CXL. ON THE PIGMENT PRODUCED BY *CHROMOBACTERIUM VIOLACEUM*.

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THE bacterial pigments in general have received very little attention and any systematic investigations in this field have been confined mainly to the pigments produced by pathogenic organisms such as *Bacillus pyocyaneus*. The small amount of pigment formed in most cases is no doubt responsible for this. The extraordinarily intense development of pigment produced in a sample of milk through the growth of *Chromobacterium violaceum*, to which our attention was directed, suggested that this was a pigment which could be produced in sufficiently large quantities for examination, and it was decided to undertake the investigation, particularly as no detailed chemical examination seemed to have been made previously. The accounts of violet bacterial pigments met with in the literature are very fragmentary and somewhat confusing, and deal mainly with their absorption spectra. Lecoq de Boisbaudran [1882] extracted a violet pigment produced by a bacterium, which he does not describe, when grown on starch paste, and records its absorption spectrum. Similar colouring matters obtained from *B. violaceus* and *B. janthinus* are described by Schneider [1895]. The pigment from *B. violaceus* was submitted to a spectroscopic analysis by Hartley [1913], and was considered by him to differ from those described by the authors mentioned inasmuch as it showed more marked absorption in the red. It is possible, however, that the pigment in all these cases was the same—for the earlier spectroscopic observations of Lecoq de Boisbaudran and Schneider were probably not exact enough for the differences to be significant—while the qualitative chemical reactions ascribed to the pigment by all these authors are in fair agreement. The organism producing it is now commonly described as *Chromobacterium violaceum* (Bergey, *Manual of Determinative Bacteriology*). Hartley prepared the pigment by extraction of slices of potato, on which the bacterium grows well, with absolute alcohol, followed by evaporation to dryness. The material obtained in this way can hardly have been very pure, but probably sufficed for the qualitative tests employed and for spectroscopic examination in the absence of any strongly coloured impurities.

For the present work the pigment has been obtained mainly by growth in a lactose broth medium. Isolation and purification presented some

difficulties as the colouring matter is very unstable in alkaline solution in which it dissolves readily, and is not very soluble in organic solvents. An analysis of the material purified as carefully as possible, in the circumstances, gave as a first approximation a formula of $(C_{10}H_{12}O_3N)_n$. Approximately one-fifth of the total nitrogen is removed by the action of nitrous acid, pointing to a value $n = 5$ (or some multiple of five). This was confirmed by a determination of the molecular weight of the substance by a modification of Rast's camphor method devised for the purpose. The value obtained, $M = 917$, agrees fairly well with that required for $n = 5$.

Preparation and isolation of the pigment.

In the earliest stage of the work sterilised skim-milk was used as a medium for growth as it possessed the advantages of being cheap and of permitting abundant pigment formation. The difficulties met with in filtering this medium and in isolating the pigment in pure form from such a complex mixture led to its eventual replacement by the simpler nutrient lactose broth, made up as follows:

5 g. lactose,
5 g. peptone,
3 g. beef extract,
1 litre of water.

Six litres of the sterile broth contained in a number of flasks were inoculated with a pure culture of *C. violaceum* and kept for about three weeks at a temperature of 20° . The pigment, which is insoluble in water, is found partly in flakes at the bottom of the flasks and partly distributed as very fine particles throughout the medium. It was dissolved out at first by repeated extractions with a mixture of alcohol and ether but this method, though suitable for skim-milk, proved unnecessarily wasteful for lactose broth. In the end it was found simplest to filter off the colouring matter by pouring the culture medium on to large filter papers. Filtration was slow but could not be improved by the use of suction, as the papers became clogged. A stream line filter was also tried but was not successful for the same reason. As soon as all the medium had run through, the residue on the paper was washed with a little water, and allowed to dry in the air. The filter papers were then cut up and extracted with absolute alcohol in a Soxhlet until no more colouring matter appeared to dissolve out. As this process usually took a long time—10 to 12 hours—a "vacuum" Soxhlet was used, to prevent possible decomposition of the pigment by prolonged heating, *i.e.* the extractor was provided with a very long Liebig condenser connected to the water-pump. The temperature of the boiling alcohol in contact with the dissolved pigment could thus be kept below 50° and practically no alcohol was lost. After the removal of as much colouring matter as possible in this way, the filter papers were further extracted by boiling for a few minutes with successive portions of fresh alcohol. These extracts were

