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Mechanism of the Antibiotic Action of Pyocyanine

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Exposure of *Escherichia coli* growing in a rich medium to pyocyanine resulted in increased intracellular levels of superoxide dismutase and of catalase. When these adaptive enzyme syntheses were prevented by nutritional paucity, the toxic action of pyocyanine was augmented. The antibiotic action of pyocyanine was dependent upon oxygen and was diminished by superoxide dismutase and by catalase, added to the suspending medium. Pyocyanine slightly augmented the respiration of *E. coli* suspended in a rich medium, but greatly increased the cyanide-resistant respiration. Pyocyanine was able to cause the oxidation of reduced nicotinamide adenine dinucleotide, with O_2^- production, in the absence of enzymatic catalysis. It is concluded that pyocyanine diverts electron flow and thus increases the production of O_2^- and H_2O_2 and that the antibiotic action of this pigment is largely a reflection of the toxicity of these products of oxygen reduction.

Pseudomonas aeruginosa is a widely distributed opportunistic pathogen. It secretes proteins which are toxic to a wide range of organisms (23), and, when grown aerobically in a phosphate-poor medium, it also secretes copious amounts of phenazine pigments (3, 8, 30). Phosphate depletion within *P. aeruginosa* has been suggested to be the initiator of phenazine biosynthesis (8, 18, 19). The major phenazine produced by this organism is pyocyanine (1-hydroxy-5-methyl phenazine), and its physiological significance has long been a mystery (35).

Interest in pyocyanine derives from its intense color, which makes its presence so obvious, from its antibiotic properties (22, 31, 34, 37), and from the correlation between its production and pathogenicity (20). Furthermore, the antibiotic cyanomycin from *Streptomyces cyanoflavus* (12) has been identified as pyocyanine (33). Pyocyanine can accept a single electron, yielding a relatively stable anion radical (28, 29), and readily undergoes a redox cycle (6, 9, 11, 26, 28, 29, 38). We have previously noted that pyocyanine induced the biosynthesis of the manganese-containing superoxide dismutase (MnSOD) in *Escherichia coli* and we inferred that it caused enhanced production of O_2^- (17, 17a; H. M. Hassan and I. Fridovich, *in* T. E. King, H. S. Mason, and M. Morrison, ed., *Oxidases and Related Redox Systems*, in press). It appeared possible that the antibiotic action of pyocyanine might actually be an expression of the toxicity of the O_2^- and of H_2O_2 produced in increased amounts in its presence. The results reported below strongly suggest this possibility.

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MATERIALS AND METHODS

E. coli B B_{12}^- (ATCC 29682) was grown aerobically at 200 rpm and at 37°C in a glucose-salts medium ± 0.5% yeast extract or in Trypticase-soy-yeast extract (TSY), as previously described (17). Growth was monitored turbidimetrically at 500 nm, rather than at 600 nm, to minimize interference from the optical absorbance of pyocyanine. Anaerobic growth was performed in cuvettes which allowed the medium to be scrubbed for 2 h with a stream of pure N_2, prior to sealing and inoculation (17). Specific growth rates and generation times were calculated as described before (14). Antibiotic effects were assessed by a filter paper disk method. Sterile Whatman no. 1 disks, loaded with graded amounts of pyocyanine, were placed onto a glucose-minimal medium seeded with *E. coli* and solidified with 0.7% agar. This soft agar rested upon a layer of glucose-minimal medium solidified with 2% agar. Test cultures were incubated for 24 h at 37°C, either aerobically or in a Coy anaerobic chamber, whose atmosphere contained less than 5 μl of dioxygen per liter.

P. aeruginosa ATCC 9027 was grown at 37°C and at 200 rpm in a phosphate-limited medium (19) which was modified to contain, per liter: DL-alanine, 8.0 g; L-leucine, 8.0 g; lithium lactate, 3.0 g; $MgSO_4$, 2.0 g; K_2HPO_4, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; and Tris, 6.1 g. When phosphate excess was desired it was achieved by adding K_2HPO_4 to 2% (wt/vol). In all cases the medium was adjusted to pH 7.1.

