IDENTIFICATION STUDIES OF A QUINYL-COUMARATE IN THE PINEAPPLE PLANT

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FEBRUARY, 1958

by
G. K. Sutherland

Hawn.
AC1
H3
no.14
cop.2

59- 1992

THESIS COMMITTEE:

Paul J. Scheuer, chairman

Willis A. Gortner

Hiromu Matsumoto

John J. Naughton

Bruce J. Cooil

TABLE OF CONTENTS

	LIST OF FIGURES	iii
	LIST OF TABLES	v
I.	INTRODUCTION	1
II.	EXPERIMENTAL	12
	A. Extraction	12
	B. Syntheses	15
	1. p-Coumaric acid	15
	2. p-Methoxy cinnamic acid	16
	3. Quinide	17
	C. Ultraviolet Spectra as a Technique in Identification	18
	D. Characterization Experiments	21
	1. Ultraviolet spectra	21
	2. Chromatography	21
	3. Preparation of derivatives	27
	4. Neutral equivalent determinations	30
	5. Conductivity experiments	31
	6. Periodate oxidations	32
	7. Infrared spectra	33
III.	RESULTS AND DISCUSSION	34
	A. Identification of a p-Coumaric Ester	34
	1. Spectral results	34
	2. Chromatography of the extract	38
	3. Hydrolysis of the extract	43
	4. Further derivatives	46

a. Methylation 46

b. Hydrogenation 49

c. Acetylation 49

d. Bromination

B. Identification of the Quinic Acid Fragment 52

1. Preliminary characterization 52

2. Chromatographic studies 53

3. Isolation of Quinide 58

4. Methylation attempts 58

C. Structural Investigation 59

1. Neutralization equivalent experiments 60

2. Titration curves in non-aqueous medium 62

3. Conductivity experiments 63

4. Periodic acid oxidation 65

5. Controlled hydrolysis 66

D. Conclusion 69

IV. SUMMARY 74

V. REFERENCES 75

VI. ACKNOWLEDGMENTS 80

LIST OF FIGURES

FIG. 1 ULTRAVIOLET ABSORPTION SPECTRA OF (1) CHLOROGENIC ACID IN pH 6.8 BUFFER OR ETHANOL, (2) p-COUMARIC ACID IN ETHANOL, (3) p-COUMARIC ACID IN pH 4.1 BUFFER. 23

FIG. 2 ULTRAVIOLET ABSORPTION SPECTRA OF (1) CAFFEIC ACID IN pH 6.8 BUFFER, (2) CAFFEIC ACID IN pH 3.5 BUFFER OR IN ETHANOL, (3) FERULIC ACID IN pH 3.5 BUFFER. 23

FIG. 3 ULTRAVIOLET ABSORPTION SPECTRA OF CHLOROGENIC ACID IN (1) pH 7.2 BUFFER, (2) pH 7.7 BUFFER, (3) pH 8.6 BUFFER. 24

FIG. 4 ULTRAVIOLET ABSORPTION SPECTRA OF 7-HYDROXY COUMARIN IN (1) pH 3.5 BUFFER, (2) pH 10.0 BUFFER. 24

FIG. 5 ULTRAVIOLET ABSORPTION SPECTRA IN ETHANOLIC SODIUM ETHOXIDE OF (1) CHLOROGENIC ACID AFTER 10 MINS., (2) FERULIC ACID. 25

FIG. 6 ULTRAVIOLET ABSORPTION SPECTRA OF p-COUMARIC ACID IN ETHANOLIC SODIUM ETHOXIDE (1) AFTER 5 MINS., (2) AFTER 20 MINS. 25

FIG. 7 ULTRAVIOLET ABSORPTION SPECTRA OF (1) INITIAL AQUEOUS EXTRACT (IN WATER), (2) FINAL EXTRACT (IN ETHANOL), (3) FINAL EXTRACT IN ETHANOLIC SODIUM ETHOXIDE (.002 M). 35

FIG. 8 INFRARED SPECTRA OF (1) AUTHENTIC p-COUMARIC ACID (2) p-COUMARIC ACID PREPARED FROM THE HYDROLYZED ESTER. 47

FIG. 9 INFRARED SPECTRA OF (1) AUTHENTIC 2,4,6-TRIBROMOPHENOL (2) TRIBROMOPHENOL PREPARED BY BROMINATION OF THE EXTRACT. 54

FIG. 10 CHROMATOGRAM OF THE QUINIDE FROM THE EXTRACT (1 AND 2) AND FROM CHLOROGENIC ACID (3) IN n-BUTANOL/ACETIC ACID/WATER (4:1:2.2). 55

FIG. 11 CHROMATOGRAM OF THE QUINIDE FROM THE EXTRACT (1 AND 2) AND FROM CHLOROGENIC ACID (3) IN ISOPROPANOL/AMMONIA/WATER (6:3:1). 55

FIG. 12 TITRATION IN AQUEOUS ETHANOL WITH 0.1N SODIUM METHOXIDE OF (1) CHLOROGENIC ACID (2) EXTRACT (3) QUINIDE. 61

FIG. 13 TITRATION IN N,N-DIMETHYLFORMAMIDE WITH 0.1 N SODIUM METHOXIDE OF (1) CHLOROGENIC ACID (2) EXTRACT (3) QUINIDE 61

FIG. 14 RATE OF PERIODATE OXIDATION OF (1) QUINIC ACID (2) QUINIDE. 70

FIG. 15 RATE OF PERIODATE OXIDATION OF (1) FERULIC ACID (2) EXTRACT BEFORE HYDROLYSIS (3) EXTRACT AFTER HYDROLYSIS. 70

LIST OF TABLES

TABLE	I.	INFLUENCE OF ALKALINE ALCOHOLIC MEDIA ON THE ULTRAVIOLET SPECTRA OF HYDROXY CINNAMIC ACIDS AND ESTERS.	22
TABLE	II.	R_f VALUES IN VARIOUS SOLVENTS.	40
TABLE	III.	COLORS DEVELOPED BY DIAZOTIZED p-NITROANILINE SPRAY.	41
TABLE	IV.	CHROMATOGRAPHY ON BORATE-IMPREGNATED PAPER.	41
TABLE	V.	ULTRAVIOLET SPECTRAL DATA ON SOME CINNAMIC ACID DERIVATIVES.	48
TABLE	VI.	R_f VALUES OF THE METHYLATED AND HYDROLYZED EXTRACT.	48
TABLE	VII.	R_f VALUES OF THE ALCOHOL (WATER-SOLUBLE) FRACTION OF THE HYDROLYZED EXTRACT.	57
TABLE	VIII.	EFFECT OF BORIC ACID ON CONDUCTANCE.	67
TABLE	IX.	EXTINCTION COEFFICIENTS OF QUINYL ESTERS IN ETHANOL.	71
TABLE	X.	CALCULATIONS OF THE MOLE RATIO OF p-COUMARIC AND QUINIC ACIDS IN THE EXTRACT.	72

I. INTRODUCTION

For quite a long time, the Pineapple Research Institute of Hawaii has been interested in bromelain, the proteolytic enzyme found in various members of the Bromeliaceae family. As early as 1928, investigations of pineapple stem colloids were carried out; and in 1949, a program was set up with the hope that a technique could be developed for isolating the enzyme commercially. During these latter investigations it was found that crude bromelain extracts when kept at neutral or alkaline pH's suffered rapid inactivation, and resulted in the formation of black pigments. It was possible to remove from stem juice by dialysis or ethyl acetate extraction, one or more compounds responsible for this discoloration of bromelain.

This discoloration of the crude enzyme solutions suggested the presence of polyphenolic materials, which were known to be widespread in plant tissues and to be susceptible to oxidation, leading to the formation of polymeric dark-brown or red compounds, often called phlobaphenes. Control of enzymic browning in industry is based on the idea that browning is due largely to interaction of a polyphenolase enzyme with molecular oxygen and a suitable phenolic substrate. Ponting and Joslyn(1) showed that polyphenolase was largely responsible for both ascorbic acid oxidation and darkening in apple tissue extracts; and it had long been recognized that

rapid browning on the outer surfaces of fresh fruits was an enzyme oxidation process, but the mechanism of this process is still not well understood. The natural phenolic substances in fruit which undergo enzymatic oxidation and polymerization to dark pigments have not been investigated to any great extent. Flavones and flavonols have been suspected of participating in such a system, since they form characteristic oxidation colors with oxidizing enzymes; e.g. quercitin was isolated from apple peel and identified(2). Szent-Györgyi(3) suggested that flavanones (hesperetin from lemon peel) served as intermediates in the oxidation chain between peroxidase and ascorbic acid. Hale(4) partially purified the chromogenic substrate of peroxidase in apples, and found a white amorphous nitrogen-free slightly acid material, autoxidizable in alkaline solution and rapidly oxidized in neutral or slightly acid solutions by peroxidase and hydrogen peroxide to form a deep brown color. Chlorogenic acid has more recently been identified as one of the substrates involved in enzymic browning of apples(5). Oxidation of 1,2-dihydroxy-or 1,2,3-trihydroxybenzene compounds in the presence of o-polyphenoloxidase or peroxidase gave rise to brown condensation products(6). The bromelain discolorations were considered sufficiently analogous to postulate the existence of flavonoid constituents in the commercially produced pineapple, (Ananas

comosus var. Cayenne).

A further suggestion for the existence of polyphenolic material in pineapple arose from other enzyme studies carried out by W. A. Gortner and M. Kent(7). Pineapple leaf and stem tissue was found to contain a strong oxidizing enzyme capable of destroying indoleacetic acid, the natural growth auxin. However, when reacting stem extract with a known quantity (ca. 200) of indoleacetic acid substrate, very little indoleacetic acid destruction was noticed if more than an optimum quantity of extract was used; while at lower concentrations very powerful oxidase activity was found. It appeared that stem extract contained a powerful inhibitor of indoleacetic acid oxidase activity. Experiments at various substrate-enzyme ratios showed, however, that low concentrations of a partially purified sample of inhibitor could stimulate, and higher levels inhibit, indoleacetic acid oxidation.

Although no isolation of this inhibitor had been carried out, certain of its characteristics had been recognized and indicated that it was possibly phenolic in nature. The ultraviolet absorption spectrum of the stem extract differed noticeably from that of a photolyzed sample; exposure to light effected a marked reduction in absorbance in the vicinity of 315 m . The curve resulting from a plot of the difference between untreated and photolyzed extract was the same curve as was obtained

by lead acetate precipitation from the aqueous extract, followed by removal of the lead with sulfuric acid.

Scopoletin (7-hydroxy-6-methoxycoumarin) had been reported(8) as a competitive inhibitor of enzymic oxidation of indoleacetic acid oxidase, but its ultraviolet absorption curve was different from that of the stem extract. Chlorogenic acid had a somewhat similar absorption spectrum to that of the partially purified inhibitor, and it also showed marked inhibition of indoleacetic acid oxidase. However, it was known to be a light-stable material, and undoubtedly distinct from the inhibitory factor in pineapple.

These data showed sufficient evidence for suspecting the presence of polyphenolic material in pineapple, quite possible of a C_6-C_3-C_6 type, which is the structural basis for flavonoid classification; or belonging to the C_6-C_3 class of compounds, of which chlorogenic acid is a commonly occurring member.

A very comprehensive survey of the occurrence of flavonoid compounds, and the biogenesis of such constituents, has been prepared by Geissman and Hinreiner(9). In the review, these authors have divided the C_{15} components of plants into the commonly occurring groups of flavonoids, coumarins and propenylbenzenes. For the purposes of the review, the flavonoids were regarded as being those compounds which possessed two aromatic rings linked by an aliphatic three-carbon chain. Further classification was determined by the state of oxidation of the C_3 fragment. This

subdivision lead to classes of compounds ranging from the highly reduced anthocyanidin group (I), through the non-heterocyclic chalcone (II), to the highest oxidation state found in flavone (III).

The hydroxylation pattern of both aromatic rings in this flavonoid series is fairly regular. Ring A, which is fused to the heterocyclic ring in most flavonoids and which is linked directly to the carbonyl group of the chalcones, is generally substituted on a phloroglucinol pattern. Ring B may be mono-, di or tri-substituted and the hydroxyl groups are found in positions 4', 3'-4', or 3'-4'-5'.

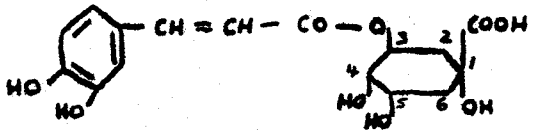
In the coumarin family the C_3 side chain may be considered esterified back to an adjacent hydroxyl group in the aromatic ring (IV).

Coumarins are widely distributed in the Umbelliferae and Rutaceae families, where they occur in roots, trunks, leaves or fruits. These compounds are mostly mono- or di- hydroxylated, in which cases the hydroxyl groups are found para to the side chain (position 7, mono-substituted coumarins), or in positions 6,7 or 7,8 for the dihydroxy members. The use of ultraviolet absorption spectra as a tool in the identification of coumarin derivatives has been suggested by Goodwin and Pollock(10). A valuable table of chromatographic data of coumarin constituents, which admirably differentiates the coumarins from the cinnamic acid group, has been prepared by Swain(11).

Among the propehylbenzene group, the commonest structures are based on a hydroxycinnamic acid type, where the double bond is in a position conjugated to the benzene ring. The C_3 side chain occurs in several oxidation states, giving rise to derivatives of cinnamyl alcohol, cinnamaldehyde and cinnamic acid. Cinnamyl alcohol has been observed as a degradation product of lignins, and dimerization of two propenylbenzene derivatives at the -carbons of the side chain would result in the nignane nucleus.

Substitution of the cinnamic acid class by hydroxyl agoups again conforms to an overall general pattern.

The position para to the unsaturated side chain is substituted in all common hydroxycinnamic acids. If this is the only substituent group the acid is known as p-coumaric acid (V, R_1=R_2=H). The dihydroxy compound caffeic acid (V, R_1=H, R_2=OH), its monomethyl ether ferulic acid (V, R_1=H, R_2=OCH_3), and the symmetrically substituted sinapic acid (V, R_1=R_2=OCH_3) have been recently recognized along with p-coumaric acid as commonly occurring phenolic constituents(12). A systematic survey of phenolic constituents in leaves of vascular plants has shown the wide incidence of these hydroxycinnamic acids, occurring for the most part as esters. In contrast, the C_{15}-flavonoids occur generally as glycosides. Esterification of a hydroxycinnamic acid with a cyclic C_6 alcohol would result in a C_6-C_3-C_6 structure similar, except for the absence of a hetero oxygen atom and the presence now of a saturated C_6 ring, to the overall C_{15} flavonoid nucleus. Such an ester is chlorogenic acid(VI), the quinic ester of caffeic acid, and which is thought to be a participant in certain biosynthetic mechanisms.



VI

The frequency of distribution of the hydroxycinnamic acids in hydrolysates of monocotyledonous and dicotyledonous leaves has been tabulated by Bate-Smith(13). The biogenetic significance of the appearance of p-coumaric and caffeic esters in the albino form of Antirrhinum majus,

and their relationship to the flavonoid constituents of the pigmented genotypes, has been emphasized by Geissman and Harborne(14). Birch(15) found that 3-hydroxy, 4-hydroxy and 4-hydroxy-3-methoxycinnamic acids were incapable of participating in flavonoid (quercitin) biosynthesis in one of the green algae, while caffeic acid was very suitable. It has been suggested(16) that the B-ring and the C_2-C_3-C_4 carbons of the C_{15} flavonoids arise from the same fundamental precursors as the cinnamic acids. That the quinic acid part of the chlorogenic acid molecule has some independent biochemical effect can be seen from the fact that, while both caffeic acid and chlorogenic acid are effective as inhibitors of indoleacetic acid oxidase (quinic acid itself does not inhibit), only chlorogenic acid is a competitive inhibitor(17) i.e. it competes with indoleacetic acid for active sites on the enzyme surface.

Evidence has been produced that members of the hydroxycinnamic acid class of compounds are involved in respiratory and protective mechanisms in plants. Kuć et al.(18) have shown that, while fungistatic phenols which are associated with disease resistance are generally present in the plant tissue prior to infection, caffeic and chlorogenic acids could be isolated from extracts of potato after the fungus Helminthosporium carbonum had been able to make limited growth. All varieties of potato tuber resistant to scab showed a strong ferric chloride test, which was taken to indicate the presence of chlorogenic

acid(19). On the basis of such tests, chlorogenic acid appeared to accumulate around areas of tissue injury, suggesting it was involved in a protective mechanism. The suggestion was made that the acid lowered the pH of the cell, thereby creating an unfavorable medium for growth of the organism.

