THE NATURE OF THE GENETIC DIFFERENCE IN THE COUMARIN METABOLISM OF BITTER AND NON-BITTER *Melilotus alba* Desr.

by

STANISLAW FREYMAN

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DIVISION
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The University of British Columbia,
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ABSTRACT

The phenylpropanoid compound, coumarin, is responsible for the bitterness and indirectly, toxicity in the forage legume sweet clover (*Melilotus alba*). Coumarin, mainly in the "bound" form as the β glucoside of o-coumaric acid is abundant in the bitter plants, but not in the shoots of the low-coumarin "Denta" and "Cumino" varieties, and in the species *M. dentata*. Substantial quantities of the coumaric acid glucoside have been found in the seed of these low-coumarin plants. The search for a compound that substitutes for coumarin in the shoots of low-coumarin plants, led to the isolation of a flavonoid, somewhat resembling quercetin.

Feeding of o and p-coumaric acids to both high and low-coumarin plants led to the methoxylation of these compounds prior to glycosidation. When cinnamic acid was fed, no difference in the metabolic products could be detected from those normally found in control plants.

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Dr. A.J.Renney, Division of Plant Science

Dr. W.D.Kitts, Division of Animal Science

Dr. K. Beamish, Department of Biology and Botany

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I INTRODUCTION

Sweet clover (Melilotus alba), a forage crop important in western Canada and in a number of other areas in the mid and high latitudes, is limited in usefulness by a measure of unpalatibility and toxicity attributed to the presence in its tissues of the chemical compound coumarin. Some success in producing low coumarin strains of the crop has attended the efforts of plant breeders. Nontheless a practical end has not yet been achieved and, it is apparent that an aid to progress would be more information on the biochemistry and genetics of coumarin and its relatives in sweet clover. To provide such knowledge was a prime objective of this study; additionally it was hoped that patterns relating to coumarin synthesis in plants, largely known from work with radio-tracers, could be confirmed by means of genetic blocks and feeding trials.

A notable feature of higher plants, and some microorganisms, is their ability to synthesize a great variety of phenolic compounds. This ability is not shared by the animal kingdom. Although coumarin itself is not a phenol, it nevertheless is closely involved in the metabolic pathways concerned with the synthesis of phenolic compounds, and coumarins with phenolic substitutions are very often formed. Knowledge of the

pathways involved in the biosynthesis of phenolic compounds has largely been gained during the last five years and they are no longer regarded as being rather remote from the main metabolic processes of the tissues. Modern methods, particularly those using radio-active tracers, have shown that acetic acid and shikimic acid can function as precursors of aromatic rings and hence phenolic compounds would appear to be closely linked to the general carbohydrate metabolism of the plant. On the other hand very little is known of the physiological functions of phenolics and coumarin in the metabolism of the plant, yet their variety and abundance suggests that they are important.

Chemical substances that play a primary role in normal metabolism are more or less common to most organisms. However, they are of lesser consequence in comparative biochemistry than the so-called secondary products, the seemingly stable end products of metabolism, such as coumarins. These compounds reveal the existence of metabolic processes peculiar to the organism in which they occur. Of all such secondary substances the most abundand and diverse are those containing phenolic hydroxl groups.

Many coumarins, probably in the bound glycosidic forms, occur in relatively large quantities in a number of plant families such as Leguminosae, Rutaceae, Umbelliferae, Solanaceae and Labiatae.

In the family Leguminosae where many species used for forage occur, the coumarins assume a very considerable practical importance. For example in the sweet clovers, especially Meliotus alba and M. officinalis, coumarin lowers palatibility, and is involved in the development of a hemorrhagic disease of livestock, attributed to the production of dicumarol, in poorly cured sweet-clover hay.

Palatable, low-coumarin sweet clover varieties have been developed from hybrids of the bitter M. alba and a non-bitter, low coumarin species M. dentata which is of little economic value. Inheritance of coumarin production in the plant is relatively simple, and is governed, most probably, by two pairs of genes.

Although much progress has been made in the development of low coumarin varieties of sweet clover, they are not free of the compound. Knowledge of the compounds or compound which substitute for coumarin in the low coumarin plants might be useful to plant breeders. Confirmation and extension of biochemical sequences relative to coumarin in particular, and phenolics in general might well prove to be of significance. Using then sweet clover as the organic subject, answers were sought to the following question. If coumarin is absent in these palatable varieties, what related compound functions as its substitute? Where do the genes exert their effect along the metabolic pathway, prior to or after the

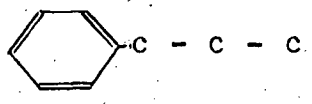
formation of coumarin? When coumarin and its chemical relatives are "fed" to non-bitter varities, in what way are these compounds metabolized?

Making use of chromatography in conjunction with other techniques of separation and identification, an attempt was made to answer these questions in the hope of adding to the knowledge of the biochemistry and genetics of coumarins in sweet clover.

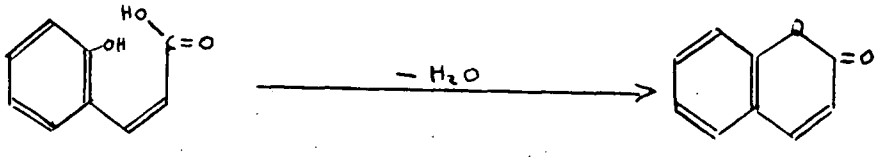
11 REVIEW OF LITERATURE

A. The nature of coumarin and its relatives.

Coumarin is one of the more important compounds belonging to the phenylpropane group, which has a nine carbon $C_6 - C_3$ skeleton, and is the most prominent structural unit among the aromatic substances in higher plants. This structure is "employed" in the chemistry of the plant in many ways.

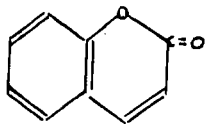


Coumarin was first isolated from tonka beans by Vogel in 1820 (90) and was initially considered to be a benzoic acid derivative. Its synthesis by Perkin (61) in 1868 from salicylaldehyde by means of the classical reaction named after him, established its relation to o-hydroxycinnamic acid. This latter compound loses a molecule of water as it cyclizes to form coumarin and the lactone ring.



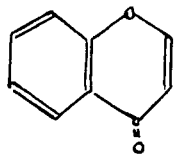
o-hydroxycinnamic acid Coumarin

Coumarin, therefore, is a heterocyclic compound belonging to a class known as benzopyrones, of which two distinct types are recognized.



Benzo α pyrone

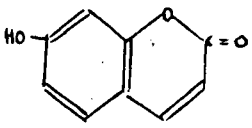
coumarin



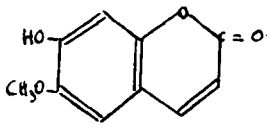
Benzo γ pyrone

chromone

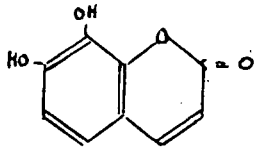
The collective term "coumarins", refers to compounds possessing the basic benzo pyrone structure; variants occur through the different hydoxyl, methoxyl or methyl substitutions. The following are representative coumarins.



umbelliferone
(7 hydroxy
coumarin)

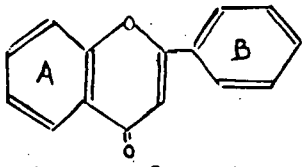


Scopoletin
(6 methoxy, 7 hydroxy
coumarin)



Daphnetin
(7,8 dihydroxy
coumarin)

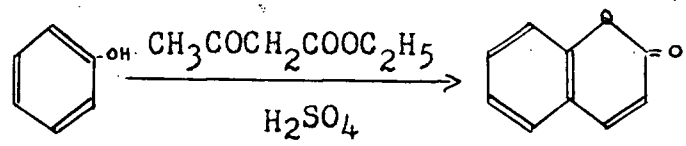
Related closely in metabolism to the coumarins are the flavanoid compounds of wide occurrence in plants and which may be regarded as "benzo" derivatives of the basic chromone structure.



Following the early pinneer work on coumarin, a certain interest was maintained, mainly because of its characteristically pleasant, sweet odor and its usefulness to the perfume and flavoring industries.

Knowledge of the chemistry of coumarin developed rapidly in the latter half of the 19th century, due mainly

to the synthetic method known as the Pechmann condensation (60), in which phenols react with B-ketonic esters in the presence of sulfuric acid.



The reaction proceeds from very simple starting materials and gives a good yield of coumarin.

Coumarins in their reactions may behave as unsaturated lactones or as substituted benzenes. The reactions which involve alkali are usually the reactions of coumaric acid. The chemistry of coumarin, therefore, consists mainly of substitution in the ring system through such well known reactions as nitration, halogenation, sulfonation, arsenation and mercuration and have been extensively reviewed by Wawzonek (92), Sethna and Shah (76) and Späth (83).

B. Coumarin in Sweet Clover.

The first person to relate the bitter taste of common sweet clover (Melilotus spp) to its coumarin content and to develop a quantitative chemical test for coumarin, was Obermayer (58) in 1913. In spite of the fact that the value of sweet clover as a soil improving crop was recognized by 1900, its use for agricultural purposes spread rather slowly

because of its low palatability. The bitter trait of the plant and its "weedy" habit made it so undesirable that certain States of America at first legislated against it. The unpopularity of sweet clover grew when it became known that feeding spoiled sweet clover hay may cause a "bleeding disease" in livestock (70, 82) due to the formation of a toxic substance, dicumarol, from coumarin (21). When such hay is eaten, dicumarol reduces the clotting power of the blood, by competing with Vitamin K, and animals may bleed to death from slight wounds or from internal hemorrhages (22, 74). It is interesting to note that in spoilage of hay of other coumarin-containing plants such as sweet vernal grass, dicumarol is produced. Ingestion of these hays also causes the hemorrhagic disease. The distribution of natural coumarins in plants, and their role in animal metabolism has been reviewed by Bradner (9).

Attempts to produce coumarin-free sweet clover varieties were initiated over 30 years ago and an extensive literature records progress. A detailed review of the subject has been presented recently by Bradner (49). Only a short review of the pertinent material is presented here. Early attempts to isolate coumarin-free plants of Melilotus alba or M Officinalis were unsuccessful (86). Kuznetsov (55) and later Brink (10, 11) reported that a certain species, found in Eastern Europe and Western Asia was entirely free of the

bitter trait. This plant was identified to be M dentata, a species of minor agricultural significance. Through interspecific crosses with the aid of grafting of the albanistic hybrids to normal plants and by subsequent back crossing to the agronomically desirable M alba (79, 12) low coumarin varieties have been developed, notably "Cumino" (36) and "Denta" (81).

The genetics involved in the inheritance of coumarin in sweet clover had attracted some interest. Among the earlier workers, Stevenson and White (85), using a colorimetic procedure, for coumarin determination, showed that the low coumarin character resulted from a single recessive gene. This conclusion was later confirmed by Horner and White (45), Rinke (67) using a slightly modified colorimetric test, concluded that high-coumarin content was inherited as a simple recessive. On the other hand, Smith (80) who successfully crossed M. dentata with M. alba, from an analysis of back cross progenies, concluded that two main genes for reduced coumarin content had been obtained from M. dentata. One of these genes was recessive to the gene for high content and the other displayed no dominance. Webster (93) using an emroyo culture technique successfully crossed high coumarin M. officinalis with low coumarin M. alba. From the analysis of the F_2 population he suggested a single major-recessive gene

for low-coumarin content was involved in his inheritance patterns. Goplen, Greenshields and Baenziger (34) have reported that two genes are concerned with the inheritance of coumarin in plants. The gene designated as "b" determines the type of coumarin present, whether free or bound, in coumarin-containing plants, while the gene symbolized as "cu" governs the production of coumarin. In the absence of "Cu", the "b" gene has no apparent effect. Thus, according to Goplen et al, in BB CuCu genotypes coumarin is free, in bb CuCu coumarin is bound and in BB cucu coumarin is absent.

C. Function of Coumarins.

In spite of the fact that the chemistry of coumarins and phenolics is well known, the functions of these compounds in the plant are still obscure (30). A notable fact is that there is still some uncertainty concerning the state in which coumarin exists in the intact plant tissue. This only emphasizes how little is known of its metabolic activities.

(i) The state of coumarin in the plant.

In early work on coumarin in sweet clover, the assumption was made that the free form of the compound predominates in the plant. Subsequent investigations demonstrated the presence of bound, in addition to free coumarin, and it was thought that both forms were normally present (75, 40) and that the relative amounts of free and bound coumarin

are gene controlled (74, 34, 35). Schaeffer et al (73) indicated that because of the uncertainty about the degree of hydrolysis of the glucoside during its isolation, ratios calculated for these two compounds would be virtually meaningless. More recently, after a reappraisal of the relationship between free and bound coumarin, Haskins and Gorz (41) substantiating the finds of Rudorf and Schwarze (71), came to the conclusion that when suitable extraction procedures are used, virtually all the coumarin is obtained in the bound form. However, Brown, (19), still more recently, presented indirect evidence that free coumarin can exist, at least ephemerally, in Hierochloa oderata.

"Bound" coumarin is a term referring to the β-glycosides of both the cis and the trans isomers of o-hydroxycinnamic acid. A controversy arose as to which isomer yields coumarin more readily on hydrolysis. The trans form would have to undergo isomerisation to the cis form for successful lactone ring formation. Brown et al. (15) earlier suggested that coumaric acid glycoside, i.e. the trans form, is also an intermediate in the formation of coumarin. Kosuge (52), on the other hand, contends that only the β-glucoside of o-coumarinic acid is identical with bound coumarin, that is the cis and not the trans isomer. The two glucosides can be distinguished by the preferential hydrolysis of the β-glucoside of coumarinic acid by β-glucosidase (52). Haskins and

Gorz (42) have also described methods of analysis which permit the cis and trans glucosides to be determined separately.

(ii) Toxicity and disease resistance.

Certain suggestions for the physiological functions of coumarins in plants have been put forward. Among the suggestions are that these compounds play an important role in disease resistance. The formation of coumarin in certain pathological conditions led Best (5) to suggest that they may be the end products of pathogen-altered pathways. Best (4) demonstrated that tobacco infected with tomato spotted wilt virus produces large amounts of scopoletin. Uritani and Hoshiya (88) suggest that the accumulation of scopoletin and umbelliferone in the sound flesh next to the injured portion of the tuber infected with Ceratostomella fimbriata, may aid in resisting the infection. Furthermore, they found the growth of C. fimbriata to be inhibited by both of these coumarins in very low dilutions. Since Coumarin per se is normally harmful to plants, the formation of a glucoside may be a mechanism of detoxification (47, 57, 63). However, it is difficult to conceive that coumarin, formed so abundantly in plants, is merely a toxic waste product, yet many phenolics applied artificially to plants are very often rapidly glycosylated (62.) Solubilization for translocation of coumarins may be an important aspect of glycoside formation

(62) and phenylpropanoids may exist in the free form only when taking part in some metabolic process, such as the formation of flavonoids or lignins.

(iii) Role in flavonoid synthesis.

The designation of flavonoid compounds by "C_6-C_3-C_6" brings out the close relationship that exists between them and the cinnamic acids and nearly related coumarins which are designated by "C_6-C_3". A number of radio--active C_6-C_3 compounds such as phenylalanine, cinnamic acid and ferulic acid, have been shown to be incorporated into the C_6(B)-C_3 portion of flavonoid compounds (24, 37, 66) examplified by the fact that caffeic acid yields quercetin in buckwheat (87.)

