86. *Blue Anthocyanin from the Flowers of Commelina, the Crystallisation and Some Properties Thereof*

*Studies on Anthocyanins. XXX**

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Despite of long studies of organic chemists,^{1,2)} the nature of the blueness in flowers has tenaciously defied their attacks. Several years ago, a series of interesting experiments was described by K. Shibata and his co-workers³⁾ in support of the hypothesis formerly presented by K. Shibata, Y. Shibata, and I. Kashiwagi,⁴⁾ according to which certain metal complex compounds of anthocyanins play an essential rôle in the manifestation of a surprising variety of flower colors in nature. Nevertheless, this important problem awaits further investigations in view of obtaining more conclusive chemical evidences. The present investigation has been conducted along this line and some notable informations, although of a qualitative nature, are described here.

Among several kinds of plant materials examined, tiny blue flowers of *Commelina communis* L. were found to be the best material for the present purpose, because of an unusual stability of the flower color; and one of the keypoints leading to the successful crystallisation lies in an application of expressed flower juice as starting material. After repeated fractionation and precipitation using pure water and ethanol alone, the pigment was obtained finally as brilliant, blue, prismatic needles. The behaviors of this substance are quite different from those of the ordinary form of anthocyanins: *e.g.* unusual stability toward acids, non-dialysability through semi-permeable membranes, electrophoretic migration towards the anode, solubility in none of the organic solvents. Especially noteworthy is that the substance contains an appreciable amount of metallic elements, of which Mg and K are inherent to the pigment molecule. This was ascertained by spectroscopic as well as paperchromatographic method.

Further chemical analyses directed in a quantitative manner are still in progress and the results will be published shortly.

Experimental. *Extraction and crystallisation of the blue pigment.* 516 g fresh petals, which were collected from *ca.* 25,000 flowers of

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Commelina, were pressed tightly in a gauze, and the residue was triturated with 45 ml water and pressed again. Combined solution was filtered (316 ml), and mixed with 9 vol. of abs. ethanol; thereby the pigment was completely precipitated as an amorphous powder, which was collected and air-dried. Yield *ca.* 1.5 g. This was dissolved in cold 30 ml water, and greenish gray insoluble matters were filtered off. On addition of further 30 ml portion of abs. ethanol and cooling, several blue micro-crystals appeared together with a large amount of colorless colloidal particles. After filtration, further 15 ml abs. ethanol was added very slowly into the filtrate; soon the blue pigment commenced to separate in crystalline form. After standing overnight in a refrigerator, the crystals, which were still accompanied by some impurities, were collected on a glassfilter and air-dried. Yield *ca.* 200 mg. Further crop of impure crystals (110 mg) was obtained from the filtrate by the addition of 30 ml ethanol.

Further purification. Throughout the whole purification process, all solvents used were purified by repeated distillation, and the filtration was done through glassfilter, washed free from soluble inorganic matters before use.

100 mg of crude crystals was dissolved in 3 ml of cold water and filtered; the resulting deep blue solution was added with 6 ml abs. ethanol to form amorphous precipitate including impurities present, and filtered quickly by suction. To the filtrate 9 ml ethanol was added so gently as to form distinct layers between two solvents, and the solution was stood overnight in the cold. During this time, the pigment separated completely in aggregates of prismatic needles, which were practically free from impurities. The process was carefully repeated again, and 50 mg of pure crystalline pigment was obtained. When precipitated with acetone in similar manner, about 45 mg of pure crystals was recovered from 100 mg of crude crystals.

