

2235

Absorption and Fluorescence of Water-Soluble Pigments Produced by Four Species of *Pseudomonas*

AARON E. WASSERMAN

Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, Pennsylvania

Received for publication 24 September 1964

ABSTRACT

WASSERMAN, AARON E. (Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, Pa.). Absorption and fluorescence of water-soluble pigments produced by four species of *Pseudomonas*. Appl. Microbiol. **13**:175-180. 1965.—Pigments produced by four species of *Pseudomonas* during growth in three media were examined for visible and ultraviolet absorption. Ultraviolet fluorescence excitation and emission spectra were obtained. In all pigments, an absorption maximum occurs at 405 m μ . Ultraviolet excitation of fluorescence occurs primarily at 400 to 410 m μ , with smaller maxima at 460 m μ , or 525 m μ , depending on pH, bacterial species, or medium. Emission maxima, after excitation at 410 m μ , occur at 390 m μ , and 455 to 475 m μ . The differences in fluorescence spectra may be used for taxonomic classification of the *Pseudomonas*.

Microorganisms of the genus *Pseudomonas* may produce a fluorescent, water-soluble pigment, or pigment complex, known classically as "fluorescin" or, more recently, "pyoverdine" (Elliott, 1958). Production of pigment has been used as one of the characters in the taxonomic classification of different species of *Pseudomonas*, although it is known that pigment formation is a variable characteristic and can be lost suddenly, as described in *Bergey's Manual* and by Rhodes (1959). The effect of the composition of the growth medium on the ability of the organisms to produce a pigment complex has been investigated (Georgia and Poe, 1931; King, Ward, and Raney, 1954; King, Campbell, and Eagles, 1948), and requirements for potassium, magnesium, and the sulfate ion have been demonstrated. It has also been established (Waring and Werkman, 1942; King et al., 1948; Totter and Mosely, 1953; Lenhoff, 1963) that an inverse relationship exists between the concentration of iron in the medium and pigment production. The oxygen tension in the growth medium influences the formation of pyoverdine, with larger amounts of pigment produced in well-aerated cultures with a high oxygen tension (Giral, 1936; Lenhoff, 1963). Elliott (1958) showed that in stationary cultures *P. ovalis* produced a blue fluorescent pigment which gradually became yellow-green, beginning at the surface and extending through the medium.

Empiric formulas for pyoverdine have been suggested by Bonde, Jensen, and Thamsen (1957), Turfreijer, Wibaut, and Boltjes (1938), and Turfitt (1937). A colorless, blue fluorescent material has been separated from pyoverdine by differential absorption (Giral, 1936; Turfreijer, et al., 1938).

The absorption spectrum of *Pseudomonas* pigment has been reported by several investigators. Turfreijer et al. (1938) and Giral (1936) showed maximal absorption at about 410 m μ ; Elliott (1958) concerned himself with a maximum at 412 m μ , although spectral absorption curves in his paper also show a maximum at approximately 270 m μ . Kraft and Ayres (1961), in studying the pigment production by two species of pseudomonads, found maxima at both 270 and 410 m μ . They also demonstrated that the composition of the medium can affect the ultraviolet-absorption spectrum of the pigment. Fluorescence emission spectra of pyoverdine were presented by Elliott (1958).

During the course of the investigation of the growth of *Pseudomonas* in maple sap in this laboratory, variations were observed in the production of pigment and in the fluorescence of the pigment under different conditions. This paper reports on the ultraviolet and fluorescence spectra of the pigments produced by four species of *Pseudomonas* growing in several media, and the effects of variation of the pH of the pigment.

MATERIALS AND METHODS

Organisms. The organisms used in these studies were *P. geniculata*, isolated in this laboratory from maple sap; *P. ovalis* NRRL B-1595 and *P. fluorescens* NRRL B-1604, both received through the courtesy of W. C. Haynes, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; and *P. aeruginosa* OSU-64, received through the courtesy of R. G. Eagon, University of Georgia, Athens. The stock cultures of the organisms were maintained on slants of either Difco Tryptone Glucose Yeast Extract agar or Difco Pseudomonas Agar F.

