Pyocyanine Biosynthesis by *Pseudomonas aeruginosa*

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SUMMARY: The formation of pyocyanine by resting *Pseudomonas aeruginosa* was studied. Glutamic acid was found to be a good substrate for this synthesis and could be replaced by several related amino acids (γ -aminobutyric acid, pyrrolidone-carboxylic acid, glutamine, aspartic acid, proline, hydroxyproline, arginine, histidine, alanine), and by some organic acids active in the tricarboxylic acid cycle (succinate, fumarate, pyruvate) when added together with ammonium ions. Magnesium ions were essential for pyocyanine synthesis and could not be replaced by other metals tested (Co, Fe). Pyocyanine formation was inhibited specifically by respiratory poisons (KCN, NaN_3); NaF, on the other hand, was not inhibitory.

Pyocyanine, the blue pigment produced by *Pseudomonas aeruginosa*, is known to possess antibiotic activity especially against the Gram-positive bacteria. Chemically, pyocyanine belongs to the phenazine group and is similar in structure to iodinine, a pigment produced by *Chromobacterium iodinum* (Clemo & Daglish, 1950), and to chlororaphin, a green pigment formed by *Pseudomonas chlororaphis* (Kögl & Postowsky, 1930). The synthesis of phenazine derivatives is, therefore, not uncommon among strictly aerobic micro-organisms.

Investigations published hitherto, including those reviewed in *Topley and Wilson's Principles* (1955), and those of Young (1947), Burton, Eagles & Campbell (1947), Burton, Campbell & Eagles (1948), and Hellinger (1951) deal with pyocyanine formation in growing cultures of *Pseudomonas aeruginosa*. The object of this investigation was to study pyocyanine formation in stationary cultures, and to determine the factors essential for its formation. A preliminary note was published previously (Halpern & Grossowicz, 1954).

METHODS

The organisms used in this work were 2 strains of *Pseudomonas aeruginosa* from the stock culture collection of this Department. The bacteria were grown in Roux bottles containing 120–150 ml. Difco Nutrient Agar and incubated at 37° for 24 hr. The organisms were taken up in saline, washed several times by centrifugation and resuspended in phosphate, borate, or 'Tris' (trihydroxy-methylaminomethane) buffers. The concentration of organisms was determined by measuring the turbidity of a suitably diluted suspension in a Coleman Junior Spectrophotometer.

Pyocyanine assay. The experiments on the formation of pyocyanine were carried out in unstoppered 12 × 75 mm. or 24 × 150 mm. test tubes containing the bacterial suspensions and appropriate substrates in a volume of 1 ml.; the pH value was adjusted to 7.8. The tubes were incubated statically at 37°. At

the end of the experiment the tubes were shaken vigorously by hand for about 2 min. in order to convert all the pigment to the oxidized (blue) state. The pyocyanine was then extracted from the reaction mixture with 2 ml. of chloroform and measured in the spectrophotometer at a wavelength of 675 m μ . A solution of crystalline pyocyanine 2 HCl (Nutritional Biochemicals Corp., Cleveland/Ohio, U.S.A.) made alkaline (pH 7.8) and similarly extracted with chloroform was used as a standard.

RESULTS

Amino acid requirements

Various amino acids were tested as possible substrates for pyocyanine synthesis. With glutamic acid as the sole carbon and nitrogen source, the yield of pigment was very good (100–250 μ g./ml.). However, even under optimal conditions not more than 2% of the glutamic acid was converted into pyocyanine, i.e. addition of 12 mg. glutamic acid to the system yielded 0.25 mg. of pyocyanine. This yield of pyocyanine was arbitrarily chosen as 100% to serve as a standard for the comparison of the amount of pyocyanine formed in the presence of other substrates. Pigment formation occurred also in the presence of alanine, arginine, aspartic acid, glutamine, histidine, hydroxyproline, proline, serine or threonine. No pyocyanine was formed in the presence of glycine, lysine, methionine, phenylalanine, tyrosine, tryptophane or valine. Asparagine, cysteine or leucine were adequate substrates for pyocyanine formation by only one of the two strains used (Table 1).

Since glutamic acid was found to be the best substrate for pyocyanine synthesis, compounds related to this amino acid were tested. Table 1 shows that pyocyanine formation in the presence of γ -aminobutyric acid was as great or slightly greater than in the presence of glutamic acid, while the yield of pigment in the presence of glutamine, proline, hydroxyproline or pyrrolidone-carboxylic (5-oxo-2-pyrrolidine carboxylic) acid was somewhat less. Of other amino acids known to be related metabolically to glutamic acid, ornithine proved better than arginine and citrulline. No pyocyanine was produced in the presence of lysine, but the decarboxylation product of lysine, cadaverine, produced 40% of the amount of pyocyanine which was formed in the presence of glutamic acid. The pyocyanine yield in the presence of aspartic acid was lower (20%) than that found in the presence of its decarboxylation products α - and β -alanine, which both yielded about 75%. Of the various aminobutyric acids only γ -aminobutyric acid was found to serve as an efficient substrate for pyocyanine synthesis (Table 1).

