STUDIES ON PIGMENTATION OF SERRATIA MARCESCENS

III. THE CHARACTERISTICS OF AN ORANGE VARIANT

ROBERT P. WILLIAMS AND JAMES A. GREEN

Department of Microbiology, Baylor University College of Medicine, Houston, Texas

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The production of color variants of Serratia marcescens by means of ultraviolet light irradiation has been reported by Labrum and Bunting (1953). These variants exhibited several shades of pink, orange, or white pigmentation, and differed markedly from the natural or wild-type red color. Rizki (1954a) demonstrated that several of these pink, orange, and white variants were capable of inducing red color in each other. This same observation was reported by us for orange and white variants (Williams and Green, 1954). The variants capable of inducing red pigmentation evidently produced a diffusible substance which caused the production of red color in reactive strains (Rizki, 1954a; Williams and Green, 1954).

The study of the relationship existing between inducing and susceptible variants is important for an understanding of the biosynthesis of prodigiosin, the natural red pigment of S. marcescens. Coincident to such a study is an investigation of the pigments of the variants. Rizki (1954b) presented ultraviolet and visible spectra for an extract of orange variant pigment. The present report describes the orange mutant produced in our laboratory, and the characteristics of its pigment.

EXPERIMENTAL METHODS

The orange variant used in this investigation was produced from S. marcescens strain Nima. Identical orange variants could be obtained by either one of two procedures. In the first method, the wild-type Nima strain was grown for 24 hr at 30 C in the glycerol-inorganic salts medium of Bunting (1940). After this period of incubation, the still growing culture was placed next to a gamma radiation source, sodium 22, and incubated an additional 12 hr at approximately 30 C. The second method utilized the usual procedure of exposing a saline suspension of organisms to ultraviolet irradiation. Following either of these treatments, appropriate saline dilutions were prepared from the irradiated cultures, and aliquots were surface plated on glycerol-inorganic salts agar. After 48 hr at 30 C, the plates were examined for color variants. The majority of the colonies were the typical wild-type red variety, but approximately 3 to 5 per cent were orange variants.

The methods employed for growth of orange variant organisms, pigment extraction, paper chromatography, and spectrophotometry were identical to those previously described (Williams et al., 1956). The petroleum ether extract of pigment was treated routinely with disodium ethylenediaminetetraacetate ("versene") to remove any possible metal contaminants (Green et al., 1956). Equal parts of ethyl ether and petroleum ether were used as the solvent for the circular paper chromatogram presented in this report. Other solvent mixtures, as indicated below, also were used in an attempt to resolve the pigment. Determination of the infrared spectrum, preparation of the hydrochloride salt, and various analyses were carried out by the procedures outlined by Green et al. (1956).

RESULTS

The organism. The orange variant is very stable, and neither red, white, nor other colored colonies arise spontaneously from the parent orange culture. White, but not red, variants can be produced by ultraviolet irradiation of a suspension of orange variant organisms. Both the orange variant and the parent, wild-type red organism are small, motile, gram negative rods. Both organisms produce acid and gas from glucose, acid from maltose and mannitol, but fail to ferment lactose or sucrose. The citrate, nitrate, Voges-Proskauer, methyl red, and urease tests are positive in both cases, but neither organism produces indole nor H2S. The two
organisms produce identical reactions in litmus milk and gelatin, the former exhibiting an acid reaction, clotting, and peptonization, whereas the latter is liquefied without formation of a pellicle. The population increase curves at both 30 and 37°C are the same for the orange and red cultures. The orange variant differs from the parent red organism by the fact that it remains colored at 37°C, whereas colonies of the red strain become white at that temperature. Acid fumes will turn colonies of the orange variant red, but growth in acid medium, to the point of acid tolerance for the culture, has no effect upon the pigment color.

The most unusual property of the orange variant is the ability of the organism to cause red pigmentation in certain white variants produced from the same parent strain of S. marcescens. These white variants, when cultured by themselves, have never become pigmented, but when the orange variant is grown close to the white variant, the portion of the latter colony adjacent to the orange colony turns red. The intensity of pigmentation and the extent of the pigmented area in the white colony is directly related to the time the cultures are grown in proximity to one another. The time of appearance of red color in the white variant, as pointed out by Rizki (1954a), is directly related to the proximity of the 2 organisms. Subculture of the white colonies, which had been induced to form red pigment by growth near the orange variant, yields only white colonies in which there is no evidence of red pigmentation.

