GENETICS OF THE FLAVONOID SYSTEM IN THE ALEURONE LAYER OF MAIZE

by

LAWRENCE THOMAS KIRBY

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L.T. Kirby

Supervisor: Assistant Professor E.D. Styles

ABSTRACT

The flavonoid system in the aleurone layer of the maize kernel provides an ideal unit for studying genetic control. This system was used as a vehicle to learn more about genetics, at the molecular level, in higher multicellular organisms.

It is known that a number of genes are involved in anthocyanin production in the aleurone layer of maize and that the genes $\underline{A}_1$, $\underline{A}_2$, $\underline{C}_1$, $\underline{C}_2$, and $\underline{R}$ must be present in the dominant form for this pigment production. Plants homozygous recessive for any of these genes (the recessive forms are designated $\underline{a}_1$, $\underline{a}_2$, $\underline{c}_1$, $\underline{c}_2$, and $\underline{r}$) will lack anthocyanin in the aleurone. A dominant allele of $\underline{C}_1$, namely, $\underline{C}^I$ also inhibits this pigment synthesis. Other genes such as $\underline{Bz}_1$, $\underline{Bz}_2$, $\underline{Pr}$, and $\underline{In}$ influence the intensity and/or nature of the pigment.

The study was approached from three directions, namely, (i) the extraction and comparison of flavonoids from various recessives, (ii) the culturing of recessive tissues, and (iii) the influence of light on gene action.

It was found that $\underline{a}_1$ and $\underline{a}_2$ recessive stocks accumulate quercetin if the genotype is $\underline{Pr}$ and kaempferol if it is $\underline{pr}$. These compounds are also found in $\underline{c}_1$ and $\underline{c}_1\underline{a}_1$ ($\underline{Pr}$ and $\underline{pr}$) genotypes after they have been exposed to light under germinating conditions.

The defect in the recessive $\underline{c}_2$ can be bypassed in tissue culture with the addition of dihydroquercetin. $\underline{a}_1$ and $\underline{a}_2$ tissues supply substances to $\underline{c}_2$, again allowing a bypass of the recessive, when they are used to cross-feed. (Cross-feeding is accomplished by pressing pieces of active aleurone together.)

Light replaces the recessive $\underline{c}_1$ under germinating conditions; approximately eleven times more pelargonidin relative to cyanidin is produced than is found in normal ($\underline{C}_1$) stock. (In $\underline{c}_1$ the ratio is 1:2.3; in $\underline{C}_1$ it is 1:25.) The consequence of forcing a system, as above, is significant because of the effects on side reactions and the effects on different ratios of end products produced.

These and other findings are discussed in terms of their general application to the genetics of higher multicellular organisms.

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INTRODUCTION

The objective of this work was to study gene action in the development of the flavonoid pigment system of maize aleurone tissue and thus learn more about genetics at the molecular level in higher organisms.

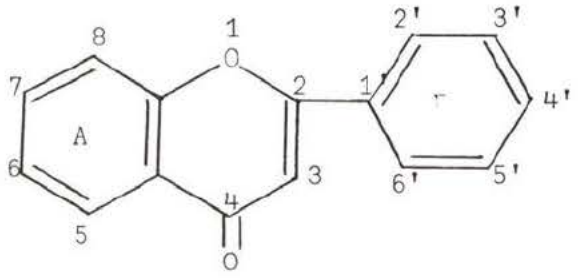
The study has been approached from three directions, namely, (i) comparison of flavonoids, (ii) culturing of aleurone tissue, and (iii) observing the influence of light on gene action. These are outlined in Parts I, II, and III of this thesis.

The aleurone layer of maize was chosen since it provides an ideal study system for a higher organism. Geneticists have worked with maize since the early part of this century and have carefully recorded almost 250 genes (26). This work has led to a definite improvement in the standard of living in many parts of the world. In the early 1960's stock with the endosperm mutant opaque - 2 was found to have twice as much lysine and tryptophan as normal stock. This finding was considered a major breakthrough in the production of high quality protein for many developing countries (23). It is also noted that the one-gene-one-enzyme hypothesis (now the one-gene-one polypeptide hypothesis) formulated by Beadle was based partly on work by Scott-Moncrieff (32) on anthocyanin biosynthesis.

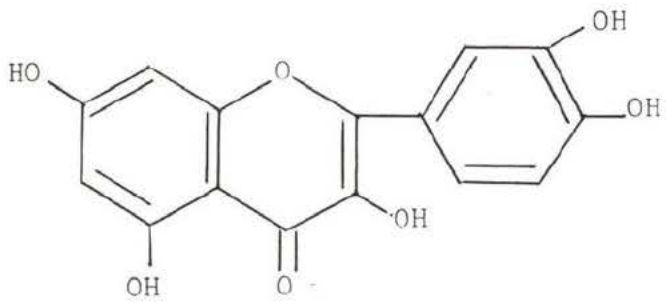
It is unlikely that a better understanding of the flavonoid system in maize aleurone will lead directly to any substantial improvement in the lot of mankind; however, the increased understanding could be of significant value when added to the developing body of genetic knowledge.

A little background information regarding flavonoids and the aleurone layer of maize is necessary to set the work in this study in the proper perspective.

Flavonoids are phenolic compounds relating to or like flavone in chemical structure. The basic structure of the flavonoid molecule is:



An example of a flavonoid found in maize aleurone is quercetin. This compound has the following structure:



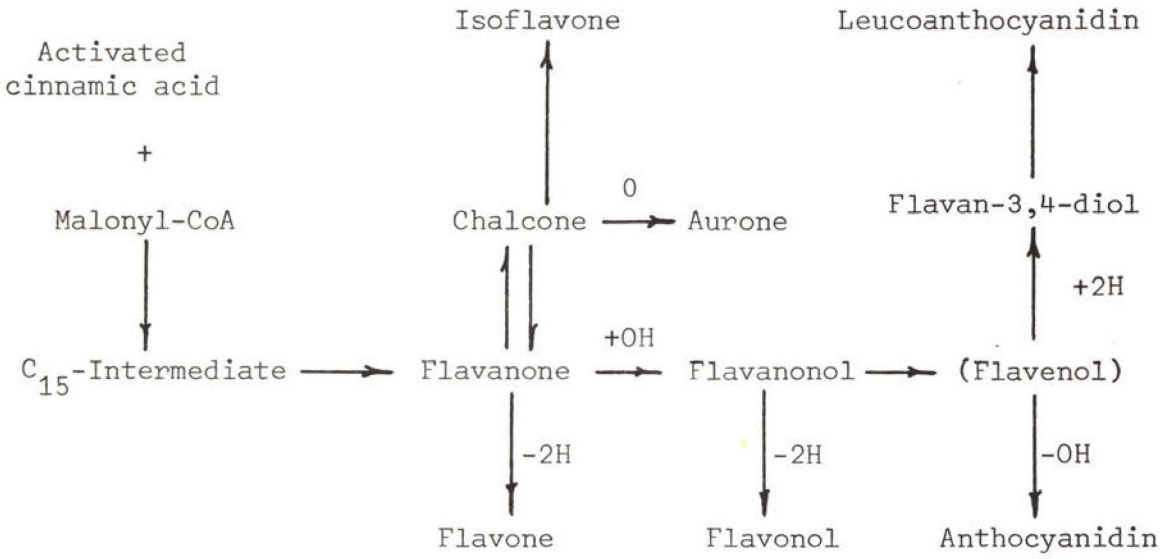
Many of these compounds occur as glycosides - the sugars appear to stabilize the molecule as far as the action of light and enzymes are concerned (16). It is known that the color of many of the flavonoid pigments can be altered by the addition or removal of hydroxyl groups

or by methylation or glycosylation of the non-sugar part (12).

A basic pattern of flavonoid synthesis appears to be common to all higher plants. As a result of many studies including tracer experiments, inheritance, physiology, and the like, the pathway for flavonoid biosynthesis as outlined in Figure 1 has been presented by Harborne (16).

FIGURE 1

BIOSYNTHETIC PATHWAYS TO THE FLAVONOIDS AS OUTLINED BY HARBORNE (16).



Figures 2, 3,and 4 illustrate the aleurone layer and its relationship to the other cells of the maize kernel. Note in Figure 2 that the aleurone cells are packed with protein grains; the pigment appears to form around these. The aleurone layer is noted for the large number of aleurone grains found in each cell. The grains are protein in nature

and provide the enzymes for starch digestion during germination (8, 20).

In the strains of maize, used in these experiments, that were grown in the greenhouse in the summer, pigment does not start to develop in the aleurone layer until at least two weeks after pollination. The pigment starts in the tip of the crown and within a week the whole kernel is usually colored. The kernel may be only one-half its mature size and be fully pigmented. When the aleurone from kernels with only crown pigment is observed under the microscope all of the cells, both pigmented and non-pigmented, are found to be packed with aleurone grains.

The maize plant is dipolid; however, the endosperm (including the aleurone) is triploid. Each nucleus contains one genome of male origin and two of female origin.

As noted above, the aleurone is colorless until about two weeks after fertilization then, depending on the genetic make-up of the stock, pigments appear. It is known that a number of genes are involved in anthocyanin production and that a recessive in any of these will block the pigment formation. The genes $\underline{A}_1$, $\underline{A}_2$, $\underline{C}_1$, $\underline{C}_2$, and $\underline{R}$ must be present in the dominant form for the production of aleurone pigment. (The recessives of these genes are $\underline{a}_1$, $\underline{a}_2$, $\underline{c}_1$, $\underline{c}_2$, and $\underline{r}$.) $\underline{C}^I$ (inhibitor), an allele of $\underline{C}_1$, will also inhibit pigment formation (3). It has been found that genes such as $\underline{Bz}_1$, $\underline{Bz}_2$, $\underline{Pr}$, and $\underline{In}$ influence the intensity and/or the nature of the pigment (1). Reddy (28) has placed the genes in sequence for the metabolic pathway to anthocyanin as follows:

$$\underline{C}^I - \underline{C}_1 - (\underline{C}_2) - \underline{R} - (\underline{In}) - \underline{A}_1 - \underline{A}_2 - \underline{Bz}_1 - \underline{Bz}_2 \longrightarrow \text{Anthocyanin}$$

(The parentheses indicate inconclusive results.)

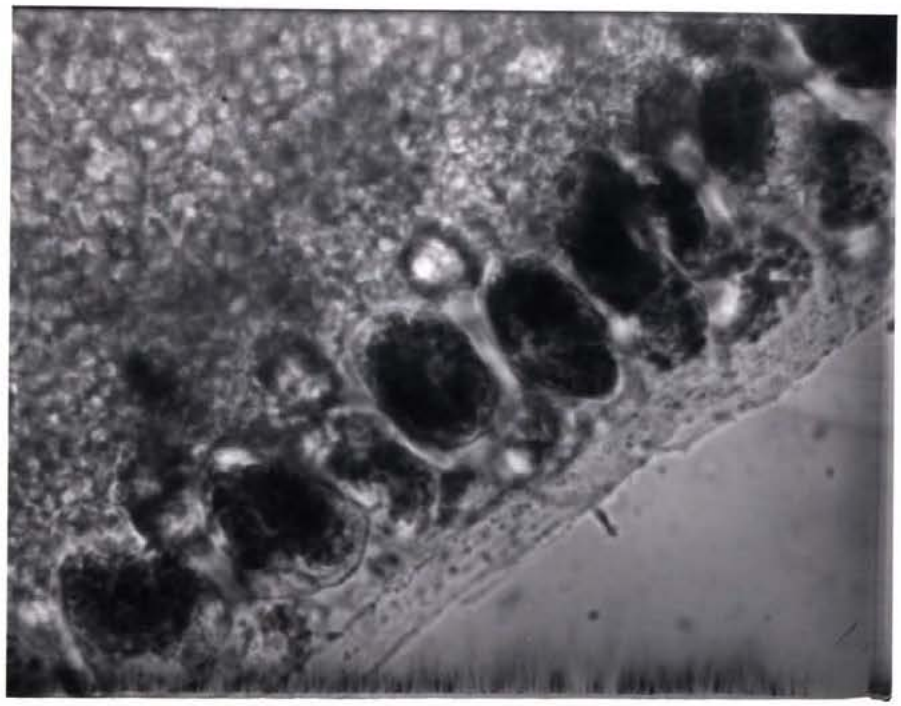


FIGURE 2. Vertical section through the aleurone region of a pigmented maize kernel. Note how the dark pigment shadows the grains in the aleurone cells. (200 X).

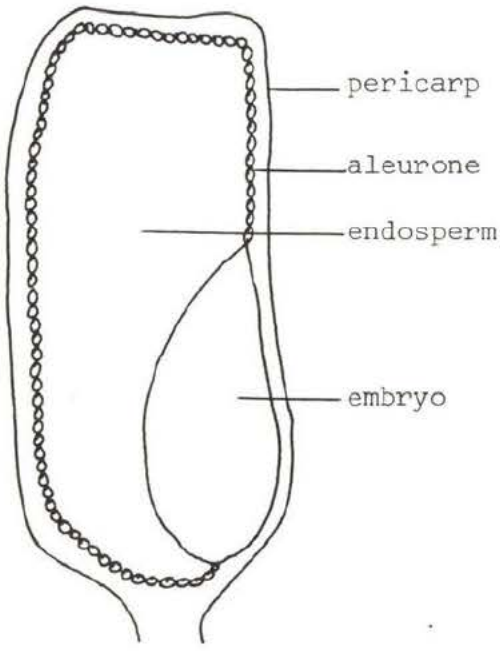


FIGURE 3. Vertical section of a maize kernel. (8 X)

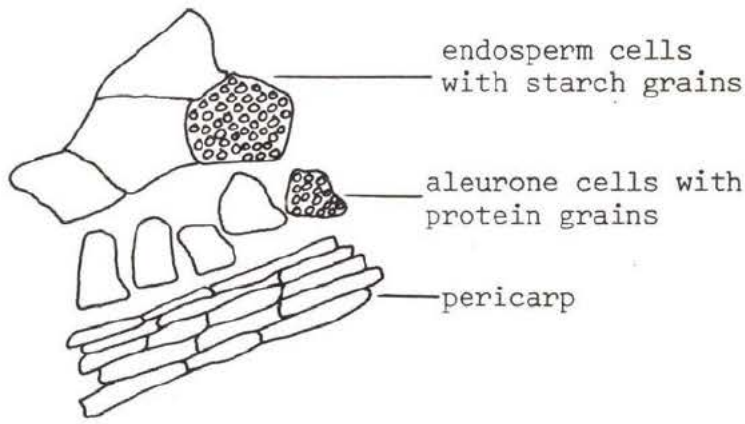


FIGURE 4. Diagrammatic representation of part of Figure 2. (100 X).

PART I

FLAVONOID COMPARISONS

INTRODUCTION

The objective of this series of experiments was to compare flavonoids from recessive and non-recessive stocks and to correlate various flavonoids with genes in the anthocyanin pathway. This should be a reasonable approach since genetic blocks would likely cause a build-up of some precursors (or other end products of alternate pathways) and a lack of others.

MATERIALS AND METHODS

Stock

Except where indicated otherwise, the stock used was W 22 - an inbred line from Wisconson. The genetic background of the material is, therefore, uniform. The specific genotypes used in these experiments were as follows:

1. $\underline{C}^{I}$*, $\underline{c}_1$, $\underline{c}_2$*, $\underline{r}_{10}\underline{r}_2$, $\underline{r}_{10}\underline{R}_2$, $\underline{a}_1$, $\underline{a}_2$
2. $\underline{Pr}\underline{Pr}\underline{Pr}$, $\underline{Pr}\underline{Pr}\underline{pr}$, $\underline{Pr}\underline{pr}\underline{pr}$, $\underline{pr}\underline{pr}\underline{pr}$
3. $\underline{C}_1\underline{C}_1\underline{c}_1$ $\underline{R}_{10}\underline{r}_{10}\underline{r}_{10}$ $\underline{Pr}\underline{pr}\underline{pr}$ $\underline{r}_2\underline{r}_2\underline{r}_2$
4. $\underline{C}_1\underline{C}_1\underline{c}_1$ $\underline{R}_{10}\underline{r}_{10}\underline{r}_{10}$ $\underline{pr}\underline{pr}\underline{pr}$
5. $\underline{bz}_1$
6. $\underline{C}_1\underline{C}_1\underline{c}_1$ $\underline{bz}_1\underline{bz}_1\underline{bz}_1$
7. $\underline{C}_1\underline{c}_1\underline{c}_1$ $\underline{bz}_1\underline{bz}_1\underline{bz}_1$
8. $\underline{c}_1\underline{bz}_1$

* These stocks were from the Maize Genetics Cooperative, University of Illinois.