Chlorogenic acid was also found in apple tissue(20), where the quinic acid was thought to make the caffeic acid fraction more sap-soluble, and render the o-dihydroxy properties more readily available. This makes the chlorogenic-caffeic relation analogous to the glycosides of insoluble flavonoids. The wide distribution of chlorogenic acid in plants is demonstrated by its identification in coffee(21), tea(22), flax(23), and sweet potato(24). The double bond in the cinnamic acid side chain presents one possible position for the existence of isomers of chlorogenic acid, and these have in fact been detected on chromatograms(22). The various possible points of esterification on the quinic acid moiety lead to other possible isomers. An amorphous material named isochlorogenic acid, and suggested to be a position isomer with the caffeic acid fraction attached to the number 5 carbon of the quinic ring, was isolated from coffee and characterized by Barnes et al.(25). This structure allows the possibility of lactonization through the C_3-hydroxyl, which is not possible with chlorogenic acid, since the C_4- and C_5- hydroxyl groups are trans to the carboxyl.

From the chlorogenic acid present in peaches, a new isomer was isolated and named neochlorogenic acid(26), but the position of attachment of the caffeoyl group has not been finalized. A further isomer of uncertain structure, pseudochlorogenic acid, was isolated by some workers in Japan(27). More recently, a dicaffeoyl quinic ester has been isolated and identified as the biologically active principle in the artichoke(28). In this material, the caffeic fractions were shown by derivatization and degradation to be attached to the C_1- and the C_4- hydroxyls.

Glycosides of the hydroxycinnamic acids are not known to occur commonly, although a recent article reports a p-coumaric ester in which the phenolic group is linked to a glucose residue(29). Virus-infected leaves of cherry and peach trees were found to be significantly higher in p-coumaric and caffeic ester content than the normal leaves(30). A few reports on the existence of p-coumaric esters are to be found in the literature of the last two years. Spectral alterations during alkaline treatment of sugar cane lignin is attributed to the hydrolysis of such an ester link(31). Chromatography of tea, apple and pear extracts led to several spots which gave rise to the appearance of p-coumaric and quinic acid spots after subsequent elution, hydrolysis and chromatography(32). Although a subsequent report referred to these p-coumaryl-quinic esters(33), no suggestion as to their structure has been made to date.

The initial object of the research was to isolate and identify, by standard chemical techniques, any flavonoid materials present in the pineapple plant. It was felt that amongst this class of compounds would occur the polyphenolic materials which were responsible for the browning of plant extracts, and the biologically active materials with the pronounced pineapple indoleacetic acid oxidase activity.

II. EXPERIMENTAL*

A. Extraction

Vegetative plants aged nine to fifteen months were provided from the experimental fields of the Pineapple Research Institute at Waipio. The leaves were removed and the lower section where root growth occurred was cut off. Leaf base material remaining was removed, and the top of the undifferentiated stump was rejected during this step. The remaining stumps, ranging from 50-150 g. were then sliced both longitudinally and laterally and macerated in a Waring Blendor with 1.4 times their weight of water for several minutes. The thick suspension from the Blendor was separated partially by use of a basket centrifuge, and the cloudy suspension obtained was heated to 90-100° for a short time to coagulate some of the protein material, cooled and filtered by suction with the aid of Dicalite filter aid. Concentrated hydrochloric acid was added to lower the pH of the solution to 1.6-1.7, since it was found that organic solvents would not remove the ultraviolet absorber from the aqueous

---

*All melting points are uncorrected. Ultraviolet spectra were taken on a Beckman Model DU Spectrophotometer fitted with a photomultiplier. Infrared spectra were taken on a Beckman Model IR-2A Spectrophotometer. Conductivity measurements were taken on a Conductivity Bridge (Industrial Instruments Co. Model RC 16-B-1). Elemental analyses were performed by the Mikroanalytisches Laboratorium im Max-Planck-Institut, Mülheim (Ruhr), Germany.

solution at neutral or slightly acid pH values, and the aqueous solution was extracted twice with one quarter its volume of ethyl acetate. During this extraction an extremely stable emulsion was formed which did not settle on standing for 24 hours, but which was largely broken by centrifuging.

The ethyl acetate layer was washed with saturated sodium bicarbonate, and the yellow alkaline solution was acidified with concentrated hydrochloric acid. This cloudy acid suspension was extracted twice with an equal volume of ether, and the pale yellow ether solution was stored in the cold until required. When an aqueous solution of the material was required, an equal volume of water was added and the ether removed either by distillation from a warm water bath, or by evaporation in vacuum while mixing the two phases with a magnetic stirrer. The aqueous remainder was filtered and lyophilized to prepare a solid sample of the extract. This final product was a yellowish white powder, which on exposure to the atmosphere became brown and resinous almost immediately. It was transferred in a low humidity atmosphere to a sample bottle, dried again in a vacuum desiccator over calcium chloride, and stored in a desiccator, where it was able to be kept for a few weeks. During each step where solutions were involved, the movement of the required material could be followed by checking the absorption spectra of the various solutions.

An alternative procedure, adopted initially for small samples, used the unheated aqueous solution from the centrifuge, from which a bulky white precipitate of an almost clay-like consistency was obtained by the addition of a saturated neutral lead acetate solution. After the solid had been separated by centrifuging, the lead was removed by stirring with 1N sulfuric acid. This acid solution was extracted with ethyl acetate as in the previous method. Paper chromatography showed that both of these methods removed the same constituents under investigation. Continuous extraction of the acid solution with ethyl acetate was attempted but abandoned because of the brown color of the organic layer and the necessity of exposing the solution to elevated temperatures for several hours. Preliminary experiments indicated that continuous extraction with ether removed only part of the material, ethyl acetate then removed the remainder, and both solutions were spectrally and chromatographically indistinguishable.

Another method involved drying the slices of the stumps in a forced-draught oven, powdering in a Wiley mill, and extracting into a suitable solvent in a Blendor or in a Soxhlet. Solutions so obtained were severly contaminated, being brown in color and showing marked spectral differences, so this approach was abandoned.

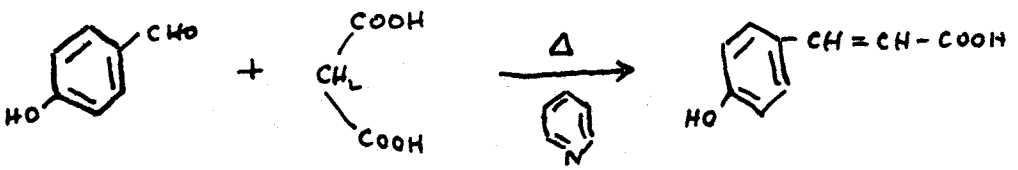
Further purification steps were investigated without success. Adsorption chromatography on Magnesol and

Florisil gave some band separation, but the material could not be recovered in the same form either by washing down the column, or by elution from the extruded column. The use of ion exchange resins (IRC-50, IRA-400), and partition chromatography on silicic acid (using 0.5 N sulfuric acid and chloroform containing 15% n-butanol as the two liquid phases), were not successful. Although some purification and separation of constituents were obtained by ascending paper chromatography on Whatman 3 MM. paper, it was found impossible to elute more than a very small fraction of the material from the chromatogram, and considerable degradation occurred during this elution. It was further established that the only way to prepare a solid extract capable of convenient handling was by freeze-drying aqueous solutions. When solutions in other solvents, from which water had been removed, were subjected to distillation at the lowest convenient temperatures, almost complete destruction of the ester took place, as indicated by the pronounced darkening of the concentrated solution, and the appearance of a viscous dark brown residue.

B. SYNTHESES

1. p-Coumaric acid: This was prepared according to the method of Pandya and Vahidy(34): 7.5 g. (0.062 moles) p-hydroxy benzaldehyde, 6.0 g. (0.058 moles) dry malonic acid, and 0.75 ml. (0.01 moles) pyridine were heated for

7.5 hours at 100°, then allowed to stand overnight. The resinous material was then extracted with 30 ml. of 20% sodium carbonate solution, and the alkaline solution acidified with 10 ml. concentrated hydrochloric acid. The pale pink-orange solid was filtered, washed with water, and recrystallized twice from hot water to give 3.0 g. of fine slightly orange powder m.p. 210.5-213.5° (dec.).

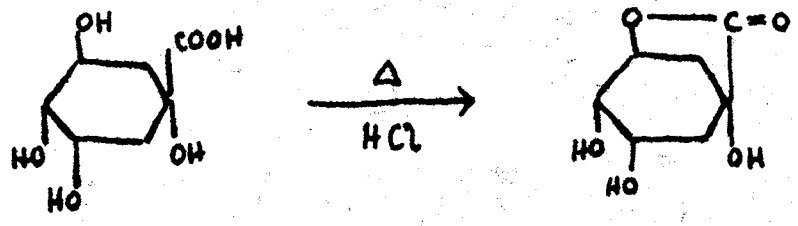


2. <u>p-Methoxy cinnamic acid</u>: This was synthsized by a similar reaction(35): 27.6 g. (0.22 moles) anisaldehyde (Eastman), 20.8 g. (0.20 moles) malonic acid (Eastman) and 30 ml. (0.4 moles) pyridine (Eastman) were heated on a steam bath together with 2 ml. piperidine for 5.5 hours. After standing overnight, sufficient concentrated hydrochloric acid (30 ml.) was added to convert the pyridine to its hydrochloride, followed by 50 ml. water. The flocculent precipitate was filtered, washed with water, then stirred with 100 ml. of 10% sodium hydroxide solution. The alkaline solution was decanted, acidified with concentrated hydrochloric acid, and the precipitate filtered, dried, and recrystallized from hot water. When the melting point was checked, the sample began to melt at 164° and by 172° had formed an opalescent liquid, which changed to a clear liquid at 187°. This agrees with

the reported liquid-crystalline properties of p-methoxy-cinnamic acid(36).

3. Quinide (quinic acid lactone) (37):

(a) From quinic acid-1 g. dry quinic acid (supplied by General Foods Laboratories) was refluxed for five hours with 50 ml. 1,4-dioxan (Eastman, purified by refluxing eight hours over sodium followed by distillation through a good fractionating column, b.p. 99°), which had been saturated with hydrogen chloride by bubbling the gas through the dioxan for one hour. The hydrogen chloride was generated from ammonium chloride and concentrated sulfuric acid. Potassium carbonate (0.5 g.) was added and the reflux continued for 20 minutes. After cooling and filtering the solvent was distilled under reduced pressure. The residue was crystallized from a small volume of boiling ethanol, washed with a few mls. of ethanol and dried at 100° for several hours. Yield 0.41 g., m.p. 194-5°. Paper chromatography showed that this preparation was free of quinic acid.



(b) From chlorogenic acid. Small amounts of quinide were prepared by boiling several milligrams of chlorogenic acid (supplied by General Foods Laboratories) with 3-5 ml. concentrated hydrochloric acid for a few minutes, and extracting

the caffeic acid formed in the hydrolysis with ether. The aqueous residue was used as a source of quinide for paper chromatography.

C. Ultraviolet Spectra as a Technique in Identification*

The presence of an ultraviolet absorption peak around 315 mμ in aqueous extracts of pineapple stumps had been shown to be connected with a material whose properties were conceivably phenolic(7). The possible relation of this material to chlorogenic acid, together with the fact that its salient characteristic in the early stages of the investigation was its absorption spectrum, prompted the accumulation of considerable ultraviolet data on commoner members of the C_6-C_3 family of plant phenols. Goodwin and Pollock have already compiled a series of ethanolic spectra of coumarins(10). The spectral changes of several hydroxycinnamic acids and their esters, caused by the influence of boric acid and sodium borate, have been used to differentiate o-dihydroxy compounds from others of this class(38). Free acids can be distinguished from their esters(39) by comparison of their spectra in the presence of sodium acetate. Where spectral data on hydroxycinnamic acids and their esters are available, these compounds have not generally been treated as a separate class of flavonoids. Since these constituents occur usually

---

*This material in Section II C has been accepted for publication in Archives of Biochemistry and Biophysics, under the title "Preliminary Classification of Some Naturally Occurring Hydroxy Cinnamic Acids Through Their Ultraviolet Spectra," by G. K. Sutherland.

in small concentrations in plant materials, provisional identification by means of their spectral characteristics is a very convenient technique.

Solutions of approximately 5 X 10^{-5} M were chosen as having appropriate absorbance values. Sodium ethoxide solvent was prepared by diluting a 0.4 M ethanolic solution of sodium ethoxide to approximately 0.002 M. Ethanol-sodium hydroxide solutions were formed by adding two drops of 6N aqueous sodium hydroxide to 3.5 ml. of an ethanolic solution of the compound under consideration. Aqueous buffers were made up from appropriate proportions of 0.1 M potassium hydrogen phosphate and 0.1N sodium hydroxide or 0.1 M phosphoric acid solutions.

The members of the C_6-C_3 family with hydroxyl substitution para to the unsaturated side chain that were studied, viz. caffeic, ferulic, p-coumaric acids and esters, and 7-hydroxy coumarin, all show absorption peaks in the region 305-330mμ. Of these, the first two disubstituted types show pronounced plateaus or shoulders at wavelengths 20-25mμ below the λmax. As indicated by Geissman and Harborne(14), the spectra of hydroxycinnamic acids are almost identical with their esters in neutral solvents, but differ considerably in strongly alkaline media.

The shape of the spectral curves of these compounds in aqueous acid buffers does not necessarily follow the shape of the ethanolic curve. Caffeic acid and ferulic acid require a much lower aqueous pH for reproduction of

their ethanolic spectra than does chlorogenic acid. Chlorogenic acid in pH 6.8 medium has an absorption curve identical with its spectrum in ethanol (Fig. 1); while caffeic acid requires a pH of 3.5 before a similar result is obtained (Fig. 2). Neither p-coumaric nor ferulic acids at similar low pH values (4.1 and 3.5 respectively) has the same absorption curve it shows in ethanol (Figs. 1 and 2), although the ferulic spectrum is quite similar. The presence of three possible isosbestic points, marking the change of the chlorogenic acid spectrum from acid to alkaline pH values, shows the necessity of choosing a suitable wavelength to measure concentrations of chlorogenic acid in aqueous solutions of uncontrolled pH (Fig. 3).

In alkaline solutions, the esters generally show greater stability than the free acids, but this is dependent both on the nature of the alkaline medium and on the cinnamic derivative. In ethanolic sodium hydroxide and aqueous alkaline buffers, caffeic and chlorogenic acids are immediately unstable, while ferulic acid and 7-hydroxy coumarin show stable bathochromic shifts of 20-65 mμ (cf. Table 1 and Figure 4).

In sodium ethoxide, chlorogenic acid shows a stable alkaline spectrum, with a large shift of approximately 60 mμ to longer wavelengths(23); the sodium ethoxide spectrum of ferulic acid is also stable with a smaller bathochromic shift (Fig. 5). p-Coumaric and caffeic acids are unstable in sodium ethoxide, and p-coumaric acid

parallels its decomposition in ethanolic sodium hydroxide with a slow shift to lower λ max. values. For this compound, a suitable spectrum can be taken immediately. Over a longer period a new peak appears at 290-295 mμ (14), (31), presumably due to decomposition at the double bond (Fig. 6). In aqueous 1N sodium hydroxide, p-coumaric acid is found to be relatively stable over a period of several days, but if such a solution is diluted with ethanol (eg. for spectral determinations), degradation begins immediately.

D. Characterization Experiments

1. Ultraviolet Spectra. All spectra were obtained by measuring the absorbance at 10 mμ intervals, except in the regions of maxima and minima, when readings were taken every 5 mμ , and if necessary every 1 mμ . 1 cm. Silica cells were used and a hydrogen lamp covered the range 205-400 mμ .

