

Studies on the Biosynthesis of Pyocyanine. (IX)

On the Effect of Pyocyanine on Respiration of Bacterial Cells of *Pseudomonas aeruginosa*

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The present study was instituted to explain a physiological significance of pyocyanine, and it was discussed mainly from a viewpoint of bacterial respiration to reason why such a pigment is usually produced independently, in appearance, of the bacterial population, according to the following fact found in this work: The bacterium, *Pseudomonas aeruginosa* can respire even when the cytochrome system, which is regarded as a main respiration pathway, may be blocked, and therefore can also acquire its energy in the presence of pyocyanine even under anaerobic condition, through a coupled oxidation-reduction process, as for example between glucose oxidation and fumaric reduction.

INTRODUCTION

Pyocyanine formed by *P. aeruginosa* is most well known among various biological pigments to be reversibly reducible and oxidizable, and can therefore be used for a biochemical technique as an indicator in comparison with other artificial dyes such as methylene blue. Pyocyanine has, since a long time ago, been recognized as producing an increase in cell respiration^{1,2)}, and a number of reports have hitherto been presented on physiological as well as biochemical properties. Most of these investigations, however, have dealt mainly with animal tissues as experimental materials, for instance blood cells³⁻⁶⁾, tumor tissues⁷⁻¹⁰⁾, sea-urchin eggs¹¹⁻¹⁴⁾ or cerebral cortex of rabbit¹⁵⁾, and seemed to examine the physiological effect as a biological pigment of reversible oxidation-reduction property, instead of ordinary synthetic dyes. And therefore very little has been known of an essential significance of pyocyanine in the bacterium producing it. Freedheim²⁾ has regarded this pigment to be an accessory enzyme in bacterial physiology, probably from the fact that this is not necessarily required for the bacterial metabolism. Runnström and Michaelis⁵⁾ stated in the metabolism of hemolyzed blood cells that pyocyanine might relate to an esterification of hexose.

As well known, on the other hand, pyocyanine reveals an antibiotic property, and several reports have been presented in comparison with other antibiotics produced by *P. aeruginosa*¹⁶⁻¹⁸⁾.

In the present paper the following abbreviations are used. DPN: diphosphopyridine nucleotide, ATP: adenosine triphosphate, FMN: flavin mononucleotide, FAD: flavin-adenine dinucleotide, Mb: methylene blue, DNP-hydrazone: 2,4-dinitrophenylhydrazone, EDTA: ethylenediamine tetraacetate, G-6-P: glucose-6-phosphate, BCG: bromocresol green

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A principle aim of the present work is to understand why such a remarkable pigment is usually produced, whereas the bacterial growth is normally brought about even when pigmentation does not occur.

MATERIALS AND METHODS

Pyocyanine. This was a crystalline material prepared according to the procedure previously reported by the author¹⁹⁾.

Bacterial strain and culture. Two strains of *P. aeruginosa* isolated in this laboratory were used, of which one strain was very much inferior in its capacity for pigmentation. Bacterial culture was carried out as follows: 200 ml of the cultural medium composed of 2% glycerol, 1% peptone, 0.2% urea, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025% K_2HPO_4 and 3.5% agar (pH 7.4) were placed in 1 liter Erlenmeyer flask, and autoclaved at 140° for 30 minutes. The glycerol of carbon source was replaced with 1% glucose plus 1% glycerol or with 2% glucose plus 5% CaCO_3 , and K_2HPO_4 was increased up to 0.1%, if necessary. The solidified medium was incubated at 37° for 48 hours, after being inoculated with the bacterial cell suspension by brush.

Preparation of acetone-dried cells. The cells harvested were suspended in 0.01 *M* phosphate buffer of pH 7.0, and incubated on 3% agar plate containing 2% active charcoal at 37° for 16 hours to obtain pyocyanine-free cells. The collected cell suspension was slowly poured with vigorous stirring into acetone, not less than 10 volumes cooled previously to -20° . After a brief stirring the cells were allowed to settle, the supernatant was decanted and the residual solvent was removed on a Büchner filter. The filtered cake of cells was washed on the Büchner with 5 volumes of the chilled acetone, followed by finally washing with cooled dry ether to remove the residual acetone, and dried over NaOH in a vacuum desiccator in the ice box, after crushing the cells wrapped in a filter paper.

Preparation of cell-free extract. Cell-free extract was prepared from acetone-dried cells as follows: the ground acetone-dried powder suspended at a concentration of 50 mg per ml of 0.05 *M* Tris buffer of pH 7.0, was allowed to stand overnight in ice box, followed by centrifugation at 9000 rpm for 30 minutes. The cell-free supernatant thus obtained was stored in a freezer, if not used immediately.

Dialysis. 20 ml of cell-free extract or of aqueous solution of its precipitate formed by treating repeatedly with saturated ammonium sulfate, was dialyzed against demineralized water in a cellophan pouch of about 30 ml in capacity, being kept in ice box (below 5°), until fluorescence or sulfuric ion was no longer recognized.

Measurement of oxygen consumption. This was performed by Warburg manometric technique. Each Warburg cup containing 3 ml of reaction mixture was incubated at 37° .

