PIGMENT PRODUCTION BY *PSEUDOMONAS AERUGINOSA* ON GLUTAMIC ACID MEDIUM AND GEL FILTRATION OF THE CULTURE FLUID FILTRATE

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Since pyocyanin was first isolated in crystalline form and its structure clarified, other phenazine pigments with a similar structure, such as the green chlororaphin(1), purple iodinin(2), and yellow phenazine-1-carboxylic acid(3), have been isolated from *Pseudomonas chlororaphis*, *Ps. iodinum* and *Ps. aureofaciens*, respectively, and their structures were thus established. The knowledge of pigments, other than pyocyanin, produced by *Ps. aeruginosa* is, however, still incomplete.

The coloration produced by *Ps. aeruginosa* in a peptone-containing medium is generally complex(4), and a difference in the coloration produced in the presence of different types of peptones occurs even when an identical strain is used(5). Therefore it is difficult to understand the pigment producing ability of the organism. With the use of glutamic acid medium, developed by the authors (1962), pigment and fluorescence are promptly produced, and the tone of the color is far more distinct than that developed in the King A and B media(6), which are considered to be appropriate for the measurement of pigment production by *Pseudomonas*.

The difference in coloration depending upon the strain employed, is also clearer, and the composition of the medium is simpler. Therefore it is believed that this medium is valuable not only in the isolation of *Pseudomonas* but also for the clarification of the mechanism involved in the production of pigment.

In the present study, the production of pigment by *Pseudomonas*, mainly *Ps. aeruginosa*, on glutamic acid medium was examined, and the culture filtrate was fractionated on a column of cross linked dextran gel for the initial isolation of pigments other than pyocyanin and fluorescent substances.
MATERIALS AND METHODS

1. Bacterial Strain

On the 120 or more strains of Pseudomonas maintained in our laboratory, 4 strains of *Ps. aeruginosa*, Nos. 134, 145, 117 and 58, were selected as the representative pigment-producing strains. Strain No. 117 was isolated from the blood of a case of sepsis, while Nos. 58, 134 and 145 were variants originating from No. 117. All of the 4 strains produced different pigments on glycerol-agar medium, No. 117 produced a brown color, melanin, No. 134 pyocyanin, No. 145 an intermediate and No. 58 did not produce any color. The ability to produce pigment by each of the strains was stabilized, and each did not show any variation upon subculture for about 7 years.

2. Culture Medium

The glutamic acid medium(?) developed by the authors, was used. The basic medium was composed of 0.2 g of KH₂PO₄, 0.01 g of MgSO₄ and 0.5 g of NaCl in 100 ml of distilled water. Sodium glutamate was added in a concentration of 1%, and the pH of the solution was adjusted to 7.2. Solid medium was also prepared by adding 1.5% agar (glutamic acid-agar medium).

3. Method of Cultivation

Fresh bacteria grown for 18 hours at 37°C in glutamic acid medium was used as the inoculum.

For gel filtration, the organism was cultured in a 16 cm diameter, 5 cm deep, flat-bottom flask containing 200 ml glutamic acid medium. The depth of the fluid layer was about 1.5 cm, since pigment production is delayed when the fluid layer is deeper. The flaked medium was inoculated with 0.5 ml of actively growing fluid culture of each of the strains and incubated at 37°C for 1~7 days during which time pigment was produced. When the anticipated color was observed, the bacterial cells were removed by filtration through a Seitz filter.

The filtrate thus obtained was not heated or treated with chemicals.

4. Fractionation Method

Dextran gel was used as the column material and distilled water as the eluent. Sephadex G-25 M was washed with distilled water and packed in a glass column 1.5×25.0 cm. Fifteen ml portion of the filtrate was added and 2 ml fraction were collected. For obtaining larger quantities, column of 3.5×45.0 cm and filtrate of 150 ml portion were used.

