

PYOCYANINE, AN ACCESSORY RESPIRATORY ENZYME

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In a previous study (1) it has been shown that pyocyanine in mixture with its leuco compound is a reversible oxidation-reduction system that at pH > 6 behaves like any one of the known reversible dyestuffs of quinoid structure. How this property may manifest itself under biological conditions is shown in the following experiment:

A culture of *Bacillus pyocyaneus* in which the blue pigment has been formed becomes colorless on the exclusion of air. On agitation with air the blue color is restored. The bacteria reduce the pigment to the colorless leuco base, which then by the oxygen of the air is re-oxidized to the colored dye. Pyocyanine acts here as an autooxidizable acceptor for labile hydrogen of the microorganisms. This experiment shows that pyocyanine may play a rôle in dehydrogenative oxidation of bacteria in that it undergoes a cycle from the oxidized to the reduced and back to the oxidized form. As potentiometric studies (1) have shown that this cycle is also reversible thermodynamically, *i.e.*, in the course of this cycle the entropy of the system, pyocyanine-leucopyocyanine, remains constant, all the theoretical requirements are given which would allow pyocyanine to serve as an ideal catalyst for bacterial oxidation processes. But before pyocyanine can be so considered, proof is required that this pigment actually accelerates the oxygen consumption, *i.e.*, the respiration of bacteria. This proof is submitted in what follows:

Outline of the Experiment

The Warburg manometric method was applied to measure the respiration of *B. pyocyaneus* with and without the addition of pure recrystallized pyocyanine, using bacilli that had been cultivated under conditions impeding the formation of pigment and that had been washed free from all traces of nutritive media and suspended in a phosphate buffer.

Procedure.—The following strains of the American Type Culture Collection were used: (1) *Pseudomonas aeruginosa* No. 97, Army Medical School, Washington, D. C.; and (2) No. 256 E. O. Jordan, University of Chicago (Catalogue of Cultures, 1928). The two strains behaved in every respect identically. Cultures free from pigment, or producing only traces of the green fluorescent pigment were obtained at 30°C. in Blake bottles on lemco agar (0.3 per cent lemco, 0.5 per cent NaCl, 1 per cent Witte peptone, 2 per cent agar, pH 7.6). Cultures 15 hours to 5 days old were used.

The bacteria were swept off the medium and washed three times in the centrifuge with distilled water, and finally suspended in phosphate buffer (Sørensen). The suspension was made thoroughly homogeneous by filtration through paper and by shaking in a machine. A count of the bacterial suspension was not made as all measurements were comparative and hence the absolute quantity of bacteria was irrelevant. Therefore not the measured absolute values of respiration, but only the relative increases, are comparable for different experiments. 1 to 2 cc. of the bacterial suspension were placed in the main compartment of Warburg's micro-respiration vessels. The small inner vessel was filled with 0.2 cc. of 6 per cent NaOH. Pyocyanine was purified by recrystallization out of chloroform and stocked in N/20 HCl. Before each experiment the necessary amount of stock solution was adjusted to the proper pH (around 7.0) by the addition of secondary sodium phosphate. This dye solution was then added to the bacterial suspension in the proportion of about 1 to 10. The final molar concentration of pyocyanine was 1/5,000 M in all experiments. All the work was carried out under aseptic conditions. Aerobic and anaerobic test cultures produced at the end of the experiments showed, firstly, that the bacilli were still living and capable of reproducing, and, secondly, that no contamination with foreign microorganisms had occurred.

RESULTS

Effect of Pyocyanine on the Respiration of B. pyocyaneus.—All experiments showed unequivocally that the bacilli, suspended in an indifferent phosphate buffer, consume a small but definitely measurable amount of oxygen. Addition of pyocyanine increases this amount up to many hundred per cent as can be seen in Table I.

Fig. 1 represents the results of a typical experiment. Control tests prove that under the conditions pyocyanine itself does not consume any oxygen. Only at strongly alkaline reaction, beyond pH about 9.4, does pyocyanine oxidize spontaneously and irreversibly.

Effect of Temperature.—The figures given above were found at temperatures ranging from 18–24°C. Elevation of temperature up to 37°C. increases the basic oxygen consumption of the bacteria alone.

