A METHOD FOR MEASURING BACTERIAL PIGMENTS
BY THE USE OF THE SPECTROPHOTOMETER AND
THE PHOTOELECTRIC COLORIMETER

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Received for publication April 21, 1941

The pigments produced by bacteria have received the attention of many investigators. In recent years, emphasis has been placed especially on the chemical nature of the pigments and their probable roles in the metabolism of the bacterial cells. Considerable progress has been made in the solution of these problems.

Relatively few workers have published results of research designed to determine the effects of the physical and chemical environment upon the production of pigments by various species of bacteria. When investigations have been reported they often concerned but one species or a small group of bacteria.

Previous investigators have measured pigment by a gross visual comparison of the amount of pigment of a culture grown upon the test medium with the pigment produced upon a control nutrient agar. Reid (1936) classified differences in shade or tint of pigment by comparing with the color standards of Maerz and Paul (1930).

An attempt was made to use this method of comparison but it was found that often, because of varying amounts of growth on the two slants, it was difficult to estimate relative amounts of pigment. A very heavy growth may appear to have a greater amount or more intensity of pigment merely because of the mass of material present. There are also technical factors such as type and direction of light, color of background, and eye fatigue which make difficult an accurate determination. The method has not proven of sufficient accuracy. Because of these difficul-
ties it was thought desirable to devise a method which would yield more accurate and reproducible results.

EXPERIMENTAL

By far the most accurate method of color comparison is the use of the spectrophotometer and the recording of the complete absorption spectra data. A study was made of several bacterial pigments by the use of a Bausch and Lomb spectrophotometer.

![Graph](image_url)

**Fig. 1. Absorption Spectra of Some Bacterial Pigments**

- *Chromobacterium violaceum*
- *Serratia marcescens*
- *Flavobacterium suaveolens*
- *Flavobacterium arborescens*

A portion of these data are shown in figure 1. The pigments of *Flavobacterium suaveolens* and *Flavobacterium arborescens* were extracted from the bacterial cells with hot methyl alcohol; the pigments of *Chromobacterium violaceum* and *Serratia marcescens* were extracted with a slightly acidified acetone-ether mixture. It has been found that the carotenoid pigments may be extracted from moist bacterial cells with cold methanol or ethanol by grinding with an abrasive such as alundum.
The availability of photoelectric colorimeters as compared with spectrophotometers makes it desirable to study the spectrophotometric curves with the object of applying their data to the photoelectric colorimeter. In figure 1 it will be noted that the various pigments observed, with the exception of the one from *Chromobacterium violaceum*, show no absorption in the extreme red portion of the spectrum (660 m\(\mu\)). A colorless suspension of bacteria would show approximately equal absorption across the entire spectrum and, hence, light of wavelengths outside the range of pigment absorption may be used to measure the turbidity of pigmented bacterial suspensions.

It can be demonstrated that the filter for optimum measurement of the color values of a solution will be that with a maximum of transmission at the absorption maximum of the solution under observation (Brode, 1939). Since both the turbidity and the color of a bacterial suspension affect the amount of light transmitted, the amount of light absorbed at a wave-length within the absorption band is equal to the turbidity absorption plus the pigment absorption. The true pigment absorption is obtained by subtracting the turbidity absorption from the total absorption. The turbidity absorption may be accepted as the total absorption at a wavelength outside the region of the pigment absorption band.\(^1\) Filters were chosen, with transmission values as indicated in table 1, for the photoelectric measurement of the pigment and the turbidity of suspensions of bacteria.

Since the turbidity may be taken as an index of the number of bacterial cells present, the average amount of pigment per unit of cells may be calculated and used for purposes of comparison. The readings of the colorimeter may be converted to photometric densities by use of a table which is supplied with the instrument. In this investigation an Evelyn photoelectric colorimeter (Evelyn, 1936) was used. Table 2 gives typical data for a suspension of *Serratia marcescens*.

The photometric density 0.1973 represents the turbidity and

\(^1\) Although the absorption of light due to the turbidity resulting from the bacteria in the suspension will increase somewhat with decreasing wave length, this increase will be so slight as not to invalidate the method.
0.3010 represents both turbidity and pigment. Subtracting the smaller from the larger value gives 0.1037 which is the photometric density due to the pigment alone.

