1963

An experimental investigation of coloring matter in flowers of vinca major

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AN EXPERIMENTAL INVESTIGATION OF COLORING MATTER IN FLOWERS OF VINCA MAJOR

A Thesis

Presented to

The Faculty of the Department of Chemistry

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

in the

Department of Chemistry

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by

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June 1963

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Dated June 8, 1963

ACKNOWLEDGMENT

The writer wishes to express her appreciation to the members of the faculty of the Chemistry Department of the University of the Pacific for their assistance during the period of study and especially to express her gratitude to Dr. Emerson G. Cobb for his guidance and criticism in this work.

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INTRODUCTION

Even though early man did not know the nature of coloring matter in flowers, he used the coloring matter as dye for centuries. There are a variety of reasons for the interest of chemists in natural and artificial coloring matters. One of these is the color pleasure, another is the commercial importance. The visible color facilitates the experimental work in the search for methods of separation, purification and determination of organic structures.

Search for the knowledge of coloring matter goes far back in history. Man has dyed his textiles with the help of mordants from the most ancient times up to the time of Perkin's discovery of mauve. Then a new era in dyestuff chemistry commenced. In the first half of the nineteenth century with the rise and development of the study of Organic chemistry much attention was directed to the extraction and characterization of pure coloring matters from natural sources--usually the bark, leaves, fruits, or sap of trees or plants. During the twentieth century--and just a little before that--great progress has been made in the synthesis of many typical natural coloring matters and the improved technique and novel synthetical methods which will bear upon the general progress of chemistry.

This research was done with the purpose of isolating and identifying the coloring matter in the flowers of Vinca Major, with

the common name of periwinkle. This was done by extracting the pigment of the flowers with methanol, amyl alcohol, and crystallizing the pigment as a chloride. For this pigment, solubility, color reactions, spectra and other physical properties were obtained. [1]

PLANT COLORING MATTERS

It would be very difficult to describe the pigments which are responsible for all the varied color effects in plants, flowers, fruits, leaves, etc. The anthocyans are an important class of soluble or sap pigments and present a great interest. The anthocyan pigments create some of the most beautiful color effects in flowers. [1]

The term "anthocyan" is derived from the Greek roots signifying respectively "flower" and "blue." It was introduced by the botanist Marquart in 1835 to designate the blue pigments of flowers. Shortly thereafter the belief developed that the red and blue pigments were merely different forms of the same substance and their different colors were due to variations in the character of the cell sap; consequently, the use of the term was extended to include all the soluble pigments of this group. After it was found out that these pigments were always combined with sugars and thus occurred as glycosides, the ending "in" was attached. [6] As from time to time evidence accumulated to show that red, purple, and blue pigments differed among themselves, the present use of the term anthocyan gradually became to designate a large class of naturally occurring plant pigments. In 1836 Hope, as a result of some extensive study and experiments, concluded that by a variety of changes the pigments, or chromules present were formed from faintly colored chromogenes.

According to him there were two types of chromogens: Erythrogen and Xanthogen.

Next year, 1837, Berzelius suggested the name Erythrophyll for red leaf pigments. He was not successful in his investigations but his method for precipitation and regeneration was used later on and successful results received. Berzelius did not think that all these pigments could be considered as the same blue substance changed by variation in the cell sap.

In 1849-50 Morot unsuccessfully attempted to synthesize the blue pigment of the cornflower by repeated precipitation of its aqueous solutions by means of alcohol. This method with newer equipment was later on used by Willstatter and Everest with good results.

Fremy and Cloez decided that there was no relationship between chlorophyll and the blue and yellow pigments. They suggested that all anthocyans were one and the same substance which they called cyanin, and that the color variations were due to the properties of the particular plant sap. Filhol, 1854, with some more experiments agreed with them.

In 1855 Martens decided that yellow and red pigments came from a faintly yellow substance produced in the sap of all plants which by oxidation produces the different yellow pigments from which

by further action of light and oxygen the red pigments were formed.

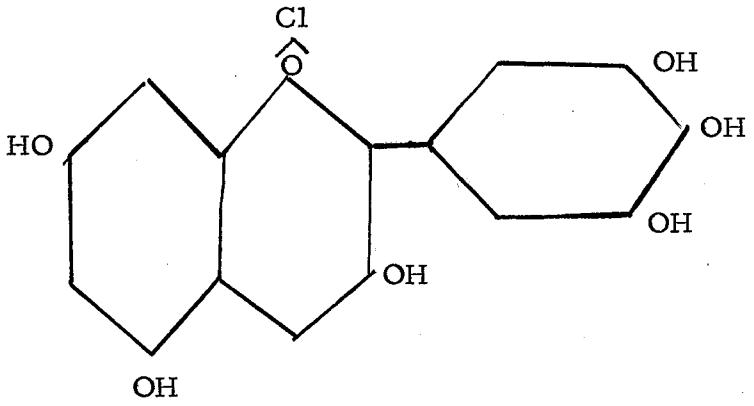
Morren, 1859, said that blue flower pigments were the alkali salts of acids which in the free state are red.

In 1858 Glenard, in 1892 Glan, and in 1894 Heise tried to prepare pure anthocyan pigments. Molisch, 1905, and later on Grafe (1906, 1909, 1911) considered the question of glucosides. In 1912, 1913 Keeble, Armstrong and Jones published papers about the formation of anthocyans and agreed with Miss Wheldale's assumptions (1911).

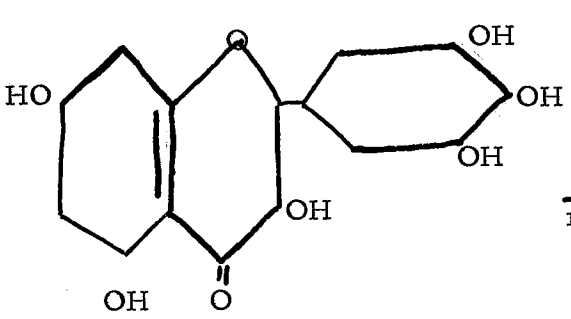
In 1913 Willstatter and Everest came to some important conclusions. It was proved that the blue form of corn flower pigment was a potassium salt, the free compound being violet in color, whereas the red form was not this latter, but an oxonium salt in which the pigment was combined with an equivalent of some mineral or plant acid. The anthocyans were found to be most stable when in the form of these oxonium salts. It was also proved that the decolorization in solution, so often mentioned by other workers, was not due to reduction. They proved that the cornflower pigment was a disaccharide and upon hydrolysis two molecules of glucose were split off from each molecule of pigment.

To prevent confusion these authors proposed the terms anthocyanins and anthocyanidins for the glucoside and non-glucoside pigments respectively, and in agreement with this assigned to the glucoside present in the corn flower the name introduced by Fremy and Cloez: cyanin, whereas to the sugar-free pigment obtained by hydrolysis the name cyadin was given. [10]

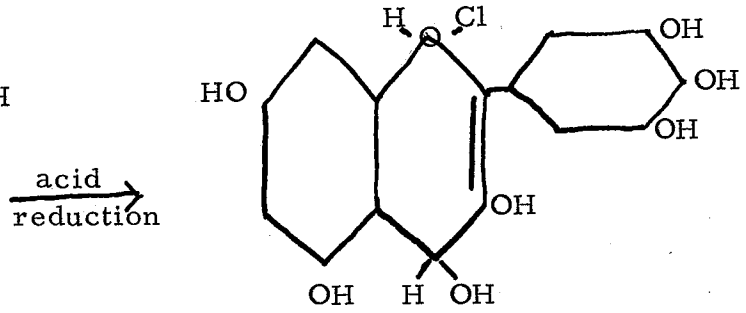
The structural formula now generally accepted for the anthocyans was suggested by Everest in 1914:



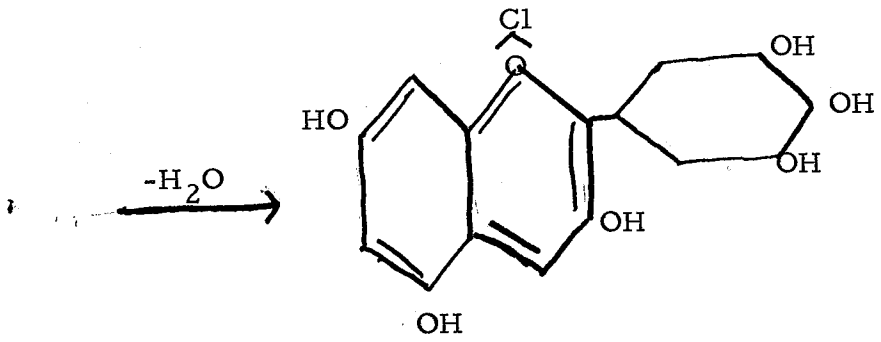
And the passage from a flavonol pigment to one of anthocyan series:



(1) flavonol derivative



(2) colorless or faintly colored imtermediate



(3) typical anthocyan

COLORING MATTER IN FLOWERS

According to their solubilities and to the manner in which they occur in the plant, pigments of the chlorophyll bearing plants may be classified as:

I. Ether soluble or plastid pigments

  A. Chlorophyll (green)

  B. Carotenoids (orange)

    1. Carotenes

    2. Xanthophylls and Xanthophyl derivatives

II. Water soluble or vacuolar pigments

  A. Anthocyanins (red, blue, purple)

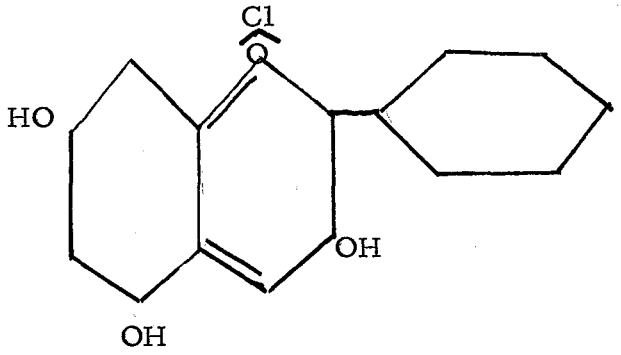
  B. Anthoxanthins (yellow)[8]

The anthocyanins, which are usually dissolved in the cell sap but in some cases occur in an amorphous or crystalline state in the plant, comprise a group of glycosidic pigments responsible for the innumerable shades of blue, purple, mauve, maroon, magenta and various shades of red and pink found in flowers, fruits and stem and leaves of plants. The aglycones, the sugar-free substances, are the anthocyanidins and all are found to be variations of a relatively simple structure differing in the state of hydroxylation, oxidation or methylation and in the case of the anthocyanins, in the position of the sugar residue or residues.[2]

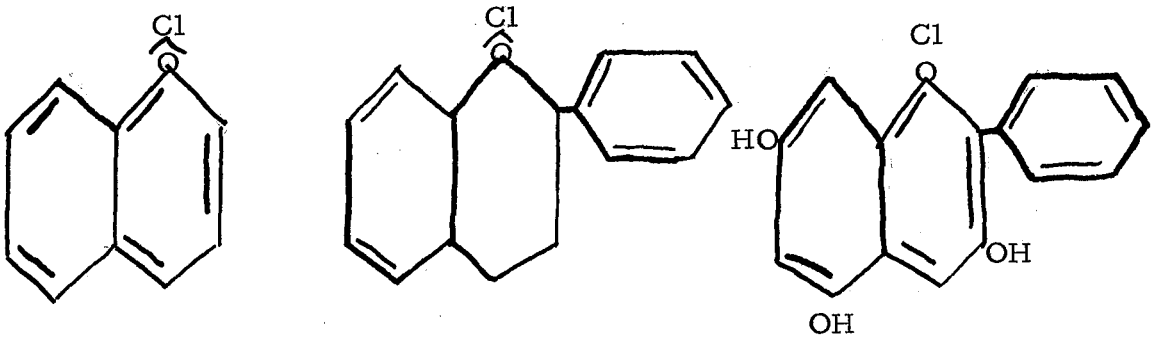
Anthoxanthins are other water soluble plant pigments related structurally to the anthocyanins. These yellow or orange pigments are derivatives of flavone. Anthoxanthins produce only pale yellow colors and are therefore not as conspicuous as anthocyanins. Often the anthoxanthins occur in colorless form, as in white flowers. By treatment with alkalies, the colorless compounds may be converted to a yellow color. [5] If a red flower turns green when treated with alkali, it indicates that anthoxanthins too are present.

