SOME ASPECTS OF THE BIOCHEMICAL GENETICS OF COUMARINS IN FORAGE LEGUMES

by

NORMAN RICHARD BRADNER

B.S.A., University of British Columbia, 1959

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN AGRICULTURE
in the Division of
PLANT SCIENCE

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL 1961

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

DIVISION
~~Department~~ of PLANT SCIENCE

The University of British Columbia,
Vancouver 8, Canada.

Date May 9, 1961

ABSTRACT

Coumarin compounds responsible for bitterness, and indirectly, toxicity, in the forage legume sweet clover (Melilotus alba) do not occur in the non-bitter "Cumino" variety of M. alba or in the species M. dentata. Nevertheless, investigation showed that compounds which are very probably coumarins do occur in the non-bitter strains.

A search for coumarins in alfalfa (Medicago sativa), closely related to sweet clover taxonomically, did not lead to the discovery of coumarins but did lead to the characterization of a new flavonoid tentatively characterized as 3:3':4' trihydroxy -5:7 dimethoxy flavone.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

My appreciation and thanks are accorded the following persons and institutions:

Dr. V. C. Brink, Division of Plant Science, for interest and guidance

Dr. J. J. R. Campbell, Division of Animal Science, for the use of equipment

Dr. H. McLean, Dr. J. A. F. Gardner, Mr. G. M. Barton, all of the Forest Products Laboratory, Canada Dept. of Northern Affairs and National Resources, for assistance in chemical characterization

Mr. Jan Brussel, Mr. Don Pearce, technicians, Division of Plant Science, for help in laboratory and field

The National Research Council of Canada for financial support

Thanks are accorded to my thesis committee:

Dr. V. C. Brink, Division of Plant Science, (Chairman).

Mr. W. E. P. Davis, Canada Dept. of Agriculture, Agassiz, B.C.

Dr. C. A. Hornby, Division of Plant Science.

Dr. N. A. MacLean, Division of Plant Science.

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

Dr. G. Rouse, Dept. of Botany.

I INTRODUCTION

Coumarin, as the substance primarily responsible for the unpalatability of common sweet clover (Melilotus alba and Melilotus officinalis), and for the toxicity, indirectly, of spoiled sweet clover hay, has claimed the interest of forage crop breeders. The absence of coumarin in Melilotus dentata and in related forage legumes such as alfalfa, and the development, through hybridization and selection of the coumarin-free, highly palatable "Cumino"-variety[1] of sweet clover provides encouragement to breeders interested in the genetical modification of the chemical composition of forage crops.

Despite the evident progress in the selection of a coumarin-free sweet clover, several questions remain unanswered about coumarin in forage legumes viz. (1) If coumarin per se is absent in coumarin-free sweet clover, are other coumarin relatives present? In other words, what are the bio-genetic implications and (2) Are coumarin derivatives of importance present in forage legumes other than sweet clover? Coumarins are known to be physiologically active in a variety of ways when fed to animals. The coumarins may be important in plants although their physiological role is not known. It is worthy of record that coumarin itself is known to induce epinasty and to prevent germination of seed of certain non-coumarin containing plants.

---

1 A variety developed at the forage laboratory, Canada Department of Agriculture, Saskatoon, Saskatchewan.

Coumarin and its chemical relatives are heterocyclic organic compounds of the C_6C_3 "type" and are not "favoured" by very specific tests for their recognition. Perhaps this is one of the major reasons for the lack of knowledge about these compounds in plants. However, many coumarins fluoresce brilliantly in ultra-violet light and use of this fact may be of value in their recognition in screening tests. To-day, moreover, chromatographic techniques can be used to assist in their recognition. Separation and identification is difficult but when chromatography is used in conjunction with other techniques such as, infra-red spectra, ultra-violet spectra, and colour tests, paper chromatography provides a means of identifying many different though related compounds.

It was decided therefore, that, with the common occurrence of fluorescence, in ultra-violet light, of coumarins, and, with the powerful tool of chromatography, a study of these substances in forage legumes was warranted. This study could prove to be useful to the forage breeder and to the plant physiologist.

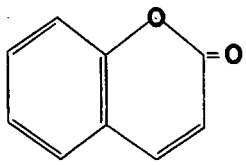
II REVIEW OF LITERATURE

A The natural coumarins

Natural coumarins have been studied for many years by chemists and a number of workers have reviewed their distribution (29, 94, 95). Coumarins are recorded in legumes, citrus crops, grasses and orchids; and Späth (103) stated that the parent compound, coumarin, has been isolated from approximately eighty different species of plants. Despite the fact that there is a widespread interest and considerable literature on the occurrence of these compounds in plants, in only a relatively few instances have these compounds in leaf, seed, and seedling been precisely characterized. Notable is the work of Späth (103) with umbelliferous species and Seshadri (94) with plants native to India. Scopoletin has been isolated in oats (43), in tobacco (10, 11), in potatoes (1, 2), and coumestrol in ladino clover and alfalfa (12, 13, 14, 33, 113). Goodwin and Kavanagh (42) demonstrated the presence of compounds in many plants exhibiting a fluorescence similar to that of scopoletin; blue, bluish or bluish-white fluorescence was demonstrated in 109 species out of a total of 135 species belonging to 126 genera and 69 families.

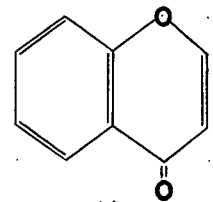
From the foregoing discussion it would seem that coumarins are found primarily in plant tissue. However, one occurrence of coumarins in animal tissue is on record; Lederer (60) has shown that 3:4 benzo-coumarins are constituents of the scent gland of the beaver.

In plants too, there is a class of compounds very similar to the coumarins - the chromones. These differ from the coumarins in the position of the carbonyl group in the heterocyclic ring.



Benzo-α-pyrone

coumarin



Benzo-γ-pyrone

chromone

Compounds in plants of such wide occurrence as flavones, isoflavones, flavonols, flavanones and flavanolones may be regarded as "benzo" derivatives of this basic chromone structure. These and other related compounds, the water-soluble plant pigments, the anthocyanins, chalcones and aurones, are widespread. (7, 9, 15, 36, 40, 50, 76, 88, 120). Because their chemical structure is similar to that of the coumarins, they can be easily confused with the coumarins.

B Coumarins in animal metabolism

As earlier stated many coumarins profoundly modify animal metabolism. In feeding trials with animals, it has been observed that coumarin itself acts as a narcotic for rabbits, frogs and earthworms and many other animals (54). It is also a sedative and hypnotic for mice (95). Wasicky (122) found that the coumarins, pimpinellin and osthruthin, have a slight toxic effect on rats, mice, and guinea pigs. Späth and Kuffner (104) demonstrated that many natural coumarins have

powerful effects on fresh water fish; the fish gradually lose their balance, and remain steady or swim on their backs, movement is then suspended, and finally they die.

Because of the physiological importance of coumarins in animal metabolism, considerable interest has centered on those coumarin-containing grasses, e.g. sweet vernal grass, and legumes, e.g. sweet clover, ladino clover, used as stock feed. Specifically, the sweet clovers (M. alba and M. officinalis), used as forage crops, are characterized by the presence of a "bitter principle" which affects their palatability as stock feed, and which imparts to the plant a distinct, but not unpleasant, vanilla-like odour. This property has long been associated with the presence of coumarin or its derivatives in the tissue of the plant. In addition to rendering plants of this crop relatively unpalatable to herbivorous animals, the coumarin is the parent material from which the anticoagulant "dicumarol" is derived in spoiled sweet clover hay (102, 105, 123). A coumarin isolated by Bickoff et al (12, 13, 14, 64) from ladino clover, alfalfa, and other legumes and called by him "coumestrol" induces oestrus. Other compounds such as genistein and biochanin A (81, 82), formononetin (8), though reacting like coumestrol are not coumarins but isoflavones.

C Coumarins in plant metabolism

Not only are coumarins widely distributed in the plant kingdom but they may be physiologically active in plants.

Although the role of coumarins in plants is not understood, coumarins applied artificially to plants have given many interesting effects. Coumarin per se favours cell enlargement in such plant tissue as oat coleoptile and leaf blade at low concentrations but checks cell enlargement at higher concentrations (69, 112, 118). Similar checking effects upon root elongation have been found for coumarin and certain derivatives (5, 46, 47, 80, 92). Thus coumarins appear to act as "anti-auxins" and offset or counteract the usual growth enhancing effect of auxins in the plant (117). Coumarin also inhibits mitosis in onion, lily (28) and oats (47).

Coumarins, and many other chemically unrelated compounds inhibit germination of seeds (55, 56). Evenari (34) has stated that germination inhibitors are widespread in plants and appear in fruit pulp, fruit coats, endosperm, seed coats, embryo, leaves, bulbs, and roots. The main known inhibitors active in the aforementioned tissues are the following substances or belong to the following chemical groups: hydrogen cyanide, ammonia, ethylene, mustard oils, organic acids, unsaturated lactones (some of which are coumarins), aldehydes, essential oils, and alkaloids. Special attention has been given to inhibitors in seed of iris, wild oats, lettuce, beets, and cabbage. Juices of lemon (97) and peach fruits (93) also have marked effects on the germination of the seed contained within their respective fruits. It is probable that germination inhibitors are present in the seeds and fruits of most species and that they are involved in a widespread mechanism of

dormancy (34).

A search for special chemical configurations relating to dormancy phenomena has been undertaken and it has been stated that compounds having the structure of unsaturated lactones possess the property of inhibiting seed germination and other growth phenomena (3, 4, 66, 74, 117). Coumarin and parasorbic acid are compounds in this category.

D Plant breeding for coumarin freedom in sweet clover

When one considers the widespread distribution of coumarins and their physiological activity in plants and animals, it is understandable that a substantial literature is devoted to coumarin and its relatives in forage grasses and legumes. Most of the literature however, relates to species containing large quantities of coumarin per se, such as sweet clover and sweet vernal grass, but very little to species which may contain little coumarin per se but, possibly, substantial quantities of other coumarins.

The literature on the development of coumarin-free sweet clover is extensive since so many workers in various fields contributed directly, or indirectly, to the successful release of M. alba variety "Cumino". Because of the importance of this work a short review of the subject is presented here. The existence of coumarin per se in plants has been known for a long time. The first recorded evidence of the isolation of coumarin from plant tissue occurred in 1820 when it was isolated from Tonka beans (119). Since that time coumarin has

been used in a variety of ways e.g. as a perfume base, as an adulterant in vanilla extract. Interest in coumarin in sweet clover developed when it was thought that this crop could be used as an industrial source of coumarin. Further interest was aroused in the early 1920's when the unpalatability of sweet clover was attributed to coumarin. Thereafter, it occurred to two Canadian workers Kirk (57), Kirk et al (58), and Stevenson et al (106), that a sweet clover free of coumarin could be bred in order that the palatability of the crop might be improved. Early attempts to select low coumarin plants were tedious (41, 108) until Clayton and Larmour (27) introduced a rapid method to detect free coumarin. Subsequently varieties low in free coumarin were selected but, later it was found that the selections still contained coumarin as a glycoside. In these strains the bound coumarin was not detected by Clayton and Larmour's test. However, simple autolysis of the plant tissue before the test was conducted, overcame this obstacle and total coumarin could then be measured (86, 87). In the search for non-bitter sweet clovers a coumarin-free species of sweet clover, M. dentata, originating in Asia Minor, was found (18, 19) and plants from this species were utilized in future breeding programmes (26, 48, 52, 85, 101, 107, 121). In crossing M. alba and M. dentata another problem arose; it was found that the hybrid contained a chlorophyll lethal which prevented the plants from growing to maturity. This problem was overcome when Brink (20) and Smith (100) demonstrated the feasibility of grafting the chlorophyll deficient seedling onto normal M. officinalis plants.

Further interest in sweet clover was created when it was realized that not only was coumarin responsible for the unpalatability of the crop but it was also involved in the formation of "dicumarol" in spoiled sweet clover hay (22, 23, 63, 67, 83, 89, 102, 105, 123). After many years of selection a few seeds of a coumarin-free variety were collected and these seeds were sent to the forage laboratory at Saskatoon, Saskatchewan. After a number of years of intensive selection M. alba variety "Cumino" was released, a notable achievement made possible by many workers.

E Analysis of coumarin

Before terminating this topic a short discussion of the chemical methods for coumarin measurement is in order since the success of the sweet clover breeding programme depended on them to a large extent: it was essential that inexpensive and rapid tests be developed for screening large numbers of plants. Early investigators (30, 31, 32) used the steam distillation method outlined by Obermayer (75) but this method was too cumbersome to screen large populations. The development of rapid colour tests, referred to above, by Clayton and Larmour (27) overcame the difficulty of this problem. However, this method only measured the free coumarin and failed to detect bound coumarin. Roberts and Link (86, 87) reported that with autolysis of the leaf material prior to extraction of the coumarin, the colorimetric method measured total coumarin content. A few years later, utilizing the methods of

Ufer (115, 116) a fluorometric procedure was developed which measured coumarin content quantitatively on a photofluorometer (98), or semiquantitatively (99, 124) by visual assessment of the fluorescence when samples were placed in ultra-violet light. For a rapid determination of the presence or absence of total coumarin, the fluorometric method described by White, Savage, and Johnston (125) and recently modified by Goplin, Greenshields, and White (49), is currently being used.

