BIOSYNTHETIC PRECURSORS OF PELARGONIDIN-3-GLUCOSIDE IN THE STRAWBERRY FRUIT

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Henry Co

1965

This is to certify that the

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Henry Co

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Pericles Markakis
Major professor

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ABSTRACT

BIOSYNTHETIC PRECURSORS OF PELARGONIDIN-3-GLUCOSIDE IN THE STRAWBERRY FRUIT

by Henry Co

^{14}C-labelled compounds were fed into maturing strawberries at the pink-white stage. The pelargonidin-3-glucoside was extracted and purified. The relative effectiveness of these compounds as precursors of the pigment was assessed by calculating the % ^{14}C converted. The order of decreasing effectiveness is as follows: cinnamic acid > shikimic acid > fructose > glucose > phenylalanine > quinic acid > sodium acetate > tyrosine.

The pelargonidin-3-glucoside was degraded into sugar, phloroglucinol and p-hydroxybenzoic acid by acid hydrolysis and alkaline degradation. Determination of these 3 degradation products showed that acetate contributed almost exclusively to ring A. Glucose and fructose contributed to both ring A and B. ^{14}C from shikimic acid, cinnamic acid-3-^{14}C, quinic acid, phenylalanine (ring labelled), and tyrosine was incorporated into p-hydroxybenzoic acid. Phenylalanine, tyrosine, quinic acid, and cinnamic acid did not label the sugar moiety. Cinnamic acid-2-^{14}C and phenylalanine-1-^{14}C labelled the pigment but not any of the 3 degradation products, suggesting that the C_6-C_3 skeleton might be incorporated as a unit.

The mode of attachment of glucose to the pelargonidin could be through sugar nucleotide, as shown by the labelling of the sugar in pelargonidin-3-glucoside when UDPG (glucose-UL-^{14}C) was fed. Glucose-

1-phosphate was almost as good a sugar donor as the UDPG. ^{14}C-glucose was not incorporated into the pigment even after 3 hours of metabolism. The pelargonidin was not labelled in all cases.

When ^{14}C-pelargonidin-3-glucoside was fed to strawberries, the cyanidin-3-glucoside was not labelled. The reverse was also true. This indicates that there might not be any interconversion between the 2 pigments.

The two-dimensional chromatogram of the ethyl acetate extract of the strawberry fruit, the reactions of the spots to various detection methods, and the UV absorption spectra of some of the compounds were presented.

Leucoanthocyanins, catechin, kaempferol and quercetin were present in the ethyl acetate extract of the strawberry fruits. They were all labelled when cinnamic acid-3-^{14}C was fed to the detached strawberry fruit.

BIOSYNTHETIC PRECURSORS OF PELARGONIDIN-3-GLUCOSIDE IN THE STRAWBERRY FRUIT

By

Henry Co

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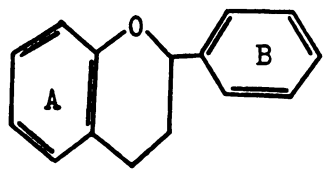
INTRODUCTION

Anthocyanins are one of the classes of flavonoid compounds which are characterized by a C_6-C_3-C_6 skeleton (I). They are responsible



(I)

for most of the red, violet and blue coloration in plants (54).

The biosynthesis of anthocyanins has been of interest to geneticists for a long time. Earlier speculations on biosynthetic routes to flavonoid substances were based on structure analyses (93). Geissman and Hinreiner (33) assumed that the common flavonoid precursor was a chalcone.

The objective of this study was to test a number of compounds which may serve as precursors of pelargonidin-3-glucoside in an effort to elucidate the biosynthetic pathway of the pigment in the strawberry fruit.

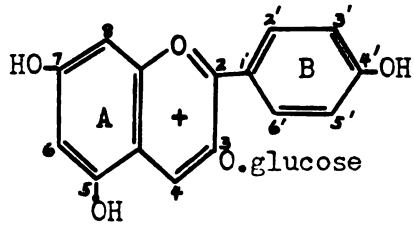
Strawberry fruits were chosen as the experimental material for the following reasons:

1. The anthocyanins of the fruit have not been studied biosynthetically.
2. The plants can be grown under green-house conditions. With light supplements, an almost continuous supply of strawberries is possible, with 2 major seasons, one in summer, and the other one in winter.
3. The major anthocyanins in strawberry fruits have been identified

as pelargonidin-3-glucoside and cyanidin-3-glucoside (72, 95, 105, 106).

4. The production of anthocyanins in strawberry is very fast. The fruit changes from pink-white to fully red in 48 hours. This is very suitable for feeding experiments with ^{14}C-labelled compounds.

5. The pelargonidin-3-glucoside (II) was chosen as the anthocyanin



(II)

to be studied, because it constitutes more than 90% of the total anthocyanins in the strawberry.

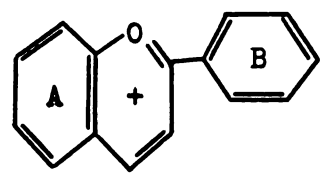
REVIEW OF LITERATURE

The term anthocyanin was coined in 1835 by Marquart (78) to denote the blue pigment of the corn flower. Later, this term was used to include the whole group of similar pigments soluble in the cell sap of flowers, fruits and other plant organs (118).

Bate-Smith (8), Blank (16, 17), Geissman (30), Harborne (45, 46), Hayashi (54), Karrer (68) and Paech (85) have review articles on anthocyanins. The two most recent books on this field are "Biochemistry of Phenolic Compounds", edited by J. B. Harborne (51), and "Chemistry and Biochemistry of Plant Pigments", edited by T. W. Goodwin (37).

A. Chemistry of anthocyanins

The chemical investigation of anthocyanins was pioneered by Willstätter and his co-workers during the period 1913-1916. The consitution common to all anthocyanins was first established by Willstätter (118), and was later confirmed by Robinson (87, 94) in an extensive series of organic synthesis experiments to be the 2-phenylbenzopyrylium, or flavylium structure:



There are 6 basic anthocyanidins commonly found in nature (17): pelargonidin, cyanidin, peonidin, delphinidin, malvidin and petunidin, all of which are hydroxylated in the 3-, 5-, and 7- positions. Peonidin, malvidin and petunidin are the methylated derivatives of cyanidin and

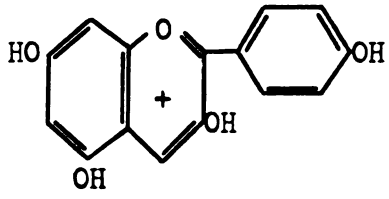
delphinidin. The 6 common natural anthocyanidins are shown in Fig 1.

The pigments generally occur as anthocyanidin glycosides, anthocyanins, containing one or more sugar residues and occasionally various organic acid residues. The relative frequencies with which the various monosaccharides occur in bound form in anthocyanins are as follows: glucose > galactose > rhamnose > xylose > arabinose (50). The attachment of sugars is found in the majority of cases to be at position 3 and less frequently at position 5 of the anthocyanidin. Only one pigment has sugar at position 7 (52). The sugar at position 5 is always glucose (50). Our present knowledge of the position of sugar attachment in the molecule is due to a series of investigations by Robinson and coworkers (11, 74, 91, 96, 97) and by Harborne (50).

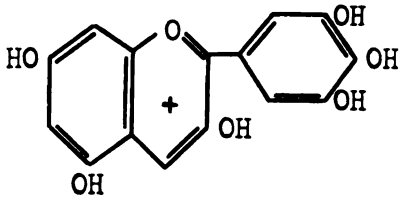
The organic acid residues commonly found are p-coumaric acid, p-hydroxybenzoic acid, malonic acid, sinapic acid and ferulic acid (50, 54). They could be attached to any of the hydroxyl groups of the anthocyanidin or the sugar residue.

The hydroxyl groups attached to the B-ring affect the color of anthocyanins. A deepening of visual color is brought about by an increase in the number of hydroxyl groups attached to the B-ring, as illustrated by the orange-red pelargonidin, deep-red cyanidin, and bluish-red delphinidin derivatives. Methyl substitution gives the anthocyanin a more or less dull bluish shade, as in peonidin and malvidin glycosides. The number, position and nature of sugar does not affect the shade of the anthocyanin color (54).

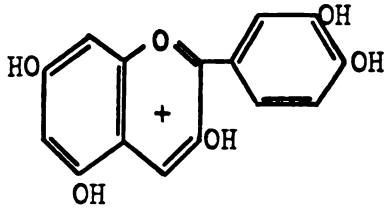
The colors of anthocyanins and anthocyanidins undergo conspicuous changes with alkali and inorganic salts. They are red in acid, and blue



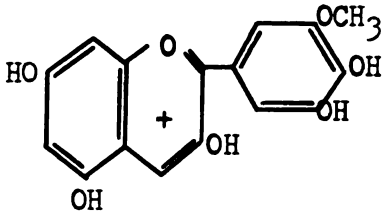
Pelargonidin



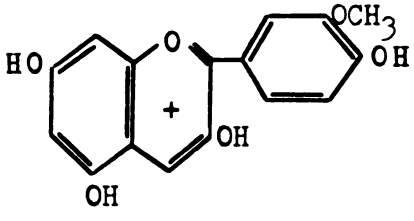
Delphinidin



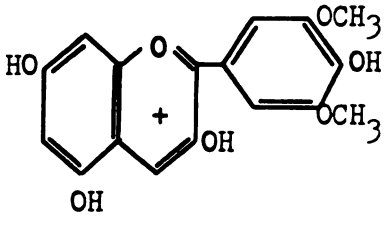
Cyanidin



Petunidin



Peonidin



Malvidin

Fig. 1. The common natural anthocyanidins

in alkaline solution. Their colors fade at pH near neutrality. Anthocyanins containing free vicinal hydroxyl groups as in cyanidin, delphinidin, petunidin and their glycosides give blue color with inorganic salts. Pelargonidin, peonidin, malvidin and their glycosides, which have only one free hydroxyl group in the B-rings, do not react with inorganic salts to give any noticeable color change.

