STUDIES ON
THE MOLECULAR ASSOCIATION OF ANTHOCYANINS

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CONTENTS

GENERAL INTRODUCTION

Water-soluble pigments of the flavonoid group such as anthocyanins, flavonols and flavones are widely distributed in higher plants. The precise importance of the flavonoids in nature cannot be easily evaluated. The most significant function of the flavonoids is their ability to impart a variety of colors to the plants (flowers, leaves, fruit and tubers). Flower colors have given immense aesthetic pleasure to man from ancient times. They also have an important role in attracting bees, butterflies and other animals to ensure fertilization. Since the use of synthetic food additives has been restricted because of possible toxicity, naturally occurring colored matters have been recently attentioned.

Chemists have intensively investigated the colored substances. The term "anthocyanin" was coined by Marquart in 1835. Since then many chemical structures of this class of pigments were clarified mainly by Willstätter, Karrer and their schools. Robinson and co-workers were the first to synthesize the natural anthocyanins. Later, Harborne

and Hayashi extracted many anthocyanins from natural plant sources and determined their chemical structures.

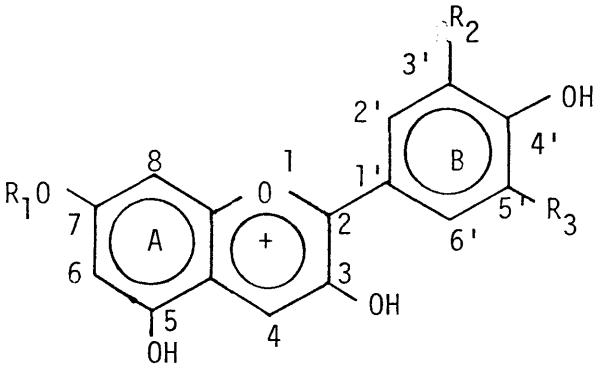
Chemical Structure of Anthocyanins

The anthocyanins are glycosides of anthocyanidins. Structures of naturally occurring anthocyanidins are shown in Fig. 1. The fundamental skeleton is 2-phenylbenzopyrylium (flavylium) ion. The OH or OCH_3 group(s) is substituted at the different position of the flavylium ion. Ordinary anthocyanins have sugar(s) substituted at 3 and/or 5-positions. Acylated anthocyanins which have an acylated sugar moiety at 3-position of anthocyanidin ring have been found.

In order to determine the structures of anthocyanins, anthocyanins have generally been isolated under strongly acidic media. This method changes flower colors to only red tone even if the colors are mauve or blue. Flowers of different colors possess the same anthocyanins and flowers of similar colors contain different anthocyanins.
These facts suggest that a variety of color tone is not due only to the differences of chemical structures of anthocyanins as shown in Fig. 1.

Structural Transformation of Anthocyanins

Naturally occurring anthocyanins are transformed as shown in Fig. 2: Flavylium ion (red) in acidic, anhydrobase



1) pelargonidin: $R_1=R_2=R_3=H$

2) cyanidin: $R_1=R_3=H$, $R_2=OH$

3) peonidin: $R_1=R_3=H$, $R_2=OCH_3$

4) delphinidin: $R_1=H$, $R_2=R_3=OH$

5) petunidin: $R_1=H$, $R_2=OCH_3$, $R_3=OH$

6) malvidin; $R_1=H$, $R_2=R_3=OCH_3$

7) hirsutidin: $R_1=CH_3$, $R_2=R_3=OCH_3$

Fig. 1 Structures of natural anthocyanidins

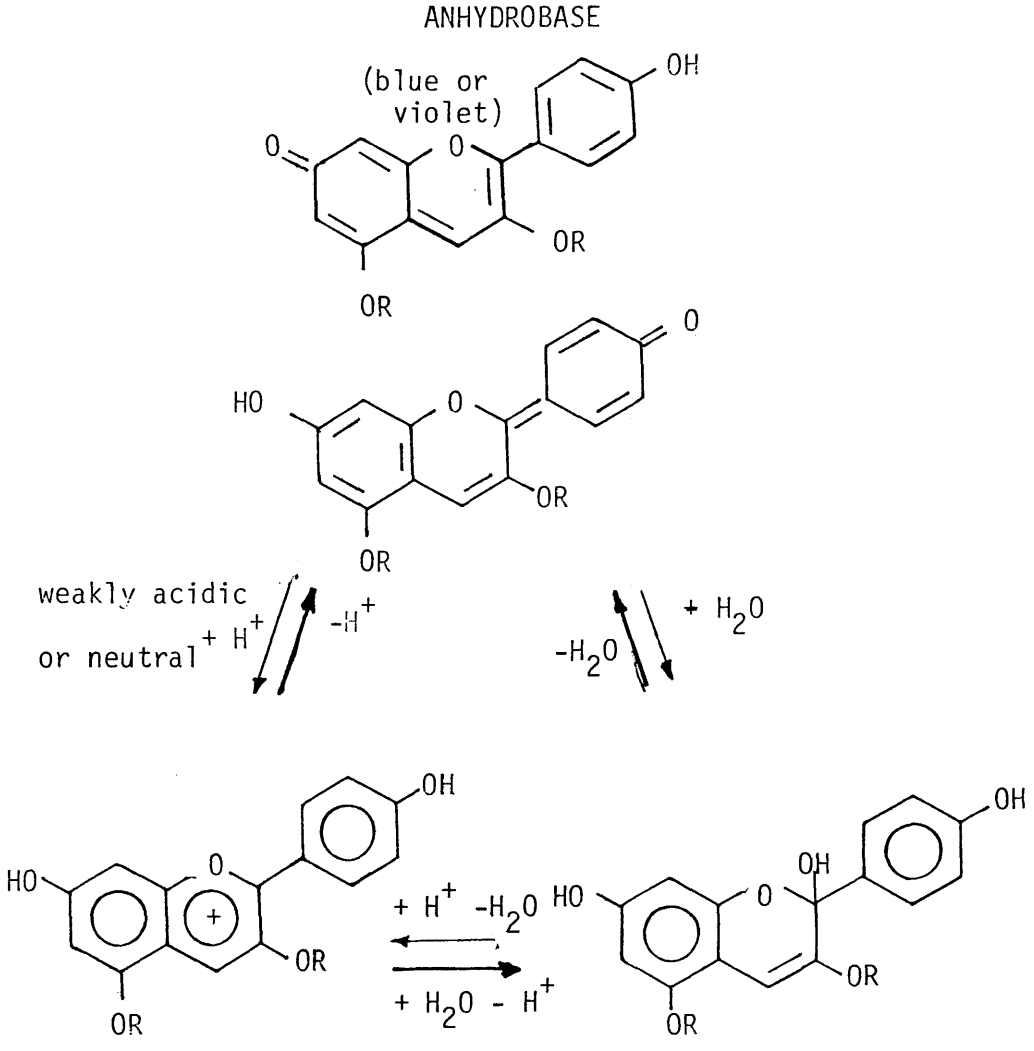


Fig. 2 Structural transformation of anthocyanin

(blue or violet) in neutral media and these structures are converted to colorless pseudobase by hydration. Accordingly, anthocyanins act as natural pH indicator.

Because the transformation is caused by the concentration of H^+, the pH of cell sap was once considered to be an important factor in flower color (proposed by Willstätter). However, a survey of 200 plants by Shibata[1] showed that all flowers were weakly acidic (about pH 5.5), irrespective of different colors. Asen et al.[2] have recently found that the pH of cell sap of morning glory (Heavenly Blue) is higher (about pH 7.5), which leads to the blue color of the plant. In any case, it is not considered that pH of cell sap is strongly acidic, which indicates that the structure of anthocyanins in cell sap is anhydrobase form. Recently Hayashi and Saito et al.[3] extracted colored anthocyanins by using a mild neutral aqueous organic solvent. Isolated colored anthocyanins in this way are named "genuine anthocyanins", which is now considered to be nearly identical with anhydrobase form.

Whether the pH of cell sap is higher or weakly acidic, anthocyanin-containing flower would be expected to be colorless, because of its conversion to colorless pseudobase (Fig. 2). Irrespective of this unstability of anthocyanins, why flowers are colored and exhibit a wide range of color (orange to blue) ? Attempts to clarify these

points have been carried out by many workers.

Factors of Flower Color Variation and Stability

Factors which may be involved in the formation of blue pigments have been extensively studied. One of them is metal chelation of anthocyanins, which was first presented by Shibata and Shibata[4], one of whom was a pioneer of the chemistry in co-ordinated complex salts in Japan. They maintained that higher stability of anthocyanins can be explained in terms of magnesium and calcium chelation with anthocyanins. Extensive studies on the metal chelation were carried out by Bayer[5]. He synthesized metal chelated anthocyanins, and observed that the Fe- or Al-chelated anthocyanins have a relatively high stability and blue color, but Mg or Ca ion is incapable of formation of stable complexes with anthocyanins which have o-dihydroxyl group in B-ring.

Second factor of color variation is co-pigmentation effect which was proposed by Robinson and Robinson[6]. By combination with flavones or tannins, flower color is shifted to longer-wave absorption and stability is increased. The co-pigmentation effect is now considered to be significantly important in different colors and stability. Asen et al.[7] have mentioned that the phenomenon of co-pigmentation offers a more logical explanation of the infinite variation in red

to blue flower colors that exist in a pH range where anthocyanins alone are virtually colorless, and that by varying the factors (pH, anthocyanin concentration and molar ratio of anthocyanin to co-pigment) they described it is possible to obtain similar absorption spectra with different anthocyanins.

Robinson and Robinson[8] also suggested that macromolecules such as polysaccharides or polypeptides bind with anthocyanins to produce change of stability and color.

Recently, unique acylated anthocyanins have been isolated which have high stabilty and blue color without aid of metal chelation, co-pigmentation or macromolecules. For example, platyconin from platycodon[9], cinerarin from cineraria[10] and anthocyanins from Lobelia or Tradescantia relexa[11]. These anthocyanins are acylated pigments with 2 or 3 aromatic acyl groups. Their corresponding deacylated anthocyanins rapidly decolorize similarly with the ordinary anthocyanidin 3,5-diglucosides, which suggests that aromatic acyl groups of their anthocyanins interact with their anthocyanidin rings, that is, the intramolecular interactions are considered to impart high stability and blue color.

Living active substances do not exist independently in living organisms. Many interactions of living matters with a variety of substances such as low molecular weight compounds or macromolecules are very important in vital functions. As typical examples are considerably known enzyme-coenzyme

complex, biological cell membrane systems, nucleic acid-protein complex and hybridization of nucleic acid etc. Hydrogen bonding, hydrophobic effect, metal chelation or charge transfer etc. are well known to become driving force for these complex formation in vital functions. Extensive studies on the molecular association of living matters have offered a immense recognition and knowlege of vital reactions, but correlation between flower colors and complexed structures of molecular association of anthocyanins remains obscure.

As previously described, anthocyanins rapidly decolorize , but addition of flavones makes the rate of decolorization slow. The author has investigated the complexed structures of anthocyanins from the point of stabilizing mechanism by noticing the differences in chemical structures of monomeric anthocyanins. The author is the first to apply circular dichroism to anthocyanins and showed that circular dichroism is most effective probe of conformational change during the molecular association of anthocyanins. The present paper deals with the molecualr association of anthocyanins. This paper is divided into two sections; part I: self-association of anthocyanins, part II: molecular complexes of anthocyanins with flavones (co-pigmentation). The author has established the occurrences of "self-association of anthocyanins", which have not been appreciated by the chemists engaged in the

studies on flower colors. As an example of molecular complexes of anthocyanins with flavones, commelinin, which is obtainable from Commelina (Japanese name: tsuyu-kusa), was investigated.

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PART I

SELF-ASSOCIATION OF ANTHOCYANINS

I. INTRODUCTION

As described in 'General Introduction', metal chelation , co-pigmentation and polymer carrier theories have been for a long time appreciated as factors of flower color variation. However, the concept of self-association of anthocyanins has not been appreciated, because no convincing evidence has so far been presented[1]. S. Asen et al.[2] were the first to discuss the possibility of the self-association, but they exhibited little concrete demonstration. Little is known of the self-association of anthocyanins.

An effective method to demonstrate the occurrences of the self-association has been required. The author has for the first time applied circular dichroism to anthocyanins. Circular dichroism is intrinsically most sensitive to molecular asymmetry and, therefore, is an effective probe of conformational change during the molecular association of anthocyanins. The author has found that monomeric anthocyanins have little optical activity, whereas self-associated anthocyanins have large molecular ellipticities and demonstrated that an extraordinarily large CD was observed in the aged aqueous solution of cyanin anhydrobase, indicating self-association of the pigment, which was further supported by the large θ value of the anhydrobase precipitated

from the aged solution. Hypochromic effect (a decrease in ε value) and extremely high stability of the pigment were further presented as the evidence of the self-association. The author further examined the possibility of the self-association of other anthocyanidin 3,5-diglucosides* such as delphin, malvin and pelargonin. A change in a shape of the visible absorption spectrum, hypsochromic or bathochromic shift, was observed.

The driving force for the self-association would be mainly the hydrophobic interactions among the aromatic nuclei stacked parallel to each other, which was presumed from dissociating mechanism by urea or dimethylsulfoxide. The author is the first to propose the structure of self-associated anthocyanins.

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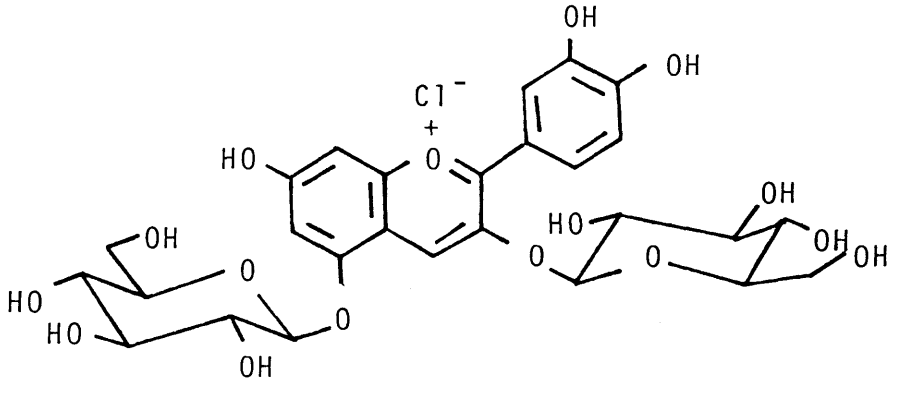
* unpublished results, T. Hoshino (in preparation for publication).

II. EVIDENCE OF THE SELF-ASSOCIATION OF ANTHOCYANINS

ANHYDROBASE OF ANTHOCYANIDIN 3,5-DIGLUCOSIDE, CYANIN ANHYDROBASE

When cyanin chloride (1) was added in phosphate buffer (0.1M, pH 7.0), the anthocyanin dissolved rapidly as its anhydrobase. The visible λ_{max} of cyanin anhydrobase varied from 572 to 566 nm by increasing the concentration from 2.5×10^{-5} to 5×10^{-4}M, when measured immediately after dissolving. Low concentration (2.5×10^{-5}M) offered no appearance of circular dichroism (CD), where little interactions of each anthocyanin molecules occurs. At high concentration (5×10^{-4}M), a blue pigment gradually precipitated as floccules after 60 min. Time courses of visible absorption spectra and optical ellipticities of the solution are shown in Fig. 1. The θ value increased gradually after dissolution, which suggests slow conformational change of the anhydrobase. Within 2 min after dissolution no orientation of cyanin anhydrobase molecules was observed. From 5 min on, the molecules having fixed conformational arrangement were progressively increased (larger θ) in spite of the decrease in anhydrobase concentration (vis. spectr.



CYANIN CHLORIDE (1)

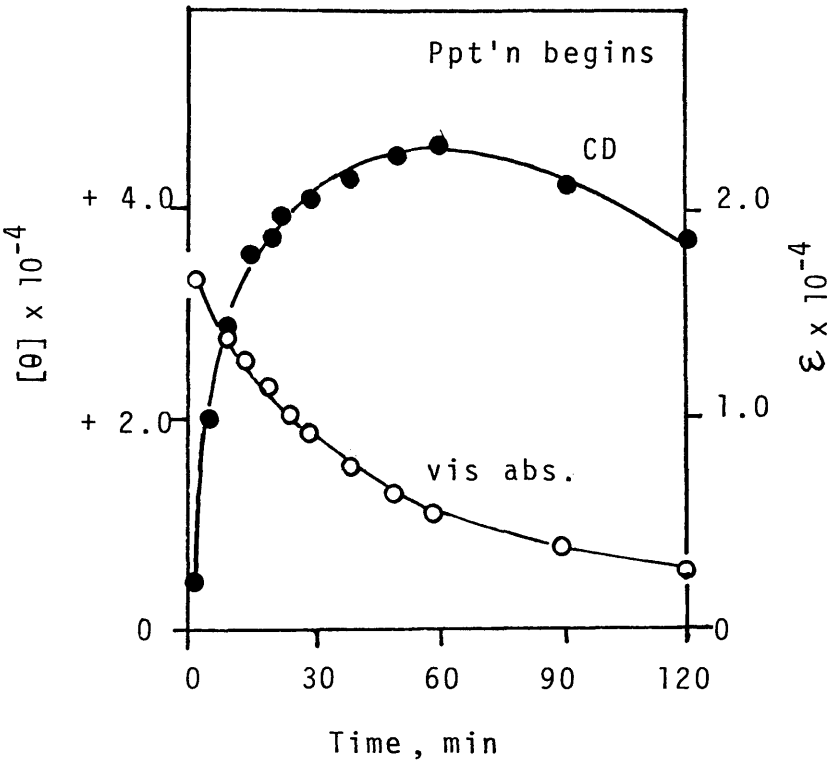


Fig. 1 Time course of visible absorption and CD at λ_{max} of cyanin chloride (5×10^{-4}M) in pH 7.0 phosphate buffer (0.1M).

in Fig. 1), after 60 min the blue precipitates came into sight, indicating the aging effect on the self-association of the anthocyanin anhydrobase to form aggregates. Thus, the flocculent precipitates must be highly self-associated aggregates of the pigment. Indeed, when the precipitates were dissolved in water (pH 7.0) the solution showed an exceptionally large value of $[\theta]$, 260,000 at 630 nm (Fig. 2, dotted line). Stability of aggregates in the solution is greatly high; the ε value at 630 nm was reduced only less than 5 % when the solution left at room temperature for 2 hours (Fig. 3). These results suggest that the aggregates formation and the decolorization (hydration) of the monomeric anthocyanin anhydrobases are competitive processes.

