Ph.D.

Chemistry

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BIOLOGICALLY ACTIVE SUBSTANCES IN BIRCH LEAVES: FLAVONOIDS AS GROWTH REGULATORS

ABSTRACT

Green leaves of Betula papyrifera were extracted with aqueous methanol. Thin-layer chromatograms of the acidic fraction of the extract revealed a flavonoid-containing segment which inhibited the endogenous growth of oat coleoptile sections. Chemical fractionation of the original extract yielded quercetin, ellagic acid and their methyl ethers, as well as methyl ethers of myricetin and gallic acid. A wide variety of flavonoids, including those isolated, were tested for biological activity. Flavone and flavanone were strong growth inhibitors, as were naringenin, fisetin and datiscetin. Although current literature explains the inhibitory action of flavones by their apparent accelerating effect on indoleacetic acid oxidase, the present results suggest that the theory is untenable in a number of specific cases. The relationship between structure and activity is complex and cannot be determined from the data available, but the activity of flavanone and flavone suggest that the carbon skeleton itself can play a significant role. The inhibitory action of flavanone was reversed by added indoleacetic acid, while that of flavone was not.

BIOLOGICALLY ACTIVE SUBSTANCES IN BIRCH LEAVES: FLAVONOIDS AS GROWTH REGULATORS

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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November 1967

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to those who have helped in the completion of this project. He is particularly indebted to:

Dr. H.I. Bolker, Research Director, for his guidance, advice and assistance throughout the investigation;

The fellow students of the Organic Chemistry Division of the Pulp and Paper Research Institute of Canada, for their interest and suggestions;

The National Research Council of Canada for a studentship during the period 1965 - 1967;

McGill University for demonstratorships during 1964 - 1966;

The Pulp and Paper Research Institute of Canada for a summer grant during 1964.

The author also wishes to acknowledge Dr. G.L. Lowry, Woodlands Research Department of the Pulp and Paper Research Institute and Mr. Z. Zdrazil of the Petawawa Forest Experiment Station, who made possible the collection of the leaves.

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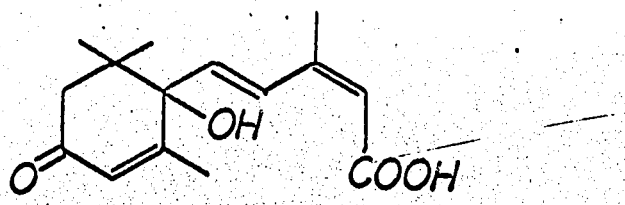
GENERAL INTRODUCTION

GENERAL INTRODUCTION

Although the growth promoting activities of the naturally occurring auxins, gibberellins and cytokinins have been studied for a number of years, endogenous inhibitors of plant growth have only recently been identified. In 1963, Wareing and co-workers (1) published a brief note describing the isolation of a small quantity of a compound which inhibited the growth of coleoptiles and was believed to have a role in the control of dormancy in woody plants. This substance, "dormin", was a colorless, non-crystalline solid, whose infrared spectrum suggested that it was a non-aromatic ketoacid. However, the quantity isolated was too small for a complete elucidation of structure.

Wareing's discovery suggested that an attempt to isolate "dormin" or a similar compound from a Canadian species of tree might be a fruitful line of research. It seemed especially worthwhile to attempt such an isolation from a very large batch of leaves, so that the quantity of product isolated would be adequate for complete determination of structure.

Exploratory work was begun on a small batch of leaves from white birch (Betula papyrifera) to establish whether inhibitory substances could be found. Since previous workers had detected active materials in the acid fraction of plant extracts, that fraction was the starting point of the present work. The first new departure was to use thin layer chromatography to separate the components of the acid fraction. Then, in the conventional way, segments of the chromatogram were subjected to an oat coleoptile bioassay procedure. A zone of strong growth inhibition was



Abscisin II

present at high R_f, and elution of this active zone yielded an impure material. The infrared spectrum of this substance indicated that the major fraction was aromatic, in contrast to that isolated by Wareing. Based on the infrared data, a procedure was devised for isolating larger quantities of the inhibitory material. While this work was in progress, Addicott (2,3) reported the identification of a cotton leaf abscission accelerating compound, abscisin II. Shortly afterwards Wareing found that "dormin" was identical to abscisin II (4), and confirmed the structure by synthesis.

In a sense, the original objective of the present thesis had been fulfilled. Yet, there remained the observation that the active fraction from birch leaves was unlike the material of Addicott. Therefore, it was decided to continue this investigation.

Ultimately, the compounds isolated, other than gallic and ellagic acid, proved to be flavones, and none were of exceptional chemical interest. Nevertheless, the literature (5) indicated that flavones might play a role in regulating the growth of plants. In addition, comparison of the infrared spectrum of the original inhibitory fraction isolated from the birch leaves with those of several flavones indicated

that the active material was very similar to quercetin. Therefore, a limited study was undertaken to determine the activity of a number of flavonoid compounds.

This thesis begins with a review of the non-polymeric substances which have been detected or isolated from three common hardwoods, birch, maple and poplar. These genera provide a sample of the types of compounds found in hardwoods in general and, thus, the review reveals the significance of the compounds isolated in this work with respect to both taxonomical and chemical interest. The second chapter describes the actual procedures which led to the isolation and identification of several natural products from the birch leaf extract. The third chapter outlines the bioassays performed on some twenty flavonoid compounds and evaluates the results in the light of current hypotheses concerning the mechanism of the biological activity of flavonoids, and the relationship between their structures and their activity.

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CHAPTER I

REVIEW OF NON-POLYMERIC EXTRACTIVES FROM BIRCH, MAPLE AND POPLAR

In the study of the composition of trees, the particular interest of the investigator often governs the approach he takes. For example, in the early part of this century, the sap of maple trees was analyzed intensively because of its commercial value. Commercial value also underlies the work done by chemists associated with the Pulp and Paper industry. These latter workers in the past have investigated mainly the polymeric constituents of wood, but more recently have begun to give equal attention to the low-molecular weight extractives, perhaps in the belief that these too will help explain differences in physical and chemical properties of various species. To many other organic chemists, the extractives of certain species simply provide a rich source of new compounds for investigation of structure and of chemotaxonomy. Other compounds of low molecular weight are of concern to plant physiologists as intermediates in metabolic processes, while nutritionists have looked to leaves and needles as a source of vitamin-rich fodder. Even the mineral content of trees has engaged the attention of geologists who hoped that such information would be an aid in prospecting. Thus, results of work on the composition of trees are scattered through many different branches of the scientific literature, and to assemble and correlate them would be a task of major magnitude. Therefore, the scope of this review was deliberately limited. Its purpose is to examine the available data on the extractives of only three genera, birch (Betula), maple (Acer), and poplar (Populus), and to present a sampling of the principal kinds of compounds to be found in hardwoods in general. However, it must be recognized that a review of this type has two limitations. First, the study of only three species as a sample contains

the inherent disadvantage that, in many cases, especially those involving the biogenetic or metabolic role of an individual compound, investigations often involve the use of a wide range of plants. Hence, although many points appear to be unanswered here, it may be that problems have been pursued more intensively with some species other than birch, maple or poplar, and the answers lie outside of this sample. The pursuit of such points was considered to be beyond the scope of this review.

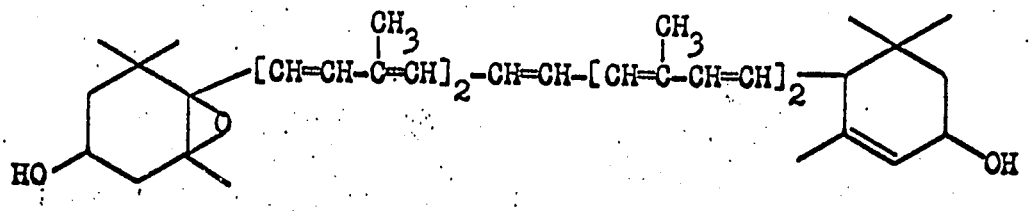
Second, the variety of methods used in the isolation of compounds is nearly as wide as the variety of materials so isolated. Often the crude extracts are subjected to somewhat harsh conditions which could conceivably cause degradation or rearrangement. Usually the authors have commented upon this point, but, in some cases, conclusions have to be accepted only with reservations.

A number of reviews of a less general nature have been published. These include an early review of birch extracts (Grasser, 1), maple sap (Aires, 2), the chemical composition of birch bark (Jensen, 3), plant pigments (Karrer and Walker, 4) and a brief review of the phenolic glycosides of Salix (willow) including their discovery, the elucidation of their structure and their synthesis (Thieme, 5).

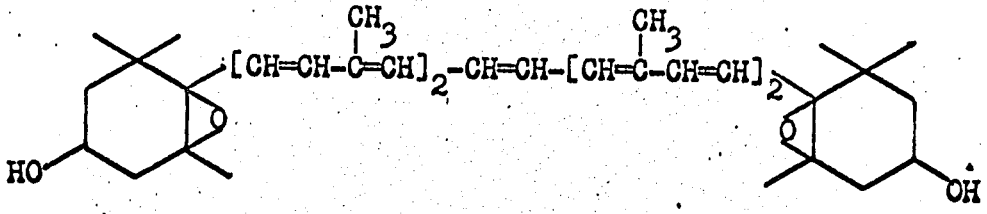
The possibility of using leaves as a source of food for animals is quite appealing and has prompted a number of workers to measure the ascorbic acid (vitamin C) levels in the various parts of trees. The results have been compiled in several papers (6-9). From the point of view of plant biochemistry, it has been found that the ascorbic acid content of the yellow parts of autumn maple leaves was higher than

elsewhere in the leaf (8). The authors suggested that the yellow pigments may play a part in the synthesis of ascorbic acid, but presented no supporting evidence.

With only one or two exceptions, the carotenoids and related compounds in birch, maple and poplar have been found exclusively in the leaves. Carotene itself has been the subject of extensive quantitative analysis, both in comparative studies among trees and as a seasonal variable (10-15). For example, L'vov and co-workers (16) found that, as maple leaves turned yellow and underwent abscission, the carotene content gradually diminished. A relationship between plastin plant pigments and the protein carriers was suggested by the authors. They hypothesized that the breakdown of the carotene in the fall is conditioned by the breakdown of the protein complexes. Goodwin (17) obtained some similar results in the leave of Prunus nigra, Quercus robur and Acer pseudoplantanus. He found that between June and November, the carotenoid levels fell almost to zero in Quercus and Acer but the decrease in Prunus was only to 50% of its original value. In all cases, however, β-carotene and neoxanthin, the 5'-hydroxy (monoepoxide) derivative of violaxanthin (II), disappeared first and their disappearance was accompanied by the formation of lutein-5,6-epoxide (I). Another compound which formed only in the fall has been tentatively identified by Grob and Eichenberger (18) as all trans-3 or 3'-hydroxy-α-carotene. It was isolated by column chromatography from the extract of yellow maple leaves but was not detected in the green summer leaves. The development of thin-layer chromatography later enabled these workers to investigate the autumn pigments of maple in more detail. They

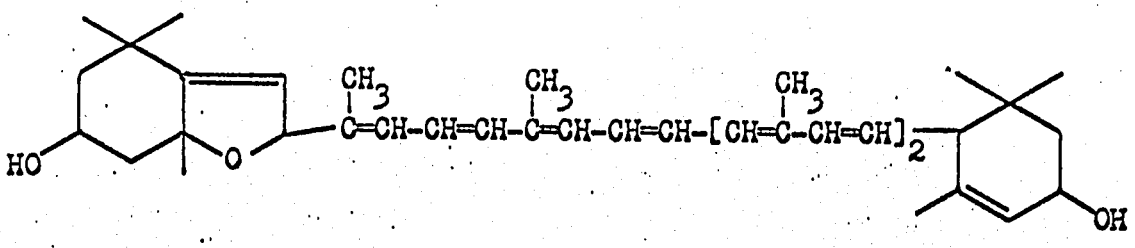


I Xanthophyllepoxide
Lutein-5,6-epoxide



II Violaxanthin

found that lutein(xanthophyll)and its epoxide (I), α- and β-carotene, neoxanthin and violaxanthin (II) were present in the green leaves and their relative proportions were essentially constant. In fall, α- and β-carotene disappeared and the esters of both violaxanthin and xanthophyll were formed (19,20). The esterifying acid of xanthophyll was later found to be linoleic acid. (21). This result conflicts with Goodwin's (17) since Grob and Eichenberger claim that neoxanthin remains in the autumn leaves. Both xanthophyll and its epoxide have been detected in maple by Karrer (4,12) who also noted that the epoxide was readily converted to flavoxanthin (III) in a very weakly acidic medium. This latter compound has not been detected in the hardwoods under consideration here but these results would lay some doubt upon other reported isolations of flavoxanthin, especially if the workup involved acidic conditions of any kind. Conceivably, other acidic componants in the same extract



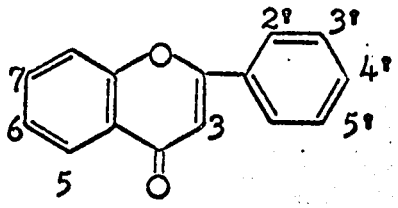
III Flavoxanthin

could also catalyse the conversion of xanthophyll epoxide to flavoxanthin. Violaxanthin also readily rearranges and it, too might be a source of "naturally occurring" flavoxanthin.

A compound very closely related to xanthophyll, abscisin II, has been isolated from the leaves of A. pseudoplantanus by Wareing and co-workers (126). (See General Introduction and Chapter II). It has also been detected in a wide variety of plant tissue, including cabbage leaves, potato tuber, avacado seed, lemon fruit and birch leaves, by optical rotatory dispersion (127). The structure of abscisin II infers that it is probably a decomposition product of the carotenoids. It reaches maximum concentrations in plants late in the growing season and then decreases during the winter until growth resumes and is believed to have a vital role in the control of dormancy.

The only report of the occurrence of carotenoids in the non-leafy parts of trees is that of Euler (22) who detected carotene in the pollen of birch and poplar.

The distribution and seasonal changes of the chlorophylls follows that of the carotenoids quite closely, and in many instances the compounds have been studied together (17,24). For example, Moore (15) found that during senescence in maple leaves, there was a direct correlation between



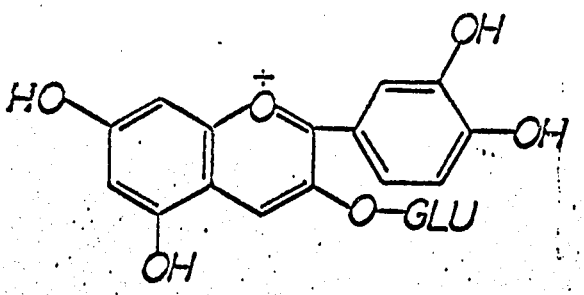
Flavone

the decrease in the contents of carotene and chlorophyll, but he did not present further evidence to show that these decreases were caused by the same processes.

Although the chlorophylls occur almost exclusively in the leafy parts of trees, they have also been observed in very low concentrations in the pith and xylem rays of young shoots of poplar, birch and alder (25). Even in these tissues, the chlorophylls appeared only in winter, thus supporting the hypothesis that chlorophyll is stored in the wood during that season. However, the transport of this substance within the plant has not yet been demonstrated.

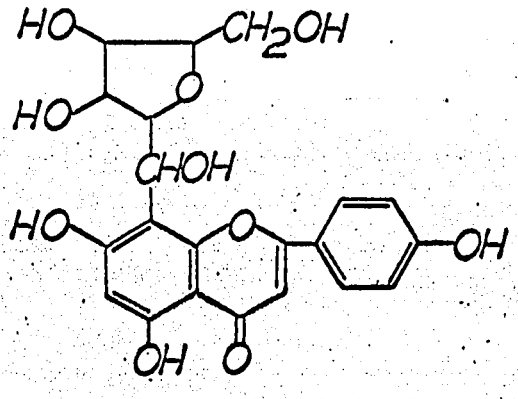
Among the genera under review, references to the leucoanthocyanidins and related compounds are few and concern only their detection, not isolation. Michaluk (26) chromatographed extracts of birch leaves and bark and identified some of these substances by their color reactions. Racz and Fuzi (27) ran similar tests on the various parts of 154 common plants including birch, oak, poplar and willow. Positive results were obtained throughout the plants, and hence, these compounds were probably not confined to only one part. A similar survey was made by Hayashi and Abe (28). Two of the compounds detected were cyanidin monoglucoside and chrysanthemin (IV).

Although the flavonoids constitute one of the largest classes of pigments in nature and are found in nearly every known plant, very little

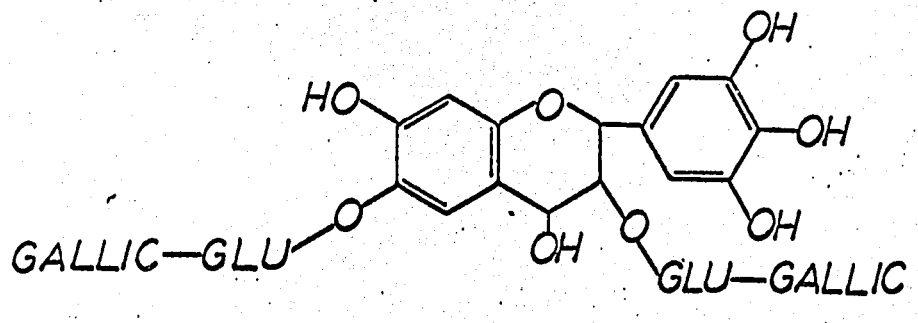


IV Chrysanthemin

is known about their physiological functions. There is some evidence that they play a significant role in the control of plant growth, and this will be discussed at length in Chapter III. A number of reviews have been written on the flavonoids, of which the most extensive are those by Harborne (29) and Dean (30). Somewhere between 200-300 flavonoids have been reported to date. Very few are known to occur in the wood and bark of trees, but are generally found in the leaves and flowers. In fact, with respect to birch, maple and poplar, the only report of the flavonoids being found in tissues other than the leaves comes from Whemer (31) who detected 5,7-dihydroxyflavone (chrysin) and its 7-O-methyl ether in the bark of several species of poplar. The flavonoids which have been isolated from the leaves of the three hardwoods consist mainly of the glycosides of 3,3',4'5,7-pentahydroxyflavone (quercetin) and 3,4',5,7-tetrahydroxyflavone (kaempferol) (32-36). Vitexin (V), saponaretin (the corresponding open ring form of vitexin), myricetin-3-digalactoside (3,3',4',5,5',7-hexahydroxyflavone) and orientin (3',4',5,7-tetrahydroxyflavone-8-glucoside) have also been detected (37-42). From the data available, no generalizations can be made about the occurrence of these compounds. Certainly no exhaustive study on the subject has been done, but it does generally appear that most, if not all, flavones exist in nature



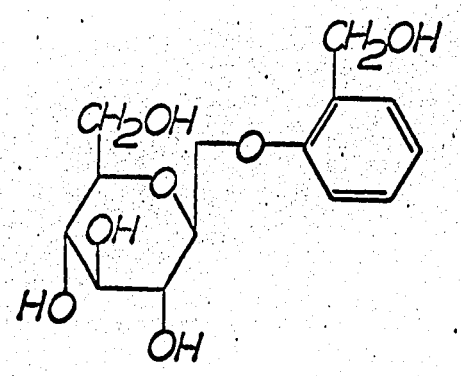
V Vitexin



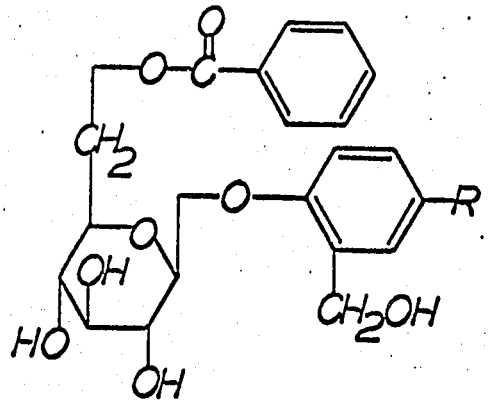
VI

in their glycosidic form. A compound closely related to the flavones was recently isolated from the leaves of Acer airzuense and it was assigned the tentative structure VI (43).