2. Chromatography. All chromatograms were run by suspending the paper strip from the glass cover of a tank of rectangular cross section. No equilibration of solvents was found necessary unless stated. Whatman No. 1 or 3 MM paper was employed, and the irrigated chromatograms were dried in an air-draught hood. The solvent systems most generally employed were those indicated in earlier references dealing with paper chromatography of flavonoid-type compounds(11), (12), (13). Sprays were

TABLE I

INFLUENCE OF ALKALINE ALCOHOLIC MEDIA ON THE ULTRAVIOLET SPECTRA OF HYDROXY CINNAMIC ACIDS AND ESTERS

	λ max. (mμ)		
	EtOH	EtOH/NaOH	EtOH/NaOEt
Chlorogenic acid	328	Unstable	385
Caffeic acid	324	Unstable	Unstable
Ferulic acid	324	343	345
p-Coumaric acid	312	Unstable	Unstable
7-Hydroxycoumarin	330	377	377

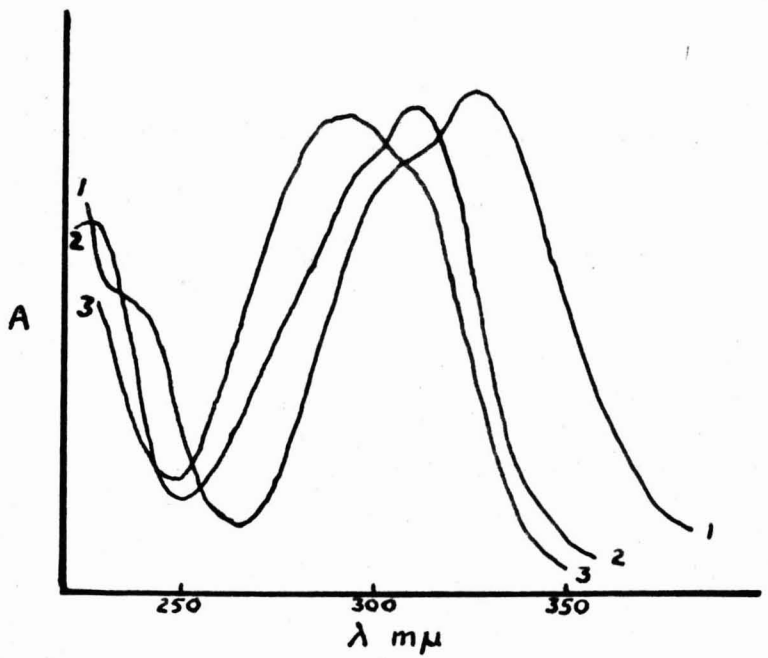


Fig. 1

Ultraviolet absorption spectra of (1) chlorogenic acid in pH 6·8 buffer or in ethanol (2) p-coumaric acid in ethanol (3) p-coumaric acid in pH 4·1 buffer.

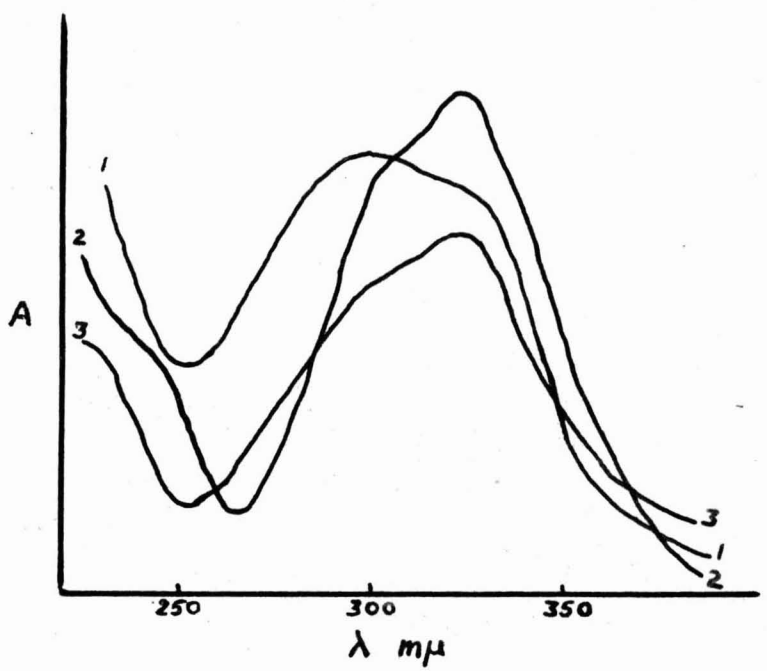


Fig. 2

Ultraviolet absorption spectra of (1) caffeic acid in pH 6·8 buffer (2) caffeic acid in pH 3·5 buffer or in ethanol (3) ferulic acid in pH 3·5 buffer.

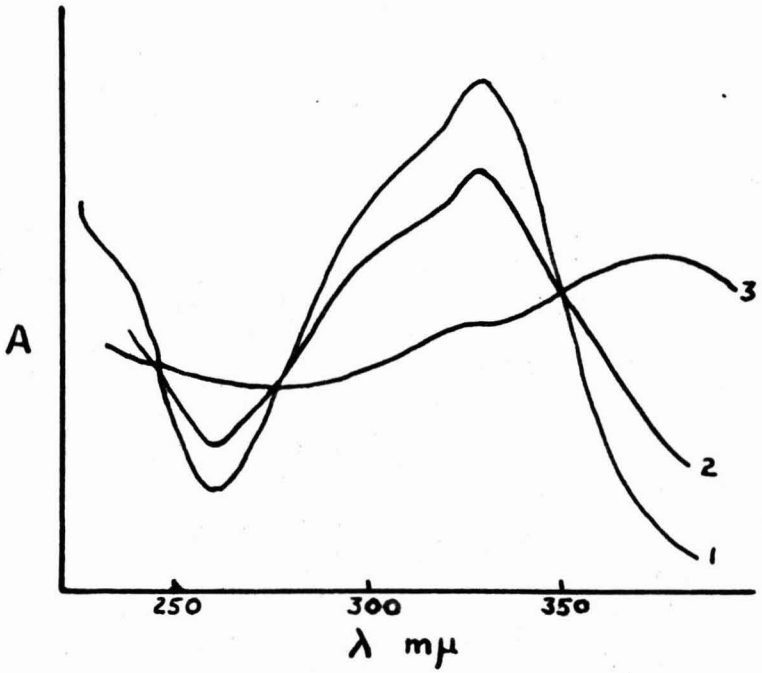


Fig. 3

Ultraviolet absorption spectra of chlorogenic acid in (1) pH 7·2 buffer (2) pH 7·7 buffer (3) pH 8·6 buffer.

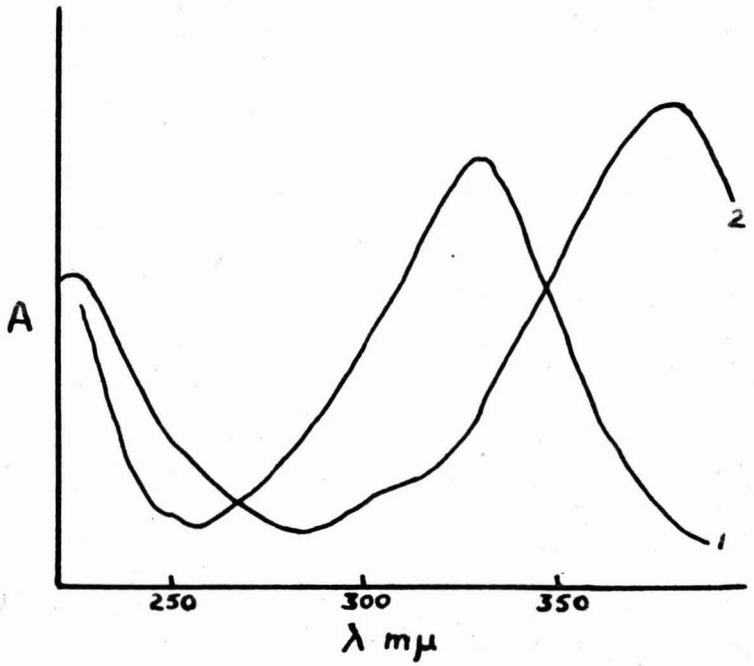


Fig. 4

Ultraviolet absorption spectra of 7-hydroxy coumarin in (1) pH 3·5 buffer (2) pH 10·0 buffer.

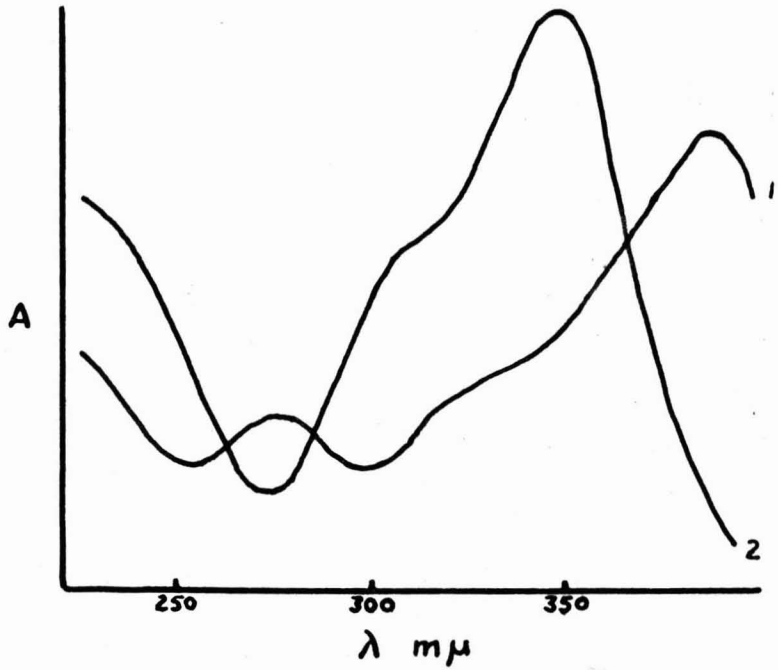


Fig. 5

Ultraviolet absorption spectra in ethanolic sodium ethoxide of (1) chlorogenic acid after 10 mins. (2) ferulic acid.

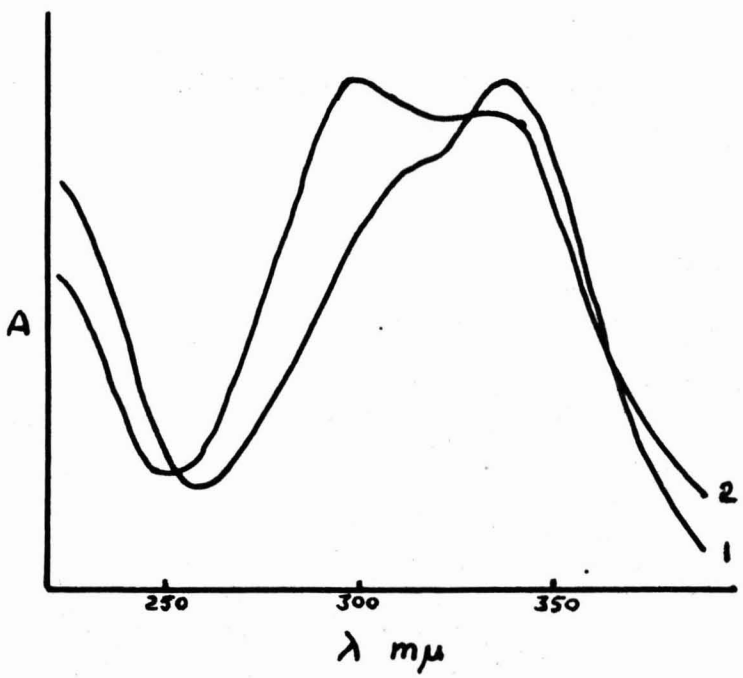


Fig. 6

Ultraviolet absorption spectra of p-coumaric acid in ethanolic sodium ethoxide (1) after 5 mins. (2) after 20 mins.

used extensively for locating phenolic spots on chromatograms. These sprays included:

(a) diazotized p-nitroaniline(11): - 5 ml. of 0.5% solution of p-nitroaniline (Eastman) in 2N hydrochloric acid, 0.5 ml. fresh 5% aqueous sodium nitrite (Baker's Analyzed C. P.), and 15 ml. of a 20% sodium acetate solution (Merck, Reagent); followed after drying by 20% aqueous sodium carbonate (Baker Analyzed).

(b) diazotized sulfanilic acid(40): - 1.5 ml. of a solution of sulfanilic acid (Eastman, 0.9 g. in 100 ml. of 1.2N hydrochloric acid), 1.5 ml. fresh 5% sodium nitrite solution; after 5 minutes a further 6 ml. of nitrite solution was added, and after standing 5 minutes the solution was diluted to 100 ml. with water.

(c) bis-diazotized benzidine(41): - 1.25 g. benzidine (Eastman), 3.5 ml. concentrated hydrochloric acid and 250 ml. water; 25 ml. of this solution was mixed with 25 ml. of 10% sodium nitrite solution, stirred until clear pale yellow, and used within 10 minutes. These diazonium sprays couple readily with phenolic compounds.

Sprays utilized for locating glycol materials on chromatograms included:

(a) periodate-benzidine(42): - saturated aqueous potassium periodate, followed within a few minutes by a solution containing 10 volumes of a benzidine solution (0.1 M in 50% aqueous ethanol), 2 volumes of acetone, and 1 volume of 0.2N hydrochloric acid. Glycols appeared as white spots

on a blue background which fades to brown, due to the removal of the periodate at the glycol spot preventing reaction with the benzidine.

(b) periodate-nitroprusside-piperazine(43): - saturated aqueous potassium periodate diluted with 2 volumes of water, followed after 20 minutes with a solution of 50 mg. sodium nitroprusside (Baker and Adamson, Reagent) and 150 mg. piperazine hydrate (Eastman, Practical) in water (2 ml.) and ethanol (10 ml.). After 5 minutes' heating at 100°, quinic acid and quinide show as yellow spots, which turn sky-blue after 24 hours.

Borate-impregnated paper was prepared by spraying sheets of Whatman No. 1 paper with 0.1 M sodium borate (Baker's Analyzed C.P.) without flooding the paper, and drying the paper by suspending it in an air-draught hood.

3. Preparation of Derivatives

(a) Hydrolysis: rapid hydrolyses were carried out by refluxing dilute solutions in 2N hydrochloric acid or 1N sodium hydroxide. Room temperature hydrolysis to prevent degradation of alkali-sensitive material was performed by allowing a dilute solution of the ester to stand in 1N sodium hydroxide under nitrogen for 6 hours.

(b) Methylation: The extract was successfully methylated after the method of Panizzi and Scarpati(44). It was refluxed in anhydrous acetone with dimethyl sulfate in the presence of anhydrous potassium carbonate. By dissolving the ester in a small volume of ethanol with a similar

volume of 20% aqueous sodium hydroxide, and heating for a short time with dimethyl sulfate, the ester could be similarly methylated. However, an ethereal solution of diazomethane was ineffective in producing methylation at room temperature. Treatment with diazomethane under reflux, followed by refluxing with methyl iodide and silver oxide, produced no crystalline derivative either before or after subsequent hydrolysis.

(c) Reduction: The extract was reduced readily with hydrogen in any of several ways. In ethanol solution in the presence of sodium amalgam (prepared by dissolving carefully 0.5 g. sodium, freshly cut into small pieces, in approximately 50 g. mercury), the material was reduced by allowing to stand overnight. Reduction of the extract caused a pronounced decrease in ultraviolet absorbance with a shift in the λ max., so the extent of reduction was readily followed. In methanol solution in a hydrogen atmosphere, and in the presence of a small amount of platinum oxide catalyst or a 10% palladium-charcoal catalyst, reduction was also effected. The palladium-charcoal catalyst was prepared according to the method described by Cheronis(45). The reduced material was recovered by diluting the solution with water, distilling the methanol under vacuum, and lyophilizing the residual aqueous solution. As with the original lyophilized extract, this reduced material shows an immediate tendency to become resinous and semi-liquid while being transferred from the drying bottle.

(d) Acetylation: An acetylated derivative was prepared very inefficiently by allowing 0.21 g. lyophilized extract to stand overnight with 2 ml. acetic anhydride and several drops of concentrated sulfuric acid. The suspension was then poured into 25 ml. ice-water and filtered, leaving a resinous residue. After evaporation to dryness in vacuum, a few minute colorless crystals were left amongst a black resinous material smelling strongly of acetic acid. No purified material was recovered in attempted crystallizations.

(e) Bromination: A sample of the lyophilized extract had shown a tendency to discolor slowly and become tacky even though it was stored over calcium chloride, so the solid (1.57 g.) was dissolved in warm water (60°-70°) and filtered to give a pale yellow solution. Sufficient bromine water (100 ml.) was added to complete the precipitation of a pale yellow flocculent material, which appeared after several minutes. After filtering, washing with cold and warm water and drying in a vacuum desicator, 0.19 g. of crude material was obtained. It dissolved readily in hot ethanol and was precipitated by the addition of water. It could be recrystallized readily by adding water to a warm ethanolic solution almost to the point of cloudiness and allowing to cool, when a voluminous mat of needle-like crystals separated. After five recrystallizations, 48 mg. of very fine white needles was obtained, m.p. 93.5°-96°, and the material was identified as tribromophenol (m.p. 94.5° from aqueous ethanol).

Calculated for $C_6H_3OBr_3$:

21.75%C; 0.91%H; 4.84%O; 72.51%Br.

Found: 22.32%C; 1.13%H; 5.05%O; 71.50%Br,

22.28%C; 0.98%H; 4.96%O; 71.69%Br.