Anthoxanthins were once very important as commercial dyes but coal tar dyes have now largely replaced them.

The result of research upon naturally occurring coloring matters has been that the anthocyans are derivatives of the benzopyranol complex. All the products of this group as yet investigated are derived from the following nucleus by the introduction of further hydroxyl groups.



By substituting a phenyl residue in position 2 of the benzopyrylium chloride (I), 2 phenyl benzopyrylium chloride or flavylium chloride (II) is obtained. The placement of hydroxyl groups in positions 3, 5, and 7 yields 3, 5, 7 trihydroxy-flavylium chloride (III). The simplest intact structural unit of anthocyanins:

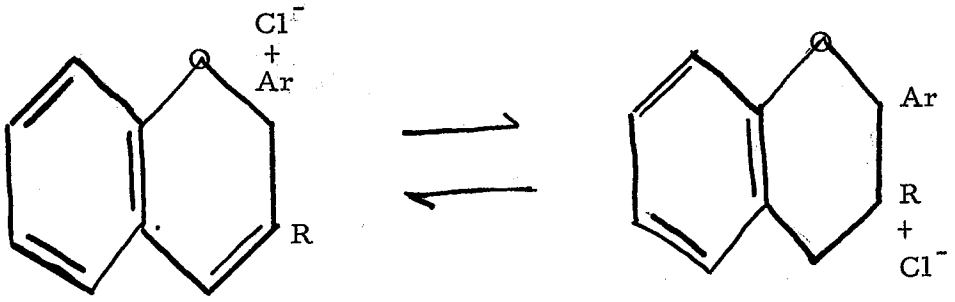


I. Benzopyrylium chloride

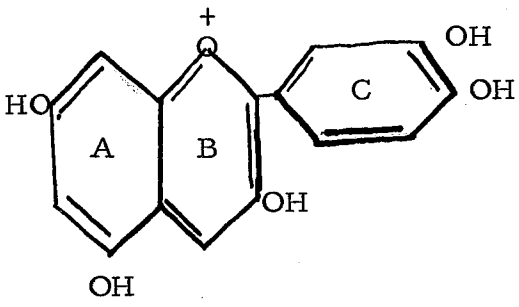
II. 2 phenyl benzopyrylium chloride

III. 3, 5, 7 trihydroxy flavylium chloride

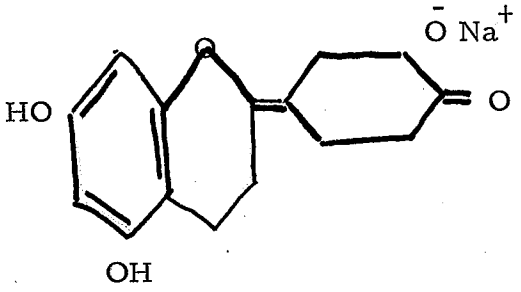
Shriner and Moffett present evidence against the above quinoid oxonium salt structure. They suggest that carbon 2, 3, 4 of the heterocyclic ring constitute a mobile allylic system through which the flavylium salts may tautomerize or resonate between structures I and II:



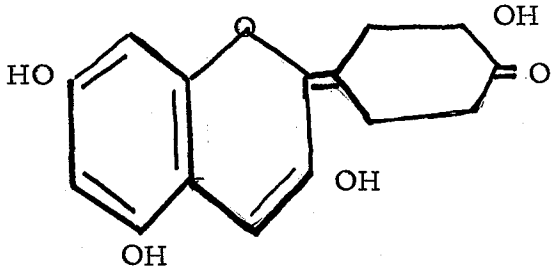
All anthocyanins contain the 2 phenyl benzopyrylium (flavylium) ion and are derivatives 3, 5, 7-trihydroxyflavylium hydroxide. The assignment of the positive charge to the oxygen atom in the oxonium salt shown below is arbitrary since the flavylium ion is a resonance hybrid.



oxonium salt in acid solution (red)



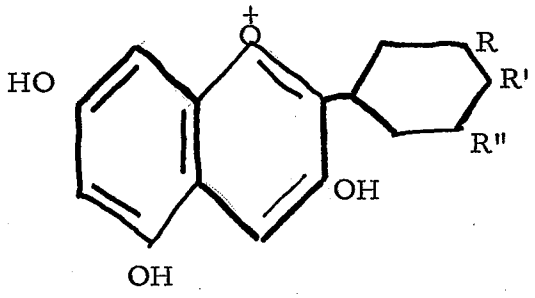
salt of basic quinone in alkaline solution (blue)



free base in neutral solution (violet)

On the basis of the extent of substitution in ring C, three main groups of anthocyanidins are differentiated. A great variety of anthocyanins may exist, since in the anthocyanidin moiety, the number of hydroxyl groups may vary from 4 to 6, and any number of these hydroxyls may be methylated. The number of carbohydrate units in the glycosides may be either 1 (in which case it is generally linked to the 3-hydroxyl of the aglycone) or 2 (linked to the hydroxyls at the 3 and 5 positions of the anthocyanidin.[5]

Anthocyanidins:



	R	R'	R''
Pelargonidin	H	OH	OH
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Peonidin	OCH_3	OH	H
Malvinidin	OCH_3	OH	OCH_3

Anthocyanins and anthoxanthins are similar in structure, both being glycosides. The non-sugar portion of the glycoside molecule

accounts for the color. The sugar molecules split off and the color of the solution is usually intensified when these glycosides are heated with hydrochloric acid. The aglycone portion of these pigments consists of one pyrone and two benzene rings. They are therefore constructed from flavones, flavonols or flavonones. [8]

According to R. Robinson, anthocyanins, flavones, flavonols, and related substances are all formed from C_6-C_3-C_6 structural framework, since the aglycon portion of the mentioned glycosides consists of 15 carbon atoms. The two C_6 rings (A and C) are phenol in nature. Ring A usually occurs as phloroglucinol and ring C as catechol. (See oxonium salt diagram on page 11.) It is assumed in this theory that both rings are built of hexoses and are linked together by a triose by means of aldol condensation, with the formation of a hypothetical intermediate. It is pointed out that substances with a C_6-C_3 structural framework are of wide occurrence in plants. Typical examples are coniferyl alcohol and eugenol.

Anthocyanins are glucosides. The sugar components are attached to the hydroxyl groups of the benzopyrilium nucleus. Mono and diglucosides and mixed diglucosides may occur. A large variety of sugars may be involved; therefore a great variety of anthocyanins may be derived from each anthocyanidin. [8] The glucosides are formed from the anthocyanidins and either dextrose, rhamnose or galactose.

Dextrose is the principal sugar found. Galactose occurs only in a single instance. The wide variety of the anthocyans is dependent upon variations in the nature and number of the sugar residues and the difference in their position in the complex molecule--in their points of attachment to the anthocyanidin nuclei. [1]

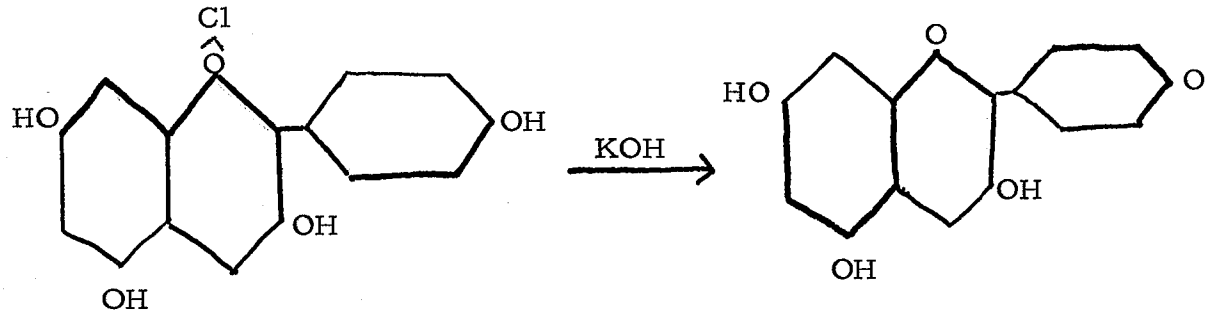
Anthocyanins have amphoteric nature. They are capable of forming salts with both acids and bases: acid salts, oxonium salts, flavilium salts.

Anthocyanins are soluble in water and hydroxylic solvents but they are insoluble in such non-hydroxylic solvents as ether, benzene or chloroform. [6]

In the presence of acids or alkalies, anthocyanins may serve as pH indicators. If no interfering substances are present, anthocyanins will appear bluish red in acid, violet in neutral solution and blue in alkaline solution. [8] The pigment of the blue corn flower and the rose, cyanin for example, is red in solutions of pH-3.0 or less, violet at pH-8.5 and blue at pH-11.0. [6]

Anthocyanins, on boiling with acids, decompose into a sugar and anthocyanidins which are of the nature of pyrilium salts. [7] The salts of anthocyanidins with acids all have a more or less red color; pelargonidin--a yellowish red, cyanidin--red tinged with violet, delphinidin--blue red. The free anthocyanidins, which are formed

from the salts by the addition of the calculated amount of alkali and which very probably have a quinonoid structure in the benzene nucleus, are violet to blue. The alkali salts of the anthocyanidins, phenates, are blue.