Associated with the fluorometric measurement of coumarin is the use of fluorescence of compounds when they are viewed under ultra-violet light. Fluorescence of coumarins on filter paper chromatograms can be quite characteristic (9, 44, 45, 109, 110). All heterocyclic substances absorb ultra-violet light and many fluoresce in it; many authors have compiled tables of compounds with their fluorescent colours (7, 25, 35, 36, 50, 51, 77, 110). Because many excellent reviews and texts have been written on the subject of paper chromatography (16, 61, 65, 114) this topic will not be discussed.

In addition to the purely physical means to measure or identify coumarins, there are chemical methods. These consist of two main methods to distinguish the coumarin ring; namely those based on the hydrolysis of the compound by alkaline reagents (59, 62, 78, 127) and those methods based on the preparation of some special derivatives (24).

In order to conduct tests with coumarins, special attention should be paid to the means of isolating the compounds in a pure form. Many coumarins can be extracted from plant

materials by suitable solvents (e.g. ether, benzene, acetone). However, care should be taken that the extractants do not cause changes in the chemical makeup of the compounds. For example, Duncan and Dustman (31), proved that destruction of coumarin takes place in a prolonged ether extraction.

If coumarins are present as glycosides, the aglycones may be isolated after preliminary hydrolysis; alternatively, the glycoside may be extracted using alcohol or water. Natural coumarins are either neutral or slightly acid and appear in the corresponding fractions of the plant extract (29). In the isolation it is usual to take advantage of the fact that the coumarin ring is opened by warm dilute alkali with the formation of the coumarinate salt, which allows the removal of neutral material (29). On decomposition of the salt with acid, the coumarinic ring cyclises spontaneously, regenerating the coumarin which is thus easily separated from acids formed by the hydrolysis of the other constituents; however, the method is not ideal because it is difficult to avoid degradation of the coumarins (29).

F Coumarin biosynthesis

It was earlier mentioned that little is known of the function of the natural coumarins in plants or, in fact, of the benzenoid compounds generally (71). One way to learn more of their functions or function would be to trace the pathways or pathway by which they are synthesized in the organism.

Several approaches may be taken to the study of the biosynthesis of coumarins and benzenoid compounds generally. Possibly the most powerful is through the use of radiotracer compounds. Neish (71), in a recent review, has given a concise statement on the present status of knowledge obtained through this approach. Another approach not adequately treated, as yet, is that of "injection or feeding" trials in living tissues of possible relatives and precursors of coumarins and related benzenoids followed by analysis of chemical changes as indicated by disappearance, accumulation etc. Yet another approach is to isolate enzymes involved in the biosynthetic pathways and the study of the enzymatic reactions in vitro. Closely related to the latter two approaches is that of the use of genetic blocks and the study of gene-controlled reactions in related species and varieties. The genetic approach has contributed substantially to the knowledge of flavonoid biosynthesis (40) but no contributions have been forthcoming as yet in the biosynthesis of coumarins: a beginning is made in the investigations to follow.

III INVESTIGATION I

A comparative study of extracts from coumarin-free sweet clover, and from coumarin-containing sweet clover.

The principal aim of this investigation was to ascertain if coumarin relatives replaced coumarin, in coumarin-free sweet clover. One means by which differences might be detected between non-bitter M. dentata, non-bitter "Cumino" sweet clover, and bitter common white sweet clover would be by chromatographing suitable extracts. The approach assumes that the solubility characteristics of the coumarins are similar. By this method, clues regarding the pathway of substitution for coumarin might be recorded in differences observed on paper chromatograms.

A 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. R. Goplen. Forage laboratory, Canada Dept. of Agriculture, Saskatoon, Saskatchewan.

c) Canadian common, bitter white sweet clover from Buckerfield's Ltd., Vancouver. B.C.

B Methods

EXTRACTION

Establishment of specific chemical constituents occurring in non-bitter sweet clovers, which do not occur in the bitter strains, as has been stated, rests in the expectation that differences may be found by chromatographing comparable tissue extracts. Materials from many extraction procedures were compared; only two, which appeared to be reasonably satisfactory, are given below.

a) Green leaf and stem tissue.

1. Slice tissue into small pieces and immerse immediately in hot 70% ethyl alcohol; then grind material in a Waring Blendor.
2. Shake for 12 hours; extract twice more with equal volumes of solvent.
3. Filter the combined extract; concentrate in a flash evaporator to approximately 10% of the original volume @ 35°C.
4. Extract the solution with diethyl ether in a liquid-liquid extractor and repeat as necessary.
5. Concentrate the ether extract to approximately 5% of the volume @ 35°C.
6. Stripe the ether solution on Whatman #3 filter paper and develop the chromatogram eight hours with glacial acetic acid: water solvent (1:1), (A:W) at about room temperature 22°C.
7. Stripe the residue left in the liquid-liquid extractor and develop it in n-Butyl alcohol: glacial acetic

acid: water (4:1:5 organic phase) (B:A:W).

8. All chromatograms are scanned under ultra-violet light (3660 Å and 2537 Å).

b) Seed

1. Grind the seed in a Wiley mill using 20 mesh screen.
2. Shake the material with 70% ethyl alcohol for 12 hours; extract twice more with equal volumes of solvent.
3. Filter the combined extract, and concentrate in a flash evaporator to approximately 10% of the volume @ 35°C.
4. Extract the solution with diethyl ether in a liquid-liquid extractor and repeat as necessary.
5. Reduce the volume of the combined ether extracts and treat as above.
6. Take the residue, or residues left in the liquid-liquid extractor and treat with basic lead acetate, centrifuge and collect the precipitate. Suspend the precipitate in 70% ethyl alcohol and bubble through H_2S gas; centrifuge and collect the solution; repeat this procedure on the solution from the first centrifugation using neutral lead acetate.
7. The alcoholic solutions of neutral lead and basic lead acetate "operations" are striped on #3 papers and developed in B:A:W solvent.
8. The chromatograms are scanned as described before.

SPOT TESTS

Many colour tests are available to aid in the iden-

tification of unknown heterocyclic compounds. A number of the tests that proved useful in this investigation are presented here.

a) NH_3 test - the chromatogram containing the purified spot was exposed to NH_3 vapours and immediately observed in light and under ultra-violet light.

b) NaOH test - a few drops of 1 N. NaOH solution was applied to the purified spot on the filter paper chromatogram and the colour of the spot was noted (91).

c) HCl test - a few drops of concentrated HCl were applied to the spot on the filter paper chromatogram and the colour of the spot was noted.

d) Diazotized-p-nitro aniline test - The diazonium solution was prepared as outlined by Roberts and Link (86). The test solution was spotted on filter paper and a few drops of 10% Na_2CO_3 added. The formation of colours, on the addition of a few drops of the diazonium reagent, was noted.

The examination of the ultra-violet absorption spectra of purified compounds is an excellent means of substantiating suspected differences between compounds.

e) Ultra-violet absorption spectrum - The compound was dissolved in 95% ethyl alcohol (2mg./l.) and the ultra-violet spectra noted using 1 ml. quartz cuvettes in a Beckman D U spectrophotometer.

C Observations and results

The principle fluorescing and absorbing spots, seen, when comparable chromatograms were examined under ultra-violet are given with their approximate Rf values, in the tables to follow. Their colours and intensities for various reasons are useful only in a very general way, and are recorded in an abbreviated form.

v.l.B.F.	-	very light Blue Fluorescence
l.B.F.	-	light Blue Fluorescence
B.F.	-	Blue Fluorescence
b.B.F.	-	bright Blue Fluorescence
B.W.F.	-	Bluish White Fluorescence
l.W.F.	-	light White Fluorescence
W.F.	-	White Fluorescence
Y.W.F.	-	Yellowish White Fluorescence
Y.F.	-	Yellow Fluorescence
l.M.F.	-	light Mauve Fluorescence
M.F.	-	Mauve Fluorescence
l.O.F.	-	light Orange Fluorescence
b.B.G.F.	-	bright Blue Green Fluorescence
v.l.B.G.F.	-	very light Blue Green Fluorescence
v.l.Ab.	-	very light Absorbance
l.Ab.	-	light Absorbance
d.Ab.	-	dark Absorbance

Spots, fluorescent and absorbing, seen when non-bitter and bitter sweet clover leaf extracts were compared under ultra-violet illumination (3660Å).

TABLE 1 - A comparison of procedural ether extracts

	Common sweet clover		"Cumino" sweet clover		Dentata sweet clover	
Spot	colour	Rf(A:W)	colour	Rf(A:W)	colour	Rf(A:W)
1	-	-	-	-	B.W.F.	.19
2	-	-	-	-	v.l.B.F.	.26
3	l.B.F.	.37	-	-	v.l.B.F.	.36
4	l.M.F.	.49	Y.W.F.	.49	v.l.B.F.	.49
5	M.F.	.59	-	-	b.B.F.	.57
6	-	-	-	-	d.Ab.	.62
7	l.W.F.	.65	W.B.F.	.70	b.B.F.	.69
8	-	-	-	-	d.Ab.	.76
9	b.B.F.	.82	-	-	Y.F.	.82
10	W.F.	.88	b.B.F.	.86	l.B.F.	.86
11	-	-	W.F.	.94	Y.F.	.92

TABLE 2 - A comparison of residues left in the liquid-liquid extractor

	Common sweet clover		"Cumino" sweet clover		Dentata sweet clover	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	B.W.F.	.05	B.W.F.	.05	B.W.F.	.06
2	b.B.F.	.07	b.B.F.	.09	-	-
3	l.Ab.	.11	l.Ab.	.12	Y.F.	.11
4	b.B.F.	.15	b.B.F.	.16	l.B.F.	.18
5	l.Ab.	.19	d.Ab.	.21	l.Ab.	.22
6	Y.W.F.	.24	Y.W.F.	.26	v.l.B.F.	.25
7	l.M.F.	.27	v.l.B.F.	.31	M.F.	.29
8	-	-	-	-	v.l.B.F.	.32
9	-	-	-	-	v.l.B.F.	.41
10	l.Ab.	.52	v.l.Ab.	.47	l.Ab.	.47
11	v.l.B.G.F.	.60	v.l.B.F.	.57	b.B.G.F.	.54
12	v.l.B.F.	.75	v.l.B.F.	.75	-	-

TABLE 3 - A comparison of materials precipitated by neutral lead acetate

	Common sweet clover		"Cumino" sweet clover		Dentata sweet clover	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	b.B.F.	.04	b.B.F.	.04	b.B.F.	.04
2	b.B.F.	.10	b.B.F.	.10	b.B.F.	.09
3	l.Ab.	.16	l.Ab.	.16	l.Ab.	.13
4	b.B.F.	.20	b.B.F.	.22	b.B.F.	.18
5	v.l.B.F.	.31	v.l.B.F.	.34	l.B.F.	.29
6	v.l.B.F.	.47	v.l.B.F.	.45	l.B.F.	.53
7	v.l.B.F.	.77	v.l.B.F.	.79	v.l.B.F.	.72

TABLE 4 - A comparison of materials precipitated by basic lead acetate

	Common sweet clover		"Cumino" sweet clover		Dentata sweet clover	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	l.B.F.	.04	v.l.B.F.	.04	M.F.	.06
2	l.O.F.	.16	l.O.F.	.16	Y.F.	.14(2537Å)
3	-	-	-	-	d.Ab.	.16(2537Å)
4	-	-	-	-	l.B.F.	.20
5	v.l.B.F.	.28	v.l.B.F.	.28	d.Ab.	.25(2537Å)
6	v.l.B.F.	.47	-	-	-	-
7	-	-	-	-	v.l.B.F.	.60

Spots, fluorescent and absorbing, seen when non-bitter and bitter sweet clover seed extracts were compared under ultra-violet illumination (3660Å).

TABLE 5 - A comparison of materials precipitated by neutral lead acetate

	Common sweet clover		"Cumino" sweet clover	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	d.Ab.	.14	d.Ab.	.13
2	v.l.Ab.	.22	l.Ab.	.24
3	d.Ab.	.28	d.Ab.	.34
4	v.l.B.F.	.60	v.l.B.F.	.53
5	v.l.B.F.	.78	v.l.B.F.	.74

TABLE 6 - A comparison of materials precipitated by basic lead acetate

	Common sweet clover		"Cumino" sweet clover	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	d.Ab.	.24	d.Ab.	.25
2	v.l.B.F.	.82	v.l.B.F.	.84

TABLE 7 - A comparison of ether extracts

	Common sweet clover		"Cumino" sweet clover	
Spot	colour	Rf(A:W)	colour	Rf(A:W)
1	d.Ab.	.23	v.l.Ab.	.16
2	v.l.B.F.	.55	v.l.B.F.	.51
3	v.l.B.F.	.75	v.l.B.F.	.72
4	v.l.B.F.	.80	l.B.F.	.82
5	d.Ab.	.88	v.l.Ab.	.88
6	l.B.F.	.95	B.B.F.	.96

General and specific qualitative tests, physical and chemical, indicative of flavonoids and coumarins in more or less pure state have appeared from time to time over a period of nearly a century and are now quite numerous. Swain (109), and Geissman (36), and others have provided summary statements on the usefulness of many of these tests and have recorded the behavior of a long list of coumarins and flavonoids with these tests. Furthermore, they have recorded their reaction on paper chromatograms.

