# Isolation and Crystallisation of the Red Anthocyanins in Natural State.*

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## A). Crystalline red anthocyanin from the cornflower

### *Introduction*

The problem[1] of flower color variation has been discoursed for many years. Recentyl, K. Hayashi and his co-workers[2,4] have succeeded in isolation of the blue pigments, commelinin and protocyanin, and stated that the blue color of these pigments is due to the complexes, which are composed of some metals and anthocyanins. On the other hand, the real nature of red anthocyanins in flowers is still open to further investigation.

At first, red cornflower was used as material for the preparation of red anthocyanin in its natural state. Powdered, dry petals were extracted with cold 70% methanol in the absence of hydrochloric acid; subsequent process was made in a manner as described below. Fotunately, the red pigment was obtained in a crystalline form. Analysis have shown that neither organic acid, polysaccride nor N-substance is contained in this red pigment. According to the result of elementary analysis, the pigment should have a composition that corresponds to [pelargonin]-OH.

Here, it must be noted that in a curde methanolic extract three kinds of pigment have been detected on the chromatogram, i. e., pelargonin itself and two kinds of p-hydroxybenzoic acid esters of the same anthocyanin. In red flowers, these 3 components are contained nearly in a ratio of 7 : 10 : 4. When the red flowers are immersed in 2% methanolic hydrochloric acid, all three components are converted into pelargonin chloride. The same is true in saponification test.

### *Experimentals*

1) Preparation of flower material and prelimary test on pigment components.

For prompt dehydration, fresh flowers (250 g) were immersed in acetone for several minutes, filtered and dried. 50 g of powdered material were extracted with 500 ml 70% methanol for 40 minutes at 60°, until the residue became almost colorless. The color of the filtered extract was purplish red, in which three pelargonidin-glucosides, Cent-R-A, Cent-R-B, and Cent-R-C, were detected by paperchromatography (c. f., Table 1). With these pigment spots, which were obtained by irrigation with 1% HCl for 3 hours (ascending method, on Tôyô No. 51 filter paper), comparative amount was measured by

* Preliminarily reported in Proc. Japan Aead., 40, 516, (1965)

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means of densitometer, using optical density as a measure. Thus, the three pigments, Cent-R-A/Cent-R-B/Cent-R-C, were present approximately in a ratio of 7 : 10 : 4. These pigments were thoroughly converted into pelargonidin on standing overnight in methanolic hydrochloric acid (e. g., 5 % acid or higher). After saponification with 7 % aqueous sodium hydroxide in an atmosphere of nitrogen (for 40 minutes at room temperature), three pigments were converted into a single substance, which was identified as pelargonin. The organic acid liberated by this treatment proved to be p-hydroxybenzoic acid by paper-chromatography.

2) Crystallization of the pigments as chloride.

Powdered red petals (8 g) were extracted twice with cold 2 % methanolic hydrochloric acid (200ml and 100ml). The combined orange red extract (250ml) was concentrated *in vacuo* up to 50ml at 30-40°, and the concentrate was stood overnight in the cold, whereupon some needle-shaped crystals separated out. These were collected by suction, the filtrate was mixed with ether (50ml), and stored in the cold to complete the separation. By this process 0. 18g of crude pigment were obtained. The whole crop of brownish red pigment was dissolved in 0. 5% aqueous hydrochloric acid (10ml) at 80-100°, and the filtered solution was stood for 0. 5 hours at room temperature. Some grayish precipitates were filtered off, and the filtrate was stood overnight in the cold, whereupon needle-shaped crystals of the chloride separated out. Yield 0. 1g. For further purification, this crop was recrystallized from hot 0. 5 % aqueous hydrochloric acid (ca. 10ml). By this means, pelargonin-Cl was obtained in the form of carmin-red needles (0. 08g), as shown in Fig. 2.

**Fig. 1**
Crystals of *Centaurea*-red-anthocyanin: Cent-R-A (crystallized from ethanol-water) ×ca. 1200

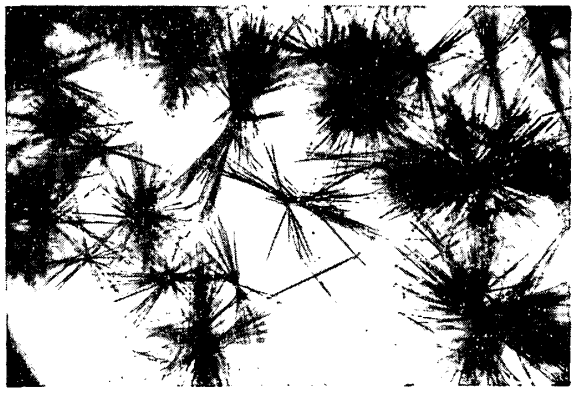


**Fig. 2**
Crystals of *Centaruea*-red-anthocyanin: pelargonin chloride (crystallized from 5% MeOH-HCl). ×ca. 1200

The identification of the crystalline pigment as pelargonin chloride was effected by paper-chromatographic examination as usual. Rf-values obtained with several solvent mixtures showed the identity of this pigment with an authentic specimen of pelargonin chloride, as shown in Table 2.