However, the blue phase of an anthocyanin does not necessarily denote an alkali medium. Recent evidence (1933) indicates that the anthocyanin may be colloidal when blue. The most important factor in flower color for a given anthocyanin is condition rather than the pH of the medium. [12]

Factors affecting the colors of the anthocyanin pigments are (1) nature and concentration of the anthocyanins and other colored substances present; (2) state of aggregation of the anthocyanin in solution, which is determined in part by the pH of the cell sap and the presence or the absence of protective colloids of the polysaccharide groups (the pentosans); (3) presence or absence of co-pigments (the tannin and flavone glycosides) and possibly the effect of alkaloids, of

traces of iron and other metals that form complex combinations; (4) colloidal condition of the pigment. [8]

Stability of certain anthocyanins and anthocyanidins in the presence of dilute solutions of $FeCl_3$:

In all cases the substances which have an OH group in position 3 rapidly lose their color, and the substances which have OH groups in position 3 absent or modified have relative stability. [11]

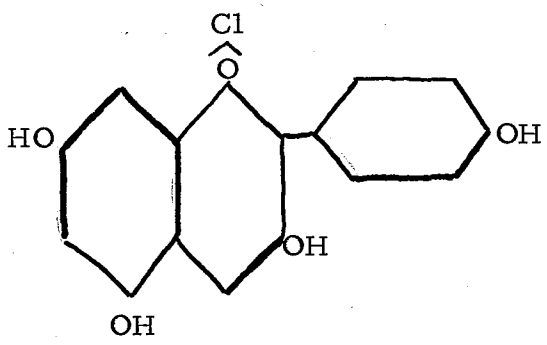
According to G. M. Robinson and R. Robinson, the co-pigments with the anthocyanins in solution break up the associated complexes. Natural co-pigments, more readily soluble in water than in amyl alcohol, behave similarly and greatly reduce the distribution number.

The relation between co-pigment effects and distribution properties: If an anthocyanin (m. mg.) is distributed between V ml, each of equilibrated isoamyl alcohol and 0. 5% HCl, D being the distribution number (% passing to the isoamyl alcohol), then if the anthocyanin is associated to double molecules in the aqueous layer, remaining unassociated in the isoamyl alcohol, we have

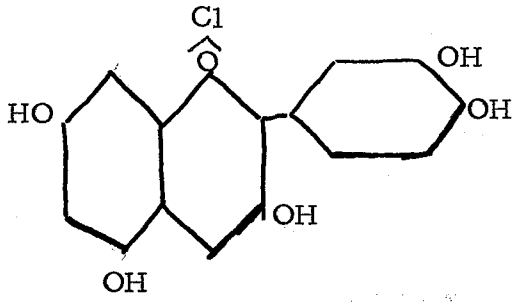
$$mD^2 + kVD - 100\,kV = 0$$

in which m and D are variables under standard experimental conditions. The condition laid down is satisfied if the curve log C_w/log C_{aa} is linear with a slope of 2. Knowing D for some particular value of m, well inside the region of agreement with the formula, it is possible to find the concentration at which the double molecules break up in the aqueous solution to a significant extent.[13]

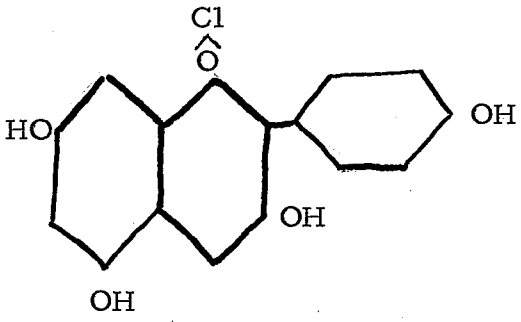
At present there are four types of anthocyanidins which are recognized: pelargonidin, cyanidin, delphinidin, and apigenin--neutral formulation.



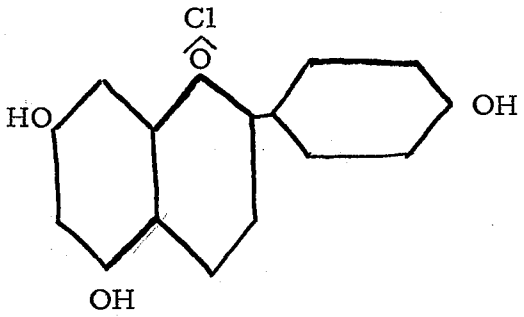
pelargonidin chloride



cyanidin chloride



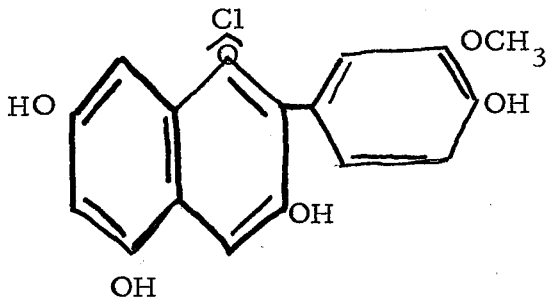
delphinidin chloride



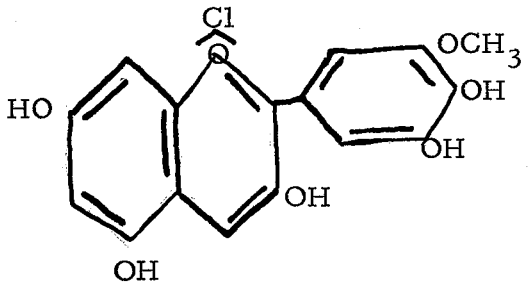
apigenidin chloride
(gesneridin)

Since anthocyanins are commonly isolated as chloride salts, it is customary to write the flavylium ion in the form of its chloride.

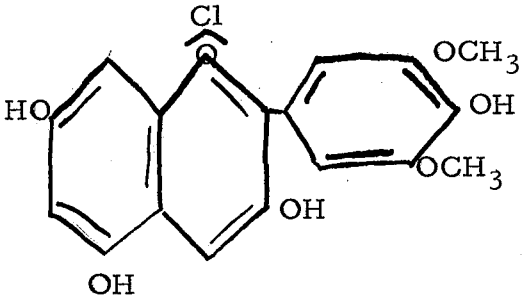
From these three anthocyanidins the following methoxylated anthocyanidins may be derived:



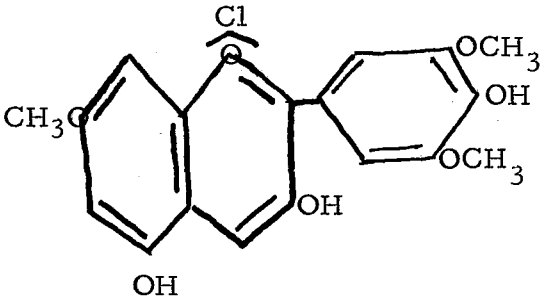
peonidin



petunidin



malvidin

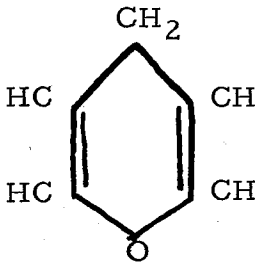


hirsutidin

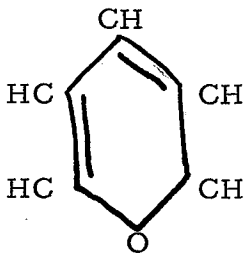
The flavones are yellow plant pigments which occur naturally as glycosides, usually of glucose or rhamnose. They are also known in this form as anthoxanthins.[2] Flavones have high melting points

and are soluble in water, alcohol, dilute mineral acids and alkalies. They may be precipitated by lead acetate from their solutions, the precipitate being yellow, orange, or red. With ferric chloride a dull green or sometimes a red brown coordination results. The solubility of the flavones in acids is due to the basic properties of the oxygen atom in the -pyron nucleus. The oxygen atom by becoming tetravalent can form additive compounds with acids producing oxonium salts. The salts are more highly colored than the bases from which they are derived and are generally very unstable in the presence of water. The flavones differ in this respect from the anthocyanidins which yield oxonium salts and frequently occur as such in the plant. [1]

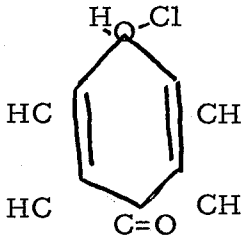
From γ-pyran and α-pyran are derived the α and γ pyrons, also salt-like compounds which are called pyryllium or pyroxonium compounds. In these compounds the basic function is exercised either by the oxygen or a carbon atom. They therefore form a definite group of oxonium or carbonium salts. The majority of red and blue flowers and berry pigments are derived from the pyrylium radical.



γ -pyran



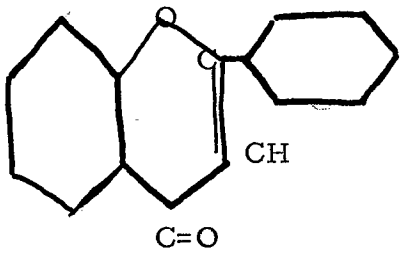
α-pyran



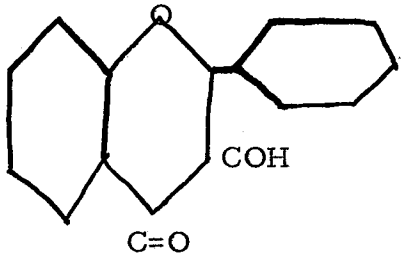
γ -pyrone-HCl

The intensity of the shade of the hydroxyl derivative of flavones depend upon the number and the position of the hydroxyl groups in the flavone molecule.

The flavonols differ from the flavones only in the possession of a hydroxyl group in place of the hydrogen in the γ pyron ring. [1]

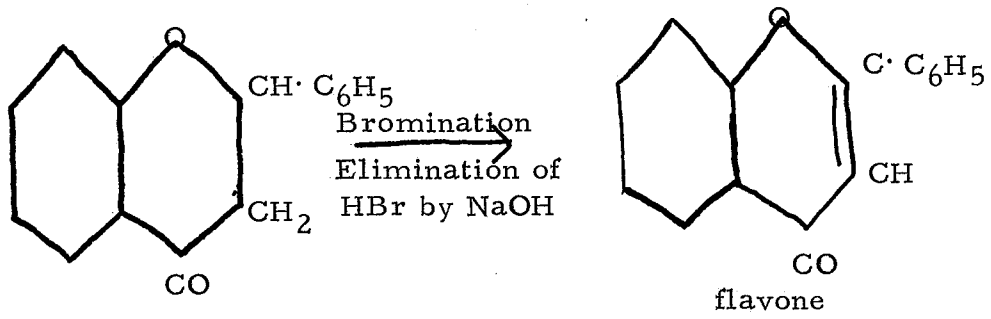


flavone



flavonol

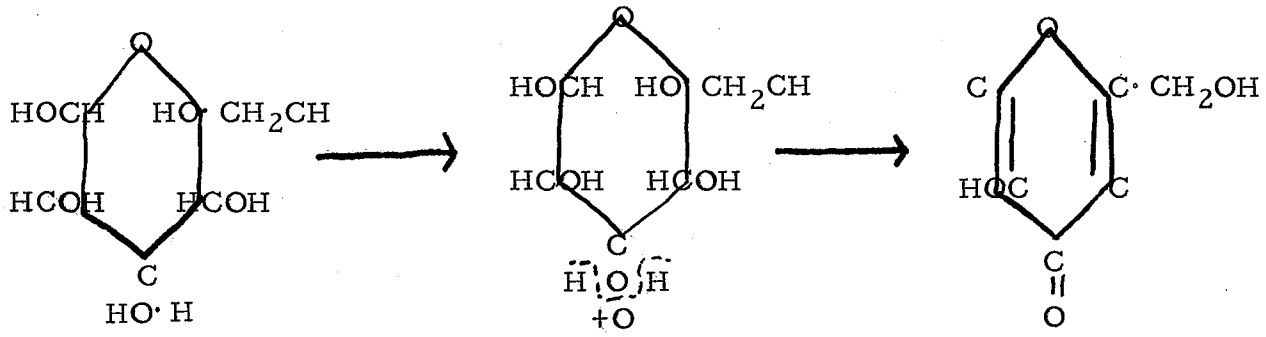
The dehydrogenation of flavanone to flavone can be carried out in one operation if the flavanone is treated with phosphorous pentachloride.