Carbon sources for pyocyanine formation

Succinate, fumarate, pyruvate and other organic acids were tested as sole sources of carbon in the presence of NH_4Cl as nitrogen source. Pyocyanine was formed in the presence of some of these substances, while others were inactive (Table 1). No pyocyanine was formed when glucose was used as the only source of carbon. Addition of malate inhibited pyocyanine production in presence of glutamate, succinate + NH_4^+ or fumarate + NH_4^+ .

Table 1. *Effect of various substrates on the formation of pyocyanine*

Total volume, 1 ml.; organisms equiv. 6.7 mg. dry wt./ml. Final concentration of substrates added: 0.1 M; MgSO_4 , 0.02 M; phosphate buffer, pH 7.8, 0.02 M. Incubation at 37° for 24 hr.

Substrate	Pyocyanine formation %*	Substrate	Pyocyanine formation %*
L-Glutamic acid	100	DL-Tyrosine	5
γ -Aminobutyric acid	110	Anthranilic acid	0
Pyrrolidonecarboxylic acid	75	α -Aminobutyric acid	5
L-Glutamine	70	α -Aminoisobutyric acid	5
DL-Proline	80	DL- β -Aminobutyric acid	15
L-Hydroxyproline	90	2,4-Diaminobutyric acid	0
DL-Ornithine	55	Cadaverine	40
DL-Citrulline	20	Betaine	10
L-Arginine	40	Tyramine	5
DL-Aspartic acid	20	Tryptamine	5
L-Asparagine†	100	Ethylamine	0
L-Alanine	85	Triethylamine	0
β -Alanine	70	Tri-aminopropanol	0
Glycine	0	Ethanolamine	0
DL-Serine	55	α -Ketoglutaric acid‡	55
DL-Threonine	35	Succinic acid‡	30
DL-Isoleucine	0	Fumaric acid‡	25
L-Leucine†	30	Pyruvic acid‡	10
DL-Valine	5	Acetic acid‡	35
DL-Methionine	0	Citric acid‡	10
L-Cysteine†	20	Malic acid‡	0
L-Cystine	0	Maleic acid‡	10
DL-Lysine	0	Malonic acid‡†	25
DL-Histidine	40	Oxalic acid‡	0
L-Tryptophane	0	Glutaric acid‡	0
DL-Phenylalanine	0	Pimelic acid‡	5

* Pyocyanine formation from glutamic acid was chosen arbitrarily as 100 %. The actual amount of pigment formed was about 0.25 mg.

† Active only with one strain.

‡ NH_4Cl (0.04 M) was added.

Mineral salt requirements

Examination of the role of metals in the pyocyanine synthesis was carried out with the aid of the chelating agent ethylenediamine tetra-acetic acid (EDTA). The addition of 0.3 % EDTA prevented the formation of pyocyanine with glutamic acid as the substrate (Table 2). However, pyocyanine formation inhibited by 0.2 % EDTA was completely restored by the addition of Mg^{++}

Table 2. *The effect of MgSO_4 on formation of pyocyanine in the presence and absence of ethylenediamine tetra-acetate (EDTA)*

Organisms equiv. 6.8 mg. dry wt./ml.; glutamic acid, 0.06 M; phosphate buffer, pH 7.8, 0.02 M. Incubation at 37° for 24 hr.

EDTA (0.3 % w/v)	—	—	+	+
MgSO_4 (0.01 M)	—	+	—	+
Pyocyanine formed ($\mu\text{g./ml.}$)	144	185	8	227

ions (0.002–0.01 M) (Fig. 1). On the other hand, $\text{Co}(\text{NO}_3)_2$ and FeCl_2 (at concentrations of 0.0002 M) inhibited pyocyanine formation (Table 3). Phosphate was not necessary for pigment synthesis in the resting system. In growing cultures phosphate seems also to be dispensable (Jordan, 1899, as quoted from *Topley & Wilson*, 1955); however, the newer work of *Burton et al.* (1948) claims the essentiality of phosphate for pyocyanine formation.

Table 3. *The influence of various cations on pyocyanine production*

Organisms equiv. 4.3 mg. dry wt./ml.; phosphate buffer, pH 7.8, 0.02 M; total volume, 1 ml. Incubation at 37° for 24 hr.