Media. Three media were used to grow the organisms. Asparagine broth, as described by Elliott (1958), contained 0.1% asparagine, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.05% K_2HPO_4 in distilled water. Malate-phosphate medium (M-P) contained 0.34% KH_2PO_4 , 0.67% Na_2HPO_4 , 0.5% DL-malic acid, 0.04% $MgSO_4 \cdot 7H_2O$, and 0.2% $(NH_4)_2HPO_4$ in distilled water; the pH was adjusted to 6.8 before sterilization. The third medium was Difco Pseudomonas Agar F.

Pigment production. Cells of the four organisms were washed with sterile distilled water from slants of Pseudomonas Agar F on which they had been growing for 24 hr at 29 C. All four species produced green pigment with yellow-green fluorescence in the agar slant. A 1-ml amount of each suspension was added to a set of flasks containing 50 ml of medium. The flasks were incubated at 29 C for 48 hr. The cultures were treated in an autoclave at 15 psi for 15 min to kill the bacteria; preliminary experiments showed that the fluorescent material was heat-stable. After cooling, the liquids were clarified by centrifugation at $20,000 \times g$ and filtration through membrane filters.

Petri plates containing Pseudomonas Agar F were heavily seeded with suspensions of the four organisms and incubated at 27 C for 18 hr. The cells were scraped from the agar surface into sterile distilled water; the agar pads were broken into pieces that could be handled conveniently, and each pad was extracted with 25 ml of distilled water. The agar suspensions were clarified by centrifugation, and the supernatant pigmented solution was filtered through membrane filters. The pH of the pigment solutions was adjusted with 0.1 N HCl and NaOH by use of a Beckman pH meter.

The absence of pyocyanin and other chloroform-soluble pigments produced by *P. aeruginosa* that might confuse the study was demonstrated by extracting acidic and basic portions of the pigment solution with chloroform.

Absorption spectra. The absorption curves were obtained with a Bausch & Lomb Spectronic-505 colorimeter in the visible and ultraviolet ranges (650 to 200 $m\mu$); the appropriate medium adjusted to the pH of the sample was used as balance control.

Fluorescence spectra. Preliminary studies and the development of fluorescence in the culture media were followed by use of a Chromatavue

Blacklight with a filter with maximal transmission at 366 $m\mu$. Qualitative observations of the color and intensity of the emission fluorescence were made visually. The spectrum curves were obtained with an Aminco-Bowman Spectrophotofluorometer (varying the wavelengths from 800 to 200 $m\mu$). A preliminary scan of the emission spectrum at an excitation wavelength of 366 $m\mu$ showed that maximal emission occurred at 470 $m\mu$. With the instrument set at this emission wavelength, the curves for the excitation spectra of the pigment solutions were obtained. These spectral curves were not corrected for variation in the source of the excitation radiation with a wavelength or for the response characteristics of the photo tube and grating for the emission spectra (White, Ho, and Weimer, 1960).

RESULTS

In asparagine medium, all organisms except *P. fluorescens* produced a visible blue-green color with fluorescence in daylight. Under ultraviolet light, the pigments fluoresced yellow-green; the colorless *P. fluorescens* solution fluoresced dull blue. In M-P medium, only *P. ovalis* produced pigment (yellow with no daylight fluorescence); under ultraviolet light, all the solutions fluoresced either blue or yellow-green.

On the Agar F plates, only *P. fluorescens* produced no pigment; the other bacteria formed strong green pigments. These were soluble in water, forming yellow or yellow-green solutions with blue-green fluorescence in daylight. In the ultraviolet light, *P. aeruginosa* and *P. geniculata* fluoresced an intense yellow-green, and *P. ovalis* and *P. fluorescens* fluoresced a dull blue-green.

The lack of visible color production by *P. fluorescens* in the three media emphasizes the variability in pigment production. The cells used to inoculate these media had been taken from a slant of Pseudomonas Agar F in which they produced a strongly colored pigment, and in subsequent studies with the same cell suspensions pigment was formed. However, even in the media that were colorless to the eye, *P. fluorescens* did produce a compound that fluoresced blue when excited at a wavelength of 366 $m\mu$.

