ignited sand in a small cylindrical polyethylene container. When shoots were used they were cut under water and transferred to and maintained in a small vial containing tap water which was replenished from time to time to prevent desiccation.

Experiments with older wheat plants, cultivated by Method B were carried out by placing whole potted plants in chamber Y.

Light was provided by means of four 150 watt, flood light bulbs arranged at equal distances from the photosynthetic chamber giving an average intensity of 1200 f.c. at the outer surface of the chamber. Temperature was maintained at 26 ± 2° C by means of a controlled current of cold water passing through the cooling jacket of chamber X. With chamber Y, temperature was maintained at 24 ± 2° C by placing the illuminated chamber in a refrigerator at -5° C.

Mechanism of $^{14}CO_2$ generation and administration:

A measured volume of $Na_2{}^{14}CO_3$ solution (in 0.1 N NaOH), containing a known amount of activity was pipetted carefully into flask a (see Figure 9). Both stopcocks c and d were kept closed and sidearm b was filled with 2N HCl. The photosynthetic chamber was partially evacuated through stopcock e which was turned off thereafter. A vacuum was generated in the flask a by opening stopcock d and the acid in the sidearm b was sucked through stopcock c which was closed immediately after. Complete $^{14}CO_2$ evolution was ensured by gentle shaking of flask a. The remaining $^{14}CO_2$ was flushed into the photosynthetic chamber by allowing CO_2-free air to be sucked, through the $^{14}CO_2$ generator, to equalize the internal pressure with that of the atmosphere. Stopcock d was then closed and the CO_2 generator disconnected. Plants were allowed to metabolize for a certain period after which the non-utilized $^{14}CO_2$ was trapped as $Ba^{14}CO_3$. The rate of $^{14}CO_2$ utilization during the experiment was measured by means of a monitor connected to an Anton Model 222 Geiger-Müller tube incorporated into the system.

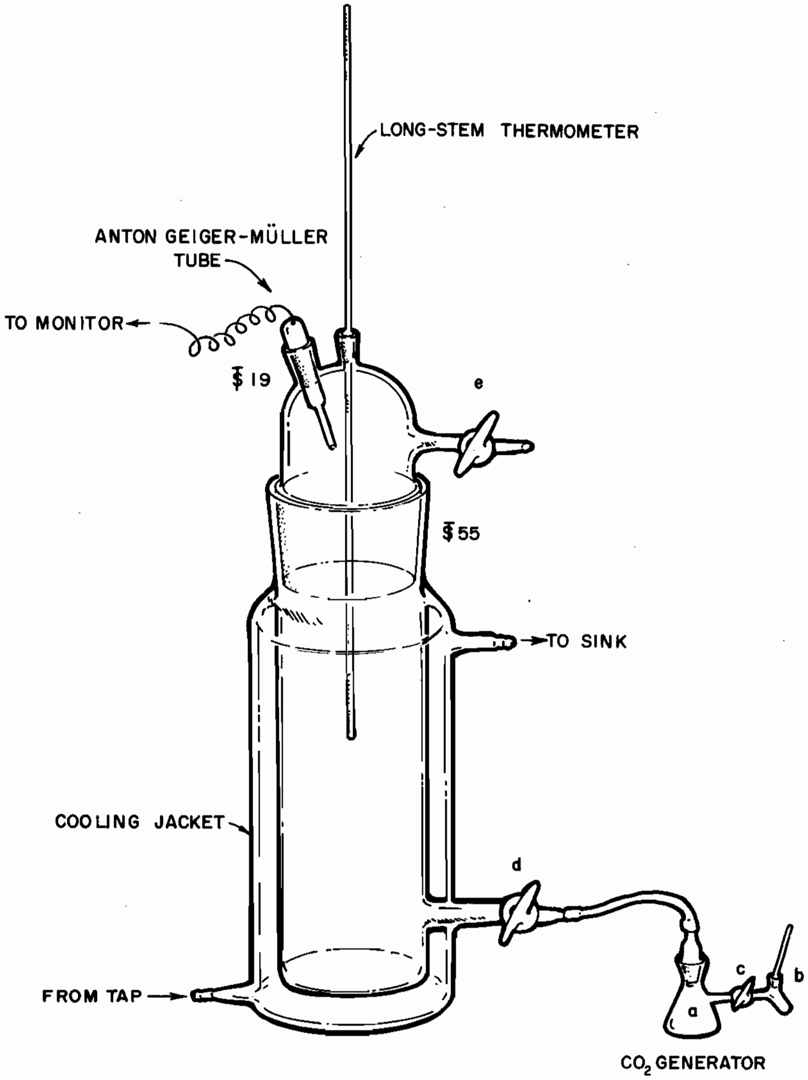


Figure 9. Photosynthetic chamber X.

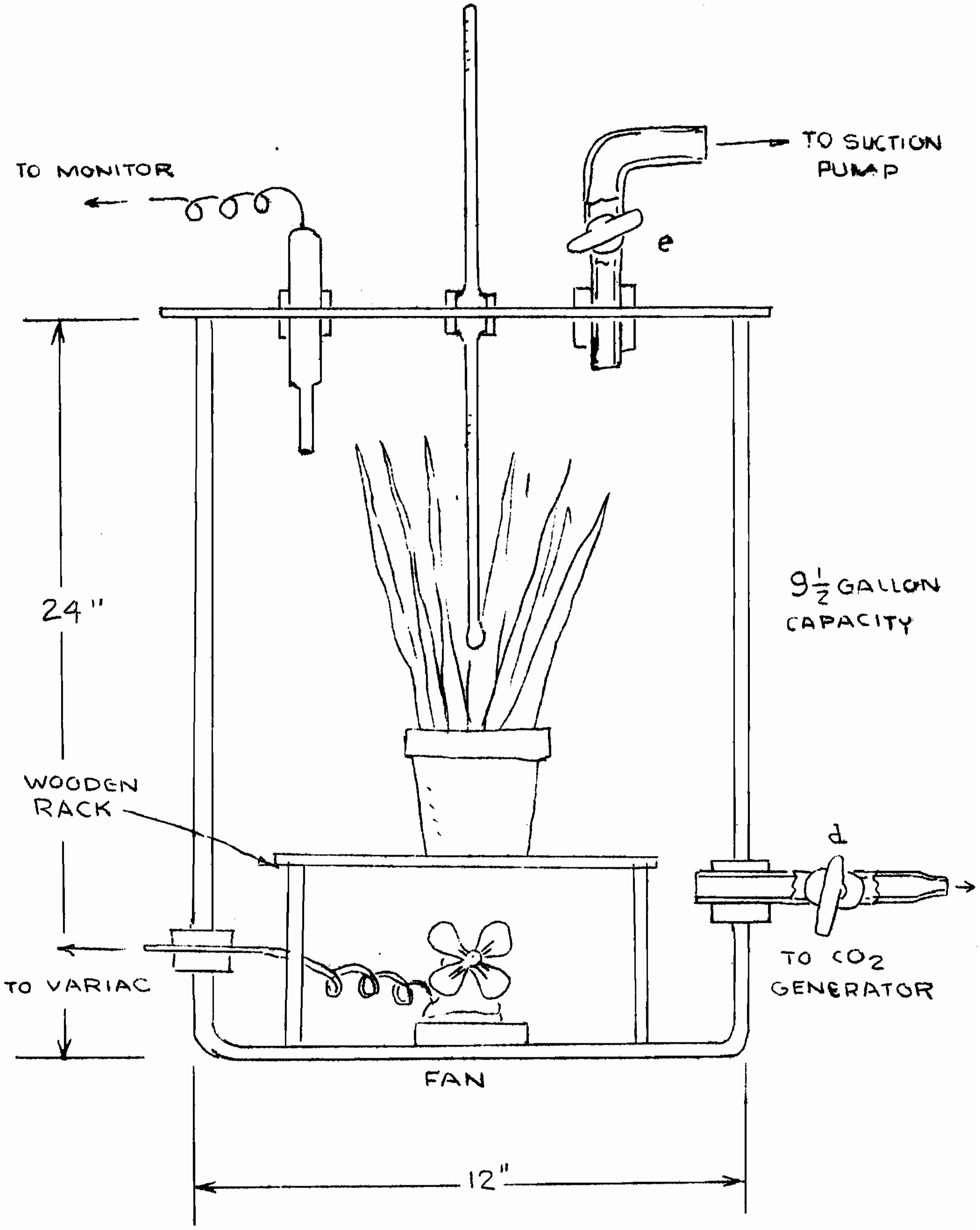


Figure 10. Photosynthetic chamber Y.

B. Shikimic and Phenylpropanoid Compounds

L-Phenylalanine, L-tyrosine and cinnamyl alcohol were administered in aqueous solutions. Shikimic, cinnamic, p-coumaric, caffeic, ferulic and sinapic acids were fed as their sodium salts in neutral aqueous solutions.

In all cases, shoots of wheat plants were cut under water and held in small vials containing known concentrations of radioactive or non-radioactive solutions. They were allowed to metabolize under a bank of fluorescent and incandescent lights (1000 - 1200 f.c.) at room temperature for certain periods during which time the solutions were completely absorbed by the plants. Either water or more of the same solution was added from time to time thereafter to prevent desiccation of the shoots. The dose, activity, and the metabolic period were varied according to the experiments as will be mentioned under Experimental.

V. PREPARATION OF PLANT EXTRACTS

A. Ethanol-Soluble Fraction

Fresh plant material was cut into 1 to 2" pieces with scissors and homogenized in boiling absolute ethanol (10 ml/g fresh wt.) in a Waring Blendor. The homogenate was extracted for 15 minutes, under reflux, on the steam bath and filtered. The residue was further extracted with 3 equal portions of 80% ethanol (10 ml/g fresh wt.) on the steam bath and the combined filtered extracts were reduced in volume *in vacuo* at 50° C. The concentrated extract was made to a measured volume (50 to 100 ml) and aliquots were taken for duplicate determinations of radioactivity and chromatographic-autoradiographic analyses. This step was omitted in the later experiments. The rest of the ethanolic extract was taken to dryness *in vacuo* at 50° C. The residue, thus obtained, was extracted with boiling distilled water (5 to 6 ml/g fresh wt.) on the steam bath for 20 minutes and filtered through a bed of Celite (Analytical Filter Aid) under suction. The filter cake was washed with boiling water and the filtrate was treated as follows:

1. One aliquot was dried *in vacuo* at 50° C and the residue taken up in a minimum amount of 70% ethanol and chromatographed directly for the naturally-occurring derivatives of phenolic acids.

2. Another aliquot was acidified to about pH 4 (litmus paper) and continuously extracted with ether, in a liquid-liquid extractor of suitable volume, for 20 hours. The ether extract was concentrated and chromatographed for free phenolic acids.

3. A third aliquot was treated with conc. HCl to make a 2*N* solution with respect to the acid. The solution was heated under reflux on the steam bath for 30 minutes and the cooled hydrolyzate extracted with ether for 20 hours. The ether extract contained those phenolic acids originally present in the form of glycosides and/or acid-sensitive esters.

4. To a fourth aliquot was added 10N aqueous NaOH to give a normality of 2 and the solution kept in a refrigerator at 5° C for 14 hours. The solution was acidified to about pH 4 with conc. HCl and continuously extracted with ether for 20 hours for those phenolic acids initially present as esters.

B. Ethanol-Insoluble Residue

Half a gram of air-dried cell wall residue, obtained as mentioned under A, was treated with 20 ml 1N aqueous NaOH in a test tube at 30° C for 4 hours. It was acidified to a pH of 4, filtered and the filtrate continuously extracted with ether for 20 hours. The ether extract contained those phenolic acids present originally as ethanol-insoluble esters. In some cases the products of hot alkaline hydrolysis, i.e. digestion on the steam bath with 1N aqueous NaOH for one hour, were also examined.

C. Alkaline-nitrobenzene Oxidation of Ethanol-Insoluble Residue

The micromethod of Stone and Blundell (1951), as modified by Towers (1951), was used. Three hundred mg of finely divided ethanol-insoluble residue were placed in a stainless steel bomb to which were added 0.7 ml of nitrobenzene and 10 ml of 2N aqueous NaOH. The bomb was tightly sealed and kept for 2-1/2 hours in a preheated, thermostatically controlled, paraffin oil bath at 160° C. Contents of the bomb were shaken vigorously for a few seconds at approximately 30-minute intervals. At the end of the heating period, the bomb was removed, rapidly cooled in crushed ice and opened. The contents were quantitatively transferred, with about 50 ml distilled water, to a one litre separatory funnel and shaken successively with four, 500 ml portions of absolute ether and the ether extracts were discarded. The aqueous portion was acidified with ice-cold conc. HCl to a pH of 4 (litmus paper) and continuously extracted with ether for 20 hours. The ether extract contained the alkaline nitrobenzene oxidation products of lignin.

D. Isolation of Klason Lignin

The Klason lignin was prepared by a modified method scaled down from that described by Brauns (1952, p. 56). To 100 mg of finely powdered, air-dried ethanol-insoluble residue was added 1 ml of 72% sulphuric acid (W/W) in a small beaker. The digestion was allowed to proceed for 2 hours at room temperature with frequent stirring by means of a glass rod after which time it was poured into a 250 ml round-bottomed flask together with 70 ml of distilled water. The suspension was boiled gently under reflux for 12 hours, cooled and filtered through a tared disc of Whatman No. 1 filter paper in a small Buchner funnel. After thorough washing, the disc was dried in a vacuum desiccator for 24 hours and its weight determined. The difference in weight represented the amount of Klason lignin. This was subsequently combusted for analysis of radioactivity.

VI. CHROMATOGRAPHIC TECHNIQUES AND EQUIPMENT

A. Paper Chromatography

1. Paper: Large sheets (18-1/2 x 22-1/4") of Whatman No. 1 chromatography grade filter paper were used for two-directional separation of plant extracts fractionated as mentioned under V. For one-directional chromatography strips, 2" wide, of the same grade paper were used.

2. Tanks: Air-tight, arborite-lined, wooden tanks containing glass troughs suitable for development of large chromatograms by the descending methods were employed.

3. Solvents: The following solvents were prepared with analytical grade reagents and distilled water:

a. Phenol-water, 4:1 W/W. (Consden et al., 1944): This solvent was used for irrigation of the first direction (parallel to the long axis of the paper) for separation of the total ethanolic extract into its individual components. Eighty ml of solvent per trough per two chromatograms were used. Development time was 25 to 28 hours at 70° F. Chromatograms were subsequently dried for 2 to 3 days in a fume hood.

b. n-Butanol-acetic acid (glacial)-water, 4:1:1.8 by volume: This one phase solvent required saturation of the tank prior to use. It was used as a second solvent, running parallel to the short axis of the paper, for chromatography of the total ethanolic extracts. Seventy-five ml of solvent were used to irrigate two chromatograms placed in the same trough in a period of 12 to 14 hours at 70° F. The chromatograms were subsequently dried in the fume hood for 4 to 6 hours.

c. Benzene-acetic acid (glacial)-water, 10:7:3 by volume: This two phase solvent required saturation of the tank with the aqueous phase for a minimum of 4 hours prior to use. The solvent had to be prepared at the same temperature at which the chromatograms were to be developed. It was used for the first direction for chromatographic separation of the phenolic acids as well as for lignin aldehydes. Saturation of the tank was facilitated by irrigation of 4 blank sheets with the organic phase of the solvent prior to use. In a well saturated tank, 70 ml of the solvent were found adequate to irrigate two chromatograms at 70° F in 4 to 4-1/2 hours. Complete removal of the solvent from chromatograms required 2 to 3 hours in the fume hood.

d. Aqueous formic acid, 2% by volume: (Bohm and Towers, 1962): This solvent does not require presaturation of the tank although it was usually practised. The solvent was used for development of chromatograms of phenolic acids in the second direction. Development time was 3 to 3-1/2 hours using 65 ml of solvent for irrigation of two chromatograms at 70° F. Chromatograms were dried subsequently for 4 to 6 hours.

e. n-Butanol-conc. NH_4OH-water, 4:1:5 by volume: This two phase solvent required presaturation of the tank for a minimum of 4 hours with the aqueous phase. Solvent (organic phase) amounts and development conditions are similar to the butanol-acetic acid-water solvent (b above).

4. Preparation of samples for chromatography: Samples, to be chromatographed, were dissolved in absolute or 80% ethanol and made to a measured volume. A known amount of this ethanolic solution representing 1 to 2 g fresh wt. of plant material was applied to a chromatogram by means of a Hamilton microsyringe. Partial drying between successive drops, to avoid spreading over of the spot, was facilitated with a cold stream of air provided by a hair-dryer.

B. Thin Layer Chromatography "TLC"

1. Plates: Smooth flat glass plates measuring 200 x 200 mm and 200 x 50 mm were used.

2. Adsorbent (Stationary phase): Silica gel G, prepared by Merck, Germany, according to the specifications of Stahl, having a grain size of 5 - 25µ and containing 13% $CaSO_4$ as binder was used.

3. Tanks: Rectangular glass tanks, with removable ground glass covers, suitable for development of the above described plates were used.

4. Solvents: The following solvents were used:

a. Ethylacetate-methylethylketone-formic acid-water, 5:3:1:1.

b. n-Butanol-acetic acid (glacial), 4:1 by volume.

These two solvents were used for two directional chromatography of extracts containing the naturally-occurring phenolic acid conjugates (prepared as mentioned under 1 page 70).

c. Benzene-acetic acid (glacial), 9:1 by volume.

d. n-Butyl ether.

Solvents c and d were used for chromatography of phenolic acid extracts as well as for lignin oxidation aldehydes.

5. Preparation of a TLC chromatoplate: To ensure that the glass plates were as clean as possible, and particularly free of grease, they were soaked in a solution of Alconox detergent, washed thoroughly with water and finally rinsed with acetone and allowed to dry.

When coating the plates with the adsorbent, the hand method of Lees and DeMuria (1962) was used. Five large plates were placed on the surface of a clean bench (4 small plates were used in place of one large plate) close together in a straight row. The two long opposite edges of the row were taped to the bench surface with 2, 3 or 4 successive layers of 1" wide masking tape extending approximately 5 to 8 mm, on the glass surface. The number of layers of tape determined the thickness of the coating layer on the plates, for instance, 2 tape thicknesses gives a layer of adsorbent of 0.6 - 0.8 mm thickness. A uniformly viscous slurry of the adsorbent was prepared by mixing, for 20 seconds in a stoppered flask, 36 g silica gel G and 72 ml distilled water. This was poured immediately on the open end of the first plate in the row and spread smoothly over the entire surface by means of a glass rod drawn in a direction parallel to the taped edges. The plates were allowed to stand for 10 minutes to set after which time the tape was removed and the plates were placed in a metal drying rack and dried in an oven at 100 to 110° C for one hour.

The sample to be chromatographed was applied across a line parallel to, and 1" above, the lower edge of the plate in the form of small overlapping drops by means of a Hamilton microliter syringe. For two-directional chromatography the plates were dried thoroughly before being run in the second direction. The development time for the butanol-acetic acid solvent was approximately two hours and one hour in the case of the other solvent systems listed above.

VII. SPRAY REAGENTS

The following spray reagents were used for detection of phenolic acid conjugates, phenolic acids and aldehydes on both paper chromatograms and thin layer chromatoplates. Colors were better developed on paper chromatograms.

A. Fast Bordeau Salt B.D. "Stabilized diazo salt of 4-amino-2,5-dimethoxybenzonitrile". (Pearl and McCoy, 1960a).

Chromatograms were sprayed with a freshly prepared 0.1% aqueous solution of this salt and oversprayed with a saturated solution of Na_2CO_3. On TLC chromatoplates, heating by means of hot air (supplied by a hair-dryer) was found necessary for full development of the color. This spray reagent was the one most frequently used throughout the course of this study.

B. Diazotized Sulfanilic Acid Spray. (Evans _et_ _al_., 1949).

The spray reagent was prepared from the following stock solutions:

1. Sulfanilic acid solution prepared by dissolving 9 g of sulfanilic acid in 90 ml concentrated HCl and diluting to one litre with distilled water.

2. Sodium nitrite solution prepared by dissolving 25 g in 475 ml distilled water.

3. Sodium hydroxide solution prepared by dissolving 100 g in 400 ml distilled water.

The spray reagent was freshly prepared prior to use by mixing the 3 stock solutions respectively in a 2:1:2 ratio by volume.

C. Diazotized _p_-Nitroaniline (Bray _et_ _al_., 1950)

The following stock solutions were prepared:

1. _p_-Nitroaniline: prepared by dissolving 1.5 g in 500 ml of 8% HCl.

2. Sodium nitrite solution: the same solution as used for sulfanilic acid spray.

3. Sodium acetate solution prepared by dissolving 100 g in 400 ml distilled water.

4. Sodium hydroxide solution, 50 g were dissolved in 950 ml distilled water.

Chromatograms were sprayed with a mixture of stock solutions 1, 2 and 3 respectively in a 5:1:15 ratio by volume followed by over-spraying with solution 4.

D. Ferric Chloride Solution (Bray *et al.*, 1950)

A 1 to 2% aqueous or ethanolic solution was used. It was particularly useful for the detection of *ortho*-dihydroxyphenolic compounds.

E. 2,4-Dinitrophenylhydrazine (DNPH) Solution

Chromatograms were sprayed with a saturated solution in 2*N* HCl. It was used particularly for distinguishing the phenolic acids from the phenolic aldehydes. Since the other spray reagents used are phenolic reagents, this solution was used for the detection of the aromatic aldehydes formed on alkaline-nitrobenzene oxidation of cell wall material.

VIII. SPECTROPHOTOMETRIC TECHNIQUES

A. Ultraviolet Spectra

The solvent used for the determination of the ultraviolet absorption spectra of the phenolic acids was 0.05N aqueous NaOH (Neish, 1961) and for the phenolic aldehydes was absolute ethanol containing 4% of 0.2% KOH in 95% ethanol (Towers, 1951). The spectra were determined of solutions containing approximately 4 to 6 µg/ml phenolic acid or aldehyde against a blank of the solvent alone in a matched pair of 1 cm silica cuvettes. A Bausch and Lomb, model 505 spectronic recording spectrophotometer was used.

For purposes of quantitative determination, standard calibration curves were prepared, for some of the phenolic acids and aldehydes, in the following manner: 10 mg of a recrystallized and dried authentic sample of each compound was dissolved in the appropriate solvent and made up to 100 ml volume. Five dilutions in the range of 2 to 10 µg/ml were made of this solution. The optical densities were determined at the wavelength of maximum absorption of each compound in a Beckman DU Spectrophotometer. The solutions were found to obey Beer's Law and a straight line relationship existed between optical density and concentration in each case.

B. Infrared Spectra

The infrared spectra of some of the phenolic acids were prepared for purposes of identification by Mr. C. W. Glennie of the National Research Council, Halifax. The spectra were obtained for the compounds as their KBr pellets (1 mg/120 mg KBr) using a Perkin-Elmer 239 Infrared Spectrophotometer.

IX. ANALYSES FOR PHENOLIC COMPOUNDS

A. Tentative Methods of Identification

On chromatograms, phenolic compounds were identified by their R_f values in two or more solvent systems and by their characteristic fluorescence under ultraviolet light (Goodwin and Kavanagh; 1950; Ibrahim and Towers, 1960) before and after exposure to ammonia vapour. The identification was confirmed by their color reactions with one or more of the chromogenic spray reagents described above. The presence of phenolic compounds known to have a restricted distribution in higher plants was confirmed by eluting spots corresponding to the suspected compound from unsprayed chromatograms and cochromatographing the eluate with an authentic sample in two solvent systems. Movement of both substances as an undifferentiated single spot was taken as a confirmation of the identity.

B. Isolation of Phenolic Acids

The residue of the dried ether extract of alkaline hydrolyzates of the ethanol-soluble fraction of wheat shoots, was dissolved in a small volume of absolute ethanol (ca 5 ml) and streaked on several sheets of Whatman No. 1 chromatography paper. The papers were developed in the benzene-acetic acid-water solvent, dried and examined under ultraviolet light. The band corresponding to the compound was cut out and eluted from the chromatograms with 80% ethanol at room temperature. The eluate was concentrated in vacuo and rebanded in a similar manner in the formic acid solvent. The slower of the two bands corresponding to the compound (the trans-form) was eluted in the same way and concentrated in vacuo. It was then transferred to a small beaker and blown down to dryness under a fine stream of dry, filtered air in the fume hood. The gummy, brownish residue, thus obtained was dissolved in a minimum volume of distilled water, treated with a pinch of Darco G. charcoal and filtered, hot, with suction through a fine sintered glass funnel.

The filtrate was reduced in volume and the compound crystallized out upon cooling slowly to room temperature. The melting point of the compound was determined with a Thomas-Hoover capillary melting point apparatus. A mixed melting point was also determined on a mixture of equal amounts of the isolated substance and an authentic sample. The infrared spectrum of the isolated compound was compared with that of an authentic sample.