Spots then, on the chromatograms of the sweet clover extracts obtained above, were screened using a number of the qualitative tests recommended as useful by Swain (109) and Geissman (36). Only ultra-violet fluorescence, in acid and alkaline media, and diazonium tests are recorded below. However, it should be pointed out that only materials eluted and paper chromatographed several times were tested for the following records.

TABLE 8 - A comparison of two spots of possible interest isolated from "Cumino" sweet clover and Common sweet clover

Test	"Cumino" sweet clover spot 10 Table 1 colour	Common sweet clover spot 9 Table 1 colour
Untreated(U.V.)	Blue	Blue
N H_3(Visual)	colourless	colourless
N H_3(U.V.)	bright Blue	bright Blue
2N.NaOH(U.V.)	Blue Green	Blue green
2N.HCl.(U.V.)	bright Blue	lighter Blue
Diazotized-P-nitro-aniline and Na_2CO_3	light Purple	light Purple

TABLE 9 - A comparison of two additional spots of possible interest isolated from "Cumino" sweet clover and Common sweet clover

Test	"Cumino" sweet clover spot 7 Table 1 colour	Common sweet clover spot 7 Table 1 colour
Untreated(U.V.)	Bluish white Fluorescence	Bluish White Fluorescence
NH_3(Visual)	Yellow	Yellow
NH_3(U.V.)	Yellow Green	light yellow Green
2N.NaOH(U.V.)	Green	b.yellow(also visual)
2N.HCl.(U.V.)	bright Blue	Blue
Diazotized-P-nitro-aniline and Na_2CO_3	inconclusive	inconclusive

TABLE 10 - A comparison of additional spots isolated from "Cumino" sweet clover and Common sweet clover

Test	"Cumino" sweet clover spot 4 Table 1 colour	Common sweet clover spot 5 Table 1 colour
Untreated(U.V.)	light Yellow Fluorescence	Mauve Fluorescence
N H_3(Visual)	No change	No change
N H_3(U.V.)	No change	Violet
2N.NaOH(U.V.)	bright-yellow	Blue
2N.HCl.(U.V.)	Fluorescence quenched	Violet
Diazotized-P-nitro-aniline and Na_2CO_3	inconclusive	inconclusive

TABLE 11 - A comparison of diethyl ether extracts of non-bitter and bitter sweet clover leaf when the developed chromatograms are treated with sodium carbonate and diazotized-p-nitro aniline

	Common sweet clover		"Cumino" sweet clover		Dentata sweet clover	
Spot	colour	Rf(A:W)	colour	Rf(A:W)	colour	Rf(A:W)
1	-	-	-	-	nil	.19
2	-	-	-	-	nil	.26
3	nil	.37	-	-	nil	.36
4	nil	.49	nil	.49	nil	.49
5	nil	.59	-	-	nil	.57
6	-	-	-	-	nil	.62
7	nil	.65	nil	.70	Blue	.69
8	-	-	-	-	Mauve	.76
9	nil	.82	-	-	Mauve	.82
10	nil	.88	light purple	.86	Mauve	.86
11	-	-	nil	.94	Mauve	.92

TABLE 12 - A comparison of ultra-violet absorption spectra of compounds of possible interest isolated from diethyl ether extracts (Table 1)

Common sweet clover			"Cumino" sweet clover		
Spot	minima	maxima	Spot	minima	maxima
9	245	270	10	250	270
7	245	270	7	253,303	285,327
4	254,265	258,271			

See Figures 1, 2, 3, 4.

Examination of Tables 1, 2, 3, and 4 reveals the following:

a) that procedural neutral lead acetate precipitates from bitter and non-bitter sweet clovers have a very similar composition.

b) that procedural basic lead acetate precipitates, from bitter and non-bitter sweet clovers, show very small compositional differences.

c) that procedural diethyl ether extracts from bitter and non-bitter sweet clovers, as indicated by ultra-violet fluorescence and Rf. values showed marked differences.

d) that residues from bitter and non-bitter sweet clovers, as indicated by ultra-violet fluorescence and Rf. values, showed marked differences.

Substance, roughly isolated by paper chromatography, eluted, and further purified by paper chromatography, and examined by comparative spot tests, supported the fact that certain differences in bitter and non-bitter sweet clovers in

Figure 1 - U.V. absorption spectrum of "Cumino" sweet clover spot 10 Table 1

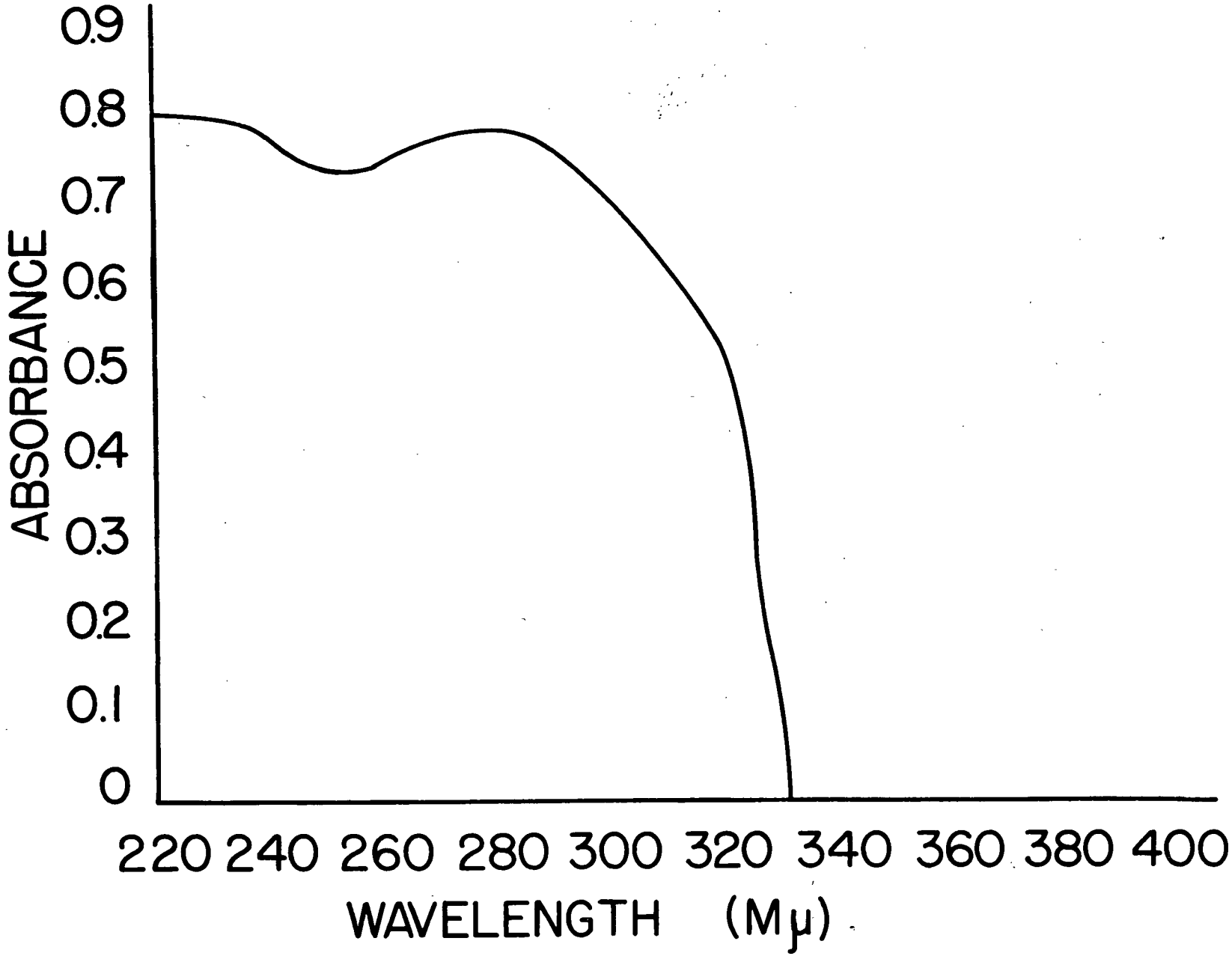
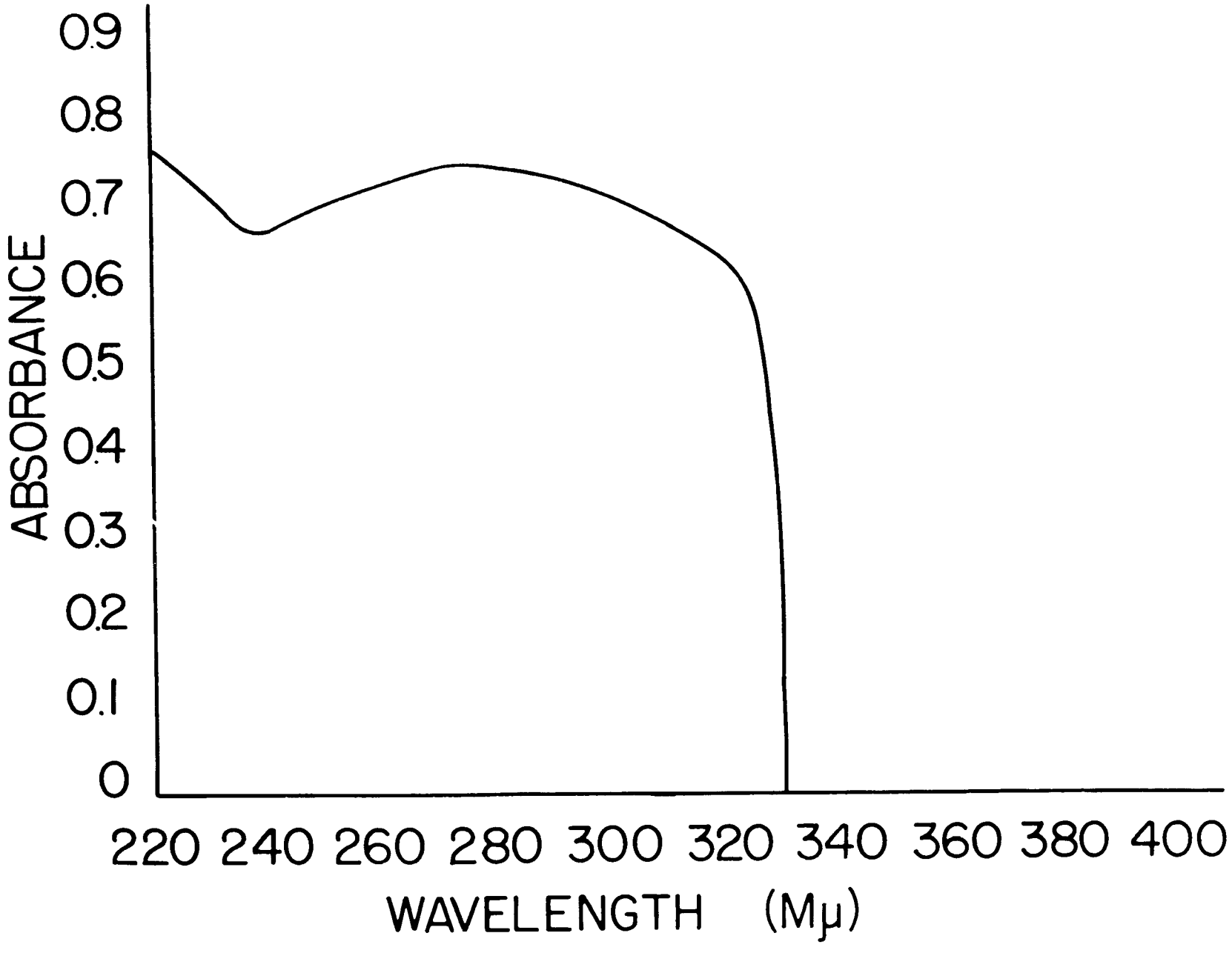

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220 240 260 280 300 320 340 360 380 400
WAVELENGTH (Mμ)

Figure 2 - U.V. absorption spectrum of Common sweet clover spot 9 Table 1


ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220 240 260 280 300 320 340 360 380 400
WAVELENGTH (Mμ)

the procedural diethyl ether soluble fraction are to be attributed to different, but possibly related, compounds. However the behaviour of compounds or compound represented by spots 7 in Common and "Cumino" sweet clovers (Table 1) suggests caution, for in neutral media differences in Rf. values and fluorescence were very slight; in 2 N.NaOH, though a marked difference in fluorescence occurred. Closer study of spots 9 - 10 (Table 1) (Table 8) did not support a possible difference at this chromatographic locus. On the other hand a difference in the region of spots 4 and 5 (Table 1) (Table 10) seemed to be substantiated. Support for a difference in the nature of the compounds in the region of spot 7 (Table 1) was found in the ultra-violet absorption spectra (Table 12).

Comparisons of chromatograms of diethyl ether extracts from Dentata and Common sweet clover, treated with sodium carbonate and diazotized-p-nitro aniline, (a general test for coumarins and many other compounds) showed differences in colour in the "Rf region" where many coumarins are found (Table 11). Comparative chromatograms also showed many fluorescence differences at loci which not necessarily showed differences in the diazo reaction (Table 1).