The pigments produced by the four species of *Pseudomonas* were affected by variation in pH; both visible color and ultraviolet fluorescence underwent changes. In general, the shifts in color were approximately the same for the four species on both the asparagine and M-P media; although the color range was similar, there were some differences in the pH levels at which the changes occurred. Under alkaline conditions (pH 9.8), the ultraviolet fluorescence was yellow-green or blue-green; as the pH became more acidic, the colors ranged through blue, blue-white and

white, to a whitish-orange, or orange, at pH 2.0. The color changes were reversible and, on the addition of sufficient alkali, an orange solution became blue or yellow-green. Upon reacidification, however, the color changes did not necessarily occur at the same pH as originally observed. Although acidification also affected the visible color of the pigment solutions, which became lighter and lost the ability to fluoresce, the strongest effect was noticed on the ultraviolet fluorescence. Both Elliott (1958) and Giral (1936) noted similar color changes with variation in pH, although neither reported the orange fluorescence observed at pH 2.0 to 3.0 in these experiments.

Ultraviolet-absorption curves were made of the solutions of pigments produced by the four species of *Pseudomonas* growing in the three media. The pigment solutions were adjusted to pH 9.8, 7.7, 6.6, and 3.1. Representative curves shown in Fig. 1 for the pigments produced during growth in M-P medium are the same for all four pH levels. The pigments produced by all four of the *Pseudomonas* species had a maximal absorption peak of 255 m μ ; only the pigment of *P. ovalis* had an additional maximum at 405 m μ . Only this organism produced a visible color in the medium.

Representative curves of the pigments produced during growth in asparagine medium (Fig. 2) were similar in shape for the four pH levels.

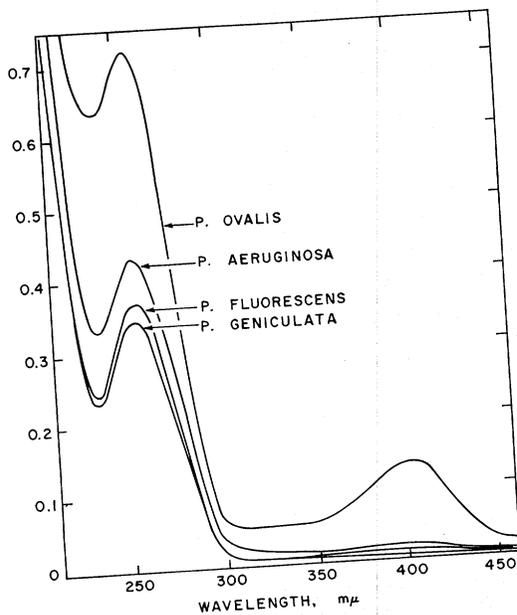


FIG. 1. Absorption spectra of the pigments produced by four species of *Pseudomonas* growing in a malate-phosphate-salts medium.

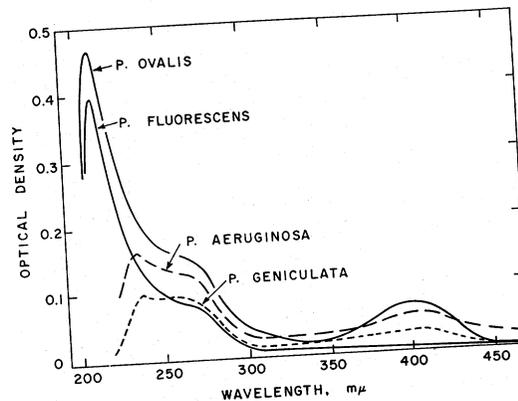


FIG. 2. Absorption spectra of the pigments produced by four species of *Pseudomonas* growing in asparagine medium.

