Amide-Linked Conjugates of Ferulic Acid in Wheat

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AMIDE-LINKED CONJUGATES OF FERULIC ACID IN WHEAT

By: Frederick Graham Kosmolak

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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To Norma

for her encouragement, support and understanding.

ABSTRACT

Two metabolites closely related to N-feruloyl-2-hydroxyputrescine were isolated from the ethanol-soluble fraction of resistant and susceptible reacting wheat leaves infected with rust. The isolated conjugates contain ferulic acid, conjugated in amide-linkage with either hydroxyputrescine, hydroxyornithine, or hydroxyproline, and yet another moiety. The tri-acetate derivative of one of the isolated conjugates has a molecular weight of 494. The isolation and characterization of these new amide-linked conjugates of ferulic acid are reported.

Feeding of D-arginine and D-glutamate did not increase the levels of the newly isolated conjugates or of N-feruloyl-2-hydroxyputrescine in wheat leaves. The amino acid γ-hydroxyarginine, purified from seeds of Vicia sativa, effected a substantial decrease in the accumulation of N-feruloyl-2-hydroxyputrescine when fed to rust-infected resistant wheat leaves, whereas the amino acids D-ornithine and γ-hydroxyglutamate effected an increase. The hydroxy amino acids could not be detected as normal constituents in the amino acid fraction of rusted wheat leaves. An examination of the ethanol-insoluble residue revealed an absence of amide-linked ferulic acid but suggested the presence of amide-linked p-coumaric acid.

Amide-linked conjugates of ferulic acid were tested for biological activity and compared to that of free ferulate. The conjugates had no effect on the germination of stem rust uredospores at ferulate concentrations causing 100% inhibition. Plant injection bioassay studies

demonstrated the inability of N-feruloyl-2-hydroxyputrescine to induce necrotic lesions in susceptible rust-infected wheat leaves at a concentration of 10^{-3} moles/liter. The biogenesis and metabolic role of N-feruloyl-2-hydroxyputrescine in resistant reacting rusted wheat leaves is discussed.

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INTRODUCTION

In plants, cinnamic acid derivatives generally occur in combined form as esters or glycosides (1,2), however they also occur as conjugates with an amide linkage. The occurrence of amide-linked conjugates of C_6C_3 and other phenolic acids is described in the literature review. The most common non-phenolic moieties in these conjugates are putrescine (3), spermidine (4) and agmatine (5).

It is well established that agmatine and putrescine accumulate in potassium-deficient barley leaves (6) which exhibit characteristic necrotic symptoms. Coleman and Richards (7) found that a necrosis characteristic of potassium deficiency could be induced by feeding putrescine to normal barley leaves. Samborski and Rohringer (8) have described the accumulation of N-feruloyl- and N-p-coumaroyl-2-hydroxyputrescine in wheat leaves showing a high degree of chlorosis and necrosis. Their biogenesis study indicated that the origin of the aromatic moieties, ferulate and p-coumarate, was consistent with current views (9,10) on the origin of phenyl-propanoid constituents in plants. The origin of the hydroxyputrescine moiety remained unsettled. In rust-infected, resistant wheat leaves that contained the 2-hydroxyputrescine amides, activity from arginine-U-^{14}C, ornithine-5-^{14}C, and proline-U-^{14}C was incorporated with low efficiency into the abnormal metabolites. No free hydroxyputrescine was detected in the tissue containing the amides, and the role of these amides in wheat is still uncertain.

A strain of Pseudomonas lacking spermidine is the only other source in which free hydroxyputrescine has been detected as a natural metabolite (11). In this organism, hydroxyputrescine is bound to ribosomes in an amount inversely proportional to bound Mg^{2+}. The diamine, which has the same number of hydrogen binding sites as spermidine may play a more specific role than magnesium or putrescine in protein synthesis in vivo, possibly by acting as a polyfunctional ligand in aligning the components of the protein synthesizing mechanism. Cadaverine-(1-5^{14}C) (12), putrescine-^{14}C (13), and ornithine-^{14}C (13) have been reported to be effective precursors to other amide-linked conjugates of phenolic acids. Amide-linked conjugates of phenolic acids with proven biological activity in a host/parasite complex are p-coumaroylagmatine and the hordatines isolated by Stoessl (14). They exhibit antifungal activity by inhibiting the germination of Monilinia fructicola.

The origin of the basic moiety of N-feruloyl-2-hydroxyputrescine was investigated to gain additional information as to the metabolism of phenolic acids, amines, and their conjugates in relation to chlorosis and necrosis in wheat.

LITERATURE REVIEW

The biochemistry of naturally occurring phenolic compounds has been reviewed extensively by several authors in recent years (15,16,17, 18,19,20,21,22). The reviews describe the occurrence, metabolism and physiological function of many aromatic metabolites such as phenolic acids, coumarins, flavonoids, alkaloids, and lignins.

Some of the more common metabolic reactions of C_6C_3 phenolic acids are shown in Fig. 1. Many phenolic acids, such as hydroxy- and methoxycinnamates occur in conjugation as glycosides or esters (1,2,23, 24,25). The formation and possible function of phenolic glycosides has been discussed by Pridham (1), but little is known about their metabolic role. Towers (26) has suggested that future studies may indicate whether certain phenolic glycosides function as detoxication products, food reserves, or respiratory substrates. Phenolic acid esters such as the "insoluble esters" (27) may be natural intermediates in the biosynthesis of lignin. Other esters may function as antioxidants (28), cell wall components (29), metabolic control factors (30), or they may have a role in host-parasite interactions (31).

The concept of amide-linked phenolic acids as a class of phenolic acid conjugates is relatively recent. The possibility of this was first mentioned by Towers (26) and later elaborated by Gross and Zenk (32,33). A survey of the literature revealed the occurrence of at least fifty-six amide-linked conjugates of aromatic acids. Of these, thirty-one are from plant sources (Table I), the remaining conjugates from both animal and bacteria (Table II).

The differences between conjugates of plant, animal, and bacterial origin are as interesting as their similarities of linkage. Most of the conjugates occuring in plants are of the cinnamic acid type, while in animals this type of conjugate is represented by only two examples. Conjugates of animal and bacterial origin commonly contain benzoate as the aromatic moiety. Conjugates isolated from these two types of sources also usually have different non-aromatic moieties. In plants, the most common non-aromatic moieties of conjugates are amines. This is in direct contrast to the amino acid conjugates isolated from animals and bacteria.

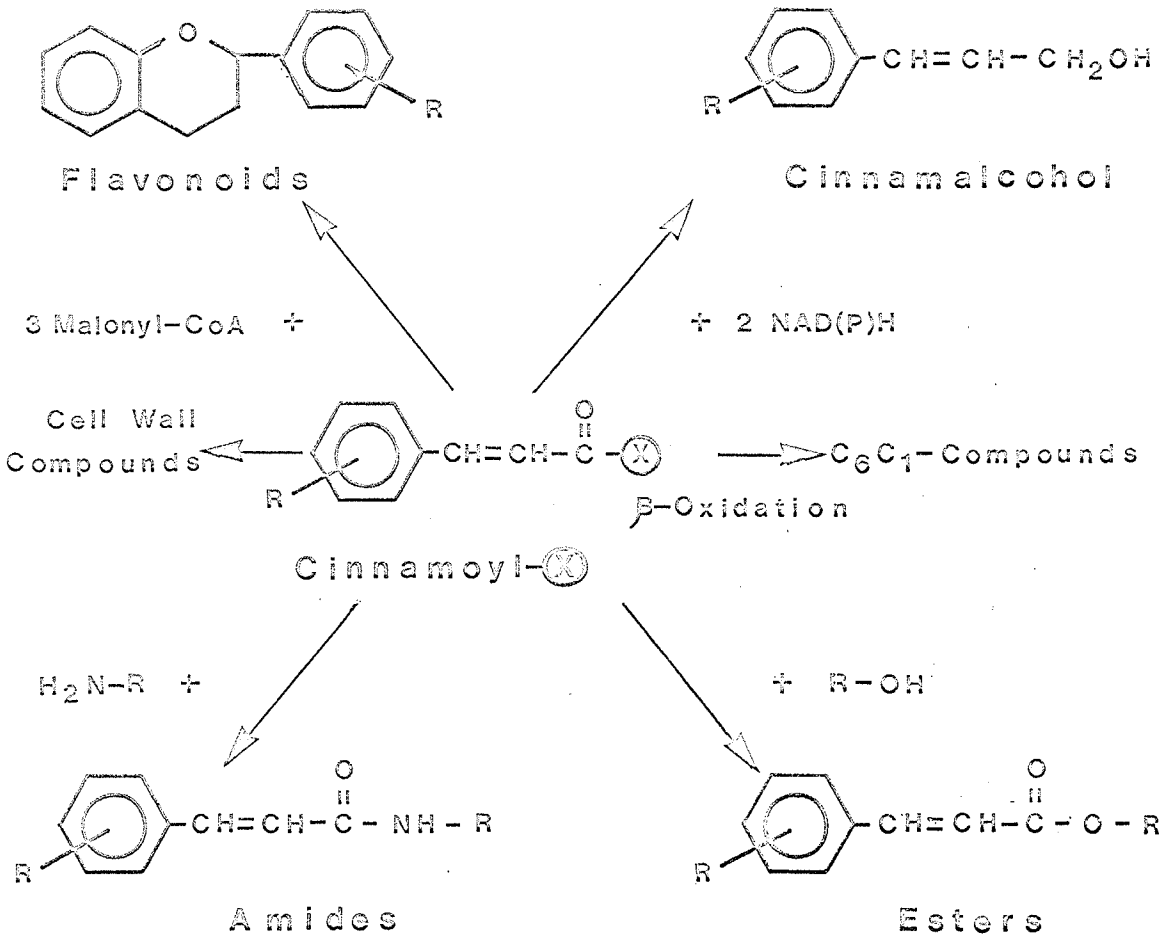
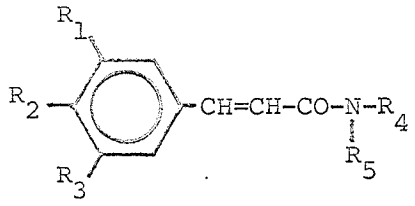


FIGURE 1. Metabolic reactions of C_6C_3 phenolic acids.

TABLE I

AMIDE-LINKED CONJUGATES OF AROMATIC ACIDS IN PLANTS



1. trans-N-Feruloylputrescine; subaphylline; trans-N-(4-aminobutyl)-4-hydroxy-3-methoxycinnamamide.

   $R_1=R_4=H$, $R_2=OH$, $R_3=OCH_3$, $R_5=(CH_2)_4NH_2$

   Source: Salsola subaphylla (34), citrus (3), tobacco (13)

2. trans-N-p-Coumaroylputrescine; trans-N-(4aminobutyl)-4-hydroxycinnamamide.

   $R_1=R_3=R_4=H$, $R_2=OH$, $R_5=(CH_2)_4NH_2$

   Source: tobacco (13)

3. trans-N-Caffeoylputrescine; trans-N-(4-aminobutyl)-3,4-di-hydroxycinnamamide.

   $R_1=R_2=OH$, $R_3=R_4=H$, $R_5=(CH_2)_4NH_2$

   Source: tobacco (13,36,37)

4. trans-N-p-Coumaroyl-2-hydroxyputrescine; trans-N-(4 amino-2-hydroxybutyl)-4-hydroxycinnamamide.

   $R_1=R_3=R_4=H$, $R_2=OH$, $R_5=CH_2CHOH(CH_2)_2NH_2$

   Source: wheat (35)

TABLE I - cont'd.

5. trans-N-Feruloyl-2-hydroxyputrescine; trans-N-(4 amino-2-hydroxybutyl)-4-hydroxy-3-methoxycinnamamide.

$R_1=R_4=H$, $R_2=OH$, $R_3=OCH_3$, $R_5=CH_2CHOH(CH_2)_2NH_2$

Source: wheat (35)

6. trans-N-p-Coumaroylagmatine; 1(trans-4-hydroxycinnamoylamino)-4-guanidinobutane.

$R_1=R_3=R_4=H$, $R_2=OH$, $R_5=(CH_2)_4NHC(=NH)NH_2$

Source: barley (5)

7. Maytenine; di-trans-cinnamoylspermidine

$R_1=R_2=R_3=R_4=H$, $R_5=(CH_2)_3NH(CH_2)_4NHOCCHCHC_6H_5$

Source: Maytenus chuchuhuasha (4)

8. trans-N-Feruloylglycyl-L-phenylalanine.