flavone

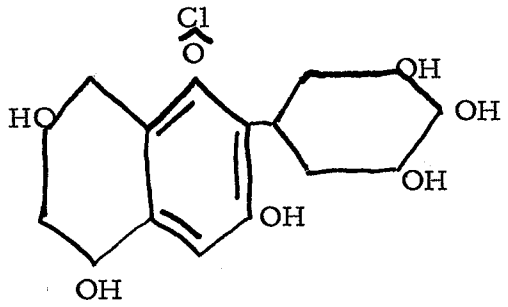
Flavone forms colorless needles, which melt at 99-100°C. It is almost insoluble in water. Its solution in concentrated sulfuric acid shows a violet fluorescence. [7]

It has been suggested that a simple change in the 6 membered hexose ring may give rise to the pyrone and pyrane nuclei of the flavone and anthocyanin pigments:

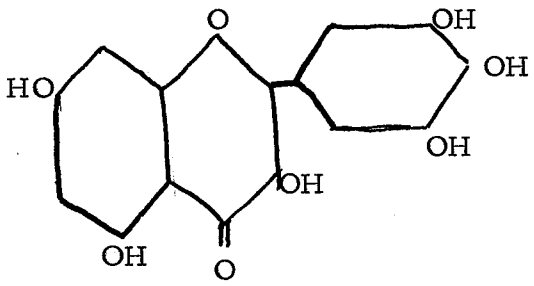


A further condensation of the hexose ring with certain hydroxy-benzoic acids might then be a possible line of synthesis of the pigments themselves.[2]

The relationship between the anthocyans and the flavonones:



delphinidin chloride



flavonol derivative[1]

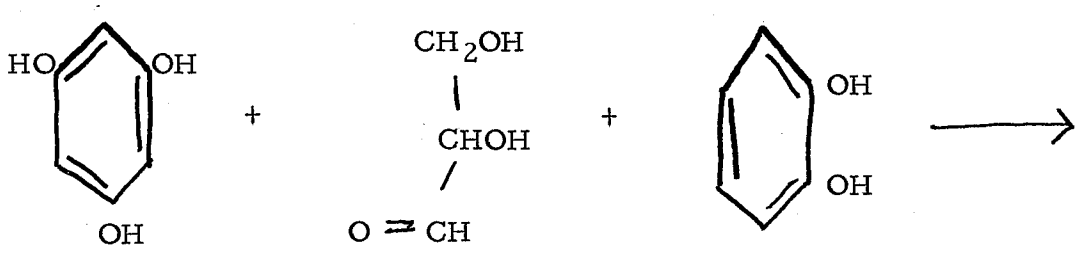
Anthocyanin pigments often cause trouble in the canning industry on account of the change in color of the fruit in the canning process or sometimes the actual perforation of the tin cans. In a study of this problem it was found that the increased corrosion of the metal cans and the color change were due to the affinity of the anthocyanins for metals, e.g., tin or iron. The metal salts being essentially insoluble, may precipitate on the inside of the can. The color of the metal salts is dull and muddy, not bright as in the original fruit or juice. The color is shifted toward the violet end of the spectrum. The reason why fruits of very low acid content but with

large amounts of pigment, such as black cherries and blueberries, bring about more extensive corrosion of plain tin or earlier perforation of enameled tin than do more acid, less deeply colored fruits, such as red raspberries or sour cherries, is also clear. In the presence of large amounts of anthocyan, salts of tin with the acids of the fruits can have only momentary existence since they are immediately decomposed with transfer of the tin to combination with the anthocyan and a liberation of the free acid to attack the metal again. This may continue until all the tin has been removed, the acid being used over and over again. With low anthocyanin and higher acid, the initial attack on the tin may be greater but it soon stops, for the anthocyanin is not present in sufficient amount to act as a reservoir for any large amount of tin. [8]

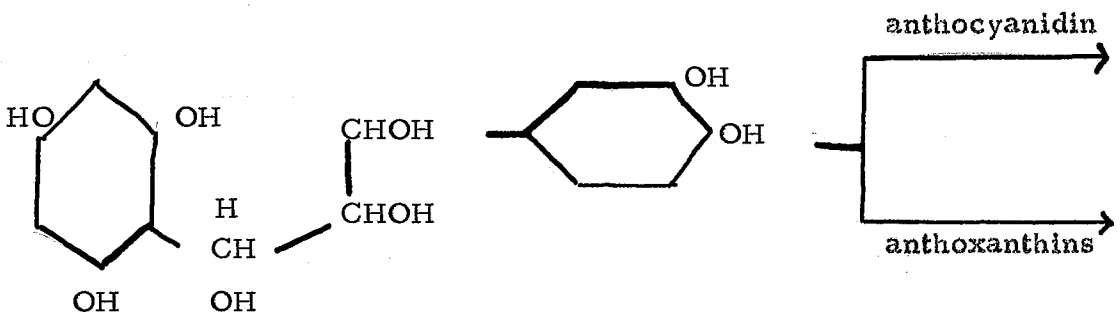
The formation of anthocyanin pigments and the development of color in plant tissues is dependent upon both heredity and environment. Increase in the number of hydroxyl groups which have been substituted on the benzene ring increases the blueness. Substitution of these hydroxyl groups is brought about by particular genes on the chromosomes in the nucleus of the living cell. Development of the red color in the anthocyanins results from conversion of the OH to OCH_3 groups. This type of change is accomplished also by particular genes. [8] In a number of species, for example, certain

genes effect the replacement of pelorgonidin anthocyanins with cyanidins. This transformation involves the introduction of an additional phenolic hydroxyl group. Other genetic factors may influence the relative quantities of anthocyanin and anthoxanthin pigments, the blocking of one or the other of these two groups, or the pH of the cell contents.[32]

Each gene controls a particular step in pigment production. The relationships between anthocyanins and anthoxanthins can be supposed to be owing to the derivation of both from a common precursor. Thus in Lathyrus genes c and r both block production of anthocyanin, and anthoxanthin accumulates, while genes m and k block production of anthoxanthin so that anthocyanin accumulates. In general, phloroglucinol, catechol and some three carbon compound, such as glyceraldehyde, may join to produce the C_6-C_3-C_6 carbon skeleton through some such intermediate as this:



phloroglucinol glyceraldehyde catechol



This intermediate, by ring closure and reduction, would yield the leuco substance as a possible intermediate in both anthocyanidin and anthoxanthin synthesis. [3]

Even though the necessary genes are present in the cells of a plant, anthocyanin formation will not take place unless environmental factors such as light, temperature, mineral deficiencies, are optional. The response to these factors seems to vary with the species. [8]

Mendel's law of inheritance has been found to apply to the inheritance of color in flowers. However, with the anthocyanins we do not always have a simple genetic formula, i. e., a one factor difference. Studies of the inheritance of color in the sweet pea have shown interesting results. The original wild pea was in all probability a chocolate and purplish blue flower. By breeding, various colors have been selected and these can be recombined to the

original color. Two whites may give purple; this occurs in certain crosses of sweet pea varieties. Red color is therefore due to two factors, A and B, and the loss of either produces a white flower. A third factor, R, is necessary to produce the blue color which when combined with red produces purple, but R has no color when alone, only when combined with A and B. Thus, flowers containing only A, B, AR, BR, or R are colorless. Flowers containing AB are red, and flowers containing ABR are purple. From such data, deductions regarding the chemical factors which are involved have been drawn. [8]

The name leuco-anthocyanin is given to the colorless substances which have been found in a variety of woods, seeds and other parts of plants; the prefix usually connotes reduction of a dyestuff and in this case it is probable that the leuco-anthocyanidin and anthocyanin are in the same state of oxidation. The leuco-anthocyanins are normally stable in the presence of 10-15% aqueous hydrochloric acid in the cold, although alcoholic hydrogen chloride of similar concentration or even weaker, brings about gradual formation of anthocyanidin. This degree of stability to the action of a mineral acid excludes the hypothesis of Rosenheim that the leuco-anthocyanins are saccharides of the anthocyanidin, or substituted anthocyanidin, pseudo-bases. Substitution of the hydrogen of the carbinol hydroxyl by alkyl groups

does not protect the pseudo-bases derived from flavylium salts against the action of even quite weak acids. [15]

The leuco-anthocyanins are divided into three classes: (a) those that are insoluble in water and the usual organic solvents, or give any colloidal solutions; (b) those readily soluble in water and not extracted from the solution by means of ethyl acetate; (c) those which are extracted from aqueous solutions by means of ethyl acetate. [14]

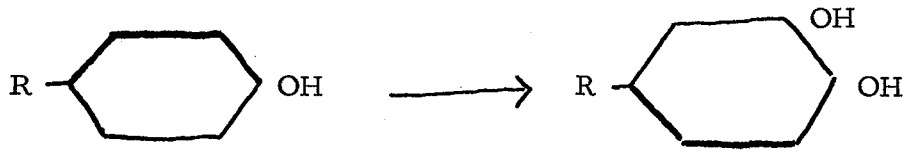
Absorption spectra: Anthocyanins have a strong absorption power over the range of 6000 to 2000 Å units. A maximum absorption, the cause of color, lies in the visible spectrum. [6] The green light transmitted by chlorophyll is absorbed by the pigment. Pelargonidin, cyanidin, and delphinidin have their absorption maxima between 500 and 550 mμ, the region of greatest transmission by chlorophyll. Leaves colored with anthocyanins may be as much as $2C^{\circ}$ warmer than other leaves on the same plant which contain only the green pigment and which have the same light exposure. [8]

Svend Aage Schou has worked on the quantitative absorption in the visible and ultra violet spectra of a group of anthocyanidins and anthocyans. The substances were studied in solutions of 0.0001 - 0.00004 molar. The solvent was alcohol, which was 0.001 m with

respect to HCl and which as purified by oxidation with iodine and repeated distillation over zinc powder.