The increase brought about by pyocyanine over this already increased respiration, is not so large as at the lower temperatures, but is still quite important: 29 per cent to 127 per cent.

Effect of pH.—Fig. 2 shows that the increase of respiration induced by pyocyanine is about constant between the tested limits of pH, 5.3

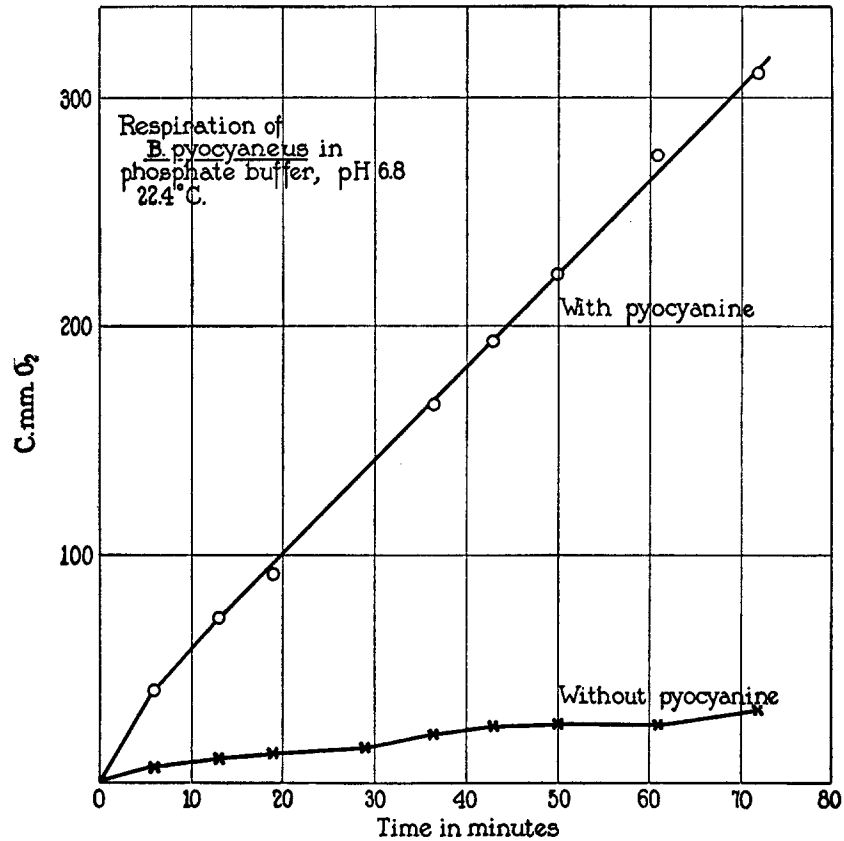


FIG. 1

and 7.7. The increase of the pyocyanine respiration observed between pH 5.3 and 7.2 parallels approximately the increase in the basic respiration of the bacteria alone.

The Oxygen Consumption of Killed Bacilli.—Does the excess consumption of oxygen brought about by pyocyanine really signify an

increase in respiration of the living bacteria or does the extra oxygen serve only for the oxidation of some bacterial product, in the sense of

TABLE I
Oxygen Consumption of Washed B. pyocyaneus Suspended in Phosphate Buffer

No.	pH	Temp. °C.	Oxygen consumed		Increased respiration <i>per cent</i>
			With pyocyanine	Without pyocyanine	
			<i>c. mm.</i>	<i>c. mm.</i>	
1	7.4	21.4	- 4	- 51	1,275
2	7.4	21.4	- 4	- 41	1,025
3	6.8	22.3	- 32	-310	970
4	6.8	22.2	-527	-290	550
5	6.9	20.9	- 4	- 96	2,400