$R_1=R_4=H$, $R_2=OH$, $R_3=OCH_3$, $R_5=CH_2CONHCH(COOH)CH_2C_6H_5$

Source: barley seeds (38)

9. trans-N-Cinnamoylhistamine.

$R_1=R_2=R_3=R_4=H$, $R_5=(CH_2)_2CCHNHCHN$

Source: Leguminosae (39)

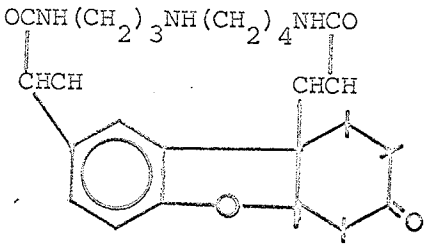
TABLE I - cont'd.

10. Casimiroedine; trans-N^{α}-cinnamoyl-N^{α}-methylhistamine-N-glucoside.

$R_1=R_2=R_3=H$, $R_4=CH_3$, $R_5=(CH_2)_2\underset{NHCH}{\overset{|}{C}}CHNC_6H_{11}O_5$

Source: Casimiroa edulis (40,41,42)

11. Lunarine.



Source: Lunaria biennis, L. rediviva (43)

12. Aegelin; egelin; trans-N(p-methoxyphenyl)α-hydroxy-β-ethyl-cinnamamide.

$R_1=R_2=R_3=R_4=H$, $R_5=CH_2CHOHC_6H_5OCH_3$

Source: Aegle marmelos (44)

13. trans-N-(p-hydroxyphenyl)β-ethyl-4-hydroxcinnamamide.

$R_1=R_3=R_4=H$, $R_2=OH$, $R_5=(CH_2)_2C_6H_5OH$

Source: Evodia belahe (45)

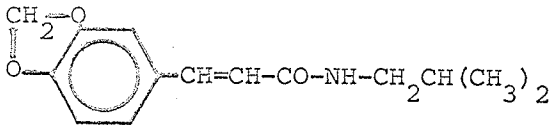
14. Herclavin; trans-N-(p-methoxyphenyl)-β-ethyl-N-methylcinnamamide.

$R_1=R_2=R_3=H$, $R_4=CH_3$, $R_5=(CH_2)_2C_6H_5OCH_3$

Source: Rutaceae (46,47)

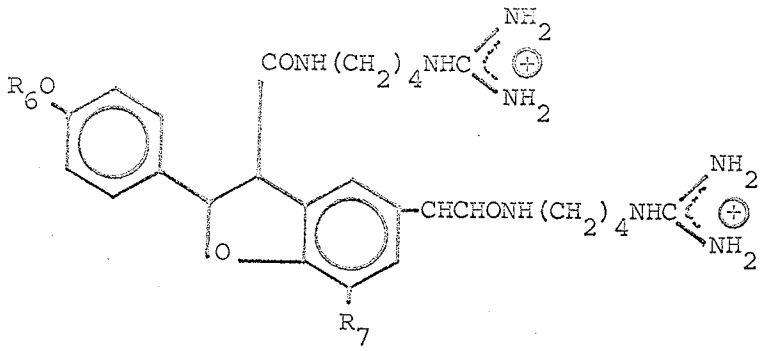
TABLE I - cont'd.

15. Fagaramide; trans-N-isobutyl-3,4-methylenedioxycinnamamide.



Source: Fagara xanthoxyloides (48), Zanthoxylum maccophyllum (49)

16. Hordatine A: $R_6=R_7=H$; Glucoside R_6=-D glucopyranosyl

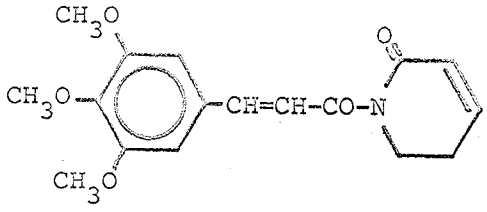


Source: barley (50)

17. Hordatine B: as hordatine A except $R_7=OCH_3$

Source: barley (50)

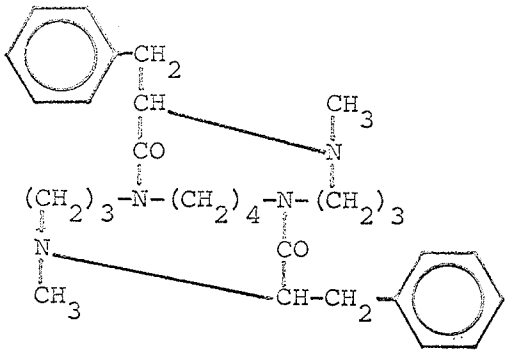
18. Piplartine;



Source: Piper longum (51,52)

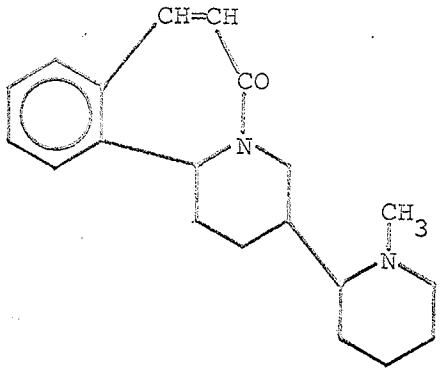
TABLE I - cont'd.

19. Homaline:



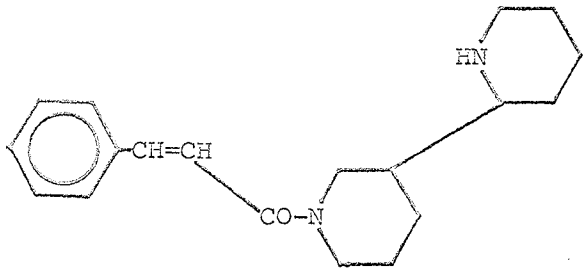
Source: Homalium sp. (53)

20. Astrocasine:



Source: Astrocasia phyllanthoides (54)

21. Astrophylline:

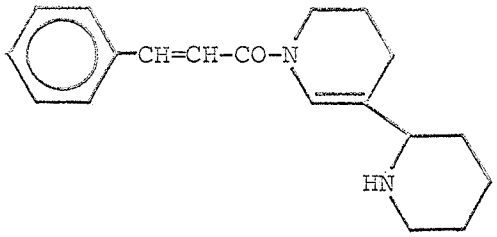


Syn. cis-N-cinnamoyl-3-(S)-[2'(R)-piperidyl]-piperidine

Source: Astrocasia phyllanthoides (55)

TABLE I - cont'd.

22. L-Adenocarpine:



Syn. 1'[trans-cinnamoyl]-2',3'-dehydrobipiperidyl-(2,3');
1[trans-cinnamoyl]-Δ^2-tetrahydroanabasine

Source: Papilionaceae (56)

23. Orensin: trans-cinnamoyl racemate of (+) and (-) adenocarpine

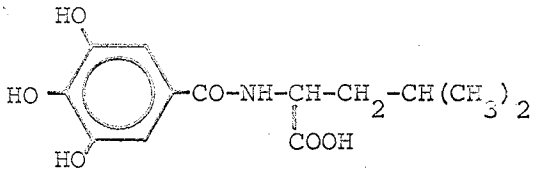
Source: Papillionaceae (57,58)

24. Isoorensin: cis-cinnamoyl racemate of (+) and (-) adenocarpine

Syn. Neoorensin

Source: Papillionaceae (57,58)

25. N-Galloylleucine:

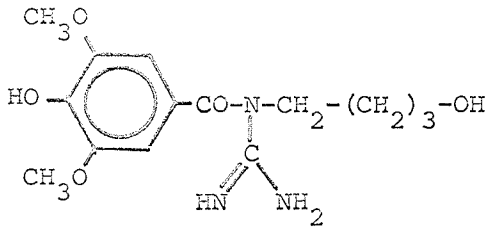


Syn. N-3,4,5-trihydroxybenzoylleucine

Source: Quercus aegilops (59,60)

TABLE I - cont'd.

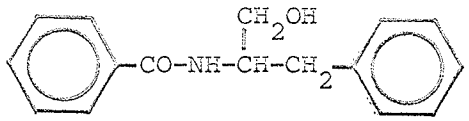
26. Leonurine:



Syn. N-syringeoyl-δ-hydroxy-N-butylguanidine

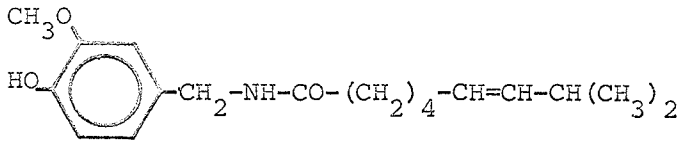
Source: Leonuras sibiricus (61)

27. N-Benzoyl-L-phenylalaninol:



Source: Catharanthus pusillus (62)

28. Capsaicin:

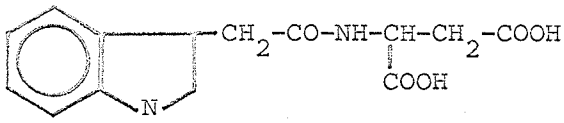


Syn: trans-N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-trans-6-enamide

Source: red pepper (63)

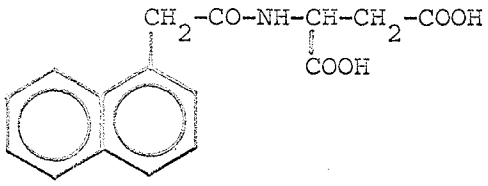
TABLE I - cont'd.

29. N-Indolylacetylaspartic acid:



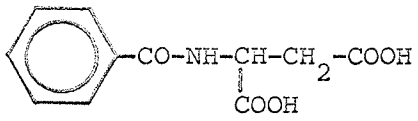
Source: pea (64)

30. N-(1-Napthaleneacetyl)aspartic acid:



Source: pea (65)

31. N-Benzoylaspartic acid:



Source: pea (65,66,67)

TABLE II

Amide-Linked Conjugates Of Aromatic Acids
In Animals And Bacteria

Name(s):	Source
1. Hippuric acid; N-benzoylglycine	mammals, insects, pigeons, reptiles (68,cf.69)
2. Ornithuric acid; N-2,5-di-benzoylornithine	domestic birds, reptiles (cf.69)
3. Phenylacetylglutamine	man (70), chimpanzee (71)
4. N-(3-Indolylacetyl)glutamine	man (72)
5. 3,4,Dihydroxy-5-methoxyphenyl-glutamine	man (73)
6. N-(4-Hydroxy-8-monoglucuronyl-quinoline-2-carboxyl)serine	rats (74)
7. N-(4,8,Diglucuronylquinoline-2-carboxyl)serine	rats (74)
8. δ-Nicotinylornithine	chick (75)
9. α-Nicotinylornithine	chick (75)
10. 2,5,Dinicotinylornithine	chick (75)
11. Disalicyloylornithine	reptiles (76)
12. p-Aminohippuric acid; N-p-aminobenzoylglycine	reptiles (76)
13. N^5-p-Aminobenzoylornithine	reptiles (76)
14. N-p-Coumaroylglycine	man (77), rats (78)
15. N-Feruloylglycine	man (77), rats (78)
16. N-Vanilloylglycine	man (77), rats (78)
17. N^2-Benzoylornithine	domestic birds (79)

TABLE II - cont'd.