The decrease in ε value is remarkable (Fig.3). Dotted line shows the spectrum of the monomeric cyanin anhydrobase. Solid line exhibits the spectrum of highly aggregated cyanin anhydrobases and the spectrum is structured. The hypochromism suggests that the aromatic nuclei (anthocyanidin rings) are stacked parallel to each other by aggregates formation.

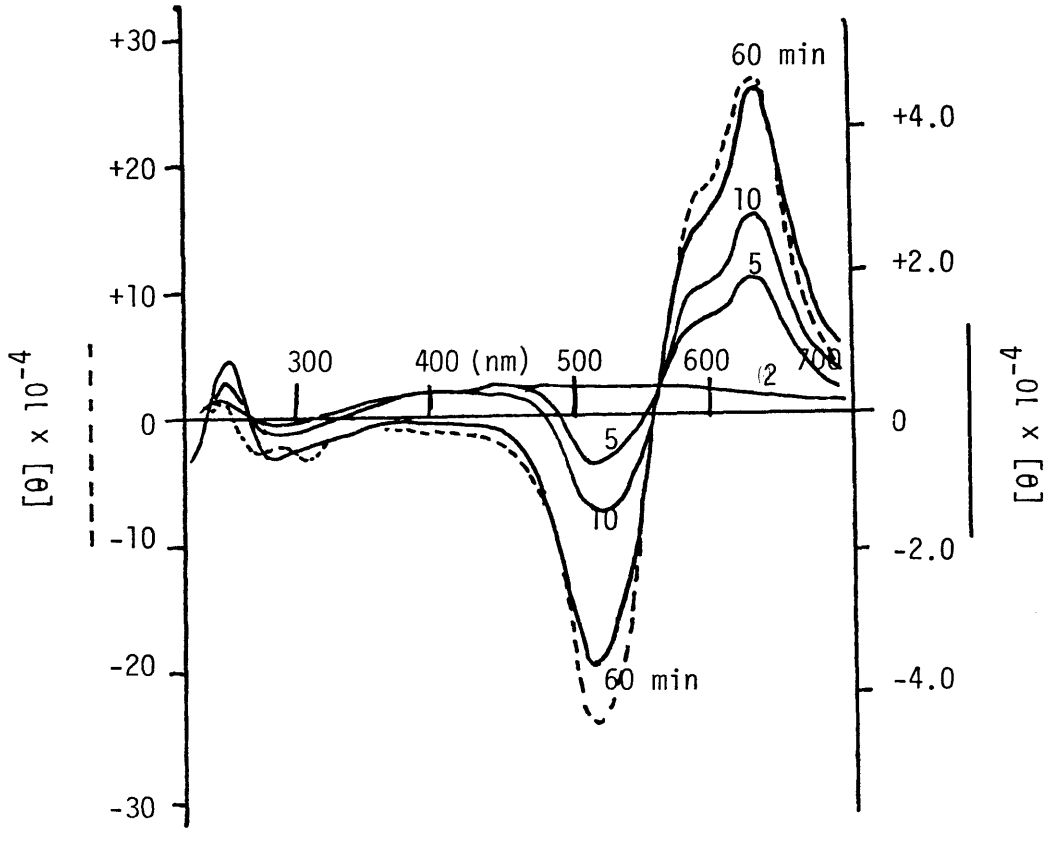


Fig. 2 ——— cyanin chloride (5×10^{-4}M) in phosphate buffer (pH 7.0, 0.1M). 2, 5, 10, 60 min after dissolving.

----- cyanin anhydrobase (aggregated precipitates, 4.6×10^{-4}M) in water (pH 7.0).

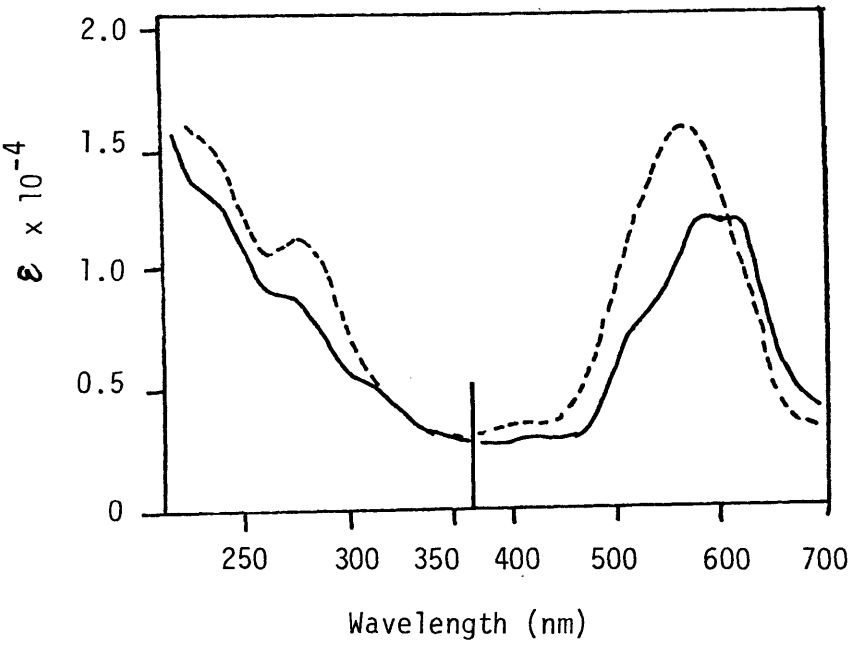


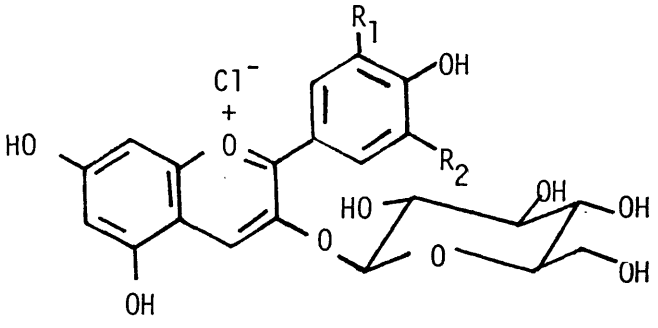
Fig. 3 ----- within 2 min after dissolving cyanin chloride ($5x10^{-4}$M) in pH 7.0 phosphate buffer (0.1M).

——— aggregated precipitates of cyanin anhydrobase ($4.6x10^{-4}$M) dissolved in water (pH 7.0).

EFFECT OF GLUCOSE MOIETY AT 3- OR 5-POSITION ON THE SELF-ASSOCIATION OF ANTHOCYANIDIN 3,5-DIGLUCOSIDES

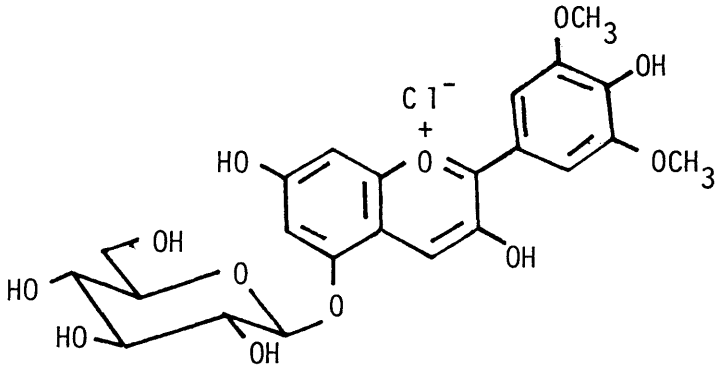
Cyanidin 3,5-diglucoside (cyanin) anhydrobase forms higly self-associated aggregates. To examine the effect of the glucose moiety on the self-association of anthocyanidin 3,5-diglucosides, cyanidin 3-monoglucoside (2), malvidin 3-monoglucoside (3) and malvidin 5-monoglucoside (4) were utilized. Cyanidin 3-glucoside and malvidin 3-glucoside shows little concentration dependence of their $[\theta]$ values and stability. Therefore anthocyanidin 3-monoglucosides are incapable of formation of high self-association. Malvidin 5-glucoside, at dilute concentraion (5×10^{-5}M), rapidly decolorized and shows little CD in neutral solutions. At high concentration (5×10^{-4}M) a red precipitates were settled out of neutral solutions. The precipitates dissolved in water shows characteristically large optical activities (Fig. 4) and greatly high stability (Fig. 5); the ε value at 494 nm was reduced only 17 % when the solution left at room temperature for 2 hours. These results indicates that malvidin 5-glucoside forms highly self-associated aggregates.

In conclusion, the glucose moiety at 5-position has an important role in the self-association of anthocyanidin



cyanidin 3-monoglucoside (2): R_1=OH, R_2=H

malvidin 3-monoglucoside (3): R_1=R_2=OCH_3



malvidin 5-monoglucoside (4)

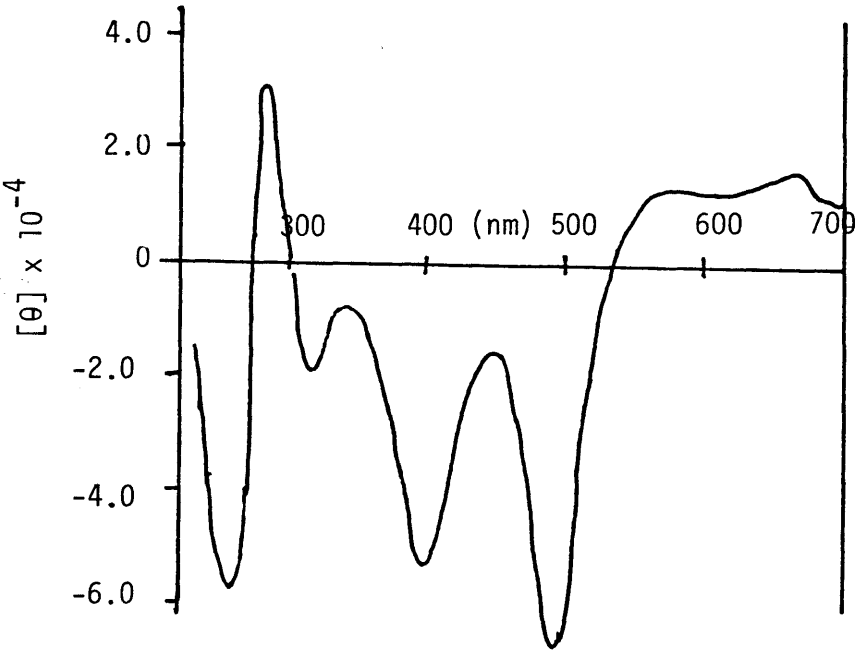


Fig. 4 CD spectrum of malvidin 5-glucoside anhydrobase (aggregated precipitates, 1×10^{-3}M) in water (pH 7.0).

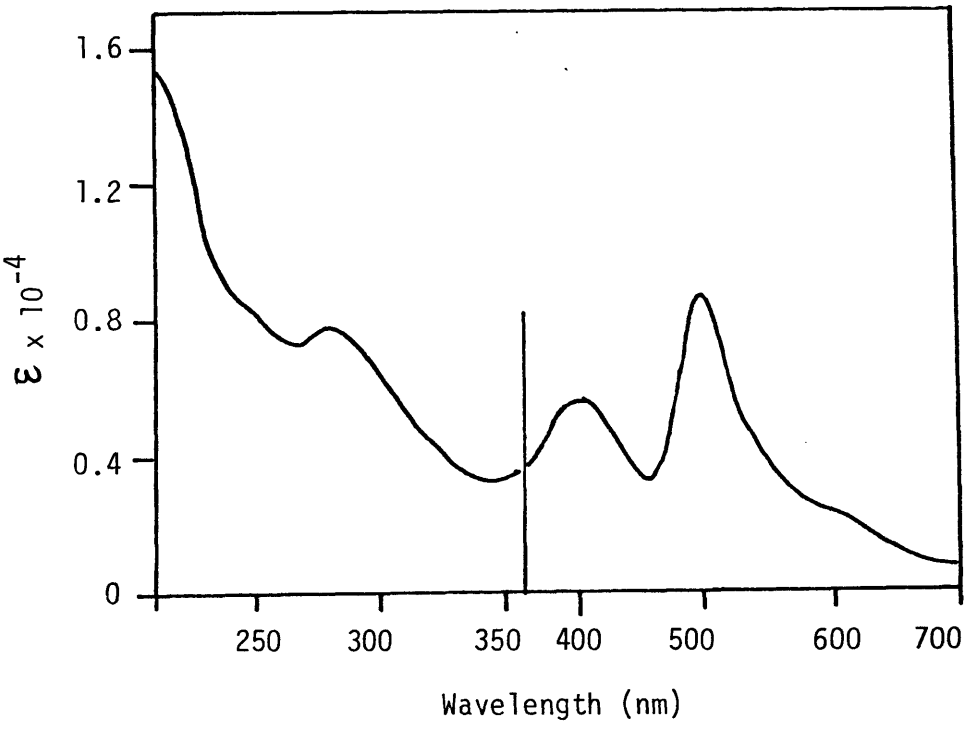


Fig. 5 aggregated precipitates of malvidin 5-glucoside anhydrobase ($1x10^{-3}M$) dissolved in water (pH 7.0).

3,5-diglucosides.

III. DISCUSSION

The concept of the self-association of anthocyanins has not been so far introduced, but should be widely accepted as one of factors of flower color variation. Why flowers are colored without decolorization, irrespective of a rapid conversion of colored anhydrobases to colorless pseudobases ? It would be well grounded that, in the natural state of cell sap, anthocyanins are present in a self-associated form.

For example, the anthocyanin pigment, malvin, was examined to be in a self-associated form. The pigment is found in the red petals of Lespedeza Thunbergii Nakai[3] (japanese name: miyaginohagi, one of aki-no-nanakusa in Japan). The phenomena that the petals are colored without decolorization and have red tone would be explained in terms of the self-association of the anthocyanin.

When the visible spectra were measured immediately after dissolution, in a dilute solution (5×10^{-5}M) the color was blue, while λ_{max} at higher concentration (5×10^{-3}M) was close to that of flavylium ion (red color) (Fig. 6).

The dramatic change of CD spectra was dependent on concentration(Fig. 7): both blue shift of λ_{max} shown in visible region and characteristically enhanced CD value.

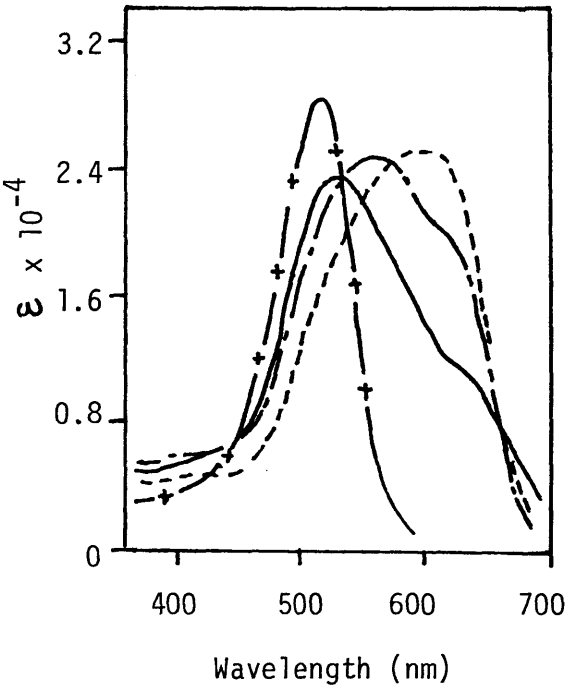


Fig. 6 Visible spectra of malvin chloride measured immediately after dissolution in phosphate buffer (pH 7.0, 0.1M) ——— : $5x10^{-3}$M, —·— : $5x10^{-4}$M, – – – : $5x10^{-5}$M

— + —: flavylium ion in N HCl ($5x10^{-5}$M)

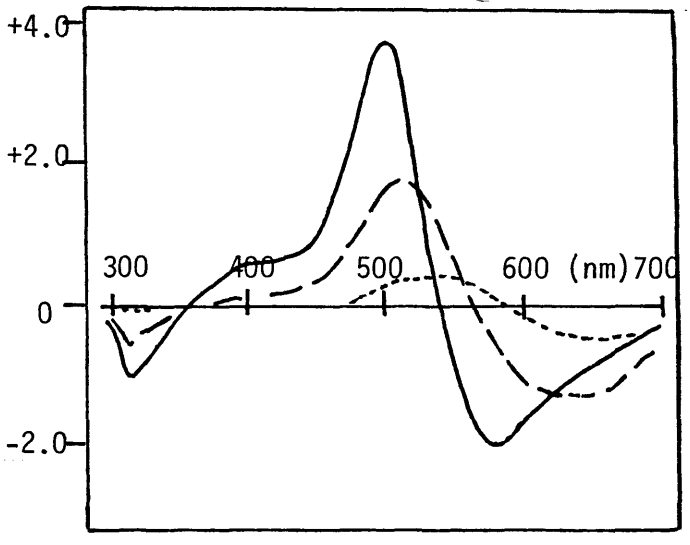


Fig. 7 CD spectra of malvin chloride measured immediately after dissolution in phosphate buffer (pH 7.0, 0.1M).
——— : 5×10^{-3}M, — — — : 5×10^{-4}M,
------- : 5×10^{-5}M.