(iv) Role in lignin synthesis

Although the Plant can utilize a number of variously substituted C_6-C_3 units for synthesis of the different building units for lignin, the highest efficiency is shown with those compounds which have the same substitution patterns as the units themselves. A series of differently substituted p-hydroxlated cinnamic acids are extremely good precursors, and play a very important role in lignification (13, 14, 17, 95). Little is known about the succeeding steps, although it is assumed that the carboxylic acid is reduced to the corresponding alcohol prior to polymerization (18).

(v) Fate of coumarin in the plant.

In order to identify metabolic products formed from coumarin, Kosuge and Conn (53) administered coumarin $3C^{14}$ to excised shoots from matured plants of Melilotus alba, and found that only very small quantities of the isotype collected in the plant tissue. Instead the coumarin was rapidly converted to melilotyl glucoside, melilotic acid and at least two other unidentified compounds. Under the same conditions radioactive o-coumaric acid was converted primarily to o-coumaryl glucoside and in small amounts to coumarin, melilotic acid, melilotyl glucoside and at least two other unidentified coumpounds. Brown (19) fed coumarin $2C^{14}$ to sweet vernal grass and recovered extremely small amounts of the C^{14} in coumarin and in the aglycone of the cis glycoside.

(vi) Intact plant responses to coumarin.

When coumarins are applied artificially to plants they have many interesting visible effects. Coumarin per se favors cell enlargement in some plants at low concentrations, but has an inhibitory effect on growth and seed germination at high concentrations. This field has been reviewed in greater detail (9). Other than an indication that coumarin may act as an "anti-auxin" and counteract the usual growth, enhancing effect of auxins in the plant (89), these studies

throw very little light on the metabolic function of coumarin at a chemical level.

D. Biosynthesis of coumarins.

Despite the fact that the widespread distribution of coumarins in higher plants has attracted attention for many years, information concerning the origin of these compounds is very recent, but surprisingly, very much progress has already been made.

Earlier theories have been critically reviewed by Reppel (65). Among the earlier schemes was one suggested by Haworth (43) in 1942 for the formation of 7-hydroxycoumarins through the oxidation of 4-hydroxycinnamic acid. It is interesting to note that all the members of the coumarin family synthesized by plants, except coumarin itself, have a phenolic oxygen at position 7, that is <u>para</u> to the side chain (28, 44). Since the families in which coumarins are predominant are also rich in the less complex C_6-C_3 structures, suggesting close biogenetic relationship between the two classes of compounds (32), p-coumaric acid should play an important role in these syntheses. Haworth's interesting suggestion has been supported by Birch (7) and it was subsequently more generally assumed that coumarins are derived from phenyl-propanoid acids of the cinnamic acid type (6, 30). Brown (16), using radio-

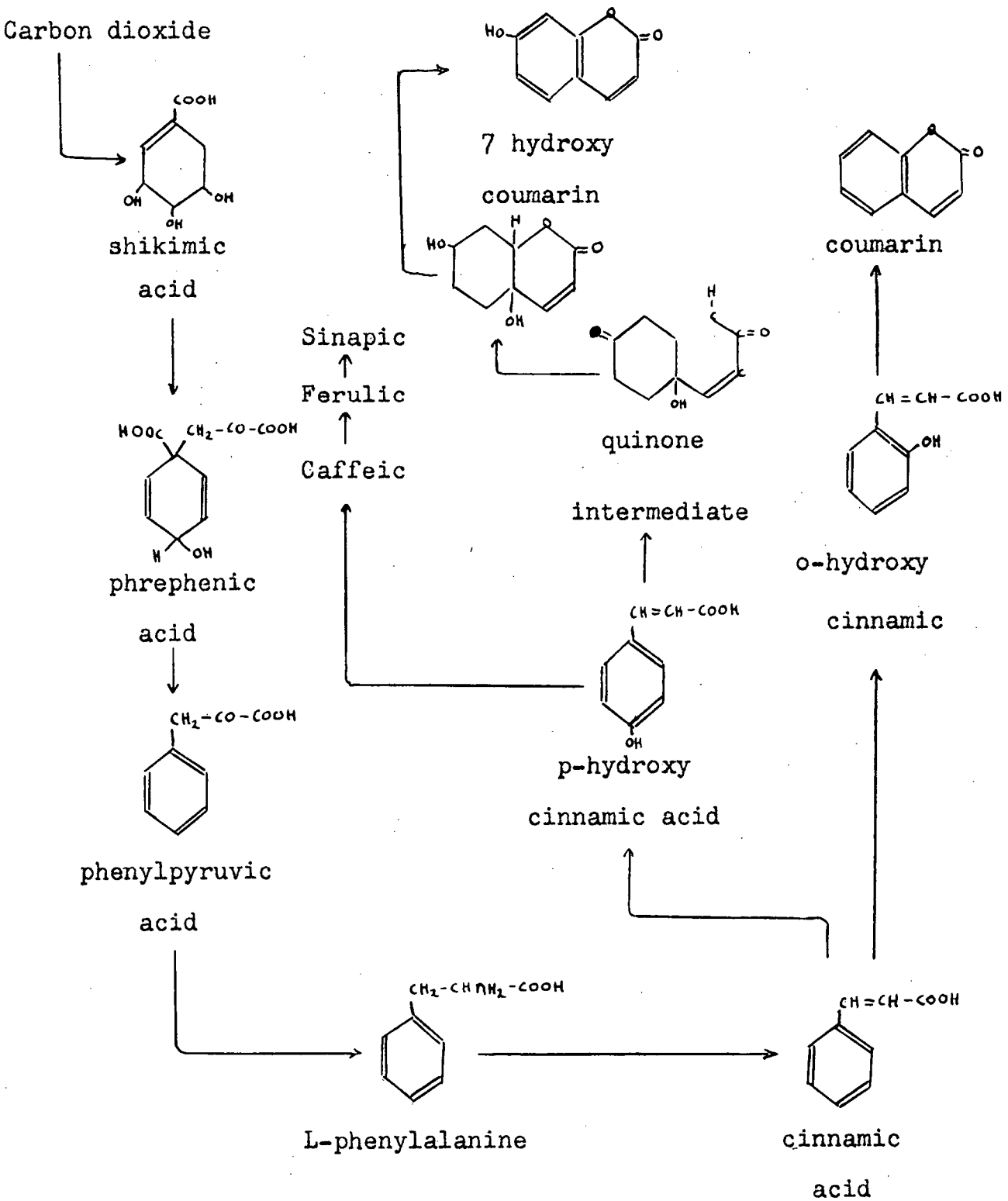
active isotopes, has shown that p-coumaric acid is over 70 times less efficient than cinnamic acid as a precursor of coumarin in Hierochloa odorata, and thus p-coumaric acid is not significantly involved in the biosynthesis of coumarin itself. It appears that in higher plants, cinnamic acid is a common precursor of all coumarins, and that ortho or para hydroxylation of this compound leads subsequently to the formation of coumarin and the 7-hydroxycoumarins respectively. Different enzyme systems may be required for the formation of the lactone ring and the 7-hydroxycoumarins. The mechanism by which the lactone ring of coumarin is formed is still not fully understood, and theories have been published which do not involve orthohydroxylation of a precursor (59). However the formation o-coumaric glucoside before the formation of coumarin supports the belief that orthohydroxylation is an essential feature of coumarin biosynthesis. In this connection it is of interest that Buhler and Mason (20) have shown that in the presence of dihydroxyfumarate, peroxidase can hydroxylate cinnamic acid in both the ortho and para positions.

Recently there has been considerable interest in the occurrence and function of the hydroxylated cinnamic acids in plants (2, 3, 25, 29, 31, 49, 56) since they are involved not only in coumarin, but also in flavonoid synthesis. These compounds fall into the phenolic category and two biosynthetic routes have been established as those usually responsible for

the formation of aromatic rings which may be ragarded as phenolic, enolic or oxygen heterocyclic. These two routes involve:

a) A poly-β-keto acid intermediate produced by head-to-rail condensation of acetate units (26, 7, 8, 28). Acetate has been shown to be a very effective source of the C_6 unit comprising ring A in flavonids (38, 48, 77, 91).

b) Formation of C_6-C_3 intermediates through the shikimic-phrephenic acid pathway (26, 27, 51). The detailed study of the biosynthesis of the coumarins almost certainly follows the shikimic-prephenic acid pathway, and not by the head-to-tail condensation of of acetate units. The following is the most accepted scheme for the biosynthesis of coumarins:


Carbon dioxide
COOH
OH
OH
OH
shikimic
acid
HOOC
CH2-CO-COOH
H
OH
phrephenic
acid
CH2-CO-COOH
phenylpyruvic
acid
CH2-CH NH2-COOH
L-phenylalanine
CH=CH-COOH
cinnamic
acid
CH=CH-COOH
OH
p-hydroxy
cinnamic acid
intermediate
quinone
Caffeic
Ferulic
Sinapic
HO
O
7 hydroxy
coumarin
H
HO
O
OH
H
O
OH
CH=CH-COOH
OH
o-hydroxy
cinnamic
O
coumarin

An interesting feature of the biosynthesis of coumarins is the known participation of glycosides in the process. When carbon-dioxide-C^{14} was administered to Hierochloa odorata, Brown (15) found that the total C^{14} in o-coumaric acid, recovered after the glucoside was hydrolyzed, reached a maximum in about 4 days. The peak in the total C^{14} of coumarin was not reached until 8 to 17 days after activation. Both showed a decline after reaching the peak, indicating that o-coumaryl glucoside, the trans form, and coumarin are both metabolic intermediates rather than end products, and that the glucoside is formed first. The specific activity of coumarin liberated by emulsin hydrolysis from the glucoside was consistently lower than that of "free" coumarin from coumarinic acid glucoside. In addition Brown (19) indicated that there is currently no tracer evidence to support the view that coumarinic acid glucoside is hydrolyzed to coumarin in vivo and suggests that coumarin is an intermediate in the formation of coumarinic acid glucoside from o-coumaryl glucoside or that coumarin and coumarinic acid glucoside are formed independently from a common precursor, the separate pool idea. Kosuge and Conn (54) in similar studies using Melilotus alba found that when radioactive o-coumaryl glucoside was fed to plants, it was converted to coumarin. They suggest that through isomerization, the cis form acts as an intermediate in the conversion. However, the turnover of the coumaric acid

glucosides to coumarin is very slow, and the same applies to melilotic acid glucoside, which may hydrolyze to melilotic acid only slightly more readily.

The activity of β-glucosidase in the biosynthesis of coumarin is also of some significance. Unlike emulsin, it shows specificity in that it hydrolyzes the cis preferentially to the trans isomer. Schaefer et al. (26) suggest that the formation of β-glucosidase in sweet clover is genetically controlled. It is of interest that "Cumino", a low coumarin variety apparently deficient in o-coumaric acid glucosides, has very high β-glucosidase activity (54.)

111 INVESTIGATIONS AND RESULTS

A Analysis of seed

An attempt was made to detect the existence of a compound which may act as a substitute for coumarin in coumarin-free sweet clover varieties, by a comparative study of extracts. A basis for the attempt lay in the assumption that the coumarin substitute was closely related to coumarin and that, like coumarin, it existed in the coumarin-free varieties in the glycosidic water soluble state. Aqueous extracts, therefore, of the seed of non-bitter M. dentata, non-bitter "Cumino" and "Denta" sweet clover varities and common, white blossom M. alba were chromatogrammed comparatively in order to establish any major differences in the occurrence of specific chemical constituents.

(i) Materials

The species and varieties of sweet clover used were as follows:

a) non--bitter, coumarin-free Melilotus dentata from A.E. Hallowell, U.S.D.A., Beltsville, Md.

b) non-bitter, coumarin-free white sweet clover, variety "Cumino" from R. Greenshields, and B.P.Golpen. Forage Laboratory, Canada Dept. of Agriculture, Saskatoon, Saskatchewan.

c) non-bitter, coumarin-free, white sweet clover, variety "Denta" from W.K.Smith, Forage and Range Research, A.R.S., C.R., U.S.D.A., University of Wisconsin, Madison, Wisconsin.

d) Canadian common, bitter, white sweet clover from

Buckerfield's Ltd., Vancouver, B.C.

e) Medicago sativa alfalfa, variety "Rhizoma" from the University of British Columbia, Vancouver, B.C.

(ii) Methods.

The extraction procedure followed somewhat that of Charaux (23) who first extracted glucosides of "coumarin" from sweet clover viz:

a) 30 g. seed was ground in a Wiley-Mill using a 40 mesh screen.

b) The ground seed was immersed in 250 ml. boiling water, and allowed to boil for 5 minutes.

c) The hot mixture was then filtered through cheese-cloth.

d) The aqueous filtrate was extracted in a separatory funnel with an equal volume of diethyl-ether for further purification, and, also, to remove any free coumarin.

e) The aqueous layer was filtered through Whatman # 1 filter Paper.

f) The extract was applied for chromatography to Whatman #3 paper, and the solvents used were: chloroform; methanol; water (5:5:1) of Jenson (50) 10% methanol, and water.

g) The chromatograms were scanned under u.v. light (both 3660°A and 2537°A max.)

The Charaux (ibid) extraction procedure included further purification steps using neutral lead acetate precipitation, followed by basic lead acetate treatment of the filtrate,

resuspention of the formed precipitate in water and bubbling H_2S gas through to form an insoluble black lead sulfide. The glycosides remained in solution after filtration or centrifugation.

Since these purification steps eliminated compounds which were not normally found in the region of the chromatograms occupied by the compounds which were of interest in this study - and were therefore of no interference - the simplified procedure described on the previous page was favored.

A few colour tests were used to assist in the identification of the spots on the chromatograms.

a) NH_3test - the chromatogram was exposed to NH_3 vapours and was immediately observed in visible light and under ultra-violet light.

b) NaOH test - a drop of 1N NaOH was applied to the spot of interest or else the sheet was sprayed with the NaOH solution.

c) HCl test - the sheet was sprayed with 1N HCl.

d) Diazotised-p-nitroaniline test - the diazonium solution was prepared as outlined by Roberts and Link (69). 10% Na_2CO_3 is first sprayed onto the filter paper in order to open any lactone ring present. It is followed by the diazonuim solution. The formation of colours is noted.

(iii) Results

Of the three glycosidic solvents used for chromatography

chloroform: methanol: water (5:5:1) proved to be most satisfactory and henceforth, unless otherwise stated, its use may be presumed.

All three varities of sweet clover as well as M. dentata yielded a very conspicuous blue non-fluorescent u.v. absorbing spot or band with an Rf.84: this spot or band is not found in the corresponding region of alfalfa. This rather heavy concentration attracted attention and further investigation was pursued.

There was good reason, from the extraction procedure used, to believe that the blue absorbing region included a glycoside or glycosides. Accordingly bands and spots from all sources were separately eluted with 10% Methanol, the volume reduced to ca. 10 ml, and boiled for 5 min. in 3N HCl. The materials were then extracted with diethyl-ether and portions of the ether solutions were chromatogrammed.

Also autolysed seed flour was extracted with ethanol. The extracts should contain both glycosides and many aglycones released by the enzymatic hydrolysis. Portions of alcoholic extracts from the separate sources were chromatogrammed.

A summary of the results is presented in Fig. 1 as a semi-diagramatic chromatogram.