Name(s):	Source
18. N^2-p-Aminobenzoylornithine	chicken (80)
19. 2,3,Dihyroxybenzoylglycine	B.subtillus (81)
20. 2,3,Dihyroxybenzoylserine	E.coli K_{12} (82)
21. 2-N,6-N,Di(2,3,dihydroxybenzoyl)-L-lysine	A. vinelandii (83)
22. Cinnamamide	Streptomyces (84,85)
23. Indolepropionylserine	B.megaterium (86)
24. Indolepropionylalanine	B.megaterium (86)
25. Indolepropionylthreonine	B.megaterium (86)

Amide-linked Conjugates Of Plant Origin

N-Feruloylputrescine was first isolated from Salsola subaphylla where it constitutes 0.087% of the plant (34). No mention to its biogenesis or biological function was made. Wheaton and Stewart (3) found N-feruloylputrescine in all commercial grapefruit and orange varieties examined, but not in any of the tangerine or lemon varieties. In four samples of grapefruit juice the concentration of N-feruloylputrescine ranged from 22 to 34 mg/liter of juice. This is an unusually high concentration, considering the relative rare occurrence of amide-linked conjugates of phenolic acids. Mizusaki et al (13) reported that considerable amounts of the radioactivity from administered ^{14}C-putrescine or ^{14}C-ornithine were incorporated into N-feruloylputrescine, N-p-coumaroylputrescine, and N-caffeoylputrescine in callus tissue cultures of Nicotiana tabacum L. Although the N-caffeoylputrescine content of the tissue samples varied from 0.2 to 1 mg/g fr. wt. (others were present in lesser amounts), none of these conjugates could be detected in normal tobacco tissue. When tobacco callus tissue was grown without the addition of exogenous putrescine or ornithine, N-caffeoylputrescine was not present in detectable amounts (37). The conjugate was detected only in the apex of the vegetative plant, and later in the flowers. It was not present in fully expanded leaves of the plant under normal growing conditions.

N-Feruloyl-2-hydroxyputrescine and N-p-coumaroyl-2-hydroxyputrescine were isolated in milligram amounts by Samborski and Rohringer (8), from rust-infected seedlings of wheat. This was the first report on the occurrence of these conjugates, and of hydroxyputrescine in a natural

source (35). Since then, Rosano and Hurwitz (11) have found free hydroxyputrescine in a species of Pseudomonas lacking spermidine, while no free hydroxyputrescine had been detected in the wheat tissues containing the hydroxyputrescine conjugates. These conjugates are abnormal metabolites of wheat, and conditions favoring conjugate production in wheat did not elicit conjugate formation in oats or barley. Production of the conjugates in wheat was correlated with chlorosis- or necrosis-producing treatments, and the largest amounts were produced under conditions of sustained stress. Although the conjugates were formed in wheat leaves as a response to a variety of treatments, the largest amounts were produced in rust-infected, resistant-reacting leaves. The amount of N-feruloyl-2-hydroxyputrescine in these leaves 8 days after infection was 0.49 μ mole ferulic acid equivalents/g fr.wt. Tyrosine-U-^{14}C did not give rise to activity in either of the conjugates. More activity from phenylalanine-U-^{14}C was recovered in the p-coumaroyl conjugate than in that of ferulic acid. Activity from arginine-U-^{14}C, ornithine-5^{14}C and proline-U-^{14}C was incorporated with very low efficiency into the conjugates. Putrescine-1-^{14}C, although metabolized, was not incorporated into the conjugates. The origin of the hydroxyputrescine moiety and possible metabolic function of the conjugates was not settled.

Stoessl (5) isolated N-p-coumaroylagmatine from young barley shoots. It exhibited weak antifungal activity, causing 24% inhibition of germination of Monilinia fructicola at 56 ppm. The hordatines and their glucosides, are other amide-linked conjugates of phenolic acids and were also isolated by Stoessl (50). They have greater antifungal activity than

N-p-coumaroylagmatine, causing 100% inhibition at concentrations as low as 6 ppm. (14). Hordatine has a similar action as streptomycin by inhibiting protein synthesis in pea stem segments while affecting ribonucleic acid synthesis and leucine uptake only slightly or not at all (87). This inhibition, as well as the antifungal activity of these compounds in a standard spore germination assay, is largely overcome by the addition of various divalent cations, e.g. Ca^{2+}, Mg^{2+}, Mn^{2+}. Stoessl (88) has suggested that the hordatines are synthesized by oxidative dimerization of two identical residues of N-p-coumaroylagmatine. He has also suggested that since conjugation of p-coumaric acid with agmatine renders the former very water soluble, the conjugation could possibly facilitate their translocation within the plant. Thus, the possibility that they have a role in lignification, either as precursors or as shunt metabolites, appears to merit investigation.

van Sumere and co-workers (38) isolated N-feruloylglycyl-L-phenylalanine from barley globulins, and proposed that N-feruloylglycine could function, in seed or embryo, as a protein synthesis initiation factor, similar to the role played by formylmethionine in E.coli. The feruloyl-conjugate was found to be a very useful substrate for the ultra-microdetermination of carboxypeptidase A activity. The resistance of amide-linked conjugates of phenolic acids to degradation is exemplified by the identification of di-trans-cinnamoylspermidine after it had been stored for approximately 35 years (4).

The leaves of Acacia argentea Maid. (formerly A. leptostachya Benth.) (Leguminosae) and the bark of A. polystacha A. Cunn. yielded

about 0.4% of N^{α}-cinnamoylhistamine (39). Work on this conjugate may provide information about the precursor or breakdown product of casimiroedine isolated from Casimiroa edulis (40,41,42). Rondest et al (45) have suggested that N-(p-hydroxyphenyl)-β ethyl-p-hydroxycinnamamide may be a precursor to isoquinoline alkaloids. The close structural relationship of astrocasine (54), astrophylline (55), orensin (57,58) and isoorensin (57,58) also suggest a common precursor such as cinnamic acid and the amine cadaverine (12).

Lunarine was identified by Tamura et al (43) as the principal alkaloid of Lunaria biennis and L. rediviva. They gave no indication as to its function but stated that biogenetically, the conjugate can arise from p-coumaric acid by oxidative phenolic coupling followed (or preceded) by amidation with spermidine and reduction of a double bond.

The literature describing the isolation and occurrence of the remaining amide-linked conjugates of phenolic acids listed in Table I does not contain information as to their function or biogenesis. Other amide-linked conjugates of aromatic acids derived from plants are thought to be detoxication products or biologically less active storage forms of active metabolites. N-Indolylacetylaspartic acid has approximately 1/1000 the activity of indoleacetic acid (64). At concentrations less than 20 mg/liter practically all of the fed indoleacetic acid which escapes degradation is conjugated with aspartic acid to form the much less active indolylacetylaspartic acid (66). Naphthalylacetylaspartic acid also appears to be a detoxication product (65). The system responsible for benzolylaspartate production in pea was induced by indoleacetic acid (67).

Other Plant Conjugates

In addition to the list of identified amide-linked conjugates of aromatic acids in Table I, there are other examples of aromatic moieties conjugated in unknown linkage. A bound form of ferulic acid in rice grain was isolated by Yoshizawa *et al* (89). The conjugate consists of two ferulate, three glucose, one glutamate and two calcium moieties. Although the moieties are tightly linked, the extraction procedure and identification give no evidence as to the linkage for the conjugation of ferulic acid. Phenolic metabolites very similar to this conjugate have also been detected in rice, barley, and corn. Some of these have marked biological effects on the growth of micro-organisims such as lactic acid bacteria (90). A dark brown pigment, identified as an iron-protein-chlorogenic acid-rutin complex was isolated from the soluble, nondialyzable fraction of aged Burley tobacco (91). Bulen and LeComte (92) isolated a yellow-green fluorescent peptide from *Azotobacter* medium. Protein-phenolic acid complexes have been isolated from leaves of *Quercus pedunculata* (93). The procedure used for isolating the above mentioned phenolic acid-protein complexes have not always safe-guarded against the possibility of artifacts as outlined by Synge (94).

Amide-linked Conjugates Of Animal And Bacterial Origin

The concept that amide-linked conjugates of aromatic acids function as detoxication products is more evident when one considers some of the conjugates of animal and bacterial origin listed in Table II. For example, ingestion of 10g of "instant" powdered coffee (equivalent to

approximately 2 g of chlorogenic acid), or of an equal amount of "caffeine-free" coffee, led to increased excretion in human urine of m-hydroxy-N-benzoylglycine and the glucuronide of m-coumaric acid (78). 3,4-Dihydroxy-5-methoxyphenylacetylglutamine is a metabolite of mescaline in man (73). The formation of the three indole conjugates in bacteria is clearly an example of detoxication (86).

Conditions of reduced iron availability enhance the formation of 2,3-dihydroxybenzoic acid and markedly stimulate the formation of the lysine conjugate of Azotobacter vinelandii (83). The glycine and serine conjugates of 2,3-dihydroxybenzoic acid were formed by Bacillus subtilis (81) and Escherichia coli K_{12} (82) in iron deficient media. A simple relationship between iron deficiency and the formation of these conjugates is not evident from the available information, but the conjugates are all formed under abnormal conditions of stress. The biological function of cinnamamide in species of Streptomyces is unknown (84,85).

Non-phenolic Moieties Of Plant Conjugates

The common non-phenolic amide-linked moieties are putrescine, hydroxyputrescine, spermine, spermidine, and agmatine. Putrescine and cadaverine have been regarded as catabolic products arising from protein degradation that can be further catabolized via intermediates leading to the Kreb's cycle. However, the most probable pathway of putrescine metabolism in plants is arginine→agmatine→N-carbamyputrescine→putrescine→spermidine→spermine (95). Thus, accumulation of amines or conjugation with phenolic acids may suggest that the further metabolism of these

compounds is blocked. If this is the case, phenolic acid-amine conjugation may be an alternate detoxication process. An alternative is to suggest a new anabolic pathway for phenolic acids and amines. This would imply that amide-linked conjugates of phenolic acids have biological functions of their own or that they function as precursors for larger molecules such as alkaloids.

As discussed by Smith (96), di- and polyamines have been found in many organisms, and they fulfil important regulatory roles, which have become apparent only in the last few years. Some are known to function as growth factors for bacteria, fungi and higher plants. Growth-stimulatory properties of the polyamines are probably due to the interaction of the compounds with nucleic acids, in particular with ribosomal ribonucleic acid (97). Although several lines of investigation have elucidated definite spermine-nucleic acid interactions in vitro, the role of the polyamines in vivo is still unclear. Tabor and Tabor (98) have discussed the stabilizing action of polyamines on bacterial spheroplants, protoplasts, and nucleic acids. With purified ribonuclease (RNase) and deoxyribonuclease, the spermine effect has ranged from slight activation to inhibition, for example, spermine inhibits RNase at pH 7, but stimulates at pH 5.

In potassium-deficient barley there is an accumulation of agmatine and putrescine, and increased activities of arginine decarboxylase and N-carbamylputrescine amido-hydrolase, the enzymes catalyzing their formation (6). The activity of these enzymes is also increased after the feeding of inorganic acids to barley seedlings. Smith (6) considered it

possible that amine production is a response to internal conditions of increasing acidity. The production of amines may restore the normal hydrogen ion concentration, but the amines may increase to levels at which they themselves become toxic. Coleman and Richards (7) found that a necrosis characteristic of potassium deficiency could be induced by feeding putrescine to normal barley leaves. The relationship between the production of necrosis in barley and the levels of putrescine is very similar to the relationship of necrosis in wheat and accumulation of N-feruloyl- and N-p-coumaroyl-2-hydroxyputrescine.

Another possible role of amines in plants is as precursors to alkaloids of the types listed in Table I. Schütte and co-workers (12) have demonstrated the high rate of incorporation of cadaverine-(1,5-^{14}C) into the cinnamic acid alkaloid adenocarpin. Yoshida and Mitake (99) have shown that agmatine-G-^{3}H and N-carbamylputrescine-1,4-^{14}C were effectively incorporated into nicotine of tobacco plants. The spontaneous conversion of the oxidation product of racemic hydroxyputrescine to pyrrole was demonstrated by Macholán et al. (100). This pyrrole derivative may in turn serve as a precursor to the pyrroloquinazdine alkaloids found in Peganum harmala (101).

Smith (96) has suggested that 2-hydroxyputrescine may be related biosynthetically to γ-hydroxyarginine which has been demonstrated in Vicia (102). The possibility also exists that 2-hydroxyputrescine may be derived from γ-hydroxyglutamate via reactions analogous to those for glutamate-ornithine-proline interconversions (103). γ-Hydroxyglutamate, a constituent of many Liliaceae, may be more widely distributed in the plant kingdom (104).

The amino acid γ-hydroxyglutamate can undergo oxidative deamination and form γ-hydroxy-α-ketoglutarate. This substance is a potent inhibitor of the Kreb's cycle enzymes: aconitase, isocitric dehydrogenase, and α-ketoglutaric dehydrogenase (105). Preliminary investigations by the author have also demonstrated an inhibition of the Kreb's cycle in wheat leaves which have been fed γ-hydroxyglutamate. Thus, the inhibition by γ-hydroxyglutamate, could be removed if the amino acid was further metabolised and detoxified by conjugation with ferulic acid to form N-feruloyl-2-hydroxyputrescine.

