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A practical method for studying the reversible phases of pyocyanin

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INTRODUCTION

During the author's residence in England, a series of investigations were made into the possibility of applying a method of paper chromatography to the pigments from a number of chromogenic bacteria. The original method was described for the separation of intracellular respiratory pigments from the leaves of Angiosperms (Conroy 1959), and the technique was slightly modified for the extraction of pyocyanin from *Psudomonas aeruginosa*. The results of the work with bacterial carotenoid pigments, e. g. *Rhodococcus*, etc., will be described in detail in due course.

By means of the method outlined herein, it was possible to study the different phases of the oxidation process by which pyocyanin is converted to pyoxanthose, as originally described by Boland. This worker discovered that a solution of pyocyanin in chloroform developed a yellow coloration when exposed to direct sunlight. He believed the chloroform to be broken down to produce free chlorine, the action of which oxidises the pyocyanin to pyoxanthose. When hydrochloric acid is added to a culture of *Ps. aeruginosa* in which pyocyanin is present, a red colour is formed. Similarly, the addition of dilute (33%) sulphuric acid to an oxidised solution produces a reddish-yellow coloration. This

work was further confirmed by Wrede (1930), who found in addition that a red coloured salt is formed by the action of several acids upon pyocyanin. These findings were borne in mind in the current work.

EXPERIMENTAL METHODS

Isolates of *Pseudomonas aeruginoso* were obtained from the Laboratory type culture collection, labelled as W. A./1 - W. A./12. They were first sub-cultured into nutrient broth, from which they were streaked onto nutrient agar slopes and incubated at 25° C. When an appreciable amount of pigment had developed, the cultures were tested with chloroform, phenol, and 20 % hydrochloric acid, to verify personally the reactions described by earlier workers.

In the test, 2.5 ml. chloroform were added to each slope culture, and the whole gently agitated and set aside until the pigment had been dissolved. Strips of Whatman No. 1. filter paper measuring 2 cm. × 8 cm. were cut, and placed individually in the tubes. The cotton wool plug was replaced to prevent excessive evaporation.

When the solution had travelled approximately 2/3 rds. of the distance up the filter paper, the latter was carefully removed with a pair of fine forceps, and quickly dried in the air. Immediately upon drying, it was placed in a quantity of acetone. The acetone gradually ascended up the paper, carrying with it the blue coloured pyocyanin.

It was noted that the colour of the pyocyanin gradually changed to yellow, through several intermediates of blue-green and yellow-green. The final colouration given when the acetone had ascended to the maximum height, was yellow. The colour was retained for some minutes after drying, when it gradually became more faint.

To determine whether this substance was pyoxanthose, the paper was finally placed in a little 20 % hydrochloric acid, which was similarly allowed to travel up it. On reaching the pigment band, the yellow colour rapidly turned to reddish-yellow. The same reaction was seen to occur if the paper was placed over the bottle mouth of a container of fuming hydrochloric acid.

DISCUSSION

By the method described, it has been possible to study the oxidation of pyocyanin to pyoxanthose by chemical means. The work of previous authors has been verified in connection with the reactions given to chemical substances.

Pyocyanin is a naturally occurring phenazine derivative produced by the greater majority of strains of *Ps. aeruginosa*. The fact that pyocyanin is an extra-cellular pigment is shown by the rapidity of its diffusion into the surrounding medium. It is a substance capable of acting in a hydrogen carrying role in certain of the dehydrogenase systems, although *Ps. aeruginosa* has been shown to possess the cytochromes a, b, and c.

Ferramola and Monteverde (1939), in their studies of strains of *Ps. aeruginosa* occuring in water, found that the organism may produce a blue or blue-green pigment, which is soluble in chloroform. They consider temperatures above 42° C. to inhibit completely all chromogenesis. The method used by these workers to obtain pyocyanin in the pure state, was by the preliminary alkalisation of the culture by dilute ammonium hydroxide, followed by extraction with chloroform. If the solution is subsequently washed with dilute hydrochloric acid, an intense rose colour is formed. Further alkalisation, and extraction with chloroform, gives a pure solution of pyocyanin.

During the course of the investigations herein described, no studies have been effected on the reversability of this reaction, but Ferramola and Monteverde have shown that this can occur by alternate alkalisation and acidification of the solution. Gale (1951) regards this reversión from the reduced to the oxidised state, and vice versa, to be an established fact. The conclusions drawn from the current work are in agreement, and it is suggested that the process may be expressed as a reversible reaction, taking the following pattern:

PYOCYANIN

(intermediate substance A)

PYOXANTHOSE

Associated with pyocyanin is a second extra-cellular pigment known as fluorescin. This substance is insoluble in chloroform, although it is soluble in water. With acid it is colourless. Further work is being continued to determine whether this may possibly be the intermediate substance A shown in the scheme above. If this line of investigation is fruitful, it may be permissible to assume that those strains of *Ps. aeruginosa* in which the pigment pyocyanin has not been recorded, may merely have lost their original pyogenic properties, and are therefore unable to complete the full reversal between the oxidised and the reduced states.

Whilst the technique used in this work has little value in accurate experimental work, it may be of interest to University demonstrators wishing to illustrate the changes occurring in the reduction-oxidation system of *Ps. aeroginosa*

REFERENCES

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3) Ferramola, R., and Monteverde, J. J.: «Organismos del género *Pseudomonas* en las aguas del país», Boletín de Obras Sanitarias de la Nación, 27, pp. 272-285 (1939).

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SUMMARY

Cultures of *Pseudomonas aeruginosa*, are incubated in agar Slopes at 25º. C. 2,5 ml. choloroform are added to each tube to dissolve the pigment and Strips of Whatman N.º 1 filter paper are entroduced, letting the solution travel up the Strips. The latter are taken out and, being quickly dried in the air, are put into acetone which rises, carrying with it the blue pigment which gradually turn yellow, through bluegreen and yellow-green. To test if it is pyoxanhose, the strips are put into a 20 %. Hydrochloric acid solution, this being an easy and very illustrative method.

SUMARIO

Cultivos de *Pseudomonas aeruginosas* en agar inclinado a 25º C. Añade a cada tubo cloroformo para disolver el pigmento e introduce tiras de papel Whatman núm. 1 dejando subir por ellas la solución: secado al aire. Introduce la tira en acetona y la deja que ascienda, arrastrando el pigmento azul. el cual cambia al amarillo, pasando por verde azulado y verde amarillento. Comprueba que se trata de Pioxantosa por el color rojo que toman las tiras del papel con a. clorhídrico al 20 %. Método fácil y muy demostrativo.