Blue anthocyanins have been isolated from natural sources (9, 10, 55). They are metal complexes of anthocyanins associated with high molecular weight compounds of unknown structures.

B. Biosynthesis of anthocyanins and other flavonoid compounds

The literature on the occurrence and biosynthesis of flavonoid compounds has been covered in several reviews and books (12, 19, 31, 33, 37, 51, 85, 99, 109).

In 1899, Overton (84) proposed the idea that anthocyanins originated from carbohydrates, and suggested that the red coloring in normal plants could be correlated with excessive sugar in those tissues. Onslow (83) suggested that the pigment was derived from flavone or flavonol pigments.

a. Relationships of different flavonoid compounds

The different classes of C_6-C_3-C_6 compounds are related by oxidation-reduction reactions of the C_3-portion, but so far, inter-transformation has not been shown in any biological system (14). Birch (14) proposed 2 alternate pathways to the production of anthocyanins, Fig. 2.

Route (i) through flavonol derivatives is a very early postulate (83). Birch (14) suggested that it is quite possible that the whole series of processes occur in one enzyme system and that no free intermediates are released.

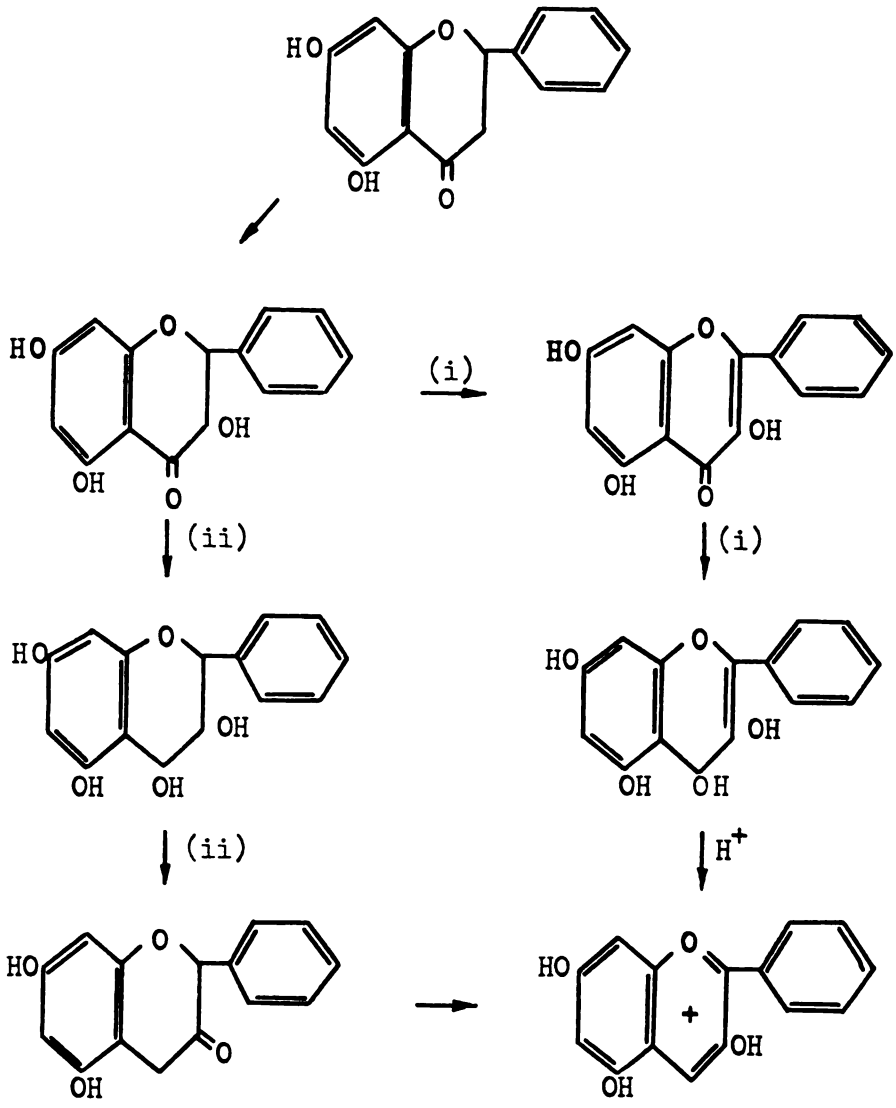


Fig. 2. Hypothetical derivation of anthocyanins from related flavonoids (14).

Among polyphenolic compounds, leucoanthocyanins have been considered to be the precursors of the anthocyanins (3, 29, 103). However, there are indications from genetic studies that the biosynthetic pathway of these two classes of compounds diverge at a fairly early stage in synthesis (2, 63). Aurone (32, 63) and flavanone (49) have also been found by genetic studies to diverge from the main flavonol-anthocyanidin pathway fairly early in synthesis. Anthocyanidins, flavonols and flavones are reported to be closely related biosynthetically (49).

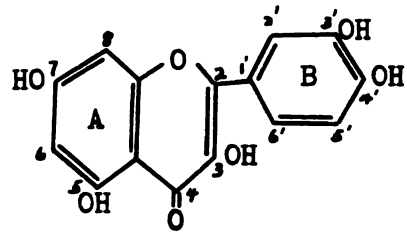
Our present knowledge on the biosynthesis of flavonoid compounds is chiefly derived from feeding experiments with ^{14}C-labelled compounds and from the genetic studies of flower colors.

b. Biosynthesis of C_6-C_3-C_6 skeleton

Earlier speculations on biosynthetic routes to flavonoid substances were based on structural analyses. It was pointed out by Robinson (93) that the C_6-C_3-C_6 skeleton (I) of flavonoid compounds could be related in part to the C_6-C_3 skeleton in substances such as phenylalanine, tyrosine, and other natural products.

The oxygenation pattern of the A-ring of several flavonoid compounds such as anthocyanins and quercetin is typically that of phloroglucinol or resorcinol and the former in particular was considered as a possible intermediate. Geissman and Hinreiner (33) has published a review on the earlier work and ideas.

The work of Underhill *et al* (111), Watkin *et al* (115), and Geissman and Swain (36) on the biosynthesis of quercetin (III) in buckwheat (Fagopyrum) showed that the ring A was formed with about equal efficiency



(III)

from either 1-^{14}C- or 2-^{14}C- acetate, but very little ^{14}C from acetate was incorporated into ring B. The ring B was derived from a C_3-C_6 compound such as phenylalanine, cinnamic acid or p-coumaric acid. These compounds were incorporated without loss or randomization of carbons to give ring B and the central C_3 unit of the heterocyclic ring (111).

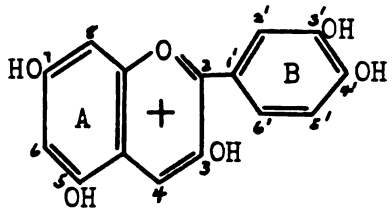
Zaprometov (121) reported that acetate was incorporated mainly into ring A and shikimic acid was incorporated mainly into ring B of catechin in young tea shoots.

Comte et al (27), also showed that β-^{14}C-cinnamic acid was incorporated into the carbon atom at position 2 of the catechin nucleus.

Hutchinson et al (58) reported that phenylalanine was incorporated into ring B and acetate into ring A of phloridzin (Phloritin glucoside) in leaf disks of Malus. Avadhani and Towers (4) showed that α-^{14}C-cinnamic acid was also readily incorporated into phloridzin.

Although quercetin might arise from the acylation of phloroglucinol by phenylpropanoic acid, this path does not seem to operate in buckwheat, since ^{14}C-phloroglucinol was not incorporated into quercetin (79).

Grisebach (38) studied the biosynthesis of cyanidin (IV) in red cabbage seedlings. Cyanidin occurs here as a complex anthocyanin. Two of the major pigments are the mono- and di-feruloyl esters of cyanidin-



(IV)

5-glucoside-3-sophoroside (50). Acetate-1-^{14}C was found to incorporate mainly into ring A. Degradation of the phloroglucinol obtained by alkaline fusion showed the ^{14}C to be concentrated in the oxygen-bearing carbon which showed that ring A was formed by head to tail condensation of acetate units. Grisebach (39) also found that phenylalanine-U-^{14}C was incorporated into ring B and the attached carbons. So far, acetate was shown to be the best precursor of ring A (40, 116).

Grisebach and Bopp (42) reported that both quercetin (III) and cyanidin (IV) were formed simultaneously from 1-^{14}C-phenylalanine in etiolated buckwheat seedling - there was no evidence that one was a precursor of the other. Both flavonoids reached their maximum specific activity at the same time and then declined. Grisebach and Patschke (43) have obtained evidence that a chalcone was a precursor of both cyanidin and quercetin. When 2', 4, 4', 6'-tetrahydroxychalcone-2-β-^{14}C-glucoside was administered to Fagopyrum, both quercetin and cyanidin were labelled. This chalcone was also converted to cyanidin in red cabbage, without randomization of the carbons.

c. Glycosidation of flavonoid compounds

Barber (5) has described the enzymic glycosylation of quercetin (III) to form rutin [quercetin-3-O-α-L-rhamnosyl-(1→6)-β-D-glucoside].