A wide variety of non-flavonoid glycosides has been found distributed throughout the leaves, bark and wood of the three hardwood species. Four of the most common are salicin (VII), populin (6-benzoylsalicin) (VIII), tremuloidin (2-benzoylsalicin) and salireposide (IX). Salicin and populin have been known for a very long time, having first been discovered in the bark of P.alba by Braconnot in 1830 (44), while the other two have only recently been isolated and characterized (45,46). Since then, although these four compounds have been reported in the literature on numerous occasions,



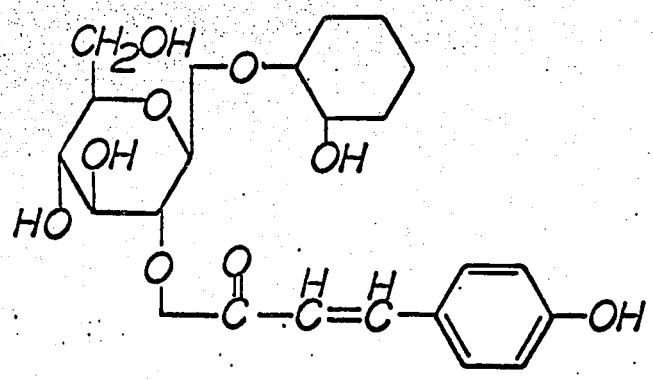
VII Salicin



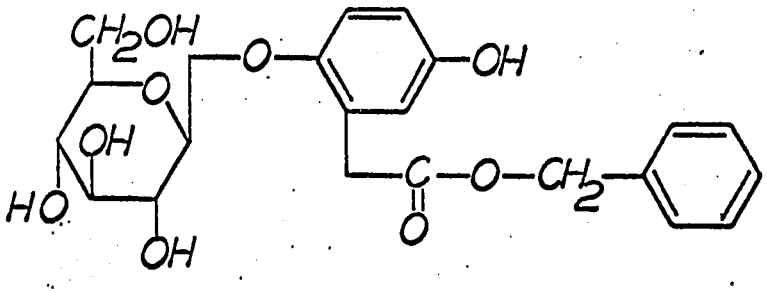
VIII Populin: R = H

IX Salireposide: R = OH

their occurrence has been limited to poplar species (31,35,36,45-54). The majority of the work in this field has been done by Pearl, Darling and their co-workers. Generally, their experimental procedure involves extraction of the bark and leaves with hot water and then treatment of this extract with basic lead subacetate to remove the free acids. The lead salts are filtered and then the filtrate, after removal of excess lead, is concentrated and subjected to solvent fractionation and chromatography. However, subsequent work indicated that, under mild basic conditions, including those used in the workup, tremuloidin rearranges to populin (55,56). In order to clarify this situation, a series of experiments was conducted with extracts from P.tremuloides, and Pearl found that treatment of the extract with a minimum of lead subacetate in the cold yielded tremuloidin, but at elevated temperatures, yielded populin (58). Thus, it was tentatively postulated that populin was an artifact of isolation. Later, this idea was rejected (53) because similar extracts involving no hydrolysis or basic conditions yielded both populin and tremuloidin. It might be noted that populin was also isolated by Faber (48) from the wood of P.tremuloides in an extract involving neither hydrolysis nor basic conditions. Other glycosides from the bark of poplar include grandidentatin (X) (58) and trichocarpin (XI) (59,60). Other closely related glycosides, including salicoside (61,62), salipopulin (63), triploside (54), and salicyloylpopuloside (61) also have been isolated from the buds and leaves of poplar. Theime (5), in addition to his review on the glycosides of Salix, has described the detection and isolation of a large number of phenolic glycosides



X Grandidentatin



XI Trichocarpin

(including those mentioned above) from poplar.

In conjunction with the studies on glycosides, a large number of low-molecular weight acids and associated derivatives has been detected in various extracts. These acids generally occur as glycosides and are liberated only after saponification, although very small amounts of the free acids exist, probably as a result of hydrolysis within the tree itself. Pearl (50) extracted poplar bark with hot water and, after saponification, found p-coumaric acid, and salicyl and genisyl

alcohols. The two alcohols could be formed by the hydrolysis of salicin and salireposide. However, in some cases, genisyl alcohol has been found when no salireposide was present, possibly because the bark contained one or more other glycosides of this alcohol. Other acids reported by Pearl include vanillic, syringic, p-hydroxybenzoic and ferulic acids, again all in low concentrations as free acids but present in much larger amounts as glycosides (51,52). From the experimental procedure used by Pearl, it is not possible to determine the manner in which these acids are bound in their natural state, i.e., whether as esters, such as the benzoyl group in salireposide, or through their phenolic groups. Basic hydrolysis of both acid and neutral fractions have yielded all of these acids and this finding suggests that there is, in fact, a liberal distribution of both kinds of linkages. The same fractions were found also to contain some of the sugars to which these acids had been linked. The sugars included galactose, arabinose, mannose, xylose and rhamnose as well as glucose (64).

The role of the low-molecular weight acids and their glycosides in the formation of lignin has been the subject of considerable investigation. It is postulated that, in trees, the various glycosides, including coniferin and syringin, are actually formed outside the zone of lignification and are then transported into this zone via the inner bark. Mugg (65) found several low-molecular weight phenolic materials in newly formed aspenwood, and these phenols, one of which was sinapaldehyde, might be considered as precursors of lignin. Subsequently, Faber (48) investigated the inner bark of P.tremuloides and found both coumaric and ferulic acids. These findings are in harmony with the Freudenberg biosynthetic pathway

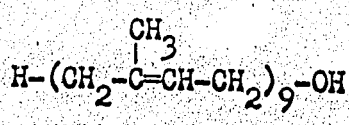
to the formation of lignin and the acids would represent an earlier stage in the scheme than sinapaldehyde. Neither coniferin nor syringin was found in the inner bark.

The investigation of low-molecular weight acids has been carried out along other lines as well. Kaurichev and co-workers (66) measured the seasonal changes in the acid content of birch and aspen leaves. The acids, including oxalic, citric, fumaric and formic, were generally found to be present in higher concentrations in aspen than in birch; they reached a maximum in spring and then decreased steadily until September. The wound exudates of B.alba were analyzed by Wolf (67) and were found to contain citric and malic acids. Unfortunately, this line of study has not been pursued further with these trees although similar work has been done with other types of plants. Abramovitch, in one of a series of papers on aspen heartwood (68), reported the isolation of some steam-volatile compounds from a benzene extract of the wood. These compounds included phenol, benzyl alcohol, β-phenylethanol, o-ethylphenol and methylbenzoate. Since no saponification steps had been included in the procedure, these compounds probably exist in the plant in this free state, although some hydrolysis possibly could have occurred during the distillation. Other volatile fractions from oak, birch, poplar and cherry contained a variety of acids, aldehydes and ketones (69). Chubinidze (70) showed that the levels of these compounds increased in the trees with increasing temperature and exposure to light.

Like these low-molecular weight compounds, the fatty acids, alcohols, paraffins, etc. are also widely scattered through the various parts of trees. Most of the work with these substances has been done with the

wood and relatively little with the bark and leaves of birch, maple and poplar. This does not, of course, necessarily reflect their actual distribution. In one of a series of studies on plant plastids, Eichenberger and Grob (71) analyzed the variations of the fatty acid content of maple leaves. They found that, of the three major components, linolenic acid was the most abundant, and linoleic and palmitic acids were present in lesser amounts. In autumn, the levels of linolenic acid decreased considerably but this decrease was countered by an increase in the C_{12} - C_{14} saturated acids. In order to investigate metabolic processes, Hossfield and Hunter (72) divided aspen bark into four layers and extracted each with petroleum ether. The authors measured the total extractives from each layer but, unfortunately, in isolating individual components, they used the whole bark. Thus, no comparative study could be made. They identified linoleic and lignoceric acids and ceryl alcohol. The workup involved saponification to free the acids, and, therefore, did not permit these workers to determine the manner in which the acids had been bound in the natural state. Again by saponification, Jensen (73, 74) found that extracts of the suberin from birch bark contained eicosanedicarboxylic, β- and ω-hydroxybehenic, 9,10,18-trihydroxystearic, 18-hydroxy-Δ^9-octadecanoic and Δ^9-octadecanedicarboxylic acids.

As mentioned previously, wood has been a much richer source of these materials than leaves, perhaps because of the function of the fatty acids in wood pulping. Generally, the chief fatty acid components of aspen and birchwood have been found to be oleic and linoleic acids, with much smaller amounts of the even-numbered saturated acids C_{14} - C_{26} also present (75-80). Two reports mention hentriacontane, the C_{31} -



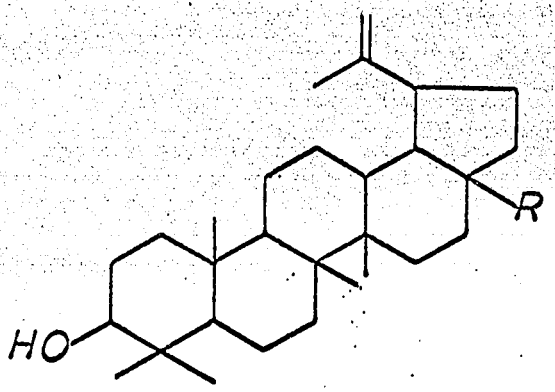
XII

paraffin (81) and a substance tentatively identified as XII (82). Perlia (80) examined the seasonal variations of some acids in birchwood and found that palmitic, stearic, arachidic and behenic acids all reached maximum levels between January and June and then decreased to a minimum in September. Propionic acid did the opposite, while butyric acid increased from a minimum in March to a maximum in May. Since the free acids exist only in very low concentrations, these experimental procedures also involved saponification. There has been some work done to determine the substances to which these acids are bound. As mentioned previously, Grob and Eichenberger (21) established that linoleic acid was the esterifying acid of xanthophyll. Buchanan (77) detected glycerol among the fatty acids in their wood extract, and it was present in a proportion corresponding to the existence of triglycerides and not mono-or diglycerides. The nature of the individual glycerides of fatty acids in wood, as in any other biological tissue, is inherently a very difficult problem to solve. Fats normally consist of mixed triglycerides and, thus, fats with say eight acidic componants could contain up to 288 individual substances. Even with just a frattion of these substances present, their separation presents a formidable task.

Two papers, one by Abramovitch (83) on the extractives of the heartwood of P.tremuloides, and one by Pearl (84) on the extractives of the whole wood of the same species, provide some interesting comparisons.

Abramovitch extracted the heartwood with petroleum ether, separated the free acids with alkali and then methylated them. Pearl extracted the whole wood with benzene and saponified the neutral fraction. He detected all the saturated fatty acids C_{12} - C_{28} except C_{27}, whereas Abramovitch reported the C_{27} acids in addition to most of the others. In both cases, these are the first reports of odd-numbered saturated fatty acids occurring in plants. Similarly, Abramovitch detected the C_{24}, C_{26}, and C_{28} saturated alcohols while Pearl found the C_{24}, C_{26} and C_{27} alcohols. It is difficult to judge if these differences with respect to finding the C_{27} and C_{28} compounds are significant, for they may be due to differences in experimental technique.

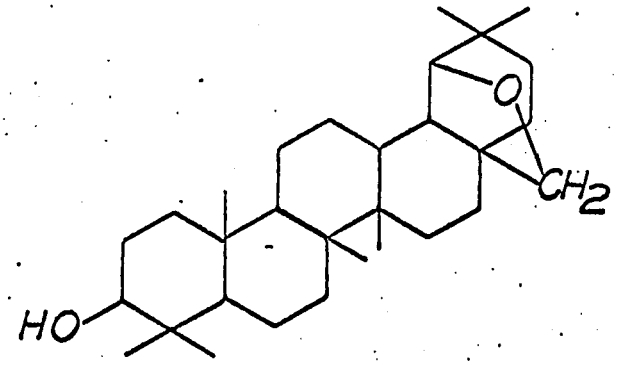
Like the long chain compounds, the terpenes and steroids are also widely distributed within the various parts of trees. Betulin (XIII) was the first to be reported, having been isolated in 1963 by Steiner (85) from the bark of a number of species of birch. Shortly thereafter Pasich (86, 87) and Jarolim reported the isolation of betulin from the same sources, and, in addition, found also lupeol (XIV), allobetulin (XVI) and an unidentified ester of betulinic acid (XV). Lupeol also occurs in the wood of P.tremula (89) and in the heartwood of P.tremuloides. The sterol β-sitosterol (XVII) is apparently more common than the substances already mentioned. It has been detected in aspen bark by Hossfield and Hunter (72), in leaves of A.amoenum (81) and in birchwood (82). Lindgren and Svahn (89) isolated both α- and β-sitosterol, and a glycoside of one of these has been reported by Perlia (78), but this compound was not completely characterized. Pearl and Harrocks (84) have commented on this work of Perlia in conjunction with their own research. They isolated a sterol from the unsaponifiable fraction of a benzene extract



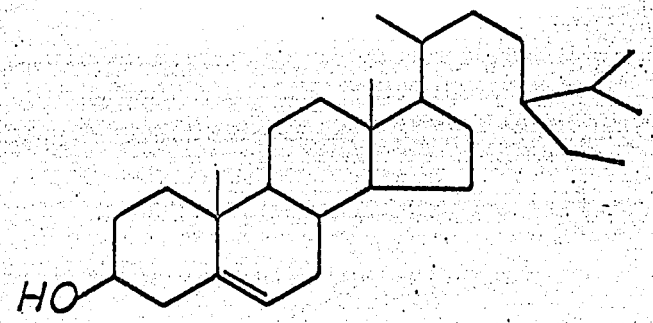
XIII Betulin: R = CH_2OH

XIV Lupeol: R = CH_3

XV Betulinic Acid: R = COOH



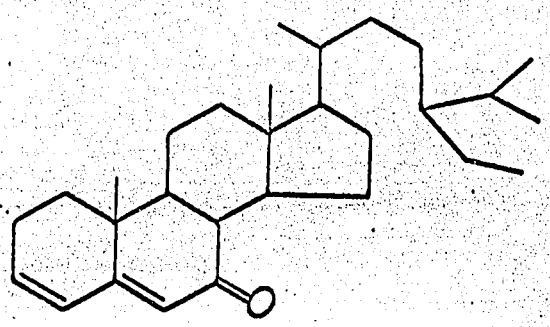
XVI Allobetulin



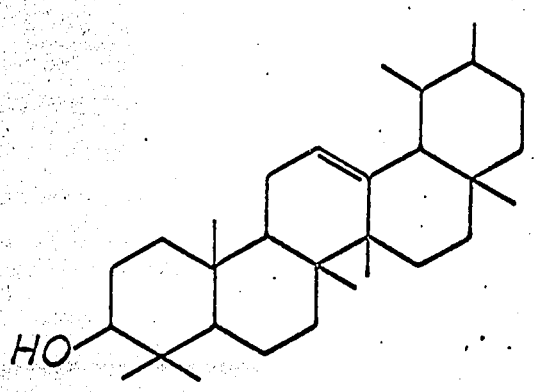
XVII β - Sitosterol

of aspenwood. This compound and its benzoate had optical rotations and infrared spectra similar to those of authentic β-sitosterol and its benzoate. However, the physical properties of the acetate were not in accord with the authentic compound. Pearl and Harrocks concluded that this material was identical to that of Perlia but that it was not β-sitosterol. Abramovitch (92) isolated a sterol from the acetone extract of aspen heartwood and found it to be identical in all respects with β-sitosterol. He then pointed out that the material which Pearl had found was probably β-sitosterol also, because the melting point and infrared spectrum of the acetate can vary considerably depending on the method of crystallization. Abramovitch also showed that the discrepancies in Pearl's elemental analysis data were due to water of crystallization. Subsequently, Pearl isolated β-sitosterol from the bark of P. grandidentata (55).

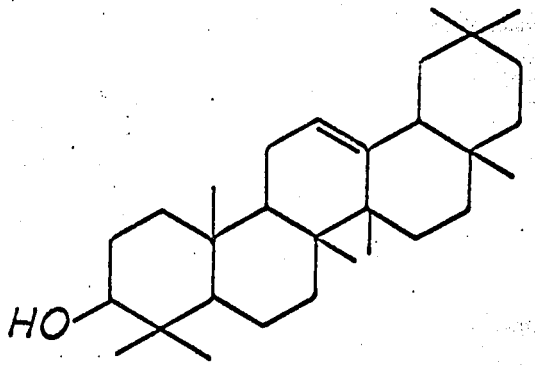
Further work by Abramovitch with various extracts of aspen heartwood yielded tremulone (XVIII) (93), α- and β-amyrin (XIX, XX), α-amyrenonol (XXI), butyrospermol and 24-methylenecycloartanol (XXII) (90, 91). However, Abramovitch questions whether tremulone and α-amyrenonol are really naturally occurring substances or artifacts of isolation. Tremulone was isolated from a nonsaponifiable fraction, but it is known that passing oxygen through aqueous colloidal suspensions of sterols containing the 5-ene-3-ol system gives rise to the 3,7-diol and 3-ol-7-one derivative. Such an oxidation could have occurred during the saponification step. Similarly, it is possible that α-amyrenonol arises from the oxidation of α-amyrin. A compound which has properties resembling tremulone had been isolated much earlier by Mitsui (94) from sugar cane



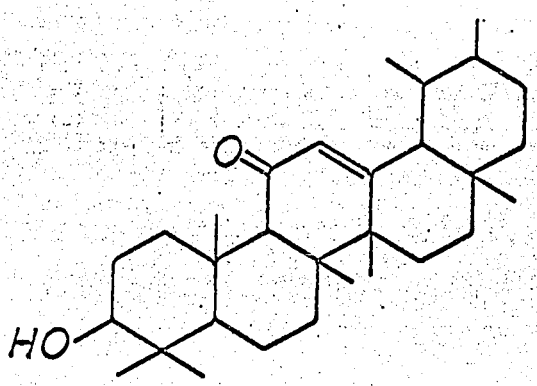
XVIII Tremulone



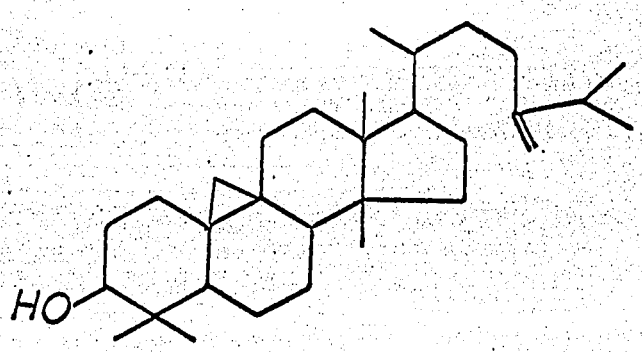
XIX α - Amyrin



XX β - Amyrin



XXI α - Amyrenonol

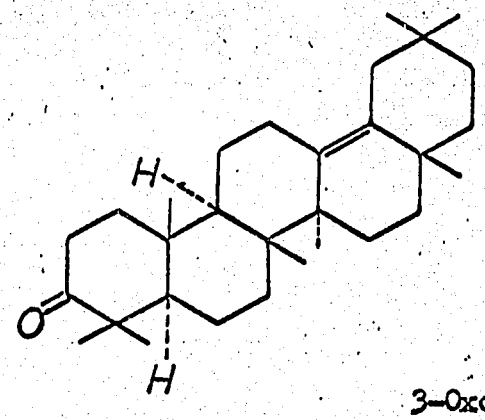


XXII 24-Methylenecycloartenol

wax in yields up to 0.2%. According to Abramovitch, it would seem unlikely that 0.2% of an artifact should have been formed under Mitusi's conditions. It is possible, therefore, that this compound was, in fact, tremulone and existed as such in the sugar cane wax. The work of Abramovitch was essentially duplicated by Lindgren and Svahn (89) and they reported the detection of cycloartenol, but not tremulone. This argument is still unresolved.

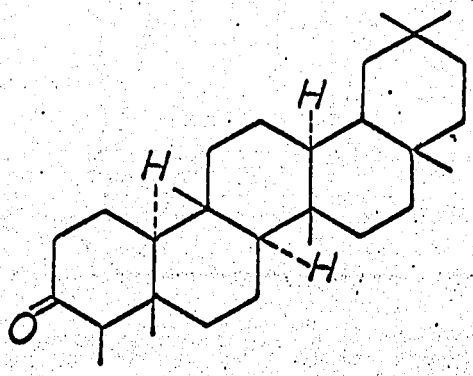
Leaves have yielded a number of other sterols and terpenes. Folientriol (XXIII), folientetraol (the corresponding 3α,12β,17α,20β-compound) from birch leaves (95,96), and 12β,20β-dihydroxy-3-oxo-25 dammarene (3-oxo-folientriol) and 3-oxo-13(18)-oleanene (XXIIIa) from black alder leaves (97) have been reported by Fischer and Seiler. Kojima (81) isolated friedelin and stigmasterol (XXIIIb,c) from the leaves of A.amoenum and Holub (98) identified ylangene (XXIIId) (the structure of which was subsequently determined by Hunter and Brogden, 128), α-betulenol acetate, α- and β-betulenol (XXIV) and caryophyllene and its monoxide from the oil of B. alba buds. Holub expressed the belief that the monoxide or similar compounds may be intermediates in the biogenesis of the betulenols from caryophyllene.

Again the question arises as to how these compounds exist in the natural state. Some have been isolated as free materials, such as those of Kojimo (81) who used alumina chromatography, but most have been isolated from saponified extracts. Some headway has been made quite recently by Paasonen (82) who used acetone to extract birchwood. He then investigated this extract with gas and thin layer chromatography and found mostly the esters of the sterols and triterpenoid alcohols.