As the absorption curves in Figure I and Table I, which describe the maxima and minima, show, the pelargonidin gives a modulated spectra. Other than the two bands in the visible, there are also three sharp bands in the ultra violet. Earlier, Willstatter had observed that pelargonidin absorbs more discontinuously in the visible than the rest of the anthocyanidins and that other than the normal absorption bands in the visible, which the color determines, there is another band in the shortwave visible. Willstatter gave the limits of 4480 - 4420 Å, to which one can find no analogies with the remaining anthocyanidins. Schou's investigations showed that this discontinuity also existed in the ultra violet range and it was easily explained by the para configuration between R and OH.

The transition of pelargonidin to cyanidin is done by the introduction of a second hydroxyl group in the ortho-position.

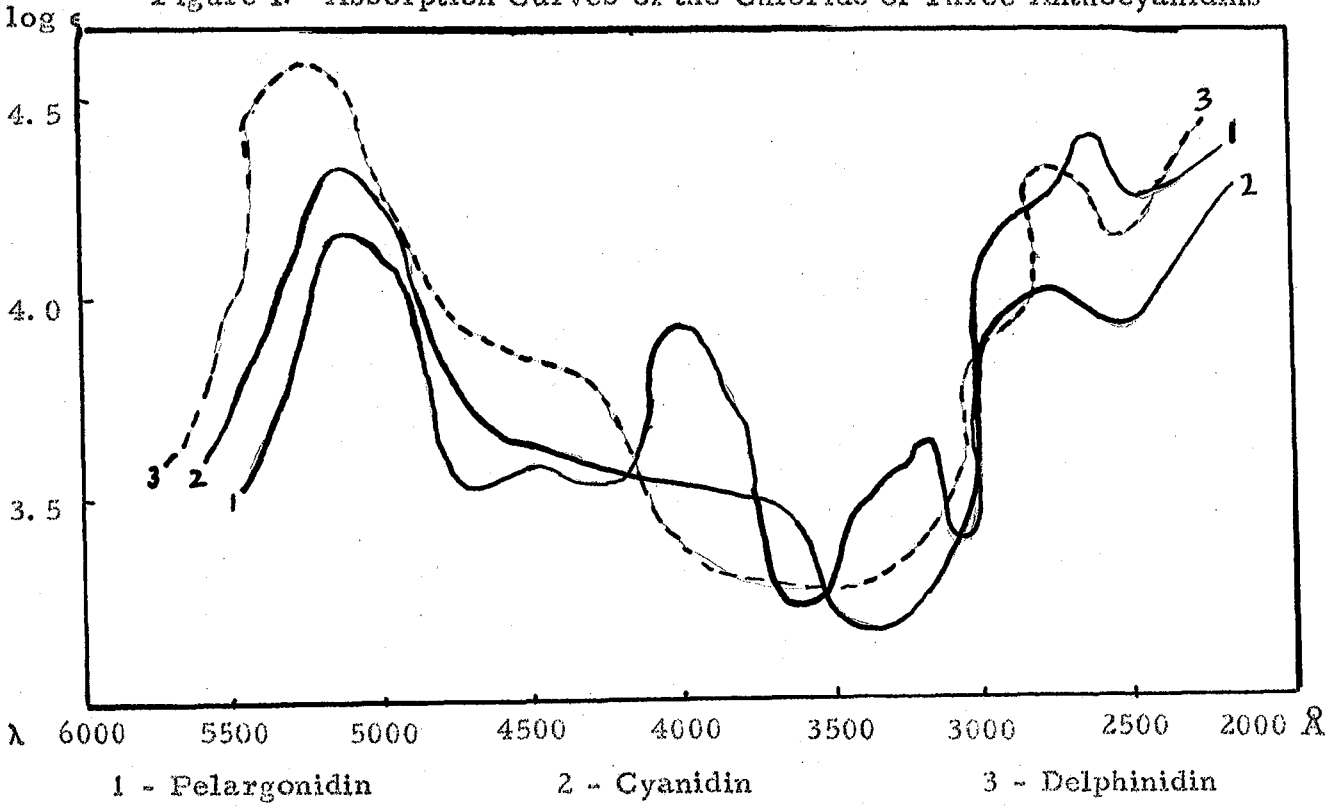


pelargonidin cyanidin

Table I. Absorption Spectra of the Chlorides of Three Anthocyanidins

Substance	Band	Maximum Å	ε	Log ε
1. Pelargonidin	I	5045	17, 800	4. 25
	II	4540	4, 450	3. 65
	III	4005	8, 700	3. 94
	IV	3310	4, 350	3. 64
	V	2670	21, 900	4. 34
2. Cyanidin	I	5105	24, 550	4. 39
	II	2695	10, 700	4. 03
3. Delphinidin	I	5225	34, 650	4. 54
	II	2750	1, 580	4. 20

Figure I. Absorption Curves of the Chloride of Three Anthocyanidins



1 - Pelargonidin 2 - Cyanidin 3 - Delphinidin

ortho position to the phenol has a coalescing effect on the spectrum and a shift of the spectrum 60 Å towards the red results.

The transition of phenol to resorcinol where an OH group is introduced to the meta position gives the same results.

The transition of phenol to pyrocatechol corresponds to the transition of pelargonidin to cyanidin and the corresponding shifts in the spectrum are also the same: a blotting out and a red-shift exactly the same as pyrocatechin.

In the methylated bands, changes in the spectra are very small. (Refer to Table II and Figure II.)

The position of the maximum as well as the size of the absorption coefficient in comparison to the unmethylated bands is almost unchanged.

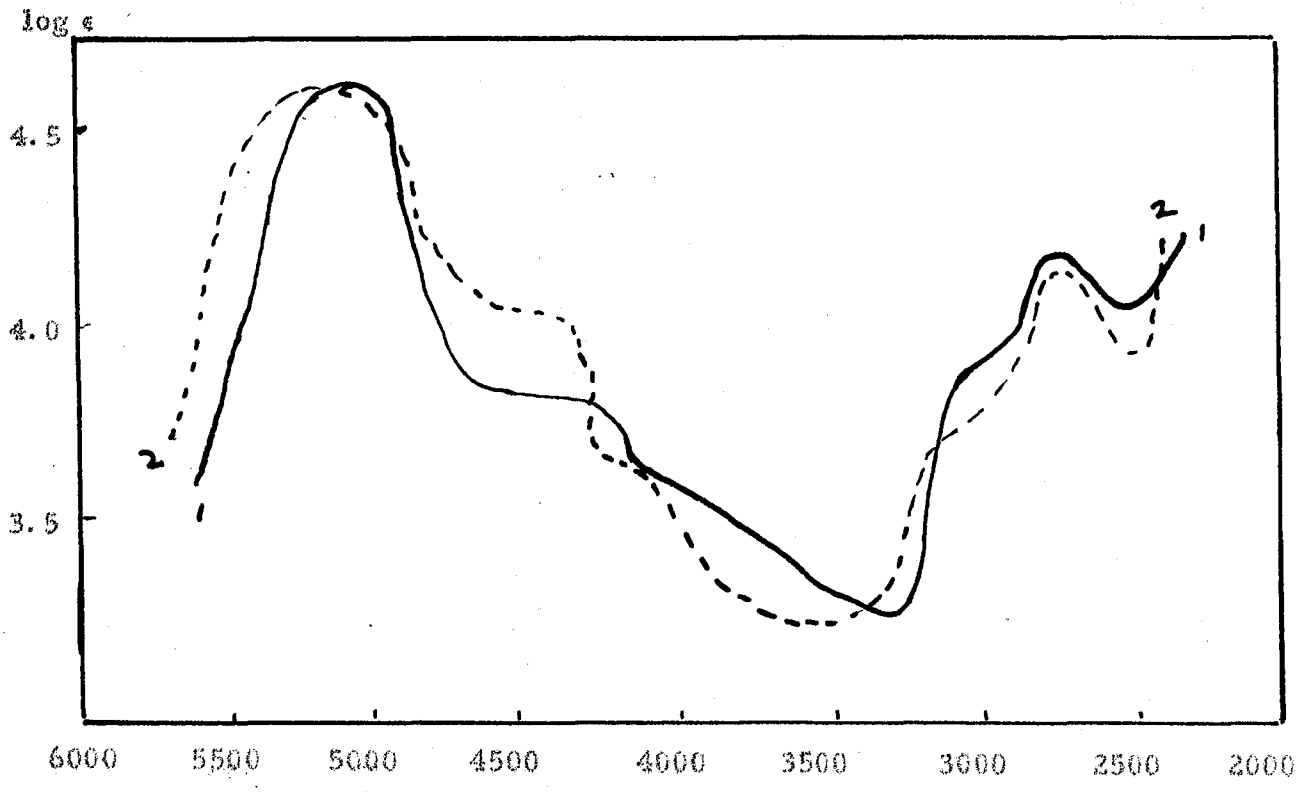
Steiner studied the spectrum of veratrol and compared it with pyrocatechol.

He showed that the number of the bands as well as the positions of the maxima and the absorption coefficient in both bands are almost the same.

Table II. Absorption Spectra of the Methylated Anthocyanidins

Substance	Band	Maximum Å	ε	Log ε
Peonidin	I	5110	37, 150	4. 57
	II	2740	15, 850	4. 20
Syringidin (malvinidin)	I	5200	37, 150	4. 57
	II	2735	14, 450	4. 16

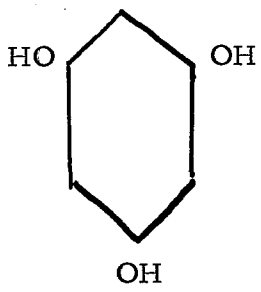
Figure II. Absorption Curves of the Methylated Anthocyanidins



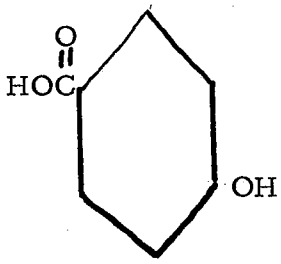
1 - **Peonidin** 2 - Syringidin

DEGRADATION PRODUCTS OF ANTHOCYANIDINS

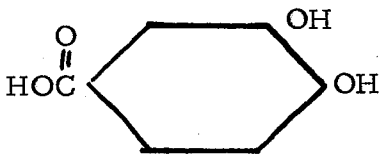
The sugar-free anthocyanidins, pelorganidin, cyanidin, and delphinidin, degrade upon fusion with potassium hydroxide into two simple products: a phenol and a phenolcarboxylic acid.[20] The three anthocyanidins give phloroglucinol as the phenol, but they give different phenolcarboxylic acids which differ from each other by the number of hydroxyl groups present. Pelargonidin upon degradation gives p-hydroxybenzoic acid as its phenolcarboxylic acid, cyanidin gives protocatechuic acid and delphinidin will give gallic acid.