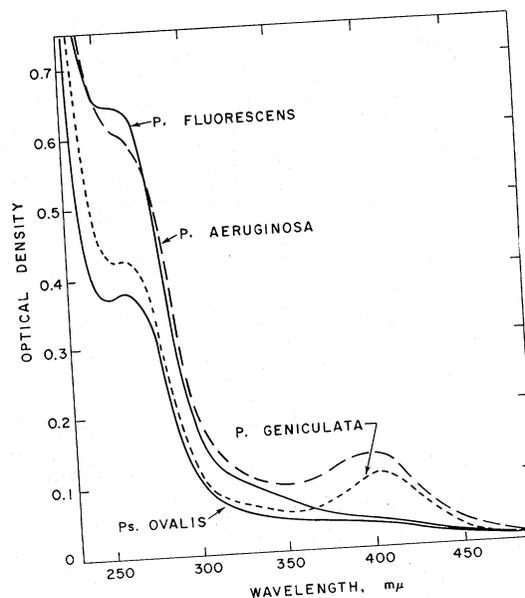


FIG. 3. Absorption spectra of the pigments produced by four species of *Pseudomonas* growing on *Pseudomonas* Agar F.

Small maxima at 405 m μ were shown by all the pigments except the one produced by *P. fluorescens*. However, all have a shoulder on the curve at approximately 250 m μ . The curves in Fig. 3 are the ultraviolet-absorption spectra of the pigments produced by growth on *Pseudomonas* Agar F. These are recorded for the unadjusted solutions only, which were in the range pH 8.0 to 8.4. An absorption peak is indicated at 263 m μ , but only *P. aeruginosa* and *P. geniculata* have one at 405 m μ .

To obtain the extinction coefficient of the strong absorption bands, material from liquid media had to be diluted 1:10, and pigments from growth on *Pseudomonas* Agar F were diluted 1:20 with distilled water. The visible color disappeared from some of these solutions, but fluorescence on excitation at 366 m μ was still obtained. The absence of absorption at 405 m μ undoubtedly is related to the disappearance of the visible color and is a function of the pigment concentration. The strong absorption at 260 m μ may be due to nucleic acids liberated into the culture media by the bacteria as a result of the degradation of non-viable cells. This was confirmed provisionally by the method of Hotchkiss (1957) in which the addition of NaOH to produce a pH of 11.8 in the pigment solution brought about the degradation of the nucleic acids and an increase in the absorption at 260 m μ .

At pH 11.8, the pigment solution was yellow and fluoresced yellow-orange under ultraviolet

light. The maximal optical density was shifted from 405 m μ to about 415 m μ .

The fluorescence characteristics of pigment solutions grown on *Pseudomonas* Agar F were determined over the range 200 to 800 m μ , and both excitation and emission spectra were obtained. With the emission spectrum set at 470 m μ , the effect of pH on the excitation spectra of the pigment produced by *P. geniculata* is shown in Fig. 4. When the pH of the pigment was made either more acidic or more alkaline than pH 6.5, a bathochromic shift occurred. On the acid side (pH 3.1) the intensity of the major peak decreased considerably and a change in shape occurred.

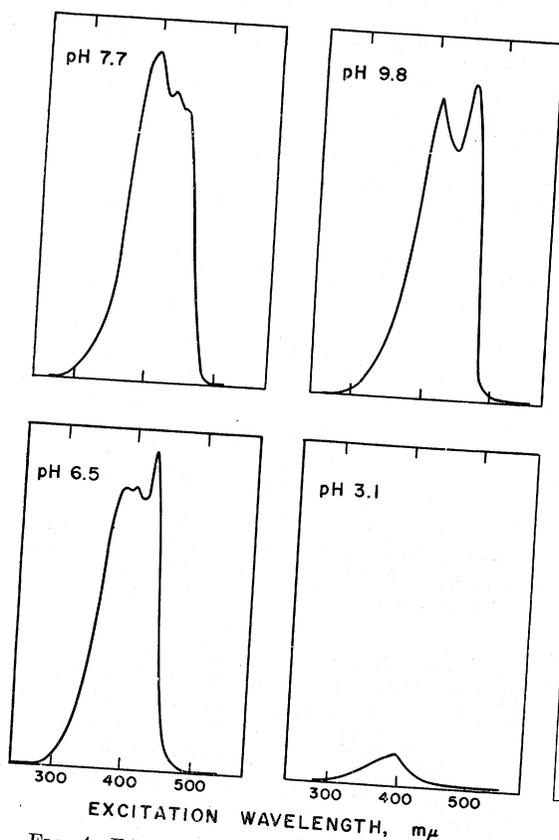


FIG. 4. Effect of pH on the excitation spectra of pigment produced by *Pseudomonas geniculata* grown on *Pseudomonas* Agar F. Emission measured at 470 m μ . Excitation spectra measured from 200 to 800 m μ .