filtered, added to the Soxhlet extract, and the whole evaporated to small volume under reduced pressure. From this, the pigment was precipitated by the addition of 3–4 times its bulk of water, filtered under pressure, washed with water, dried on the pump and extracted several times with chloroform to remove traces of fatty substances, etc. The chloroform extract was always coloured greenish-brown, apparently by a second pigment present in small amounts. The residual pigment was now re-dissolved in alcohol, filtered, reprecipitated by addition of water, filtered off, dissolved again in alcohol and concentrated under reduced pressure until only a few cc. remained, and the greater part of the pigment had separated out. The solid was filtered off, dried in a vacuum desiccator and used for analyses and other estimations. A further supply of a presumably less pure material was obtained by the addition of water to the alcoholic filtrate. The total yield of pigment from 6 litres of lactose broth was about 60 mg.

Properties. The solid pigment is a purple-black amorphous substance which decomposes on heating without melting. It is insoluble in water, chloroform and other chlorinated solvents, light petroleum, carbon disulphide and acetic acid. It dissolves in ethyl alcohol to a beautiful bluish-violet solution, in ether (especially ether containing some alcohol), and in acetone to give purple solutions. Although these solutions are strongly coloured, the concentration of dissolved substance for saturation is low—probably not more than about 0.1 %. Pyridine—the best organic solvent observed—contained when saturated at ordinary temperature about 0.5 % of the pigment. Alkalis dissolved the pigment readily to green solutions, which, on heating, rapidly turned blood red, and then slowly passed through reddish brown shades to brown. Some structural change seems to occur on solution in alkali, as addition of acid does not restore the original colour.

Heating a pyridine solution of the pigment with acetic anhydride gave a red solution which was fairly stable to further heating. On the grounds that the pigment might contain a reactive NH_2 group, attempts were made to prepare derivatives by the action of formaldehyde and of toluene-*p*-sulphonyl chloride in pyridine solution respectively. No change was observable although in the latter case boiling was continued for several hours.

A number of tests were carried out on alcoholic solutions of the pigment. These appeared to be fairly stable to reducing agents, remaining unchanged when acted upon by sulphur dioxide, zinc and dilute sulphuric acid, and stannous chloride. Potassium permanganate instantly gave a brownish-yellow solution, while bromine water brought about immediate decoloration.

The action of nitrous acid on the pigment gives a reddish brown solution from which a flocculent brown precipitate, very difficult to filter, eventually settles out. After destruction of the excess of nitrous acid by urea and acidification with dilute sulphuric acid, the brown substance was extracted by *n*-butyl alcohol, washed free from acid with sodium bicarbonate solution, and isolated by removal of the alcohol. The product did not respond to the

Liebermann nitroso-reaction, so that the original pigment does not seem to possess a secondary amine group.

The pigment dissolves in concentrated nitric acid to give an orange solution which becomes more intense in colour on addition of alkali. It does not respond to Millon's test.

Analysis:

Found for pigment	Calc for $(C_{10}H_{12}O_3N)_n$	Calc. for $C_{50}H_{59}O_{15}N_5$
C = 62.07 %	C = 61.86 %	C = 61.92 %
C = 6.04 %	H = 6.18 %	H = 6.09 %
N = 7.14 %	N = 7.22 %	N = 7.23 %
O = 24.75 % (by diff.)	O = 24.74 %	O = 24.76 %

From the content of amino-nitrogen and the molecular weight it appears (as will be seen later) that $n = 5$ which would make the formula for the substance $C_{50}H_{60}O_{15}N_5$. The slightly differing formula, $C_{50}H_{59}O_{15}N_5$ is suggested for the pigment as it is in somewhat better agreement with the analytical data.

Action of nitrous acid on the pigment.