	1	2	3	4	5	6	7	8	9	10	11	12
FeCl_3 (0.0002 M)	—	+	—	—	—	+	+	+	—	—	—	—
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.0002 M)	—	—	+	—	—	—	—	+	+	+	—	—
MnCl_2 (0.0002 M)	—	—	—	+	—	—	+	—	—	+	+	—
MgSO_4 (0.05 M)	—	—	—	—	+	+	—	—	+	—	+	—
Glutamic acid (0.02 M)	+	+	+	+	+	+	+	+	+	+	+	—
Pyocyanine formed ($\mu\text{g./ml.}$)	59	10	24	64	69	21	26	6	24	25	73	10

Fig. 1

Fig. 2

Fig. 1. Dependence of pyocyanine synthesis on the concentration of MgSO_4 in the presence of ethylenediamine tetra-acetate (EDTA). Total volume, 1 ml.; glutamic acid, 0.06 M; EDTA 0.2 % (w/v); organisms equivalent to 6.5 mg. dry wt. bacteria; phosphate buffer, pH 7.8, 0.02 M; incubation at 37° for 24 hr.

Fig. 2. Effect of pH value on pyocyanine formation. Total volume, 3 ml.; glutamic acid, 0.067 M; MgSO_4 , 0.01 M; organisms equivalent to 6.5 mg. dry wt. bacteria/ml.; buffers: pH 7.2 and below, McIlvaine's buffer; above pH 7.2, 'Tris' buffer.

Effect of pH value on pyocyanine formation

Fig. 2 shows that the synthesis of pyocyanine took place over a wide range of pH values with optimum synthesis at pH 7.0–8.0. At pH values of 6.0 and 10.0 pyocyanine formation was only about 30 % of that found at pH 7–8.

The rate of pigment formation

The synthesis of pyocyanine was rather slow; the minimal time required for measurable amounts of pigment to be formed was *c.* 6–8 hr. (Fig. 3). Experiments were carried out with the aim of enhancing the rate of synthesis of pyocyanine. Since oxygen is essential for its formation, the effect of aeration on the incubation mixture was investigated. When wider test tubes (24 × 150 mm.) were used instead of the smaller ones, the final yield of pyocyanine was doubled, but the rate of synthesis was unchanged. Similar results were obtained when the reaction vessels were shaken in a Ross-

Fig. 3

Fig. 4

Fig. 3. Pyocyanine synthesis as a function of time. Total volume: 10 ml.; glutamic acid, 0.1 M; MgSO_4 , 0.02 M; organisms equivalent to 7 mg. dry wt. bacteria/ml.; phosphate buffer, pH 7.8, 0.02 M; incubation at 37°.

Fig. 4. Dependence of pyocyanine synthesis on the concentration of organisms. Total volume: 1.5 ml.; glutamic acid, 0.067 M; MgSO_4 , 0.01 M; phosphate buffer, pH 7.8, 0.02 M; incubation at 37° for 24 hr.

Kershaw shaking apparatus. Stronger shaking in a reciprocating type of shaker inhibited pigment formation; in fact no pyocyanine at all was formed on vigorous shaking.

In view of the possibility that the slow synthesis was due to the lack, or insufficient formation, of some essential co-factor, the reaction mixture was enriched with yeast extract (0.2 %, w/v), tomato juice (3 %, v/v), or a mixture of vitamins. The vitamin mixture contained the following substances (µg./ml.): thiamine, 2.0; riboflavin, 1.0; nicotinamide, 15; Ca-pantothenate, 12.0; biotin, 0.2; pyridoxal-HCl, 2.0; pyridoxamine 2HCl, 2.0; choline, 3.0; inositol, 150.0; folic acid, 2.5; vitamin B_{12} , 0.01; *p*-aminobenzoic acid, 0.1. This was added in 0.1 ml. volumes to the 1 ml. incubation mixture.

In order to exclude the possibility that pyocyanine was synthesized by the bacteria multiplying during the incubation of the system, the following experi-

ment was designed. A series of reaction mixtures, containing a constant amount of glutamic acid, MgSO_4 and varying amounts of bacteria was set up. Fig. 4 shows that pyocyanine formation was proportional to the quantity of organisms added, indicating that the synthesis was not influenced by bacterial growth. In another experiment the concentration of organisms was kept constant while the concentration of glutamic acid varied from 0.15 to 2.4% (w/v) (Fig. 5). The results obtained were essentially similar to those presented in Fig. 4. In other experiments colony counts and turbidity measurements were made; no evidence for bacterial growth during incubation for pyocyanine formation was found. On the contrary, a considerable decrease in colony count and some decrease in turbidity was noted.