The enzyme was prepared by ammonium sulfate precipitation from a buffer extract of mung bean leaves. While the different preparations varied in their respective abilities, all but one preparation catalyzed the following two reactions involving different types of nucleoside diphosphate glucose (NDPG) or its rhamnose counterpart.

$$\text{NDPG} + \text{Quercetin} \longrightarrow \text{Quercetin-3-}\beta\text{-D-glucoside} + \text{NDP}$$

$$\text{NDP-L-rhamnose} + \text{Quercetin-glucoside} \longrightarrow \text{Rutin} + \text{NDP}$$

The reactions were followed by measuring the incorporation of radioactivity into the products from nucleoside diphosphate sugars labelled with ^{14}C in their sugar moieties. Some recent results have shown that UDP-rhamnose was a somewhat more effective donor than TDP-rhamnose for rutin synthesis (6).

The work on rutin formation indicates that the pattern for enzymic glycosylation of phenols discovered by Yamaha and Cardini (119, 120) may be widespread, i. e., phenols are first converted to the β-monosides and then successively to di-, tri-, and tetra-saccharides.

Mansell (75) reported on the glucosylation of anthocyanins in detached petals of Impatiens balsamina. Para-coumaryl-3,5-diglucoside was produced by the developing petals of a genetically white strain when pelargonidin-3-monoglucoside was fed.

d. Biosynthetic pathway based on genetic evidence

A pathway of flavonoid biosynthesis based on genetic studies was drawn up by Harborne (49). Glycosylation was considered to be the final steps in synthesis (33). Methylation and acylation of the anthocyanins were also considered to take place toward the end of the synthesis since

the co-occurring and related flavones are unaffected. The introduction of the 3-hydroxyl group was assumed to take place before ring closure of the central pyran ring since it is a common feature of both flavonols and anthocyanidins. Finally, hydroxylation of the B ring was assumed to take place at the C_{15} level rather than at the C_9 level, because there is a closer correlation between the hydroxylation pattern of the different classes of flavonoids, than between flavonoids and cinnamic acids and other C_9 compounds.

C. Methods of analyses of flavonoid compounds

a. General references

Methods of identification of flavonoid compounds were reviewed by M. K. Seikel (102) in the book "Biochemistry of Phenolic Compounds", edited by Harborne. Seshadri (100), Seikel (101), Venkataraman (112) and Jurd (65) each has a chapter on the isolation and purification of flavonoid compounds in Geissman's book (31). Harborne (45, 46, 47, 48) and Geissman (30) both have review articles on the identification of flavonoid compounds.

More recent books on flavonoid compounds include "The Chemistry of Flavonoid Compounds", edited by T. A. Geissman (31), "Biochemistry of Phenolic Compounds", edited by J. B. Harborne (51), "Biogenesis of Natural Compounds", edited by P. Bernfeld (12), "Chemistry of Natural Phenolic Compounds", edited by W. D. Ollis (82), "Methods in Polyphenol Chemistry" (90, "Enzyme Chemistry of Phenolic Compounds" (89) both edited by J. B. Pridham, and "The Chemistry and Biochemistry of Plant Pigments", edited by T. W. Goodwin (37).

b. Isolation

The flavonoid compounds are extracted from the plant materials with acetone, ethanol, methanol, or water (100, 102). The chlorophylls, carotenoids, and other waxy materials are usually removed by extraction with petroleum ether, hexane or benzene.

c. Purification

Initial purification of flavonoid compounds can be carried out by extraction with ether, ethyl acetate, or butanol (102), or by precipitation with neutral or basic lead acetate (54).

Column chromatography can be used for the separation of flavonoid compounds. Cation-exchange resins (60, 117), Magnesol (a synthetic hydrated magnesium acid silicate) (59), silicic acid (105), cellulose powder (113) and polyamide (24, 25, 80) were some of the absorbents used.

Paper chromatography is the most important method for separating complex flavonoid mixture. The techniques of one-dimensional, two-dimensional, and preparative chromatography have been covered in several books on chromatography (18, 56, 104). The developing solvents (45, 47, 101, 102) and spraying reagents for detecting flavonoid compounds on paper chromatograms (101, 102) have been summarized in several publications.

Thin-layer chromatography (13, 15, 57) and paper electrophoresis (77, 88) are some of the other methods for purification of flavonoid compounds.

d. Identification

Preliminary identification is carried out on the paper chromatogram. Anthocyanins, chalcones and aurones are visible under ordinary light.

The other flavonoid compounds, except flavanones, isoflavones, catechins and leucoanthocyanins (47), can be revealed with UV light, with or without the help of NH_3 fumes. Catechins and leucoanthocyanins can be located with p-toluene sulfonic acid (101). Flavanones turn magenta with sodium borohydride spray (101).

Micro methods are available for complete identification of flavonoid compounds. They are based on paper chromatographic study of the glycoside in several solvent systems and of the aglycone, the sugar, and the cinnamic acid derivatives (if present) formed on hydrolysis (45), followed by a spectral study of the glycoside and aglycone.

The unknown glycoside is co-chromatographed with known compounds in at least 3 different solvent systems and the R_f values compared with the published data (8, 30, 45, 47, 101). The absorption spectra of the glycoside (46, 65), and the shift of absorption maximum upon addition of aluminum chloride (34, 44, 108), sodium ethylate (76), sodium acetate (66, 67), and boric acid-sodium acetate (64) are determined and compared with those in literatures.

Infra-red spectroscopy has been used to investigate the flavones, flavonols, flavanones and isoflavones (61, 71) and to identify different anthocyanidins (92). It is especially useful in confirming the presence or absence of methoxy group in the pigment.

Several methods are available for the determination of the nature (1, 23, 52, 86) and the position of attachment (23, 24, 45, 46) of the sugar residues to the aglycone.

The acyl group, if present, can be readily removed by cold basic hydrolysis, isolated and identified (18, 101).

The aglycone, produced after complete acid hydrolysis, is identified by paper chromatography (45, 46, 54, 101) and spectral methods (46, 48, 98) as with the glycoside.

The aglycone can also be degraded into smaller fragments by alkaline reagents (27) and the resulting phenolic compounds identified with paper chromatography and chromogenic reagents (21, 107).

MATERIALS AND METHODS

A. Anthocyanins

a. Materials

1. Plant material

Strawberries of the Ever-bearing type were grown in pots in the green-house.

2. ^{14}C-labelled compounds

D-glucose-UL-^{14}C

D-fructose-UL-^{14}C

Sodium acetate-1-^{14}C

DL-phenylalanine-1-^{14}C

DL-phenylalanine-^{14}C (ring labelled)

L-tyrosine-UL-^{14}C

Shikimic acid-GL-^{14}C

Quinic acid-GL-^{14}C

Cinnamic acid-3-^{14}C

Cinnamic acid-2-^{14}C

D-glucose-1-phosphate-UL-^{14}C

Uridine-diphospho-glucose (glucose-UL-^{14}C)

^{14}C-cyanidin-3-glucoside

^{14}C-pelargonidin-3-glucoside

Cinnamic acids were purchased from Merck, Sharp and Dohme of Canada, Ltd., Montreal, Canada. D-glucose-1-phosphate-UL-^{14}C and uridine-diphospho-glucose (glucose-UL-^{14}C) were purchased from International Chemical and Nuclear Corporation, City of

Industry, California. ^{14}C-cyanidin-3-glucoside and ^{14}C-pelargonidin-3-glucoside were isolated from strawberries fed with cinnamic acid-3-^{14}C. The rest were purchased from New England Nuclear Corporation, Boston, Mass.

b. Methods

1. Administration of ^{14}C-labelled compound to strawberries

At least 10 strawberries were used in each experiment. Maturing strawberries in the pink-white stage were removed from the plant by cutting the stems under water with a razor blade so that about one inch of stem remained attached to each berry. Individual berries was placed in an aqueous solution of the desired radioactive compound in a tube which was prepared from a 5 mm I.D. glass tubing by sealing off one end and cutting the tubing to make a one inch tube. The tube with the berry was placed in a beaker, and covered with a watch glass to minimize dehydration of the fruit. The cinnamic acid was administered as its sodium salt. About 1-3 μc of the radioactive compound was administered to each strawberry depending on its size. The ^{14}C solution (50-100 μl) was taken up within 3-4 hours. Water was added every time the tube was about to dry out so that the last trace of the ^{14}C compound was washed into the plant and that a continuous supply of water was available to the strawberries thus preventing dessication.

The strawberries were allowed to metabolize for 2 days at room temperature until they turned very red. The pigments were then extracted.

In the case of the experiments on glucosylation, the D-glucose-UL-^{14}C, D-glucose-1-phosphate-UL-^{14}C, or uridine-diphospho-glucose (glucose-UL-^{14}C) was injected into the strawberries receptacle (70) with a 100 μl syringe and #23 hypodermic needle. The fruits were allowed to metabolize for 1 or 3 hours before the extraction of the pigments.

In the experiments on the interconversion of pelargonidin-3-glucose and cyanidin-3-glucose, the ^{14}C pigments previously isolated and purified from strawberries fed with cinnamic-3-^{14}C were injected into the receptacles of strawberries. The fruits were allowed to metabolize for 24 hours before the extraction of the pigments.

2. Isolation and purification of the pigments

The fully ripened strawberries were extracted thoroughly with several portions of hot butanol containing 0.1% HCl. The anthocyanins were precipitated from the combined butanolic extracts by addition of about 10 volumes of petroleum ether (b.p. range 30-60°). The pigments were then dissolved in a small volume of methanol and the solution was streaked on Whatman #3MM paper and developed with BAW: n-butanol-acetic acid-water (4:1:1.25 v/v/v), in descending direction for 16 hours. The chromatograms were dried and the pigments, cyanidin-3-glucoside and pelargonidin-3-glucoside, were each cut from the paper and extracted several times with 0.01% methanolic HCl. The combined methanolic extracts were concentrated under reduced pressure in a flash evaporator at 38°C. The pelargonidin-3-

glucoside was again purified by descending paper chromatography using 2% acetic acid as developing solvent. Further purification was made using paper electrophoresis. The Durrum type paper electrophoresis cell was used, with a voltage of 480 volts in 0.5N acetic acid, on Whatman #3MM paper. Another descending paper chromatography was carried out using n-butanol-acetic acid-water (4:1:1.25 v/v/v), as developing solvent.