The interactions of each of anthocyanin molecules are little in a dilute concentration, whereas larger in a higher concentration. Fig. 8 shows that in more diluted concentration of anthocyanins the pigment is more hydrated to colorless product, which suggests that the amount of hydration is concentration dependent of anthocyanins. The stability of 5×10^{-3}M suddenly became enhanced when compared with that of 5×10^{-4} or 5×10^{-5}M, which is significant from the view of the fact that anthocyanin concentration in cell sap is generally higher than 10^{-2}M.[4] These results suggest that malvin anhydrobase exists in a self-associated form in the flower petals. Other anthocyanidin 3,5-diglucosides are also capable of the self-association. Cyanin and pelargonin have the properties of aging effect (slowly fixed conformation), positive cotton effect, and blueing effect of the aggregates for the self-associtions, whereas malvin, delphin and hirsutin have a rapidly fixed conformation after dissolving, negative cotton effect and reddening effect (hypsochromic shift) of the aggregates for the self-association. It is of interest and significance that the two types for the self-association of anthocyanins are reversed in the characterization. Acylated anthocyanins such as awobanin, tibouchinin and violanin etc. are also capable of the self-association, because variations of Θ values (a decrease

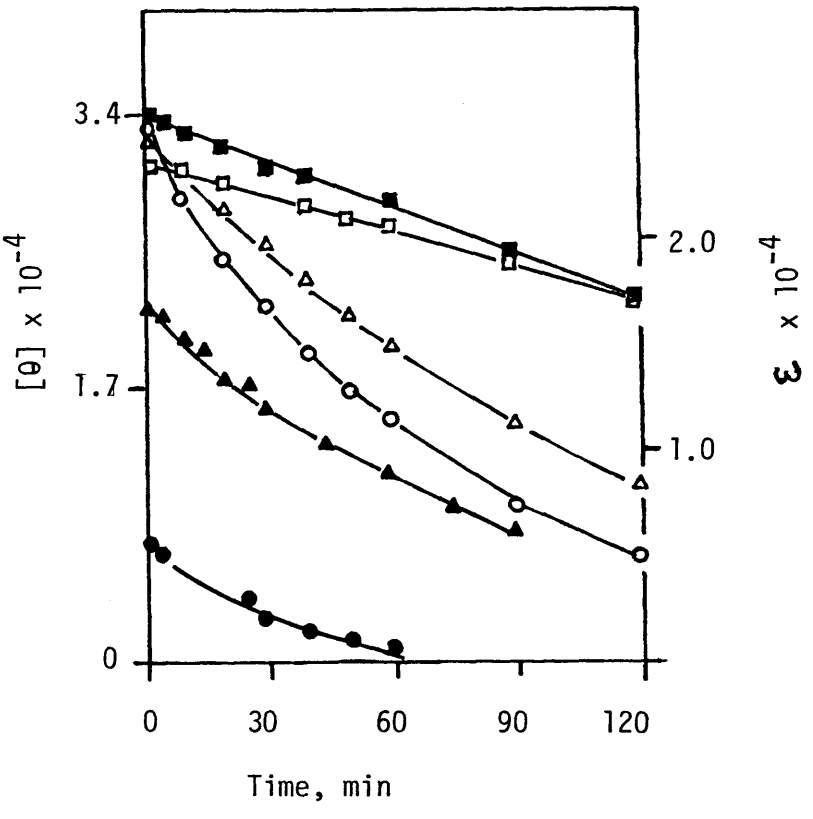
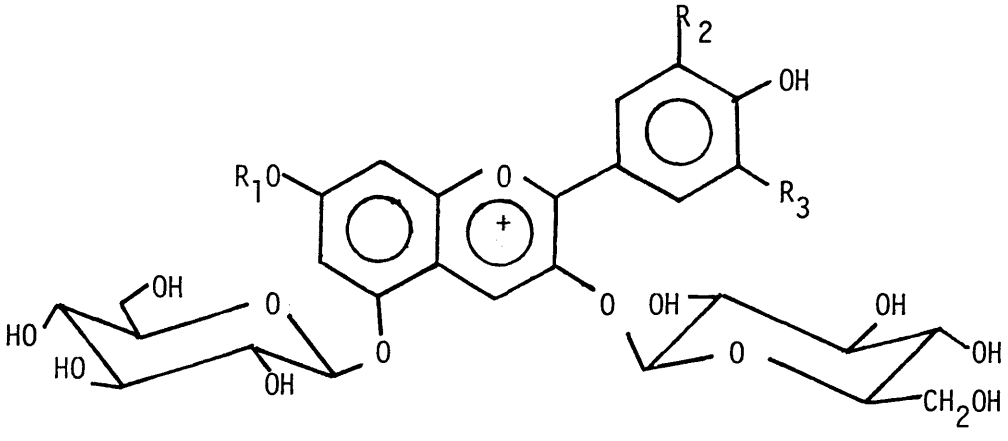


Fig. 8 Time course of visible absorption and CD at λ_{max} of malvin chloride in pH 7.0 phosphate buffer (0.1M).

white: visible abs., black: CD

□—■ : 5×10^{-3}M, △—▲ : 5×10^{-4}M, ○—● : 5×10^{-5}M



1) malvin	R_1=H, R_2=R_3=OCH_3
2) delphin	R_1=H, R_2=R_3=OH
3) pelargonin	R_1=R_2=R_3=H
4) hirsutin	R_1=CH_3, R_2=R_3=OCH_3

Chemical structures of anthocyanidin 3,5-diglucosides

for awobanin, an increase for tibouchinin and violanin) occurred by increasing anthocyanin concentration. These results further support the occurrences of the self-association of anthocyanins. In conclusion, the self-association of anthocyanins is spreading in nature.

As forces to attract anthocyanin molecules for the self-association, hydrogen bonds, van der Waals forces and hydrophobic interactions etc. would be supposed. Water is a highly structured liquid, with hydrogen bonds linking the individual molecules to each other. The structured arrangements of water molecules would have to be disrupted by any solute dissolved in water. Some hydrogen bonds would have to be broken: if the solute is polar, new hydrogen bonds between water and the solute would be formed, but if the solute is non-polar, such as hydrocarbon, and unable to form hydrogen bonds, the net result expected would be the formation of "structured water" surrounding dissolved hydrocarbon molecules or groups. Urea, although capable of participating in hydrogen bond formation, acts mainly to reduce the intermolecular order of water surrounding hydrocarbons. DMSO (dimethylsulfoxide), an aprotic solvent, also acts similarly with urea. The characteristic CD of cyanin aggregates precipitated from neutral solution was lost by the addition of urea or DMSO. As shown in Figs. 9 and 10, addition of urea or

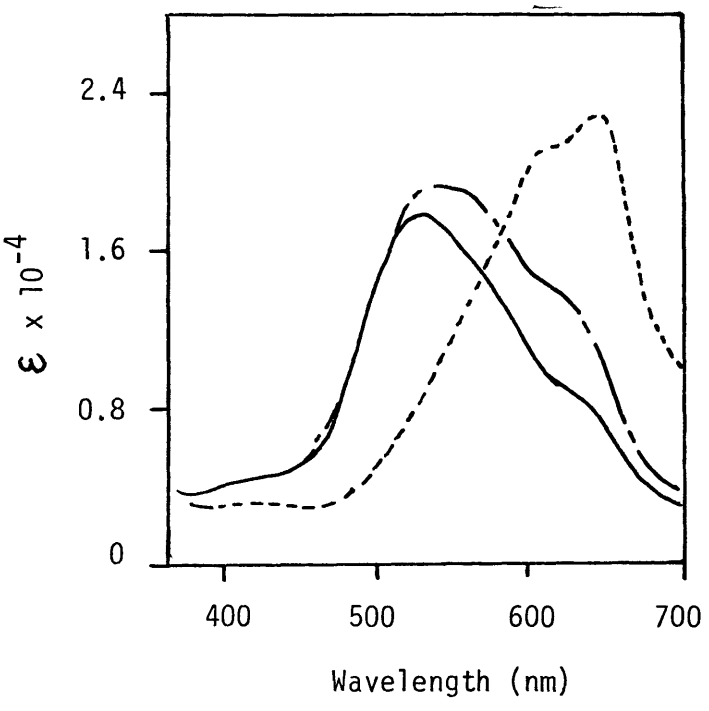


Fig. 9 Effect of urea and dimethylsulfoxide on the absorption of malvin in phosphate buffer, pH 7.0.
——— : 5×10^{-3}M, —·— : + 4M urea,
----- : + DMSO (50%).

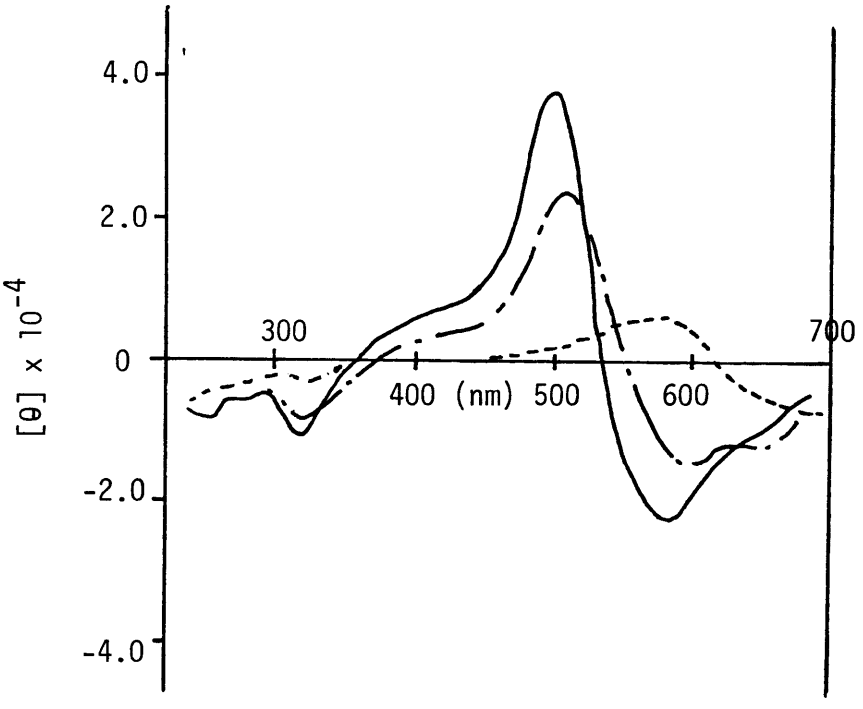


Fig. 10 Effect of urea and DMSO on the CD spectra of malvin in phosphate buffer, pH 7.0.

——— : 5×10^{-3}M, —- — : + 4M urea,

------ : + DMSO (50%).

DMSO gave a red shift and an increase of ε value in visible absorption, and gave a decrease of θ value, which are indicative of disaggregation of the anthocyanin anhydrobase. In addition, malvin anhydrobase at 5×10^{-4} M is stable for 1 hour in 4M $MgCl_2$, but in the solvent of 4M $MgCl_2$ containing 8M urea the λ_{max} absorbs at longer-wavelength and the stability becomes lower (Fig. 11). DMSO and urea disrupted "structured water" surrounding the aggregates to weaken the attractive force for the self-association. The disaggregating mechanism involved in the process would support that the self-association would arise from hydrophobic interactions.

Changes of optical properties observed in visible absorption and CD with increments of anthocyanin concentration are hypochromism, characteristic appearance of CD and shift of λ_{max} to shorter wavelength (malvin and delphin) or longer wavelength (cyanin and pelargonin). These changes are generally interpreted in terms of dimers, trimers, and larger polymeric species at higher anthocyanin concentration. These optical properties would be due to the interactions of each of anthocyanidin nucleus (interactions of each chromophores). Driving force for the self-association is mainly hydrophobic effect, which was infered from the disaggregating mechanism of urea or DMSO. Anthocyanins are aromatic compounds with

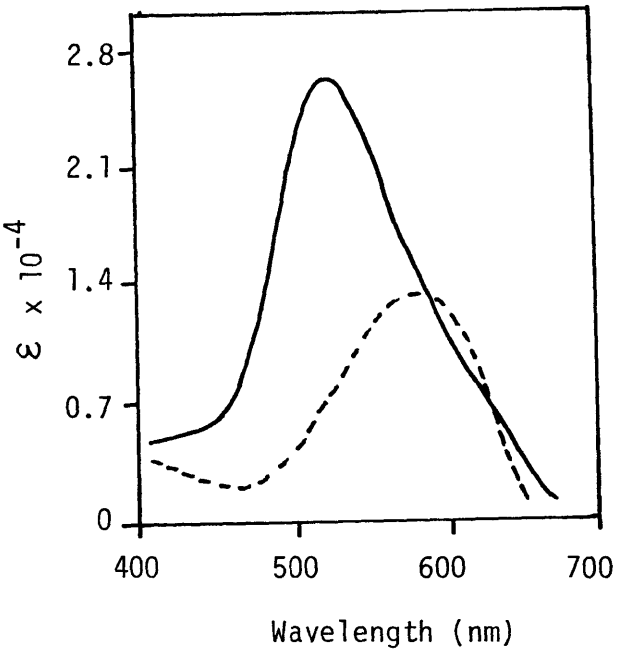


Fig. 11 Effect of 8M urea on the absorption of malvin anhydrobase (5×10^{-4}M).
in 4M $MgCl_2$ (stable): ———
in 4M $MgCl_2$ containing 8M urea (60 min after dissolution): ------

glucose moiety(ies) surrounding anthocyanidin nucleus. Thus, aggregates of the self-association would be stacked anthocyanidin molecules. This concept is widely appreciated in the formation of stacked base-base pairs of nucleic acids. It is well known that the formation of the base-base pairs causes hypochromism and an increase in CD.[5] Indeed, the aggregates formation of cyanin and delphin anhydrobases causes a remarkable decrease in ε value (Figs. 3 and 12) and exceptionally large molecular ellipticities (Fig. 2 for cyanin aggregates, -270,000 at 540 nm for delphin aggregates). Other anthocyanin, such as malvin, was examined to have a hypochromic effect by the self-association. As shown in Figs. 6 and 8, a decrease in ε value measured immediately after dissolution was slight when malvin anhydrobase concentration increased. Since anthocyanins, generally, are rapidly transformed from colored anhydrobases to colorless pseudobases, it is difficult to evaluate whether a change of ε value caused by the self-association occurs or not. The author et al.[6] have found that concentrated solutions of some neutral salts such as 4M $MgCl_2$ or 4M NaCl strongly stabilize anthocyanin anhydrobases. Malvin chloride was dissolved in MeOH, a few drops of 0.5N NH_4OH were added to the solution to change the red flavylium ion to purple anhydro-base, and then the solution was quickly dried over P_2O_5

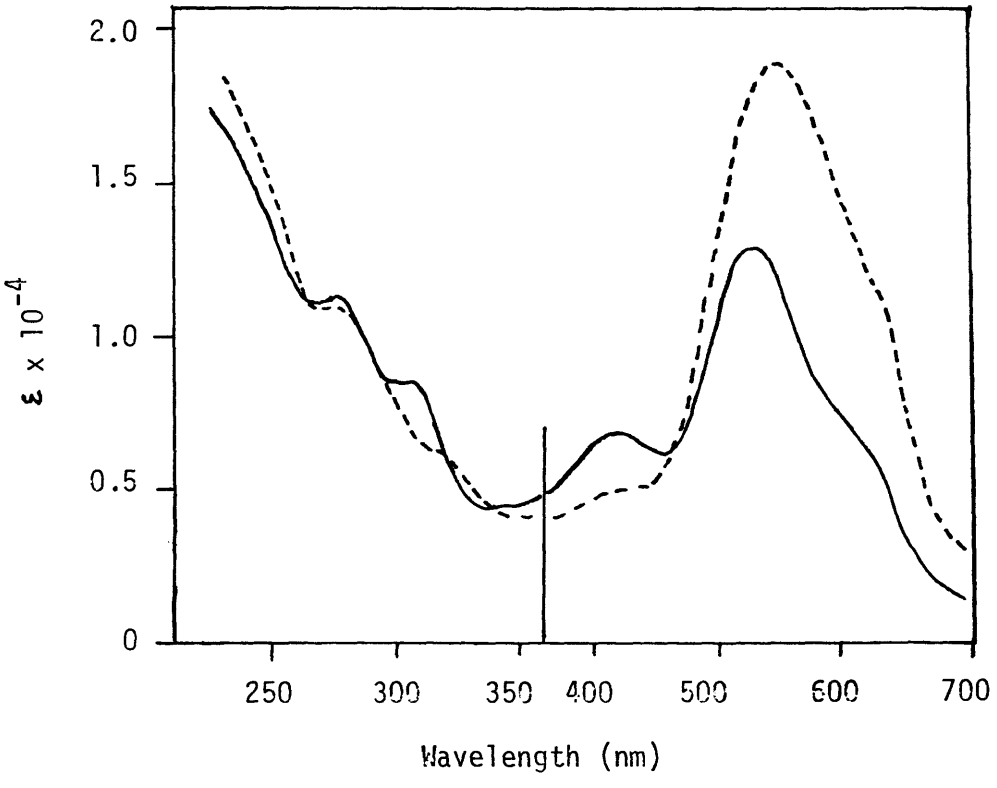


Fig. 12 ------ within 2 min after dissolving delphin chloride ($5x10^{-4}$M) in pH 7.0 phosphate buffer (0.1M).

——— aggregated precipitates of delphin anhydrobase ($9.6x10^{-4}$M) dissolved in water (pH 7.0).

in vicuo. Malvin anhydrobase obtained in this way was dissolved in 4M $MgCl_2$. The visible absorption spectra of malvin anhydrobase in 4M $MgCl_2$ are shown in Fig. 13. The visible absorption of dilute concentration (5×10^{-5}M) was not down for 30 min., which suggests that the anhydrobase is not transformed to colorless product in 4M $MgCl_2$ for that time. Fig. 13 shows that the self-association of malvin brings about a hypochromic effect.
In conclusion, the phenomenon of the hypochromism is generally found for the self-association of anthocyanins.

The author will propose trimeric structure of cyanin anhydrobase shown in Fig, 14. This model allows the overlaping of the aromatic systems (hydrophobic parts) surrounded by glucose moieties (hydrophilic portion). The concept of the stacking structure would be further supported by the comparison of the degree of the self-association of delphin with that of malvin anhydrobase. Because the slope of [θ]/molar concentration of delphin is larger than that of malvin anhydrobase (Fig. 15), the degree of the self-association of delphin is higher than that of malvin. To that order, steric hindrance of CH_3 groups of malvin to the stacking would be introduced (Fig. 16). Methyl groups do not allow the close approach of hydrophobic parts of neighboring anthocyanin molecules.