9. $\underline{a_1}\underline{c_1}$

10. $\underline{a_1}\underline{r_2}\underline{r_{10}}$

11. $\underline{a_2}\underline{r_2}\underline{r_{10}}$

12. $\underline{a_1}\underline{c_1}\underline{r_2}\underline{r_{10}}$

13. $\underline{bz_1}\underline{pr}$

14. $\underline{a_2}\underline{pr}$

The following should be noted:

(i) In the 23 stocks just outlined the other genes in the anthocyanin pathway are dominant (except $\underline{C^I}$ and $\underline{in}$), for example, $\underline{a_1}$ would be $\underline{C_1}\underline{C_2}\underline{R_2}\underline{a_1}\underline{A_2}\underline{in}\underline{Bz_1}\underline{Bz_2}\underline{Pr}$.

(ii) In those cases where only one allele has been shown the other two are identical, for example, $\underline{c_1}$ would be $\underline{c_1}\underline{c_1}\underline{c_1}$.

(iii) $\underline{R_{10}}$ is assumed to be in the recessive form, that is, $\underline{r_{10}}$ except where indicated otherwise.

(iv) $\underline{R_2}$ and $\underline{R_{10}}$ denote aleurone color factors on chromosomes 2 and 10 respectively.

Extraction

The amount of solvent used, the number of extractions made, the time of extraction, and other procedures were all varied; the following outline represents the most satisfactory procedure found.

The seeds were soaked in water for approximately 30 minutes and the pericarps then removed. The aleurone layer was scraped with a scalpel and the resulting tissue placed in a 5 ml freeze-drying bottle[1].

[1]. VirTis screw cap vac vials from Fisher Scientific Company.

Methanolic - HCl solvent (1% concentrated HCl) was used. The tissue was covered with 1 ml of solvent and allowed to stand for 45 minutes. Three more extractions were usually made but with the time increasing to 1½, 3, and 8 hours respectively. The volume of extract was reduced to 1 ml by freeze-drying[1]. and, except where glycosides were desired, 1 ml of 2M HCl was added. The tube was securely stoppered (with a rubber stopper and a metal cap) and hydrolyzed in a boiling water bath for 1 hour (15). The resulting solution was cooled and (i) freeze-dried to remove the methanol, or (ii) freeze-dried to dryness. In the former, an iso-amyl alcohol extraction was made and in the latter an ether extraction (12, 14, 16, 41). Ether extracts were usually made for the colorless tissue and iso-amyl alcohol for the anthocyanin colored tissue); however, both tissues were extracted in ether and both in iso-amyl alcohol as checks for consistency.

Four ether extracts were made each of 30 minutes duration with constant agitation (1 ml of ether was used each time). Four iso-amyl extracts (each of 0.3 ml) were made. The solutions were agitated vigorously and the iso-amyl alcohol removed with a pasteur capillary pipette. The flavonoid extracts were then freeze-dried and either stored in a freezer for future use or applied directly to chromatogram sheets by dissolving in methanolic-HCl (1% concentrated HCl) and applying with a pipette.

If glycosides were desired, the extract direct from the tissue was freeze-dried and the resultant reduced volume applied to cellulose chromatogram sheets.

It should be noted that all extracts were kept in the dark where

[1]. Unitrap VirTis freeze-drying apparatus from Fisher Scientific Company.

ever possible. The materials were originally kept under a nitrogen atmosphere; however, this was later found to be unnecessary.

Isolation

Thin layer chromatography (TLC) was used to isolate components in the various extracts. Eastman chromagram cellulose sheets # 6064 without fluorescent indicator were used quite successfully. (Sheets with fluorescent indicator were tried but not found satisfactory.) The sheets were run in an Eastman Chromagram Thin Layer Chromatography Developing Apparatus.

The main task was to find the best solvent systems for the various extracts. Chromatograms were first run in one direction; when a good separation was obtained two-dimensional work was undertaken to see if better resolution could be obtained. After this check, it was found that for this work the one-dimensional technique was usually satisfactory. It should be noted, that a great deal of time was saved by using established solvent systems - the problem was to find the best system. In the case of anthocyanin aglycone separation this problem did not present itself since Mullick (25) had worked out some very good solvents. After checking the anthocyanin aglycone extracts using formic-acid - 4M HCl (2:1 v/v) in the first direction and MeOH - HCl - H_2O (190:1:10 v/v) in the second direction only the one-dimensional technique using formic-acid 4M HCl was employed.

Most of the work was done with colorless aleurone, that is, non-anthocyanin pigmented; compounds such as those indicated in Figure 1 could, therefore, be expected. With this in mind, solvents that separated the maximum number of these materials were chosen (12, 14, 40). As

indicated previously, this work was mainly concerned with aglycones. Glycosides were run only to (i) check to see if aglycones were present in the glycoside extracts and (ii) to see if differences were obvious that had not been seen with the aglycones.

Isolations were made from all of the stocks listed previously. See Table 1 for a summary of the solvents used, for the extracts and standards.

The following solvents were tested on the extracts from the recessive stock: acetic acid 15%, 30%, 40%, and 60%; Forestal (acetic acid- concentrated HCl - H_2O 30:3:10 v/v); BAW (butanol-acetic acid - H_2O 4:1:5 v/v upper layer); ethyl acetate - formic acid - H_2O 10:2:3 v/v; benzene - ligroin - methanol - H_2O 50:50:1:50 v/v; xylene - acetic acid - H_2O 3:1:3 v/v; BeAW (benzene - acetic acid - H_2O 125:72:3 v/v); formic acid - 4M HCl 2:1 v/v; and ethyl acetate - H_2O saturated. These solvents had all been used successfully on paper with flavonoids (11, 12, 16, 40).

Identification

The identification techniques used for isolated flavonoids were (i) RF values, (ii) visible color, (iii) color under U.V. light, (iv) reagents, and (v) U.V. spectra. Nuclear magnetic resonance was attempted; however, a large enough quantity of material could not be feasibly extracted to give readings. For example, at least 3 mg. of pure material were required and only .021 mg. of a compound like quercetin could be obtained from 25 seeds. (See Appendix I). The same was true for infra red spectra.

Standards (after having been chromatographed as were the extracts) were used for comparison purposes. (See Appendix II). The reagents

TABLE I

SOLVENTS TESTED WITH VARIOUS ALEURONE EXTRACTS AND STANDARDS

Stock	Solvents[1]
$\underline{a}_1$ (aglycones)	FA-4M HCl, Forestal, 30% A.A., BeAW
(glycosides)	BAW, 30% A.A.
(2-D aglycones)[2]	FA-4M HCl + 30% A.A., 30% A.A. + BeAW
$\underline{a}_2$ (aglycones)	FA-4M HCl, Forestal, 30% A.A., BeAW.
(glycosides)	BAW, 30% A.A.
$\underline{c}_1$ (aglycones)	FA-4M HCl, 30% A.A.
(glycosides)	BAW, 30% A.A.
(2-D aglycones)	FA-4M HCl + 30% A.A., 30% A.A. + BeAW
$\underline{r}_{10}$ (aglycones)	FA-4M HCl, 30% A.A.
(glycosides)	BAW, 30% A.A.
(2-D aglycones)	FA-4M HCl + 30% A.A., 30% A.A. + BeAW
$\underline{c}_2$ (aglycones)	Forestal, 30% A.A.
(glycosides)	BAW, 30% A.A.
$\underline{C}^I$ (aglycones)	FA-4M HCl, 30% A.A.
(glycosides)	BAW, 30% A.A.
PrPrPr (aglycones)	FA-4M HCl, 30% A.A.
(glycosides)	BAW, 30% A.A.
(2-D aglycones)	FA-4M HCl + MeOH - HCl - H_2O 30% A.A. + BeAW
PrPrpr (aglycones)	FA-4M HCl
Prprpr (aglycones)	FA-4M HCl
prprpr (aglycones)	FA-4M HCl
(glycosides)	BAW
(2-D aglycones)	FA - 4M HCl + MeOH - HCl - H_2O
$\underline{a}_1\underline{c}_1$ (aglycones)	FA-4M HCl, 30% A.A., Forestal
$\underline{a}_1\underline{c}_1\underline{r}$ (aglycones)	FA-4M HCl, 30% A.A., Forestal
$\underline{a}_1\underline{r}$ (aglycones)	FA-4M HCl, 30% A.A., Forestal
$\underline{a}_2\underline{r}$ (aglycones)	FA-4M HCl, 30% A.A., Forestal
$\underline{a}_2$-$\underline{a}_2$pr (mix-aglycones)	FA-4M HCl, 30% A.A., Forestal
$\underline{b}_1$ (aglycones)	FA-4M HCl, 30% A.A., Forestal
(glycosides)	FA-4M HCl

TABLE I (continued)

Stock	Solvents
$\underline{bz_1pr}$ (aglycones)	FA-4M HCl, 30% A.A. Forestal
$\underline{c_1r}$ (aglycones)	30% A.A.
(glycosides)	BAW, 30% A.A.

Standards[3.]	Solvents
cyanidin chloride	FA-4M HCl
pelargonidin chloride	FA-4M HCl
delphinidin chloride	FA-4M HCl
flavone	30% A.A., BAW
D-catechin	Forestal, BAW
dihydroquercetin	30% A.A., BAW
quercetin	30% A.A., BAW
kaempferol	FA-4M HCl, Forestal, 30% A.A., BeAW, BAW
quercetin	FA-4M HCl, Forestal, 30% A.A., BeAW, BAW
chalcone	30% A.A., BAW
apigenin	30% A.A., BAW
flavanone	30% A.A., BAW
cinnamic acid	30% A.A., BAW
naringenin	30% A.A., BAW
caffeic acid	30% A.A., BAW
shikimic acid	30% A.A., BAW
phenylalanine	30% A.A., BAW
naringin	30% A.A., BAW

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1. Solvent Symbols:
   FA-4M HCl = formic acid - 4M HCl (2:1 v/v)
   Forestal = acetic acid - con. HCl - H_2O (30:3:10 v/v)
   BeAW = benzene - acetic acid - H_2O (3:1:3 v/v)
   BAW = butanol - acetic acid - H_2O (4:1:5 v/v upper layer)
   A.A. = acetic acid

2. 2-D = two-dimensional

3. See Appendix II for details of the standards.

p-toluene sulfonic acid, Na_2CO_3, $AlCl_3$, Rhodamine B, and NH_3 were used and observations made of the visible color and color under ultra violet light (12, 14). These were first used on the chromatograms on which standards had been run - the results were characteristic of those outlined in the literature (12, 14).

The spectral work was the largest and most important part of the identification procedure. Both qualitative and quantitative data were obtained and techniques were used to identify micro-amounts of material.

The reflectance attachment for the Unicam SP 700 spectrophotometer was used to obtain U.V. spectra of compounds without eluting the compounds from the cellulose chromatograms. The technique was based on a paper by Kirk et al (21). (See Appendix III.)

Solution spectra were obtained by using the Unicam SP 800 spectrophotometer with semi-micro cells. The isolated compounds were scraped (along with the cellulose) off the polyethylene backing and placed in 10 ml freeze-dryer bottles. For anthocyanidin elution MeOH - HCl (0.01% concentrated HCl) was used and for other flavonoids 95% EtOH was used. The scrapings were covered with solvent (approximately 1 ml); this was done a number of times until the eluate remained colorless. Depending on the amount of compound to be eluted, as many as 8 extracts were made - the time intervals being 15 to 60 minutes. (Note that the extracts from the recessives are almost colorless but the same number of extracts are made.) The eluates were centrifuged then freeze-dried. The resultant residue was either stored in a freezer or dissolved immediately in solvent (the same as used for eluting) and a spectrum run. The minimum amount of eluate required is 1 ml - with this small amount a 5 mm lift has to be placed under the semi-micro cells in the spectrophotometer. If the solution is

too concentrated it is diluted until a spectrum can be obtained.

The reagent $AlCl_3$ (16) was used to see if a spectral shift was obtained with the magenta and orange-pink isolates from Pr and pr stock; H_3BO_3/NaOAc (12) was used with yellow isolates from a_1, a_2, and $c_1$pr stock.

Tables II and III outline the spectra that were run.

OBSERVATIONS

Chromatograms

The following solvents were found most satisfactory:

(i) For two-dimensional anthocyanin aglycones FA-4M HCl + MeOH - HCl - H_2O

(ii) For one-dimensional anthocyanin aglycones FA-4M HCl

(iii) For two-dimensional flavonoid aglycones 30% A.A. + BeAW and 30% A.A. + Forestal

(iv) For one-dimensional flavonoid aglycones Forestal or 30% A.A.

The other solvents tested were rejected since the compounds moved with the solvent front or remained at the origin and there was often excessive tailing. (No data or photos have been included on these solvents.)

The following observations were made when comparing chromatograms of the various stocks:

(i) c^I, c_1, c_2, r_{10}, c_1a_1, a_1r, a_2r, and c_1a_1r showed no differences.

(ii) a_1 and a_2 had yellow compounds (visible under U.V. light). The compound of $a_2$pr had a higher RF than that of a_2 and a_1. (See Table II and Figures 5, 6, and 7).

(iii) Some Pr and pr developing stock, that is, stock that was approximately one-half pigmented showed yellow pigment as in $a_2$pr and a_2 and a_1. The Pr and pr mature stock in some cases showed this (See Table II).

(iv) The PrPrPr, PrPrpr, and Prprpr genotypes showed small amounts of orange-pink pigment and large amounts of magenta pigment. (See Table III for quantitative values).

(v) A compound was isolated from bz_1 stock. (See Table II and Figure 8).

(vi) Standards, namely, quercetin and kaempferol were run which had the same RF values and color under U.V. light as the yellow compounds in (ii) and (iii) above. (See Table II and Figure 7).

(vii) The reagents previously outlined did not prove to be of any use in identification.

Note: The photographs of all chromatograms were taken under short wave ultra-violet light with a high speed Ektachrome daylight film.

Spectroscopic Examination

Spectra, on the Unicam SP 800, were run for the yellow compounds, quercetin and kaempferol standards, the orange-pink and magenta compounds, and cyanidin, pelargonidin and delphinidin standards. See Tables II and III for the peaks, spectral shifts with reagents, and quantitive data. See also Figures 9 to 13 inclusive for examples of the appearance of various spectra.

See Appendix III for a discussion of the usefulness of reflectance work, on the Unicam SP 700, and an outline of the spectral data.

Aleurone Color

The visible color of the aleurone from the various stocks was as follows:

(i) C^I, c_1, c_2, r, c_1a_1, a_1r, a_2r, and c_1a_1r are all colorless

(ii) a_1 and a_2 are colorless to a slight gray color

Figure 5. Chromatogram of an $\underline{a}_1$ extract run in two directions. The first was left to right with 30% A.A. and the second was upward with FA - 4M HCl. The spot in the lower left was identified as quercetin.

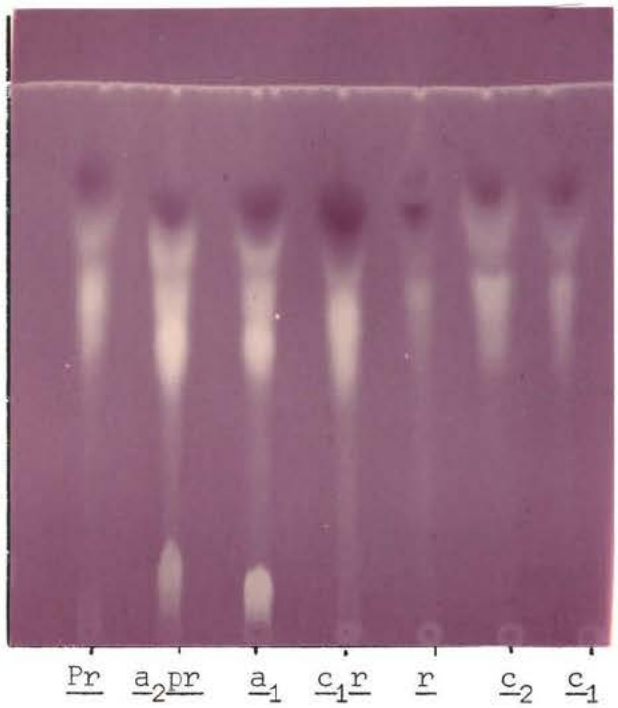


Figure 6. Chromatograms of extracts from 7 tissues, as indicated, run in 30% A.A. The spot near the origin in $\underline{a}_2\underline{pr}$ was identified as kaempferol and the spot near the origin in $\underline{a}_1$ was identified as quercetin.