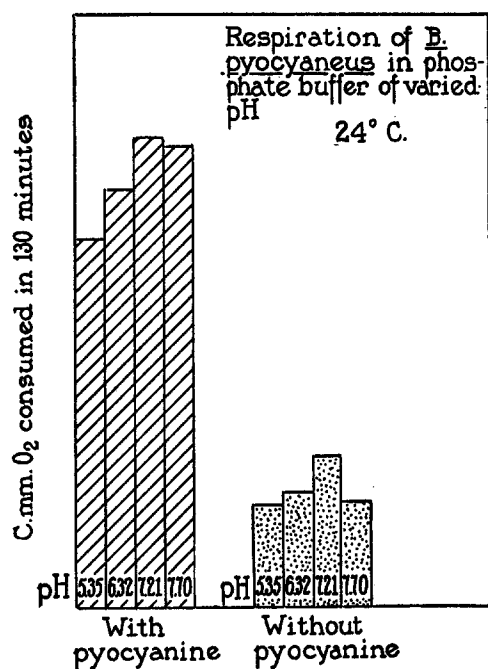


FIG. 2

the accessory respiration (*Nebenatmung*) of Batelli and Stern (2)? Bacteria killed by 1 hour exposure to 56°C., or by the addition of tolu-

ene did not show any oxygen consumption at all, either with or without pyocyanine (see Fig. 3). Therefore it may be concluded that pyocyanine affects actually the respiration (*Hauptatmung*) of the living bacteria.

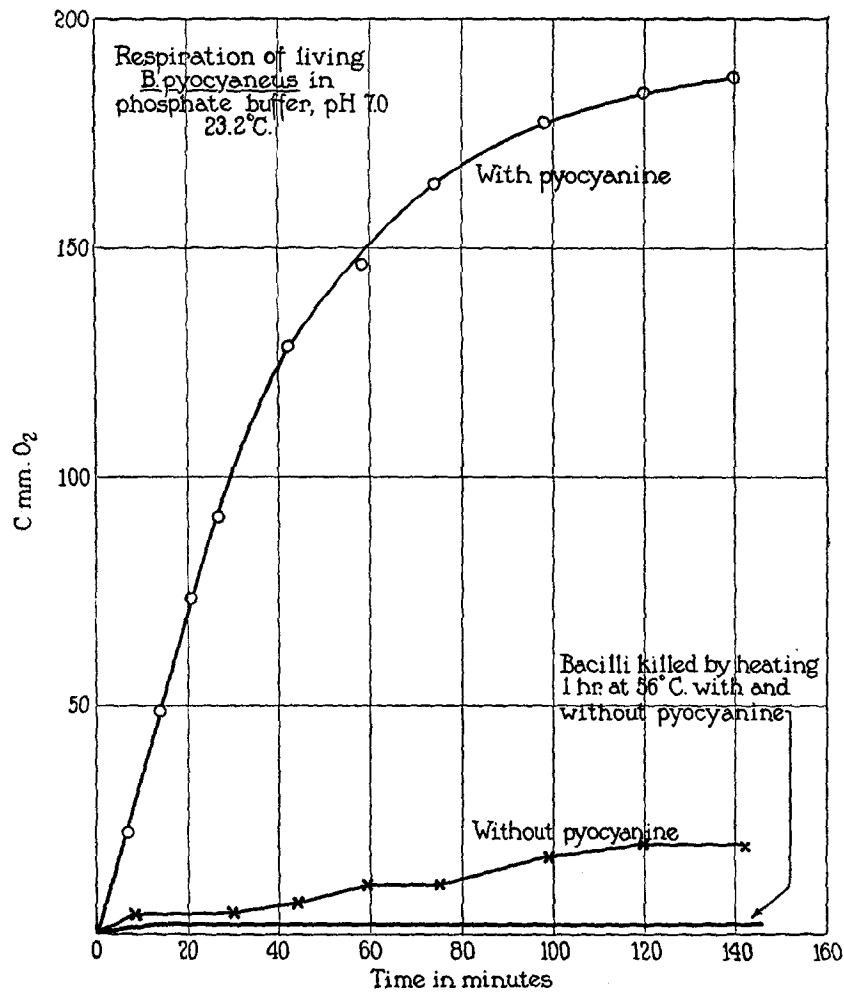


FIG. 3

Effect of Pyocyanine on the Respiratory Quotient.—Is the increase of respiration induced by pyocyanine purely quantitative or does pyocyanine affect also the process of oxidation qualitatively? With a view

to elucidating this question the respiratory quotient was determined by the method of Warburg and Yabusoe (3). As shown in Table II, it was found that without pigment and even when appreciable amounts of oxygen are consumed, the formation of carbon dioxide is very small; but it is enhanced by the addition of pyocyanine to such extent that the respiratory quotient increases many times and approaches 1.0. It appears thus that the oxidations brought about by the bacteria without the pigment are to a great extent incomplete, but that they can be completed with its assistance.