The absorption curves was also sufficient to show the identity of both pigments (Fig3). An anhydrous pigment melted at 179°C under decomposition, and no depression was

observed in practice on admixing with the authentic specimen.

Hydrolysis of the crystalline pigment was made with 20% HCl by boiling for 3 minutes. The hydrolysates that were produced were pelargonidin chloride and glucose alone. This was ascertained by paper-chromatography in the usual way. Furthermore, elementary analysis gave the results corresponding to the diglucoside of pelargonidin chloride, as shown in Table 3.

3) Isolation of the red anthocyanin in its native form.

Although a vast amount of informations has been accumulated on the structure of anthocyanins, nothing is known about the intrinsic form of this pigment, that really occure in the cell sap of red flowers. On this account, the isolation of the red pigment from red cornflower was attempted in this experiment.

Powdered petals (39g) of red cornflower was immersed in 60% methanol (1.051), and stood in the cold for an hour, then at 60-70° for further 20 minutes. Pink-orange extract (900ml) was evaporated *in vacuo* up to a syrup. This was washed

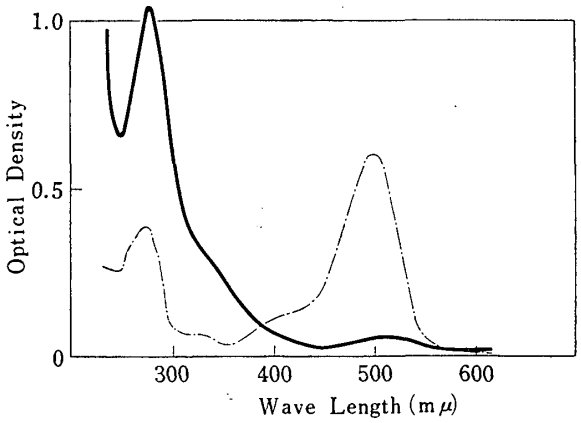



**Fig. 3** Absorption spectra of

——— crystalline *Centaurea*-red-anthocynin (Cent-R-A) and pelargonin-Cl, in 0.1 mol K-acetate buffer (pH 4.75, $8\times10^{-5}$ mol)

– – – – crystalline *Centaurea*-red-anthocyanin (Cent-R-A) and Pelargonin-Cl, in 5% HCl (aqueous) : $2\times10^{-5}$mol

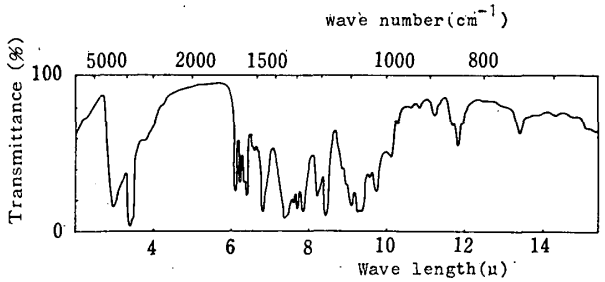



**Fig. 4**
Infra-red absorption spectrum (nujol mull).
—— pelargonin-Cl

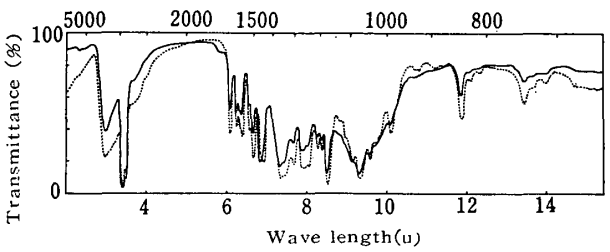



**Fig. 5**
Infra-red absorption spectra (nujol mull).
—— pelargonin-OH
····· pelargonin (Cl-free)[5]

**Table 1**

Rf values of *Centaurea*-Red-anthocyanins crystallized from 70% methanol at 60 (by ascending method, on Tôyô No. 51 filter paper at ca. 15°)

| Solvent | Cent-R-A | Cent-R-B | Cent-R-C | Cent-R-S* | pelargonin chloride | Raphanusin-Cl |
|---|---|---|---|---|---|---|
| 1% HCl | 0.23 | 0.36 | 0.50 | 0.24 | 0.24 | 0.65 |
| AcOH/HCl/$H_2O$ (15 : 3 : 82) | 0.43 | 0.53 | 0.63 | 0.43 | 0.43 | 0.69 |
| n-BuOH/HCl/$H_2O$ (7 : 2 : 5 : ) | 0.14 | 0.23 | 0.23 | 0.14 | 0.14 | — |
| N-BuOH/ 2 N-HCl (1 : 1) | 0.09 | 0.24 | 0.24 | 0.09 | 0.09 | 0.08 |
| n-BuOH/AcOH/$H_2O$ (4 : 1 : 5) | 0.28 ** | 0.35 ** | 0.35 ** | 0.23 | 0.23 | 0.15 |
| Visible color | Orange red | orange red | orange red | orange red | orange red | orange red |
| Color in ultra Violete | Yellow | yellow | yellow | yellow | yellow | yellow |

