STUDIES ON PIGMENTATION OF *SERRATIA MARCESCENS*

V. Accumulation of Pigment Fractions with Respect to Length of Incubation Time[1]

ROBERT P. WILLIAMS, CORA LEE GOTT, AND JAMES A. GREEN

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

Received for publication July 29, 1960

In 1930 Amako reported that as cultures of *Serratia marcescens* grew old their color changed from red to dark violet. He postulated that the change was associated with the pathway of pigment formation in the microorganism. Weiss (1949) observed a similar color change. Colonies growing on agar were orange-red in color at the end of 24 to 48 hr, but as they grew older the color changed to red-purple. He attributed the color change to certain pigment components which accumulated in the cells with age, and which, together with what he termed the major pigment fraction, contributed to the altered color. We have observed similar color changes, and have demonstrated that the pigment of 5-day-old cultures of *S. marcescens* may be separated into a blue fraction and a red fraction, the latter composed of three components (Green, Rappoport, and Williams, 1956). Chemical data presented in the report suggested that the blue pigment might be formed by aggregation of the red fractions. The combined blue and red pigments could account for the dark violet and red-purple colors of older colonies described by Amako (1930) and Weiss (1949).

The experiments now reported were undertaken to determine whether the blue pigment fraction increased with the age of the cultures, and thus could contribute to the color change of older colonies. In addition, if the blue component were demonstrated to develop later than the red fraction, this fact would suggest that the blue pigment might be produced from the red.

MATERIALS AND METHODS

The Nima strain of *S. marcescens* employed was the same one utilized in previous investigations (Williams, Green, and Rappoport, 1956). The organism was grown on a modification of the medium of Bunting (1940) containing glycerol, 1.0 per cent; ammonium citrate, 0.5 per cent; dipotassium phosphate, 1.0 per cent; ferric ammonium citrate, 0.005 per cent; magnesium sulfate, 0.05 per cent; and sodium chloride, 0.05 per cent. The medium was made up in water double distilled from glass and adjusted to pH 7.0. Five-liter Povistsky diphtheria toxin bottles were filled with 200 ml of media, and then sterilized by autoclaving. Inoculation was carried out by adding to the bottles 10 ml of a 48-hr culture grown in the same medium. The cultures were incubated at 27 C in the dark with the bottles placed on their sides.

Cells were harvested by centrifugation, washed with water, and pigment extracted by acetone treatment (Williams et al., 1956). During the early stages of growth when pigmentation was slight, the pigment fractions were separated by paper chromatography using a solvent of ether-petroleum ether (1:1). If the chromatographic paper was not treated with ammonia fumes prior to application of the sample, this solvent separated the pigment into the blue fraction and a single red fraction containing the three unresolved components (Williams et al., 1956). The pigments were eluted from the paper with chloroform. Column chromatography (Green et al., 1956) was employed to separate the two fractions when larger amounts of pigment were available. No attempt was made to separate the red fraction into its components.

Purification of the pigments was carried out by repeated solvent transfer or by solvent precipitation. The red fraction was transferred from petroleum ether to ethanol and back again until no blue pigment could be detected by paper chromatography. The blue component was purified by repeated precipitation with petroleum ether from an ethanol-chloroform solution. The fraction was considered pure if no red pigment could be detected by paper chromatography. Final purification of the pigments was achieved by converting them to the hydro-

[1] This investigation was supported by research grant E-670(C5) from the National Institutes of Health, U. S. Public Health Service.

chlorides. Dry pigment samples were dissolved in petroleum ether, then precipitated as the hydrochlorides by bubbling dry HCl gas through the solutions.

Quantitative measurement of the pigments was carried out by using their absorption coefficients. Weighed samples of the hydrochlorides were dissolved in either acidified chloroform or ethanol, and the optical density of the samples determined at 537 mμ employing a Beckman spectrophotometer, model DU. The absorption coefficient of the blue pigment was 5.9×10^3 cm^2 per mg; that of the red, 46.9×10^3; and that of the combined fractions, 51.5×10^3. These values compare to the figure of 7.07×10^4 reported by Castro et al. (1958) for the extinction coefficient of purified prodigiosin hydrochloride. The latter compound is presumably derived from the major component of the red pigment fraction. It is not surprising that the absorption coefficient of our red fraction is lower than the value reported for purified prodigiosin hydrochloride since the fraction contains at least two other pigment components. These components were present in too small quantities for individual examination. No value for the absorption coefficient of the blue component has been reported. As indicated by the absorption coefficient data the absorbance of the red pigment is about 8 times that of the blue.

Viable counts were carried out in duplicate by standard plate count methods employing the medium described above to which was added 0.1 per cent yeast extract, 0.2 per cent Sheffield Farms "N-Z" casein hydrolyzate, and 2.0 per cent agar. The plates were counted after 48 hr incubation at 27 C. Dry weights of the organisms were determined by centrifuging the cells from the medium, washing them three times with deionized water, and then drying the cells to constant weight at 60 C in a desiccator. The values reported are the average of duplicate samples.