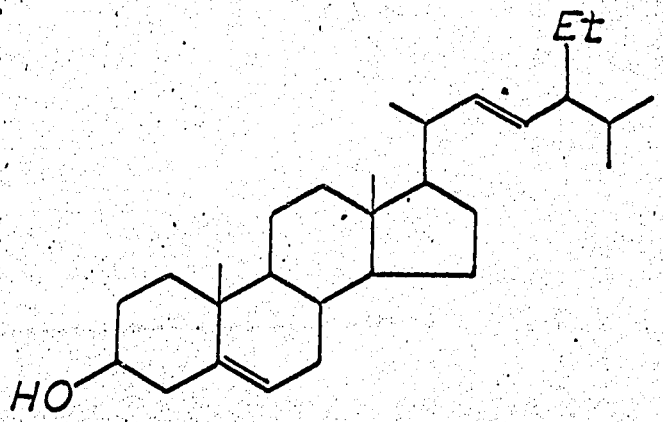
XXIII a

3-Oxo-13(18)-oleanene



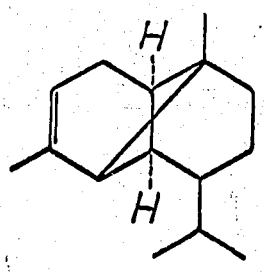
XXIII b

Friedelin



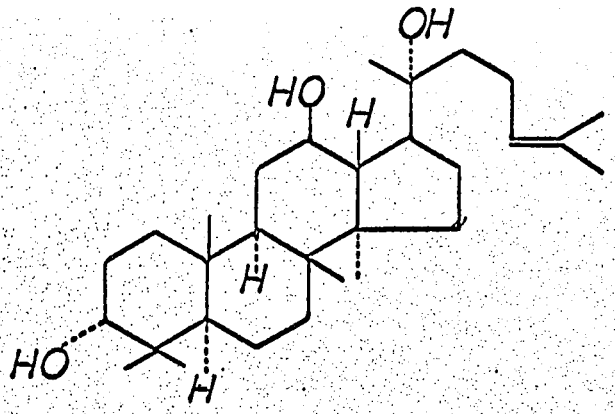
XXIII c

Stigmasterol

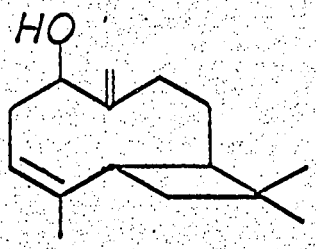


XXIII d

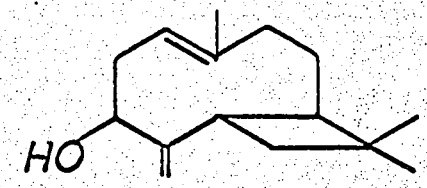
Ylangene



XXIII Folientriol



α

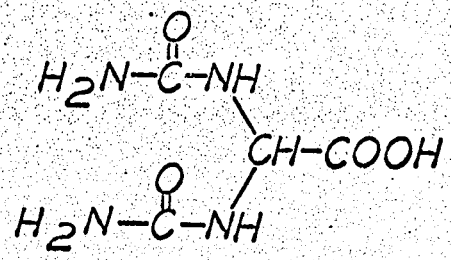


β

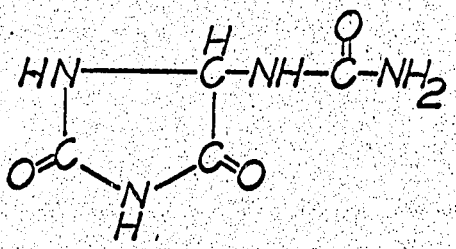
XXIV Betulenol

He was able to determine that the solid esters, at least, were formed from linoleic acid. However, this is the only such study to date.

A wide variety of nitrogen-containing compounds has been reported to occur in trees but their occurrence seems to be restricted to the sap, leaves and pollen. A number of studies have been made on the seasonal variations of amines and amino acids. In analyzing maple sap, Clements (99) found, that during the early tapping season, there were traces of aspartic and glutamic acids, valine and leucine. After mid-season, the concentration of glutamic acid increased sharply



XXV Allantoic Acid

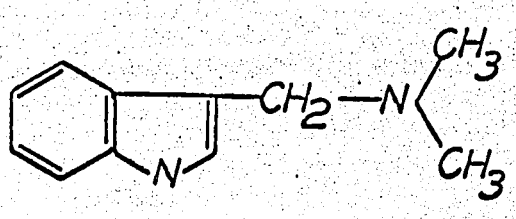


XXVI Allantoin

then decreased as glutamine appeared. The late-season sap contained large amounts of glutamine and increasing amounts of valine and leucine. Paralleling this study is a report by Plaisted (100) on changes in the aminoacids of maple leaves. The amount of protein per leaf increased during late spring and early summer, dropped to a constant level in summer and then decreased rapidly during senescence. This pattern was also followed by β-aminobutyric acid, serine and glycine. Aspartic and glutamic acids, as well as alanine, were found to be at a maximum before spring and decreased steadily throughout the growing season, while glutamine, phenylalanine, threonine, leucine and valine increased. These changes appear to fit well with the results of Clement's work. Other amines and aminoacids which have been found include aspargine in the buds of P.basanifera (62), α-aminoadipic acid in both the green and yellow leaves of A. platanoides (101), and methylamine and isoamylamine, also from maple leaves (102).

The first report of allantoic acid (XXV) in birch, poplar or maple came from Fosse (103-105). Later, Echevin (106) found that the flower and fruit of A. pseudoplantanus contained both allantoic acid and allantoin (XXVI). The acid was present in higher concentrations than allantoin before maturity but the reverse was true at complete maturity. These two compounds accounted for 25% of the total N in the leaves. In a study comparing the types of nitrogen compounds transported by trees, Wolffgang and Mothes (107) reported that maple sap contained allantoin and allantoic acid while alder sap contained mostly citrulline, and elm had primarily asparagine. Virtanen and Kari (108) compared the aminoacids in pollen with those in the leaves and needles of a number of species of trees. Much more free proline was found in the pollen than in the green parts of the trees, while hydroxyproline and pipecolic acid were found exclusively in the pollen. The first (and so far the only) report of alkaloids occuring in the three genera under review comes from Patcher, who isolated gramine (XXVII) from the leaves of A. saccharides (109,110). This compound was previously known to occur only in grasses.

A few other miscellaneous compounds have been isolated from birch, maple and poplar. "Acertanin" was first mentioned in 1922 by Perkin and Uyeda (111) who isolated it from the leaves of A.ginnala. However, the structure was not fully elucidated until 1952. Kutani (129) proved that this substance was 3,6-di(galloyl)-1,5-anhydro-D-glucitol. Quebrachitol (XXVIII) is a compound which is widely distributed in maple. It has been either detected or isolated in a number of species (112-115), but there

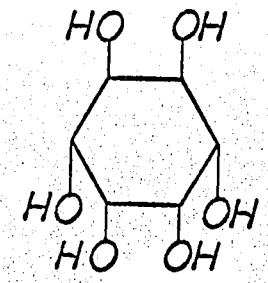


XXVII Gramine

is no mention of its occurrence in birch or poplar. This difference may be significant, although perhaps its detection has been inadvertently missed by workers in this field. Very little has been reported on the occurrence of saponin in these hardwoods. Heine (116, 117) detected saponins in the leaves of A. saccharides and the bark of A. platanoides but did not isolate them.

Most of the work discussed in this review so far has involved the investigation of the non-polymeric constituents of trees. This in no way reflects the emphasis of the work done to date. Perhaps equal attention has been focused on both the general chemical composition of trees and the study of the polymeric materials. However, since most of this latter work is of interest to industrial and polymer chemists alone, only brief mention of it will be made.

Maple sap was studied intensively at the beginning of this century and the literature contains many reports on standardized procedures for the analysis of turbidity, flavor, purity, etc. In addition, investigations have been made on the sugar levels in saps (118, 119, 123)



XXVIII Quebrachitol

and the seasonal fluctuations of these levels (120-122). Bark extractives, of course, have received considerable attention from the Pulp and Paper industry. Usually, measurements have been made on the total material extracted by ether, alcohol, water, etc., the total pentosans, hexosans, cellulose and the total acids and "ash" contents. Wood, too, has received the same sort of treatment. Most of the fundamental research has involved the polysaccharides and oligosaccharides in the various parts of the wood. Timell (124) and Sharkov (125) have produced the majority of the work in this field.

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CHAPTER II

ISOLATION OF BIOLOGICALLY ACTIVE SUBSTANCES FROM BIRCH LEAVES

INTRODUCTION

The nature of endogenous growth regulators has been a subject of considerable interest to plant physiologists for many years. Numerous studies have been made with auxins, gibberellins and kinins. However, it was not until quite recently that the study of natural growth inhibitors became widespread. One of the first reports of endogenous growth inhibiting substances came from Hemberg in 1949 (1). The ether extracts of dormant potato peelings were partitioned into neutral and acidic fractions, which were then chromatographed on paper. With an Avena curvature bioassay, growth inhibiting material was detected in both fractions. Subsequently, Hemberg found that there was a strong correlation between the levels of inhibitory material in the acidic fraction and the state of dormancy of the potato plant. The content of the inhibitor decreased regularly with the resumption of growth in spring (2-4). Similar studies were made by Buch and Smith (5) and they found that the relationship invariably held. Luckwill (6) extended this work to a number of other plants, again using paper chromatography in conjunction with bioassays. He detected both auxins and inhibitors. These techniques subsequently became quite standard. In 1953, Bennet-Clark and Kefford (7) referred to an "inhibitor-β complex", a growth inhibitory material they found in acidic fractions of extracts from a variety of etiolated seedlings and roots. Shortly thereafter, a number of other workers reported the detection of "growth inhibitors" in a wide range of plant tissue. These include buds (8-13), leaves (14-17),

fruit (16, 18-22), seeds (23-25) and sap (26). Some of the procedures used were quite similar to that of Bennet-Clark and Kefford and, thus, the various workers claimed to have detected the same material. The term "growth inhibitor" might be misleading. That a compound is classified as such means only that it inhibited the growth of some plant tissue in bioassays, and in no way implies that the material is a naturally occurring growth regulator. Only in some of the cases mentioned above (8, 11-13, 17, 24, 25) was the "growth inhibitor" detected on the chromatogram shown to be related to the state of dormancy of the plant. Some controversy as to the nature of these inhibitory materials began to arise. Housley and Taylor (27), in examining potato peelings, isolated a substance chromatographically which corresponded to inhibitor-β. However, when large quantities of the peeling extracts were worked-up, the fraction which had the strongest inhibitory action and which corresponded to the inhibitory zone on the chromatogram was found to be simply a mixture of fatty acids. The authors claimed that the growth inhibition of "β" was really the toxic effect of these acids. Koves and Varga (22, 28) and Varga (19, 20, 29, 30) reported "β" to be a mixture of coumarin and phenolic acids, including salicylic, cinnamic, ferulic and o- and p- coumaric acids. Jones and co-workers (31-33) subjected paper chromatograms of peach extracts to various spot tests and found inhibitor-β to consist of organic cyanides.

It is quite possible that some of the compounds which are claimed to be "inhibitor-β" do, in fact, act as growth retardants, but there was also considerable evidence for the existence of a true, endogenous dormancy inducer. Lane and Bailey (34) detected an inhibitor

corresponding to "β" in the buds of silver maple, and then, in an extensive series of tests aimed at identifying this material, eliminated as possibilities most of the previously mentioned substances. Barlow (35) extracted the shoots of plum and showed that the inhibitor isolated by paper chromatography acted on wheat coleoptiles in a reversible manner, i.e. growth resumed after the sections had been washed free of the inhibitor.

Wareing and his co-workers have done considerable work with a growth inhibitor from the leaves of sycamore. The inhibitor was first detected in 1958 (36) and subsequent investigations showed that this material was very closely associated with the onset of dormancy in the plant (37, 38). The inhibitor, which chromatographically corresponded to "β", was finally isolated as a pure compound in sufficient quantity for identification (39). It was found to be identical with abscisin II, an abscission accelerator isolated recently from cotton plants by Addicott and his co-workers (40, 41).

Another compound, naringenin (4', 5, 7- trihydroxyflavanone) has also been closely associated with dormancy. It was isolated in 1959 by Hendershott and Walker (42) from dormant peach buds, and is one of the few flavonoids for which growth regulatory activity has been demonstrated convincingly.

In view of these findings, the acidic componants of birch leaves have been examined for the possible existence of other endogenous growth regulators, and to study further the physiological role of the flavonoids. White birch trees, being one of the most populous species in Canada, were selected for the work because they are easily accessible.

METHODS AND RESULTS

The green leaves of Canadian white birch (Betula papyrifera) were extracted with aqueous methanol. The methanol from this solution was removed by evaporation and the remaining aqueous mixture was acidified and extracted with butanol. This, in turn, was extracted with sodium bicarbonate, acidified and re-extracted with butanol. It was originally intended to investigate this acidic fraction for growth regulatory substances by means of thin layer chromatography (TLC) and bioassays. Silica gel TLC is a simple but powerful separatory technique. A systematic search was initiated to find a solvent system which would cleanly separate a maximum number of componants of the acid mixture. Over 200 individual systems were tested. The best one was benzene: ethylacetate: water: acetone::1:1:1:5. To test the various fractions for biological activity, the chromatogram was divided into twenty sections and either eluted with methanol or tested directly on the oat coleoptile bioassay as described by Nitsch and Nitsch (43). Two problems were immediately encountered. The recovery of material from the silica gel was very low (~50-75%), and the silica gel itself (Silica gel H), or some contaminant within it, had a toxic affect on the coleoptile sections. These factors made such an approach unsuitable. Chromatography on a silica gel column was likewise unsuitable. The silica gel was thoroughly washed before use and the eluates were filtered extensively. This partially removed the toxicity problem, but again, the recovery of material was low and the resolution, even with solvent modifications, was too poor for the results of the bioassays to be conclusive. Thin layer chromatography on micro-

crystalline cellulose (44) gave significantly better results. It is non-toxic to stem growth, and similar to, but more convenient than, paper chromatography. The search for a suitable solvent system was based on those used with paper. The best was found to be n-butanol: i-propanol: water: NH_4OH (conc) ::5:5:1:1. This gave two distinct inhibitory fractions, at R_f 0.55 - 0.65, and R_f 0.70 - 0.80 (fig.1). The material in these two zones was eluted from the chromatogram with methanol. The substances so obtained were impure but their infrared spectra were recorded, as shown in figs. (2) and (3). The spectrum of the first fraction, R_f 0.55 - 0.65, is similar to that of an unsaturated fatty acid (O-H stretching, 3250cm^{-1}, carbonyl stretching, 1705cm^{-1}, conjugated C=C stretching, 1615cm^{-1}). The spectrum of the second fraction, R_f 0.70 - 0.80, suggested that the major portion was a flavonoid. The spectrum of quercetin is shown in fig. (4) for comparison. The two are not identical but the similarity is close.

An attempt was made to isolate larger quantities of this substance by chromatography of the acidic mixture on a cellulose column. However, the resolution deteriorated considerably even with solvent modifications and the inhibitory fraction corresponding to the flavonoid substance could not be detected by bioassay. It was subsequently decided to examine the ketonic fraction of this acidic mixture by other physical and chemical methods and to test the various components for biological activity.

The ether-soluble fraction of the mixture was treated with Girard's Reagent-P to remove the non-ketonic constituents. The water-soluble

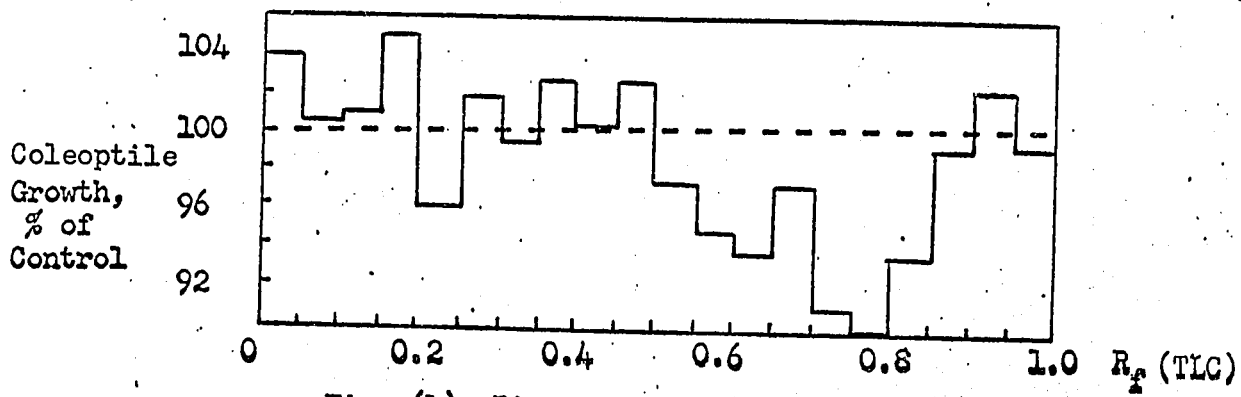


Fig. (1) Bioassay: Acidic Fraction (A)