D Discussion of results

The search for differences in coumarin metabolism in bitter and non-bitter sweet clover is like looking for the proverbial "needle in the haystack." The differences may be

Figure 3 - U.V. absorption spectrum of "Cumino" sweet clover spot 7 Table 1

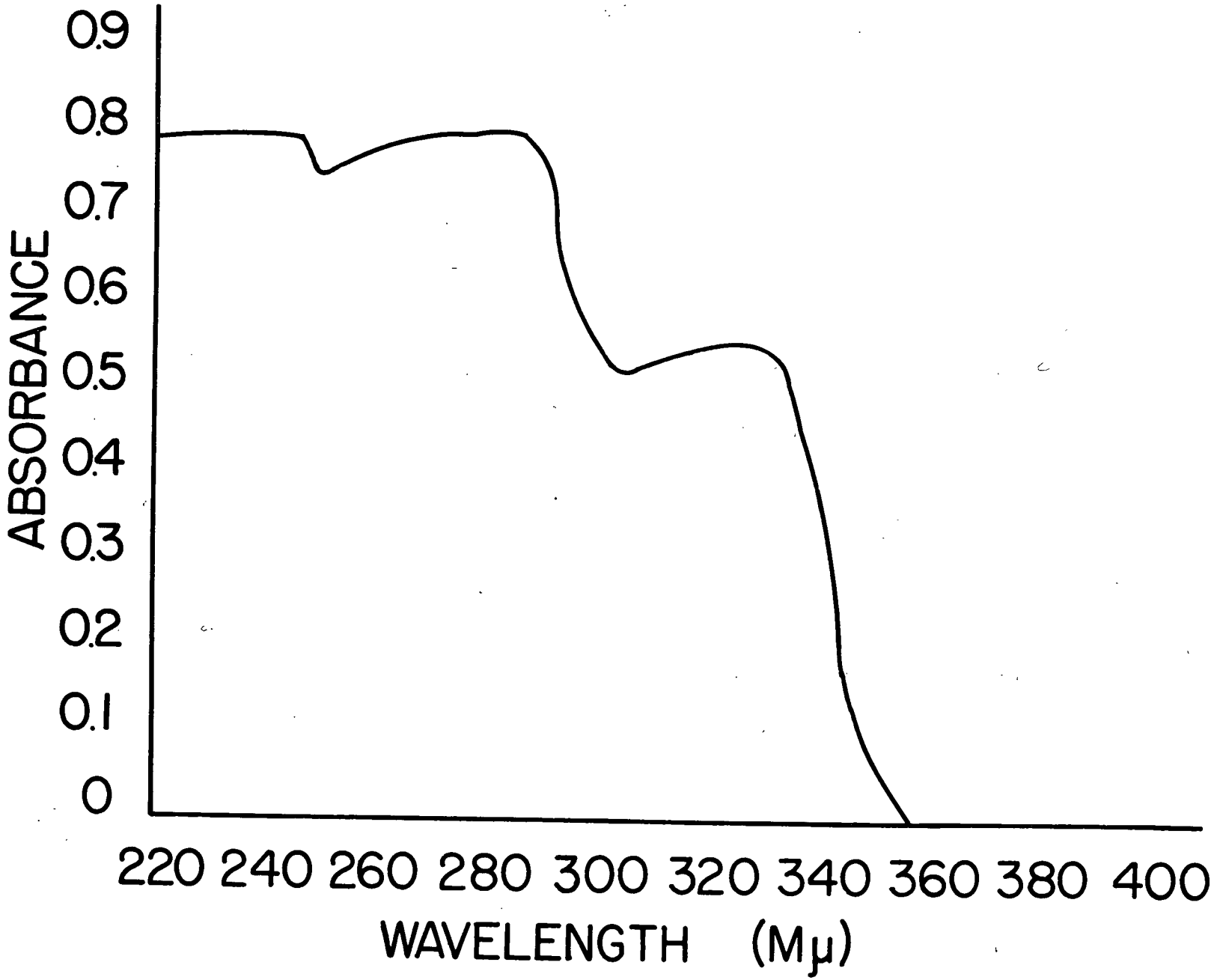
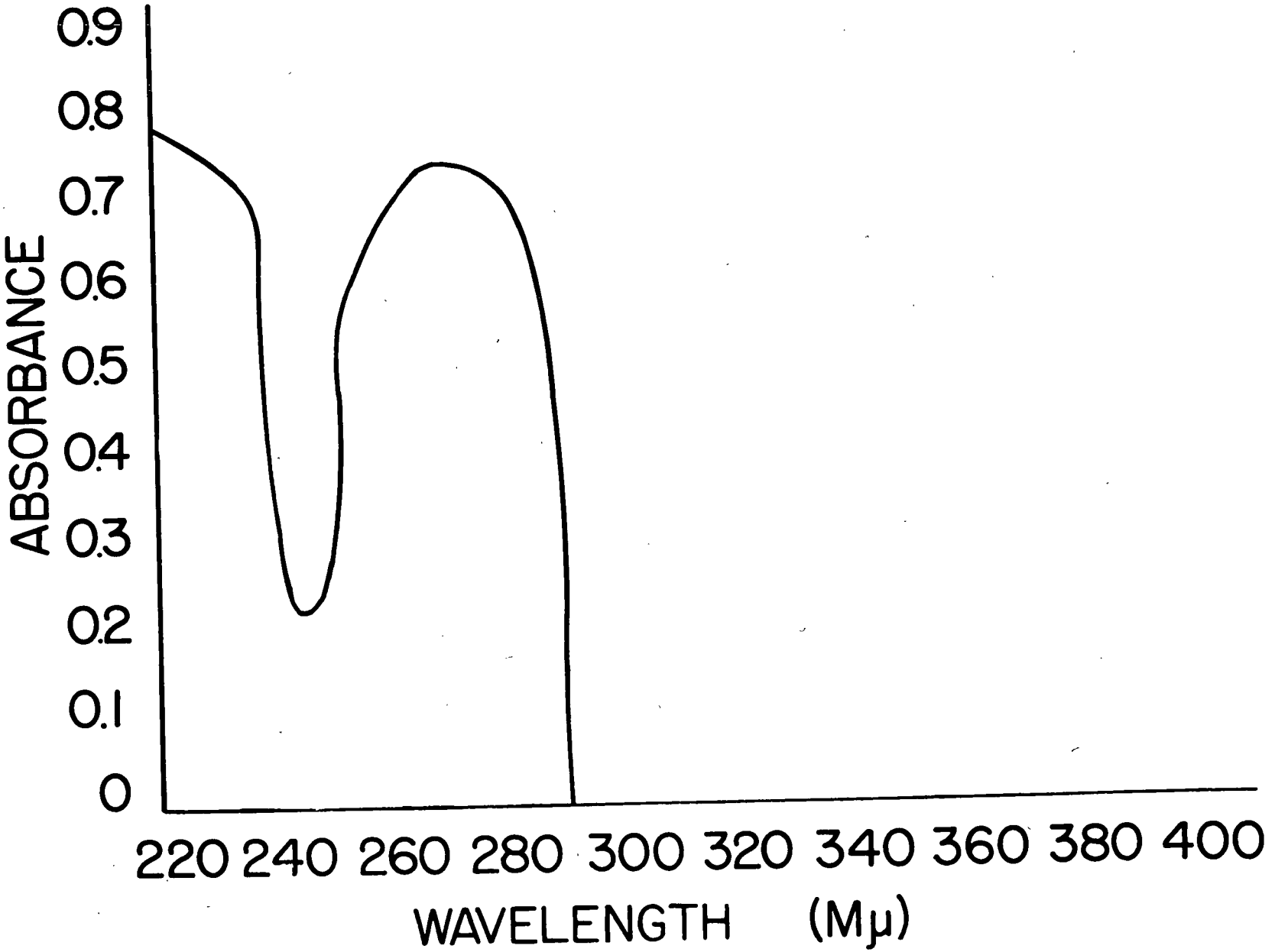

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220 240 260 280 300 320 340 360 380 400
WAVELENGTH (Mμ)

Figure 4 - U.V. absorption spectrum of Common sweet clover spot 7 Table 1


ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220 240 260 280 300 320 340 360 380 400
WAVELENGTH (Mμ)

established early in a metabolic sequence, in which case one might expect the bitter coumarin of Common sweet clover to be "replaced" by compounds in non-bitter sweet clover which differ quite markedly chemically from coumarin. On the other hand, as has been pointed out, the inheritance of "non-bitterness" is fairly simple and, in general, in other metabolic changes related to simple gene differences, the chemical change is fairly simple, too, e.g. addition or loss of -OH groups, addition or loss of $-OCH_3$ groups. Moreover, the "Cumino" "non-bitter character" was transfered to Common sweet clover and then repeated backcrossing to Common sweet clover followed. Thus, "Cumino" should genetically be very similar to "Common" except for the "non-bitter" character; it follows then that the chemical differences revealed on chromatograms etc. are very likely to be associated with the coumarin metabolism of these legumes.

On the other hand many differences in morphology and physiology exist between non-bitter species M. dentata and bitter species M. alba and M. officinalis. Nevertheless the non-bitter character in M. dentata is the source of the non-bitter character in "Cumino". Therefore, it is reasonable to suppose that the common metabolism in "Cumino" and "dentata" is much the same.

The coumarin metabolism of Common sweet clover is fairly complex even though the literature and breeders tend to emphasize only coumarin per se. However, it should be remembered that even in Common sweet clover the closely related

melilotic acid occurs with coumarin. Coumarin is also found as the aglycone and glycoside, and coumaric acid or acids are concomitant compounds. Knowledge of the coumarin metabolism of Common sweet clover is very probably incomplete. However the metabolic changes associated with the "non-bitter gene or genes" are probably revealed in the production of new coumarins; some support for this view is the appearance of coumarins in other legumes e.g. coumestrol in alfalfa and ladino clover; angelicin and psoralin in Psoralea corylifolia; p-Coumaric acid in Dariesia latifolia and Trifolium pratense.

In conclusion it would appear that diethyl ether extracts are well worth studying since differences in the sweet clovers have been recorded for these extracts and further, these differences appear to be coumarin compounds. Additionally, it would be of interest in future investigations to examine these differences as individual isolates.

IV INVESTIGATION II

A search for coumarin metabolism in alfalfa.

The genus Medicago, to which alfalfa belongs, and the genus Melilotus, to which sweet clover belongs, are closely related taxonomically. The closeness of the relationship would be accepted on almost any basis of scrutiny. Both genera contain species of major economic importance; in the one genus, Melilotus, as has already been discussed, coumarin and its metabolites are of very considerable significance; on the other hand, as yet, no firm evidence of a coumarin metabolism has been recorded for alfalfa. An indication that a coumarin metabolism exists in the alfalfas (and other legumes), as well as in the sweet clovers, comes with the discovery of the oestrogenically active "coumestrol" in alfalfa, by Bickoff and associates, which may involve a coumarin in its biosynthesis.

To determine whether or not other coumarins occur in alfalfa became the objective of this second study. Although coumarin per se occurs in Common sweet clover to the extent of .5 to 1.5% of the dry forage, a coumarin counterpart for alfalfa does not seem to be on record. If it does exist it may not be easy to recognize for as has been pointed out, specific chemical and physical tests for coumarins, as distinct from a number of other heterocyclic compounds, are not available.

An entrée to a possible coumarin metabolism of alfalfa seemed to offer itself in the record of a brilliantly fluorescent, but reasonably stable compound, occurring in alfalfa

seeds (21). Qualitative tests, other than fluorescence also gave some support to the belief that the compound fell into the general category of the compounds to be sought for.

The report to follow is essentially the record of the isolation from alfalfa and partial characterization of this one compound of probable interest. Adding somewhat to the importance to be attached to the isolation and characterization of the fluorescent compound in alfalfa is the fact that other forage legumes, such as red clover, white clover, etc. also carry brilliantly fluorescent compounds. These differ, very little, probably, from the alfalfa compound and it is fair to surmise that characterization of the one will tend to make easy the characterization of the others. It is understandable, that some data comparing alfalfa isolates and red clover and sweet clover isolates are presented; principle interest, nonetheless, focuses on the isolation and characterization of the major fluorescent compound of alfalfa.

A Materials

The species and varieties of forage used were as follows:

a) Medicago sativa variety "Rhizoma" from the University of B.C.

b) Trifolium pratense variety "LaSalle" from Macdonald College, P.Q.

c) Melilotus alba, Canadian Common from Buckerfields Ltd., Vancouver, B.C.

B Methods

Method I

Approximately one hundred seeds of alfalfa, red clover, and sweet clover were placed on filter paper in petri plates. The paper was moistened with distilled water and the petri plates incubated at 26°C. for a period of 48 hours. The colour of the fluorescent material if any, was then noted under long and short wave ultra-violet light (3660Å and 2537Å respectively).

Method II

In order to compare the fluorescent compounds present in several forage legumes, parallel extractions were conducted on germinated and ungerminated seed of Rhizoma alfalfa, LaSalle red clover and Common sweet clover. In the extracts it was hoped that the main compounds responsible for the difference in fluorescence could be crudely separated.

One hundred grams of the seed of each species were germinated for 48 hours at 26°C., then ground in a Waring blendor, autolyzed briefly, and extracted three times with 200 ml. of hot 70% ethyl alcohol. The solutions were then filtered and concentrated in a flash evaporator at 35°C. to approximately 5% of the total volume. The concentrate was then striped on No. 3 Whatman filter paper and developed in B:A:W solvent. Colours and Rf values were then noted under ultra-violet lamps.

Methods III and IV

Two methods are listed below since procedures for handling small quantities of plant material differ rather markedly from those for handling large quantities of plant material.

