Chromatographic Separation of the Pigment Fractions from a *Serratia marcescens* Strain

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A procedure was developed for the separation of pigment fractions in a wild-type *Serratia marcescens* strain. Separation was achieved by column chromatography and elution with several organic solvents. At least six pigment fractions were obtained from the alumina columns by this technique, whereas only four fractions had been reported previously. Spectral and elemental analyses indicate that, in *S. marcescens*, prodigiosin is a complex of six fractions, differing in absorption spectra while retaining the general characteristics of the whole pigment.

It has been reported that prodigiosin, the red pigment of *Serratia marcescens* (6), contains a maximum of four fractions. Weise (5) noted three red fractions upon column chromatography with magnesium oxide and acetone-petroleum ether mixtures. We found, by further separation procedures, a total of six fractions, readily separable by column chromatography.

**Materials and Methods**

Pigment from *S. marcescens* strain 75, grown on a medium developed by Bondarenko (1) and obtained from the Midwest Culture Service (Terre Haute, Ind.), was initially extracted by an acetone treatment (6). A column was prepared by adding alumina (Alcoa, chromatographic grade) to a Pyrex cylinder (1.5 × 26 cm) filled with petroleum ether (boiling point, 38.3 to 52.1 C). The alumina was allowed to settle while petroleum ether was slowly passed through the column, and the alumina was then tamped until the proper consistency was obtained. Pigment dissolved in petroleum ether was applied to the top of the column and was eluted stepwise with the following solvents: chloroform, acetone, and 95% ethyl alcohol. Rechromatography of the initial fraction, eluted with chloroform, was obtained with a second alumina column (1.5 × 15 cm) by use of chloroform and acetone. The fraction remaining at the top of the first column was cut out and washed in acid alcohol (8 ml of concentrated HCl in 92 ml of 95% ethyl alcohol); the slurry was filtered. Each fraction eluted from the column was concentrated in vacuo and stored in vials covered with aluminum foil for further analysis.

Initial elution with chloroform, on an alumina column, resulted in the separation of a purple fraction (If), then an orange-red fraction (Ia), which subsequent rechromatography showed to be comprised of two readily separable fractions; a pink fraction (Id and le) remained at the top of the column. After moving 5 to 10 cm down the column, the purple fraction (II) formed a stationary while the orange-red fraction (Ia) moved steadily down the column. The two orange-red fractions were collected as one fraction, dried in vacuo, resuspended in a small volume of petroleum ether, and rechromatographed on a second and shorter alumina column. Elution of this column with chloroform separated a pink fraction (IIa) initially; subsequent elution with acetone resulted in the separation of a second, slow-moving yellow fraction (IIb).

After elution of the combined orange-red fraction, continued elution of the initial alumina column with chloroform separated a third orange fraction (Ib). Treatment of the pigment fractions remaining on the alumina column (Ic) with acetone resulted in the further elution of a purple-red fraction (Id). When the purple-red fraction was eluted with 95% ethyl alcohol, the separation of a pink fraction (Ie) resulted. A purple fraction (If) remained about 5 to 10 cm from the top of the column. This fraction was cut out of the column, washed in acid alcohol, filtered, and dried in vacuo. (It should be noted that the color of these fractions is solvent-dependent and may change from solvent to solvent.) Figure 1 summarizes the separation procedure.

Spectral analyses of the whole pigment and the fractions were carried out in chloroform (spectrophotometric grade, Mallinckrodt Chemicals, St. Louis, Mo.). Visible and ultraviolet analyses were performed on a Cary 14 spectrophotometer with a 1-cm path length. Infrared analysis was conducted with a Beckman IR8 Infrared Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) at various concentrations. (Figure 11 shows less intensity due to the small amount of this pigment fraction available for analysis.)