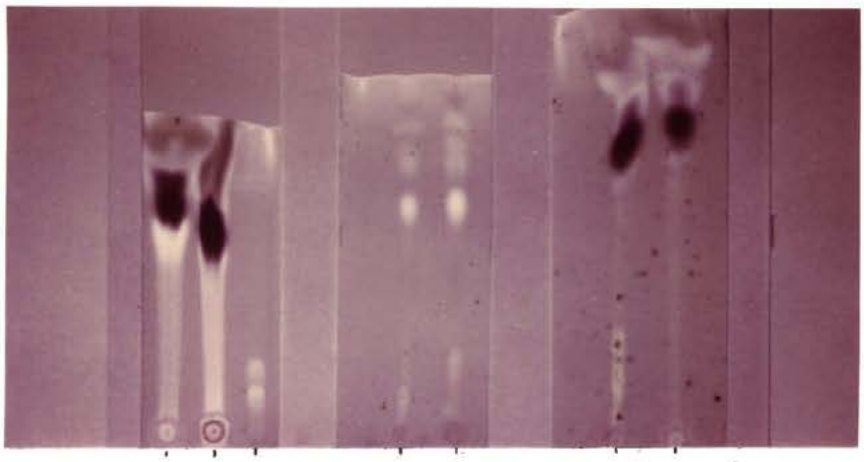


Figure 7. Chromatograms of extracts from 6 tissues and of a mixture of 2 standards, as indicated, run in FA-4M HCl. Note that the kaempferol (K) is slightly above the quercetin (Q). The spot near the origin in a_1 was identified as quercetin and the spot slightly higher in a_2pr as kaempferol. c_1a_1 germinated in light shows 2 spots - the lower is quercetin and the higher is kaempferol.

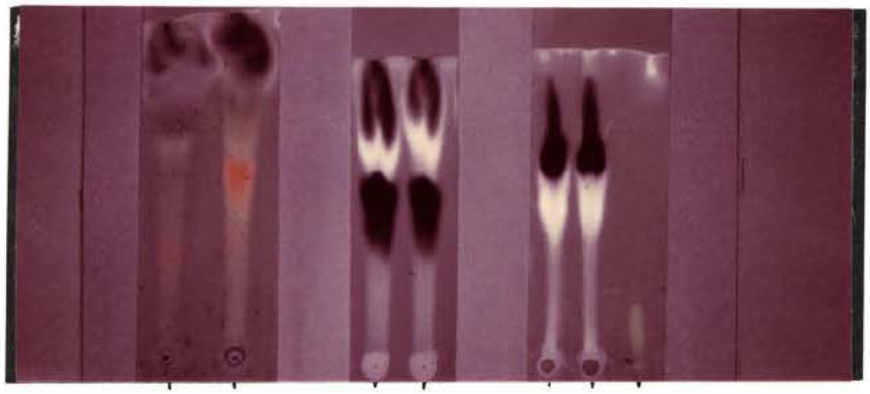


Figure 8. Chromatograms from 6 tissues and of a mixture of 2 standards run in the solvents as indicated. The FA-4M HCl chromatograms are of iso-amyl alcohol extracts; therefore, the cynidin spot for bz_1 and pelargonidin spot for bz_1pr are present. Note the dark blue compound associated with bz_1 and bz_1pr.

TABLE II

CHARACTERISITICS OF ISOLATED YELLOW PIGMENTS AND OF STANDARDS[1].

Material	Color U.V.	RF Values (a) 30% AA	RF Values (b) FA-4M HCl	RF Values (c) Forestal	λ max. mμ 95% EtOH	λ max. mμ H_3BO_3/NaOAc	Number of Chromograms (a)	(b)	(c)	Number of Spectra
Quercetin	yellow	.12	.16	.48	372, 257	385,260	3,	2,	3,	3
Kaempferol	"	.19	.26(5)	.65	368,268	368,268	3,	2,	3,	3
$\underline{a}_1$	"	.11(5)	.15(5)	.46	373,257	384,260	4,	2,	2,	3
$\underline{a}_2$	"	.13	.15(5)	.47(5)	372.5,258	384.5,260	2,	2,	2,	2
$\underline{a}_2\underline{pr}$	"	.16	.25	.62	368,270	368,270	3,	2,	2,	3
$\underline{c}_1\underline{a}_1$ + light	"	.17 .12(5)	.25 .16	-	-	-	2,	2,		-
$\underline{c}_1\underline{pr}$ + light	"	-	.16 .27	.52 .67	368,269	368,268		3,	3,	3
$\underline{pr}$	"	-	.23	.64	-	-		2,	2,	-
$\underline{c}_1$ + light(e)	"	-	.24 .15	.64 .46	-	-		2,	2,	-
$\underline{c}_1$ + light(n.e.)	"	-	.14	.45	-	-		2,	1,	-
$\underline{Pr}$	"(faint)	-		-	-	-		1 (5 checked)		-

Note: 1.If the concentration of pigment is great enough a visible yellow color is seen.
2.A few drops of the reagent H_3BO_3/NaOAc in 95% EtOH are added.
3.FA-4M HCl is formic acid 4M HCl (2:1 v/v)
4.Forestal is acetic acid (AA) - concentrated HCl - H_2O (30:30:10 v/v)
5. Spectra from Geissman (12) are as follows: quercetin 371, + H_3BO_3/NaOAc 389
kaempferol 367.5 + H_3BO_3/NaOAc 367.5
Spectra from Harborne (16) are as follows: quercetin 374, 255
kaempferol 368, 268
6.(e) = embryo is present (n.e.) = embryo was removed.
7.An extract from 25 seeds was usually enough for a U.V. spectrum.
8.A dark blue compound (under U.V.) was isolated from $\underline{bz}_1$ and $\underline{bz}_1\underline{pr}$ stock. The RF values were as follows: (i) FA-4M HCl .84, (ii) Forestal .60, and (iii) 30% AA .70.

1. See Appendix IV for details of the individual values.

TABLE III

CHARACTERISTICS OF ISOLATED ANTHOCYANIDIN PIGMENTS AND OF STANDARDS[1].

Material	Visible Color	RF in FA-4M HCl	(a) λ max. mμ MeOH-HCl	(b) λ max. mμ $AlCl_3$ added	Quantity of Pigment Absorbance	Quantity of Pigment Ratio	No. of Chromograms	No. of Spectra (a)	No. of Spectra (b)
Pr	magenta	.44	536	548			5	6	3
	orange-pink	.58(5)	524	523			5	4	4
pr	magenta	-	535	547			-	3	3
	orange-pink	-	523	523			-	6	3
c_1 + light	magenta	.43					4		
	orange-pink	.57					4		
b_{1}	magenta	.41					1		
b_{1}pr	orange-pink	.57					1		
cyanidin	magenta	.44	535	553			2	2	2
pelargonidin	orange-pink	.59	520	520			2	2	2
$C_1c_1c_1$Rrr					5.2 (total pigment)		1	1	
$C_1c_1c_1$Rrrprprpr					4.6 (total pigment)		1	1	
PrPrPr	magenta				17.4) 18.1	24		2	
	orange-pink				.7)	1		2	
PrPrpr	magenta				16.0) 16.8	19		2	
	orange-pink				.8)	1		2	
Prprpr	magenta				15.8) 16.6	19		2	
	orange-pink				.8)	1		2	
prprpr	magenta				2.3) 20.6	.13		2	
	orange-pink				18.3)	1		2	

Note: 1. Cyanidin and pelargonidin spectral data are from Harborne (16).
2. MeOH-HCl is methanol containing .01% concentrated HCl.
3. The quantitative data were checked with other seeds grown at the same time (See Part III).
4. Twenty five seeds were used for each extract.

1. See Appendix IV for details of the individual values.

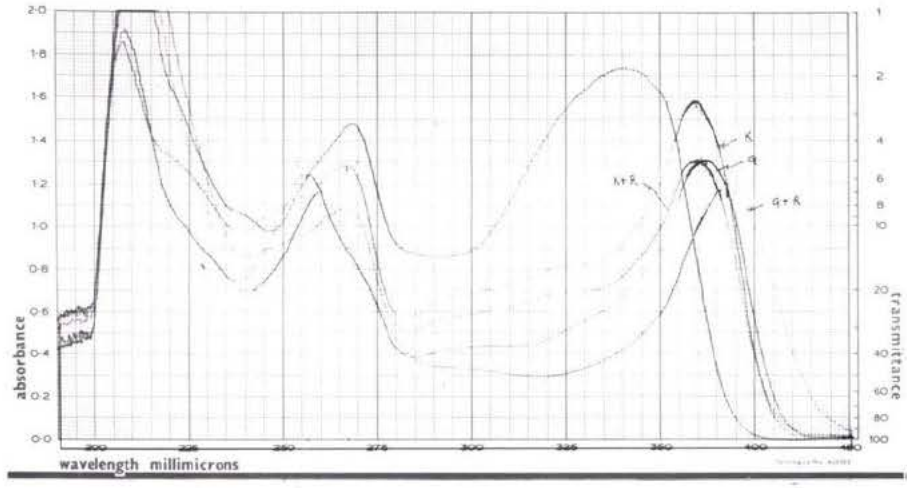


Figure 9. Spectra of flavonoid standards in 95% EtOH. K = kaempferol, Q = quercetin, R = H_3BO_3/NaOAc reagent.

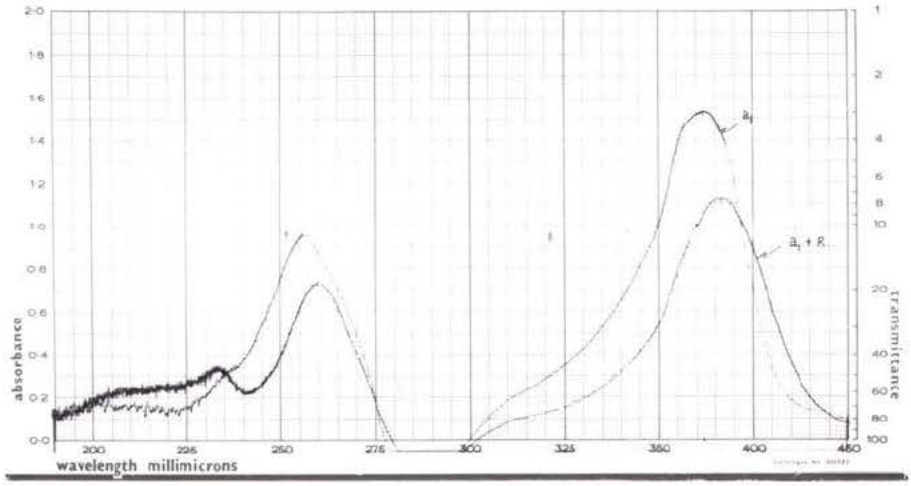


Figure 10. Spectrum of the yellow pigment in 95% EtOH, isolated on a chromatogram from an a_1 extract of 25 seeds. R = a few drops of H_3BO_3/NaOAc.

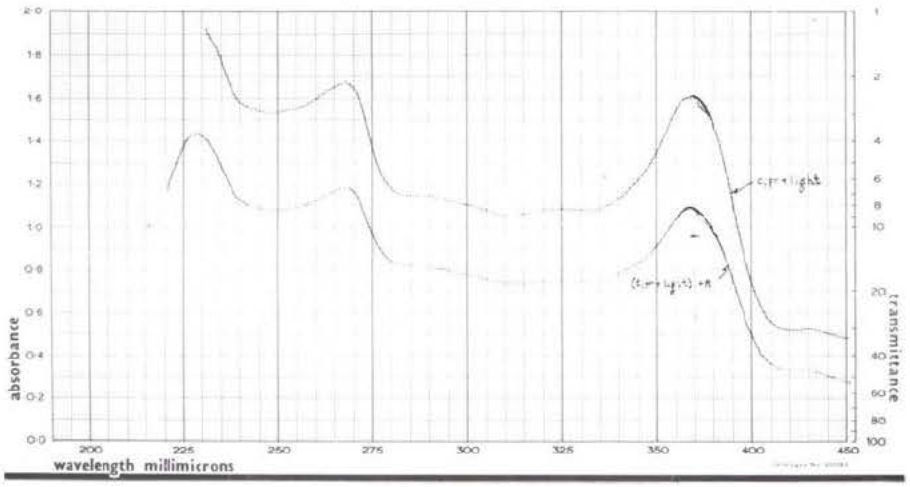


Figure 11. Spectrum of the yellow pigment, in 95% EtOH, isolated on a chromatogram from a c<u>1</u>pr extract of 25 seeds germinated in light. R = a few drops of H_3BO_3/HaOAc.

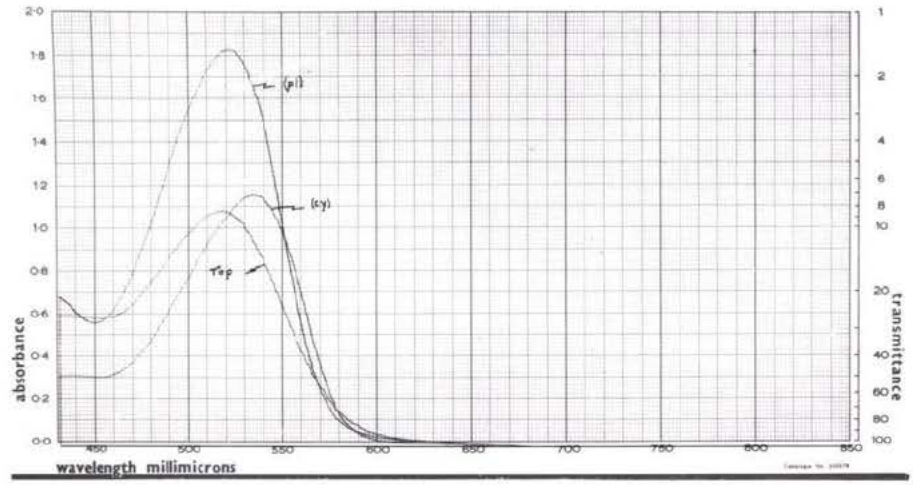


Figure 12. Spectra of the pelargonidin (pl) and cyanidin (cy) pigments isolated on a chromatogram from 25 prprpr seeds. The solvent is MeOH - .01% concentrated HCl. The pelargonidin was diluted 10X and the cyanidin 2X.

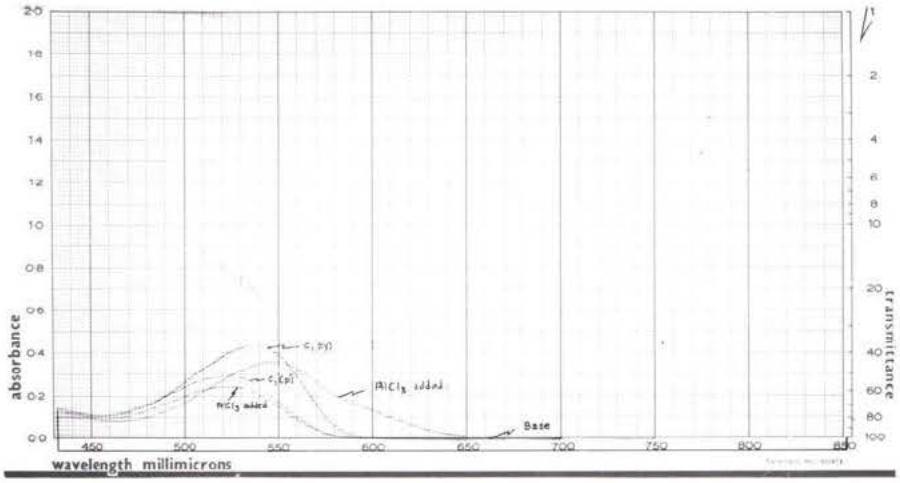


Figure 13. Spectra of pelargonidin (pl) and cyanidin (cy) pigments isolated on a chromatogram from PrPrPr stock. The solvent is MeOH - .01% concentrated HCl. Note the consequence of adding a few drops of 5% $AlCl_3$.

(iii) $\underline{Pr}\underline{Pr}\underline{Pr}$, $\underline{Pr}\underline{Pr}\underline{pr}$, and $\underline{Pr}\underline{pr}\underline{pr}$ are deep purple-red.

(iv) $\underline{pr}\underline{pr}\underline{pr}$ is red.

(v) $\underline{bz}_1$ is gold-brown.

(vi) $\underline{bz}_1\underline{pr}$ is bronze.

(vii) $\underline{c}_1\underline{c}_1\underline{c}_1\underline{bz}_1\underline{bz}_1\underline{bz}_1$ is colorless.

(viii) $\underline{C}_1\underline{c}_1\underline{c}_1\underline{bz}_1\underline{bz}_1\underline{bz}_1$ is light gold-brown.

(ix) $\underline{C}_1\underline{C}_1\underline{c}_1\underline{bz}_1\underline{bz}_1\underline{bz}_1$ is deeper gold-brown.

(x) $\underline{C}_1\underline{C}_1\underline{c}_1\underline{R}_{10}\underline{r}_{10}\underline{r}_{10}\underline{r}_2\underline{r}_2\underline{r}_2\underline{Pr}\underline{pr}\underline{pr}$ is light purple and

$\underline{C}_1\underline{C}_1\underline{c}_1\underline{R}_{10}\underline{r}_{10}\underline{r}_{10}\underline{r}_2\underline{r}_2\underline{r}_2\underline{pr}\underline{pr}\underline{pr}$ is red. Each has colorless areas.