The purified pigment was dissolved in methanol containing 0.1% HCl.

3. Measurement of the yield of pelargonidin-3-glucoside

The optical density of the pigment in 0.1% methanolic HCl was measured with a Beckman DU spectrophotometer at 506 mμ (46). The concentration was obtained from a standard curve prepared with crystalline pigment, Fig. 3.

4. Measurement of radioactivity

A measured amount of the pigment or sugar solution was transferred into a planchet and the radioactivity of the pigment or sugar was determined with a Nuclear Chicago Corporation's Decade Scaler (model 181B) with automatic sample changer (model C-110-B) and printing timer (model C-111-B). A gas mixture of 98.7% helium and 1.3% butane was used as the carrier gas.

5. Acid hydrolysis of the pigment

Six normal HCl was added to the aqueous solution of a known amount of the pigment to make a 10% HCl solution, and the mixture was placed in a boiling water bath for 30 minutes. The mixture was cooled quickly. The pelargonidin was separated from the sugar

by extraction with a few drops of isoamyl alcohol (69).

6. Sugar moiety of the anthocyanin

After removal of the isoamyl alcohol layer, a portion of the hydrolysate was spotted on a Whatman #1 paper and developed descendingly for 48 hours in n-butanol-acetic acid-water (4:1:1.25 v/v/v) solvent. Authentic glucose was run side by side with the sugar. The sugar was further purified with a second descending paper chromatography using acetone-butanol-water (7:2:1 v/v/v) (56).

After the paper was thoroughly dry, the portion containing the sugar was cut as a strip and passed through an automatic chromatogram scanner (model 880) by Vanguard Instrument Company, La Grange, Ill., to determine if the sugar was labelled or not. The helium-butane mixture mentioned above was also used as the carrier gas.

7. Alkaline degradation of the aglycone

The pelargonidin was precipitated from the isoamyl alcohol extract by addition of about 10 volumes of benzene. The resulting pelargonidin was subjected to alkaline degradation by the procedure of Comte et al (27): The pigment was placed in a test tube and heated with KOH pellets under N_2 to 180-190°F for 3 minutes, then the temperature was raised rapidly to 245-250°F and maintained at this temperature for $3\frac{1}{2}$ minutes. The solution was cooled down as quickly as possible by first blowing air on the outside of the tube by means of a hair dryer, then by placing the tube in ice water when the temperature was low

enough not to cause breakage of the tube. The solidified mixture was dissolved in distilled water and acidified with 6N HCl to a pH of 5. The resulting solution was extracted 4 times with 3 ml portions of diethyl ether. The combined ether extracts were evaporated at room temperature almost to dryness by blowing a stream of N_2 over the solution. The resulting ether solution was transferred quantitatively to a spot on Whatman #1 paper and developed in a descending direction for 4 hours with a n-butanol-benzene-acetic acid-water (1:5:1:0.5 v/v/v/v, upper phase) solvent mixture (27). A mixture of authentic phloroglucinol and p-hydroxybenzoic acid was run next to the mixture. The 2 phenolic compounds were further purified first by thin-layer chromatography on silica gel G as adsorbent and absolute methanol as the developing solvent, and then by another descending paper chromatography using benzene-acetic acid-water (2:2:1 v/v/v, upper phase) as the solvent (21). The portion of the chromatogram containing the 2 phenolic compounds was cut as a strip and run under the chromatogram autoscanner to locate the radioactivity.

The phloroglucinol and p-hydroxybenzoic acid were visualized by spraying with diazotized sulfanilic acid (28, 35) (25 ml of freshly prepared 5% $NaNO_2$ + 5 ml of sulfanilic acid solution [0.9 gm sulfanilic acid + 9 ml concentrated HCl, diluted to 100 ml]), followed by 15% Na_2CO_3. Phloroglucinol gives a reddish brown spot and the p-hydroxybenzoic acid gives an orange spot.

B. Phenolic compounds

a. Materials

1. Plant materials

Strawberries as in section A.

2. ^{14}C-labelled compounds

Cinnamic acid-3-^{14}C.

b. Methods

1. Administration of ^{14}C-compounds

The cinnamic acid-3-^{14}C solution was injected into both green and pink-white strawberries. After 24 hours of metabolism, both pink-white and red strawberries were obtained.

2. Extraction of phenolic compounds

The strawberries were dropped into boiling 80% ethanol and kept there for 3 minutes (90) to inactivate the enzymes. After filtering, the residue was extracted twice with methanol. The combined alcoholic extracts were washed with petroleum ether (b.p. range 30-60°) to remove chlorophylls, carotenoids and waxy materials. The washed extract was then concentrated under vacuum at 38°C in a flash evaporator until all alcohol was evaporated. The aqueous solution was extracted 10 times with ethyl acetate. The combined ethyl acetate extracts were dried with anhydrous sodium sulfate, filtered and concentrated to a small volume.

3. Paper chromatography

The ethyl acetate extract was chromatographed descendingly in two dimensions on Whatman #1 paper, using n-butanol-acetic

acid-water (6:1:2 v/v/v) (81) as the first solvent for 15 hours and then 2% acetic acid as second solvent for 4 hours.

4. Examination of the paper chromatograms and location of the phenolic compounds

The air-dried chromatograms were observed in the visible, and under UV radiation, before and during fuming with NH_3. The papers were examined in both 254 and 336 mμ of UV radiation, and after spraying with 5% $AlCl_3$ in ethanol.

Another chromatogram was sprayed with 0.5% $FeCl_3$ + 0.5% $K_3Fe(CN)_6$ in aqueous solution. All phenolic compounds give blue spots. A permanent record could be obtained by dipping the chromatogram in 2N HCl solution followed by thorough washing in distilled water (7).

Another spray used was 15% Na_2CO_3, followed by diazotized sulfanilic acid.

Three percent p-toluene sulfonic acid in ethanol was used to locate the leucoanthocyanins and catechins. Leucoanthocyanins give orange to red spots, catechins give brown spots.

5. Purification of the compounds

The spots containing the compounds were cut from the chromatograms, eluted, and concentrated. The compounds were purified by paper chromatography using different solvent systems.

6. Identification of the compounds

i. Leucocyanidins

The portions of the paper (spots L1 to L5) containing leucoanthocyanins were each cut and eluted with 80% methanol.

The eluates were concentrated under vacuum. Each concentrate was transferred to a test tube, 0.6N butanolic HCl (110) added, and the tube was placed in a boiling water bath for 40 minutes.

The converted pigments were purified twice with descending paper chromatography using Forestal solvent (22): acetic acid-concentrated HCl-water (30:3:10 v/v/v) and n-butanol-2N HCl (1:1 v/v) (45) as the developing solvents.

The purified pigments were used for spectra determinations.

ii. Flavonols

The spots (Q, K,) which fluoresced yellow under UV in NH_3 fumes, were each cut and eluted with 80% methanol. The eluates were concentrated and hydrolyzed in 2N HCl for 20 minutes in a boiling water bath. The aglycone was extracted with ethyl acetate, and chromatographed twice using Forestal solvent and n-butanol-acetic acid-water (6:1:2 v/v/v) as developing solvents.

iii. Catechin

Spot C was cut from the chromatograms, eluted, and purified by paper chromatography using three different solvents: water, 2% acetic acid, and n-butanol-acetic acid-water (6:1:2 v/v/v).

7. Absorption spectra

The UV and visible absorption spectra of the purified compounds in 95% ethanol were obtained with a Bausch and Lomb spectronic 505 recording spectrophotometer. The spectra were

also obtained after addition of $AlCl_3$ (108), NaOAc, and NaOAc + H_3BO_3 (64, 67).

8. Measurements of radioactivity

The radioactivities of the purified spots were measured as described in the section on anthocyanins.

RESULTS AND DISCUSSION

A. Extraction and purification of the pigments

Several solvents could be used to extract the anthocyanins from fruits (54). One percent HCl in methanol was found to be the best solvent for extracting anthocyanins from Bing cherries (73). But in these experiments, hot 0.5% butanolic HCl was used to avoid the extraction of excess sugars which will interfer in the separation of the pigments on paper.

In order to ensure that the percent recoveries of the pigments from each experiment are as close to each other as possible, the isolation and purification procedures were standardized. It was found that 2 paper chromatographic runs, one paper electrophoresis and another paper chromatography were sufficient to give a pigment with constant specific activity. The paper should not be over or underloaded because overloading will result in inefficient separation and underloading will result in poor recovery of the pigments. The R_f values of the 2 strawberry pigments are presented in Table 1.

Table 1. The R_f values of strawberry pigments

	Paper chromatography		Paper electrophoresis
	BAW (4:1:1.25)	2% HAc	(0.5N HAc, 480 volts)
Pelargonindin-3-glucoside	.36	.34	Moved 3.3cm in 5 hrs to cathode
Cyanidin-3-glucoside	.29	.27	Moved 1.1cm in 5 hrs to cathode

B. Efficiency of various ^{14}C-compounds as precursors of pelargonidin-3-glucoside.

About 1-3 μc of the ^{14}C-labelled compound was administered into each strawberry depending on its size. Since the ^{14}C compounds administered had a sufficiently high specific activity (1-10 mc/m moles), there was no danger of overloading to a point which would affect the metabolism of the strawberries.