Soluble extracts were prepared (13) and assayed for superoxide dismutase (SOD) (27) and for SOD isoenzymes (13) as previously described (1). Catalase was assayed by the method of Beers and Sizer (2), and *E. coli* hydroperoxidases were separated by polyacrylamide gel electrophoresis and visualized as described

before (16). Protein was estimated by the Lowry method (24). Respiration of cell suspension was measured polarographically with and without pyocyanine ± cyanide (17a). Rates of O_2^- production were measured in terms of SOD-inhibitable reduction of nitro blue tetrazolium (1). Pyocyanine from Pharm-Eco-Labs, Inc. of Simi Valley, Calif., was twice recrystallized according to Frank and DeMoss (8) and was assayed based upon $E^{1\%}_{1\,cm}$ = 117 at 520 nm in 0.2 N HCl (25).

RESULTS

Nutritional modification of pyocyanine toxicity. Paraquat causes enhanced production of O_2^- within *E. coli*, and the cells adapt by increased synthesis of MnSOD (15, 17). Any condition that interfered with rapid protein synthesis prevented this adaptive enzyme induction and enhanced the lethality of paraquat (17). *E. coli* cells were thus much more susceptible to paraquat lethality in a glucose-salts medium than they were in richer media (17). If pyocyanine action also depends upon increased production of O_2^- and H_2O_2, then *E. coli* should be more susceptible to its antibiotic action in a minimal medium which limits the rate of MnSOD synthesis. Figure 1 demonstrates that the growth of *E. coli* was suppressed by pyocyanine in a glucose-minimal medium and that addition of yeast extract markedly diminished this growth suppression. The toxicity of pyocyanine was similarly minimized in a TSY medium. Waksman and Woodruff (34) had previously noted that the antibiotic action of pyocyanine was dependent upon the growth medium, but did not offer an explanation.

The ability of pyocyanine to induce the synthesis of SOD and of catalase in *E. coli* growing in a glucose-minimal medium ± 0.5% yeast extract was explored. There was no induction of SOD in the minimal medium (Table 1). Supplementation with yeast extract permitted marked inductions of both SOD and catalase activities, with the catalase level going up more dramatically than the SOD level at the higher levels of pyocyanine. As expected, the effect of pyocyanine on the generation time (*G*) was the inverse of its effects on SOD and catalase. Thus, when induction of these protective enzymes was prevented by the nutritional paucity of the medium, pyocyanine markedly increased the generation time. In contrast, when enrichment of the medium with yeast extract permitted large induction of the protective enzymes, the growth rate was much less affected by pyocyanine.

Dioxygen dependence of pyocyanine toxicity. Biological production of O_2^- and H_2O_2 is possible only in the presence of dioxygen. If the antibiotic action of pyocyanine is due to increased production of O_2^- and H_2O_2, then its adverse effects on *E. coli* should be dioxygen

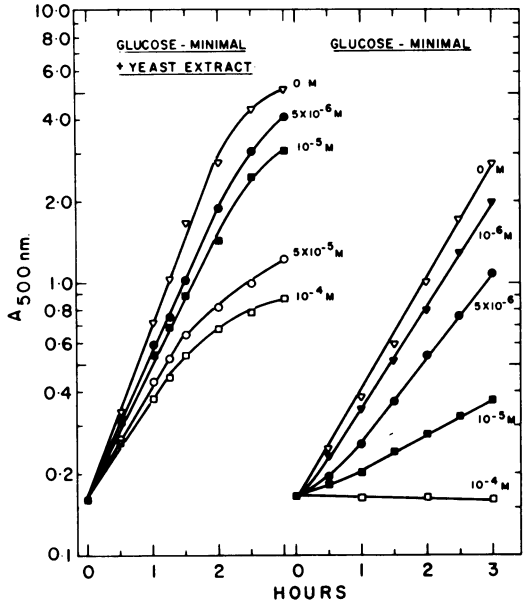

FIG. 1. *Effects of media composition on pyocyanine toxicity. E. coli B cells, grown aerobically to late logarithmic growth phase in a glucose-minimal medium, were diluted to an initial absorbance of 0.16 at 500 nm into fresh media containing the indicated concentrations of pyocyanine. Yeast extract (0.5%) was added to the glucose-minimal medium where indicated. The cultures were incubated at 37°C on a rotary water-bath shaker at 200 rpm. Growth was monitored in terms of absorbance at 500 nm. Absorbances greater than 1.0 were measured upon diluted samples with correction for the dilution factor.*