C. Isolation of Lignin Oxidation Aldehydes

Isolation of p-hydroxybenzaldehyde, vanillin and syringaldehyde from the products of alkaline-nitrobenzene oxidation of cell wall material was accomplished by thin layer chromatography on silica gel G plates in the following way.

The dry residue of the ether extract of the acidified lignin oxidation mixture was dissolved in 2 ml of absolute ethanol. It was applied as narrow (5 mm) bands on several silica gel plates and developed in the benzene-acetic acid solvent. After the plates were dried, a 2 cm band along the edge of the plate parallel to the course of solvent movement was sprayed with the DNPH reagent, the rest of the plate being protected by a glass sheet. The band corresponding to each of the three aldehydes was scraped off into a small clean beaker with a sharp-edged stainless steel spatula. The bands were eluted twice with absolute ethanol at room temperature and filtered. The filtrate was reduced in volume under vacuum at 30° C and poured into a microsublimation apparatus. Complete drying of the eluate left behind a smooth, thin film coating the round bottom of the apparatus. Each of the three aldehydes was sublimed using either methanol or water as the heating bath. The sublimate was scraped off the cold finger and its melting point was determined.

D. Isolation of Radioactive Hydroxybenzoic Acids

To the dried residue of the ether extract of an alkaline hydrolyzate of the ethanol-soluble fraction of wheat shoots administered p-coumaric acid-3-^{14}C, 10 mg of p-hydroxybenzoic acid were added and the mixture was dissolved in 2 to 3 ml of absolute ethanol. The ethanolic solution was chromatographed and subsequently treated as described previously (see B, page 82). Location of the band corresponding to the acid on chromatograms was achieved by means of spraying a narrow strip of the chromatogram and cutting out the band matching the colored spot of the acid on the sprayed strip.

p-Hydroxybenzoic acid, eluted from chromatograms, was crystallized three times from water after the addition of 40 mg of non-radioactive p-hydroxybenzoic acid as carrier. The crystalline material was filtered through a very small sintered glass funnel of fine porosity, washed with cold water and dried at 40° C in a heated vacuum desiccator for 24 hours.

In the same manner, vanillic and syringic acids were isolated from extracts of wheat shoots administered ferulic acid-3-^{14}C and sinapic acid-3-^{14}C respectively.

E. Decarboxylation of the Hydroxybenzoic Acids

The copper chromite method of Ruggli and Brandt (1944) was used. Fifty to 100 mg of the radioactive hydroxybenzoic acid were transferred to a conical flask together with 3 ml of freshly distilled quinoline and 25 mg of copper chromite. Nitrogen was bubbled through slowly during the reaction period and the mixture boiled for 30 minutes using a microflame. The $^{14}CO_2$ evolved was trapped in 2 tubes containing $Ba(OH)_2$ solution. After cooling, the suspension of $BaCO_3$ was filtered through a sintered glass funnel and the precipitate of $BaCO_3$ was washed rapidly with copious amounts of water. The funnel and contents were dried in an oven at 105° C overnight after which they were transferred to a vacuum desiccator. The specific activity of the $BaCO_3$ was determined, after weighing, by means of the Liquid Scintillation Spectrometer. Corrections were made for the amount of $BaCO_3$ by determinations of blank runs.

X. RADIOACTIVITY, DETECTION AND MEASUREMENT METHODS

A. Autoradiography

Autoradiography was used for detection of radioactive spots on two-directional chromatograms of plant extracts after administration of carbon-14 compounds. Chromatograms were developed in such a way that the solvent fronts in both directions were confined to an area measuring 14 x 17" which approximates the size of the X-ray film used (Kodak, no screen, rapid X-ray film). Dried chromatograms were cut to this size and marked at their corners with radioactive ink. They were placed in immediate contact with the film in a Kodak, X-ray exposure holder for a period of 2 to 6 weeks depending on the degree of radioactivity in the chromatographed extract. After the required exposure period had elapsed, the film was developed using Kodak rapid X-ray developer for 5 minutes followed by washing in tap water for 1 minute and fixing in a Kodak fixative solution for 10 minutes. The developed autoradiograph was washed in tap water for 2 to 3 hours and hung to dry. The dark spots on the autoradiograph represented radioactive substances on chromatograms.

B. The Strip Scanner

Strips of Whatman No. 1 chromatography paper having a width of 1-1/2" were used for one-directional chromatography by the descending method. The dried chromatostrip was passed, at a regulated speed, under a windowless Geiger-Müller tube attached to an actigraph 11 (Nuclear Chicago Model 1620 B Analytical Count Ratemeter). The radioactivity was recorded on a Texas recorder (Model PRR, Texas Instruments Incorporated). This method was particularly useful for checking the purity of carbon-14 compounds before administration as well as for a quick determination of the distribution pattern of radioactivity in plant extracts.

C. Oxidation of the Radioactive Material to $^{14}CO_2$ and Measurement of $^{14}CO_2$ Activity

A measured sample (10 to 25 mg) of the dried residue, obtained from either ethanol-soluble or insoluble fractions, was oxidized to CO_2

with Van Slyke reagent (Van Slyke et al., 1951). The oxidation was carried out in a special pyrex glass vial connected to a collecting system leading to an evacuated stainless steel ion chamber. Heat was applied to the combustion vial by means of a small electric heater (hot spotter) for 20 minutes during which complete oxidation of the sample was achieved. The heat source was removed and the remaining $^{14}CO_2$ in the collecting system was flushed into the ion chamber with a slow stream of non-radioactive CO_2 from a cylinder. The radioactivity of the collected $^{14}CO_2$ was measured in the gas phase with a Dynacon (Nuclear Chicago Model 600 dynamic condenser electrometer) equipped with a recorder. Samples were combusted in duplicates with an error of less than 4%.

D. Liquid Scintillation Spectrometry

This method was used for counting radioactivity of individual compounds isolated from plant extracts by means of chromatographic procedures.

1. Scintillation liquids: The following solutions were used (Harlan, 1961).

a. consisted of 3000 ml toluene, 12 g PPO (2,5-diphenyloxazole) and 0.9 g POPOP [1,4-bis-2-(5-phenyloxazolyl)-benzene].

b. consisted of 2400 ml toluene, 1512 ml absolute ethanol, 16 g PPO and 0.6 g POPOP.

2. Counting of samples: Direct counting of radioactive spots cut

out from paper chromatograms was achieved by placing the paper spot in a counting vial and adding 15 ml of Liquid a. Spots eluted from chromatograms were made to a measured volume with absolute ethanol. A known amount was carefully pipetted into a counting vial and dried under a fine filtered stream of dry air and 15 ml of Liquid b were added.

$^{14}CO_2$ collected after administration of $^{14}CO_2$ in photosynthesis, as well as CO_2 produced by decarboxylation of the hydroxybenzoic acids was trapped and counted as $Ba^{14}CO_3$. A known weight of $Ba^{14}CO_3$ was placed in a counting vial. The vial was loosely filled with Cab-o-sil and 15 ml of solution "a" were added. The vials were tightly capped immediately after preparation and whenever Cab-o-sil was used, they were shaken gently to ensure a stable uniform suspension of the radioactive material in a transparent gel free of air bubbles. All vials were cooled down in a freezer for a minimum period of 30 minutes before counting.

Radioactivity measurements were carried out in a Tri-Carb, Series 314A, Liquid Scintillation Spectrometer. Vials were counted at a high voltage setting of maximum efficiency which was found to vary depending on the contents of the counting vials. In every case sample counting was preceded by a standardization of the machine and determination of the counting efficiency as well as the background count. Duplicate samples were counted twice and the mean of the 4 readings was corrected for the efficiency of the machine after subtraction of the background count.

EXPERIMENTAL AND RESULTS

I. DEVELOPMENT OF THE QUANTITATIVE PROCEDURE

An essential preparatory step in studying the biosynthesis of the phenolic acids was the development of a method for the quantitative determination of these compounds in extracts of plant tissues. A chromatographic-spectrophotometric method was developed and a brief presentation of the main steps which were carried out for development of the method is described below.

A. Preparation of Standard Curves for the Phenolic Acids and Phenolic Aldehydes

The standard curves were prepared as described previously (see page 80) for ferulic, sinapic, p-coumaric, 2-hydroxy-3-methoxycinnamic, p-hydroxybenzoic, vanillic and syringic acids as well as for p-hydroxybenzaldehyde, vanillin, and syringaldehyde. These phenolic acids were found, in preparatory experiments, to be the major phenolic acids in wheat plants. The aldehydes are obtained together with other products from alkaline nitrobenzene oxidation of lignin of wheat (Brown, 1961b). The standard curves of the abovementioned compounds are shown in Figure 12 and the wavelength of maximum absorption of each compound is shown below:

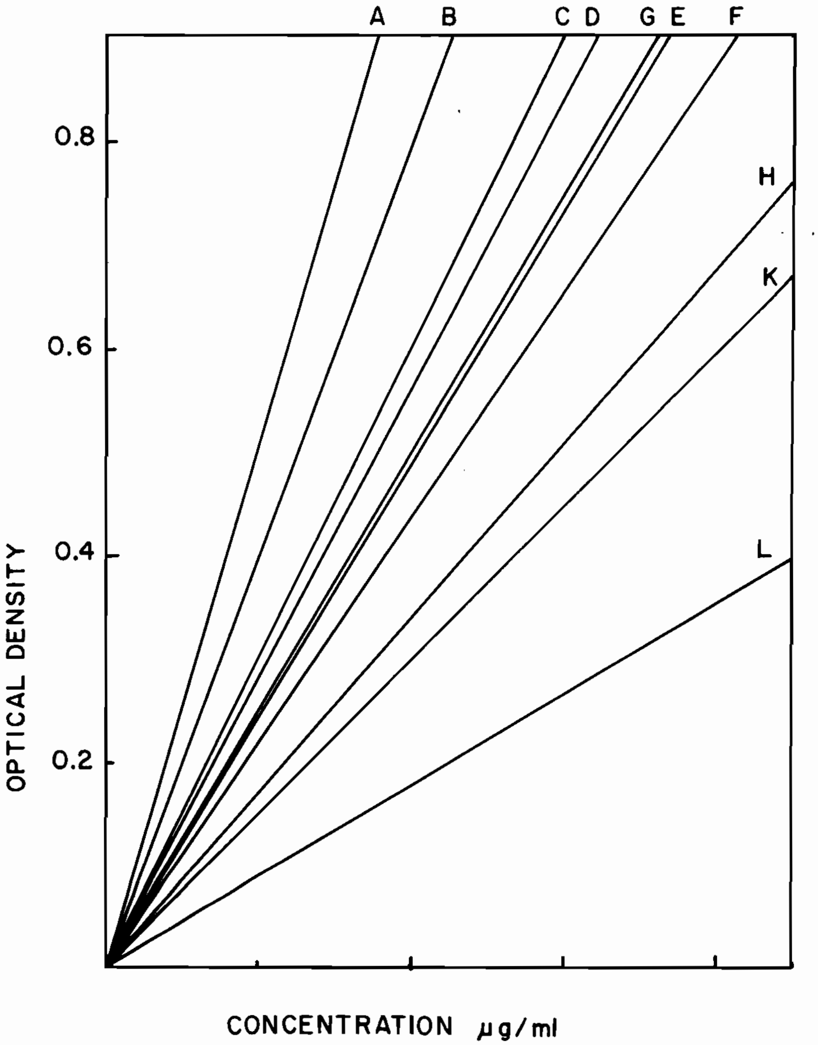


Figure 12. Standard curves of some phenolic acids and aldehydes.

A = p-Hydroxybenzaldehyde
B = Vanillin
C = Syringaldehyde
D = p-Coumaric Acid
E = Ferulic Acid
F = Sinapic Acid
G = p-Hydroxybenzoic Acid
H = Vanillic Acid
K = Syringic Acid
L = Orthoferulic Acid

Phenolic compound	Wavelength of maximum absorption (mμ)
p-Coumaric acid	333
Ferulic acid	345
Sinapic acid	354
2-Hydroxy-3-methoxy-cinnamic acid	369
p-Hydroxybenzoic acid	282
Vanillic acid	298.5
Syringic acid	302
p-Hydroxybenzaldehyde	336
Vanillin	354
Syringaldehyde	370

B. Determination of the Percentage Recovery from Chromatograms

Solutions containing 200 μg/ml of authentic samples of ferulic, sinapic, p-coumaric, vanillic and syringic acids were made in absolute ethanol. Measured volumes of each solution (0.05 to 0.5 ml) were chromatographed two-directionally on large sheets of Whatman No. 1 paper in the benzene-acetic acid-water and formic acid solvents. The spots corresponding to the compounds were cut out, eluted and their optical densities were determined as described previously.

A total of twelve determinations for each acid representing four levels of concentrations (5 μg to 100 μg per chromatogram) were carried out. The percentage recoveries for each acid were calculated and the averages were 97% for p-coumaric, ferulic, and sinapic acids and 92% for vanillic and syringic acids.

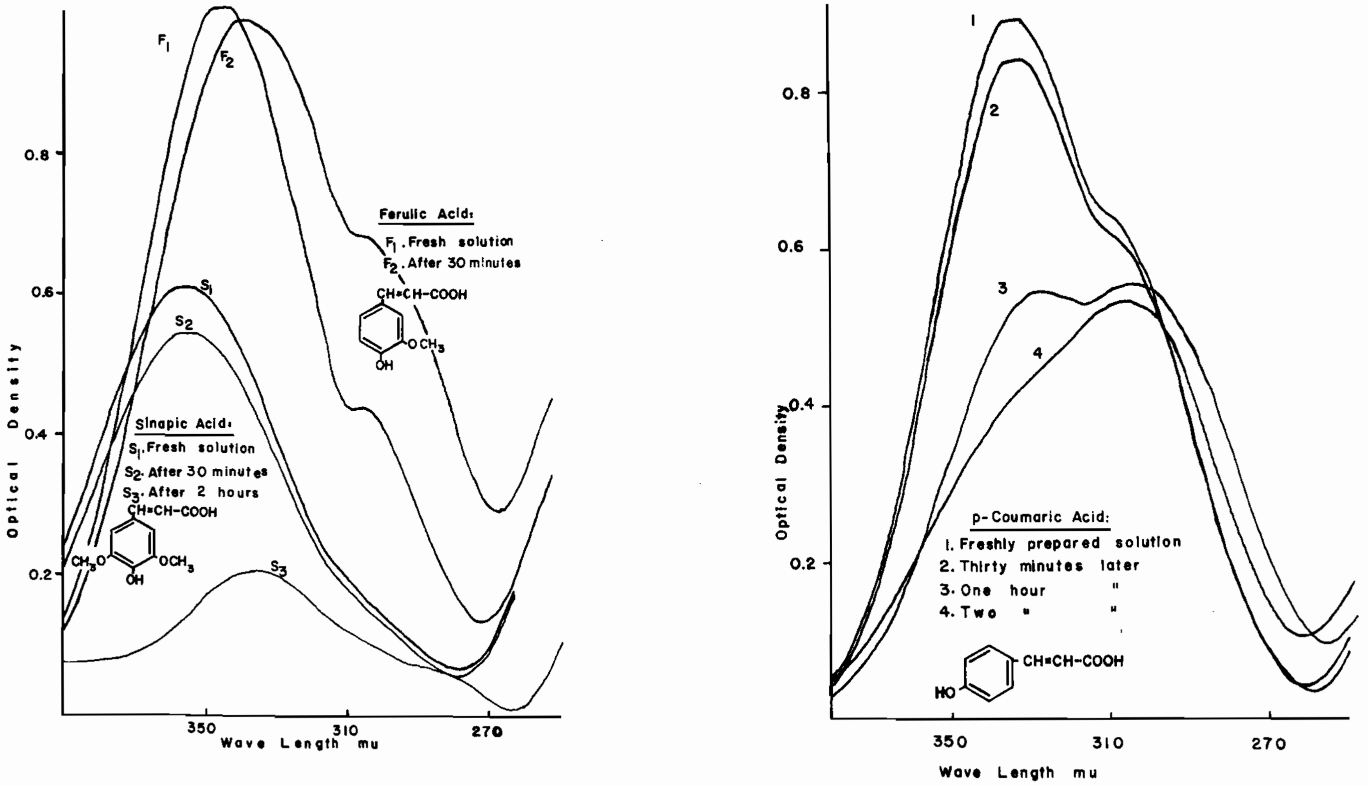
C. The Effects of Ultraviolet Irradiation in the Presence of NH_3 Fumes on Absorption Spectra of the Phenolic Acids

During the development of the quantitative procedure, chromatograms of phenolic acids were usually examined under ultraviolet light before and after exposure to ammonia vapours since p-coumaric acid is fluorescent only under the latter conditions. A spectrophotometric study of compounds eluted from chromatograms treated with ammonia fumes while exposed to ultraviolet light revealed great variations in the absorption spectra of the hydroxycinnamic acids whereas the hydroxybenzoic acids were apparently stable to this treatment.

A gradual hypsochromic shift of the wavelength of maximum absorption of p-coumaric acid was found to take place from 333 mμ to 296 mμ. The transformation began within thirty minutes following irradiation of chromatograms exposed to ammonia vapours and was complete in two hours. The absorption spectrum of non-irradiated p-coumaric acid was also found to undergo this change albeit at a much slower rate. The transformation became apparent within two to three hours and it took about twelve hours for the new absorption maximum at 296 mμ to fully develop. Similar changes were also shown by sinapic acid. Ferulic acid was the least affected of the three hydroxycinnamic acids. However, the absorption maximum at 345 mμ was shifted and replaced by another maximum at 339 to 340 mμ with considerable decrease in optical density. Normal and transformed absorption spectra of these three acids are shown in Figure 13.

As a consequence of the aforementioned observations, the exposure of chromatograms to ammonia vapours was completely eliminated. p-Coumaric acid was henceforward detected by its quenching behaviour under ultraviolet light of 2537 Å. Irradiation of chromatograms under non-alkaline conditions was proven to be ineffective in inducing these changes. Further studies along these lines were considered beyond the scope of the present investigation and were not attempted.

Figure 13. Spectral changes of hydroxylated cinnamic acids after irradiation with ultraviolet light and exposure to ammonia fumes.



D. Outlines of the Quantitative Procedure

The method for the extraction of phenolic acids from plant materials has already been described (see Material and Methods). Ether extracts of phenolic acids were chromatographed two-directionally in the benzene-acetic acid-water solvent followed by the formic acid solvent. The acids were eluted from chromatograms and their optical densities were determined. The amount of each acid was calculated from standard curves and corrected for losses in chromatography. Results were expressed in micrograms per gram of extractive free dry weight.

II. SURVEY OF THE PHENOLIC ACIDS IN WHEAT PLANTS

A. The Phenolic Acid Conjugates in Ethanolic Extracts of Wheat Shoots

Of the well-known phenolic acids only trace amounts of p-coumaric and ferulic acids could be detected as occurring in the free state in ethanolic extracts of wheat shoots. Chromatograms of non-hydrolyzed extracts indicated the presence of a complex mixture of fluorescent and non-fluorescent compounds of which many gave color reactions for phenols. A two dimensional thin layer chromatoplate of a hot water extract is illustrated in Figure 14.

Six hundred grams of fresh shoots obtained from 25-day old wheat plants grown by Method A were homogenized and extracted thoroughly with ethanol. The ethanolic extracts were evaporated and the residue was taken up in boiling water and filtered through Celite. The hot water extract was concentrated in vacuo at 50° to a thick brown syrup.

Fig 14

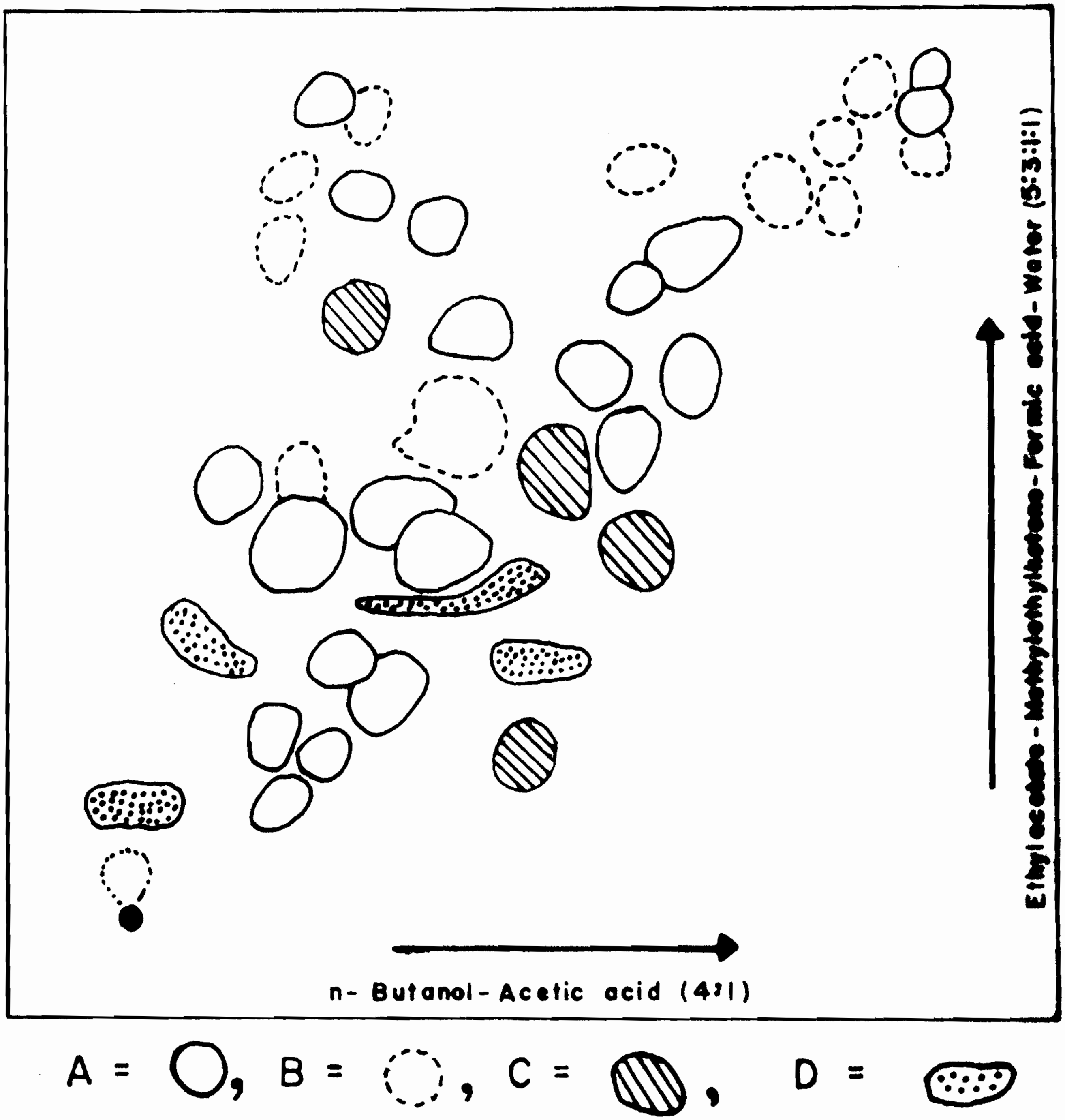


Figure 14. Map of a two-directional chromatoplate of an unhydrolyzed ethanolic extract of wheat shoots.

Key:

A = Fluorescent spots under UV, giving color reaction for phenols.
B = Fluorescent spots under UV but not giving color reactions for phenols.
C = Non-fluorescent spots giving color reactions.
D = Flavonoid compounds.

This was subsequently streaked on several sheets of Whatman No. 3 paper and chromatographed in the butanol-acetic acid-water solvent. Examination of the developed chromatograms under ultraviolet light indicated the presence of altogether nine fluorescent and three absorbing bands. Each band was eluted twice with 80% aqueous ethanol at room temperature. The combined eluates of each band were dried in vacuo at 50° C and redissolved in a small volume of 80% ethanol, purified by chromatography once more in the same solvent system and eluted. An aliquot of each band was subjected to alkaline hydrolysis. The hydrolyzates were acidified and extracted by shaking with small volumes of ether and the ether extracts were chromatographed two-directionally for their phenolic acid content.

The results indicated the possibility that some of these compounds were still mixtures. This was confirmed by carrying out two-directional chromatography of an unhydrolyzed aliquot of each band in the butanol-acetic acid-water solvent for the first direction followed by the formic acid solvent in the second direction. In all cases from one to three, and sometimes as many as five, compounds were detectable on these chromatograms. The individual major spots were cut out and eluted with 80% ethanol at room temperature. The ultraviolet absorption spectra of some of the eluates were determined before and after the addition of two drops of 2 N NaOH solution. Alkaline hydrolysis of the eluates was also carried out and the hydrolyzates were chromatographed for their phenolic acids. The results are summarized in Table III and a map of a two-dimensional chromatogram containing the major spots is illustrated in Figure 15.