RESULTS

The relationship between length of incubation time and the accumulation of red and blue pigment is shown in Fig. 1. Visible pigmentation was first apparent at about 12 hr in cells grown at 27 C in the glycerol-mineral salts medium. At this time the cells were light pink in color, but it was impossible to extract enough pigment for reliable assay. Pigment could be extracted and assayed by paper chromatography from 16-hr-old cells. Only the red fraction was present, and weighed 120 μg per g dry cells. The red fraction comprised the major portion of total pigment until 144 hr.

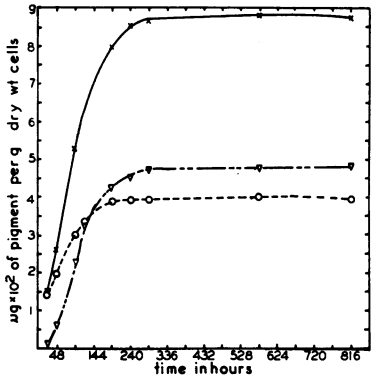

Fig. 1. Weight of whole pigment, the red, and the blue fractions plotted against length of incubation time. ×——× = Curve for whole pigment; O-----O = curve for the red fraction; ▽--▽ = curve for blue fraction.

Blue pigment, detectable by paper chromatography, first appeared at 24 hr. The total weight of pigment at this time was 155 μg per g dry cells of which the blue fraction composed 8 μg or about 5 per cent. Maximal increase in both red and blue pigments occurred between 24 and 120 hr. During this interval the red fraction increased about 2½ times whereas the blue increased over 21 times. Thus the latter was accumulating at almost 10 times the rate of the former. However, it should be noted that the only pigment extracted from cells at 16 hr was the red fraction, and presumably synthesis of this fraction must have been taking place even prior to this time. At about 144 hr the weight of the two fractions is almost equal. From this time to the termination of the experiment the blue fraction becomes the major constituent, and after 288 hr comprises about 55 per cent of the total weight of pigment.

Comparison between the rate of pigment accumulation and the population increase curve can be made by referring to Figs. 1 and 2. The curves of the latter figure represent changes in viable cell count and total dry weight of organisms during the incubation period covered by the pigment accumulation experiments. In Fig. 2 the time interval for the first period of growth

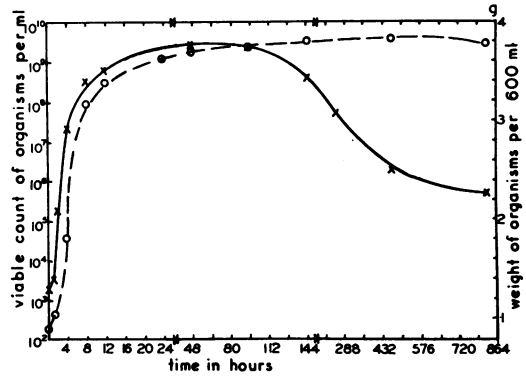

Fig. 2. Viable count and total dry weight of organisms of *Serratia marcescens* strain Nima plotted against length of incubation time X——X = Curve for viable count; O- - -O = curve for dry weight.

has been expanded so that early changes in cell population can be depicted. The maximal rate of cellular growth occurred between 2 and 12 hr, whereas maximal pigment production was between 24 and 120 hr (Fig. 1). At 12 hr, when visible pigment was first apparent, the increase in rate of cell growth had already begun to slacken, and at 18 hr, when the blue fraction first appeared, the cells were almost in their stationary phase.

The time of maximal pigment production coincided with the stationary phase of the growth cycle. Increase in pigmentation did not cease until after 288 hr, at which time the culture was well into its phase of decline. It is interesting to note that pigmentation was increasing from 98 to 288 hr although the viable count was decreasing. During the decline and senescent phases of the culture the amount of pigment in the cells parallels the total cell mass as represented by the dry weight rather than the viable count.

DISCUSSION

These results substantiate the postulates of Amako (1930) and of Weiss (1949) that the pigment composition of *S. marcescens* varies with the age of the culture. When grown in glycerol-mineral salts medium the Nima strain first has a pink color which rapidly becomes red, and at 4 days begins to turn deep purple. As the cells continue to grow older, the purple cultures develop a metallic sheen not unlike that of *Escherichia coli* grown on eosin-methylene blue medium. These changes in visible color parallel the variation in pigment accumulation shown in Fig. 1. It seems reasonable to attribute the purple color of older cells to the increase in blue pigment and its admixture with the red. However, it should be emphasized that the color and pigment changes we have described apply only to glycerol-mineral salts medium. Bunting (1940) pointed out that variation in media composition caused a change in pigmentation, and we have noted that *S. marcescens*, when grown on peptone medium, develops with age an orange rather than a purple color.

