

INFLUENCE OF THE CONCENTRATION OF IRON ON THE PRODUCTION OF FLUORESCIN BY PSEUDOMONAS AERUGINOSA¹

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Numerous studies have been made to determine the specific stimuli for pigment production in *Pseudomonas aeruginosa* and related species, but the process still appears to be incompletely understood (Sullivan, 1905; Young, 1947; King *et al.*, 1948).

Since pyocyanine is thought to be a respiratory pigment (Friedheim, 1931), it seemed likely that it or fluorescin, a closely related substance, might appear as a partial substitute for iron containing cytochrome and related systems when some precursor of the latter compounds is limiting. We have studied quantitatively the relation of the concentration of iron in the medium to the production of fluorescent pigment by *P. aeruginosa*. Preliminary observations have been extended also to *P. fluorescens* which behaved in a manner very similar to *P. aeruginosa*.

A reciprocal relation between available iron and fluorescent pigment was found, and use was made of the observation to study possible relationships of several bacterial growth inhibitors to the metabolism of iron. Cultures in broth containing chloromycetin, streptomycin, penicillin, or 8-hydroxyquinoline together with sufficient iron to suppress the formation of pigment were compared with standard cultures containing graded levels of iron under pH conditions in which the fluorescent pigment is readily visible.

King and co-workers (1948) have studied mineral requirements for the production of fluorescin using *P. aeruginosa*. They also found a relation of iron content to the production of fluorescin, but some of our results are not fully in agreement with theirs.

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EXPERIMENTAL METHODS

The organism used was *Pseudomonas aeruginosa*, strain A.T.C.C. 10145. Two basal media were employed: one contained asparagine, 0.5 per cent; MgSO₄, 0.01 per cent; K₂HPO₄, 0.05 per cent. The other consisted of glycerol, 1 per cent; (NH₄)₂SO₄, 0.1 per cent; K₂HPO₄, 0.4 per cent; and MgSO₄·7H₂O, 0.1 per cent. The procedure of Waring and Werkman (1942) was followed for the preparation of the iron low media, water, and glassware. Thirty ml quantities of media in 125 ml Erlenmeyer flasks were autoclaved at 15 lb for 15 minutes. Where required, a ferrous sulfate solution sterilized by filtration through a Seitz EK filter pad was added aseptically to the autoclaved media. The final volume was the same in all flasks. After inoculation the flasks were incubated at 32 C for 48 hours. Growth was determined by making turbidity measurements with a Coleman Universal spectrophotometer. Interference from the fluorescent pigment was found to be unimportant at 650 m μ wavelength. Fluorescence was measured with the same instrument using the ultraviolet attachment. Comparison of an appropriate dilution of supernatant medium was made with a solution of 0.075 mg quinine bisulfate in 10 ml of water.

RESULTS AND DISCUSSION

The results indicate that the production of fluorescin in the media employed is related inversely to the logarithm of the concentration of iron over a rather wide range. Table 1 gives a summary of typical findings. Of particular interest is the fact that good production of fluorescin was obtained on a glycerol-ammonium sulfate medium. King *et al.* (1948) found no fluorescent pigment with a similar medium. Two possible explanations for this difference suggest themselves. It was noted that at pH 6 to 7 fluorescence was no longer visible and might be overlooked if

examination was not made by ultraviolet light. Alternatively, if extreme precautions are not used to remove all iron contamination, there may be sufficient available iron, at a pH below 7, to pre-

TABLE 1
Influence of the Fe⁺⁺ content of media on "fluorescin" production by Pseudomonas aeruginosa

ASPARAGINE FINAL pH 8.3 TO 8.5			GLYCEROL FINAL pH 6.0 TO 6.6	
Fe ⁺⁺ added mg/liter	Growth (O.D.)*	Relative flu- orescence†	Growth (O.D.)	Relative fluorescence
0.0	0.56	300	4.5	370
0.023	0.62	340	4.8	290
0.050	0.62	270	5.0	240
0.100	0.64	210	4.8	165
0.167	0.62	110	5.1	110
0.667	0.98	70	5.8	10
1.667	0.98	20	5.8	5

* Optical density

† 0.075 mg quinine bisulfate in 10 ml = 100.