Table III

Phenolic Acid Conjugates in Unhydrolyzed Extract of Wheat Shoots

Spot* number	Fluorescence U.V.		U.V. spectra (mμ)		Hydrolysis products
	Without NH_3	With NH_3	Ethanol	Plus NaOH	
1	blue	green	326	343	ferulic acid
2	blue	green	324	342	ferulic acid
3	blue	brightens	330	349	ferulic acid
4	none	none	263	285	p-hydroxybenzoic acid
5	none	absorbs	272	301	vanillic acid
6	none	none	285	306	syringic acid
7	none	dark blue	310	335	p-coumaric acid
8	none	blue	316	338	p-coumaric acid
9	blue green	brightens	322	366	caffeic acid
10	blue	brightens	332	359	sinapic acid
U	-	-	-	-	unidentified mixture

* See Figure 15.

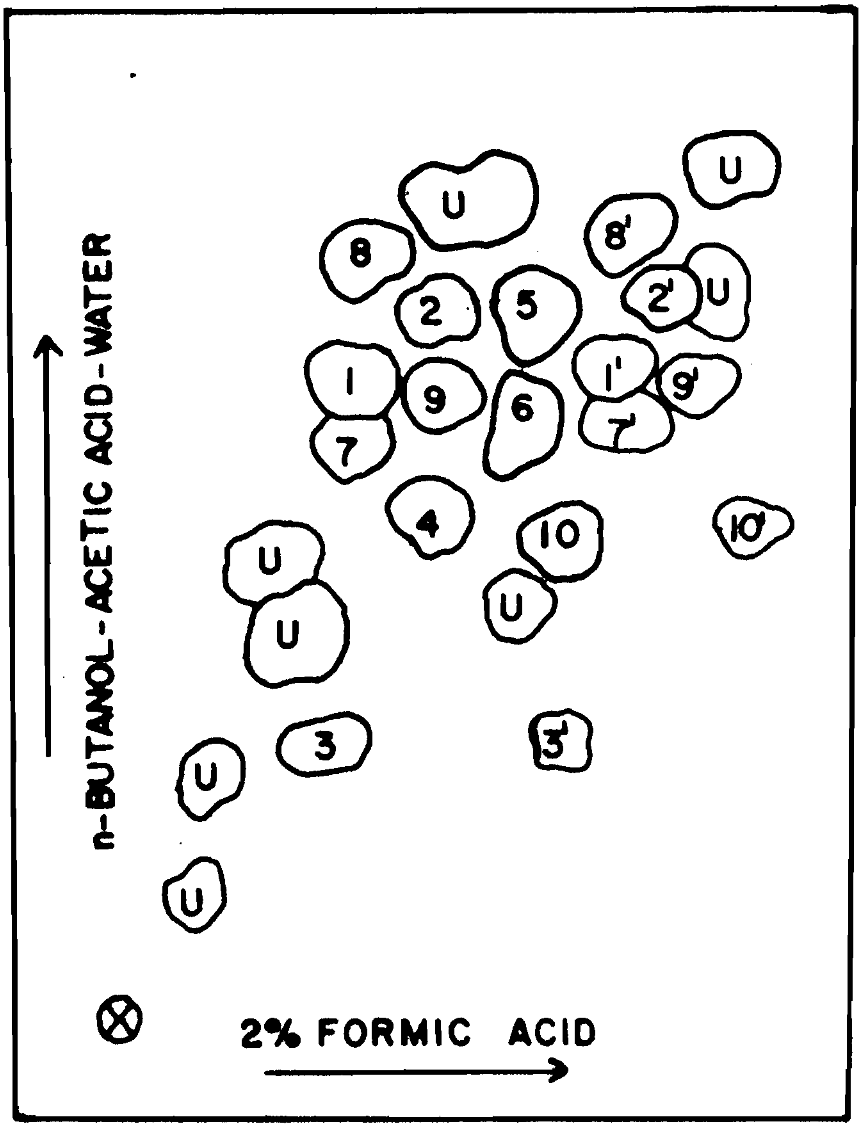


Figure 15. Map of two-directional chromatogram showing the major spots in unhydrolyzed extract of wheat shoots.
Key: See Table III

In this way, ferulic acid was identified as a component acid of three different conjugates, p-coumaric acid of two conjugates and each of sinapic, caffeic, p-hydroxybenzoic, vanillic and syringic acids as well as vanillin of one conjugate. Identification of the nonphenolic moiety(ies), however, was not attempted due to the small amounts isolated. The possibility that some of these acids occur as components of still other conjugates not described in Table III cannot be excluded. There were indications that some of these acids were present in the "unidentified mixture" in Table III, although the amounts did not allow for critical identification.

A second attempt was made, with the aim of isolating some of these compounds in reasonable quantities for identification, using twice the weight of plant material. A preliminary fractionation of the concentrated hot water extract was carried out by chromatography on a charcoal-Celite column (25 x 300 mm). The non-aromatic compounds were eluted with one litre of water and discarded. Subsequently the following fractions were collected in approximately 200 ml volumes of each; 20% and 50% aqueous ethanol, absolute ethanol, 50% ethanol in benzene and absolute benzene. Individual fractions were subjected to repeated thin layer chromatography on silica gel plates employing the butanol-acetic acid and the ethylacetate-methylethylketone-formic acid-water solvent systems.

This attempt resulted in the isolation of a total of 53 apparently different bands containing phenolic compounds. The amount of material obtained from each band was again no better than in the previous attempt and consequently this type of analysis was abandoned.

B. The Phenolic Acids in Hydrolyzates of Ethanolic Extracts of Various Organs of the Wheat Plant

Germinating and non-germinated grains, whole embryos (8 to 10 mm in length), green influorescences and roots and shoots at various stages of growth and development of the plant were analyzed and surveyed for their phenolic acid contents. Except where otherwise stated, 400 embryos, 20 g fresh weight of roots and 40 g fresh weight of shoots, green influorescences or grains were used for each analysis.

A total of thirty-four phenolic compounds could be detected on chromatograms of alkaline and acid hydrolyzates of ethanolic extracts of tissues of various organs. Most of the prominent ones were identified as hydroxylated benzoic and cinnamic acids in addition to the flavonoid compound tricin. Many of these compounds could only be detected at certain stages of growth and development of the plant, and furthermore their distribution appeared to have been influenced by the method of cultivation used for growing the experimented plants. Figure 16 illustrates a chromatographic map of the readily detectable phenolic compounds in hydrolyzates of ethanolic extracts of wheat tissues.

The distribution of the identified phenolic acids and their related compounds in shoots of various ages obtained from plants grown by the two methods of cultivation is given in Tables IV and V. The distribution of those identified in all the other organs together with the behaviour of these compounds in ultraviolet light and their color reactions with Fast Bordeau Red B.D. (FBR) are shown in Table VI.

Many unidentified phenolic compounds were encountered in various analyses and sometimes in relatively large quantities and there are indications that some of them were phenolic acids. A brief

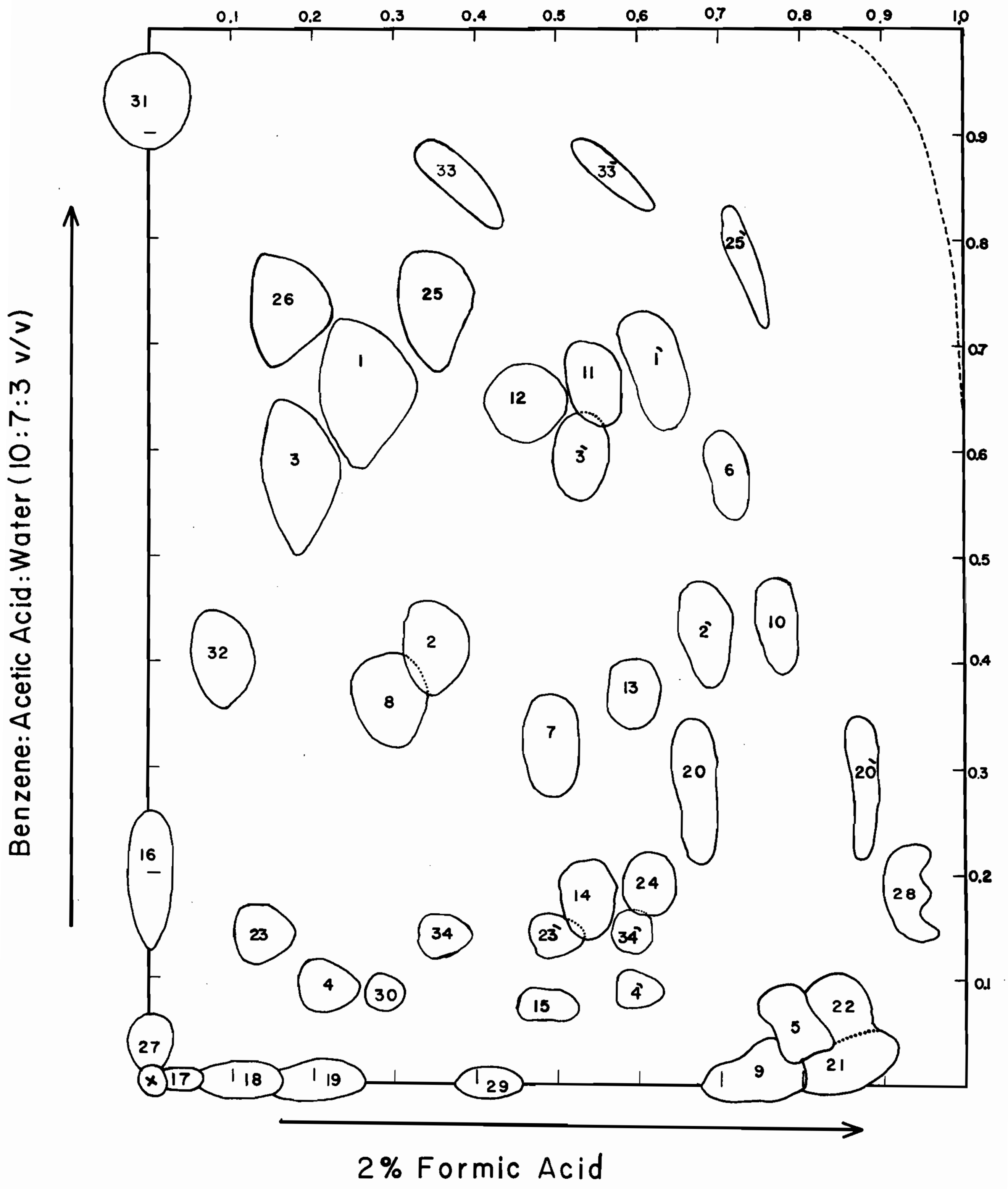


Figure 16. A chromatographic map of phenolic compounds in hydrolyzates of ethanolic extracts of wheat tissues.

Table IV

Phenolic Acids and Related Compounds Identified from Shoots of Wheat Plants Grown by Method A (Nutrient Solution)

No.	Phenolic compound	Embryos 8-10 mm in length	Shoots (days)						
			5	9	15	25	35	47	57
2	*p*-Coumaric acid	+	+	+	+	+	+	+	+
4	Caffeic acid	+	t	t	t	t	t	t	t
1	Ferulic acid	+	+	+	+	+	+	+	+
3	Sinapic acid	+	+	+	-	+	+	+	+
5	*p*-Hydroxyphenyl-lactic acid	+	+	+	+	x	x	x	x
7	Umbelliferone	-	-	+	+	-	-	-	-
8	Scopoletin	-	-	-	+	-	t	-	+
10	*p*-Hydroxyphenyl-acetic acid	-	-	-	-	-	-	-	+
9	Homogentisic acid	+	+	+	+	+	+	+	+
13	*p*-Hydroxybenzoic acid	t	+	+	+	+	+	+	+
15	Protocatechuic acid	+	-	-	-	-	-	-	-
14	Gentisic acid*	+	+	+	+	+	+	+	+
11	Vanillic acid	+	+	+	+	+	t	+	+
12	Syringic acid	+	+	+	+	+	t	+	+
16	Tricin	-	t	+	t	+	+	+	+

Note: +, detected on chromatograms; -, not detected on chromatograms; t, detected in trace amounts; *, obtained on acid hydrolysis only; x, its presence could not be ascertained.

Table V

Phenolic Acids and Related Compounds Identified from Shoots of Wheat Plants Grown by Method B (Soil)

No.	Phenolic Compound	Shoots (days)						
		25	35	45	55	65	75	100
2	*p*-Coumaric acid	+	+	+	+	+	t	+
4	Caffeic acid	t	t	t	-	-	t	t
1	Ferulic acid	+	+	t	t*	t	t	+
3	Sinapic acid	+	-	-	-	-	-	+
6	Melilotic acid	-	-	-	-	-	-	+
8	Scopoletin	-	+	+	-	t	-	-
10	*p*-Hydroxyphenyl acetic acid	-	-	-	-	+	-	+
9	Homogentisic acid	-	+	+	+	+	t	-
13	*p*-Hydroxybenzoic acid	+	+	-	-	-	-	t
14	Gentisic acid*	+	+	+	-	-	+	+
11	Vanillic acid	+	+	+	+	+	+	+
12	Syringic acid	+	+	+	-	-	+	+
16	Tricin	+	+	+	-	-	+	+

Note: *, obtained on acid hydrolysis only; +, detected on chromatograms; -, not detected on chromatograms; t, detected in trace amounts.

Table VI

Phenolic Acids and Related Compounds Identified in Various Tissues of Wheat

Phenolic Compound	Fluorescence in ultraviolet light		Color reaction with FBR	Grains	Inflor-escence	Roots (days)[A]	
	Without NH_3	With NH_3				7	24
p-Coumaric acid	Absorbs	dark blue	violet-purple	+	+	t	+
Caffeic acid	Blue	brightens	bleaching to green	-	t	-	+
Ferulic acid	Blue	brightens	sky blue	+	+	t	t
Sinapic acid	Blue	brightens	light green, fades	+[Ⱥ]	-	-	-
Melilotic acid	none	none	reddish brown	-	+	-	-
p-Hydroxyphenyllactic acid	none	none	dull pink	-	-	-	-
Umbelliferone	bright blue	very bright	light pink	+	-	-	-
Scopoletin	blue	brightens	pink	+	-	-	+
o-Hydroxyphenylacetic acid	none	none	reddish brown	-	+	-	-
Homogentisic acid	none	none	light brown	+	+	-	t
Salicylic acid	pale blue	same	yellow-orange	-	-	t	+
p-Hydroxybenzoic acid	none	"	orange	+	-	+	+
Gentisic acid[Ⱥ]	bright blue	brightens	bleaches	+	+	+	t
Protocatechuic acid	none	none	bluish-gray	-	-	-	+
Vanillic acid	"	"	bright pink	+	+	+	+
Syringic acid	"	"	violet	+	+	-	-
Tricin	Absorbs	golden	brown	+	t	-	t

Note: +, detected on chromatograms; -, not detected on chromatograms; t, detected in trace amounts;

Ⱥ, obtained on acid hydrolysis only; A, taken from plants grown by method A (nutrient solution).

description of some of these compounds and their distribution in tissues of various organs of the wheat plant are given in Table VII. One of them was successfully isolated and identified as a new cinnamic acid derivative as will be described below (see page).

C. The Phenolic Acids in Alkaline Hydrolyzates of the Ethanol-Insoluble Residue of Wheat Shoot Tissues

The ethanol-insoluble residue of wheat shoot tissues was subjected to alkaline hydrolysis at 30° C (cold alkaline hydrolysis) or on the steam bath (hot alkaline hydrolysis) as described previously. Only three phenolic compounds were detected in appreciable amounts on chromatograms of these alkaline hydrolyzates. Two of the compounds were identified as p-coumaric and ferulic acids. The third major compound (compound No. 20 in Figure 16 and Table VII) could not be identified. Hot alkaline hydrolysis released sinapic, p-hydroxybenzoic, vanillic and syringic acids as well as vanillin and p-hydroxybenzaldehyde in apparently higher yields than did cold alkaline hydrolysis. Syringaldehyde could not be detected in either hydrolyzate. In some analyses a small spot with the characteristic blue fluorescence in ultraviolet light of caffeic acid and giving the same R_f values was detected. A confirmation of the identity of this spot was not possible since it produced no color reaction with FBR or ferric chloride solution. Caffeic acid is readily detectable by these reagents. However, the possibility that the non-reactivity of this spot was due to the small amount present on chromatograms could not be excluded.

Table VII

Description of Some Unidentified Phenolic Compounds in Hydrolyzates of Ethanolic Extracts of Tissues of Wheat*

Compound No.	Fluorescence in ultraviolet light		Color reaction with FBR	Distribution					Remarks
	Without NH_3	With NH_3		Embryos	Shoots	Roots	Inflorescence	Grains	
17, 18 and 19	absorb	golden	brown	+	+	t	+	+	Yellow color, may be flavonoids
20	blue	brightens	pink	+	+	-	+	+	See text
21 and 22	absorb	same	dark brown	-	+	-	+	-	A major compound in shoots
23	blue	brightens	no color	+	+	+	-	-	Two spots, possibly isomers
34	blue	brightens	no color	-	+	-	+	-	"
28	yellow	golden	violet-brown	-	+	-	-	-	
26	green	brightens	no color	-	+	-	-	-	
33	dark blue	brightens	purple	-	+	-	-	+	Two spots, possibly a cinnamyl derivative

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* See Figure 16.

III. QUANTITATIVE CHANGES IN PHENOLIC ACIDS OF BOTH ETHANOL-SOLUBLE AND INSOLUBLE FRACTIONS OF WHEAT SHOOTS

In order to evaluate the reproducibility of the quantitative procedure with plant material, and to determine the variability of the phenolic acid content of plants exposed to identical environmental conditions the following experiment was performed. Four replicates of seeds were germinated by method A under identical conditions at the same time and the shoots were harvested after five days. The amounts of p-coumaric, ferulic, sinapic, vanillic and syringic acids present in alkaline hydrolyzates of the ethanol-soluble fraction were determined. The means and standard error, expressed in μg/g dry weight of extractive-free plant material were calculated and found to be: ferulic acid 1140 ± 45; p-coumaric acid 1030 ± 35; sinapic acid 420 ± 7; vanillic acid 88 ± 3 and syringic acid 81 ± 4. This established that the differences due to the variability in content in these plants together with those due to experimental errors do not exceed 3 to 5% and consequently that the method would be useful.

Quantitative determinations of the five major phenolic acids, ferulic, p-coumaric, sinapic, vanillic and syringic, obtained on alkaline hydrolysis of the ethanol-soluble fraction as well as of the two major acids, ferulic and p-coumaric, released on alkaline hydrolysis of the ethanol-insoluble fraction were made of the following tissues of wheat plants: non-germinated grains, germinating grains after removal of the embryos, whole embryos (8 to 10 mm in length) and shoots of various ages over a period of 57 days following germination. The shoots were from plants grown by method A. Table VIII shows that the grain contained

Table VIII

Quantitative Determinations of Some Phenolic Acids Obtained on Alkaline Hydrolysis of Tissues of Wheat

Phenolic acid	Ungerminated whole grains		Germinating grains (48 hours)				5-day old shoots	
			Grains		Embryos			
	µg per 100	µg per g. dry wt.	µg per 100	µg per g. dry wt.	µg per 100	µg per g. dry wt.	µg per 100	µg per g. dry wt.
Ferulic acid								
Ethanol-soluble	8	3	57	26	44	310	331	1140
Ethanol-insoluble	-	-	396	181	182	1290	139	478
p-Coumaric acid								
Ethanol-soluble	2	1	18	8	37	261	300	1030
Ethanol-insoluble	-	-	0	0	0	0	99	341
Sinapic acid								
Ethanol-soluble	0	0	15	7	14	95	122	420
Syringic acid								
Ethanol-soluble	5	2	18	8	28	197	24	81
Vanillic acid								
Ethanol-soluble	40	14	22	10	38	272	26	88

Note: -, not determined.

very small amounts of the acids, with vanillic acid being the major constituent. Ferulic, p-coumaric, sinapic, vanillic and syringic acids increased in amount in the ethanol-soluble fraction of the embryo as well as in the shoot tissues after germination proceeded. The level of these acids increased with time (Figures 17 and 18), and, with the exception of sinapic acid which was highest in concentration in 5-day old shoots, reached a maximum nine days after germination. Thereafter a marked decrease was apparent.

Although one-day old embryos were found to have synthesized a very large quantity of ferulic acid in the ethanol-insoluble fraction, p-coumaric acid could not be detected (Table VIII). The largest amounts of these two acids, however, were obtained from the ethanol-insoluble fraction of shoots with considerable changes in their levels at various stages of growth and development (Figure 19).

IV. ISOLATION AND IDENTIFICATION OF A NEW MONOHYDROXYMONOMETHOXY-CINNAMIC ACID DERIVATIVE

Among the unidentified phenolic compounds a spot, giving a bright blue-green fluorescence in ultraviolet light and with R_f 0.75 in the benzene-acetic acid-water solvent, was observed on chromatograms of acid- or alkaline hydrolyzates of ethanolic extracts of some wheat shoots (compound No. 25 in Figure 16). The fluorescence was intensified with NH_3 vapours and the spot gave a red color with FBR spray reagent. Chromatography of this compound in the formic acid solvent gave two spots, a major one with R_f 0.36 and a minor one with R_f 0.73. Both spots were

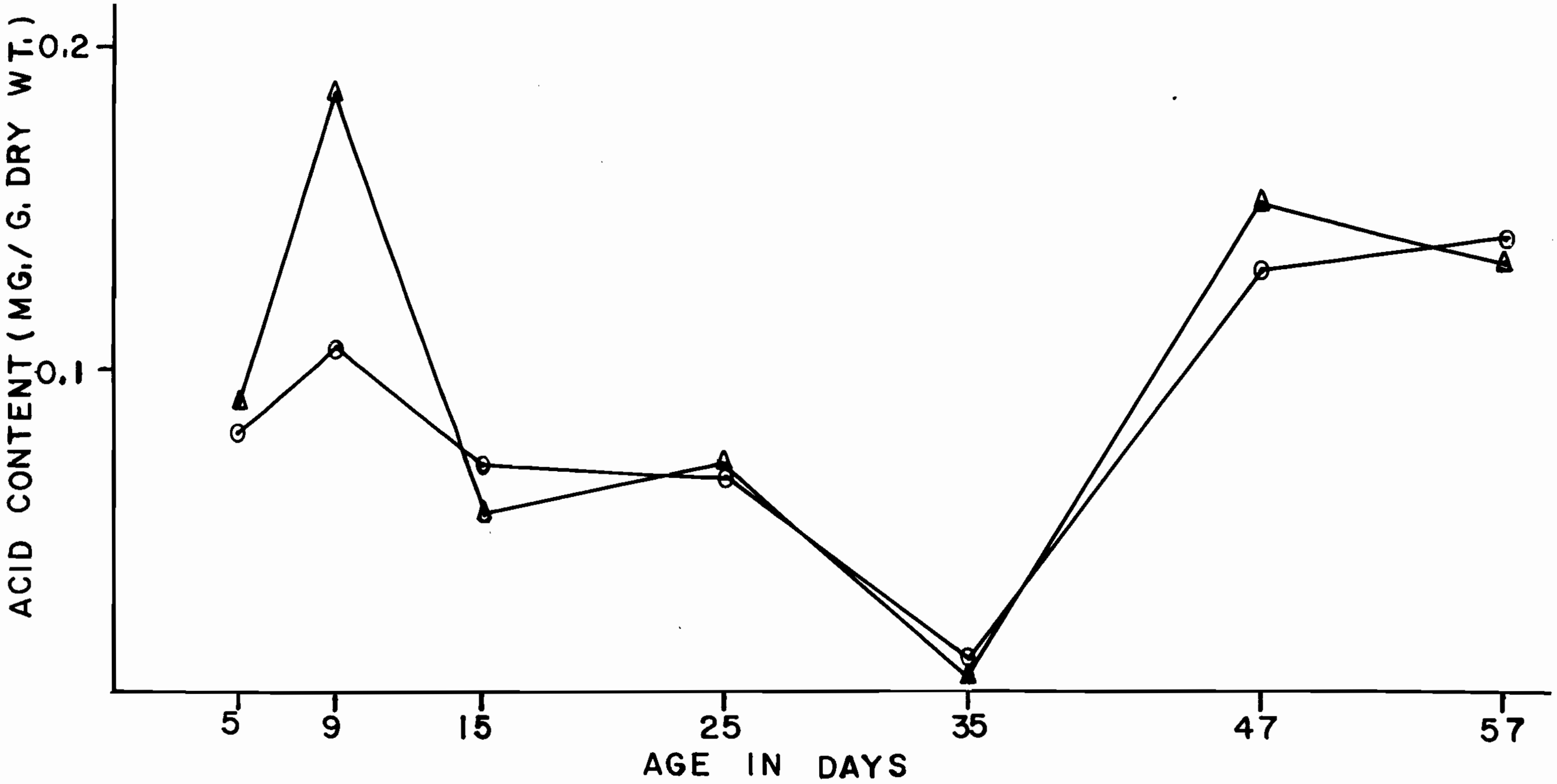

Figure 17. Changes with time in concentration of hydroxylated benzoic acids in the ethanol-insoluble fraction of wheat shoots. △, vanillic acid; ○, syringic acid.