DISCUSSION

Both one-dimensional and two-dimensional chromatography were used. It is noted that the two-dimensional work is superior for isolation and resolution; however, for this work one-dimensional was found to be satisfactory. (The extracts were tested with two-dimensional systems as a check but no other compounds were separated.) FA-4M HCl was used for anthocyanin aglycone work and Forestal was best for the separation of quercetin and kaempferol. In the latter case, 30% acetic acid gave good results for quercetin or kaempferol alone and worked well for the separation of the yellow pigments in the recessives. Forestal in this case was superior if a mixture of $\underline{a}_2$ and $\underline{a}_2\underline{pr}$ yellow pigments were to be separated.

From the color in U.V. light, visible color, RF values, and U.V. spectral data - all being compared with the kaempferol and quercetin standards - it appears that the yellow pigment in $\underline{a}_2$, $\underline{a}_1$, and $\underline{Pr}$ stock is quercetin and that in $\underline{a}_2\underline{pr}$ and $\underline{pr}$ stock is kaempferol. Sands (31) found isoquercitrin in the plants of $\underline{a}_1$ stock. Coe (personal communication) indicates that isoquercitrin may be present in the aleurone of $\underline{a}_1$, $\underline{a}_2$, $\underline{bz}_2$, and $\underline{Pr}$ stock

but quercetin in $\underline{bz}_1$ material. It should be noted that isoquercitrin upon hydrolysis gives rise to quercetin and glucose and quercitrin to quercetin and rhamnose (12).

The above finding appears reasonable since pr stock gives rise to pelargonidin and Pr stock to cyanidin. The only difference between cyanidin and pelargonidin is the number of hydroxyl (OH) groups on the B ring, namely, two and one respectively. The only difference between quercetin and kaempferol is again the number of OH groups on the B ring, namely, one and two respectively. (See Appendix V for the formulas of these compounds.) It is observed in the flavonoid pathway that quercetin and kaempferol are end products on a side branch (flavonol line) of this system and could reasonably be formed if there were a genetic block close to but beyond the precursor flavanol. (See Figure 1.) These materials could also be formed if the major line, that is, the path to anthocyanin could not handle all of the precursors. Shunts could be opened (flavonol being a main one in this case) to provide a release until the system shuts down. In the case of $\underline{a}_2$ stock, leucoanthocyanidin has also been isolated (2) and, indeed, was also noted in the $\underline{a}_2$ stock used in this work when the extract was heated with MeOH-HCl. This would indicate that the $\underline{a}_2$ block is beyond (below) "flavenol" as indicated in Figure 1. No leuco substance has been observed in $\underline{a}_1$; therefore, the $\underline{a}_1$ block would be before "flavenol". It appears that the flavonol pathway is opened fairly easily since (i) at least as much flavonol is formed in $\underline{a}_2$ stock as in $\underline{a}_1$ even though the leuco pathway is open in $\underline{a}_2$, (ii) no other extra amounts of side products were noted in the extracts, for example, of flavone and the like, and (iii) some flavonol may form in Pr and pr stock.

Another genetic block in the flavonoid system of maize aleurone in which flavonol would perhaps be expected to accumulate is in $\underline{bz}_1$ stock.

According to studies by Reddy (29), $\underline{bz}_1$ acts after $\underline{a}_2$. A very small amount of anthocyanin is produced with the $\underline{bz}_1$ recessive; however, much more of a gold-brown pigment is produced as is noted by the aleurone color. (See Figure 8.) A compound was isolated from $\underline{bz}_1$ extract and preliminary studies indicate that it is not any of the standards listed in Appendix II. No flavonol was observed in the extracts; therefore,the precursors must go into the formation of the gold-brown pigment. This block is perhaps too far from the flavonol path for any influence to be noted.

From the work that has been done, the position of the genes on the flavonoid path appear to be as follows:

(i) $\underline{A}_1$ acts between flavanonol and "flavenol".

(ii) $\underline{A}_2$ acts below "flavenol".

(iii)$\underline{C}^I$, $\underline{C}_1$, $\underline{C}_2$, $\underline{R}$,and $\underline{Pr}$ act before flavanonol.

The extracts from the $\underline{Pr}\underline{Pr}\underline{Pr}$, $\underline{Pr}\underline{Pr}\underline{pr}$, $\underline{Pr}\underline{pr}\underline{pr}$,and $\underline{pr}\underline{pr}\underline{pr}$ stocks provided some interesting findings. These stocks were all grown at the same time and are of the same background - the $\underline{Pr}\underline{Pr}\underline{pr}$ and $\underline{Pr}\underline{pr}\underline{pr}$ genotypes were produced by crosses of the $\underline{Pr}\underline{Pr}\underline{Pr}$ and $\underline{pr}\underline{pr}\underline{pr}$ material. The data in Table III indicate that the total amount of pigment produced in all the stocks is approximately the same. It is interesting to note that a small amount of pelargonidin is produced in the $\underline{Pr}\underline{Pr}\underline{Pr}$ material and a small amount of cyanidin in the $\underline{pr}\underline{pr}\underline{pr}$ stock. This has previously been reported (11, 35). The $\underline{Pr}\underline{Pr}\underline{pr}$ and $\underline{Pr}\underline{pr}\underline{pr}$ stock both produce a very small amount of pelargonidin - much less than would be expected according to the genotype and compared with the amount produced in $\underline{pr}\underline{pr}\underline{pr}$ stock. It appears that the $\underline{Pr}$ gene in this stock is very dominant and is almost as effective in one dose as in three. This indicates that only one gene may be active in $\underline{Pr}\underline{Pr}\underline{Pr}$ stock. The $\underline{Pr}$ - $\underline{pr}$

system does not take the form of a dominant - recessive relationship in the conventional sense due to the pigment produced by prprpr stock. It seems reasonable in this situation that if Pr is present then the precursor has another OH added to the B ring and if it is not present the precursor continues and is not changed from the one OH on the B ring. Some pelargonidin could form even if the genotype were PrPrPr either by later losing an OH group or having missed the OH addition - perhaps in a period of very rapid pigment formation. The formation of cyanidin in prprpr stock is a little more difficult to explain especially since a relatively large amount of it is formed compared with pelargonidin formed in PrPrPr. One explanation is that the Pr gene is not completely inactive. If it is assumed that the one-gene-one-polypeptide hypothesis applies to this situation then the difference between the viable and non-viable polypeptide must be slight. Even though the genotype is prprpr, some active enzyme is produced, and cyanidin is, therefore, formed.

That the Pr - pr system differs in basic operation from the other genes such as R - r and C_1 - c_1 is indicated by (i) visible aleurone pigmentation and (ii) by quantitative comparisons of total pigment formed. As noted in the observations, the intensity of bronze or gold-brown pigment formation in bz_1pr and bz_1 stock increases from zero in $c_1c_1c_1$ stock to light, medium and full in $C_1c_1c_1$, $C_1C_1c_1$, and $C_1C_1C_1$ stock respectively. The same is true for the intensity of pigment in $C_1C_1c_1R_{10}r_{10}r_{10}r_2r_2r_2$PrprPr and $C_1C_1c_1R_{10}r_{10}r_{10}r_2r_2r_2$prprpr - the main factor in these genotypes appears to be the R_{10} gene. Quantitative data for the latter materials are listed in Table III. If these data are compared with those from the stock discussed in the Pr - pr system it is seen that there is approximately five times more pigment in the complete stock. (Approximately the same

quantity of aleurone was extracted in all cases.) Genes such as R and C_1, if recessive, give rise to substantially less total pigment formation and are complete in their lack of action, that is, materials do not "leak" through as is indicated by the stock which is completely recessive.

Before leaving this section, it should be noted that the extracts were hydrolyzed; the sugars were, therefore, removed from the compounds. This was done to simplify the work since sugars can form many different complexes with a single type of flavonoid molecule (12, 14, 16). Some of these compounds are very labile during extraction and isolation and a number of artifacts could be formed. Also, unhydrolyzed extracts of maize recessives have been made by many workers, for example, Coe (2), Reddy (28), and Gavazzi (17). It was, therefore, felt unnecessary to repeat this work in detail especially when the objective was to find the correlation between the recessives and the flavonoid aglycones.

PART II

TISSUE CULTURES

INTRODUCTION

The objectives of this work were (i) to identify gene products and precursors by adding various chemicals (mainly flavonoids) to bypass recessives and (ii) to bypass recessives by cross-feeding.

SECTION 1. PRECURSOR FEEDING

MATERIALS AND METHODS

Stock

Except where indicated, the stock used was W22 - an inbred line from Wisconson. Stocks recessive for the following color factors were used in these experiments: $\underline{c}_1$, $\underline{c}_2$, $\underline{r}_{10}$, $\underline{a}_1$, and $\underline{a}_2$. Materials with the dominant $\underline{C}^I$ and with $\underline{r}_{10}\underline{R}_2$ were also used. (Unless indicated otherwise $\underline{r}_2$ is assumed for all genotypes.)

Seeds were sown in the greenhouse during June and July - the days to pollination varied from 70 to 80. Since, under these conditions the pollen often matures before the silks, it was necessary to sow an additional set of seeds for each genotype approximately one week after the initial planting.

Medium

The culture medium was made in two parts: (i) Tris buffer - sucrose solution and (ii) precursor solution. The Tris buffer was 0.05 M and the sucrose was 0.05 M. The pH of this was adjusted to 6.0 with 1 M NaOH. The precursor solutions were made in the following concentrations: 1×10^{-4},

1×10^{-5}, and 1×10^{-6} gm/ml. All of the dry chemicals were dissolved in glass distilled water. (See Appendix II for a list of the 15 precursors used.)

Each culture tube (10 ml test tube with a styrofoam cap) consisted of 2 ml of Tris buffer - sucrose solution and 4 ml of precursor solution. All of the materials except the precursors were autoclaved.

Culture Techniques

Each set of cultures consisted of 3 tubes of each of the precursors plus 3 controls - which consisted of all components except the precursor. The tests were carried out 15, 20, 25, and 28 days after pollination. One test was run on $\underline{a}_1$ stock 32 days after pollination since this material did not mature as rapidly as the others. Four sets of tests were undertaken with $\underline{c}_2$ stock at 28 days.

Kernels were removed from the plant and brought directly to the laboratory where the pericarps were removed and the aleurones peeled off. Aleurone from a different kernel was placed in each tube. The cultures were then placed in a dark cupboard at room temperature ($21^{\circ}C$). Tests were also made with $\underline{c}_1$ and $\underline{c}_2$ tissue under light.

The cultures were kept for a maximum of 72 hours under the conditions outlined and before being discarded were tested with HCl. The tissue was dipped in the HCl for a few seconds then observed for any increase in color intensity. (This is a test for anthocyanin (12)).

OBSERVATIONS

The $\underline{c}_2$ tissue in dihydroquercetin developed red pigment 25 and 28 days after pollination. No pigment developed with any of the other precursors or in the controls. The pigment developed best in the 10^{-4}

Figure 14. Photograph of two pieces of c_2 aleurone from a dihydroquercetin culture. Note the patches (now faded) of red pigment. (3X).

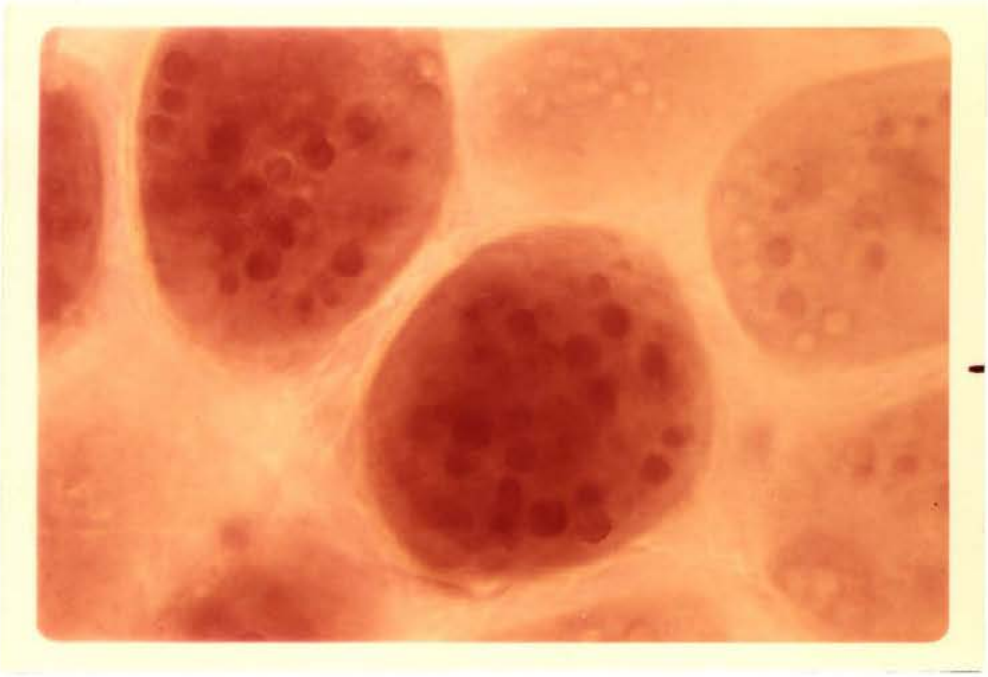


Figure 15. Photograph of cells of c_2 aleurone from a dihydroquercetin culture. Note the deeply pigmented aleurone grains in the cells. (400X).

and 10^{-5} gm/ml concentrations. Tubes which had been set up with a saturated solution of dihydroquercetin showed no more pigment than the material in the 10^{-4} and 10^{-5} concentrations. The pigment developed in the dark or in the light and signs of development could be seen within two hours after placing the tissue in the precursor solution. It was observed that the pigment formed in patches - this development is illustrated in Figures 14 and 15.

When the pigmented tissue was placed in MeOH-HCl (1% concentrated HCl) the red color became very bright - this is a test for anthocyanin (12).

DISCUSSION

Over 1,400 culture tubes were set-up and of these 300 were of $\underline{c}_2$ tissue. Pigment did not develop in any controls but pigment did develop in all 45 of the dihydroquercetin tubes when the stock was 25 and 28 days old.

It may at first seem strange that pigment did not form in any of the other recessive tissues - especially those in dihydroquercetin since this precursor is on the main line of the flavonoid pathway. (See Figure 1.) It should be recalled, however, that $\underline{a}_1$ and $\underline{a}_2$ stock would not be expected to produce pigment since the precursors used are found before these recessives. This is shown by the accumulation of kaempferol and quercetin with $\underline{a}_1$ and $\underline{a}_2$ recessive genes.

There are hints that the dominant inhibitor $\underline{C}^I$ and the recessives $\underline{c}_1$ and $\underline{r}_2$ act to repress the whole flavonoid system - this thought will be expanded in a later section. If this is not the case then one is led to the hypothesis that dihydroquercetin is the direct product of the

action of the c_2 gene and for the flavonoid pathway to be opened beyond a recessive the immediate product of that gene must be provided. This does not follow from work that has been done in many other systems where pathways can be opened by adding a precursor that is found some distance beyond a recessive, for example, in bacteria and Neurospora.

It is interesting to note how rapidly pigment forms in the c_2 tissue and how age of tissue and concentration of precursor influence the pigment. If pigment is going to form it will do so within a few hours and carrying the culture for a number of days serves no useful purpose. The tissue, however, must be at a stage when pigment could normally form - the length of the stage may be quite long, for example, ten days.

Increasing the concentration of precursor beyond a certain level does not lead to increased pigment formation - the system can only handle a given amount of material. On the other hand, there is a critical level below which pigment formation cannot be observed - there may well be some formation; however, it is too small to be observed by the techniques used.

The last point that should be discussed concerns the reasoning behind the culture technique. The simplest method which would work was desired; this was a culture of the tissue at the stage when it would normally produce pigment. Many of the problems defining a complete medium for the tissue were, therefore, eliminated. The sucrose was added since Straus (35) found that anthocyanin production increased significantly with the addition of sugar. He also found that pH 6.0 was optimum; therefore, Tris buffer with pH 6.0 was used. The levels of

precursors used covered the normal range of concentrations found in the literature (9, 27, 34, 36, 38).

SECTION 2. CROSS-FEEDING

METHODS AND MATERIALS

Stock

The genotypes used in Section 1 were also used for this experiment.

Culture Techniques

Table IV outlines the age, light conditions, and number of cultures established.

Kernels were removed from plants and immediately brought to the laboratory where under sterile conditions the pericarps were removed and aleurones peeled off. The tissue was placed on agar slants - 0.8% sterile agar in 10 ml test tubes with styrofoam caps. For the controls, only the aleurone from one kernel was used per tube; whereas, for the cross-feeding tests the aleurone layers from two different recessive seeds were pressed together by the inner surfaces and placed on the agar slant.

The tubes to be exposed to light were placed in a growth chamber with two 20 watt cool-white General Electric fluorescent lamps. The tubes for the dark test were placed in a cupboard. (The temperature was 21°C in both situations.)

In some tests mineral oil or corn oil was placed over the tissue in the tube and in others one piece of tissue was subjected to heat or different surfaces were pressed together.

The characteristic HCl - anthocyanin test was carried out on each piece of tissue.

