


Some Properties of Pyoverdine, the Water-soluble Fluorescent Pigment of the Pseudomonads

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The fluorescent water-soluble pigment complex produced by pseudomonads has been variously called "bacterial fluorescein" or "fluorescin." These terms are unfortunate for they are synonymous with resorcinolphthalein and resorcinolphthalin, respectively (Merek Index, 1952). The bacterial pigment is not related to either of these compounds. However, the term "pyoverdine," suggested by Turfreijer (1941), specifically identifies the bacterial pigment complex and will be used here.

Pyoverdine has interested bacteriologists for years, but few intensive studies have been done on it. Some investigators have studied media for maximum pigment production. This work was adequately reviewed by Seleem and Stark (1943) except for the important contribution of Sullivan (1905), who was the first to describe a simple synthetic medium composed of asparagine, magnesium sulfate, and potassium hydrogen phosphate. King et al. (1948), Baghdiantz (1952), and Totter and Moseley (1953) studied the stimulation of pigment production by added minerals. King et al. (1954) described simple solid media.

No one has yet determined the exact chemical nature of pyoverdine or its fractions, nor has anyone yet succeeded in crystallizing it. Various authors have used activated carbon to remove it from aqueous solution. When thus adsorbed it can be eluted with aqueous acetone or alcohol (Giral, 1936; Turftt, 1937; Turfreijer, 1941). Turfreijer succeeded in separating four fractions based on solubilities of phosphotungstic acid precipitates, and described many properties of these fractions. Empirical formulae suggested by Turftt (1937), Turfreijer et al. (1938), and Bonde et al. (1957) were probably determined on mixtures, and therefore not valid. Giral (1936), Chodat (1951), and Naves (1955) all claim to have separated blue and yellow fractions by chromatographic means. Giral (1936) considered pyoverdine to be a lyochrome or a pterine. Birkhofer and Birkhofer (1948) reported that it was composed in part of riboflavin.

Giral (1936) found pyoverdine to be soluble in water, formic acid, aqueous alcohol, aqueous acetone, and 90 per cent pyridine. Turftt (1937) found it was also soluble in phenol and acetic acid. Giral (1936), Turftt (1937), and Turfreijer (1941) have presented absorption spectra of pyoverdine in various reagents. Meader et al. (1925) described the indicator characteristics of pyoverdine, and Giral (1936) described the effect of a large number of reagents on its appearance. He stated that air favored its production, but that light had the opposite effect.

The object of the present study was to obtain enough information about the properties of pyoverdine to form a basis for determining it in frozen whole egg. The resultant method is described in the second paper of this series (Elliott, 1958).

Experimental

Equipment and materials. The Pseudomonas ovalis and Pseudomonas fluorescens cultures used in the experiments had been isolated earlier from fluorescent eggs (Elliott, 1954) and identified by the method of Haynes (1953). These cultures have been deposited at the Northern Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois, and have been numbered B1595
and B1613, respectively. Asparagine broth for the production of pyoverdine consisted of asparagine, 0.1 per cent; MgSO4·7H2O, 0.05 per cent; and K2HPO4, 0.05 per cent in distilled water. The filtrates of fluorescent cultures used in several experiments were prepared by inoculating asparagine broth with one of the above organisms, incubating it in the dark for 1 to 3 weeks at room temperature, and filtering it through a Seitz pad or a Mandler filter, previously washed free of soluble fluorescent materials.

Absorption spectra were determined on a Beckman1 model DU spectrophotometer, and pH measurements on a Beckman1 model H2 pH meter. Fluorescence emission spectra were obtained using the equipment described by French (1955). All emission spectra were corrected for the instrument response to a standard light source. Fluorescence measurements were made on a Coleman2 model 12B photofluorometer. Visual fluorescence of materials was determined in subdued lighting or in the dark using a Vogelite3 model 6W101 hand fixture previously described (Elliott, 1954). A Vogel blue to green fluorescence color comparison chart was used for recording small changes in black-light fluorescent colors (Benson and Vogel, 1955). Reagents and glassware were tested frequently for the presence of foreign fluorescent materials. In general, chemically pure reagents were nonfluorescent, whereas technical grades had to be purified. Darco G604 was the activated carbon used for adsorption of pyoverdine.

Solvents. As a means of separating pyoverdine from frozen whole egg magma, a pyoverdine solvent immiscible in water would be ideal. A large number of such solvents was tried, but no suitable one was found since pyoverdine has a strong affinity for the aqueous phase in any extraction system. Water and aqueous alcohol were found most practical for the present work.