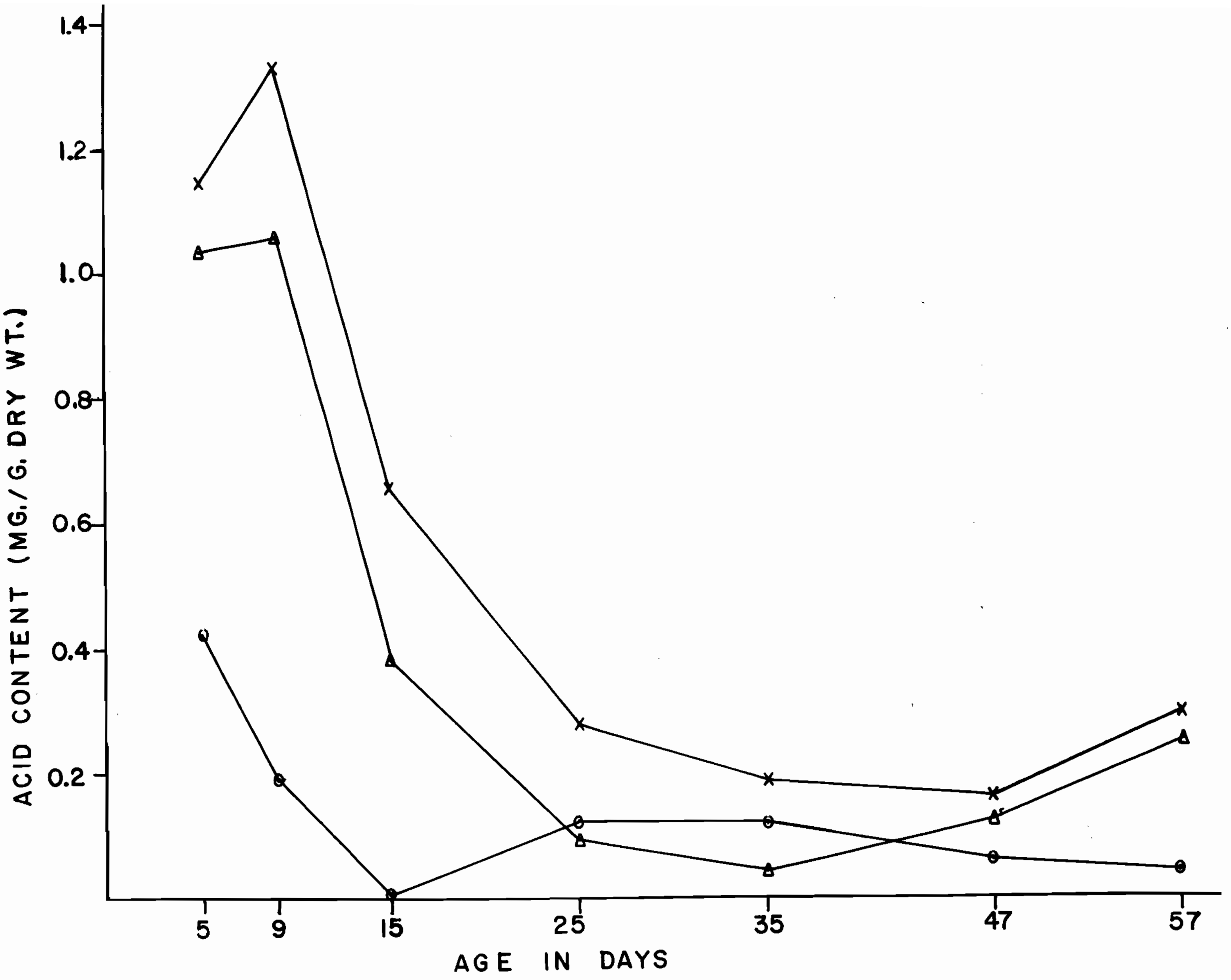

Figure 18. Changes with time in concentration of hydroxylated cinnamic acids in the ethanol-soluble fraction of wheat shoots. X, ferulic acid; Δ, p-coumaric acid; o, sinapic acid.

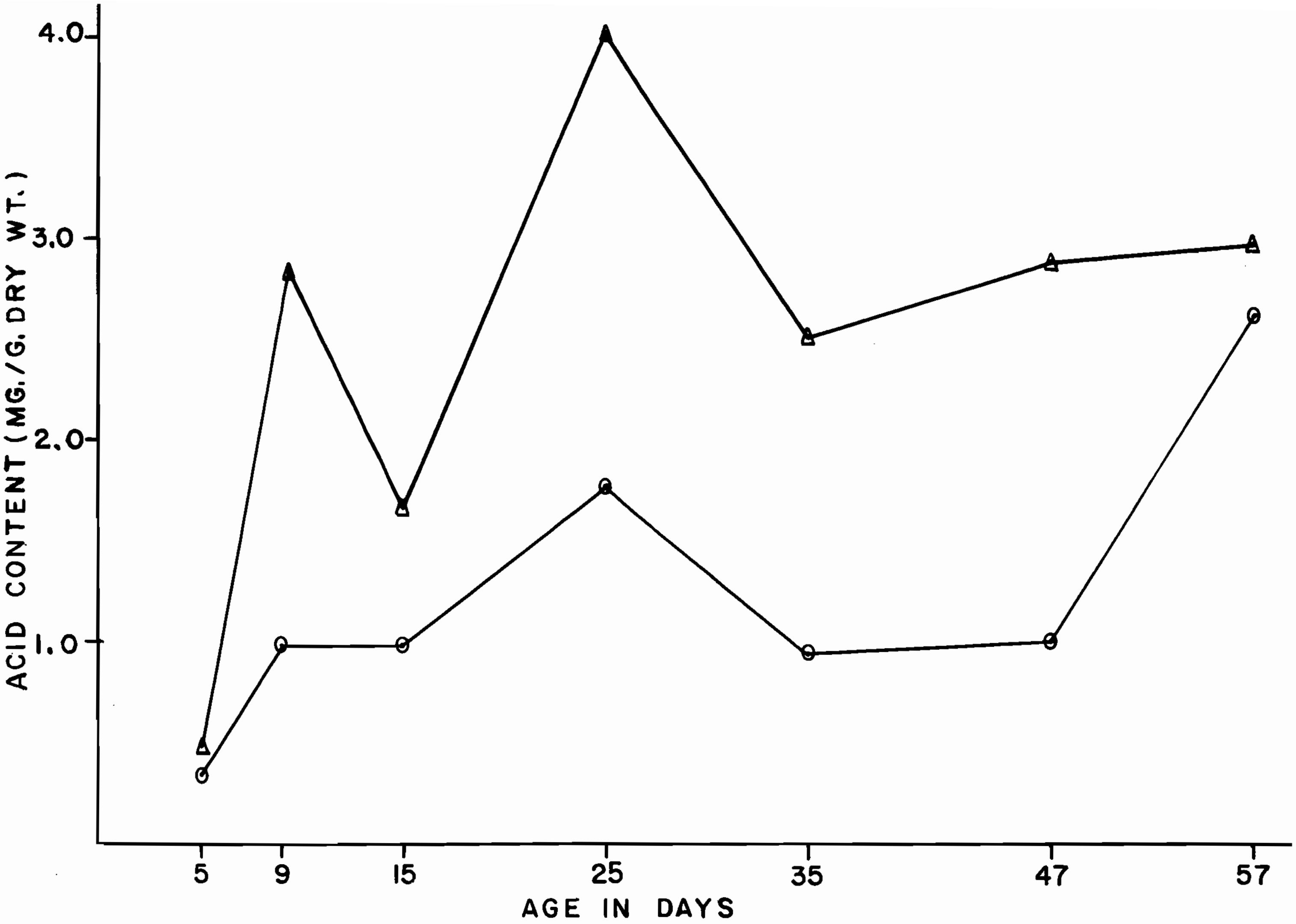

Figure 19. Changes with time in △, ferulic and ○, p-coumaric acids in the ethanol-insoluble fraction of wheat shoots.

fluorescent in ultraviolet light and gave the same color reaction with the FBR reagent. The resolution of the compound into two spots giving an identical color reaction with the phenol reagent suggested that it was a cinnamyl derivative. The cis- and trans-isomers of cinnamic acid as well as of its derivatives obtained by ring substitution are resolved very strikingly in the formic acid or in other aqueous solvents.

Three hundred grams of shoots obtained from 19-day old plants grown by method A were homogenized in hot ethanol in a Waring Blendor and thoroughly extracted with ethanol under reflux on the steam bath. The combined extract was processed and the compound was isolated as described previously (see page 82). The brown residue isolated by chromatography was crystallized from hot water, after the addition of a pinch of charcoal to give almost 5 mg of needles m.p. 175 to 176.

The similar mobility of the compound to ferulic and isoferulic acids on chromatograms (Figure 20) suggested that it could be a monohydroxymonomethoxycinnamic acid. Its ultraviolet absorption spectra in ethanol and in ethanol plus NaOH (Table IX and Figure 21) also indicated that it is closely related to these compounds. It was compared with authentic orthoferulic (2 hydroxy-3-methoxycinnamic) acid and the two compounds were found to be indistinguishable chromatographically in the benzene and the formic acid solvents. Authentic orthoferulic acid melted at 183 to 184° and a mixed melting point of 181 to 182° was obtained. Further purification of the isolate was not attempted but an infrared spectrum was obtained. The spectrum was essentially identical to that of orthoferulic acid (Figure 22) thus establishing its structure.

Orthoferulic acid was detected only in hydrolyzates of wheat shoots. The amounts at different stages of growth are shown in Table X.

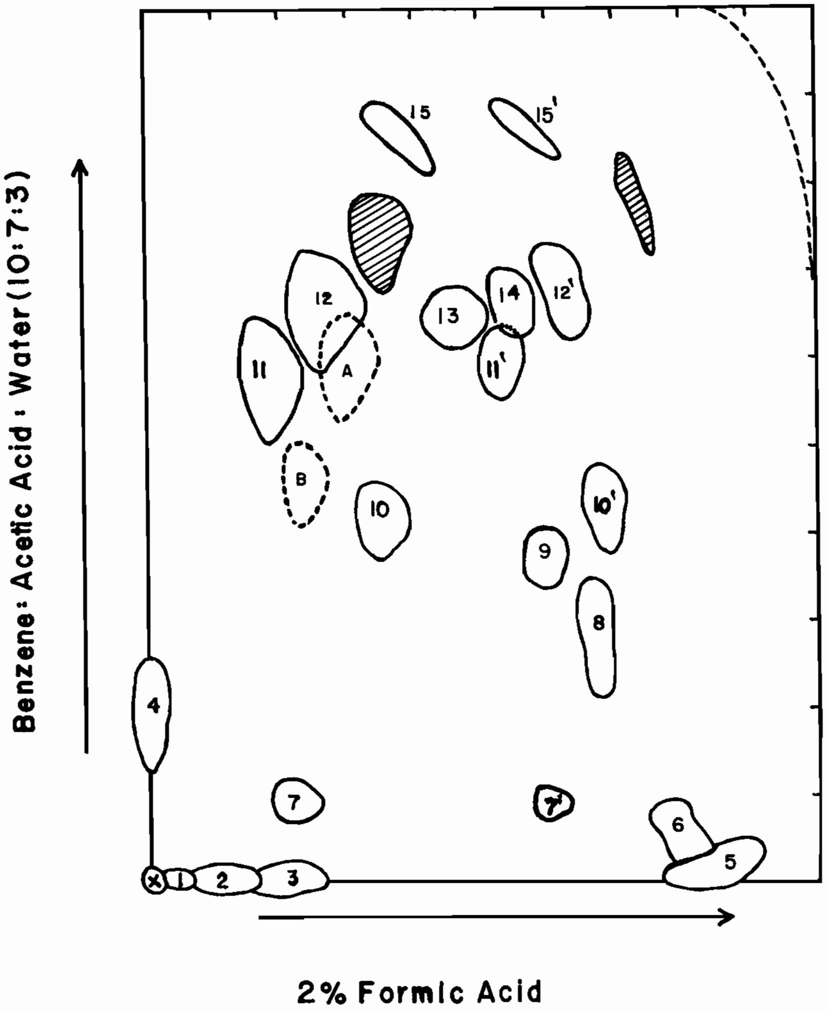

Figure 20. A chromatographic map showing the relative positions of some monohydroxymonomethoxy cinnamic acids to other phenolic constituents of wheat shoots.
Key: 12, Ferulic acid; large shaded area, orthoferulic acid; small shaded area, 8-methoxycoumarin, A and B, added isoferulic acid and 2-hydroxy-4-methoxycinnamic acid respectively.

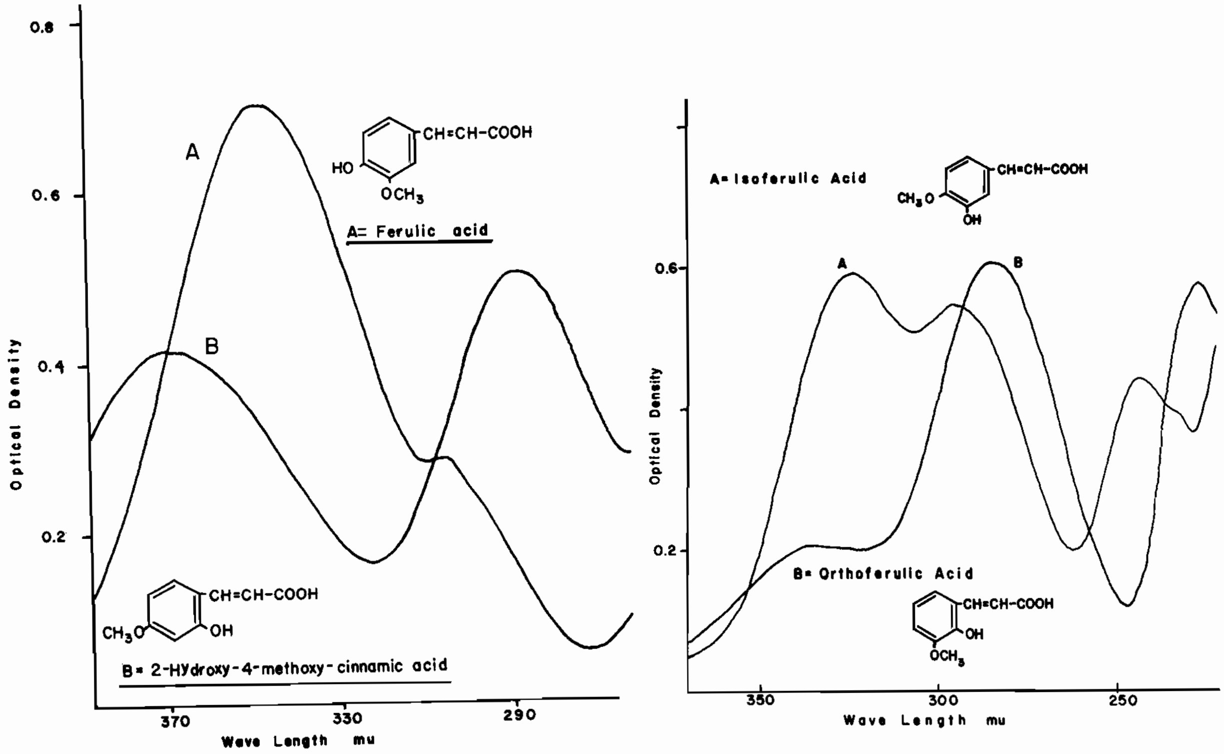

Figure 21. Ultraviolet absorption spectra of some monohydroxymonomethoxycinnamic acids. Solvent = 0.05N aqueous NaOH.

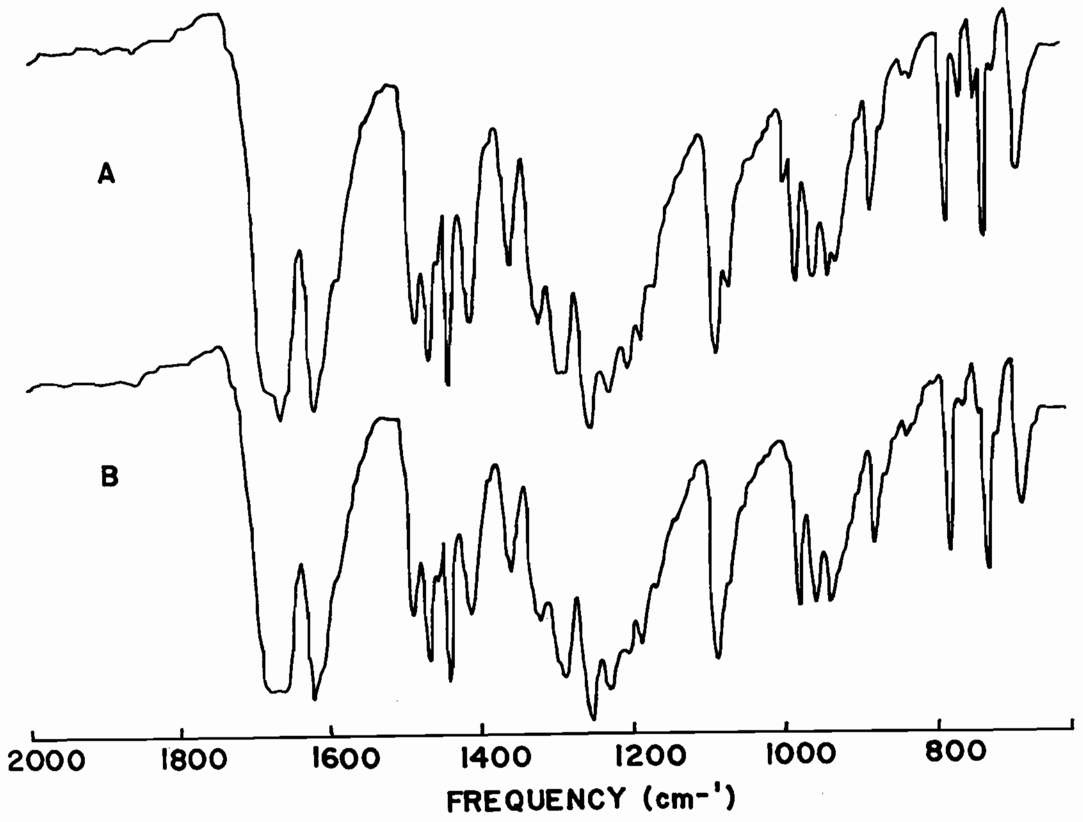

Figure 22. Infrared spectra of A, authentic orthoferulic acid; B, compound isolated from wheat extracts.

Table IX

Ultraviolet Absorption Spectra of Some Monohydroxymonomethoxy Cinnamic Acids

Compound	Ethanol		NaOH - Ethanol	
	λmax (mμ)	λmin. (mμ)	λmax (mμ)	λmin (mμ)
Orthoferulic acid	227, 285, 332	246, 321	242, 287, 369	265, 320
Isolated compound	227, 285, 332	246, 321	242, 287, 309	265, 320
2-Hydroxy-4-methoxy-cinnamic acid	220, 290, 329	258, 305	233, 387, 362	269, 313
Isoferulic acid	243, 295, 324	262, 306	259, 292, 344	279, 318
Ferulic acid	236, 299*, 324	262	241, 306*, 348	271

* Inflection

Table X

Orthoferulic Acid Content of Wheat Shoots

	A									B			
Age of plants (days)	2	5	7	15	19	25	35	47	57	65	75	85	100
Amounts mg/g.d.w.	0	t	0.15	0.30	0.41	0.23	t	t	0	0	0	0	0

Note: t, indicates a trace on chromatograms; A, plants germinated by method A (nutrient solution);

B, plants germinated by method B (soil).

The faster moving fluorescent spot (R_f 0.73 in formic acid) was usually detected on chromatograms in which orthoferulic acid was present. It was tentatively identified as 8-methoxycoumarin, the lactone of the cis-isomer of orthoferulic acid, by its color reaction with FBR, cochromatography with authentic 8-methoxycoumarin and by its formation from orthoferulic acid. This spot was detected on chromatograms which were exposed to ultraviolet light only after being developed in the two solvent systems (benzene-acetic acid-water and formic acid). It was unlikely therefore that it was an artifact resulting from ultraviolet irradiation as may be suspected.

STUDIES ON THE BIOSYNTHESIS OF THE PHENOLIC ACIDS IN WHEAT

I. FORMATION IN PHOTOSYNTHESIS FROM $^{14}CO_2$

A. In Young Wheat Plants

A series of fourteen experiments were conducted on intact wheat seedlings grown by method A. In each experiment, 120 to 150 seedlings (comprising 8 to 10 g fresh wt. of shoots) were administered 20 µc of $^{14}CO_2$ and allowed to photosynthesize in chamber X for periods of time which varied according to the experiment. The plants were either directly extracted or were permitted to photosynthesize in atmospheric CO_2 for an additional period before being extracted. The extracts were fractionated and chromatographically analyzed for each of the three phenolic acid fractions, i.e. the free acids, those obtained on acid hydrolysis and those obtained on alkaline hydrolysis. The phenolic acids of each fraction were identified on chromatograms and assayed for their radioactivity by means of autoradiography. The results are summarized in Table XI.

At the time when this series of experiments, as well as those immediately following, were carried out the author was not aware of the comparatively large amounts of phenolic acids obtainable upon alkaline hydrolysis of the ethanol-insoluble fraction. Consequently the analyses for phenolic acids in the earlier series of experiments were limited to the ethanol-soluble fraction.

Aliquots of the ethanol-insoluble residue (100 mg) were subjected to alkaline nitrobenzene oxidation. The oxidation mixture was acidified and extracted with ether. The ethereal extract was chromatographed for its aldehyde content and autoradiographed for detection of radioactivity in any of the three substituted benzaldehydes produced on lignin oxidation. The results are incorporated in Table XI.

In general, the incorporation of radioactivity from $^{14}CO_2$ was detected only in p-coumaric and ferulic acids. Nevertheless the radioactivity levels in these two compounds were either very low or not measurable. Furthermore, in some experiments (for instance Nos. 4, 5 and 7 in Table XI) the lignin aldehydes were radioactive whereas the corresponding cinnamic acids, p-coumaric, ferulic and sinapic acids, were not radioactive as indicated by the same method of assay, namely autoradiography.

Table XI

Formation of Hydroxycinnamic Acids and Lignin from $^{14}CO_2$ in Photosynthesis

Experiment Number	Period of photosynthesis in hours			Age of plants (days)	Total activity uptake (μc)	Radioactivity in products		
	In $^{14}CO_2$	In $^{12}CO_2$	Total			*p*-Coumaric acid	Ferulic acid	Lignin aldehyde
1	1	-	1	10	12.6	-	-	-
2	2	-	2	10	10.8	-	-	-
3	3	-	3	12	14.3	-	-	-
4	3	2	5	12	13.7	-	-	+
5	4	5	9	14	17.5	-	-	+
6	2	4	6	21	14.8	-	-	-
7	3	18	21	18	13.9	-	-	+
8	1	25	26	22	15.4	+	+	+
9	2	25	27	11	16.2	+	+	+
10	2	30	32	14	12.4	-	-	±
11	5	43	48	15	12.6	±	±	+
12	3	69	72	17	10.4	±	±	+
13	5	91	96	19	8.3	±	±	+
14	7	17	24	20	13.1	+	+	+

B. In Older Wheat Plants

The possibility that the lack or rather weak incorporation of radioactivity into the hydroxycinnamic acids from $^{14}CO_2$ was due to a slow rate of synthesis, because of the low rate of lignification in young wheat plants (Stone et al., 1951), was examined by conducting a similar series of experiments with older, greenhouse grown, intact wheat plants. The experiments were carried out with 35-day old plants and thereafter at ten-day intervals with successively older plants up to and including 75-day old material.