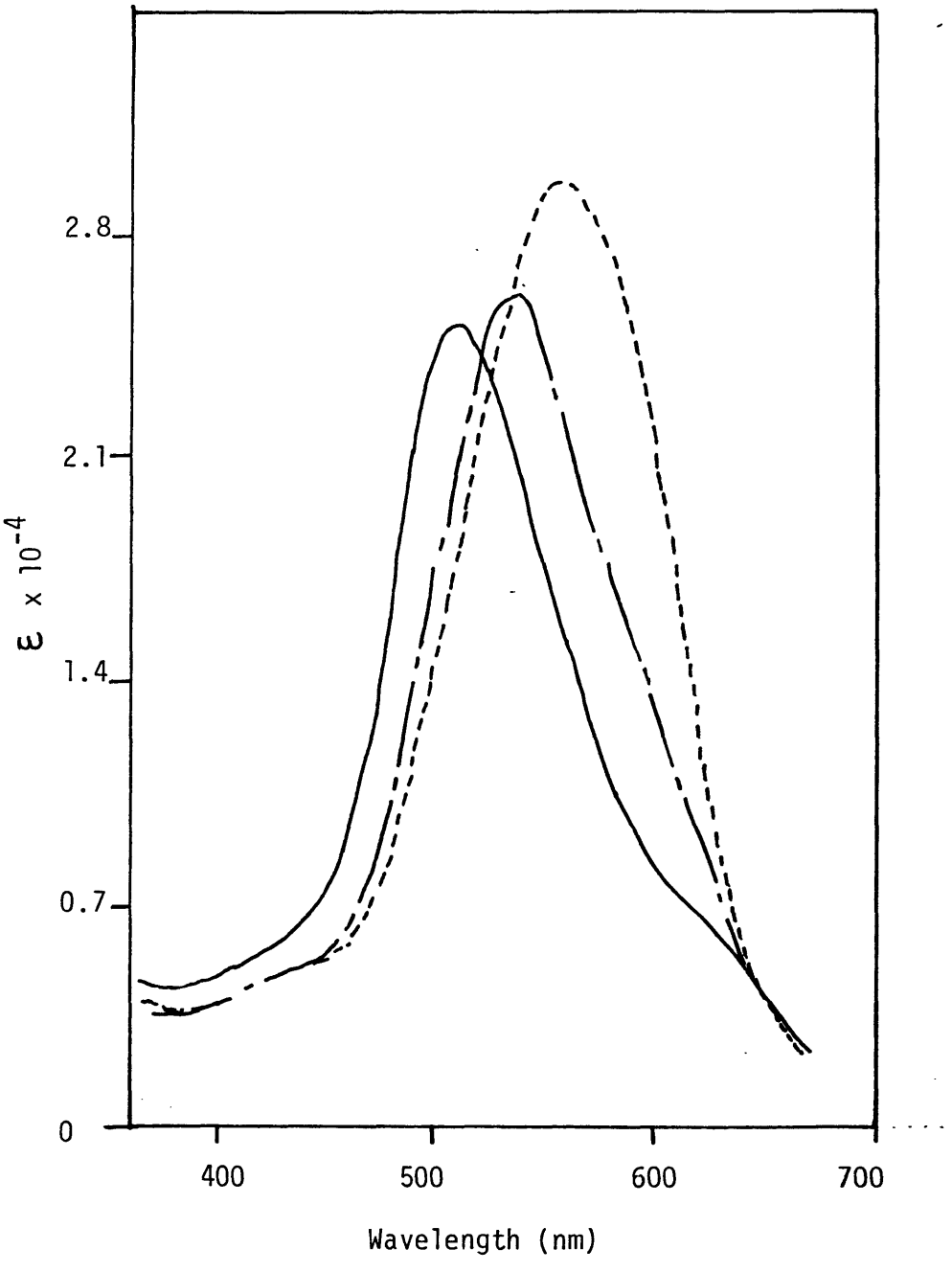


Fig. 13 Visible spectra of malvin anhydrobase in 4M $MgCl_2$.
——— : $5x10^{-3}M$, —·— : $5x10^{-4}M$, ------ : $5x10^{-5}M$.

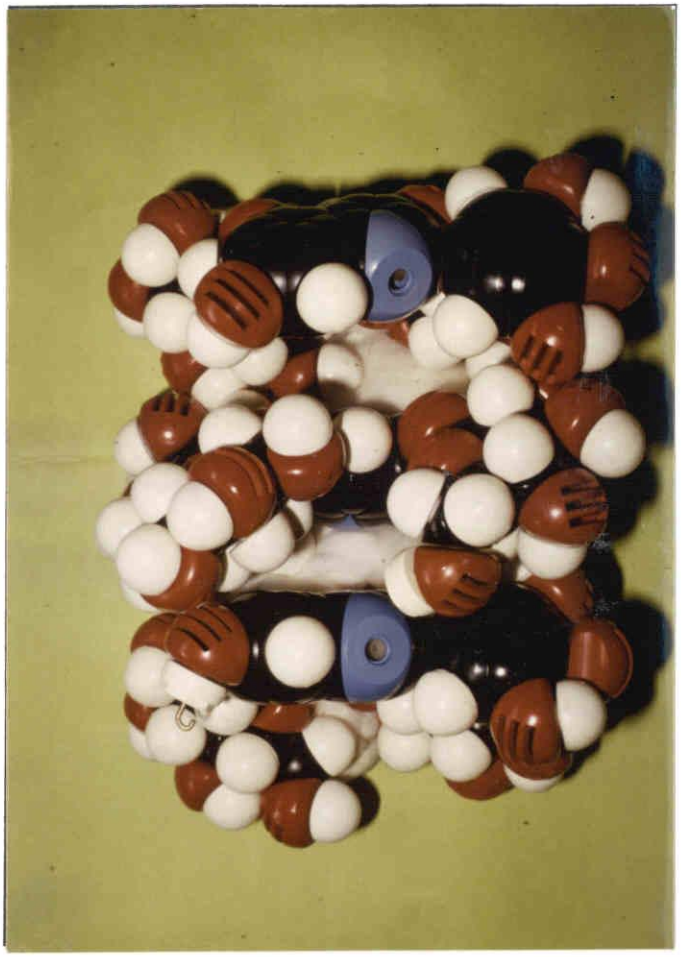


Fig. 14 Trimeric structure of cyanin anhydrobase

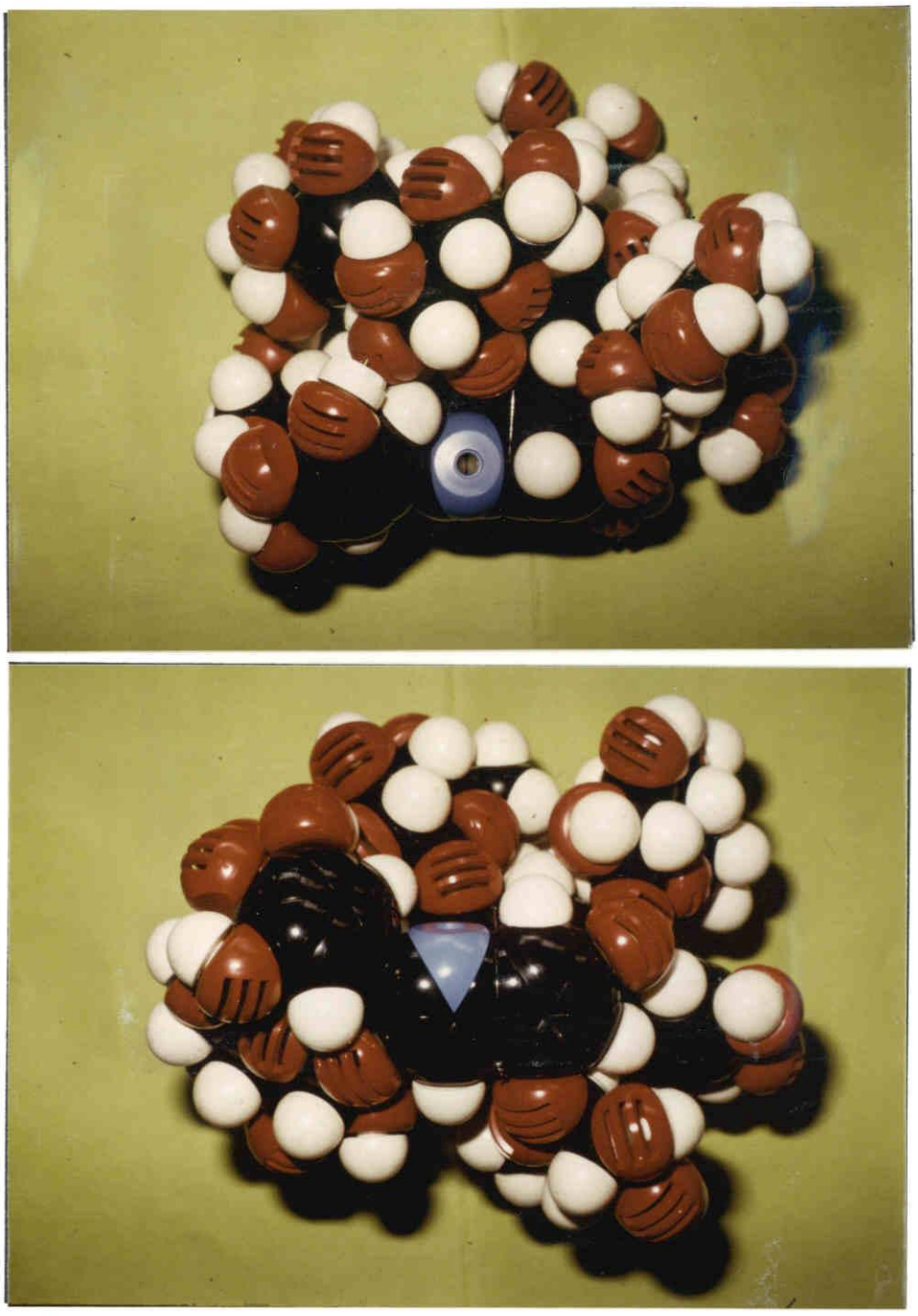


Fig. 14

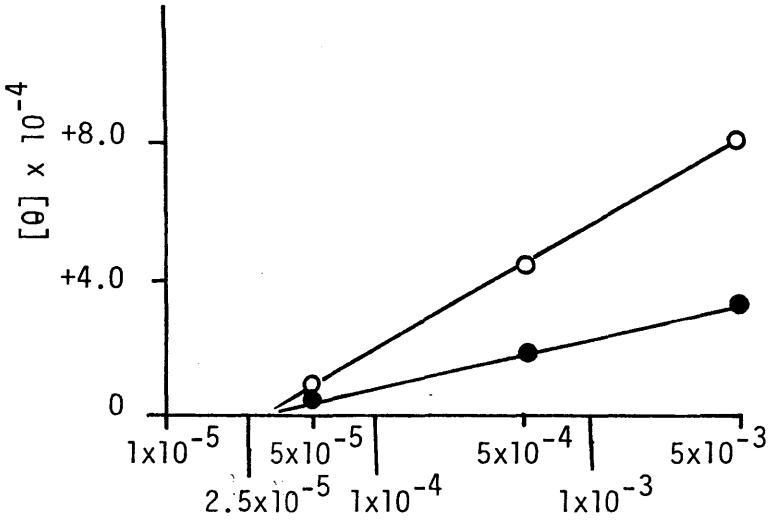


Fig. 15 CD values at λ_{max} measured immediately after dissolution in pH 7.0 versus molar concentration of anthocyanins.

○——○ : delphin, ●——● : malvin.

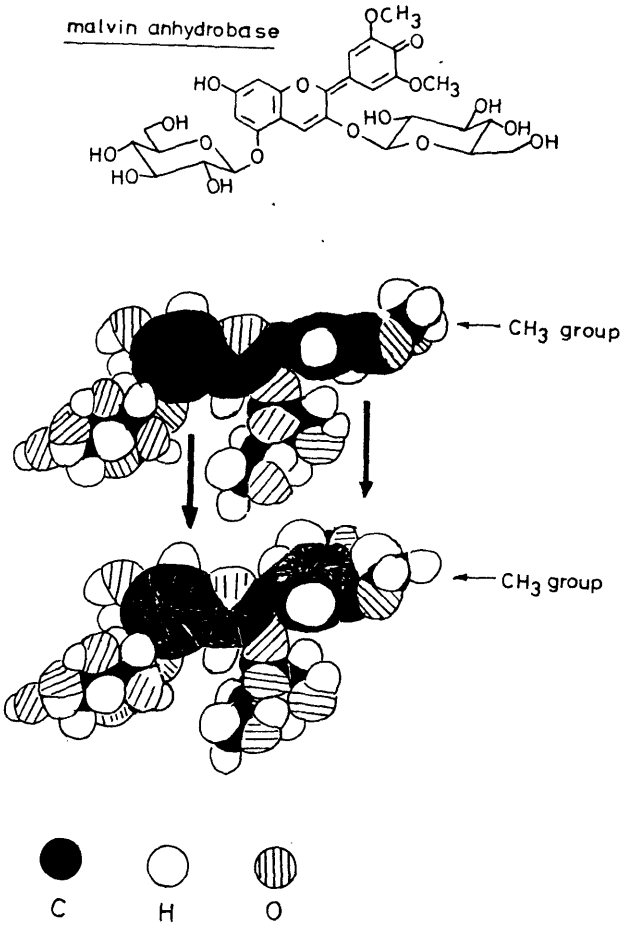


Fig. 16 CH_3 groups of malvin anhydrobases do not allow the close approach to have a tight complex formation.

The glucose moiety at 5-position of anthocyanidin nucleus has an important role in the self-association. The glucose moiety may be necessary for anthocyanin molecules to attain the ingenious arrangement of the stacking of anthocyanidin 3,5-diglucosides.

Origin of the large optical ellipticities would come from molecular dissymmetry of each of the anthocyanin molecules whose conformation is fixed in some way, or a helical conformation of thread-likely aggregated anthocyanin molecules. Chirality induced by the self-association would be attributable to the reduction in planarity of anthocyanidin rings: twist of phenyl groups in some way (Fig. 16). The highly aggregated cyanin anhydrobases have an exceptionally large molecular ellipticities (Fig. 12). Although the association degree of the cyanin aggregates is not determined, it is considered to be greatly high because of its large [θ]. Accordingly, the cyanin aggregates possibly aquire a helical conformation in which anthocyanin molecules become thread-likely stacked. This concept must be further demonstrated by other experimental evidence.

Concentrated solution of some neutral salts such as 4M NaCl strongly stabilizes anthocyanin anhydrobases.[6] It was examined whether the stabilizing mechanism would come from the enhanced degree of the self-association

of anhydrobases caused by inorganic salt effect (high ionic strength). Addition of 4M NaCl to malvin anhydrobase gave larger θ value, hypsochromic shift and higher stability, which suggests that sodium chloride acts as the promoting agent for the self-association of anthocyanins, and the resulting higher stabilization occurs.

The occurrence of the self-association of flavylium ions are considered to be impossible for the sake of electrostatic repulsion, in contrast with non-charged anhydrobases. The author has found the surprizing phenomenon that malvidin 5-monoglucoside gradually decolorizes even in a strongly acidic medium when dissolved at dilute concentration (1×10^{-5}M). At higher concentration ($> 5 \times 10^{-5}$M) this pigment gradually precipitates as the red floccules. It was examined whether the protection of decolorization is due to the self-association of malvidin 5-monoglucoside in strongly acidic media. CD specrtra was dependent on the concentration of the pigment in strongly acidic media, which is suggestive of the occurrences of the self-association of the flavylium ions. Other anthocyanins are also capable of the self-association in strongly acidic media, because θ values increased with increments of concentration of flavylium ions.

IV. EXPERIMENTAL

INSTRUMENTS

Uv-visible absorption spectra were measured with a Hitachi 323 spectrophotometer.

CD spectra were determined with a Jasco J-500C spectropolarimeter equipped with a model DP-500 data processor. Generally, anthocyanins are rapidly transformed in neutral aqueous solutions from colored anhydrobases to colorless pseudobases. Therefore, for rapid measurements of CD spectra in the range of 200-700 nm within 60 seconds, the J-500C type was employed.

MATERIALS.

The purity of all isolated anthocyanins was examined spectrophotometrically.

CYANIN (cyanidin 3,5-diglucoside)

Cyanin chloride was obtained as follows: shisonin[7.8.9] (cyanidin 3-p-coumaroylglucoside-5-glucoside) isolated from Perilla ocimoides was partially hydrolyzed by a mild acid treatment and chromatographed on an Avicel column with n-BuOH-2N HCl (1:1) as an eluent.

SHISONIN (cyanidin 3-p-coumaroylglucoside-5-glucoside)[7.8.9]

The leaves of Perilla ocimoides were soaked in Et_2O

to remove yellow pigments. On separating of the yellow solution, it was then treated with methanolic HCl. The solution was evaporated under reduced pressure and precipitated by addition of Et_2O. From the aqueous solution of the chloride, the pigment was precipitated as its lead salt by basic lead acetate. The lead salts were washed well with MeOH, and dissolved in 5% HCl-MeOH and filtrated. The supernatant was precipitated by the adddition of Et_2O. The aqueous solution of the flavylium chloride was adsorbed on a polyamide column, and then washed well with HCl-H_2O (1 ml conc HCl/l) to remove impurities. Subsequently shisonin was eluted with HCl-MeOH (1 ml conc HCl/l). Further purification was attained by use of Avicel column chromatography with n-BuOH-2N HCl (1:1).

CYANIDIN 3-MONOGLUCOSIDE

Red leaves were soaked overnight in 0.3% HCl-MeOH and separated by filtration, and then the solution was evaporated to a small quantity of the solution, and allowed to stand overnight. Yellow precipitates were separated out. Filtration was carried out by use of Cellite. The supernatant was evaporated to dryness. Precipitation from the methanolic solution of the dried matters by the addition of Et_2O was carried out three times. From the aqueous solution of the precipitates, its lead salt was collected

by addition of basic lead acetate. The lead salts were dissolved in 10% HCl-MeOH and subjected to filtration and chromatography on Avicel column with AcOH-HCl-H_2O (5:1:40) as an eluent. Repetition of this chromatography was carried out to obtain higher purity of the anthocyanin. The absorption spectrum of the isolated anthocyanin (1% HCl-MeOH) lacked an absorption band at 300-310 nm and had a distinct shoulder in the 410-440 nm region. This is characteristic of an unacylated anthocyanin with a sugar at 3-position.[10] Addition of $AlCl_3$ to the solution offered longer-wavelength shift, which is indicative of the occurrence of o-dihydroxyl group in B-ring.[10] The complete structure was determined by the PMR spectrum according to the method of Goto et al.[11] (1 mg in 0.3 ml CD_3OD containing 2 µl of 20% DCl in D_2O at 70°, measured with JEOL JNM-FX 100 FT-NMR spectrometer, 100 MHz) δ 9.02 (1H,s, H-4), 8.22 (1H, q, J=2 and 9Hz, H-6'), 8.06 (1H, d, J=2Hz, H-2'), 7.04 ((1H, d, J=9Hz, H-5'), 6.94 (1H, d, J=2Hz, H-6), 6.72 (1H, d, J=2Hz, H-8), 5.29 (1H, d, J=7Hz, Glc anomeric H).

MALVIDIN 3-MONOGLUCOSIDE AND 5-MONOGLUCOSIDE

Malvin chloride (purchased from Aldrich) was partially hydrolyzed with aqueous 2N HCl in a water bath. The reaction mixture was subjected to Avicel TLC with

H_2O-HOAc-HCl (87:10:3), to monitor the reaction. The hydrolyzates were chromatographed on Avicel column with an eluent of the solvent to separate the mixture of malvidin 3- and 5-glucoside from both the starting materials and the aglycon. The fraction of the mixture of malvidin 3- and 5-glucoside was concentrated to a small quantity and allowed to stand overnight. Only malvidin 5-glucoside was gradually separated out and centrifuged. Pure malvidin 5-glucoside was finally attained by crystallization from 1% HCl. Malvidin 5-glucoside has the pink fluorescence under ultra-violet light.[12] The supernatant was subjected to Avicel column chromatography with an eluent of n-BuOH-2N HCl (1:1). The fraction of malvidin 3-glucoside was evaporated to dryness. Malvidin 3-glucoside lacks the fluorescence under UV light.[12]

UV-VISIBLE ABSORPTION AND CIRCULAR DICHROISM FOR THE SELF-ASSOCIATION

The weight of anthocyanin chlorides was measured on a microbalance. All anthocyanin chlorides to be investigated were dissolved in phosphate buffer (pH 7.0, 0.1M) so that the concentration of anthocyanins was 5×10^{-5}, 5×10^{-4} or 5×10^{-3}M. The spectra were determined at room temperature using cells having a path length of either 0.1, 1 or 10 mm which was employed at 5×10^{-3},

5×10^{-4} or 5×10^{-5}M of anthocyanin concentration, respectively. Variation of ε or Θ with times after dissolution was determined.