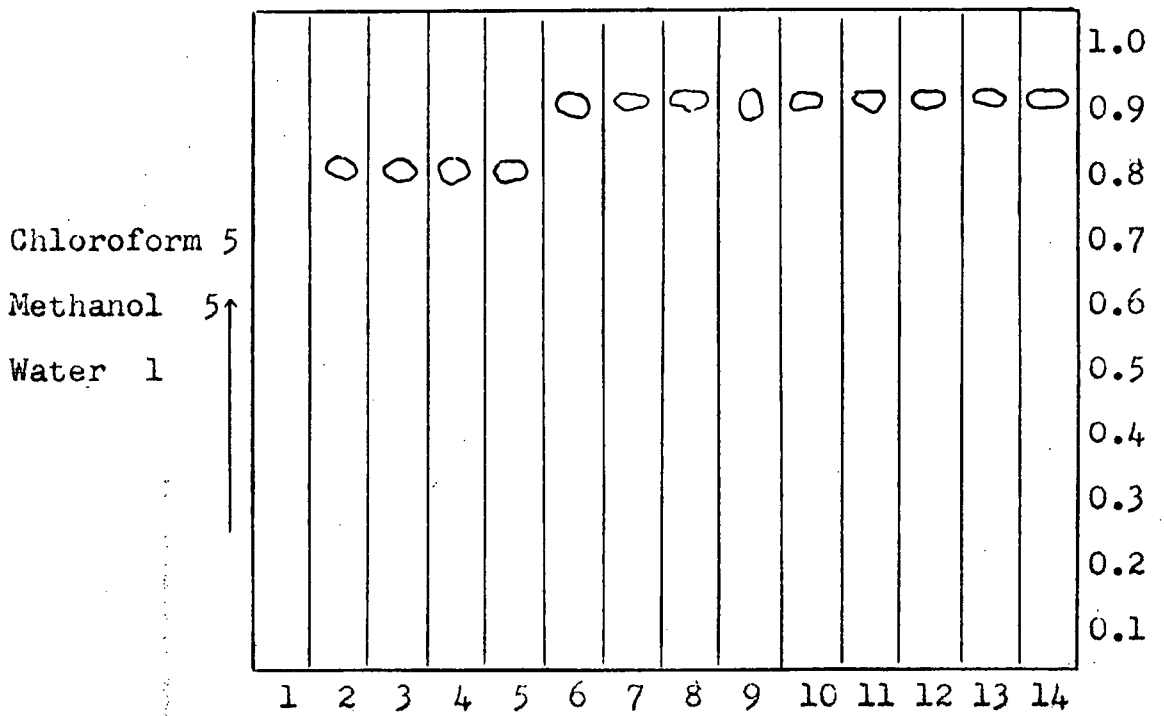


Fig. 1. Semi-diagramatic chromatogram showing conspicuous non-fluorescent, u.v. absorbing spots, of similar nature in alfalfa and bitter and non-bitter sweet clovers obtained from seed extracts, solvent: chloroform, methanol, water (5:5:1)

1. ex aqueous, alfalfa seed

2, 3, 4, 5, ex aqueous common sweet clover, "Cumino", "Denta", and M. dentata respectively.

6. Coumarin

7, 8, 9, 10 acid hydrolysis, ether extraction

11, 12, 13, 14 autolysis, ethyl alcohol extraction.

Colour reactions, supra vide, for the chromatospots

depicted in the diagram of Fig. 1 were obtained and are summarized in Table 1. The observations are consistent with the view that water extraction yields glycosides which on hydrolysis or autolysis yield coumarin. Apparently seed of both bitter M. alba and non-bitter "Cumino", "Denta" and M. dentata contain glycosides of coumaric acid.

Table 1. Colour reactions of chromatospots numbered as in Fig.1.

Spot	Spray reagent			
	p-nitroaniline	NH_3	NaOH	HCl
1.	-	-	-	-
2.	-	-	-	white fl
3.	-	-	-	"
4.	-	-	-	"
5.	-	-	-	"
6.	red	fl	bright y.fl u.v.	-
7.	red	fl.	"	-
8.	red	fl.	"	-
9.	red	fl.	"	-
10.	red	fl.	"	-
11.	red	fl.	"	-
12.	red	fl.	"	-
13.	red	fl.	"	-
14.	red	fl.	"	-

uv - ultra violet y.fl yellow fluorescence
w.fl white "

B Analysis of sugar

It was noted that in the literature reviewed, reference was made consistently to "free" and "bound" coumarin. It was tacitly accepted that bound coumarin exists in the glycosidic form and no mention was made of the sugar or sugars actually involved. In very recent literature, authors tend to be somewhat more specific and the β-glucoside of o-coumaric acid has been synthesized.

An attempt was made to determine whether or not the blue absorvent band, from the chromatogrammed seed extracts, is a glycoside; if this proved to be fact, as might be supposed, it became a matter of interest to know the identity of the associated sugar or sugars.

(i) Materials

Common white sweet clover seed and green tissue was used.

(ii) Method

The seed was extracted in the same manner as previously stated.

The green tissue was extracted as follows:

a) Fresh plant material was placed in hot 95% ethanol and allowed to boil for 5 min.

b) The contents were then transferred to a Waring Blendor where the tissue was ground in ethanol.

c) The extract was filtered, and the filtrate transferred to a flash evaporator where the alcohol was evaporated.

d) The remaining residue was then taken up in water and any insoluble matter, which included most of the chlorophyll, was filtered off. If any chlorophyll remained in the solution, it precipitated out after the filtrate stood over-night in a freezer.

e) Both the seed and the green tissue extracts were applied to Whatman #3 filter paper and developed using the descending method of chromatography with chloroform: methanol: water (5:5:1) solvent.

After elution of the blue band, hydrolysis with 3N HCl and scrubbing with an equal volume of ethyl ether, the aqueous layer was concentrated in a flash evaporator and applied to Whatman # 3 filter paper. Alongside the unknown, an aqueous solution of glucose, mannose, arabinose, xylose and galactose was spotted. The chromatogram was developed by letting ethyl acetate: pridine: water (8:2:1) solvent move for 48 hours down the sheet which has had its lower edge cut with pinking scissors to facilitate solvent run-off.

The sugar spots were detected on the paper by first spraying 3% p-anisidine hydrochloride in moist butanol (46), and then by placing the sheet for ca. 5 min. in an oven at 100°C for colour development.

(iii) Results

The same blue, coumarin yielding compound present in the seed, was found in the green tissue of bitter common sweet clover.

Following hydrolysis, the aqueous layer yielded a spot, which, on spraying with p-anisidine hydrochloride and subsequent development at 100°C turned a greenish brown colour in the same way as glucose. This colour reaction is typical of all aldohexoses. The spot also moved exactly the same distance as glucose and has thus an R_G value of 1.

The blue band, therefore, is the β-glucoside of o-coumaric acid, and is present in the green tissue and seed of bitter M. alba and in the seed but not in the green tissue of the non-bitter varieties "Cumino" and "Denta" and in M. dentata.

C. A comparative study of green shoots.

Since the seed of both the bitter and non-bitter sweet clover plants contain the β-glucoside of o-coumaric acid, it is not suited for a comparative study; attention was, therefore, turned to the green shoots of the plants in the search of a compound serving as a substitute of coumarin in non-bitter plants. This study was carried out on a comparative basis, with a somewhat similar approach to that of Alston et al (1), who, by chromatographic analyses of leaf extracts of

hybrids in the genus Baptisia demonstrated a patter of inheritance in which components of parent species were present. For a strict comparison of the chemical constituents, the collecting, extracting and chromatography of the bitter and non-bitter plants, was normally performed simultaneously under identical conditions.

(i) Soxhlet extraction.

Bitter M. alba, non-bitter variety "Cumino" and M. dentata shoots were collected, allowed to wilt for enzymatic hydrolysis of the glycosides, air dried at 40°C and ground in a Wiley Mill. The ground material was then extracted for 12 hours in a soxhlet extracor using chloroform, acetone and methanol as solvents. Since so many plant constituents are soluble in these solvents, chromatographic separation of the extracts was highly unsatisfactory and no difference could be detected.

(ii) Steam distillation

Since coumarin per se is highly volatile, steam distillation was used in an attempt to isolate a related compound with similar physical properties, which might exist in coumarin-free varieties. Aqueous extracts of common white blossom sweet clover and "Cumino" variety were obtained as described previously, for the isolation of o-coumaric glucoside from the green tissue. The aqueous extracts were hydrolyzed by

adding 10 ml. of conc. HCl to 100 ml. boiling extract, and refluxing for about 5 minutes. It was noted that on addition of the acid a flaky brown precipitate was formed. The hydrolyzed extract was steamdistilled until about 100 ml. of the distillate was collected. In both cases some insoluble material was distilled over with the water and a very strong difference in odor between the bitter and non-bitter steam distillates was noted. However, an attempt to isolate any of the volatile constituents by ethyl ether extraction, followed by chromatography proved unsuccessful.

(iii) Liquid-liquid extraction.

Hydrolyzed and unhydrolyzed aqueous extracts of bitter and non-bitter plant shoots were extracted for 12 hours in a continuous liquid-liquid extractor, using ether, benzene and petroleum ether as solvents. The extracts were applied to Whatman #3 filter paper and the solvent used for separation was descending chloroform: methanol: water (5:5:1)

Some differences, in the appearance of bands, could be detected; however, they were mostly inconsistent and rather confusing. Ether removed from the hydrolyzed non-bitter plant extracts, a dull fluorescent compound, which did not appear in the corresponding region of a chromatogram of similarly treated bitter plant extracts. Instead large quantities of coumarin were recovered from the "bitter" extracts,

The dull fluorescent compound was later found to be identical with one isolated and investigated in a manner described in the following section.

(iv) A comparison of aqueous extracts.

(a) Materials

Fresh green field grown shoots of common bitter M. alba, non-bitter strains "Cumino" and "Denta", and non-bitter species M. dentata were used.

(b) Methods

Isolation procedures.

The extraction procedure followed was exactly the same as described previously, with exceptional care being given to minimize hydrolysis of the glycosides, by placing the plants in boiling alcohol as soon as possible after they had been cut.

The two chromatography solvents used most extensively for isolation, were chloroform: methanol: water (5:5:1) and 1% acetic acid.

Bands of interest were cut out and eluted with 10% ethanol.

Analytical procedures

A number of glycosidic and phenolic solvents were used for chromatography and for Rf value measurements the chromatograms were run on Whatman #1 filter paper in a solvent-saturated atmosphere at 20°C ± 1°C.

Many colour tests are available to help in the identification of unknown heterocyclic and phenolic compounds. A number of tests which proved useful in this investigation are as follows.

a) $FeCl_3/K_3Fe(CN)_6$ - 1 gm. of each compound is dissolved in 100 ml. H_2O. If phenolic hydroxyl group is present, a blue colour appears.

b) $FeCl_3$ - Production of colour with 1% aqueous $FeCl_3$ solution, is general with all classes of poly-hydroxy flavonoid compounds (30).

c) Diazo reactions;

A few diazonium salts were used, which under proper conditions couple with aromatic compounds containing a powerfully electron-releasing group; e.g.-OH. Substitution usually occurs <u>para</u> to the activating group. Azo compounds formed by the coupling reaction are usually strongly coloured.

(i) Diazotized benzidine hydrochloride.

Solution (1): 5 gms. benzidine added to 14 ml. 37% HCl and the suspension is dissolved in 980 ml. water.

Solution (2): 10% $NaNO_2$ - freshly prepared.

Two volumes of solution (2) were mixed with 3 volumes of solution (1) and sprayed after 10 minutes.

(ii) Diazotized p-nitroaniline - This test was conducted as outlined in the analysis of the seed.

(iii) Diazotized sulfanilic acid - 0.1g diazotized sulfanilic acid was dissolved in 20 ml. 10% Na_2CO_3.

d) Mg-HCl test - This test devised by Shinoda (78) is specific for flavonoid compounds. To a small amount of the test material in aqueous solution, a small piece of Mg. ribbon was added and then a few drops of conc. HCl. The formation of colour was noted for a period of 15 min. This test was also carried out by spraying the spot on chromatography paper first with an ethanolic suspension of fine Mg. powder, immediately followed by conc. HCl.

e) NH_3 fumes - After the spot was exposed to the fumes it was observed under visible and u.v. light. This is not a very specific test but is an indication of the degree of methoxylation of flavonoid and phenolic compounds.

f) Conc. HNO_3 - The filter paper was sprayed with the concentrated acid. Some phenolic compounds dissolve in concentrated nitric acid to give a brilliant blue colour.

g) HCl - Spots or bands on the chromatography paper were exposed to HCl fumes.

h) 1% Boric acid - 1% oxalic acid. Flavonols with a free 5 hydroxy group react with boric acid in the presence

of organic mineral acids to give bright yellow or greenish yellow fluorescence.

i) Ammoniacal $AgNO_3$ - 14 gms. $AgNO_3$ were dissolved in 100 ml. H_2O and 6N NH_4OH was added until the silver oxide formed just dissolved. With this reagent, o-dihydroxy compounds give a colour reaction.

j) Vanillin-p-toluene sulfonic acid - 2 gms. vanillin and 1 g. p-toluene sulfonic acid was dissolved in 100 ml. absolute ethyl alcohol. After spraying, the chromatogram was heated in an oven at 80 - 100°C. for five to ten minutes. A positive reaction indicates the presence of a phloroglucihol or catechol type of compound.

k) Sodium molyhydate - 1 g. was dissolved in 100 ml. water. This reagent reacts with o-dihydroxy compounds.

l) $KMnO_4$ - 1% aqueous $KMnO_4$, when sprayed on paper, leaves a red film except where a redox reaction has taken place, and the area of the spot remains white or turns slightly yellow.

m) Neutral PbAc - 5% aqueous solution of neutral lead acetate.

n) Basic PbAc - 5% aqueous solution of basic lead acetate

o) Alcoholic $AlCl_3$ - 5 g. of $AlCl_3$ was dissolved in 100 ml. 95% ethanol. After spraying, the paper was allowed to dry and was then exposed to ammonia fumes.

p) Neutral $AgNO_3$ - The acetone-silver nitrate reagent was prepared by adding 50 ml. of saturated aqueous $AgNO_3$ to 1 liter of acetone and then adding just enough water to dissolve the precipitate.

Absorption spectra of compounds from bands of possible interest in the visibile and ultra violet light range were obtained using 3 ml. silica cuvettes in a Beckman DK-2 spectrophotometer; the compounds were dissolved in 95% ethanol or ethyl acetate.

For infra-red absorption spectra, 0.5 mg. of precipitated material was mixed with 0.6 g. anhydrous potassium bromide and put into a pelleting unit. The pellet was prepared using a pressure of 20,000 lbs. per square inch, and inserted in a Beckman JR-3 spectrophotometer and a recording obtained.

(c) Results.

First attempt.

When the aqueous extracts of the bitter and non-bitter plants were applied to Whatman #3 filter paper and a series of different glycosidic solvents were used for separation by the descending method, a white fluorescent band appeared consistently on chromatograms of the non-bitter plants in exactly the same region as that occupied by o--coumaric acid glucoside on the corresponding chromatograms of the bitter plants. No o-coumaric acid

glucoside was detected in the coumarin-free plants, and the white fluorescent compound appeared to be present in the M. alba varities "Cumino" and "Denta" as well as in M. dentata. While the β-glucoside of o-coumaric acid was found to be abundant in the bitter common white blossom sweet clover, the fluorescent compound appeared to be present in far smaller quantities in the coumarin-free plants. The solvents with the corresponding Rf values are represented in Table 11.