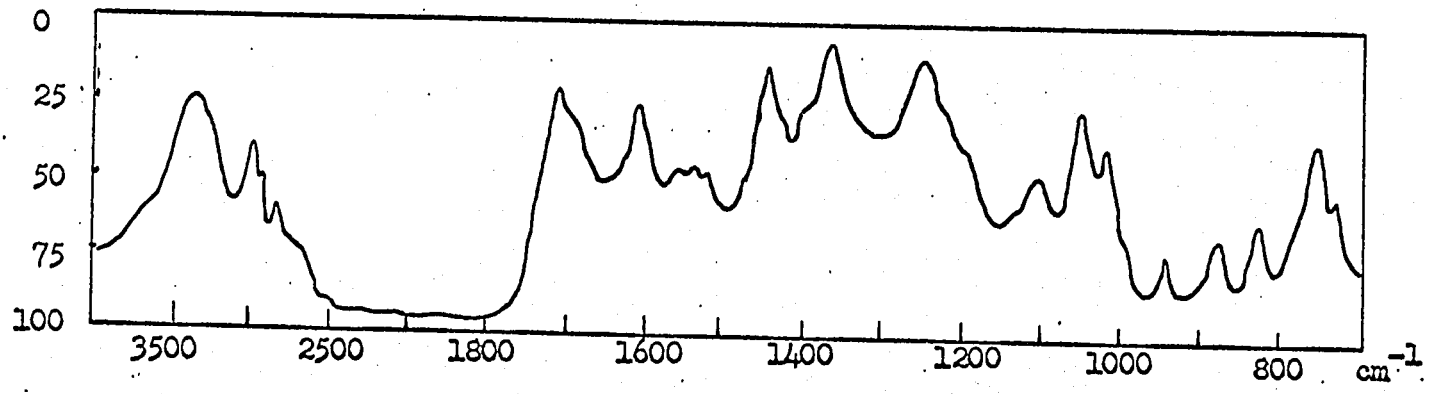


Fig. (2) R_f 0.55-0.65

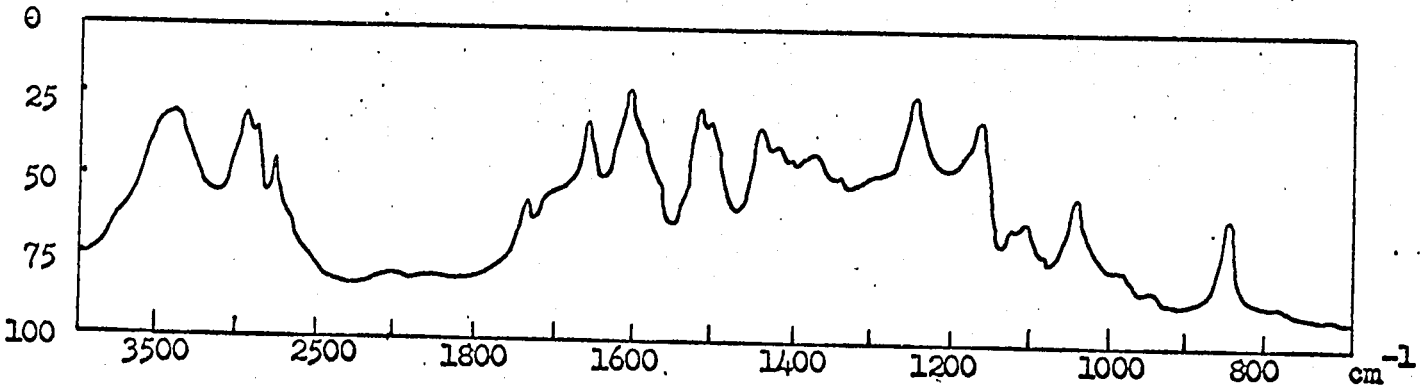


Fig. (3) R_f 0.70-0.80

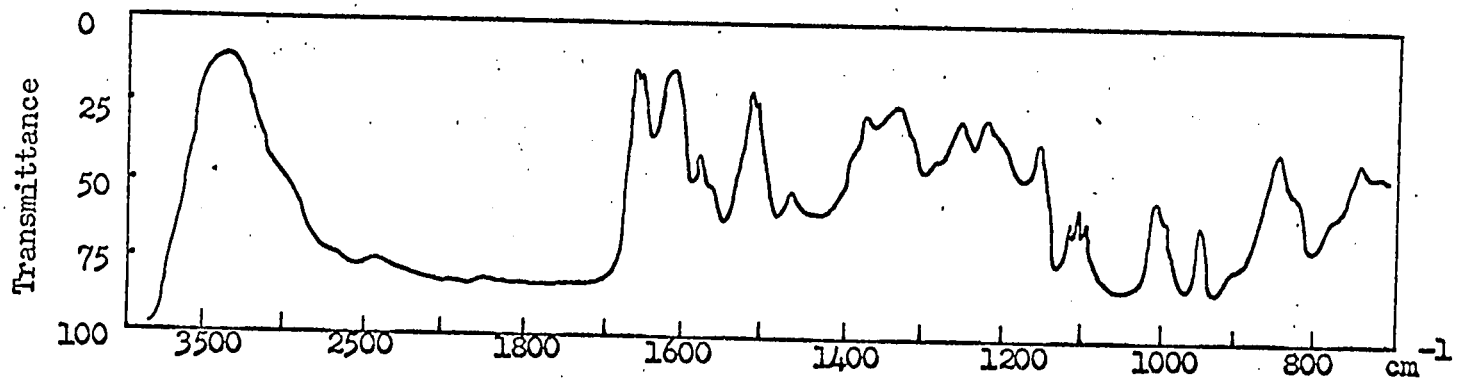
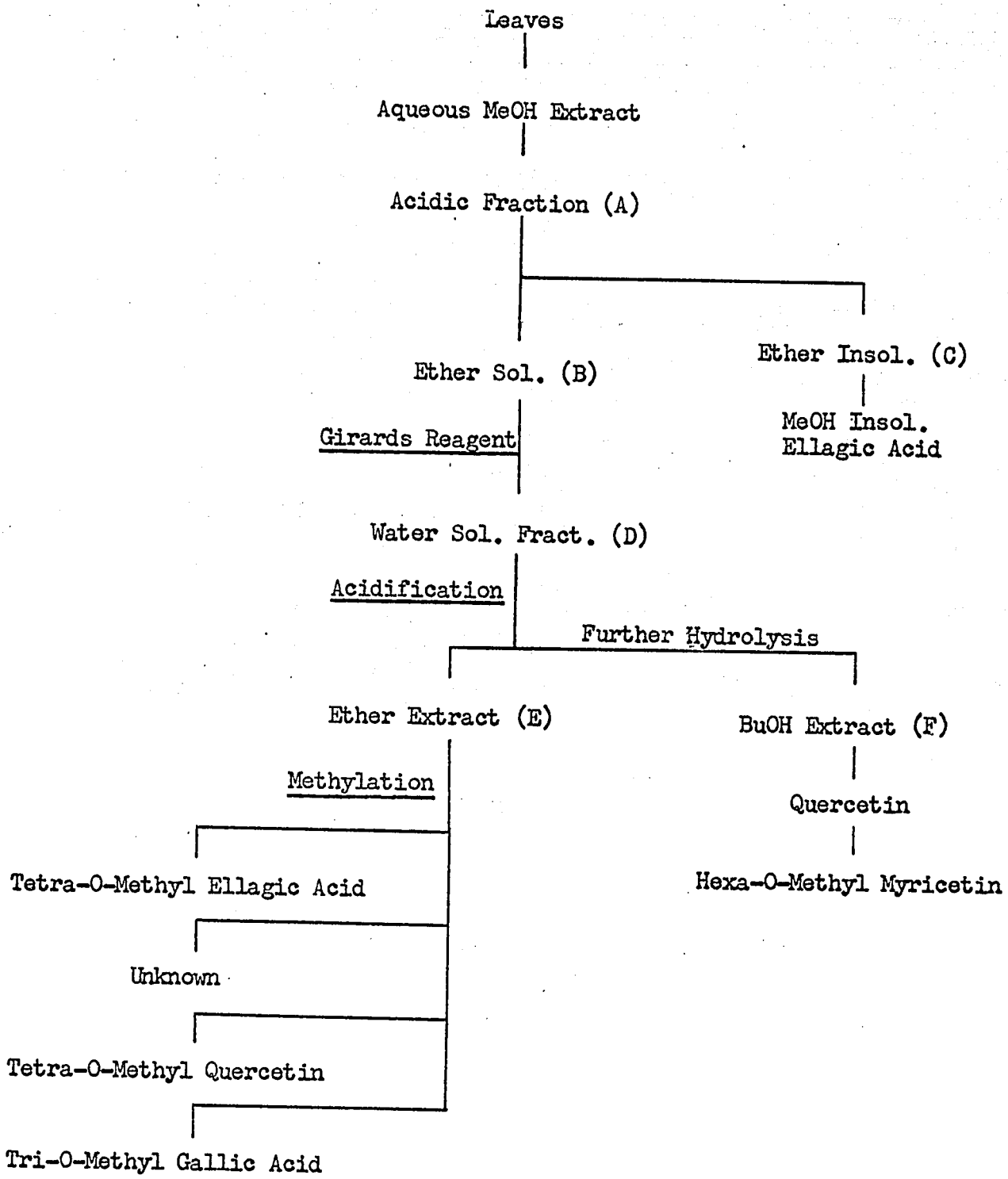


Fig. (4) Quercetin

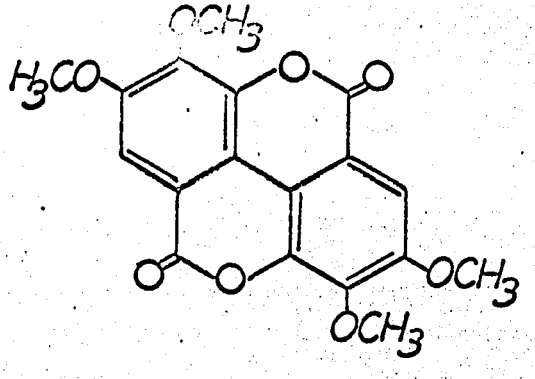
WORKUP OF BIRCH LEAF EXTRACT



ketonic mixture was hydroyzed under mild conditions and then extracted with ether. This extract was methylated with diazomethane and, after it had stood at room temperature for a short time, a white solid precipitated. This material was quite difficult to purify due to its insolubility in most solvents. It was crystallized from a large volume of dichloromethane. Mass spectroscopy (fig.5) indicated that it had a molecular weight of 358 and isotopic analysis fit the empirical formula $C_{18}H_{14}O_8$. This was confirmed by elemental analysis, and a search of the literature suggested that this compound was the tetra-O-methyl ether of ellagic acid (I). A sample of this compound was prepared by treating authentic ellagic acid with diazomethane, and it proved to be identical with the isolated compound.

Evaporation of the supernatant liquid from which the ellagic acid derivative had been obtained, yielded a bright yellow substance which was easily crystallized from alcohol. Again, mass spectroscopy (fig.6) and elemental analysis suggested the formula $C_{15}H_6O_3(OCH_3)_4$. The material corresponded to 5-hydroxy-3,3',4',7-tetramethoxyflavone as reported in the literature and this was confirmed by synthesis.

When the mother liquor from this compound was evaporated, an oily mixture was obtained from which nothing more could be crystallized. This oil was chromatographed on silica gel giving one fairly well resolved spot. It was eluted with dichloromethane, but attempts at crystallization were not successful, because of its low melting point. The infrared spectrum indicated that this compound was the methyl ester of a carboxylic acid. It was saponified with sodium hydroxide and the reaction product was purified by column chromatography and crystallization from ether. It was identified, by



I Tetra-O-Methyl Ellagic Acid

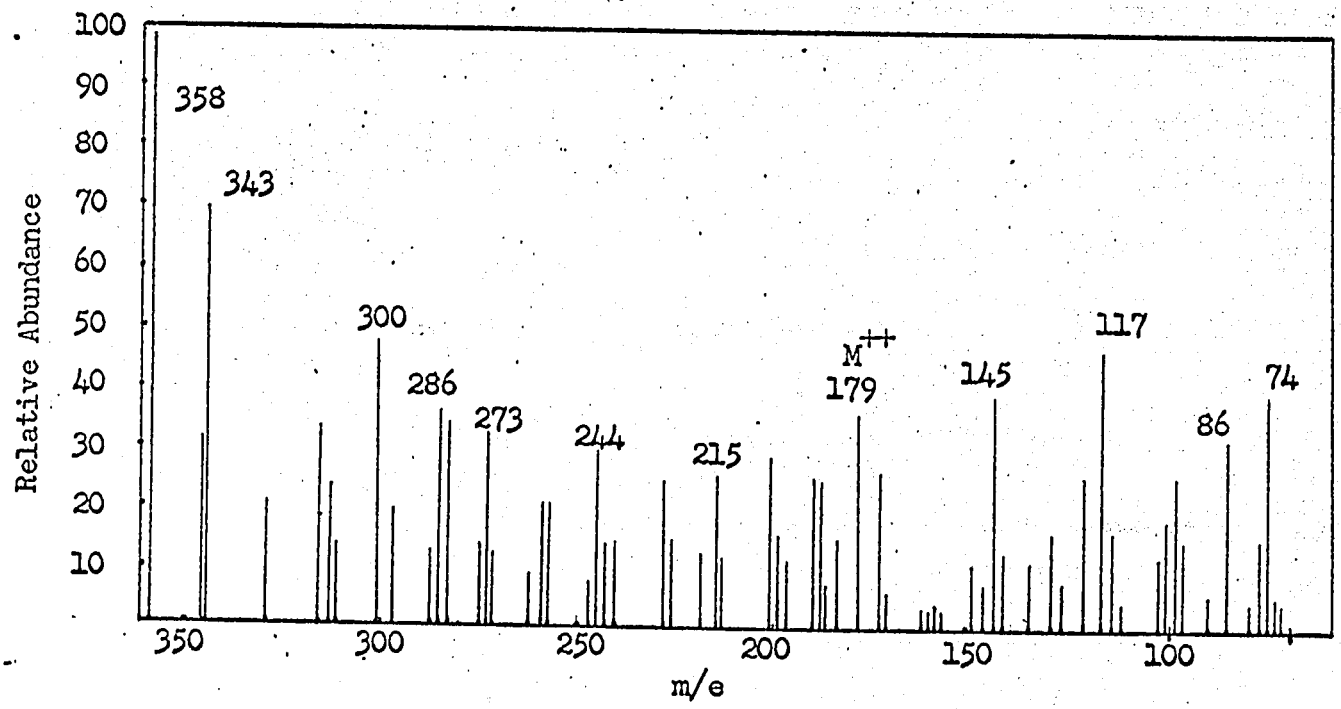


Fig. (5) Tetra-O-Methyl Ellagic Acid

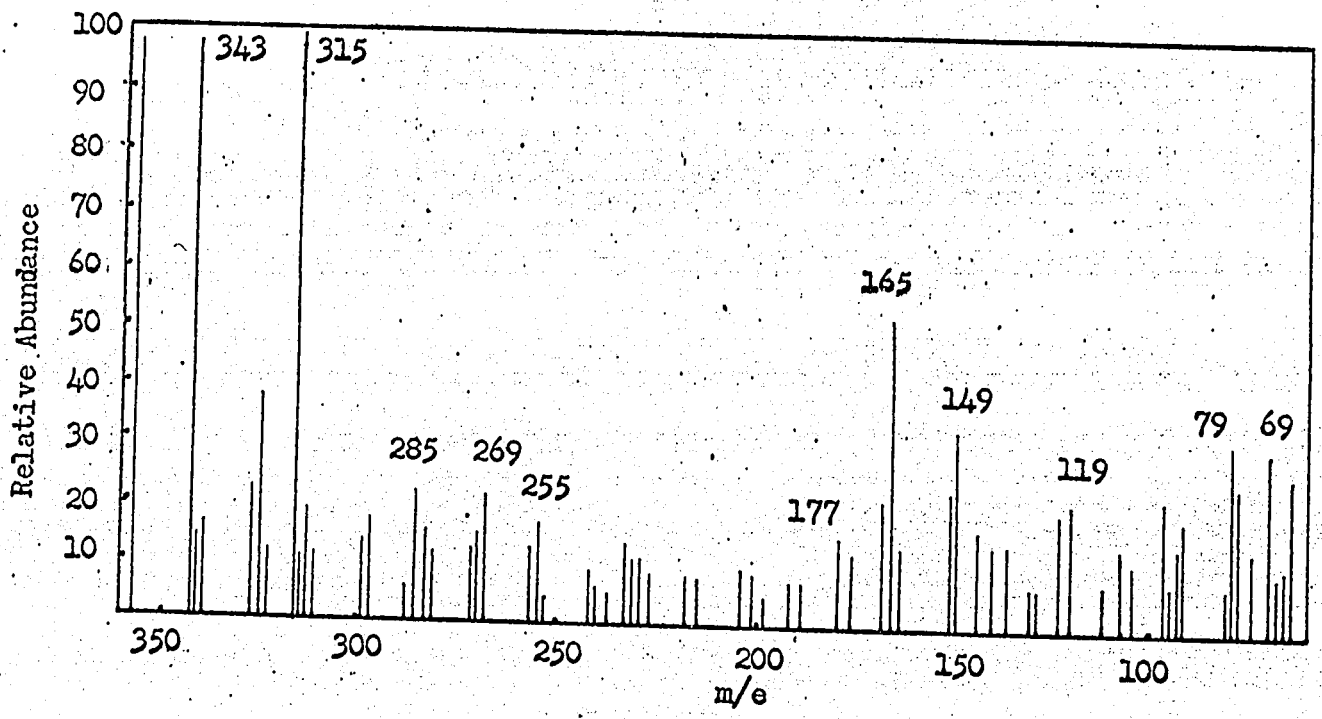


Fig. (6) 3,3',4',7-Tetra-O-Methyl Quercetin

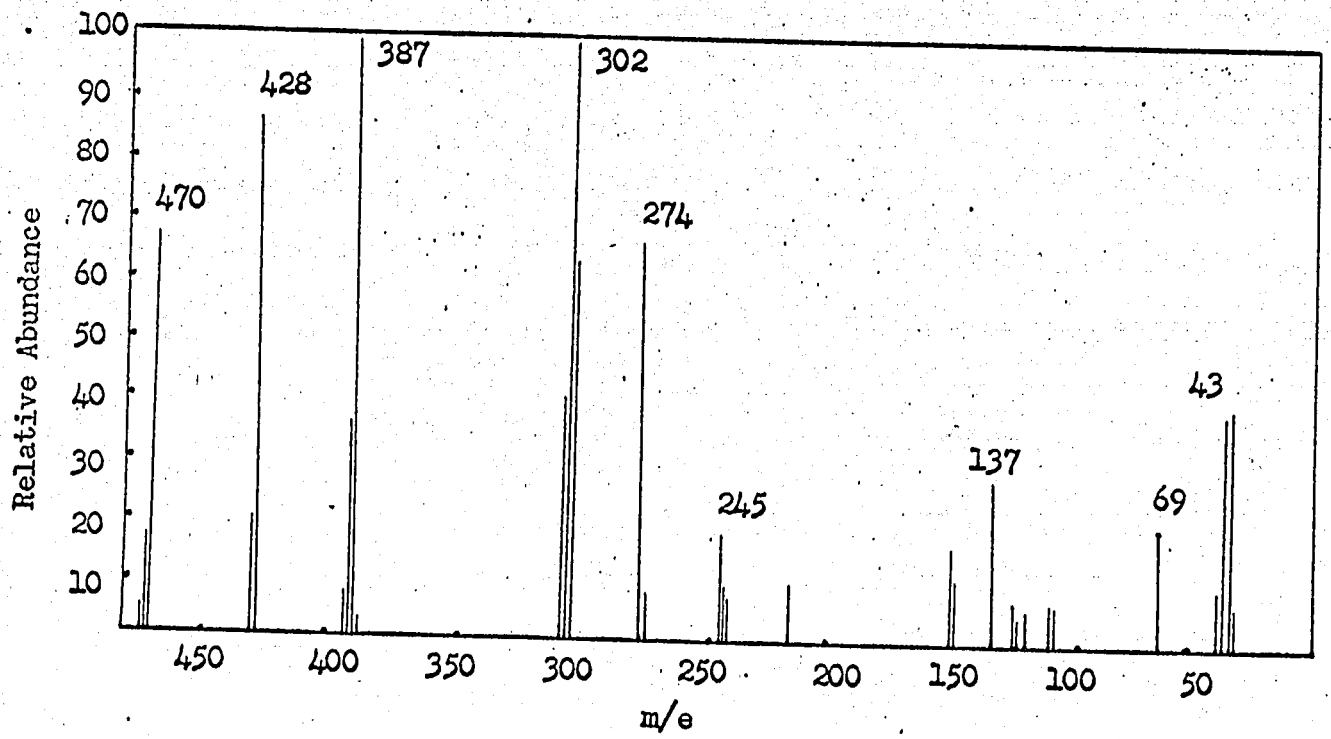


Fig. (7) 3,3',4',7-Tetra-O-Acetyl Quercetin

its infrared spectrum and mixed melting point, as tri-O-methylgallic acid.

The water-soluble ketonic mixture was hydrolyzed further and extracted with butanol. The ether-soluble portion of this fraction yielded a yellow, solid substance which could not be purified. However, the infrared spectrum of the crude substance indicated that it was quercetin itself. It was treated with acetic anhydride and sodium acetate to give an easily crystallized white solid. Similar treatment of authentic quercetin yielded an identical compound which was shown by mass spectroscopy (fig. 7) to be the tetra-acetate of quercetin.

The mother liquor of the quercetin fraction was evaporated to dryness and shaken with water. This extracted a white suspension from the oily residue. Mass spectral (fig. 8) and elemental analysis data gave the compound a tentative formula $C_{15}H_4O_2(OCH_3)_6$. The infrared spectrum was found to be identical with that of Hergert (57) for the hexa-O-methyl ether of myricetin (3,3',4',5,5',7-hexamethoxyflavone).

The ether-insoluble portion of the original acidic fraction was shaken with methanol, dissolving most of the material. After several washings with methanol, a light yellow residue remained undissolved. This substance could not be purified by crystallization, chromatography or sublimation. Consequently, it was acetylated to give a white solid which was purified by successive precipitations from boiling chloroform. Infrared spectrum showed that this was ellagic acid itself.

After treatment of the acidic fraction with Girard's Reagent and diazomethane, another compound was isolated from the same fraction as 5-hydroxy-3,3',4',7-tetramethoxyflavone. Unfortunately, the amount of

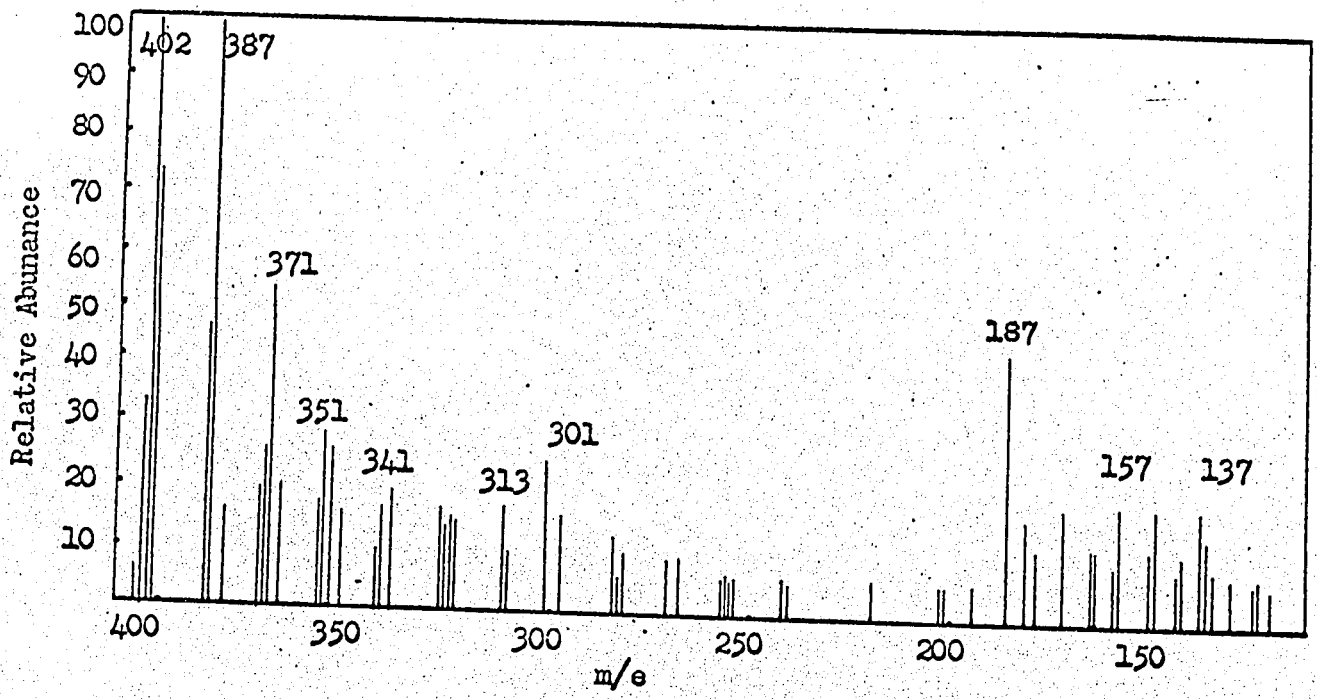


Fig. (8) Hexa-O-Methyl Myricetin

material obtained (~60mg) was too small for a complete elucidation of structure, and attempts to isolate more from a second sample of leaves were not successful. However, the samples were not collected at exactly the same time of year; failure to find the compound in the second sample may suggest that its level in the leaves varies significantly. Infrared and mass spectra are shown in figs. (9) and (10). The mass spectrum shows a parent peak at m/e = 388 with slight impurities at m/e = 402 and 374. Isotopic analysis indicates an empirical formula $C_{20}H_{20}O_8$. This is substantiated by elemental analysis, which also indicates the presence of 5 methoxyl groups. These data correspond to a hexahydroxyflavone. Methylation of the sixth hydroxyl group would account for the impurity at m/e = 402. The infrared spectrum shows hydroxyl absorption at 3385cm^{-1} which is too high for a 3- or 7-hydroxyflavone. A 5-hydroxyl