4. Neutral equivalent determinations: Because of the apparently weak acidic nature of the extract a neutral equivalent determination was run in a non-aqueous medium, according to the method of Fritz(46). Approximately 1N sodium methoxide was prepared by dissolving about 0.5 g. freshly cut sodium in 10 ml. absolute methanol (Baker Analyzed), adding 15 ml. further methanol followed by 150 ml. benzene (Baker and Adamson), and storing the solution in a glass stoppered reagent bottle. This solution was standardized against dry benzoic acid (Merck, primary standard) in N,N-dimethylformamide (Eastman) using a Beckman Model G pH meter equipped with saturated calomel and glass micro-electrodes. A 5 ml. burette was used to add the sodium methoxide solution, and a magnetic stirrer employed. After the end-point was reached during the standardization, an unexplained tailing off in meter readings values persisted, but the jump in meter readings was easily sufficient to calculate the end-point.

After allowing the extract and sample bottle to reach equilibrium in the micro-balance, a small sample was removed and dissolved in 5 ml. N,N-dimethylformamide and the cap was immediately replaced on the sample bottle. The weight of sample removed was measured by difference

(7.88 mg., 12.32 mg.). The solution was treated in the same manner as in the standardization of sodium methoxide, except that a 1 ml. burette replaced the 5 ml. burette to measure the volume of sodium methoxide solution added.

5. Conductivity Experiments: All measurements were made in a constant temperature bath set at 25°. Electrodes for the conductivity cell were chosen so as to give suitable resistance values in 10^{-3} M chlorogenic acid solutions. The platinized electrodes were soaked in 12% hydrochloric acid for 30 minutes, and before use in any solution they were carefully washed with distilled water, then with redistilled 95% ethanol, and allowed to dry. Standard potassium chloride was prepared by dissolving 0.74625 g. (Merck, Reagent) in 1 l. of water, to prepare a solution which at 25°C should have a specific conductance κ = .001408. The resistance of the cell, read on the conductivity bridge, was 306 ohms, and this corresponded to:

$$\text{Cell constant} = R \times \kappa$$
$$= 306 \times 0.001408$$
$$= 0.4311$$

A 0.1 M boric acid solution (Baker's Analyzed C. P.) had a much higher resistance than the 10^{-3} M solutions of the controls. The appropriate weight of the organic acid to give a 10^{-3} M concentration was dissolved in 25 ml. of water, and after measuring the resistance 0.1546 g. boric acid was dissolved in the solution to make it 0.1 M with respect to boric acid.

wt. chlorogenic acid	9.1 mg.
wt. quinic acid	4.8 mg.
wt. p-coumaric acid	4.1 mg.
wt. caffeic acid	4.5 mg.
wt. lyopilized extract	8.23 mg.

The extract was removed from the dry sample after equilibrium had been established in the semimicro-balance, in a bottle which was immediately recapped and weighed. The material removed was dissolved immediately in 25 ml. water. The resistances measured reached equilibrium within a few minutes.

6. Periodate Oxidations: The method of Pohle et al.(47) was adapted to the needs of the present semi-micro quantities as follows. Standard (approximately 0.1 M) sodium thiosulfate was prepared and standardized against potassium iodate according to the method outlined by Willard and Furman(48). Periodate reagent was prepared by dissolving approximately 0.6 g. periodic acid (H_5IO_6, G. Frederick Smith Chemical Co.) in 20 ml. water and adding 80 ml. glacial acetic acid; the solution was stored in a dark bottle. To 5 ml. of the acidified hydrolysate was added 2 ml. of the periodate reagent and the oxidation allowed to proceed at room temperature for 10 minutes. 1 ml. of 20% potassium iodide solution was added, and the liberated iodine titrated with the standard thiosulfate until a light yellow color appeared, when 0.1 ml. of starch solution was added and the titration continued to the disappearance

of the blue starch-iodine color. A blank was run with each determination, and the concentration of quinic acid in the original aqueous solution of extract was given by:

$$(V - x) \times \frac{M}{4} \times 10^{-3} \text{ moles of quinic acid/ml.}$$

where V = volume of thiosulfate solution required for the blank

x = volume of thiosulfate solution required for the solution

M = molarity of the thiosulfate solution

7. Infrared Absorption: Because of the pronounced hygroscopic nature of the lyophilized extract, no infrared spectra were obtained. During preparation of a mineral-oil mull, the solid began to agglomerate on the salt plates and no peaks could be identified in the resultant spectrum. Other spectra were taken of samples prepared as a Nujol mull. Attempts were made to prepare pellets of the extract in potassium bromide (Harshaw, spectral grade), but again no useful spectra resulted. Similar properties of the reduced extract again prevented any spectrum from being taken.

III. RESULTS AND DISCUSSION

A. Identification of a p-Coumaric Ester*

Preliminary flavonoid tests, as described by Geissman(49), were carried out on the fruit (core, flesh and skin) and on the vegetative stumps, and the failure to observe positive reactions pointed to the absence of flavonoid materials of the general C_6-C_3-C_6 pattern. This is in agreement with the results of a recent preliminary investigation for flavonols in pineapple(50), when a flavonol-like material was separated which failed to reduce with magnesium and hydrochloric acid, which is a characteristic test for flavonols.

1. Spectral Results: The first aqueous stem extract obtained, i.e. by extraction from the plant with water followed by removal of the solid, showed a characteristic aqueous absorption spectrum with λ max. 213, 278, mμ, λ min. 256 mμ, and a strong inflection around 315 mμ (Fig. 7). Solutions obtained by similar treatment of the fruit showed the absence of this inflection, and extraction from the plant with hexane and ligroin also failed to remove the material responsible for this inflection. Soxhlet-extraction from dried and powdered stump material

---

*A preliminary report on the occurrence of a p-coumaric ester has been submitted for publication under the title "Ferulic and p-Coumaric Acids in Pineapple Tissue as Modifiers of Pineapple Indoleacetic Acid Oxidase," by Willis A. Gortner, Martha J. Kent and G. K. Sutherland.

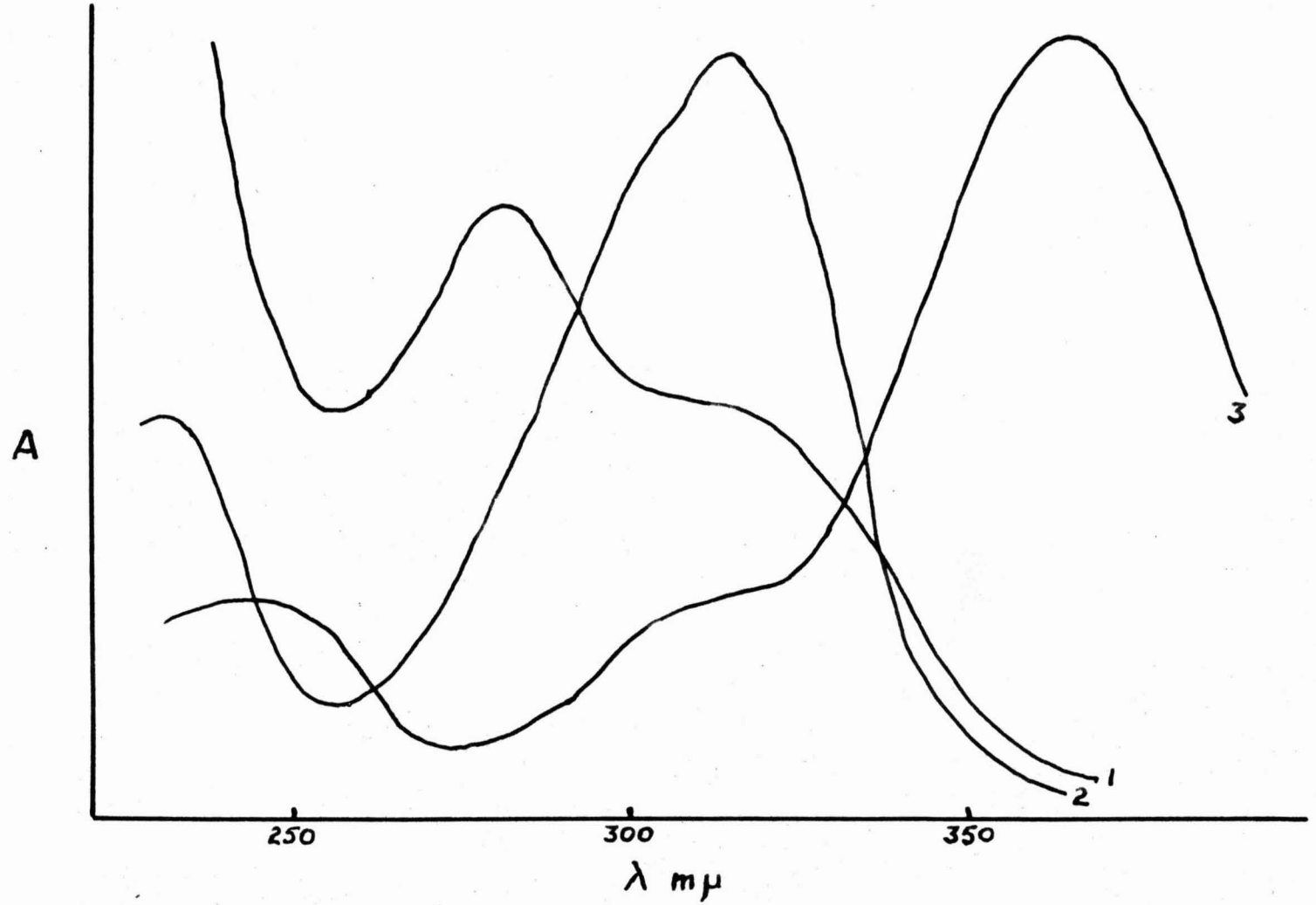


Fig. 7

Ultraviolet absorption spectra of (1) initial aqueous extract in water (2) final extract in ethanol (3) final extract in ethanolic sodium ethoxide.

with ethyl acetate and ether was similarly unsuitable. Extractions from various sections of the stump showed that this spectral pattern was not confined solely to any particular section. Exposure of this preliminary extract to strong sunlight resulted in the gradual disappearance of the inflection portion of the absorption curve, but temperatures of the order of 100° for short times left it unaffected.

The precipitate obtained with lead acetate was regenerated with sulfuric acid, and an ethyl acetate-soluble material was extracted. It was found to be also soluble in ether and n-butanol, insoluble in carbon tetrachloride and hexane. However, several ether extractions of the sulfuric acid solution removed only part (approximately 50%) of the material responsible for the absorption in the vicinity of 315 mμ. The residual absorber was then immediately soluble in ethyl acetate. The ethyl acetate-soluble material had the same spectral characteristics as the lyophilized material obtained from the alternative extraction. The ethanolic absorption spectrum of the extract had maxima at 215 mμ and at 314 mμ, a minimum at 254 mμ (Fig. 7). This same spectrum was shown in water and in aqueous acid buffers. In aqueous alkaline buffers above pH 9.6, in ethanolic sodium ethoxide (approximately .002 M) and in aqueous ethanolic sodium hydroxide, a bathochromic shift of 51 mμ was observed (Fig. 7). The

alkaline curve had λ max. 365 mμ. In aqueous 1N sodium hydroxide, a slow reversal of this shift was noticed over a six hour period, during which time the λ max. shifted slowly down to 337 mμ. The curve formed after this interval was stable over 24 hours in the aqueous alkaline medium (λ max. 337 mμ, λ min. 261 mμ, inflection 315 mμ), but if diluted with ethanol, a further rapid shift to λ max. 290-295 mμ occurred. The change from acid (λ max. 314 mμ) to alkaline (λ max. 365 mμ) absorption occurred rather suddenly between pH 8.8 and 9.6, with an isosbestic point near 330 mμ.

Treatment of the extract with hot hydrochloric acid or sodium hydroxide and examination of the resultant acidified solution showed very little alteration in the ethanolic spectrum. However, the large bathochromic shift in alkaline solutions was replaced by a smaller shift to 337 mμ, the same absorption spectrum as was obtained by prolonged alkaline treatment at room temperature.

The spectral curves of the extract in ethanol and ethanolic sodium hydroxide were essentially the same as the absorption spectra reported for methyl p-coumarate in these solvents(30). In addition, the alkaline or ethanolic spectra which arose after acid or alkaline treatment of the extract had the same shape as p-coumaric acid in these solvents, and showed the same rapid shift to lower wavelengths in ethanolic sodium hydroxide as did this acid. These spectral characteristics strongly suggested the

presence of an ester of p-coumaric acid. The large shift to longer wavelengths in alkaline solutions was attributed to a free phenolic group in a para-position to the cinnamic acid side chain, and to an esterified carboxyl group in the side chain. The presence of an ionizable carboxyl group in the side chain would interfere with the resonance structure in the phenoxide ion, so that a large bathochromic shift in alkaline solvents would point to the absence of a free carboxyl group.

2. Chromatography of the Extract: When a sample of the extract was chromatographed on Whatman No. 1 paper with the organic layer from a butanol-acetic acid-water (4:1:5) mixture, two overlapping spots were seen after drying and inspecting under ultraviolet light. One spot (A, R_f 0.86) was deep blue and almost invisible, the slower moving spot (B, R_f 0.82) was a bright light blue. Two-dimensional chromatography, with butanol-acetic acid-water or amyl alcohol-acetic acid-water (4:1:5, organic layer) as the first solvent followed by irrigation with 2% acetic acid at right angles, did not bring out any more spots, nor did it completely separate the two spots already mentioned. The only solvent which gave any real separation of the spots was phenol-water (73;27 w/v), but it was found difficult to remove this solvent from the paper. Other R_f values found in various solvent systems are listed in Table 2.

Colors developed by chromogenic sprays are reported

in Table 3. Because of the general overlapping of the two spots, the colors of the extract were due to a combination of the individual spot colors, but it was noticed that the strongest color coincided with the dark fluorescing spot A, while the colors developed by spot B were relatively insignificant.

That the dark fluorescent material of spot A was mainly responsible for the total absorption shown by extract solutions was demonstrated by cutting from the chromatogram a horizontal strip of paper containing the spot, fixing it behind the spring clip in the cell carrier of the spectrophotometer, and running its absorption spectrum, using as a blank a strip of paper from an adjacent horizontal section of the chromatogram. This technique has been used by Bradfield and Flood for various plant phenols(51). The dark area showed the strongest absorption, and the shape of the curve obtained was, in general, the same as that obtained from ethanolic solutions of the extract. The adjacent light blue area also showed this same curve, but the intensity of absorption was much less. Elution from these areas was possible with difficulty using 70% ethanol, and these eluates behaved spectrally in the same manner as the original unchromatographed extract. Chromatography on Whatman 3 MM paper could not be used as a purification technique because of discoloration of the chromatogram during drying and the difficulty of elution, even with methanol or ethanol in a Soxhlet extractor.

TABLE II

R_f VALUES IN VARIOUS SOLVENTS

Solvent	Ester A	Ester A hydrol.	p-Coum. acid	Ester B	Ester B hydrol.	Ferulic acid
1. n-Butanol/acetic acid/water (4:1:5)	0.86	0.92	0.92	0.82	0.90	0.90
2. n-Amyl alcohol/acetic acid/water (4:1:5)	0.74		0.88	0.70		
3. Phenol/water (73:27)	0.30	0.80	0.84	0.40		0.81
4. Aqueous phase from solvent 1.	0.80			0.83		
5. Ethyl acetate/conc. ammonia (1:1)	0	0.07	0	0	0	0
6. Ethyl acetate/conc. HCl (1:1)		0.87	0.87		0.90	0.90
7. Ethyl acetate/water (1:1)		0.92	0.92			0.55
8. m-Cresol/acetic acid/water (50:2:48)		0.78	0.81	0.38		
9. Water	0.91	0.88	0.81	0.91		
10. Toluene/acetic acid/water (4:1:5)	0	0.09	0.10	0	0.47	0.50
11. Acetic acid/conc. HCl/water (30:3:10)	0.89		0.88	0.92		

TABLE III

COLORS DEVELOPED BY DIAZOTIZED p-NITROANILINE SPRAY

	With sodium acetate	With sodium acetate and sodium carbonate
Extract spot A	pale yellow	red-brown
p-Coumaric acid	yellow-orange	blue-violet
Ferulic acid	pink	blue-green
Caffeic acid	brown	brown
Chlorogenic acid	brown	yellow

TABLE IV

CHROMATOGRAPHY ON BORATE-IMPREGNATED PAPER

	R_f			
	Plain paper		Borate paper	
	1[a]	2[b]	1[a]	2[b]
p-Coumaric acid	.88	.96	.29	.03
Chlorogenic acid	.20	.11	.02	0
Ester spot A	.09	Streaks .09-.49	.01	0

[a]Solvent: n-butanol/water (1:1)
[b]Solvent: ethyl acetate/water (1:1)

The formation of colored spots with the diazonium sprays again indicated the presence of a free phenolic group. The relatively high R_f values of both spots in butanol and amyl alcohol solvents suggested the absence of any glycosidic linkage. The relatively low R_f values in phenolic solvents suggested that neither spot was a coumarin, since these were known to move generally close to the solvent front(11). A comparison with reported R_f values of p-coumaric acid and esters(14) suggested that the dark blue fluorescent material was not a simple alkyl ester. Consideration of the values in Table 2 indicated that the alcohol moiety rendered the ester more water-soluble than the free acid.