MATERIALS AND METHODS

Plant Material And Fungus

Flag leaves of field grown rust-infected resistant and susceptible wheat (Triticum aestivum L.) were used as source material for the preparative isolation of the conjugates.

In precursor studies we used seedlings of the resistant line of Chinese spring wheat containing the temperature sensitive gene Sr6 from Red Egyptian spring wheat. The temperature sensitive gene Sr6 conditions resistance to race 56 of stem rust at mean temperatures below 20°C. Plants were grown for the first five days in a growth chamber maintained at 20°C with a photoperiod consisting of 16 h light at 1400 ft-c (approx. 15,000 lux) and 8 h darkness. On the sixth day the temperature was raised to 25°C, rendering the plants susceptible to race 56. When seven days old, the primary leaves were inoculated with uredospores of stem rust (race 56), the pots containing the plants were capped with plastic hoods to maintain 100% relative humidity, and incubated at 25°C in darkness for 18 h. During this period of darkness uredospores germinated, and formed appressoria and substomal vesicles, but no infection hyphae. Upon return to the light period, infection hyphae developed and host cells were infected. After the first light period of 16 h the plastic hoods were removed and the plants were maintained at 25°C for another day to allow good rust development. On the eleventh day the temperature was dropped to 20°C inducing the expression of resistance in the Sr6-containing plants. This treatment

elicited the formation of chlorotic and necrotic lesions, symptomatic of a resistant reaction. The number of chlorotic and necrotic cells/site area was substanially increased by allowing the fungus to invade many cells at 25°C before inducing the expression of resistance in the Sr6-containing plants. The plants were used for precursor studies six days after inoculation.

Primary leaves of the variety "Little Club" used for injection bioassays, and Sr6-containing plants of Chinese spring wheat used for γ-hydroxyamino acid studies were grown under conditions previously described (106).

Wheat stem rust uredospores of race 56 (Puccinia graminis (Pers.) f.sp. tritici Erikss. & Henn.) were collected from greenhouse-grown wheat (Triticum aestivum) and stored in liquid nitrogen until needed (107). Before use in plant injection and germination assays the spores were exposed to a 40°C heat shock for 6 min immediately upon removal from liquid nitrogen.

Bioassays With Rust Uredospores

Rust uredospores (5 mg) were shaken with 200 ml quartz double distilled water for 5 min to remove endogenous germination inhibitors. On hundred milliliters of this suspension were passed, with suction through a Millipore membrane filter (SCWP 8 µ, 47 mm, Millipore Ltd., Montreal, Quebec). The membranes with evenly deposited spores were razor cut into 25 mm square pieces and each of these was floated on 50 µl testing solution in a petri dish (35 x 10 mm). All lids were lined with Whatman No. 1 filter paper soaked with 0.1 ml water and 1 µl of 10 µl nonanol in 10 ml acetone. The buffer of Allen and co-workers (108) was used as a control and as a solvent for the substances to be tested. After a germination

period of 1 h at 20°C in the dark, the samples were stained with 0.2% cotton blue in lactophenol and the extent of germination was estimated microscopically. Spores were considered to have germinated if their germ tubes exceeded 200 microns in length.

Bioassay With Rust-Infected Wheat Leaves

Primary leaves of wheat of the variety "Little Club" were obtained from 6-day old plants grown under conditions previously described (106). The upper side of a 2-cm segment located 6 cm from the tip was inoculated with uredospores of race 56 of stem rust suspended in 1.0% aqueous gelatin (containing 0.001% Tween 20). The spore concentration was 5 mg/ml. The plants were then placed into the dark for 16 h at 100% relative humidity. After a light period of 24 h, the marked segments were flooded (109) with solutions of various metabolites dissolved in the buffer of Allen et al (108) which was also used as a control. The inoculated leaf segments were excised 6 h after flooding and fixed in Farmer's fixative (abs. ethanol/glacial acetic acid, 3:1). Cell necrosis produced as a consequence of flooding was visualized by fluorescence microscopy (110).

Synthesis Of Model Compounds

The following model compounds were prepared and used for comparison with newly isolated, naturally occurring conjugates.

Feruloylproline: 31 mg of O-acetylferuloylproline methyl ester (gift from Dr. A. Stoessl) was shaken with 5 ml N/10 NaOH for 3 h. The product was then pased through a small column (0.5 x 3 cm) of Amberlite IRC-50 (H^+) followed by 10 ml water and 50 ml 80% methanol. The solvents used for elution

were evaporated *in vacuo* and 18 mg of product was obtained.

Feruloylproline amide: 28 mg of O-acetylferuloylproline methyl ester was treated with 1 ml concentrated NH_4OH overnight. The product was evaporated *in vacuo* and purified by chromatography on two short columns (0.5 x 4 cm) of silica bedded in chloroform. The sample was eluted with 20% methanol in chloroform, and isolated by preparative thin-layer chromatography on layers of Silica Gel GF_{254} developed with 10% methanol in chloroform. The amide was filtered through cotton wool and taken to dryness (10.6 mg).

Feruloylpyrollidine: 100 mg ferulic acid ethyl ester (Dr. A. Stoessl) was dissolved in 1 ml pyrollidine and was kept overnight at room temperature. The mixture was taken to dryness *in vacuo* and the residue was repreatedly washed with methanol to remove traces of free pyrollidine. The product (32 mg) was purified by thin-layer chromatography on layers of Silica Gel GF_{254} using ether/methanol/acetic acid (90:10:2) as a solvent.

Identities of the above 3 products were confirmed by nuclear-magnetic-resonance spectroscopy. In addition to these compounds, the following samples (synthesized and authenticated by Dr. A. Stoessl) were used as comparative models in characterizing the isolated conjugates: acetylferuloylproline methyl ester, feruloylproline methyl ester, acetylferuloylproline, acetylferuloylproline amide.

Isolation And Purification Of γ-Hydroxyarginine

γ-Hydroxyarginine was needed as a possible precursor to the 2-hydroxy-putrescine moiety of the conjugates isolated from wheat. Since it is not commercially available it was prepared from the seeds of *Vicia sativa*.

Seeds of Vicia sativa L. used for the isolation of γ-hydroxyarginine were from a collection of four lines, Krasnader IIs and Krasnadar 7 grown at Melfort, Sask., a line from Romania and a fourth line from Poland. The procedure of Bell and Tirimanna (102) was used with the following modifications as their method of purifying hydroxyarginine by crystallization was unsuccessful in our hands.

The charcoal filtrate was chromatographed at 40°C on a (3 x 55 cm) jacketed column of Bio Rad ^{50WX8} [H^+], 200-400 mesh (Bio Rad Laboratories, Richmond, Cal., U.S.A.). A linear gradient elution (pH 7.0 to pH 1.0) of distilled water to 5N HCl at a flow rate of 1 drop/6sec was used to fractionate the extract. Fractions showing a positive reaction with Sakaguchi's reagent were pooled and concentrated in vacuo. Thin-layer chromatography revealed that hydroxyarginine and arginine were the only components in this fraction. γ-Hydroxyarginine was preparatively separated from arginine by 4 successive chromatographic runs on columns (0.9 x 100 cm) of the same resin at a flow speed of 1 drop/min using the technique of Witkop and Beiler (111). The purified γ-hydroxyarginine was freed from inorganic ions by gel filtration on a (1.5 x 50 cm) column of Sephadex G-10 embedded and eluted with distilled water.

The isolated compound showed identical color reactions when tested with thin-layer detection reagents as that of the γ-hydroxyarginine described by Bell and Tirimanna (102). Nuclear-magnetic-resonance spectroscopy and two-dimensional thin-layer chromatography in many systems coupled with high voltage thin-layer electrophoresis revealed that the γ-hydroxyarginine was contaminated with a trace of arginine. A

fluorometic scan (Turner Model 111 Fluorometer) of the thin-layer separation of the Dansylated isolate demonstrated the composition to be 98% γ-hydroxyarginine and 2% arginine. Thin-layer chromatographic separation of γ-hydroxyarginine from arginine was best achieved by chromatographing the Dansyl derivatives on MN-Polygram sheets of polyamide-6 (Mondray Ltd., Montreal, Quebec) in water. Dansyl derivatives were prepared by dissolving an aliquot of the sample in 25 µl 0.1 M $NaHCO_3$ and reacting overnight with 25 µl of 10% Dansyl chloride in acetone (Pierce Chemical Co., U.S.A.) diluted 1:50 with acetone.

Arginine and γ-hydroxyarginine could also be separated on MN cellulose 300 layers by high voltage electrophoresis (Desaga apparatus, 100 V/cm, 20 min) in 0.02 M acetic acid adjusted to pH 10.5 with concentrated NH_4OH. Yield as determined by the method of Yemm and Cocking (112) was 188 mg γ-hydroxyarginine from 1420 g of seeds.

Analytical Separation And Hydrolysis Of Conjugates

The chromatographic properties of the isolated conjugates were compared with those of model compounds with and without exposure to ultra-violet light prior to development on MN cellulose 300 plates with 2% formic acid or high voltage electrophoresis on MN cellulose 300 (Desaga apparatus, 100 V/cm, 20 min) using various buffers (0.02 M acetic acid, adjusted to desired pH with concentrated NH_4OH).

Aliquots (<1 mg) of amide-linked conjugates of phenolic acids were dissolved in 0.5 ml of double distilled (constant boiling) 6N HCl in a clean vial. The samples were flushed with nitrogen, frozen in an ethanol-liquid nitrogen bath and sealed *in vacuo*. After heating for 17 h at 110°C the

samples were transferred with water to small flasks and taken to dryness *in vacuo*. The hydrolysis products were dissolved with 100 µl N/10 HCl, subjected to thin-layer chromatography and thin-layer electrophoresis, and visualized with spray reagents used for detecting phenolic acids, amino acids, organic acids and sugars. Aliquots of model compounds were treated and analyzed in the same manner.

Feeding Studies

In studies to determine the effect of possible precursors on the accumulation of *N*-feruloyl-2-hydroxyputrescine, wheat leaves were excised, cut to four-inch lengths and were allowed to stand, with their cut ends, in solutions of precursor for a metabolic period of 22 hours in the light (ca. 10,000 lux) at 19-21°C. The compounds and their concentrations were as follows: D-arginine (0.3 mM), D-glutamic acid (0.3 mM), D-ornithine (0.3 mM), γ-hydroxyarginine (1.7 mM), γ-hydroxyglutamate (1.0 mM). Each of these substances was dissolved in 0.01 M potassium phosphate, pH 7.0, containing 0.0001 M $Ca(H_2PO_4)_2$. This buffer was also used as a control. At the end of the metabolic period the conjugates were extracted as described previously for *N*-feruloyl-2-hydroxyputrescine (8).

Methods Used For Detecting The Possible Occurrence Of Free γ-hydroxyamino Acids In Wheat Leaves

γ-Hydroxyglutamic acid and γ-hydroxyarginine are two amino acids which, if present as normal wheat leaf constituents, could possibly serve as precursors to the basic moiety of *N*-feruloyl-2-hydroxyputrescine. To

investigate their presence, the following methods were adopted, because conventional thin-layer chromatographic analysis of amino acid fractions will not separate the hydroxy amino acids from their parent amino acids.

The extraction procedure of Rohringer et al (106) was used to isolate a water-soluble fraction from wheat leaves. Acidic amino acids were then isolated by passing the water-soluble fraction through a strong cation exchange resin (Amberlite IR-120 [H^+], prepared according to Awapara et al (113). The occurrence of free γ-hydroxyglutamic acid in this fraction was investigated by thin-layer electrophoresis (Desaga apparatus, 100 V/cm, 18 min) in pH 2.0 buffer (57 ml acetic acid, 17 ml formic acid, made to 1 liter with water). In this system γ-hydroxyglutamate is clearly separated from glutamate.

To isolate the basic amino acids, the water-soluble fraction (106) was purified by the method of Splittstoesser (114). The occurrence of free γ-hydroxyarginine in this fraction was investigated using the analytical techniques described previously for γ-hydroxyarginine from seeds of Vicia sativa.