Paper chromatography. Glucose and its reaction product, or other organic acids were detected by paper chromatography using the following solvent sys-

tems: For the former, *n*-butanol-methanol-2% ammonium hydroxide (4:4:2); for the latter, *n*-butanol-methanol-water (4:4:2).

Locating reagent for glucose and keto acids was prepared according to a modified method of Trevelyan *et al.*²⁰⁾ in which the solvent, acetone for silver nitrate and NaOH was replaced by methanol, and the other organic acids were located by spraying with a methanolic solution of BCG. On the other hand, aniline phthalate was found to be the most favorable reagent for the detection of 2-ketogluconate: When heated at 100° for 2 minutes after being sprayed with this reagent and with diluted methanolic solution of NaOH, the spot was colored to pink which was characterized by 2-ketogluconate in the present reaction system.

RESULTS AND ITS DISCUSSION

Effect of Pyocyanine on Respiration of Living Cells

The cells grown on glycerol or glucose peptone agar for 48 hours were collected, suspended at a level of 30 mg wet cells per ml of 0.01 *M* phosphate buffer of pH 7.2, allowed to stand on agar plate containing charcoal for 16 hours to obtain pyocyanine-free cells, and was estimated their auto-respiration in the

Table 1. Effect of pyocyanine on auto-respiration of intact cells.

Minutes (Pyocyanine)	Bacteria							
	<i>P. aeruginosa</i>				<i>P. fluorescens</i>		<i>S. aureus</i>	
	B_{16}		B_a					
	-	+	-	+	-	+	-	+
5	24.8	28.0	27.8	27.4	26.9	24.6	24.0	18.4
10	46.0	53.4	55.0	56.2	56.4	34.0	36.4	24.6
15	64.4	76.2	74.2	76.6	80.2	52.2	54.6	35.4
20	86.6	90.6	90.3	98.4	102.8	66.4	60.3	42.4

Bacterial cells were suspended in 0.01 *M* phosphate buffer of pH 7.2, containing 0.001 *M* pyocyanine. Each Warburg cup contained 3 ml of reaction mixture. Figures show oxygen consumption (μ l).

Table 2. Effect of pyocyanine on respiration of resting cells.

Minutes (Pyocyanine)	Substrates								
	Lactate		Glycerol		Glucose		Gluconate		Nil
	-	+	-	+	-	+	-	+	-
10	8.7	8.4	16.0	16.2	6.0	6.8	4.8	4.6	4.8
20	19.3	20.8	34.8	32.4	23.4	23.9	12.4	12.0	14.0
30	28.9	32.4	49.6	44.0	33.4	36.3	18.8	17.2	15.6
40	37.0	42.6	65.7	56.8	40.8	52.2	26.4	26.0	23.4
50	52.4	52.4	81.2	70.2	61.8	69.3	31.2	30.8	28.0

Bacterial cells harvested from glycerol medium were suspended in 0.05 *M* phosphate buffer of pH 7.0, containing 0.005 *M* substrate and 0.001 *M* pyocyanine, at the concentration of 30 mg/ml. Bacteria used were *P. aeruginosa*, strain B_{16} .

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presence of 0.001 *M* pyocyanine. Results are shown in Table 1. Pyocyanine did not reveal any increasing effect on respiration of the intact cells of the present bacteria, while some inhibitory effects were observed with other bacteria such as *P. fluorescens* or *S. aureus*. No increasing effect was also observed with resting cells of *P. aeruginosa* (Table 2).

However, the increasing effect of pyocyanine was observed on autorespiration in the presence of KCN which produced an inhibitory action at a higher concentration, although appreciable effect was not recognized at a lower level as well as in the case of EDTA, 8-hydroxyquinoline and other metal chelators (Fig. 1 and Table 3).

In general, pyocyanine revealed no effect on respiration of the pigment producer, *P. aeruginosa*, differing from the results by other workers or with other materials. For instance, Freedheim²⁾ showed that pyocyanine increased to a

Table 3. Effect of pyocyanine on auto-respiration in the presence of inhibitors.

Minutes (Pyocyanine)	Inhibitors							
	Nil		KCN		EDTA		8-Hydroxyquinoline	
	-	+	-	+	-	+	-	+
10	64.8	75.0	65.4	64.2	68.6	75.2	62.4	60.3
20	100.2	110.0	110.2	108.5	98.0	112.8	89.2	102.0
30	146.8	150.3	142.4	134.3	146.2	151.7	148.2	161.2
40	176.4	178.2	173.0	160.2	172.0	170.9	168.6	172.4

Inhibitors were used at the concentration of 0.001 *M*, and other experimental method was the same as in Table 1. Bacterial strain: B₁₆.

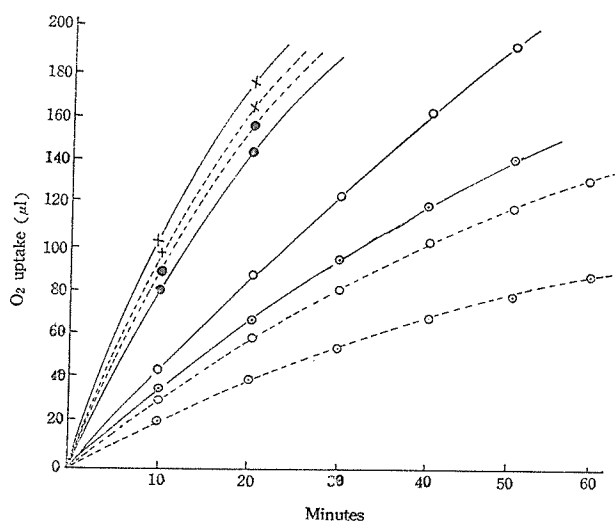


Fig. 1. Effect of pyocyanine on auto-respiration of intact cells in the presence of KCN.