THE SPECTRA OF HIGHLY SELF-ASSOCIATED ANTHOCYANINS

CYANIN ANHYDROBASE AGGREGATES

Precipitates of cyanin anhydrobase was collected by centrifugation of the solution (2 ml of 5×10^{-4}M of cyanin chloride in phosphate buffer, pH 7.0, 0.1M) after allowing to stand for 2 hours. To the precipitates was added 0.5 ml of water and the mixture centrifuged. The pH of the supernatant was determined to be 7.0 with a Beckman model 72 pH meter. The concentration of the anthocyanin was determined spectrophotometrically after dilution of the supernatant with 3N HCl.

MALVIDIN 5-GLUCOSIDE ANHYDROBASE AGGREGATES

The similar procedure to cyanin anhydrobase aggregates was carried out. Precipitates of the pigment anhydrobase was collected by centrifugation of the solution (5×10^{-4}M in phosphate buffer, pH 7.0, 0.1M) after allowing to stand for 2 hr. To the precipitates was added 1 ml of water and centrifuged. The concentration of the anthocyanin was determined spectrophotometrically after dilution of the supernatant with 3N HCl. The acidified solution was examined whether the absorbance decreases or not.

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PART II

MOLECULAR COMPLEXES OF ANTHOCYANINS WITH FLAVONES

I. INTRODUCTION

A wide variety of blue colored flower species has been intensively investigated by many workers. The blueing of anthocyanin color in vivo by flavones and related substances, co-pigmentation, has been explained as a major factor of blue flowers. Bayer[1] and Hayashi et al.[2] have isolated natural blue pigments, i.e., protocyanin from blue corn flowers and commelinin from Commelina communis. These typical blue pigments contain anthocyanins combined with both metals and organic substances such as flavone or macromolecules. The typical blue pigments have strongly suggested to chemists the necessity of metal ion such as Mg^{2+}, Fe^{3+} or Al^{3+} to form the stable blue color. Indeed, blue corn flowers contain a metal complexed anthocyanin, cyanin.[3] Red corn flowers, on the other hand, are red presumably because their anthocyanins (pelargonidin glycosides) are incapable of complexing metals, although they contain adequate flavone and flavonol co-pigments.[4] Thus, metal complexing would still appear to be effective in blue corn flowers. It has been considered that both chelation of anthocyanins with some metal ions and co-pigmentation are responsible for the

blueing of anthocyanins of higher plants. The phenomenon of co-pigmentation, which is one of major factors to cause bluer color, is widely spreading. However, little is chemically known of the mechanism and the structure of co-pigmentation.

Commelinin is one of the blue pigments which has made chemists accept the concept of metal complexing. However, the author's studies on commelinin have clarified that the metal ion, magnesium, is not necessary for the formation of the blue color of commelinin. The author also proposed that the color is due to a stacked molecular complex of the anthocyanin and the flavone.

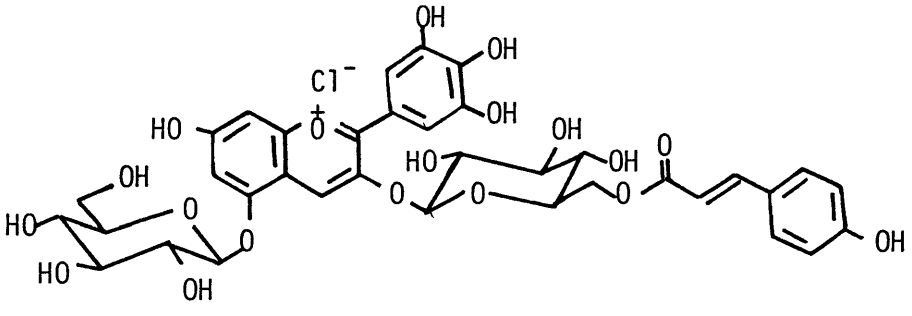
Origin of the extraordinary stability and blueness of commelinin was investigated. Awobanin, one of components of commelinin, is a unique acylated anthocyanin with p-coumaroyl group. Little is known of the effects of the acyl groups of anthocyanins on co-pigmentation. The author demonstrated that the acyl groups of anthocyanins have an important role in the stability and blueing effect of anthocyanin-flavonoid co-pigment complexes and reported that the stability and blue color of commelinin is ascribed to the effect of the aromatic acyl group of awobanin and found that the acylglucose moiety at the 3-position of the anthocyanidin links the flavone to the anthocyanin and with its hydrophobic nature, it tightens the bonding

between the two flavonoid units.

II. CONSTITUTION OF COMMELININ

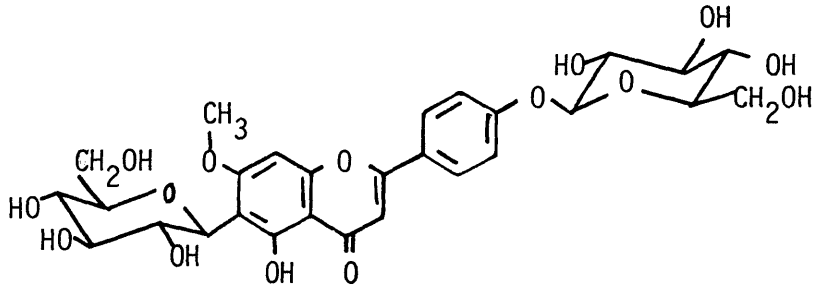
Commelinin, which is obtainable from petals of Commelina (japanese name: tsuyukusa), is one of the typical sky-blue flower pigments.[5,6] It contains no high molecular components such as peptides or polysaccharides. Hayashi et al.[7] assumed that it consists of two molecules each of an anthocyanin, awobanin (1)[8], and a flavone, flavocommelin (2)[9], and one atom each of Mg and K. They explained its color and stablity in terms of co-ordinated complex of Mg and four molecules of the flavonoids; K being not an essential component (Fig. 1). Bayer[10] opposed this explanation, however because in general Mg^{2+} does not form stable chelates with anthocyanins; he suggested the presence of Fe^{3+} or Al^{3+} in this pigment. No such trivalent metal ions, however, are involved as is evident from the analysis of Hayashi et al.[7] Natural commelinin does contain Mg as reported by Hayashi et al.,[7] but no evidence has been given whether Mg is essential for the formation of the complex. To clarify this point, the author has examined reproduction of the pigment from awobanin and flavocommelin under the complete absence of Mg^{2+}.

Freshly prepared awobanin chloride was treated with



AWOBANIN CHLORIDE (1)

delphinidin 3-(6-O-p-coumaroyl)glucoside-5-glucoside



FLAVOCOMMELIN (2)

4'-glucosylswertisin

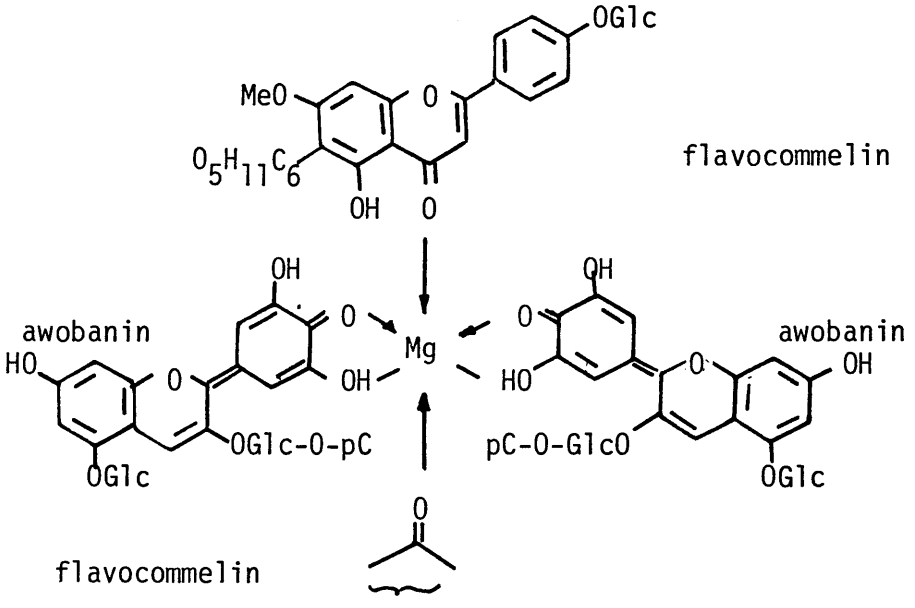


Fig. 1 STRUCTURE OF COMMELININ PROPOSED BY HAYASHI & TAKEDA , 1970

aq. ammonia to produce awobanin anhydrobase. To a concentrated aqueous solution of awobanin anhydrobase was added crystalline flavocommelin and the mixture was stirred at room temperature. Although the flavone is almost insoluble in water, it rapidly dissolved in the solution by formation of a complex. The resulting mixture was passed through a column of Sephadex G-10 to remove the starting materials, and the blue pigment was precipitated from the eluates by addition of ethanol. Commelinin thus prepared showed UV (Fig. 2), IR (Fig. 3), and CD spectra (Fig. 4) superimposable to those of natural commelinin. The characteristic CD spectrum (Fig. 4) has a strong diagnostic value for the formation of complex, since only weak CD is observed with the components, awobanin and flavocommelin. Mg content of this synthetic pigment was given by atomic absorption spectroscopic analysis to be 0.013%, which is far less than that expected from the theoretical value of 1 atom Mg in the complex (calculated value 0.87%; Mg content in natural commelinin 0.5-0.7%). Thus, evidently Mg is not an essential component to produce the blue color of commelinin.

To remove Mg from natural commelinin, dialysis in 4M NaCl was carried out, in which commelinin does not decompose.[11] Mg contents by the dialysis were as follows: no dialysis (0.51%), after 12 hours (0.26%),

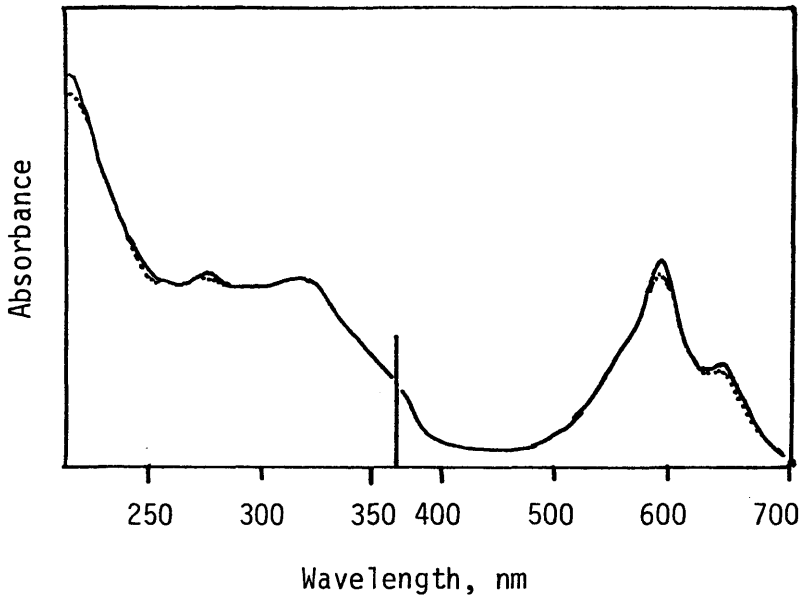


Fig. 2 Uv-visible spectra of natural and synthetic commelinin in water, path length 0.1 mm

——— : natural commelinin

----- : synthetic commelinin

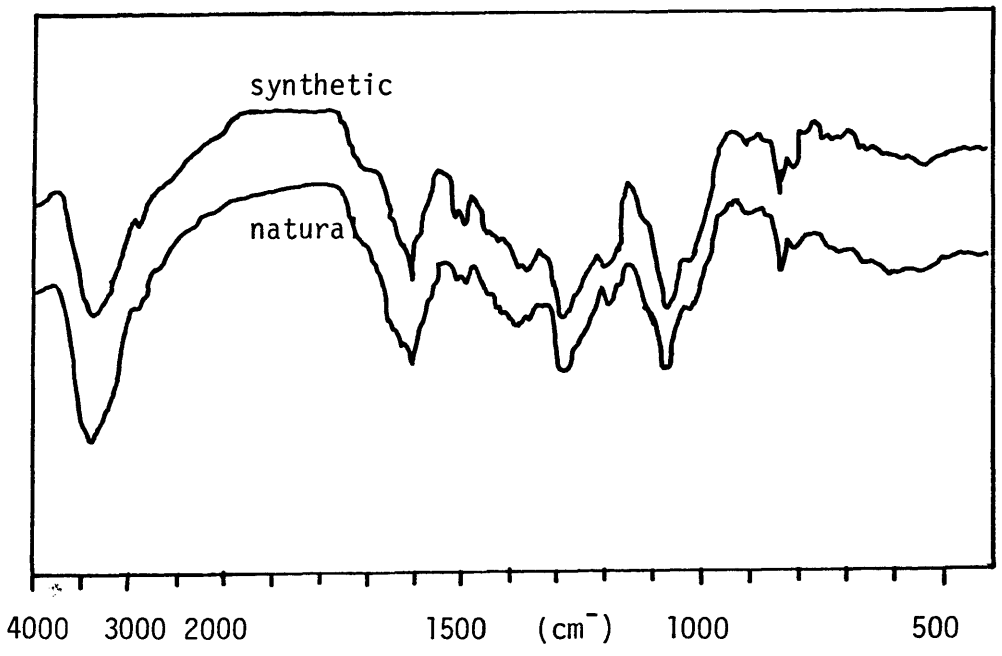


Fig. 3 IR spectra of natural and synthetic commelinin (KBr disc)

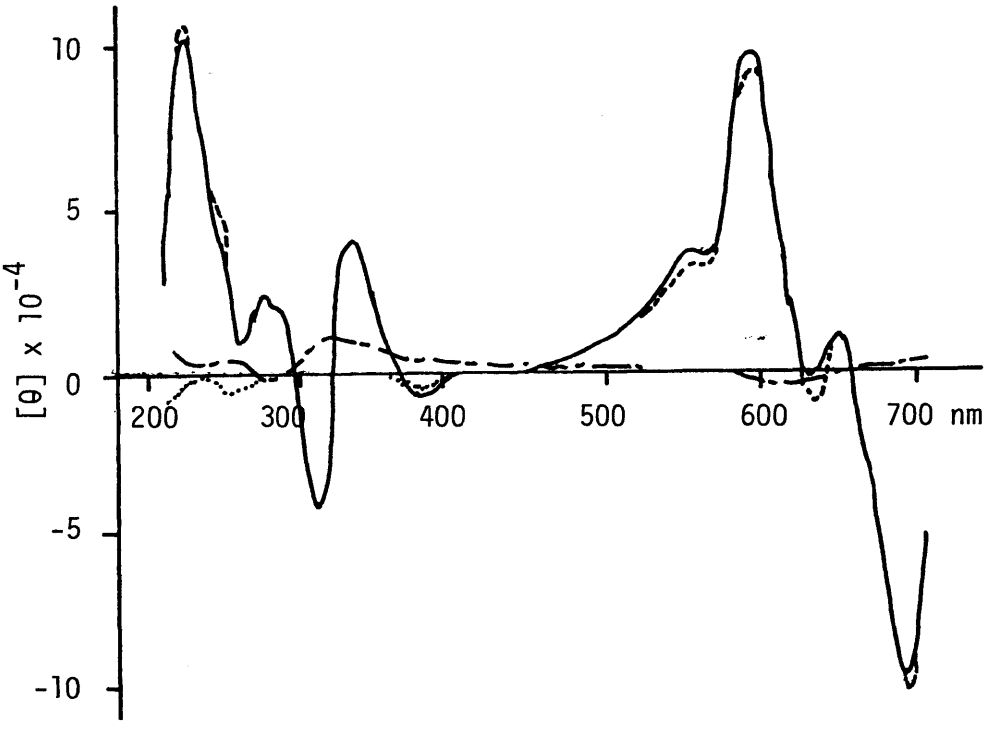


Fig. 4 CD spectra of natural and synthetic commelinin in water, pathlength 0.1 mm.

———— : natural commelinin

——— : synthetic commelinin

—·— : awobanin anhydrobase

····· : flavocommelin

after 24 hours (0.25%). Mg in the pigment was decreased to about half content. Further reduction in Mg content by the dialysis was unsuccessful, possibly because of the contamination of magnesium ion in the concentrated aqueous solution of NaCl.

Commelinin tends to include magnesium ion. Natural commelinin dissolved in 1M $MgCl_2$ was precipitated by addition of ethanol (1). The pigment thus obtained was dissolved in water and again precipitated with ethanol (2). Further purification was attained by the repetition of the precipitation (3). Mg contents thus prepared were as follows: (1) 1.7%, (2) 1.4%, (3) 1.4%.

Although commelinin has the nature for Mg^{2+} to be involved in the complex, Mg^{2+} is not essential component for the complex formation and possibly would form a salt similarly with K ion. Indeed, commelinin has negative charge(s) in aqueous solution; it moves electrophoretically toward the anode at weakly-acidic-neutral pH rang where the color of the pigment does not change from blue to red.