Extraction Of Alcohol-Insoluble Residue Of Wheat Leaves

A search was made to detect the possible occurrence of amide-linked conjugates of phenolic acids in the alcohol-insoluble residue of plants that are known to contain such conjugates in the alcohol-soluble fraction. The alcohol-insoluble residue (100 g) of rust-infected wheat leaves was extracted (1 h at room temperature) with 3 liters water containing 0.1% acetic acid and 0.1% mercaptoethanol. The aqueous extract was

separated from the residue by centrifugation (10 min, 2000 x g). This extract was concentrated *in vacuo*. The residue was then extracted again with 0.1 M potassium phosphate buffer pH 6.0, containing 0.1% mercaptoethanol. The buffer-insoluble residue was further extracted for 30 min at 90°C with 15% trichloroacetic acid (TCA) containing 0.1% mercaptoethanol. The TCA was removed by continuous overnight extraction with ether and the TCA-soluble fraction was concentrated *in vacuo*.

The acetic acid-soluble, buffer-soluble, TCA-soluble and TCA-insoluble residues were hydrolyzed for 6 h at room temperature with 2N NaOH, adjusted to pH 4 with 6N HCl, and extracted continuously with peroxide free ether overnight to remove ester-bound phenolic acids. The ethereal phase was discarded and the aqueous phase was concentrated *in vacuo*. All fractions were examined by thin-layer chromatography and thin-layer electrophoresis for the presence of amide-linked conjugates of ferulic acid These fractions were then dialysed against several changes of distilled water and the non-dialyzable portions were examined as above.

Aliquots from each non-dialyzable fraction were incubated with pronase (115), trypsin (116), emulsin (117), and used for partial acid hydrolysis (38) and alkaline hydrolysis in 2N NaOH on a steam bath for 2 h. The hydrolysates were subjected to thin-layer chromatography and thin-layer electrophoresis and examined for both free ferulate and amide-linked conjugates of ferulic acid.

RESULTS AND DISCUSSION

Isolation Of Amide-Linked Conjugates Of Ferulic Acid

Preliminary investigations of an ethanol-soluble fraction of wheat leaves in which N-feruloyl-2-hydroxyputrescine occurs (8) suggested the presence of another ferulic acid conjugate (or conjugates) at a concentration approximately 1/10 of N-feruloyl-2-hydroxyputrescine. From a cursory analysis of the initial, relatively crude extracts it was not immediately clear how many ferulic acid conjugates there were, since large numbers of other extractives interfered with chromatography and detection. The following detailed investigation centered on compounds that were not retained by the cation exchanger Amberlite IRC-50 (H^+) (Rohm and Hass, Philadelphia, Pa., U.S.A.) and thereby differed from the behaviour of N-feruloyl-2-hydroxyputrescine, which was retained by this resin. As purification progressed, it became clear that we were not isolating one ferulic acid conjugate, but two, both occurring as cis and trans isomers. The similarity of their chromatographic and electrophoretic properties indicated that they were closely related, and that they had certain properties in common with N-feruloyl-2-hydroxyputrescine. The possibility that these unknown conjugates were biogenetically related to N-feruloyl-2-hydroxyputrescine was of interest.

The conjugates could be separated from many other extractives by two-dimensional chromatography on MN cellulose 300 with propanol/ammonium hydroxide/water (6:3:1) in the first direction and 2% formic acid in the second (tank with paper liner) or by high voltage electrophoresis (Desaga apparatus, 100 V/cm, 30 min) with either of two

buffers (57 ml acetic acid and 17 ml formic acid, made to 1 liter with water, pH 2.0: or 0.02 M acetic acid, adjusted with concentrated ammonium hydroxide to pH 6.1 followed in the second direction by chromatography with 2% formic acid. The unknown conjugates reacted with reagents usually used for detecting phenolic compounds, and colors of the products were characteristic for ferulate. Fluorescence in ultra-violet (365 nm) was the most sensitive method of detection and conjugate content was estimated routinely on chromatograms by visual comparison of the size and intensity of fluorescing spots or by measurement of the fluorescing light (Zeiss spectrophotometer PMQII with chromatogram scanning attachment; activation and fluorescence maxima at 365 nm and 466 nm, respectively).

Because the unknown wheat metabolites occurred in relatively small amounts, it was necessary to start from large quantities of source material. Flag leaves of wheat (7 kg) of various genetic backgrounds were collected in the field from plants exhibiting natural rust infections. An effort was made to select leaves with extensive areas of rust-induced chlorosis and necrosis since preliminary tests had shown that these contained ferulic acid conjugates in higher concentration than healthy leaves. As older flag leaves contain much higher amounts of phenols and pigments than the primary leaves used previously (8), a new large-scale fractionation procedure was developed. This procedure is schematically represented in Fig. 2 and described in detail as follows.

Step 1. Extraction. All leaves were cut into pieces of approximately 1 cm length and boiled briefly with 96% ethanol (1 liter/100 g fr. wt.). After

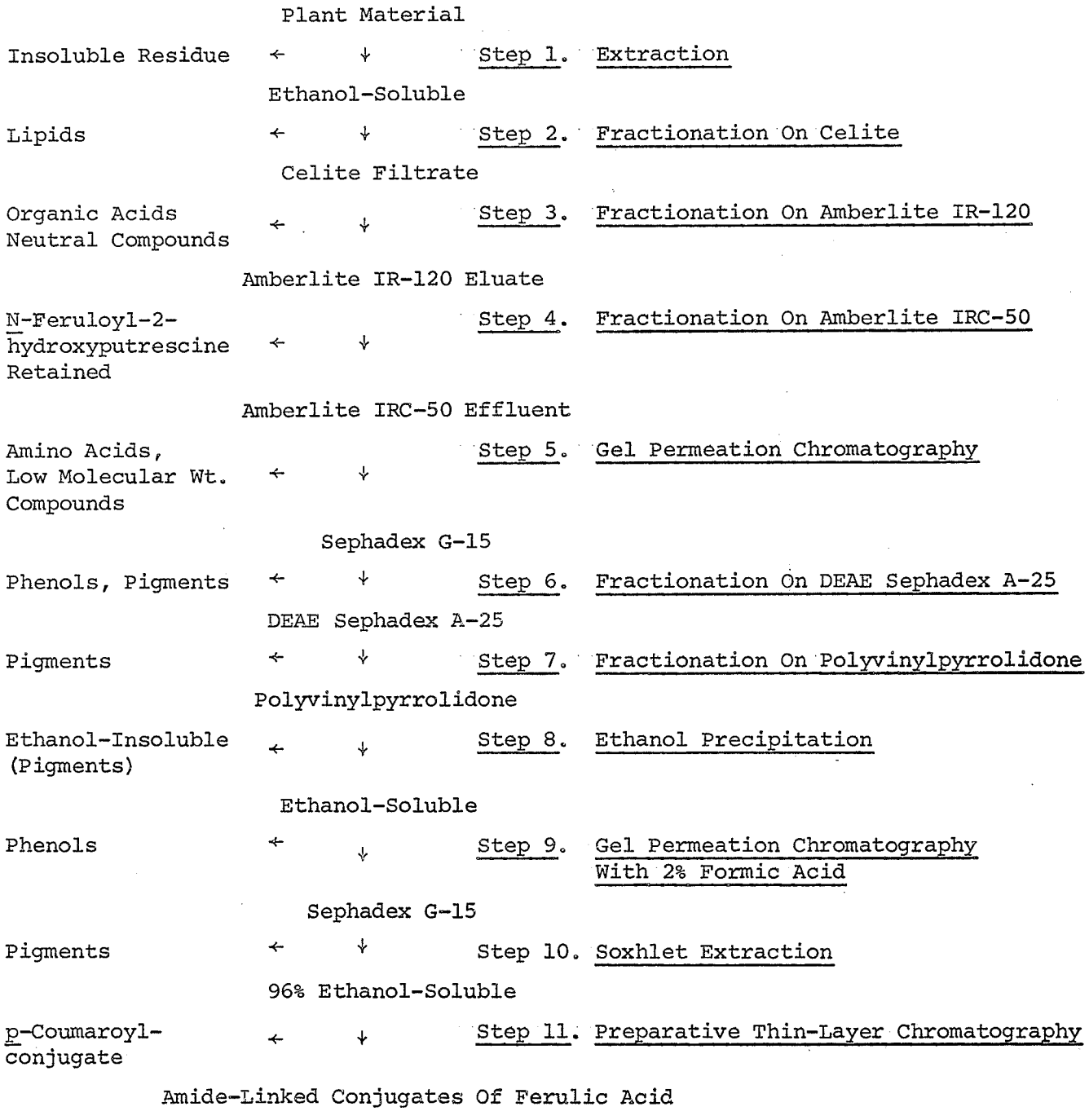


FIGURE 2. Isolation flow diagram.

filtration through Whatman No. 1 filter paper, batches of leaf pieces were recovered and homogenized in a blender with 90% methanol (total of 80 liters), the suspensions were filtered and the filter cakes percolated with 80% ethanol (50 liters) and acetone (30 liters). The insoluble residue was stored frozen until further use. All extracts were combined and dried *in vacuo* in 400-ml batches, each containing 2 g Celite analytical filter aid (Canadian Johns-Manville Co., Port Credit, Ontario). This step provided the initial solvent soluble and insoluble fraction.

Step 2. Fractionation On Celite. Batches of Celite were suspended in a total of 20 liters of water, heated to 50°C, and filtered through Celite and Whatman No. 5 filter paper. The filter cakes were percolated with a total of 21 liters of hot water (80°C). All aqueous extracts were combined, concentrated *in vacuo* and made to 3 liters with water. Lipids and chlorophylls were retained on the Celite filter cakes and discarded.

Step 3. Fractionation On Amberlite IR-120. The aqueous extract was divided into equal portions and passed through 33 columns, each containing 50 ml of Amberlite IR-120 (H^+) (Rohm and Haas, Philadelphia, Pa., U.S.A.). The use of 33 short columns in contrast to a fewer number of larger columns shortened the time in which the conjugates were in contact with the resin and therefore was expected to minimize degradation. A similar procedure was adopted for subsequent chromatographic separations. Each column was washed with 250 ml water and the effluents were discarded. The resin was adjusted to pH 9 with 5N ammonium hydroxide in an ice-bath, the columns

were repacked, and each was eluted with 400 ml 5N ammonium hydroxide. The columns were further eluted, during two successive days, with several batches of 5N ammonium hydroxide, totalling 150 ml/column and the combined eluates were dried *in vacuo*. Because of their behaviour on the weak cation exchange resin Amberlite IRC-50, the unknown conjugates were expected in the Amberlite IR-120 effluents. Instead, they emerged in the eluate, together with *N*-feruloyl-2-hydroxyputrescine. This unexpected behaviour may have been due to adsorption of the unknowns on the resin matrix, a characteristic that has been observed with many other aromatic metabolites (118).

Step 4. Fractionation On Amberlite IRC-50. The residue was dissolved in 1 liter of water and equal portions were passed through 26 columns, each containing 25 ml Amberlite IRC-50 (H^+). Each column was washed with 250 ml water. The combined effluents contained the unknowns and were concentrated *in vacuo*. *N*-Feruloyl-2-hydroxyputrescine and other cationic aromatic compounds were retained by the resin.

Step 5. Gel Permeation Chromatography. The concentrated effluent was adjusted to 400 ml with water, divided into equal portions and passed through 6 columns (5 x 80 cm) of Sephadex G-15 (Pharmacia (Canada) Ltd., Montreal, Quebec) embedded in de-aerated water. The metabolites were eluted with water at 65 ml/h. The eluate was continuously monitored for ultra-violet absorbing substances (Pharmacia UV Monitor) and various fractions were examined by thin-layer chromatography (Fig. 3). Fractions containing the desired conjugates were pooled, concentrated *in vacuo*, and taken up in 200 ml water.