Experimental method was the same as in Table 1.

- (···○···) 4 mM KCN was added, (—○—) 4 mM KCN plus 1 mM pyocyanine,
- (···○···) 2 mM KCN, (—○—) 2 mM KCN plus 1 mM pyocyanine,
- (···○···) 1 mM KCN, (—○—) 1 mM KCN, plus 1 mM pyocyanine,
- (···×···) Control, (—×—) 1 mM pyocyanine.

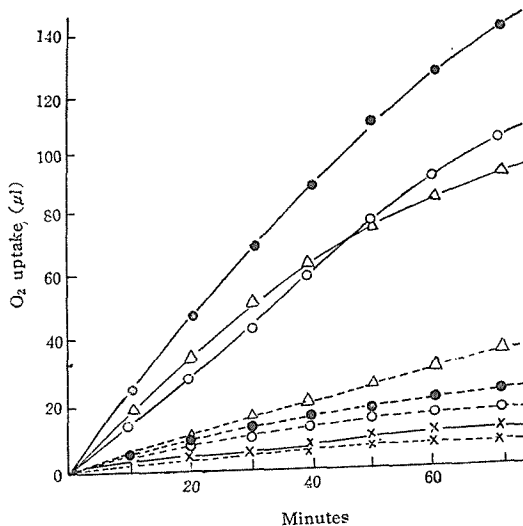


Fig. 2. Effect of pyocyanine on respiration of cell autolysate. Experimental method and material were the same as in Table 2.
 (···●···) Glucose, (—●—) glucose plus pyocyanine,
 (···○···) lactate, (—○—) lactate plus pyocyanine,
 (···△···) succinate, (—△—) succinate plus pyocyanine,
 (···×···) glycerol, (—×—) glycerol plus pyocyanine.

great degree the respiration of living cells of *P. aeruginosa* and other bacteria, being paralleled with an increase in RQ (respiratory quotient), and attributed this effect to the assumption that the oxidation of endogenous materials such as lipid, polysaccharide and decomposition products of cell components was accelerated by pyocyanine. On the other hand, Dickens⁹⁾ stated in the experiment with tumor tissue that pyocyanine was less toxic and caused a great increase in respiration, but inhibited aerobic glucolysis, differing from the case of methylene blue which accelerated both respiration and aerobic glucolysis.

It appears likely that pyocyanine does not play any role on aerobic metabolism in the present bacteria, unless a decreasing respiration is caused because of an injury of the cytochrome system which should be a normal respiration pathway, or of another factor. (See results shown in the following section).

When washed cells obtained from 48 hours' culture were suspended in 0.01 *M* phosphate buffer of pH 7.4, and further incubated with shaking at 37° for 48 hours, they were almost autolyzed into an eggwhite-like fluid, liberating a small amount of ammonia gas. This enzyme sample did not show any oxidation of glucose, glycerol or of organic acids as well as auto-respiration. There could be observed in this case a remarkable effect of pyocyanine on oxidation of the added substrate (Fig. 2). The same effect was also observed with acetone-dried cells, as will be illustrated in the following.

Effect on Oxidation of Glucose and Organic Acids by Acetone-dried Cells

Although acetone-dried cells were found to be preservable for a fairly long

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time, the sample which was expected to respond to pyocyanine could be prepared only by careful procedure under suitable conditions. Therefore, drying must be carried out immediately at low temperature. Table 4 shows the result on the oxidative metabolism of sugar and organic acid by acetone-dried cells prepared from the culture on glucose medium. In the absence of pyocyanine, O₂ consumption could hardly occur as in the case of autolysate. As will be illustrated later, acetone-dried cells revealing O₂ consumption should, of course, accompany a decoloration of pyocyanine in anaerobic condition, and their velocities were paralleled with each other. With the sample obtained from glucose medium, gluconate was, in general, observed to be best dissimilated, and the reaction was fast enough to occur without any phosphorylation. On the contrary, glucose-6-phosphate was hardly metabolizable, even in the presence of pyocyanine, and glycerol by itself was found to be not decomposed by any acetone-dried preparation, differing from the case of resting system (Table 5).

Table 4. Effect of pyocyanine on respiration of acetone-dried cells.

Minutes (Pyocyanine)	Substrates						
	Succinate		Lactate		Glucose		Nil
	—	+	—	+	—	+	
10	11.2	20.0	—	17.5	4.5	28.6	—
30	13.4	27.4	—	28.2	5.1	58.2	—
60	15.7	31.7	—	40.0	5.5	102.2	—
80	19.0	40.0	—	51.2	6.1	131.0	—

Acetone-dried cells prepared from the culture on glucose medium containing CaCO₃, were suspended in 0.05 M Tris buffer of pH 7.0 at the concentration of 10 mg/ml. Other experimental material and method were the same as in Table 2.