TABLE 1. *Correlation between growth of E. coli B in different media in the presence of pyocyanine with SOD and catalase synthesis*[a]

Pyocyanine (M)	Glucose-minimal medium			Glucose-minimal medium + YE		
	G (min)	SOD (U/mg)	Catalase (U/mg)	*G* (min)	SOD (U/mg)	Catalase (U/mg)
0	43	6.9	12.0	28	9.6	20.1
1×10^{-6}	49	4.6	9.7	—	—	—
5×10^{-6}	60	3.4	12.3	33	11.2	22.4
1×10^{-5}	145	4.1	26.4	38	13.0	27.1
5×10^{-5}	∞	—	—	42	20.8	257.8
1×10^{-4}	∞	—	—	48	34.2	358.4

[a] Cells were from the experiment reported in Fig. 1. Cells were sonicated, and dialyzed cell-free extracts were assayed for SOD and catalase activities. YE, Yeast extract; *G*, generation time. —, No data.

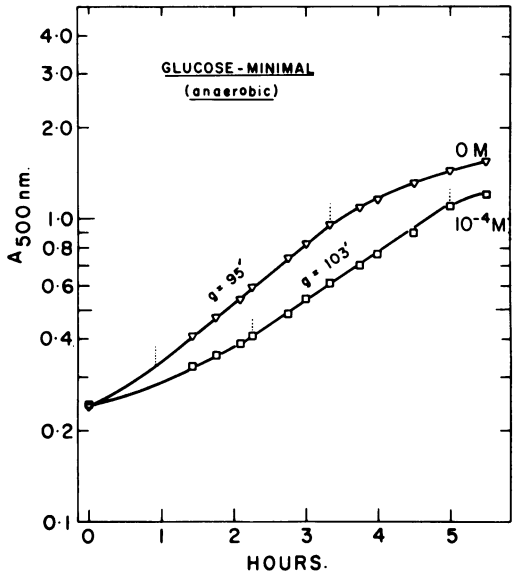

FIG. 2. *Effects of anaerobiosis on the toxicity of pyocyanine. Special anaerobic cuvettes, filled with the glucose-minimal medium plus the indicated concentrations of pyocyanine, were flushed with a stream of oxygen-free nitrogen for 2 h prior to sealing and inoculation by tipping in the inoculum from the side arm. The inocula were from a late-log culture grown anaerobically in glucose-minimal medium. The cuvettes were incubated at 37°C, and growth was monitored at 500 nm. g, Generation time in minutes (').*

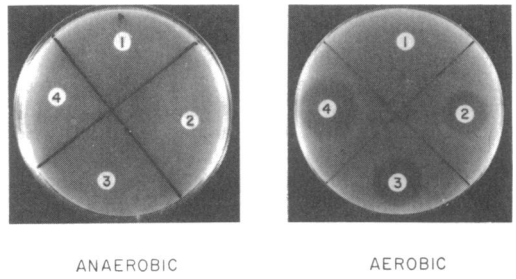

FIG. 3. *Effect of anaerobiosis on the antibiotic action of pyocyanine. E. coli B cells from a 24-h culture (stationary-phase cells) growing in glucose-minimal medium were incorporated into the soft agar overlay and used to seed the glucose minimal agar plates. Sterile filter paper disks containing (disk 1) 0, (disk 2) 4.2, (disk 3) 8.4, or (disk 4) 21 μg of pyocyanine were placed on the top of the solidified soft agar. One set of plates was incubated aerobically, and another set of plates was incubated anaerobically in a Coy chamber. The plates were incubated for 24 to 48 h at 37°C before the results were recorded.*

dependent. That this was the case is documented by the data in Fig. 2 and 3. Figure 2 presents the growth of *E. coli* in anaerobic glucose-minimal medium in the absence and in the presence of 0.1 mM pyocyanine. This level of pyocyanine, which completely suppressed growth in aerobic minimal medium (Fig. 1), caused only a slight increase in the generation time in the anaerobic medium. Indeed, most of the effect of pyocyanine seen in the anaerobic cuvettes (Fig. 2) was probably due to residual traces of dioxygen. In Fig. 3 the antibiotic action of pyocyanine, by the filter paper disk assay, was compared aerobically and anaerobically. The antibiotic activity, seen aerobically, vanished in the absence of dioxygen.

