Characterization of a Pigment from a Pseudomonad

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The chloroform-soluble pigments of various pseudomonads have been found to be phenazine derivatives (Clemo & Daglish, 1950; Hillemann, 1938; Kogl & Postowsky, 1930; Kluyver, 1956; Haynes *et al.* 1956), whereas the constitutions of water-soluble fluorescent pigments of various pseudomonads are not well known. This has been mainly due to the failure of previous attempts to obtain the pigments in a highly purified or in a crystalline state (Elliott, 1958). By using an adsorbent different from those of previous workers and finally by electrophoresis over a cellulose bed, it has been possible to isolate in crystalline state a water-soluble yellow-green fluorescent pigment from a pseudomonad and this compound appears to be a pteridine derivative. The pteridine nature of such water-soluble fluorescent pigments was also suggested by Giral (1936). The present paper is concerned with the method of isolation and the chemical nature of the fluorescent pigment.

EXPERIMENTAL

Organism and media. The organism is a strain of *Pseudomonas fluorescens-putida* intermediate as described by Ganguly (1955). Its growth conditions have been described by Chakrabarty & Roy (1964). It was maintained on 1% casein hydrolysate-agar slants and subcultured fortnightly. The pseudomonad could produce its characteristic yellow-green fluorescent pigments only in casein hydrolysate or synthetic media, but not in nutrient broths.

The strain of *Crithidia fasciculata* (*Culex pipiens* strain) ATCC 12857, used for assaying pteridines, was a kind gift from Dr H. N. Guttman of Haskins Laboratories, New York. The basal and maintenance media used in carrying out the assay with this strain were those recommended by Nathan & Cowperthwaite (1954). Only rigorously cleansed test tubes were used for such assays (Guttman, 1962), where 2.0 ml. of double-strength basal medium was added followed by the addition of standard solutions of folic acid, biopterin and the present pigment (compound P) in desired concentrations, singly or in combination. The final volume in each case was adjusted to 4.0 ml. with glass-distilled water. The media were autoclaved at 15 lb./in.² for 15 min., cooled and finally inoculated with 2 drops of a water suspension of *C. fasciculata*. This was previously grown for 5 days in the synthetic basal medium containing 1 mg. of beef extract (Difco)/ml., centrifuged, washed once with cold sterile 0.9% NaCl and finally suspended in cold sterile distilled water. After 7 days at 25° growth of the organism was measured turbidimetrically in a Klett-Summerson

photoelectric colorimeter fitted with a 660 m μ filter. An uninoculated medium served as a control for setting the instrument, and a blank was maintained which did not contain folic acid, compound P or biopterin.

Production and isolation of the pigment. To isolate the pigment in bulk, large-scale fermentation was carried out in 1% (w/v) casein hydrolysate medium dispensed in 1 l. Fernbach flasks. The inoculum volume was 2%, with a culture grown on the medium. Fermentation was carried out at room temperature (30–32°) for 5 days in the dark. Cells were then centrifuged out (at 1500g for 20 min.) and the broth was concentrated from 12 l. to 1 l. under vacuum. Salts that separated out at this stage were removed by centrifugation.

The concentrated broth was then treated according to the method of Crammer (1948). To 1 l. of the broth was added about 250 g. of solid ammonium sulphate. After thorough mixing the solution was extracted at least thrice in the dark with 100 ml. of water-saturated phenol. The separation of phases was achieved by centrifugation. To about 300 ml. of the combined phenol extracts an equal volume of water was added and phenol was removed by repeated extraction with ether. The water layer contained inorganic salts and the pigment, which was dark brown with green fluorescence. It was then subjected to the following operations:

(1) Adsorption on Norit. About 10 g. of Norit A (Pfanstiehl Chemical Co., Ill., U.S.A.) was added to 100 ml. of the solution and the mixture was shaken vigorously for 40–45 min. and passed through a Buchner filter. The charcoal was washed with 3 l. of water before the actual elution of the fluorescent compounds with aq. 10% (v/v) pyridine.