Table 5. Effect of pyocyanine on respiration of acetone-dried cells cultured on glucose medium.

Minutes (Pyocyanine)	Substrates								
	Glucose		Gluconate		G-6-P		Glycerol		Nil
	—	+	—	+	—	+	—	+	—
20	8.0	43.7	6.8	55.5	7.5	14.0	8.8	9.2	7.8
40	14.4	57.0	13.5	107.4	15.1	23.9	16.4	16.8	14.2
60	19.2	78.4	18.9	149.2	21.0	30.9	22.6	24.3	19.6

Experimental method was the same as in Table 4.

It was observed that glucose takes up O₂ to be about twice as much as do gluconate, although the reaction velocity of the latter was much more rapid than the former, and CO₂ was not liberated (Fig. 3 and Table 6). From these observations, 2-ketogluconate accumulation could be suggested in both cases, as was presented by other investigators²¹⁻²³). The fact that phosphate is not inhibitory in this enzyme system also suggests that this behavior differs from glucose-6-phosphate dehydrogenase of Dickens²⁴), and agrees with the statement

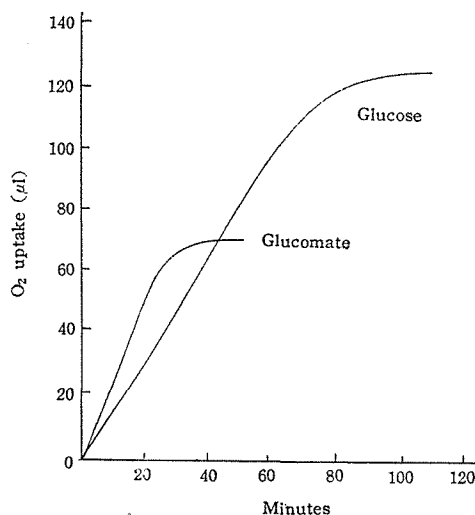


Fig. 3. Comparative experiments on oxidation of glucose and gluconate in the presence of pyocyanine. Each Warburg cup contained 0.3 ml of 0.02 *M* substrates, 0.3 ml of 0.01 *M* pyocyanine, 1.5 ml of the suspension of the concentration of 20 mg of acetone-dried cells per ml of 0.5 *M* Tris buffer of pH 7.2, and 0.9 ml of demineralized water.

Table 6. Comparative experiments on glucose oxidation of resting and acetone-dried cells in the presence of pyocyanine.

Minutes (KOH)	Resting cells			Acetone-dried cells		
	—	+		+	—	
20	75.6	41.4	(37.5)	37.4	37.2	(—)
30	118.2	63.3	(60.6)	53.0	52.0	(—)
50	168.9	91.2	(86.1)	80.6	80.0	(—)

Each cell preparation was obtained from the medium containing 1% glucose plus 1% glycerol. Concentration of glucose used as substrate was 0.002 *M*. Figures in parenthesis show μ l of CO₂ liberated. + Data in presence of KOH as absorbent of CO₂, — KOH was absent.

Table 7. Effect of pyocyanine on respiration of acetone-dried cells cultured on glycerol medium.

Minutes (Pyocyanine)	Substrates								
	Glucose		Gluconate		G-6-P		Glycerol		Nil
	—	+	—	+	—	+	—	+	—
20	7.2	40.2	7.6	7.0	6.8	42.3	6.0	8.2	7.0
40	13.0	52.6	12.4	13.2	14.8	58.4	14.9	16.6	12.6
60	16.0	72.8	16.8	18.4	17.8	79.2	20.8	24.8	15.8

Experimental method was the same as in Table 4.

of Campbell *et al.*²³⁾ that no esterification of glucose is involved in the present reaction system.

Thus, in general, glucose-6-phosphate dehydrogenase system has not been

recognized in the metabolism of glucose in *P. aeruginosa*. However, the author could point out the existence of glucose-6-phosphate dehydrogenase system even in the present bacteria, because pyocyanine, in the presence of glucose-6-phosphate, can rapidly be reduced by the acetone-dried cells or by the cell-free extract prepared from the culture on glycerol medium, as shown in Table 7 and will be illustrated later. This phenomenon lead the author to suppose that hexose phosphate would be formed from triose phosphate.

In the experiment instituted to ascertain why acetone-dried cells hardly reveal any O₂ consumption, the following fact was found: when DPN and FAD were supplemented, O₂ uptake could considerably be recovered, even in the absence of pyocyanine (Figs. 4a and 4b). It follows from this fact that the lack

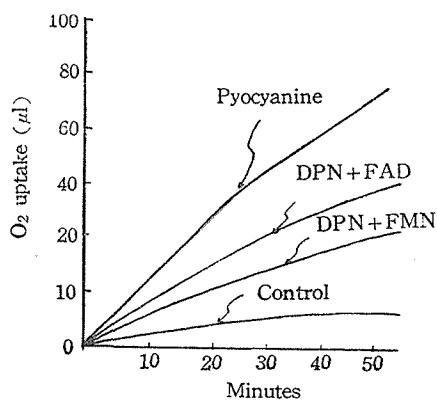


Fig. 4a.