Of What Substances Is the Oxidation Catalyzed by Pyocyanine?— Since in all the experiments described above the bacteria were freed through washing of all traces of the nutritive media, only such substances as are part of the microorganisms can come under consideration. This conclusion is sustained by the analysis of the curves show-

TABLE II
Respiratory Quotient of Washed B. pyocyaneus Suspended in Phosphate Buffer

With pyocyanine	Without pyocyanine	Temp.	pH
		°C.	
0.77	0.17	22.2	6.8
0.9	0.1	20.9	5.8

ing the oxygen consumption as a function of time. As Fig. 3 shows, the curve for oxygen consumption under the influence of pyocyanine progresses during the 1st hour in a straight line, *i.e.*, the consumption is directly proportional to time, but thereafter it falls off more and more, the curve tending to become parallel to the abscissa. But, in the presence of an assimilable substance, such as glucose, asparagin, or pyruvic acid, the curve of oxygen consumption continues to progress in a straight line (Fig. 4). It follows that the flattening of the curve just mentioned cannot be caused by a diminution of the partial oxygen pressure, but is the consequence of an exhaustion of oxidizable substances. Furthermore it cannot be that the oxidation of these added substances is catalyzed by pyocyanine because if they are added without pigment to the washed bacteria, the respiration also increases many times. Under such circumstances the subsequent addition of

pyocyanine does not appreciably increase the respiration which has already been enhanced. This falls in with the findings on the influence of higher temperature reported in a previous paragraph. It may