An attempt was made to determine the nitrogen liberated from the pigment by the action of nitrous acid in the Van Slyke micro-apparatus. Several difficulties presented themselves. The only solvents in which the pigment was sufficiently soluble to permit the use of amounts even as great as 10 mg. were pyridine and sodium hydroxide solution. The use of the former seemed seriously to affect the method even when a sufficient excess of acetic acid was subsequently introduced to neutralise it, giving low results and much slower evolution of nitrogen when added to a known solution of tyrosine. An alkaline solution of the pigment had therefore to be introduced, although it was almost certain that permanent chemical changes had been produced in the substance by solution in alkali. It was furthermore obvious that if only about one-fifth of the nitrogen could be removed by the action of nitrous acid (as appeared to be the case), the volume of nitrogen obtained from about 10 mg. of pigment would not much exceed 0.2 cc. In these circumstances it was of the utmost importance to see that the blanks were as small as possible. Blank estimations were carried out with a number of samples of sodium nitrite and found, however, to be surprisingly large, in all cases amounting to about 0.5 cc. for an evolution of 25 cc. of nitric oxide. Some values obtained from a sample of Kahlbaum's nitrite were, *e.g.* 0.46, 0.46, 0.59, 0.44, 0.48, 0.45 cc. It was hoped that the gas found in the blanks might be nitrous oxide, or at least contain a definite percentage of nitrous oxide, so that a gas analysis might be used to determine in an actual estimation how much of the total volume was due to the blank and how much to the substance under examination, but an analysis of the "blank" gas in a Bone and Wheeler apparatus proved it to consist entirely of nitrogen. Recrystallisation of the sodium nitrite led to a slightly lower value for the blanks, which were, however, still considerable.

At this stage it was found that Van Slyke [1913] had already remarked on this matter as follows: "The substance in the nitrite which gives the small amount of gas obtained on blank determination we have never been able to identify or remove...." To test the point further we prepared a solution of nitrite by the method recommended by Divers [1899], namely by passing the gases obtained by heating arsenious acid with nitric acid into sodium carbonate solution. The blank from a sample of nitrite prepared in this way was 0.25 cc. for a nitric oxide evolution of 35 cc. In view of these facts it seems possible that part at least of the blank is due to a secondary decomposition of nitrous acid with formation of nitrogen, and consequently cannot be avoided in these determinations. The procedure which had to be adopted finally was to make a determination of amino-nitrogen and follow it immediately by a blank estimation, taking care that the volume of nitric oxide evolved in the two cases should be exactly the same. No great accuracy could be expected for the result in view of the relative proportions of blank and estimation, so that the agreement found for duplicates below is very satisfactory.

(a) 8.725 mg. pigment dissolved by warming with 3 cc. *N* NaOH and introduced into the reaction vessel.

Shaken, allowed to stand for a half-hour, and then shaken vigorously for five minutes.

Vol. of moist N = 0.66 cc.; vol. of blank N = 0.46 cc. Temp. 16° Bar. 770 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 19.0 \%$$

(b) 8.255 mg. pigment. Vol. of moist N = 0.352 cc. Blank = 0.17 cc. Temp. 16°. Bar. 767 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 18.2 \%$$

These values indicate that approximately one-fifth of the total nitrogen is removable by the action of nitrous acid.

Molecular weight. The first attempts to determine the molecular weight of the pigment were made with the Pregl ebullioscopic apparatus, using alcohol as solvent. Although the solution was very deeply coloured, no appreciable rise of boiling point could be detected on addition of the pigment on account of its low solubility and apparently high molecular weight. Acetone, and methyl alcohol, both of which dissolve the pigment to give highly coloured solutions, were also tried as solvents but with as little success. An attempt to use pyridine had to be abandoned on account of the impossibility of obtaining a steady temperature. Fortunately the pigment showed slight solubility in camphor, though only sufficient to give a freezing point depression of about 0.5° by the ordinary Rast method for molecular weight determination. A modification of this method was, however, devised by the authors (and is elsewhere described [1927]), which allowed of the accurate determination of molecular weights from such small depressions of freezing point. The following estimations have been made by its use.

(a) 3.77 mg. pigment in 434 mg. camphor. $\Delta = 0.38^\circ$.

$M = 923$.

(b) 3.14 mg. pigment in 348 mg. camphor. $\Delta = 0.40^\circ$.

$M = 910$. Mean value $M = 917$.

$(C_{10}H_{12}O_3N)_4$ requires $M = 776$.

$C_{50}H_{59}O_{15}N_5$ requires $M = 969$.

The molecular weights found point to the latter formula. Although the results are somewhat low, this is not unnatural in view of the very great influence of traces of simpler impurities on the freezing point, and the unavoidable inclusion in the weight of the solvent of camphor on the sides of the containing vessel.

SUMMARY.

The pigment produced by *Chromobacterium violaceum* has been isolated and examined. Determinations have been made of the empirical formula, molecular weight, and nitrogen removable by the action of nitrous acid. The formula $C_{50}H_{59}O_{15}N_5$ is provisionally suggested for the pigment.

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