Attempts at purification. A crude preparation of pyoverdine was obtained by adsorption on activated carbon from a culture filtrate of P. ovalis, followed by elution with aqueous alcohol. This material dried to a brown amorphous residue but did not crystallize. On heating, it began to soften at 230 C, but even at 252 C it did not melt completely. Although this indicates that pyoverdine is a mixture, it will be considered a single entity in further discussion here. Unsuccessful attempts were made to fractionate this material by paper and column chromatography.

Antibiotic properties. Brief experiments using Micrococcus pyogenes var. aureus and Escherichia coli with the agar-cup-plate method showed a concentrated aqueous solution of crude pyoverdine to diffuse rapidly into the medium surrounding the cup. With neither organism was there any evidence of antibacterial action.

Absorption spectra. The absorption spectrum offered a possible means of identification and measurement of the pigment. Five mg of crude pyoverdine, prepared as described above, when dissolved in 25 ml of water presented the absorption curve shown in figure 1. Similar spectra were obtained on the fluorescent filtrates of cultures (figures 2-5) and on this same pyoverdine preparation dissolved in 0.5 per cent AlCl3. The curves obtained agreed well with those presented by Giral (1936), Turffitt (1937), and Turfreijer (1941).

Fluorescence emission spectra and the effect of pH change. Fluorescence emission spectra were determined at three pH values on a fluorescent culture filtrate of Pseudomonas ovalis and on pure riboflavin solutions. Absorption spectra then were run on the same solutions. Results are plotted in figure 2. The pH shift in absorption maxima in pyoverdine solutions as described by Turffitt (1937) is evident here. Also, when the pH is either raised or lowered, the emission maximum shifted toward the red. Total emission is lessened by such pH changes, the greater decrease occurring in alkali. The same pH variations cause nearly complete quenching of fluorescence emission of riboflavin, though little change in its absorption spectrum.

That pyoverdine is a sensitive pH indicator was shown by adjusting the pH values of an asparagine broth culture of P. ovalis using HCl and NaOH. The colors noted are shown in table 1. All color changes were reversible.

Riboflavin content. If riboflavin were a free component of pyoverdine, as described by Birkhofer and Birkhofer (1948), the identification and measurement of the pigment could be accomplished easily by established methods. Riboflavin was found to be absent when a P. ovalis culture was analyzed by the official method (AOAC, 1955). Furthermore, the absorption spectrum
of pyoverdine is not that of riboflavin, and its pH indicator characteristics are different. It was concluded that riboflavin is not a free component of pyoverdine. Naves (1955) came to this same conclusion. It is of interest to note, however, that species of *Pseudomonas* that produce riboflavin are known (for example, Ganguly, 1955; Landenburger, 1952.) It is possible that Birkhofer and Birkhofer isolated one of these. Separation of pyoverdine from riboflavin is not easy.

**Beer's law.** Measurements made on a *P. ovalis* fluorescent filtrate at 412 m\(\mu\) at various stages of dilution showed optical density to be proportional to concentration. Similarly, the fluorescence of a culture filtrate when diluted was proportional to concentration, using the B1 and PC2 filters of the photofluorometer. An extract of fluorescent eggs, prepared in accordance with the quantitative method described in the second paper of this series, was also diluted quantitatively. Fluorescence of this material was likewise proportional to concentration. Thus pyoverdine obeys Beer's law in he concentrations expected in egg spoilage.

**Pyoverdine formation and oxidation-reduction potential.** When a culture of the facultative organism *Pseudomonas ovalis* is grown in a colorless asparagine broth, then examined under black light, pyoverdine formation begins with a light blue color throughout the medium, then the top few millimeters become yellow-green. Finally the yellow-green color diffuses throughout the medium. This more rapid production of pyoverdine at the surface must be related to the higher oxygen tension there. It is not related to pH, as the following data show:

<table>
<thead>
<tr>
<th>Portion</th>
<th>Color under black light</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>Green BG 60</td>
<td>7.5</td>
</tr>
<tr>
<td>Bottom</td>
<td>Blue BG 80.8</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Giral described greater production of pyoverdine during aeration. An experiment was conducted which confirmed his findings. Results, presented in figure 3, show the rapid production of pyoverdine in the culture aerated during growth with a continuous bubbling of sterile air. On the other hand, the culture held aerobic without this flow of air produced much less, and the

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*Figure 2.* Absorption and fluorescence emission spectra of a filtrate of a fluorescent culture of *Pseudomonas ovalis* and of pure riboflavin.
one in which anaerobic conditions were maintained by a layer of oil on the surface produced almost none of the pigment.

This study shows that air is necessary in the production of pyoverdine. Subsequent studies showed a cessation of pyoverdine production in spoiling liquid egg by the time the oxidation-reduction potential had fallen to $-0.3 \text{ v}$. This observation is illustrated in table 2; however, these data do not indicate the exact voltage at which production ceases.