(2) Adsorption on Dowex 50. Pyridine was removed from the eluate by repeated extraction with chloroform, and the eluate was concentrated to a small bulk. It was then poured over a column (35 cm. $\times$ 1.5 cm.) of Dowex 50 (200–400 mesh). The fluorescent compounds were adsorbed and appeared in a single brown band. The column was washed with about 2 l. of water, and the fluorescent compounds were finally eluted with aq. 10% (v/v) pyridine.

(3) Electrophoresis over a cellulose bed. Since the eluate showed at least three bands (one violet, one green and the other bluish green, all moving towards the anode in 0.05 M-acetate buffer, pH 5.6), and owing to failure of attempts to adsorb and elute preferentially one or more compounds from a number of adsorbents, separation of the components by cellulose-bed electrophoresis was finally adopted. The powdered cellulose (Van Waters and Rogers Inc., San Francisco) was equilibrated with 0.05 M-acetate buffer, pH 5.6, for 12 hr. at 4° in position on the electrophoresis bed, and was joined to the buffer components of cathode and anode by filter-paper strips. A concentrated solution of the fluorescent compounds in water was streaked in, and electrophoresis was carried out in the dark for 48 hr.

at 4–5°. The separated bands were marked under ultraviolet light, cut out and eluted with a small volume of water.

(4) Second adsorption on Dowex 50. The main green fluorescent band in water solution was again passed through two successive Dowex 50 columns, and was eluted in each case with aq. 10% (v/v) pyridine. The final eluate was light brown and gave a green fluorescence under ultraviolet light.

(5) Crystallization from methanol. The above pyridine eluate was evaporated to dryness under vacuum, taken up in 2 ml. of hot water, filtered, and finally 8 ml. of hot methanol was added. On cooling, light-brown crystals separated out, which were recrystallized from 80% (v/v) methanol. The final yield was about 25 mg. from 12 l. of broth. This pigment has been given the trivial name 'compound P' in the absence of other details of chemical characterization.

Paper chromatography and paper electrophoresis of the pigment. The homogeneity of compound P was tested in a number of chromatographic and electrophoretic systems with different solvents and buffers at different pH values. For chromatography, butan-1-ol-ethanol-water (4:1:7, by vol.), butan-1-ol-pyridine-water (4:3:7, by vol.), butan-1-ol-acetic acid-water (4:1:5, by vol.) etc. were generally employed, in all of which it gave a single spot. For electrophoresis, 0.03 M-citrate buffer, pH 4.6, 0.05 M-acetate buffer, pH 5.6, or 0.05 M-tris buffer, pH 7.0, was generally employed, where again compound P always moved with a single band.

Degradation studies. Since preliminary characterization of the pigment suggested it to be a pteridine, and since most naturally occurring pteridines are 2-aminopteridines, the pigment was subjected to oxidation with chlorine water and subsequent hydrolysis with HCl as reported by Forrest & McNutt (1958). The final hydrolysate was tested for the presence of guanidine by adding Weber's nitroprusside reagent as described by Andes & Myers (1937).

Alkaline hydrolysis of the compound was done by heating 8 mg. of compound P in 2 ml. of 1 N-NaOH at 100° for 30 min. The solution was then saturated with CO₂, evaporated under vacuum, the residue was extracted with hot ethanol and the extract was chromatographed. Urea was detected in the chromatogram by its characteristic yellow colour with Ehrlich reagent or as its mercury salt as described by McNutt (1956). Analytical periodate oxidation of compound P was carried out by the method of Dixon & Lipkin (1954). Periodate consumption was, however, measured at 260 mμ instead of at 223 mμ to avoid the intense absorption of compound P in that region.

Photolysis. Preliminary experiments showed that, in the presence of strong sunlight, the fluorescence of compound P in solution was destroyed. Photolysis of compound P was therefore effected by keeping an alkaline solution (15 mg. in 2 ml. of 0.1 N-NaOH) under a 100 w lamp for several days. The fluorescence disappeared and crystals appeared in the original solution of compound P. They were centrifuged, washed with water and dried. Recrystallization could not be carried out because of the small amount. The light-grey crystals were insoluble in water, ethanol etc., and the absorption spectrum in 1 N-HCl was taken in a Beckman model DU spectrophotometer.