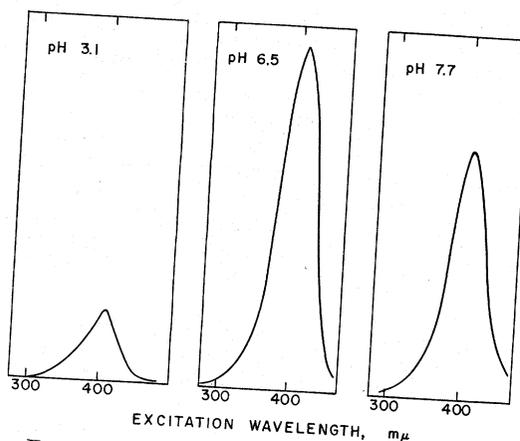


FIG. 5. Effect of pH on the excitation spectra of pigment produced by *Pseudomonas ovalis* grown on *Pseudomonas* Agar F. Emission measured at 470 m μ . Excitation spectra measured from 200 to 800 m μ .

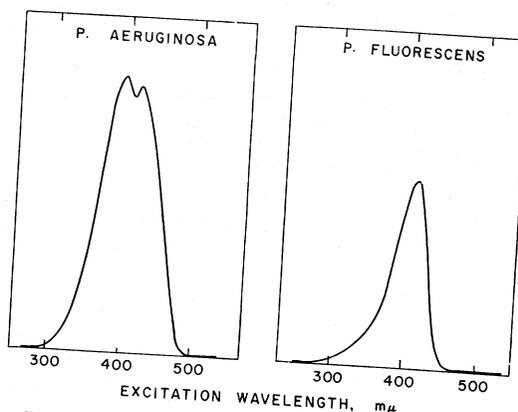


FIG. 6. Excitation spectra of pigments produced by *Pseudomonas aeruginosa* and *P. fluorescens* grown on *Pseudomonas* Agar F. Emission measured at 470 m μ . Excitation spectra measured from 200 to 800 m μ .

With pigment produced by *P. ovalis* grown on Pseudomonas Agar F (Fig. 5), a bathochromic shift does not occur when the pH is varied from 6.5. However, at pH 3.1, the spectrum is similar to that of *P. geniculata* at the same pH.

The pigments produced by *P. aeruginosa* and *P. fluorescens* during growth on Pseudomonas Agar F were examined only at pH 8.4 (Fig. 6). The spectrum of *P. aeruginosa* resembled that of *P. geniculata*, and the spectra of *P. fluorescens* and *P. ovalis* were similar.

DISCUSSION

The production of a pigment by an organism, or a group of organisms, is a characteristic of taxonomic importance. The water-soluble fluorescent pigment of some species of *Pseudomonas*, however, is produced erratically, depending on the composition of the medium, aeration, and possibly other factors. In these studies, the greatest amount of color, both visible and under ultraviolet light at 366 m μ , was produced by *Pseudomonas* growing on Pseudomonas Agar F, a formula designed for maximal formation of this pigment. Some visible color was produced in asparagine medium, and no visible pigment was produced in a malate-phosphate-salts medium by three of the four organisms tested. If intensity of color is related to concentration, then the quantity of pigment produced by the cells on the Pseudomonas Agar F is greater than in the liquid media. However, in the liquid media that had no visible color, and even with the colorless growth of *P. fluorescens* on Pseudomonas Agar F, there was an ultraviolet fluorescence ranging from dull to intense blue. Elliott (1958) indicated this to be a concentration effect, claiming that dilution of a concentrated pigment fluorescing yellow-green produced a blue fluorescence. The blue fluorescence, however, can also be produced by adjustment of the pH to the neutral range. Furthermore, diluting a solution of pigment 1:100 with water after adjusting to pH 11.8, where it fluoresced yellow-orange, changed the fluorescence to a barely discernible yellow-green; additional dilution resulted in the disappearance of fluorescence.