TABLE IV

CULTURES OF ALEURONE TISSUE ON AGAR

Stock	No. of Cultures	Days from Pollination	Light Conditions	Area of Red Pigment	Remarks
$\underline{c}^I$	5	31-35	light	-	
	2	31-35	dark	-	
$\underline{c}_1$	10	25-34	light	0.75	
	4	18-22	light	-	
	6	25-34	dark	-	
$\underline{c}_2$	20	28-38	light	-	(one showed slight signs of pigment)
	4	28-38	dark	-	
$\underline{r}_{10}$	10	25-38	light	-	
	2	25-38	dark	-	
$\underline{a}_1$	10	29-33	light	-	
	2	29-33	dark	-	
$\underline{a}_2$	10	29-35	light	-	
	2	29-35	dark	-	
$\underline{a}_1\underline{r}_{10}$	4	22-26	light	-	
$\underline{c}_1\underline{r}_{10}\underline{R}_2$	15	25-40	light	0.5 - 0.75	
	5	25-40	dark	-	
$\underline{a}_1:\underline{c}_2$	6	22-29:28-31	light	-	
	14	29-37:28-38	light	0.25)	in $\underline{c}_2$ only
	4	29-37:28-38	dark	0.25)	
$\underline{a}_2:\underline{c}_2$	18	27-34:28-35	light	0.75)	in $\underline{c}_2$ only
	4	27-34:28-35	dark	0.75)	
	5	18-24:28-35	light	-	
$\underline{c}_1:\underline{c}_2$	5	28-30:28-32	dark	-	
$\underline{r}_{10}:\underline{c}_2$	8	25-38:28-34	light	-	
	2	25-38:28-34	dark	-	
$\underline{c}^I:\underline{c}_2$	9	31-35:31-34	light	-	
$\underline{c}_1\underline{r}_{10}\underline{R}_2:\underline{c}_2$	6	32:31-34	dark	-	
$\underline{a}_2:\underline{c}_1$	12	29-35:28-30	dark	-	
$\underline{a}_1:\underline{c}_1$	4	29-33:28-30	dark	-	

TABLE IV (continued)

Stock	No. of Cultures	Days from Pollination	Light Conditions	Area of Red Pigment	Remarks
$\underline{r}_{10}:\underline{c}_1$	4	26-30:28-30	dark	-	
$\underline{a}_2:\underline{c}_1\underline{r}_{10}\underline{R}_2$	10	29-35:25-40	dark	-	
$\underline{a}_2:\underline{a}_1$	20	29-35:29-33	light	-	(oil used in 10 tests)
	4	29-35:29-33	dark	-	(oil used in 2 tests)
$\underline{a}_1:\underline{C}^I$	3	29-33:31-35	light	-	
$\underline{a}_2:\underline{C}^I$	8	29-35:31-35	light	-	
$\underline{a}_2:\underline{r}_{10}$	3	29-35:28-30	light	-	
$\underline{a}_2:\underline{a}_1\underline{r}_{10}$	2	29-35:22-26	light	-	

Note: (i) The area of pigment refers to the fraction of the aleurone surface.

(ii) Light was from two 20 watt fluorescent lamps. A 60 watt tungsten bulb was also effective.

(iii) "Days from Pollination" refers to the range within which culture tubes were set-up, e.g., 5 tubes of $\underline{C}^I$ aleurone were established in the period 31 to 35 days after pollination.

OBSERVATIONS

The following observations were made: (See Table IV also)

(i) $\underline{c}_1$ and $\underline{c}_1\underline{r}_{10}\underline{R}_2$ produce pigment if they are exposed to light.

(ii) The only combinations in which pigment was produced were $\underline{a}_1:\underline{c}_2$ and $\underline{a}_2:\underline{c}_2$ - red pigment formed in the $\underline{c}_2$ tissue in both tests.

(iii) The tissues produce pigment regardless of the surfaces in contact, that is, inner, inner-outer, or outer surfaces.

(iv) When $\underline{a}_2$ was heated to 80°C for one and one-half hours $\underline{c}_2$ did not produce pigment when in combination with that $\underline{a}_2$ tissue.

(v) Pigment was not produced by the $\underline{c}_1$ aleurone if it was younger than 22 days after pollination, and pigment did not develop in the $\underline{a}_1:\underline{c}_2$ or $\underline{a}_2:\underline{c}_2$ combinations if the $\underline{a}_1$ tissue was younger than 28 days and the $\underline{a}_2$ younger than 24 days.

(vi) Pigment was never seen in the $\underline{c}_1$ tissue in less than 30 hours; however, if pigment was going to be produced in the $\underline{c}_2$ aleurone it could be observed within 3 hours.

(vii) The application of oil to the $\underline{a}_1:\underline{a}_2$ combination did not give rise to pigment formation.

(viii) When the tissues with red pigment were placed in MeOH-HCl (1% concentrated HCl) the red color became very bright. Spots of pigment barely visible then became very obvious.

DISCUSSION

The technique of cross-feeding maize tissue was developed by Reddy (28). He hypothesized that if two pieces of active aleurone, each with a different recessive, were pressed together one of the pieces should develop pigment if a precursor accumulated at one gene block (recessive).

This would then diffuse to the other tissue thus providing a product not available in that tissue since it had a recessive also. Reddy pressed together all possible combinations of tissues and from this work put forward the following gene order for flavonoid synthesis: $\underline{C}^{I}$-$\underline{C}_1$-($\underline{C}_2$)-$\underline{R}$-($\underline{In}$)-$\underline{A}_1$-$\underline{A}_2$-$\underline{Bz}_1$-$\underline{Bz}_2$-Anthocyanin. Part of this order, namely, the positions of $\underline{A}_1$, $\underline{A}_2$, $\underline{Bz}_1$, and $\underline{Bz}_2$ was also suggested in work done by Coe (2).

An experiment similar to Reddy's was undertaken in this laboratory initially to test the $\underline{c}_1\underline{r}_{10}\underline{R}_2$ stock and to elucidate a little more the precursor work as outlined in the previous section. It has been suggested by Styles (personal communication) that the $\underline{R}_2$ gene is a duplicate of the $\underline{R}_{10}$ gene. If this were the case then $\underline{r}_{10}\underline{R}_2$ stock would be expected to behave like the $\underline{r}_2\underline{R}_{10}$ stock in Reddy's experiment. If the flavonoid pathway could be opened fairly easily, as indicated by Reddy, then it would be difficult to explain why only $\underline{c}_2$ produced pigment with the dihydroquercetin precursor. It was thought that a duplication of Reddy's experiment would indicate when the flavonoid pathway was active in the various recessives; the tissue at that specific stage could then be fed precursors.

The first interesting point noted was the production of pigment in $\underline{c}_1$ tissue if it were exposed to light. This meant that all cultures with $\underline{c}_1$ had to be kept in complete darkness if meaningful results were to be obtained. Secondly, whereas Reddy found the action of his $\underline{c}_2$ stock erratic the $\underline{c}_2$ used in this experiment was found to be quite consistent, that is, the controls did not pigment and the $\underline{a}_1$ and $\underline{a}_2$ recessives were the only ones which reacted favourably with $\underline{c}_2$. Thirdly, no other combinations ever produced pigment. This was indeed strange since $\underline{a}_1$:$\underline{a}_2$ combinations

were made when they were known to be active with $\underline{c}_2$. Reddy reported that he had no success with the $\underline{a}_1$:$\underline{a}_2$ combination unless it had been coated with mineral oil. This was then done but with no better success.

It should be noted that Reddy had exposed all of his cultures to a tungsten light. At this point the $\underline{c}_1$ tissue was again checked and found to produce pigment when exposed to either tungsten or fluorescent light. Could the stage of development of the tissue used in this laboratory be the reason for the inability to duplicate Reddy's results or was the stock of different background and, therefore, causing the problem? There is definitely a stage below which pigment will not form as is illustrated with the $\underline{a}_1$, $\underline{a}_2$,and $\underline{c}_1$ material. (Note that the term stage is preferable to age since depending on the growing conditions the stage of development can vary greatly regardless of age - see Appendix VI). Once the pathway is active, however, it appears to remain open for a relatively long period. This period is until the aleurone can no longer be peeled from the underlying endosperm - up to ten days in some cases! Since a fairly wide range of stock maturity was used, stage of development does not appear to be the problem. This leaves the background of the stock as the last obvious factor. Since there is no easy way of checking this, Reddy's work will be left to stand on its own and an attempt will be made to explain the results obtained with the stock used in this laboratory. It may be noted that Coe (personal communication) has also been unsuccessful in a number of attempts to repeat the experiment.

There is little question about the transfer of a substance from the $\underline{a}_1$ and $\underline{a}_2$ tissue to the $\underline{c}_2$. This is indicated since (i) the other recessives when in contact with $\underline{c}_2$ do not produce pigment, (ii) the $\underline{c}_2$ control does not

produce pigment, and (iii) when $\underline{a}_2$ is heated and pressed with $\underline{c}_2$ the $\underline{c}_2$ is not activated. The $\underline{a}_1$ and $\underline{a}_2$ genes almost certainly act after $\underline{c}_2$ (see the work in Part I). It is also noted that the $\underline{c}_2$ system was the only one activated by feeding precursors.

It appears that the genes beyond the genetic block in the recessives other than $\underline{c}_2$ are in some way repressed or at least are not as easily activated as those in the $\underline{c}_2$ system. It is well known that controlling units and inhibitors are present in the flavonoid system of maize aleurone (1, 4, 5, 22). The $\underline{c}_1$ gene could cause a repression since the phytochrome system appears to be active here - this is discussed in Part III.

The question of no pigment production in the $\underline{a}_1$:$\underline{a}_2$ combination is a formidable one. It was obvious that Reddy also had problems with this pair. Mineral oil, as noted previously, appeared to solve his dilemma; however, in this laboratory neither mineral oil nor corn oil had any influence. No clear reason can be suggested at the present time for the inaction in the $\underline{a}_1$:$\underline{a}_2$ combination.

PART III

LIGHT AND GENE ACTION

INTRODUCTION

It has been observed that the aleurone of $\underline{c}_1$ kernels when germinated in the presence of light produces a red pigment. With this in mind, the objective of this section of the work was to study the influence of light on gene action.

MATERIALS AND METHODS

Stock

The following stocks, all W22 inbred material, were used in this work: $\underline{c}_1$, $\underline{c}_1\underline{pr}$, $\underline{c}_1\underline{r}_{10}\underline{R}_2$, $\underline{c}_1\underline{r}_{10}\underline{R}_2\underline{pr}$, $\underline{c}_1\underline{bz}_1$, $\underline{c}_1\underline{a}_1$, and $\underline{c}_1\underline{a}_1\underline{r}_{10}$. As usual, all of the other genes in the flavonoid pathway were in the dominant form, namely, $\underline{C}_2$, $\underline{R}_{10}$, $\underline{A}_1$, $\underline{A}_2$, $\underline{Bz}_1$, $\underline{Bz}_2$ except $\underline{C}^I$ and $\underline{in}$.

Factors Varied

The factors light, temperature, age, and condition of tissue were varied as follows for the $\underline{c}_1$ stock:

(i) Light

Both tungsten (25 and 60 watt G.E.) and fluorescent (cool white) lights were used. (See Appendix VI for the spectral output from these lamps.)

An attempt was made to determine the wave lengths of light which were critical by (i) using Wratten filters and (ii) using the Spectronic 20 machine. The filters shown in Table V were placed between two pieces of glass and this was cemented onto the top of a glass petri-dish. The remainder of the dish was completely blackened so that only light passing

through the filter was able to reach the kernels. The distance of the containers from the light source was then adjusted so that an approximately equal intensity of light fell inside each container (65 Foot candles). Seeds were placed inside the dishes (in water) and observed at time intervals for pigment formation.

The Spectronic 20 was used to obtain specific wavelengths of light for the tissue. For each test a seed was placed on a piece of wet styrofoam inside a cuvette. It was necessary to use the styrofoam to elevate the seed and thus have the aleurone exposed to the maximum amount of light. The machine had to be placed in a growth chamber in which the temperature was 6°C in order to maintain the seed temperature at 21°C. If the machine were run in the open room the temperature of the seed environment elevated to over 38°C which inactivated the tissue. The influence of light at specific wavelengths is outlined in Table VI.

(ii) Temperature

Ten seeds were placed in each petri-dish and exposed to temperatures ranging from 0°C to 45°C (See Table VII). This was done in a growth chamber under constant cool white fluorescent light.

(iii) Age

Kernels at different stages of development were placed under germinating conditions in the growth chamber. Constant cool white fluorescent light was used and a temperature of 21°C was maintained. (See Table VIII).

(iv) Condition

The seeds were mechanically manipulated in the following ways and observations made:

(a) The husks were removed from the cobs of plants growing in the greenhouse at 15, 20, 25, and 30 days after pollination.

(b) Ten kernels each with (i) the embryo removed, (ii) the pericarp removed, (iii) the embryo and pericarp removed, and (iv) only the pericarp over the embryo removed were placed in the growth chamber under germinating conditions in light.

(c) Aleurone was removed from ten seeds and placed on agar and placed in the growth chamber. The tissue was exposed to fluorescent light.

Extractions

Extractions and isolations were made from the aleurone of $\underline{c_1}$, $\underline{c_1 pr}$, $\underline{c_1 a_1}$, and $\underline{c_1 a_1 r}$ as follows: (i) when germinated in the dark for 4 days, and (ii) when germinated in the light. Spectra were also run on $\underline{c_1}$ and $\underline{c_1 pr}$ isolates. (See Part I for the techniques used.)

Quantitative comparisons were made between isolates of $\underline{Pr}$, $\underline{pr}$, $\underline{c_1}$, and $\underline{c_1 pr}$ - all having been germinated in the growth chamber in light for 80 hours.

$\underline{c_1}$, $\underline{c_1 r_{10} R_2}$, $\underline{c_1 r_{10} R_2 pr}$, and $\underline{c_1 bz_1}$ Genotypes

The influence of light on these stocks was observed. Twenty seeds of each were placed in germinating conditions under fluorescent light and ten seeds of each were placed under the sameconditions in the dark.

OBSERVATIONS

(i) Light

The aleurone layers of 20 kernels tested were fully pigmented when observed after 72 hours under (i) cool white fluorescent lamps, and (ii) tungsten lamps. (See Figures 16 and 17) The aleurone from a duplicate

experiment in the dark showed spots of pigment on 4 seeds.

As noted in Table V the aleurone under filter 89B produced no pigment in fluorescent light but was over one-half pigmented if exposed to a tungsten lamp. This is reasonable since the fluorescent lamp emits very little light above 700 mμ. (See Appendix VII). The stock under the #70 filter was approximately one-quarter pigmented under both the tungsten and fluorescent light. There was over half pigmentation with the other filters in both fluorescent and tungsten light.

The Spectronic 20 data indicate that pigment is produced in the wavelength range 450 mμ to 700 mμ. Note that only a very small amount of pigment was produced in all cases. (See Table VI).

(ii) Temperature

Pigment does not start to form until 6 - 9°C and stops at 39 - 42°C. (See Table VII).

(iii) Age

Pigment did not form in the tissue until 25 days after pollination. (See Table VIII).

(iv) Condition

Pigment did not develop in any of the kernels on cobs with the husks removed. Table IX outlines the pigment formation in kernels that were mechanically changed in various ways. Note that the same experiment was repeated with the seeds kept in the dark - only the odd spot of pigment developed in these seeds.

(v) Extractions

See Figures 18, 19, and 20 for chromatograms of the extracts; see

Figure 16. Maize kernel of genotype c_1 germinated in light for 72 hours. Note the development of the red pigment. (4X).