Chromatography on borate-impregnated paper was investigated in the hope of finding further information on the alcohol moiety of the ester. This had been utilized by other workers(11) to indicate the presence of adjacent hydroxyl groups. Table 4 shows the very considerable reduction in R_f values on borate paper. However, in the light of other data on conductivity (q.v.), and in view of the fact that the R_f for free p-coumaric acid was lowered very considerably, it is doubtful whether these figures can be used to suggest the presence of borate-reactive groupings. There were some indications that chromatography on borate paper with butanol-acetic acid-water (4:1:2.2) was able to separate the two spots, which up to this stage had not been resolved.

3. Hydrolysis of the Extract: Further spectral data on the hydrolyzed extract pointed to the existence of a p-coumaric acid. Prolonged reflux in alkaline solution resulted in the disappearance of the λ max. of 314 mμ in ethanol solution and the new λ max. occurred at 270 mμ. This might be expected from degradation at the double bond of a cinnamic acid side chain. Extensive heating with acid caused the formation of dark resinous material. p-Coumaric acid is known to decarboxylate under the influence of hot acid, and a possible polymeric degradation product has been reported from a methyl p-coumarate derivative(29).

Hydrolysis, followed by acidification if necessary, resulted in a cloudy solution and much of this cloudiness was removed by extraction with ether. When this ether solution was evaporated and the residue dissolved in aqueous buffers from pH 4.1 to 9.8, the absorption spectra of these solutions were identical with the spectra of p-coumaric acid in the same solvents. These spectra were not the same as the ethanolic or aqueous spectra of p-coumaric acid. As the pH rose, there appeared to be a slow movement from λ max. 292 mμ at pH 4.1 to λ max. 267 mμ at pH 9.2. In more alkaline buffers and in .002 M ethanolic sodium ethoxide, the typical alkaline p-coumaric curve was obtained.

Accompanying the spectral changes resulting from acid or alkaline treatment of the extract was the appearance of two new spots on chromatograms.

After the hydrolysis, the materials responsible for these new spots were recovered from the acidified reaction mixture by extraction into ether. Under ultraviolet light these new spots had the same colors as the dark blue (A) and light blue (B) fluorescing spots from chromatograms before hydrolysis, and moved in most solvents with very similar R_f values. However, their values (q.v. Table 2) were different from those of the unhydrolyzed extract. Furthermore, when the chromatogram was sprayed with diazotized p-nitroaniline, the two generally overlapping spots became yellow, and sodium carbonate formed a deep blue color centered mainly on the spot which showed dark blue fluorescence under ultraviolet light.

It was found possible to separate these two spots by chromatography with toluene-acetic acid-water (4:1:5, organic layer), after equilibrating the chromatogram overnight in the presence of the aqueous phase of this solvent. The dark spot (R_f 0.07) gave the colors reported above for the diazotized p-nitroaniline-sodium carbonate spray, while the light blue spot (R_f 0.47) turned pink and blue-green in turn with this spray. The R_f values and colors from these dark blue and light blue spots agreed very well with the appropriate data found for p-coumaric and ferulic acids respectively (Tables 2 and 3).

To illustrate further the identity of these spots with p-coumaric and ferulic acids, the chromatograms were sectioned and the absorption spectrum of each spot run

directly on the paper. Although the curves obtained were not the usual ethanolic curves for these acids (λ max. 270 mμ, λ min. 250 mμ, inflection 300 mμ for both spots), they were almost identical with the absorption curves of authentic p-coumaric and ferulic acids taken on paper after chromatography with this toluene-acetic acid-water solvent.

Large samples of extract were hydrolyzed in 1N alkali at room temperature to obtain recoverable amounts of p-coumaric and ferulic acids. From 3 kg. fresh stumps, only 9 mg. of white crystalline material was obtained after one crystallization from hot water, m.p. 197.5°-199°. No difference could be detected between the infrared absorption spectrum of this material and that of authentic p-coumaric acid, m.p. 210°-213° (See Fig. 8)*. However, chromatography of this white solid in toluene-acetic acid-water showed the existence of a ferulic spot as well as a p-coumaric spot. Because of the extremely similar properties of these two acids, no means were found to obtain p-coumaric acid in a pure form. Even vacuum sublimation did not succeed in separating them. Chromatography on Whatman 3 MM paper with toluene-acetic acid-water separated them, but after elution from the paper with methanol in a small Soxhlet extractor, no solids were

---

*The strong absorption peaks around 4.1 and 14.9 in Fig. 8 (1) are due to carbon dioxide present in the spectrophotometer system.

recovered.

4. Further Derivatives

(a) Methylation.—Methylation with an ethereal solution of diazomethane did not alter the alkaline absorption spectrum of the extract, as would be expected by the formation of an ether linkage at the phenolic group. However, after treatment of the extract with methyl sulfate, the bathochromic shift of the absorption curve in the presence of sodium ethoxide was replaced by a smaller hypsochromic shift. After hydrolysis of this preparation, a curve was obtained similar to the curve from the unhydrolyzed preparation, with λ max. 305 mμ, λ min. 251 mμ in ethanol and λ max. 280 mμ, λ min. 248 mμ in ethanolic sodium ethoxide. When the spectrum of p-methoxycinnamic acid was taken, it proved almost identical with that of the methylated hydrolyzed extract, with λ max. 305, 225 mμ, λ min. 247 mμ in ethanol and λ max. 283, 228 mμ, λ min. 244 mμ in ethanolic sodium ethoxide (see Table 5). Chromatography of the ether-soluble material from the methylated hydrolyzed extract yielded only one spot which could be located under ultraviolet light. This spot was very light blue, and could also be located with a bromophenol blue spray. In the solvents chosen, this spot moved with the same R_f values as did p-methoxycinnamic acid (see Table 6), and although some of these values were very close to that for p-coumaric acid, the failure to form a colored spot with the diazotized p-nitroaniline spray clearly indicated the

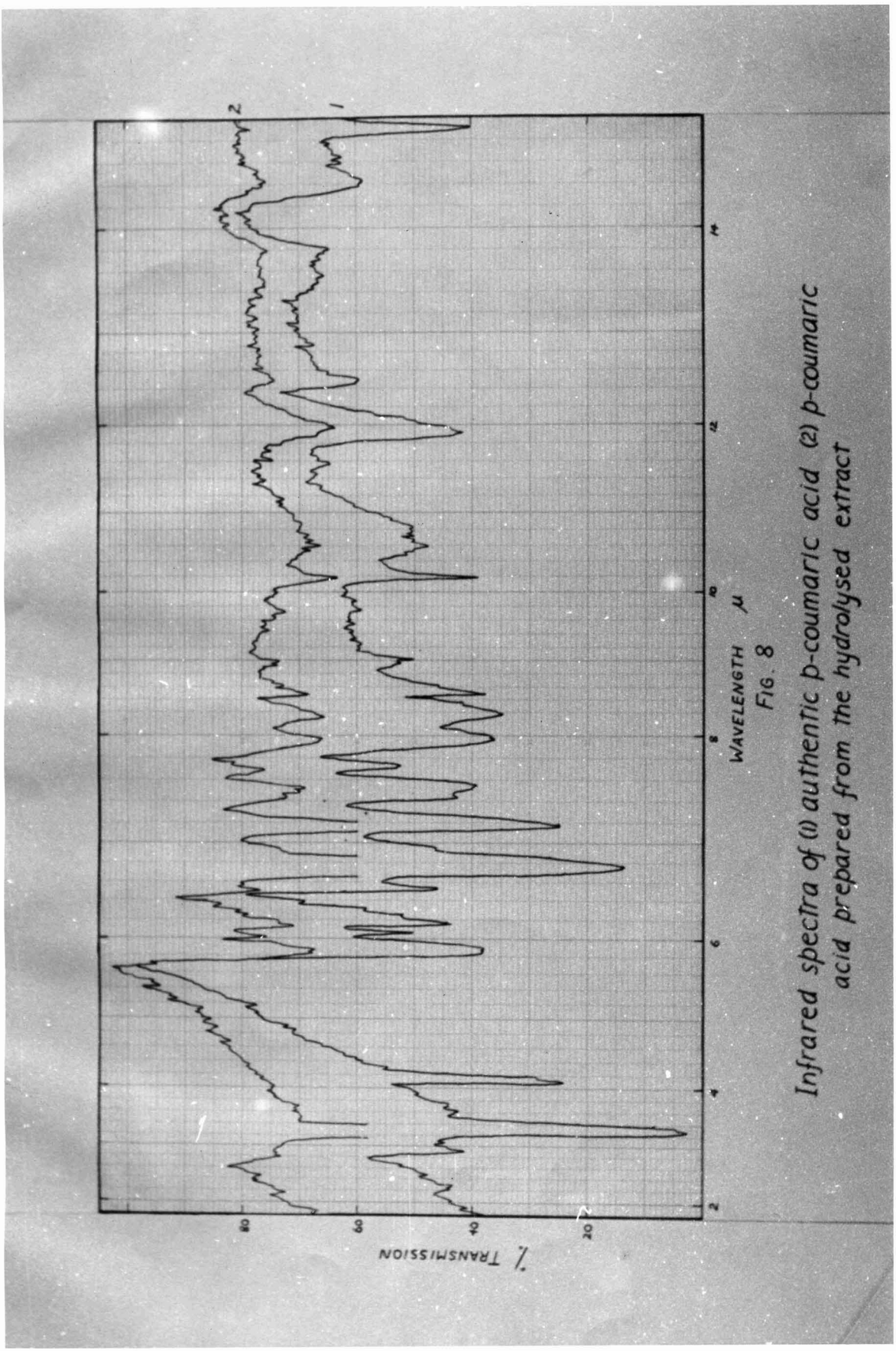


Fig. 8

Infrared spectra of (1) authentic p-coumaric acid (2) p-coumaric acid prepared from the hydrolysed extract

TABLE V

ULTRAVIOLET SPECTRAL DATA OF SOME CINNAMIC ACID DERIVATIVES

	Ethanol		Ethanol/NaOEt	
	$\lambda_{max.}$	$\lambda_{min.}$	$\lambda_{max.}$	λ_{min}
p-Hydroxy hydrocinnamic acid	280, 224	248	283	
Reduced extract	282	250		
Reduced, hydrolyzed extract	280, 223	250	282	
p-Methoxy cinnamic acid	305, 225	247	283, 228	243
Methylated extract			282	247
Methylated, hydrolyzed extract	305	251	280	247
Cinnamic acid	271			
Acetylated extract	285	250		

TABLE VI

R_f VALUES OF THE METHYLATED AND HYDROLYZED EXTRACT

	Meth. extr.	Meth. hydr. extr.	p-OMe cinn. acid	p-Coumaric acid
Ethyl acetate/2N HCl (1:1)	0.96	0.96	0.96	0.87
n-Butanol/acetic acid/water (4:1:5)	0.90	0.93	0.93	0.92
n-Propanol/conc. ammonia (7:3)	0.27	0.27	0.29	0.51

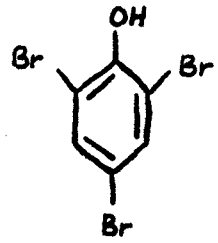
absence of a free phenolic group.

(b) Hydrogenation.—Catalytic hydrogenation of the extract resulted in a most pronounced reduction in absorbance of the solution, coupled with a shift to lower wavelengths (λ max. 280 mμ , λ min. 254 mμ). This change was readily interpreted as the reduction of a double bond conjugated to a benzene ring. 7-hydroxy-4-methyl coumarin showed a parallel change in its ultraviolet absorption spectrum after similar treatment. Because of the strong tendency to become resinous, it proved impossible to take an infrared spectrum of the reduced material after lyophilization. The reduced material could be conviently hydrolyzed with hot hydrochloric acid, and ether extraction removed a material with an ultraviolet spectrum almost identical with that of the unhydrolyzed reduced extract (λ max. 280, 223 mμ , λ min. 252 mμ in ethanol). This curve was almost identical with the absorption spectrum for p-hydroxy hydrocinnamic acid (λ max. 280, 224 mμ, λ min. 248 mμ in ethanol), which had similar low absorbance values for comparable dilutions. See Table 5 for spectral data.

(c) Acetylation.--After acetylation, the ethanolic absorption spectrum indicated a reaction had occurred which resulted in a new λ max. 285 mμ , λ min. 250 mμ . While this was not the same as the λ max. for cinnamic acid (271 mμ), it could be interpreted best as an acetylation of the phenolic group. Such a reaction to a large extent removes the possibility of participation by that group

in the resonance structure, and the spectrum tends to resemble that of the parent compound(52).

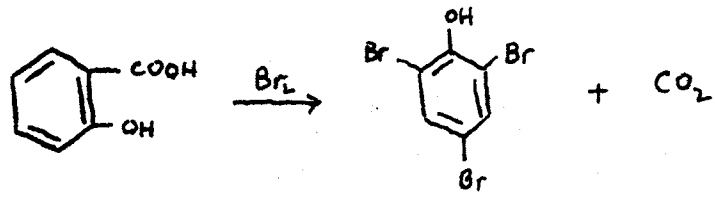
(d) Bromination.--The unsaturated and phenolic character of the extract, as suggested by its spectra and ready hydrogenation, prompted the use of bromine in an attempt to form a new derivative. The white needle-like crystals obtained after numerous recrystallizations from aqueous ethanol appeared identical with the brominated derivative obtained from p-coumaric acid, m. p. 95.5°-96.5°. Both compounds possessed identical ultraviolet and infrared spectra, and the absence of a carbonyl peak in the infrared spectrum was noticed. An absorption peak at 11.7μ was evident, and this was interpreted as a 1,2,3,5-tetrasubstituted benzene(53). The absence of the carbonyl absorption and the presence of the other peak pointed to the replacement of the complete side chain. Analytical results agreed with the values calculated for 2,4,6-tribromophenol(VII).



VII

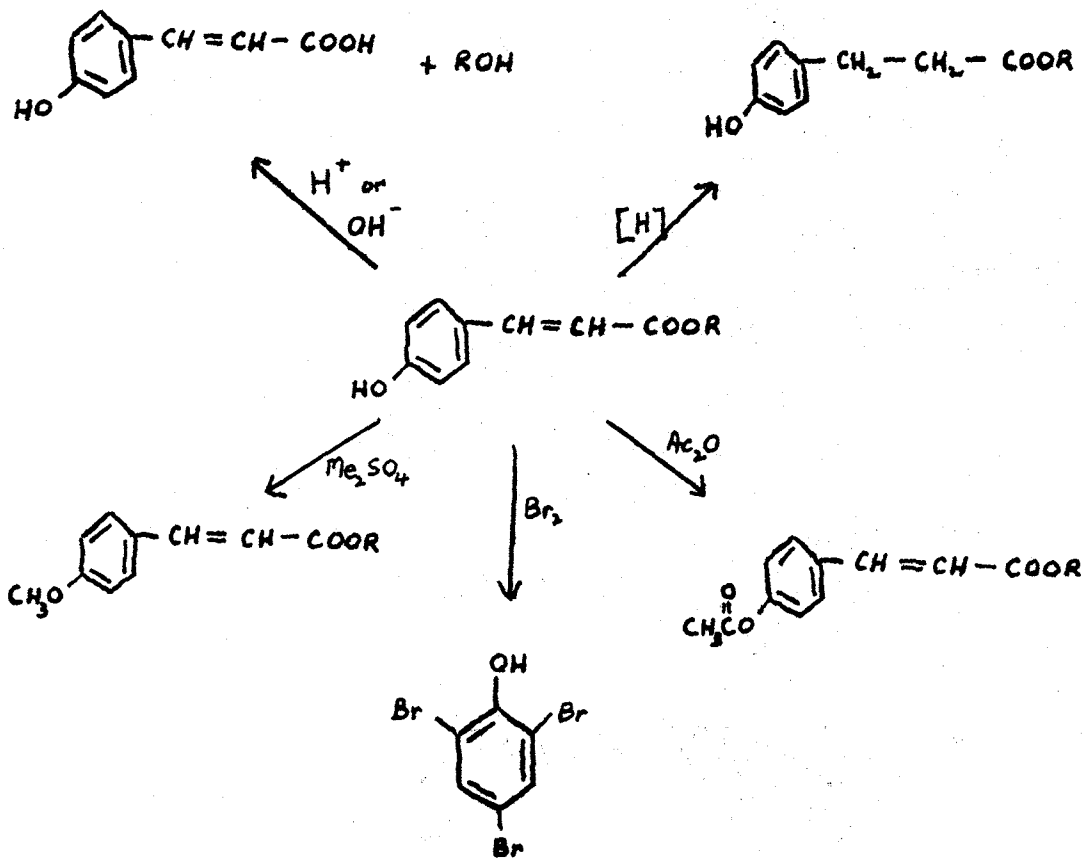
The infrared spectrum of recrystallized 2,4,6-tribromophenol (Eastman, Technical) proved to be identical with the spectra of the brominated derivatives of the extract and of p-coumaric acid (Fig. 9). No brominated

derivative of ferulic or chlorogenic acids could be prepared by this method, but salicylic acid under the conditions of this reaction is known to yield 2,4,6-tribromophenol with quantitative evolution of carbon dioxide(54).