The efficiency of the various ^{14}C-labelled compounds as precursors of pelargonidin-3-glucoside was expressed as % ^{14}C converted. It was suggested (114) that the percentage conversion value is the most useful way for recording the efficiency of a precursor in a tracer experiment in those cases where reliable analyses of the amount of end product are feasible.

The efficiency of the various ^{14}C-labelled compounds as precursors of the pigment is shown in Table 2.

Table 2. Efficiency of various compounds as precursors of pelargonidin-3-glucoside of strawberries

Compound fed	% ^{14}C converted
Sodium acetate-1-^{14}C	.29
L-tyrosine-UL-^{14}C	.15
DL-phenylalanine-1-^{14}C	.85
D-glucose-UL-^{14}C	1.59
D-fructose-UL-^{14}C	1.66
Quinic acid-GL-^{14}C	.37
Cinnamic acid-3-^{14}C	3.32
Shikimic acid-GL-^{14}C	2.19

Shikimic acid and cinnamic acid were the best precursors among the compounds tested as shown by higher % ^{14}C converted into pelargonidin-3-glucoside. Glucose and fructose came next, followed by phenylalanine. Tyrosine was the poorest of them all. Sodium acetate and quinic acid were a little better than tyrosine.

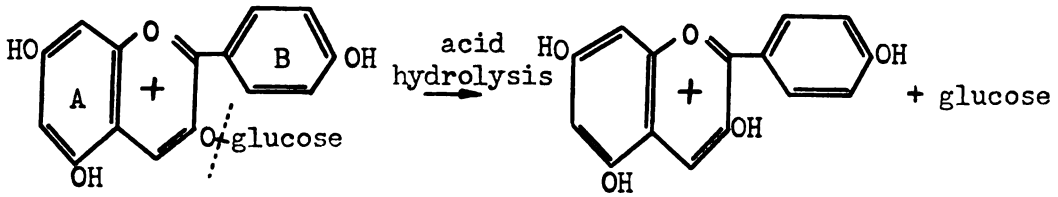
C. Distribution of ^{14}C in the pigment

In order to locate the position of the ^{14}C, the pigment was degraded into glucose, phloroglucinol, and p-hydroxybenzoic acid by acid hydrolysis and alkaline degradation, Fig. 4, as described in the experimental section.

After purification, the chromatograms were scanned for radioactivities. The results are shown in Table 3.

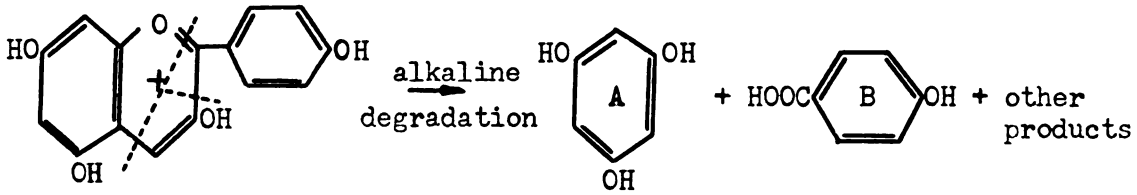
Table 3. Distribution of ^{14}C in pelargonidin-3-glucoside of strawberries with administration of various ^{14}C-labelled compounds

Compound fed	Phloro-glucinol	p-hydroxy-benzoic acid	glucose
Sodium acetate-1-^{14}C	+	trace	+
L-tyrosine-UL-^{14}C	-	+	-
DL-phenylalanine-^{14}C (ring labelled)	-	+	-
D-glucose-UL-^{14}C	+	+	+
D-fructose-UL-^{14}C	+	+	+
Quinic acid-GL-^{14}C	-	+	-
Shikimic acid-GL-^{14}C	trace	+	+
Cinnamic acid-3-^{14}C	-	+	-
DL-phenylalanine-1-^{14}C	-	-	-
Cinnamic acid-2-^{14}C	-	-	-



Pelargonidin-3-glucoside

Pelargonidin



Pelargonidin

Phloro-glucinol

p-hydroxy-benzoic acid

Fig. 4. Chemical degradations of pelargonidin-3-glucoside of strawberry fruit

Phenylalanine, tyrosine, cinnamic acid and quinic acid did not label the sugar moiety of the pigment.

Acetate contributed almost exclusively to ring A. Glucose and fructose contributed to both ring A and B. ^{14}C from shikimic acid, quinic acid, cinnamic acid-3-^{14}C, phenylalanine-^{14}C (ring labelled) and tyrosine was incorporated into p-hydroxybenzoic acid. Cinnamic acid-2-^{14}C and phenylalanine-1-^{14}C labelled the pigment but not any of the three degradation products. Therefore the ^{14}C must be in C_3 or C_4 of the pigment. This means that the cinnamic acid and phenylalanine should be incorporated as a unit. Figs. 5, 6, 7, and 8 show the incorporation of various ^{14}C compounds into pelargonidin.

From the results of these experiments, and those of Grisebach (38), a scheme of the synthesis of the C_6-C_3-C_6 skeleton of anthocyanin can be drawn. Fig. 9 shows such a scheme.

Ring B and the 3 carbon atoms (2,3,4) of the heterocyclic ring are derived from a phenylpropane compound such as cinnamic acid, phenylalanine or tyrosine. These compounds are incorporated as a unit. Ring A originates from acetate. Grisebach (38) found that when acetate-1-^{14}C was fed to red cabbage seedlings, the ^{14}C was concentrated in the oxygen-bearing carbons of phloroglucinol, and when he fed acetate-2-^{14}C, the ^{14}C was found in the non-oxygen-bearing carbons. This led him to assume a head-to-tail condensation of the acetyl units.

Phenylalanine or tyrosine was considered not to take part directly in the condensation to the C_6-C_3-C_6 compound (41).

Since shikimic acid and quinic acid were not as good precursors of the pigment as cinnamic acid, cinnamic acid or one of its derivatives

