Studies on "Bluing Effect" in the Petals of Red Rose VI.
Further observations on the development of blue color
of the spherule

Hitoshi Yasuda
Department of Biology, Faculty of Science, Shinshu
University, Matsumoto, Japan

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In the previous papers (Yasuda 1970, Yasuda et al. 1978), it was reported that
the blue spherule appeared in the upper epidermal cells of petals could be adduced as
one of the causes of bluing effects in red roses. The histochemical observations pro-
vided that the spherule was composed of tannic substances, anthocyanin, iron and
calcium for the most part (Yasuda 1970, 1973, 1974, 1976). At the same time, it
was suggested that the blue color of this spherule could be displayed by the co-opera-
tion of the following three components: tannic substances, anthocyanin, and iron.

The present study was designed to gain a better understanding about this co-
operation of three components.

Materials and methods

With the exception of the histophotometric observation, the plants used in the
present investigation were cl-Crimson Glory grown outdoors in the field in the cam-
pus of the Faculty of Science, Shinshu University (Asahi-3, Matsumoto, Japan).

For the electron microscopic observations of the spherule, the epidermis were
peeled off from the petals exhibiting the bluing effect sufficiently and were fixed in
2.5% glutaraldehyde for 5 hours or 2% of potassium permanganate 1 hour in phos-
phate buffer (pH 7.0). During the fixation, the temperature was about 5°C in the
refrigerator. After the fixing solution was removed from the materials by washing
them several times with the same buffer solution, the materials were dehydrated in
ascending grades of ethylalcohol and propylene oxide, and embedded in Epon.
The sections were cut with a glass knife and poststained with an uranyl acetate solu-
tion and a lead acetate solution. Observations were made with a Hitachi HS-8
electron microscope.

To observe the color change of spherule upon heating it, the fresh petals con-
taining the blue spherule in their upper epidermal cells were heated to 70–80 de-
grees using the micro-melting point apparatus which is a microscope with an elec-
tric heating device and a thermometer in its stage.

The test solutions described in Table 1 were prepared, and color changes of blue
spherules with them were observed microscopically. The pH of the solutions were
adjusted to the values shown in Table 1 respectively by adding dilute hydrochloric
acid to them. After the treatment of the fresh epidermis with these solutions, the
Prusian blue reaction was applied histochemically to the epidermis according to
the procedures described Tarao (1957) and Yasuda (1973).

The blue spherules were decolored by soaking the upper epidermis peeled off freshly from the bluing petals in 1% methanolic hydrochloric acid for about two hours at room temperature. The detail of this decoloration procedure was indicated previously (Yasuda 1970). The decolored spherules obtained thus were stained with cyanin solution (about 100 µg in 1 ml) and 1% ferric sulphate according to the following two types of treatments respectively:

Treatment 1 Decolored spherule→Stained with ferric sulphate→Stained with cyanin solution.
Treatment 2 Decolored spherule→Stained with cyanin solution→Stained with ferric sulphate.

The spectral measurements of the intact upper epidermal cells showing bluing effect in various degrees were made with the histophotometer designed by Asen et al. (1971). For these measurements the fresh epidermis were stripped off from the bluing petals of a rose cultivar, Crimson Glory, grown in the green house in the US Department of Agriculture, Agricultural Research Center (Beltsville, Maryland, U. S. A.).

Results

Electron micrographs of the spherule were shown in Figs. 1a and b. In the previous observation with a light microscope (Yasuda 1974) it was indicated that three layers could be distinguished in the spherule fixed in Kaiser’s solution. These layers are demonstrated clearly also in the electron micrograph (Fig. 1a). A very interesting feature is the presence of the several cytoplasmic strands crossing between

Figs. 1a–b. Electron micrographs of the spherule. a, a cross section of spherule, indicating the three layers in its internal structure. Arrows denote the cytoplasmic strands connecting between the parietal cytoplasmic layer and the spherule. b, a portion where the spherule is quite near to the cell wall, showing the parietal cytoplasmic layer taking the direction to the surface of the spherule (arrows). Abbreviations: cw, cell wall; sp, spherule; cy, cytoplasmic layer.
the body and the parietal cytoplasmic layer. These strands were failed to be recognized in the previous observation with the light microscope.