III A micro isolation procedure

1. One hundred grams of alfalfa seed was ground in a Wiley mill using a 20 mesh screen and shaken with 200 ml. of hot 70% ethyl alcohol for 12 hours. The extraction was repeated with two portions of alcohol.
2. The crude extract was then concentrated in a flash evaporator to approximately 10% of the total volume and then extracted with 200 ml. of diethyl ether in a liquid-liquid extractor for 12 hours. The ether extract was then reduced to approximately 5% of the total volume in a flash evaporator at 30°C.
3. The ether extract was then striped on Whatman No. 3 filter paper and developed in B:A:W solvent. The papers were dried and observed under ultra-violet lamps to locate the blue-fluorescence.
4. The blue-fluorescent bands were then cut out and eluted with 70% ethyl alcohol. The eluate was rechromatogramed and eluted three times to purify.
5. After the final elution the alcohol solution was dried and the material taken up in a minimum quantity of hot water. The water solution was then treated with concentrated hydrochloric acid, concentrated, cooled, and the fine yellowish crystalline precipitate collected.

(Diagrammatic presentation of procedure, Figure 5.)

IV A macro isolation procedure.

1. Two kilograms of alfalfa seed were ground in a Wiley mill using a 20 mesh screen and immediately extracted with approximately 16 liters of hot water for three hours. The crude water extract was reduced in volume to approximately 4 liters.
2. The concentrated extract was then extracted with a total of 800 ml. of diethyl ether in a large liquid-liquid extraction unit.
3. The crude crystalline product formed during the extraction procedure was collected.
4. The volume of the ether extract was reduced to approximately 10% of the total volume and left in a freezer for several weeks. At the end of this time fairly pure crystals were obtained.

(Diagrammatic presentation of procedure, Figure 6.)

Method V

Colour tests and reactions.

Many colour tests are available to aid in the identification of unknown heterocyclic compounds. A number of the tests that proved useful in this investigation are presented here.

a) Ferric chloride test - 1.0 mg. of test material was dissolved in 0.5 ml. of 70% ethyl alcohol and an alcohol solution of ferric chloride was added and colours noted(17).

Figure 5 - Micro method for seed

Grind - (20 mesh)

Extract - (70% EtOH)

Reduce volume (in vacuo @ 35°C.)

Continuous Liquid-Liquid extraction (H_2O-Et_2O)

H_2O phase

discard

Et_2O phase - reduce volume

Chromatograph (Bu:HAc:H_2O solvent)

Elute (EtOH 70%)

Remove EtOH

Dissolve residue in minimal hot H_2O

Add conc. HCl

Reduce volume, cool

Collect crystals of the hydrochloride

Figure 6 - Macro method for seed

Grind - (20 mesh)

Extract - (Hot water)

Reduce volume (in vacuo @ 35°C.)

Continuous Liquid-Liquid extraction (H_2O-Et_2O)

H_2O phase	Et_2O phase	Volume reduction in vacuo
	Collect crystals	Collect crystals in freezer
		Recrystallization

b) Neutral and basic lead acetate test - a saturated aqueous solution of both reagents was added to a dilute alcoholic solution of the test material and colours of the precipitate noted (35).

c) NaOH test - a few drops of 1.0 N. NaOH solution were added to an alcoholic solution of the test material and the colour of the resulting solution noted (91).

d) Na_2CO_3 test - a few drops of 10% Na_2CO_3 solution were added to the alcoholic test solution and the colour noted.

e) HCl test - a few drops of concentrated HCl were added to the alcoholic test solution and the colour noted.

f) H_2SO_4 test - a few drops of concentrated H_2SO_4 were added to the alcoholic test solution and the colour noted (53, 70).

g) HNO_3 test - a few drops of concentrated HNO_3 were added to the alcoholic test solution and the colour noted (84).

h) Mg - HCl test - 1.0 mg. of the test material was dissolved in 1.0 ml. of 70% ethyl alcohol; a small piece of magnesium ribbon was added and then a few drops of concentrated HCl. The formation of colours was noted for a period of fifteen minutes (79, 90, 96).

i) Zn - HAc - HCl test - 0.50 grams of screened, graded zinc was added to 1.0 ml. of a water solution of the test material (0.5 mg. per ml.). To this mixture 2.0 ml. of acetone was added, followed by 1.0 ml. of glacial Acetic acid. The mixture was then stirred at 65°C. for 40 minutes. 1.0 ml. of concentrated HCl was added slowly and the

colour formed in 15 to 20 minutes noted (6, 37, 38, 39, 79).

j) Diazotized - p - nitroaniline test - this test was conducted as outlined in Investigation I (Methods).

k) Boric-citric acids test - the boric acid and citric acid solutions were prepared as outlined by Wilson (126) and Taubock (111). 0.5 mg. of the test material was dissolved in 1.0 ml. of dry acetone and half put in the boric-citric acid solution and half put in the citric acid solution. The colour in the boric-citric acid solution was compared with that in the citric acid solution.

l) Choline-tetraphenyldiboroxide test - some aqueous choline solution was placed on a watch glass and a few drops of tetraphenyldiboroxide (as prepared by Neu (72)) was added. Then a few drops of the test solution (0.25 mg. in 1 ml. of methanol) was added and the colour in daylight and in ultra-violet light noted (73).

m) NH_3 test - the compound on a chromatogram was exposed to ammonia fumes and the colour in daylight and in ultra-violet light noted.

Method VI

pH fluorescence

A rapid method to assess the reaction of a compound to changes in the hydrogen ion concentration is presented below.

An array of solutions of different hydrogen ion concentrations as outlined by Goodwin and Kavanagh (44, 45) was

prepared and a few mls. of each solution was placed in petri plates. Pieces of No. 3 Whatman filter paper containing the test compound were cut up and floated on the pH solutions. The reaction as observed under ultra-violet light was noted immediately.

Method VII

Analytical data derived from the purified crystalline substance.

a) Melting point determination - a few micrograms of the crystalline test material were spread on a slide, covered and observed on a "Kofler Micro Hot Stage".

b) Ultra-violet absorption spectrum - the compound was dissolved in spectral grade isopropyl alcohol (3 mg./l.) and the ultra-violet spectrum measured using 3 ml. silica cuvettes in a Beckman DK-2 spectrophotometer.

c) Infra-red absorption spectrum - 1.0 mg. of crystalline material and 1.0 gram of potassium bromide (anhydrous) were thoroughly mixed and put into a pelleting unit. The pellet, prepared using a pressure of 20,000 lb. per square inch, was a circular translucent sheet of K Br and the crystalline material. The pellet was then inserted in a Beckman I.R.3 spectrophotometer and the recording made.

d) Rf data - the Rf of the individual spots was measured using a chromatogrid distributed by the California Foundation for Biochemical Research, Los Angeles 63, California, U.S.A.

e) Carbon-hydrogen analysis - eight mg. of the purified

crystalline product was sent to Clark Microanalytical Laboratories, 104½ West Main St., P.O. Box 17, Urbana, Illinois, U.S.A., for this analysis.

f) Nitrogen, Sulphur and Halogens - these tests were conducted according to McGookin (68).

C Observations and results

The results are tabled (Tables 13 - 19) and need little supplementation or complementation.

The occurrence of a distinctive brilliantly-fluorescing compound or compounds associated with germinating alfalfa seed is substantiated in Table 13: germinating red clover and sweet clover seed, by contrast, did not fluoresce. Nonetheless, it is notable that, through the method II (Table 14), the presence of alcohol-soluble fluorescent compounds in the living or autolyzed tissues of red clover and sweet clover is established. Table 14, furthermore, establishes the occurrence of fluorescent compounds in hydrolysate and autolysate of germinated seed which do not occur in the non-germinated seed.

The recognition of the major fluorescent compounds diffusing to filter paper or other media from germinating alfalfa seed presents no special difficulty; they appear to be two in number and one of these is the most brilliant and probably the most abundant.

The principle fluorescent and absorbing spots seen,

when comparable chromatograms were examined under ultra-violet light are given with their approximate Rf. values in Tables 13 - 16.

TABLE 13 - Colour of diffusates from germinating legume seed under visible and U.V. light

Seed	colour in Visible light	colour in Ultra-violet light
alfalfa	yellow exudate	bright bluish-fluorescence
sweet clover	yellow exudate	no fluorescence
red clover	yellow exudate	no fluorescence

TABLE 14 - A comparison of fluorescent spots in germinated and ungerminated alfalfa seed extracts from method II

	Germinated		Not Germinated	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	d.Ab.	.08	l.B.F.	.07
2	W.F.	.13	Y.W.F.	.12
3	d.Ab.	.20	-	-
4	l.B.F.	.25	-	-
5	d.Ab.	.37	d.Ab.	.35
6	b.B.F.	.60	b.B.F.	.64
7	d.Ab.	.79	d.Ab.	.83

TABLE 15 - A comparison of fluorescent spots in germinated and ungerminated Common sweet clover seed extracts from method II

	Germinated		Not Germinated	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	v.l.B.F.	.05	Y.W.F.	.07
2	-	-	d.Ab.	.14
3	Y.F.	.24	v.l.Y.F.	.18
4	d.Ab.	.26	d.Ab.	.25
5	l.B.F.	.35	l.B.F.	.37
6	l.Y.F.	.40	-	-
7	l.Ab.	.42	-	-
8	l.B.F.	.48	l.B.F.	.47
9	l.B.F.	.61	l.B.F.	.69
10	l.B.F.	.82	-	-
11	d.Ab.	.90	d.Ab.	.90

TABLE 16 - A comparison of fluorescent spots in germinated and ungerminated red clover seed extracts

	Germinated		Not Germinated	
Spot	colour	Rf(B:A:W)	colour	Rf(B:A:W)
1	d.Ab.	.09	d.Ab.	.08
2	-	-	l.B.F.	.18
3	l.Ab.	.21	-	-
4	l.Y.F.	.28	l.Y.F.	.25
5	l.B.F.	.32	l.B.F.	.37
6	l.B.F.	.48	-	-
7	-	-	l.B.F.	.64
8	l.B.F.	.91	l.B.F.	.85

For the most brilliant compound, which was isolated and crystallized by several methods, aforementioned, the pH-fluorescence data are given in Table 17. The wide range of pH over which the compound fluoresces in the ultra-violet and the attendant colour changes are notable.

TABLE 17 - pH - ultra-violet fluoresce response of alfalfa isolate

pH	Materials	Fluorescence
-1.6	8.0 N. H_2SO_4	Y.G.F.
-0.7	4.0 N. H_2SO_4	l.Y.G.F.
0.3	1.5 N. H_2SO_4	l.Y.G.F.
1.3	0.1 N. H_2SO_4	B.F.
2.2	98 ml. 0.1 M. citric acid and 2 ml. 0.2 N. Na_2HPO_4	B.F.
3.0	79.45 ml. 0.1 M. citric acid and 20.55 ml. 0.2 N. Na_2HPO_4	B.F.
4.0	61.45 ml. 0.1 M. citric acid and 38.55 ml. 0.2 N. Na_2HPO_4	B.F.
5.0	48.5 ml. 0.1 M. citric acid and 51.5 ml. 0.2 N. Na_2HPO_4	B.F.
6.0	36.85 ml. 0.1 M. citric acid and 63.15 ml. 0.2 N. Na_2HPO_4	B.F.
7.0	17.65 ml. 0.1 M. citric acid and 82.35 ml. 0.2 N. Na_2HPO_4	l.Y.G.F.
8.0	2.75 ml. 0.1 M. citric acid and 97.25 ml. 0.2 N. Na_2HPO_4	Y.G.F.
9.45	0.02 M. Na_2HPO_4	Y.G.F.
10.4	0.2 N. Na_2HPO_4 and 2 M. Na_2CO_3	b.Y.G.F.
11.0	0.2 M. Na_2CO_3	b.Y.G.F.
11.7	0.01 N. NaOH	Y.G.(quenched)
12.6	0.1 N. NaOH	Y.G.(quenched)

B.F. - Blue Fluorescence

l.Y.G.F. - light Yellow Green Fluorescence

Y.G.F. - Yellow Green Fluorescence

b.Y.G.F. - bright Yellow Green Fluorescence

Characterization of the crystalline product was materially aided by a series of spot tests, the record of which is given in Table 18. The flavonoid nature of the compound is indisputably established; furthermore the compound yields tests very characteristic of polyhydroxy flavones, viz.

1) Mg -HCl test - this test is one of the most useful qualitative tests for the study of flavonoid compounds. The appearance of a pink to magenta colour indicates the presence of flavonols, flavanones and flavanolones. Flavones which lack the 3 - hydroxyl group do not usually respond to this test. In addition, the shade of the colour that develops is fairly characteristic and three general categories are noted

flavones	-	Orange	-	red
flavonols	-	red	-	crimson
flavanones	-	crimson	-	Magenta

2) NH_3 test - the development of a bright yellow spot when a compound is exposed to NH_3 is a fairly reliable test for certain polyphenolic carbonyl compounds represented by the flavones, flavanones, chalcones and xanthones. This test is very useful as a rapid means to determine the structures mentioned.

3) $FeCl_3$ test - the production of colours with ferric chloride is a general property of all classes of polyhydroxy flavonoid compounds. Ortho-dihydroxyl groups often give

green colours but this generalization is of limited value because green colours are also given by many compounds which do not contain the ortho-dihydroxyl grouping. However, the presence of a 3, 4, 5 trihydroxy grouping in the B ring can be detected because deep blue to black colours are produced with this reagent.