TABLE 11 - Rf values given by a number of solvents of a fluorescent compound isolated from aqueous extracts of non-bitter plants.

Solvent	Rf
chloroform: methanol: water 5 5 1	0.83
n-butanol: acetic acid: water 4 1 5	0.63
n--butanol: ethanol: water 40 11 19	0.55
ethyl acetate: acetic acid: water 9 2 2	0.62
15% acetic acid	0.90

The fluorescent band, which had been separated by descending chloroform: methanol: water (5:5:1) was eluted from Whatman #3 filter paper with 10% ethanol.

Part of the eluate was hydrolyzed by boiling under reflux for ten minutes with 3N HCl. On hydrolysis a small precipitate was formed and a very strong, characteristically pleasant odor was emitted. When NaOH was added to a portion of the hydrolyzed solution, in order to neutralise the acid, the colour changed to yellow exactly at the neutral point and the odor could no longer be detected. The compound formed on acid treatment, presumably the aglycone, was removed with ethyl ether. The aqueous layer was run for 48 hours with ethyl acetate; pyridine; water (8:2:1) solvent and sprayed with anisidine hydrochloride, however, no sugar could be detected. The ethyl ether layer was also spotted and the chromatogram developed using descending chloroform: methanol: water (5;5:1). The Rf value of the compound, which appeared as a dull light fluorescent spot on the paper, is 0.83, and is identical to the compound extracted in a continuous liquid-liquid extractor with ether, from the hydrolyzed aqueous extracts of non-bitter plants. A number of spray reagents were used and the reactions are presented in Table 111.

TABLE 111 - Colour reactions produced by a compound formed on acid treatment of a fluorescent compound isolated from non-bitter plants.

Reagent	Reaction
HCl	-
HNO_3	-
$KMnO_4$	-
$FeCl_3$	-
$AlCl_3$ NH_3 vapour	-
Na_2CO_3	-
NH_3 vapour	-
diaz.-p-introaniline	-
diaz. benzidine hydrochloride	red

The volume of the unhydrolyzed eluate was considerably reduced in a flash evaporator,(removing practically all the ethanol present), and was placed in a refrigerator. After a few days a flaky white precipitate appeared, which was separated by centrifugation and dried at 40°C. in a

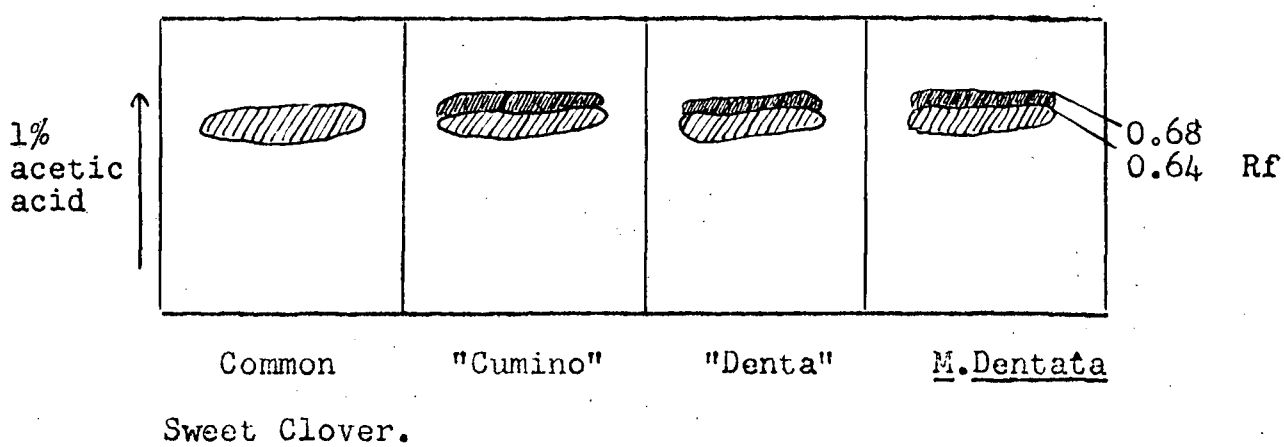
vacuum oven.

An infra-red absorption spectrum of the precipitated compound indicated that no aromatic ring is involved, and the spectrum resembled that of a fatty acid ester. Since an aliphatic compound is inconsistent with what would be expected to serve as a substitute for coumarin in non-bitter plants, further characterization was not attempted.

Second attempt

The search for a closely related compound to coumarin, in non-bitter plants, was continued on a comparative basis, using aqueous extracts. A larger variety of chromatography solvents were tested, and a very interesting separation was obtained, with 1% acetic acid, a solvent used by Brown (19).

Figure 11 - Chromatographic comparison of extracts from bitter and non-bitter plants.



A blue absorbent band common to both the bitter and non-bitter plants, differentiated into a very dark

absorbent region with a slightly higher Rf value in the coumarin-free plants. This dark region was especially conspicuous on chromatograms of extracts from young non-bitter shoots, where little interference from yellow pigments was encountered, and the compound was found to be at least as abundant as o-coumaric acid glucoside in bitter sweet clover plants. The type of resolution is best represented diagramatically (Fig.11)

The dark compound is highly unstable, even in the dry form, and will degrade turning yellow on the filter paper within three to four days, much sooner in the presence of light.

The darker upper region of the absorbent band was eluted with 10% ethanol, the volume was reduced in a flash evaporator and it was immediately stored in a freezer. On thawing the aqueous eluate, a white precipitate was formed. The precipitate was found to be only slightly soluble in water, sparingly soluble in ether, practically insoluble in o.5N HCl, and, turning yellow, was highly soluble in alkali.

For measurement of the absorption spectrum in the I.R. light range, the precipitate was washed a few times with 0.5N HCl, dried in a vacuum oven at 40°C., mixed with anhydrous KBr and pelleted. For a u.v. absorption spectrum the washed compound was dissolved in 95% ethanol. The absorption spectra are presented in Figures 111 and 1V.

For chromatography, the ethanolic solution was spotted on Whatman # 1 filter paper and the compound was run in a series of solvents. (See Table 1V) The spots were then sprayed with a number of colour test reagents.(See Table V)

TABLE 1V - Rf values of dark absorbent compound isolated from non-bitter plants.

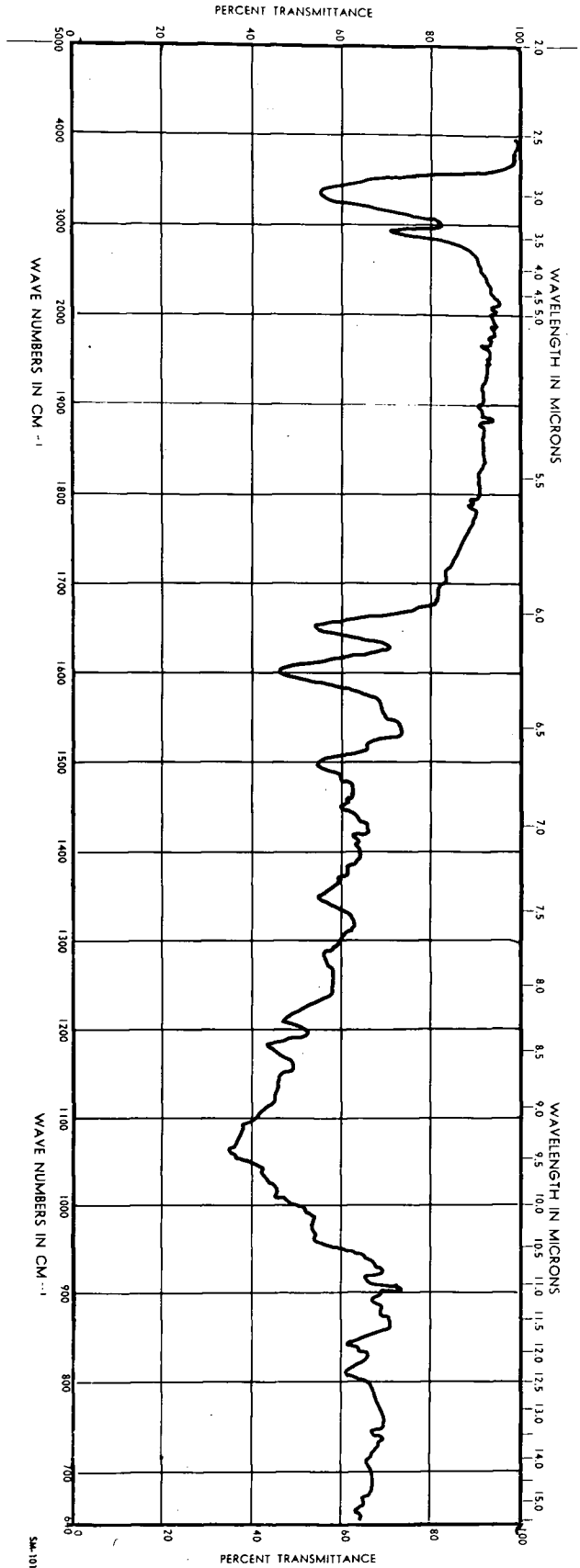
Solvent	Rf values ascending	Rf values descending
15% acetic acid	0.61	0.74
2-butanol sat. water	0.64	-
n-butanol: acetic acid: water 4 1 5	0.47	0.46
water	-	0.60
butanol: pyridine: water 10 3 3	-	0.47
n-butanol: ethanol: formic acid:water 5 1 1 1	0.26	-
chloroform, 2 propanol: acetic acid; water 1 2 1 1	0.52	-
1% acetic acid	-	0.68

TABLE V - Colour reactions with different spray reagent given by a dark absorbent compound isolated from non-bitter sweet clover plants.

Reagent	Reaction
$FeCL_3/K_3Fe(CN)_6$	blue
1% $KMnO_4$	slightly yellow-no red in region of spot
1% $FeCl_3$	light brown
p-nitroaniline	orange ½ - 1 hour after spraying
diazotized sulfanilic acid	yellow immediately
diazotized benzidine	orange immediately
NH_3 fumes	yellow immediately
$AgNO_3$ acetone	-
$AgNO_3$ ammoniacal	yellow
vanillin p-toluene sulfonic acid	light yellow
Na-molybdate	
NaOH	yellow immediately*

* On spraying the yellow spot with HCl, the colour disappeared and the spot again appeared dark blue absorbent under u.v. light.

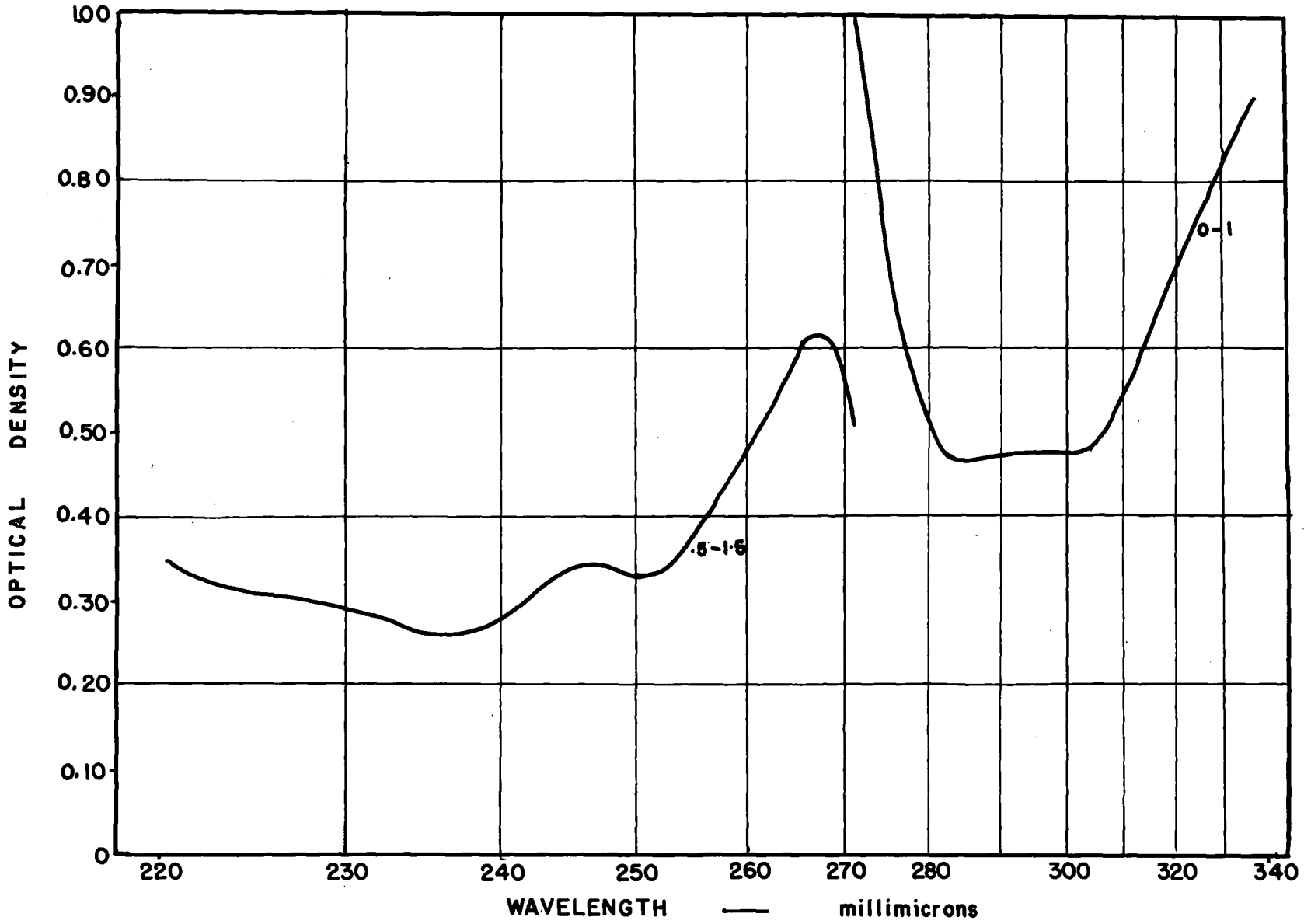
Figure III - I.R. absorption spectrum of dark absorbent compound isolated from non-bitter sweet clover plants.



NOTE CHANGE OF SCALE AT 2000 CM⁻¹

Figure IV - U.V. absorption spectrum of dark absorbent compound isolated from non-bitter sweet clover plants.

The increase in density range from 0 - 1 to 0.5 - 1.5 was necessary because of the high concentration of the solution in the u.v. region.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
OPTICAL DENSITY
220
230
240
250
260
270
280
300
320
340
WAVELENGTH —— millimicrons
·5-1·5
0-1

An attempt was made to hydrolyze the dark blue absorbing compound with emulsin and HCl. Emulsin was added to an aqueous solution of the compound, and allowed to stand for a few hours at 37°C., after which it was stored in a refrigerator for one week. A white fluorescent compound, identified as o-coumaric acid, was liberated in small quantities. On hydrolysis by boiling under reflux for ten minutes in 3N HCl, a brown precipitate was formed. The precipitate was separated by centrifugation, dried for an IR absorption spectrum, and dissolved in ethyl acetate for a u.v. and visible light range absorption spectrum, and for chromatographic application. The absorption spectra of the brown precipitate and quercetin , for comparison, are presented in Figures V, VI, VII and VIII.