The supernatant from the photolysis experiment was chromatographed after being concentrated to a small volume, and periodate-oxidizable substances were detected by the method of Buchanan, Dekker & Long (1950).

RESULTS

Since there is not much information in the literature about the nature of the water-soluble fluorescent pigments elaborated by various pseudomonads, attempts were made to evaluate the properties and to find out the chemical nature of the pigments. After crystallization the pigment appears as a light-brown microcrystalline solid with a distinct X-ray-diffraction pattern. It does not melt below 300°. It is homogeneous in a number of chromatographic and electrophoretic systems (Table 1). The absorption spectra of the compound in water, 0.1 N-hydrochloric acid and 0.1 N-sodium hydroxide are shown in Fig. 1.

The compound is highly soluble in water, pyridine and phenol, and almost insoluble in ethanol, chloroform, butanol, pentanol, ether, benzene, cyclohexanol, pentyl acetate etc. It thus differs substantially from the phenazine group of pigments. Its aqueous solution is light brown and gives a green fluorescence under ultraviolet light. The

Table 1. *Electrophoretic mobility and R_F values of compound P in acetate buffer and different solvent systems*

Buffer or solvent system used	R_F value of compound P	R_F value of riboflavin
Butan-1-ol-pyridine-water (4:3:7, by vol.)	0.56	0.72
Butan-1-ol-ethanol-water (10:3:7, by vol.)	0.21	0.36
Butan-1-ol-acetic acid-water (4:1:5, by vol.)	0.17	0.28
2-Methylpropan-2-ol-water (3:2, by vol.)	0.54	0.45
0.05 M-Acetate buffer, pH 5.6	Mobility: - 40 mm. towards the anode	Mobility: + 10 mm. towards the cathode

Fig. 1. Absorption spectra of the pigment (compound P) in water (—), acid (---) and alkali (- - - -). The concentration of compound P was 30 μg./ml.

fluorescence is quenched by the addition of traces of sodium dithionite, strong acids etc., and is enhanced by the addition of alkali.

The compound contains C, H, O and N, but no S or P. Calculated after drying the sample at 140° over P_2O_5 for 8 hr. under a pressure of 1 mm. Hg, the elementary composition was as follows: C, 44.83, H, 6.25, N, 13.05 %. The molecular weight of the compound, determined by Rast's method, was roughly 210.

The aqueous solution of the compound on filter paper, when sprayed with aq. 5 % (w/v) silver nitrate, developed a yellow spot. It also gave a brownish precipitate with 5 % silver nitrate solution, suggesting that it is possibly a 2-amino-4-oxo- or 2,4-dioxo-pteridine (Masuda, Kishi & Asai, 1957). That it was not a 2-aminopteridine was proved by the fact that, on oxidation with chlorine water and subsequent hydrolysis with hydrochloric acid, it did not produce guanidine, which is normally formed by all 2-amino-4-oxopteridines (Stokstad *et al.* 1948).

Prolonged hydrolysis of the compound with 1N-sodium hydroxide showed with Ehrlich reagent only a faint spot of urea in the chromatogram, indicating that urea is produced in minute amount. This suggests the possible presence of the 2,4-dioxopteridine structure.

Photolysis of the compound under alkaline conditions gave a product with the same absorption spectrum in 1N-hydrochloric acid as that of the parent compound, suggesting the presence of the original ring within the molecule. The supernatant on chromatography showed the presence of periodate-oxidizable compounds, as was the case with another fungal pteridine pigment (Masuda *et al.* 1957). Compound P, on periodate oxidation, consumed approx. 1 mole of periodate/mole. The presence of a polyhydroxy compound having two vicinal hydroxyl groups attached to the side chain of the parent pteridine structure can be inferred from such observations.

An aqueous 0.5 % (w/v) solution of the compound has pH 3.47, and its electrophoretic behaviour suggests that it is acidic. The titration curve of an aqueous solution of the compound with 0.1N-sodium hydroxide presents a typical neutralization curve of a weak acid with pK 4.6 (Fig. 2). The infrared-absorption spectrum of the compound shows some resemblance to that of other pteridine pigments (Masuda, 1955; Wolf, Jones & Nathan, 1958). However, a strong absorption at 6.9μ suggests that it possibly contains a carbonyl group. The nuclear-magnetic-resonance spectrum of the substance shows that the protons of the compound rapidly exchange with deuterium of the D_2O medium, suggesting the presence of ionizable hydrogen atoms in the molecule.