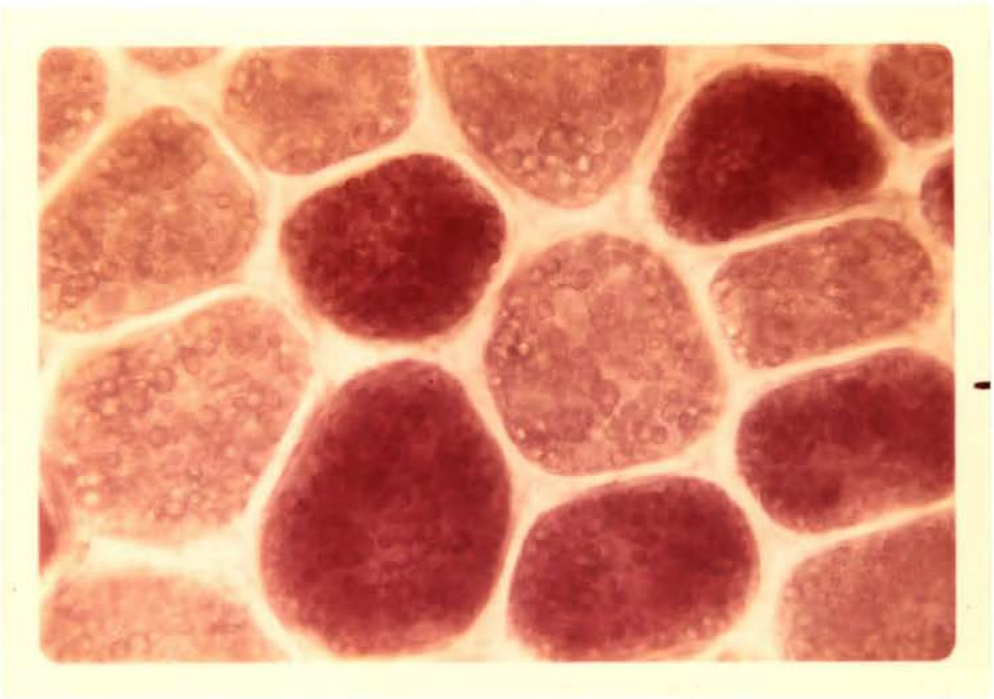
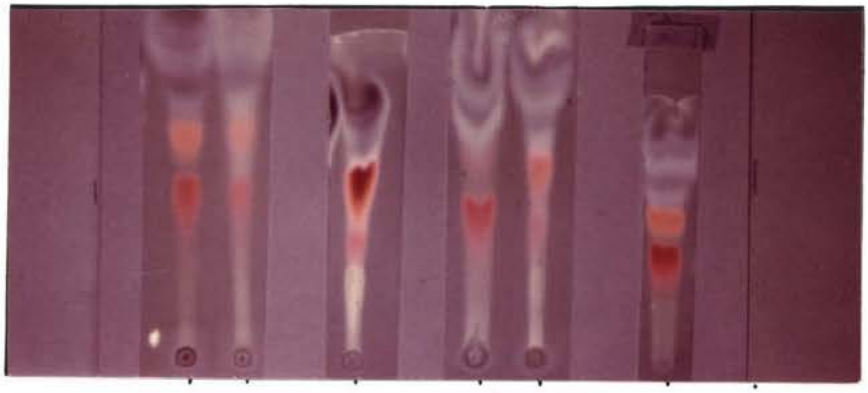
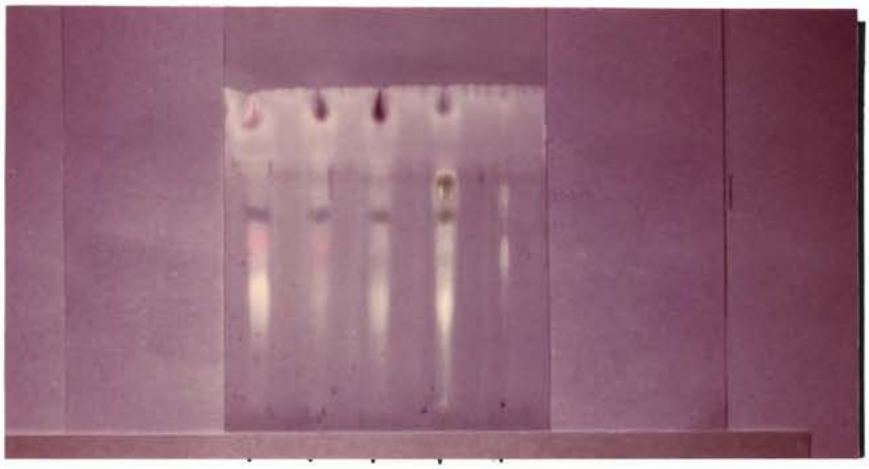


Figure 17. Cells from the aleurone of a maize kernel of genotype c_1 germinated in light for 72 hours. Note the formation of pigment only in certain cells and that each cell is packed with aleurone grains (300X).



$\underline{c_1}$ $\underline{c_1}$ $\underline{c_1pr}$ $\underline{Pr}$ $\underline{pr}$ $\underline{c_1}$
(n.e.)

Figure 18. Chromatograms of extracts from 6 tissues, as indicated, run in FA-4M HCl. All kernels were germinated in light. Note the high content of pelargonidin (top orange spots) - the lower magenta spots are cyanidin. Note also the pigment between the origin and cyanidin - this is most obvious in $\underline{c_1pr}$. The pigment was identified as kaempferol and quercetin. (n.e. = embryo was removed).



$\underline{Pr}$ $\underline{c_1}$ $\underline{pr}$,$\underline{c_1pr}$,K+Q

Figure 19. Chromatograms of extracts from 4 tissues germinated in light and a mixture of 2 standards, as indicated, run in Forestal. The extracts were first chromatographed in FA-4M HCl and the yellow pigments eluted. The yellow pigment eluates were the extracts run for these chromatograms (K = kaempferol and Q = quercetin).

Figure 20. Chromatogram, run in 2 directions, of an extract from c_1 stock germinated in light. The first direction was left to right with FA-4M HCl and the second was upward with MeOH-HCl-H_2O. The lower magenta spot is cyanidin and the orange-pink spot to the right and upward is pelargonidin.

TABLE V

DEVELOPMENT OF PIGMENT IN c_1 ALEURONE TISSUE USING WRATTEN FILTERS

Filter	Transmission Characteristics[1.]	Area of Red Pigment	
		Fluorescent Light[2.]	Tungsten Light[2.]
glass		1.0 (fully pigmented)	.75
89B	only above 690 mμ transmitted	0 (no pigment)	.50
70	only above 655 mμ transmitted	.25	.25
47B	360 - 500 and above 738 mμ transmitted	1.0 (fully pigmented)	.50
38A	320 - 640 mμ transmitted	1.0 (fully pigmented)	.50
29	only above 590 mμ transmitted	.50	.50
22	only above 550 mμ transmitted	.50	.50
8	only above 460 mμ transmitted	.50	.50
2E	only above 415 mμ transmitted	.75	not observed
dark		(1 spot of pigment)	1 spot of pigment

1. These data are from The Kodak Wratten Filters Booklet, The Eastman Kodak Company.

2. See Appendix VII for the spectral emission characteristics.

TABLE VI

c_1 KERNELS IN THE SPECTRONIC 20

Wave-length mμ	Red Pigment
350	-
400	-
450	along part of the crown ridge
500	along part of the crown ridge
550	along part of the crown ridge
600	small patches in crown
660	small patches in crown
700	small patches in crown
730	-
750	-
800	-

Note: The maximum pigment ever formed covered only 5% of the area of the aleurone.

TABLE VII

INFLUENCE OF TEMPERATURE ON PIGMENT FORMATION IN c_1 KERNELS

Temperature (^{o}C)	Time (Hours)	Red Pigment
0	300	no pigment
3	300	no pigment
6	200	3 spots
9	150	starting to form
12	100	starting to form
15	70	starting to form
18	50	starting to form
21	35	starting to form
24	35	starting to form
27	35	starting to form
30	35	starting to form
33	35	starting to form
36	35	starting to form
39	100	2 spots
42	100	no pigment
45	100	no pigment

Note: There was full pigment in the material in the 21 to 33^{o}C range after 72 hours

TABLE VIII

INFLUENCE OF AGE ON PIGMENT DEVELOPMENT IN c_1 TISSUE

Age (days)	No. of Samples	Red Pigment in Aleurone	
		Aleurone On Seed	Aleurone Culture
15	10	-	-
18	10	-	-
22	10	-	-
25	10	pigmented	pigmented
28	10	pigmented	pigmented
30	10	pigmented	pigmented
32	10	pigmented	pigmented
34	10	pigmented	pigmented

TABLE IX

INFLUENCE OF MECHANICAL MANIPULATIONS ON PIGMENT FORMATION IN c_1 TISSUE

Type of Change	No. of Seeds	Time (Hrs.)	Area of Red Pigment
none (full seed)	10	24	-
		32	0.10
		48	0.25
		72	0.75
no pericarp over embryo	10	24	0.10
		32	0.25
		48	0.75
		72	1.0 (full)
pericarp completely removed	10	24	0.15
		32	0.50
		48	1.0 (full)
no embryo	10	24	0.15
		32	0.75
		48	1.0 (full)
no embryo, no pericarp	10	24	0.15
		32	0.80
		48	1.0 (full)

TABLE X

COMPARISON OF CHROMATOGRAMS OF STOCKS GERMINATED IN LIGHT [1].

Stock	Orange-Pink Spot RF FA-4M HCl	Magenta Spot RF FA-4M HCl	RF of Yellow Pigments FA-4M HCl		Forestal		30% A.A.	
$\underline{c}_1$	.57	.43	.15	.24	.46	.64	-	
$\underline{c}_1\underline{pr}$	.57	.43	.16	.27	.52	.67	-	
$\underline{c}_1\underline{a}_1$			.16	.25	-		.13	.17
$\underline{c}_1\underline{a}_1\underline{r}_{10}$			-		-		-	

Note: The same comparisons for stock germinated in the dark indicated no differences in these stocks.

1. See Appendix IV for details of the individual values.

TABLE XI

COMPARISON OF SPECTRA OF PIGMENTS FROM Pr, pr, $\underline{c}_1$, AND $\underline{c}_1\underline{pr}$ STOCKS ISOLATED ON CHROMATOGRAMS

Stock	No. of Seeds	Absorbance Units Orange-Pink Spot	Magenta Spot	Total	Ratio
$\underline{c}_1$	25	2.8	6.4	9.2	1:2.3
	25	3.1	6.8	9.9	1:2.2
	30 (very small)	.70	1.7	2.4	1:2.4
$\underline{c}_1\underline{pr}$	40	4.1 (5)	.48	4.6	1:.12
	40	4.6	.52	5.1	1:.11
Pr	25	.58	15.7	16.3	1:27
pr	25	16.1	2.0	18.1	1:.12

Note:
1. All of the seeds were germinated in light.
2. A spectrum was run for the yellow pigment isolated in $\underline{c}_1\underline{pr}$ stock. The λ max. was 368 and there was no spectral shift when H_3BO_3/NaOAc was added.
3. See Table III for more Pr and pr values. The totals in this Table are less than those in Table III since not as much aleurone could be removed due to the nature of the germinated seed.
4. The ratio is the ratio of orange-pink to magenta material.

TABLE XII

PIGMENT FORMATION IN VARIOUS GENOTYPES WHEN GERMINATED IN LIGHT

Stock	Time (Hrs.)	Pigment Formation
$\underline{c}_1$	48	0.7 of area pigmented red.
	72	0.9 of area pigmented red.
$\underline{c}_1\underline{r}_{10}\underline{R}_2$	48	0.1 of area pigmented red.
	72	0.1 of area pigmented red.
$\underline{c}_1\underline{r}_{10}\underline{R}_2\underline{pr}$	48	0.1 of area pigmented red.
	72	0.1 of area pigmented red.
$\underline{c}_1\underline{bz}_1$	48	0.5 of area pigmented gold-brown.
	72	0.5 of area pigmented gold-brown.

Note: 1. The red pigment when tested with HCl became very bright.

2. The gold-brown pigment when tested with HCl showed no signs of red color.

3. The stocks when germinated in the dark showed no pigment.

Tables X and XI for RF and spectral data.

(vi) $\underline{c_1}$, $\underline{c_1 r_{10} R_2}$, $\underline{c_1 r_{10} R_2 pr}$, and $\underline{c_1 bz_1}$ Genotypes

See Table XII for a description of the pigmentation.

DISCUSSION

The influence of temperature on pigment formation can probably be explained by an over-all inactivation of the kernel metabolism at the high and low temperatures. The seeds at these temperatures also did not germinate.

As was observed in the previous work on culturing, there is a stage of development which the aleurone must achieve before it will normally develop pigment. Once this stage is reached with $\underline{c_1}$ tissue the flavonoid pathway can be opened at any time by simply exposing the seed to germinating conditions in light.

It was interesting to note how various physical changes made on the seed influenced the formation of pigment. When the husks were removed from the developing cobs, thus exposing the kernels to light, no pigment formed. However, if the seeds were later removed and placed under germinating conditions in light pigment did develop. It appears that the aleurone must be placed in an environment which would at least "trick" the tissue into "thinking" it was to start preparations for germination. Only then is the $\underline{c_1}$ gene activated, or at least the flavonoid system is stimulated. It should be noted that germinating conditions are not the only factors since, if in the dark only the very odd spot of pigment is ever seen on some seeds. The odd spot of pigment when in the dark does indicate that the uptake of water and tissue activation set the stage but light is the main factor.

The removal of various parts of the seed, namely, the embryo and pericarp

significantly increases the rate of pigment formation. As noted in Table IX the removal of the embryo and/or the pericarp gives rise to full pigment formation in 48 hours; whereas, the normal seed is less than half pigmented by this time. The mechanical manipulations, needless to say, give rise to a more rapid stimulation of aleurone - perhaps more rapid water uptake is involved. Similar tissues in the dark still produced no pigment; therefore, it was not simply an injury that caused the pigment development.

That the same flavonoid pathway is stimulated in the $\underline{c}_1$ system with light as is normally active in non-recessive stock is indicated by studies with $\underline{c}_1\underline{a}_1$, $\underline{c}_1\underline{a}_1\underline{r}_{10}$, and $\underline{c}_1\underline{bz}_1$. When $\underline{c}_1\underline{a}_1$ is germinated in light and the tissue extracted, yellow pigments are isolated that appear to be quercetin and kaempferol (See Figure 7.) These are not present if the stock is germinated in the dark. The pathway in light appears to be opened as far as the $\underline{a}_1$ block and as with the regular $\underline{a}_1$ stock, flavonol accumulates. In the case of $\underline{c}_1\underline{bz}_1$ again the pathway is opened but this time to the $\underline{bz}_1$ block and the compound characteristic of that recessive accumulates. When $\underline{c}_1\underline{a}_1\underline{r}_{10}$ stock was tested no yellow pigment could be isolated. Since no compound characteristic of the $\underline{r}$ recessive has been isolated it is difficult to determine if $\underline{r}$ blocked the path after $\underline{c}_1$ or before $\underline{c}_1$ - thus eliminating the supply of precursor. Whatever is the situation, the characteristic action (inaction) of $\underline{r}$ was observed.

Two very significant observations were made when extracts from the $\underline{c}_1$ and $\underline{c}_1\underline{pr}$ stock germinated in light were compared with extracts from $\underline{Pr}$ and $\underline{pr}$ stock. Definite yellow pigments were isolated from the $\underline{c}_1$ and $\underline{c}_1\underline{pr}$ stock - enough from the $\underline{c}_1\underline{pr}$ to obtain a very good U.V. spectrum. From color, RF, and spectral comparisons with kaempferol and quercetin

the main isolate from $\underline{c_1 pr}$ appeared to be kaempferol with a much lesser amount of quercetin. The main isolate from $\underline{c_1}$ appeared to be quercetin with a lesser amount of kaempferol. There was a small quantity of yellow compound in three out of four extracts from $\underline{pr}$ but in only one out of four $\underline{Pr}$ extracts was any sign of a yellow pigment observed. At best, there was almost nothing compared with that in $\underline{c_1}$ and $\underline{c_1 pr}$. The second observation concerned the ratio of cyanidin to pelargonidin produced in the $\underline{c_1}$ compared with the $\underline{Pr}$ stock. In $\underline{c_1}$ it is approximately 2.3:1; whereas, in $\underline{Pr}$ it is 25:1.

A comparison was also made between extracts from $\underline{c_1}$ when the embryo and pericarp had been removed and when no seed parts had been removed. Observations of the chromatograms indicated that more yellow pigment was present in the former extracts. (See Figure 18.)

It is noted from the chromatograms (Figure 19) that more of the yellow pigment is formed in $\underline{c_1 pr}$ than in $\underline{c_1}$ stock. This is also true when comparing $\underline{Pr}$ and $\underline{pr}$, that is, the $\underline{pr}$ will show the yellow pigment; whereas, $\underline{Pr}$ may show nothing or only very faint signs.

(It should be noted that for all comparisons, extracts from equal volumes (and numbers) of seeds were made. The stock in most cases was grown under the same conditions and usually at the same time.)

It is pertinent to recall at this time the formation of flavonols in $\underline{a_1}$, $\underline{a_2}$, $\underline{a_2 pr}$, and $\underline{c_1 a_1}$ stock and also to recall the discussion of the functioning of the $\underline{Pr}$ and $\underline{pr}$ genes. $\underline{Pr}$ directs a modification of a precursor; whereas, its allele $\underline{pr}$ does not.

A picture of the action of the flavonoid system considering $\underline{Pr}$, $\underline{pr}$, $\underline{c_1}$, $\underline{a_1}$, and $\underline{a_2}$ seems to be as follows: The $\underline{pr}$ gene is not modifying its

precursor; therefore, under normal development more materials are being pushed along the flavonoid path than can be handled at the end. The flavonol shunt is, therefore, opened. When this system is forced to produce pigment very rapidly, as in $\underline{c_1}\underline{pr}$ germinated in light, much more material is sent to the shunt. The $\underline{Pr}$ gene on the other hand modifies its precursor (the same one as $\underline{pr}$'s) and, therefore, regulates the release of its product. The pathway under normal conditions can handle most of the material and little if any flavonol is formed. Under the influence of light $\underline{c_1}$ develops pigment very rapidly and the system is, therefore, forced. There is not enough time for the $\underline{Pr}$ gene to modify all of its precursor, for example, perhaps not enough enzyme is produced; an abnormally high level of pelargonidin is, therefore, produced. The flavonol shunt is also opened to accept some of the excess of materials.