TABLE 2
Influence of bacterial growth inhibitors on fluorescin production by Pseudomonas aeruginosa in presence of 1.667 mg Fe⁺⁺/liter asparagine medium

SUBSTANCE ADDED TO 30 ML MEDIUM	GROWTH (O.D.)	RELATIVE FLUORESCENCE
None	0.85	6.5
Chloromycetin 1 mg	1.0	6.1
Streptomycin 1 mg	0.96	9.5
Penicillin 1,000 units	0.49	31
500 units	0.67	24
200 units	0.66	17
8-Hydroxyquinoline 0.052-5.2 mg	0.9-0.1	<10

vent pigment formation. At a pH above 7 the iron, if oxidized, presumably would be removed by precipitation as Fe(OH)₃ and not interfere with the formation of pigment. The pH of the ammonium succinate and similar media of King

and co-workers probably rose above pH 7 as did the asparagine medium in the present work. Therefore there would be a copious visible production of pigment under conditions of iron concentration which might suppress production with higher acidities. On the other hand, with a glycerol medium the pH at 48 hours was between 6.0 and 7.0 and the fluorescent pigment was not visible, although present.

The glycerol medium proved capable of supporting much greater growth in 48 hours than did the asparagine medium although the total production of pigment was approximately the same in both media.

From the evidence presented it appears likely that the main factor involved in the accumulation of fluorescin and, as shown by Burton *et al.* (1948), of pyocyanine in pseudomonad cultures is the concentration of iron and its availability to the organism.

The effects of chloromycetin, streptomycin, penicillin, and 8-hydroxyquinoline are shown in table 2. Penicillin was the only one of these to increase the production of fluorescin in the presence of excess Fe⁺⁺. This may indicate that penicillin exerts its effect on *P. aeruginosa* by interfering with an iron containing enzyme or enzymes. Whether a similar effect is exerted on other organisms is not certain. At the very high concentrations used in the present study penicillin may influence different enzymes from those affected in other organisms which are inhibited at lower concentrations.

Surprisingly 8-hydroxyquinoline, an iron chelating substance, did not increase the production of fluorescin. It must be concluded that this material produces inhibition by some mechanism other than that of rendering iron unavailable for utilization by the organism. From this it may be inferred that the removal of 8-hydroxyquinoline inhibition by addition of a metal does not necessarily indicate the participation by the metal in a vital function.

SUMMARY

At a final pH between 6 and 8.5 production of fluorescin by *Pseudomonas aeruginosa* was found to be related inversely to the concentration of iron in the media.

Penicillin elicited production of fluorescin by *P. aeruginosa* in the presence of excess iron, whereas

chloromycetin, streptomycin, and 8-hydroxyquinoline in the concentrations tested failed to increase the production of pigment.

REFERENCES

- BURTON, M. O., CAMPBELL, J. J. R., AND EAGLES, B. A. 1948 The mineral requirements for pyocyanine production. *Can. J. Research*, **26C**, 15-22.
- FRIEDHEIM, E. A. H. 1931 Pyocyanine; an accessory respiratory enzyme. *J. Exptl. Med.*, **54**, 207-221.
- KING, J. V., CAMPBELL, J. J. R., AND EAGLES, B. A. 1948 The mineral requirements for fluorescin production. *Can. J. Research*, **26C**, 514-519.
- SULLIVAN, M. X. 1905 Synthetic culture media and the biochemistry of bacterial pigments. *J. Med. Research*, **14**, 109-160 (1905-1906).
- WARING, W. S., AND WERKMAN, C. H. 1942 Growth of bacteria in an iron free medium. *Arch. Biochem.*, **1**, 303-310.
- YOUNG, G. 1947 Pigment production and anti-biotic activity in cultures of *Pseudomonas aeruginosa*. *J. Bact.*, **54**, 109-117.