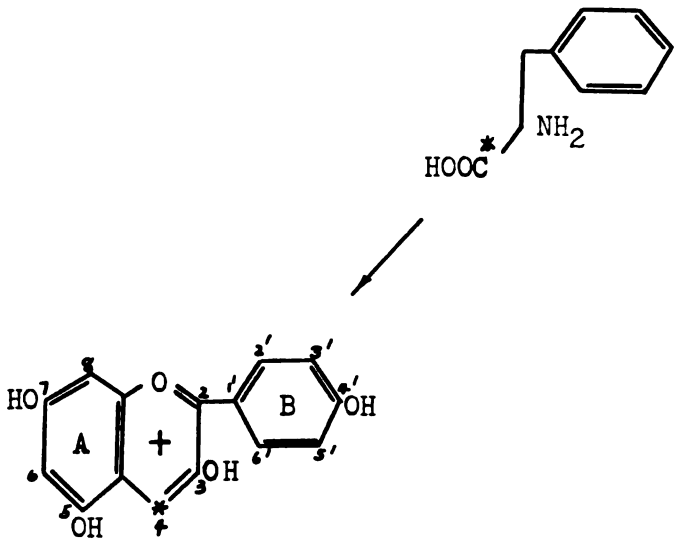


Fig. 5. Incorporation of phenylalanine-1-^{14}C into pelargonidin

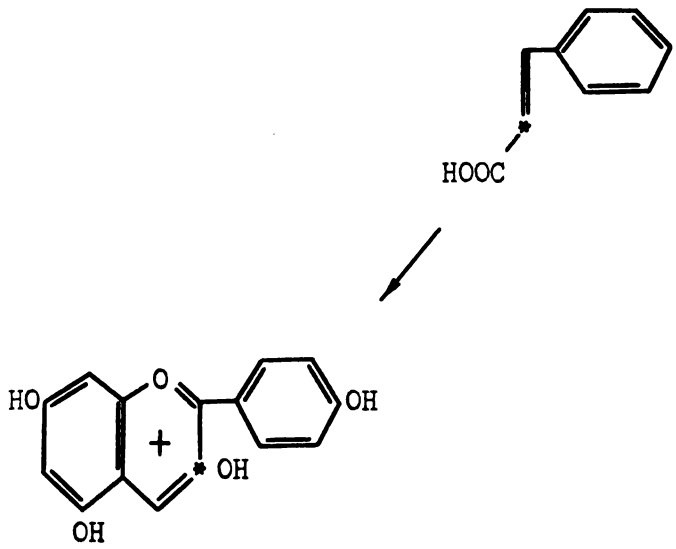


Fig. 6. Incorporation of cinniamic acid-2-^{14}C into pelargonidin

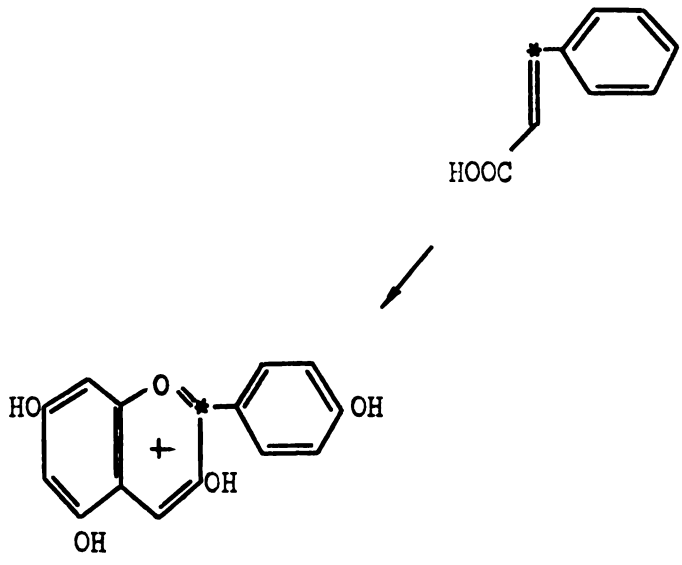


Fig. 7. Incorporation of cinnamic acid-3-^{14}C into pelargonidin

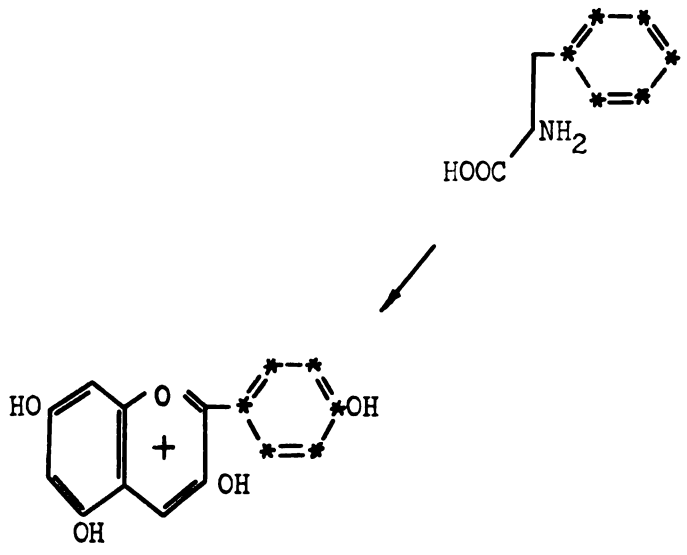


Fig. 8 Incorporation of phenylalanine-^{14}C (ring labelled) into pelargonidin

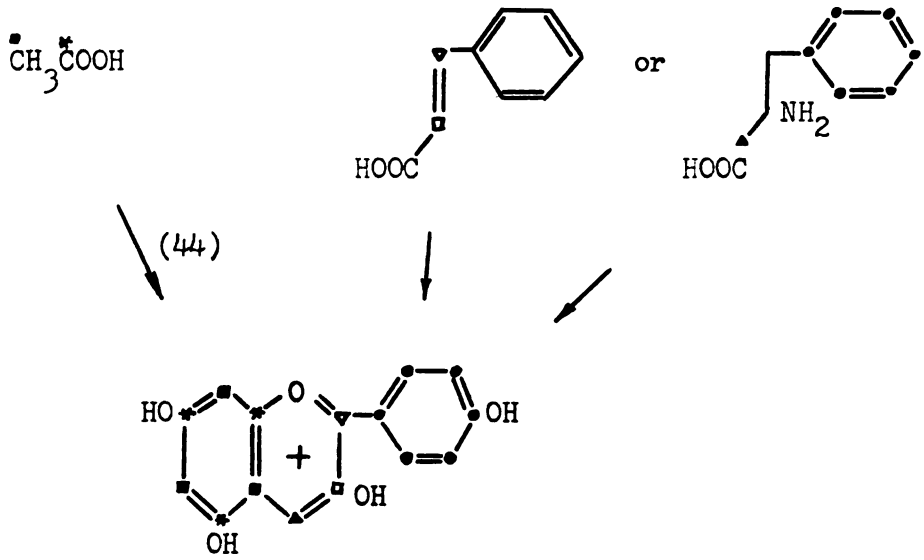


Fig. 9. Origin of the carbon atoms in anthocyanidin

is probably the phenylpropane intermediate which condenses with acetate to form the C_6-C_3-C_6 compound.

In the experiment to be shown later, when cinnamic acid-3-^{14}C was fed to green strawberry fruit and the phenolic compounds isolated, no cinnamic acid-3-^{14}C was found in the extract. Most of the radioactivities ended up in a phenolic compound which was extractable with ethyl acetate. This indicates that cinnamic acid is metabolized very readily by strawberry fruit. Since it disappears before the anthocyanin is formed, an intermediary phenolic compound, such as the highly radioactive one detected here, would be an intermediate in the anthocyanin formation.

It seems that anthocyanins are synthesized in a similar manner as the other flavonoid compounds such as quercetin in buckwheat and catechin in tea leaf. Various C_3-C_6 compounds were found to be incorporated into ring B, and acetate was found to be incorporated into ring A of quercetin (111, 115) and catechin (121). The C_3-C_6 compounds were also found to be incorporated into the flavonoid compounds as a unit (111).

D. Glucosylation of pelargonidin-3-glucoside

The mode of glucosylation of pelargonidin-3-glucoside was investigated by injecting solution of ^{14}C-labelled D-glucose, D-glucose-1-phosphate or uridine diphospho glucose into maturing strawberries.

Table 4 shows the results of the glucosylation experiments.

^{14}C-glucose did not label the sugar in pelargonidin-3-glucoside even in 3 hours. Glucose-1-phosphate was shown to be a better glucose donor to the pigment. UDPG was only a little better as glucose donor.

The pelargonidin was not labelled by any of the sugars fed, even up to 3 hours of metabolism.

Table 4. Efficiency of various sugar compounds as precursors of glucose in pelargonidin-3-glucoside of strawberry

Compound fed	Feeding time (hour)	% ^{14}C converted
D-glucose-UL-^{14}C	1	0
	3	0
D-glucose-1-phosphate-UL-^{14}C	1	trace
	3	.12
Uridine-diphospho-glucose (glucose-UL-^{14}C)	1	trace
	3	.18

It should be noted that there was no possibility of contamination of the isolated anthocyanin by the sugars fed as shown by the differences in R_f values of the various sugars and pigment in the different purification steps, Table 7.

E. Interconversion of the 2 pigments in strawberry

Experiments were carried out to see whether there is interconversion taking place between the pelargonidin-3-glucoside and cyanidin-3-glucoside.

^{14}C-pelargonidin-3-glucoside and ^{14}C-cyanidin-3-glucoside isolated from strawberries fed with cinnamic acid-3-^{14}C were used. The cinnamic acid-fed pigments were used because they had the highest specific activities and because the sugar moieties were not labelled, Table 3. Labelled sugar moiety in the compound fed is undesirable because the sugar might be detached from the aglycone and be used for the synthesis of new aglycone.

The radioactivity of the purified pigments was measured. Table 5 shows the results of the interconversion experiments.

Table 5. The radioactivity of the 2 major strawberry pigments fed with ^{14}C-pigments

Fed	Pigment isolated	Total cpm
^{14}C-pelargonidin-3-glucoside	Pelargonidin-3-glucoside	1110
	Cyanidin-3-glucoside	0
^{14}C-cyanidin-3-glucoside	Pelargonidin-3-glucoside	0
	Cyanidin-3-glucoside	730

There was no apparent interconversion of the 2 major pigments of strawberry. Maybe the specific activities of the compounds fed were not high enough to show any interconversion.

E. Phenolic compounds in strawberry

In an attempt to find the other precursors of the pigment, the phenolic compounds in the fruit were investigated.

a. Paper chromatogram

A drawing of the two-dimensional chromatogram of the ethyl acetate extract of the strawberry fruit fed with cinnamic acid-3-^{14}C is shown in Fig. 10. All are phenolic compounds except spots 8, 9, 10 and 12. The latter spots are useful in locating phenolic compounds in comparative paper chromatography.

The reactions of the spots to the detection methods are given in Table 6. The absorption spectra of some of the spots are shown in Figs. 11, 12, 13 and 14.

b. Identification of some of the phenolic compounds

1. Leucocyanidins

Spots L-1 to L-5 were each eluted from two-dimensional paper chromatogram, purified, and their UV absorption spectra deter-

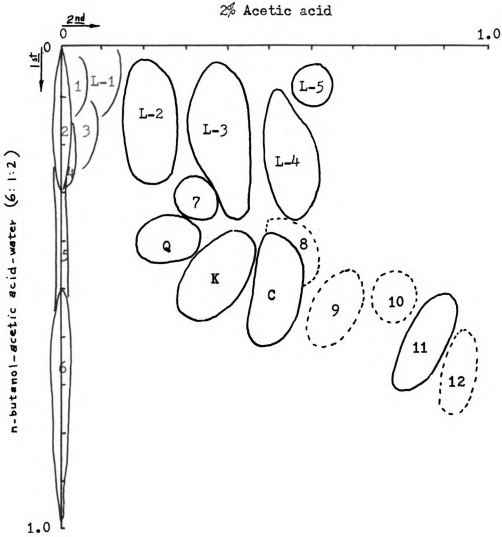


Fig. 10. Two-dimensional paper chromatogram of the ethyl acetate extract of strawberry fruit

Table 6. Reaction of spots on chromatogram to the detection methods

Detection method	Appearance	Spots reacting
visible light	yellow green	6
UV (long wavelength)	purple	2
	light yellow	1
	yellow	4, 7, 8
	blue	5, 9, 10, 12
	yellow green	6, K
	dark	Q
UV (short wavelength)	dark	11, C
NH_3 in UV (long wavelength)	light yellow	1, 2, 3
	yellow	4, 7, 8, Q
	yellow green	6, 9, K
	blue	5, 10, C
NH_3 in visible light	light yellow	Q, K
15% Na_2CO_3	yellow	2, Q, K
15% Na_2CO_3 + diazotized sulfanilic acid	orange	1, 2, 3, 5, L-1 to L-5, C, Q
	red violet	6
3% p-toluene sulfonic acid	red purple	1, 2, L-1 to L-5, C
	grey black	6
5% $AlCl_3$	yellow brown	2, 5, 6
	yellow	Q, K
	light	C
0.5% $FeCl_3$ + 0.5% $K_3Fe(CN)_6$	blue	1, 2, 3, 4, 5, 6, 11, L-1 to L-5, Q, K, C

mined in 95% ethanol (one peak at 280 mμ) and with added $AlCl_3$. No shift was detected upon addition of $AlCl_3$. This indicated that they might be related to one of the flavans (119).