Following hydrolysis, no sugar could be detected in the aqueous mother-liquor, the dark compound, therefore, does not seem to be glycosidated.

The brown precipitate gives an infra-red and visible light range absorption spectrum very similar to that of quercetin, and in addition somewhat resembles quercetin in its appearance and physical properties such as solubilities. Both quercetin and the brown compound are soluble in alkaline aqueous solutions with a strong yellow colour.

When applied to chromatography paper, the brown

compound appeared as a yellow spot, which, when run in a series of flavonoid solvents, gave Rf values distincly different to quercetin.(See Table VI). A series of spray reagents were used, and in addition, the Shinoda colour test carried out on an aqueous solution of the compound, produced a light orange colour within ten minutes. (See Table VII).

TABLE VI - Rf values of yellow spot obtained on acid treatment of a dark absorbent compound isolated from non-bitter "Cumino" plants compared to quercetin Rf values(descending.)

Solvent	Yellow spot	Quercetin
butanol: 27% acetic acid 1 1	0.94	
m.cresol:Acetic acid:water 50 2 48	0.70	.28
phenol: 73	0.76	
butanol: acetic acid: water 4 1 5	0.99	.80
water	0	0
Acetic acid:hydrochloric acid: 30 3 water 10	0.69	
1% acetic acid	0.10	.05

TABLE VII - Colours produced by different spray reagents on a yellow spot obtained on acid treatment of a dark absorbent compound isolated from non-bitter "Cumino" plants.

Reagent	Reaction
NH_3 fumes	yellow intensified
$AlCl_3$	greenish yellow
diazotized benzidine	slightly brownish red
$FeCl_3/K_3Fe(CN)_6$	blue
$FeCl_3$	-
p-nitroaniline	greenish yellow
diazotized sulfanilic acid	yellowish brown
$AgNO_3$ acetone	-
Mg-HCl	very slightly orange
PbAc. basic	yellow intensified
PbAc. neutral	yellow intensified
HNO_3	-
boric acid	-
HCl fumes	increased fluorescent under U.V.
vanillin p-toluene sulfonic acid	-

Figure V - I.R. absorption spectrum of yellow compound obtained on acid treatment of a dark absorbent compound isolated from non-bitter "Cumino" plants.

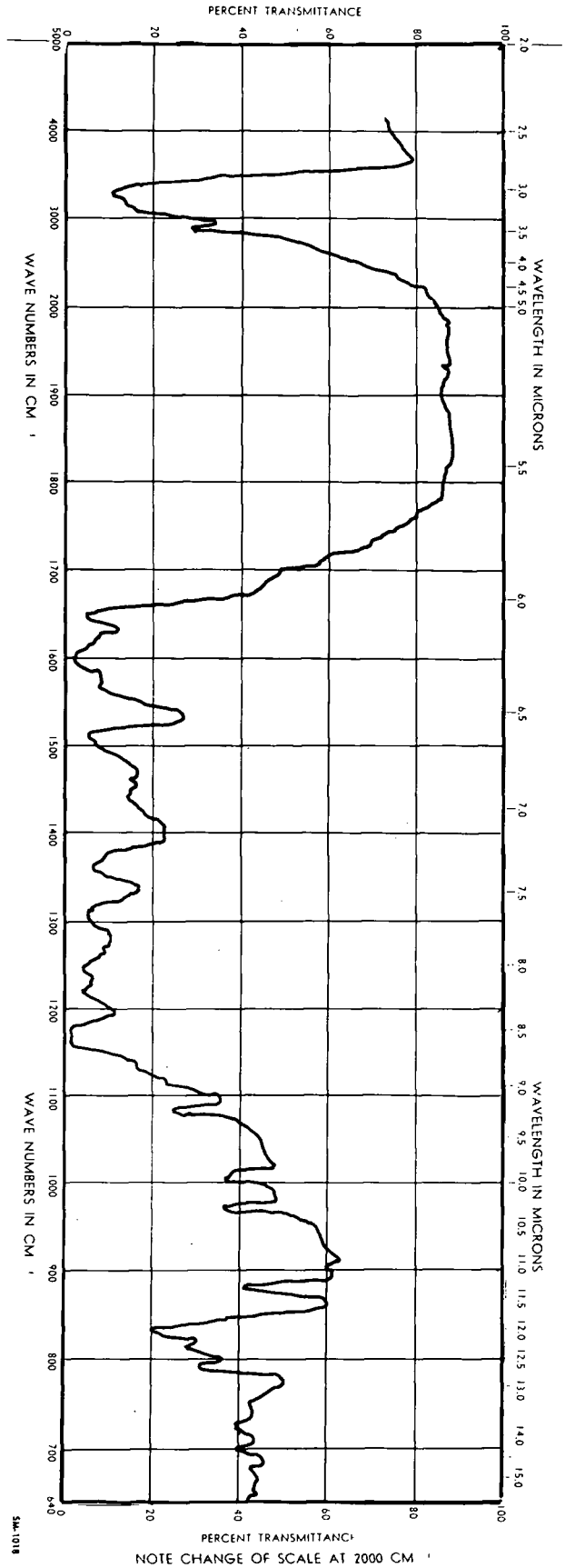

PERCENT TRANSMITTANCE
WAVELENGTH IN MICRONS
WAVE NUMBERS IN CM⁻¹
WAVELENGTH IN MICRONS
WAVE NUMBERS IN CM⁻¹
PERCENT TRANSMITTANCE
NOTE CHANGE OF SCALE AT 2000 CM⁻¹
SM-1018

Figure VI - I.R. absorption spectrum of quercetin

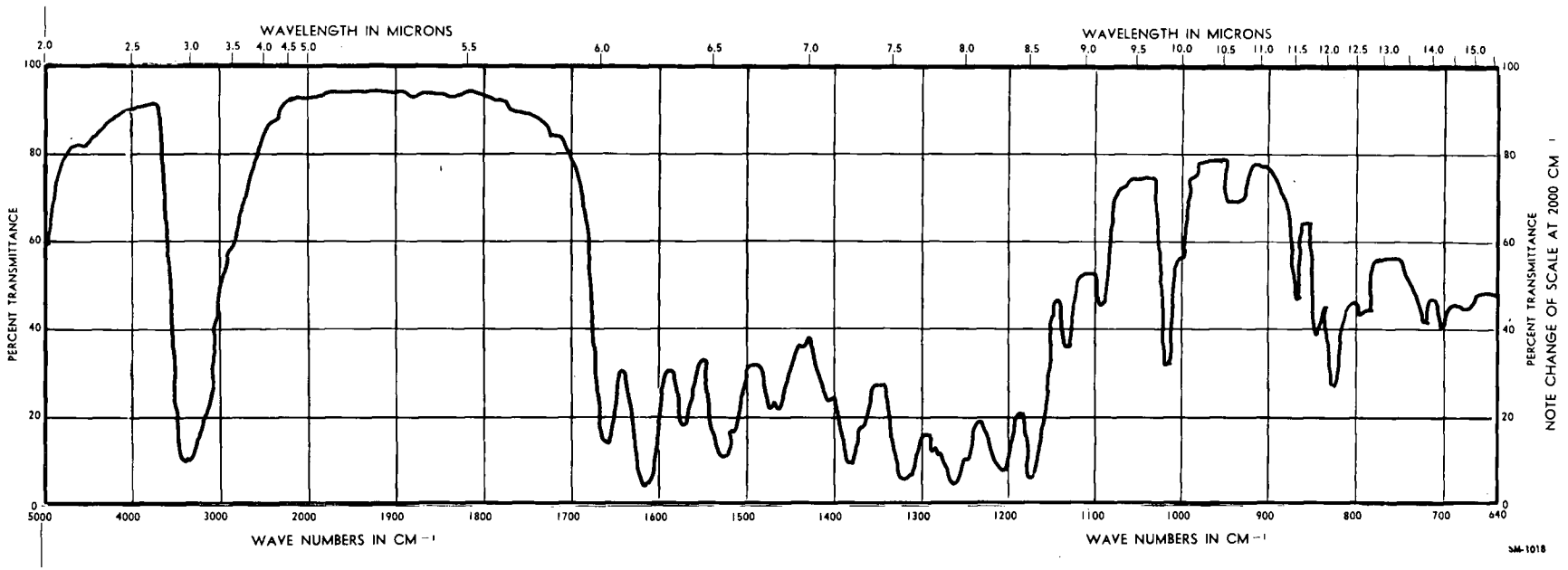

WAVELENGTH IN MICRONS
2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 14.0 15.0
WAVELENGTH IN MICRONS
PERCENT TRANSMITTANCE
100 80 60 40 20 0
5000 4000 3000 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 640
WAVE NUMBERS IN CM⁻¹
WAVE NUMBERS IN CM⁻¹
PERCENT TRANSMITTANCE
NOTE CHANGE OF SCALE AT 2000 CM⁻¹
3M-1018

Figure VII - U.V. absorption spectrum of yellow compound obtained on acid treatment of a dark absorbent compound isolated from non-bitter "Cumino" plants

The increase in density range from 0 - 1 to .5 - 1.5 was necessary because of the high concentration of the solution in the u.v.region.

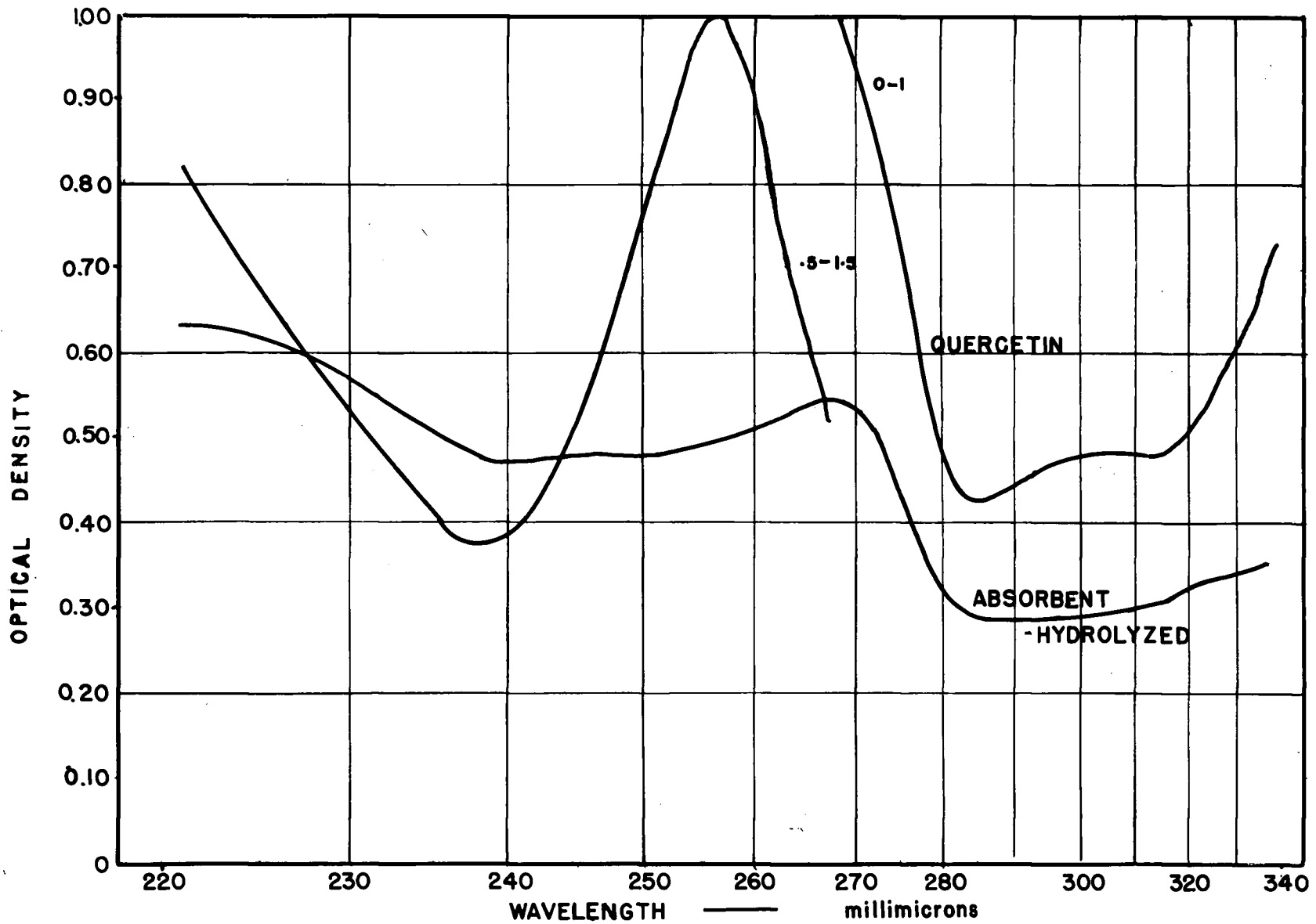
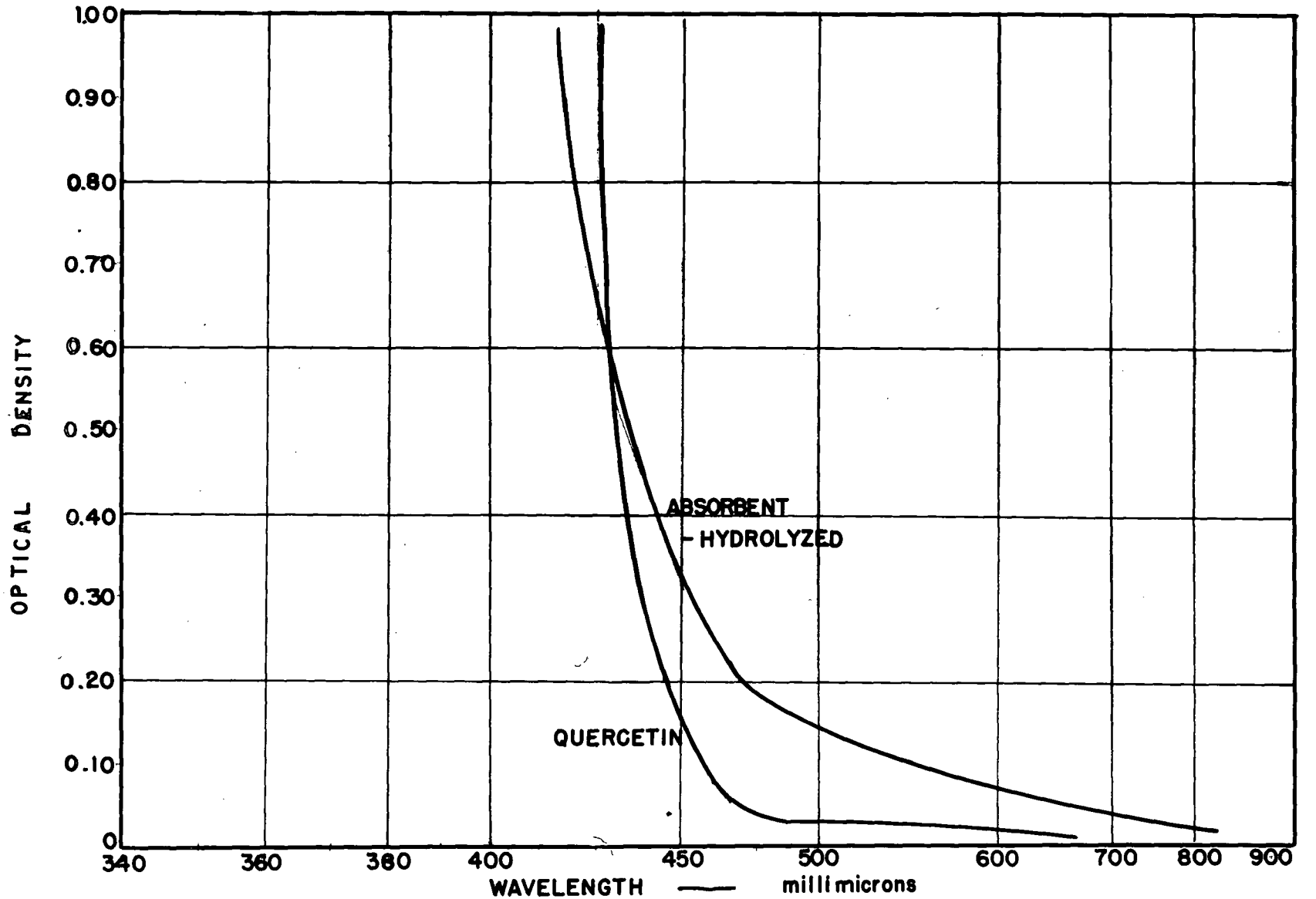

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
OPTICAL DENSITY
220
230
240
250
260
270
280
300
320
340
WAVELENGTH —— millimicrons
0-1
·5-1·5
QUERCETIN
ABSORBENT
-HYDROLYZED

Figure VIII - Absorption spectrum in the visible light range of a yellow compound obtained on acid treatment of a dark absorbent compound isolated from non-bitter "Cumino" plants.