Fig. 1b is the photograph of the portion where the spherule is quite near to the cell wall. From this feature it is evident that the parietal cytoplasmic layer which is lying along the cell wall takes the direction to the surface of the spherule at this portion, running round the spherule.

No changes could be perceived in the color and in the shape when the blue spherules were warmed to about 70-80°C with the micromelting point apparatus.

The results of color observation about spherules treated with various reagents are summarized in Table 1. When the intact spherules were treated with citric acid, EDTA and o-phenanthroline in the pH values of 3, 4 or 7, they retained the original blue color. However, their blue color turned red by these reagents in pH 2. Its blue color was also changed to red with 0.1 N hydrochloric acid (pH 1). In all treatments it was clearly demonstrated that iron was detected in the spherules showing blue color, but not in the spherules colored in red, after the treatments of reagents mentioned above.

Table 1. Color changes in blue spherule with various reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>pH</th>
<th>Color of spherule</th>
<th>Prussian blue reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N Citric acid</td>
<td>4.0</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>Red</td>
<td>-</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>1.0</td>
<td>Red</td>
<td>-</td>
</tr>
<tr>
<td>0.02 M EDTA</td>
<td>4.5</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>Red</td>
<td>-</td>
</tr>
<tr>
<td>o-Phenanthroline (Saturated solution)</td>
<td>7.5</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>Blue</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>Red</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. The stainings of decolored spherules in Treatment 1 and Treatment 2

<table>
<thead>
<tr>
<th>The first staining</th>
<th>The second staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agent</td>
<td>Color</td>
</tr>
<tr>
<td>Treatment 1</td>
<td>Feric sulphate</td>
</tr>
<tr>
<td>Treatment 2</td>
<td>Cyanin</td>
</tr>
</tbody>
</table>

In the previous investigation (Yasuda 1970), it was reported that the decolored spherules were stained red by cyanin alone, while blue by the complex staining of cyanin and ferric sulphate. In the present study, two types of staining, Treatment 1 and Treatment 2, were carried out against the decolored spherules. As shown in Table 2, the second staining brought the same coloration both in the Treatment 1 and Treatment 2.

Fig. 2 is the absorption curves of the epidermal cells providing the bluing effects in various intensities. The curve obtained from the young cell, the cell sap of which showing red tinge and containing little bluish spherule, has a sharp wave length of maximum absorption at 530 nm (Curve A). However, the old cell, its cell
Fig 2. Absorption curves of the epidermal cells providing the bluing effects in various intensities.  
A: The curve obtained from the young cell, the cell sap of which showing red tinge and containing little bluish spherule.  
B: The curve obtained from the old cell, its cell sap being purplish.  
C: The curve obtained from the older cell having the blue spherule.

sap being purplish (Curve B) or the older cell having the blue spherule (Curve C), showed rather wide range of absorption from 470 nm to 680 nm.

Discussion

In the previous observation (Yasuda 1974), the following three possibilities for the relationship between the spherule and cytoplasmic layer, were proposed:

1. The spherule is covered perfectly with the cytoplasmic layer.
2. The spherule is covered partially with the cytoplasmic layer.
3. The spherule is not covered at all with the cytoplasmic layer.

The electron micrographs presented in Fig. 1 supports the first and second possibility, however not the third one. From the developmental observation about this spherule (Yasuda 1974), it was suggested that this spherule bears some resem-
blane to the tannin vacuole of morter cell in *Mimosa* (Toriyama 1955, 1966). Toriyama *et al.* (1968) clearly showed that the tannin vacuole of *Mimosa* is enveloped with the cytoplasmic layer. So, it can be said from the observation presented above that a further similarity between the spherule and tannin vacuole exists also with respect to the surface structure.