In each case, a pot containing 6 to 12 plants was placed in chamber Y. The plants were administered 50 µc of $^{14}CO_2$ and allowed to photosynthesize for 2 hours after which the chamber was flushed with air and opened. Approximately half the number of the plants was taken out for analysis with the rest being left for a further 6-hour period of photosynthesis in atmospheric CO_2 under the same conditions. Analyses for phenolic acids in various fractions of the ethanolic extracts and alkaline nitrobenzene oxidation of the ethanol-insoluble residues were carried out. Chromatograms and autoradiograms of the various fractions were also prepared.

In these plants, the phenolic acids were found to be present in much lower amounts than in the younger plants and in some cases certain acids were completely absent. Autoradiographic analyses revealed no activity in either the detectable phenolic acids or in the lignin aldehydes although the total uptake of activity by these plants was found to be between 40 to 60% of the administered $^{14}CO_2$. Elution of the spots, corresponding to vanillin, from chromatograms of lignin oxidation extracts was carried out at a later stage of this study when the author had access to the use of the Liquid Scintillation Spectrometer, however, and this aldehyde was found to be radioactive (0.3 to 0.4 μc/mmole).

C. In Other Plant Species

For purposes of comparison, experiments with $^{14}CO_2$ similar to those already described for wheat, were performed on other plant species including three dicotyledons and a gymnosperm. In each experiment, a young shoot, weighing 8 to 12 g (fresh weight) was administered 50 μc of $^{14}CO_2$ and allowed to photosynthesize for a period of 7 hours in chamber X. Photosynthesis in atmospheric $^{12}CO_2$ was allowed to proceed for a subsequent period of 17 hours. The plant material was analysed for its free phenolic acids as well as for the phenolic acids released on acid or alkaline hydrolysis of the ethanol-soluble fraction. Alkaline nitrobenzene oxidation was carried out on 100 mg dry wt. aliquots of the ethanol-insoluble residues and the extracts were chromatographed for their aldehyde content. The radioactivity in various spots on chromatograms was detected by means of autoradiography. The results were as follows:

1. Malus species

No phenolic acids occurring in the free state could be detected in this plant. However, several phenolic acids were identified in both alkaline and acid hydrolyzates of the ethanolic extract. These acids were shown to be ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, phloretic, *p*-hydroxyphenylacetic and traces of caffeic acids. In addition, umbelliferone and a number of unidentified phenolic compounds were detected. No sinapic or syringic acids could be detected in these extracts. Only ferulic and p-coumaric out of all the identified phenolic acids on chromatograms of both fractions were found to be radioactive (Figure 23). The lignin aldehydes, i.e., p-hydroxybenzaldehyde, vanillin and syringaldehydes were also found to be radioactive.

2. Hydrangea macrophylla

Of the known phenolic acids, only caffeic acid was detected on chromatograms of the free acid fraction. Ferulic, p-coumaric, caffeic and protocatechuic acids as well as traces of p-hydroxybenzoic and vanillic acids were detected in both alkaline and acid hydrolyzates with caffeic acid being the most prominent compound. No sinapic or syringic acid could be detected. Umbelliferone, 7-hydroxy-8-methoxycoumarin and the phenylisocoumarin, hydrangenol, were also detectable on chromatograms of both hydrolyzates. All of the compounds mentioned above, with the exception of p-hydroxybenzoic and vanillic acids were found to be radioactive. However, the major radioactive compound was caffeic acid and to a slightly lesser extent p-coumaric and ferulic acids. The radioactivity levels in the two coumarins and in hydrangenol were generally low. The three aldehydes obtained on alkaline nitrobenzene oxidation of the ethanol-insoluble fraction were also radioactive.

3. Gaultheria procumbens

No known phenolic acids could be detected in the free acid fraction in this species. p-Hydroxybenzoic, vanillic, salicylic, gentisic, o-pyrocatechuic and p-coumaric acids as well as traces of ferulic and caffeic acids were detectable on chromatograms of both alkaline and acid hydrolyzates. No sinapic or syringic acid could be

Figure 23. Print of an autoradiogram of the chromatographed phenolic acids in alkaline hydrolyzates of an ethanolic extract of *Malus sp.* administered $^{14}CO_2$ in photosynthesis.

detected. Although the hydroxylated benzoic acids were the dominant phenolic acids on these chromatograms, no activity was detected in any of them on autoradiograms. However, p-coumaric acid and ferulic acid, despite the presence of the latter in only trace amounts, were found to be radioactive. Similarly p-hydroxybenzaldehyde, vanillin and syringaldehyde were radioactive.

4. Pinus resinosa

p-Coumaric acid was found to be the major phenolic acid present in both alkaline and acid hydrolyzates. In addition, ferulic, p-hydroxybenzoic, vanillic and phloretic acids were readily detectable on chromatograms of both fractions. Acids occurring in the free state were lacking also in this species. Slight activity was detected in p-coumaric and ferulic acids only. Vanillin, produced from alkaline nitrobenzene oxidation of the ethanol-insoluble residue was appreciably radioactive. No syringaldehyde could be detected and a trace of activity as well as a weak color reaction were detectable in a position of the chromatogram corresponding to p-hydroxybenzaldehyde. The identity of the latter compound could not be verified due to the small amount present.

II. FORMATION IN PHOTOSYNTHESIS FROM $^{14}CO_2$ USING THE ISOTOPE COMPETITION AND TRAPPING TECHNIQUE

A. Photosynthesis in $^{14}CO_2$ with the Simultaneous Administration of Non-radioactive Phenylalanine

Wheat shoots obtained from 65-day old greenhouse-grown plants were cut under water and divided randomly into two sets of thirty shoots each. The cut ends of the first set were immersed in distilled water in a glass vial whereas the second set was held in another vial containing a 0.05% aqueous solution of phenylalanine. The two sets of plants were placed together in chamber X and administered 50 μc of $^{14}CO_2$. Photosynthesis was allowed to proceed for 9 hours after which time the remaining $^{14}CO_2$ was flushed away and the plants were allowed to photosynthesize in atmospheric CO_2 for a further 17 hours. The analysis was limited to the phenolic acids released on alkaline hydrolysis since this fraction had been shown in previous experiments to yield by far the largest amount of phenolic acids from the ethanolic extracts. The total activity acquired by each of the two sets of plants was determined. A known aliquot of each of the ethanol-soluble and insoluble fractions was combusted and the CO_2 was collected and its activity determined with the Dynacon. The total activity acquired by the phenylalanine fed plants was found to be 16.8 μc whereas that of the other plants was 15.9 μc. Thus the total activity of both sets represented approximately 65% of the activity of the administered $^{14}CO_2$.

In this experiment the phenolic acid content was found to be very low even in the plants administered phenylalanine. Ferulic and p-coumaric acids were detected only in trace amounts and their radioactivities could not be ascertained even after autoradiography. Alkaline nitrobenzene oxidation of the ethanol-insoluble residues yielded weakly radioactive p-hydroxybenzaldehyde, vanillin and syringaldehyde from plants maintained on distilled water. The aldehydes obtained from plants administered phenylalanine did not contain measurable amounts of radioactivity.

An identical experiment was performed on 25-day old shoots obtained from greenhouse-grown plants. The results of this experiment showed clearly that ferulic and p-coumaric acids, released on alkaline hydrolysis of the ethanolic extract of plants maintained on water, acquired appreciable amounts of radioactivity. No activity could be detected on autoradiograms of the other set of plants. The radioactivity in the three lignin aldehydes was also considerably higher in the case of plants maintained on water. It should be noted that the total radioactivities acquired by both sets of plants were almost equal (19.2 μc in plants kept in water and 19.8 μc in the other plants). The total uptake by both sets of plants represented 78% of the radioactivity administered as $^{14}CO_2$.

B. Photosynthesis in $^{14}CO_2$ with the Simultaneous Administration of Shikimic Acid or a Phenylpropanoid Compound

Wheat shoots, obtained from 27-day old greenhouse-grown plants, were divided into five sets, each consisting of fifteen shoots. Each set of plants was held with the cut ends immersed in a vial containing either water or a 0.1% aqueous solution of shikimic, cinnamic or p-coumaric acid or a 0.05% solution of cinnamyl alcohol. The plants were maintained in the light for six hours when they were transferred to other vials containing fresh solutions and placed in chamber X. They were allowed to photosynthesize in $^{14}CO_2$ (200 μc) for a period of 9 hours followed by a 17-hour period in atmospheric CO_2. Plants administered cinnamyl alcohol were observed turning yellow, and for this reason, they were killed and extracted after a total period of only 11 hours (9 hours in $^{14}CO_2$ + 2 hours in $^{12}CO_2$). A good portion of each shoot had turned completely yellowish in color presenting an etiolated appearance.

After a total period of 26 hours, the other 4 sets of plants were killed and extracted. The total activities acquired by both the ethanol-soluble and the ethanol-insoluble fractions of each set were determined by means of the Dynacon. Samples of the ethanolic extracts were chromatographed two-directionally in the phenol-water solvent followed by the butanol-acetic acid-water solvent. Autoradiograms of these chromatograms are shown in Figure 24.

Klason lignin was isolated from two 100 mg aliquots of each ethanol-insoluble residue. The isolated lignin was weighed, and wet combusted to CO_2 which was assayed for radioactivity with the Dynacon. Ten to 12 mg of Klason lignin were obtained in each determination. Alkaline nitrobenzene oxidation of the ethanol-insoluble residues was also carried out on 100 mg aliquots. The aldehydic products were chromatographed and the spot corresponding to vanillin was eluted in each case and its specific activity determined. In the alkaline hydrolyzates of the ethanolic extracts only ferulic and p-coumaric acids were detected as being radioactive. The spots corresponding to each of these two acids were eluted from chromatograms and their specific activities were determined. Similarly, the specific activities of p-coumaric and ferulic acids, released on alkaline hydrolysis of the ethanol-insoluble residue, were determined. The results are shown in Table XII.

A marked decrease, in the total uptake of $^{14}CO_2$, occurred in plants administered cinnamyl compounds making it difficult to draw comparisons between the results of the experiments. Shikimic acid was very effective in reducing the incorporation of radioactivity in Klason

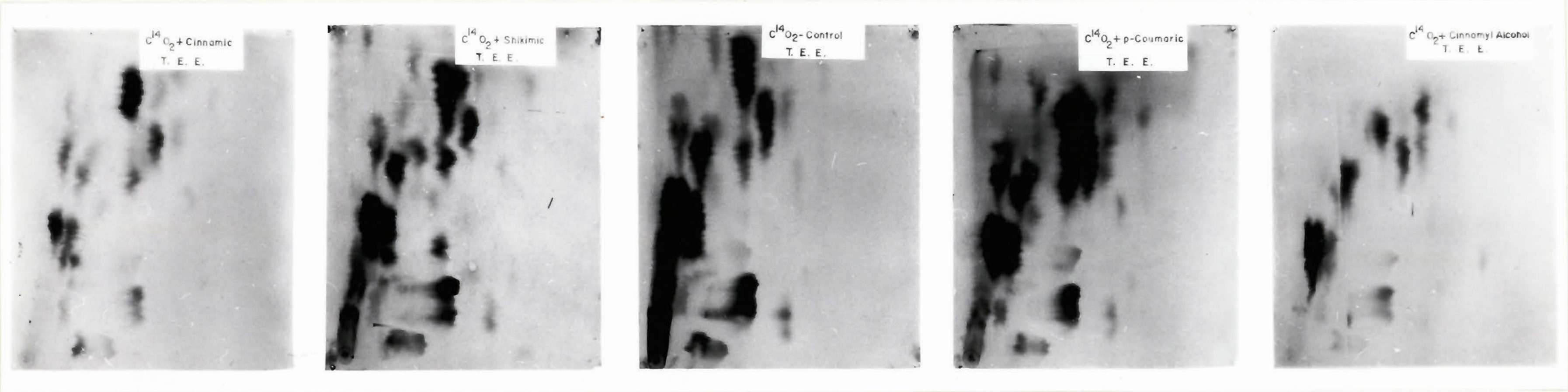

Figure 24. Print of autoradiograms of the chromatographed unhydrolyzed extracts of wheat shoots administered $^{14}CO_2$ and non-labeled shikimic acid or a phenylpropanoid compound in photosynthesis.

Table XII

Formation of Hydroxycinnamic Acids and Lignin in Wheat Administered $C^{14}O_2$ in Light Together with Non-Labeled Lignin Precursors

	Total activity (µc/g. dry wt.)					Specific activity (µc/mmole)				
				Klason lignin		p-Coumaric acid		Ferulic acid		
Experiment	Ethanol soluble fraction	Ethanol insoluble fraction (residue)	Total uptake	Total activity	Percent Conversion	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Lignin vanillin
$^{14}CO_2$ (Control)	40.0	6.2	46.2	0.60	1.3	0.90	1.96	2.76	5.82	2.28
$^{14}CO_2$ + Shikimic acid	37.3	8.1	45.4	0.36	0.8	0.54	1.24	2.16	4.35	1.63
$^{14}CO_2$ + Cinnamic acid	26.4	2.7	29.1	0.30	1.0	0.07	--[A]	0.69	1.72	0.57
$^{14}CO_2$ + p-Coumaric acid	30.2	5.8	36.0	0.45	1.2	0.06	1.08	1.15	2.98	1.10
$^{14}CO_2$ + Cinnamyl alcohol	10.8	0.9	11.7	0.08	0.7	0.004	--[A]	0.39	0.64	0.27

A = sample was lost.

lignin as well as in diluting the specific activities of both ethanol-soluble and insoluble forms of p-coumaric and ferulic acids and of vanillin. Similarly, cinnamic and p-coumaric acids and cinnamyl alcohol caused dilution in activity of all of the aforementioned compounds. An interesting observation in the last 3 feedings, however, is that although the specific activity of the ethanol-soluble esters of p-coumaric acid was extremely low, that of the corresponding esters of ferulic acid was appreciable. Furthermore, in plants fed with non-labeled p-coumaric acid the specific activity of the ethanol-insoluble esters did not seem to be affected whereas that of the soluble esters was considerably diluted.

III. BIOSYNTHESIS FROM CARBON-14 LABELED PHENYLPROPANOID PRECURSORS AS COMPARED WITH $^{14}CO_2$

Wheat seedlings, 8 days old, were obtained from 14 trays germinated, at the same time, by method A. Uniform plants with an average height of 6 in. were selected. Shoots were cut under water and randomly grouped into 8 sets, each consisting of 200 shoots. The plants were subsequently treated as follows:

A. One set was placed in distilled water and administered $^{14}CO_2$ (100 μc) in chamber X. Photosynthesis was allowed to proceed in $^{14}CO_2$ for 5 hours and in atmospheric CO_2 for a subsequent period of 19 hours, thus bringing the total period of photosynthesis to 24 hours.

B. To each of the remaining sets a ^{14}C-labeled phenylpropanoid compound was administered. L-Phenylalanine-U-^{14}C and L-tyrosine-carboxyl-^{14}C were administered as their aqueous solutions whereas cinnamic, p-coumaric, caffeic, ferulic and sinapic acids were administered as their sodium salts in aqueous solutions of neutral pH. Shoots were held in small vials containing 5 mg of the radioactive compound in 10 ml water. The total and specific activities of each of the administered compounds are listed in Tables XIII and XIV. The plants were allowed to metabolize in light (1200 f.c.) for 24 hours during which time the solutions were completely absorbed. Small volumes (3 ml) of water were added to each vial to prevent desiccation of the shoots. At the end of the metabolic period each set of plants was washed with distilled water and analyzed as described previously.

Quantitative determinations were made of ferulic, p-coumaric and sinapic acids released on alkaline hydrolysis of the ethanol-soluble and insoluble fractions (cell wall residue) as well as of p-hydroxybenzoic, vanillic and syringic acids in the ethanol-soluble fraction. Quantitative determinations were also made of vanillin, syringaldehyde and p-hydroxybenzaldehyde obtained on alkaline nitrobenzene oxidation of the ethanol-insoluble residues. The radioactivity incorporated into each of the abovementioned compounds was measured using the Liquid Scintillation Spectrometer and the specific activities were calculated.

For ease of reference to the hydroxycinnamic acids obtained from various fractions the terms "soluble esters" and "insoluble esters" will be used hereafter, respectively, for ethanol-soluble and ethanol-insoluble derivatives which release hydroxycinnamic acids on alkaline

Table XIII

Total Activities of Hydroxycinnamic Acids Isolated from Wheat Shoots after Administration of Labeled Phenylpropanoid Compounds and of $^{14}CO_2$ in Photosynthesis

Compound administered	Total activity (mμc/g. dry wt.)					
	p-Coumaric acid		Ferulic acid		Sinapic acid	
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters
$^{14}CO_2$ = 60 μc	9.6	30.0	36.0	144.0	2.4	9.6
L-Phenylalanine-U-^{14}C = 5 μc	6.30	20.0	27.5	120.0	3.0	14.0
L-Tyrosine-1-^{14}C = 5 μc	5.8	27.0	14.0	130.0	1.5	15.0
Cinnamic acid-3-^{14}C = 3.1 μc	173.6	14.3	269.7	74.4	15.2	10.5
p-Coumaric acid-3-^{14}C = 0.96 μc	42.2	3.7	18.2	15.4	1.7	0.5
Caffeic acid-3-^{14}C = 1.1 μc	1.4	0.7	6.6	9.0	1.7	0.3
Ferulic acid-3-^{14}C = 2.2 μc	1.8	1.1	83.6	39.6	3.7	1.3
Sinapic acid-3-^{14}C = 1.4 μc	11.1	2.1	9.4	4.1	40.2	18.6

Table XIV

Specific Activities of Hydroxycinnamic Acids Isolated from Wheat Shoots after Administration of Labeled Phenylpropanoid Compounds and of $^{14}CO_2$ in Photosynthesis

Compound administered and its specific activity (μc/mmole)	Specific activity (μc/mmole)					
	p-Coumaric acid		Ferulic acid		Sinapic acid	
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters
$^{14}CO_2$ 800	2.35	5.93	6.67	22.53	3.60	4.65
L-Phenylalanine-U-^{14}C 165	1.22	3.11	3.13	10.38	1.91	3.38
L-Tyrosine-1-^{14}C 181	1.38	3.95	2.50	11.75	1.10	4.12
Cinnamic acid-3-^{14}C 92.2	16.18	4.10	27.94	11.11	8.78	4.04
p-Coumaric-3-^{14}C 31.5	4.50	1.13	2.46	1.59	1.04	0.32
Caffeic acid-3-^{14}C 39.6	0.21	0.09	0.93	0.85	1.12	0.15
Ferulic acid-3-^{14}C 87.1	0.26	0.14	14.52	6.50	6.65	0.55
Sinapic acid-3-^{14}C 63.5	1.39	0.27	1.90	0.54	57.73	6.80

hydrolysis. The results of these experiments are presented in Tables XIII to XXI inclusive.

It is noteworthy in Tables XIII and XIV that $^{14}CO_2$ and the aromatic amino acids were incorporated more readily into the insoluble esters of hydroxycinnamic acids than into the soluble esters whereas the reverse was true for precursors such as cinnamic, p-coumaric, caffeic, ferulic and sinapic acids. It is also evident that although the soluble and insoluble esters of p-coumaric acid were present in smaller amounts than the corresponding esters of ferulic acid (Table XV), nevertheless, they had considerably lower specific activities (Table XIV). This had been a general observation in all experiments regardless of the precursor administered. The only exception was in plants fed with p-coumaric acid in which case only the soluble esters of p-coumaric acid acquired higher specific activity than the corresponding esters of ferulic acid. The specific activities of the insoluble esters, however, reflected the same general picture obtained with other precursors. It is interesting that these findings are opposite to what would be expected if the p-coumaric acid derivatives were acting as precursors of the ferulic acid derivatives.

In order to compare the efficiency of incorporation by the labeled precursors into the soluble and insoluble esters of the hydroxycinnamic acids the dilution values and the per cent conversion were calculated and are presented in Tables XVI and XVII. The dilution value is obtained by dividing the specific activity of the administered compound by that of the product, both being calculated in terms of μc per mmole. It has been generally assumed that lower dilution values indicate higher efficiencies of precursors involved. It is clear in Table XVI

Table XV

Total Amount of Hydroxycinnamic Acids Isolated from Wheat Shoots Administered Labeled Phenylpropanoid Compounds and $^{14}CO_2$ in Photosynthesis

Compound administered	Amount of acid found (µmole/g. dry wt.)					
	p-Coumaric acid		Ferulic acid		Sinapic acid	
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters
$^{14}CO_2$	4.09	5.06	5.40	6.40	0.67	2.06
L-Phenylalanine-U-^{14}C	5.12	6.43	8.79	11.56	1.57	4.14
L-Tyrosine-1-^{14}C	4.20	6.84	5.60	11.06	1.36	3.64
Cinnamic acid-3-^{14}C	10.73	3.48	9.65	6.70	1.73	2.61
p-Coumaric acid-3-^{14}C	9.39	3.31	7.41	9.66	1.66	1.50
Caffeic acid-3-^{14}C	6.81	7.33	7.10	10.61	1.47	2.20
Ferulic acid-3-^{14}C	6.77	7.86	5.76	6.09	0.56	2.40
Sinapic acid-3-^{14}C	7.96	7.78	4.94	7.52	6.96	2.74

Table XVI

Dilution Value of Hydroxycinnamic Acids Isolated from Wheat Shoots Administered $^{14}CO_2$ in Photosynthesis and Labeled Phenylpropanoid Compounds

(Calculated from Data Presented in Table XIV)

Compound administered	Dilution value					
	p-Coumaric acid		Ferulic acid		Sinapic acid	
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters
$^{14}CO_2$	340	135	120	35.5	222	172
L-Phenylalanine-U-^{14}C	135	53	52.7	15.9	86.4	48.8
L-Tyrosine-1-^{14}C	130	45.8	72.4	15.4	165	43.9
Cinnamic acid-3-^{14}C	5.7	22.5	3.3	8.3	10.5	22.8
p-Coumaric acid-3-^{14}C	7.0	28.0	12.8	19.8	30.3	97.2
Caffeic acid-3-^{14}C	188.6	440	42.6	46.6	35.4	264
Ferulic acid-3-^{14}C	335	622	6.0	13.4	13.1	158.4
Sinapic acid-3-^{14}C	45.7	235	33.4	117.6	1.1	9.34

Table XVII

Percent Conversion of $^{14}CO_2$ in Photosynthesis and of Labeled Phenylpropanoid Compounds to Hydroxycinnamic Acids in Wheat Shoots

(Calculated from data presented in Table XIII)

Compound administered	Per cent conversion					
	p-Coumaric acid		Ferulic acid		Sinapic acid	
	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters	Soluble esters	Insoluble esters
$^{14}CO_2$	0.016	0.05	0.06	0.24	0.004	0.016
L-Phenylalanine-U-^{14}C	0.125	0.40	0.55	2.40	0.06	0.28
L-Tyrosine-1-^{14}C	0.116	0.54	0.28	2.60	0.03	0.30
Cinnamic acid-3-^{14}C	5.60	0.46	8.70	2.40	0.49	0.34
p-Coumaric acid-3-^{14}C	4.40	0.39	1.90	1.60	0.18	0.05
Caffeic acid-3-^{14}C	0.13	0.06	0.60	0.82	0.15	0.03
Ferulic acid-3-^{14}C	0.08	0.05	3.80	1.80	0.17	0.06
Sinapic acid-3-^{14}C	0.79	0.15	0.67	0.29	2.87	1.33

that the lowest dilution values in the soluble and insoluble esters of p-coumaric and ferulic acids were obtained with the feeding of cinnamic acid. Cinnamic acid appeared to be a more efficient precursor of esters of sinapic acid than was ferulic acid, although the latter has been shown to be a direct precursor of sinapic acid (McCalla and Neish, 1959b).

Phenylalanine and tyrosine were equally good precursors of the esters of p-coumaric and ferulic acids (Table XVI). Caffeic acid was generally a poor precursor. Sinapic acid was converted to ferulic acid and even to p-coumaric acid with low enough dilution to suggest that this might have occurred by removal of one or two methoxyl groups, respectively, rather than by degradation to carbohydrate and recycling of the isotopic carbon. However, no further studies were done to confirm or disprove this. Caffeic and ferulic acids were not converted to esters of p-coumaric acid as readily as was sinapic acid.