* saponified anthocyanin
** red in visible light
dull red under UV-light

**Table 2**

Rf values of crystalline *Centaurea*-Red-anthocyanin (Cent-R-A) :
(by ascending method, on Tôyô No. 51 filter paper, at ca. 15°)

| Solvent | Cent-R-A | Crystal as chloride | Pelargonin-Cl |
|---|---|---|---|
| 1% HCl | 0.24 | 0.24 | 0.24 |
| AcOH/HCl/$H_2O$ (15 : 3 : 5) | 0.43 | 0.43 | 0.43 |
| n-BuOH/HCl/$H_2O$ (7 : 2 : 5) | 0.14 | 0.14 | 0.14 |
| n-BuOH/2N-HCl (1 : 1) | 0.09 | 0.09 | 0.09 |
| N-BuOH/AcOH/$H_2O$ (4 : 1 : 5) | 0.28 * | 0.23 | 0.23 |
| Colors in visible light | orange red | orange red | orange red |

* red in visible light
dull red in UV-light

**Table 3**

Elementary analysis of pelargonin-Cl isolated from red cornflower : C-H determination

| Subst. (air-dried) | $CO_2$ | $H_2O$ | C% | H% |
|---|---|---|---|---|
| 3.092mg | 5.405mg | 1.541mg | 47.70 | 5.58 |
| 3.065mg | 5.365mg | 1.485mg | 47.77 | 5.42 |
| Calc. for $C_{27}H_{31}O_{15}Cl \cdot 2\frac{1}{2} H_2O$ | | | 47.97 | 5.37 |

with ether (100ml), and ethanol (100ml), in order to remove all soluble matters present.

The insoluble portion dissolved in warm water (30ml), filtered, and added with ethanol (30ml). After standing about 4 hours in the cold, dark grayish precipitaet was filtered off, and the filtrate was evaporated to dryness *in vacuo* at 30-40°. The dried red pigment was dissolved in cold water (25ml), mixed with ethanol (50ml), and stood overnight in the cold, whereupon the dark red pigment separated out as a powder. Yield ca. 0.8 g. Being examined by paper-chromato graphy, this crystalline crop was nothing but Cent-R-A, one of the three pigment components in flowers as described in the previous section. The other two in the residue solution were components, Cent-R-B and Cent-R-C, were remained in solution, and could not be crystallized.

Crystallization of the red powder was achieved by the following process. Red powder (0.8 g) was dissolved in 50% methanol (50ml) at 60°. The solution was filtered free from insoluble matters, and concentrated a small volume (30ml) at 30-40° *in vacuo*. On standing overnight in the cold, reddish purple pigment was precipitated (0.18g). This was dissolved in hot 50% ethanol (40ml), and insoluble impurities were filtered off. On standing in a refrigerator for several hours, the red pigment commenced to separate in the form of needle crystals (Fig 1). Yield 0.05g. Further crop was obtained from the mother liquor in a yield of 0.04g.

4) Qualitative and quantitative analysis of the red anthocyanin.

The crystalline red pigment is soluble in hot water and aqueous ethanol, but insoluble in the cold. The color of an aqueous solution is purplish red.

The pigment is soluble in dilute aqueous or methanolic in an orange-red color. That is characteristic for pelargonin choloride. The following behaviors have been noted with this pigment:

| solvent | solubilty | color |
|---|---|---|
| water {hot | soluble | purplish red |
| water {cold | insoluble | — |
| ethanol {hot | soluble | purplish red |
| ethanol {cold | insoluble | — |
| dil. HCl (aqueous) | soluble | orange red |
| 2 % HCI (methanolic) | soluble | orange red |

Color reaction of aqueous solution with

| | |
|---|---|
| $FeCl_3$ (aqueous) | no change |
| NaOH (aqueous) | blue-violet; rapidly fading into yellowish color. |

The crystals undergo gradual color change into black at ca. 255°C, but do not melt below 300°C. On the other hand, pelargonin chloride melts at 180° under simultaneous decomposition. The result of elementary analysis is shown in Talbe 4. The crystals do not contain N-substance. The analytical figure obtained shows that the

**Table 4**

Elementary analysis of Centaurea-red-anthocyanin (Cent-R-A).

(a) Water of crystallization

| Subst. (air-dried) | Loss in weight (115°, $P_2O_5$, 1mmHg) | Found |
|---|---|---|
| 3.677 mg | 0.183 mg | 4.98% |
| 3.761 mg | 0.160 mg | 4.25% |
| Calc. for $C_{27}H_{31}O_{15} \cdot OH. 1\frac{1}{2} H_2O$ | | 4.22% |

(b) C-H determination

| Subst. (anhydr.) | $CO_2$ | $H_2O$ | C% | H% |
|---|---|---|---|---|
| 3.601mg | 6.964mg | 1.622mg | 52.77 | 5.04 |
| 3.489mg | 6.780mg | 1.576mg | 53.03 | 5.05 |
| Calc. for $C_{27}H_{31}O_{15} \cdot OH$ | | | 52.94 | 5.27 |

(c) N-estimation

| Subst. (anhydr.) | $N_2$ (25°, 785 mmHg) | Found |
|---|---|---|
| 4.995mg | 0.005cc | 0.0% |

crystalline pigment must have an elementary composition just corresponding to [pelargonin]-OH.