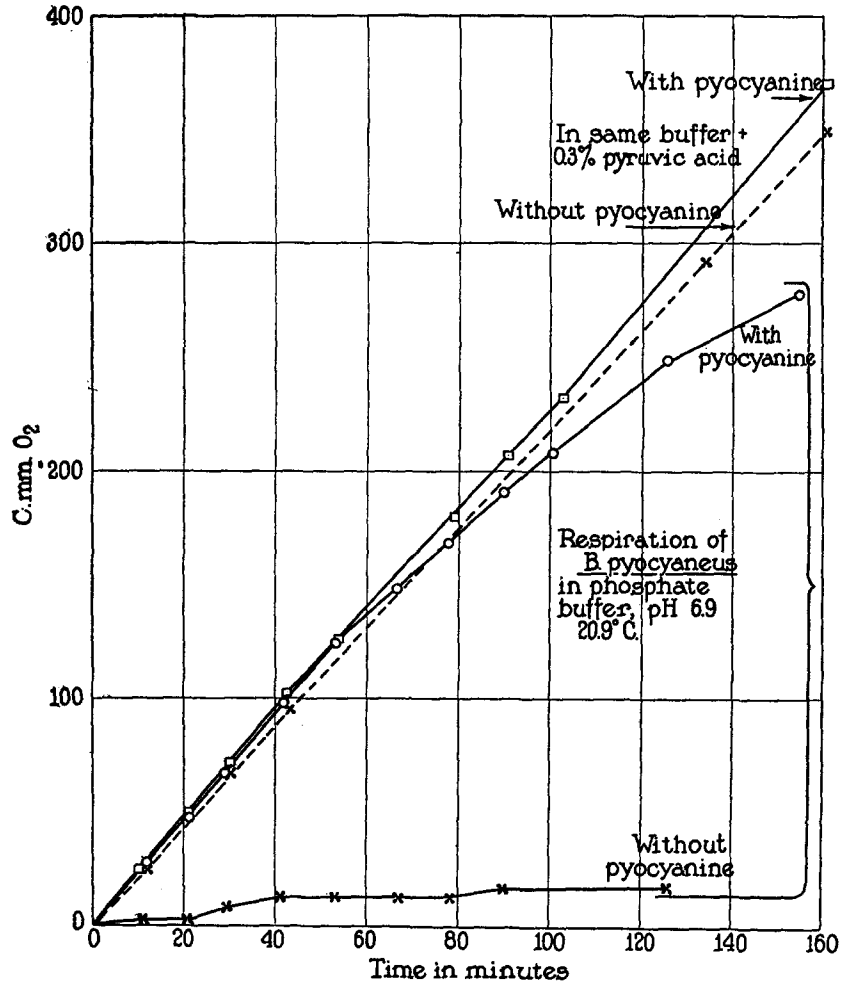


FIG. 4

be concluded that pyocyanine does not enhance respiration under all circumstances, but that it only catalyzes the oxidation of certain unknown substances closely associated with the bacteria, perhaps bac-

terial lipoids and polysaccharides, or certain products of their disintegration.

The Significance of Pyocyanine as a Respiratory Enzyme.—The fact that *B. pyocyaneus* has an appreciable oxidative metabolism even in the absence of pyocyanine tends to indicate that the latter does not play the main rôle in the respiration of the bacteria. Also further experiments show that pyocyanine is an accessory ferment, the effect of which is dependent upon the presence of one or several other respira-

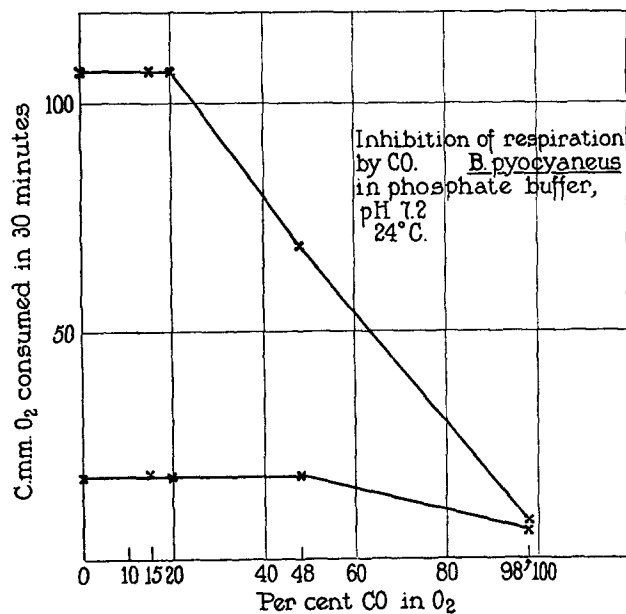


FIG. 5

tory ferments, amongst which must be considered the cytochrome shown spectroscopically by Yaoi and Tamiya (4) to be present in *B. pyocyaneus*. Their observation has been fully confirmed in the course of these experiments.

In a test-tube experiment at pH > 5, the oxidation and reduction of leucopyocyanine is in no way impeded by carbon monoxide or by potassium cyanide. But, as shown in Figs. 5 and 6, these substances, known readily to form complexes with heavy metals, impede not only the basic respiration of the bacteria alone, but also the respiration due

to pyocyanine. Figs. 5 and 6 further reveal the curious phenomenon that the pyocyanine enhanced respiration is affected at a lower potassium cyanide and carbon monoxide threshold-concentration than is the basic respiration. As the association of different hemochromogens, known as cytochrome, does not combine with carbon monoxide