The crystalline specimen sinters at about 230° slightly, but does not melt below 290°. The substance contains neither methoxyl nor halogen. After drying in a vacuum over P₂O₅ at 100°, it loses the water of crystallisation corresponding to 10.66, 11.24, and 10.90%, respectively. Elemental analyses based on anhydrous substance gave the following results; C 51.87, 50.93; H 5.07, 5.02; ash (as oxide) 9.27, 10.17; N 0.76% (after Dumas-Pregl). In order to know whether N is inherent to the molecule or not, further recrystallisations were repeated twice, and the homogeneous, short-cut, prismatic needles thus obtained were analysed again. Found: C 50.60, H 4.22, ash (as oxide) 9.57, and N 0.46%. As this lowest range of N-value was also encountered on blank tests using N-free authentic samples under the same experimental conditions, the N-value found above may well be negligible

in our further criticism on pigment constitution.

Notes on some important properties. The pigment crystals dissolve in water very easily with deep blue color indistinguishable from that of *Commelina*-flowers. The substance is neither acid nor base, because no change of pH-value could be observed before and after dissolution of the crystals in distilled water. Among various organic solvents tested, ethylene glycol is only one, in which the pigment dissolves with blue coloration; but the color fades gradually into brown on standing.

The following reactions were noted with an aqueous solution of the pigment:

- Picric acid rose-red color, but no precipitate;
- Gallic acid no color change, but the original blue fades into purple on standing;
- Ammonium sulfate . . . neither color change nor precipitation;
- Phosphotungstic acid . . same as above;
- Lead acetate complete precipitation in flocculent form;
- Ferric chloride green, followed by decolorization and decomposition.

Stability against hydrochloric acid. The pigment is very stable in aqueous hydrochloric acid below 1% concentration. For instance, a concentrated aqueous solution was prepared, and every two drops thereof were added in small test tubes containing each 1 ml hydrochloric acid of 0.5, 1, 2, 5, and 10%, respectively, and color change of each solution was carefully observed. Although a violet to reddish tinge appeared in solutions of more than 2% acid, no color change occurred in acids of 1% or below. Despite of these initial differences, conversion of blue into red took place within 2 to 3 hours in all cases.

After standing for 3 weeks, each of the reddened solutions obtained above was shaken with equal volume of *iso*-amyl alcohol, whereby all of them showed nearly the same distribution corresponding to those of ordinary acylated diglycosidic anthocyanins. Accordingly, it follows that under these experimental conditions the hydrolytic breakage takes place not in glycoside bonds but probably in sites of metal chelation within a complex pigment molecule.

Paperchromatographic test. Irrigation with commonly used solvent mixture brought about decolorization or tailing. Only the one available was 50% ethanol. With this solvent the pigment gave a blue distinct spot, R_f-value of which was found as 0.77 by one-dimensional, ascending method at 20°.

Paper-electrophoresis. The experiment was carried out with M/20 sodium acetate buffer (pH 6.0) on Tôyô No. 50 filter paper. The pigment solution was streaked on a paper sheet just halfway between

the electrodes, and 0.5 mA/cm electric current was applied at room temperature; the pigment spot began to move at once towards the anode. After 3 hours, the distance of migration was about 15 mm. Neither breakdown of the pigment nor liberation of organic substances took place during the electrophoresis. However, the electrophoretic behavior was quite reverse in the case of ordinary red form of anthocyanin; it tends to migrate towards the cathode. This clearly indicates the existence of essential structural difference between the blue and red forms of anthocyanin.

Quantitative tests on products of complete hydrolysis. 1.1 mg of air-dried crystals was dissolved in 0.5 ml 20% hydrochloric acid, boiled for 2 minutes, and cooled quickly in running water. With this hydrolysate the following tests were carried out:

(a) *Ether-soluble component.* The above hydrolysate was shaken twice with equal volume of ether. After evaporation of the ethereal extract, colorless needle-shaped crystals remained, which were identified as *p*-coumaric acid by paperchromatography. $R_f=0.87$ was found after irrigation with *n*-butanol/acetic acid/water (4:1:5, v/v) by one-dimensional, ascending method at 20°.