Absorption curves of the red pigment and pelargonin-Cl in 1/10 mol K-acetate buffer solution (pH 4.75) was carefully compared with each other. As can be seen from Fig. 3, both pigments are identical at the pH value applied in this experiment. Also IR absorption curves were estimated (Fig. 4, 5).

Paper-chromatographic analyses have shown that Rf values of the crystalline pigment are identical with those of pelargonin-Cl, so far as HCl-containing solvents are concerned. However, in the case of n-buthanol/acetic acid/water (4:1:5:, v/v), both pigments are quite different from each other in Rf-values as well as in color of the spots (Table 2).

Paper-electophoresis was also run in 1/20 mol potassium acetate buffer (pH 4.5) on Tôyô No. 51 filter paper, whereby electric current of 0.5 mA/cm was applied at room temperature. The red pigment does not move, so that interposition of metallic elements in the molecule may well be excluded in this case.

## *Discussion*

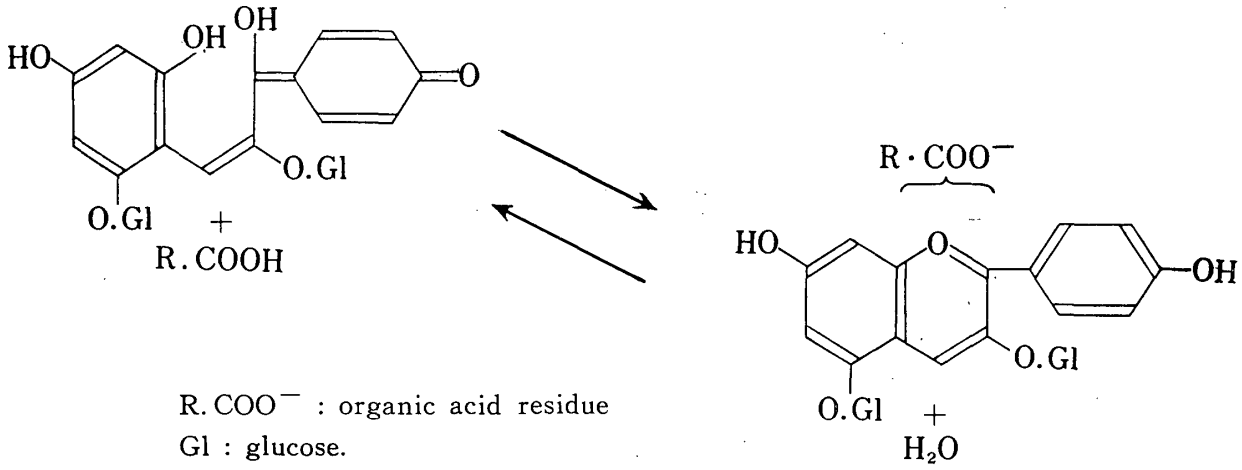
The problem of flower color variation was first studied by R. Willstätter and his co-workers[1] between the blue and red flowers of the cornflower. They stated that the pigment of the blue flower is due to alkaliphenolate of cyanin, while the red flower owes its color to oxonium salt of pelargonin. Later, G. Robinson[1] detected much chlorogenic acid in red cornflower petals, and suggested that this acid may play a role of co-pigment in the formation of a red color. Recently, E. Bayer[3] has also studied on

this problem, and described that in cornflower some co-pigment may participate in the formation of red color in flowers, since any simple pelargonin is scarcely detectable on the paper-chromatograms.

According to the results of the present experiment, a main pigment of red cornflower, which was crystallized in an unmmured state, has none of the associate substances such as organic acids, inorganic substances, an others. In an aqueous solution, this pigment exhibits a purplish red color, that is quite similar to the color of natural flowers. On acidification with hydrochloric acid, the color of the solution turns into orange-red, exhibiting an intense eosin-like fluorescence. No doubt, this is nothing but the color of pelargonin chloride. On the other hand, a solution of the crystalline pigment in acetic acid is purplish red, and it does not show fluorescence. Therefore, it seems likely that a purplish red color appearing in the cornflower is reduced to interactions between the oxonium cation of pelargonin and some mobile organic acid anions in the cell sap.

To sum up the results obtained above, the present author would like to propose a tentative formula for the crystalline pigment, which undergoes a reversible transformation in the presence of fatty acids or some anoido substances in the cell sap, as shown in the following:

Probable structure of crystalline red anthocyanin and its reversible interconversion in the cell sap.




$R.COO^-$ : organic acid residue
Gl : glucose.

Here, it must be added that number of free organic acids may play a major role, if any, in the formation of red color of flowers, since it exsits in free state in an appreciable amount in the petals of the same color.