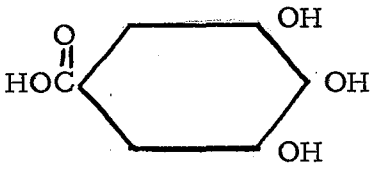
phloroglucinol



p-hydroxybenzoic acid

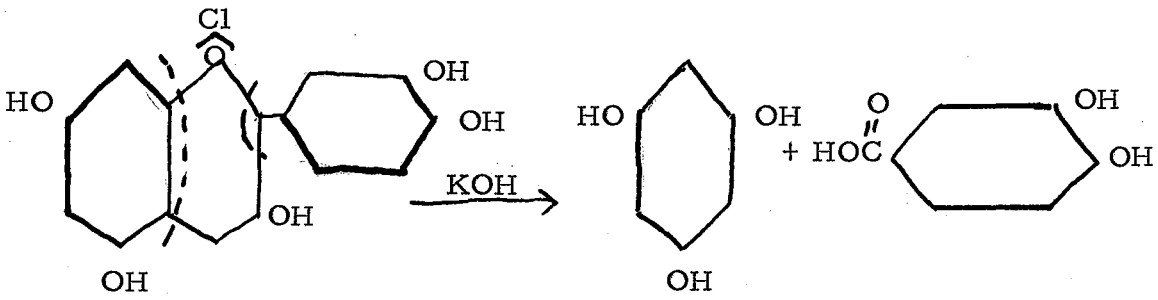


protocatechuic acid



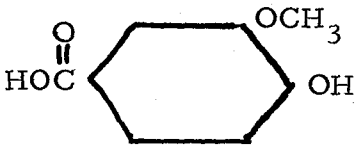
gallic acid

The degradation of cyanidin:

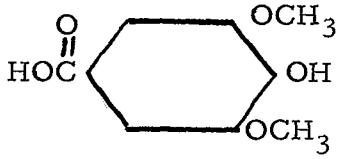


cyanidin phloroglucinol protocatechuic acid

In an atmosphere of hydrogen, to get the phenols and the phenolcarboxylic acid, the degradation of anthocyanins was carried out by Karrer, with 10% barium or sodium hydroxide.[7] The use of dilute alkali was better than potassium hydroxide because this did not remove the methoxyl groups present. Also the use of hydrogen peroxide to degrade the anthocyanins was an improvement because this did not remove the sugar residues or the methoxyl groups and yet opened the ring structure between the 2 and 3 carbon atoms. This method helped to establish the positions of the methoxyl groups of peonidin, malvinidin, and hirsutidin. Karrer degraded peonidin which gave phloroglucinol and vanilic acid, and syringidin which gave phloroglucinol and syringic acid.



vanilic acid



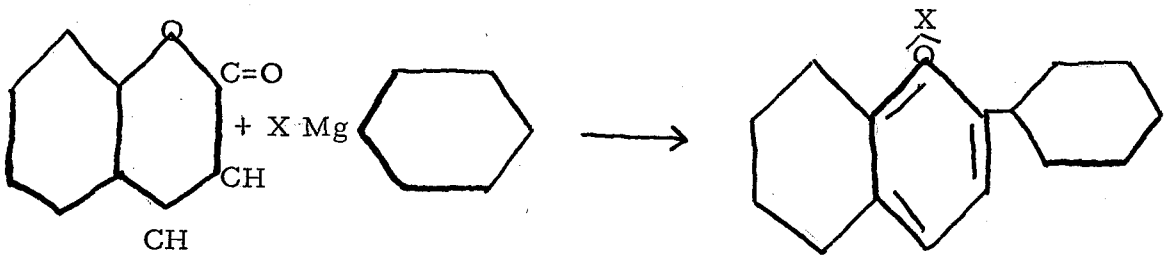
syringic acid

Karrer also found the position of the sugar on the anthocyanins by methylation of the anthocyanins followed by the removal of the sugar group and the identification of the unmethylated position that the sugar had originally occupied. Through this latter method, he found out that the sugar residue of the monoglucosides was always at the same location: 3 hydroxyl position of the anthocyanin nucleus. In the case of diglucosides the second sugar residue is attached generally to the 5 position, though not always.

Today, most of the investigators degrade the anthocyanidin with 10% barium hydroxide in an inert atmosphere, to avoid side oxidations.

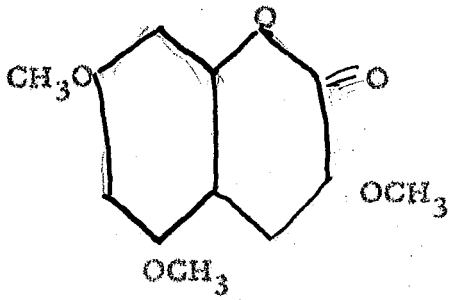
SYNTHESIS OF ANTHOCYANINS AND ANTHOCYANIDINS

First Willstatter, later on Robinson were the first ones to try to synthesize anthocyanins and anthocyanidins. Willstatter[20] used Grignard reagents to synthesize anthocyanidin, with 3 methoxy coumarins to produce the desired anthocyanidins. The following equation shows the general method in an equation form:

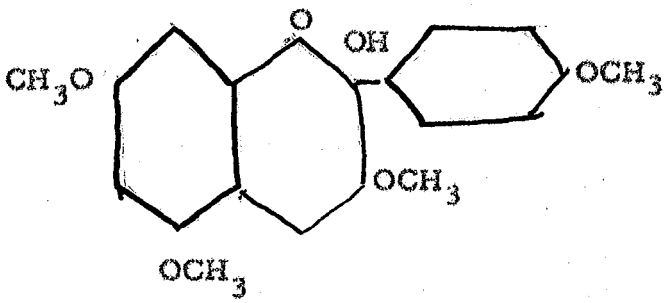


The synthesis of pelargonidin that Willstatter employed is as follows: P-anisylmagnesium bromide reacts with 3, 5, 7 trimethoxycoumarin producing the carbonol base, pelargonidin tetramethyl ether. This is acidified to the chloride of pelargonidin tetramethyl ether, which is heated with concentrated hydrochloric acid in a sealed tube in which the methoxy groups hydrolyze off, giving pelorganidin chloride.

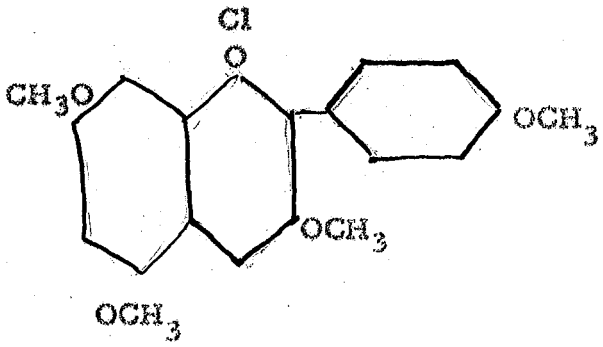
p-anisylmagnesium bromide



3, 5, 7 trimethoxy coumarin



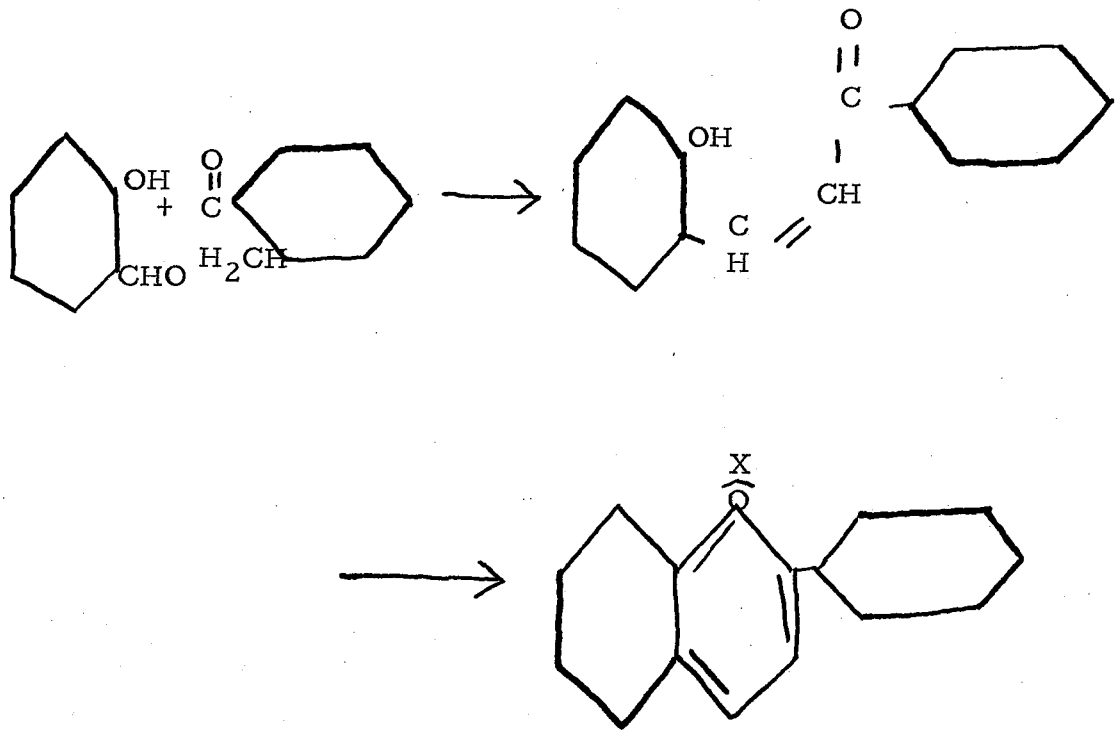
pelargonidin tetramethyl ether



chloride of pelargonidin tetramethyl ether

Robinson,[16] who has done a great amount of work on anthocyanins and anthocyanidins, used a different method than Willstatter's method in synthesizing them. He employed the condensation of 0-hydroxy benzaldehydes with appropriate ketones to form 2-hydroxychalcone which is converted to the anthocyanidin with hydrochloric acid, causing ring closure.

The following equation shows the overall reaction:



This synthesis was much better, since Robinson, who did more work on synthesis of anthocyanidins than any other investigator, was able to synthesize all of the anthocyanidin types and even several of the naturally occurring anthocyans. This was a big achievement because this method proved that anthocyanins could be prepared in the manner made in nature instead of being in the form of a salt. By these two methods, Robinson and Willstatter confirmed the parent types of the anthocyanidins: pelargonidin, cyanidin, and delphinidin.

In 1914 Everest[4] attempted to synthesize several anthocyanins by the reduction of flavones with zinc dust and hydrochloric acid. But these experiments were merely qualitative and the salt of the anthocyanin was never isolated.

In the same year Willstatter and Mallison reduced quercetin in methylalcoholic hydrochloric acid with magnesium, zinc dust or sodium amalgam as the source of hydrogen, to cyanidin chloride. The product was difficult to isolate because the yield was very small.

Asahina and Inubuse in 1928[19] used the alkaline reductions of flavones to synthesize the corresponding anthocyanidins. They were not very successful either.

OCCURRENCE, PROPERTIES, AND ISOLATION OF THE ANTHOCYANINS

Occurrence: Over twenty glycosidic combinations of the various anthocyanidins have been isolated from the flowers and fruits of plants. The varying attachment of the sugar molecule yields different compounds of varying color and shades of color. The anthocyanins usually occur as mixtures and the amount in the various flowers varies over a wide range. In red dahlias, for example, this pigment comprises over 20% of the dry weight of the petals, while the cyanin of the blue cornflower represents 0.75% of the weight of the dry petals. In the dark blue pansy the anthocyanin content (violanin) is approximately 33.0%.