1 Many of the data presented in this paper are taken from a thesis submitted by T. E. Worthy in partial fulfillment of the requirements for the M.S. degree at Northern Illinois University.
**RESULTS AND DISCUSSION**

Absorption spectra of prodigiosin and the pigment fractions are shown in Fig. 2–8. Prodigiosin is seen to possess a band at approximately 2,750 A, and the presence of this band is indicative of conjugated unsaturation in the molecule. The displacement of this band (B-band) from approximately 2,400 to 2,750 A is characteristic of the presence of a chromophore, a covalently unsaturated group responsible for electric absorption, attached to the heteroaromatic nucleus. A heteroaromatic nucleus without a chromophore absorbs in the 2,300 to 2,400 A range. If the molecular formula postulated by Wasserman (4) is correct, the shift in the B-band is probably due to the presence of the methene group. The B-band shift may also be the result of the presence of the additional pyrrole rings.

The absorption spectrum of prodigiosin found in this study is similar to those reported in the literature. In the visible spectrum, a major peak is noted between 5,350 and 5,400 A, with a small hump at about 5,000 A on the descending slope of this peak. These peaks are in good agreement with those found by Williams et al. (6), Moesi (3), and Burgova et al. (2).

Examination of the absorption spectra of the various fractions shows a degree of dissimilarity. All fractions, other than Ib, show a distinct peak at about 5,400 A. Fractions IIa, IIb, Id, Ie, and If contain the small hump at 5,000 A. Table 1 summarizes the absorption characteristics.

Examination of the ultraviolet range is not as fruitful as examination of absorption spectra owing to a strong tendency of the solvent, chloroform, to absorb at 2,500 A. A small hump is noted, nonetheless, in fractions IIb, Ib, Id, Ie, and If. This corresponds to the peak at about 2,750 to 2,800 A in prodigiosin, suggesting structural similarities of these fractions with the original prodigiosin. Some further study in the ultraviolet range, by use of a nonabsorbing solvent, is feasible.

Infrared spectra of prodigiosin and of the various fractions are shown in Fig. 9–15. Analysis of prodigiosin shows peaks at 3,000, 1,750, 1,350, 1,200, 750, and 660 cm\(^{-1}\), which is in good agreement with the spectral data of Burgova et al. (2). Burgova indicated that absorption in the ranges of 1,000 to 1,500 and 3,000 to 3,500 cm\(^{-1}\) is characteristic of the pyrrole ring; for-
FIG. 3. Absorption spectrum of fraction IIa in chloroform.

FIG. 4. Absorption spectrum of fraction IIb in chloroform.

FIG. 5. Absorption spectrum of fraction Ib in chloroform.
**Fig. 6.** Absorption spectrum of fraction 1d in chloroform.

**Fig. 7.** Absorption spectrum of fraction 1e in chloroform.

**Fig. 8.** Absorption spectrum of fraction 1f in chloroform.
absorption at 2,800 to 2,900 cm\(^{-1}\) was reported to be characteristic of methylene and methyl groups, whereas absorption at 3,100 cm\(^{-1}\) may be related to the presence of a methene group.

**TABLE 1. Absorption characteristics of prodigiosin and its fractions (max \(\text{A}\)) in chloroform**

<table>
<thead>
<tr>
<th>Compound</th>
<th>(\lambda_{\text{max}}) (A)</th>
<th>(\text{a}_{\text{max}}) (A)</th>
<th>(\lambda_{\text{max}}) (A)</th>
<th>(\text{a}_{\text{max}}) (A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prodigiosin...</td>
<td>2,750</td>
<td>560</td>
<td>5,400</td>
<td>48</td>
</tr>
<tr>
<td>IIa.............</td>
<td>—</td>
<td>—</td>
<td>5,400</td>
<td>20</td>
</tr>
<tr>
<td>IIb.............</td>
<td>2,800</td>
<td>700</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Id.............</td>
<td>2,650</td>
<td>400</td>
<td>5,400</td>
<td>30</td>
</tr>
<tr>
<td>le.............</td>
<td>2,650</td>
<td>400</td>
<td>5,400</td>
<td>30</td>
</tr>
<tr>
<td>If.............</td>
<td>2,900</td>
<td>320</td>
<td>5,450</td>
<td>16</td>
</tr>
</tbody>
</table>

\(\lambda_{\text{max}}\) and \(\text{a}_{\text{max}}\) are given in \(\text{cm}^{-1}\) and absorbance, respectively.