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
OPTICAL DENSITY
ABSORBENT
- HYDROLYZED
QUERCETIN
340
360
380
400
450
500
600
700
800
900
WAVELENGTH —— millimicrons

Investigations carried out so far suggest that both the u.v. absorbent compound found in non-bitter plants and also the brown precipitate formed on acid treatment, are flavonoids. An attempt was made to break the acylated linkage and split the A ring from the B ring. The B ring, theoretically should be closely involved in the metabolic pathways responsible for phenylpropane compounds, and thus is closely related to coumarin in bitter plants. An equal volume of 4N NaOH was added to a solution of the absorbent compound, and a constant stream of nitrogen was bubbled through, while the solution was kept in the dark for two hours. The alkali was neutralized with 4N HCl, and the whole aqueous mixture extracted with ethyl acetate and chromatogrammed. Due to unsatisfactory results, the procedure was repeated with a slight modification in that the alkali solution was heated for one hour. The ethyl acetate extract and the water layer was applied to Whatman # 1 filter paper and developed in descending 15% acetic acid, 1N NaOH and 1N HCl solvents. Rather confusing and inconsistent results were attributed to impurities that may have been included on elution of the original band. Due to the instability of the dark compound, it could not be further purified by repeated chromatography and elution. As previously described, purification of the sample for absorption spectra was achieved by precipitation, followed by repeated washing

of the precipitate in a weak acid with some loss of material. The problem posed by this method, is that rather large quantities are required for precipitation; these are not easily obtained by ordinary paper chromatography.

Since the solubility of the absorbent compound in different solvents had been determined, an attempt was made to extract and precipitate the compound from non-bitter plants, without the use of chromatography. The alcoholic extract of the shoots was evaporated to dryness in a flash evaporator and the residue was taken up in 1N NaOH, so as to dissolve all of the dark absorbent compound. The solution was then neutralized with an equi-equivalent amount of acid. The final solution was made slightly acidic and stored in the refrigerator, in the hope that the compound would precipitate out. After two weeks a precipitate formed; however, the dark compound was heavily contaminated with other plant constituents.

D Feeding Experiments.

The investigation pursued so far involved a comparative study of the normal products of metabolism in bitter and non-bitter plants. In order to throw more light on the synthesis of phenylpropane compounds in coumarin-free plants, an attempt was made to determine in what way coumarin

and its immediate precursors, o-coumaric acid and cinnamic acid, are metabolized when applied to normally coumarin-free sweet clover plants.

(i) Materials

Both seed and field grown plants of bitter M. alba and non-bitter varieties "Cumino" and "Denta" and non-bitter species M. dentata were used.

For seed a saturated solution of coumarin, o-coumaric acid and cinnamic acid in 1% ethanol was used. On feeding the shoots a saturated solution of coumarin, o- and p-coumaric acids and cinnamic acid in 2% ethanol was used.

(ii) Method

(a) Seed

The seeds were scarified with sand paper, soaked for one minute in 6% H_2O_2, then germinated and permitted to grow for six days on filter paper saturated with the different solutions. (Fig. IX.

(b) Shoots

The method used was basically that of Roach and Roberts (68). Leaves were removed from shoot tips and these were cut back about a half-inch. The ends were then inserted for four days in vials filled with the solution. Since the tip of the shoot turned chlorotic and wilted within about sixteen hours after insertion in o- and p-coumaric acid, it was cut back about a half-inch every day. (Fig. X).

Figure 1X - Technique for feeding seed

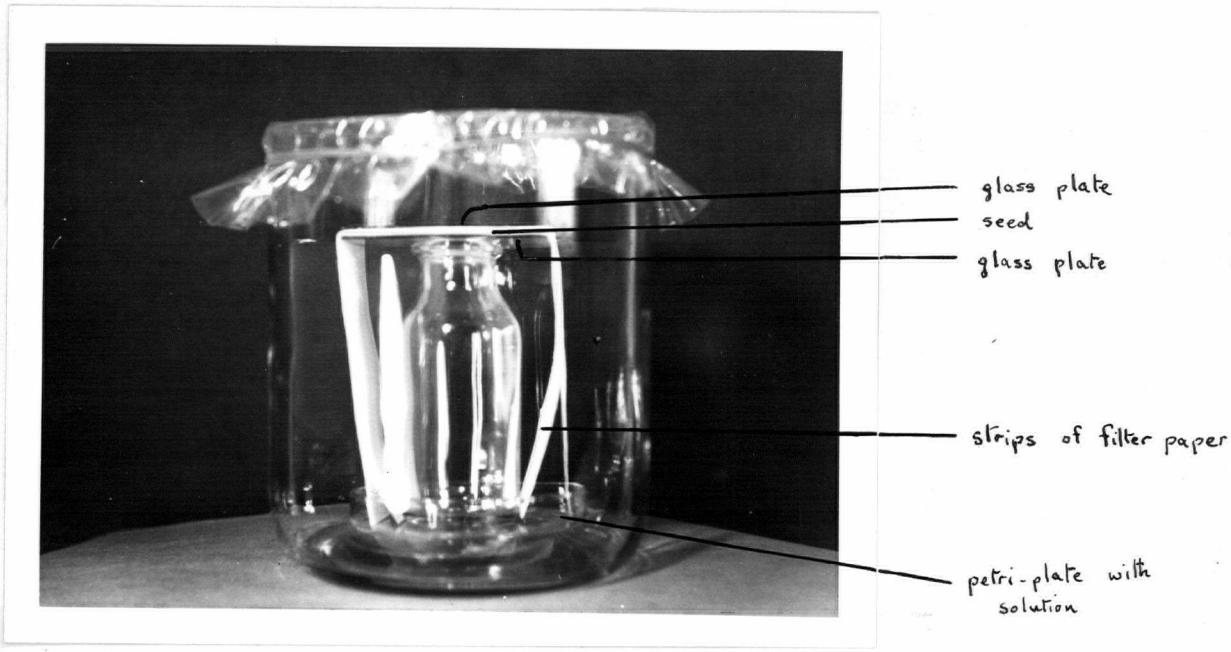


Figure X - Technique for feeding shoots.

On the fourth day the shoots were cut eight to ten inches from the tip and extracted in the manner described previously.

The following is a slightly different extraction procedure used with the seedlings:

a) The harvested seedlings were placed in a beaker of distilled water so as to remove any compound from the surface.

b) The complete seedlings were then ground in a mortar with ca. 15 ml. 95% EtOH.

c) The extract was filtered and the mortar rinsed with a further 5 ml. 95% EtOH.

d) The residue on the filter paper was washed with 10 ml. 80% EtOH.

e) The combined filtrate was concentrated to a volume of ca. 5 ml. in a flash evaporator, so as to precipitate the chlorophyll and other fat--soluble material.

f) The concentrated extract was passed over a celite column and washed down with a 80% EtOH.

g) The volume was once again reduced to ca. 5 ml.

Plants used as controls received exactly the same treatment, with the only difference that no compound was dissolved in the 1% ethanol, in the case of the seed; and in the 2% ethanol in the case of the shoots.

The extracts, both treated and control, were applied to Whatman #1 filter paper. Both ascending and descending chromatography, and phenolic and phenolic glycoside solvents were employed.

(iii) Results

(a) Coumarin

Coumarin first of several compounds used in the feeding trials, proved to be an extremely difficult compound to work with. It inhibited germination to such an extent, even when used in very dilute solutions, that the extracts of the few seedlings which were recovered, were insufficient for any conclusive results. Similarly, when feeding the shoots with coumarin it was found that within a day the stem became chlorotic and shrivelled as far as eight inches from the inserted tip.

"Free" coumarin seems to be equally toxic to the high-coumarin and to the low-coumarin plants.

(b) o-Coumaric Acid

Attention was next given to the metabolism of the applied o-coumaric acid.

All shoot and seedling extracts following o-coumaric acid feedings, when applied to Whatman #1 filter paper, yielded a distinct white fluorescent band at an Rf value of 0.61, when the chromatogram was developed with descending 1% acetic acid. Extracts of control plants did not give this band in the corresponding region of the chromatogram. The difference was very conspicuous.

In addition, small quantities of the β-glucoside of o-coumaric acid were detected in the non-bitter varieties which had been fed; of cource in the bitter varieties there was a great deal of the o-coumaric acid glycoside present. The difference is particularly conspicuous in shoot extracts, since absolutely no trace of the o-coumaric glucoside was found to be present in the control plants. The fact that shoots of non-bitter plants can be induced to form very small quantities of the o-coumaric acid glucoside is rather interesting.

The fluorescent band supra vide was eluted using 10% ethanol, and the eluate was concentrated in a flash evaporator. The fluorescent compound was then hydrolyzed by adding 0.5 mg. emulsin to 5 ml. of the eluate and letting it stand at 37°C. for ten minutes. In addition, acid hydrolysis as described previously was also carried out, and in both cases the same aglycone was liberated. The sugar attached was identified as glucose. Rf values and colour reactions for both the glucoside and the aglycone are presented in Tables VIII, IX and X.

TABLE V111 - Rf values for the aglycone of a white u.v. fluorescent glucoside, obtained on feeding of o-coumaric acid to bitter and non-bitter sweet clover plants. All chromatograms run at 20°C. ± 1°C.

Solvent	Rf values ascending	Rf values descending
n butanol: acetic acid: water 4 1 5	0.86	0.87
15% acetic acid	0.46	0.51
n-butanol: ethanol: formic acid:water 5 1 1 1	0.89	
chloroform: 2-propanol: acetic acid: 1 2 1 water 1	0.85	
2-butanol sat. water	0.90	
water	-	0.35
n-butanol: 27% acetic acid 1 1	-	0.89
ethyl acetate: acetic acid: water		0.88
acetic acid: hydrochloric acid: 30 3 water 10	-	0.86

TABLE 1X - Rf values for a u.v. fluorescent glucoside obtained on feeding of o-coumaric acid to bitter and non-bitter sweet clover plants. All chromatograms prepared by descending method at 20°C. ± 1°C.

Solvent	Rf value
n- butanol: pyridine: water 6 4 3	0.58
n--butanol: ether: water 40 11 19	0.58
ethyl acetate: acetic acid:water 9 2 2	0.47
1% acetic acid	0.61
chloroform: methanol: water 5 5 1	0.70
n- butanol: acetic acid: water 4 1 5	0.50
15% acetic acid	0.68
n-butanol: pyridine: water 10 3 3	0.35
chloroform: 2-propanol: acetic acid: water 1 2 1 1	0.54

TABLE X - Colour reactions produced with different spray reagents by aglycone of the fluorescent glucoside obtained on feeding of o-coumaric acid to bitter and non-bitter sweet clover plants.

Reagent	Colour reaction
$FeCl_3/K_3Fe(CN)_6$	blue
$AgNO_3$ Acetone reagent	light brown 2 - 3 hrs.
$AgNO_3$ Ammoniacal	reddish brown
diazotized sulfanilic acid	-
p-nitroaniline	-
1% $FeCl_3$	-
1% $KMnO_4$	faint yellow

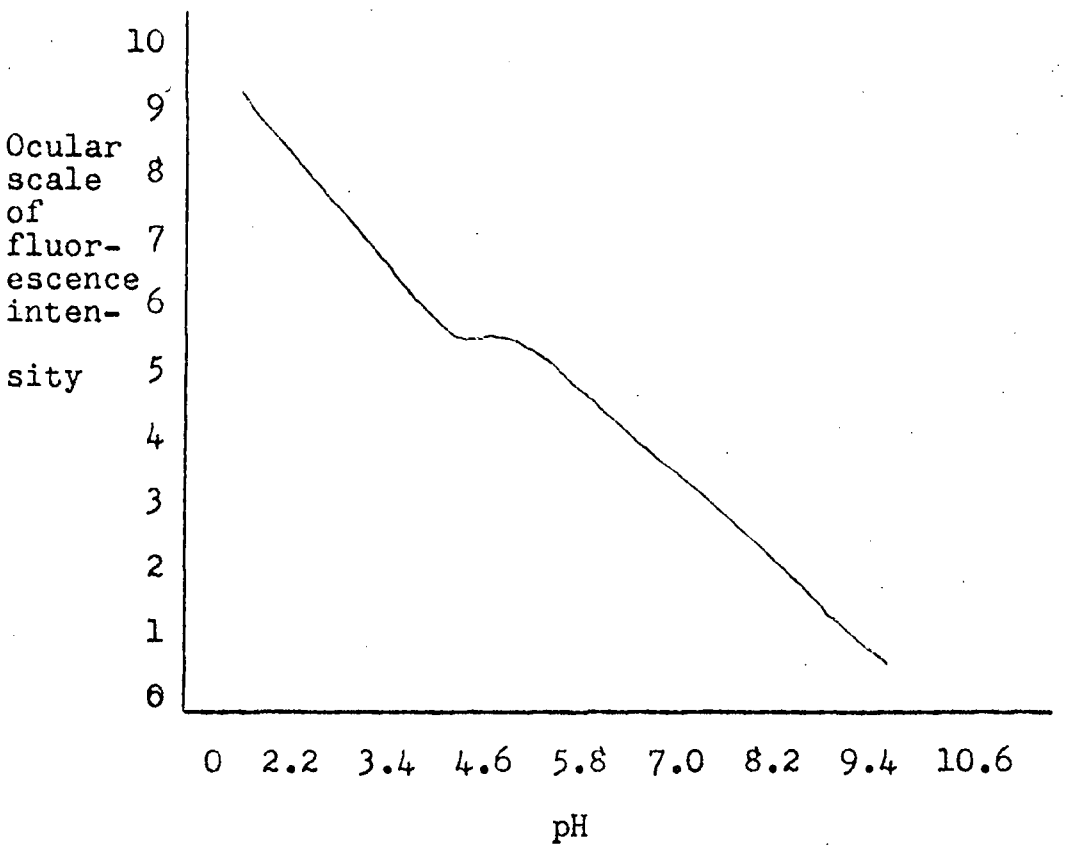
The reactions indicate that the compound has a phenolic hydroxyl group and that the position para to the hydroxyl group is substituted since no coupling with the diazonium reagent takes place.