Fig. 5 shows the stability of commelinin (2.56×10^{-5} M as MW 1400) in aqueous solution. Commelinin rapidly decomposes in a dilute concentration and gradually goes into an equilibrium state between association and dissociation of the components, but the complete equilibrium is

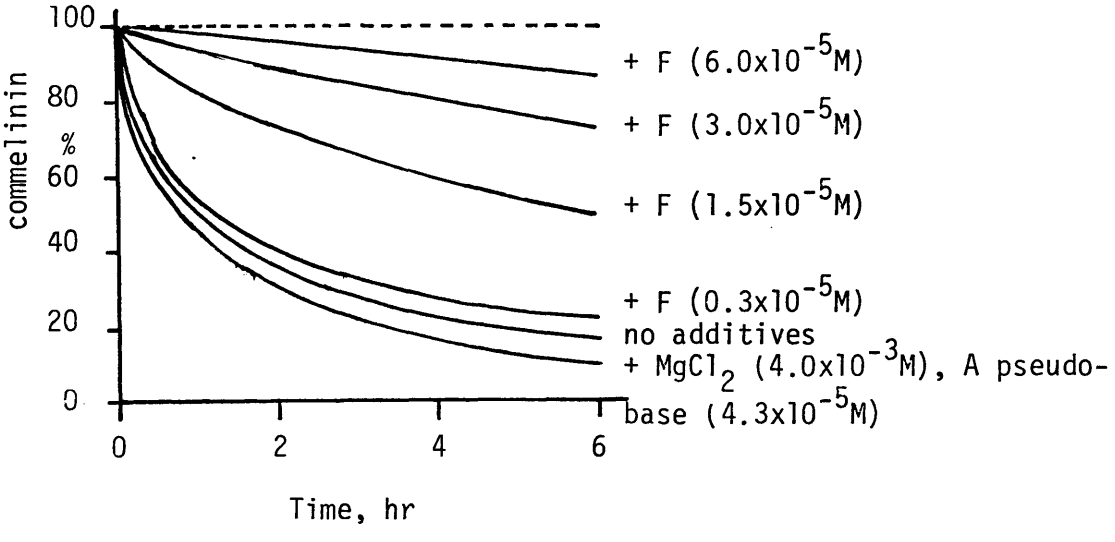


Fig. 5 Effect of flavocommelin, awobanin, and Mg^{2+} on the stability of commelinin

commelinin (as AF M. W. 1400) 2.56x10^{-5}M in 0.01M phosphate buffer pH 6.5 at 20°

not accomplished, which suggests that hydration rate of the anhydrobase to form the pseudobase is rapid, that is, the equibrium between the formation of pseudobase and that of anhydrobase leans toward the formation of pseudobase, or suggests that further decomposition of pseudobase to an unknown compound[12] occurs when left for a long time.

These mechanism are shown as

$$(AF)_n \rightleftharpoons nA + nF$$

$$nA \rightleftharpoons n\text{-}AOH$$

$$AOH \longrightarrow \text{unknown compound}$$

Here, A, F, and AOH represent awobanin anhydrobase, flavocommelin, and awobanin pseudobase, respectively. Addition of awobanin pseudobase (AOH, 4.3×10^{-5}M) or magnesium chloride (4.0×10^{-3}M) gave no effect on the stability of commelinin. That is to say, the pseudobase or magnesium ion does not promote the association of the components to form the complex. This evidence further suggests the unnecessariness of magnesium for the blue complex formation. In addition, the rate of decolorization of synthetic commelinin (Mg content: 0.013) was completely identical to natural one, which strongly supports no ability of Mg^{2+} to associate two flavonoid unit. On the other

hand, addition of flavocommelin strongly retards the dissociation of commelinin, which suggests that awobanin anhydrobase molecules tend to trap the flavocommelin to form the tight complex. This mechanism is shown as

$$A + F \rightleftharpoons AF$$

$$AF + F \rightleftharpoons FAF$$

$$(AF)_n + F \rightleftharpoons (AF)_n\text{-}F$$

Commelinin is considered to be monomeric, dimeric, or larger polymeric AF unit(s). The association degree was investigated by the determination of both dissociation constant and molecular weight by centrifugation method.

Fig. 6 shows concentration dependence of dissociation of commelinin in water. Completely reversible reaction between association and dissociation was not attained because of rapid hydration rate of the anhydrobase. If their points of 3 hours after dissolution are regarded as the equilibrium,

$$(AF)_n \rightleftharpoons nA + nF$$

$$K = \frac{[A]^n [F]^n}{[AF]_n} \cdot M$$

here, n=1, 2, 3,$\cdots$,n and [A]=[F]

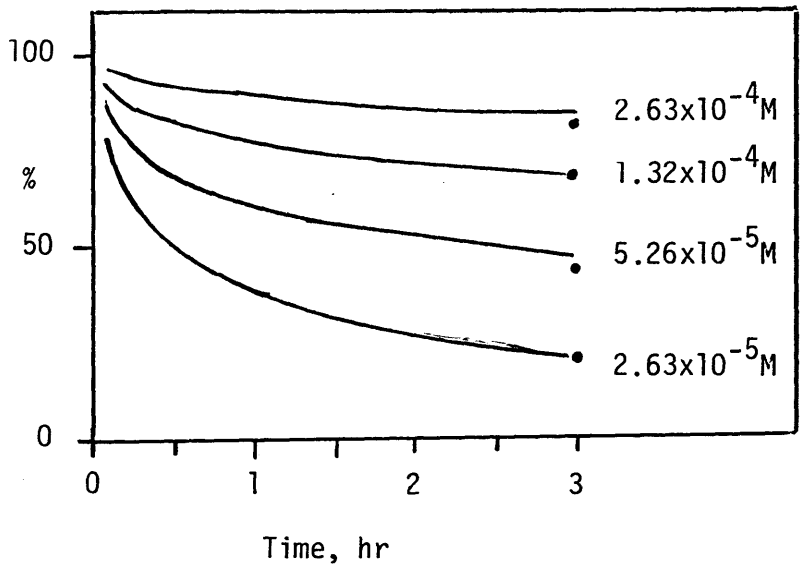


Fig. 6 Concentration dependence of dissociation of commelinin in water.

the number of n yields 2 in order that founded values may be satisfied with calculated ones. Details of the calculation are shown in Chapter 6 of my paper for the degree of Master.

Determination of molecular weight of commelinin by centrifugation method is shown in Fig. 7. Commelinin does not decompose in 3M NaCl.[11] Molecular weight in 3M NaCl was measured to be 1400-5600, mainly 4100 ± 400. Details are shown in Chapter 6 of my paper for the degree of Master. The different methods show that the composition of commelinin is around $(AF)_2$-$(AF)_3$.

In conclusion, commelinin is composed of only organic compounds, awobanin (an anthocyanin) and flavocommelin (a flavone), and constructed from $(AF)_2$-$(AF)_3$ units. The extraordinary stability and blueness of commelinin must be explained only by the terms of copigmentation. The origin is discussed in chapter 3.

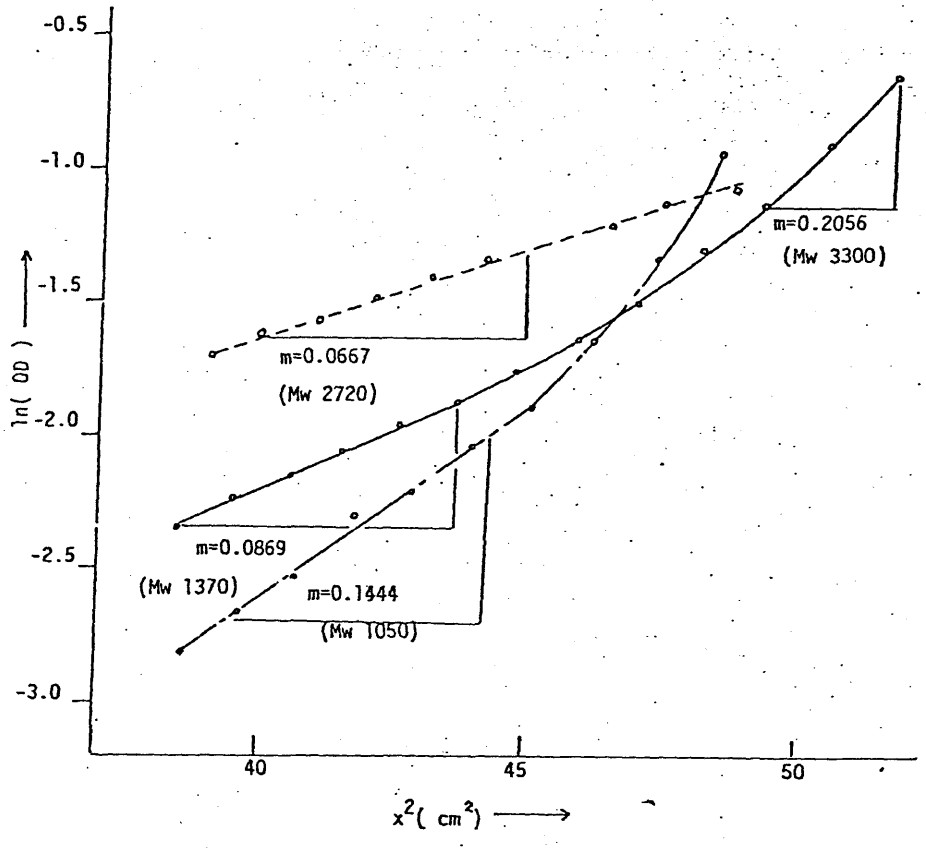


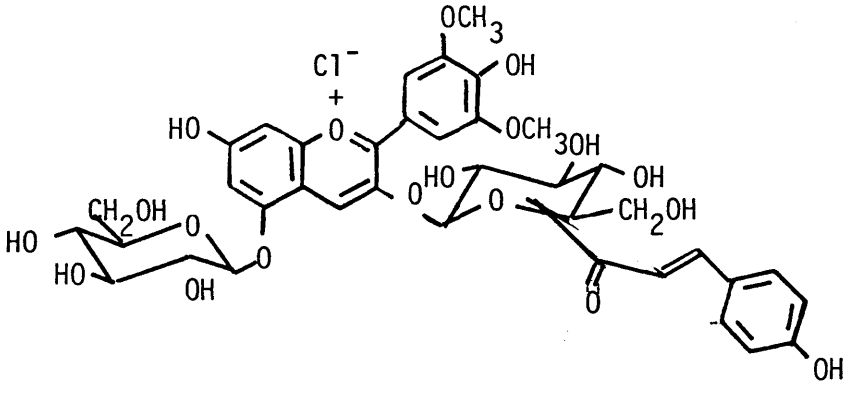
Fig. 7: Molecular Weight determination

$$m = \frac{d\ln C_x}{dx^2}$$

III. EFFECT OF ACYL GROUP OF AWOBANIN ON THE STABILITY AND BLUENESS OF COMMELININ

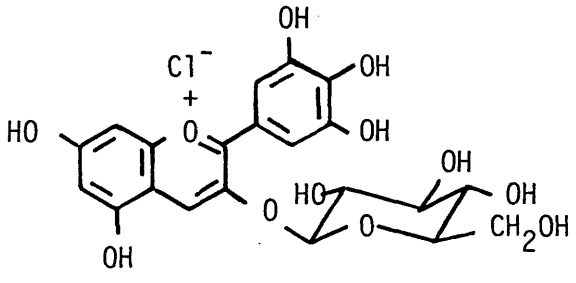
Awobanin, one of components of commelinin, is a unique and different anthocyanin from the ordinary 3,5-diglucosides; which is a monoacylated anthocyanin with p-coumaroyl group. Little has been known of the effects of the acyl groups of anthocyanins on co-pigmentation and no reports on the effect has been appeared. Commelinin has the extraordinary stability in aqueous solutions, compared with other anthocyanin complexes. Hayashi et al. explained its stability in terms of the magnesium chelation. As discussed in chapterII, the author has clarified that magnesium ion does not contribute to the stability. The author has found that the origin of the stability and blueness is attributable to the effect of acyl group of the anthocyanin on the co-pigmentation.

Acylated anthocyanins which have a p-coumaroylglucose moiety at the 3-position of the anthocyanidin nucleus and their deacylated anthocyanins were used to examine the effect of the acyl group on co-pigmentation. Anthocyanins used in this experiment were: awobanin (delphinidin 3-p-coumaroylglucoside-5-glucoside) (A) (1), tibouchinin (malvidin 3-p-coumaroylglucoside-5-glucoside) (T) (3),



TIBOUCHININ CHLORIDE (3)

malvidin 3-p-coumaroylglucoside-5-glucoside



DELPHINIDIN 3-MONOGLUCOSIDE (Dp-G) (4)

delphin (delphinidin 3,5-diglucoside) (D), delphinidin 3-monoglucoside (Dp-G) (4), malvin (malvidin 3,5-diglucoside) (M). Flavocommelin (4'-glucosylswertisin) (F) (2) was utilized as the co-pigment. Malvidin-based anthocyanins are incapable of metal chelation, because of the absence of o-dihydroxyl system in B-ring.

Fig. 8 shows the visible absorption spectra of TF and MF complexes measured 2 hours after dissolving in phosphate buffer (pH 6.0) at a variety of molar ratios of co-pigment to anthocyanin (conc. 5×10^{-4}M). Both malvidin-based anthocyanins, T and M, were stabilized with F, the acylated anthocyanin (T) being much more strongly stabilized than the other (M). In the presence of a large excess of co-pigment, both anthocyanins gave almost identical spectra, suggesting that while the acyl group in T has the ability to stabilize the complex it has almost no effect on color variation.

Fig. 9 shows the maximal absorbance of TF or MF complexes against time after solution in phosphate buffer (pH 6.0). Rapid decolorization occurred in the absence of the co-pigment; M and T were almost colorless after 2 hours. Addition of the co-pigment F delayed conversion of the anhydrobases to the colorless pseudobases. T anhydrobase was stabilized to the same extent as M anhydrobase but a concentration ca five times lower.

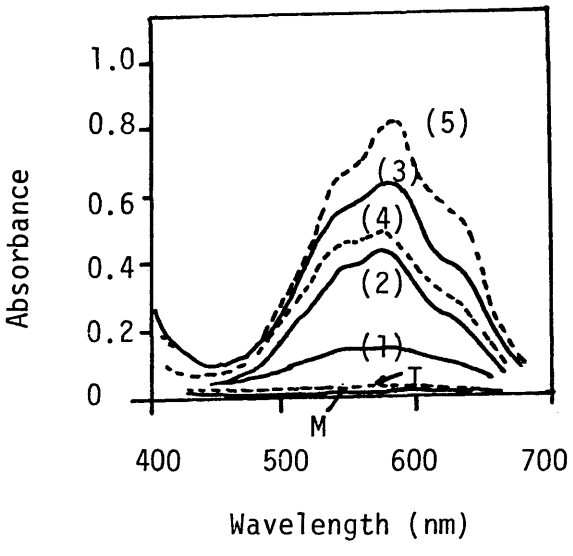


Fig. 8 Visible spectra of MF or TF complexes after 2 hr in phosphate buffer, pH 6.0 (anthocyanin concentration 5×10^{-4}M, path length; 1mm). Molar ratio of F to M (malvin) or T (tibouchinin) is as follows: —— MF complex, (1) 4.6:1, (2) 7.7:1, (3) 12.8:1, ----- TF complex, (4) 1.5:1, (5) 8.6:1.

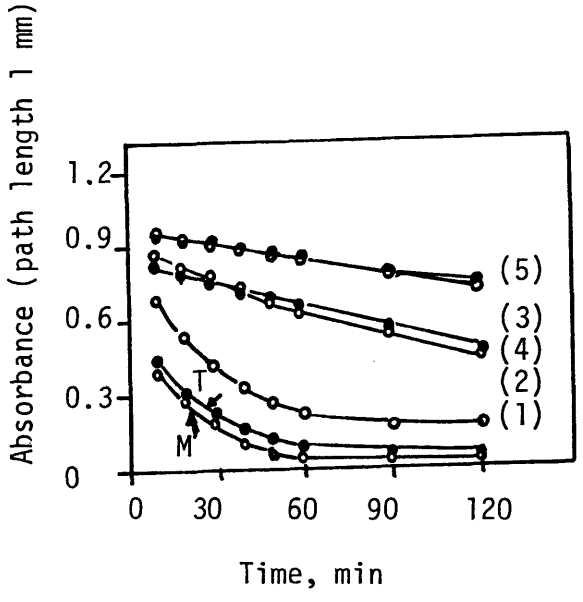


Fig. 9 Absorbance at λ_{max} of TF or MF complexes against time. Molar ratio of F to M or T is as follows:

O—O MF complex, (1) 4.6:1, (2) 7.7:1, (3) 23.5:1;

●—● TF complex, (4) 1.5:1, (5) 4.6:1.

Thus, the acyl group of T had a stabilizing effect on complex formation.

At a fixed concentration of anthocyanin (5×10^{-4}M), an increase in co-pigment concentration resulted at first in a rapid rise in the visible absorbance (Fig. 10). As the co-pigment concentration continued to increase, however, the increase in absorbance slowed down and eventually no further change in absorbance was observed. From Fig. 10, the co-pigment equivalents required to yield half-maximal can be determined. This is defined as the co-pigmentation constant (Kc). The reciprocal, 1/Kc, can be considered as an index of the affinity of an anthocyanin for its co-pigment. The Kc value changes with the concentration of anthocyanin, and Kc values, therefore, must be compared at the same molar concentration of anthocyanins. Kc values of TF and MF complexes were found to be 1.6 and 8.0, respectively, at 5×10^{-4}M anthocyanin. Thus, the p-coumaroyl residue in T increases the attraction between M and F five-fold. At a higher anthocyanin concentration (5×10^{-3}M), the MF compex gives a considerably lower Kc value (1.5), which is nearer to the value (1.1) of the TF complex. Thus, complex formation is much more dependent on the anthocyanin concentration in the case of the unacylated complex (MF), than for the acylated anthocyanin complex (TF). Thus, the acyl moiety

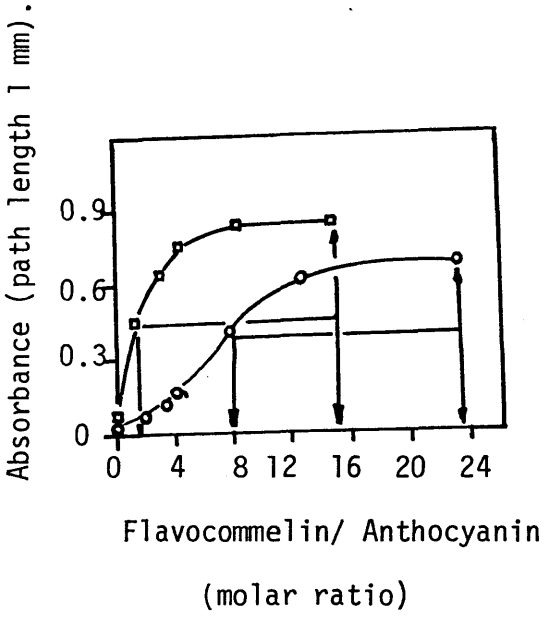


Fig. 10 Effect of flavocommelin concentration on absorbance of malvidin-based pigments ($5x10^{-4}M$) after 2 hr at pH 6.0.

—□— T, —○— M.

increases the strength of the complex formed and also supresses its dissociation, even in dilute solution.

Similar experiments were carried out in the delphinidin series using A, D, and Dp-G in order to determine the stability of the commelinin complex. In the presence of F, A showed a clearly structured and intense absorption maximum in the visible region, which closely resembled that of natural commelinin in spite of the complete absence of magnesium ion (Fig. 11). The corresponding unacylated anthocyanins, D and Dp-G, formed only weak complexes with F and failed to produce structured absorption.

