XXV. BACTERIOLOGICAL AND BIOCHEMICAL RELATIONSHIPS IN THE *PYOCYANEUS-FLUORESCENS* GROUP

II. INVESTIGATIONS ON THE GREEN FLUORESCENT PIGMENT

By GEORGE EDGAR TURFITT

From the Department of Biochemistry, University College, Nottingham

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In a previous paper [Turfitt, 1936] an attempt was made to justify the classification of the *pyocyaneus-fluorescens* group of bacteria on the basis of their distinctive property of producing a diffusible, green fluorescent pigment in the culture medium. It was shown that this chromogenic character was remarkably constant on certain specified media, and further, that the fluorescent organisms were very closely related both morphologically and culturally.

The isolation of the fluorescent pigment is a problem of very considerable difficulty as a result, not only of the very minute amounts of the colouring matter present in the culture, but also of the nature of the compound itself. The present publication describes the most successful of the many methods investigated to effect this isolation and includes a comparative account of the pigments from various fluorescent organisms.

Experimental

Culture of the fluorescent bacteria

The organisms employed were stock cultures of *B. pyocyaneus*, *B. fluorescens liquefaciens*, and *B. fluorescens non-liquefaciens* obtained from the National Collection of Type Cultures, Lister Institute, London (Catalogue Nos. 1999, 964, 3247 respectively) and in addition certain selected strains isolated from nature, viz. No. 3 from canal water; No. 37 from human faeces; No. 69 from uncultivated soil; No. 81 from snow. In each case, growth on ordinary nutrient media was accompanied by the diffusion of a green or greenish blue fluorescence throughout the culture.

On account of the insoluble nature of the pigments in all ordinary organic solvents it was decided, for ease of manipulation in extraction, to employ a liquid medium for the mass cultures, and preferably one of a definite chemical composition in order that identical conditions might be repeated.

(1) Medium for *B. pyocyaneus* strains (stock *B. pyocyaneus*, and strains Nos. 3, 37):

NH_4NO_3	0·1 %
K_2HPO_4	0·025 %
$MgSO_4, 7H_2O$	0·025 %
EtOH	0·1 %
Distilled water	—

The medium was sterilized by steaming in a Koch sterilizer at 100° during 1 hour on each of 3 successive days, the alcohol (freshly boiled) being added subse-

quently by means of a sterile pipette. (*B. fluorescens* strains were devoid of colour when cultured upon this medium.)

(2) Medium for *B. fluorescens* strains (Stock *B. fluorescens*, and strains Nos. 69, 81):

Asparagine	...	...	0·3 %
K_2HPO_4 ...	...	...	0·05 %
$MgSO_4, 7H_2O$	...	...	0·05 %
Distilled water	...	...	—

Sterilization was carried out in this case by autoclaving for 20 min. at 110°. (*B. pyocyaneus* strains, when cultured on this medium, gave good yields of fluorescent pigment, but very occasionally there was a slight tendency for traces of pyocyanine to be formed.)

Mass cultures of each organism were prepared in 32 l. quantities, the medium being contained in eight 5 l. flat-bottomed flasks. After inoculation from 24-hour agar slants, the cultures were incubated at 25° for a period of 15 days. At the end of this time the medium showed a green fluorescence and a cloudiness due to bacterial growth.

Extraction of pigment

Although the ultimate object of these experiments, the determination of the identity or otherwise of the green pigments produced by the *pyocyaneus-fluorescens* bacteria, has been satisfactorily achieved, the insolubility of the pigments in all organic solvents tried (with the exception of phenol and acetic acid, in which they dissolve as readily as in water) rendered their isolation in a state of purity a matter of extreme difficulty.

The very minute amounts of colouring matter present in the media necessitated, as a preliminary to the actual isolation process, the use of some method of concentration in order to obviate working with inconveniently bulky solutions. Adsorption of the pigment upon some suitable medium, followed by its subsequent elution therefrom, seemed an attractive process for the concentration, and a considerable amount of work has been done with a view to determining the most satisfactory adsorbent (Table I). In each case 50 ml. samples of culture media were treated with 2 g. of adsorbent and rapidly stirred for 30 min. at a temperature of 20°.

Table I. *Adsorption experiments*

Adsorbent	*p*H	Adsorption	Eluted by
Alumina	4·0	Nil	—
	10·0	,,	—
Silica gel	4·0	,,	—
	10·0	,,	—
Kaolin	4·0	Considerable	Alkali, phenol, acetic acid
	10·0	Nil	—
Norite (HCl purified)	4·0	Considerable	Phenol, acetic acid
	10·0	Nil	—
Blood charcoal (B.D.H.)	4·0	Complete	Phenol, acetic acid
	10·0	,,	,,
"Suma-Carb"	4·0	,,	,,
	10·0	,,	,,

Activation of the adsorbents (by heating *in vacuo* in a quartz flask at 500° during 2 hours) increased the adsorptive power in all cases with the exception of "Suma-Carb".