Many phenolic compounds have Rf values similar to those indicated in Table VIII . A number of controls were spotted alongside the unknown on the chromatogram and run in a series of solvents. Some compounds with extremely similar Rf values, but in no case identical are ferulic, isoferulic, caffeic and sinapic acids, o-, m- and p-coumaric

acids, 34 dimethory, 2 hydroxy, 3 methoxy, 2 hydroxy, 5 methoxy cinnamic acids, herniarin, umbelliferone and scopoletin. Using pH fluorescence curves, Goodwin and Kavanagh characterized many coumarins and relatives (33)

In order to obtain an indication of the intensity of fluorescence at different pH values, the aglycone was sprayed on chromatography paper with a series of buffers. Since no fluoremetric instrument was available, the fluorescence was observed rivually under u.v. light and numbers were allotted according to the intensity. The approximate pH fluorescence curve of the unknown compound is given in Fig. 111

Figure XI - Relative intensities of fluorescence at different pH values of an unknown compound obtained on feeding trials.



In addition an absorption spectrum in the u.v. light range was obtained using a Beckman D.U. spectrophotometer. Fig. X11).

(c) p-Coumaric acid

p-Coumaric acid was fed to both bitter and non-bitter plant shoots in a further attempt to determine the type of hydroxylation or methoxylation that takes place in the plant when cinnamic acid relatives are fed. A few synthetic p-hydroxylated cinnamic acid relatives were available for comparative purposes. It was hoped that by feeding of p-coumaric acid more light might be shed on the nature of the phenolic aglycone isolated from plants fed with o-coumaric acid.

The feeding, extracting and chromatography procedures were exactly the same as followed previously. Extracts from both bitter and non-bitter plants yielded a very faintly visible band on chromatographic separation with 1% acetic acid solvent. This band fluoresced a bright blue under u.v. light following sparying with 1N NaOH. To facilitate in the marking of the band for elution, the edges of the sheets were sprayed with NaOH. The strips were cut out, eluted with 10% ethanol and acid hydrolyzed. The ethyl ether layer, following extraction, was applied to Whatman #1 filter paper alongside a series of substituted cinnamic acids. A number of bands were

liberated on hydrolysis, probably due to impurities which may have been included with the rather complicated manner of marking out the band for elution. However, the Rf values and the colour reactions of one rather prominent spot gave every indication that the compound is sinapic acid. (Table

TABLE X1 - Rf values and colour reactions for sinapic acid and unknown aglycone isolated from plants fed with p-coumaric acid. Chromatograms prepared by ascending method at 20°C ± 1°C.

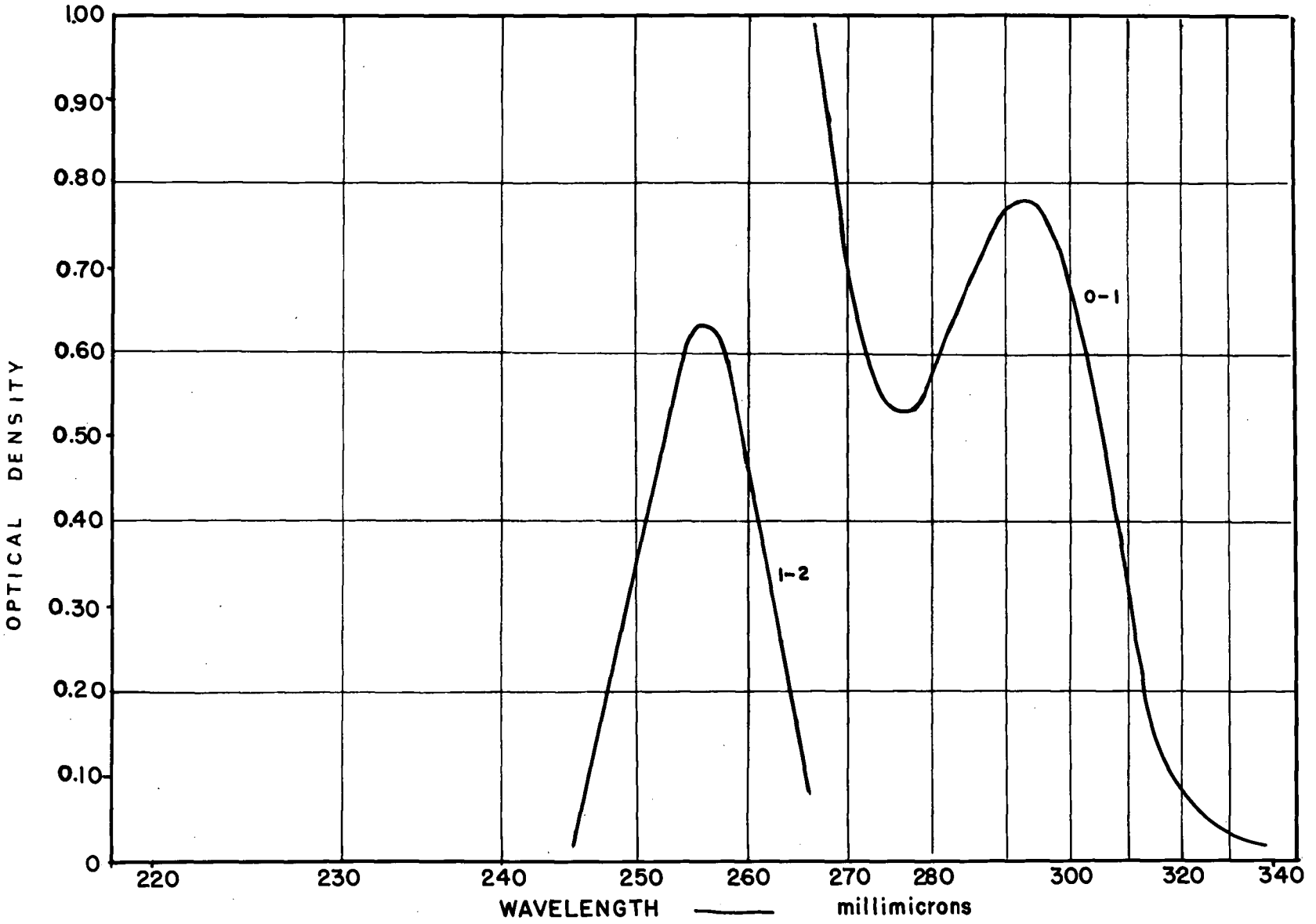
Solvent	Rf values Aglycone	Rf values Sinapic acid
n-butanol: acetic acid: water 4 1 5	0.87	0.87
n-butanol: ethanol:formic acid: water 5 1 1 1	0.92	0.92
15% acetic acid	0.50	0.50
Reagent	Colour Reaction	
$FeCl_3/K_3Fe(CN)_6$	blue	blue
p-nitroaniline	-	-
neutral $AgNO_3$ in acetone	light brown 2 hrs.	light brown 2 hrs.

(d) Cinnamic acid

Cinnamic acid was also applied to both seeds and shoots of bitter and non-bitter varieties and species. However, on chromatographic examination of extracts from treated and control plants, no difference could be detected.

Figure XII - U.V. absorption spectrum of aglycone of the fluorescent glucoside obtained on feeding o-coumaric acid to bitter and non-bitter sweet clover plants.

The increase in density range from o - 1 to .5 - 1.5 was necessary because of the high concentration of the solution in the u.v. region


1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
OPTICAL DENSITY
220
230
240
250
260
270
280
300
320
340
WAVELENGTH —— millimicrons
0-1
1-2

IV. DISCUSSION & CONCLUSION

Discussion

As early as 1896, Wilson, (94) in his book "The Cell in Development and Inheritance" stated that inheritance is the recurrence, in successive generations, of like forms of metabolism. Genetics, as a field of study, came to existence at the turn of the century , and for a number of decades, geneticists overlooked this metabolic aspect. They dealt to a great extent with genes and characters, but gave very little attention to the problems of how genes act in the metabolism and development of organisms. Geneticists were concerned primarily with how genes are inherited rather than with how they act, or the effects of their action.

More recently, a slightly different approach led to a reconsideration of the basic tenets of classical genetics and also the earlier notion or concept of the gene. Genetics, today, is approaching a time when it can specify what a given gene or a set of closely linked genes is doing, as accurately as biochemistry can specify what an enzyme such as pepsin is doing, even if the precise details of what is happening in either case is not known. Biochemical genetics may be approached from two directions. One is the study of the chemical structure of the genetic material itself, namely D.N.A. The other approach, from an opposite direction, involves the study of the metabolic pathways controlled by

genes. In higher plants, there are few examples of the latter approach and relatively little is known of gene controlled processes. Studies of gene-controlled reactions in the plant may be useful in ordinary genetics and biochemistry, and also may be of considerable practical significance, especially in breeding programs.

The production of low-coumarin varieties of M. alba, involved back-crossing for five to seven generations, to the bitter common sweet clover parent. With each generation low - coumarin plants were selected. Thus, varities such as "Cumino" and "Denta" have, very probably, a genic complement the same as bitter M. alba, excepting for a one or two gene difference involved in the production of coumaric acid glucoside (34). Since the genetic difference is rather simple, it was assumed that the pathway, normally leading to the production of glucoside of o-coumaric acid in the bitter plants, might be only slightly altered in the non-bitter plants, and that the end product might be closely related to o-coumaric acid glucoside.

The application of paper chromatography to genetic research is relatively recent. Because of its simplicity, versatility and cheapness, the technique has been effectively used in many kinds of biological investigations, and an extensive biochemical background is not essential for its successful application. For an example, Hadorn and Mitchell (39) working with the sepia mutant of Drosophila melanogaster

chromatographically isolated a yellow pigment from the mutant, and showed that its chemical structure was extremely closely related to that of the red pigment, normally found in wild type flies. The technique of feeding, in conjunction with chromatography, of chemical components, especially when "marked" with radio-active tracers, is useful in the study of the metabolism of these components in a plant.

With this background an attempt was made to determine what compound substitutes for coumarin in the non-bitter plants, and also where the gene for low-coumarin exerts its effects along the shikimic-prephenic acid pathway.

A comparative study of the seed of sweet clover revealed that the β -glucoside of o-coumaric acid is present in both the bitter and non-bitter plants. This would suggest that a development aspect is also involved in the pathways concerned with the synthesis of phenylpropanoid compounds. It appears that the normally "leaky" genes for low-coumarin lose "control" in the seed, which results in the appearance in the tissue of o-coumaric acid glucoside. The β -glucoside of o-coumaryl and o-coumaric acids could not be differentiated; however, on hydrolysis the glucosidic band could not be detected on the chromatograms, but only coumarin which appeared in quantity.

A search for a compound that substitutes for coumarin in the shoots of non-bitter plants, was based on the

supposition that the compound is closely related to coumarin itself, with the difference based only on possible substitutions in the benzene ring, and thus has very similar properties such as solubilities and reaction to stray reagents. Since coumarin exists in the bound glycosidic form, it was assumed that its substitute in the non-bitter plants would also be glycosidated, and thus water soluble. This search proved to be an extremely lengthy task, and was only complicated by the fact that there are no coumarin specific reagents available, in spite of the extensive knowledge of coumarin chemistry. No close relative to coumarin could be found in abundance in the green shoots of non-bitter plants, but instead a compound was isolated from the shoots of all the non-bitter plants, which appears to be a flavonoid, with properties somewhat similar to quercetin. Many workers have already demonstrated that phenylpropanoid compounds, of which coumaric acid is a representative, act as flavonoid precursors (91).

Cinnamic acid when fed to sweet clover, is apparently taken up in the metabolism and "disappears" in both the bitter and non-bitter plants. Together with the isolation of a flavonoid type compound from non-bitter plants, the results from cinnamic acid feeding, would suggest that the compound is a common precursor in both the bitter and non-bitter plants. In the bitter plants, the cinnamic acid would undergo <u>ortho</u> hydroxylation and then glycosidation to form the β-glucoside of o-coumaric acid. In the non-bitter plants, the cinnamic

acid might be incorporated to form ring B of a flavonoid compound. The ring A of the flavonoid compound would be formed by head-to-tail condensation of acetate units.

The genes for low and high coumarin content might exert their effect just after the formation of cinnamic acid, or even earlier. It appears that the genes, instead of bringing about the small change by a hydroxylation or methoxylation in the benzene ring of coumaric acid, carry the synthesis with a slight alteration "farther" on the pathway, with the formation of a flavonoid; also glycosidation is hindered.

Feeding of o-coumaric acid led not to the production of the β-glucoside of o-coumaric acid, as reported by Kosuge and Conn (54), but instead significant quantities of a glycoside of an unidentified phenolic compound, resembling isoferulic acid. In addition Kosuge and Conn, ibid, did not report any toxic effects which were experienced in this study on feeding o- and p-coumaric acid and coumarin to both bitter and non-bitter plants. The formation of an unidentified phenolic glucoside following o-coumaric acid feeding in all the plants fed, irrespective of variety or species, only further supports the supposition that the genetic difference between low- and high-coumarin strains is initiated "earlier" in the shikimic acid-phenylpropanoid pathway. However, since no radio-active tracers were used, it cannot be stated with certainty that the phenolic

glucoside produced on feeding of o-coumaric acid is due to a disruption of a normal metabolic pathway, or, as a result of the metabolism of the fed compound.

Feeding of p-coumaric acid led to the production, of a glycoside of sinapic acid in all the plants to which it was applied. The formation of sinapic acid involved a 3,5 methoxylation of the p-coumaric acid. A similar methoxylation of the o-coumaric acid would produce 2 hydroxy, 3,5 methoxy cinnamic acid. Unfortunately no synthetic sample of this compound was available for comparison with the unidentified aglycone from o-coumaric acid feeding.

Conclusions

1. The β-glucoside of o-coumaric acid, which on hydrolysis yields coumarin, was found to be present in the seed of both the non-bitter varieties "Cumino" and "Denta", in the seed of non--bitter M. dentata, and in the bitter common M. alba.
2. The search for a compound that substitutes for coumarin in the shoots of non-bitter plants, led to the isolation of a compound from non-bitter plants, which appears to be a flavonoid, and resembles quercetin in many respects. This compound did not appear in the green tissues of bitter sweet clover.
3. Feeding of o-coumaric acid to shoots of bitter and non-bitter plants, led to the formation of a phenolic glucoside in all the plants, irrespective of varieties or species. Hydrolysis yielded an aglycone, similar to isoferulic acid. The aglycone might be 2 hydroxy, 3.5 methoxy cinnamic acid.
4. Feeding of p-coumaric acid to shoots of bitter and non-bitter plants, led to the formation of sinapic acid glycoside.
5. When cinnamic acid was fed to plants, no difference in the metabolic products could be detected from those normally found in control plants.
6. The gene or genes for low coumarin, apparently exert their effect before the formation of coumarin, and o-coumaric

acid most probably is not a precursor to a flavonoid compound isolated from non-bitter sweet clover plants. Cinnamic acid might be a common precursor to both coumarin and the flavonoid, and the low-coumarin genes might carry the cinnamic acid "farther" along the acetate pathway.