The Kc values of AF and DF complexes were 1.0 and 12.0, respectively, (Fig. 12), at an anthocyanin concentration of 5×10^{-4}M. This result shows that A has a much greater affinity for the co-pigment than D. This may be because flavocommelin, F, is tailored by nature to fit perfectly with awobanin, A. Since the Kc value of delphin is similar to that of delphinidin 3-glucoside, the glucose moiety at the 5-position of D has little effect on the stability of the complex. Addition of magnesium acetate (5×10^{-3}M) stabilized the DF complex slightly, but produced a complex which was much less stable than the AF complex without magnesium ion. Thus, the p-coumaroyl group of A is important in stabilizing commelinin

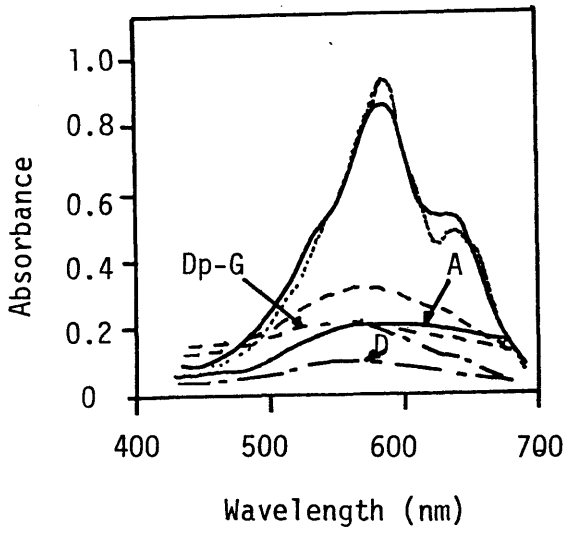


Fig. 11 Visible spectra of awobanin, delphin and delphinidin 3-monoglucoside complexed with flavocommelin after 2 hr at pH 6.0. Anthocyanin concentration 5×10^{-4}M; molar ratio of F to anthocyanin is in parentheses. ——— AF complex (2:1), ——·—— DF complex (4:1), — — — Dp-G·F complex (3.5:1), ------- natural commelinin (5×10^{-4}M as MW 1400 ; path length 1 mm).

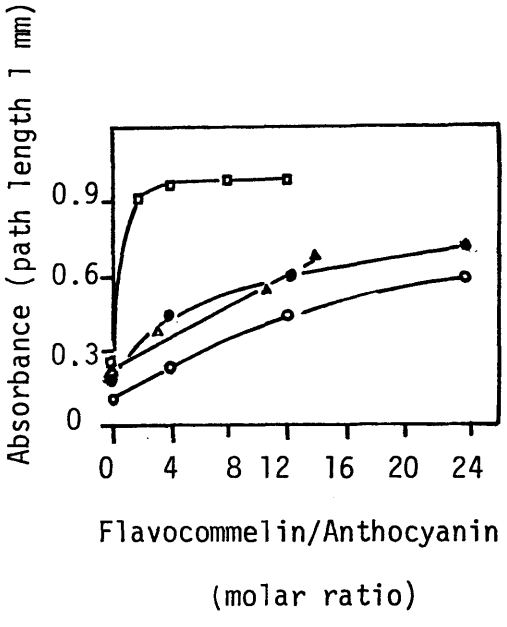


Fig. 12 Effect of flavocommelin concentration on absorbance of delphinidin-type compounds (5×10^{-4}M) after 2 hr at pH 6.0. □—□ A, ○—○ D, ●—● D + 5×10^{-3}M $Mg(OAc)_2$, △—△ Dp-G.

in vivo.

The flavylium ions are also capable of complex formation with co-pigment.[13] In a strongly acidic solution (pH 0.8), malvin and tibouchinin also form complexes with F, as indicated by their absorption maxima, which are shifted to longer wavelengths with increasing F concentration (Fig. 13). The shift with the acylated anthocyanin is larger than that with the unacylated one, at lower molar ratios (< 10) of F/anthocyanin. A similar effect is found in the case of awobanin and delphin. These facts indicate that the acyl moiety increases the size of the bathochromic shift shown by the co-pigment complex in strongly acidic media. Although the flavylium chlorides of M and T are difficult to dissolve at pH 0.8 to more than 5×10^{-4}M, addition of molar equivalent of the co-pigment causes them to go into solution immediately; thus the co-pigment can interact with the flavylium ion in acidic solution.

In conclusion, the hydrophobic acyl group in awobanin and tibouchinin greatly stabilizes the molecular complex formed between anthocyanins and the flavone in a dilute aqueous solution. It is suggested that the acylglucose moiety at the 3-position of the anthocyanidin links the flavone to the anthocyanin and with its hydrophobic nature, it tightens the bonding between the two flavonoid

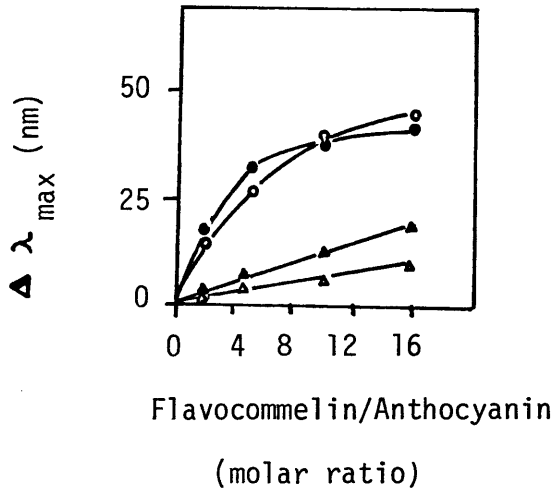


Fig. 13 Effect of flavocommelin on λ_{max} of anthocyanin in HCl-KCl buffer, pH 0.8. ○—○ M ($5x10^{-4}$M), △—△ M ($2.5x10^{-5}$M), ●—● T ($5x10^{-4}$M), ▲—▲ T ($2.5x10^{-5}$M).

units.

IV. DISCUSSION

The stabilizing effects of some concentrated salts solutions on the molecular complexes were found by the author et al. [11] The stabilizing effect of 4M NaCl on commelinin is shown in Fig. 14. Commelinin is rapidly decomposed when its aqueous solution is diluted to the concentration about 10^{-5}M: reduction in visible absorbance is about 80% when left for 3 hours, whereas no decomposition occurs in 4M NaCl (Fig. 14). $MgCl_2$(4M) has also the stabilizing effect; content of commelinin can be quantitatively measured by using 4M $MgCl_2$, because a relationship was observed between concentration of the pigment and intensity of the absorption maximum, but when water is used for dilution of the pigment solution, deviation of Beer's plots was found (Fig. 15). Accordingly, even in a dilute concentration (ca 10^{-5}M) amount of molecular complexes can be quantitatively measured when concentrated salt solutions such as 4M NaCl and 4M $MgCl_2$ are used as the solvent. The effects of some neutral salts on the stability of commelinin are shown in Fig. 16. The figure shows that sodium chloride has the stronger stabilizing effect on commelinin (a molecular complex of anthocyanin with

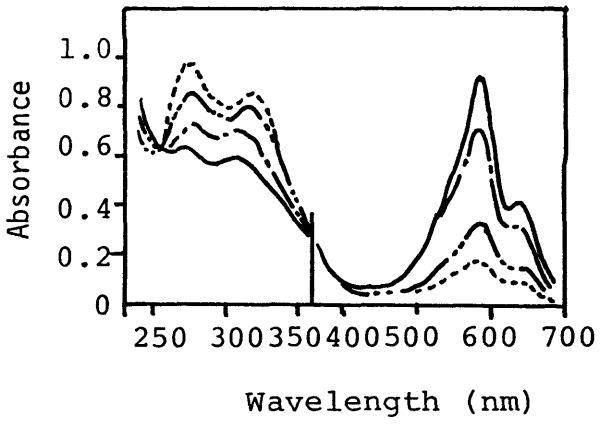


Fig. 14 Uv-visible spectra of commelinin (concn. 31.3 mg/liter; measured at 20°).

—— in 4M NaCl (stable for a few hours);

—·— in water (2 min after dissolution);

—·—·— in water (1 hr after dissolution);

------ in water (3hr after dissolution).

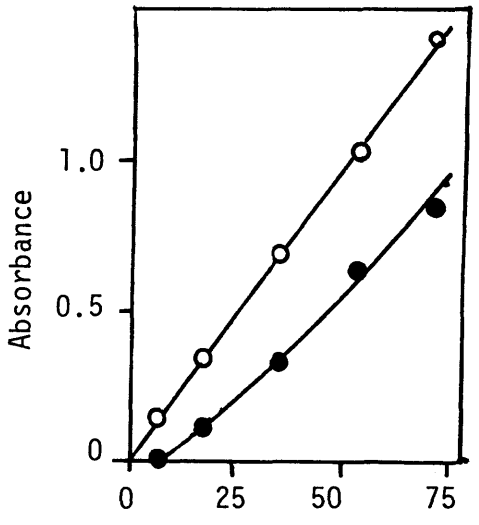


Fig. 15 Plots of the absorbance at 590 nm versus concentration of commelinin

○—○ in 4M $MgCl_2$ (0.5 hr after dissolution)

●—● in water (0.5 hr after dissolution)

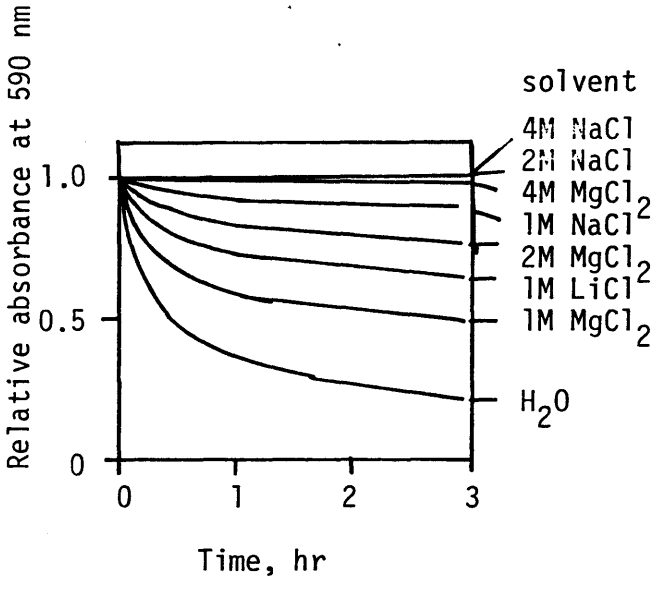


Fig. 16 Stability of commelinin in aqueous salt solutions (concentration: 31.3 mg/liter; path length 10 mm; 20°)

flavone) than magnesium chloride.

In proposition of the complexed structure of anthocyanin with flavone, it is significant to determine the composition ratio of acylated and unacylated anthocyanin complexes. To clarify this point, gel filtration method, which has been used for the purification of natural and synthetic commelinin, was applied to TF, MF, and DF complexes. Malvin anhydrobase (10 mg) and F (molar ratio ca 1:2) were dissolved in a minimum amount of acetate buffer (pH 5.5), since the anthocyanin complex is stable at a higher anthocyanin concentration. The solution was passed through a Sephadex G-15 column (0.8 x 12.5 cm); a purplish blue band was rapidly eluted with water, while excess starting materials remained on the column. This blue fraction was quickly dried in vacuo, and its composition determined by measuring its spectrum (Fig. 17) in a strongly acidic medium (ca 0.8). The spectrum was almost identical to the sum of the spectra of M and F (molar ratio 1:1) in the same solvent, indicating that the molar ratio of M and F in the blue complex is 1:1. This method was applied to TF and DF complexes (Figs. 18 and 19). In the case of DF, 4M NaCl was used as the eluent in place of water, because the DF complex is otherwise too unstable; concentrated salt solutions such as NaCl and $MgCl_2$ stabilize the

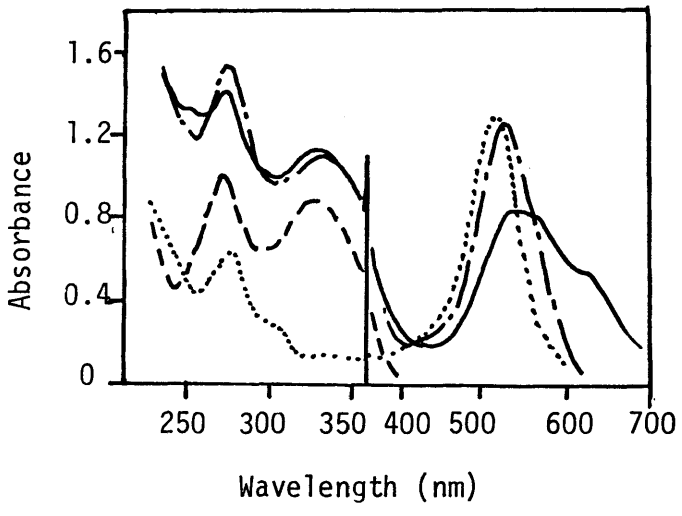


Fig. 17 Uv-visible spectra of MF compoex isolated by gel filtration. Path length 1 mm; ——— MF in 4M NaCl (concentration 6.3×10^{-4}M as MW 1300); ——·——MF in 4M NaCl containing 0.05 ml conc HCl; ..... M in 0.2N HCl-4M NaCl (6×10^{-4}M); – – – F in 0.2N HCl-4M NaCl (6×10^{-4}M). In the ratio of absorption intensity at UV-visible region, isolated MF complex is almost identical to the mixture of M and F (molar ratio 1:1).

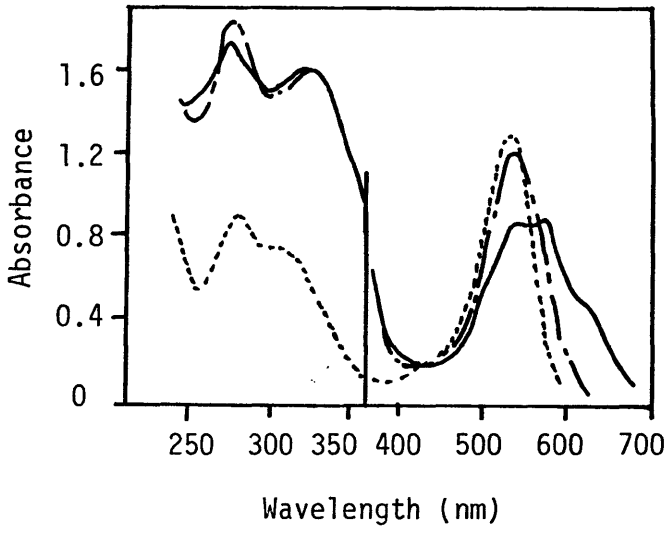


Fig. 18 UV-visible spectra of isolated TF complex. Path length 1 mm; ——— TF in phosphate buffer, pH 6.0 ($6x10^{-4}$M as MW 1400); —·— TF in buffer (3ml) containing 0.2 ml concHCl, pH 0.8; ------ T in HCl-KCl buffer, pH 0.8 ($6x10^{-4}$M).

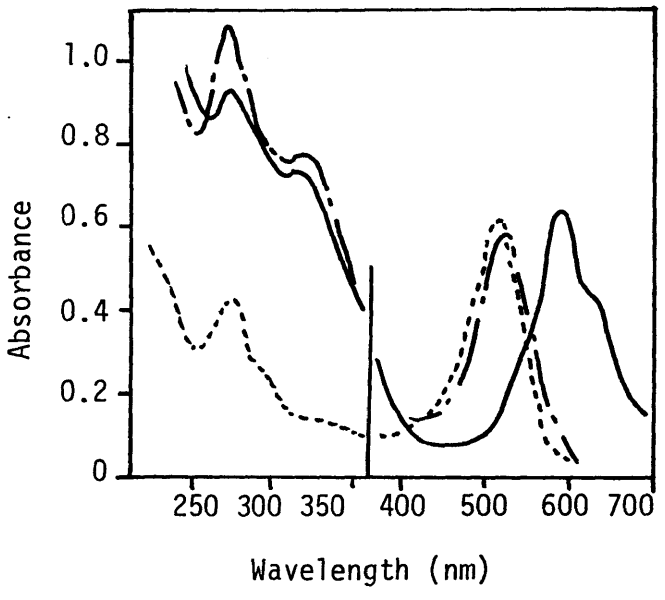


Fig. 19 UV-visible spectra of purified DF complex by gel filtration with 4M NaCl. Path length 10 mm; ——— DF in 4M NaCl (concentration unknown); —·—·— DF in 4M NaCl (3 ml) containing 0.1 ml conc HCl; ---------- D in 0.2N HCl-4M NaCl (2.8×10^{-5}M).

anhydrobases of anthocyanins. The component ratio of the isolated TF complex by gel filtration was 1:1 (Fig. 18). Although the component ratio of the isolated DF complex was ca 1:1.5 (Fig. 19), a 1:1 ratio would presumably have resulted if the complex could have been further purified. These results suggest that both acylated and unacylated anthocyanins always associate with flavocommelin at the fixed molar ratio of 1:1.

The stablizing effect of aromatic acyl group on co-pigmentation elucidated that the driving force for the association between anthocyanins and flavones is hydrophobic interactions. Indeed, commelinin dissociates in organic solvents such as 80% dimethylsulfoxide and loses its large CD; the hydrophobic interactions weaken in such aprotic solvents. Thus, the interactions between anthocyanidin rings and flavone nuclei would be postulated and the interactions of π-electrons would cause blueing effect, possibly by charge transfer complex formation.

As mentioned in part I, the self-association of anthocyanins takes place in aqueous solutions and the aggregates are present in a staked form of anthocyanin molecules. Addition of flavocommelin to the anthocyanin-containing solution exhibits longer wavelength absorption (blueing effect). This bathochromic shift of anthocyanins by co-pigmentation accords with the

phenomenon of less self-association of anthocyanins, because higher self-association gives shorter wavelength absorption as shown in malvin and delphin. At a high concentration of delphin, the anthocyanin anhydrobase rapidly precipitates as the consequence of high self-association, but addition of molar equivalent of the co-pigment makes the precipitates readily soluble and their color bluer. These phenomena indicate that the co-existence of flavones weakens the degree of the self-association of anthocyanins in aqueous solutions. The formation of molecular complex of anthocyanins with flavones and that of the self-association would be competitive, but the formation constant for the co-pigmentation is far larger than that for the self-association, that is, affinity strength of the flavone for anthocyanin very exceeds that of individual anthocyanin molecules, by judging from the evidence mentioned above.