Table XVIII shows that the hydroxybenzoic acids, p-hydroxybenzoic, vanillic and syringic acids, became labeled when $^{14}CO_2$ or any of the C^{14}-labeled phenylpropanoid compounds was administered (see also Figures 25 to 30). The lowest dilution value for each of the three benzoic acids was obtained when its corresponding hydroxycinnamic acid was administered. Thus p-coumaric, ferulic and sinapic acids were converted, respectively, to p-hydroxybenzoic, vanillic and syringic acids with almost no dilution of the isotopic carbon. In these latter feedings, the hydroxybenzoic acids were isolated by chromatography, diluted with inactive carrier and after repeated crystallization decarboxylated (pages 84 and 85). The carboxyl-CO_2 was collected as $BaCO_3$ and its radioactivity was determined. The results, presented in

Table XVIII

Specific Activities (μc/mmole), Dilution Values and Total Amounts (μmole/g. dry wt.) of Hydroxybenzoic Acids Isolated from Wheat Shoots Administered Either a C^{14}-Labeled Phenylpropanoid Compound or $^{14}CO_2$ in Photosynthesis

Compound administered and its specific activity μc/mmole	p-Hydroxybenzoic acid			Vanillic acid			Syringic acid		
	Total amount	Specific activity	Dilution value	Total amount	Specific activity	Dilution value	Total amount	Specific activity	Dilution value
$^{14}CO_2$ 800	0.28	12.5	64	0.19	19.6	41.2	0.17	14.45	55.4
L-Phenylalanine-U-^{14}C 165	0.30	4.6	36	0.14	18.7	8.8	0.19	7.4	22
Cinnamic-3-^{14}C 92.2	0.26	26	3.6	0.28	46.3	2	0.13	38.8	2.4
p-Coumaric-3-^{14}C 31.5	0.37	26.8	1.2	0.18	13	2.4	0.17	4	7.9
Caffeic-3-^{14}C 39.6	0.35	2.8	14	0.21	9	4.4	0.19	8.1	4.9
Ferulic-3-^{14}C 87.1	N.D.*	N.D.*	N.D.*	0.21	48	1.8	0.13	13.8	6.3
Sinapic-3-^{14}C 63.5	0.44	2.8	23	N.D.*	N.D.*	N.D.*	0.33	61	1

* Not determined.

Figure 25. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered $^{14}CO_2$.

Figure 26. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered L-phenylalanine-U-^{14}C.

Figure 27. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered L-tyrosine-1-^{14}C.

Figure 28. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered cinnamic acid-3-^{14}C.

Figure 29. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered *p*-coumaric acid-3-^{14}C.

Figure 30. Print of an autoradiogram of the chromatographed phenolic acids in an alkaline hydrolyzate of an extract of wheat shoots administered ferulic acid-3-^{14}C.

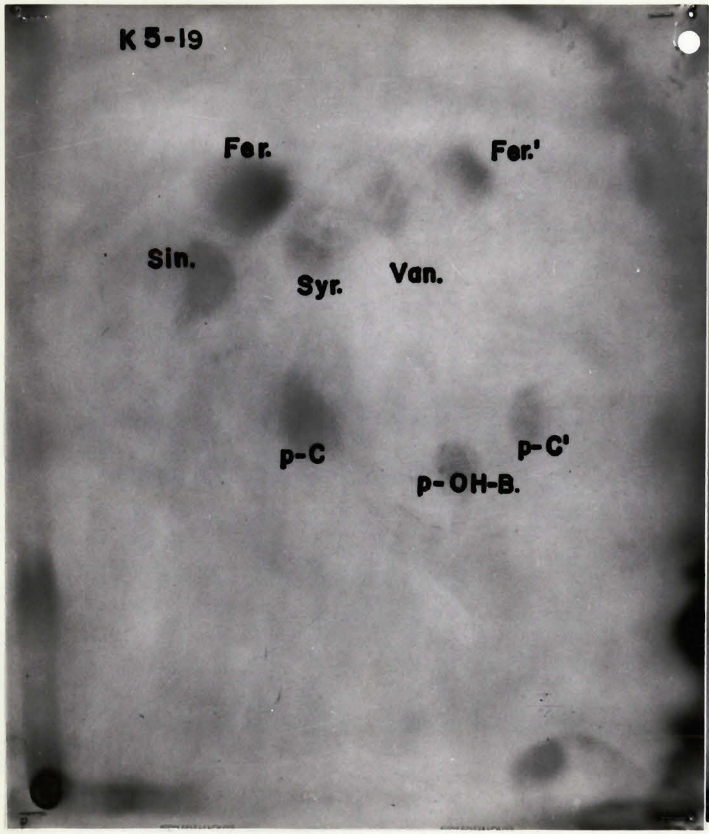

Figure 25

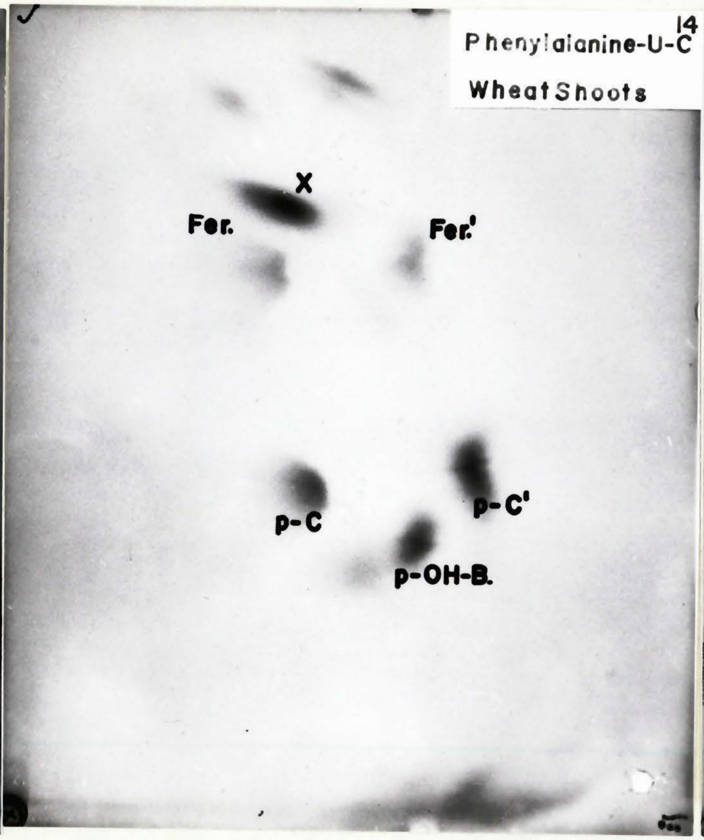

Figure 26.

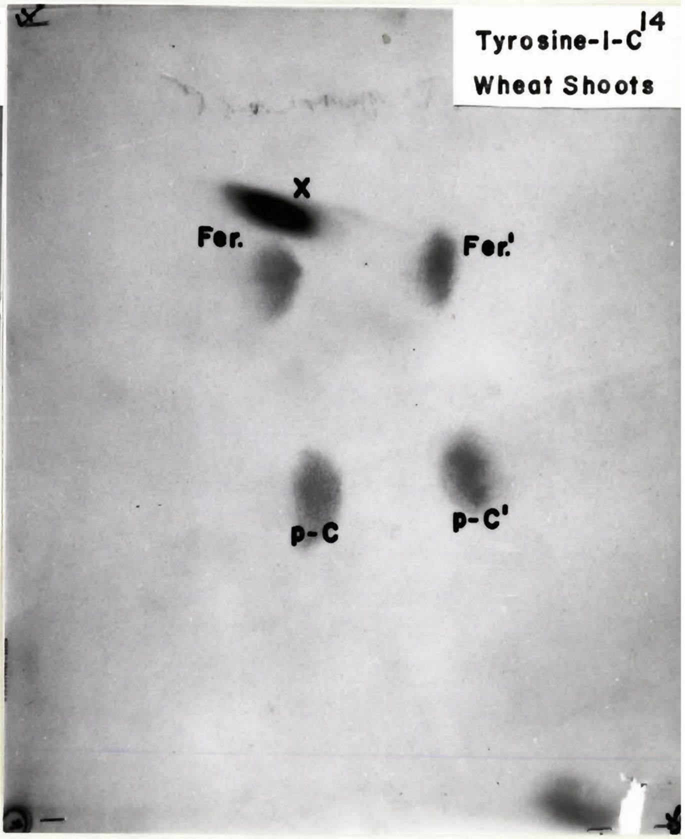

Figure 27.

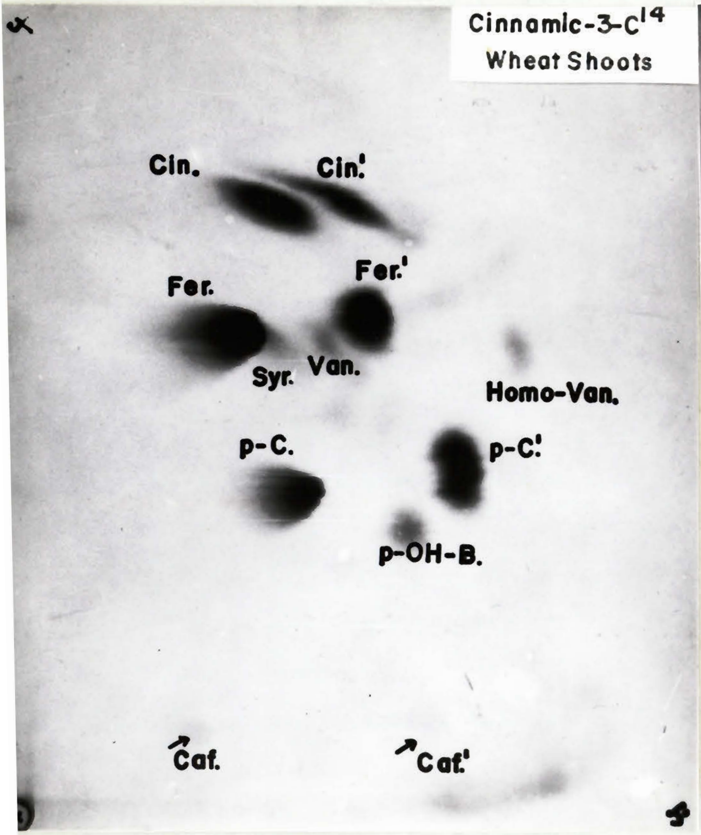

Figure 28.

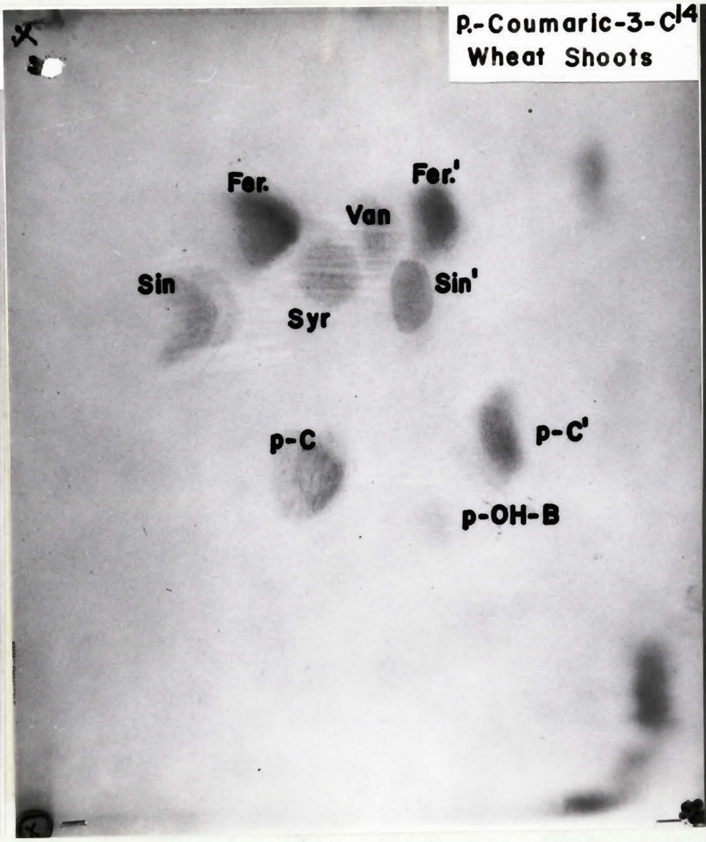

Figure 29.

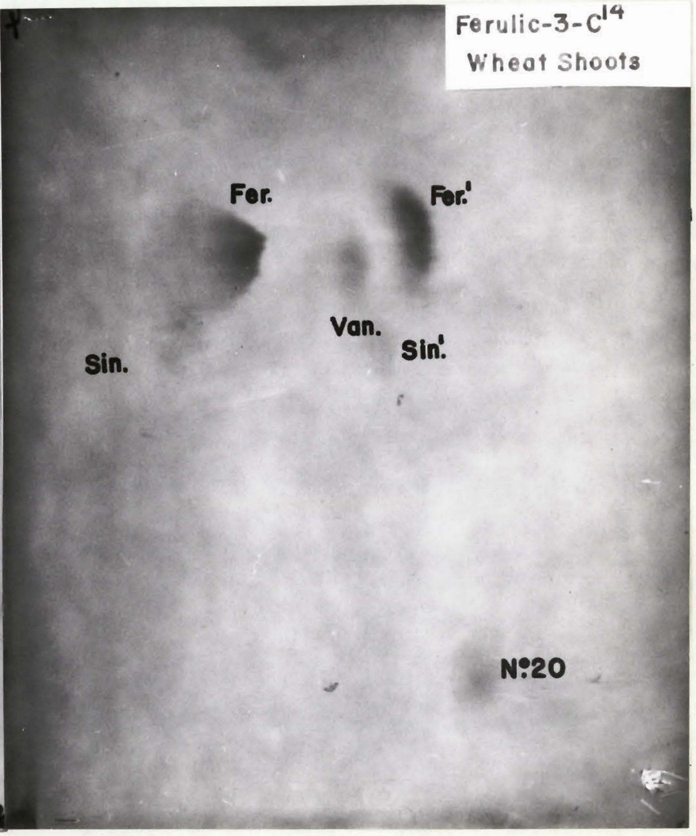

Figure 30.

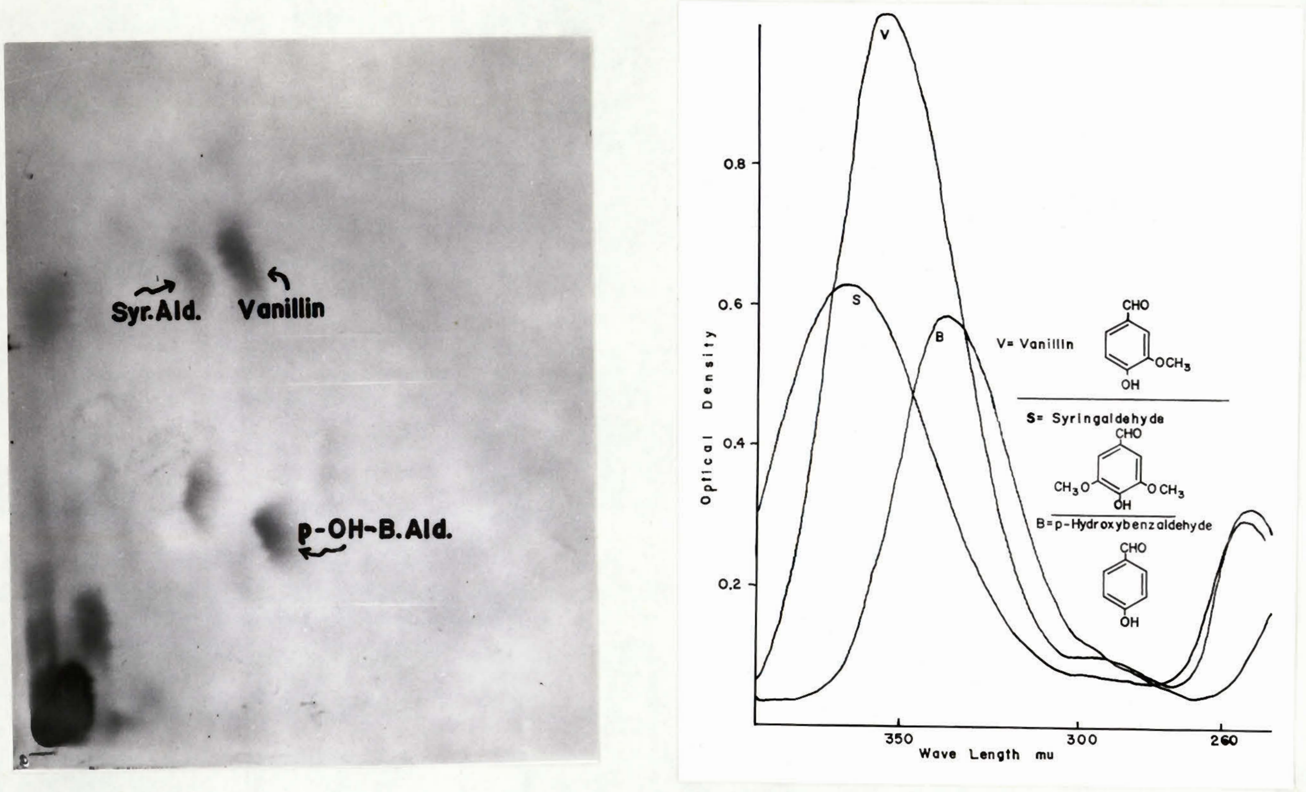

Figure 31. A, Print of an autoradiogram of the chromatographed lignin aldehydes formed from $^{14}CO_2$ in photosynthesis; B, Ultraviolet spectra of these aldehydes.

Table XIX show clearly that the radioactivity has been retained in the same position relative to the ring.

Cinnamic acid again was clearly the most efficient general precursor for all three hydroxybenzoic acids (Table XVIII). The specific activities of p-hydroxybenzoic acid were usually lower than those of vanillic acid in all feeding experiments except in the one with p-coumaric acid. Caffeic and sinapic acids were converted to p-hydroxybenzoic acid presumably by dehydroxylation and demethoxylation respectively and by removal of two carbons of the side-chains of the two acids. As it was not determined whether the activity of p-hydroxybenzoic acid, in these instances, was confined to the carboxyl carbon this assumption may not be valid.

The hydroxybenzoic acids, in any one particular feeding even when $^{14}CO_2$ was administered, displayed specific activities that were considerably higher than those of their hydroxycinnamic acid analogues. This is readily observed by comparing the data represented in Tables XIV and XVIII.

The data pertaining to p-hydroxybenzaldehyde, vanillin and syringaldehyde, obtained on alkaline nitrobenzene oxidations of the ethanol-insoluble cell wall residues, are summarized in Tables XX and XXI.[*] In the experiment with tyrosine alkaline nitrobenzene oxidation was not carried out since tyrosine-$^{14}COOH$ was used.

Table XX shows that there was a greater conversion to lignin, as represented by the aldehydes, of cinnamic, p-coumaric, ferulic and sinapic acids than of phenylalanine or $^{14}CO_2$. Caffeic acid was not efficiently utilized for lignin formation as compared with the other

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[*] See Figure 31.

Table XIX

Decarboxylation of Radioactive Hydroxybenzoic Acids Isolated after the Administration of C^{14}-labeled Hydroxycinnamic Acids to Wheat Shoots

Compound administered	Compound isolated (B)	Specific activity of (B) (mμc/mmole)	Specific activity of $BaCO_3$ from decarboxylation of (B) (mμc/mmole)
p-Coumaric acid-3-^{14}C	p-Hydroxybenzoic acid	187	161
Ferulic acid-3-^{14}C	Vanillic acid	224	219
Sinapic acid-3-^{14}C	Syringic acid	279	277

Table XX

Total Activities (mμc/g. dry wt.) and Total Amounts (μmole/g. dry wt.) of Lignin Aldehydes Isolated from Wheat Shoots Administered Either a Labeled Phenylpropanoid Compound or $^{14}CO_2$ in Photosynthesis

Compound administered and its total activity	p-Hydroxybenzaldehyde			Vanillin			Syringaldehyde		
	Total activity	Percent conversion	Total amount	Total activity	Percent conversion	Total amount	Total activity	Percent conversion	Total amount
$^{14}CO_2$ 60 μc	102	0.17	25.50	342	0.57	60.30	84	0.14	7.94
L-Phenylalanine-U-^{14}C 5 μc	95	1.90	32.10	155	3.10	79.49	65	1.30	7.25
Cinnamic-3-^{14}C 3.1 μc	155	5.00	29.75	217	7.00	69.77	71.3	2.30	7.43
p-Coumaric-3-^{14}C 0.96 μc	77.8	8.10	23.71	58.6	6.10	70.55	23.04	2.40	9.03
Caffeic-3-^{14}C 1.1 μc	8.8	0.80	25.88	39.6	3.60	66.00	15.40	1.40	8.37
Ferulic-3-^{14}C 2.2 μc	13.2	0.60	23.57	282	12.8	69.19	27.39	1.25	8.48
Sinapic-3-^{14}C 1.4 μc	32.06	2.29	27.17	30.8	2.20	59.23	99.40	7.10	11.43

Table XXI

Specific Activities (μc/mmole) and Dilution Values of Lignin Aldehydes Isolated from Wheat Shoots Administered Either a Labeled Phenylpropanoid Compound or $^{14}CO_2$ in Photosynthesis

Compound administered and its specific activity μc/mmole = A	p-Hydroxybenzaldehyde		Vanillin		Syringaldehyde	
	Specific activity B	Dilution value A/B	Specific activity C	Dilution value A/C	Specific activity D	Dilution value A/D
$^{14}CO_2$ 800	4.00	200	5.67	141	10.58	75.6
L-Phenylalanine-U-^{14}C 165	2.96	55.7	1.95	84.6	8.97	18.4
Cinnamic-3-^{14}C 92.2	5.21	17.7	3.11	29.6	9.60	9.6
p-Coumaric-3-^{14}C 31.5	3.28	9.6	0.83	38.0	2.55	12.4
Caffeic-3-^{14}C 39.6	0.34	117	0.60	66.0	1.84	21.5
Ferulic-3-^{14}C 87.1	0.56	156	4.07	21.4	3.23	27.0
Sinapic-3-^{14}C 63.5	1.18	53.8	0.52	122	8.70	7.30

hydroxycinnamic acids. Moreover, p-coumaric, ferulic and sinapic acids were incorporated preferentially into the parts of lignin which yield the corresponding aldehydes on oxidation. This has been indicated by a greater conversion and a lower dilution value in each of the aldehydes when the analogous acid was administered.

In these experiments there was apparently an active synthesis of "syringyl lignin", judging from the low dilution values for syringaldehyde (Table XXI). These dilution values were 2 to 4 times lower than the corresponding values for vanillin from all precursors except ferulic acid in which case it was higher. However, it is clear from the data represented in Table XXI that the ratios of specific activities of the aldehydes depended very markedly on the precursor. Cinnamic acid was the most efficient precursor for all three aldehydes.

IV. TIME-COURSE TRACER STUDIES ON FORMATION OF HYDROXYCINNAMIC ACIDS AND OF LIGNIN IN WHEAT

As the results of the abovementioned experiments showed clearly that the insoluble esters of the hydroxycinnamic acids do not represent an inactive reserve of phenylpropanoid units but that they are being constantly produced in the light in young wheat plants, time-course studies of their formation were carried out.

A. From $^{14}CO_2$ in Photosynthesis

Seven sets, each consisting of 160, 8-day old seedlings, were placed, with their roots completely immersed in Hoagland's nutrient solution in polyethylene containers, in a large vacuum desiccator. $^{14}CO_2$ (1 mc) was generated and introduced into the vacuum desiccator. The plants were allowed to photosynthesize for 2 hours (1200 f.c.) after which the remaining $^{14}CO_2$ was trapped as $Ba^{14}CO_3$ by repeated cycles of suction-flushing with CO_2 free air and one set of plants was removed for analysis. The remaining sets were allowed to metabolize for further periods in cycles of 18 hours light and 6 hours dark. Sets were removed, one at a time, for analysis at the following intervals: 6, 10, 24 and 48 hours, 7 and 14 days.

Quantitative determinations and radioactivity measurements were made of soluble and insoluble esters of p-coumaric and ferulic acids, of soluble esters of sinapic acid and of vanillin and syringaldehyde obtained on alkaline nitrobenzene oxidations of the ethanol-insoluble residues.