\(\text{a}_{\text{max}}\) Concentration (mg/ml) with a 1-cm path.

Comparison of Fig. 9 to the infrared data of Williams et al. (6) shows somewhat more variation than that shown by Burgova. This may be due to the use of different strains of *S. marcescens*, although Burgova did not mention a specific strain.

Examination of the infrared spectra of the fractions indicates a general similarity to the basic structure proposed by Wasserman (4). Fraction IIa shows, in addition to the absorption for the pyrrole ring, the presence of an alkane group (740 cm\(^{-1}\)) and the presence of a conjugated carbon-carbon double bond (640 cm\(^{-1}\)). Fraction IIb lacks the peaks at 3,000 cm\(^{-1}\) and at 1,350 cm\(^{-1}\). The presence of a much reduced peak at 1,200 cm\(^{-1}\) may be indicative of a decreased number of pyrrole rings. The peak at 650 cm\(^{-1}\) is characteristic of a vinyl group which would include a methene group if bonded to a
FIG. 11. Infrared spectrum of fraction IIb in chloroform.

FIG. 12. Infrared spectrum of fraction Iib in chloroform.

FIG. 13. Infrared spectrum of fraction IId in chloroform.
Pyroline ring. Fraction Ib shows all the characteristic peaks of prodigiosin with the exception of the absorption at 1,700 and 1,350 cm\(^{-1}\). Fraction Ib appears to be the fraction that Burgova reported to be most responsible for the characteristics of prodigiosin. Fraction Id and Ie are very similar; both lack peaks at 1,700, 1,350, and 650 cm\(^{-1}\). Fraction If is the most characteristic of prodigiosin. This fraction was not reported by Burgova and the blue fraction mentioned by Williams differs from If in spectral properties. The presence of a peak in If at 2,800 cm\(^{-1}\), which is absent in the whole pigment, may be explained by the strong absorbance of prodigiosin in the 3,000 cm\(^{-1}\) range.

Table 2 summarizes the results of elemental analyses performed on the six fractions (Micro Tech Laboratories, Skokie, Ill.). It can be seen that these analyses indicate some degree of difference in composition among the six fractions, and these differences are based on the varying amounts of the elements present. A comparative analysis of the ash content of the unseparated pigment and the pigment separated on an alumina column.

**Table 2. Elemental analysis of the six fractions of prodigiosin**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>IIa</td>
<td>70.78</td>
<td>10.80</td>
<td>0.87</td>
<td>11.36</td>
</tr>
<tr>
<td>IIb</td>
<td>69.80</td>
<td>10.13</td>
<td>1.99</td>
<td>7.22</td>
</tr>
<tr>
<td>Ib</td>
<td>62.29</td>
<td>10.03</td>
<td>1.18</td>
<td>9.97</td>
</tr>
<tr>
<td>Id</td>
<td>68.63</td>
<td>10.00</td>
<td>1.13</td>
<td>11.85</td>
</tr>
<tr>
<td>Ie</td>
<td>73.04</td>
<td>11.67</td>
<td>0.58</td>
<td>14.78</td>
</tr>
<tr>
<td>If</td>
<td>71.37</td>
<td>11.50</td>
<td>0.25</td>
<td>5.46</td>
</tr>
</tbody>
</table>
showed a change in residue composition. The unseparated pigment gave a negative test for aluminum, whereas the separated fractions gave a strong positive reaction. The residue from the unseparated pigment was not identified but it was thought to be a metallic element. This indicates the distinct possibility of chelation in prodigiosin and its fractions.

On the basis of the data presented, it can be seen that prodigiosin, as it occurs in bacteria, is a complex of at least six fractions, having different absorption spectra but the same general characteristics of the whole pigment.

LITERATURE CITED