The Robinsons[16] have made an extensive survey on the occurrence of anthocyanins and have developed means of detecting qualitatively the type of anthocyanidin derivative that an extract of the plant tissue in question might contain. The bases for the methods are the characteristic color reactions given by the anthocyanins with alkalies and ferric chloride and the distribution coefficient of the anthocyanin between immiscible solvents.[6]

The various anthocyanidins exhibit the following behavior: Pelargonidin in amyl alcohol, sodium acetate gives a violet-red color; there is no change upon addition of a trace of ferric chloride. It is

largely extracted by the cyanidin reagent (cyclohexanol-toluene 1:5) and completely by the delphinidin reagent (picric acid- anisol- ethyl amyl ether). It is not destroyed in the oxidation test (the addition of 10% aqueous NaOH to a dilute solution of the pigment which is then shaken in the presence of air). Pelargonidin is recognized by the color of its acid solution and by the color reactions of the anthocyanins derived from it.

Peonidin differs chiefly from pelargonidin in the color of its acid solutions and in the color reactions of related anthocyanins.

Cyanidin gives a reddish violet solution when sodium acetate is added to amyl alcohol extract over water and ferric chloride changes the violet to a bright blue color. This is apt to be confused with malvidin containing a trace of ferric- reacting anthocyanidin. Cyanidin is fairly stable in the oxidation test; it imparts a rose red color to the cyanidin reagent and its extraction by the delphinidin reagent is not complete from dilute solutions. In order to distinguish cyanidin from impure malvidin, it is best to perform the ferric reaction without sodium acetate using a carefully washed amyl alcohol solution diluted with ethyl alcohol. It is only when the anthocyanin is a 3:5 dimonoside that confusion with malvidin is a possibility; the reactions of cyanidin 3-glycosides are characteristics.

Malvidin gives a slightly bluer violet in the amyl alcohol-sodium acetate test than cyanidin. Ferric chloride does not change it. Pure malvidin is, however, of rare occurrence. The oxidation test leaves malvidin largely unchanged and it is not extracted by the cyanidin reagent but completely by the delphinidin reagent.

Petunidin gives a violet blue in the amyl alcohol- sodium acetate test and pure blue after the addition of ferric chloride. It is destroyed in the oxidation test, not extracted by the cyanidin reagent, and has a lower distribution than cyanidin in the delphinidin reagent. It is best recognized by successive extractions of a solution with small portions of the delphinidin reagent.

Delphinidin gives a blue solution in amyl alcohol on the addition of sodium acetate. It is destroyed in the oxidation test, not extracted by the cyanidin reagent or by the delphinidin reagent.

Color reactions of the anthocyanins: Pelargonin gives a violet blue coloration with aqueous sodium carbonate and this becomes greenish-blue on the addition of acetone. Decisive confirmation is obtained by addition of 1/4 volume of concentrated HCl to the solution and boiling for 1/2 - 1 minute; then on extracting with amyl alcohol a green fluorescence due to pelargonin will be observed. Pelargonidin 3-glycosides, for example, callistephin, give a violet-red coloration

with sodium carbonate and this is rather stable towards sodium hydroxide. No other anthocyanin type gives a similar reaction.

Peonin (3:5 type) gives a blue coloration with sodium carbonate. Peonidin 3-glycosides do not occur in the sequel but the sodium carbonate reaction is a rich violet unchanged by sodium hydroxide. Cyanin gives a rich pure blue coloration with sodium carbonate, unstable to sodium hydroxide, whereas mecocyanin (chrysanthmin) gives a blue violet with sodium carbonate changing to pure blue with sodium hydroxide.

Malvin (3:5 type) gives a bright greenish-blue with sodium carbonate, while oenin (malvidin 3-glycoside) gives a blue violet unchanged by sodium hydroxide.

Isolation: The pigment is first extracted from the plant material with methyl or ethyl alcohol containing HCl. The crude chloride is then precipitated with ether. It is purified by re-dissolving in aqueous HCl, a suitable quantity of alcohol is added and then ether to effect a re-precipitation of the salt. The final re-crystallization may be done with alcoholic HCl or aqueous alcoholic HCl.

Anthocyanin is sensitive to oxygen; therefore all operations are carried out in an atmosphere of hydrogen or nitrogen.

A large number of naturally occurring flavonoid pigments have been separated by paper chromotography and identified by a study of R_f values in various solvents. [21]

Table III

R_f Values of Anthocyanidins

(Bate-Smith, 1956)

	Solvents[a]	
Compound	1	2
Pelargonidin	0.80	0.68
Cyanidin	0.69	0.50
Paeonidin	0.72	0.63
Delphinidin	0.35	0.30
Petunidin	0.45	0.45
Malvidin	0.53	0.60

[a] Solvents: (1) n Butanol: 2N HCl = v/v
(2) Water: acetic acid: concent. HCl = 10:30:3 v/v

Table IV. Anthocyanidin Color Reactions and Tests

	Pelargonidin	Cyanidin	Delphinidin	Peonidin	Malvidin or Syringidin
Color of aqueous solution	Red	Violet red	Bluish red	Violet red	Violet red
Solubility of chloride in water	Readily soluble	Only slightly soluble	Soluble	Readily soluble	Slightly soluble
Ferric chloride reaction	Not definite	Intense blue	Blue	Not definite	No reaction
Behavior toward Fehling's solution	Reduces when warmed	Reduces in the cold	Reduces in the cold	Reduces when boiled	Reduces when boiled
Color change in soda solution	Violet then blue	Violet then blue	Violet then blue	Violet then blue	Violet then greenish blue
Behavior in aqueous solution	Color fades on standing	Color disappears on heating	Fading slow in cold; rapid when heated	Color disappears on heating	In very dilute sol. color disappears when heated
Extraction into anisol-ether	Completely extracted	Partially extracted	Not extracted	Completely extracted	Completely extracted
Extraction into toluene-cyclohexanol	Extracted	Slightly extracted	Not extracted	Extracted	Not extracted
Stability toward NaOH in air	Stable	Stable	Destroyed	Stable	Stable

RESEARCH

Petals of flowers of vinca major were picked from plants in the Memorial Stadium at the University of the Pacific campus. The bluish-violet petals with a slight yellow color in the middle were dried in the open air for two weeks. The petals were too small to be separated from the yellow pollen center. Therefore the whole petal was dried. When thoroughly dry, a small portion of them were mixed with dry methanol in a Waring blendor. Dry hydrogen chloride gas was bubbled into the methanol petal mixture until saturation. Color of the mixture, a dull red at the beginning, became a bright red color as the hydrogen chloride was added, indicating the formation of an anthocyanidin. The bright red color deepened as the bubbling was continued. The methanol solution of the anthocyanidin was filtered under reduced pressure and the extracted petals were discarded as waste. The addition of dry ether to the methanol solution of the anthocyanidin caused the formation of a somewhat dull reddish jel-like precipitate. Excess methanol was driven off by heating, leaving a supersaturated solution of anthocyanidin. Evaporation was carried out under reduced pressure, slightly above room temperature to avoid decomposition. The supersaturated methanol solution was cooled and the crystallized anthocyanidin separated by filtration. The steps were done continuously up to this point. The crystals were

further dried in a vacuum desiccator to constant weight. Color of the crystals was purplish brown.

The crystals were tested for solubility in water and various other solvents. They were found to be very slightly soluble in water. The light bluish red color slowly faded on standing. The crystals were soluble in methanol, ethanol and amyl alcohol, insoluble in ether, benzene, carbon tetrachloride and other nonpolar solvents. In 10% sodium hydroxide solution there was a color change indicating that the anthocyanidin was destroyed. The addition of dilute barium hydroxide solution caused a precipitate to settle out. This was assumed to be the barium salt of vinca major anthocyanidin.

Fehling's solution reduced the vinca major anthocyanidin in cold.

A second extraction was carried out with methanol. This time the amount of petals was about three times more than the first extraction. Dry hydrogen chloride gas was bubbled into the methanol petal mixture until saturation. The color of the mixture, which was dull brownish red at the beginning, turned bright red and again then darker red.

The methanol containing the anthocyanidin was filtered out and dry ether added. No jel-like precipitate formed. It was assumed that enough hydrogen chloride gas was not bubbled in and the chloride

salt had not formed yet. The ether and the methanol were evaporated in hot water in open air. More hydrogen chloride gas was bubbled in. Ether was added a second time and the jel-like precipitate formed though the separation layer between the precipitate and the solution was not very distinct. The excess methanol was driven off by heating in hot water and the supersaturated solution of anthocyanidin was cooled, the crystals separated out by filtration, and dried in a vacuum desiccator to constant weight. This second extraction was done much more slowly and in the open air over a period of three to four days. The crystals were very dark brown. The anthocyanidin was at this stage a mass with undefined crystalline structure. Therefore a re-crystallization was carried out with the hope of purification. Knowing that anthocyanins are sensitive to oxygen and according to Karrer and Robinson much better results are received if all the steps are continuous and one after another in the shortest time possible, it was assumed that the vinca major anthocyanidin had decomposed.

The mean percentage of anthocyanidin in the dry petals of vinca major flowers was calculated to be approximately 20%.

A third extraction was carried out with a soxhlet extracter and methanol. The extraction was continued for about ten hours. The resulting extraction was brownish red, which became a bright red upon bubbling hydrogen chloride gas into the methanol extract.

Passing ether into the mixture resulted in the formation of dull red colored jel-like precipitate. Excess methanol was driven off and the supersaturated solution allowed to cool. This time all the steps were carried out continuously and effort was made to avoid contact of anthocyanidin with oxygen. Filtration was carried out in an atmosphere of nitrogen, and the crystals dried in a vacuum desiccator. According to Robinson, again (J. Chem. Soc. 1937) anthocyanins retain water of solvation under 110° high vacuum. It was assumed that this was the reason for the anthocyanidins being in the form of precipitate rather than real dry crystals; the precipitate therefore was scraped off the container and put in the vacuum desiccator again. The resulting crystals were dry.

Paper chromotography: A drop of concentrated anthocyanidin methanol solution was placed on chromotographic paper strips and the ascending technique employed with n-butanol:2N HCl-v/v and water: acetic acid; concentrated HCl - 10:30:3 v/v as solvents. The chromotography was carried on for 10 hours. The mean R_f value was 0.33 for solvent 1 and 0.32 for solvent 2. Delphinidin has an R_f value of 0.35 for solvent 1 and 0.30 for solvent 2. Paper chromotography with two different solvents was carried out three times. The R_f values were almost the same. There was a separation of a slight yellow color. Since this did not correspond to any of the R_f values of

anthocyanidins, it was assumed that flavone was extracted with the petals and was causing this second layer. The same problem arose with the absorption spectra. A Beckman Model B spectrophotometer was used. The absorption spectra was studied in the visible and in the ultraviolet regions. The anthocyanidin was studied in solutions from 0.0001 to 0.00004 molar. The solvent was alcohol which was 0.001 molar with respect to HCl. It was clear from the absorption spectra that there was another substance besides the vinca major anthocyanidin. In the ultraviolet region the maximum absorption was 2700 Å while in the visible region the maximum absorption was a wider band: 5500-5000 Å.