Pigmentation has often been regarded as a manifestation of older or senescent bacterial cultures. Harris (1950) reported that the pigment, pyocyanine, of *Pseudomonas aeruginosa* was produced late in the culture cycle of the organism after maximal growth had occurred. However, the fluorescent pigment produced by the same organism was formed at a rate similar to that of cellular proliferation. Frank and DeMoss (1959) have reported similar data for pyocyanine synthesis. As has been pointed out, the data presented in Figs. 1 and 2 demonstrate that pigment synthesis in *S. marcescens* occurs late in the culture cycle after the cells have passed the logarithmic phase of growth. There can be no doubt that the blue fraction is characteristic of older cells since its accumulation occurs exclusively after maximal growth. Although the greater portion of the red fraction also is produced after the cells have reached maximal growth, it is apparent that some synthesis of the pigment has occurred earlier since there are 120 μg per g of cells of the substance present in 16-hr-old cultures. In experiments utilizing a different, somewhat deficient medium containing less phosphate and tris(hydroxymethyl)aminomethane as a buffer, red pigment has been observed after about 4 hr incubation (Green, 1956). Thus red pigment is being formed in actively growing cells although it does not reach an extractable concentration until a later phase of growth.

The fact that much of the increase in pigment occurred during the senescent phase of growth when the organisms were dead or dying gives rise to a paradox in which a substance contained within the cells is increasing in spite of a decrease in viable cells. It appears that the pigment-forming system is not exclusively a property of

viable cells, but is capable of producing pigment in either viable or nonviable cells as long as the precursors are present. Although the cellular function of the pigment of *S. marcescens* is unknown, it is doubtful that a substance which increases to a large extent during the senescent phase of growth can be of very great physiological importance to the cell.

From the data presented in this report we cannot state definitely that the blue pigment is formed from the red. The red fraction can be detected earlier than the blue, and during the time when the rate of pigment accumulation is greatest the blue fraction increases more rapidly than the red. These facts suggest that the blue fraction might be formed from the red, but further investigations utilizing other techniques will have to be carried out before any synthetic relationship between the two fractions can be stated to exist.

SUMMARY

The accumulation of the red and blue fractions of the pigment of *Serratia marcescens* has been measured with respect to length of incubation time. Detectable amounts of the red fraction appear at 16 hr, and the pigment increases until about 192 hr. Blue pigment first appears at 24 hr, and reaches a maximum at 288 hr. From 144 hr until termination of the experiment, the blue fraction was the major component of the pigment. Maximal increase of cells occurred between 2 and 12 hr. The time of maximal pigment production coincided with the stationary phase of the growth cycle, and increased pigmentation was still occurring after the cultures had entered the phase of decline. During the later stages of the growth cycle, the amount of pigment in the cells paralleled the dry weight of organisms and not the viable count. The results confirmed the hypothesis that the color changes occurring in cultures of *S. marcescens* as they grow older are due to accumulation of different pigment fractions.

REFERENCES

AMAKO, T. H. 1930 Beiträge zur Kenntnis der Farbstoffbildung des *Bacillus prodigiosus*. Zentr. Bakteriol. Parasitenk. Abt. I, Orig., **116**, 449–499.

BUNTING, M. I. 1940 A description of some color variants produced by *Serratia marcescens* strain 274. J. Bacteriol., **40**, 57–68.

CASTRO, A. J., J. F. DECK, M. T. HUGO, L. R. WILLIAMS, AND M. R. ZINGG 1958 Prodigiosin hydrochloride. J. Org. Chem. **23**, 1232.

FRANK, L. H., AND R. D. DEMOSS 1959 On the biosynthesis of pyocyanine. J. Bacteriol., **77**, 776–782.

GREEN, J. A. 1956 Studies on pigmentation and metabolism in *Serratia marcescens*. Ph.D. Thesis, The Rice Institute, Houston, Texas.

GREEN, J. A., D. A. RAPPOPORT, AND R. P. WILLIAMS 1956 Studies on pigmentation of *Serratia marcescens*. II. Characterization of the blue and combined red pigments of prodigiosin. J. Bacteriol., **72**, 483–487.

HARRIS, J. O. 1950 The relationship between stage of growth and pigment production by *Pseudomonas aeruginosa*. Trans. Kansas Acad. Sci., **53**, 494–499.

WEISS, C. M. 1949 Spectrophotometric and chromatographic analyses of the pigment produced by members of the genus *Serratia*. J. Cellular Comp. Physiol., **34**, 467–492.

WILLIAMS, R. P., J. A. GREEN, AND D. A. RAPPOPORT 1956 Studies on pigmentation of *Serratia marcescens*. I. Spectral and paper chromatographic properties of prodigiosin. J. Bacteriol., **71**, 115–120.