(b) *Anthocyanidin.* The above aqueous layer was diluted with an equal volume of water, and after removal of the last trace of ether *in vacuo*, was extracted twice with each 0.5 ml portion of *iso*-amyl alcohol. The anthocyanidin was transferred thoroughly into the alcoholic layer, and qualified by paperchromatography; thereby a single spot was obtained after irrigation with acetic acid/conc. hydrochloric acid/water (5:1:5, v/v) by ascending method. $R_f=0.2$ (at 20°). By means of co-chromatography and parallel run, this was identified as delphinidin chloride.

(c) *Sugar component.* Colorless aqueous solution obtained above was evaporated to dryness *in vacuo* over solid sodium hydroxide. The residue was taken up in several drops of water, and paperchromatographed using butanol/acetic acid/water (4:1:5, v/v) as solvent and 1% alcoholic aniline hydrochloride or ammoniacal silver nitrate as spray reagent. One sugar spot alone could be detected on the chromatogram; $R_f=0.36$ (by ascending method at 20°). This agrees very well with that of glucose ($R_f=0.35$).

To sum up, it seems that the organic components of the blue pigment molecule are delphinidin, glucose, and *p*-coumaric acid. This result is quite same as previously shown by C. Kuroda⁵⁾ in the case of 'awobanin', an ordinary red form of anthocyanin obtained from the same plant source.

Identification of metallic components. After incineration in a platinum boat, 19.4 mg blue crystals gave 1.6 mg white ash (8.25%).

When added with a few drops of 10% aqueous hydrochloric acid, the ash dissolved completely under slight bubbling. This indicates the presence of carbonates of certain alkali metals. The solution was diluted with water (*ca.* 2 ml), and applied to paperchromatographic test. As control, chlorides of heavy metals, Mo, Co, Cu, Ni, and Fe, were run in parallel, using *n*-butanol/acetic acid/water (4:1:5, v/v) on one hand, and acetone/*n*-butanol/hydrochloric acid (10:4:2, v/v) on the other. Development of the characteristically colored spots was effected by rubenic acid and pyrocatechol, and also by potassium ferrocyanate reagent. In these cases, none of the metals tested could be detected in ash substance in question. The same negative results were also ascertained by colorimetric tests on a solution of ash by means of the following reagents: ammonium rhodanate or α,α' -dipyridyl for Fe, potassium xanthogenate for Mo, chromotropic acid for Ti, benzidine for Mn and benzoin oxime or dithizone for Cu.

As regards alkaline earth metals, a solution of ash in dilute hydrochloric acid was spotted on Tôyô No. 50 filter paper and irrigated with acetone/*n*-butanol/conc. hydrochloric acid (10:4:2, v/v) at $25 \pm 2^\circ$ according to the ascending method. The detection of the spot was accomplished by means of dilute aqueous solution of plasmocorinth B,^{***)} with which Ca gives a red, and Mg a blue-violet color. The sample derived from the blue crystals gave well-defined single spot, showing blue-violet color and $R_f = 0.10$. By parallel run, the R_f -value of Mg was found to be somewhat higher ($R_f = 0.12$). Despite of this slight disagreement in R_f -value, the color of spot was quite identical in both cases. No red spot due to Ca could, however, be observed on the chromatogram. Therefore, magnesium seems to be one of the chief components of ash substance.

Emission-spectral analysis. Further qualification of the metallic elements contained in the blue crystals was performed by means of emission spectrum, whereby the blue pigment itself was used as materials on one hand, and the white ash therefrom on the other. In both experiments the results were quite identical, and have revealed the presence of the following elements: Mg, K, and Na as main components, and Ca, Mn, Cu, Ti as trace elements. Among them, two elements, Mg and K, take part in the formation of blue anthocyanin, while the others are due to contamination during the course of preparation, as will be shown in subsequent communication.

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***) Disodium salt of 1-hydroxy-4-chloro-2,2-diazobenzene-1,8-dihydroxynaphthalene-3,6-disulfonic acid (Sumitomo Chemicals). Cf. H. Kikkawa and Z. Ogita: Kagaku (Chemistry) (in Japanese), **10**, 77/81 (1955).

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