The phenomenon of induced pigmentation was strain specific. Although several orange variants caused red pigmentation in white variants when both were produced from the parent Nima strain of S. marcescens, these same orange variants would not react with white variants produced from 3 other strains (B, Hy, or Nim). The parent

![Figure 1. Paper chromatogram of orange variant pigment](image)
red Nima strain does not have the ability to induce red pigmentation in white variants.

The pigment. A circular paper chromatogram of extracted orange pigment developed with the ethyl ether-petroleum ether solvent is shown in figure 1. The star-shaped appearance of the chromatogram, apparently due to uneven weighting of the glass plate used to hold the paper, renders difficult the calculation of a specific Rf value. An average of several determinations gives a value of Rf 0.48, with a range from Rf 0.28 to 0.83.

In an effort to resolve the pigment into possible components, circular paper chromatograms were prepared using several solvent mixtures such as chloroform : petroleum ether, acetone: chloroform : petroleum ether, ether : butanol : petroleum ether, and heptane : butanol : ethylene dichloride : petroleum ether. The pigment moved as a single band in all of the solvents employed. These results indicate that the orange variant pigment is a single substance, in contrast to natural pigment (Williams et al., 1956).

Orange pigment was eluted from the paper chromatograms (figure 1) and ultraviolet and visible spectra were determined under acid, alkaline, and neutral conditions. The results of these experiments are presented in figure 2. In an acid solution the pigment exhibits a sharp spectral peak at 500 mμ, which shifts to a broader peak at 460 mμ under alkaline conditions. The most marked characteristic of the spectra is the strong absorption in the ultraviolet region beginning at 350 mμ.

Figure 3 presents the infrared spectrum of the orange pigment. For comparison, the infrared spectrum of the natural, unfractionated red pigment is included. There is a gross similarity between the 2 spectra, but many differences are evident. These differences can be seen in figure 3, particularly between the 1,700 cm⁻¹ and 900 cm⁻¹ bands.

The results of chemical analyses carried out on the hydrochloride salt of the orange pigment are given in table 1. Comparison of these values with those reported by Green et al. (1956) for the natural pigment fractions demonstrates that the orange variant pigment is a different substance.

DISCUSSION

As demonstrated in this report, the orange variant is similar to the wild-type red S. marcescens strain Nima from which it was derived except for its orange color, and its production of a diffusible substance which causes red pigmentation in certain white variants produced from the same parent strain.

If the results of the orange-variant pigment
analyses, presented in figures 1, 2, and 3, and table 1, are compared to similar results obtained from analyses of natural red pigment (Green et al., 1956; Williams et al., 1956), it is apparent that the 2 pigments are markedly different. The orange variant pigment moves as a single substance on paper chromatograms, whereas natural red pigment is fractionated into four components by the same procedure. The spectral properties of orange variant pigment differ from those of natural pigment as well as from the spectral properties of any of the components of the latter substance. In the visible and ultraviolet regions, orange variant pigment has an acid maximum at 500 mμ and exhibits much greater absorption in the ultraviolet spectrum than in the visible. Natural pigment and its blue and red components have acid maxima at or near 540 mμ and exhibit greater, or nearly equivalent, absorption in the visible range as compared to the ultraviolet (Williams et al., 1956). The orange component of natural prodigiosin has an acid maximum at 500 mμ, but has less absorption in the ultraviolet than in the visible range. A more detailed comparison of the orange variant pigment to the orange component of natural red pigment is presented below.

Figure 3 demonstrates that the infrared spectra of the orange variant and the natural red pigments, although grossly similar, differ in many details. The same statement can be made for the infrared spectra of the blue and the combined red components of natural pigment (Green et al.,

<table>
<thead>
<tr>
<th>Chemical Determination</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Chloride</td>
<td>6.19%</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>8.68%</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>373</td>
</tr>
</tbody>
</table>

All values are the average of several determinations.
1956). These comparisons indicate that the orange variant pigment, the natural pigment, and the components of the latter are all somewhat similar substances, but that they possess certain characteristics which differentiate the compounds from one another.

When the data in table 1 are compared to similar chemical analyses for the blue and combined red components of natural prodigiosin (Green et al., 1956), it is evident that these substances and the orange pigment differ in their chemical composition. The chloride content of orange variant pigment is greater than that of the blue fraction, but less than that of the combined red fraction. The nitrogen content of both the blue and the combined red fractions of natural pigment is greater than that of the orange pigment. Most striking is the difference in molecular weights of the pigments, which are 775, 460, and 373 for the blue fraction, the combined red fraction, and the orange pigment, respectively.