Effects of pyocyanine on SOD and catalase isoenzymes. *E. coli* contains three forms of SOD (13) and two hydroperoxidases (16). Thus, there are superoxide dismutases based upon iron (FeSOD) (36) and upon manganese (MnSOD) (21), and there is a hybrid which, although containing iron, is composed of one subunit from FeSOD and one subunit from MnSOD (7). Previous work has shown that the FeSOD is constitutive, being present even in anaerobically grown cells, whereas the MnSOD is under repression control and is made in response to intracellular O_2^- production. Both of the hydroperoxidases of *E. coli* are effective catalases, but hydroperoxidase I is also an active general peroxidase (4). Aerobic growth in the presence of pyocyanine led to a marked increase in MnSOD with a concomitant slight decrease in FeSOD (Fig. 4A). This is consistent with previous work in which the intracellular O_2^- production was raised by a variety of conditions (17a). The slight decrease in FeSOD probably results from a partial depletion of FeSOD, due to greater production of the hybrid SOD in the presence of higher levels of the MnSOD. Both hydroperoxidases were increased during aerobic growth in the presence of pyocyanine (Fig. 4B; Table 1). Pyocyanine was thus qualitatively similar to paraquat in its effects on SOD and catalase in *E. coli*. There were, however, quantitative differences. Paraquat caused a relatively greater induction of SOD (15), whereas pyocyanine caused a relatively greater induction of hydroperoxidases.

Effects of pyocyanine on cell respiration. *E. coli*, grown for 2 h in aerobic TSY medium, was collected by centrifugation, suspended in 50 mM potassium phosphate–0.1 mM $MgSO_4$ at pH 7.0, and then placed under a Clark oxygen electrode. Such cells respired vigorously when TSY medium was added, and the respiration was largely inhibited by 2 mM cyanide (Fig. 5A). Pyocyanine, added after the cyanide, relieved that inhibition. Figure 5b shows the rate of

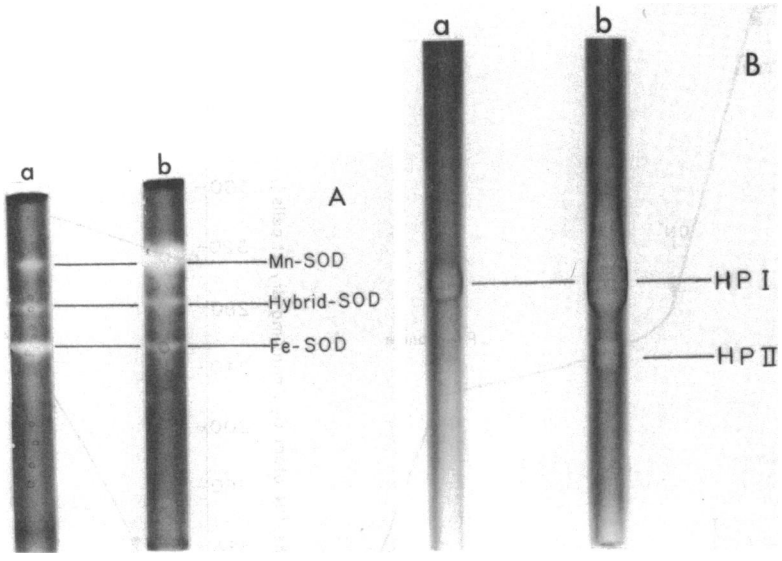

FIG. 4. *(A) Effect of pyocyanine on the synthesis of SOD isozymes. E. coli B was grown aerobically for 3 h in TSY medium with and without 0.1 mM pyocyanine. Dialyzed cell-free extracts were prepared, and samples containing equal amounts of proteins (250 μg) were applied to 10% polyacrylamide gels. After electrophoresis the gels were stained for activity. Gel a was obtained from the extracts of cells grown in the absence of pyocyanine, whereas gel b was from the corresponding culture in its presence. (B) Effect of pyocyanine on the synthesis of catalase isozymes. E. coli B was grown for 3 h in glucose-minimal medium containing 0.5% yeast extract with and without 0.1 mM pyocyanine. Gel a, 500 μg of protein from extracts of cells grown in absence of pyocyanine; gel b, 250 μg of protein from the corresponding culture grown in its presence.*