## *Summary*

1. Red anthocyanin was isolated in a crystalline form from red cornflower petals.
2. The crystals contain neither organic acid nor inorganic matter. Association of N-substance and carbohydrate may also be excluded. Therefore, co-pigment does not participate in the production of red color.
3. The absorption spectrum of the red crystalline pigment was measured in potassium-

acetate buffer at pH 4.7; it was shown to be identical with that of pelargonin-Cl in the same solvent.

4. Red crystalline pigment is easily converted into pelargonin-Cl in dilute hydrochloric acid. Together with the analytical data the structure of the red pigment is presumed to be [pelargonin]-OH.

5. Besides, two acylated pelargonin are detected by paper-chromatography of the flower extract. Organic acid is common to both pigments, and is p-hydroxybenzoic acid.

6. A tentative structure of the red anthocyanin and its inter-conversion in the cell sap are discussed.

## *References*

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## B) Red anthocyanin from the rose

### *Introduction*

As is well known, the anthocyanin of red rose has been known to be cyanin. This pigment was first isolated by R. Willstätter and J. Nolan[1] in the form of chloride by the use of hydrochloric acid. Strictly speaking, this form of pigment is nothing but an artifact. Correct understanding of the native form of red anthocyanin must be brought about by the preparation processed under natural conditions.

For this purpose, experiments were carried out in order to obtain crystalline specimen of red pigment, using deep red garden varieties of rose as starting material. Of course, the use of acidic solvents was avoided throughout the whole process.

### *Experimentals*

1) Materials: The following four garden varieties of red rose (***Rosa gallica L.***) were used for preliminary tests: Josephine Bruce, Tassin, Charles Mallerin (cultivated at Keisei Rose Garden Co.) and Happiness (cultivated at Gotô Rose Garden Co.). Among them, ***Tassin*** and ***Josephine Bruce*** were most suitable for preparative purpose, because of their higher pigment content and availability of material, (Table 5 and 6).

2) Isolation and crystallization of the red pigment.

(a) Tassin: Fresh flower (790g) were dehydrated quickly by immersing in acetone (12l) for some time, and dried in a vacuum. The powdered material (130g) was

**Table 5**

Rf values of Rose-red-anthocyanins crystallized from 50% methanol
(by ascending method, on Tôyô No. 51 filter paper at room temp.)

| Solvent | Tassin | Josephins Bruce | Happiness | Charles Mallerin | Cyanin-Cl (control) |
|---|---|---|---|---|---|
| 1% HCl | 0.21 | 0.22 | 0.22 | 0.21 | 0.21 |
| AcOH/HCl/$H_2O$ (15: 3: 82) | 0.35 | 0.35 | 0.34 | 0.34 | 0.35 |
| n-BuOH/HCl/$H_2O$ (7: 2: 5) | 0.17 | 0.16 | 0.16 | 0.16 | 0.17 |
| n-BuOH/2N-HCl (1: 1) | 0.04 | 0.04 | 0.03 | 0.04 | 0.04 |
| n-BuOH/AcOH/$H_2O$ (4: 1: 5) | 0.30 * | 0.30 * | 0.30 * | 0.30 * | 0.29 |
| Colors in visible light | magenta | magenta | magenta | magenta | magenta |
| Colors in UV-light | bright red | bright red | bright red | bright red | bright red |

* mauve in visible light, rose purple in UV-light.

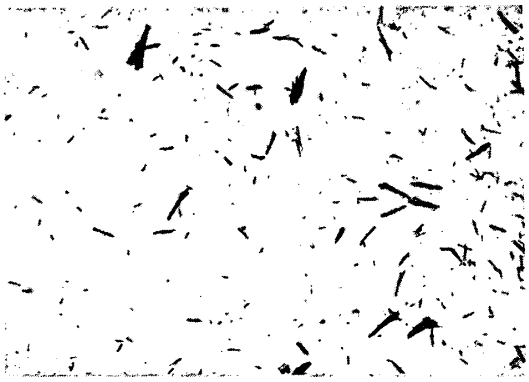


**Fig. 6**
Crystals of Rose-red-anthocyanin from Tassin (crystallized from a mixture of acetone, ethanol and water).
× ca. 1200

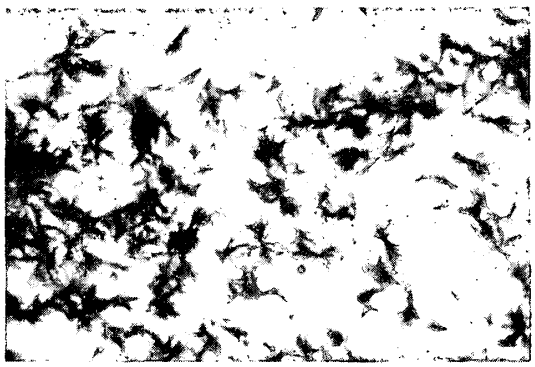


**Fig. 7**
Crystals of Rose-red-anthocyanin from Josephine Bruce (crystallized from a mixture of acetone ethanol and water).
× ca. 1200