The last gene to be discussed in this part of the work is $\underline{R_2}$ which has been suggested by Styles (personal communication) as being a duplicate of $\underline{R_{10}}$. $\underline{R_2}\underline{r_{10}}$ stock has aleurone pigment as does $\underline{R_{10}}\underline{r_2}$ material. $\underline{R_2}\underline{r_{10}}\underline{c_1}$ aleurone on agar in light produces pigment in quantity that is not observably much different from that of $\underline{c_1}\underline{r_2}\underline{R_{10}}$, that is, the area covered with pigment is approximately the same in both tissues. The mature seeds of these stocks when germinated in light, on the other hand, show vast differences in pigment production. The $\underline{c_1}\underline{r_{10}}\underline{R_2}$ aleurone has only a few patches of pigment; whereas, the $\underline{c_1}\underline{r_2}\underline{R_{10}}$ aleurone is completely pigmented. The backgrounds of the stocks are similar - the only difference being the number of dominant and recessive $\underline{R_2}$ and $\underline{R_{10}}$ genes. It appears from this work that the $\underline{R_2}$ gene is as active as $\underline{R_{10}}$ during aleurone development; however, the $\underline{R_2}$ locus action is reduced as the aleurone nears maturity. When the aleurone is mature, $\underline{R_2}$ can be only partially activated. The $\underline{R_{10}}$ gene does not

close down in this way and can be activated quite readily in $\underline{c}_1\underline{r}_1\underline{R}_{10}$ tissue under germinating conditions in light. A controlling unit could easily be suggested as either working with $\underline{R}_2$ or perhaps $\underline{R}_2$ is a controlling unit itself. Controlling elements are known to exist in the flavonoid pathway in maize (22). It has been suggested by Gavazzi (11) that the $\underline{R}_{10}$ locus may consist of a structural and a controlling unit; therefore, the idea of a repressor or at least a regulator with the $\underline{R}_2$ system is not mere speculation.

How can the action of $\underline{c}_1$ with light be explained? It is well known that the chromo-protein phytochrome is active in light absorption in tissues that develop anthocyanin pigment when exposed to light (6, 7, 13, 24, 37). Indeed, maize seedlings have been used previously in phytochrome studies. The approach has been mainly the influence of different wavelengths and intensities of light and the influence of other environmental conditions. The action of phytochrome is rather complicated and the original hypothesis of

$$\text{P660} \underset{\text{far red}}{\overset{\text{red}}{\rightleftharpoons}} \text{P730}$$

is much too narrow to explain the absorption of light at many other wavelengths (6, 7,13). Depending on the species, light of different wavelengths has been found to be most effective in promoting anthocyanin production. In general there is a maximum between 400 and 500 mμ and between 600 and 800 mμ(7). A wavelength that partially inhibits in one species may stimulate in another. Correll et al (6) have found that pure phytochrome from rye absorbs light at 580, 660, 670, and 730 mμ - perhaps more will be found in the future. The studies undertaken in this laboratory with maize aleurone indicate that a wide range of wavelengths are involved. This fits with the general (expanded) view of the action of the phytochrome.

Regardless of the specifics, which no one seems to be sure about, light is absorbed and the $\underline{c}_1$ locus is stimulated, derepressed, or is bypassed altogether. Due to the lack of success in stimulating the flavonoid pathway of $\underline{c}_1$ stock either with precursors or by cross-feeding (see Part II) there is the suggestion that with $\underline{c}_1$ the path beyond this locus cannot be opened. Light removes this inhibition - perhaps indirectly. Mohr (24) in his work with mustard seedlings has suggested a histone relationship - these were in vogue in the early 1960's - and that light through phytochrome acts on regulator genes which in turn influence DNA-histone relationships. The suggestion, that $\underline{c}_1$ is a regulator is an interesting one and seems to fit relatively well with the observations made in this laboratory.

GENERAL IMPLICATIONS

The observations made in each of the three parts of this thesis have been discussed with respect to maize aleurone tissue. The more general implications will be outlined here.

In the introduction it was noted that hopefully some of the information gained from this study would have general application to the genetics of complex multicellular organisms. Indeed, this is the only way a genetic study of flavonoid synthesis in maize aleurone can be justified. Keeping in mind the many limitations that of necessity are a part of any generalizations the following may be found applicable to other higher organisms:

1. A genetic block in a pathway may lead to a shunting of compounds prior to the block along other paths. Some shunts appear to be more easily opened than others. This has been noted in other plants, for example, in the flavonoid pathway in clover (41), and snapdragons (16). It has also been observed in microorganisms, for example, the histidine biosynthesis pathway in Salmonella (18), and the pathway of arginine biosynthesis in E. coli (39).
2. A single dominant allele may give rise to much less end product than if both alleles (or 3 in the case of a triploid) were dominant. There is, therefore, a dosage effect. In other systems, however, the same amount of end product is produced whether only one or all three (for a triploid) alleles are dominant. In humans, a homozygous situation will give rise to twice the quantity of the enzyme galactose - 1-P-uridyl transferase as will a heterozygote(33). In Drosophila an individual homozygous for the bar locus has eyes only half as wide as individuals heterozygous for bar (10). Human females with their two X chromosomes show the same dosage effects as males with only

one X (10, 19, 33).

3. Precursors (in the form of specific chemicals or cross-feeding sources) can be added to some systems to bypass recessive genes. These are effective only if the genes beyond the block are not also "closed" due to the recessive containing a general repressive unit. Much work has been done in bypassing genetic blocks in microorganisms, for example, in the pathway of arginine biosynthesis in E. coli (39) and tryptophan biosynthesis in Neurospora (18). Cross-feeding work has been done in Drosophila by transplanting organs from one individual to another (19).

4. There is not always complete dominance with all dominant genes or complete recessiveness with all recessives. For example, some viable polypeptides (enzymes) may be produced by a recessive and often not enough is produced by the dominant. A genotype completely dominant could, therefore, produce some "recessive" product and a recessive could produce some "dominant" product. This situation has been found quite common for eye pigment mutants in insects, for example, Drosophila and for growth-factor requiring mutants of microorganisms such as bacteria and Neurospora (18).

5. Some gene systems can be activated if a critical environmental factor, for example, light is supplied. There appears to be the release of an inherited repressing unit in the system. Examples of this general observation are numerous in plants. For example, one species of primrose has red flowers at moderate temperatures and white at high temperatures (10); light will stimulate anthocyanin synthesis in many plant species (16). A moist hatching environment

may give rise to an abnormal abdomen in Drosophila and the temperature at a specific stage in development determines the number of eye facets (10).

6. If a system is forced beyond its normal rate of production, then often the path cannot handle all of the precursors and alternate pathways are used as shunts. It is, therefore, observed how side-reactions could occur. Also, in the situation where a dominant allele influences a precursor but its recessive does not, and the precursor, modified or not, continues along the path, there may be a large amount of the "recessive" product relative to the "dominant" compared to the ratio under normal production even though the genotype is completely dominant. This was the finding with germinated c_{-1} stock; no finding similar to this was located in the literature.

7. In systems containing double recessives, if the first gene can be activated then the path may be opened as far as the second recessive and alternate paths activated between the first and second recessive. Similar situations exist in some of the feeding experiments with bacteria and Neurospora since side reaction materials have been isolated (18).

8. A similar gene may be available to replace the function of a recessive. The replacement may, however, not be complete. An example similar to this has been found in E. coli where duplicate enzymes are present to convert chorismate to prephenate. That two pathways with the same enzyme were present was shown since one path can be inhibited by tyrosine and the other by phenylalanine (18).

9. Many genes are active only at a certain stage in development of a tissue. This implies the functioning of regulators and other controlling

units. A controlling gene for 9 other genes in the biosynthesis of histidine in Salmonella has been suggested (10); Jacob and Monod's operon hypothesis for E. coli is well known (39). Regulators have also been suggested for Drosophila (19).

It is noted that a number of the points have been suggested and confirmed by other workers for other organisms; in those cases this work is another confirmation and an extension of the general findings.

SUMMARY

1. $\underline{a}_1$, $\underline{a}_2$, $\underline{c}_1\underline{a}_1$ (germinated in light), and $\underline{c}_1$ (germinated in light) accumulate quercetin with traces of kaempferol; $\underline{a}_2$ $\underline{pr}$, $\underline{c}_1\underline{pr}$ (germinated in light), and $\underline{pr}$ accumulate kaempferol with traces of quercetin. These are glycosylated in the natural state.

2. A compound was isolated, but not identified, from $\underline{bz}_1$ aleurone. $\underline{c}_1\underline{bz}_1$ when germinated in light produces the $\underline{bz}_1$ compound.

3. Approximately equal amounts of pigment are produced in both $\underline{Pr}$ and $\underline{pr}$ aleurone. Other genes, however, such as $\underline{R}$ and $\underline{C}_1$ give rise to significantly reduced quantities of end product if some of the alleles are recessive, for example, $\underline{Rrr}$.

4. Dihydroquercetin will replace the $\underline{c}_2$ mutant in the flavonoid system in tissue cultures.

5. $\underline{a}_1$ and $\underline{a}_2$ aleurone will supply material to activate $\underline{c}_2$ aleurone in cross-feeding tests.

6. $\underline{c}_1\underline{r}_2\underline{R}_{10}$ seeds germinated in light will become fully pigmented; however, $\underline{c}_1\underline{r}_{10}\underline{R}_2$ will form only a few spots of pigment. The aleurone from the developing kernels shows approximately the same amount of pigment in both tissues.

7. The ratio of pelargonidin to cyanidin in $\underline{c}_1$ stock germinated in light is approximately eleven times greater than the ratio in normal ($\underline{C}_1$) stock.

8. A number of different wavelengths of light in the range 400 to 800 mμ appear to be involved with the production of anthocyanin in the aleurone of $\underline{c}_1$ germinating kernels. The role of phytochrome is discussed.

9. Different mechanical manipulations of $\underline{c}_1$ seed, for example, embryo removal were found to increase the rate of anthocyanin synthesis in $\underline{c}_1$ tissue germinated in light.

10. The location of pigment within the aleurone cells and the pigmenting of only certain cells was noted.

11. The positions of a number of genes on the flavonoid path in maize aleurone have been suggested.

12. It was found that reflectance spectroscopy could be used in identifying micro-amounts of flavonoids without eluting from the cellulose chromatograms.

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APPENDIX I

CALCULATION OF THE AMOUNT OF PIGMENT EXTRACTED FROM VARIOUS GENOTYPES

The calculations have been made according to the following equation:

$$M = \frac{A}{El}$$

M = Molarity
A = Absorbance
E = Molar extinction coefficient
l = path length

Genotype	Pigment	M.W.	E[1.]	A	No. of Seeds	Mg. of Pigment
$\underline{a_1}$	quercetin	302	21,800	1.54	25	.021
$\underline{c_1 pr}$ + light	kaempferol	286	22,100	1.61	25	.021
$\underline{Pr}$	cyanidin (Cl)	323	31,200	17.4	25	.18
$\underline{pr}$	pelargonidin(Cl)	307	31,600	18.3	25	.18

Note: The volume of solution used in each case was 1 ml.

The path length is constant at 1 cm.

1. The E values are from Harborne (16) and Geissman (12).

APPENDIX II

CHEMICAL STANDARDS

Precursor Experiments

Compound	Control No.	Source[1.]	M.W.	Solubility
Dopa (L-Dihydroxphenylalanine)	8555	NBC	197.2	
D-Catechin (Flavan)	7242	K-K	362.3	
Dihydroquercetin (Flavanonol)	3990	K-K	302	
D-L-Phenylalanine	3062460	BDH	165.2	
Chalcone	1254	EOC	208.3	poor
Flavanone	10,203-2	ACC	224.3	fair
Cinnamic Acid	57B-2380	SCC	148.2	
Naringenin (Flavanone)	95B-0500	SCC	272.2	fair
Caffeic Acid	98B-2210	SCC	180.2	
Shikimic Acid	127B-1570	SCC	174.2	
L-Cystine	16B-0830	SCC	240.3	poor
L-Aspartic Acid	106B-0560	SCC	133.1	
L-Phenylalanine	66B-0400	SCC	165.2	
Riboflavin (Vit B_2)	16B-0080	SCC	376.4	
Naringin (Flavone-glycoside)	66B-2350	SCC	580.5	fair

Chromatography Experiments

Compound	Control No.	Source	M.W.
Flavone (2 Phenylchrome)	8093	NBC	222.2
Delphinidin Chloride	25575	CAL	338.7
Quercitrin (Flavonol-glycoside)	S1606	MRL	448
Kaempferol (Flavonol)	S2892	MRL	286
Cyanidin Chloride	R3042	MRL	323
Pelargonidin Chloride	S2419	MRL	307
Quercetrin (Flavonol)	P1635	EOC	302.2
Apigenin (Flavone)	A9185	ACC	270.2

1. NBC = Nutritional Biochemical Corporation
   K-K = K and K Laboratories Inc.
   BDH = British Drug Houses Ltd.
   EOC = Eastman Organic Chemicals
   ACC = Aldrich Chemical Co. Inc.
   SCC = Sigma Chemical Company
   CAL = Calbiochem.
   MRL = Mann Research Laboratories.

APPENDIX III

SP-700 REFLECTANCE WORK

Since only micro-amounts of material are often available for analysis from systems such as the aleurone layer of maize it was decided to undertake some reflectance work on flavonoid isolations from this layer. The reflectance procedure enables a spectrum of the isolated flavonoid to be run without having to elute the compound from the chromatogram and thus losing some of the material.

The chromatogram (TLC) is run as usual on cellulose but the isolated compound is cut from the sheet along with a section of cellulose on which only the solvent is present (this is used for a reference). The reference is placed in one side of the reflectance device and the sample compound in the other side. A spectrum is then run. The data listed on the next page arerepresentative of materials run in this way. Note that the peaks are approximately 10 units higher than those obtained for solution spectra - these values compare very favourably with those outlined by Roux (30) who did similar work using paper.

Very little was done on quantitative measurements, indeed, the method does not lend itself to accurate quantitative data as is obvious from the procedure (21).

In summary, this method is useful if the amount of material isolated is so small that elution and solution spectra would not be possible. The technique has the disadvantages of (i) being more difficult to set up, that is, to cut the cellulose, fit the machine, and adjust the machine, and (ii) being much less accurate for quantitative data than solution procedures.

APPENDIX III (continued)

Reflectance Data

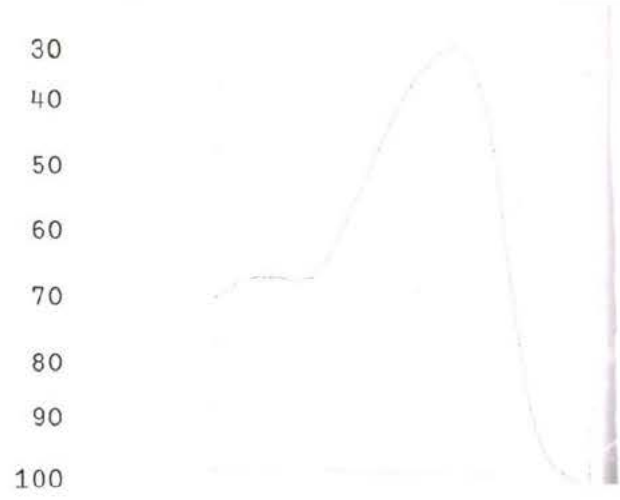
Stock-Standards	λ. max.cm^{-1}	λ max.mμ
cyanidin chloride	18,350	546
	18,375;35,825	545;279
	18,350	545
pelargonidin chloride	18,950	529
	18,900;36,650	530;273
	18,850	531
delphinidin chloride	18,100	553
	18,000	556
flavone	33,700	297
kaempferol	26,450;36,750	378;272
flavanone	30,600;38,000	327;264
D-catechin	35,500	282
c_1 + light (cyanidin)	18,250	548
Pr	18,250	548

Spectral Data from Various Sources

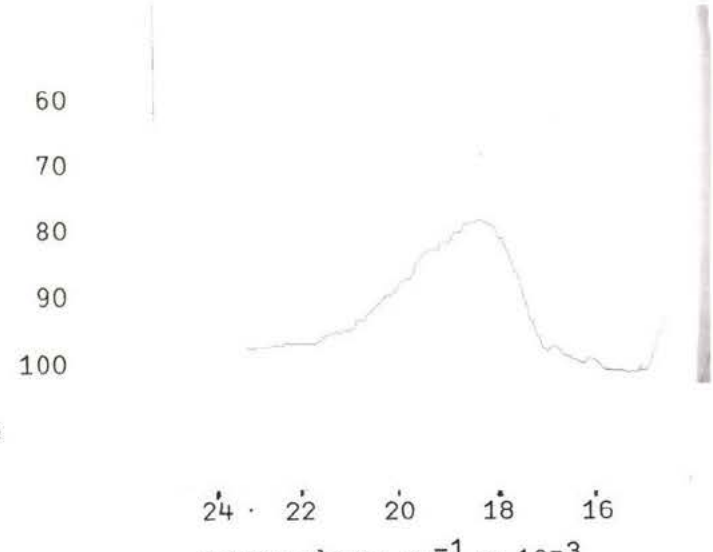
Compound	λ. max.mμ(16)	λ max.mμ(30)
cyanidin chloride	535, 277	545
pelargonidin chloride	520, 270	530
delphinidin chloride	546, 277	555
D-catechin	280	
flavanone	320, 250	
kaempferol	368, 268	
flavone	297	

APPENDIX III (continued)

Examples of Spectra from the SP 700 Reflectance Accessory



Reflectance spectrum of cyanidin chloride on cellulose



Reflectance spectrum of cyanidin (chloride), on cellulose, from $\underline{c_1}$ tissue germinated in light.