The changes in specific activities of the abovementioned compounds up till 48 hours are shown in Figure 32. Similar changes were also noted in the total activities of these compounds and are summarized in Table XXII. It is clear from these results that the insoluble esters of p-coumaric and ferulic acids were labeled quickly and then gradually lost isotopic carbon as the soluble esters and lignin picked it up (Figure 32). In other words the insoluble esters acted kinetically as if they were precursors of lignin and the soluble esters. Even in plants allowed to metabolize for 7 and 14 days after administration of $^{14}CO_2$, radioactivity still could be detected in both soluble and insoluble esters of p-coumaric and ferulic acids although at a very low level (12 and 23 mμc per g dry wt. of plant material, respectively, in soluble

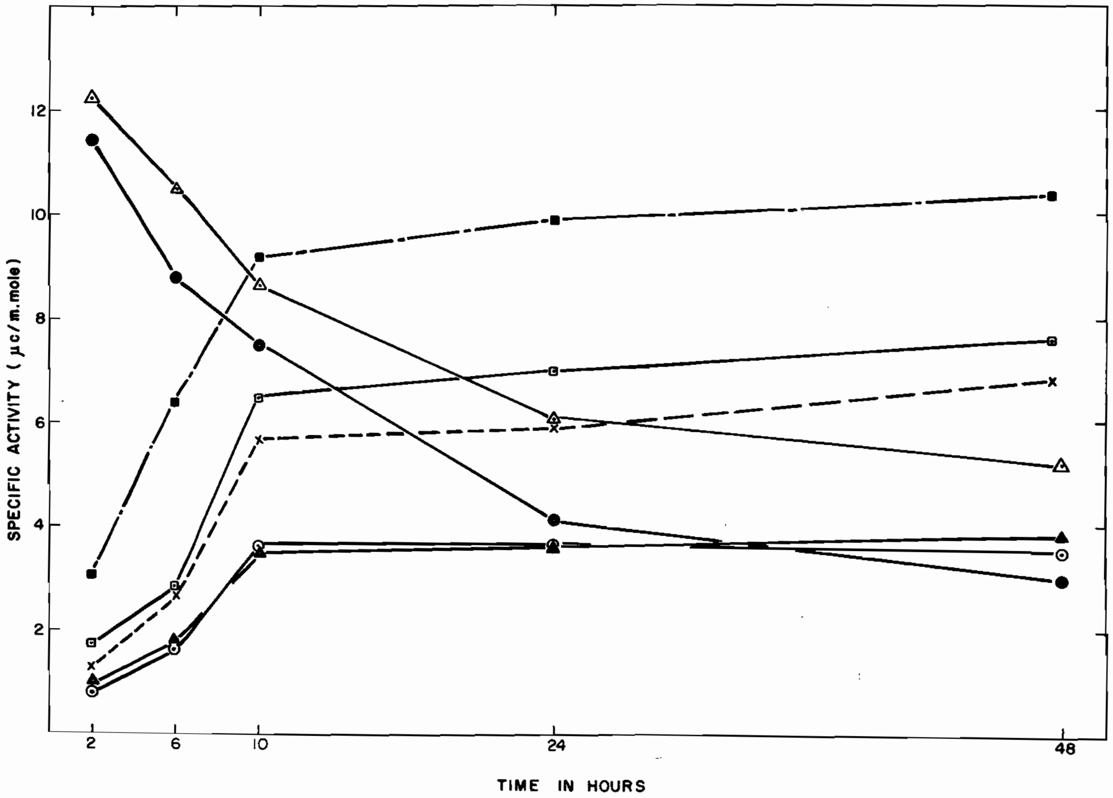


Figure 32. Changes with time in specific activities of hydroxycinnamic acids and lignin aldehydes after photosynthesis in $^{14}CO_2$.

Table XXII

Total Amounts and Total Activities of Hydroxycinnamic Acids Isolated after Administration of $^{14}CO_2$ to Wheat Plants in Time-Course Studies

Total amount (μm per g. dry wt.) and total activity (mμc/g. dry wt.) of hydroxycinnamic acid isolated	Metabolic period (hours) after administration of $^{14}CO_2$ in photosynthesis				
	2	6	10	24	48
p-Coumaric acid					
Soluble esters					
Total amount	6.00	6.56	6.11	5.69	5.27
Total activity	4.86	10.76	22.30	21.11	17.13
Insoluble esters					
Total amount	6.99	6.91	7.08	6.41	6.59
Total activity	80.1	60.8	53.2	26.7	19.8
Ferulic acid					
Soluble esters					
Total amount	6.49	6.80	6.58	6.08	5.69
Total activity	11.55	19.58	42.77	42.86	43.24
Insoluble esters					
Total amount	20.00	19.79	21.44	17.99	18.35
Total activity	244.4	208.2	185.2	109.7	95.4
Sinapic acid					
Soluble esters					
Total amount	1.16	1.34	1.30	1.19	1.10
Total activity	1.18	2.45	4.56	4.31	4.22

and insoluble esters of ferulic acid and much smaller but detectable amounts in the corresponding esters of p-coumaric acid).

B. From Carbon-14-labeled Ferulic and Cinnamic Acids

Thirty-five-day old plants with an average height of 18 in. were used. The shoots were cut under water and divided into two equal sets of 48 plants each. Sodium cinnamate-3-^{14}C was administered to one set and sodium ferulate-3-^{14}C to the other through the cut ends of the shoots in the light. The radioactive solutions were completely absorbed within the first two hours after which 12 plants from each set were removed for analysis. Subsequent treatment was as follows: the remaining plants of each set, after washing of their immersed ends, were transferred to a non-radioactive solution of the administered compound at the same concentration. Twelve plants from each set were removed for analysis after 4 hours and another 12 after 8 hours. The remaining 12 plants from each set were transferred to distilled water and analyzed after a further 14 hours. Plants were maintained in light (1200 f.c.) for the duration of the experiment.

Quantitative determinations and radioactivity measurements were carried out, for the hydroxycinnamic acids and the lignin aldehydes, in the usual manner. The results follow:

1. From cinnamic acid

Changes with time in the total activities of soluble and insoluble esters of p-coumaric and ferulic acids, of soluble esters of sinapic acid and of lignin aldehydes are shown in Figure 33. By far the greatest amount of [^{14}C] was incorporated into both types of esters of ferulic acid. The total activity of the insoluble esters of p-coumaric

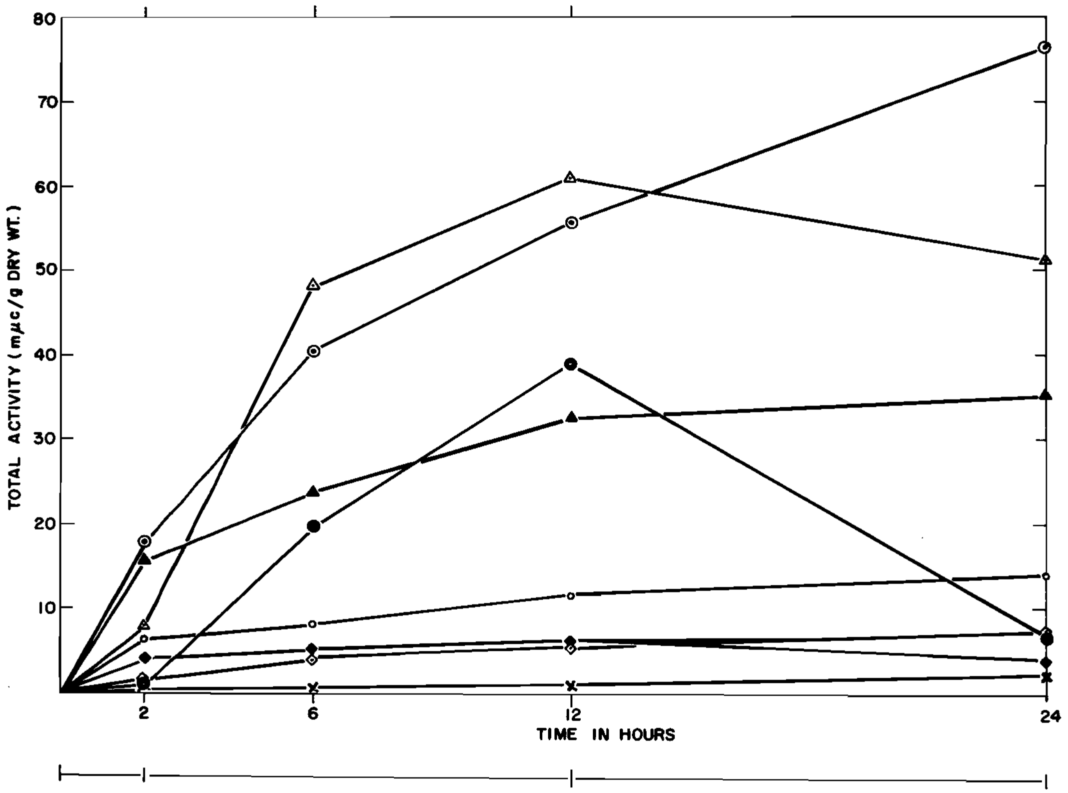


Figure 33. Changes with time in total activities of hydroxycinnamic acids and lignin aldehydes after administration of cinnamic acid-3-^{14}C.

acid was much greater, after two hours, than that of the soluble esters. The situation, however, was reversed after 4 hours where the total activity of the soluble esters was almost 4 times as much as that of the insoluble esters and yet a more marked continued increase was evident after 12 hours. This was followed by a decrease in the total activity of both fractions which was very pronounced for the soluble esters (from 39 mμc to 6.7 mμc).

Similar, but not identical, changes were evident in the corresponding fractions of ferulic acid with the exception of the insoluble esters where a slow build-up of radioactivity was still taking place even after the removal of the exogenous supply of cinnamic acid and the transfer of plants to distilled water. Because cut shoots were employed it was not considered useful to prolong the duration of the experiment although it would be of interest to know how long it would take to reduce the activity in the insoluble esters of ferulic acid as was the case with p-coumaric acid. Changes in the specific activity of these compounds are shown in Table XXIII and it can be seen that they reflect the same picture obtained with the total activity. It is interesting, however, that whereas after 2 hours there was almost no detectable activity in the soluble esters of p-coumaric acid, an appreciable amount was found in their counterparts of ferulic acid. The same observation can also be made with respect to the specific activity.

No sinapic acid could be detected after 2 hours and even in plants analyzed after 6 hours only a trace amount was detectable on chromatograms. Subsequent changes in the total amounts and in the

Table XXIII

Total Amounts and Specific Activities of Hydroxycinnamic Acids and Lignin Aldehydes Isolated after Administration of Cinnamic Acid-3-^{14}C to Wheat Shoots

Total amount (μm per g. dry wt.) and specific activity (μc/mmole) of compounds isolated	Metabolic period (hours) after administration of cinnamic acid-3-^{14}C			
	2	6	12	24
<u>p-Coumaric acid</u>				
Soluble esters				
Total amount	0.48	1.18	1.26	1.11
Specific activity	< 1	15.95	31.01	6.02
Insoluble esters				
Total amount	8.80	8.66	10.76	7.49
Specific activity	0.45	0.63	0.64	0.53
<u>Ferulic acid</u>				
Soluble esters				
Total amount	0.97	4.01	4.24	5.38
Specific activity	7.79	12.06	14.66	9.31
Insoluble esters				
Total amount	28.41	33.91	33.22	35.97
Specific activity	0.634	1.20	1.69	2.12
<u>Sinapic acid</u>				
Soluble esters				
Total amount	Nil	traces	0.75	1.55
Specific activity	Nil	< 1	1.73	1.49
<u>p-Hydroxybenzaldehyde</u>				
Total amount	23.80	24.90	25.20	26.10
Specific activity	0.27	0.33	0.46	0.53
<u>Vanillin</u>				
Total amount	58.95	59.47	59.87	60.92
Specific activity	0.27	0.40	0.55	0.58
<u>Syringaldehyde</u>				
Total amount	10.4	10.5	11.3	12.0
Specific activity	0.15	0.4	0.52	0.59

specific activities of the ethanol-soluble esters of this acid are shown in Table XXIII, and in the total activity in Figure 33. In general the amount present was very small and the radioactivity level was extremely low.

The radioactivity of the three lignin aldehydes (Table XXIII and Figure 33) increased throughout the duration of the experiment.

2. From ferulic acid

The results of this experiment are shown in Figure 34 and in Table XXIV. Figure 34 illustrates a rapid initial increase in radioactivity of the soluble esters of ferulic acid although these may include the administered free acid. However, after the exogenous supply of the radioactive acid was removed the total activity of the soluble esters fell rapidly in the first 2 hours and thereafter at a slower rate. The same pattern is obvious with respect to specific activity (Table XXIV). A slow but steady build-up of radioactivity in the insoluble esters of ferulic acid and in the lignin aldehydes, vanillin and syringaldehyde, is clearly evident in these results. It is noteworthy that, in this experiment, the insoluble esters of ferulic acid did not achieve activities comparable to those of the soluble esters whereas in the cinnamic acid time-course experiment it did so almost immediately (Figure 33).

A steady decrease in the specific and total activities of sinapic acid was displayed throughout the experiment.

The results of these two experiments are clearly different from those obtained with the $^{14}CO_2$ time-course study (Figure 32 and Table XXII) since in the former experiments the insoluble esters of ferulic acid did not act like intermediates in lignin biosynthesis.

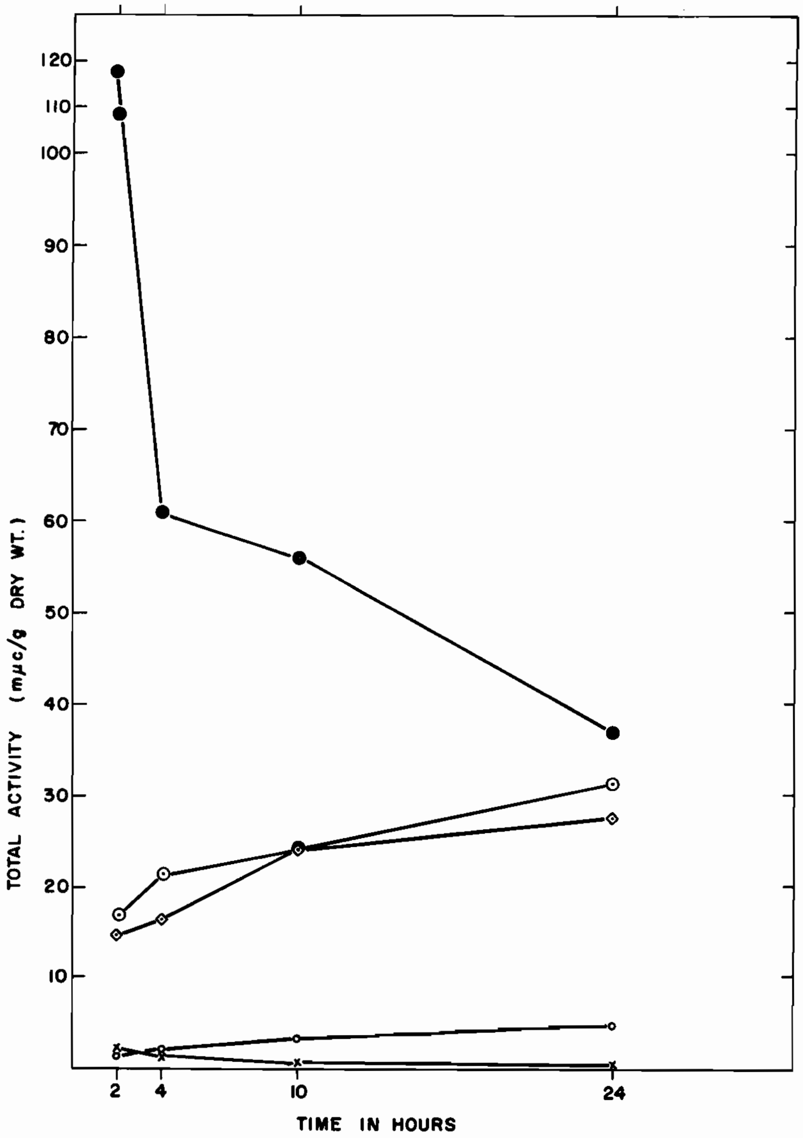


Figure 34. Changes with time in total activities of hydroxycinnamic acids and lignin aldehydes after administration of ferulic acid-3-^{14}C.

Table XXIV

Total Amounts and Specific Activities of Hydroxycinnamic Acids and Lignin Aldehydes Isolated after Administration of Ferulic Acid-3-^{14}C to Wheat Shoots

Total amount (μm per g. dry wt.) and specific activity (μc/mmole) of compounds isolated	Metabolic period (hours) after administration of ferulic acid-3-^{14}C			
	2	4	10	24
Ferulic acid				
Soluble Esters				
Total amount	6.48	5.32	5.19	4.81
Specific activity	16.75	11.87	10.83	9.18
Insoluble Esters				
Total amount	30.13	29.43	28.87	26.94
Specific activity	0.49	0.57	0.84	1.02
Sinapic acid				
Soluble Esters				
Total amount	1.75	1.53	1.47	1.07
Specific activity	1.34	0.90	0.50	0.49
Vanillin				
Total amount	60.66	61.45	61.18	65.66
Specific activity	0.28	0.35	0.39	0.48
Syringaldehyde				
Total amount	12.1	12.7	13.1	16.5
Specific activity	0.14	0.16	0.26	0.30

DISCUSSION

I. THE QUANTITATIVE PROCEDURE

The need for a quantitative procedure, for determination of the phenolic acids in plant extracts, became evident shortly after the present study had been initiated. A search in the literature revealed that there are very few methods which have been tried and none of these appeared to be suitable for the purposes of this study. Most of these are colorimetric methods and require a relatively high concentration of the compound to be determined. In addition, these methods are based on the reaction of the phenolic compound with a suitable chromogenic reagent, such as a diazonium salt, to form a highly colored complex from which the original phenolic compound is practically non-recoverable. Hence, subsequent utilization of the compound for other purposes, such as determination of its radioactivity, is obviously precluded. This is particularly so when it is remembered that breakdown of the compound and loss of some of the breakdown products are liable to occur. Furthermore, it has been reported (Neish, 1961) that determination of p-coumaric acid is more accurate by spectrophotometric rather than by colorimetric means.

The chromatographic technique of Ibrahim and Towers (1960) as modified by Bohm and Towers (1962) was found to be satisfactory for the separation of most of the more commonly occurring phenolic acids in plant extracts. However, there were a few difficulties encountered in the application of this chromatographic method which are pointed out below. Sinapic acid, particularly when present in large amounts, tends to overlap with ferulic acid. This also applies to the corresponding benzoic acids, syringic and vanillic acids respectively. In order to achieve clear separation of these compounds it was essential to maintain a proper saturation of the chromatographic tanks, to utilize freshly prepared solvents and to delay the development of chromatograms in the second solvent until all traces of the first solvent were removed.

The resolution of the geometric isomers of members of the cinnamic acid series in the second solvent, although useful in the identification of these compounds, makes it a tedious task when a large number of quantitative determinations have to be carried out as was the case in the biosynthetic studies.

An alkaline solvent was used for spectrophotometric determinations since under these conditions a bathochromic shift in the wavelength of maximum absorption, of each acid, is obtained. A considerable increase in the absorption is usually associated with this bathochromic shift (Jurd, 1957) thus allowing for the detection of a much smaller amount of the acid. This is very important when dealing with small amounts of plant extracts.

Unlike ferulic acid, p-coumaric and caffeic acids were reported by Sutherland (1956) to be unstable in ethanolic-sodium hydroxide solutions. A slow shift of the maximum absorption of p-coumaric acid to a lower wavelength is immediately apparent and, over a longer period, a new peak develops at 290 to 295 mμ, presumably due to decomposition associated with the double bond (Smith, 1955a). Sutherland (1956) further reported that this acid is relatively stable in 1 N aqueous sodium hydroxide solution over a period of several days. It has been indicated, however, in the present study that decomposition is induced by ultraviolet irradiation of p-coumaric acid in the absence of ethanol (Figure 13) under alkaline conditions. Therefore in applying the quantitative method, caution must be observed when locating the spots on chromatograms by means of ultraviolet light.

The quantitative method, as it presently stands, was found to give reproducible results within less than 5% of error. Amounts as low as 4 μg of any of the acids were successfully determined with a reasonable degree of accuracy by this method.

II. THE PHENOLIC ACIDS IN WHEAT

The phenolic acids in wheat tissues were found to be largely in a bound form, that is in ester or glycosidic linkages. Only trace amounts of p-coumaric and ferulic acids were detected in the free state and the possibility that these may have been released as a result of partial hydrolysis during the extraction procedure cannot be excluded. The results represented in Figure 14 illustrate the diversity of phenolic compounds occurring in shoots of this plant. It was estimated that kilogram quantities of fresh plant material would be needed in order to isolate some of these conjugates in amounts that would permit of their purification and structural elucidation.

Despite the fact that extensive studies have been carried out on the metabolism of cinnamic and hydroxycinnamic acids in relation to lignin biosynthesis in the wheat plant, there are no reports in the literature of previous attempts to identify the naturally-occurring forms of these acids in wheat tissues. The fact that these compounds occur in small amounts in an extremely complex mixture possibly explains why this work has not been attempted previously. Sahai and Shaw (1961) reported the isolation from wheat leaves of a Salkowski-positive compound which, on hydrolysis, gave rise to both p-coumaric and ferulic acids. Although 4 kilograms of plant material were used in this work, no positive identification of this compound could be achieved. These authors suggested that the unknown compound is either a mixture of p-coumaric and ferulic acid derivatives having identical chromatographic behaviour in the solvent systems used or that it is a derivative of both p-coumaric and ferulic acids. Reznik and Urban (1957) reported the presence of chlorogenic acid in wheat and that administration of labeled ferulic acid resulted in incorporation of activity into this compound. This meant that demethylation of ferulic acid had occurred. Recently, doubt has been cast on these results by the findings of Runeckles (1963b) that the incorporation of radioactivity from ferulic acid into chlorogenic acid by tobacco leaf discs is negligible. Runeckles presented evidence that feruloyl-quinic acid possesses similar R_f value to chlorogenic acid in the solvent system used by Reznik and Urban in their studies (1957), and pointed out the possibility that the compound identified by these authors as chlorogenic acid may have been, in fact, a feruloylquinic acid. The results of this study support Runeckles' views since caffeic acid was detected in only trace amounts in wheat tissues no matter what method of cultivation was used.

The formation of glucose esters of hydroxycinnamic acids has been reported by Higuchi and Brown (1963a) to occur in wheat plants from exogenously supplied labeled hydroxycinnamic acids but not from phenylalanine. However, in this work there was no indication as to the extent to which these esters were formed. Since more than one derivative of the hydroxycinnamic acids (e.g. ferulic and p-coumaric acid in Table III) is present in wheat tissues it will be important to identify these compounds and to establish their relationships.

The survey conducted on the distribution of the phenolic acids in hydrolyzates of extracts of various tissues of wheat revealed many interesting points which are worthy of comment. Ferulic, p-coumaric and vanillic acids were detected consistently in various tissues of this plant. All other acids accumulated in detectable amounts only at a certain stage or stages of growth and development. Furthermore, the method of cultivation appeared to have influenced, qualitatively and possibly quantitatively, the phenolic acid content of shoots. This is readily deduced from comparing the results represented in Tables IV and V. The last 4 columns of Table IV should be compared with the first 4 columns of Table V since they represent plants of almost equal chronological age. In greenhouse, soil-grown plants (Table V) sinapic acid was detectable only in 25-day old plants; ferulic acid displayed a continuous decrease in amount with increasing age of plants and in 45-day old plants was present exclusively in the form of a glycoside(s); caffeic and syringic acids disappeared from 55-day old plants. All of these compounds were consistently detectable in plants cultivated in nutrient media (Table IV) under controlled environmental conditions.

One or more of various explanations may be offered to account for these variations. The plants grown in nutrient media possessed thinner stems and narrower leaves than those grown by the other method. Furthermore there was no tillering whatsoever produced by the former plants whereas in the latter, 2 to 6 shoots were produced by each grain. These differences indicate that although both plants were of the same chronological age, nevertheless, they must have varied in their physiological age. In this respect, a distinction should be made between the synthesis of primary plant metabolites, such as sugars and amino acids, common to all living organisms, and secondary plant products, such as the phenolic acids considered here. Very often, changes in environment influence the synthesis of the latter substances in a more pronounced way than that of the primary metabolites. Such changes have often been noted but seldom fully exploited for a clearer understanding of the control of synthesis and metabolism of these compounds.

Since no artificial light supplement was used for plants grown in the greenhouse, their low phenolic acid content may have been caused by the filtering out by glass of ultraviolet light. Frey-Wyssling and Bäbler (1957) reported that tobacco plants grown under glass (in the greenhouse) produced only 14% of the chlorogenic acid normally produced under conditions in which the plant is exposed directly to sunlight. Furthermore, ultraviolet irradiation, for 1-1/2 hours daily, resulted in a slightly more than four fold increase in chlorogenic acid content and an 8-hour daily irradiation period brought it up to its normal level.

Flavonoid compounds were also shown to be equally sensitive to variations in environmental conditions surrounding plants. Rutin could not be detected at all in greenhouse-grown tobacco plants whereas the content of this flavonoid compound in field-grown plants was appreciable (Frey-Wyssling and Bäbler, 1957)].

Another environmental factor affecting the content of phenolic compounds in plants was reported by Gortner (1963) who showed that the total phenolic content of stem tissues of pineapple (which consists largely of p-coumaric and ferulic acid derivatives) is inversely related to the soil moisture content. However, the results of the present work are in contradiction to those reported by Gortner. The plants grown in nutrient media and thus exposed to higher moisture conditions were found to contain more phenolic acids than those grown in soil.