Bromination of the extract in glacial acetic acid, in an attempt to get addition to the cinnamic double bond without replacement of the side chain, gave no product.

The results as outlined above pointed to the existence of an ester of p-coumaric acid in the vegetative plant. This ester could be hydrolyzed without much difficulty and the p-coumaric acid fragment removed by extraction from the acidified solution with ether. The double bond of the side chain could be easily reduced, and the phenolic group methylated or acetylated by standard techniques. Bromination of the molecule resulted in the replacement of the cinnamic acid side chain, with the formation of a tri-substituted phenol.



B. Identification of the Quinic Acid Fragment.

1. Preliminary Characterization: The alcohol fragment involved in the ester linkage had next to be identified. After the p-coumaric acid was removed by ether extraction of the acidified hydrolysate, the aqueous fraction remaining gave a positive ceric nitrate test for alcohols(55), indicating that the alcohol fragment of the ester was rather water-soluble. This solution showed no ultraviolet absorption spectrum above 210 mμ. Preliminary chromatography in n-butanol-formic acid-water (4:1:5, organic layer) showed that a spot which had travelled some distance (R_f 0.50)

gave a positive reaction with a ceric nitrate spray (20 g. in 50 ml. 2N nitric acid, white spot on a yellow background after several minutes), and gave an acidic reaction with bromophenol blue and bromcresol green sprays. Although this R_f value was not the value reported(56) for quinic acid in this solvent, it was the same as the R_f for a spot which appeared after treatment of quinic acid with hot hydrochloric acid. The chromatographed alcohol fragment was consequently sprayed with the periodate-benzidine spray which had been successfully used with carbohydrates(42), and the alcohol spot immediately colored white with a broad deep blue edge.

2. Chromatographic Studies: Because of the glycolic and acidic properties exhibited by the alcohol (water-soluble) fraction of the ester, a preparation of quinic acid was attempted, since this material (1,3,4,5-tetra-hydroxy cyclohexane-carboxylic acid) contains similar groupings, and is an important constituent of certain plant esters. After acid hydrolysis of chlorogenic acid a water-soluble material remained after removal of the caffeic acid formed on hydrolysis. This solution was chromatographed in several solvents along with the alcohol fraction from the acid-hydrolyzed extract, and both spots behaved identically with respect to R_f values and spray colors (see Figs. 10, 11). The R_f values of these spots were substantially different from those reported in the literature for acidic solvents(43), (57), but agreed with the R_f value found for quinide,

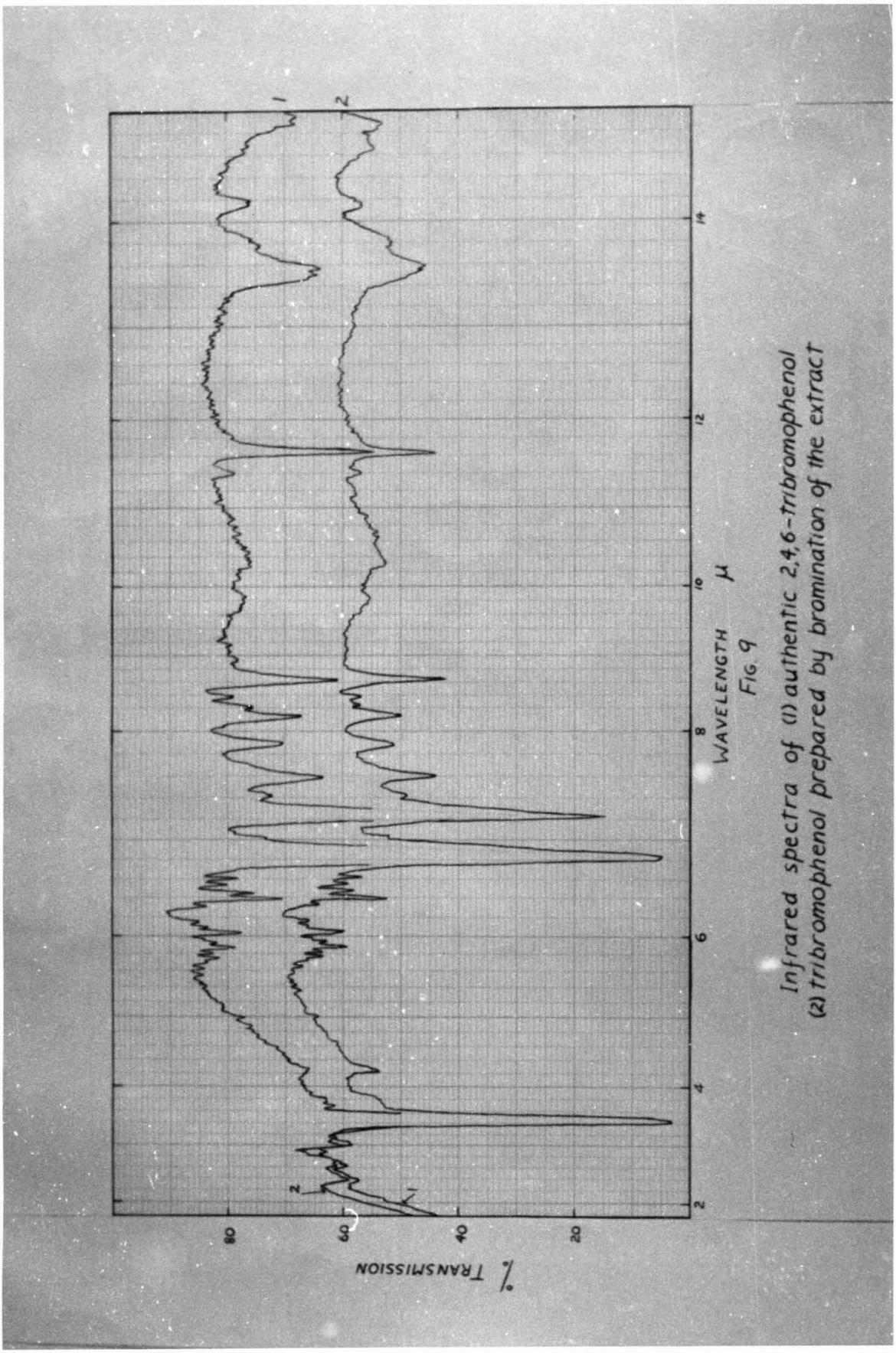


Fig. 9

Infrared spectra of (1) authentic 2,4,6-tribromophenol
(2) tribromophenol prepared by bromination of the extract

FIG. 10. Chromatogram of the quinide from the extract (1 and 2) and from chlorogenic acid (3) in butanol/acetic acid/water (4:1:2.2).

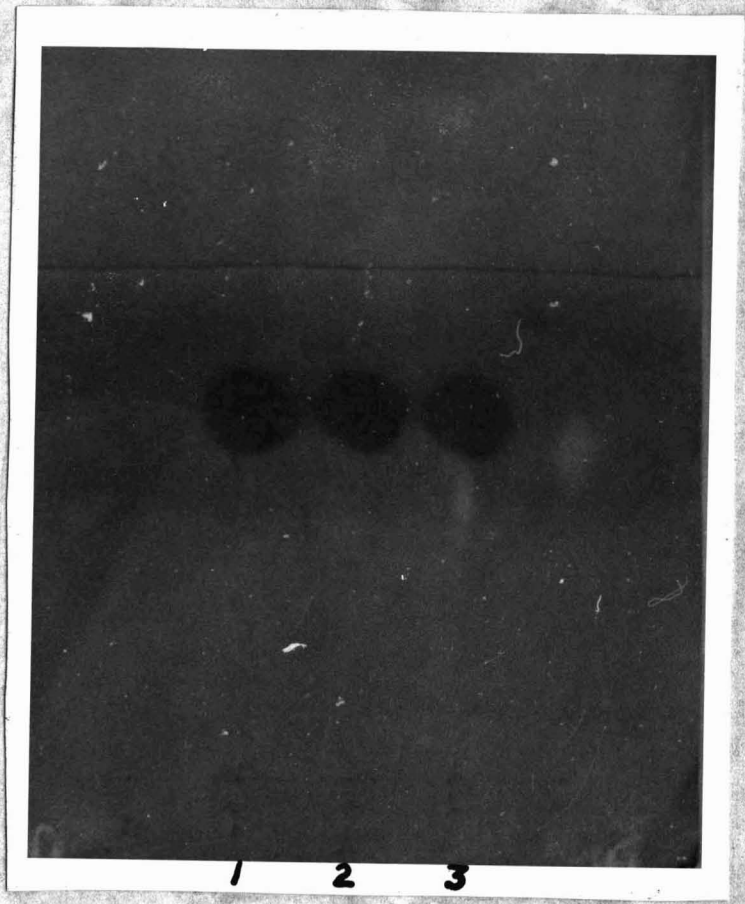


FIG. 11. Chromatogram of the quinide from the extract (1 and 2) and from chlorogenic acid (3) in isopropanol/ammonia/water (6:3:1).

the γ-lactone of quinic acid. In addition a faint spot appeared at R_f 0.77 in butanol-acetic acid-water (4:1:2.2), which is also reported(43) for acid-treated quinic acid. When chromatograms were run for the acid-hydrolyzed extract and authentic samples of quinide and quinic acid (Nutritional Biochemicals Co.), the alcohol fragment from the extract behaved identically with quinide (see Table VII). In alkaline solvents, the quinide and quinic spots move to the same position on the chromatogram.

If quinic acid is esterified with p-coumaric acid in the extract, the unlactonized acid would be expected after alkaline hydrolysis of the ester. However, in no solvents could free quinic acid be shown to occur after such hydrolysis. This was presumably due to a very large salt effect, since acidification of the alkaline medium after hydrolysis left large amounts of sodium chloride in solution. Opaque patches of the wet chromatogram in certain solvents (e.g. the solvent mixture t-butanol-benzyl alcohol-isopropanol-water) from an alkali-hydrolyzed sample coincided with the periodate-reactive spot, but moved very slowly and increased considerably in area. When these salt-contaminated solutions were passed over a column of Dowex-50-X cation exchange resin (Dow Chemical Co.), prepared in the acid form by washing with hydrochloric acid followed by water, the solution recovered from the bottom of the column gave only chromatographic evidence for quinide (see Table VII). Quinic acid itself, when exposed

TABLE VII

R_f VALUES OF THE ALCOHOL (WATER-SOLUBLE) FRACTION OF THE HYDROLYZED EXTRACT

Solvent	Ester A acid-hydrol.	Quinic acid	Quinide	Ester A alkal.-hydrol.
n-Butanol/acetic acid/water (4:1:2.2)	0.54	0.38	0.53	0.54
Isopropanol/ammonia/water (6:3:1)	0.71	0.66	0.66	0.69
n-Butanol/formic acid/water (4:1:5)	0.48	0.41	0.48	0.49
n-Propanol/ammonia/water (6:3:1)	0.54	0.52	0.52	0.52
Benzyl alcohol/t-butanol/isopropanol/water (3:1:1:1 plus 1.8% formic acid)	0.32	0.31	0.32	

to alkaline conditions, acidified and desalted in the same manner, also gave only quinide spots in subsequent chromatograms. Desalting is apparently necessary on the alkali-hydrolyzed ester for reproducible chromatograms, but under the conditions employed, the lactone ring appears to close very readily.

3. Isolation of Quinide: After reduction or methylation with dimethyl sulfate and subsequent hydrolysis of the extract, no difference could be found in the quinide spot on chromatograms. From an extract sample prepared from 2.5 kg. stumps, and which had been hydrolyzed at room temperature by 1N aqueous sodium hydroxide for six hours under nitrogen, minute white crystals were recovered after desalting on a Dowex-50-X resin. These crystals, m.p. 188-192°, were tentatively identified as quinide (cf. m.p. 193°-195° for quinide), but insufficient material was available for any further physical data. From the reduced extract an extremely small quantity of minute crystals was similarly recovered after hydrolysis, m.p. 151°-158°, apparently an impure sample of quinide. There was insufficient material for a recrystallization.

4. Methylation Attempts: Complete methylation of the ester was attempted by the use of dimethyl sulfate in acetone, followed by reflux with methyl iodide and silver oxide. An amorphous material resulted in very poor yield, but after hydrolysis neither the ether-soluble nor the water-soluble fractions gave any derivative.

On the basis of the relative amounts which appear on chromatograms after hydrolysis, the above evidence was interpreted as indicating the presence of a quinyl p-coumarate. Although ferulic acid does appear in chromatograms of the hydrolyzed material, it does so in smaller quantities. Furthermore, the absorption spectrum of the extract in ethanol resembles that of p-coumaric acid, while a ferulic ester in any concentration would be expected to have a noticeably different spectrum. It may be that the ferulic acid also occurs esterified with quinic acid, but the relatively low amounts of the ferulic ester present in extracts have prevented any data being accumulated for the alcohol moiety of this ester. Undoubtedly, the major ester component in the extract is a p-coumaric-quinic ester. This still leaves the question of the structure of this ester to be answered.

C. Structural Investigation

Although the evidence pointed to the presence of a quinic acid ester of p-coumaric acid in the plant, the presence of more than one hydroxyl group in the quinic acid molecule required the accumulation of further data. The mole ratio of the constituents of the ester had yet to be determined. The ready availability of esterification positions on the quinic acid fragment required a decision amongst the various position isomers that could arise. Experiments were designed to yield quantitative data which

could be used to interpret the structure of the ester.

1. Neutralization Equivalent Experiments: Several unsuccessful determinations were run on the solid remaining after lyophilization. It had been demonstrated that a neutral equivalent of chlorogenic acid could be obtained in aqueous methanol with reasonable accuracy on a 10-15 mg. sample. When a similar determination was carried out on an extract sample, a value of 170 was found (Fig. 12). This value, which was lower than expected from any quinyl-coumarate containing only one quinic acid moiety (i.e. only one free titratable acid group) could be explained by assuming a considerable amount of acidic contaminant. Since the lyophilized solid had shown a strong tendency to alter after short exposures to the air, a sample of the solid used for the neutral equivalent determination was chromatographed, and considerable quantities of free p-coumaric acid were found. Since this neccessarily means the accompanying presence of quinic acid, it is not surprising that the experimentally determined value for the neutral equivalent was low.