From Table I it is clear that charcoal has the highest adsorptive capacity of the substances tried, and extensive use has been made of both activated B.D.H. blood charcoal and "Suma-Carb". Adsorption was in general carried out from alkaline solution, because the completion of the process was then readily observed by the disappearance of the green colour.

Earlier work on the isolation was based solely upon the elution of the pigment from the charged adsorbent by phenol and by glacial acetic acid. Unfortunately, however, such experiments suffered from the common defect that the products invariably contained a considerable, though variable, percentage of inorganic matter.

A projected attempt to effect a purification by direct electrodialysis of the contaminating salts through cellophane membranes was frustrated by the fact that the pigment itself migrated, under the influence of the electric current, to both anode and cathode compartments. In the method finally adopted (Table II) this difficulty is surmounted by performing the electrodialysis on the entire adsorbate; under these conditions, the pigment is retained upon the charcoal whilst the ionized saline impurities migrate towards the electrodes.

Table II

CULTURE

Rendered alkaline with ammonia, and "Suma-Carb" added; allowed to settle. Decanted; filtered.

FILTRATE discarded

CHARCOAL

Washed; extracted repeatedly with warm dilute HCl until washings phosphate-free. HCl washed out with distilled water. Charcoal suspended in water and subjected to electrodialysis. Suspension filtered; charcoal dried and thoroughly extracted with acetic acid. Solvent removed by distillation *in vacuo* and solid finely ground and dried.

PIGMENT

The selection of "Suma-Carb" as the adsorbent in this process was decided on two main considerations, viz. its exceedingly high adsorbent properties, and the ease with which the spacing agent (chiefly $Ca_3(PO_4)_2$) could be removed, after adsorption of the pigment, by simply washing with dilute HCl. It was found that if electrodialysis was attempted without this previous removal of the spacing agent, the process was inconveniently prolonged. On the other hand, removal by means of HCl prior to the adsorption caused a very marked reduction in the adsorptive capacity of the charcoal.

The dialyses were conducted in a wax-impregnated wooden cell with compartments each measuring $12{\cdot}5 \times 10 \times 7{\cdot}5$ cm., separated by membranes of cellophane, No. 400. The outer compartments, containing the platinum foil electrodes each of dimensions 7×7 cm., were maintained by the use of cooling coils at a temperature of 22°. The charcoal suspension in the centre compartment was stirred mechanically throughout the experiments.

During the course of the dialysis the current, which initially was 10 mA., rose gradually to 450 mA. After remaining almost constant at this value for 3·5 hours, there was a very gradual decrease down to 90 mA. over a period of 2 days. This stage seemed to represent the limit of purification possible by the method, since further passage of the current over a considerable period of time did not cause any reduction below 90 mA.

The above process constitutes a great advance over previous methods, not only in increased yield (0·78–0·85 g. per 32 l.), but also in simplicity of operation. Although, admittedly, the ash content of the product is not reduced to zero, it is nevertheless brought to a very low order. Quantitative tests on the ash have shown it to consist almost entirely of $CaCO_3$, so that the actual metal content of the product is practically negligible.

Comparison of the pigments

Thumm [1894] is reported [Lehmann & Neumann, 1931] to have described various properties of the pigments and to have concluded that the identical colouring matter was produced by all the common green fluorescent organisms. The proof or disproof of this statement by the use of more exact methods has been the main object of the present work.

As prepared by the author, the various green pigments possess identical general properties. They are amorphous, greenish brown powders, readily soluble in water, phenol and acetic acid, but insoluble in all other organic solvents tried. Sullivan [1905] has stated that the green pigment is insoluble in chloroform, but dissolves in alcohol and ether. This, however, is undoubtedly erroneous, although solution of the colouring matter occurs in dilute aqueous alcohol.

A dilute aqueous alkaline solution shows a green fluorescence, but on acidification there is obtained a colourless, non-fluorescent solution. More concentrated alkaline solutions, however, have a red colour whilst at the same time exhibiting an intense green fluorescence. A similar red colour is observed in ageing agar slant cultures of all *pyocyaneus-fluorescens* organisms, and in this connexion the report of Meader *et al.* [1925] on the red colouring matter, "pyorubrin", is of considerable interest. This substance, they affirm, occurs in all cultures of *B. pyocyaneus*, whether the strains are capable of producing the blue pyocyanine or not, and they regard it as being as characteristic of *B. pyocyaneus* as pyocyanine itself. They further remark that the "pyorubrin" in ageing cultures containing pyocyanine is of a dull red shade and never shows the brilliant red obtained in non-pyocyanigenic strains. This substance is considered to be a definite pigment, distinct from the green fluorescent colouring matter, but they state that they were unable to separate them one from the other. It seems, indeed, that all the evidence advanced by these workers, in support of their claim for a distinct colouring matter, can actually be explained on the assumption that the red colour is due merely to an increased concentration of green fluorescent pigment. The dull red shade encountered in cultures containing pyocyanine is probably due to the concentrated fluorescent pigment together with some modified form of pyocyanine produced perhaps by oxidation processes. Gessard [1917], Mamelle [1918] and others, describe strains of *B. pyocyaneus* (var. *erythrogenes*) which produce a brilliant red pigment, but the nature of the colouring matters of these exceptional strains has as yet not been investigated.