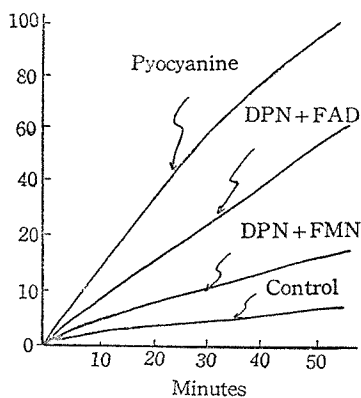


Fig. 4b.

Fig. 4a. Effect of DPN, FAD and FMN on glucose oxidation.

Fig. 4b. Effect of DPN, FAD and FMN on gluconate oxidation. Acetone-dried powder of the cells grown on glucose was suspended at the concentration of 10 mg/ml of 0.05 M Tris buffer of pH 7.0, containing 0.002 M substrate and the requisite amounts of pyocyanine, DPN, FAD and FMN which were 0.001, 0.001, 0.0005 M, respectively.

of respiration by acetone-dried cells may be attributable to the block of the enzyme in some steps to their cytochrome system. Similar mechanism may also be anticipated with the cell autolysate, although no effect of the coenzymes mentioned above was recognized, suggesting degeneration of the enzyme protein of FAD or of the further steps in the cytochrome system. It is suggested from these facts that pyocyanine can become a direct hydrogen acceptor from the substrate without being mediated by DPN and flavoprotein, and also that yellow enzyme assigns its prosthetic group to FAD, because of an inferior effect of FMN, which might be revealing the reaction: $\text{FMN} \xrightarrow{\text{ATP}} \text{FAD}$.

Comparative Experiments on the Effect of Respiration between Pyocyanine and Methylene Blue

As is familiar, Mb is most usually employed on a biochemical technique as an oxidation-reduction indicator, and therefore should be tested in expectation of

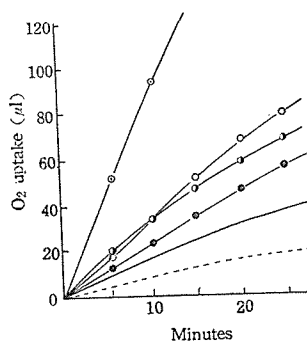


Fig. 5a.

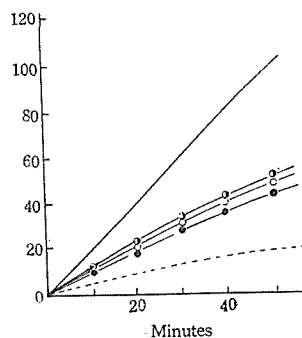


Fig. 5b.

Figs. 5a, 5b. Comparative experiments on the effect of pyocyanine and methylene blue on glucose oxidation.

Reaction mixture was composed of 0.004 *M* glucose, 1% acetone powder and requisite amount of pyocyanine or of methylene blue.

Fig. 5a : (—●—) With 5 *mM* pyocyanine, (—○—) 1 *mM* pyocyanine, (—◐—) 0.5 *mM* pyocyanine, (—●—) 0.1 *mM* pyocyanine, (—) 5 *mM* Mb, (.....) control.

Fig. 5b : (—○—) 1 *mM* Mb, (—◐—) 0.5 *mM* Mb, (—●—) 0.1 *mM* Mb, (—) 1 *mM* pyocyanine, (.....) control.

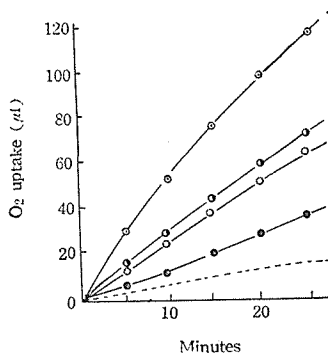


Fig. 6a.

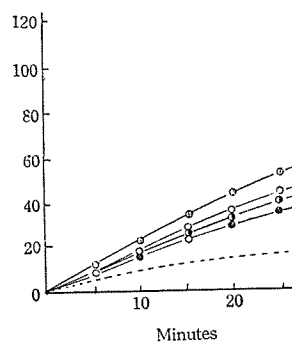


Fig. 6b.

Figs. 6a, 6b. Comparative experiments on the effect of pyocyanine and methylene blue on lactic acid oxidation.

Experimental method was the same as in Figs 5a and 5b.

Fig. 6a : (—●—) With 5 *mM* pyocyanine, (—○—) 1 *mM* pyocyanine, (—◐—) 0.5 *mM* pyocyanine, (—●—) 0.1 *mM* pyocyanine, (.....) control.

Fig. 6b : (—⊕—) With 2 *mM* Mb, (—○—) 1 *mM* Mb, (—◐—) 0.5 *mM* Mb, (—●—) 0.1 *mM* Mb, (.....) control.

the same effect as pyocyanine. Figs. 5 and 6 show their comparative data on the oxidations of glucose and lactate. There was observed a remarkable difference in their promoting effects on O_2 consumption, whereas a measurable difference could not be recognized in the velocity of their decoloration. From these facts, it is suggested that there exists another quite different mechanism in increasing effect of pyocyanine on respiration, besides the analogous role with Mb of a hydrogen acceptor, because the stimulating effect was more markedly produced at an extremely high concentration of pyocyanine which far exceeds its physiological limit in the bacteria, differing from the case of Mb in which a

noticeably different acceleration could not be pointed out in various concentrations. These phenomena may not be enough to explain the mechanism of the increasing respiration only by the role of hydrogen acceptor.