When spots L-1 to L-5 were each heated in 0.6N butanolic HCl in a boiling water bath, red pigment was produced. This placed the compound in the general classification of leucoanthocyanins.

The red pigments were then chromatographed with the Forestal solvent. A red spot was detected in all fractions at R_f=.47. The spot corresponded to cyanidin as determined by co-chromatography in the same solvent system. They were also chromatographed in n-butanol-2N HCl (1:1 v/v) solvent. Their R_f=.73 also corresponded to that of cyanidin by co-chromatography.

The visible absorption spectra of all the red pigments, Fig. 11, showed a peak in 95% ethanol at 545 mμ , and shifted to a longer wavelength upon addition of $AlCl_3$, indicating the presence of a O-dihydroxyl group (34). The red pigments had the same spectral characteristics as cyanidin (46).

Therefore, spots L-1 to L-5 are leucocyanidins. They might be the same leucocyanidin with various degree of polymerization.

2. Flavonols

Spots K and Q fluoresced yellow under UV with NH_3 fumes, indicating that they might be flavonols. The spots were cut from chromatograms, eluted, concentrated and hydrolyzed with acid. The aglycones were extracted with ethyl acetate and chromatographed with the Forestal solvent. Spot Q, R_f=.42, was

quercetin, and spot K, R_f=.58, was kaempferol as shown by co-chromatography with kaempferol from instant tea extract (26) and quercetin from a commercial source.

Their R_f values in n-butanol-acetic acid-water (6:1:2 v/v/v) were also the same as the known kaempferol, R_f=.83, and quercetin, R_f=.64.

They also had the same UV absorption spectra, Figs. 12 and 13, as the known compounds.

3. Catechin

Spot C is catechin. It appeared dark under short wavelength UV, showed blue fluorescence under long wavelength UV after exposure to NH_3 fumes, and had the same R_f as known catechin in several solvent systems. It had R_f of .39 in distilled water, .48 in 2% acetic acid, and .62 in n-butanol-acetic acid-water (6:1:2 v/v/v). It also had the same absorption spectrum as catechin, Fig. 14.

c. Determination of radioactivity

The spots (L-1 to L-5, Q, K, C, and 11) were each cut from the chromatogram, eluted and the eluates concentrated. They were purified once more with descending paper chromatography using 20% acetic acid as solvent. The radioactivity of each compound was counted. Catechin, kaempferol, quercetin, and leucocyanidin were all radioactive, with a leucocyanidin (L-4) and catechin having the second highest radioactivity. Spot 11 was the most radioactive spot.

d. Comparison of the chromatograms of red and green strawberry

extracts

The spots corresponded to leucocyanidin (L-4 and L-5) and catechin (C) were higher in color intensity in the green than in red strawberry fruits. Since they were radioactive, they might be anthocyanin precursors.

However, catechin has been considered as a metabolic end product, because radioactive d-catechin which was taken up by Chamaecyparis pisifera cuttings was not transformed into other flavonoids such as quercetin or taxifolin (53).

Leucoanthocyanins have also been considered as metabolic end products (41). Bopp and Matthiss (20) attempted to show that direct transformation of leucoanthocyanins to anthocyanins can take place. With the feeding of carboxyl-^{14}C-phenylalanine to Impatiens balsamina, and plotting the specific activities of anthocyanin and leucoanthocyanin against time, they showed that the decreasing portion of the leucoanthocyanin curve intersected the anthocyanin curve approximately at its maximum. However, these authors offered no proof that the leucoanthocyanins in Impatiens were identical with flavan-3,4-diols. There are numerous substances (pro-anthocyanidins) (62) in plants which are transformed into anthocyanidin by the action of acids.

It can be stated that there is no conclusive evidence that catechin and leucoanthocyanins are not precursors of anthocyanins.

SUMMARY AND CONCLUSIONS

1. ^{14}C-labelled compounds were fed into maturing strawberries at the pink-white stage. The pelargonidin-3-glucoside was extracted and purified. The relative effectiveness of these compounds as precursors of the pigment was assessed by calculating the % ^{14}C converted. The order of decreasing effectiveness is as follows: cinnamic acid > shikimic acid > fructose > glucose > phenylalanine > quinic acid > sodium acetate > tyrosine.
2. The pelargonidin-3-glucoside was degraded into sugar, phloroglucinol and p-hydroxybenzoic acid by acid hydrolysis and alkaline degradation. Determination of these 3 degradation products showed that acetate contributed almost exclusively to ring A. Glucose and fructose contributed to both ring A and B. ^{14}C from shikimic acid, cinnamic acid-3-^{14}C, quinic acid, phenylalanine (ring labelled), and tyrosine was incorporated into p-hydroxybenzoic acid. Phenylalanine, tyrosine, quinic acid, and cinnamic acid did not label the sugar moiety. Cinnamic acid-2-^{14}C and phenylalanine-1-^{14}C labelled the pigment but not any of the 3 degradation products, suggesting that the C_6-C_3 skeleton might be incorporated as a unit.
3. The mode of attachment of glucose to the pelargonidin could be through sugar nucleotide, as shown by the labelling of the sugar in pelargonidin-3-glucoside when UDPG (glucose-UL-^{14}C) was fed. Glucose-1-phosphate was almost as good a sugar donor as the UDPG. ^{14}C-glucose was not incorporated into the pigment even after 3 hours of metabolism. The pelargonidin was not labelled in all

cases.

4. When ^{14}C-pelargonidin-3-glucoside was fed to strawberries, the cyanidin-3-glucoside was not labelled. The reverse was also true. This indicates that there might not be any interconversion between the 2 pigments.
5. The two-dimensional chromatogram of the ethyl acetate extract of the strawberry fruit, the reactions of the spots to various detection methods, and the UV absorption spectra of some of the compounds were presented.
6. Leucoanthocyanins, catechin, kaempferol and quercetin were present in the ethyl acetate extract of the strawberry fruits. They were all labelled when cinnamic-3-^{14}C was fed to the detached strawberry fruit.
7. On the basis of the information obtained in this work, it appears that a) acetic acid is a very likely precursor of ring A of pelargonidin-3-glucoside of strawberries, b) cinnamic acid, shikimic acid or phenylalanine may serve as precursor of ring B, c) cinnamic acid and phenylalanine are incorporated intact, d) UDPG and glucose-1-phosphate are mediating in the glycosidation of anthocyanidin.

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APPENDIX

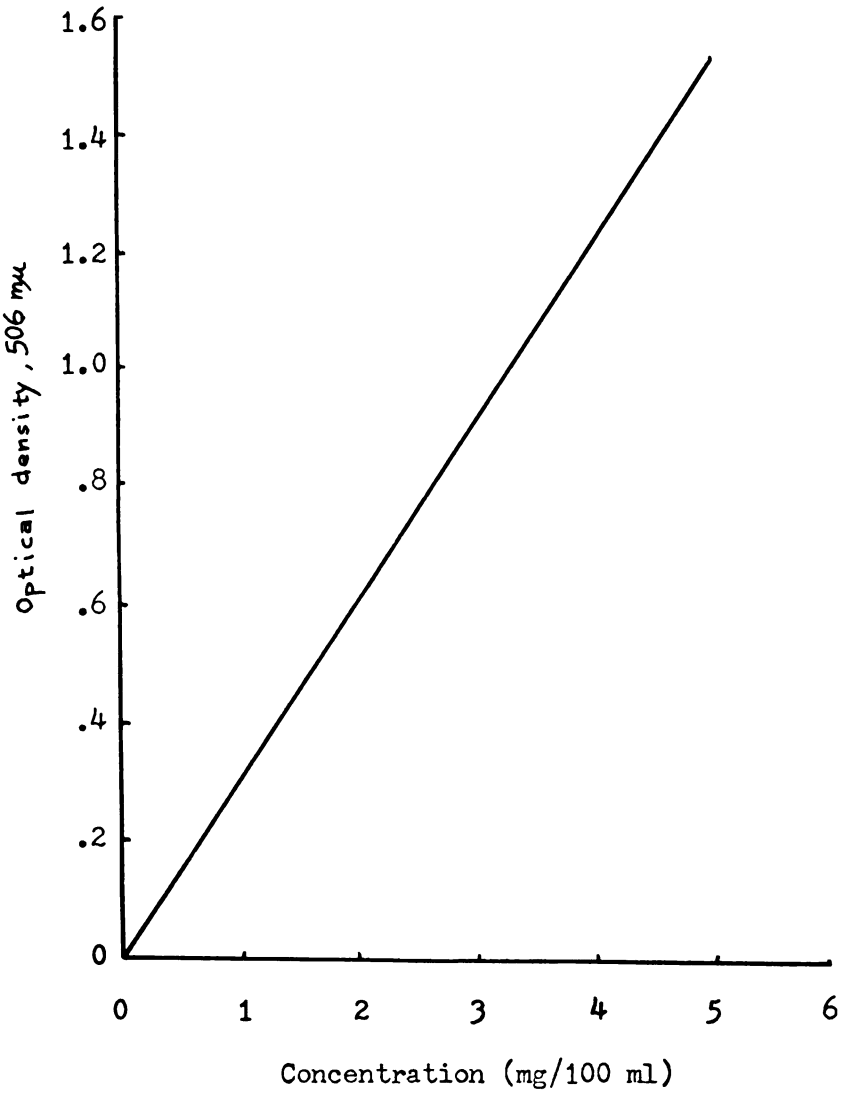

Fig. 3. Standard curve of pelargonidin-3-glucoside in .1% methanolic HCl.