The growth-promoting capacity of the pigment towards the organism *Crithidia fasciculata* is seen from the results in Table 2. This protozoan responds to 2-amino-4-hydroxy-6-substituted or 2,4-dihydroxy-6-substituted pteridines, where the substituent in the 6-position is a methyl, hydroxymethyl or formyl group (Wolf *et al.* 1958; Broquist & Albrecht, 1955). The addition of $1\mu g.$ of compound P/ml. to the broth enhances growth to a considerable extent only in the presence of low concentrations ($1\mu g./ml.$) of folic acid, but not in the presence of low concentrations of biopterin. This signifies that the pigment may be an unconjugated pteridine with a substituent at the 6-position.

A rational inference from the above observations is that the compound is possibly a 2,4-dioxopteridine with a carboxyl or hydroxyl group in the ring which confers on it its acidic properties. The

Fig. 2. Titration curve of the pigment (compound P) with 0.1 N-NaOH against pH.

Table 2. Effect of compound P on the growth response of *Crithidia fasciculata* in the presence of folic acid or biopterin

The method of assay is as described in the Experimental section.

Additions	Growth (Klett units)		
	Alone	Folic acid added ($1\mu g./ml.$)	Biopterin added ($1\mu g./ml.$)
None	5	—	—
Folic acid ($1\mu g./ml.$)	14	—	120
Biopterin ($1\mu g./ml.$)	13	128	—
Compound P ($1\mu g./ml.$)	8	43	11
Compound P ($5\mu g./ml.$)	10	40	16

presence of a polyhydroxy moiety, containing at least two vicinal hydroxyl groups, and attached possibly to the pyrazine ring of the molecule, can also be inferred from the photolysis and periodate-oxidation studies.

DISCUSSION

The genus *Pseudomonas* comprises a group of organisms that are unique in producing a variety of water-soluble or water-insoluble pigments. The chemical nature of such pigments has been investigated in some cases and they have mostly been found to be phenazine derivatives. The pteridine nature of the pigment elaborated by the present *P. fluorescens-putida* intermediate strain again points out the diverse nature of the pseudomonads in their general behaviour. Although a variety of insect and fungal pigments have been found to be pteridine in nature (Forrest & Mitchell, 1955; Wolf *et al.* 1958; Maley & Plaut, 1959; Forrest & McNutt, 1958; Masuda *et al.* 1957), reports on bacterial pteridine pigments like the present one are rare. However, a notable difference of this pigment from the well-characterized pteridines of insects is that the latter ones are 2-aminopteridines and the present one and most of the fungal pigments are 2,4-dioxopteridines. All these naturally occurring pteridines have close similarities in their fluorescent nature, in their general methods of isolation and in their photolability and periodate-oxidizability. However, many mammalian and insect pteridines lack the polyhydroxy side chain (Forrest & Mitchell, 1955) and are therefore incapable of undergoing this photolytic reaction.

The properties of the present pigment suggest that it is similar in many respects to those reported already for other water-soluble fluorescent pigments of various pseudomonads (Giral, 1936; Turfitt, 1937; Elliott, 1958). Both Giral (1936) and Turfitt (1937) isolated the pigment by adsorption on charcoal and studied some of its properties. The last author has also shown that pigments from *Bacillus pyocyaneus*, *B. fluorescens liquefaciens* and *B. fluorescens non-liquefaciens* had nearly the same empirical formulae, and were identical in their absorption spectra. Turfitt assigned an empirical formula $C_4H_7NO_2$ to the pigment, and showed that it had an absorption maximum at $410 m\mu$ in alkaline pH, which shifted to $310 m\mu$ on acidification. Elliott (1958) extracted the pigments from the culture broth of *P. ovalis* as a brown amorphous powder which melted over a temperature range $230-250^\circ$. The absorption spectrum of this pigment was, however, identical with that described by Turfitt (1937). Both Giral (1936) and Turfitt (1937) refuted the original observation of Sullivan (1905) that the pigments were soluble in ethanol and ether, and showed that the pigment complex was soluble