On distillation of the dry pigments with KOH or zinc dust, there is a copious evolution of ammonia, and a colourless aqueous distillate is obtained, followed by red-brown oily drops soluble in alcohol and ether and possessing a strongly basic odour.

In view of the probable amphoteric nature of the compounds, attempts have been made to obtain insoluble heavy metal salts; although darkening of the solutions occurred on adding silver nitrate, ferric chloride, mercuric chloride etc., no precipitates were obtained. Attempts to prepare picrates and platinichlorides have likewise been unsuccessful.

With Millon's reagent, an immediate positive reaction was obtained in the cold, suggesting that a free *p*-hydroxyphenyl grouping may be present.

The majority of workers on the fluorescent pigments [Jordan, 1899; Meader *et al.* 1925; etc.] have stated that the green substance, like pyocyanine, is an oxidation product of a colourless leuco-base. An observation advanced in support of this theory is the initial production of green colour at the surfaces of cultures, where atmospheric oxidation can first occur; on disturbing the medium, the colour diffuses throughout the culture. Like Thumm [1894], however, the author can find no evidence for leuco-compounds. It seems probable that *B. pyocyaneus*, which is normally aerobic, would carry on its metabolic processes with more readiness at the surface where there was a plentiful oxygen supply, than in the depths of the culture where it would function as a facultative anaerobe. The ammonia produced at the surface by these processes would thus cause the development of the green colour. The following experiment demonstrates the correctness of this view. A 50 ml. quantity of colourless liquid medium was obtained by means of a pipette from the bottom of a 4-day EtOH culture of *B. pyocyaneus*, which had a definite green zone near the surface; this liquid was carefully transferred in equal 25 ml. quantities to each of two 50 ml. beakers. According to the leuco-base theory, on addition of 1 ml. hydrogen peroxide to one of the beakers, a green colour would be expected, whereas actually the liquid remained colourless. To the contents of the other beaker a little dilute ammonia was added; a green fluorescence was immediately obtained.

Samples of the pigments have been gently refluxed during many hours with zinc and HCl but no loss of colour has been observed; on rendering alkaline the fluorescence at once returned.

The name "bacteriofluorescein" has been proposed for the green pigment [Lehmann & Neumann, 1931]. Whilst this is, in the author's opinion, inappropriate in that it suggests a resemblance to ordinary fluorescein which is more apparent than real, it is not proposed to suggest an alternative until more light has been thrown upon the chemical constitution.

A. *Analyses.* The green pigments isolated by the dialysis procedure from cultures of the stock strains of *B. pyocyaneus*, *B. fluorescens liquefaciens* results and *B. fluorescens non-liquefaciens* gave closely comparable analytical (Table III). In each case, the data would appear to indicate the empirical

Table III. *Fluorescent pigments from stock strains*

	B. pyocyaneus %	*B. fluorescens liq.* %	*B. fluorescens non-liq.* %
C	48·94	49·40	49·01
H	7·02	7·38	7·15
N	13·49	13·16	13·33
Ash ($CaCO_3$)	0·43	0·48	0·39
Ca	0·17	0·19	0·16
O	30·38	29·87	30·35*

Fluorescent pigments from natural strains

Strain no.	Source	N (%)	Ash (%)
3	Canal water	13·46	0·76
37	Faeces (G.E.T.)	13·30	0·69
69	Soil	13·36	0·70
81	Snow	13·34	0·91

* Oxygen values calculated using Ca, not ash, percentages.

formula $C_4H_7O_2N$. Owing to the completely insoluble nature of the pigments in the solvents ordinarily employed for molecular weight determinations by the freezing point depression method (camphor, dioxan, etc.), this estimation was not attempted, especially as for the present purpose comparison of the empirical formulae was sufficient in itself.

In addition to the stock cultures, various strains from nature were studied, isolation of the pigment in these cases being effected by an adsorption-elution process, referred to above as one of the earlier isolation methods. Combustions were not carried out on these products, but estimations of N by the micro-Kjeldahl method gave almost identical results; as will be seen from Table III, the ash contents were considerably higher than those of pigments obtained by the dialysis technique.