**Effect of light.** A preliminary test showed that culture filtrates changed in fluorescent color from green (BG 50.8) to blue (BG 80.8) when exposed to direct sunlight and to bluish green (BG 60.8) when exposed to a black-light lamp. Figure 4 shows the drastic effect of direct sunlight in breaking down the pigment on exposure in silica cells.

Further experimentation with known wave bands has shown that pyoverdine is readily decomposed by ultraviolet light, but less readily so by visible light. In acid solution it appears to have greater susceptibility to long wave ultraviolet light; but in neutral solution it is more susceptible to the short ultraviolet bands. It is readily decomposed by laboratory daylight that has passed through window glass. Fluorescence of one solution dropped 30 per cent on 3 hr exposure to laboratory daylight out of direct sunlight.

Pyoverdine will not decompose on several hours' exposure to light from incandescent or fluorescent lamps that emit little or nothing in the ultraviolet range. It is worthy of note, however, that pyoverdine solutions will break down even in the dark if stored several weeks.

**Effect of concentration on color.** There is a correlation between pyoverdine concentration and its fluorescent color. That the yellow-green color may indicate

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**Figure 3.** Comparative production of pyoverdine by *Pseudomonas ovalis* B1595 in asparagine broth cultures (1) continuously aerated during growth, (2) aerobic but not aerated, (3) anaerobic by reason of a 5-mm layer of sterile mineral oil on its surface, and (4) anaerobic with added 0.5 per cent glucose. Only the 2-day absorption spectra are presented.

**Figure 4.** Effect of 132 min of sunlight passed through silica glass on the absorption spectrum of the sterile filtrate of a *Pseudomonas ovalis* culture.

**Figure 5.** Absorption spectra of filtrates of identically treated cultures of the "blue" *Pseudomonas fluorescens* B1613 and the "yellow-green" *Pseudomonas ovalis* B1595.
high concentration and the blue color a low concentration was shown in the following series of experiments.

A culture of *Pseudomonas ovalis* in asparagine broth fluoresced a brilliant yellow-green (BG 50.8). When diluted 1 to 50 with asparagine broth of the same pH (8.1), it fluoresced blue (BG 80.8).

*Pseudomonas fluorescens* B1613, which had been isolated from a blue fluorescent egg, produced blue fluorescence consistently when inoculated into shell eggs. On the other hand, *Pseudomonas ovalis* B1595, isolated from a yellow-green fluorescent egg, produced yellow-green fluorescence when inoculated into shell eggs. These eggs were broken out and stored as separate lots in the freezer. Subsequent analysis by the quantitative method described in a second study (Elliott, 1958) showed that the egg pulp prepared from the light blue fluorescent eggs contained one third as much pyoverdine as that from the yellow-green fluorescent eggs. Absorption spectra run on identically treated culture filtrates of these two organisms showed a similar difference in pyoverdine concentration (figure 5).

Anaerobic cultures of *Pseudomonas ovalis* in asparagine broth produced only small quantities of pyoverdine and fluoresced blue (figure 5). Likewise, artificial conditions which created a low oxygen tension resulted in blue fluorescence in eggs inoculated with *P. ovalis*. A batch of fresh eggs was immersed in a cold-water suspension of the organism. Then the eggs were divided, and half of them were immersed immediately in melted paraffin. All were held at 15 C. All resulting fluorescent eggs in the paraffin-coated lot were blue when examined with the black-light candler; all those in the uncoated lot were yellow-green.

**Acknowledgments**

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**Summary**

The term "pyoverdine" is recalled from the work of Turfreijer (1941) as the most suitable for the watersoluble fluorescent pigment of the pseudomonads. A crude preparation of pyoverdine would not crystallize, and had no precise melting point. Its absorption and fluorescence emission spectra are presented. Riboflavin is not a free component of pyoverdine. Pyoverdine is a reversible pH indicator. It is decomposed by short and by long wave ultraviolet light but not by visible light. It is produced more rapidly and in greater quantity in aerated media than in media with lesser oxygen tension. Its production by pseudomonads ceases in spoiling egg by the time the oxidation-reduction potential falls to −0.3 v. Fluorescence of pyoverdine is proportional to concentration. It has no antibiotic activity against *Micrococcus pyogenes* var. *aureus* or against *Escherichia coli*.

Some color variations in pyoverdine may be due to variation in concentration. A strain of *Pseudomonas fluorescens* produced less pyoverdine in eggs and cultures than did a strain of *Pseudomonas ovalis*. Cultures of the former fluoresced blue consistently, whereas those of the latter fluoresced yellow-green.

**REFERENCES**


Benson, R. C. and Vogel, M. J. 1955 Principles of identi-


King, J. V., Campbell, J. J. R., and Eagles, B. A. 1948 Mineral requirements for fluorescein production (by Pseudo-