The infrared spectrum of the vinca major anthocyanidin was also obtained by using a Perkin-Elmer Model 12-C Infrared spectrophotometer. The infrared spectrum showed that there were hydroxyl groups present, but there was no indication of the presence of methoxyl groups.

A small amount of petals were extracted with amyl alcohol. The extract upon addition of sodium acetate had a light blue color. The color was changed to pale greenish yellow by addition of ferric chloride.

The fifth extraction, using a small amount of petals, was made with a mixture of one volume of cyclohexanol and five volumes of

toluene. A very light pink color was seen, therefore it was assumed that the vinca major anthocyanidins were not extracted with the cyanidin reagent.

A mixture of 1% aqueous HCl and anisole (5 volumes) and ethyl isoamyl ether (1 volume) containing picric acid: The vinca major anthocyanidin was not extracted by the organic solvent layer, though there was a slightly yellow coloration.

It was also found that the extraction of delphinidin gives better results with small amounts and the tests carried out as soon as possible.

The vinca major anthocyanidin was fused with KOH (10%). The degradation products were a phenol and the potassium salt of a phenol carboxylic acid. The residue left after evaporating the added diethyl ether melted at 219-220°C. Phloroglucinol melts at 219°C.

The methyl ester of the phenolcarboxylic acid decomposed at 220-221°C. Gallic acid decomposes at 220°C.

These products indicated that the vinca major anthocyanidin is very likely 3, 5, 7, 3, 4, 5 hexahydroxy 2 phenyl benzopyrylium or delphinidin.

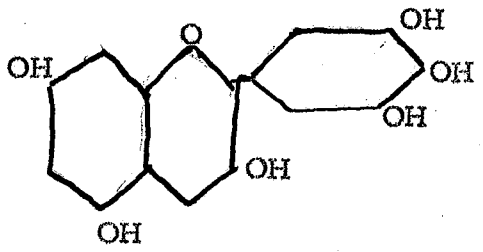


Figure III. Transmission Spectrum of Unknown Anthocyanidin between 220 and 760 millimicrons

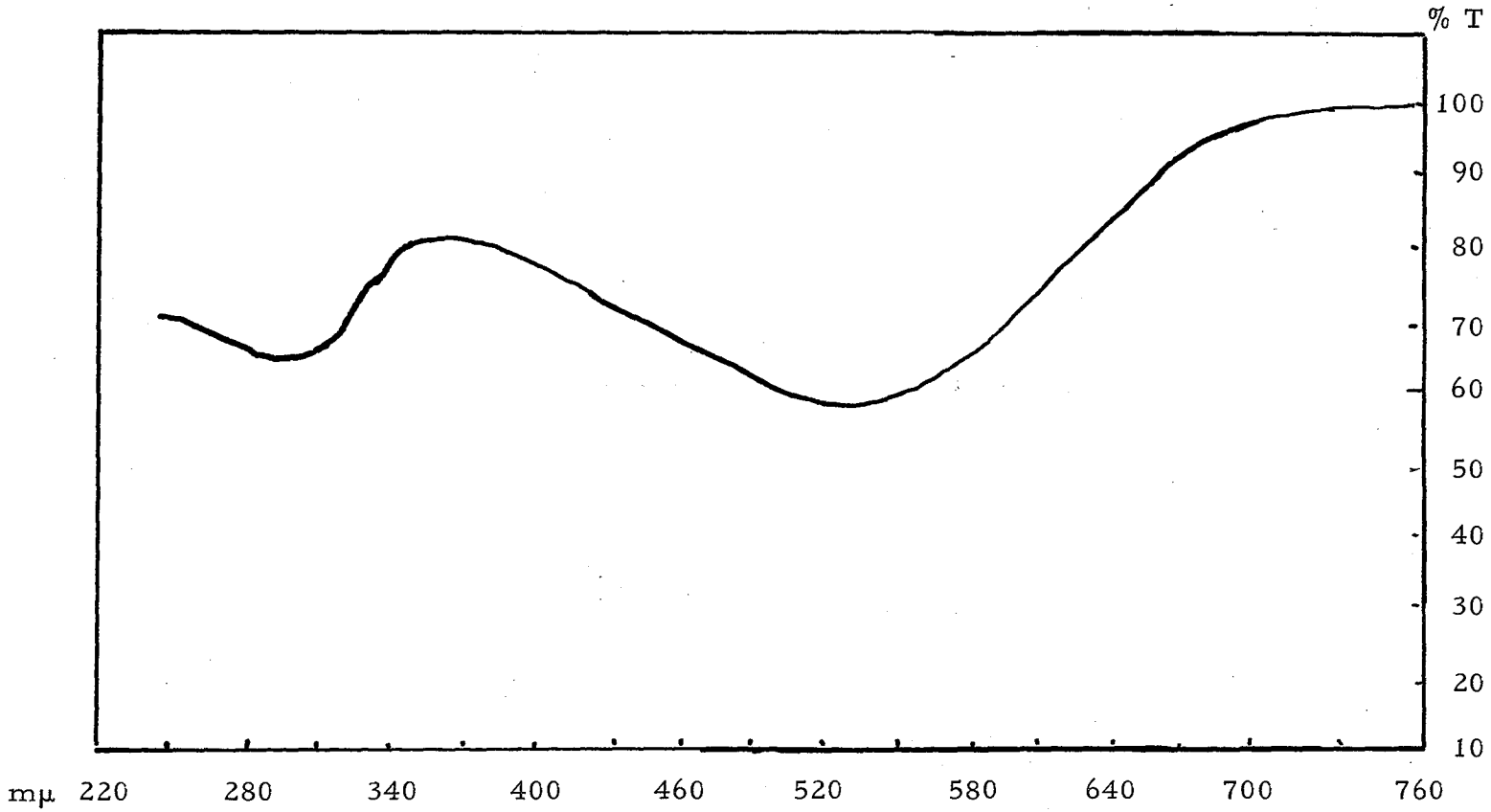
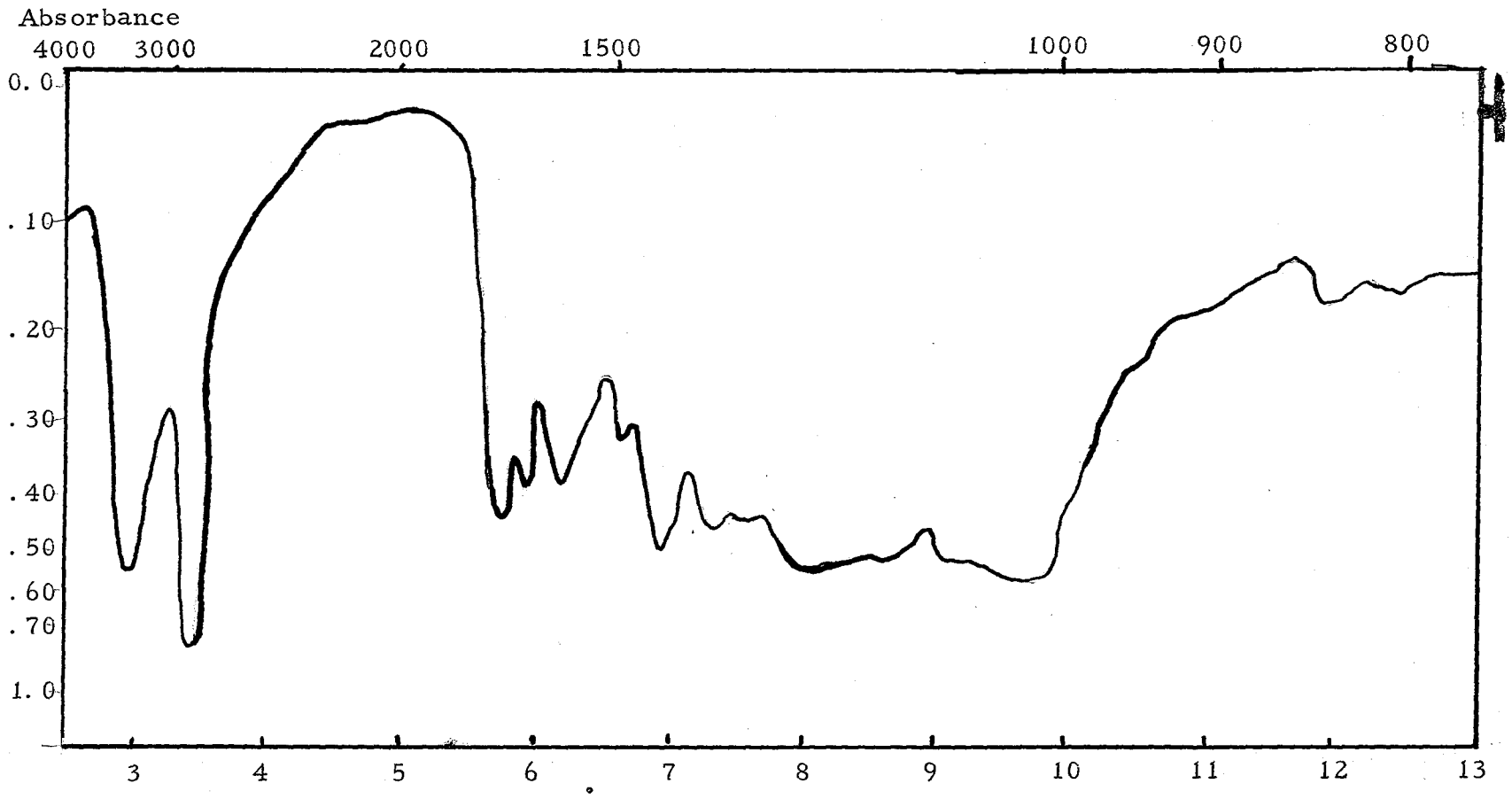


Figure IV. Infra-red Spectrum of Unknown Anthocyanidin



SUMMARY

The coloring matter in the flowers of vinca major was shown to be an anthocyanin because of the fact that it was red in acid solutions and blue in alkali. The pigment was extracted with methanol and precipitated as a chloride. The pigment was soluble in methanol and ethanol, and very slightly soluble in water. It was not soluble in nonpolar organic solvents. It was not extracted with cyclohexanol and toluene. The pigment reduced Fehling's solution when cold.

The degradation products of the pigment were phloroglucinol and gallic acid.

The infra-red spectra showed that the pigment had hydroxyl groups but no methoxyl groups present on the nucleus. The paper chromotography gave an R_f value which corresponded to delphinidin. The spectrum of the pigment in the range of 220-760 millimicrons gave a maximum absorption of 5500-5000 Å in the visible region and 2700 Å in the ultra-violet region.

The pigment in the flowers of vinca major was shown thus to be delphinidin.

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