TABLE 18 - Spot test results given by the crystalline isolate from alfalfa

Name	Test	U.V. Test	Remarks
$FeCl_3$	dark green sol.	-	++(polyhydroxy flavonoids)
neut. PbAc	yellow ppte.	-	+(flavones)
basic PbAc	yellow ppte.	-	+(flavones)
NaOH	yellow on paper	greenish yellow	+(flavones and flavonols)
Na_2CO_3	yellow on paper	greenish yellow	+(flavones and flavonols)
HCl	yellow sol.	dull yellow	
H_2SO_4	bright yellow sol.	-	+(flavones and flavonols)
HNO_3	yellowish-brown sol.	-	
Mg-HCl	yellow immediately, red in 10 secs.	-	++(flavonols, flavanones, flavanolones)
Zn.-HAc-HCl	red in 30 mins.	-	+(slight flavanone - contaminant?)
Diazo	no reaction	-	-No structure like simple coumarins
Boric-citric	bright yellow sol.	-	+possible 5-OH gp.
Choline-t.P.b.	reddish brown sol.	bright reddish orange	+possible 3-OH gp.
NH_3	yellow on paper	bright yellowish green	+(flavones, flavanones, chalcones and xanthones)

TABLE 19 - Physical and chemical data for the crystalline isolate from alfalfa

19:1 Physical data

1. Colour - pale yellow
2. Crystal form - thin needles
3. Melting point - 261° - 262°C.

19:2 Microanalysis (organic)

Carbon	- 55.9%	Sulphur	- nil
Hydrogen	- 4.18%	Halogens	- nil
Nitrogen	- nil		

19:3 Rf data (Whatman No. 1 paper) (20°C.)

Solvent	Spot centre	leading edge
B:A:W (4:1:5) organic phase	.57	.69
HAc:H_2O (1:3)	.40	.48
Isopropanol:H_2O (1:4)	.06	.11

19:4 Infra-red spectrum for the alfalfa isolate

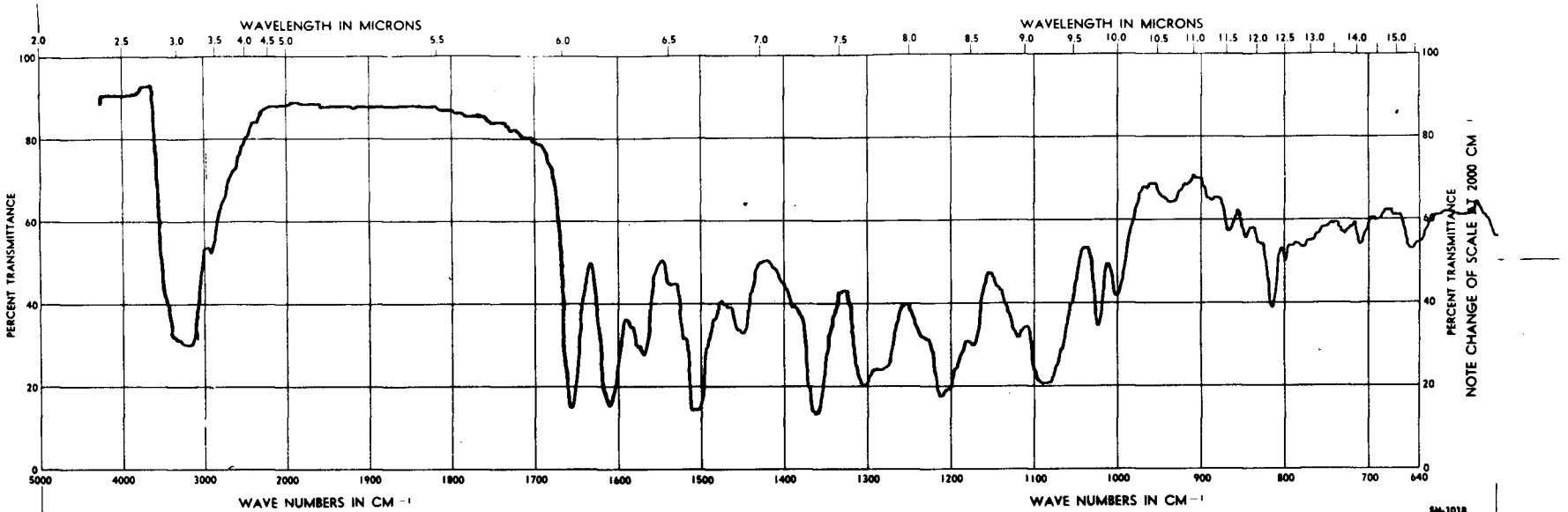
Please see Figure 7 Graph A.

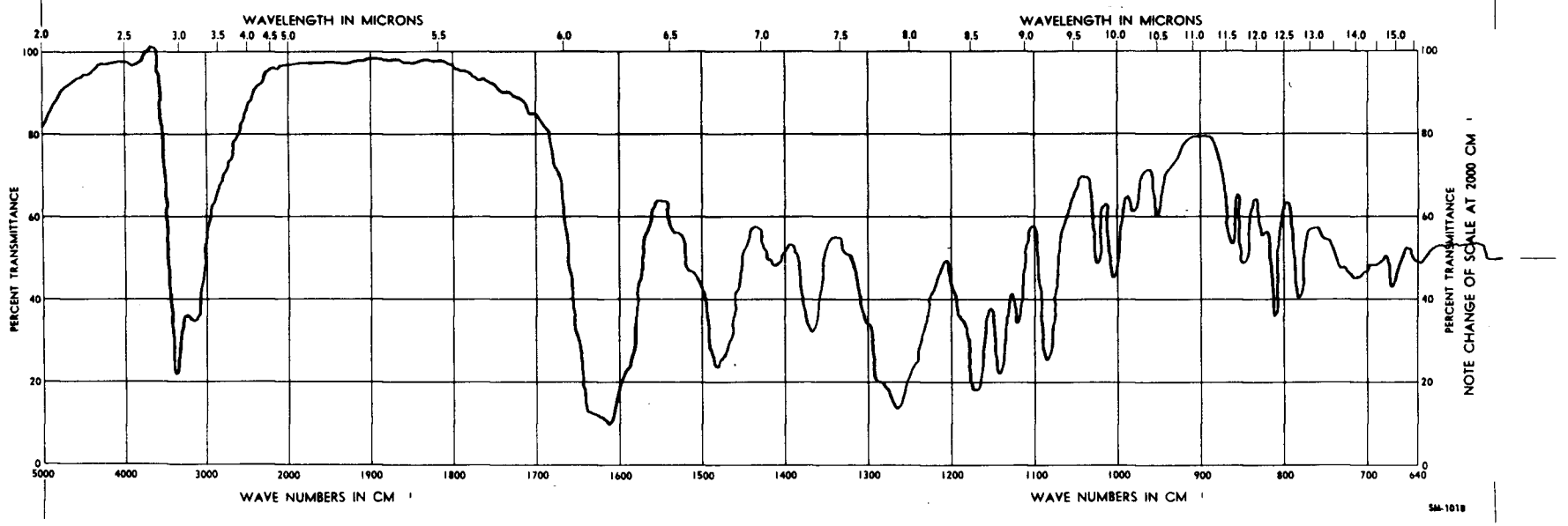
Interpretation of the spectrum indicates that the compound contains a carbonyl at the 4 position and several hydroxyl groups, similar in many respects to dihydroquercetin (Figure 7 Graph B). Additionally, there is some indication that the carbonyl is chelated with a group at the 3 position on the pryone ring.

Graph A - alfalfa isolate

Figure 7 - I.R. absorption spectra

Graph B - dihydroquercetin


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
PERCENT TRANSMITTANCE
0 20 40 60 80 100
5000 4000 3000 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 640
WAVE NUMBERS IN CM⁻¹
NOTE CHANGE OF SCALE AT 2000 CM⁻¹
SM-1018


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
PERCENT TRANSMITTANCE
0 20 40 60 80 100
5000 4000 3000 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000 900 800 700 640
WAVE NUMBERS IN CM⁻¹
NOTE CHANGE OF SCALE AT 2000 CM⁻¹
SM-1018

19:5 Ultra-violet spectrum for the alfalfa isolate

Please see Figure 8.

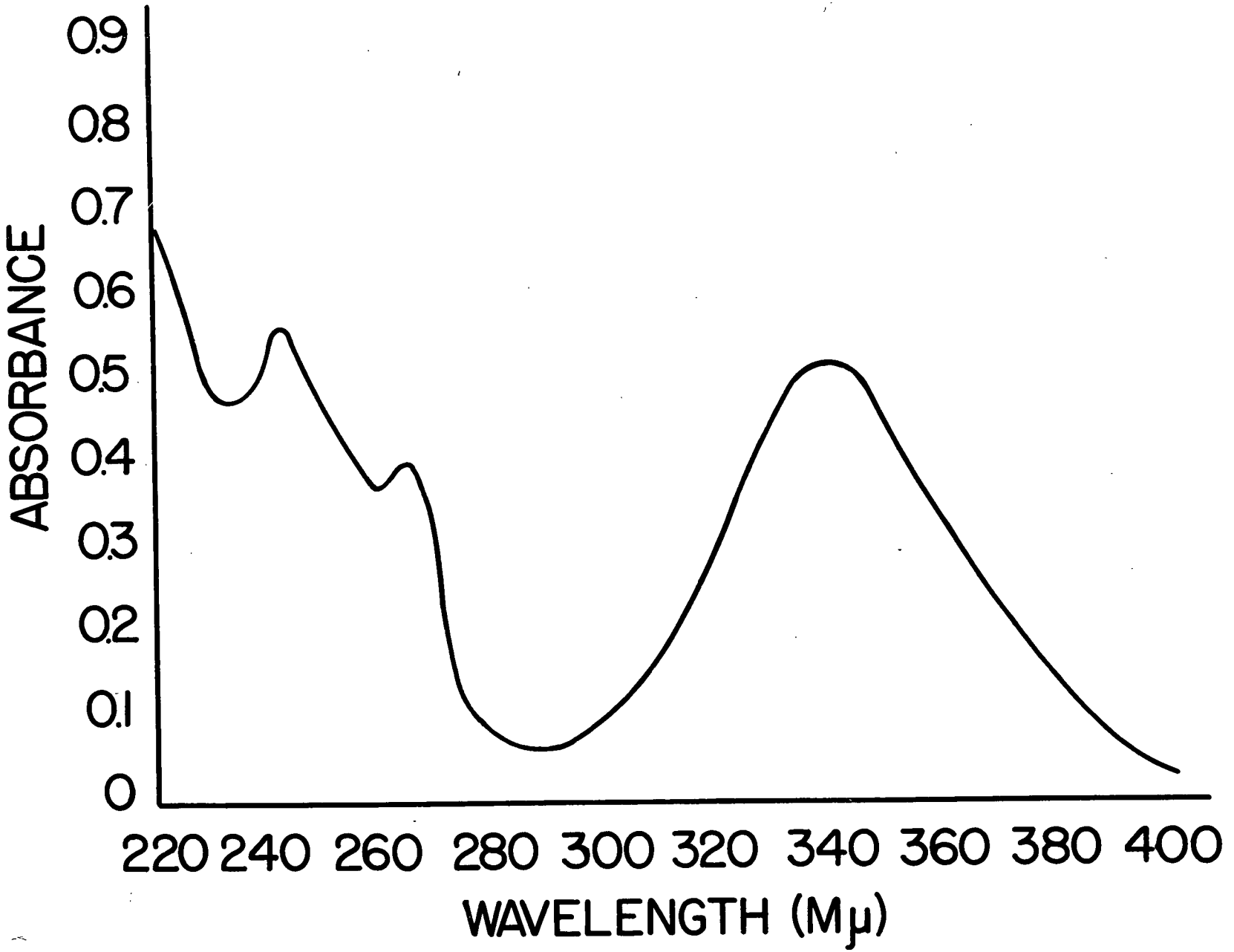
Maxima	Minima
241.5	232.5
264.0	259.0
339.5	287.0

Numerous attempts to crystallize the unknown compound in such solvents as benzene, chloroform, and ethyl alcohol proved unsuccessful since only a brown sticky residue was formed. After several attempts, approximately 3.5 mg. of a dark yellow material (a chloride salt) was precipitated from a water solution treated with concentrated HCl. Because such small yields were obtained from this method, a large scale extraction was attempted using diethyl ether since it was noted that the compound was sparingly soluble in this solvent. A crude crystalline product (approximately 30 mg.) was collected during the extraction procedure, and a pure crystalline product (approximately 15 mg.) was collected from the diethyl ether extract when it was reduced in volume and left in a freezer for several weeks.

D Discussion

An attempt to find a coumarin metabolism in alfalfa led to the study of two brilliantly fluorescing compounds which diffuse from germinating alfalfa seed. Justification

Figure 8 - U.V. absorption spectrum of alfalfa isolate


ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220 240 260 280 300 320 340 360 380 400
WAVELENGTH (Mμ)

for the attempt lay in the somewhat limited evidence of spot tests, fluorescence type, and the fact that a coumarin metabolism seemed to be widespread in the legumes.

The two compounds are undoubtedly related chemically for in various procedures they appear together; their solubilities, fluorescence, Rf values, response to spot tests are similar. If one compound is characterized very probably characterization of the other would not be difficult.