For the same reason, periodate activity demonstrated by the lyophilized solid could not be interpreted as indicating any adjacent hydroxyl groups in the unhydrolyzed ester. The very different glycol concentrations from different samples of the extract indicate that the extent of this hydrolysis is difficult to predict or control. Molecular weight calculations, based on the absorbance

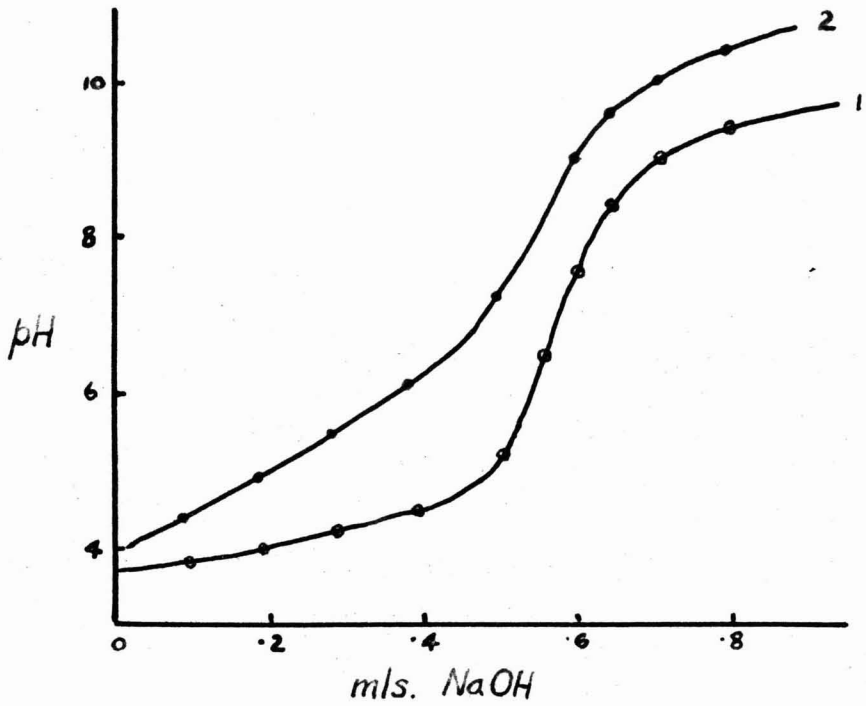


Fig. 12

Titration in aqueous ethanol with 0·1N sodium hydroxide of (1) chlorogenic acid (2) extract

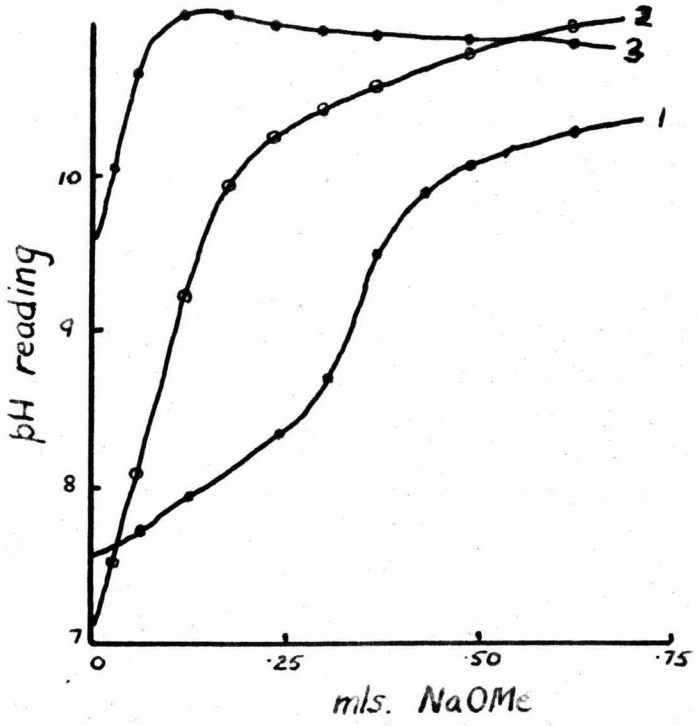


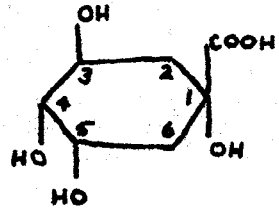
Fig. 13

Titration in N,N-dimethylformamide with 0·1N sodium methoxide of (1) chlorogenic acid (2) extract (3) quinide

at 314 mμ of solutions containing known amounts of the lyophilized solid, did not lead to any values which could be interpreted reasonably on the basis of a quinyl-coumarate ester. Presumably the contaminating material, which is either extraneous and/or due to degradation subsequent to lyophilization, interferes considerably in all the above experiments.

2. Titration Curves in Non-Aqueous Medium: Because of the weakly acid nature of quinic acid, and derivatives, and because of the strong tendency shown by the extract to hydrolyze in the presence of small amounts of water, neutral equivalent determinations were run on a freshly prepared sample of the lyophilized extract in N,N-dimethylformamide. This is a well known technique to emphasize the acidic property of weak acids. Chlorogenic acid was taken as a control and showed an easily recognizable weak acid titration curve in this solvent (Fig. 13). The extract, however, showed no slow initial rise as expected for a weak acid, and the titration curve could only be interpreted as indicating the absence of an acid-titratable group. Extraction into sodium bicarbonate solution during an initial step requires that there must be some group present that readily shows an acidic nature under rather mild alkaline conditions. These results suggested the existence of a lactone grouping, and when quinide, the γ-lactone of quinic acid, was titrated in dimethylformamide, the same type of curve resulted. This indicated that the hydroxyl group

attached to carbon atom number three of the quinic acid molecule was not involved in esterification with a p-coumaric acid molecule, since this cis-hydroxyl is the only one available for ring closure with the carboxyl group (VIII).

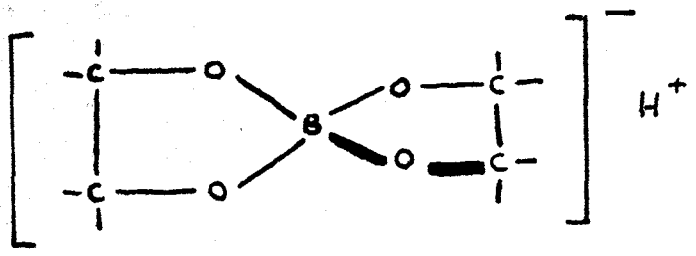


VIII

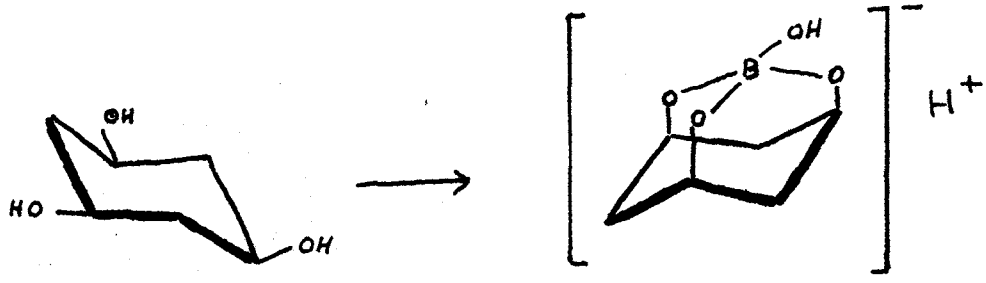
On this assumption, it is not too surprising that water is removed under the drastic drying conditions of the freeze-dryer to form a lactone, even though in the plant there is probably an equilibrium involving a major contribution by the free acid. The partial ether-solubility of the ester in the early stages of extraction can be interpreted as indicating this acid-lactone equilibrium. A similar compound, isochlorogenic acid, was isolated(25) from coffee, but neither the compound nor any derivative was obtained in a crystalline form.

3. Conductivity Experiments: The effect of the extract on the conductivity of boric acid was used to decide that at least one of the hydroxyl groups on carbon atoms number 4 and 5 of the quinic moiety must be involved in esterification. This method has been discussed rather fully by Böeseken(58). This author concluded that in saturated six-membered rings with adjacent hydroxyl groups, neither cis- nor trans-1,2-diols had any positive effect on the conductivity of

boric acid. This was attributed to the non-planar nature of the cyclohexane ring, which resulted in the hydroxyl groups being unfavorably situated for the formation of a boric acid complex of the type:



Nevertheless, experiments showed that quinic acid and chlorogenic acid had pronounced effects on the conductivity of boric acid. The considerable amount of substitution in the cyclohexane ring probably results in a suitable conformation of the hydroxyl groups for formation of a cyclic borate ester. It has recently been suggested(59) that in cyclitols, complex formation may be possible with non-adjacent hydroxyl groups, again because of the non-planar character of the ring and the possibility of inversion of the conformation to cause a more suitable spatial arrangement.



The main spatial requirements are that the (adjacent)

hydroxyl groups shall be cis to one another, as are the hydroxyl groups on carbon atoms number 4 and 5 in quinic acid or in the quinic acid fragment of chlorogenic acid. In Table VIII are listed the molar conductances and the increments in molar conductance in the presence of boric acid, obtained from the expression

$$\Delta \Lambda_m = \Lambda_m(B) - \left[\Lambda_m(C) + \Lambda_m(A) \right]$$

where $\Delta \Lambda_m$ = increment in molar conductance

$\Lambda_m(B)$ = molar conductance of the compound (10^{-3} M) in the presence of boric acid (0.1 M)

$\Lambda_m(C)$ = molar conductance of the compound (10^{-3} M)

$\Lambda_m(A)$ = molar conductance of 0.1 M boric acid

The molar conductances (Λ_m) of both quinic and chlorogenic acids were increased by very significant amounts in the presence of boric acid; neither the extract nor p-coumaric acid showed any comparable increase. This can only be attributed to the absence of adjacent cis-hydroxyl groups, which requires that there should be esterification at the 4 or 5 position of the quinic acid.

4. Periodic Acid Oxidation: The choice between these two positions was decided by the results of periodate oxidation. Because of the presence of certain amounts of free quinic acid, demonstrated chromatographically in all

samples prior and subsequent to lyophilization, it was necessary to take the ether solution of the extract and to remove any accompanying traces of quinic acid by chromatography in butanol-acetic acid-water (4:1:2.2). Both quinic acid and quinide have R_f values in this solvent sufficiently different from that of the ester to allow good separation. When the chromatogram was sprayed with the periodate-benzidine spray, the ester spot turned deep blue, as did free p-coumaric acid. The extract solution was chromatographed in other solvents, but in none of the cases was any reactivity shown with the periodate spray that could be interpreted as glycol oxidation. Since periodic acid will react with both cis- and trans-glycols, this means that the hydroxyl group at the number 4 position of the quinic acid fragment must be the one esterified with p-coumaric acid.

5. Controlled Hydrolysis: To decide the status of the hydroxyl group at position number 1 on the quinic acid fragment, the extract sample was hydrolyzed completely, without degradation of any component, and the concentrations of the p-coumaric and quinic acids so formed were determined. The ultraviolet spectrum of the extract solution after six hours in 1N sodium hydroxide under nitrogen was identical with that of p-coumaric acid (λmax. 337 mμ). The absence of the strong bathochromic shift in alkali, attributed to the ester group, indicated that hydrolysis was complete. Nor did 12 hours' hydrolysis under these conditions alter

TABLE VIII

EFFECT OF BORIC ACID ON CONDUCTANCE

	Resistance (R)	Specific[a] Conductance (κ)	Molar[b] Conductance (Λ_m)	$\Delta\Lambda_m$
Boric acid (0.1M)	63,800 ohms	6.76×10^{-6}	6.76×10^{-4}	
Chlorogenic acid	3,280	1.31×10^{-4}	131	
Chlorogenic acid and boric acid	2,180	1.98	198	67
Quinic acid	3,200	1.35	135	
Quinic acid and boric acid	2,380	1.81	181	46
Caffeic acid	8,700	0.50	50	
Caffeic acid and boric acid	7,900	0.55	55	5
p-Coumaric acid	7,200	0.60	60	
p-Coumaric acid and boric acid	7,600	0.59	59	-1
Extract[c]	3,500	1.23		
Extract and boric acid	3,550	1.21		

[a]Specific conductance, κ = cell constant/R

[b]Molar conductance, $\Lambda_m = \kappa \times 10^3/M$

[c]The sample did not dissolve completely in the 25 ml. of water used; its concentration is unknown.

the experimentally determined quinic acid and p-coumaric acid concentrations. After acidification, the quinic acid content was determined on an aliquot by reaction with periodic acid and subsequent iodometric titration. The p-coumaric acid content was determined by measuring the absorbance at 312 mμ of an ethanolic solution of an aliquot at known dilution.

Under identical alkaline condition, quinide saponifies and after acidification titrates completely as free quinic acid. Experiments showed that 10 minutes was sufficient time for the periodate reagent to completely oxidize the amounts of quinic acid in these experiments. On the other hand, quinide and the extract, both before and after hydrolysis, showed a slow increase in periodate consumption after this interval (see Figs. 14, 15). Since there was no reason to suspect the presence of any quinide, this increase was attributed to non-glycollic periodate-reactive impurities. Ferulic acid showed a similar slow consumption of periodic acid which increased with time (Fig. 15).

Under the alkaline conditions prevailing during the hydrolysis, p-coumaric acid was found to be essentially stable over a 48-hour period. After acidification, the p-coumaric acid concentration was determined from the expression: $A = \epsilon c \ell$

where

A = absorbance of the solution at 312 mμ

ϵ = extinction coefficient of p-coumaric acid

= 2.29×10^4 in ethanol(38)

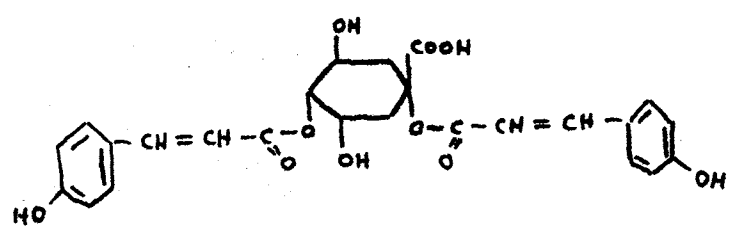
ℓ = light path (1 cm.)

The absorbance measurements were run in ethanol solutions.

Early determinations pointed to a very close p-coumaric/quinic mole ratio of 2:1. Subsequent experiments indicated that these results were not entirely reproducible. (Table X). The p-coumaric acid concentrations were seen to cover a rather wider range than would be expected from similarly prepared samples. Since the concentrations calculated for the p-coumaric acid were generally in excess of its solubility, non-representative sampling could explain the spread. Extinction coefficients were estimated for mono- and di-coumarates by analogy with the $\Delta (\log \epsilon)$ values observed for esters of caffeic acid (see Table IX). From these values, ester concentration before hydrolysis was estimated, and was found to agree best with the quinic acid concentration calculated after hydrolysis on the assumption of a quinyl dicoumarate (Table X). Since there can be only one quinic acid fragment in the ester (only one point of attachment to p-coumaric acid), this indicates the presence of two p-coumaric groups per quinic acid molecule.

D. Conclusion

The structure of the main ester in the vegetative plant is suggested to be quinyl-1,4-di-p-coumarate (IX).