The tone and fluorescence of each fraction was examined under visual light and ultraviolet rays. The absorption spectrum and the characteristic wave length of the fluorescence were measured in a spectrophotometer.
RESULTS

1. Pigment Production of Each Strain

The appearance of pigment and fluorescence was most rapid on the glutamic acid slant. Fresh yellow color spreading over the entire surface became apparent in about 10 hours with strains Nos. 134, 145 and 117, and brilliant fluorescence was observed under an ultraviolet lamp. Strain No. 134 produced pyocyanin with time, and the medium showed beautiful yellow-green coloration. The yellow tone gradually faded with a decrease in fluorescence, and at the same time, the medium turned into blue-green.

Strain No. 145, initially yellow, turned into a red color and finally the medium took a brownish tone. Strain No. 117 retained the initial yellow color and fluorescence. Strain No. 58 did not produce any pigment or fluorescence within 1-4 days of incubation.

The production of pigment in the liquid medium was delayed as compared to the solid medium especially the slant, and the brilliant yellow color was not observed. The culture of strain No. 117 showed only a light yellow color throughout, while those of Nos. 134 and 145 became yellowish green, blue-green, orange-yellow, red and red-brown, and a pronounced fluorescence was emitted.

The color of the fluid portion of the culture of strain Nos. 134 and 145 did not change after the removal of cells. In the case of No. 117, the culture was a light yellow color after 24 hours of incubation, but the filtrate was light blue. However, when incubated for 1 week, both the culture fluid and the filtrate were light yellow with a greenish tone. Strain No. 58, originally colorless, showed light yellow color in the glutamic acid-liquid medium, and egg-yolk color fluorescence was emitted after incubation of more than 1 week. Its filtrate was light blue in color. This blue pigment was relatively stable in acid and base and not transferred to the chloroform layer.

2. Gel Filtration (Fig. 1)

1) When gel filtration was carried out under ultraviolet lamp, a bluish white brilliant fluorescent substance was seen to flow out initially. This substance was observed in tube No. 5 at the earliest and in tube No. 9 at the latest, and it was present up to tubes Nos. 19-21. This fluorescent fraction was designated as Fluorescence I.

Fluorescence I appeared strongly in the filtrates of strains Nos. 134, 145 and 117, but it was almost absent in that of No. 58.

The culture fluids of strains Nos. 134 and 145 showed a strong yellow color under an ordinary light, and the latter half of Fluorescence I showed a brilliant yellow-green fluorescence after a relatively short period of incubation. The two parts of Fluorescence I, blue and yellowish green, were clearly separated in the
column. Even when they were eluted and collected into neighboring tubes, the two parts seemed not to mix. The blue part of Fluorescence I appeared as a light yellow under an ordinary light, while the greenish yellow portion of Fluorescence I of strains Nos. 134 and 145 disappeared and only a whitish blue fluorescence remained. Under an ordinary light, the greenish yellow color disappeared and a light yellow was observed.

After a prolonged incubation (more than 1 week) of strain No. 145, the fraction of Fluorescence I appeared as orange-brown color under an ordinary light, and the fluorescence became an indistinct dirty color.

The Fluorescence I of strain No. 117 showed a slight yellow color under an ordinary light but a brilliant bluish white fluorescence was visible under a UV lamp. The blue pigment of strain No. 58 appeared in the fractions corresponding to Fluorescence I of other strains and slight fluorescence was noted under an ultraviolet lamp.

2) Immediately after starting the elution schedule on the Sephadex column, a narrow egg-yolk yellow fluorescent band became apparent near the top of the column which gradually descended. This was designated as Fluorescence II. Fluorescence II was collected in tubes No. 22~26, mainly in three tubes, Nos. 23, 24 and 25. Tube No. 23 emitted an egg-yolk yellow fluorescence, No. 24 a dark yellow and No. 25 a relatively bright yellow fluorescence. This Fluorescence II appeared in the culture filtrates of all four strains. Except for the filtrate of strain No. 134, Fluorescence II appeared in the gel column and could be collected even before the culture fluid showed color or fluorescence. Fluorescence II was especially apparent in the filtrate of strain No. 58, which was originally a colorless strain.