Unquestionably the one compound chosen for study is flavonoidal. Furthermore it is well hydroxylated, but not much more can be stated with certainty.

The occurrence of a coumarin-like structure in the molecule cannot be ruled out entirely, but it is definitely not a simple, more or less, symmetrical coumarin. The ultraviolet spectral maxima fall within the ranges given by Geissman (36) for flavones; on the other hand it is to be noted that the U.V. spectral maxima for coumestrol also fall within this range and caused its discoverers much concern.

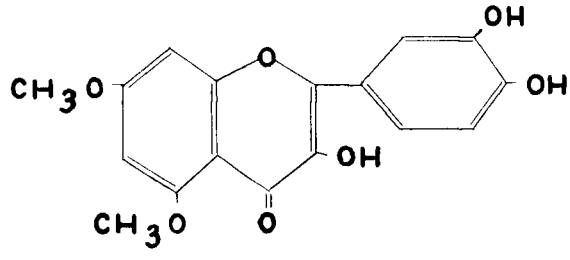
The infra-red spectrum is most interesting and as was pointed out by H. McLean and G. M. Barton of the Forest Products laboratory, very closely resembles that for dihydroquercitin, a flavanone.

Some evidence of value in characterization may be gained from the fact that the alfalfa isolate is brilliantly blue fluorescent; the fluorescence is very similar to that of the coumarins, umbelliferone, coumestrol and the flavone, 3:4 - dihydroxy flavone. Dihydroquercitin and quercitin are

not fluorescent and appear brown in ultra-violet.

Examination in the laboratory and in publications, of physical and chemical properties of large numbers of coumarins and flavonoids does not yield any whose properties are closely similar to those of the alfalfa isolate.

The sum of all available evidence, some firm and some speculative, leads to the suggestion that the alfalfa isolate is a polyhydroxy flavone with the following approximate structure and formula.



$C_{17}H_{14}O_7$

V SUMMARY AND CONCLUSIONS

1) The breeding of forage crops for modifications, losses or gains, of specific chemical constituents is a comparatively new development in forage crop breeding. One undertaking of this kind, that of breeding the forage legume, sweet clover, free from the bitter, and indirectly toxic, coumarin has met with some success. Success, the literature reveals, has attended the undertaking, only after many errors were made. Some of the mistakes could, very probably, have been avoided if a better understanding of coumarin metabolism had been known. An understanding of how genes for bitterness and non-bitterness in sweet clover act in biochemical terms would provide valuable information for the plant breeder. Little is known of the metabolism of coumarin and its relatives even in sweet clover and in related forage species such as alfalfa.

2) From a survey of the literature it was noted that a great deal is known of the chemistry of coumarins, that a great deal is known of coumarin pharmacology in animals, that a little is known of coumarin as it is applied to non-coumarin containing plants, but that very little is known of the function and metabolism of coumarin in the plant.

3) In an attempt to determine the nature of the differences in the actions of genes for non-bitterness and

bitterness (viz. coumarin free and coumarin present), "Cumino" non-bitter sweet clover and, the genotypically very similar bitter Common sweet clover were compared chromatographically. Compounds not well characterized, but which were very likely coumarin relatives appeared in "Cumino" sweet clover. It is suggested that genes for non-bitterness in "Cumino" sweet clover, and very probably in Melilotus dentata, may be responsible for methylation or hydroxylation of the coumarin per se and its close relatives which occur in Common sweet clover. The coumarins, thus elaborated in "Cumino" and M. dentata are non-bitter and are formed by simple additions to the coumarin ring or to its precursor.

4) A search for a metabolic system involving coumarins in alfalfa, a close relative, taxonomically, of sweet clover, led to the special study of two brilliantly fluorescing compounds occurring in seed and leaf, which it was thought, from preliminary examination, were coumarins. Detailed study however showed that the compounds were not likely coumarins as such, but polyhydroxy flavones, new to the literature and new to forage chemistry. The proposed formula for one of the two closely related compounds is as follows: 3:3′:4′ trihydroxy - 5:7 dimethoxy flavone.

VI LITERATURE CITED

1. Andreae, W. A.
1948 The Isolation of a Blue Fluorescent Compound Scopoletin from Green Mountain Potato Tubers, Infected with Leaf Roll Virus. Can. J. Res. C. 26 : 31-34.

2. Andreae, S. R. and Andreae, W.A.
1949 The Metabolism of Scopoletin by Healthy and Virus Infected Potato Tubers. Can. J. Res. C. 27 : 15-22.

3. Audus, L. J.
1948 Studies on the Phytostatic Action of 2,4-dichlorophenoxyacetic Acid and Coumarin. The Reversibility of Root Growth Inhibitions. New Phytol. 47 : 196-219.

4. Audus, L. J. and Quastel, J. H.
1947 Coumarin as a Selective Phytocidal Agent. Nature. 159 : 320-324.

5. Avers, C. J. and Goodwin, R. H.
1956 Studies on Roots IV. Effects of Coumarin and Scopoletin on the Standard Root Growth Pattern on Phleum pratense. Amer. J. of Bot. 43 (8) : 612-620.

6. Barton, G. M. and Gardner, J. A. F.
1958 Determination of Dihydroquercetin in Douglas Fir and Western Larch Wood. Anal. Chem. 30 (2) : 279-281.

7. Bate-Smith, E. C.
1956 The Commoner Phenolic Constituents of Plants and their Systematic Distribution. Scient. Proc. Roy. Dublin Soc. 27 : 165-176.

8. _____, Swain, T. and Pope, G. S.
1953 The Isolation of 7 - hydroxy - 4 methoxy Isoflavone (Formononetin) from Red Clover (Trifolium pratense) and a Note on the Identity of Pratol. Chem. and Ind. 1127.

9. _____, and Westall, R. G.
1950 Chromatographic Behavior and Chemical Structure 1. Some Naturally Occurring Phenolic Substances. Biochim. Biophys. Acta. 4: 427-440.

10. Best, R. J.
1944 A Fluorescent Substance Present in Plants. 2. Isolation of the Substance in a Pure State and its Identification as 6 - methoxy-7 hydroxy - 1:2 benzpyrone. Austral. J. Exp. Biol. Med. Sci. 22 : 251-255.

11. _____.
1948 Studies on a Fluorescent Substance Present in Plants. 3. The Distribution of Scopoletin in Tobacco Plants and Some Hypotheses on its Part in Metabolism. Austral. Exp. Biol. Med. Sci. 26 : 223-230.

12. Bickoff, E. M., Booth, A. N., Lyman, R. L., Livingston, A. L., Thompson, C. R., DeEds, F.
1957 Coumestrol, a New Estrogen Isolated from Forage Crops. Sci. 126 (3280) : 969-970.

13. Bickoff, E. M., Booth, A. N., Lyman, R. L., Livingston, A. L., Thompson, C. R., and Kohler, G. O.
1958 Isolation of a New Estrogen from Ladino Clover. Agric. and Food Chem. 6 (7) : 536-539.

14. Bickoff, E. M., Lyman, R. L., Livingston, A. L., Booth, A. N.
1958 Characterization of Coumestrol, a Naturally Occurring Plant Estrogen. J. Am. Chem. Soc. 80 : 3969-3971.

15. Blank, F.
1947 The Anthocyanin Pigments of Plants. Bot. Rev. 13 : 241-317.

16. Block, R. J., Le Strange, R., Zweig, G.
1952 Paper Chromatography. Academic Press, New York.

17. Briggs, L. H., and Locker, R. H.
1951 Chemistry of New Zealand Melicope species. Part VII Some Observations on the Relation Beween the Constitution of Some Flavonol Derivatives and their Acidity, Basicity, $FeCl_3$ Colours and U.V. Absorption Spectra. J. Chem. Soc. 3136-3142.

18. Brink, R. A.
1934 A Non-bitter Variety of Melilotus. Sci. 79 : 301.

19. _____, and Roberts, W. L.
1937 The Coumarin Content of Melilotus dentata. Sci. 86 : 41-42.

20. Brink, V. C.
1940-41 The Content Distribution, and Some Metabolic Aspects of Coumarin in Sweet Clover. (Melilotus alba Des). Summaries of Doctoral Dissertations, University of Wisconsin.

21. _____.
1958 Note on a Fluorescence Test to Distinguish Seeds of Alfalfa and Sweet Clover in Mixtures. Can. J. Plant Sci. 38 : 120-121.

22. Campbell, H. A., and Link, K. P.
1941 Studies on the Hemorrhagic Sweet Clover Disease. IV The Isolation and Crystallization of the Hemorrhagic Agent. J. Biol. Chem. 138 : 21-32.

23. _____, Smith, W. K., Roberts, W. L., and Link, K. P.
1941 Studies on the Hemorrhagic Sweet Clover Disease. II The Bioassay of Hemorrhagic Concentrates by Following the Prothrombin Level in the Plasma of Rabbit Blood. J. Biol. Chem. 138 : 1-20.

24. Canter, F. W., and Robertson, A.
1931 Conversion of 7-hydroxy-3:4 dimethylcoumarin into 2:4 dimethyoxy α, β dimethyl cinnamic acid. J. Chem. Soc. 1875-1876.

25. Casteel, H. W., and Wender, S. H.
1953 Identification of Flavonoid Compounds by Filter-paper Chromatography. Anal. Chem. 25 : 508-509.

26. Clayton, J. S.
1934 A Study of the "Bitter Principle" in Melilotus species. MSc. Thesis, University of Saskatchewan, Saskatoon, Saskatchewan.

27. _____, and Larmour, R. K.
1935 A Comparative Colour Test for Coumarin and Melilotic Acid in Melilotus species. Can. J. Res. C. 13 : 89-100.

28. Cornman, I.
1947 The Responses of Onion and Lily Mitosis to Coumarin and Parasorbic Acid. J. Exp. Biol. 23 : 292-297.

29. Dean, F. M.
1952 Naturally Occurring Coumarins. Fortschritte Der Chemie Organischer Naturstoffe. 9 : 225-291.

30. Duncan, I. J., and Dustman, R. B.
1934 Quantitative Determination of Coumarin in Plant Material. Ind. Eng. Chem. (Anal. Ed.) 6 : 210-213.

31. _____, and _____.
1937 Determination of Coumarin in Sweet Clover. A Comparison of the Steam Distillation and Alcoholic Extraction Methods. Ind. Eng. Chem. 9 (10) : 471-474.

32. Dworak, L.
1937 The Selection of Elite Forms of Melilotus albus with Low Coumarin Content. Herbage Abstr. 7 : 217.

33. Emerson, O. H., and Bickoff, E. M.
1958 Synthesis of Coumestrol 3,9-dihydroxy-6H-benzofuro (3,2-c) (1) benzopyran-6-one. J. Amer. Chem. Soc. 80 : 4381-4383.

34. Evenari, M.
1949 Germination inhibitors. Bot. Rev. 15 : 153-194.

35. Gage, T. B., Douglass, C. D., Wender, S. H.
1951 Identification of Flavonoid Compounds by Filter-paper Chromatography. Anal. Chem. 23 (2) : 1582-1585.

36. Geissman, T. A.
1955 Anthocyanins, Chalcones, Aurones, Flavones and Related Water-soluble Plant Pigments. Modern Methods of Plant Analysis. 3 : 450-498.

37. _____, and Clinton, R. O.
1946 Flavanones and Related Compounds. I. The Preparation of Polyhydroxy Chalcones and Flavanones. J. Am. Chem. Soc. 68 : 697-700.

38. _____, and _____.
1946 Flavanones and Related Compounds. II. The Coloured Reduction Products of Polyhydroxy-flavanones. J. Am. Chem. Soc. 68 : 700-706.

39. _____, and _____.
1946 Flavanones and Related Compounds. III. The Reduction of Luteolin and Apigenin Trimethyl-ether with Sodium Amalgam. J. Am. Chem. Soc. 68 : 706-708.

40. Geissman, T. A., and Hinreiner, E.
1952 Theories of the Biogenesis of Flavonoid Compounds. Bot. Rev. 18 (1) : 77-244.

41. Gelcinskaja, R. B., and Bordunova, M. A.
1934 Breeding Coumarin-free Sweet Clover. Trudy Prik. Bot. Genet. i Selektcii. 3 : 315-323 (Herbage Abstr. 6 : 116.1936.)

42. Goodwin, R. H., and Kavanagh, F.
1948 Fluorescing Substances in Roots. Bull. Torrey Bot. Club. 75 (1) : 1-17.

43. _____, and _____.
1949 The Isolation of Scopoletin, a Blue-Fluorescing Compound from Oat Roots. Bull. Torrey Bot. Club. 76 : 255-265.

44. _____, and _____.
1950 Fluorescence of Coumarin Derivatives as a Function of pH. Arch. Biochem. Biophys. 27 (1) : 152-173.

45. _____, and _____.
1952 Fluorescence of Coumarin Derivatives as a Function of pH. Arch. Biochem. Biophys. 36 : 442-455.

46. _____, and Pollock, B. M.
1954 Studies of Roots. I. Properties and Distribution of Fluorescent Constituents in Avena Roots. Amer. J. Bot. 41 (6) : 516-529.

47. _____, and Taves, Carolyn.
1950 The Effect of Coumarin Derivatives on the Growth of Avena Roots. Amer. J. Bot. 37 : 224-231.

48. Goplen, B. P., Greenshields, J. E. R., and Baenziger, H.
1957 The Inheritance of Coumarin in Sweet Clover. Can. J. Bot. 35 : 583-593.

