On the Anthocyanin in the Blood-red Flower of

*Tulipa Gesneriana L.*

Studies on the Physiology of Liliaceae II.

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In 1916 R. Willstätter and E. K. Bolton for the first time identified qualitatively the flower pigment of a variety of *Tulipa Gesneriana L.* with cyanidin glucoside and carotene, and later G. M. Robinson and R. Robinson (1932) with the aid of an improved qualitative method of their own found that anthocyanin in the tulip flower exists mostly in the form of pelargonidin glucoside, occasionally accompanied with a small amount of cyanidin glucoside.

In 1953 M. Shibata succeeded in obtaining in crystalline state the pigment of a garden variety of tulip, "Queen of night", and in 1954 that of another garden variety, "Eclipse". The former was determined as rhamnoglucosidyl-delphinidin, and was tentatively named "Tulipanin" (M. Shibata, 1956).

The present study has been undertaken for the purpose of investigating the chemical properties of the flower pigment of "Eclipse". The bulbs of this variety were imported into Japan from Holland in 1950. The flower is blood-red and has a blue patch at the lower part of the perianth. Early in May, 1955, the material was collected at Mr. Yonebayashi’s garden at Tonami city, Toyama Prefecture.

The process of isolation of the pigment is described in detail in the experimental part. The yield was 7.25 g, that is about 0.07% of the fresh weight of the whole supply of perianths. The crystallized pigment was a glycoside which consisted of
cyanidin, glucose and rhamnose. So "Eclipse"-anthocyanin must be either one of "keracyanins" (R. Willstätter and E. H. Zollinger, 1916, K. Hayashi, 1941, K. Hayashi et al., 1954) or prunicyanin (R. Willstätter and E. H. Zollinger, 1916). "Eclipse"-anthocyanin closely resembles hitherto known keracyanins. It slightly differs, however, from them described in the literature in the number of molecules of water of crystallization and in solubility in dilute hydrochloric acid.

It melted undepressed on admixture with an authentic specimen of keracyanin. Its Rf-value was also identical with that of keracyanin.

From these results it is concluded that "Eclipse"-anthocyanin is identical with keracyanin (3-O-rhamnoglucosidylcyanidin) (Fig. 1).

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**Experimental**

Isolation of "Eclipse"-anthocyanin as chloride.

About 10 kg of blood-red fresh perianths of "Eclipse"-flower, exclusive of pistils and stamens, were immediately immersed in 7l of cold 2 % methanolic hydrochloric acid. After being allowed to stand for 20 hours the immersed perianths were pressed and filtered. Then the residual perianths were soaked in 2l of methanol for 3 hours, pressed and filtered. This process was repeated once more. About 11 l of the combined dark red extracts obtained were mixed, under continuous stirring, with saturated solution of basic lead acetate, whereby white precipitate of lead chloride appeared first, and then bluish green precipitate of lead compound of anthocyanin was formed. The precipitate was filtered by suction, washed thoroughly with water and then with absolute ethanol and dried in a calcium chloride-containing desiccator, while the dirty purple filtrate, which no longer produced any precipitate with basic lead acetate, was discarded. The dried lead compound of anthocyanin was about 240 g in weight, inclusive of a considerable amount of lead chloride. It was then pulverized in a mortar, converted into chloride with 5 % methaolic hydrochloric acid and then filtered. The filtrate was concentrated to 1/2 volume in vacuo under 35°, chilled in a refrigerator and on addition of 3-5 volumes of ether precipitated anthocyanin chloride. After keeping in a refrigerator overnight, the supernatant liquor was decanted and the remaining hygroscopic amorphous anthocyanin was dissolved in a
minimum amount of absolute ethanol and filtered. The filtrate was added again with 2-3 volumes of ether and kept in a refrigerator overnight. The amorphous precipitate, which was no longer hygroscopic, was dissolved in a minute quantity of absolute ethanol and 1/2 volume of 20% ethanolic hydrochloric acid was added to it. It soon became muddy, an indication that the crystallization of anthocyanin started. It was kept in a refrigerator for 2 days and then filtered. The yield was about 7.5 g. Then a small amount of cold 20% ethanolic hydrochloric acid was added to the mother liquor and kept in a cold place for a few days, until a little more crystals of the red colouring principle were obtained. The total weight of the raw crystals was about 8.19 g. These crystals were dissolved in a minimum quantity of warm water and then filtered. An equal volume of cold 20% ethanolic hydrochloric acid was added to the filtrate and the mixture was allowed to evaporate slowly, till the solution began to turn opaque and deposited crystals at the bottom of a crystallizing dish. The process was repeated again and about 7.25 g of pure crystals were obtained. The yield of the colouring principle therefore was 0.07% of the fresh weight of the total perianths.

Anthocyanin chloride.

The "Eclipse"-anthocyanin, as given in Fig. 2, crystallized in red brown needles with a fine metallic luster. It melted at 177-179° (uncorr.), shrinking abruptly and turning black (decomp.). It was found to have 2 molecules of water of crystallization and to lose them in vacuo (2 mm Hg) at 105°, while the known keracyanins which it closely resembles are described to contain 6, 4, 3, or 2 1/2 molecules of water of crystallization. The solubility of "Eclipse"-anthocyanin increased with the decreasing concentration of hydrochloric acid, whereas known keracyanins are reported to be readily soluble in 1% hydrochloric acid and slightly soluble in either more or less than 1% hydrochloric acid.

No depression of melting point was observed in the mixture of keracyanin (176°) and "Eclipse"-anthocyanin (177°).