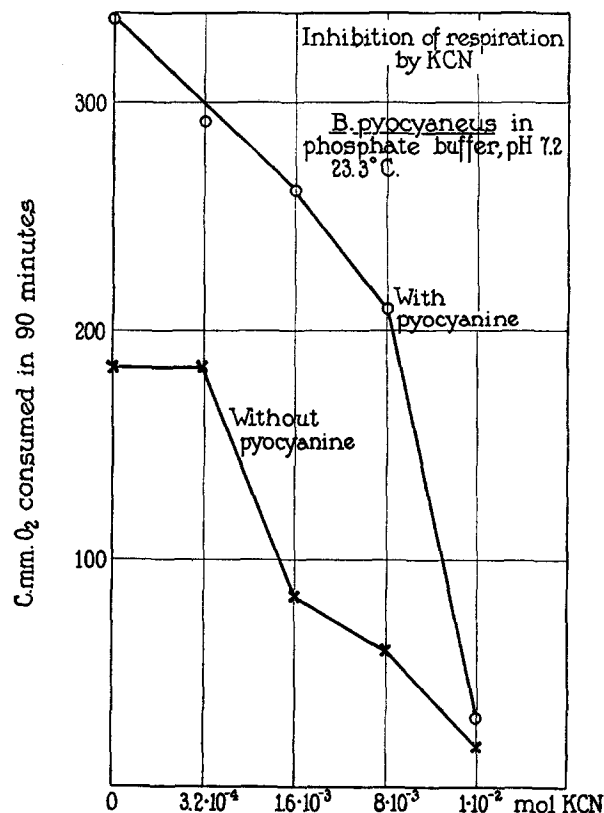


FIG. 6

(at physiological pH) the observation that the respiration of *B. pyocyaneus*, with and without pyocyanine, is impeded by carbon monoxide indicates that the cytochrome, although it may be a necessary link in the chain of bacterial oxidation catalysts, is not the final one, and that that chain is not complete without the carbon monoxide sensitive respiratory enzyme of Warburg.

The increase of respiration induced by pyocyanine is not specific for the species *B. pyocyaneus*. It has been observed in varying degree with other microorganisms containing cytochrome, *i.e.*, with staphylococci (Fig. 7) and pneumococci (Fig. 8); whereas with obligatory

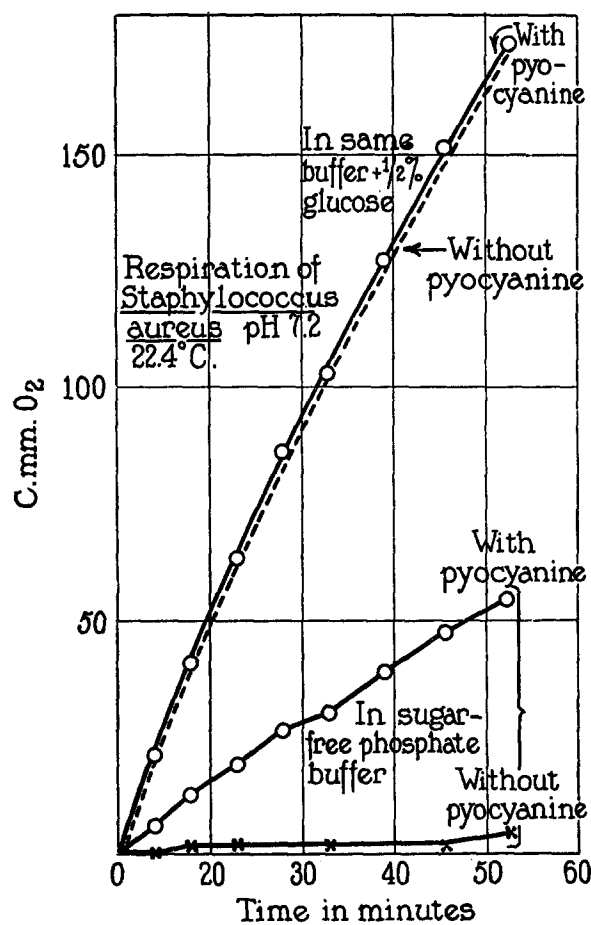


FIG. 7

anaerobics such as *B. tetani*, which do not contain any cytochrome, pyocyanine had no effect. Furthermore, its effect is not restricted to bacteria only, but may be observed also with animal cells. Fig. 9 shows the increase in respiration induced by pyocyanine in red blood corpuscles of the rabbit.

The fact that pyocyanine is to a large degree not species-specific makes reasonable the view that the characteristic properties which condition its significance as an oxidation catalyst are of a purely physi-