respiration, ±2 mM cyanide, as a function of pyocyanine. Pyocyanine slightly augmented normal respiration, but grossly increased the cyanide-resistant respiration. Pyocyanine has been reported to increase the respiration of mammalian cells (10, 32) and to reverse the cyanide inhibition of respiration (5).

Mechanism of O_2^- generation by pyocyanine. *E. coli* contains a soluble diaphorase, which catalyzes the reduction of paraquat by NADPH. This diaphorase is presumed to be the site of diversion of intracellular electron flow by paraquat (17b). Paraquat has been shown to generate O_2^- with NADPH as the electron donor only if this diaphorase is present (17b). In contrast, pyocyanine appears capable of direct interaction with NADH. Thus, pyocyanine catalyzed the reduction of nitro blue tetrazolium by NADH, and this reduction was largely inhibited by SOD (Fig. 6). Phenazine methosulfate has been reported to exert a similar effect (30).

Extracellular events in the antibiotic action of pyocyanine. Most of the paraquat reduced within *E. coli* reacts with dioxygen to generate O_2^- before it can diffuse from the cell. This is a consequence of the fact that its reaction with dioxygens is extremely rapid, i.e., $k = 7.7 \times 10^8\ M^{-1}\ sec^{-1}$. Some paraquat radical does, however, diffuse from the cell, especially when rapid respiration depletes intracellular dioxygen. Under such conditions extracellular O_2^- production occurs by reoxidation of the effused paraquat radical (17b). The *E. coli* cell envelope appears to be impermeable to O_2^-, and although extracellular O_2^- can damage the cell, it cannot cause induction of MnSOD synthesis (17b). If reduced pyocyanine were to react with dioxygen more slowly than does the paraquat radical, it would escape from the cell to a greater degree and thus lead to more extracellular O_2^- production. Extracellular O_2^- would there dismute, giving rise to H_2O_2, which could enter the cell and stimulate the synthesis of catalase. This could explain the observation that pyocyanine induces catalase more than SOD, whereas paraquat induces SOD more than catalase. Extracellular O_2^- and H_2O_2 could also make a large contribution to the lethality of pyocyanine. Either SOD or catalase added to the medium was able to partially eliminate the antibiotic effect of pyocyanine, and SOD plus catalase was even more effective (Fig. 7). These protective effects by exogenous SOD and catalase indicate that extracellular O_2^- and H_2O_2 were important factors in