Effect of Other Phenazine Compound on O_2 Consumption of Acetone-dried Cells

In order to speculate the mechanism of these different behaviors between two pigments mentioned above, some other comparative experiments have been carried out: Phenazine methosulfate ($E_0' = +0.080$ V) should perhaps behave more like methylene blue ($+0.011$ V) if the redox-potential is a critical thing, or more like pyocyanine (-0.034 V) if the chemical structure is more important. Their results are shown in Figs. 7a and 7b.

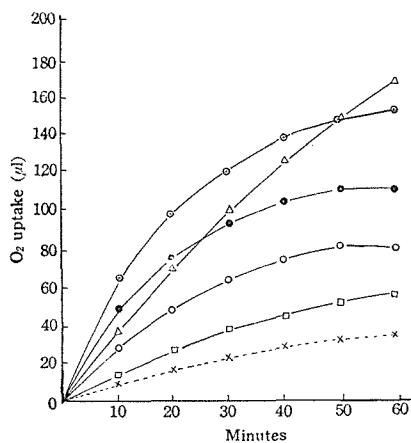


Fig. 7a.

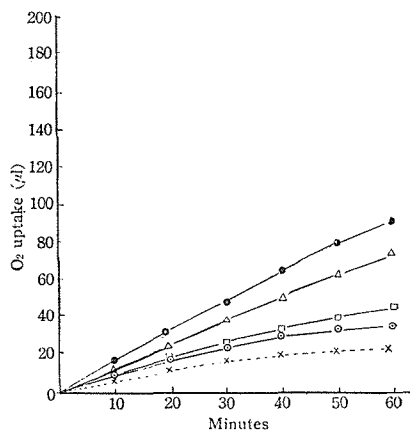


Fig. 7b.

Fig. 7a. Effect of phenazine methosulfate on glucose oxidation.

Fig. 7b. Effect of phenazine methosulfate on lactic oxidation.

Three ml of the reaction mixture, composed of 0.05 M Tris buffer of pH 7.0, 4 mM substrate, 1% acetone-dried cells and requisite amount of the pigment, was placed in Warburg cup, and incubated at 37°. (—●—) With 2 mM phenazine methosulfate, (—●—) 0.4 mM phenazine methosulfate, (—○—) 0.2 mM phenazine methosulfate, (—△—) 2 mM pyocyanine, (—□—) 2 mM Mb, (—×—) control.

Phenazine methosulfate revealed such a remarkable effect on glucose oxidation as to exceed pyocyanine. However O_2 consumption, accelerated markedly by phenazine methosulfate at an earlier stage, was observed to slow down gradually, whereas the effect of pyocyanine was shown to be of straight line. This reason appeared to be attributable to a fall in pH, since this phenomenon was not recognized with sodium lactate. On lactic oxidation, a considerably increasing acceleration was also caused by phenazine methosulfate in the behavior analogous to pyocyanine, regardless of the different potential between them. Accordingly, it seems probable that a noticeable effect of pyocyanine on respiration is ascribable rather to its chemical structure.

Reduction of Pyocyanine by Cell-free Extract

It has been experienced that an extraction of the enzymes was not necessarily

easy even in the preparation from the acetone-dried powder, for they were not only difficult to separate from the cells but also there was caused an inactivation of the enzymes during the purification procedure. And yet, however, the following enzymatic activities were successfully found in this preparation: glucose, gluconate, glucose-6-phosphate, lactate and malate dehydrogenases.

On the contrary, with glycerol and succinate, reduction of pyocyanine was hardly recognized. A reducing power of pyocyanine has well been shown in relation to oxidation-reduction potentials of the substrate as expressed below.

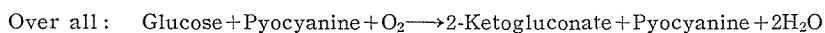
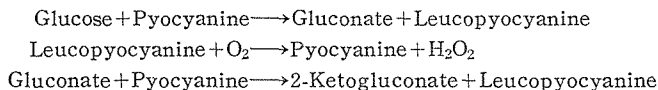
With the preparation from glucose medium:

Glucose	G-6-P	Gluconate	Lactate	Malate	Succinate	Glycerol
###	—	###	###	###	—(+)	—

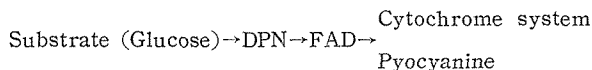
With the preparation from glycerol medium:

Glucose	G-6-P	Gluconate	Lactate	Malate	Succinate	Glycerol
###	###	—(+)	###	###	—	—

The following experiment was carried out to ascertain whether the reduction of pyocyanine occurs directly from the substrate or whether it is mediated by DPN or FAD which are linked not only with cytochrome-cytochrome oxidase system but also associable with an artificial oxidation-reduction indicator such as Mb. The cell-free preparation obtained from acetone-dried powder was precipitated by saturating with ammonium sulfate, followed by centrifugal separation of the precipitate, and dialyzed according to the method already described. The results of the present experiment suggested that gluconate could easily react directly with pyocyanine, and also with glucose to reduce pyocyanine without any intermediate carrier as was supposed before; although this could not be demonstrated by others owing to a loss of their dehydrogenase activity during the purification process. On the other hand, it may become clear by the present experiment that both glucose and gluconate are oxidizable without phosphorylation, because phosphate and ATP which are regarded to inhibit glucose-6-phosphate dehydrogenase and DPN actions, respectively, did not reveal any effects. Therefore the following scheme will be possible:



However, the following scheme may not necessarily be negated in the growing system:

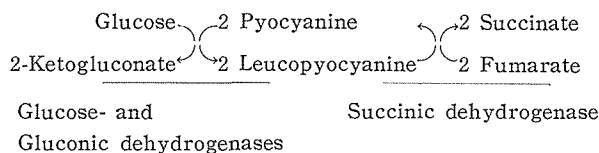


Lactate, malate and other organic acids would be catalyzed by pyocyanine through either mechanisms. The fact that glycerol hardly caused both decoloration of pyocyanine and O₂ consumption with acetone-dried cells as well as cell autolysate, would be ascribable to an injury of phosphorylation system. It was, as mentioned before, interesting to note that reducing activity of pyocyanine

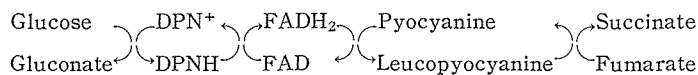
with succinate was not necessarily paralleled with O_2 consumption by any of enzyme samples. This reason might be attributable to a role of pyocyanine other than as a hydrogen acceptor, as mentioned before.

Coupling Reaction between Glucose and Fumaric Acid

Green and his co-worker²⁶⁾ have found the coupled reaction between lactic and fumaric acids, mediated by Mb or pyocyanine. There appears likely to occur usually such a reaction metabolism under anaerobic condition, according to an oxidation-reduction potential of the metabolites. It has been found in the present bacteria that besides the same reaction as above, glucose oxidation was coupled with fumaric reduction in the presence of their dehydrogenase, being mediated by pyocyanine. A similar reaction has also been found with gluconate. Accordingly, the following reaction will be presented:



As mentioned before, the following system would not also be denied to exist in the metabolism of growing state:



The above-mentioned reaction could clearly be observed to occur separately by the following technique. Since 1 *M* glucose can completely reduce 2 *M* pyocyanine, 5 ml of the reaction mixture containing 0.05 *M* Tris buffer of pH 7.2, 1 *mM* pyocyanine, 0.5 *mM* glucose and 0.5% acetone-dried powder were taken into a Thunberg tube of 50 ml in capacity, keeping 0.2 ml of 5 *mM* sodium fumarate in its side chamber, and incubated at 37° for several minutes under anaerobic condition. The perfectly decolored pyocyanine was markedly recolored after a short time when they were mixed with each other.

In order to verify further the above-mentioned reaction, the following experiment was carried out: 20 ml of the reaction mixture containing 0.01 *M* glucose (or gluconate), 0.001 *M* pyocyanine, 0.02 *M* sodium fumarate, 0.5% acetone powder and 0.05 *M* Tris buffer of pH 7.2, were placed in a 100 ml Erlenmeyer flask (or 50 ml test tube) and incubated anaerobically at 37°. The reaction could proceed even in the presence of catalytic amounts of pyocyanine, as long as a further hydrogen acceptor from leucopyocyanine, i.e. fumarate was present, owing to a sequential reversibility, although the reaction velocity varied according to the concentration of pyocyanine. After 4 hours' incubation the reaction mixture was centrifuged, saturated with ammonium sulfate and extracted with acetone and with butanol, and then the concentrated aqueous solution was obtained by transference of the butanolic solution of the product into diluted sodium bicarbonate solution, so as to subject to the chromatographic detection of the product and to test the formation of its DNP-hydrazone. Results are shown in Fig. 8.

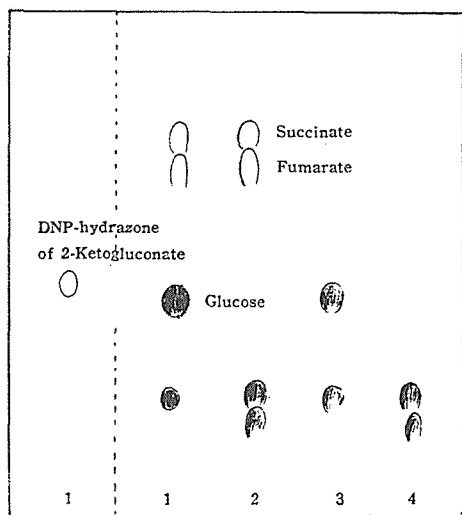


Fig. 8. Paper chromatography of the product in the coupled reaction between glucose and fumarate in the presence of pyocyanine.

1 : products from glucose and fumarate in anaerobic condition, 2 : do. from gluconate. 3 : product from glucose under aerobic condition. 4 : do. from gluconate.