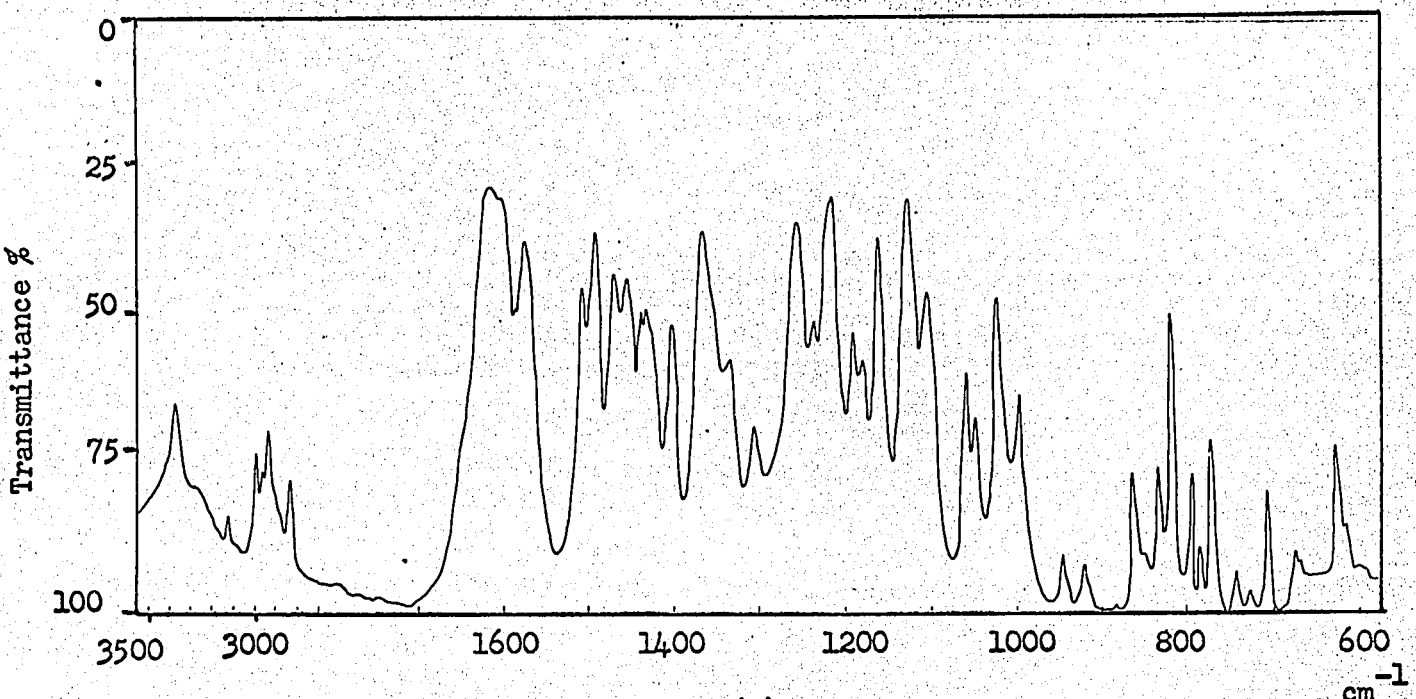


Fig. (9) Unknown

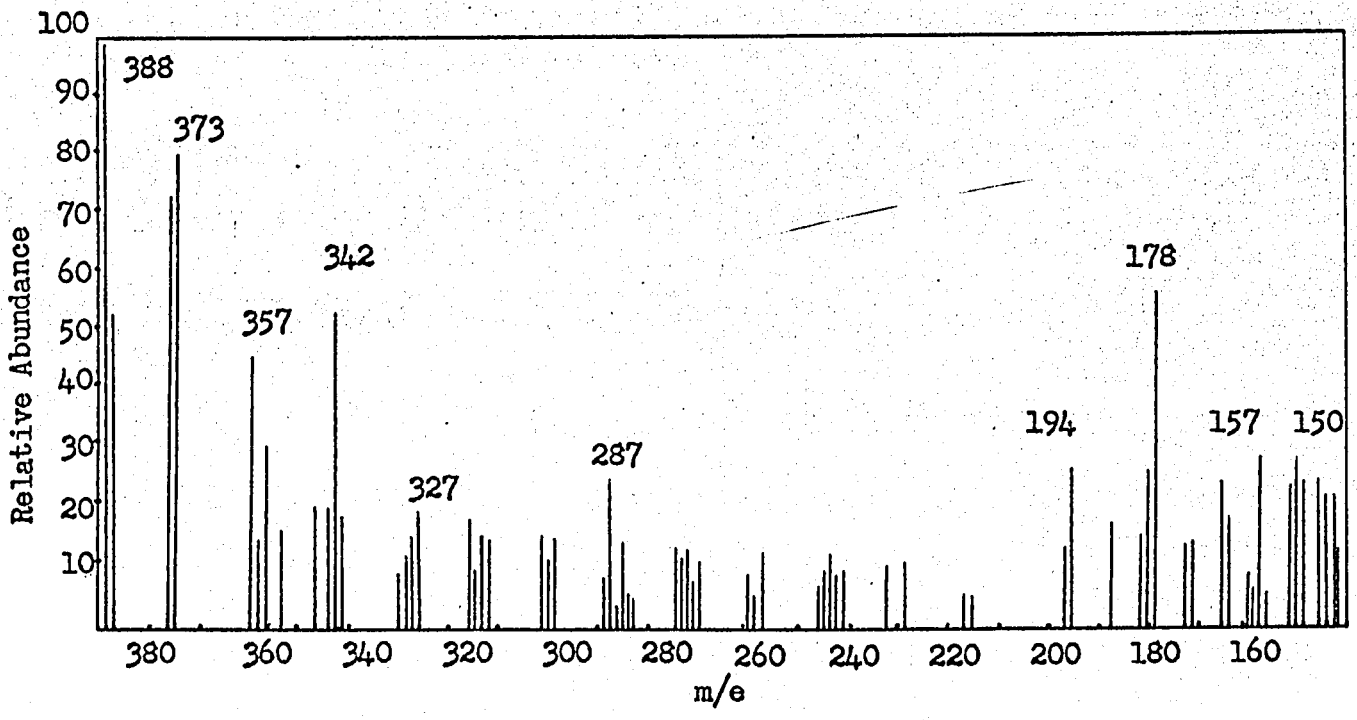


Fig. (10) Unknown

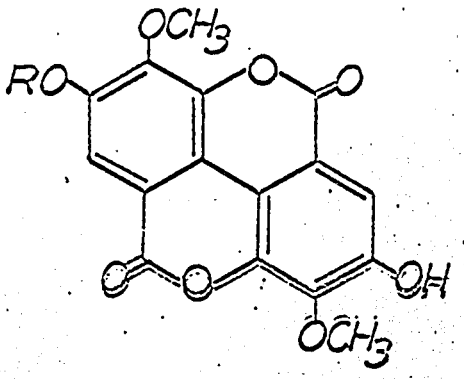
group shows no absorption. The peak, however, does fit for a hydroxyl on the "B" ring of a flavone. The absorption at 1623cm^{-1} corresponds to the carbonyl group of a 5-methoxyflavone (45). There are no appropriate infrared spectra in the literature with which this compound might be compared.

The biological activities of the flavones are discussed in Chapter III.

DISCUSSION

The isolation of hexa-O-methyl myricetin is quite interesting. Although myricetin itself is widely distributed throughout the plant world, it has been detected in birch only once, as the 3-digalactoside, in Betula verrucosa and B. pubescens (50) and has not yet been detected in poplar or maple. Methyl derivatives of myricetin are much rarer than the parent compound in plant tissue. There exists in the literature only one report of a naturally occurring 5-O-methyl ether of myricetin. Egger (51) isolated such a compound from the petals of rhododendron in 1962. This, then, is the first reported isolation of naturally occurring hexamethyl myricetin from plant material. However, the isolation of this compound perhaps is not so surprising in the light of the finding of O-methyl derivatives of both quercetin and ellagic acid from the same leaf extract.

Ellagic acid is one of the most common yellow pigments in the plant world. It generally occurs as a mono- or polyglycoside and is often found in conjunction with myricetin and gallic acid. An extensive survey of the distribution of ellagic acid has been compiled by Bate-Smith (52). Although it occurs in common trees such as alder, beech and oak, it has not previously been detected in birch, poplar or maple. Ellagic acid is essentially insoluble in ether and was correspondingly isolated from the ether-insoluble fraction of the acids mixture. However, the tetra-O-methyl ether derivative came from the ether-soluble fraction, suggesting that,



II R = H

III R = CH_3

as with quercetin, it was methylated to a certain extent to begin with. Only two methyl ethers of ellagic acid are known as natural products, the dimethylether (II) which was isolated from the roots of Euphorbia formosana (57), and the trimethyl ether (III) from the bark of Eugenia maire (58).

The isolation of a gallic acid derivative is not surprising in view of the presence of ellagic acid. Again, the original methylation pattern could not be determined. This material is believed to be a precursor of ellagic acid since oxidative coupling of the esters of gallic acid, followed by lactonization gives this compound.

EXPERIMENTAL

Melting points were determined on a calibrated Fisher-Johns apparatus. Infrared spectra were recorded on a Unicam SP100 spectrophotometer. Mass spectra were measured by the Morgan, Schaffer Corp., Montreal, on a Hitachi Perkin-Elmer RMU-60 mass spectrometer operating at 70 ev. Elemental analyses were done by Beller Mikroanalytisches Laboratorium, Gottingen, W. Germany, and Schwarzkopf Mircoanalytical Laboratory, Woodside, N.Y. Yields of the isolated compounds are expressed as percentage of dry weight of the leaves. Diazomethane was prepared from N-methyl-N-nitroso-p-toluenesulphonamide according to Vogel (53). Acetylations were done according to Shriner (54).

LEAF EXTRACT, ACID FRACTION (A)

Green leaves of Canadian white birch (Betula papyrifera), 258 kg wet weight, were collected in July, 1965 at the Petawawa Forest Experiment Station, Chalk River, Ontario. They were immediately placed in 80% aqueous methanol. After two days of intermittent agitation at room temperature, the alcoholic extract was filtered and evaporated under vacuum at 50°C until most of the alcohol had been removed. The remaining aqueous solution was acidified to pH 3 with HCl and extracted with n-butanol. The butanol fraction was extracted with 5% sodium bicarbonate and the extract was acidified to pH 3 with HCl and re-extracted with n-butanol. This butanol extract (A) contains the acidic fraction, and all subsequent work was done with this material. Dry weight, 182 kg.

CHROMATOGRAPHY AND BIOASSAYS

Thin layer and column chromatography with silica gel was done in the usual manner. TLC with microcrystalline cellulose was done as described by Wolfrom et al (44). Microcrystalline cellulose and water were blended together in proportions of 1:4 w/w. The slurry was placed under vacuum for a few minutes to remove the air, and then it was spread on glass plates, at a thickness of 1.0 mm for preparative work, and allowed to dry at room temperature for 24 hours. Fraction (A) was applied to the chromatograms as a methanol solution and the plates were developed in the system 1-butanol: 2-propanol: H_2O: NH_4OH (conc)::5:5:1:1. The chromatograms were divided into 20 equal sections and eluted with methanol. The eluates were placed in 10 ml beakers and evaporated to dryness at a slightly elevated temperature. The oat coleoptile bioassays were conducted in these beakers exactly as described by Nitsch (43).

DIPHENIC ACID, 6,6-DIHYDROXY-4,4',5,5'-TETRAMETHOXY-, Di-δ-LACTONE (TETRA-O-METHYL ELLAGIC ACID)

Extract (A) was evaporated to dryness under vacuum at 60°C and divided into ether-soluble (B) and ether-insoluble (C) fractions. Fraction (B) was evaporated to dryness and dissolved in methanol. It was treated with Girard's Reagent-P according to Vogel (55) and the reaction mixture was poured into water. The water-soluble fraction (D) was acidified with HCl to a concentration of 0.25N and allowed to stand at room temperature for 1 hour. It was then extracted four times with ether. The ether fraction (E) was dried over Na_2SO_4 and methylated with excess CH_2N_2 at -20°C and, after reaching room temperature, a white solid

precipitated. It was removed by centrifugation, crystallized from dichloromethane and identified as tetra-O-methyl ellagic acid, m.p. 330°C.

Anal: Calcd. for $C_{18}H_{14}O_8$: C, 60.39; H, 3.95; O, 35.75

Found: C, 60.45; H, 4.00; O, 34.64

Mass Spec: Req'd: P, 100; P+1, 19.91; P+2, 3.47

Found: P, 100; P+1, 19.70; P+2, 3.35

Yield, 1.96 gm, 0.0011%.

5-HYDROXY-3,3',4',7-TETRAMETHOXYFLAVONE

The supernatant fraction (E) was evaporated to a small volume and a yellow solid separated. It was filtered, crystallized from ethanol and identified as 3,3',4',7-tetra-O-methoxyquercetin, m.p. 157.0-157.5°C.

Anal: Calcd. for $C_{19}H_{18}O_7$: C, 63.67; H, 5.07; O, 31.25; 4(OCH_3), 34.62

Found: C, 63.44; H, 5.03; O, 31.24; (OCH_3), 34.20

Mass Spec: Req'd: P, 100; P+1, 21.1; P+2, 3.51

Found: P, 100, P+1, 21.2; P+2, 3.48

Yield, 7.4 gm, 0.0041%.

TRI-O-METHYL GALLIC ACID

The mother liquor from fraction (E), after removal of the quercetin derivative, was subjected to thin layer chromatography on silica gel H, using the solvent system, dichloromethane : methanol ;: 20 : 1. The fraction at R_f 0.35-0.45 (colorless; blue fluorescence under U.V. lamp) was eluted with dichloromethane. It was dissolved in methanol and heated at 50°C for two hours with 5% aqueous NaOH. The reaction mixture was acidified with HCl to pH 3 and then extracted with ether. The ether extract was chromatographed on a silica gel column, using ether as the solvent. The

fluorescent fraction was collected and the solid material crystallized from ether, giving 3,4,5-trimethoxybenzoic acid, m.p. 168-169°C. The infrared spectrum of authentic material was identical, mixed melting point was unchanged. Yield, ~60 mg.

3,3',4',5,7-PENTAHYDROXYFLAVONE, PENTAACETATE

The aqueous fraction (D) was acidified further with HCl, to a concentration of 0.5N, and then stirred for 24 hours at 40°C. It was extracted with 1-butanol and the extract was evaporated under vacuum at 50°C. The solid residue was shaken with ether, and when the ether-soluble fraction (F) was dried over Na_2SO_4 and evaporated to a small volume, a bright yellow solid precipitated. Attempts at purification were unsuccessful. Crystallization from alcohol or acetone caused apparent decomposition. However, the infrared spectrum of the crude material was similar to that of quercetin. This substance was acetylated to give a white solid (G) which was crystallized from methanol, m.p. 194-196°C. Authentic quercetin was treated in a similar manner to yield a substance identical to (G). The molecular weight peak of the mass spectrum showed that this compound was the tetraacetate of quercetin. Yield, 23 gm, 0.026%.

3,3',4',5,5',7-HEXAMETHOXYFLAVONE

The mother liquor of (F) was evaporated to dryness to give a viscous yellow-brown tar. This was shaken with water for two hours and a white suspension formed. The suspension was decanted and the solid phase centrifuged. It was crystallized from ether and identified as the hexa-O-methyl ether of myricetin, m.p. 154.5-155.0°C.

Anal: Calcd. for $C_{21}H_{22}O_8$: C, 62.67; H, 5.52; O, 29.32; 6 (OCH_3), 43.26

Found: C, 62.56; H, 5.54; O, 31.72; (OCH_3), 45.57

Mass Spec: Req'd: P, 100; P+1, 23.35; P+2, 4.21

Found: P, 100; P+1, 22.86; P+2, 4.17

Yield, 0.97 gm, 0.00053%.

6,6-DIHYDROXY-4,4',5,5'-TETRAHYDROXYDIPHENIC ACID, δ-DI-LACTONE (ELLAGIC ACID)

Fraction (C) was shaken with methanol repeatedly until only a light yellow solid residue remained undissolved. It was removed by centrifugation but all attempts at purification by crystallization, sublimation and chromatography were unsuccessful. The compound was acetylated to give a white solid which was purified by successive precipitations from boiling chloroform, and then was identified as the tetraacetate of ellagic acid, m.p. 310°C (dec).

Anal: Calcd. for $C_{22}H_{14}O_{12}$: C, 56.21; H, 3.01; O, 40.81; $4(OCH_3)$, 36.6

Found: C, 55.90; H, 2.98, O, 40.73; (OCH_3), 36.4

Yield, 1.35 gm, 0.00074%.

UNKNOWN

Fraction (E) was evaporated to a small volume and a yellow solid crystallized out (before the separation of the quercetin derivative). It was recrystallized from ethanol, m.p. 230-231°C.

Anal: Calcd. for $C_{20}H_{20}O_8$: C, 61.91; H, 5.21; $5(OCH_3)$, 38.70

Found: C, 61.19; H, 5.42; (OCH_3), 34.62

Mass Spec: Req'd: P, 100; P+1, 22.24; P+2, 3.90

Found: P, 100; P+1, 21.99; P+2, 3.81

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CHAPTER III

FLAVONOIDS AS PLANT GROWTH REGULATORS

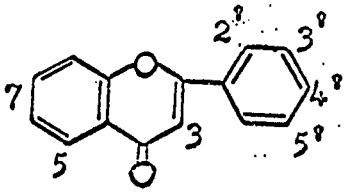
INTRODUCTION

The preliminary work described in Chapter II indicated that a flavonoid compound, possibly quite similar to quercetin, was found to be a significant inhibitor of oat colepotile growth. Naringenin reportedly has a similar effect. Little is known about the actual physiological properties of the flavonoids, but from these results it seemed possible that they might play an active role in the growth regulation of plants. Therefore, in addition to those isolated in this work, a wide variety of other flavones was tested for their biological activity.

Early work with natural plant growth regulators has been focused on determining the correlation between their action in bioassays and the fluctuations of their concentrations in the plant during the corresponding growth phenomenon. Indole-3-acetic acid (IAA) was first discovered in experiments with grass shoots. The acid was found to be responsible for localized growth promotion (e.g. curvature) in the plant. Similar investigations of dormant plant material first yielded abscisin II, believed to be a major factor in the control of dormancy. The physiological role of the flavonoids in plant growth regulation has been the subject of considerable investigation for the past decade, but no conclusive evidence has yet been advanced to show that they actually play a significant part in the growth cycle. Certainly, relationships such as those which have been demonstrated for IAA and abscisin II are scarce. Hendershott and Walker (1) isolated naringenin (table I) from the dormant flower buds of peach. This substance did act as a growth inhibitor in

TABLE I

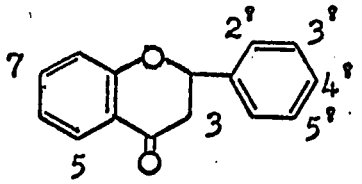
FLAVONES



	3	5	7	2'	3'	4'	5'
FLAVONE							
CHRYSIN		OH	OH				
TECTOCHRYSIN		OH	OCH_3				
APIGENIN		OH	OH			OH	
ACACETIN		OH	OH			OCH_3	
DATISCETIN	OH	OH	OH	OH			
FISETIN	OH		OH		OH	OH	
KAEMPFEROL	OH	OH	OH		OH		
QUERCETIN	OH	OH	OH		OH	OH	
RHAMNETIN	OH	OH	OCH_3		OH	OH	
MORIN	OH	OH	OH	OH		OH	
MYRICETIN	OH	OH	OH		OH	OH	OH

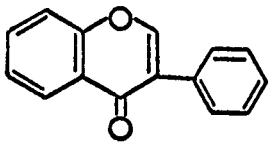
TABLE I - CONT'D

FLAVANONES



	3	5	7	2'	3'	4'
FLAVANONE						
PINOCEMBRIN		OH	OH			
NARINGENIN		OH	OH			OH
HESPERETIN		OH	OH		OH	OCH_3
PINOBASKIN	OH	OH	OH			
AROMADENDRIN	OH	OH	OH			OH

ISOFLAVONES



GENISTEIN		OH	OH			OH
BIOCHANNIN A		OH	OH			OCH_3

NARINGIN: Naringenin-7-rhamnoglucoside

HESPERIDIN: Hesperetin-7β-rutinoside

PHLORETIN: 2',4,4',6'-Tetrahydroxydihydrochalcone

PHLORIDZIN: 2'β-Glucoside of Phloretin

bioassays and its content in the plant decreased during winter until dormancy was broken. More recently, Bagni and Francassini (2) found that naringenin, when applied at concentrations of 10^{-4}M, completely blocked the formation of buds in the roots of Cichorium intybus, and even at lower concentrations the bud formation was significantly reduced. Phloridzin has also been implicated in the control of dormancy in apple (3,4). Sarapuu (5-7) studied young apple shoots throughout the winter and found that it accumulated during the summer until the onset of dormancy and then decreased steadily until growth resumed in spring. Hagan and co-workers (8) reported that, in grapefruit, a number of flavonoids, including naringenin, naringin, hesperidin and neohesperidin, reached maximum levels in July and then decreased rapidly until late November. These fluctuations in the levels of naringenin, phloridzin and the flavones in grapefruit all parallel those which have been found for abscisin II. It is interesting to note also that both naringenin and abscisin II reach maximum levels in dormant peach, so that both compounds might be closely associated with this dormancy (9). Whether or not these two compounds are interrelated and whether or not a similar relation exists with other flavonoids remains to be seen. Analogous experimental data have also associated the flavonoids with the induction of flowering (10), the movement of tendrils (11), disease resistance within plants (12), and the ability of plants to root (13).

The reported findings on the action of naringenin prompted a number of workers to investigate the growth effects of many other flavonoids. This problem has been studied from two aspects: Their in vivo action, mainly in assorted bioassays; and their in vitro action, generally with IAA-oxidase. Closely paralleling this work are a number of studies on the physiological

properties of simpler phenols and phenolic acids. Comparison of these reports reveals a significant relationship, and the two must be discussed together.

The first experiments were conducted in 1961 by Stenlid (14), who tested the effects of some flavonoids on the growth of wheat roots. It is generally believed (15) that roots contain a supra-optimal amount of IAA which acts as a growth inhibitor. Therefore, the addition of more of this auxin increases the inhibition while decreasing its level promotes root growth. The flavones were tested alone for their effect on the endogenous system, and in conjunction with additional auxins and sugars, all of which are known to inhibit root growth. Above certain concentrations, all of the flavones acted as inhibitors. Below these critical levels (unique for each substance) some compounds, pinobaskin, pinocembrin, and genistein, significantly accelerated the root growth. The others had little or no effect. With the addition of the various auxins and sugars, the inhibition normally caused by these compounds were relieved to a considerable extent by nearly all of the flavonoids. In a similar experiment with anthocyanins (16), it was found again that almost all of these compounds alleviated the auxin- and sugar-induced inhibition of root growth. The flavonoids which behave in this way are acting in a manner similar to known antiauxins and possibly some sort of antagonistic effects can be inferred. But it must be broader than a simple antiauxin effect since the sugars are also involved.