Flavones would be intercalated into the stacked self-associated anthocyanin molecules with its strong hydrophobic affinity for anthocyanins, so that formation of ordered self-aggregation would be disrupted and more systematic anthocyanin-flavonoid co-pigment complexes would be constructed. As previously mentioned, a component ratio of anthocyanin-flavone complexes is 1:1 and interactions between anthocyanidin rings and flavone nuclei have a

bearing of blueing effect. Thus, a stacked structure of anthocyanin with flavone would be proposed(Figs. 20 and 21). The stacked molecular complex allows maximum overlap of aromatic systems and the sugar moieties surrounding aromatic systems (hydrophobic portion) makes the complex relatively hydrophilic and water soluble. In this arrangement, the two flavonoid unit is further stacked side by side to form dimer, trimer, and larger polymer of the unit. In this structured orientation, phenyl groups of both of the flavonoid nuclei twist in a same way, which may cause optical activity of the complex (Figs. 4 and 21).

The concept of a stacked molecular complex would be further supported by other experimental evidence.[14] For example, it was examined whether a modified flavocommelin, 5-OMe flavocommelin (5), forms a complex with awobanin. The complex obtained a large CD, a blueing effect in visible absorption, and electrophoretic movements; the results giving the evidence of complex formation, but the stability was less than that of commelinin. This may be because methyl group at 5-position hinders ster^eochemically a tightened complex formation (Fig. 22).

In chapter 3, the author suggested that the acylglucose moiety at 3-position of the anthocyanidin embraces the flavone with its hydrophobic nature. The C. P. K. model

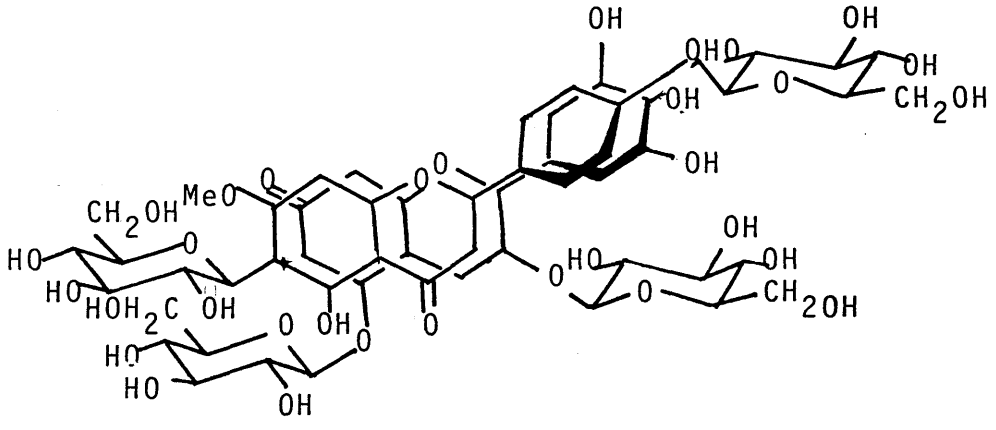
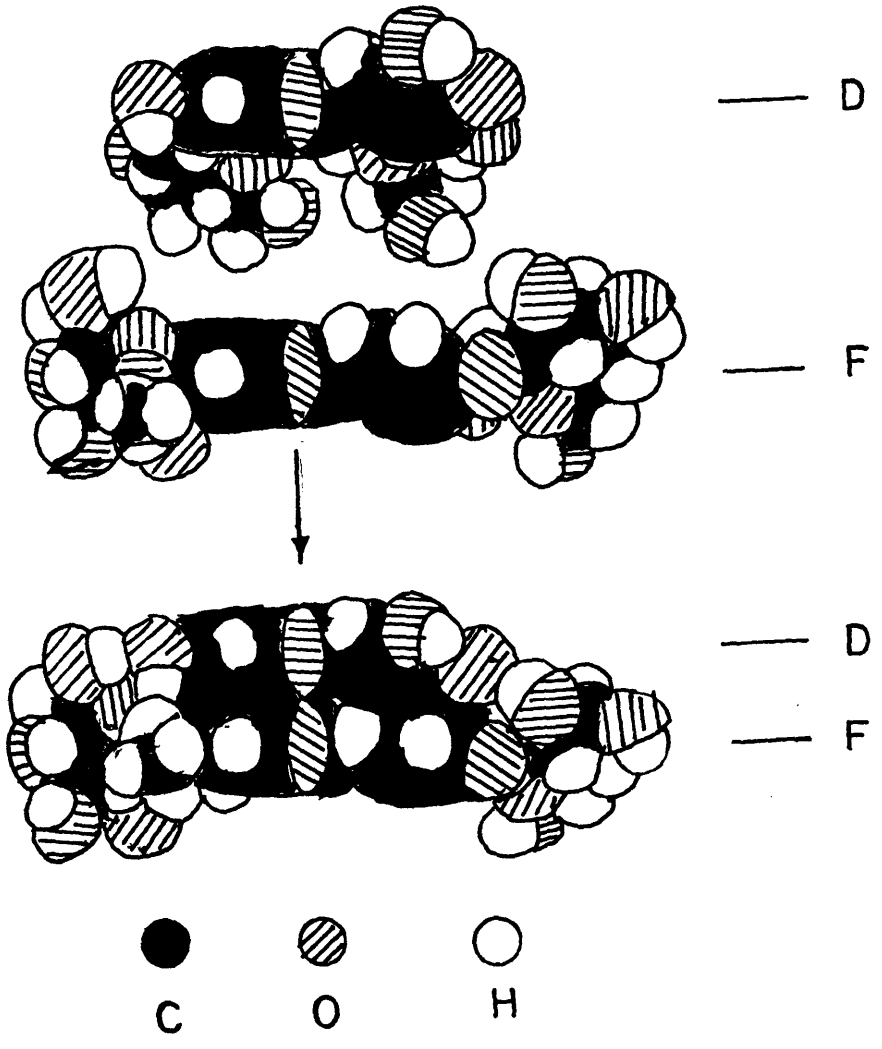
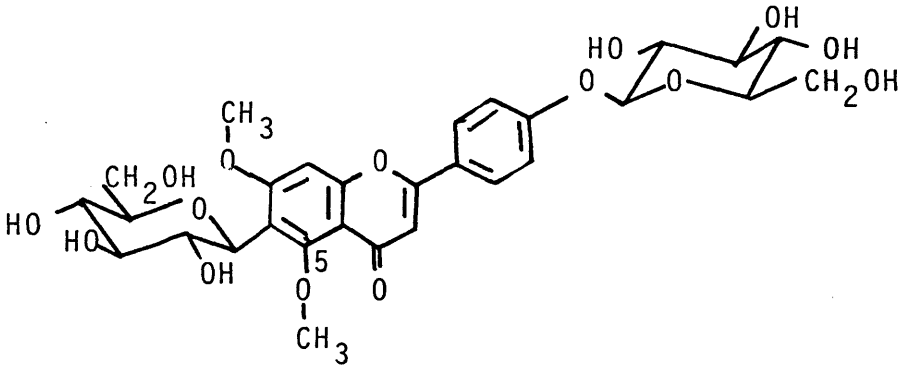


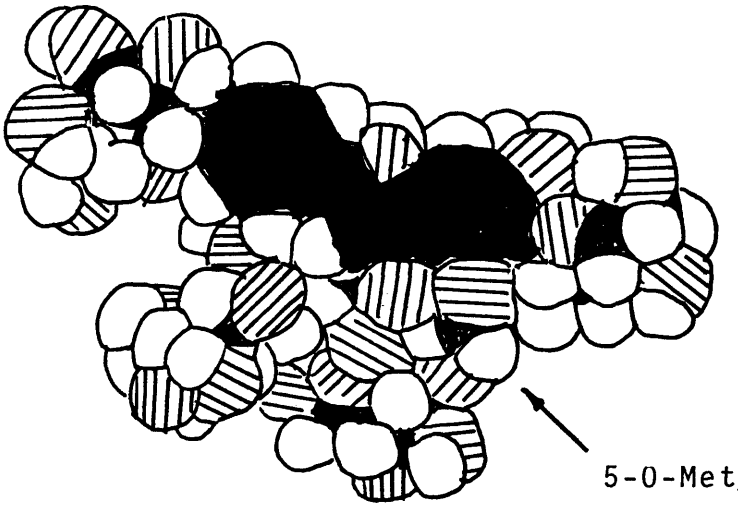
Fig. 20 Proposed structure of delphin- flavocommelin complex (a stacked molecular complex)

Fig. 21





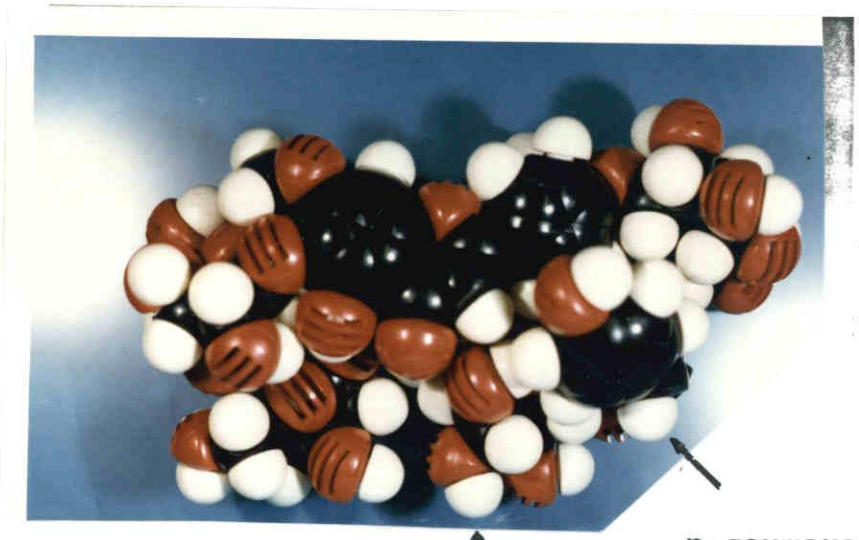
5-OMe flavocommelin (5)



COMPLEX OF AWOBANIN AND 5-O-METYL-FLAVOCOMMELIN

Fig. 22

is shown in Fig. 23. The model exhibits clearly the stabilizing effect that p-coumaroyl group suppresses the dissociation of delphin and flavocommelin. The model also shows an ingenious arrangement of AF unit; in this orientation, hydrophobic portion of p-coumaroyl group is covered between 4'-glucose moiety of flavocommelin and the glucose residue at 3-position of awobanin. The AF unit is further stacked to form dimer, trimer, and so on. Effect of 4'-glucose moiety of flavocommelin on the complex formation was examined.[14] Swertisin was able to have the stacking with awobanin; the CD is structured similarly with that of commelinin (AF unit). The stability of the complex was far less than that of commelinin. The absence of 4'-glucose moiety of flavocommelin shows the inability to cover the hydrophobic portion of the aromatic acyl group, which would cause the less stability of swertisin-awobanin complex.



glucose moiety

at 4'-position of F

p-coumaroyl group

glucose moiety at

3-position of A

F

A

Fig. 23.

V. EXPERIMENTAL

INSTRUMENTS

Uv-visible spectra were measured with a Hitachi 323 or EPS-3T spectrophotometer.

CD spectra were determined with a Jasco J-40 spectropolarimeter.

Ir was measured with a Perkin Elmer 237 spectrometer.

Contents of magnesium and other metals were analyzed with a Hitachi 208 atomic absorption spectrometer.

Molecular weight of commelinin was determined with a Hitachi UCA-1A analytical ultra-centrifuge.

MATERIALS

The purity of all isolated anthocyanins was examined spectrophotometrically.

TIBOUCHININ (malvidin 3-p-coumaroylglucoside-5-glucoside)

Tibouchinin chloride was isolated from the flowers of Tibouchina semidecandra.[15] The petals were immersed in HCl-MeOH (1 ml conc HCl/1.) at 4°. The solution was then subjected to filtration, concentration in vacuo below 30° to dryness, dissolution in a small quantity of H_2O, and centrifugation to remove insoluble materials. The supernatant was adsorbed on a polyamide column (3 x

25 cm), and then washed well with HCl-H_2O (1 ml conc HCl/l.) to remove impurities. Subsequently tibouchinin was eluted with HCl-MeOH (1 ml conc HCl/l.). Pure tibouchinin was finally purified by crystallization from MeOH-Et_2O. (Found: C, 50.95; H, 5.33. Calc. for $C_{38}H_{41}O_{19}Cl \cdot 3H_2O$: C, 51.21; H, 5.28%).

MALVIN CHLORIDE

Malvin chloride was purchased from Aldrich.

AWOBANIN (delphinidin 3-p-coumaroylglucoside-5-glucoside)

Awobanin chloride was obtained from commelinin. The solution of commelinin in 2N HCl was allowed to stand at room temperature, when the color gradually changed from blue to red. Separation of awobanin from the solution was carried out by use of Avicel microcrystalline cellulose column chromatography with HOAc-HCl-H_2O (5:1:40) as eluent. The anthocyanin fractions were evaporated to minimum volume under reduced pressure below 40° and then quickly dried over KOH.

Delphin chloride (delphinidin 3,5-diglucoside)

Delphin chloride was obtained after a solution of awobanin chloride dissolved in 20% aqueous HCl was allowed to stand at room temperature for a few days.

The dark precipitates were collected by centrifugation and washed with EtOH. Crystallization was effected by dissolution in H_2O and addition of 7% HCl-EtOH. The absorption spectrum of the isolated anthocyanin (in 1% HCl-MeOH) lacked both an absorption band at 300-310 nm and a distinct shoulder in the 410-440 nm region. This is characteristic of an unacylated anthocyanin with a sugar at both the 3- and 5-positions.[16] (Found: C, 45.58; H, 5.13. Calc. for $C_{27}H_{31}O_{17}Cl \cdot 3H_2O$: C, 45.25; H, 5.17%). FD MS: m/e 627 for $C_{27}H_{31}O_{17}^{+}$.

DELPHINIDIN 3-MONOGLUCOSIDE

Delphinidin 3-monoglucoside was isolated from blue hydrangea petals.[17] The petals were soaked overnight in HOAc and separated by filtration, and then the solution was evaporated to dryness. The residue was dissolved in 3% HCl-MeOH and allowed to stand overnight when yellow precipitates separated out. Filtration was carried out with aid of Cellite. The purified anthocyanin was obtained by means of Avicel column chromatography with n-BuOH-HOAc-H_2O (4:1:5) as an eluent. Further purification was attained by use of HOAc-HCl-H_2O (15:1:84).

COMMELININ

Commelinin was isolated from the flowers of Commelina communis according to the method of Hayashi et al.[18, 19] Fresh petals were immediately freezed with dry ice after picking the flowers. The press-juice was obtained at the room where a temperature is controlled at 4-5° and centrifuged to remove insoluble matters, and then EtOH (6 vols) was added to the supernatant, where blue precipitates separated out. The precipitates were collected by centrifugation, dried over anhydrous $CaCl_2$ in vacuo, dissolved in minimum amount of water, and then centrifuged to remove greyish, insoluble matters. To the supernatant was added EtOH slowly and gently until the precipitation begins and allowed to stand overnight at 4°. Collected blue precipitates by centrifugation was purified by addition of EtOH after dissolving in water. This procedure was repeated three times or more in the same way. Further purified commelinin was obtained by use of Sephadex G-15 column.

FLAVOCOMMELIN

Flavocommelin was prepared according to the method of Takeda et al.[9] To the press-juice of the petals of Commelina communis was added EtOH and centrifuged to collect blue precipitates. The supernatant contains

flavocommelin. The yellow supernatant was concentrated under reduced pressure, and saturated methanolic solution of lead acetate was added to remove some impurities as a greenish blue precipitate. After centrifugation, the solution was saturated with hydrogen sulfide, filtered, and then concentrated to a some small volume in vacuo. The concentrated solution was washed with chloroform, benzene, and ether. Subseqently it was shaken with a half volume of the 1:1 mixture of acetone and carbon disulfide, and allowed to stand at room temperature for a few days, when a yellow pigment gradually separated out in an amorphous state near to the boundary of the two layers. The yellow precipitates were dissolved in hot 50% MeOH, treated with some active charcoal, and filtered. The pale yellow solution was then treated with CS_2 and allowed to stand overnight at room temperature , when flavocommelin was separated as pale yellow powder. Flavocommelin was crystallized from MeOH-CS_2.

5-OCH_3-FLAVOCOMMELIN (5-methoxy-6-C-glucosyl-7-methoxy-4'-O-glucosylflavone)

The octa- and nonaacetate of flavocommelin synthesized by Ac_2O/Py were separated on TLC (silica gel) with acetone-hexane-ether (1;1;1) to obtain the octaacetate which has unacylated hydroxyl group at 5-position of the

flavone nucleus. The hydroxyl group was methylated with methyl iodide and silver oxide in chloroform under refluxing. The octaacetate of 5-O-Me-flavocommelin was hydrolyzed with saturated anhydrous ammonia-MeOH. The product was crystallized with EtOH.

SWERTISIN

Swertisin was prepared according to the method of Takeda et al.[9] Flavocommelin was refluxed in 1N H_2SO_4 for two hours, and allowed to stand overnight at 4°. The aglycon was collected by filtration, and washed with some water and crystallized from 60% MeOH.

SYNTHESIS OF COMMELININ IN THE ABSENCE OF Mg METAL

Awobanin chloride was dissolved in MeOH, a few drops of 0.5N NH_4OH were added to the solution to change the red flavylium ion to purple anhydrobase, and then the solution was quickly dried over in vacuo. The weight of the anhydrobase and flavocommelin (1.1-1.5 equivalent to the anhydrobase) was measured on a microbalance, and then a small quantity of water was added and stirred. The reaction mixture was passed through a short column of Sephadex G-10 or G-15 to remove the starting materials, and the blue compound was precipitated from the eluates

by addition of ethanol.

THE SPECTRA OF ANTHOCYANIN-CO-PIGMENT COMPLEXES

The anthocyanins were dissolved in MeOH, a few drops of aquous ammonia (0.5N) was added to the solution to change the red flavylium ions to purple anhydrobases, and then the solution was quickly dried over P_2O_5 in vacuo. The weight of anhydrobase was measured on a microbalance. The solution of flavocommelin in 0.01M phosphate buffer was added to the previously weighed anhydrobase so that the concentration of anthocyanin was 5×10^{-3} or 5×10^{-4}M. Absorption spectra were determined with a Hitachi 323 spectrophotometer at $23 \pm 2°$ using quartz cells having a path length of either 0.1, 1 or 10 mm which was employed at 5×10^{-3}, 5×10^{-4} or 2.5×10^{-5}M of anthocyanin concentration, respectively.

CORRECTION OF THE THICKNESS OF OPTICAL CELLS

The thickness of 10, 1, or 0.1 mm cell was spectrophotometrically corrected by dilution of the solutions of flavocommelin in water or methyl orange in 0.1N HCl.

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