The variability in the phenolic acid content of plants grown under different conditions was absent from plants, of the same age, which were grown in a controlled environment. Qualitative differences could not be detected in quadruplicate batches of 5-day old shoots and the quantitative variations in the major phenolic acids were found to fall within the limits of experimental error (page 107). This provides clear cut evidence for the effect of environmental factors on the accumulation of certain phenolic acids in the wheat plant. Obviously more studies are needed along these lines in order to establish what particular factors are involved. This is particularly important for the understanding of the role(s) played by these substances since a myriad of effects on biological reactions has been ascribed to them. These include their role in plant disease resistance (Farkas and Kiraly, 1962), as activators or inhibitors of enzymic reactions, particularly indoleacetic acid oxidase (Zenk and Müller, 1963), and as cofactors in photosynthetic phosphorylation (Krogmann and Stiller, 1962).

The most striking feature of the phenolic acid pattern in wheat emerging from this study, is the dynamic status of these compounds. There were significant qualitative and quantitative changes (Figures 17, 18 and 19) during and subsequent to germination indicating that these compounds are not end-products of metabolism. Similar results were reported by other workers. Urban (1959) showed that not only seasonal but also diurnal variations in the amounts of phenolic compounds occur in sunflower, corn and wheat plants. After following the changes in phenolic content of various organs of _Theobroma cacao_, Griffiths (1958b) concluded that the phenolic compounds vary with the morphology of the tissue as well as with the physiological age of the material.

Pronounced quantitative changes of metabolites as well as of enzymes appear to be characteristic of germination in plants (Miller, 1938; Oota _et al._, 1953; Stafford and Magaldi, 1954; Akagawa and Beevers, 1957; and Webster, 1959). The seemingly inconsistent changes in the levels of metabolic intermediates during this process may be a result of changes in the levels of enzymes concerned with their synthesis and degradation. This view has been expressed by Krupka and Towers (1959) in connection with the changes in α-oxo acids in germinating wheat and it was suggested that a steady level of substrates is achieved only later in germination.

Quinic and shikimic acids, intermediates implicated in the biosynthesis of plant phenolic acids, have been shown by Szymanski (1962) to undergo an increase in amounts during and subsequent to germination of wheat seedlings reaching a maximum around 6 days after germination and falling rapidly thereafter. Quinic acid was completely undetectable after 17 days whereas small quantities of shikimic acid were still detectable. Phenylalanine and tyrosine ammonia lyases have been found in 7-day old wheat seedlings although these enzymes could not be detected in wheat germ (Neish, 1961). The formation of cinnamic acid and its derivatives would be affected not only by the levels of these enzymes but also by changing concentrations of phenylalanine and tyrosine as a result of protein hydrolysis and resynthesis.

The question as to whether the decrease in the phenolic acid content is due to translocation or to catabolic activity cannot yet be answered. Translocation of phenolic compounds has not been detected under natural conditions in plants (Bate-Smith, 1962a). On the other hand, oxidation of phenolic compounds as respiratory substrates has been established for microorganisms (Evans, 1958; Towers, 1964a) and strongly indicated in higher plants (Zaprometov, 1959; Chen, 1963).

The distinctive behaviour during growth and development of ferulic and p-coumaric acids, on the one hand, and of sinapic acid on the other, is also worthy of comment (see Figure 18). The results of McCalla and Neish (1959b) with Salvia indicated that these acids are related in the following way: cinnamic → p-coumaric → caffeic → ferulic → sinapic. Some of these hydroxylations can be effected through esters of these acids (Levy and Zucker, 1960). Sinapic acid could be formed by a hydroxylation of ferulic acid to give 3,4-dihydroxy-5-methoxycinnamic acid followed by O-methylation in the 5-position. Although the postulated intermediate has not been found to occur naturally, Higuchi and Brown (1963b) have shown that C^{14}-labeled 3,4-dihydroxy-5-methoxycinnamic acid as well as ferulic acid-β-C^{14} were converted to sinapic acid-C^{14}

when administered to wheat plants. While this may be considered good evidence for the derivation of sinapic acid from ferulic acid it does not follow that sinapic acid is a precursor of the syringyl residues in lignins. The results of the present investigation reveal that very little sinapic acid occurs either in the ethanol-soluble or insoluble fractions in young or old plants in contrast to ferulic and p-coumaric acid. This could be due to a slower rate of synthesis or to a rapid turn-over. From the studies with $^{14}CO_2$ in photosynthesis p-coumaric and ferulic acids were shown to be synthesized more readily than sinapic acid. Brown and Neish (1959) found that sinapic acid, alone among the several phenylpropanoid compounds judged to be efficient precursors of lignin, was not incorporated (in maple) into lignin structures which can be reduced to dihydrosinapyl alcohol. They concluded that the role of sinapic acid as a lignin precursor is more complex than that of cinnamic or ferulic acids.

The anomalies with regard to sinapic acid could be explained if it were assumed that the origin of the syringyl residues in lignins is the result of hydroxylation and O-methylation of guaiacyl residues in the lignin polymers. Sinapic acid, therefore, although it may become incorporated into lignins when administered to plants, may not be the natural precursor of the syringyl units found therein. It is of interest that a lignan acid, plicatic acid, which contains a monomethyl pyrogallol nucleus, has been found to occur naturally (Gardner *et al.*, 1959).

III. PHENOLIC ACIDS AND CHEMICAL TAXONOMY

It has been generally assumed that the phenolic compounds present in a plant tissue are characteristic of the species, a view based on the long-held belief that phenolic compounds are metabolically inert (Bate-Smith, 1958). Consequently the phenolic compounds were regarded as a potentially useful class of compounds in problems of chemical taxonomy. In the last decade a vast number of papers has been published on the phenolic acid contents of plants in which the presence or absence of certain hydroxycinnamic acids or even, sometimes, of a particular acid has been considered to be of taxonomic value (see, for instance, Pearl et al., 1957 and Griffiths, 1959).

The results of the present investigation point out the danger inherent in adopting this view. The question arises as to the actual meaning of variation between the phenolic acid contents of two plants since this may well be merely a reflection of differences in their environmental conditions. Important factors to be taken into consideration before any taxonomic use can be made of the phenolic acid contents of plant species include the age, the physiological state of the material, and the type of tissue to be analysed.

Since the assumption that phenolic acids are metabolically inert is no longer valid, more studies are needed to determine to what extent genetical factors and environmental factors are responsible for the types and amounts of the phenolic acids accumulating in plants.

In this investigation four phenolic acids and a coumarin of unusual interest were identified in the wheat plant. The first of these is orthoferulic (2-hydroxy-3-methoxycinnamic) acid which has so far not been reported for any other plant or microorganism. This acid is probably synthesized in the wheat plant in the following way: cinnamic acid → o-coumaric acid → 2,3-dihydroxycinnamic acid → o-ferulic acid, although the intermediate acids have not been detected so far. In the many surveys of plant phenolic acids which have been carried out with one-directional chromatographic systems it may have escaped detection because it is located close to ferulic acid and gives a similar fluorescence.

The second acid which has not been reported before as a constituent of wheat is o-hydroxyphenylacetic acid. This is the major acid of members of the genus Astilbe in the Saxifragaceae although it is not present in other genera of this family (Billek and Kindl, 1962). Apart from Billek and Kindl's survey of the Saxifragaceae no other systematic investigations of higher plants have been made for this acid. It will undoubtedly be found in other species since the closely related compound, o-coumaric acid is not an uncommon constituent of plants.

The other three compounds are p-hydroxyphenyllactic acid, homogentisic acid and 8-methoxycoumarin. The last compound was detected only when orthoferulic acid was found to be present in the plant extract indicating its formation from the latter acid by ortho-hydroxylation, isomerization to the cis-configuration and lactonization. Whether these compounds have any taxonomic importance or not awaits further study.

IV. BIOSYNTHESIS OF HYDROXYCINNAMIC ACIDS IN WHEAT

A. The Hydroxycinnamic Acids

Evidence for the participation of shikimic acid in the formation of hydroxycinnamic acids is given in Table XII in which it is shown that the simultaneous administration of non-radioactive shikimic acid with $^{14}CO_2$ in light resulted in a reduction in specific activities of the soluble and insoluble derivatives of p-coumaric and ferulic acids. These results also showed clearly that the ethanol-insoluble forms of these acids attained higher specific activities than the ethanol-soluble forms from $^{14}CO_2$ in photosynthesis in all experiments. Another important feature of the results presented in Table XII is that it indicates that the relationship of p-coumaric acid to ferulic acid is not a simple one.

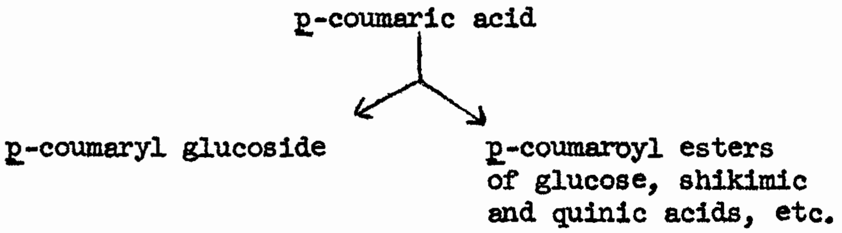
Since the specific activities of p-coumaric acid were always lower than those of ferulic acid even though the former acid was usually present in lower concentrations than the latter, only a small fraction of p-coumaric acid in the cells appears to be involved in the synthesis of ferulic acid. Whether the active p-coumarate is a soluble or an insoluble derivative could not be decided from this experiment.

When C^{14}-labeled phenylpropanoid compounds (including the aromatic amino acids) were administered (Table XIV) it was found again that the specific activities of p-coumaric acid were lower than those of ferulic acid in every case except when p-coumarate-C^{14} was administered. In this case the ethanol-soluble derivatives of p-coumaric acid had a higher specific activity than the corresponding derivatives of ferulic acid, which perhaps is not unexpected. These results show clearly that hydroxylation and O-methylation of p-coumaric acid proceeds through certain active derivatives. The identification of these active intermediates would be worthy of investigation.

These results are similar to those reported for the Krebs-cycle acids in different species of plants by McLennan et al. (1963). Great differences in the specific activities of these acids were observed after the administration of acetate-C^{14} and the differences were ascribed to the existence, in the cell, of different pools of each acid some of which are physically remote from the respiratory centres. It is apparent, therefore, that 'compartmentation' is not only characteristic of phenolic acids but also of the fundamentally more important Krebs-cycle acids.

As already mentioned, the nature of the ethanol-soluble derivatives of hydroxycinnamic acids in wheat presents a complex picture. Unless the important but difficult task of identifying the various forms of the phenolic acids is carried out further progress in our understanding of aromatic metabolism must be slow.

That cinnamic acid was the most efficient precursor of the hydroxycinnamic acids mentioned in Tables XIII and XIV may be explained by the fact that this compound lacks a free hydroxyl group and therefore cannot be glycosylated when introduced into plants. All the substituted cinnamic acids considered in this work possess at least one free hydroxyl which is readily glycosylated. A number of studies (Avadhani and Towers, 1961; Harborne and Corner, 1961; Runeckles and Woolrich, 1963) has shown that the soluble phenolic acid fraction, obtained from plants which have been administered cinnamic or hydroxycinnamic acids, consists of esters and glucosides of these acids. p-Coumaric acid, for example would, to some extent be metabolized as follows:



In tobacco, the major derivative of ferulic acid was reported to be the glucoside (Runeckles and Woolrich, 1963). Glycoside formation may explain why there was a greater per cent conversion to p-coumaric acid in the ethanol-soluble fraction from cinnamic acid-C^{14} than from p-coumaric acid-C^{14} itself (Table XVII). Similarly it may explain why there was far less activity in the ferulic acid recovered from the soluble fraction when ferulic acid-C^{14} was provided than from cinnamic acid-C^{14}. Administered ferulic and p-coumaric acids would give rise, in part, to glucosides and these would not be affected by alkaline hydrolysis. Acid hydrolyses were not carried out on the ethanol-soluble fractions in these experiments. Hence the radioactivity incorporated into these glucosides would not be included in these determinations.

B. The Hydroxybenzoic Acids

The problem of the origin of vanillic and syringic acids in wheat has been partially resolved. Their structures and their similar behaviour in wheat suggest that they are closely related metabolically. Geissman and Hinreiner (1952) have suggested that aromatic compounds of the C_6-C_1 class may be regarded as degradation products of members of the C_6-C_3 class. Thus vanillic and syringic acids could be derived from ferulic and sinapic acids respectively. Alternatively they may be formed as follows: 5-dehydroshikimic → protocatechuic → vanillic → syringic. The enzymatic synthesis of protocatechuic acid from 5-dehydro-shikimic acid has been demonstrated with Neurospora (Gross et al., 1956).

The formation of p-hydroxybenzoic, vanillic and syringic acids in wheat must have been by a fairly direct route because even from CO_2 they had much higher specific activities than their C_6-C_3 analogues in the soluble fraction (Tables XIV and XVIII). This is also indicated by the fact that when they were formed from administered hydroxycinnamic acids labeled in the β-carbon, the activity was confined to the carboxyl carbon of the C_6-C_1 acid (Table XIX).

The manner in which they may be formed from active C_6-C_3 derivatives of the hydroxycinnamic acids is indicated below:

$$\begin{tikzcd}
\text{R-CH=CH-COSCoA} \arrow[d, "+ H_2O"'] \\
\text{R-CHOH-CH}_2\text{-COSCoA} \arrow[d, "- 2H"'] \\
\text{R-CO-CH}_2\text{-COSCoA} \arrow[d] \\
\text{R-COOH} + \text{CH}_2\text{COOH}
\end{tikzcd}$$

where R = p-hydroxyphenyl, guaiacyl or syringyl nucleus.

What is the subsequent fate of these C_6-C_1 compounds which appear to be ubiquitous in plants? Although they accumulate in some species (Birkofer et al., 1961) they never appear to be present in wheat in high concentration either in the ethanol-soluble or ethanol-insoluble fraction yet they are readily formed in photosynthesis. This would suggest that they represent a stage in the breakdown of phenylpropanoid compounds leading to ring cleavage and terminating in complete oxidation to CO_2. This possibility can only be examined by the administration of ring-labeled hydroxybenzoic acids. These are not available commercially at this time.

V. PHENOLIC ACIDS AND LIGNIN FORMATION

Preliminary studies on the formation of the hydroxycinnamic acids from $^{14}CO_2$ in photosynthesis indicated that they were formed very slowly and that only the major acids present incorporated any measurable activity, e.g., p-coumaric and ferulic acids in wheat and caffeic acid in Hydrangea. Furthermore, the detection of radioactivity in lignin aldehydes but not in the hydroxycinnamic acids in short term experiments with $^{14}CO_2$ in light (Table XI) pointed to the possibility that these ethanol-soluble derivatives of the phenolic acids were not intermediates in lignin biosynthesis. Attention was directed, therefore, to investigating the ethanol-insoluble derivatives of these acids.

Evidently there is a complex relationship between intermediates of lignin biosynthesis and the soluble and insoluble esters of hydroxycinnamic acids. The time-course experiments with $^{14}CO_2$ (Figure 32), which represent the closest approach to normal physiological conditions, strongly suggest that the intermediates in lignin biosynthesis are the esters of the hydroxycinnamic acids which are not soluble in ethanol.

This would appear to eliminate the free acids or quinic acid esters as important intermediates under natural conditions. However when the free acids are administered they appear to be incorporated into lignin by way of soluble esters. This difference is probably not due to the method of administering the precursors since phenylalanine and tyrosine, although fed in the same way as the cinnamic acid derivatives, acted like carbon dioxide in labeling the insoluble esters more than the soluble esters (Tables XIII and XIV). This is good evidence that these amino acids are natural precursors of lignin in wheat.

Figure 35 outlines a working hypothesis which is an attempt to explain these results in the simplest way possible. Under natural conditions plants assimilate carbon dioxide by photosynthesis and use carbohydrate thus obtained for synthesis of phenylalanine and tyrosine by the shikimic acid pathway. In addition to contributing to protein synthesis these amino acids can undergo loss of nitrogen to form the corresponding cinnamic acid derivatives. It has been demonstrated that plants contain enzymes which can convert L-phenylalanine to cinnamic acid or L-tyrosine to p-coumaric acid *in vitro*. It is possible that in the environment of the living cell these acids are not released from the enzymes but undergo further reactions, which eventually lead to formation of lignin, without ever existing as free acids. The formation of the free acids in the *in vitro* systems may be due to the high pH (*ca* 9) or to the presence of esterases. It can be visualized that an enzyme-ester of a hydroxycinnamic acid would be insoluble in hot 80% ethanol and this may be the nature of the active "insoluble esters" of Figure 35. A considerable amount of hydroxycinnamic acids is obtained by alkaline

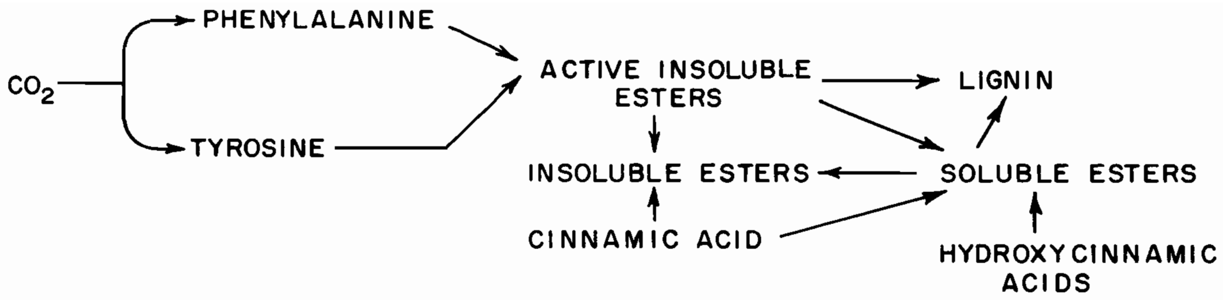


Figure 35. Hypothetical outline of lignin biosynthesis.

hydrolysis of the ethanol-insoluble fraction and it is not suggested that this is all bound to enzymes. Compounds, such as the ferulic ester of xylan (Fausch et al., 1963) may be present in greater amounts but need not be regarded as active intermediates. Obviously more information is needed concerning the exact nature of the "insoluble esters" of the hydroxycinnamic acids before this data can be interpreted fully.

When salts of cinnamic acid or the hydroxycinnamic acids are fed there is apparently an activation of the carboxyl group leading to formation of soluble esters from which lignin can be synthesized. There is also a slow exchange between the soluble and insoluble esters but the data suggest (Figures 33 and 34) that the insoluble esters formed here are not active intermediates in lignin biosynthesis. Apparently wheat plants are able to incorporate hydroxycinnamic acids into lignin by at least two routes. Under natural conditions the route involving ethanol-insoluble intermediates seems to be the more important. It has not been established whether the lignins formed by these routes are identical. The fact that the ratios of specific activities of the lignin aldehydes depended very markedly on the precursor (Table XXI) is suggestive.

Brown et al. (1958) found that p-hydroxyphenylpyruvic acid was utilized by wheat about twice as efficiently as phenylalanine for the formation of p-hydroxyphenyl lignin and only about one-fourth as efficiently for the synthesis of the other two types of lignin. As the material which was analyzed was not known to be nitrogen-free and since Stone et al. (1951) had concluded that p-hydroxybenzaldehyde could arise from the oxidation of tyrosine in the protein associated with the cell wall Brown et al. (1958) questioned the significance of their results.

In another study with wheat, however, Brown and Neish (1956) showed that there was a preferential synthesis of p-hydroxyphenyl lignin from p-coumaric acid, (which is not convertible to tyrosine in wheat) and of guaiacyl and sinapyl lignins from ferulic and sinapic acids respectively.

As we have seen from Tables XX and XXI the results with CO_2 on the distribution of activity between the lignin aldehydes is distinctly different from that obtained when hydroxycinnamic acids were used as precursors. Although the acids of the insoluble fraction would contribute to the yield of lignin aldehydes, since oxidations were carried out on non-hydrolyzed samples of cell-wall material, their contribution would not be sufficient to account for the difference.

The preferential synthesis of guaiacyl lignin from ferulic acid may be a reflection of the relative rates of two sets of competing reactions, the one leading to further ring substitution and the other to reduction and polymerization. This would apply to the preferential synthesis of p-hydroxyphenyl lignin from p-coumaric acid as well and this explanation has been proposed by Brown and Neish (1956). Alternatively it may be suggested that the exogenously supplied hydroxycinnamic acids either contribute to two different types of lignin 'building stones' or that they are converted to lignin via two different pathways one of which does not allow for further ring substitution.

SUMMARY

1. An extensive review of the literature has been made concerning the chemistry, distribution and metabolism in plants of phenolic acids and their derivatives.

2. A quantitative method, based on spectrophotometry and paper chromatography, for the estimation of phenolic acids in plant extracts, was developed and applied to the analysis of phenolic acids in wheat tissues.

3. Quantitative determinations were made of p-coumaric, ferulic, sinapic, p-hydroxybenzoic, vanillic and syringic acids which occur as alkali- and acid-hydrolyzable derivatives in ethanolic extracts of wheat tissues. The ethanol-insoluble residues also yielded phenolic acids on cold alkaline hydrolysis. Comparatively large quantities of p-coumaric and ferulic acids were obtained from this fraction, the amounts depending on the age of the tissue. Phenolic acids in the ethanol-soluble fraction reached a maximum concentration about 9 days after germination. The amounts fell off with increasing age, reaching a minimum 4 to 5 weeks later. The behaviour of sinapic acid was markedly different from that of p-coumaric and ferulic acids and it is suggested that it may not be a natural precursor of the syringyl units of lignin.

4. Orthoferulic (2-hydroxy-3-methoxycinnamic) acid was isolated in small amount from an alkaline hydrolyzate of an ethanolic extract of wheat shoots. This phenolic acid was found in shoots only at certain stages of growth and was absent from embryos, grains or roots. A number

of other phenolic compounds, hitherto not reported for wheat, were also identified. These are p-hydroxyphenyllactic and homogentisic acids and 8-methoxycoumarin. In addition, the chromatographic properties, color reactions, fluorescence and distribution of a number of unidentified phenolic compounds in hydrolyzates of wheat tissues, were described.

5. The formation from $^{14}CO_2$ in photosynthesis and from C^{14}-labeled phenylpropanoid compounds of soluble and insoluble derivatives of the hydroxycinnamic and hydroxybenzoic acids as well as of lignin aldehydes obtained by alkaline nitrobenzene oxidation in wheat tissues, was measured. Labeled CO_2, phenylalanine or tyrosine were incorporated more readily into the phenolic acids bound to the insoluble residue than into the ethanol-soluble derivatives whereas the reverse was true for labeled precursors such as cinnamic, p-coumaric, caffeic, ferulic and sinapic acids.

6. Time-course studies with labeled carbon dioxide suggested the sequence: carbon dioxide → aromatic amino acids → ethanol-insoluble derivatives of the phenolic cinnamic acids → lignin. Soluble derivatives of the hydroxycinnamic acids appeared to exchange with the insoluble derivatives and to act as intermediates in lignification especially when the free acids were administered. A scheme is suggested to explain these complex relationships.

7. The hydroxybenzoic acids in wheat were shown to be synthesized from the corresponding phenylpropanoid analogues by removal of two carbons of the 3-carbon side-chain. These C_6-C_1 compounds attained high specific activities from $^{14}CO_2$. They probably represent a stage in the catabolism of phenylpropanoid compounds.

CLAIM TO ORIGINALITY AND CONTRIBUTION TO KNOWLEDGE

This is the first study to have been made of the formation of hydroxycinnamic and hydroxybenzoic acids in any plant from CO_2 in photosynthesis and to show the relationships of the C_6-C_3 acids to each other and to lignin. A hitherto unreported pool of hydroxycinnamic acids in ethanol-insoluble residues of wheat tissues has been shown to be active in lignin biosynthesis and a new scheme of lignin synthesis, incorporating these results, has been suggested.

Studies of the phenolic acids in wheat during growth and development have been carried out by means of a newly developed quantitative method. Such a study has not been made before with any plant.

A new phenolic acid, 2-hydroxy-3-methoxycinnamic acid and a derivative of it, 8-methoxycoumarin, have been shown to be natural constituents of the wheat plant. These compounds have not been found to occur in other plants or microorganisms.

The hydroxybenzoic acids of wheat tissues were shown to be derived from their corresponding C_6-C_3 analogues. This has been suggested by others but has never been demonstrated before.

The results of this study should be of value to scientists interested in the metabolism of aromatic compounds and the process of lignification in vascular plants.

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