extracted with 700 ml of a mixture (acetone/ethanol/water, 4: 2: 3, v/v) for 6 hours, and filtered, whereby ca. 500ml extract was obtained. This was evaporated to 50ml *in vacuo* at 30-40°. Dark red concentrate was filtered free from greyish impurities, and mixed with an excess of ethanol (100ml). On cooling in a refrigerator, dark red, blackish precipitate were formed. Yield 500 mg. This was dissolved in 50% aqueous methanol (25ml) at 70-80°; some insoluble matters, were filtered off, and the filtrate was concentrated to a viscous syrup in a vacuum at 30-40°. Then, the syrupy mass was treated with cold water and acetone one after another, whereby the pigment was gradually separated as a solid. The product was collected and dried. Yield 0.19 g.

Recrystallization was effected by the following process. Dark red powder (0.19 g)

**Table 6**
Rf values of Rose-red-anthocyanins recrystallized from 2% MeOH-HCl

| Solvent | Tassin | Josephine Bruce | Happiness | Charles Mallerin | Cyanin-Cl (control) |
|---|---|---|---|---|---|
| 1% HCl | 0.21 | 0.21 | 0.22 | 0.21 | 0.21 |
| AcOH/HCl/$H_2O$ (15: 3: 82) | 0.35 | 0.35 | 0.34 | 0.35 | 0.35 |
| n-BuOH/HCl/$H_2O$ (7: 2: 5) | 0.16 | 0.17 | 0.17 | 0.16 | 0.16 |
| n-BuOH/2N-HCl (1: 1) | 0.04 | 0.03 | 0.03 | 0.03 | 0.04 |
| n-BuOH/AcOH/$H_2O$ (4: 1: 5) | 0.24 | 0.24 | 0.24 | 0.24 | 0.24 |

magenta in visible light, bright red in UV-light.

**Table 7**

Rf values crystalline Rose-red-anthocyanins.
(by ascending method, on Tôyô No. 51 filter paper, at room temp.)

| Solvent | Tassin | Josephine Bruce | Cyanin-Cl (control) |
|---|---|---|---|
| 1% HCl | 0.21 | 0.21 | 0.21 |
| AcOH/HCl/$H_2O$ (15: 3: 82) | 0.35 | 0.35 | 0.35 |
| n-BuOH/HCl/$H_2O$ (7: 2: 5) | 0.16 | 0.17 | 0.17 |
| n-BuOH/2N-HCl (1: 1) | 0.04 | 0.04 | 0.04 |
| n-BuOH/ACOH/$H_2O$ (4: 1: 5) | 0.30 * | 0.30 * | 0.29 |
| Colors in visible light | magenta | magenta | magenta |
| Colors in UV-light | bright red | bright red | bright red |

* mauve in visible light,
rose purple in UV-light.

was dissolved in 30% methanol (80ml) at 70-80°, and filtered from insoluble matters. The filtrate was evaporated to dryness *in vacuo* at 30-40°, and the residue was dissolved in 50% ethanol (50ml) at 80°, and filtered. The solution was concentrated to 10 ml, then mixed with 50% aqueous acetone (3ml), and stood in the cold for about a week, whereby the pigment was precipitated as brownish red crystals (Fig. 6). These were collected, and washed with cold water and acetone. Yield 0.07g

(b) Josephine Bruce; Fresh flowers (1000g) were dried with acetone (18 l) similar to the case of Tassin mentioned above. Extraction of the pigment was made with 1.2 ℓ of solvent mixture (acetone/ethanol/water, 11: 4: 9, v/v) for several hours. After filtration, the extract (800ml) was concentrated to 100 ml *in vacuo* at 30-40°. After removal of grayish impurities, the filtrate was mixed with ethanol 5 vols. and ether 3 vols., and stored in the cold for 5 hours. In this case, further amount of grayish brown, sticky mass was produced, which was removed by decantation. Then, the deep red solution was concentrated *in vacuo* at 30-40° up to a syrupy consistency. To this pigment syrup acetone (150ml) was added in order to effect the separation of the pigment. Purplish red precipitate obtained was collected, dissolved in 50% methanol (100ml) at 70°C, and filtered free from insoluble matters. The filtrate was concentrated to 5 ml, and added with cold water and acetone as in the case of Tassin. By this treatment, the pigment was separated in a form of semi-crystalline powder of dark red color, Subsequent operation for crystallization was made in a similar manner as described in the case of Tassin-anthocyanin. Final crop of the purest pigment were composed of homogeneous, dark red, crystalline powder, which were completely converted into fine red needles (Fig. 7) on covering with a few drops of water on microscopic slide.

Table 8

Elementary analysis of crystalline Rose-red-anthocyanins.