LITERATURE CITED

1. Alston, R.E., Turner, B.L., and Lester R.N.O
1962 Chromatographic validation of two morphologically similar hybrids of different origins.
Science 137: 1048-1049.

2. Bate - Smith, E.C.
1954 Ferulic, sinapic and related acids in leaves.
Chem. and Ind. 1457-1458

3. Bate - Smith, E.C.
1956 The commoner phenolic constituents of plants and their systematic distribution.
Sci. Proc. R.Dublin Soc. 27: 165-176

4. Best, R.J.
1948 Fluorescent substances in plants 111 Distribution of scopoletin in tobacco plants and some hypotheses on its part in metabolism.
Austral. J. Exper. Biol & Med. Sci. 26: 223-230

5. _____
1944 Studies on a fluorescent substance present in plants.
Austral J. Exper. Biol & Med. Sci. 22: 251-255

6. Birch, A.J.
1957 Biosynthetic relations of some natural phenolic and enolic compounds.
Fortschr, Chem. Org.Naturst. 14: 186-216.

7. Birch, A.J., and Smith, H.
1958 Chemical Society Special Publication London No. 12. 1

8. Birch, A.J., in Todd, A.R.
1956. Perspectives in Organic CHemistry.
Interscience Publishers Inc. New York.

9. Bradner, N.R.
1961 Some aspects of the biochemical genetics of coumarins in forage legumes.
Master's Thesis, University of British Columbia.

10. Brink, R.A.
1934 A non-bitter variety of Melilotus
Science 79: 301

11. Brink, R.A., and Roberts, W.L.
1937 The coumarin content of Melilotus dentata.
Science 86: 41.42

12. Brink, V.C.,
1941 The content, distribution and some metabolic aspects of coumarin in sweet clover (Melilotus alba Des.)
Summaries of Doctoral Dissertations, University of Wisconsin.

13. Brown, S.A., and Neish, A.C.
1955 Studies of lignin biosynthesis using isotopic carbon. 1V Formation from some aromatic monomers.
Can. J. Biochem. Physiol. 33: 948-962

14. _____
1956 Studies of lignin biosynthesis using isotopic carbon V Comparitive studies on different plant species.
Can. J. Biochem. Physiol. 34: 769-778.

15. Brown, S.A., Towers, G.H.N., and Wright, D.
1960 Biosynthesis of the coumarins. Tracer studies on coumarin formation in Hierochloe odorata and Melilotus Melilotus officinalis.
Can. J. Biochem. Physiol. 38: 143-156.

16. Brown, S.A.
1960 Ober die Lactonringbildung des Cumarins 2. Naturforschung. 15b: 768-769

17. _____
1961 Chemistry of lignification.
Science 134: 305-313

18. _____
1961 Symposium on Biochemistry of Plant Phenolic Substances.
Colorado State University, Fort Collins, Colorado.

19. _____
1962 Biosynthesis of the coumarins 111 The role of glycosides in the formation of coumarin by Hierochloe odorata.
Can. J. Biochem. Physiol. 40: 607-618

20. Buhler, D.R., and Mason, H.S.
1961 Hydroxylation catalyzed by peroxidase.
Arch. Biochem. Biophys. 92: 424-437.

21. Campbell, H.A. and Link, K.P.
1941 Studies on the hemorrhagic sweet clover disease lV The isolation and crystallization of the hemorrhagic agent.
J. Biol. Chem. 138: 21-33

22. Campbell, H.A., Smith, W.K. and Link, K.P.
1941 Studies on the hemorrhagic sweet clover disease ll. The bioassay of hemorrhagic concentration by following the prothrombin level in the plasma of rabbit blood.
J. Biol. Chem. 138: 1-20

23. Charaux, M.C.
1925 Sur le Melilotoside, glucoside generateur d'acide coumarique extrait des fleurs de Melilotus altissima Thuil. et de Melilotus arvensis /Wallr.
Bull. Soc. de Biol. Vll: 1056-1059

24. Comte, P., Ville, A., Zingelstein,G., Favre-Bonvin,J. and Mentzer,C.
1958 Biosynthese des derives flavoniques chez Prunus avium l. Incorporation du trans cinnamate de Potassium $3^{14}C$.
Bull. Soc. Chim. Biol. 40: 1117-1131.

25. Corner, J.J., and Harborne, J.B.
1960 Cinnamic acid derivatives of potato berries.
Chem & Ind. 76.

26. Davis, B.D.
1955 Intermediates in amino acid biosynthesis
Adv. Enzymol. 16: 247-312.

27. Davis, B.D. in McElroy, W.D., and Glass, H.D.
1955 A Symposium on Amino Acid Metabolism
John Hopkins Press, Baltimore, Maryland.

28. Dean, F.M.
1952. Naturally occurring coumarins.
Fortschr, Chem. Org. Naturst 9: 225

29. Gamborg, O.L., and Neish, A.C.
1959 Biosynthesis of phenylalanine and tyrosine in young wheat and buckwheat plants.
Can. J. Biochem. Physiol. 37: 1277-1285

30. Geissman, T.A.
1958 The metabolism of phenylpropane derivatives in plants.
Encyclopedia of Plant Physiol. X: 543-559

31. Geissman, T.A. and Harborne, J.B.
1955 The chemistry of flower pigmentation in Autirrhinum majus IV The albino (-mm-nn) form
Arch. Biochem. Biophys. 55: 447-454

32. Geissman, T.A., and Hinreiner, E.
1952 Theories of the biogenesis of flavonoid compounds.
Bot. Rev. 18: 77-244

33. Goodwin, R.H. and Kavanagh, F.
1952 The fluorescence of coumarin derivatives as a function of pH 11.
Archiv. Biochem. Biophys. 36: 442-455

34. Goplen, B.P., Greenshields, J.E.R., and Baenziger, H.
The inheritance of coumarin in sweet clover.
Can. J. Bot. 35: 583-593.

35. Gorz, H.J., and Haskins, F.A.
1960 Genetic blocks in the synthesis of coumarin
J. of Heredity 51: 74-76.

36. Greenshields, J.E.R.
1958 Note on Cumino sweet clover.
Can. J. Plant Science 38: 507-508

37. Grisebach, H.
1959 Biochemistry of Wood.
Pergamon, London.

38. _____
1958 Zur biogenese des cyanidins 111 Mit: Uver die herkunft des ringes B.
2. Naturforschung 13b: 335-336

39. Hadorn, E., and Mitchell, H.K.
1951 Properties of mutants of Drosophila melanogaster and changes during development as revealed by paper chromatography.
Proc. Nat. Acad. Sci. 37: 650-665.

40. Haskins, F.A., and Gorz, H.J.
1957 Fluorometric assay of free and bound coumarin in sweet clover.
Agron. J. 49: 493-497.

41. _____
1961 A reappraisal of the relationship between free and bound coumarin in Melilotus.
Crop Science 1: 320-323.

42. _____
1961 Assay of cis and trans o-hydroxy cinnamic acids in sweet clover extracts.
Biochem. Biophys. Research Commum
6: 298-303

43. Haworth, R.D.
1942 The chemistry of the lignan group of natural products.
J. Chem. Soc. 446-456.

44. Hendrickson, J.B. in Gates, M.
1960 The Biogenesis of Natural Substances.
Interscience Publisher Inc., New York.

45. Horner, W.H., and White, W.J.
1941 Investigations concerning the coumarin content in sweet clover 111. The inheritance of the low coumarin character.
Sci. Agr. 22: 85--92

46. Hough, L., Jones, J.K.N., and Wadman, W.H.
1950 Quantitative analysis of mixture of sugars by method of partition chromatography V. Improved methods for the separation and detection of the sugars, and their methylated derivatives on paper chromatography.
J. Chem. Soc.1702 - 1706.

47. Hutchinson, A., Roy, C., and Towers, G.H.N.
1958 Synthesis of phlorin and other phenolic glucosides by plant tissues.
Nature London 181: 841-842.

48. Hutchinson, A., Taper, C.D. and Towers, G.H.N.
1959 Phloridzin in Malus.
Can. J. Biocham. Physiol. 37: 901-910

49. Ibrahim, R.K. and Towers, G.H.N.
1960 The identification by chromatography of plant phenolic acids.
Arch. Biochem. Biophys. 87: 125-128

50. Jensen, K.B.
1953 Paper chromatography of cardiac glycosides and aglycones from Digitalis purpurea.
Acta. Pharmacol. et Toxicol. 9: 99-108

51. Kalam, E.B., Davis, B.D., Srinivisan, P.R. and Sprinson, D.B.
1956. The conversion of various carbohydrates to 5 - dehydroshikimic acid by bacterial extracts.
J. Biol. Chem. 223: 907-912.

52. Kosuge, T.
1961 Studies on the identity of bound coumarin in sweet clover.
Arch. Biochem. Biophys. 95: 211-218

53. Kosuge, T., and Conn, E.E.
1959 The metabolism of aromatic compounds in higher plants 1 Coumarin and o-coumaric acid.
J. Biol. Chem. 234: 2133-2137.

54. _____
1961. The metabolism of aromatic compounds in higher plants 111 The Glucosides of o-coumaric, coumarinic and melilotic acids.
J. Biol. Chem. 236: 1617--1621

55. Kuznetsov, V.A.
1927 Melilotus dentata and coumarin content
Ann. Inst. Expt. Agron. V No. 2-3

56. McCalla, D.R., and Neish, A.C.
1959. Metabolism of phenylpropanoid compounds in Salvia 111 Biosynthesis of phenolic cinnamic acids.
Can. J. Biochem. Physiol. 37: 537-547.

57. McIlroy, R.J.
1951 The Plant Glycosides.
Edward Arnold, London.

58. Obermayer, E.Z.
1913 Quantitative bestimmung des kumarins in Melilotus - Arten. 2. Anal. Chem. 52: 172-191

59. Ollis, W.D. and Grisebach, H.
1961 Biogenetic relationships between coumarins, flavonoids, isoflavonoids and rotenoids. Experientia. 17: 4-12

60. Pechmann, H. and Cohen, J.B.
1884 Uber die verbindungen der phenole mit accetessigather. Das - methylumbelliferon. Ber. 17: 2129-2138

61. Perkin, W.H.
1868 Artificial production of coumarin, and formation of its homologues. J. Chem. Soc. 21: 53

62. Pridham, J.B.
1959 Phenolics in plants in Health and Disease. Proceedings of a Plant Phenolics Group Symposium, Bristol. Pergamon Press.

63. _____
1958 Metabolism of phenolic compounds by the broad bean Vicia faba. Nature London 182: 795-796.

64. Rao, P.S. and Seshadri, T.R.
1949 (C.f. Chem. Abs. 976. 1950) Proc. Ind. Acad. Sci.. 30 (A): 30-34

65. Reppel, L.
1954 Natural coumarins. Pharmazie 9: 278-299.

66. Rezin, H.
1959 Biochemistry of Wood. Pergamon, London.

67. Rinke, E.H.
1945 Inheritance of coumarin in sweet clover. J. Am. Soc. Agron. 37: 635-642.

68. Roach, W.A., and Roberts, W.O.
1945 Further work on plant injection for diagnostic and curative purposes.
J. Pomol. 21: 108-119.

69. Roberts, W.L. and Link, K.P.
1937 A precise method for the determination of coumarin, melilotic acid and coumaric acid in plant tissue.
J. Biol. Chem. 119: 269-281.

70. Roderick, L.M. and Schalk, A.F.
1931 Studies on sweet clover disease.
N. Dak. Agr. Sta Bul. 250

71. Rudorf, W. and Schwarze, P.
1958 Beitrage zur zuchtung eines cumarin-freien steinklees. Untersuchungen uber cumarins und verwandte verbindungen.
Z.f. Pflanzenzucht 39: 245-274.

72. Schaeffer, G.W., Haskins, F.A., and Gorz, H.J.
1960 Genetic control of coumarin biosynthesis and glucosidase activity in Melilotus alba.
Biochem. Biophys. Research Commun. 3:269-271

73. _____
1961 Genetic, developmental, and within-plant variation in free and bound coumarin content of sweet clover.
Crop Science 1: 194-196.

74. Schofied, F.W.
1922 A brief account of a disease in cattle simulating hemorrhagic septicaemia due to feeding sweet clover.
Can. Vet. Record 3: 74-78

75. Schwarze, P., and Hoffschmidt, R.
Untersuchungen uber das cumarin des steinklees und methoden zur trennung des freien und "gebunden" cumarins.
Naturwissenschaften 45: 518-519

76. Sethna, S.M. and Shah, N.M.
1945 The chemistry of coumarins.
Chem. Rev. 36: 1-62

77. Shibat, S., and Yamazaki, M.
1958 The biogenesis of plant products 1. The biogenesis of rutin.
Pharm. Bul. 6: 42-45

78. Shinoda, J.
1928 (c.f. Chem. Abs. 2947,1928)
J. Pharm. Soc. Japan. 48: 214-220.

79. Smith, W.K.
1943 Propagation of chlorophyll-deficient sweet clover hybrids as grafts.
J. Heredity 34: 135-140.

80. _____
1948. Transfer from Melilotus dentata to M. alba of the genes for reduction in coumarin content.
Genetics 33: 124-125.

81. _____
1961. Denta, a new non-bitter sweet clover variety.
Crops and Soils 13: 22.

82. Smith, W.K., and Brink, R.A.
1938 Relation of bitterness to the toxic principle in sweet clover.
J. Agr. Res. 57: 145-154.

83. Spath, E.
1937. Die naturlichen cumarine.
Ber. dtsch. chem.ges. 70A: 83-117.

84. Stachmann, M.A., Huebner, C.F., and Link, K.P.
1941 Studies on the hemorrhagic sweet clover disease V. Identification and synthesis of the hemorrhagic agent.
J. Biol. Chem. 138: 513-527.

85. Stevenson, J.M., and White, W.J.
1940. Investigation concerning the coumarin content of sweet clover 1. The breeding of a low-coumarin line of sweet clover. Melilotus alba.
Sci. Agr. 21: 18-28.

86. Ufer, M.
1934. A method useful from a breeding standpoint to the selection of low coumarin forms of sweet clover (Melilotus)
Zuchter 6: 255-258.

87. Underhill, E.W., Watkin, J.E., and Neish, A.C.
1957. Biosynthesis of quercetin in buckwheat.
Can. J. Biochem. Physiol. 35: 219-237.

88. Uritani, I., and Hoshiya, I.
1953. Coumarin substances from sweet potato, and their physiology. J.Agr. Chem. Soc. Japan. 27: 161-164.

89. Veldstra, H., and Havinga, E.
1945. On the physiological activity of unsaturated lactones. Encymologia 11: 373-380.

90. Vogel, A.
1820 Gilberts Ann. 64: 161

91. Watkin, J.E., and Neish, A.C.
1960. Biosynthesis of quercetin in buckwheatlll. Can. J. Biochem. Physiol. 38: 559-567.

92. Wawzonek, S.
1951 Heterocyclic Compounds. 2. John Wiley and Sons, Inc. New York.

93. Webster, G.T.
1955 Interspecific hybridization of Melilotus alba x M officinalis using embryo culture. Agron. J. 47: 138-142.

94. Wilson, E.B.
1896. The Cell in Development and Inheritance. The Macmillan Co., New York.

95. Wright, D., Brown, S.A. and Neish, A.C.
1958. Studies of lignin biosynthesis using isotopic carbon Vl. Formation of the side chain of the phenylpropane monomers. Can. J. Biochem. Physiol. 36: 1037-1045.