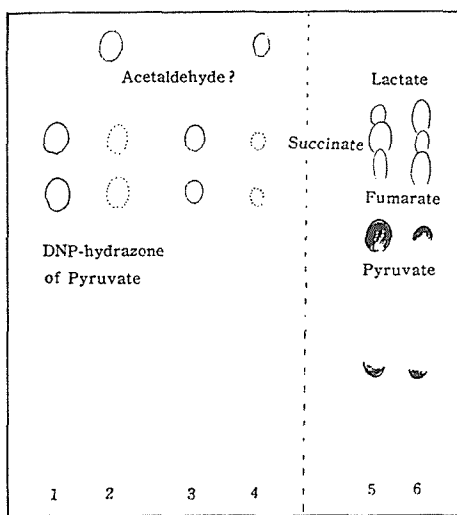


Fig. 9. Paper chromatography of the products of coupled reaction between lactic and fumaric acids in the presence of pyocyanine or of methylene blue.

1 : 2,4-dinitrophenylhydrazine of the product in the presence of pyocyanine after 4 hours' incubation, 2 : do. after 16 hours' incubation. 3 : 2,4-dinitrophenylhydrazine of the product in the presence of methylene blue after 4 hours' incubation, 4 : do. after 16 hours' incubation, 5 : products in the presence of pyocyanine after 4 hours' incubation, 6 : do. in the presence of methylene blue.

The formation of 2-ketogluconic and succinic acids was clearly demonstrated. Similar experiments with lactate, following the above-mentioned technique, have also demonstrated the formation of succinate and pyruvate which could further be converted probably to acetaldehyde after a long time's (16 hours) incubation (Fig. 9). In the present experiment it has also been possible to recognize the same effect of Mb, although its capacity was far inferior to pyocyanine. These results were never obtained in the absence of the pigment, or under aerobic condition.

As regards the problem on essential physiological role of pyocyanine in the present bacteria, such coupled oxidation-reduction process must be of great importance. The above-mentioned reaction should rather be regarded as an example, for these reactions may usually occur in the metabolism of static culture. In fact, similar reaction was also observed with glucose-6-phosphate or malate.

DISCUSSION ON PHYSIOLOGICAL ROLE OF PYOCYANINE

On physiological significance of pyocyanine, the author will bring up the following discussion, according to the experimental results already described.

The producer of pyocyanine, *P. aeruginosa* would go without assigning any physiological role to pyocyanine, as long as the cytochrome system is normally acting in its respiration, and pyocyanine would therefore become no more than an accessory. In practice, however, these ideal environments can not always be present in all of the micro-flora, especially of the present bacteria of static aerobe, even though an intermediate carrier in the cytochrome system may not essentially be blocked. In natural surroundings, there must certainly be shortage of oxygen in some spheres, differing from such an artificial circumstance given with aerating or shaking culture. Even when the bacteria are cultured in the solution of a spacious surface and of thin layer, there is always observable a reduction of pyocyanine except on the surface, suggesting an insufficient action in cytochrome system as well as active role of pyocyanine. Even in these cases, bacterial metabolism must also proceed as in the case of anaerobic bacteria, through such a coupled oxidation-reduction process as already manifested. Although pyocyanine did not reveal any increasing effect on normal respiration of intact cells of the bacteria, this is, of course, a superficial view obtained under artificial circumstance, regardless of a sight induced to support the conception that pyocyanine has its physiological role on bacterial activity even without regard to the special case in which cytochrome system might be blocked by the production of $\text{HCN}^{(26-28)}$, or by a poor phosphate metabolism which might bring about a need of DPN or of FAD as supposed before.

On the other hand, the antibiotic property of pyocyanine will be brought up together with the above, in discussing its physiological significance, because pyocyanine shows a bacteriostatic activity on the other bacteria, as reported by some workers. Even to the same genus, *P. fluorescens*, pyocyanine was found to suppress its respiration at a concentration of 0.001 *M* which is regarded to be a physiological level in *P. aeruginosa*.

SUMMARY

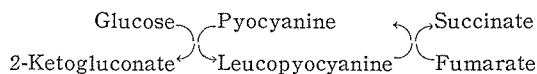
As a foundation to discuss the physiological role of pyocyanine in *P. aeruginosa*, some experiments were designed and the following results have been obtained.

1. Pyocyanine produced some recovery of auto-respiration of the living cells inhibited by high concentration of KCN, although no effect was observed on normal respiration.
2. An increasing effect was markedly observed on respiration of acetone-dried cells or cell autolysate, suggesting that some blocked steps to cytochrome system which were demonstrated to be DPN or flavoprotein, were replaced by pyocyanine.
3. Pyocyanine caused a remarkable increase in respiration especially at extremely high concentration which far exceeds its physiological limit in the bacteria, suggesting another different mechanism of stimulating effect, besides the analogous effect with Mb of hydrogen acceptor.
4. It has been shown by acetone-dried cells or cell-free preparation that pyocyanine was reduced with glucose, gluconate, glucose-6-phosphate, lactate and

malate, showing the parallelism between reducing power and redox-potential.

5. It was suggested that there also exists in the present bacteria glucose-6-phosphate dehydrogenase system when cultured in glycerol medium, whereas this dehydrogenase activity was hardly recognized with the cells grown on glucose.

6. It was found that pyocyanine could be linked directly with the substrate without any intermediate carrier, and that the following coupled reaction could occur under anaerobic condition.



These reactions, including similar ones which were also observed with glucose-6-phosphate, lactate or malate, would make it possible to acquire the bacterial energy even under anaerobic condition.

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