49. _____, _____, and White, W. J.
1956 Selection Techniques in Screening for Coumarin Deficient Sweet Clover Plants. Can. J. Bot. 34 : 711-719.

50. Harborne, J. B.
1959 Chromatography of Flavanoid Pigments. J. of Chromatography. 2 : 581-604.

51. Hayashi, K., Abe, Y., Noguchi, T., and Suzushino, K.
1953 Studien über Anthocyane. XXII. Beiträge zur papier-chromatographischen. Analyse der natürlichen Anthocyane und Anwendung derselben zur Untersuchung von Farbstoffen en der roten Impatiens-Blüten und den blutroten Pfirsich-Früchten. Pharm. Bull. Pharm. Soc. Japan. 1 : 130-134.

52. Horner, W. H., and White, W. J.
1941. Investigations Concerning the Coumarin Content in Sweet Clovers. III. The Inheritance of the Low Coumarin Character. Sci. Agr. 22 : 85-92.

53. Hunter, E. C. E., and Partington, J. R.
1933 Studies in Dielectric Polarization. Part V. Benzene Solutions of Pyrones, Thiopyrones, and Thioketones, Dioxan Solutions of Ureas and Thioureas. J. Chem. Soc. 87-90.

54. Iida, G.
1935 Chemische und Pharmakologische Untersuchungen üben das Fraxin, einen Bestandteil von Fraxinus borealis Naki. Tohoku J. Exp. Med. 25 : 454.

55. Ishkawa, S.
1955 A Note on the Effect of Coumarin on the Germination of Seeds. Kumamoto J. of Sci. 2 (B) : 97-103.

56. _____.
1958 Physiological Studies on Seed Germination. II. Affect of Coumarin on Respiration by Germinating Seeds. Kumamoto J. Sci. 4 (1) : 9-15.

57. Kirk, L. E.
1926 A Comparison of Sweet Clover Types with Respect to Coumarin Content, Nutritive Value and Leaf Percentages. J. Amer. Soc. Agron. 18 : 385-392.

58. _____, and Davidson, J. G.
1928 Potentialities of Sweet Clover as Plant Breeding Material. Sci. Agric. 8 : 446-455.

59. Kulkarni, A. B., and Merchant, J. R.
1955 J. Sci. Ind. Res. 14 (B) : 153-156.

60. Lederer, E.
1949 Chemistry and Biochemistry of Some Mammalian Secretions and Excretions. J. Chem. Soc. 1949: 2115-2125.

61. Lederer, E., and Lederer, M.
1957 Chromatography, a Review of Principles and Applications. Sec. ed. Elsevier Pub. Co. Amsterdam, New York.

62. Limaye, D. B.
1934 Synthese von 2- acyl - resorcinen mittels des. Nidhon-Prozesses. I Treil:2-Benzoyl resorcin. Ber. 67 : 12-15.

63. Link, K. P.
1943/1944 The Anti-coagulant from Spoiled Sweet Clover Hay. The Harvey Lectures. 39 : 162-216.

64. Lyman, R. L., Bickoff, E. M., Booth, A. N., and Livingston, A. L.
1959 Detection of Coumestrol in Leguminous Plants. Arch. Biochem. Biophys. 80 : 61-67.

65. Martin, A. J. P.
1947/1948 Partition Chromatography. Ann. N.Y. Acad. Sci. 49 : 249-264.

66. Mayer, A. M., and Evenari, M.
1952 The Relation between the Structure of Coumarin and its Derivatives, and their Activity as Germination Inhibitors. J. Exp. Bot. 3 : 246-252.

67. Maynard, L. A.
1947 Animal Nutrition. 2nd. Ed. McGraw-Hill Book Co. Inc. New York.

68. McGookin, A.
1955 Qualitative Organic Analysis. Reinhold Publishing Corporation. New York.

69. Miller, C. O., and Meyer, B. S.
1951 Expansion of Chenopodium album Leaf Disks as Affected by Coumarin. Plant Physiol. 26 : 661-634.

70. Murti, V. V. S., Rajogopalan, S., and Row, L. R.
1951 Proc. Ind. Acad. Sci. 34 (A) : 319-323.

71. Neish, A. C.
1960 Biosynthetic Pathways of Aromatic Compounds. Ann. Rev. of Plant Phys. 11 : 55-80.

72. Neu, R.
1954 Tetraphenyl-diboroxyd aus Natriumtetraphenyloborate. Ber. 87 : 802-805.

73. Neu, R., and Schwabe, W.
1958 The Analytical Use of Diarylboric Acids and Especially of Tetraphenyldiboroxide. Chem. Analyst. 47 (4) : 106-109.

74. Nutile, G. E.
1945 Inducing Dormancy in Lettuce Seed with Coumarin. Plant Physiol. 201 : 433-442.

75. Obermayer, E. Z.
1913 Quantitative Bestimmung des Kumarins in Melilotus-Arten. Z. Anal. Chem. 52 : 172-191.

76. Paech, K.
1955 Colour Development in Flowers. Ann. Rev. Plant Physiol. 6 : 273-298.

77. Paris, R. A.
1952 Chromatographic sur Papier de Quelques Dérivés Flavoniques. Bull. Soc. Chim. 34 : 767-772.

78. Pechmann, H., and Cohen, J. B.
1884 Ueber die Verbendungen der Phenole mit Acetessigäther. Das -Methylumbelliferon. Ber. 17 : 2129-2138.

79. Pew, J. C.
1948 A Flavanone from Douglas-Fir Heartwood. J. Am. Chem. Soc. 70 : 3031-3034.

80. Pollock, B. M., Goodwin, R. H., and Green, Susan.
1954 Studies on Roots. II. Effects of Coumarin, Scopoletin and other Substances on Growth. Amer. J. Bot. 41 (6) : 521-529.

81. Pope, G. S., Elcoate, P. V., Simpson, S. A., and Andrews, D. G.
1953 Isolation of an Oestrogenic Isoflavone (Biochanin A) from Red Clover. Chem. Ind. 1092.

82. _____, and Wright, H. G.
1954 Oestrogenic Isoflavones in Red Clover and Subterranean Clover. Chem. Ind. 1019-1020.

83. Quick, A. J.
1937 The Coagulation Defect in Sweet Clover and in the Hemorrhagic Chick Disease of Dietary Origin. Am. J. Physiol. 118 : 260-271.

84. Rao, P. S., and Seshadri, T. R.
1949 Proc. Ind. Acad. Sci. 30 (A) : 30-34.

85. Rinke, E. H.
1945 Inheritance of Coumarin in Sweet Clover. J. Am. Soc. Agron. 37 : 635-642.

86. Roberts, W. L., and Link, K. P.
1937 Determination of Coumarin and Melilotic Acid. A Rapid Micro Method for Determinating Melilotus Seed and Green Tissue. Ind. Eng. Chem. (Analyt. Edit.) 9 : 438-441.

87. _____, and _____.
1937 A Precise Method for the Determination of Coumarin, Melilotic Acid and Coumaric Acid in Plant Tissue. J. Biol. Chem. 119 : 269-281.

88. Robinson, R.
1936 Formation of Anthocyanins in Plants. Nature. 137 : 172-173.

89. Roderick, L. M., and Schalk, A. F.
1931 Studies on Sweet Clover Disease. N. Dak. Agr. Expt. Sta. Bull. 25.

90. Ross, D. J.
1950 The Chemistry of Corymbiferin, a Pigment Derived from a Glucoside Occurring in Gentiana corymbifera. New Zealand J. Sci. Tech. 32 (B) : 39-43.

91. Row, L. R., and Seshadri, T. R.
1945 Proc. Ind. Acad. Sci. 22 (A) : 215-224.

92. San Antonio, J. P.
1952 The Role of Coumarin in the Growth of Roots of Melilotus alba. Bot. Gaz. 114 : 70-95.

93. Scott, D. H., Waugh, J. G., and Cullinan, F. P.
1942 An Injurious Effect of Peach Juice on Germination of the Seed. Am. Soc. Hort. Sci. 40 : 283-285.

94. Seshadri, T. R.
1951 Biochemistry of Natural Pigments. (Exclusive of Haeme Pigments and carotenoids). Ann. Rev. Biochem. 20 : 487-512.

95. Sethna, S. N., and Shaw, N. M.
1945 The Chemistry of Coumarins. Chem. Rev. 36 : 1-62.

96. Shinoda, J.
1928 J. Pharm. Soc. Japan. 48 : 214-220.

97. Sinclair, W. B., and Eng, D. M.
1945 Organic Acids of Lemon Fruits. Bot. Gaz. 107 : 231-242.

98. Slatensek, J. M.
1947 Some Causes for Variation of Coumarin Content in Sweet Clover. J. Am. Soc. Agron. 39 : 596-605.

99. _____, and Washburn, R. L.
1944 A Rapid Fluorometric Method for the Determination of Coumarin and Related Compounds in Sweet Clover. J. Am. Soc. Agron. 36 : 704-708.

100. Smith, W. K.
1943 Propagation of Chlorophyll-deficient Sweet Clover Hybrids as Grafts. J. Heredity. 34 : 135-140.

101. _____.
1948 Transfer from Melilotus dentata to M. alba of the Genes for Reduction in Coumarin Content. Genetics. 33 : 124-125.

102. _____, and Brink, R. A.
1938 Relation of Bitterness to the Toxic Principle in Sweet Clover. J. Agr. Res. 57 : 145-154.

103. Späth, E.
1937 Die Natürlichen Cumarine. Ber. dtsch. Chem. Ges. 70 (A) : 83-117.

104. _____, and Kuffner, F.
1936 Die natürlichen Cumarine und thie Wirkung auf fische. Monatsh. Chem. 69 : 75.

105. Stahmann, W. K., 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.

106. Stevenson, T. M., and Clayton, J. S.
1936 Can. J. Res. 14 (C) : 153.

107. _____, and White, W. J.
1940 Investigations Concerning the Coumarin Content of Sweet Clover. I. The Breeding of a Low-coumarin Line of Sweet Clover - Melilotus alba. Sci. Agr. 21 : 18-28.

108. Surorov, V. V.
1936 Cultivation of Sweet Clover and its Prospects in the U.S.S.R. Trudy Prikl. Bot. Genet. i Selektcii. 20 : 21-33. (Herbage Abstr. 7 : 299. 1937.)

109. Swain, T.
1953 The Identification of Coumarin and Related Compounds by Filter-paper Chromatography. Biochem. J. 53 : 200-208.

110. _____.
1954 Spectral Studies on Phenolic Compounds. Chem. Ind. 1480-1481.

111. Taubock, K.
1942 Über Reaktionsprodukte von Flavonolen mit Borsaure und organischen Saüren und ihre Bedeutung für die Festlegung des Bors in Pflanzenorganen. Naturwiss. 3 : 439.

112. Thimann, K. V., and Bonner jr., W. D.
1949 Inhibition of Plant Growth by Protoanemonin and Coumarin, and its Prevention by BAL. (2, 3 dimercaptoproponol) Proc. Nat. Acad. Sci. (U.S.A.) 35 : 272-276.

113. Thompson, C. R., Curl, A. L., and Bickoff, E. M.
1959 Isolation of Carotenoids, Coumestrol, Chlorogenic Acid, and Antibiotics. Anal. Chem. 31 : 838-841.

114. Thompson, J. F., Honda, S. I., Hunt, G. E., Krupka, R. M., Morris, C. J., Powell Jr. L. E., Silberstein, O. O., Towers, G. H. N., and Zacharius, R. M.
1959 Partition Chromatography and its Use in the Plant Sciences. Bot. Rev. 25 (1) : 1-263.

115. Ufer, M.
1934 A Method Useful from a Breeding Standpoint to the Selection of Low Coumarin Forms of Sweet Clover (Melilotus). Zuchter. 12 : 255-258. (Eng. translation).

116. _____.
1934 Breeding Melilotus and Orithopus. Mitt Landw. 49 : 897-899.

117. Veldstra, H., and Havinga, E.
1945 On the Physiological Activity of Unsaturated Lactones. Enzymologia. 11 : 373-380.

118. _____, and _____.
1945 Untersuchungen über pflanzliche Wuchsstoffe. Vll. Rec. Trav. Chim. 62: 841-852.

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

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

121. Webster, G. T.
1955 Interspecific Hybridization of Melilotus alba and M. officinalis Using Embryo Culture. Agron. J. 47 : 138-142.

122. Werder, F. W.
1936 Merck's Jahresberichte. 50 : 88.

123. White, W. J., Greenshields, J. E. R., and Chubaty, W.
1954 The Effect of Feeding Sweet Clover Silage on the Prothrombin Time of Blood of Cattle. Can. J. Agr. Sci. 34 : 601-606.

124. _____, and Horner, W. H.
1940 Investigations Concerning the Coumarin Content of Sweet Clover. II. Sources of Variation in Tests for Coumarin Content. Sci. Agr. 21 : 29-35.

125. _____, Savage, R. G., and Johnston, F. B.
1952 A Slightly Modified Fluorometric Method of Testing for Coumarin Content in Sweet Clover. Sci. Agr. 32 : 278-280.

126. Wilson, C. W.
1939 A Study of the Boric Acid Colour Reaction of Flavone Derivatives. J. Am. Chem. Soc. 61 : 2303-2306.

127. Wittig, G..
1924 Über einfache Chromon-und Cumarin Synthesen. Ber. 57 : 88-95.