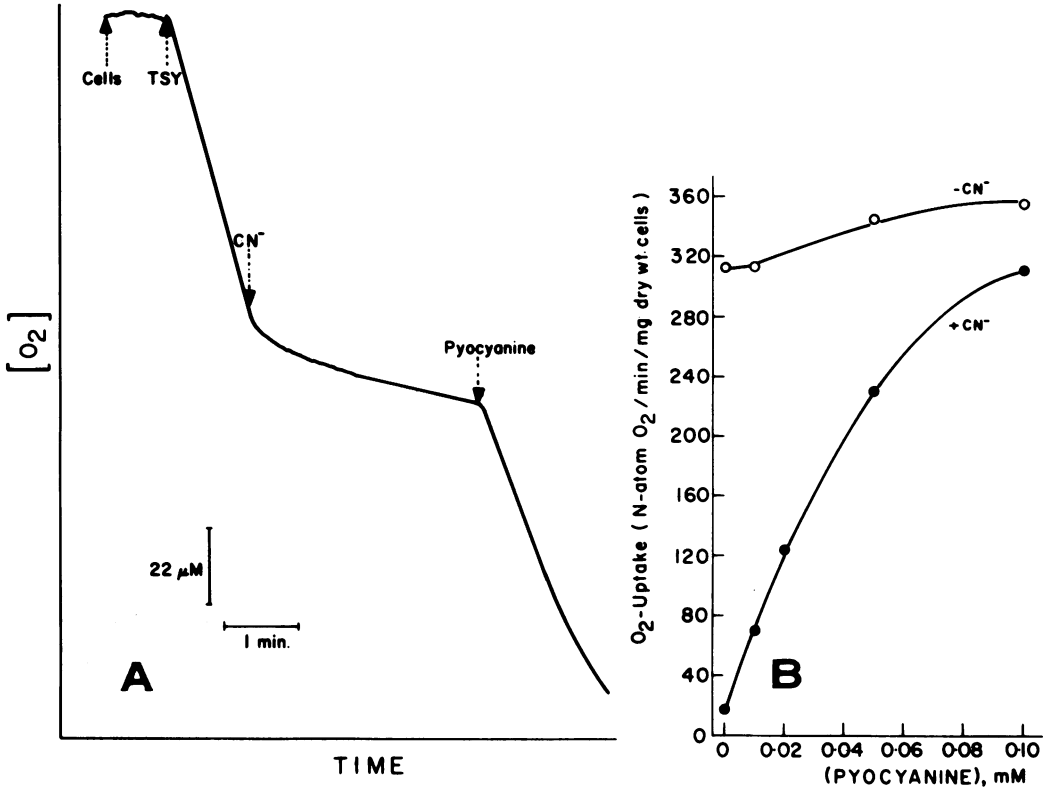

FIG. 5. *(A) Effect of pyocyanine on the rate of cyanide-resistant respiration in E. coli. Cells were grown aerobically for 2 h at 37°C in TSY medium, collected by centrifugation, and suspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$. The reaction mixture contained 0.05 ml of cells (0.56 mg [dry weight] of cells), 0.1 ml of fresh TSY, and 50 mM potassium phosphate (pH 7.0) to a final volume of 2 ml. Where indicated, cyanide was added to a 2.0 mM final concentration. Pyocyanine was added to 0.05 mM after inhibition by CN^- was established. (B) Effects of pyocyanine on cyanide-sensitive and cyanide-resistant respiration. Conditions were the same as in (A) except that different concentrations of pyocyanine were tested. Oxygen uptake is presented in the ordinate as nanoatoms of O_2 consumed per minute per milligram (dry weight) of cells.*

the antibiotic action of pyocyanine.

Another way to account for the relatively greater induction of catalase than of SOD by pyocyanine would be to propose that it undergoes a predominantly divalent redox cycle within the cell. Although this remains possible, it could not account for the protective effects of extracellular SOD and of catalase, shown in Fig. 7.

Pyocyanine and *P. aeruginosa*. If *P. aeruginosa* uses pyocyanine production to its advantage in competing with other bacteria in the same ecological habitat, it must therefore have a mechanism to insure its own protection or immunity against the bactericidal agent it produces. This immunity could be via higher concentrations of SOD and catalase or by lack of permeability. We tested these possibilities. *P. aeruginosa* made 62% higher catalase when grown under conditions conducive for pyocyanine production (Table 2). However, the level of SOD was slightly lower. We also checked the effect of pyocyanine on the rate of cyanide-resistant respiration in *P. aeruginosa* and found it to be nonresponsive to pyocyanine. The respiration of *P. aeruginosa* was generally resistant to cyanide; thus, 8 to 10 mM was required to inhibit the respiration by 91.3%, and 0.134 mM pyocyanine caused this cyanide-resistant respiration to rise from 8.7 to 14.8%. This increase is very moderate compared to that seen in *E. coli* (Fig. 5). These results tentatively indicate that *P. aeruginosa* is not as permeable to pyocyanine as is *E. coli*, and they show that the organism