Nitsch and Nitsch (17,18) tested the modifying effects of a wide variety of phenols, phenolic acids and flavones on the IAA-induced growth of oat first internode sections. They found that the monophenols

o-, m-, p-cresol and phenol itself inhibited the growth as did the metadiphenol resorcinol. The orthodiphenols catechol and protocatechuic and caffeic acids were synergistic. Methylation of the various hydroxyl groups gave no clear-cut results. With the flavones and anthocyanins, the presence of a 3-hydroxyl group gave the compounds growth-promoting affects, e.g. quercetin, kaempferol, fisetin and rhamnetin, while, without a 3-hydroxyl group, the activity depended upon the substitution in the "B" ring. In such a case, the activity paralleled the pattern of the simpler phenols. Again, methylation gave inconsistent results. The authors ascribed these effects to the action of the phenols and flavonoids on IAA-oxidase, some acting as synergists, others as antagonists of the enzyme. It is tempting to speculate that the 3-hydroxyflavones act by one mechanism, but, without the 3-hydroxyl group, act by another similar to that of the phenols. In comparing these results with those of Stenlid on IAA-induced root inhibition, naringenin, genistein, quercetin and apigenin behave as might be expected, but hesperetin, which gave a marked reduction in the root inhibition, acted as a synergist to the IAA-induced stem growth.

Other more recent studies have been made on the physiological effects of phenols. Maksimov and Radkevich (19) tested the activities of a number of phenols on the residual growth of corn coleoptiles. Over a concentration range similar to that used by Nitsch, phloroglucinol and pyrogallol had auxin-like activity, while catechol, gallic acid and hydroquinone inhibited the coleoptile growth. Bilbao (20) tested the effect of some phenolic acids on the germination of wheat and barley. He reported that p-hydroxycinnamic and ferulic acids inhibited germination,

while the effects of p-hydroxybenzoic and vanillic acids were much less pronounced.

As mentioned above, Nitsch and Nitsch hypothesized that the flavonoid and phenolic activities are due to their action on the auxin-oxidase enzyme. There has been considerable work done with this system, much of it independent of the investigations already discussed. In 1947 Tang and Bonner (21) reported that the IAA-oxidase activity in etiolated pea buds was reduced by exposure to light. Subsequently, Hillman and Galston (22) accumulated experimental data indicating that this phenomenon was due to a specific substance in the plant, and they showed that this material was present in higher concentrations in plants grown in red light than in those grown in darkness. Mumford (23) attempted to isolate such a substance and obtained 3-(4-hydroxycinnamolytriglucosyl)-kaempferol (I). It was shown that kaempferol itself was much more active than (I). Mumford suggested that this was because p-hydroxycinnamic acid is an excellent IAA-oxidase co-factor. Further work (24) showed that two major compounds present in "dark-grown" plants were a kaempferol hexaglucoside and a glucoside of p-hydroxycinnamic acid. He suggested that red light "triggered" an exchange reaction between these two species to give (I).

This work inferred that the flavonoids affect plant growth through the IAA-oxidase system. Similar results were obtained by Fuyura (25) and both he and Mumford conducted further tests with the flavones on mung-bean IAA-oxidase. Mumford found that although kaempferol is a strong inhibitor, methylation of either the 4'- or 7-hydroxyl group reduced this activity considerably. He also reported that naringenin and apigenin-7-glucoside act as enzyme co-factors. Fuyura showed, however, that kaempferol actually

exhibited synergistic properties at low concentrations (10^{-6}M) while at higher concentrations it acted antagonistically. These two reports are not directly comparable since Mumford used dichlorophenol in the control medium as a co-factor.

This work prompted Stenlid to pursue the matter further (26). He tested a wide variety of flavones, flavanones, isoflavones and anthocyanins on IAA-oxidase from pea roots, and found that almost all of them affect the enzyme in one way or another. All compounds with 3',4'-dihydroxyl groups inhibited the destruction of IAA, while those with only a 4'-hydroxyl group increased the activity of the enzyme. The only exception to this generalization was morin which acted as an enzyme antagonist. In addition, Stenlid reported that a 7-hydroxyl group strengthens the stimulatory properties of 4'-hydroxyflavonoids, but in itself, e.g. 7-hydroxy-2-phenyl-benzopyrylium chloride, did not make the compound active.

A similar study was made by Pilet and Gaspar (27) with respect to the action of hydroxybenzoic acid on the IAA-oxidase isolated from the roots of Lens culinaris. They found that p-hydroxybenzoic acid increased the rate of IAA destruction while the meta and ortho isomers had significantly less effect. Varga and Koves (28) reported very similar results. The experimental technique employed by these workers involved an enzyme system to which IAA and the test compound had been added. After set periods of time, the residual IAA was measured. Zenk and Muller (29) used a different approach, employing the endogenous oxidase system in "dark-grown" Avena coleoptiles to which was added carboxyl-labeled IAA. They measured the $^{14}CO_2$ which was subsequently evolved, and found that,

as in the results of Pilet (27) and Varga (29), phloretic, p-hydroxybenzoic and p-coumaric acids were strong enzyme synergists. Caffeic, sinapic, ferulic and isoferulic acids were antagonistic to the system.

As indicated earlier, most of the work described so far has been concerned with the hypothesis that flavonoids regulate plant growth through their action on IAA-oxidase, but the physiological role of the flavonoids in growth processes has been examined from other angles as well. Somewhat related to the work above, Schwertner and Morgan (30) have investigated the role of IAA-oxidase in abscission control in cotton, and how it is affected by various phenols and flavones. They found that, in experiments conducted both with and without exogenously applied IAA, p-coumaric acid, β-methylumbelliferone (7-hydroxy-5-methylcoumarin), resorcinol and p-hydroxybenzyl alcohol all acted as enzyme co-factors and also significantly increased the abscission of cotton leaves. Conversely, a number of substances, including catechol, caffeic acid and quercetin acted as enzyme antagonists and also decreased leaf abscission. The action of the test materials on the enzyme agree well with the other reports. These results seem to suggest that IAA-oxidase functions in vivo to regulate the abscission process and that this activity can be modified by the presence of phenols and flavones in a manner which is perhaps quite well defined by structure. Phillips (31,32) studied the effect of naringenin on several growth and dormancy systems and found that it could induce a light requirement for the germination of lettuce seed, but that this requirement, in turn, could be reversed with the addition of gibberellic acid. Since gibberellic acid alone stimulates growth, Phillips postulated that this reversal of the induced light

requirement is due to a neutralization of an inhibitory system by the gibberellin and that the state of dormancy in a plant is determined by the balance between these two factors. The inhibitory system in this case might involve naringenin. Tronchet and Vuillemin (33) measured the differences in the flavonoid content between gibberellin-treated and untreated plants. They reported that the levels of flavonoids in the treated plants were relatively high, especially in quercetin glucosides, whereas the untreated plants contained much larger amounts of polyphenols and coumarin. This line of investigation, however, has not been pursued.

Parups (34) has investigated the effects of the flavonoids on more specific growth processes. He found that some flavonols and hydroxy acids promoted lignification inplants while flavones (unsubstituted at the 3-position) and methoxycinnamic acid retarded lignin synthesis. Also, since the growth and development of plants depends, in part, on the synthesis of protein, Parups (35) attempted to determine the effect of certain flavones on this process. This was done by measuring their effect on the incorporation of leucine-^{14}C into the protein of potato tubers and Escherichia coli. He found that the flavones inhibited this process anywhere from 58-94%. As a rule, the aglycones hesperetin, naringenin, and phloretin were more effective than their corresponding glucosides. It might be expected that since the glucosides are more soluble, their entry into the cell would be easier and thus they would exert a stronger effect. However, the data infer that the unsubstituted material is the active form and the glucosidic groups must first be hydrolyzed by the plant. It is known also that the transport of amino acids through cell membranes may be inhibited by flavones. Thus, Parups conducted

similar experiments with cell-free E. coli. hoping that this would nullify permeability and transport problems. But he found that hesperetin and its glucoside actually stimulated protein synthesis, and although the glucoside of phloretin still inhibited the incorporation of leucine to the same extent as with potato, the aglycone was a strong accelerator of the process in E.coli. Again, results such as these indicate that many unknown factors are involved. The results are interesting in the light of recent work with both IAA and abscisin II. Armstrong has reviewed the literature on the mechanism of IAA activity (36). It has been found that increased synthesis of all major RNA fractions is initiated by the treatment of a number of different culture tissues with IAA, and that this auxin-induced growth is quite sensitive to specific inhibitors of RNA and protein synthesis. Abscisin II is also thought to be involved in the formation of RNA and DNA. Overbeek (37) found that the synthesis of these two substances in extracts from Lemna minor cultures was considerably suppressed by abscisin II. This work might indicate the nature of plant growth regulator activity in general, and further investigation of the physiological role of the flavonoids at this level might be worthwhile.

As mentioned earlier, it has been hypothesized that the flavonoids act as plant growth regulators through their effects on IAA-oxidase. Such a theory involves inherent assumptions. It would be expected that a substance which acts as an IAA-oxidase synergist would inhibit the IAA-induced growth of plant stems and reverse the IAA-induced inhibition of root growth, whereas an enzyme antagonist would have opposite effects. A comparison of the data presented by Nitsch (17,18) and Stenlid (14,26) does reveal that a large number of compounds behave in this way (Table II).

TABLE II

COMPOUND	EFFECT OF COMPOUND		
	IAA-OXIDASE ACTIVITY	IAA-INDUCED STEM GROWTH	IAA-INHIB. ROOT GROWTH
CATECHOL	-	+	
RESORCINOL	+	-	
CAFFEIC ACID	-	+	
SINAPIC ACID	-	+	
p-OH-BENZOIC ACID	+	-	
p-COUMARIC ACID	+	-	
FERULIC ACID	-	-	
NARINGENIN	+	-	+
GENISTEIN	+	-	+
APIGENIN	+	-	+
QUERCETIN	-	+	-
FISETIN	-	+	
KAEMPFEROL	-	+	
MORIN	-		-

(+) = Promotes the indicated activity
(-) = Inhibits or reverses the indicated activity

The list of exceptions, however, is not completely insignificant. Ferulic acid, hesperetin, pinocembrin and biochannin A do not act according to the hypothesis. Two of these are methylated derivatives of compounds which do behave as expected, and these results serve to emphasize that there are a number of factors in operation, some perhaps involved with methoxyl groups, which have not yet been fully characterized. Generally, however, the data are quite consistent with the hypothesis and give it a considerable degree of support. But there is one other contradiction. Stenlid reported (14) that the flavonoids exerted the same affects against exogenously applied sugars as they did against the IAA. Such results raise some doubts against the validity of the hypothesis by inferring that the flavonoids are acting as a simple interference factor to normal physiological processes and are not realy endogenous regulators of any kind. However, in view of the reported action of naringenin in peach and the studies of Mumford and Fuyura (23-25), it is apparent that some flavones do exert physiological affects, and the results of Stenlid with the sugars might be taken as coincidental.

Another point at which these studies are aimed is the question of relationship between structure and activity. A lack of experimental data does not permit any conclusive results, but with those available it seems apparent that a 3-hydroxyl group gives a flavone enzyme antagonistic properties regardless of the remaining substitution pattern. Beyond this, little else can be said with any degree of validity.

The next question which must be raised is the effect of flavonoids on the endogenous growth of plant tissue. To assume that such compounds control the growth through their action on the naturally occurring oxidase

system within a plant further implies that those substances which are antagonistic towards the enzyme will promote endogenous stem growth and inhibit the root growth, etc. The very existence of endogenous IAA-oxidase systems in plants has been questioned. Briggs (38) has suggested, in fact, that it might actually be an artifact of cut surfaces. However, the existence and activity of such an enzyme system has been inferred from a number of experiments, and some direct evidence also exists. As mentioned previously, Zenk and Muller (29) added carboxyl-labeled IAA to Avena coleoptile sections and measured the $^{14}CO_2$ which was evolved. They found the rate of discharge of this gas to be constant for more than 10 hours. This suggests quite strongly that labeled IAA was being oxidized, and perhaps in a specific manner. This latter fact would need to be confirmed with further experiments on IAA labeled at different positions.

Unfortunately, at this point, the experimental data on the effects of the flavonoids and phenols on endogenous plant tissue growth are insufficient for discussion. Only Stenlid (14) has investigated this aspect, using the root growth test, and the results obtained are far from conclusive. Naringenin, apigenin and biochannin A, all of which are enzyme synergists, exhibited no effects on the endogenous root growth. Hesperetin was an enzyme antagonist but also promoted root growth, while pinocembrin, also a root stimulator, did not affect the enzyme activity <u>in vitro.</u>

It might be argued that the use of a root growth bioassy is not valid. The inhibition of root growth is caused by supra-optimal concentrations of IAA, but the mechanism through which this inhibition occurs is still unknown. It has been suggested that the mechanism is significantly different from that of the growth promoting effects operative at lower concentrations. If this is the case, it does not seem unreasonable

that the results from such a bioassay would be inconsistent with those from stem growth experiments. This would apply to both residual and IAA-induced growth.

Thus, it is the purpose of this report to investigate more thoroughly the effects of the flavonoids on endogenous growth systems, and, with these additional data, to further examine the IAA-oxidase hypothesis. Also, the relationship between the structure of flavonoids and their effects on endogenous growth will be examined in detail.

MATERIALS AND METHODS

Twenty flavonoids were tested for their biological activity on oat coleoptile sections. The tests were conducted without the addition of any auxins or co-factors such that the growth system would remain completely endogenous. The flavonoids encompassed a wide range of hydroxylation patterns, from flavone itself to the hexahydroxyflavone myricetin. In a number of cases, the hydroxyl groups were methylated and the effects of these methyl groups were also studied. The compounds and their structures are listed in Table III. Of the twenty flavonoids tested, flavanone, 3-hydroxyflavone and its O-methyl ether, 7-hydroxyflavone, and the methyl ethers of quercetin and myricetin are not known to occur in nature. Flavone has been found in only one family of plants, the Primula species, and, within this group, its distribution is quite extensive. However, it is found in the plant as a heterogeneous white substance deposited on the leaves and stem of the plants and it is therefore believed to be produced only by an aberrant biosynthetic pathway. The distribution of both quercetin and myricetin is extensive, these being two of the most common flavones known. Apigenin also is distributed in a considerable number of plants. It was first isolated from parsley seed in 1901 and, to date, at least six O-glycosides as well as a larger number of C-glycosyl derivatives have been described in the literature. Acacetin, the 4-O-methyl ether of apigenin is fairly common too, having been detected or isolated from Linaria, Robinia and Ammi species and in the heartwood of various Prunus species. The 4',7-di-O-methyl derivative, however, has been reported only once, in

TABLE III

Flavone	
Flavanone	
-	3-Hydroxyflavone
-	3-Methoxyflavone
-	7-Hydroxyflavone
Chrysin	5,7-Dihydroxyflavone
Tectochrysin	5-Hydroxy-7-methoxyflavone
Apigenin	4',5,7-Trihydroxyflavone
Acacetin	5,7-Dihydroxy-4'-methoxyflavone
-	5-Hydroxy-4',7-dimethoxyflavone
Fisetin	3,3',4',7-Tetrahydroxyflavone
Datiscetin	2',4',5,7-Tetrahydroxyflavone
Quercetin	3,3',4',5,7-Pentahydroxyflavone
-	5-Hydroxy-3,3',4',7-tetramethoxyflavone
Morin	2',3,4',5,7-Pentahydroxyflavone
-	5-Hydroxy-2',3,4',7-tetramethoxyflavone
Myricetin	3,3',4',5,5',7-Hexahydroxyflavone
-	3,3',4',5,5',7-Hexahydroxyflavone
Naringenin	4',5,7-Trihydroxyflavanone
-	5,7-Dihydroxy-4'-methoxyflavanone

birch buds. Chrysin is the simplest flavone that can be formed through the shikimic acid pathway. Chrysin and tectochrysin are not widely distributed in the plant world, but have been found in the wood of a number of Pinus and Prunus species. The 2'-hydroxyflavones are rare also. Only two, morin and datiscetin, are known with certainty to exist. Morin appears in the wood of several species of Moracea and datiscetin has been detected only in Datisca cannabina. Fisetin, the 5-deoxy derivative of quercetin, has a somewhat similar distribution, having been found in Betea, Rhus, Querbracho and Gleditschia. Naringenin was first isolated from Citrus in 1885, but its distribution seems to be limited also, occurring mainly in species of Prunus.

A more complete discussion of the occurrence and properties of these flavones can be found in the literature cited earlier.

None of the compounds used in this work has previously been tested for its physiological activity on an endogenous stem growth bioassay. The only other reported use of an endogenous bioassay test is that of Stenlid (10). He used the wheat root bioassay on a few of the compounds tested in this work, including naringenin, chrysin, apigenin, morin and quercetin. Flavone, flavanone, datiscetin, 7-hydroxy- and 3-hydroxyflavone and its O-methyl ether, and the ethers of quercetin, myricetin and morin have not previously been tested in any way for their biological growth effects.

The need for a thorough series of experiments involving the effects of the flavonoids as endogenous plant tissue growth was emphasized earlier. The use of the particular bioassay employed in this work was prompted by two factors. The flavonoid growth inhibitory fraction originally separated from the extract of birch leaves was detected by means of this

assay. In addition, the oat coleoptile bioassay, as described by Nitsch and Nitsch (39) is a simple but very sensitive test, especially towards growth inhibitors.

The flavones were tested for their activity over a range of concentrations from 0.00001 mg/ml ($\sim 10^{-8}$M) to 10.0 mg/ml ($\sim 10^{-2}$M). Since most of compounds tested were insoluble in water or the buffer-sucrose medium used in the tests, the solutions of the flavones were prepared with common organic solvents, acetone, dichloromethane, methanol, pyridine, which were subsequently removed by evaporation. Control experiments were run simultaneously, both with the organic solvent residues and with the buffer-sucrose solution alone. The results with these control tests indicated that the solvents contained no residues significantly toxic towards the coleoptile sections. Therefore, this method of quantitative application of the test materials was quite satisfactory. Four to six runs were made with each flavone and the results, expressed as a percentage of the growth of the controls, were averaged. These are presented in graphical form in the next section.

Five flavones, apigenin, naringenin, fisetin, flavone and flavanone were tested also for their biological activity on oat coleoptile sections in the presence of exogenously applied IAA.

RESULTS AND DISCUSSION

Of the twenty flavones tested, only five exhibited distinct biological activity within common "physiological concentration" ranges. These are flavone, flavanone (figs. 1,2), naringenin (fig.8), datiscetin and fisetin (figs. 12,13), all of which acted as strong inhibitors of residual coleoptile growth. However, both fisetin and flavanone acted as moderate growth accelerators at very low concentrations. The activities of both flavone and flavanone have not previously been observed in any biological test of this type, and these results are viewed with some surprise. The significance of their biological activity is considerable and will be discussed in some detail later. The 3-methoxy derivative of flavone (fig.4) also appeared to give a moderate growth promoting effect at a very low concentration, but, above this level ($\sim 10^{-5}$M), it inhibited the coleoptile growth considerably. Quercetin (fig.14) had a slight inhibitory effect above 10^{-5}M and myricetin (fig.18) behaved in a similar manner, except that the inhibition was more pronounced at higher concentrations. Since these two compounds have essentially no activity when present in smaller amounts, the type of growth inhibition exhibited by these compounds probably can be attributed to a toxic effect. Tectochrysin (fig.7) gave a weak but constant growth inhibition over the entire concentration range while morin (fig.16) accelerated the growth slightly, also to a constant level over the whole range. These effects could be due either to limited solubilities of the substances in the test medium, or to their limited uptake by the plant tissue. The remaining compounds were all inactive in the bioassays.