APPENDIX IV

DETAILS OF RF VALUES AND SPECTRAL DATA FROM TABLES II AND III

Details of RF values from Table II

Material	RF 30% A.A.		RF FA-4M HCl		RF Forestal	
quercetin	.11		.15		.49	
	.13		.17		.48	
	.12		.16		.46	
	.12				.48	
kaempferol	.17		.25		.64	
	.20		.28		.67	
	.21		.26(5)		.64	
	.19				.65	
$\underline{a}_1$	.13		.15		.46	
	.12		.16		.46	
	.11		.15(5)		.46	
	.10					
	.11(5)					
$\underline{a}_2$	.13		.16		.47	
	.13		.15		.48	
	.13		.15(5)		.47(5)	
$\underline{a}_2\underline{pr}$	.17		.26		.63	
	.15		.24		.61	
	.16		.25		.62	
	.16					
$\underline{c}_1\underline{a}_1$ + light	.18	.13	.26	.16		
	.16	.12	.24	.16		
	.17	.12(5)	.25	.16		
$\underline{c}_1\underline{pr}$ + light			.16	.27	.54	.67
			.16	.25	.50	.69
			.15	.28	.52	.66
			.16	.27	.52	.67
$\underline{pr}$				.23		.65
				.23		.63
				.23		.64
$\underline{c}_1$ + light			.14	.24	.47	.66
			.16	.24	.45	.62
			.15	.24	.46	.64

NOTE: The variation in RF values can be greater than those shown - this was checked by noting the variations of RF with concentration. The applications for the standards were kept at levels which gave reasonably consistent results as the data indicate.

APPENDIX IV (continued)

Details of RF Values from Table III

Material	RF FA-4M HCl	Material	RF FA-4M HCl
c_1 + light (magenta)	.42 .41 .44 .42 .43 .43	c_1 + light (orange-pink)	.58 .57 .55 .60 .57 .57
PrPrPr (magenta)	.43	PrPrPr (orange-pink)	.58
PrPrPr (magenta)	.45	PrPrpr (orange-pink)	.59
Prprpr (magenta)	.45	Prprpr (orange-pink)	.57
prprpr (magenta)	.43 .44	prprpr (orange-pink)	.60 .58(5)
b_1 (magenta)	.41	$b_1$pr (orange-pink)	.57
cyanidin chloride (magenta)	.44 .44 .44	pelargonidin chloride (orange-pink)	.59 .59 .59

Details of Spectral Peaks from Table II

Stock	Spectral Peaks (mμ)	Stock	Spectral Peaks (mμ)
a_1	373 372 - 258 372 - 256 372 - 257 373 257	a_1 + R	383 - 260 385 - 261 384 - 260 384 260
$a_2$pr	369 - 270 367 367 - 270 368 270	$a_2$pr + R	367 - 270 368 - 270 368 368 270
a_2	372 - 258 373 - 258 372(5)258	a_2 + R	384 - 260 385 - 260 384(5)260
$c_1$pr (yellow)	368 - 269 368 - 269 367 - 268 368 269	$c_1$pr (yellow) + R	368 - 268 367 - 268 368 - 269 368 268
quercetin	372 - 257 374 - 257 373 - 257 372 257	quercetin + R	386 - 260 384 - 260 385 - 259 385 260

APPENDIX IV (continued)

Stock	Spectral Peaks (mμ)	Stock	Spectral Peaks (mμ)
kaempferol	369 - 267	kaempferol + R	369 - 267
	368 - 268		368 - 268
	368 - 268		368 - 268
	368 268		368 268

Note: 1. All solvents were 95% EtOH

2. R = a few drops of H_3BO_3/NaOAc.

Details of Spectral Peaks from Table III

Stock	Spectral Peaks (mμ)	Stock	Spectral Peaks (mμ)
Pr (magenta)	535	Pr + $AlCl_3$ (magenta)	546
	538		548
	536		549
	536		548
	537		
	536		
	536		
Pr (orange-pink)	522	Pr + $AlCl_3$ (orange-pink)	523
	524		522
	524		525
	523		522
	525		523
	524		
pr (orange-pink)		pr + $AlCl_3$ (orange-pink)	523
	522		523
	522		522
	523		523
	524		
	524		
	523		
	523		
pr (magenta)		pr + $AlCl_3$ (orange-pink)	547
	536		546
	535		548
	535		547
	535		

Note: All solvents were MeOH-HCl (.01% concentrated HCl).

APPENDIX IV (continued)

Spectral Data from Harborne (16)

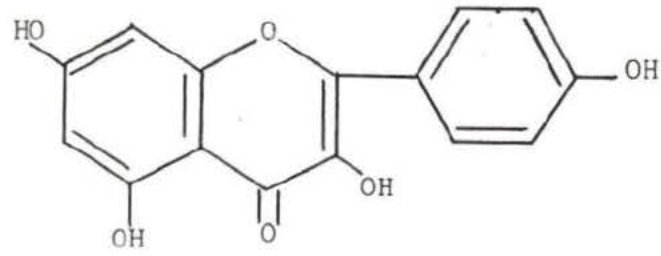
Material	λ max. mμ	Solvent
quercetin	374, 255	95% EtOH
kaempferol	368, 268	95% EtOH
cyanidin	535, 277	MeOH-HCl (.01 con. HCl)
pelargonidin	520, 270	MeOH-HCl (.01% con. HCl)
cyanidin + $AlCl_3$	553	MeOH-HCl (.01% con. HCl)
pelargonidin + $AlCl_3$	520	MeOH-HCl (.01% con. HCl)

Spectral Data from Geissman (12)

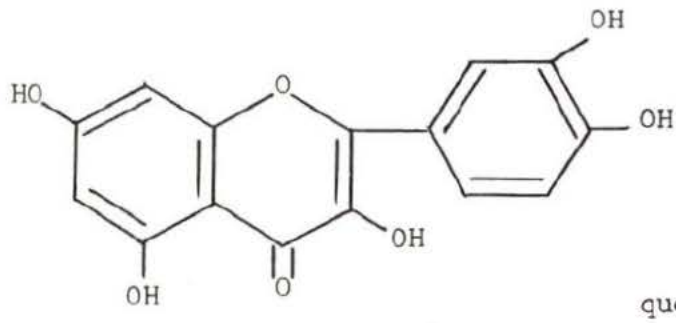
Material	λ max. mμ	Solvent
quercetin	371	95% EtOH
quercetin + H_3BO_3/NaOAc	389	95% EtOH
kaempferol	367.5	95% EtOH
Kaempferol + H_3BO_3/NaOAc	367.5	95% EtOH

APPENDIX V

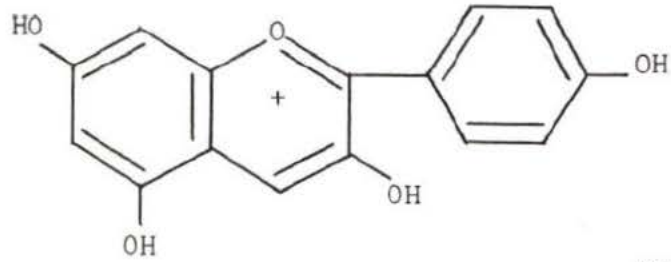
FORMULAS OF SOME FLAVONOIDS



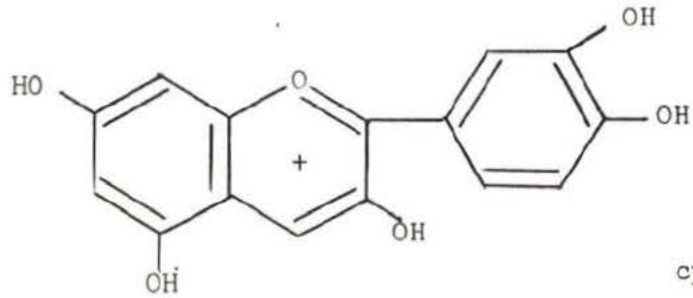
kaempferol



quercetin



perargonidin



cyanidin

APPENDIX VI

TISSUE MATURITY

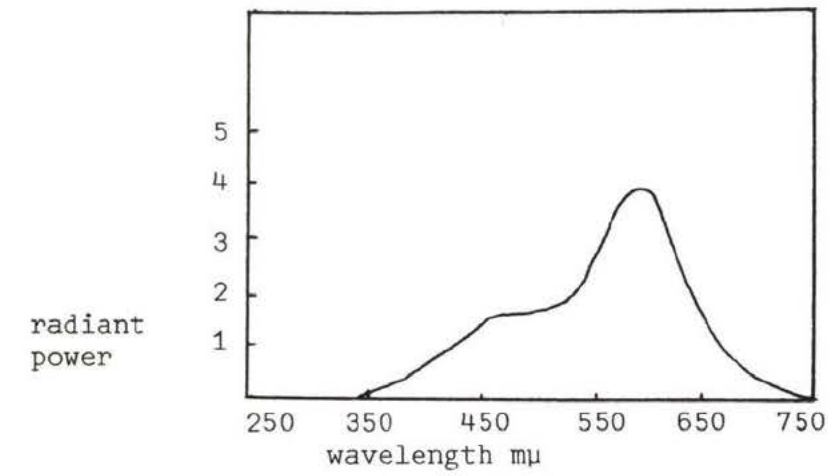
Most of the stock used in the experiments in this thesis were grown under the same environmental conditions in a greenhouse; days from pollination can, therefore, be used when comparing tissues. This, however, is not reliable when comparisons are to be made of stocks grown under different conditions as is shown between greenhouse and field grown material. It was observed that stock from the same source, and pollinated at the same time, showed as much as 15 days difference in kernel maturity depending on whether the material was field or greenhouse grown.

To overcome this problem, a short description should be given regarding the characteristics of the seed as well as the age. For example, the aleurone of the seed over 38 days from pollination used in these experiments was hard and it was almost impossible to peel it from the underlying endosperm. The material under 18 days was extremely soft and milky and again it was almost impossible to remove the aleurone.

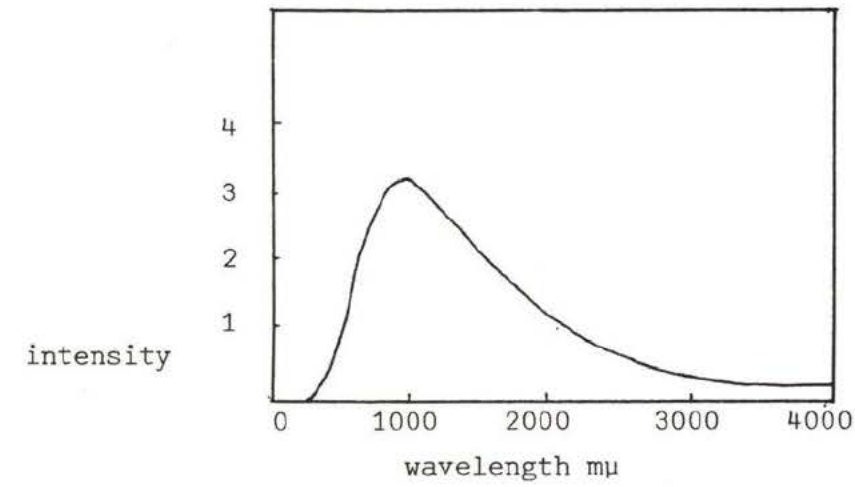
APPENDIX VII

SPECTRA FROM FLUORESCENT AND TUNGSTEN LAMPS

Cool White Fluorescent Lamp Spectrum[1].



Tungsten Lamp Spectrum[2].



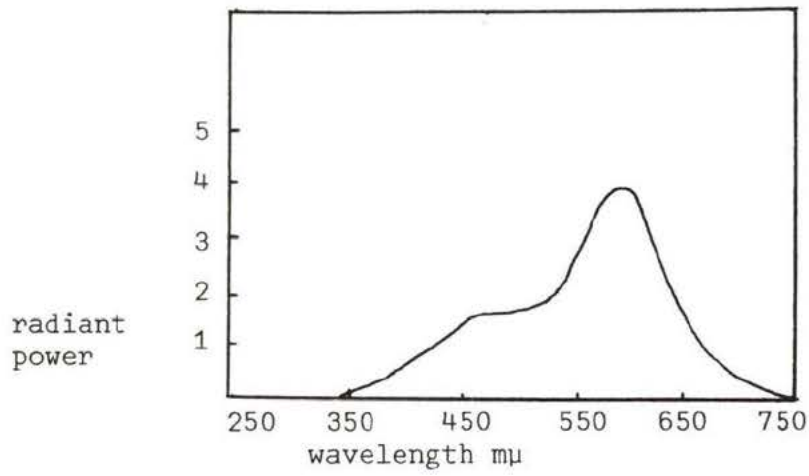
1. From The Fluorescent Lamps Booklet, 1968. The General Electric Company.

2. From Matter Energy and Radiation by J.R. Dunning and H.C. Paxton, 1941. McGraw-Hill Book Company, New York. 668 pp.

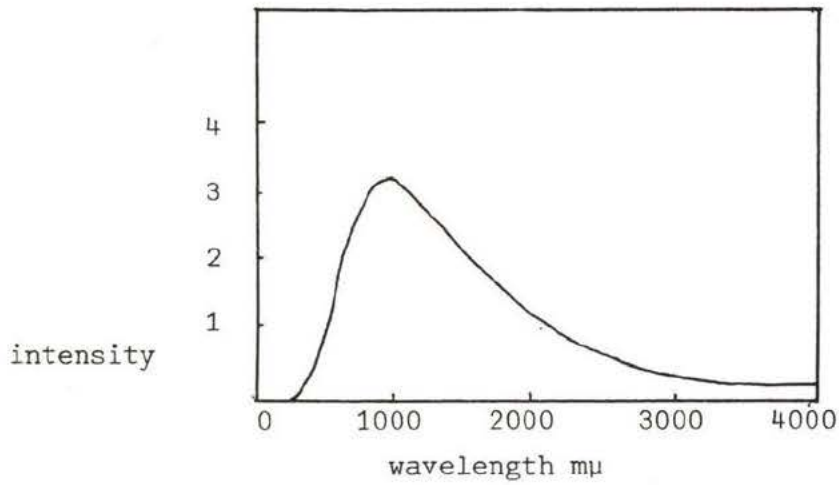
APPENDIX VII

SPECTRA FROM FLUORESCENT AND TUNGSTEN LAMPS

Cool White Fluorescent Lamp Spectrum[1].



Tungsten Lamp Spectrum[2].



1. From The Fluorescent Lamps Booklet, 1968. The General Electric Company.

2. From Matter Energy and Radiation by J.R. Dunning and H.C. Paxton, 1941. McGraw-Hill Book Company, New York. 668 pp.

VITA

Surname: KIRBY Given Names: LAWRENCE, THOMAS

Place of Birth: TORONTO, ONT. Date of Birth: JULY 31, 1938.

Educational Institutions Attended, with Dates of Entering and Leaving:

UNIVERSITY OF TORONTO, FACULTY OF FORESTRY 1959 to 1963

UNIVERSITY OF TORONTO, COLLEGE OF EDUCATION 1965 to 1966

Degrees, Diplomas, Etc., Awarded, with Dates and Names of Institutions:

B.Sc.F 1963 University of Toronto

Teaching Certificate 1966 University of Toronto

Honors and Awards:

1960 - Awarded the White Pine Bureau Scholarship

1961 - Awarded the Harold S. Edmonds Prize, and the Spruce Falls Power and Paper Co. Ltd. Scholarships (Mathematical and Biological)

1962 - Awarded the F.K. Morrow Scholarship, the R.P. Wright Scholarship and the Spruce Falls Power and Paper Co. Ltd. Scholarships (Mathematical and Biological)

1963 - Awarded the C.I.F. Gold Medal, the P.D. Leslie Scholarship and the Osmose Wood Preserving Scholarship.

1968 - Awarded a National Research Council Scholarship

1969 - Awarded a National Research Council Scholarship.

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