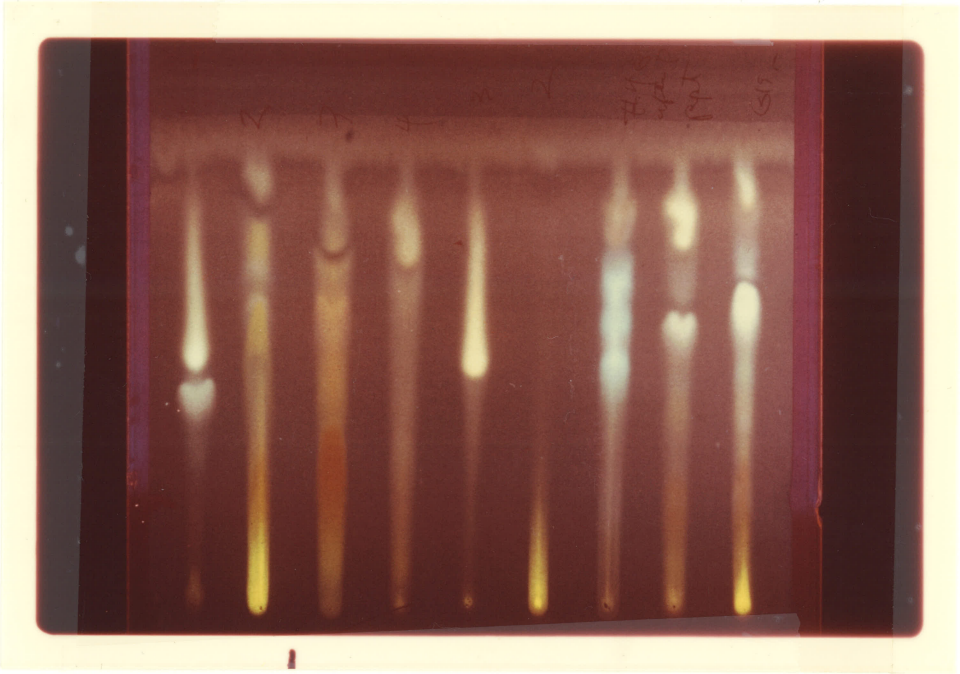


FIGURE 3. Thin-layer chromatography of Sephadex fractions (Steps 5 & 6). The photograph shows compounds fluorescing under ultra-violet light (365 nm). The unknown conjugates fluoresce blue and are visible in third lane from the right.

Step 5 was very useful in the purification because it removed the bulk of contaminating compounds. There were some losses due to irreversible adsorption on the chromatographic support, but repeated use of each Sephadex column gave better yields and more reproducible elution profiles probably because the more reactive adsorption sites had been saturated.

Step 6. Fractionation On DEAE Sephadex A-25. The extract was further fractionated by passing the sample through 3 columns (5 x 80 cm) of DEAE Sephadex A-25 (acetate form) (Pharmacia (Canada) Ltd., Montreal, Quebec) embedded in water. The eluting solvent was 30% formic acid. The conjugates were detected (Fig. 3) and combined as in Step 5. Although some purification was achieved with this chromatographic support, it was not sufficient to warrant its use in future isolations.

Step 7. Fractionation On Polyvinylpyrrolidone. The concentrate was made to 100 ml with water and equal portions were passed through 13 columns (1.9 x 20 cm) of polyvinylpyrrolidone (PVP) (Polyclar AT powder, GAF Corp. N.Y., U.S.A.) embedded in water. The conjugates were eluted with water/ethanol/formic acid (3:1:1). Fractions containing the conjugates were pooled and concentrated as before. This step removed a contaminating pigment and caused appreciable losses due to irreversible adsorption on the chromatographic support. It also introduced an ultra-violet absorbing degradation product of PVP which was removed by gel permeation chromatography on 4 columns (2.5 x 48 cm) of Sephadex G-15 as described in Step 5.

Step 8. Ethanol Precipitation. The extract from Step 7 was concentrated in vacuo to 50 ml and ethanol was added to a concentration of 80%. After standing overnight at 0°C the sample was centrifuged (10 min, 12,000 x g): the precipitate contained pigments and was discarded. The supernate was concentrated to remove alcohol and then adjusted to 100 ml and a formic acid content of 2%. In future isolations, ethanol precipitation may be used to advantage in earlier steps during the purification procedure.

Step 9. Gel Permeation Chromatography With 2% Formic Acid. The extract was fractionated eight successive times on columns (2.5 x 80 cm) of Sephadex G-15 embedded in 2% formic acid and eluted with this solvent at 60 ml/h. The eluate was continuously monitored for ultra-violet absorption at 320 nm (absorption max. of ferulate; Zeiss spectrophotometer PMQII) and for fluorescence at 465 nm (fluorescence max. of conjugates; Turner Model 111 Fluorometer with 325 nm primary filter (7-54 + 34A) and 465 nm secondary filter (110-827). This made it possible to separate the fluorescing conjugates from at least 7 non-fluorescing, ultra-violet absorbing contaminants.

Step 10. Soxhlet Extraction. The fractions containing the conjugates were pooled and concentrated in vacuo. The concentrated extract was layered on pre-extracted Silica Gel HR powder in a soxhlet thimble and dried under a jet of nitrogen. The gel was first extracted overnight with ether, then with absolute ethanol and 96% ethanol. The desired conjugates were found only in the 96% ethanol fraction.

At this stage, aliquots of the preparation were subjected to

thin-layer chromatography in more than 20 systems. The preparation appeared homogeneous in all systems except in 2% formic acid on layers of MN cellulose 300. In this system, the isolate was resolved into two components that gave identical reactions with all spray reagents tested. Exposure of the origin to ultra-violet, after spotting and before development with 2% formic acid, yield 4 components, indicating that each of the two substances existed as cis and trans isomers. A comparison of the chromatographic behaviour of the isomeric forms with ferulic acid and other model compounds confirmed that the unknowns were conjugates of ferulic acid and not free C_6C_3 acids (119).

After separation on thin-layer plates, diffuse reflectance spectra of the components were recorded (Zeiss Thin-Layer Chromatogram Spectrophotometer; 240-380 nm) and these indicated the presence of yet another impurity that co-chromatographed with one of the isolated feruloyl conjugates. The following steps were undertaken to separate the two conjugates from each other and the remaining impurity.

Step 11. Preparative Thin-Layer Chromotography. MN-Kieselgel N-HR (Mondray Ltd. Montreal, Quebec) was washed with ammoniacal 50% ethanol and 0.5 mm-thick layers (20 x 20 cm) were prepared. Before chromatography the plates were washed to the top with developing solvent I[toluene/ethylacetate/ formic acid/water (45:35:20:2.5)]. The ethanol-soluble material from Step 10 was dissolved in 12.0 ml 80% ethanol, and 2 x 80 µl were applied as a streak (Camag "Chromatocharger") on the origin of each of seventy-five plates. The conjugates were purified by developing with Solvent I.

They were located under ultra-violet light, scraped off, and eluted with ammoniacal 50% ethanol. The eluate was then re-chromatographed on 60 plates of Camag microcrystalline cellulose (Mondray Ltd. Montreal, Quebec) which had been prewashed with 2% formic acid and 80% ethanol. The layers (0.5 mm thickness) were developed at 4°C with 2% formic acid (Solvent II). The conjugates were located as above, and eluted from scrapings with 50% ethanol.

Solvent system I was used in Step 11 to free the contaminant from the desired feruloyl-conjugates. The contaminant was a conjugate of p-coumaric acid. It was not isolated because of its susceptibility to oxidation during chromatography. Preparative thin-layer chromatography in solvent II was used to separate the feruloyl-conjugates from each other. The cis and trans isomers of each feruloyl-conjugate yielded identical ultra-violet absorption profiles when scanned on MN cellulose 300 layers from 240 nm to 360 nm. The conjugates can be purified further by acetylation (3:2 mixture of pyridine/aceticanhydride, overnight at room temperature) and chromatography of the products with ether/methanol/acetic acid (90:10:2) on layers of Silica Gel GF_{254}.

Chromatography in several systems and thin-layer electrophoresis under various conditions revealed the presence of no contaminating metabolites (Figs. 4 & 5). Each conjugate was considered pure enough for structural studies. Final yield of each conjugate was approximately 7 mg.

Structural Studies With The Isolated Ferulic Acid Conjugates

The isolated conjugates were characterized by recording their ultra-violet spectra, and by comparing their chromatographic and electrophoretic

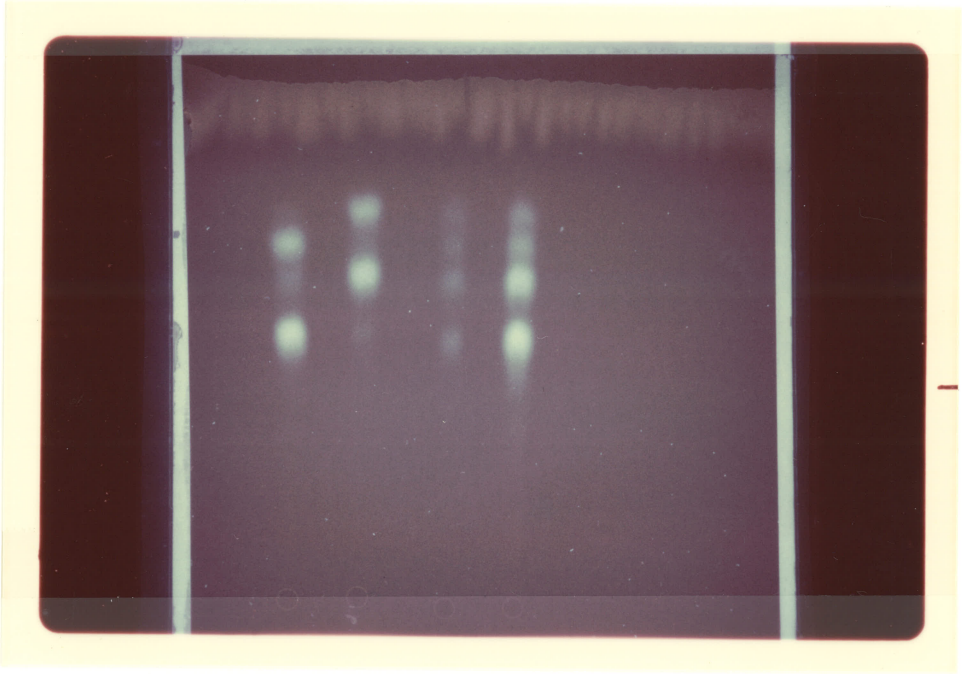


FIGURE 4. Thin-layer chromatogram of cis and trans isomers of the isolated amide-linked conjugates of ferulic acid. Photograph of conjugates fluorescing under ultra-violet (365 nm). Left to right: 1) conjugate #1 with approx. 5% conjugate #2, 2) conjugate #2 with approx. 5% conjugate #1, 3) & 4) mixture of conjugates #1 & #2.

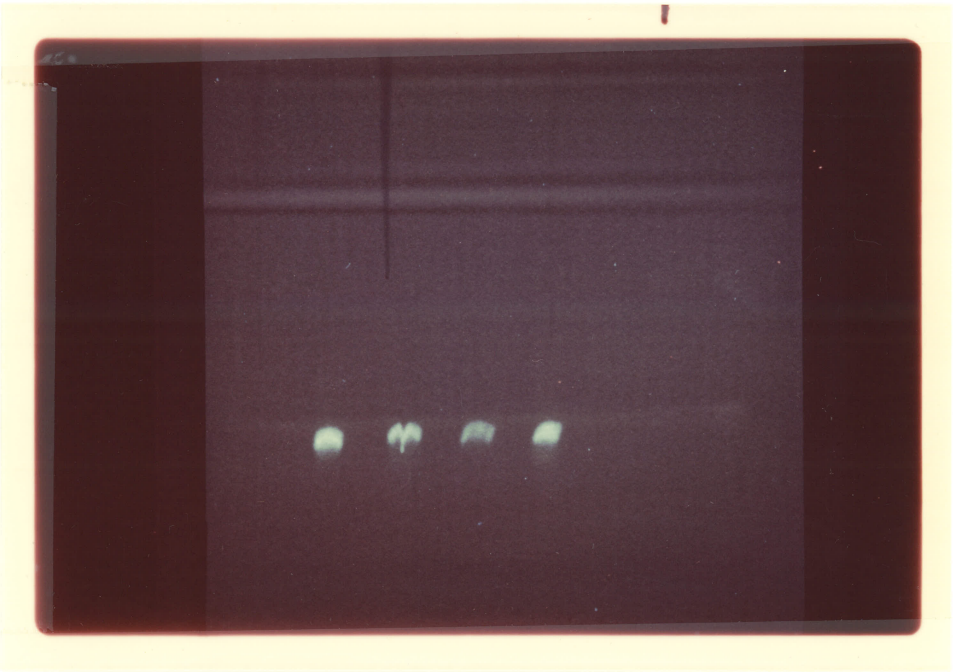


FIGURE 5. High voltage electropherogram of isolated amide-linked conjugates. Left to right: 1) conjugate #1, 2) conjugate #2, 3) & 4) mixture of conjugates #1 & #2. Photograph of conjugates fluorescing under ultra-violet light (365 nm).

behaviour with model compounds. An analysis of hydrolysis products was also attempted, but the amounts available for this study were too small to yield conclusive results, except to identify ferulic acid as one of the hydrolysis products. Nuclear-magnetic-resonance, mass and infrared spectra were determined in collaboration with Dr. A. Stoessl and Dr. M. Gordon.