3) When observed under an ordinary light, the fraction of Fluorescence I is generally light yellowish green or light blue in color. Orange-brown color was observed in the fraction of Fluorescence I of strain No. 145 when incubation was prolonged, while the fraction of Fluorescence II showed a light yellow brown color. With the disappearance of the yellowish green color of Fluorescence I in the early stage of incubation of strain No. 145, a red pigment became apparent in the column between Fluorescence I and II. This was collected in tubes Nos.
From the filtrate of strain No. 134, a blue pigment appearing later than Fluorescence II was collected in tubes Nos. 27-37. This pigment, producing a blue color in alkaline solution, was soluble in chloroform, and upon acidification with HCl, it changed into a faint red color and was expelled from the chloroform. It is believed that the blue pigment is pyocyanin.

3. Absorption Spectrum (Fig. 2 (A) - (D), Table 1)

Fluorescence I showed a maximal absorption at 400 m$\mu$ with no differentiation between the blue and yellow-green portions. The maximal absorption of Fluorescence II obtained from culture filtrates of strains Nos. 134 and 145 was
Table 1. Spectrophotometric analysis of fluorescent fractions.

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<tr>
<th>Strain No.</th>
<th>Fluorescence I</th>
<th>Fluorescence II</th>
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<tr>
<td></td>
<td>blue</td>
<td>greenish yellow</td>
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<tr>
<td>Ultraviolet absorption</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>134</td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td></td>
<td></td>
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<tr>
<td>117</td>
<td></td>
<td></td>
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<tr>
<td>58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal wave length of fluorescence</td>
<td>460</td>
<td>460</td>
</tr>
<tr>
<td>134</td>
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<td></td>
</tr>
<tr>
<td>145</td>
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<td>117</td>
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<td>58</td>
<td></td>
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<tr>
<td></td>
<td>dark yellow</td>
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at 360 mμ and it was present in tubes Nos. 23, 24 and 25. However, similar fractions obtained from the culture filtrate of strain No. 117 barely showed a distinct peak on the absorption spectrum. In the case of strain No. 58, tube No. 24 showed a maximal absorption at 300 mμ and tube No. 25 at 340 mμ.

Of the red colored fractions of strain No. 145, tubes Nos. 18~19, in which fluorescence hardly appeared under ultraviolet rays, showed an absorption spectrum with two peaks, at 360~380 mμ and at 520 mμ. The pyocyanin of strain No. 134 showed peaks at 310 mμ and 380 mμ.

4. Maximum Wave Length of the Fluorescence (Fig. 3 (A)-(D), Table 1)

The maximal wave length of Fluorescence I was 460 mμ in the case of strains Nos. 134 and 145, and 470 mμ in the case of No. 117. The maximum wave length of the yellow-green fluorescence appearing in Fluorescence I in the early stage of growth of strains Nos. 134 and 145 was 460 mμ.

Fluorescence II was measured in tubes Nos. 23, 24 and 25 of each strain, but no pronounced peaks were observed when this compound was obtained from strains Nos. 134 and 145. Maximum wave length at 460, 490 and 500 mμ was found in the fluorescent spectra of strains Nos. 117 and 58.

DISCUSSION

It has been known that Ps. aeruginosa produces blue colored pyocyanin which spreads out over the culture medium, and this has been one of the most important indices in the isolation and identification of this organism. Some strains, however, do not produce pyocyanin in an ordinary medium, and when this alone is used as an index, there is a fear of making a false evaluation.