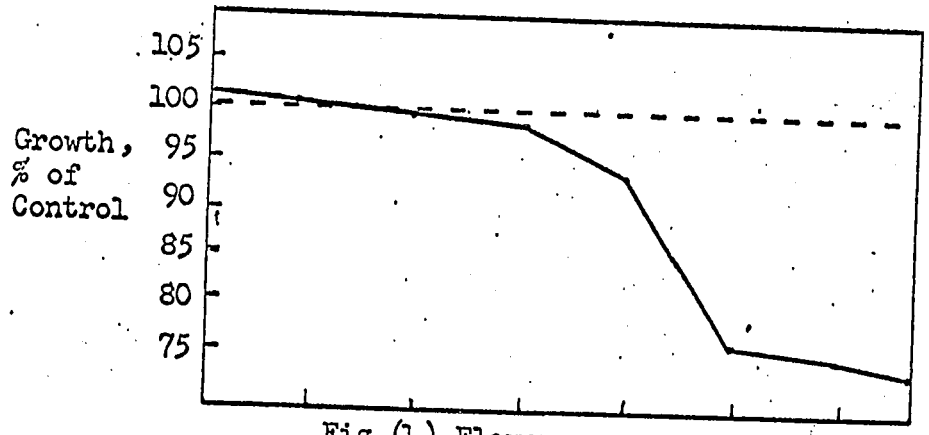


Fig (1) Flavone

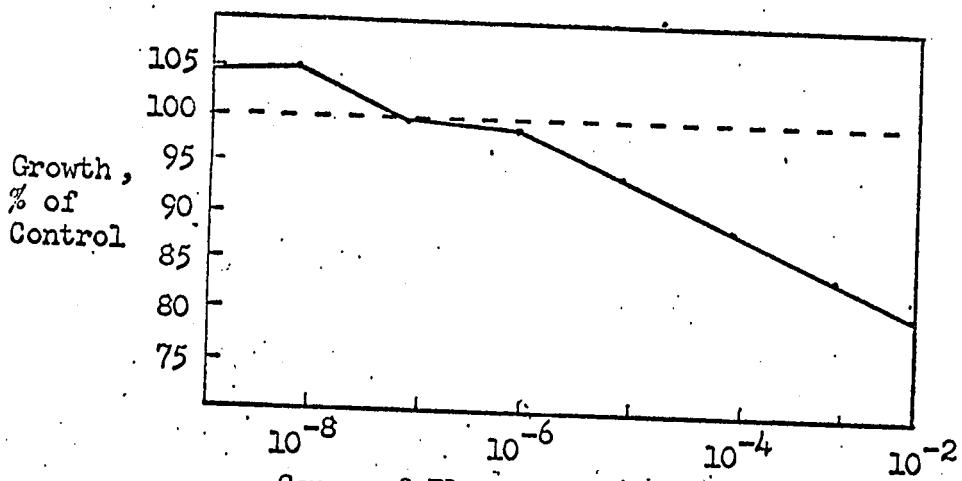


Fig. (2) Flavanone

The oat coleoptile bioassay gave results which were generally consistent from one test to another. Four tests were run for each compound when the physiological effects were large, six were run when the effects were not pronounced. Due to the high reproducibility obtained in these tests, the results can be discussed and interpreted with a reasonable degree of confidence. However, two points must be raised. Since such a large proportion of the compounds did not show any net growth effects, it could be argued that the lack of activity was due to the limited solubility or uptake of the materials in question. However, there is no reason to assume that more of these flavones should be active in the first place. Also, if any of these "inactive" compounds

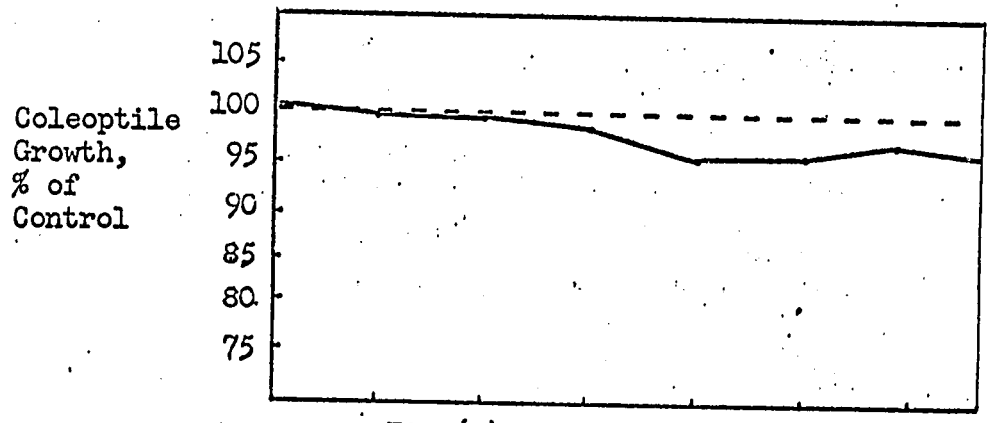


Fig (3) 3-Hydroxyflavone

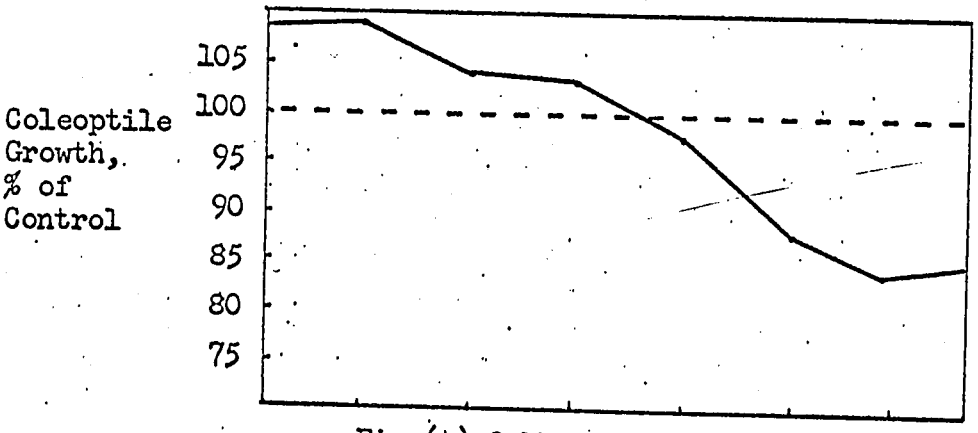


Fig (4) 3-Methoxyflavone

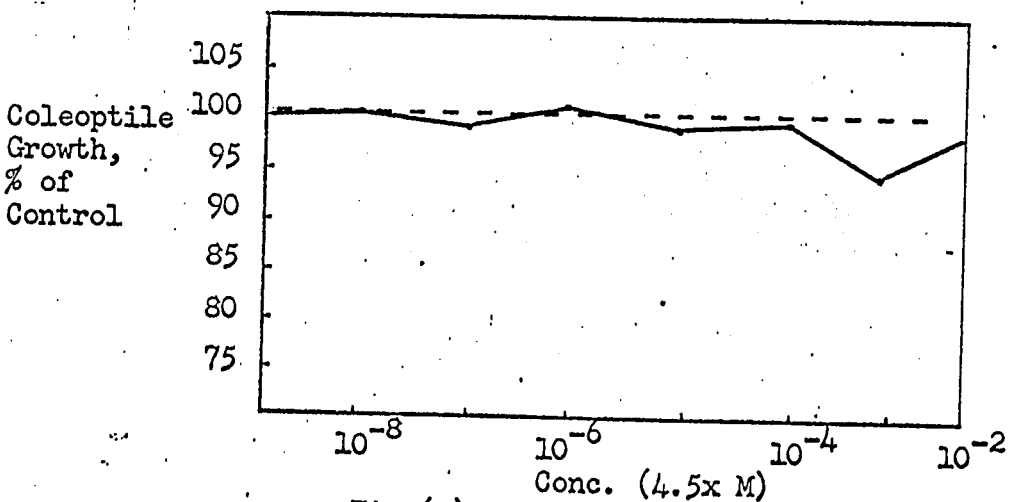


Fig (5) 7-Hydroxyflavone

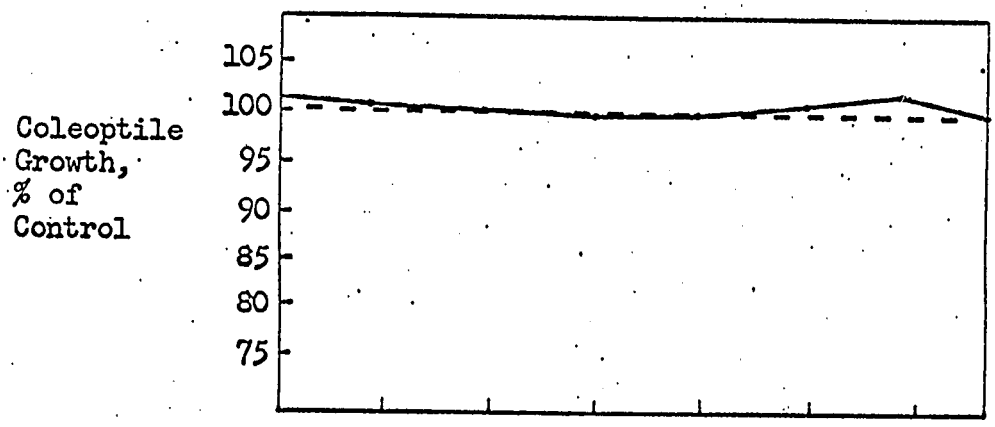


Fig.(6) Chrysin

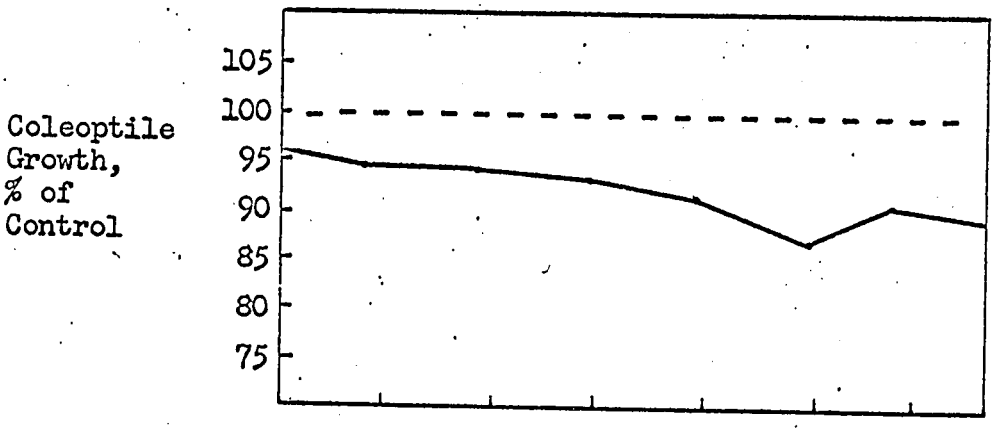


Fig.(7) 7-O-Methyl Chrysin

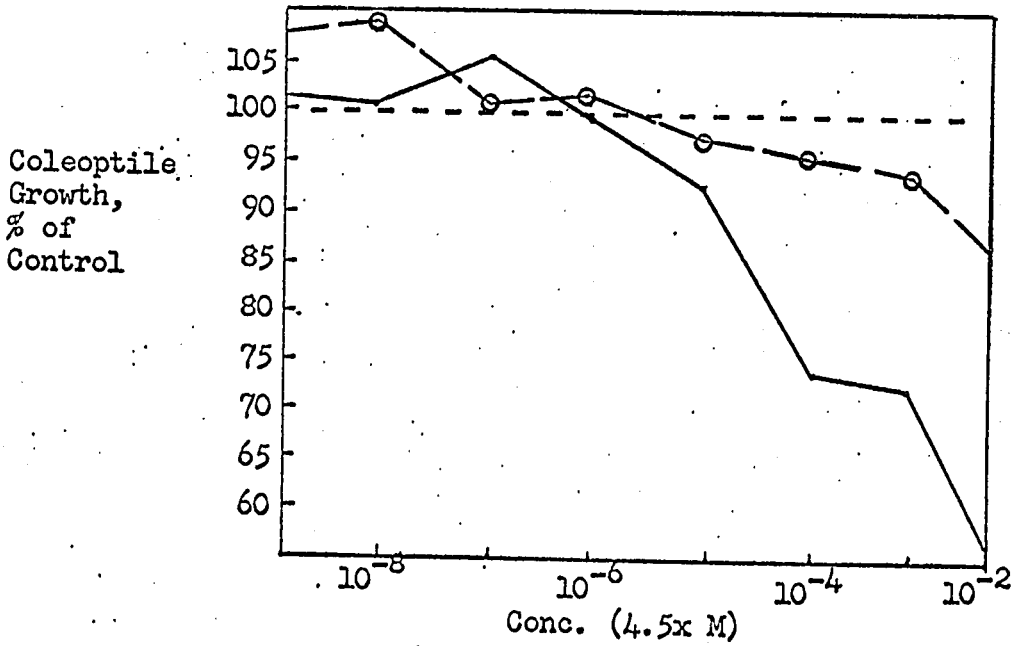


Fig.(8) Naringenin(—•—)

7-O-Methyl Naringenin(—o—)

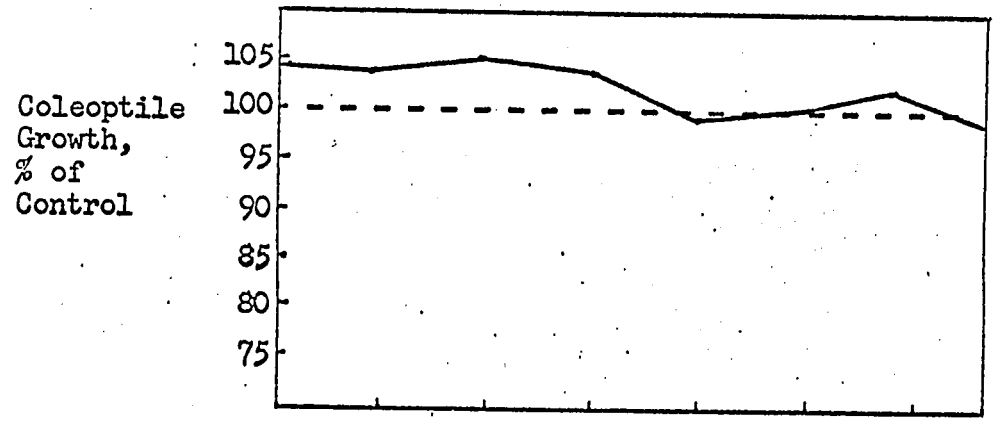


Fig.(9) Apigenin

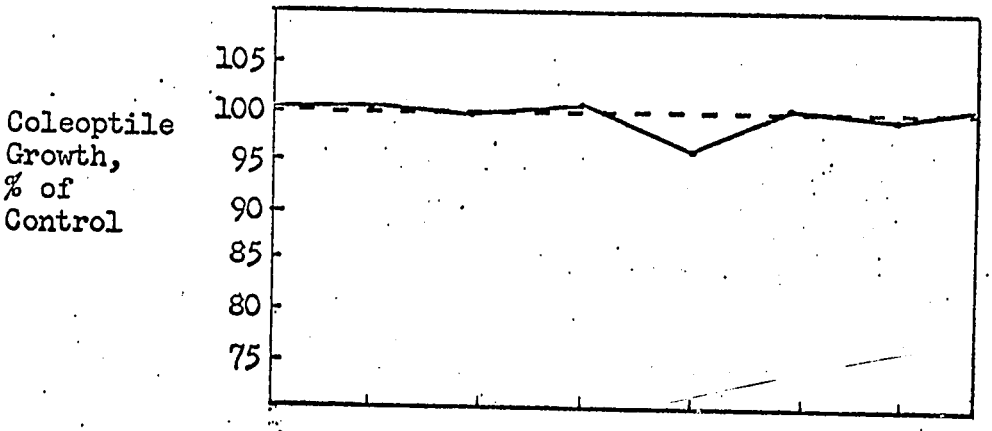


Fig.(10) 4'-O-Methyl Apigenin

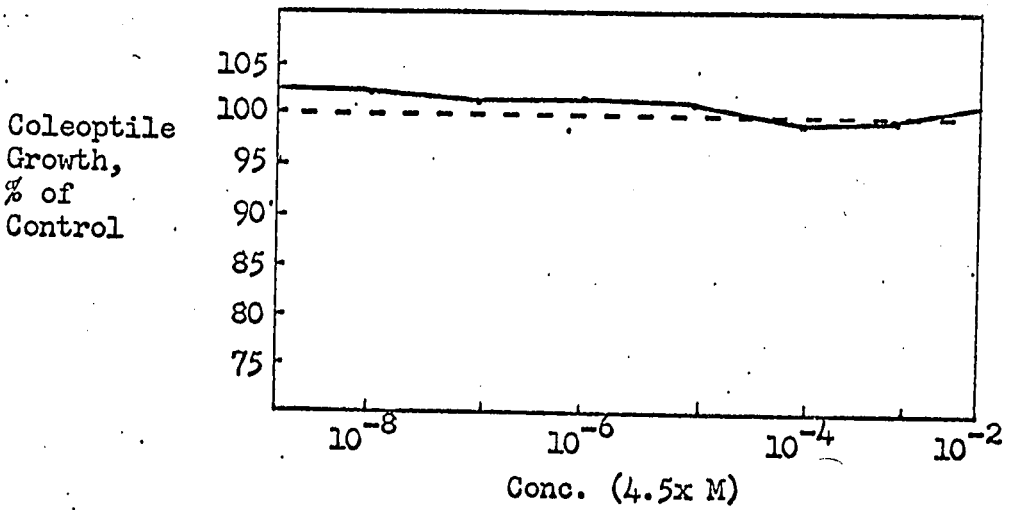


Fig.(11) 4',7-Di-O-Methyl Apigenin

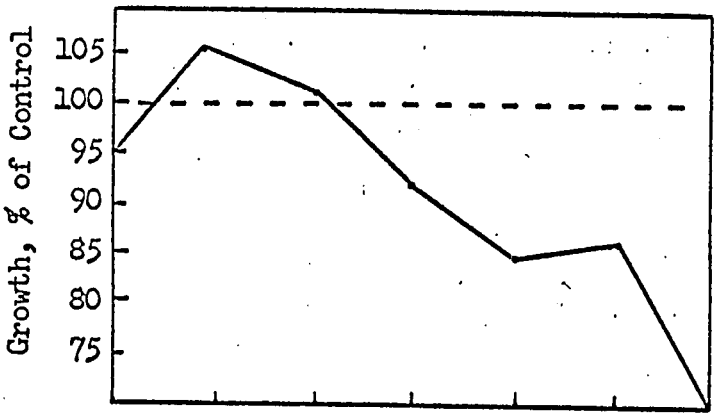


Fig.(12) Datiscetin

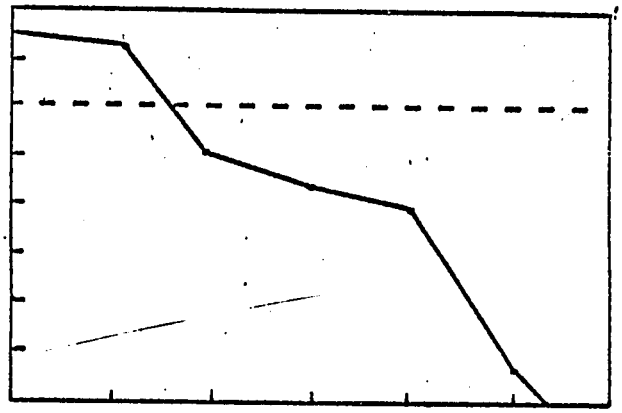


Fig.(13) Fisetin

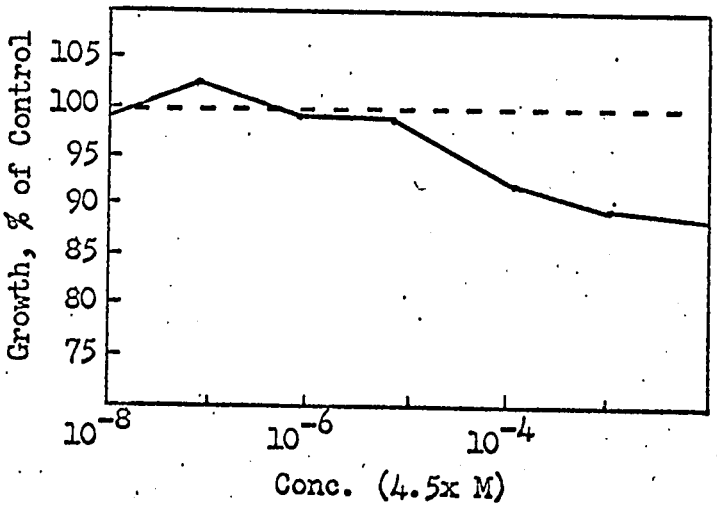


Fig.(14) Quercetin

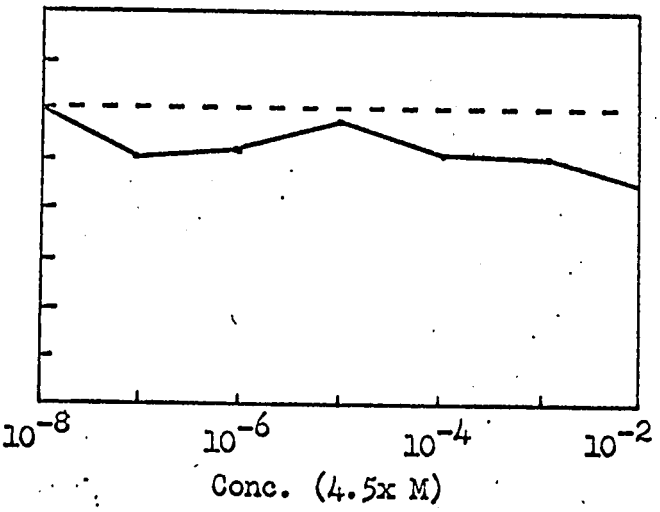


Fig.(15) 3,3',4',7-Tetra-O-Methyl-Quercetin

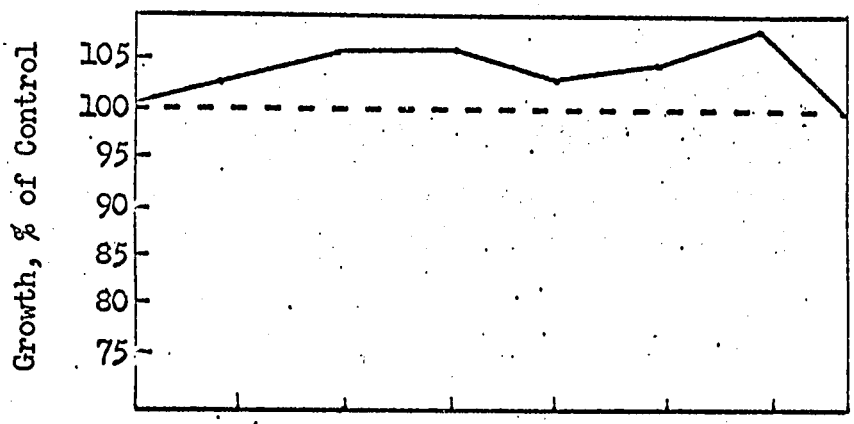


Fig.(16) Morin

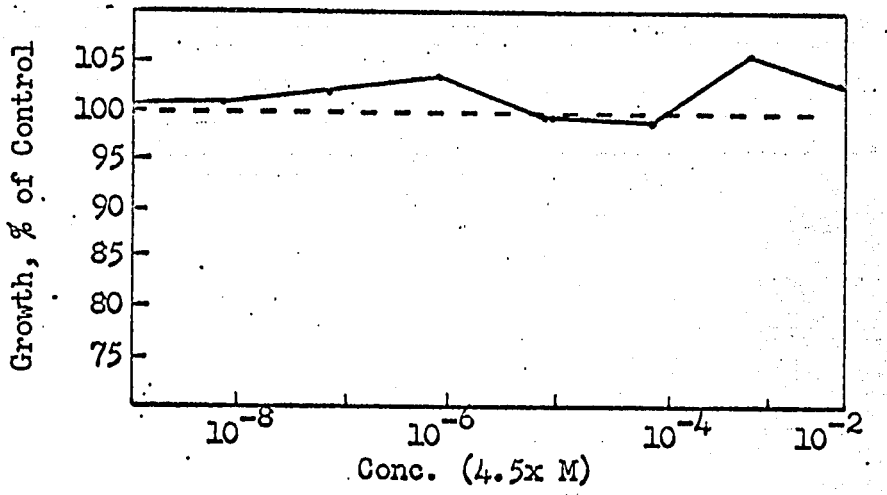


Fig.(17) 2',3,4',7-Tetra-O-Methyl Morin

had pronounced physiological activity in a naturally occurring system, the activity probably would have shown up in a manner similar to that of morin and tectochrysin. Second, the oat coleoptile test is not as sensitive to growth accelerators as the first internode test, and perhaps the growth promoting effects of some of the flavones were significantly modified. This is a valid point and emphasizes a limitation to the experimental procedure. However, the sensitivity of this coleoptile bioassay to growth promoters is quite sufficient for the purpose of this work and it will become apparent in the ensuing discussion that this