The model compound, N-feruloylproline, chromatographed nearest the isolated conjugates in all systems investigated. High voltage electrophoresis of model compounds, and of neutral compounds to measure the electro-osmotic drift, suggested the presence of a free carboxyl group, because the isolated conjugates behaved as anions with carboxylic ionic properties (Fig. 6).

Although it was apparent from analysis of different extracts that the natural isomer of the isolated conjugates was the trans form, bulk preparation showed the conjugates to be in a cis-trans equilibrium, probably because of the use of ultra-violet light in isolation. In this preparation, the isomeric equilibrium of the conjugates was determined by measuring their fluorescence on thin-layer plates of MN cellulose 300 developed with 2% formic acid as a solvent. The conjugates were in an equilibrium of 60% trans isomer and 40% cis isomer. The ultra-violet spectra (Fig. 7) of the isolated conjugates were closest to that of ferulic acid, and they displayed a bathochromic shift of 59 nm, compared with 26 nm for free ferulic acid. Assuming the molar extinction coefficient of ferulic acid to be 24,000 and the contribution of the cis isomer to absorption at 322 nm to be zero, the molecular weight of either conjugate was estimated to be between 505

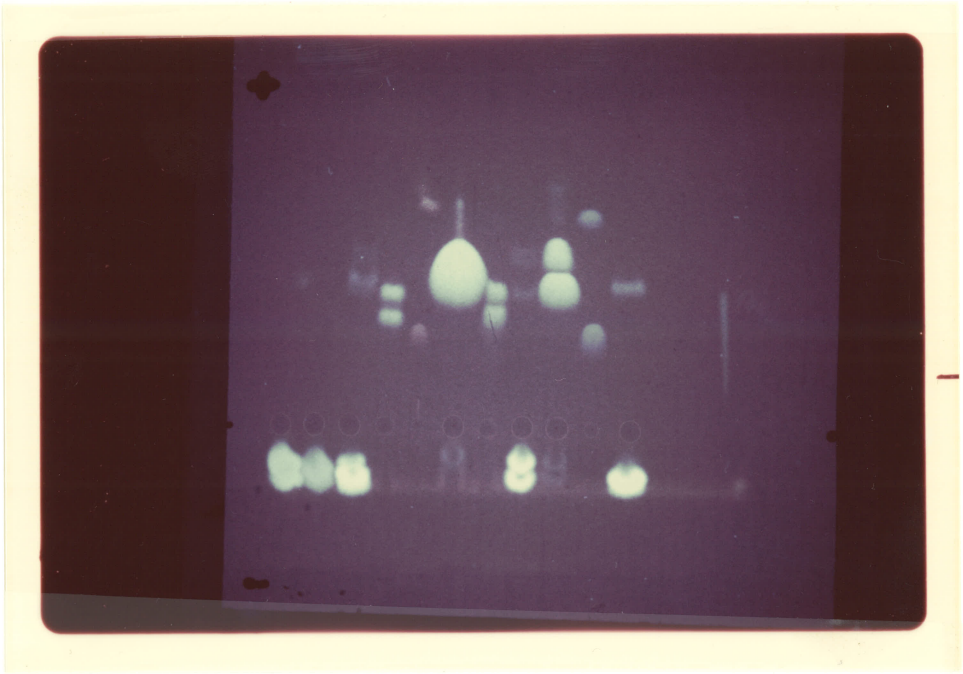


FIGURE 6. High voltage electropherogram of amide-linked conjugates of ferulic acid and model compounds. Photograph shows compounds fluorescing under ultra-violet light (365 nm). Left to right: 1) feruloylproline methyl ester 2) acetylferuloylproline methyl ester and feruloylproline amide 3) acetylferuloylproline amide 4) isolated conjugate #1 5) isoferulic acid 6) acetyl-feruloylproline 7) isolated conjugate #2 8) feruloylproline amade 9) feruloylproline 10) ferulic acid 11) acetylferuloyl-proline amide 12) glucose.

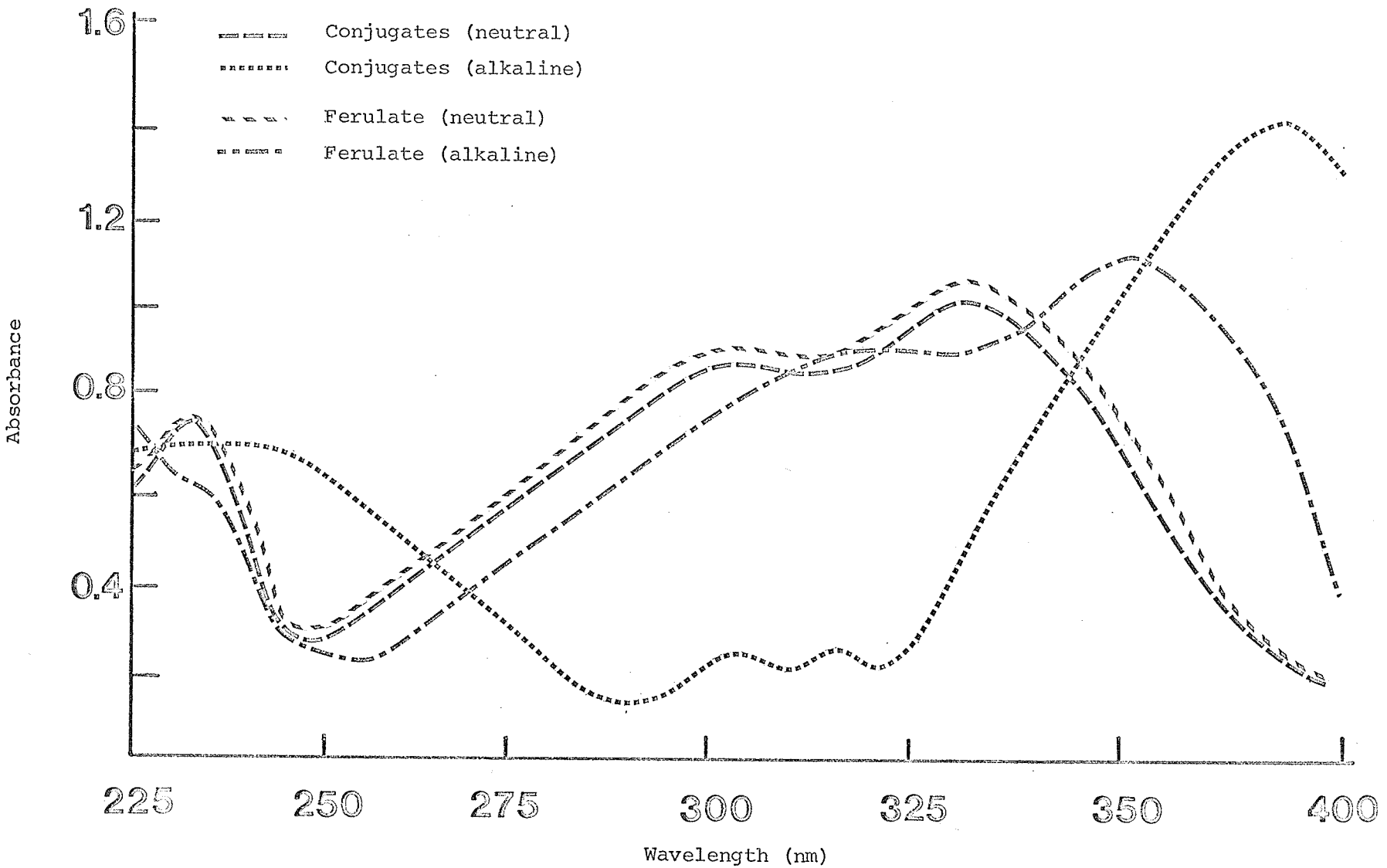


FIGURE 7. Ultra-violet absorption spectra of isolated conjugates and ferulic acid (in 96% ethanol).

and 564, but was more precisely determined from mass spectra. These revealed that the tri-acetate of conjugate #2 (Fig. 4) has a molecular weight of 494. From infrared and nuclear-magnetic-resonance studies the structure appears complex. It contains ferulic acid in amide-linkage with either hydroxyputrescine, hydroxyornithine, or hydroxyproline, in linkage with yet another moiety. Depending upon which hydroxy metabolite is amide-linked to ferulic acid, the other moiety may have a molecular weight between 42 and 86. An acetyl group which has a molecular weight of 49 appears to be one possibility for the other moiety based on these weight calculations. Structural studies are still in progress to confirm the identity of the unknown conjugates.

Occurrence Of Newly Isolated Conjugates

Since infection with rust affected the amount of N-feruloyl-2-hydroxyputrescine in wheat leaves (8), it was of interest to determine whether amounts of the newly isolated conjugates were also affected by rust infection (Table III). The newly isolated conjugates did not display the same pattern of accumulation as N-feruloyl-2-hydroxyputrescine, but they did accumulate slightly (20%) in both resistant and susceptible lines. This increase is most probably due to the stress conditions induced by the fungus rather than to the fungus itself, because the susceptible reacting leaves contained much more fungal material than the resistant reacting leaves.

TABLE III. Occurrence Of Newly Isolated Amide-Linked Conjugates Of Ferulic Acid In 14 Day Old Primary Leaves Of Near-Isogenic Lines Of Wheat

Plant Material*	Disease reaction	Amount of conjugate*** (mμ mole ferulic acid equivalents/g fr.wt.)	% Accumulation Healthy = 100%
Healthy Resistant	None	34	100
Healthy Susceptible	None	34	100
Infected Resistant**	Res.	44	129
Infected Susceptible**	Sus.	40	118

* Harvested six days after inoculation.

** Infected with race 56 stem rust.

*** Determined by measurement of the fluorescing light as mentioned earlier in Results and Discussions.

Investigation Of The Insoluble Residue

Reports on protein-bound phenolic acids (38,91,92,93), suggested to us that some amide-linked conjugates of phenolic acids may have their biogenetic origin in fractions which would be ethanol-insoluble. Therefore, the ethanol-insoluble residue of wheat leaves, which is largely composed of buffer-soluble proteins, nucleic acids, glycoproteins, and other cell wall material, was investigated for the presence of amide-linked ferulic acid. It was fractionated into acetic acid-soluble, buffer-soluble, trichloroacetic acid (TCA)-soluble and TCA-insoluble materials and all ester-bound phenolic acids were removed by extraction with ether following mild alkaline hydrolysis. Phenolic glycosides, that might have been present in the TCA-soluble and TCA-insoluble fractions, were hydrolysed by treatment with hot TCA, and extraction with ether would have also removed their phenolic aglycons.

Thin-layer chromatographic analysis of these fractions gave no evidence for amide-linked ferulate in any of them, but amide-linked p-coumarate was detected in the TCA-soluble and TCA-insoluble fractions after they had been subjected to partial acid hydrolysis (38).

Observations On Hydrolytic Conditions

During the preparative isolation of the ferulic acid conjugates, we observed conjugates of other phenolic acids. These were not isolated and identified. Most of them were probably esters and glycosides, but some may be amide-linked. A closer scrutiny of the fractions which have been called "soluble esters" and "insoluble esters" (27) may reveal the

presence of amide-linked conjugates of phenolic acids. For example, it was noted by the author that after an overnight continuous extraction with ether, the model compound N-feruloylproline was completely extracted into the acidic ether phase (pH 2.0). Previous investigators have assumed that this phase contains phenolic acids only in the free form. The hydrolysis conditions used by El-Basyouni and Neish (9) (2N HCl, steam bath, 45 min), El-Basyouni and Towers (120) (1N NaOH, steam bath, 1 h), Glass and Bohm (121) (1N NaOH or 1N HCl, heat to boil and cool), and by Ibrahim and Towers (122) (2N HCl, steam bath, 1 h) were designed to release phenolic acids conjugated as esters or glycosides. Yet these same hydrolysis conditions will effect a release of ferulic acid from amide-linked conjugates such as the newly isolated conjugates and N-feruloylproline.