(a) water of crystallization:

| Subst. (air-dried) | | Loss in weight (105°, $P_2O_5$, 1 mmHg) | Found |
|---|---|---|---|
| Tassin | 2.790 mg | 0.082 mg | 2.94 % |
| Tassin | 2.790 mg | 0.065 mg | 2.79 % |
| | Calc. for $C_{27}H_{31}O_{16}$ (-OH) · $H_2O$ | | 2.79 % |

(b) C-H determination:

| Subst. (anhydr.) | | $CO_2$ | $H_2O$ | C% | H% |
|---|---|---|---|---|---|
| Tassin | 2.708 mg | 5.100 mg | 1.179 mg | 51.39 | 4.87 |
| Tassin | 2.658 mg | 5.038 mg | 1.175 mg | 51.72 | 4.95 |
| | Calc. For $C_{27}H_{31}O_{16}$-OH | | | 51.59 | 5.13 |
| Josephine Bruce | 3.259 mg | 6.040 mg | 1.490 mg | 50.58 | 5.16 |
| Josephine Bruce | 3.622 mg | 6.705 mg | 1.669 mg | 50.87 | 5.22 |
| | Calc. for $C_{27}H_{31}O_{16}$-OH · $1/2H_2O$ | | | 50.87 | 5.22 |

(c) N-estimation

| Subst. (air-dried) | $N_2$ (28.3°, 756 mmHg) | | Found |
|---|---|---|---|
| Josephine Bruce | 3.292 mg | 0.004 cc | 0.13 % |

(d) Content of cyanin and glucose

| Subst. (anhydr.) | | cyanin-Cl | glucose | Found |
|---|---|---|---|---|
| Tassin | 1.243 mg | 1.228 mg | — | 98.79 % |
| Tassin | 9.261 mg | — | 4.000 mg | 43.19 % |
| | | | (Theo retical | 57.33 %) |

3) Qualitative and quantitive analysis of the red anthocyanins.

Crystals of the red anthocyanins are very difficultly soluble in water, ethanol, and acetone in the cold, but dissolved in hot, aqueous ethanol and acetone to form purplish red solutions. They are soluble in aqueous or alcoholic hydrochloric acid, giving a cherry red color that is characteristic for cyanin-Cl. When the pigments from Tassin and Josephin Bruce were dissolved in aqueous or methanolic hydrochloric acid and stood for several days in the cold, crystals of cyanin-Cl are tormed.

Both pigments have no melting point below 300°, but show a blackening of color at ca. 255°. Paperchromatographic data agree with those of cyanin-Cl, as shown in Table 7. In hydro-chloride, whereby no other substance can be detected even by chromatographic means.

On the analysis of paper-chromatography with the solvent, n-butanol/acetic acid/water (4: 1: 5, v/v), its Rf value did not agree with cyanin-Cl as shown in Table 7, on this reason the red pigment became a salt of acetic acid, so this difference is between cyanin chloride and cyanin acetate.

Paper-electrophoresis shows that the red pigments are neutral; that is, they move onto neither of the electrodes.

Besides the following measurements have been made for the sake of correct

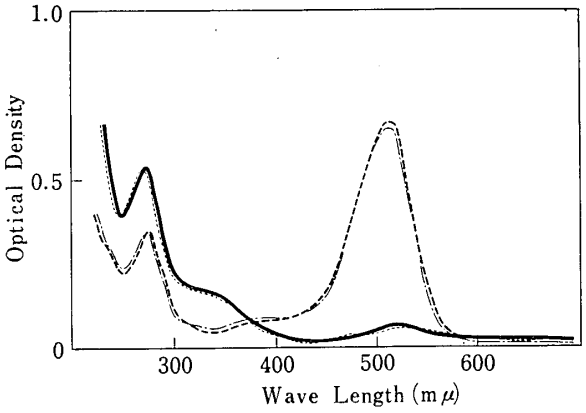



**Fig. 8** Absorption spectra of

——— Rose-Red-anthocyanins from Tassin and Josephine Bruce, each sample in 0.1 mol K-acetate buffer (pH 3.65, $4\times10^{-5}$ mol)

..... Cyanin-Cl in 0.1 mol K-acetate buffer (pH 3.65, $4\times10^{-5}$ mol)

—·—·— Rose-Red-anthocyanins from (Tassin and Josephine Bruce, each sample in 5% HCl (aqueous): $2\times10^{-5}$ mol