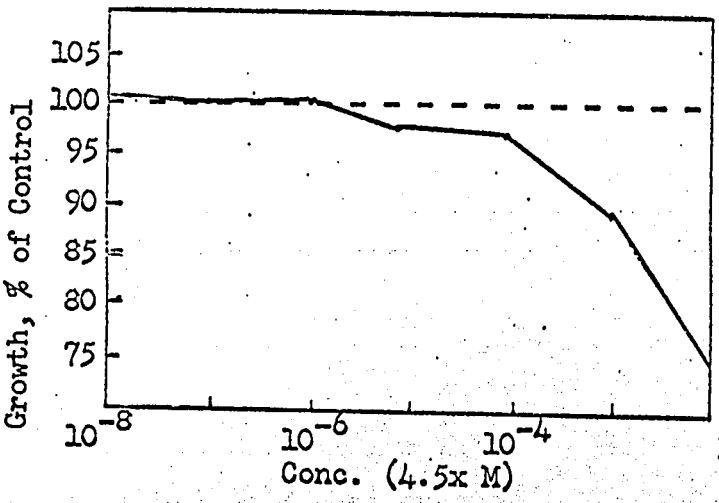


Fig. (18) Myricetin

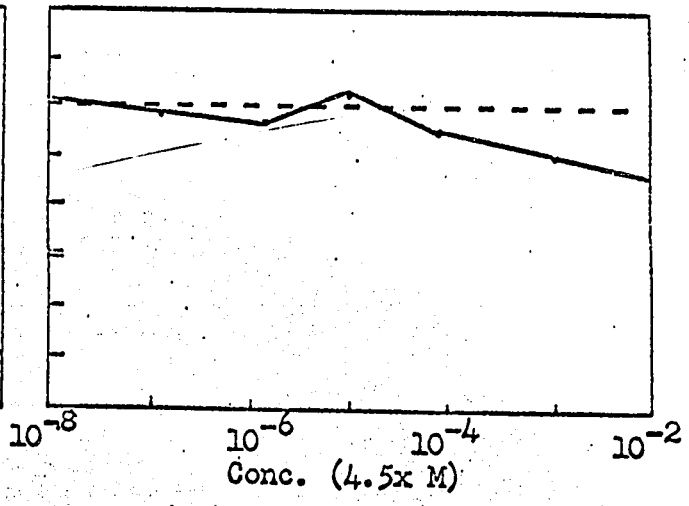


Fig. (19) Hexa-O-Methyl Myricetin

limitation has little bearing on the overall results.

The experimental results obtained from this work can be examined from two aspects: their bearing on the IAA-oxidase theory; and the relationship between the structure of the flavonoids and their effects on the endogenous growth system. The IAA-oxidase theory, as discussed previously, states that some flavonoids act as plant growth regulators and exert this influence through their activity on an IAA-oxidase enzyme system within the plant. A compound which activates this enzyme in vitro should inhibit the residual growth of plant tissue, and a compound which is an enzyme antagonist would stimulate the tissue growth. Examination of the data from the growth experiments conducted by Stenlid (26) reveals a considerable number of cases which are inconsistent with this hypothesis (Table IV). Morin, an enzyme inhibitor and naringenin, an enzyme synergist, behave in the manner predicted in the coleoptile growth assay. However, quercetin and myricetin, both enzyme antagonists, have no growth promoting effects on the coleoptiles. Fisetin, a strong enzyme inhibitor, also strongly inhibits the coleoptiles, and apigenin,

TABLE IV

SUBSTANCE	EFFECT ON: IAA-OXIDASE	EFFECT ON: ENDOGENOUS STEM GROWTH
NARINGENIN	+	-
MORIN	-	+
QUERCETIN	-	0
APIGENIN	+	0
TECTOCHRYSIN	0	0
MYRICETIN	-	0
FISETIN	-	-

an enzyme synergist, does not affect the coleoptile growth to any extent. It becomes quite evident from these results that the relationship between the action of the flavonoids on in vitro IAA-oxidase and their effect upon endogenous stem growth is obscure, if it exists at all. The studies of Mumford (23), Fuyura (25) and Zenk and Muller (29) do suggest that there is an IAA-oxidase system in growing plants and that its activity can be modified by kaempferol and its derivatives; the studies of Nitsch (18) and Stenlid (26) show quite convincingly that the behavior of several flavones towards in vitro IAA-oxidase and IAA-induced stem growth is closely related; and the experimental evidence presented here certainly does demonstrate that some flavones influence endogenous coleoptile growth. However, these data in no way support the assumption that flavones play a vital role in plant growth regulation through their effects on the auxin-oxidase enzyme.

The results of the experiments on the biological activity of flavone, flavanone, apigenin, fisetin and naringenin in the presence of additional IAA tend to agree with the above conclusions. Naringenin, flavone and apigenin were unaffected by the exogenous auxin. The activity of apigenin in this case does conflict with the results of Nitsch (18). This might be due to the absence here of the glucoside group, but this anomalous behavior has little bearing on the overall discussion. The presence of IAA removed the strong inhibitor action of fisetin and this flavone actually promoted growth to a slight extent, agreeing with Nitsch. The growth inhibition of flavanone was also destroyed. If the behaviors of flavone and flavanone in the presence of exogenous IAA reflects their probable behavior on an in vitro IAA-oxidase system, this would further

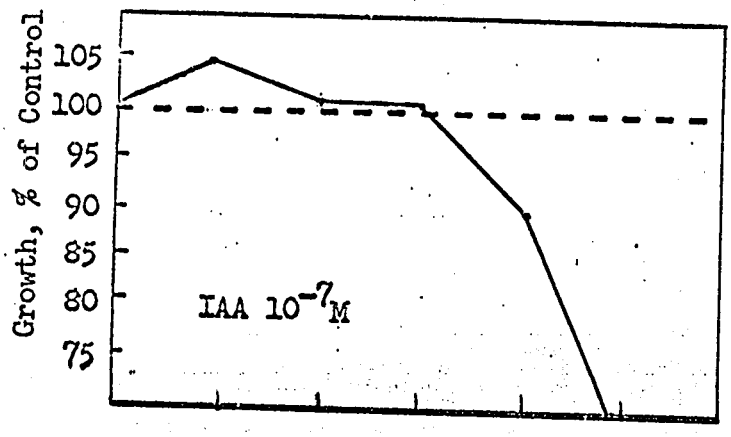


Fig. (20) Flavone

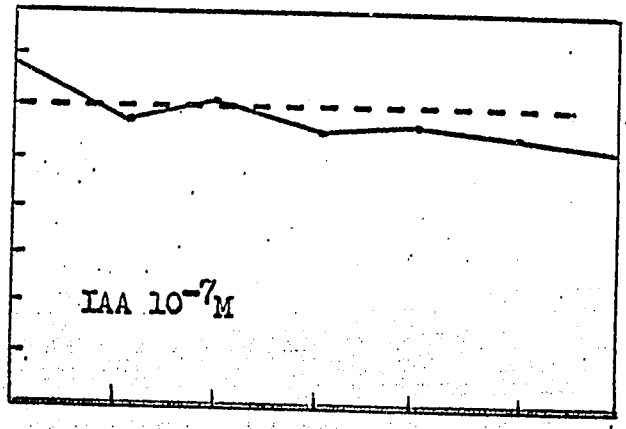


Fig.(21) Flavanone

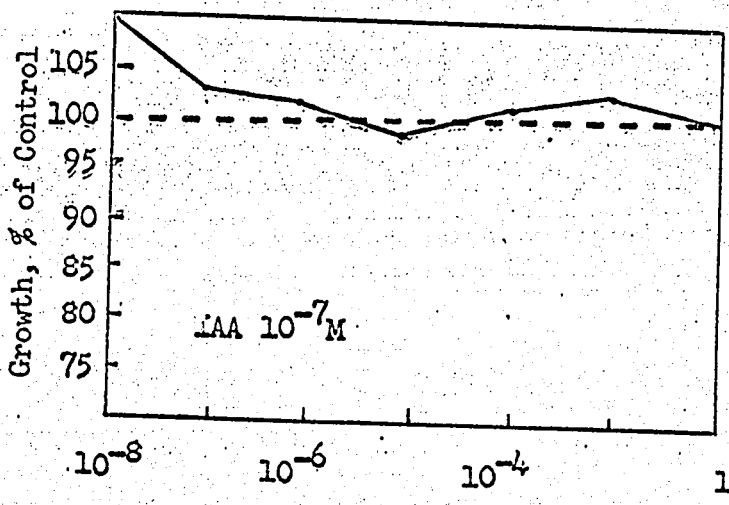


Fig.(22) Apigenin

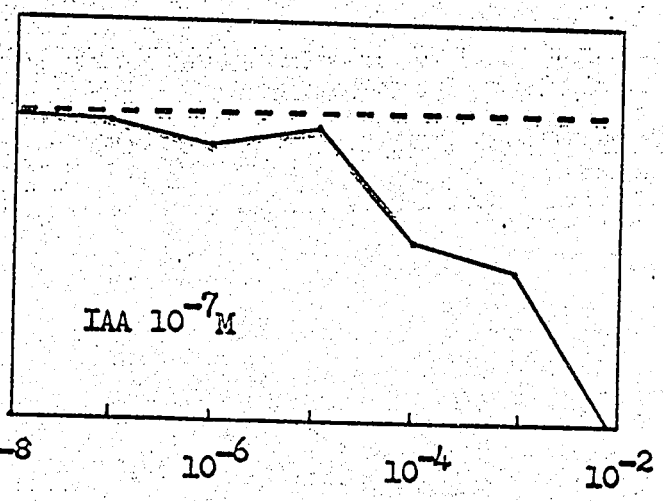


Fig.(23) Naringenin

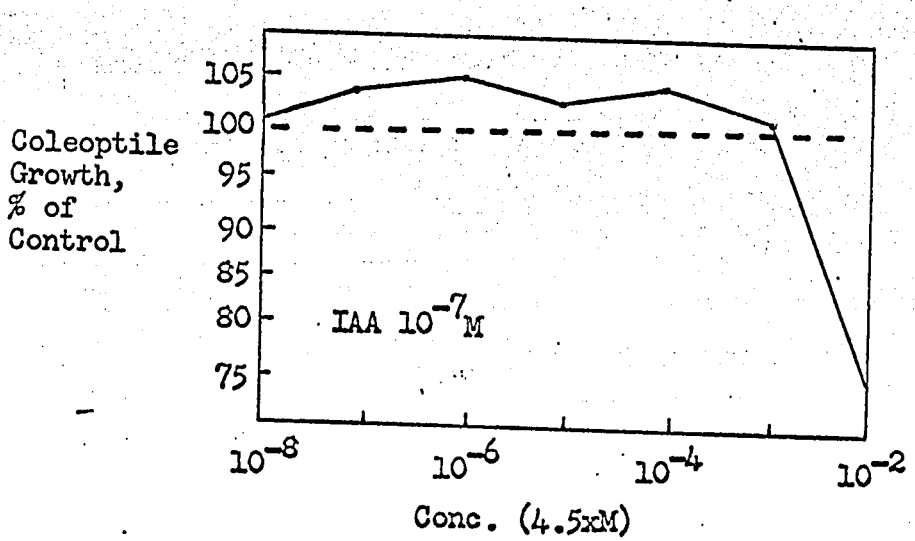


Fig.(24) Fisetin

emphasize the lack of relationship between the effects of the flavones on endogenous growth and *in vitro* enzyme activity. This latter point however is purely speculative.

One possibility does emerge from all of these studies. Stenlid (14) has suggested that the flavonoids might have two different roles in growth regulation, one which affects the activity of exogenously applied materials, e.g. as an enzyme antagonist or synergist, and the other, not necessarily related to the first, is operative only on endogenous growth systems. This latter effect could conceivably be exerted through an endogenous IAA-oxidase system, but this would yet have to be demonstrated. Such an approach is quite appealing and is worth consideration.

A detailed examination of the results from this work, the effects of the flavonoids on a purely endogenous system, reveals that few, if any, conclusions can be reached as to an explicit relationship between structure and the growth regulating effects of the flavonoids. With the three hydroxylated flavones which act as strong growth inhibitors, the only common structural feature is a 7-hydroxyl group. This particular group may be a major factor in their activities, but in itself is not sufficient, as 7-hydroxyflavone is completely inactive. The strong inhibitory effects of flavone and flavanone indicate that hydroxyl groups are not actually a necessary requirement for biological activity, and suggests quite convincingly that the molecular skeleton is intimately associated with the physiological properties of the flavonoids. That these two substances act as inhibitors within the range of "physiological concentrations" infers that the effects are not of a toxic nature. Even

at higher concentrations there was no visible evidence of toxicity. It might be argued that, since flavone and flavanone have no hydroxyl group substituents, the mechanism through which they inhibit the coleoptile growth is significantly different from mechanism of hydroxyflavone activity. One fact counters this argument. Naringenin was found to be the strongest growth inhibitor of all the compounds tested. However, apigenin, which structurally is so closely related to naringenin, has no activity at all. These findings tend to confirm that the skeletal structure of a substance plays a major role in determining its physiological activity. But the substituents play more than a simple modifying role. The presence of a 3- or 7-hydroxyl group completely destroys the activity of flavone. Although fisetin is a strong growth inhibitor, insertion of a 5-hydroxyl group, as in quercetin, also removes its effect. The substituents in the "B" ring play an equally important role. Datiscetin is a strong inhibitor but its 4'-hydroxy derivative, morin, is a weak growth promoter. The presence of an additional group at the 5' position, myricetin, in turn, destroys this growth promoting effect. The structural requirements for biological activity involve one additional factor, Methylation of the hydroxyl groups gave completely mixed results. The 7-O-methyl ether of naringenin is inactive at high concentrations and acts as a weak growth stimulator at lower concentrations. Conversely the methylation of morin removes its growth promoting ability. Although 3-hydroxyflavone is inactive, its methylated derivative is a growth promoter below 10^{-5}M but is an inhibitor above this level, perhaps due to toxicity. On the other hand, methylation of both myricetin and quercetin removes their toxic effect which are evident at high concentrations.

It is quite obvious from these facts that a number of complex and interrelated factors are involved in these growth regulatory activities. Neither skeletal structure, type of substituent nor substitution pattern are independently involved. It is impossible at this point to derive any generalizations, but this is not surprising, considering the complexity of the "simple" growth system used in these tests. The mechanism through which the flavonoids exert their effects must first be characterized, probably at a more fundamental level, before questions as to structural effects can be answered. Of course, this is true also for the other plant growth regulators, the auxins, gibberellins and kinins.

EXPERIMENTAL

Melting points were recorded on a calibrated Fisher-Johns apparatus. All solvents were redistilled before use and all test materials were recrystallized. 5-Hydroxy-3,3',4',7-tetramethoxyflavone and 3,3',4',5,5',7-hexamethoxyflavone were obtained as described in Chapter II. The other flavonoids used in this work were obtained commercially, except for those which were prepared as described below. Diazomethane was prepared according to Vogel (40).

3-METHOXYFLAVONE

One gram of 3-hydroxyflavone was dissolved in 200 mls of absolute ether (diethyl ether). The solution was cooled to -20°C and an excess of diazomethane solution was slowly added. The reaction mixture was allowed to warm to room temperature and was then evaporated to dryness under vacuum at 40°C. A solid residue remained and it was crystallized from ethanol, m.p. 113-114°, Lit. 114°C (41). Yield: 1.04 gm, 96%.

5-HYDROXY-4',7-DIMETHOXYFLAVONE

One gram of 4',5,7-trihydroxyflavone was dissolved in 25 ml of pyridine. The solution was cooled to -20°C and treated with diazomethane as described above. After evaporation, the solid residue was crystallized from ethanol, m.p. 131.5°C, Lit. 131°C (41). Yield: 1:10 gms, 93%.

4',5-DIHYDROXY-7-METHOXYFLAVANONE

Naringenin, 2.72 gms, was dissolved in CH_2Cl_2 and cooled to -75C. Diazomethane, 0.01 moles, was slowly added with vigorous stirring. The

solution was evaporated under vacuum and the solid residue crystallized from CH_2Cl_2, m.p. 151-152°C, Lit. 152°C (41). Yield: 2.22 gms, 77%.

BIOASSAYS

Seven solutions in a concentration range from 10 mg/ml to 0.00001 mg/ml were prepared with each flavone using an appropriate solvent. 1 ml of each solution was placed in a 10 ml beaker and then evaporated to dryness under vacuum for 4 hours. Three control beakers were prepared in a similar manner, using the solvent alone. Two mls of a solution containing 2% sucrose and a buffer at pH5 (k_2HPO_4 1.794 gm/ml + citric acid monohydrate 1.019 gm/l) were put into each beaker. Three other control beakers contained this sucrose-buffer solution alone.

Oat seeds, var. Brighton, were soaked in tap water for two hours and then planted on wet vermiculite in plastic trays. These were exposed to red light (39) for 4 hours, covered with a thin layer of dry vermiculite, and then grown in the covered trays at a 45° angle for 3 days in darkness. Coleoptile sections, 4 mm long, were cut 3 mm from the tip of each shoot and ten of these were placed in each beaker. The beakers were covered with "parafilm" and incubated in darkness for 20 hours with a gentle, random horizontal agitation. All work was done under green light (39) and the temperature was kept constant at 26°C. After 20 hours, the sections were placed on a calibrated transparency on an overhead projector, and the lengths of the section were measured from their projected images on a screen. Four to six runs were made with each flavone. The results were calculated as % growth of that of the controls and these were averaged for all runs. The results are plotted as molar concentrations.

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CLAIMS TO ORIGINAL RESEARCH

1. A thin-layer chromatographic method was devised to give maximum separation of the components of the acid fraction of a methanol extract from birch leaves.
2. Eluates from two zones of the chromatogram showed some inhibitory activity towards the residual growth of oat coleoptiles. One zone probably contained a fatty acid, and was presumed to inhibit growth by virtue of its toxicity. The other zone appeared to contain one or more flavonoid compounds.
3. Large-scale fractionation, using chemical techniques of an extract of birch leaves, yielded quercetin, ellagic acid and O-methyl ethers of gallic acid, ellagic acid, quercetin, and myricetin.
4. The isolation of hexa-O-methyl myricetin represents the first time it has been isolated from any plant material.
5. Evidence indicated that the ellagic acid ether isolated from the leaves had been at least partially methylated in its original state. Thus, this is the first reported isolation of a methylated derivative of ellagic acid from species of Betula.
6. Twenty flavonoid compounds were tested for their effects on the residual growth of oat coleoptiles. These included the first plant-growth bioassays of any kind conducted with the following compounds: flavone, flavanone, datiscetin, 7-hydroxyflavone, 3-hydroxyflavone, 3-methoxyflavone, and the methyl ethers of quercetin, myricetin and morin.
7. In these bioassays naringenin, fisetin, datiscetin, flavone and flavanone were found to act as inhibitors of growth. This was the

first observation of such activity by the latter three compounds.

8. When added indoleacetic acid was present in the bioassay medium, the inhibitory effect of flavanone was completely removed, and the response to fisetin was altered. The responses towards flavone, apigenin and naringenin were essentially unchanged.
9. The results of the bioassays were examined in the light of a current theory on the mode of action of flavonoids which suggests that they act on the indoleacetic acid-oxidase enzyme. The present evidence leads to the conclusion that while the theory may be valid for some active flavones, it does not hold for all; and another mechanism must be sought.
10. The biological activity of flavone and flavanone indicates that the presence of phenolic hydroxyl groups is not always necessary in compounds that are inhibitors.