On the other hand, it seems reasonable to expect that the red color of anthocyanin turns blue by forming a complex with tannic substance and iron on the surface of the spherule. Robinson *et al.* (1931) and Lawrence (1932) found that anthocyanins form some complexes with such substances as escrin, tannin, ethylgallate, 2-hydroxyxanthin and some flavonoids in the cell sap, changing the reddish tinge of anthocyanin to bluish. This phenomenon is well known as a co-pigmentation effect, and is supported as a significant hypothesis for the development of blue color in anthocyanin petals. Jurd (1966) described that some metallic components are necessary for the development of the co-pigmentation effects.

According to Robinson's description, the anthocyanin-co-pigment complex is dissociated reversibly by heating the aqueous solution of the complex. However, in the blue spherule any color changes did not occur in heating it. Accordingly, it can be said that rather strong association among the tannic substances, anthocyanin and iron occur on the surface of the spherule.

![Diagram](image)

**Figs. 3a–d.** Some proposals explaining the possible relationships among Fe, cyanin and basic substance. a, cyanin is associated directly to the surface of the base, Fe is connected to cyanin. b, Fe is associated directly to the base, and cyanin is connected to the Fe. c, the three components, Fe, cyanin and base, are connected each other. d, Fe and cyanin are associated individually to the surface of the basic substance of spherule, being given some special character by the association of Fe.

○ : Fe; ———: cyanin; ————: base.

While the three components of the blue spherule shows a comparatively stable association against the high temperature, the metallic component, iron, is easily released by the treatments with the solutions having low pH values (Table 1). The concomitant relationship between existance of iron and the blue coloration in the spherule gives a support to the hypothesis previously reported that iron is necessary for developing the blue color in the spherule.

Citric acid, EDTA and o-phenanthroline are well known as the reagents which form a chilate compound with some metallic elements such as iron, calcium and etc. As shown in Table 1, the results that iron still remained in the spherule even after the treatments with these reagents, clearly demonstrate that the iron is conjugated more strongly with the surface of the blue spherule than with these chilating agents.
The author would like to propose some possible relationship among iron, anthocyanin and basic substance as shown in Figs. 3a–d. From the fact that the decolored spherules gave the same stainings both in the Treatment 1 and 2, it seems to be difficult to expect the types of a and b in Fig. 3.

According to the absorption spectra of fresh petals measured by Saito (1967) the spectra of the petals containing a metallo-anthocyanin have a remarkable character that there are two peaks, one being at 592–630 nm and others at 640–672 nm. The absorption spectra of the blue spherule shown in Fig. 2 are quite different from those of the petals including the metallo-anthocyanin. Therefore, it seems to be unreasonable to assume that three component are associated with each other as Figs. 3c.

Fig. 3d illustrates that anthocyanin and iron connect individually with the surface of the spherule. In this case, it could be thought that iron gives to the surface of the spherule some special property that the blue color can develop when anthocyanin is absorbed on it. The present author imply that this schema is the most possible illustration for the relationship among the spherule, anthocyanin and iron.

Summary

Electron-microscopical, histochemical and histophotometrical studies were given to the blue spherule present in the upper epidermal cells of rose petals exhibiting the bluing effect. The results obtained are summarized below.

1. The observation with electron-microscope clearly demonstrated that three layers are distinguishable in the spherule, and that the parietal cytoplasmic layer which is lying along the cell wall takes the direction to the surface of the spherule running around it at the portion where the spherule is quite near to the cell wall.

2. When the intact spherules were treated with citric acid, EDTA and o-phenanthroline in the pH 3, 4 or 7, they retained the original blue color, but their blue color turned red by the reagents in pH 2 and 0.1 N hydrochloric acid (pH 1).

3. After the treatments of these reagents, iron was detected in the spherules showing blue color, but not in the spherule colored in red.

4. The complex staining of cyanin and ferric sulphate were given to the spherules decolored with 1% methanolic hydrochloric acid. The same staining features were obtained both in the Treatment 1 (decolored spherule→stained with cyanin→stained with ferric sulphate) and in the Treatment 2 (decolored spherule→stained with ferric sulphate→stained with cyanin.)

5. The absorption curves of the epidermal cells containing the blue spherule showed rather wide range of absorption from 470–680 nm.

6. The relationship among anthocyanin, iron and the surface of the spherule is discussed.

Literature cited