The bathochromic shift, or shift to the longer wavelengths, was observed in ultraviolet excitation spectra at pH 3.1 with *P. geniculata*, but not with *P. ovalis*, pigment. However, there were similarities in the behavior of the two pigments in acid solution; in both instances, there was a decrease in the intensity of the major peak. The bathochromic shift for the *P. ovalis* pigment possibly occurs at a more acidic pH, according to the fluorescence observed under ultraviolet

light at 366 m μ . At pH 3.0 to 3.5, *P. geniculata* pigment fluoresced a dull-orangeish color, whereas *P. ovalis* pigment fluoresced dull-blue. However, at pH 2.0 the pigment of *P. ovalis* also fluoresced dull-orange. That there are differences in the structure of the pigments produced by the organisms may be inferred from the difference in the excitation spectra of the pigments and the fact that the *P. ovalis* pigment did not fluoresce orange until a lower pH was reached.

The excitation spectra of the pigments produced by the four *Pseudomonas* species growing on Pseudomonas Agar F can be divided into two groups: (i) *P. aeruginosa* and *P. geniculata* and (ii) *P. ovalis* and *P. fluorescens*, even though visible color may be lacking in daylight or, if present, may appear indistinguishable among the different species. It is possible that the excitation spectra of the many species of *Pseudomonas*, grown on a single medium under standardized conditions, may be useful as a taxonomic characteristic for the differentiation of groups of related species.

LITERATURE CITED

- BONDE, G. J., C. E. JENSEN, AND J. THAMSEN. 1957. A water-soluble fluorescing bacterial pigment which depolymerizes hyaluronic acid. *Acta Pharmacol. Toxicol.* **13**:184-193.
- ELLIOTT, R. P. 1958. Some properties of pyoverdine, the water-soluble fluorescent pigment of the pseudomonads. *Appl. Microbiol.* **6**:241-246.
- GEORGIA, F. R., AND C. F. POE. 1931. Study of bacterial fluorescence in various media. I. Inorganic substances necessary for bacterial fluorescence. *J. Bacteriol.* **22**:349-361.
- GIRAL, F. 1936. Sobre los liocromos característicos del grupo de bacterias fluorescentes. *Anales Real Soc. Espan. Fis. Quim. (Madrid)* **34**:667-693.
- HOTCHKISS, R. D. 1957. Methods for characterization of nucleic acid, p. 708. *In* S. P. Colowick and N. O. Kaplan [ed.], *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
- KING, E. O., M. K. WARD, AND D. E. RANEY. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J. Lab. Clin. Med.* **44**:301-307.
- KING, J. V., J. J. R. CAMPBELL, AND B. A. EAGLES. 1948. The mineral requirements for fluorescin production. *Can. J. Res.* **C26**:514-519.
- KRAFT, A. A., AND J. C. AYRES. 1961. Production of fluorescence on packaged chicken. *Appl. Microbiol.* **9**:549-553.
- LENHOFF, H. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome and by *Pseudomonas fluorescens*. *Nature* **199**:601-602.
- RHODES, M. E. 1959. The characterization of *Pseudomonas fluorescens*. *J. Gen. Microbiol.* **21**:221-263.
- TOTTER, J. R., AND F. T. MOSELEY. 1953. Influence

- of the concentration of iron on the production of fluorescin by *Pseudomonas aeruginosa*. J. Bacteriol. **65**:45-47.
- TURFITT, G. E. 1937. Bacteriological and biochemical relationships in the *Pyocyaneus-Fluorescens* group. II. Investigations on the green fluorescent pigment. Biochem. J. **31**:212-213.
- TURFREIJER, A., J. P. WIBAUT, AND T. Y. K. BOLTJES. 1938. The green fluorescent pigment of *Pseudomonas fluorescens*. Rec. Trav. Chim. **51**:1397-1404.
- WARING, W. S., AND C. H. WERKMAN. 1942. Iron requirements of heterotrophic bacteria. Arch. Biochem. **1**:425-433.
- WHITE, C. E., M. HO, AND E. Q. WEIMER. 1960. Methods for obtaining correction factors for fluorescence spectra as determined with the Aminco-Bowman spectrophotofluorometer. Anal. Chem. **32**:438-440.