Feeding Studies

The claim as to whether certain metabolites serve as precursors to the basic moiety of N-feruloyl-2-hydroxyputrescine is best achieved by feeding radioactive metabolites, isolating the conjugate, and then determining the specific radioactivity incorporated in the 2-hydroxyputrescine moiety. However this method of investigation was not possible in these studies because the metabolites chosen for feeding studies could not be obtained in radioactive form. As a result of this, a method was adopted to investigate the effect of non-radioactive metabolites on the production of N-feruloyl-2-hydroxyputrescine and the newly isolated conjugates.

One of our objectives was to try and effect a substantial increase in the accumulation of the conjugates by feeding various substances. If this was possible, larger amounts of conjugates could be isolated from smaller samples, the conjugates would be enriched relative to other phenolic extractives, and this would facilitate the purification process. Although the most sensitive method of determining conjugate content is by measuring their fluorescing light (Zeiss spectrophotometer PMQII with chromatogram scanning attachment; activation and fluorescence maxima at 365 nm, and 466 nm, respectively), the per cent change in accumulation must be interpreted with caution. This is not because the changes are not real, but because the changes in per cent accumulation may reflect not only substrate availability but degradation as well as the actual process of conjugation.

Studies were undertaken to investigate the possibility that γ-hydroxyarginine may affect the accumulation of N-feruloyl-2-hydroxyputrescine. This amino acid had been isolated previously by Bell and Tirimanna (102) from Vicia sativa and by Fujita (123) from the sea cucumber Polycheira refescens. Fujita studied its reaction with enzymes known to catalyse metabolic reactions of arginine. The work of Makisumi (124) supports the view that γ-hydroxyarginine can be a substrate in many of these reactions. Thus, hydroxyputrescine may well be formed from γ-hydroxyarginine via hydroxyagmatine and N-carbamylhydroxyputrescine. This is further supported by identification of the intermediate hydroxyagmatine in another organism, the sea-anemone Anthopleura japonica (125). The work of Lindstedt and Lindstedt (126) is also of interest here because in studying a homologous reaction with lysine decarboxylase from Bacillus cadaverine they identified hydroxycadaverine as the decarboxylation product of naturally occurring hydroxylysine.

Free γ-hydroxyarginine could not be detected in rusted wheat leaves containing N-feruloyl-2-hydroxyputrescine, but it should be pointed out that detection of this compound, if present in a plant extract, would be extremely difficult. The author found only two systems which could separate hydroxyarginine from arginine (thin-layer chromatography of Dansyl derivatives and thin-layer electrophoresis at pH 10.5). Although evidence for its natural occurrence was lacking, it was fed as a possible precursor as described in Materials and Methods. Feeding with γ-hydroxyarginine decreased the amount of N-feruloyl-2-hydroxyputrescine in the tissue by at least 50% (Table IV). It is difficult to explain these results by assuming that exogenous γ-hydroxyarginine stimulated the degradation of the conjugate. Since arginine is known to inhibit ornithine formation from glutamic acid in Escherichia coli (127) and in Neurospora lacking arginase (128), it is more likely that hydroxyputrescine formation, and therefore synthesis of the conjugate, is inhibited by γ-hydroxyarginine. If γ-hydroxyarginine is present in wheat leaves and functions in the same manner as arginine in Escherichia coli (127) and Neurospora (128), then the formation of N-feruloyl-2-hydroxyputrescine may be dependent on the conversion of glutamate to ornithine. The data in Table IV also show that feeding with γ-hydroxyarginine had no effect on the levels of the newly isolated conjugates in contrast to its effect on the amount of N-feruloyl-2-hydroxyputrescine. This can be explained by assuming that N-feruloyl-2-hydroxyputrescine is functionally separated in the cell from the newly isolated conjugates, and that the precursor does not have equal access to both. The author is not aware of any reports of

TABLE IV. Effect Of Various Amino Acids On The Accumulation Of N-Feruloyl-2-hydroxyputrescine And Of The Newly Isolated Conjugates In Rust-Infected Resistant Wheat Leaves

Feeding solution*	Amount of conjugate (mμ mole ferulic acid equivalents/g fr. wt.)**		% Accumulation (Buffer control=100%)	
	N-feruloyl-2-hydroxyputrescine	Newly isolated conjugates	N-feruloyl-2-hydroxyputrescine	Newly isolated conjugates
Buffer control	458	44	100	100
D-arginine (0.3 mM)	451	42	98	96
D-glutamic acid (0.3 mM)	463	43	101	98
D-ornithine (0.3 mM)	541	45	118	102
γ-hydroxyarginine (1.7 mM)	211	45	46	102
γ-hydroxyglutamic acid (1.0 mM)	552	43	121	98

* All precursors were dissolved in 0.01 M potassium phosphate, pH 7.0, containing 0.001 M $Ca(H_2PO_4)_2$, and this buffer was also used as a control.

** Determined by measurement of the fluorescing light as mentioned earlier in Results and Discussion.

functionally separate arginine pools in wheat leaves although there are two arginine pools in Escherichia coli (129). Another explanation would be that γ-hydroxyarginine does not serve as a precursor at all, but affects the accumulation of the conjugates in some other way and that this effect differs, depending on the conjugate .

If N-feruloyl-2-hydroxyputrescine is synthesized via the glutamate to ornithine pathway, introduction of an hydroxyl group would be necessary somewhere in this pathway. The hydroxyl group could arise from γ-hydroxyglutamic acid, a constituent of many Liliaceae which may be more widely distributed (104). It is not known whether γ-hydroxyglutamate may serve as a substrate of glutamic-γ-semialdehyde dehydrogenase, the first enzyme in the glutamate to ornithine pathway, but in some animals and micro-organisms it does serve as a substrate for the synthesis of hydroxyproline (103). Because of the possible interrelationship between glutamic acid, γ-hydroxyglutamic acid, and hydroxyproline, it was of interest to feed γ-hydroxyglutamic acid as possible precursor for the basic moiety of N-feruloyl-2-hydroxyputrescine. Indeed γ-hydroxyglutamic acid increased the level of N-feruloyl-2-hydroxyputrescine by 21% but it had no effect on the amount of the newly isolated conjugates (Table IV), although these are evidently closely related. Again, no single explanation can be given, and the results can be discussed in similar terms as those obtained after feeding of γ-hydroxyarginine. However, the data are consistent with the view that formation of N-feruloyl-2-hydroxyputrescine depends on the operation of the glutamate→ornithine→hydroxyproline pathway.

It is well known that many conjugates are detoxication products of D-amino acids. For example, N-malonyl-D-β-methionine (130) and N-succinyl-D-valine (131) are formed from the respective exogenously supplied D-amino acids. Rosa and Neish (132) have demonstrated the formation of N-malonyl conjugates of 8 different D-amino acids in barley shoots. Since activity from L-arginine-U-^{14}C, L-ornithine-5-^{14}C and L-proline-U-^{14}C was incorporated with very low efficiency into N-feruloyl-2-hydroxyputrescine, D-arginine, D-glutamate and D-ornithine were fed, because N-feruloyl-2-hydroxyputrescine may be the end product of a detoxication process for these D-amino acids. Indeed, D-ornithine increased the amount of N-feruloyl-2-hydroxyputrescine by 18% (Table IV). In all these experiments, D-amino acids were fed at concentrations equivalent to the estimated pool sizes of the respective L-amino acids (133) but below their phyto-toxic levels. The D-amino acids arginine and glutamate effected no change in the accumulation of the N-feruloyl-conjugates.

The origin of the hydroxyputrescine moiety of N-feruloyl-2-hydroxyputrescine still remains a mystery. The formation of the conjugate can be affected by feeding γ-hydroxyarginine, γ-hydroxyglutamate, or D-ornithine. This may be because they affect the process of conjugation rather than synthesis of the basic moiety . The conjugate N-feruloyl-2-hydroxyputrescine may arise from (or serve as a precursor to) a more complex structure such as the newly isolated conjugates. It may be possible to speculate further once the structural studies on the newly isolated conjugates are completed.

Experiments On The Possible Biological Activity Of Conjugates

Experiments were conducted to test the newly isolated ferulic acid conjugates, N-feruloyl-2-hydroxyputrescine, N-feruloylproline, and free ferulate for possible inhibitory effects during germination of stem rust uredospores. Free ferulate, in a cis-trans equilibrium, effected 100% inhibition at a concentration of 200 µg/ml (Table V). The tested conjugates and the model compound N-feruloylproline were without effect at this concentration. Controls displayed close to 100% germination with an average germ tube length of 700 µ. Evidently, conjugation renders ferulic acid less inhibitory.

Since it is well established that putrescine accumulates in potassium-deficient barley leaves (6) which exhibit characteristic necrotic symptoms, and since it was shown that this characteristic can be induced by feeding putrescine to normal barley leaves (7), it was of interest to determine whether N-feruloyl-2-hydroxyputrescine can produce necrosis in wheat leaves. The conjugate was unable to induce necrotic lesions at a concentration of 10^{-3}M when tested as described in Materials and Methods. Free ferulate and putrescine also lack the ability to induce necrosis when tested at this concentration. However, this concentration (10^{-3}M) is far below the putrescine concentration (75×10^{-3}M) used by Coleman and Richards (7) to induce necrosis in barley leaves. It was unfortunate that we lacked sufficient amounts of N-feruloyl-2-hydroxyputrescine and newly isolated conjugates to test them at concentrations closer to those used by these authors.

TABLE V. The Effect Of Amide-Linked Conjugates Of Ferulic Acid And Of Free Ferulic Acid On The Germination Of Stem Rust Uredospores

Test Solution* (200 µg/ml)	% Germination**
Control	>98
Ferulic acid	< 1
Newly isolated conjugate #1	>98
Newly isolated conjugate #2	>97
N-Feruloyl-2-hydroxyputrescine	>97
N-Feruloylproline	>98

* All conjugates were dissolved in 0.01 M potassium phosphate, pH 7.0, containing 0.0001 M $Ca(H_2PO_4)_2$, and this buffer was also used as a control.

** Spores were considered to have germinated if their germ tubes exceeded 200 microns in length.

SUMMARY AND CONCLUSIONS

The biogenesis and metabolic role of N-feruloyl-2-hydroxyputrescine in rusted wheat leaves was investigated. The observations and conclusions of this investigation are summarized as follows:

1. A technique is described for the preparative isolation of essentially pure amide-linked conjugates of ferulic acid from the ethanol soluble fraction of rusted wheat leaves.
2. The isolated conjugates were characterized by ultra-violet, nuclear magnetic-resonance, infrared, and mass spectra, thin-layer chromatography and high voltage thin-layer electrophoresis.
3. The isolated conjugates contain ferulic acid, conjugated in amide-linkage with hydroxyputrescine, hydroxyornithine, or hydroxyproline, and yet another moiety. The tri-acetate derivative of one of the isolated conjugates has a molecular weight of 494.
4. A new method is reported for the preparative isolation of γ-hydroxyarginine from Vicia sativa.
5. New methods are reported for the separation of arginine and γ-hydroxyarginine by thin-layer chromatography of the Dansyl derivatives and high voltage thin-layer electrophoresis of the natural compounds.
6. A new method is reported for the separation of glutamic acid and γ-hydroxyglutamic acid by high voltage thin-layer electrophoresis.
7. γ-Hydroxyarginine and γ-hydroxyglutamic acid were not detected in wheat leaves.

8. γ-Hydroxyarginine, when supplied exogenously to wheat leaves effected a substantial decrease in the amount of N-feruloyl-2-hydroxyputrescine, while feeding with γ-hydroxyglutamate and D-ornithine had the opposite effect.

9. A micro-bioassay for testing the effect of small amounts of metabolites on the germination of rust uredospores demonstrated no biological activity for N-feruloyl-conjugates at a concentration at which free ferulate effected 100% inhibition.

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