With the use of the glutamic acid medium slants developed by the authors, a majority of the strains produce a bright yellow color and a brilliant fluorescence after incubation for 10~13 hours. Under a prolonged cultivation this initial
yellow color is maintained or changes to green or red depending on the strain. It was also found that strains which do not produce color visible under ordinary light, produce a beautiful fluorescence when observed under an ultraviolet lamp.

This glutamic acid medium is, therefore, valuable for the isolation of *Ps. aeruginosa* from clinical material. It was possible to isolate *Ps. aeruginosa* by smearing stool specimens of patients on glutamic acid medium plates, observing the production of color and fluorescence or only fluorescence and verifying the findings with the oxidase reaction.

More than 140 strains of pseudomonas maintained in our laboratory or newly isolated were comparatively studied using the King A, King B and glutamic
acid media. It was found that the glutamic acid medium was superior to the 
former from the standpoint of rapidity and clarity of the production of pig-
ment and fluorescence.

Though the glutamic acid medium is a very simple synthetic medium, the 
pigment and fluorescence produced by *Ps. aeruginosa*, depending upon the 
ability of each strain, may serve in the clarification of the mechanism involved 
in the production of pigment other than pyocyanin.

Pigments and fluorescent substances other than pyocyanin are not extracted 
by organic solvents such as chloroform, benzine, benzol, xylol, ether, amyl 
alcohol and ethyl acetate, thus isolation is not too easy. The cross linked dextran 
gel, Sephadex G-25 M, used in the present study with water as the eluent pro-
vided a method in fractionating the substance from the culture filtrate to some 
extent. The fluorescent substance produced by pseudomonas has been called 
Fluorescin, but it has been pointed out previously by us that two or three sub-
stances are included from the standpoint of color tone. As shown in the present 
study, there are at least two substances, Fluorescence I and II, which can be 
separated by gel filtration. From the manner of their descent in the Sephadex 
column, it is believed that the latter is of smaller molecular weight than the 
former.

Fluorescence II appears in the column very early stage of cultiavtion even 
before the appearance of other pigments and Fluorescence I. This is true for all 
culture filtrates of strains tested except for No. 134. Therefore in the case of 
strain No. 134, Fluorescence I appears first. Strain No. 145 produces an orange 
colored and a red colored pigment besides Fluorescence I and II, and No. 58, 
which was believed to be a colorless strain, produced a bluish pigment similar 
to pyocyanin. It is interesting to note that the blue pigment produced by strain 
No. 58 is relatively stable, differing from the pyocyanin produced by *Ps. aeru-
ginosa*.

As described above several fractions of pigment and fluorescent substances 
were isolated from the culture filtrate of *Ps. aeruginosa* by gel filtration. It is 
assumed, however, that each of these fractions is not pure and may be composed 
of several substances. This is supported by the findings from the absorption 
spectra and the pattern of the characteristic wave length curves of fluorescence. 
Studies on the purification of these various pigments and fluorescent substances 
are currently in progress.

**SUMMARY**

1) Four strains of *Pseudomonas aeruginosa*, Nos. 134, 145, 117 and 58, with 
different pigment producing ability on glycerol-agar medium, were cultivated on 
 glutamic acid medium to observe their mode of pigment production.

2) The glutamic acid medium developed by the authors is of a simple
composition, and the production of pigments and fluorescent substances in this medium is so rapid and brilliant that it is valuable in the study of pigment production by the Pseudomonas.

3) Four strains of *Ps. aeruginosa* were grown in the glutamic acid medium, and gel filtration was carried out on their culture filtrate. The following results were obtained.

a) The fluorescent material produced by *Ps. aeruginosa* can be separated into at least two portions on the dextran gel column. They were designated as Fluorescence I and Fluorescence II.

b) It is not clear whether the greenish yellow part of Fluorescence I appearing in the early stage of cultivation of strains No. 134 and 145 is or not identical to Fluorescence I.

c) The red and orange pigments of strain No. 145 and the blue pigment of No. 58 are believed to be of different substances.

REFERENCES