····· Cyanin-Cl in 5% HCl (aqueous): $2\times10^{-5}$ mol

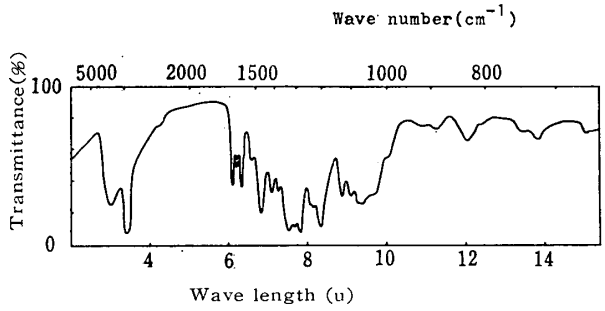



**Fig. 9**
Infra-red absorption spectrum (nujol mull).
—— cyanin-Cl

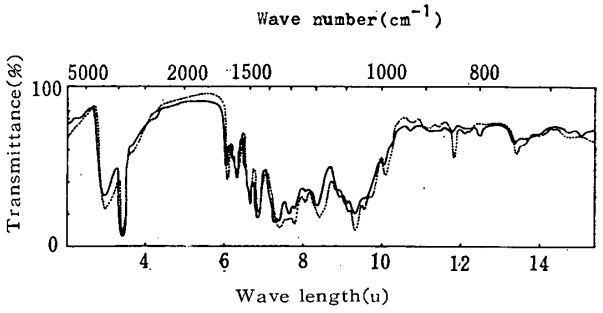


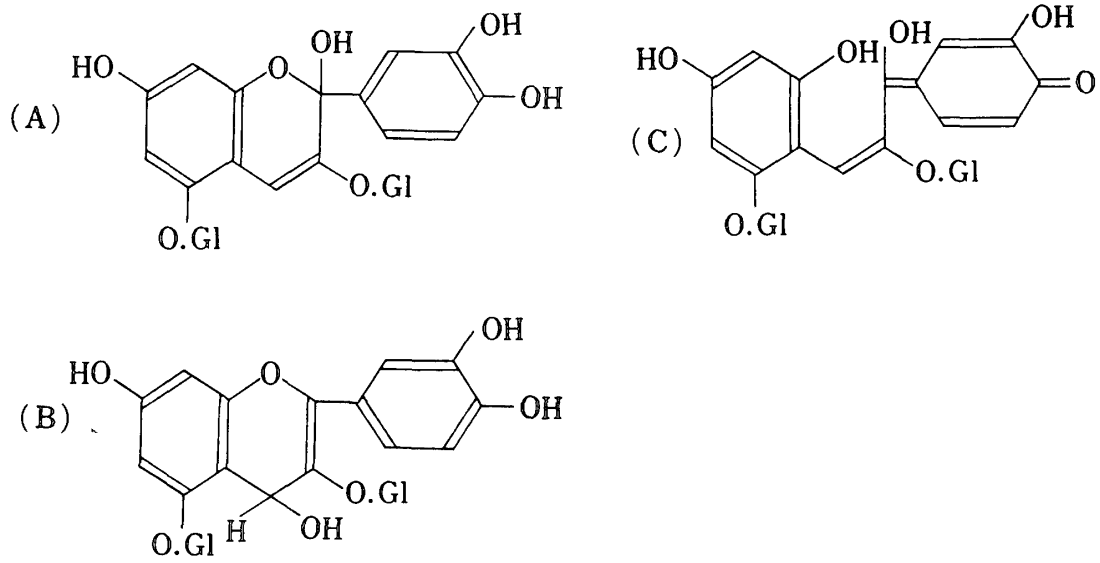

**Fig. 10**
Infra-red absorption spectra (nujol mull).
—— cyanin-OH (Tassin)
····· cyanin (Cl-free)[3]

evaluation of the pigments: namely, elementary analysis (Table 8), absorption spectra (Fig. 8), and IR-absorption curves (Fig. 9, 10).

Examination on the products of complete hydrolysis. Experiments for this purpose were carried out in the same way as described in the case of Tassin-anthocyanin.
The results are shown in Table 8, from which it is concluded that the red pigments obtained from both Tassin and Josephin Bruce are composed of cyanidin and glucose alone.

## *Discussion*

The two red anthocyanins obtained from the deep red garden varieties of rose are identical with each other in respect to IR- and UV-spectra and elementary analysis. Also it has proved that the UV absorption spectra of this pigment are identical with those of cyanin chloride within a wider range of pH value. However, a fundamental difference lies in that the red pigment seems to contain one more hydroxyl group in place of chlorine in cyanin molecule. To determine the position of this hydroxyl is a basic problem, that requires further investigation. For the sake of convenience, the following formula may be taken into consideration:




The structures, (A) and (B), have already been assigned to the pseudobase or carbinol base of anthocyanin. According to R. Willstätter,[2] the substances having these structures are colorless, whereas the crystalline pigment from rose exhibits a purplish red color in an aqueous solution. Accordingly, it is likely that the pigment in question may correspond to the structure (C). On the basic of this assumption, further investigations are planned at present.

## *Summary*

Crystalline red anthocyanins are isolated from two garden varieties of *Rosa gallica L.* (Tassin and Josephine Bruce) without the use of mineral acid. The other two garden varieties (Charles Mallerin and Happiness) also contain the same pigment.

A series of analytical experiments showed that both pigments are identical with each other, and that the pigment may be reduced to a diglucoside of hydroxylated cyanidin, [cyanin]-OH.

Finally, a tentative structure is proposed for the red anthocyanin obtained.

## *References*

1) R. Willstätter und J. Nolan: Liebigs Ann. Chem. **408**, 1 (1915).
2) R. Willstätter und A. E. Everest: Liebigs Ann. Chem. **401**, 189 (1913).
3) K. Hayashi, K. Takeda, and N. Saitō: Proc. Japan Acad. in the press.