IX

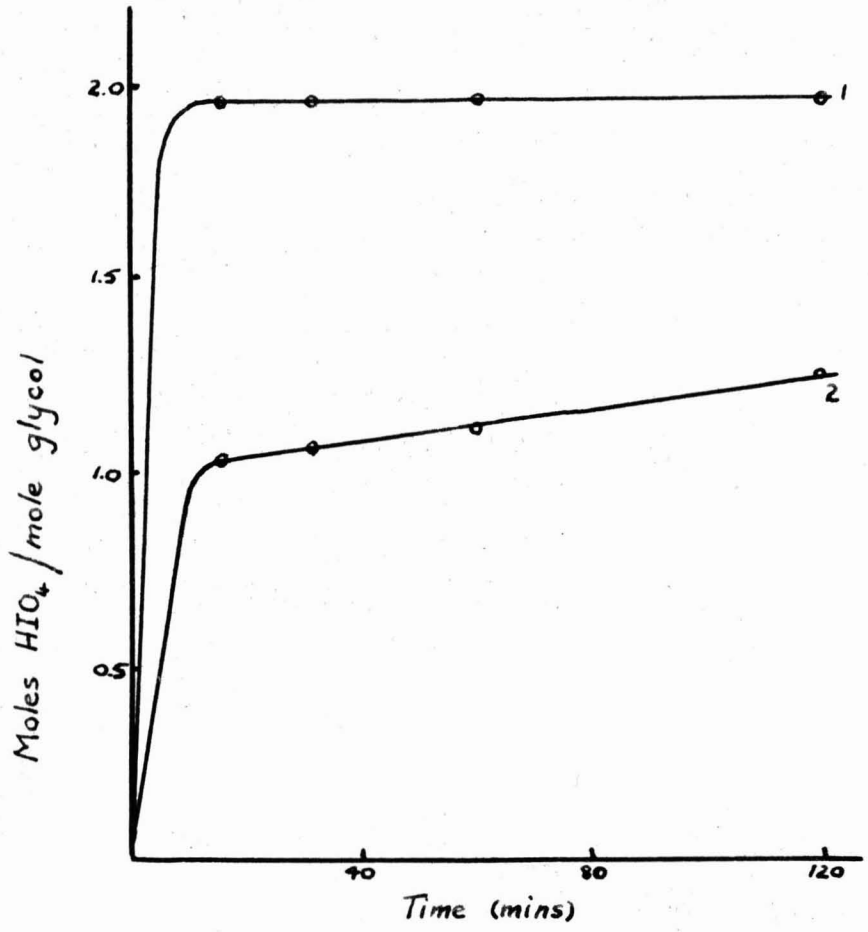


Fig. 14

Rate of periodate oxidation of (1) quinic acid (2) quinide

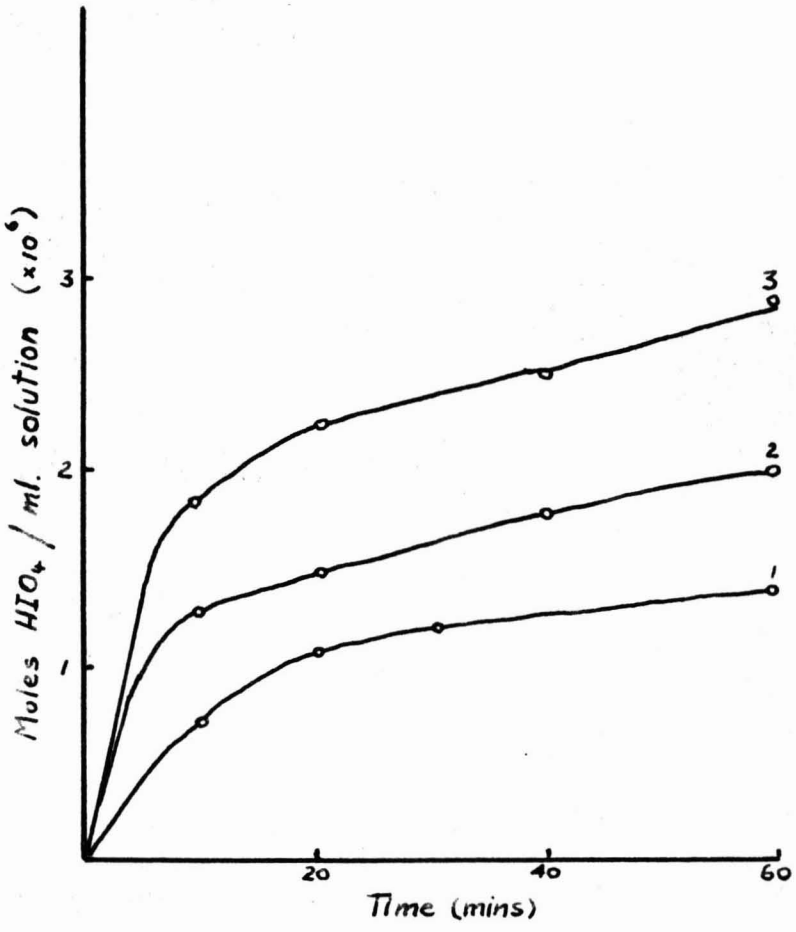


Fig. 15

Rate of periodate oxidation of (1) ferulic acid (2) extract before hydrolysis (3) extract after hydrolysis

TABLE IX

EXTINCTION COEFFICIENTS OF QUINYL ESTERS IN ETHANOL

	log ϵ	Δ (log ϵ) for esterification	$\epsilon_{est.}$	Reference
Caffeic acid	4.27			(38)
Ethyl caffeate	4.35	0.08		(38)
Chlorogenic acid	4.29	0.02		(20)
p-Coumaric acid	4.36			(38)
Methyl p-coumarate	4.40	0.04		(38)
Quinyl p-coumarate	4.37(est)	0.01(est)	2.34×10^4	
Quinyl di-p-coumarate			4.68×10^4	

TABLE X

CALCULATIONS OF THE MOLE RATIO OF p-COUMARIC AND QUINIC ACIDS IN THE EXTRACT

Moles ester/ml.[a] before hydrolysis	Moles quinic/ml. after hydrolysis	Moles p-coumaric/ml. after hydrolysis	Remarks
1.2×10^{-6}	1.0×10^{-6}	2.5×10^{-6}	12 hrs. hydrolysis
1.2	0.9	2.3	6 hrs. hydrolysis
0.7	0.9	0.9	Low p-coumaric estimate, possibly some loss during extraction.
0.5	0.4	1.0	This extract stood several days in acid solution.
2.4	1.0	3.4	
——	0.5	2.1	The ester was purified chromatographically.

[a]These figures based of the estimated extinction coefficient for a quinyl di-p-coumarate.

Such a compound is completely analogous to cynarine, in which quinic acid is esterified through the 1 and 4 positions with caffeic acid. This compound was isolated(28) by some Italian workers from artichoke, Cynara scolymus, and possesses chloretic and cholesterinolytic activity. The possibility of attachment of a group through position number 5 on the quinic acid fragment cannot be ignored. But in view of the evidence for the existence of a p-coumarate linkage at the adjacent carbon atom, it is suggested that steric factors would tend to make existence of any bulky group at the number 5 position unlikely. Partial lactone formation either before or during extraction, might explain the incomplete solubility of the material in ether. Final elucidation of the structure must await further work.

Assays carried out at the Pineapple Research Institute have shown that both p-coumaric and ferulic acids, and possibly their esters as they occur in the plant, act as modifiers of pineapple indoleacetic acid oxidase. In the absence of suitable phenolic material, no enzyme activity occurred. p-Coumaric acid or its naturally occurring ester acted as a coenzyme for pineapple indoleacetic acid oxidase. Ferulic acid at low concentration levels stimulated partial enzyme activity when little or no p-coumaric acid was present; at higher levels, ferulic acid or its ester was a powerful enzyme inhibitor.

IV. SUMMARY

A material was extracted from pineapple plants which is responsible for the absorption around 314 mμ of aqueous extracts of the plants. A technique was developed for preliminary classification of hydroxycinnamic acids and esters through their ultraviolet absorption spectra. The material which is extracted from the plant stumps, and which does not occur in the fruit, shows two chromatographically similar components. The component present in greatest concentration was shown to be an ester of p-coumaric acid; the accompanying material was a ferulic ester. Hydrolysis showed the lactone of quinic acid to be readily identifiable, in such concentrations as to suggest that quinic acid represents the alcohol moiety of the p-coumaric ester. Neutral equivalent experiments showed the presence of a lactone ring and thus the existence of a free hydroxyl group at position number 3 of the quinic acid fragment. Conductivity data indicated the absence of free adjacent cis-hydroxyl groups in position number 4 and 5. Lack of susceptibility to periodate activity indicated an attachment through the hydroxyl at position number 4. Gentle hydrolysis and estimation of the quinic acid and p-coumaric acid formed pointed to the existence of a dicoumarate. Steric reasons suggested the structure of the ester to be a quinyl-1,4-di-p-coumarate. Assays indicate that this naturally occurring ester acts as a coenzyme for pineapple indoleacetic acid oxidase.

V. REFERENCES

1. Enzyme-catalyzed oxidative browning of fruit products. M. A. Joslyn and J. D. Ponting. Advances in Food Research 3, 1, (1951).

2. The isolation and identification of quercitin from apple peels. C. E. Sando. J. Agr. Research 28, 1243, (1924).

3. Reference 1, p. 12.

4. Peroxidase in the darkening of apples. A. K. Balls and W. S. Hale. Ind. Eng. Chem. 27, 335, (1935).

5. Chlorogenic acid and enzymic browning of apples and pears. C. Weurman and T. Swain. Nature 172, 678, (1953).

6. Enzymatic oxidation of catechols, protocatechuic and hydroxy cinnamic acids. Karl Herrmann. Naturwissenschafter 42, 465, (1955).

7. Indoleacetic acid oxidase and an inhibitor in pineapple tissue. Willis A. Gortner and Martha Kent. J. Biol. Chem. 204, 593, (1953).

8. Effect of scopoletin on indoleacetic acid metabolism. W. A. Andreae. Nature 170, 83, (1952).

9. Theories of the biogenesis of flavonoid compounds. Part I. T. A. Geissman and Elly Hinreinher. Botan. Rev. 18, 77, (1952).

10. Ultraviolet absorption spectra of coumarin derivatives. Richard H. Goodwin and Bruce M. Pollock. Arch. Biochem. and Biophys. 49, 1, (1954).

11. Identification of coumarins and related compounds by filter paper chromatography. T. Swain. Biochem. J. 53, 200, (1953).

12. Commoner phenolic constituents of plants and their systematic distribution. E. C. Bate-Smith. Sci. Proc. Roy. Dublin Soc. 27, 165, (1956).

13. Ferulic, sinapic and related acids in leaves. E. C. Bate-Smith. Chemistry & Industry 1954, 1457.

14. The chemistry of flower pigmentation in Anthirrhinum majus. IV. The albino (-mm-nn) form. T. A. Geissman and J. B. Harborne. Arch. Biochem. and Biophys. 55, 447, (1955).

15. Biogenesis of flavonoids in Chlamydomonas eugametos. A. J. Birch, F. W. Donovan and F. Moewus. Nature 172, 902, (1953).

16. Biosynthesis of flavonoid compounds in higher plants. T. A. Geissman and T. Swain. Chemistry & Industry 1957, 984.

17. Chlorogenic acid as a competitive inhibitor of indoleacetic acid oxidase. Ruth S. Rabin and Richard M. Klein. Arch. Biochem. and Biophys. 70, 11, (1957).

18. Chlorogenic and caffeic acids as fungistatic agents produced by potatoes in response to inoculation with Helminthosporium carbonum. J. Kuć, R. E. Henze, A. J. Ullstrup and F. W. Quackenbush. J. Am. Chem. Soc. 78, 3123, (1956).

19. Relation of chlorogenic acid to scab resistance in potatoes. Gestur Johnson and Lawrence A. Schall. Science 115, 627, (1952).

20. Isolation of chlorogenic acid from apple. A. C. Hulme. Biochem. J. 53, 337, (1953).

21. Organic acids in brewed coffee. H. H. Mabrouk and F. E. Deatherage. Food Technol. 10, 194, (1956).

22. Chlorogenic acids of tea and maté. E. A. H. Roberts. Chemistry & Industry. 1956, 985.

23. Study of phenolic compounds in oil-flax (Linum usitatissimum). I. A. M. Cruickshank and T. Swain. J. Exp. Botany 7, 40, (1956).

24. Chlorogenic acid and respiration of sweet potatoes. George O. Rudkin and J. M. Nelson. J. Am. Chem. Soc. 69, 1470, (1947).

25. Isochlorogenic acid-isolation from coffee and structure studies. H. M. Barnes, J. R. Feldman and W. V. White. J. Am. Chem. Soc. 72, 4178, (1950).

26. A new isomer of chlorogenic acid from peaches. Joseph Corse. Nature 172, 771, (1953).

27. Derivatives of caffeic acid in sweet potatoes attacked by black rot. Ikuzo Uritani and Masateru Miyano. Nature 175, 812, (1955).

28. Constitution of cynarine, the active principle of the artichoke. Luigi Panizzi and Maria Luisa Scarpati. Nature 174, 1062, (1954).

29. The constitution of linocinnamarin. Harold J. Klosterman, F. Smith and C. O. Clagett. J. Am. Chem. Soc. 77, 420, (1955).

30. The flavonoid constituents of normal and virus-infected peach and cherry leaves. T. A. Geissman. Arch. Biochem. and Biophys. 60, 21, (1956).

31. Ester groups in lignin. D. C. C. Smith. Nature 176, 267, (1955).

32. The suspected presence of p-coumaryl-quinic acids in tea, apple and pear. R. A. Cartwright, E. A. H. Roberts, A. E. Flood and A. H. Williams. Chemistry & Industry. 1955, 1062.

33. The phenolic substances of manufactured tea. 1. Fractionation and paper chromatography of water-soluble substances. E. A. H. Roberts, R. A. Cartwright and (Miss) M. Oldschool. J. Sci. Food Agr. 8, 72, (1957).

34. Condensation of aldehydes with malonic acid in the presence of organic bases. VI. Condensation of p-hydroxy-benzaldehyde. K. C. Pandya and Toqir Ahmad Vahidy. Proc. Indian Acad. Sci. 4A, 140, (1936).

35. The mobility of symmetrical triad (prototropic) systems. Part IV. Mobility in the simple three -carbon systems terminated by aryl groups. Christopher Kelk Ingold and Charles William Shoppee. J. Chem. Soc. 1929, 447.

36. Purification and physical properties of organic compounds. VIII. A contribution to the thermal study of liquid crystal formation. Evald L. Skau and Harry F. Meier. Trans. Faraday Soc. 31, 478, (1935).

37. Synthesis of cynarine, the active principle of the artichoke. Luigi Panizzi and Maria L. Scarpati. Gazz. chim. ital. 84, 806, (1954). Original paper not translated. C. A. 50, 882, (1956).

38. A spectrophotometric method for the detection of o-dihydroxyl groups in flavonoid compounds. Leonard Jurd. Arch. Biochem. and Biophys. 63, 376, (1956).

39. The detection of aromatic acids in plant extracts by the ultraviolet absorption spectra of their ions. Leonard Jurd. Arch. Biochem. and Biophys. 66, 284, (1957).

40. Richard J. Block, Emmett L. Durrum and Gunter Zweig. "A Manual of Paper Chromatography and Paper Electrophoresis," Academic Press Inc., New York, 1955, p. 95.

41. Separation of phenolic heartwood constituents by paper partition chromatography. Gösta Lundstedt. Acta. Chem. Scand. 4, 448, (1950).

42. Detection of glycosides and other carbohydrate compounds on paper chromatograms. J. A. Gifonelli and Fred Smith. Anal. Chem. 26, 1132, (1956).

43. Theogallin as a galloyl ester of quinic acid. R. A. Cartwright and E. A. H. Roberts. Chemistry & Industry 1955, 230.

44. Isolation and constitution of the active principle of the artichoke. Luigi Panizzi and Maria L. Scarpati. Gazz. chim. ital. 84, 792, (1954). Original paper not translated. C. A. 50, 880, (1956).

45. Nicholas D. Cheronis, Micro and Semicro Methods, in Arnold Weissberger ed., "Technique of Organic Chemistry," Interscience Publishers Inc., New York, 1954, Vol. 6, p. 240.

46. James S. Fritz, "Acid-Base Titrations in Nonaqueous Solvents," the G. Frederick Smith Chemical Co., Columbus, Ohio, 1952, pp. 28-29.

47. Determination of monoglyceride in fats and oils by oxidation with periodic acid. W. D. Pohl, W. C. Nehlenbacher and J. H. Cook. Oil and Soap 22, 115, (1945). This method has been adapted in "Quantitative Organic Analysis via Functional Groups," by Sidney Siggia (John Wiley and Sons Inc., New York, 1949) pp. 8-9.

48. Hobart H. Willard, N. Howell Furman and Clark E. Bricker, "Elements of Quantitative Analysis, Theory and Practice," D. Van Nostrand Company Inc., Princeton, New Jersey, 1956, pp. 264-267.

49. T. A. Geissman, Anthocyanins, Chalcones, Aurones, Flavones, and Related Water-Soluble Plant Pigments, in K. Paech and M. B. Tracey, eds. "Modern Methods of Plant Analysis," Springer-Verlag, Berlin, 1955, Vol. 3, pp. 463-474.

50. Preliminary investigation of flavonol content in pineapple. B. L. Williams and S. H. Wender. Proc. Oklahoma Acad. Sci. 33, 249, (1952).

51. Direct measurement of absorption spectra of plant phenols on paper strip chromatograms. A. E. Bradfield and A. E. Flood. J. Chem. Soc. 1952, 1470.

52. Reference 47, p. 491.

53. R. Norman Jones and Camille Sandorfy, The Application of Infrared and Raman Spectrometry to the Elucidation of Molecular Structure, in Arnold Weissberger, ed. "Technique of Organic Chemistry," Interscience Publishers Inc., New York, 1956, Vol. 9, p. 577.

54. Bromine substitution in salicylic acid. I. M. Kolthoff. Pharm. Weekblad 69, 1159, (1932). Original paper not translated. C. A. 27, 280, (1933).

55. Reference 43, p. 457.

56. Isolation of l-quinic acid from apple. A. C. Hulme. J. Exp. Botany 2, 298, (1951).

57. Amino acids, fruit acids and polyols of Myrobalans. D. E. Hathaway. Biochem. J. 63, 380, (1956).

58. The use of boric acid for the determination of the configuration of carbohydrates. J. Böeseken. Advances in Carbohydrate Chem. 4, 189, (1949).

59. Cyclitols. Part V. Paper ionophoresis, complex formation with borate, and the rate of periodic acid oxidations. S. J. Angyal and D. J. McHugh. J. Chem. Soc. 1957, 1423.

VI. ACKNOWLEDGMENTS

The writer is conscious of his great debt to Dr. Willis A. Gortner, Pineapple Research Institute of Hawaii, for first suggesting the problem, for his continued interest, and for his constant encouragement and advice. Thanks are also due to the committee members for their willingness to discuss difficulties as they arose; to the Pineapple Research Institute of Hawaii for a research grant during the course of the investigation; and to the United States Educational Foundation in Australia for a Fulbright travel grant to study in the United States.