Table 7. R_f values of different sugar compounds and pelargonidin-3-glucoside

Compound	Paper chromatography BAW (4:1:1.25)	Paper chromatography 2% HAc	Paper electrophoresis (0.5N HAc, 480 volts)
Glucose	.18	.34	0
Glucose-1-phosphate	.04	.94	moved 10.1 cm in 3 hours to anode
Uridine-diphospho-glucose	.00	1.00	moved 16.8 cm in 5 hours to anode
Pelargonidin-3-glucoside	.36	.34	moved 3.3 cm in 5 hours to cathode

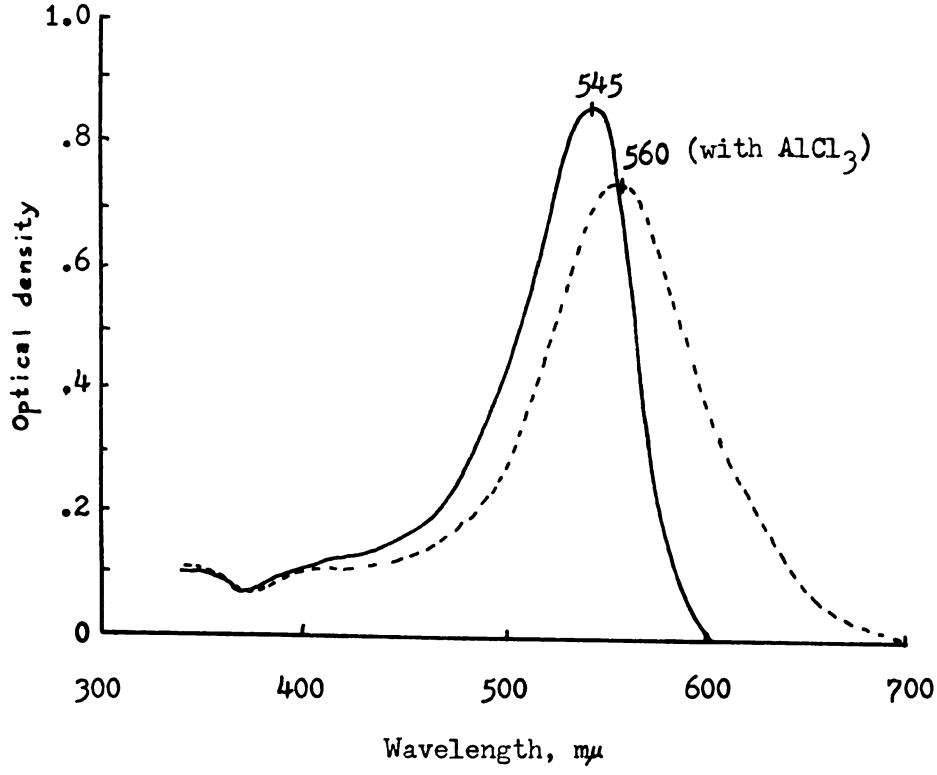

Fig. 11. Absorption spectra of cyanidin in .1% ethanolic HCl.

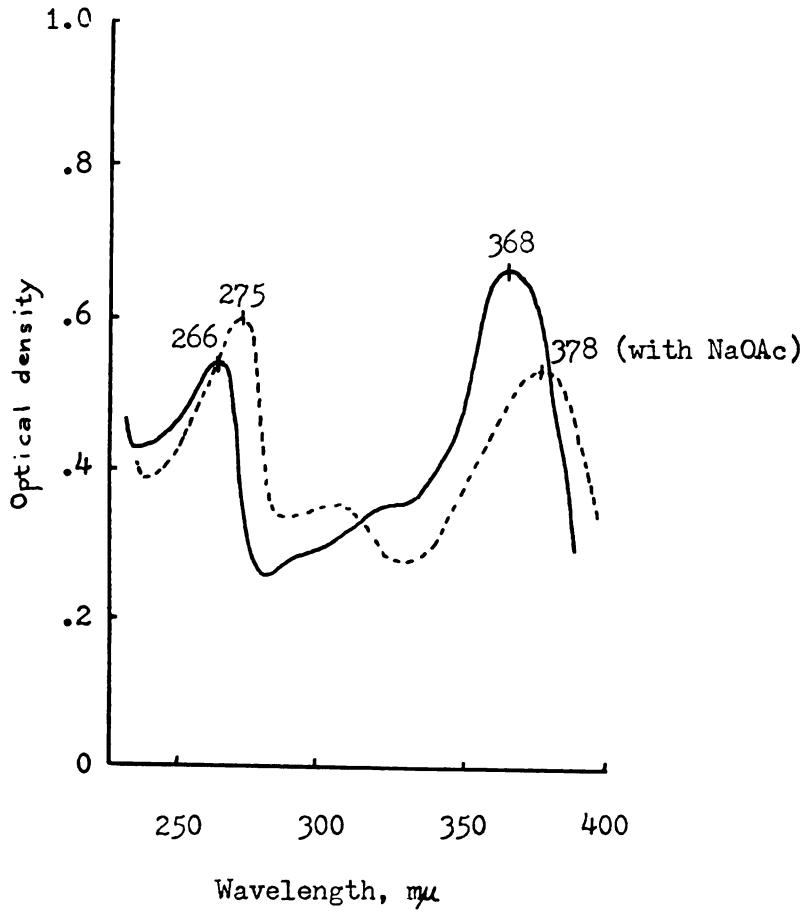

Fig. 12. UV absorption spectra of kaempferol in 95% ethanol.

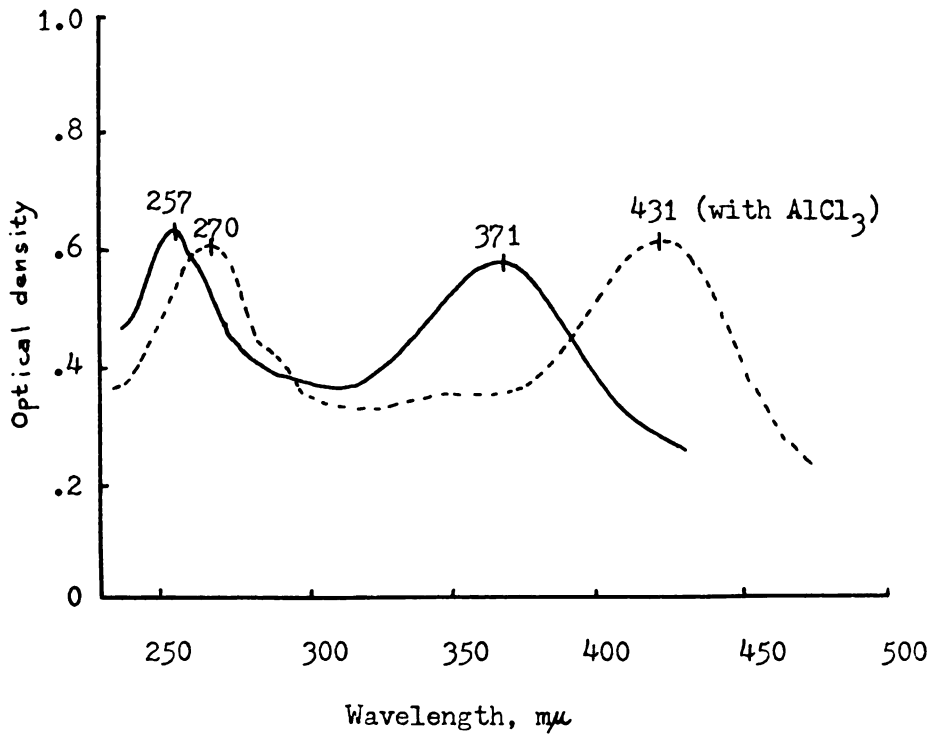

Fig. 13. Absorption spectra of quercetin in 95% ethanol.

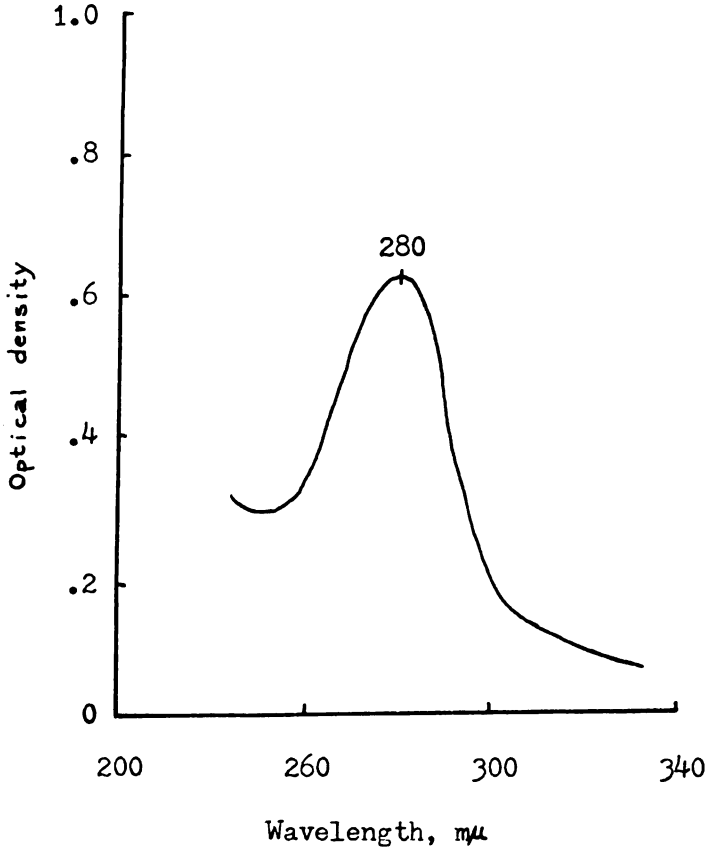

Fig. 14. UV absorption spectrum of catechin in 95% ethanol.