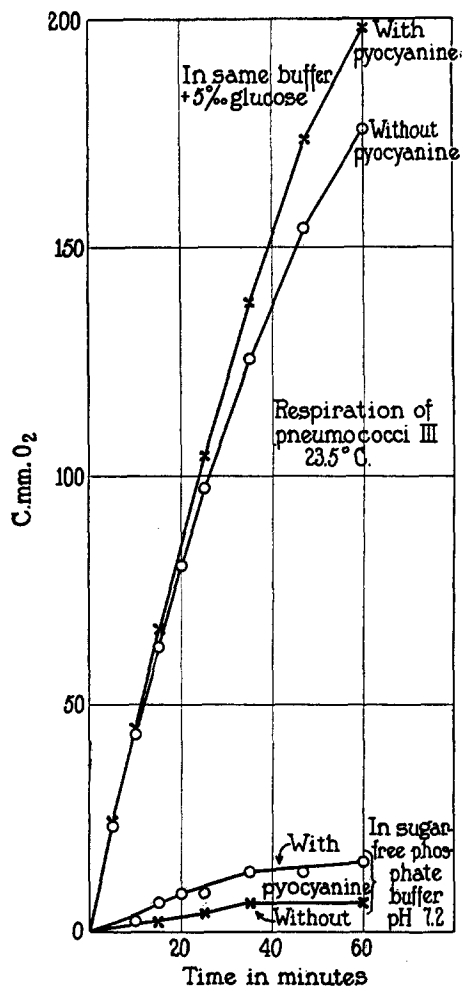


FIG. 8

cochemical nature, the decisive feature being reversibility. If this is so one may expect that any other reversible system of the same potential range would have the same biological effect, granting that it has no other properties, such as solubility, permeating propensity,

toxicity, which might act as counteracting influences. Pyocyanine seems to be favored in respect to these secondary chemical properties by its low affinity for proteins and its high affinity for lipoids. Of the

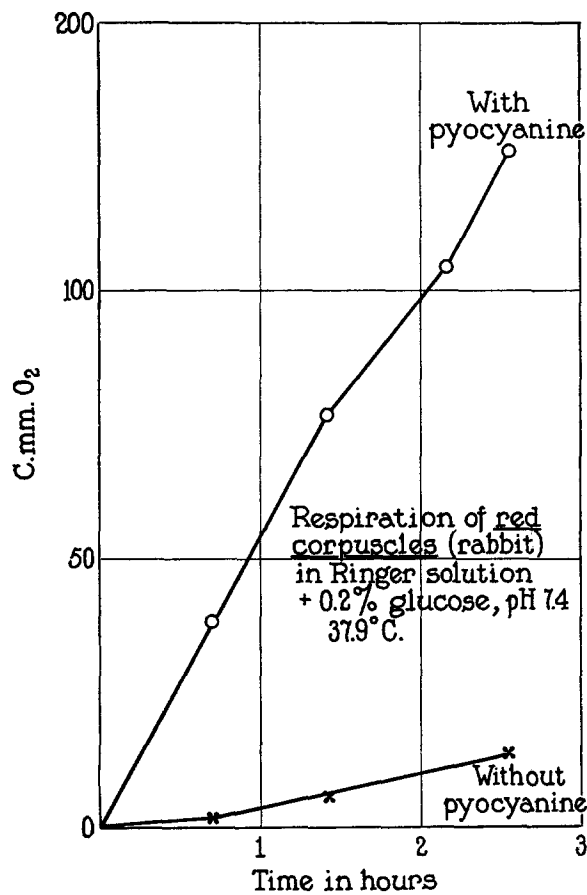


FIG. 9

synthetic organic oxidation-reduction systems methylene blue and indigo tetrasulfonate are closest to pyocyanine in their potential range. At pH 7.0 and 30°C. the normal potentials are:

	mv.
Indigotetrasulfonate.....	-46
Pyocyanine.....	-34
Methylene blue.....	+11

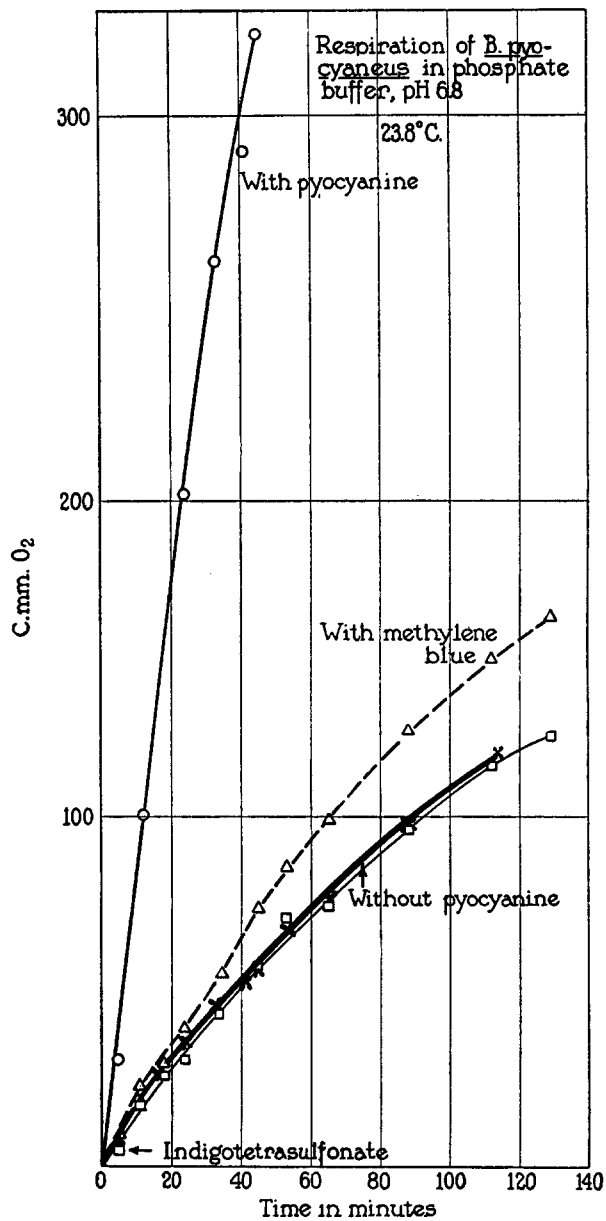


FIG. 10

Fig. 10 shows the comparative effect exerted by these three substances in equal molecular concentrations $\left(\frac{\text{Mol}}{5,000}\right)$ on the respiration of *B. pyocyaneus*. Whereas indigotetrasulfonate with a potential range more negative than that of pyocyanine is devoid of effect the more positive methylene blue induces an increase of 30 per cent, and pyocyanine itself an increase of 440 per cent. In connection with the significance to be attributed to the value of the normal potential of the catalyst there is one obvious condition: It must be more positive than the reduction potential of the compound of which it catalyzes the oxidation. The findings just given would seem to indicate that the potential range of pyocyanine represents the lower limit of the potential range in which hydrogen can be accepted from the bacteria.

The experiment with methylene blue links this work with earlier observations showing that methylene blue enhances not only the respiration of acetone-treated yeast and staphylococci (Meyerhof (5)), but also the respiration of living cells as *e.g.* red and white blood cells, sea urchin and star fish eggs, and cancer cells (Barron and coworkers (6)). These observations disclosed the possibility of biological oxidations being catalyzed by autooxidizable hydrogen acceptors. The search for substances in the living cell which act like the artificial model, methylene blue, led to the isolation of two kinds of substances; First, a group having the character of reversible systems (echinochrome, hermidin) but not as yet proved to play an actual rôle as respiratory enzymes of the living cell; second, the respiratory supplement discovered by Michaelis and Salomon (7) in extracts of various organs, especially liver, which has a mode of action not yet shown to be that of a reversible system.

SUMMARY

Pyocyanine, the blue pigment of *B. pyocyaneus*, can increase the respiration of living cells to a great degree (maximum observed increase 24-fold). The reversibility of its oxidation and reduction is responsible for this. The effect is non-species-specific and has been observed in varying degrees with *B. pyocyaneus*, *Staphylococcus aureus*, *Pneumococcus* Type III, and the red blood corpuscles of rabbits.

The effect of pyocyanine is dependent on the presence of another

respiratory ferment sensitive to potassium cyanide and carbon monoxide.

The increase of respiration induced by pyocyanine is paralleled by an increase in the respiratory quotient. The pyocyanine catalysis is not indiscriminately effective in all oxidations, but only in the oxidation of certain substances closely associated with the bacterial body.

I wish to express my sincere thanks to Dr. L. Michaelis, in whose laboratory this work was carried out, for many helpful suggestions and criticisms.

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