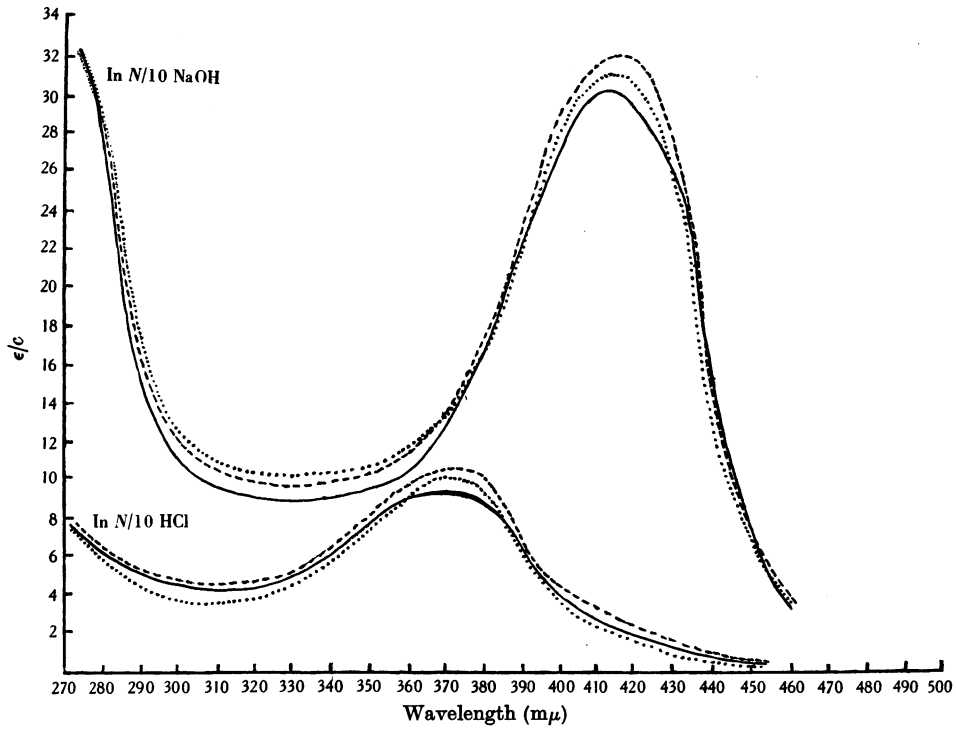

Fig. 1. --- *B. pyocyaneus.* —— *B. fluorescens liquefaciens.* ··· *B. fluorescens non-liquefaciens.* ϵ = extinction coefficient. c = concentration (g./100 ml.).

B. *Absorption spectra.* Quantitative spectrophotometric examinations of the pigments isolated from the three stock strains have furnished absorption curves which, for all practical purposes, are identical (Fig. 1). The very slight differences in the curves may be due to small variations in the inorganic content as shown by the ash values.

The data were obtained using a Hilger All-metal Quartz Spectrograph (E 316) in conjunction with a Hilger Spekker Photometer; the light source consisted of a condensed spark between tungsten-steel electrodes.

The concentrations of the solutions were of the order of 0·001 %, and to give a complete comparison of the pigments absorption measurements were made on

both acid and alkaline solutions. Acidification had the effect of shifting the absorption maximum towards the lower wave-lengths.

A previous investigation of the ultraviolet absorption spectrum of the green pigment of *B. pyocyaneus* is described by Cluzet *et al.* [1921]. These workers isolate their product by filtering a broth culture of a non-pyocyanigenic strain through a Chamberland candle and evaporating the filtrate to dryness. They record a large band extending from 358·0 to 425·0 mμ, with its centre at 391·5 mμ.

Discussion

In view of the evidence advanced above, there can be no reasonable doubt as to the identical nature of the green fluorescent pigments of the *pyocyaneus-fluorescens* organisms. Attempts to ascertain the chemical constitution of the substance have been hindered by the extremely small amounts available. The introduction of the dialysis isolation process should, however, do much to overcome this difficulty.

Summary

1. Methods are described for the isolation of the green fluorescent pigment of the *pyocyaneus-fluorescens* bacteria; although complete purification from inorganic salts has not been effected, the purest specimens give an ash value ($CaCO_3$) of less than 0·4 %.

2. Considerations of properties and analytical and spectrophotometric data point to the identical nature of the green pigment from the various organisms of the group selected at random for these experiments.

3. The empirical formula of the pigment is found to be $C_4H_7O_2N$.

4. A well-defined band with absorption maximum 410 mμ is obtained in alkaline solution; on acidification of the solution the band becomes less marked (maximum 370 mμ), and is shifted towards the shorter wavelengths.

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