Paper chromatography by the ascending one dimensional procedure was achieved at room temperature with acetic acid-36% hydrochloric acid-water (3:1:8, v/v) and with use of the filter paper (Toyo, No. 52, 40×40 cm). The paper chromatogram was shown in Fig. 3 and the Rf-values were as follows: keracyanin (K)=0.47, "Eclipse"-anthocyanin (E)=0.46 and the mixture of keracyanin and "Eclipse"-anthocyanin (E+K)=0.46. The fact that the keracyanin from Canna generalis (K. Hayashi et al., 1954) and "Eclipse"-anthocyanin gave the same Rf-value with the same developing solvent shows that they are the same compound.

The distribution number of "Eclipse"-anthocyanin, as measured after R. Will-
stätter and E. H. Zollinger (1916), was 6.7 and 7.1, while that of keracyanin from *Canna generalis* has been reported 6.7 (K. Hayashi et al., 1954).

The “Eclipse”-anthocyanin showed the absence of methoxyl group after Zeisel-Pregl method.

Anal. Calcd. for C_{27}H_{31}O_{15}Cl: C, 51.33; H, 4.92. Found: C, 51.08; H, 4.94.

Water of crystallization. Calcd. for C_{27}H_{31}O_{15}Cl. 2H_{2}O: H_{2}O, 5.82. Found: H_{2}O, 5.41.

The absorption spectrum of “Eclipse”-anthocyanin was compared with that of the authentic specimen of keracyanin from *Canna generalis* with use of 5/10,000 mol of each colouring principle in 60 %ethanol containing 0.1 % hydrochloric acid.

As seen from Fig. 4, the absorption curves coincide almost completely.

On the basis of these data, it seems reasonable to assume that keracyanin from *Canna generalis* and "Eclipse"-anthocyanin are the same compound.

Hydrolysis of anthocyanin chloride.

In 6 ml warm water were dissolved 284.0 mg of pure crystals of “Eclipse”-anthocyanin and the solution added with an equal volume of 36 % hydrochloric acid was boiled for 3 minutes. The solution, when allowed to stand for a day in a refrigerator, deposited a dark chocolate-brown crystalline substance (aglycone). The aglycone was collected, washed with 10 % hydrochloric acid and dried over sodium hydroxide in a desiccator. The aglycone obtained was 144.9 mg in weight, the yield being 51.02 % of the anthocyanin used.

The rose-coloured mother liquor was shaken thoroughly with iso-amyl alcohol.
to remove the remaining aglycone. It was then shaken with ether to remove the small amount of iso-amyl alcohol and then neutralized with cold sodium hydroxide solution. The results of the qualitative determination by the orcinol and the phlorogruccinol tests and the diphenylamine test after Ihl-Peckmann-Jolles as well as by the paper chromatography indicated that the sugars in the solution were glucose and rhamnose.

In addition to the above mentioned tests, the mixed melting point determinations of the osazones of the sugars were carried out with the authentic specimens of rhamnosazone and glucosazone. The osazones of the sugars prepared by the usual method were treated with acetone: from the acetone soluble fraction crystalline yellow bushes (m.p. 179°) were obtained and from the acetone insoluble fraction fine yellow needles (m.p. 204°). On mixing the former with rhamnosazone (m.p. 180°) and the latter with glucosazone (m.p. 206°), no depression of melting point was observed.

It was concluded therefore that the sugars which combine with “Eclipse”-anthocyanidin are glucose and rhamnose.

Aglycone (cyanidin chloride)

The above mentioned crystals of the aglycone were recrystallized twice from 2 % ethanolic hydrochloric acid in characteristic red brown needles. They presented the same appearance as described in detail in the report of R. Willstätter and E. K. Bolton (see Fig. 5). They lost a molecule of water of crystallization in vacuo (2 mm Hg) at 105° and showed the following characteristics: they were easily soluble in ethanol and methanol; insoluble in water, dilute hydrochloric acid and concentrated sulphuric acid, either warm or cold; fairly soluble in 7 % sulphuric acid and crystallized out as the sulphate of aglycone. The aglycone dissolved in hydrochloric acid was perfectly epiphasic with iso-amyl alcohol. The purple ethanolic solution of aglycone on addition of 2 volumes of water became colourless (pseudobase formation); on addition of ferric chloride solution, turned blue and then dirty green; turned reddish purple with 50 % sodium acetate; blue with 50 % sodium carbonate; when 20 % lead acetate was added, formed a blue precipitate.


The properties and the results of the analyses coincide perfectly with those of cyanidin, indicating that the aglycone of “Eclipse”-anthocyanin is none other than cyanidin.
Summary

The anthocyanin in blood-red flower of "Eclipse", a garden variety of tulip (*Tulipa Gesneriana* L.) has been studied and it has been proved that it is none other than keracyanin (3-O-rhamnoglycosidylvianidin 2H₂O) by chemical analysis, absorption spectroscopy and paper chromatography.

We are indebted to Prof. K. Hayashi (Tokyo University of Education) for the valuable advice and the kind supply of the authentic specimen of keracyanin and to Mr. Yonebayashi for the donation of "Eclipse"-flower used in the experiment.

References


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Observational and Experimental Studies of Sensitive Plants

IX. On the Canaliculated Intercellular Spaces of Primary Pulvinus*

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In one of the previous papers of this series of investigation, the author has confirmed the presence of canaliculated intercellular spaces in the motor tissue of *Mimosa pudica* (1). In the present report, the secretion of tannin substance into the canaliculated intercellular spaces were demonstrated in the fixed material. Here, the canaliculated spaces of the upper side and the lower side of primary pulvinus are chiefly dealt with.

Material and Method

The primary pulvinus of *Mimosa pudica* was employed for material. The plants were raised in pots exposed to the open air in the field. Anthocyanin was found to

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