Of particular interest is a comparison between the orange variant pigment and the orange fraction of natural pigment. Williams et al. (1956) have demonstrated that the orange fraction is the most rapidly moving component of prodigiosin, traveling almost at the solvent front. The orange variant pigment does not move as rapidly, and, as is shown in figure 1, stays relatively nearer the origin than the solvent front. This difference in paper chromatographic properties is more evident when the two pigments are compared on the same chromatogram (Williams and Green, 1955).

Table 2 compares the ultraviolet and visible spectral properties of the 2 orange pigments. Although both pigments have maxima at 500 μm and minima at 420 μm in the acid curves, the remainder of the comparison points are different. Particularly notable are the 2 optical density ratios, the isosbestic point, and the marked absorption of the orange variant pigment in the ultraviolet region which precludes the measurement of any values beyond 350 μm. On the basis of chromatographic and ultraviolet and visible spectral evidence, we believe the orange variant pigment to be a different compound than the orange fraction of natural pigment.

The most unusual property of the orange variant is its ability to cause red pigmentation in certain white variants. Williams and Green (1955) have analyzed the induced red pigment, and have demonstrated that it is identical to natural prodigiosin, being composed of the same 4 fractions. Orange variant pigment per se did not appear to be part of the induced pigment. Thus it seems that the orange variant pigment itself does not cause pigmentation, but that the orange variant produces a diffusible substance which in turn causes red pigmentation in white variants. Such a conclusion is justified by the results presented in this report with respect to the degree and time of appearance of the induced pigment. Since the spectral data of this report indicate that the orange variant is grossly similar to natural prodigiosin, we propose the hypothesis that both the natural and the orange variant pigments are synthesized by the same metabolic pathway, but that this pathway is changed in the orange variant preventing the conversion of a precursor(s) of the pigment into natural prodigiosin. As a result of the metabolic change, the precursor(s) of the pigment accumulates in the milieu of the orange variant. The precursor(s) then diffuses through the medium and causes the production of natural prodigiosin in susceptible white variants. The latter organisms, once furnished with the required precursor(s), can

**TABLE 2**

Comparison of spectral properties of the orange variant pigment and the orange fraction of natural pigment

<table>
<thead>
<tr>
<th>Points for Comparison</th>
<th>Pigment</th>
<th>Orange variant</th>
<th>Orange fraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mμ</td>
<td>mμ</td>
<td></td>
</tr>
<tr>
<td>Acid curve</td>
<td>Maxima</td>
<td>500, 390</td>
<td>500, 370, 275</td>
</tr>
<tr>
<td></td>
<td>Minima</td>
<td>420, 375</td>
<td>420, 320</td>
</tr>
<tr>
<td>Alkaline curve</td>
<td>Maxima</td>
<td>460, 400</td>
<td>470, 380, 275</td>
</tr>
<tr>
<td></td>
<td>Minima</td>
<td>420, 370</td>
<td>375, 310</td>
</tr>
<tr>
<td>Isoebestic point</td>
<td></td>
<td>480</td>
<td>480</td>
</tr>
</tbody>
</table>

| O. D. ratio of acid maximum to alkaline maximum | 1.3 | 1.0 |
| O. D. ratio of acid maximum at 500 mμ to that at 270 mμ | <0.5† | 2.5 |

* Data from Williams et al. (1956).
† Due to strong absorption in the ultraviolet region, no exact value could be obtained for a 270-mμ maximum.
complete the formation of pigment. Santer and Vogel (1956) have isolated such a precursor from another variant of *S. marcescens*. This mechanism would be similar to the syntrophic mechanism demonstrated by Davis (1950) for certain biochemical mutants involved in aromatic ring biosynthesis. Hence, induced pigmentation should be termed syntrophic pigmentation. This hypothesis is under investigation.

SUMMARY

An orange variant of *Serratia marcescens* has been produced by irradiation of the wild-type organism. The orange variant appears to be morphologically, culturally, and biochemically identical to the wild-type organism. However, it has the unusual ability to produce or accumulate a diffusible substance which brings about red pigmentation in certain white variants produced from the same parent strain. The pigment of the orange variant has been analyzed. It moves as a single substance upon paper chromatography. The orange pigment has very strong absorption in the ultraviolet spectrum, and in the visible range, in acid solution, has a maximum at 500 m\(\mu\). Evidence is presented from ultraviolet, visible, and infrared spectral data, and from chemical analyses for chloride, nitrogen, and molecular weight indicating that the orange variant pigment differs from the natural red pigment and its fractions. But the infrared data suggest that the orange variant pigment is related to the natural pigments. The importance of this fact relative to the induced pigmentation ability of the orange variant is discussed.

REFERENCES