Action of various metabolic inhibitors

It was hoped that by addition of metabolic inhibitors further information about the biosynthetic pathways in pyocyanine synthesis might be obtained. Several agents were also tested with a view to securing bacteriostatic conditions for the system studied without impairing pyocyanine formation. Penicillin, aureomycin or chloromycetin did not selectively inhibit bacterial growth or pyocyanine formation. At the lower concentrations of the antibiotics used both processes continued at normal rates, whereas higher concentrations, when inhibitory, affected both the growth of *Pseudomonas aeruginosa* and pyocyanine synthesis to the same extent. KCN or NaN_3 at 0.001M inhibited pyocyanine synthesis by about 60%, and at 0.01M the inhibition of synthesis was complete. Iodoacetate (0.01M) also prevented the formation of pyocyanine. At the given concentrations cyanide and azide inhibited growth as well, whereas iodoacetate did not. Thus iodoacetate inhibited selectively pyocyanine formation. NaF was not inhibitory of growth or synthesis even at 0.01M (Table 4). Thiomersalate at a concentration of 1:30,000 completely prevented pyocyanine formation. Cysteine, glutathione and thioglycolic (mercaptoacetic) acid produced a marked decrease in the rate of pyocyanine synthesis.

Table 4. *Effect of KCN, NaN_3 , and NaF on pyocyanine synthesis*

Glutamic acid, 0.081M; MgSO_4 , 0.01M; organisms equiv. 4.3 mg. dry wt./ml.; phosphate buffer, pH 7.8, 0.02M; total volume, 1 ml. Incubation at 37° for 24 hr.

	Pyocyanine formed ($\mu\text{g./ml.}$)	Inhibition of pyocyanine formation (%)
Control (no inhibitor added)	54	0
NaF (0.01M)	53	0
KCN (0.0001M)	48	11
KCN (0.001M)	24	56
KCN (0.01M)	0	100
NaN_3 (0.0001M)	45	17
NaN_3 (0.001M)	23	57
NaN_3 (0.01M)	0	100
Mono-iodo-acetate (0.01M)	0	100

Fig. 5

Fig. 6

Fig. 5. Influence of glutamic acid concentration on the formation of pyocyanine. Total volume: 1 ml.; organisms equivalent 4.7 mg. dry wt. bacteria; phosphate buffer, pH 7.8, 0.02M.

Fig. 6. The relationship between substrate disappearance and the formation of pyocyanine. Total volume: 1 ml.; amino acids, 0.03M; MgSO_4 , 0.02M. Phosphate buffer, pH 7.8, 0.02M. Incubation at 37°. x = γ -aminobutyric acid; O = L-glutamic acid; ● = L-hydroxyproline. Solid line = pigment formation; broken line = disappearance of substrate.

DISCUSSION

Glutamic acid and some compounds closely related to it were thus found to be very good substrates for pyocyanine formation by non-proliferating suspensions of *Pseudomonas aeruginosa*. The greater production of pyocyanine in the presence of γ -aminobutyric acid than in the presence of glutamic acid raises the question as to whether the γ -aminobutyric acid serves as a suitable precursor in the biosynthesis or whether it enters the organisms more rapidly than glutamic acid and is there carboxylated to glutamic acid. The high yield of pyocyanine in the presence of β -alanine (the carboxylation of which to aspartate is not known to occur in this organism), suggests that the decarboxylated amino acids (β -alanine, γ -aminobutyric acid) may function as such and not via glutamic or aspartic acids. On the other hand, the appreciable activity of α -alanine might be explained by the ability of this compound to enter the Krebs cycle via pyruvic acid, and subsequently to undergo transformation to glutamic acid. The same mechanism might explain the effect of acetic, fumaric and succinic acids. This possibility appears plausible in view of the occurrence of the tricarboxylic acid cycle in various species of *Pseudomonas* (Barrett & Kallio, 1953; Campbell, Smith & Eagles, 1953; Claridge &

Werkman, 1954). The present work supports the view that glutamic acid holds a key position in pyocyanine synthesis and may be linked with the tricarboxylic acid cycle. Some insight into the sequence of the biosynthetic steps of pyocyanine formation may be gained by studying the relationship between the disappearance of the substrate and the formation of pyocyanine. Fig. 6 shows that the bulk of the substrates disappeared before measurable amounts of pyocyanine were formed and its synthesis continued after complete exhaustion of the added substrates. This relationship points towards the formation, from glutamic acid, of some unknown intermediary prior to its incorporation to form the molecule of pyocyanine.

The role of magnesium ions in pyocyanine formation was demonstrated in a magnesium-deficient system. The minimal effective amount of Mg^{++} ions was lower with glutamic acid than with any of the other substrates tested. The steep ascent of the curve resulting from the addition of Mg^{++} beyond 5–6 μ mole/ml. (Fig. 1) suggests that this ion participates in more than one reaction step.

The rate of pyocyanine synthesis was rather slow. Attempts to enhance its rate of formation by increasing aeration, or by supplying missing nutrients, have so far failed. It might perhaps be assumed that some essential intermediary of unknown nature, which is synthesized at a very slow rate, acts as a limiting factor in the formation of pyocyanine. The specific sensitivity of the system to respiratory poisons suggests a function of respiratory enzymes in pyocyanine biosynthesis.

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