in water, phenol, acetic acid, formic acid etc. The present pigment (compound P) has a marked similarity to this pigment complex with regard to its solubility behaviour, although it differs in its absorption spectrum, melting point and some other physical characteristics. This pigment also gives a brownish precipitate with 5% (w/v) silver nitrate solution, a reaction that Turfitt (1937) found to be negative with his preparation of the *P. fluorescens* pigment. This difference in the behaviour of these two pigments may be due to a number of factors. First, the strains producing these pigments are widely different, and the media used for fermentative production of the pigments are not identical. Secondly, the pigment reported by Giral (1936), Turfitt (1937) and Elliott (1958) was a complex mixture containing non-ignitable ash, whereas the present pigment is much more purified chemically. It may also be that the present pigment is somewhat different structurally from the reported pigments of Giral (1936) and Turfitt (1937), whereby such difference in properties of the present pigment from those reported above can be explained.

Although compound P was found to be homogeneous in a number of systems of paper chromatography and paper electrophoresis, rigorous tests of its purity by Craig's countercurrent distribution did not prove successful because of its insolubility in all water-immiscible organic solvents.

The physicochemical properties of compound P suggest the presence of a pteridine nucleus. This has been confirmed by the ultraviolet-absorption and infrared-absorption spectra of the pigment, and the assay with *C. fasciculata*. The enhancement of fluorescence with increase in pH, a reaction exhibited strongly by compound P, is a common characteristic of the pterins (Forrest, Hanly & Lagowski, 1961). The pteridine nature of compound P has also been confirmed by nutritional studies, where purines like adenine, xanthine and to some extent guanine stimulate its formation, but uracil is without any effect. This is consistent with the observations of Albert (1954) and of Esposito & Fletcher (1961) demonstrating purines as the biological precursors of pteridines. The fluorescence of the compound is destroyed by reducing agents, like dithionite, that possibly cause the formation of a reduced colourless 'leuco' derivative as is the case with a number of other pteridine fluorescent pigments (Masuda, 1955; Maley & Plaut, 1959). The growth-promoting activity of compound P towards *C. fasciculata* and its consumption of 1 mole of periodate/mole suggest that the pteridine nucleus might contain two *cis* vicinal hydroxyl groups in the 6-position with the N^8 -position being presumably free, a reaction that might explain the formation of the minute quantity of urea on alkaline hydrolysis (Kuhn, Rudy & Wagner-Jauregg, 1933).

SUMMARY

1. A water-soluble greenish fluorescent pigment from the culture broth of a strain of *Pseudomonas fluorescens-putida* intermediate has been crystallized after its extraction with water-saturated phenol, successive adsorption on Norit A and Dowex 50, and finally separation from other associated components by electrophoresis over a cellulose bed.

2. The pigment has been shown to be a brownish microcrystalline low-molecular-weight high-melting solid with a distinct powder-X-ray-diffraction pattern. Its different physicochemical properties have been described.

3. Chemical degradative studies and the assay of the pigment with *Crithidia fasciculata* suggest that the pigment is a pteridine derivative with a polyhydroxy moiety in the side chain.

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The Metabolism of Fructose Polymers in Plants

4. β -FRUCTOFURANOSIDASES OF TUBERS OF *HELIANTHUS TUBEROSUS* L.*

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The Jerusalem artichoke (*Helianthus tuberosus* L.) contains in its stems and tubers a series of β -D-fructofuranosides, each member of which has the general formula $\text{Glc} \sim \text{Fru-Fru}_n$, where $\text{Glc} \sim \text{Fru}$ represents a sucrosyl group and n may be any number from zero (sucrose) to about 35 (inulin);

the fructose residues appear all to be (2 $\rightarrow$ 1')-linked, giving straight chains (Haworth & Learner, 1928); a similar situation occurs in other members of the Compositae. A chromatogram of this series of compounds from an extract of artichoke tubers is shown in Fig. 1. Each spot on paper chromatograms seems to represent a single substance (Bacon, 1960), and glucose-free fructose chains

* Part 3: Edelman, Recaladin & Dickerson (1963).