It is necessary to calculate the results to a standard turbidity in order to be able to compare one set of measurements with another. It was found experimentally, using the photoelectric colorimeter, that for fairly dilute suspensions of pigmented bacteria the photometric density due to pigment was directly proportional to the photometric density due to turbidity; slight deviations were observed with very turbid suspensions. It has been our practice to dilute the suspensions to a photometric density due to turbidity of 0.2000 or less before the readings are taken. Results are calculated for convenience to a standard turbidity of 0.1000. The following proportion is used for this calculation.

\[
\frac{\text{Amount of pigment for 0.1000}}{0.1000} = \frac{\text{Measured pigment}}{\text{Measured turbidity}}
\]

### TABLE 1

Maximum transmission of filters for colorimetric estimation of bacterial pigments

<table>
<thead>
<tr>
<th>BACTERIUM</th>
<th>WAVELENGTH OF FILTER FOR TURBIDITY</th>
<th>WAVELENGTH OF FILTER FOR TURBIDITY PLUS PIGMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromobacterium violaceum........</td>
<td>Red 690</td>
<td>Green 540</td>
</tr>
<tr>
<td>Flavobacterium suaveolens........</td>
<td>Red 660</td>
<td>Blue 420</td>
</tr>
<tr>
<td>Flavobacterium arborescens........</td>
<td>Red 660</td>
<td>Blue 420</td>
</tr>
<tr>
<td>Serratia marcescens................</td>
<td>Red 660</td>
<td>Green 520</td>
</tr>
<tr>
<td>Staphylococcus aureus.............</td>
<td>Red 660</td>
<td>Blue 420</td>
</tr>
</tbody>
</table>

### TABLE 2

Typical data for pigment determination

<table>
<thead>
<tr>
<th>FILTER</th>
<th>GALVANOMETER READING</th>
<th>GALVANOMETER CORRECTION</th>
<th>CORRECTED READING</th>
<th>PHOTOMETRIC DENSITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>mμ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>520</td>
<td>50½</td>
<td>-½</td>
<td>50</td>
<td>0.3010</td>
</tr>
<tr>
<td>660</td>
<td>64</td>
<td>-¼</td>
<td>63½</td>
<td>0.1973</td>
</tr>
</tbody>
</table>
METHOD FOR MEASURING BACTERIAL PIGMENTS

In the example given in table 2, the calculations are as follows:

\[ \frac{x}{0.1000} = \frac{0.1037}{0.1973} \]
\[ x = \frac{0.1037 \times 0.1000}{0.1973} = 0.0525 \]

The amount of pigment for turbidity of 0.1000 in this example is 0.0525. Calculation to the same turbidity is made for each determination in order to obtain comparable results.

The suspensions of bacterial cells, which are used in the pigment determination, are prepared as follows. Agar slants of the test media are inoculated in a uniform manner and incubated at the temperature and for the time desired. For most of our work 20°C and 7 days incubation were used. At the end of the incubation period, the cells are scraped off gently into distilled water. The suspension is shaken and then filtered through several layers of cheese-cloth to obtain homogeneity. The estimation of pigment is finally made on the bacterial suspensions prepared as explained.

The method described in this paper is simple and rapid. Reproducible values are obtainable if reasonable care is exercised in preparing the suspensions of bacteria and in making the colorimeter readings. The method is being applied in this laboratory to studies of the effect of physical and chemical factors on the production of pigments by bacteria. Results will be published in subsequent papers.

SUMMARY AND CONCLUSIONS

A method is described for measuring the comparative amounts of pigment produced by bacteria under various environmental conditions. A spectrophotometer is used to secure the complete absorption spectra data for each pigment. These data are then applied in the use of a photoelectric colorimeter for the measurement of the pigment in a bacterial suspension. One filter is chosen which transmits the light waves which are unabsorbed by the bacterial pigment; a second filter transmits the light
which is affected most by the pigment. The colorimeter reading with the first filter represents turbidity and with the second filter, turbidity plus pigment. Calculations are made to obtain the photometric density due to pigment for a standard turbidity.

By this method varying amounts of growth do not confuse the amount or intensity of pigment because the values are adjusted to a standard turbidity.

The method is simple and rapid. Reproducible values are obtained if reasonable care is exercised. Much greater accuracy is obtainable than by the method of gross visual observation.

REFERENCES