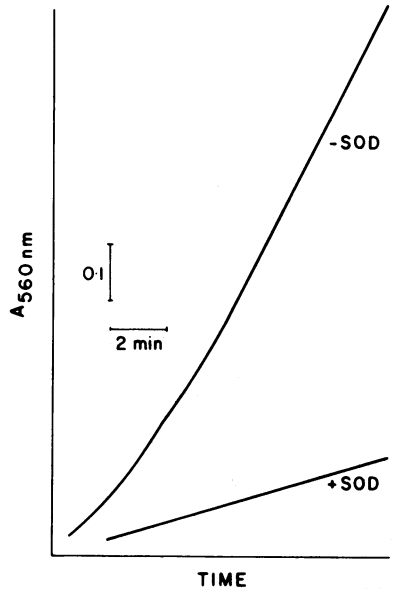

FIG. 6. *Pyocyanine and the generation of superoxide radical (O_2^-). The ability of pyocyanine to generate O_2^- was estimated in terms of SOD-inhibitable reduction of nitro blue tetrazolium. Reaction mixtures contained 0.44 mM NADH, 0.16 mM nitro blue tetrazolium, 40 μM pyocyanine, ±10 μg of the bovine copper-zinc SOD, and 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA, to a total volume of 3 ml. nitro blue tetrazolium reduction was followed at 560 nm and 25°C in a double-beam spectrophotometer in cuvettes with 1-cm path.*

makes higher catalase to protect against H_2O_2 that might be generated outside the cells via extracellular autooxidation of pyocyanine. We presume that *P. aeruginosa* actively secretes pyocyanine while keeping its intracellular level low by a combination of low permeability and active extrusion.

DISCUSSION

Pyocyanine increases the cyanide-resistant respiration of *E. coli* and causes increases in the biosynthesis of SOD and of catalase. We infer that pyocyanine can divert the electron flow within this organism from the normal cytochrome pathway to an O_2^-- and H_2O_2-producing pathway. Since pyocyanine, per se, catalyzes the SOD-inhibitable reduction of nitro blue tetrazolium by NADH, it appears that direct reduction of pyocyanine by NADH provides the means for this diversion of the electron flow. The antibiotic action of pyocyanine appears to be largely due to the toxicity of the O_2^- and H_2O_2 it engenders. Thus, (i) molecular oxygen is essential for expression of this antibiotic action; (ii) rapid induction of SOD and catalase in rich media markedly decreases the toxicity of aerobic pyocyanine; and (iii) addition of SOD and catalase to the suspending medium provides protection against the antibiotic effect.

TABLE 2. *SOD and catalase activity in P. aeruginosa ATCC 9027*[a]

Growth condition	SOD (U/mg)	Catalase (U/mg)
Phosphate limited	38.1	541.8
Phosphate unlimited	43.3	333.8

[a] *P. aeruginosa* were grown for 36 h in minimal medium (4) at 37°C and 200 rpm.

The quantitative differences between pyocyanine and paraquat can be explained in terms of differences in rates of reaction of their univalently reduced forms with dioxygen. If the pyocyanine radical reacted more slowly than the paraquat radical it would, to a greater extent, escape from the cell before giving rise to O_2^-. Hence a greater fraction of O_2^- production would be extracellular with pyocyanine than was the case with paraquat. Since *E. coli* appears to be impermeable to O_2^-, this would have the consequence that H_2O_2, generated by dismutation of O_2^- in the medium, would reenter the cell and cause induction of catalase. In toto, one would then see greater induction of catalase than of SOD with pyocyanine and the reverse with paraquat. Because the paraquat radical reacts with O_2^- at an almost diffusion-limited rate, it is easy to imagine that the corresponding reaction of the pyocyanine radical is slower. Alternatively, one could propose that the autooxidation of reduced paraquat gives rise mostly to O_2^-, whereas the autooxidation of reduced pyocyanine gives rise mostly to H_2O_2. We do not, at present, have the data for definitely distinguishing between the explanations, but the marked protection offered by exogenous SOD and catalase certainly supports the first proposal.

What advantages are gained for *P. aeruginosa* by the secretion of pyocyanine? When growing in the presence of other cells this organism could gain nutrients by eliminating competition and could even gain access to the nutrients already locked up inside the other cells, by killing them. The fact that starvation for phosphate triggers pyocyanine production supports this interpretation. Dioxygen, which is essential for the mechanism of antibiotic action supported by our work, is also needed for production of pyocyanine. *P. aeruginosa* thus makes pyocyanine when phosphate deficiency indicates the need to eliminate competing cells and when dioxygen is

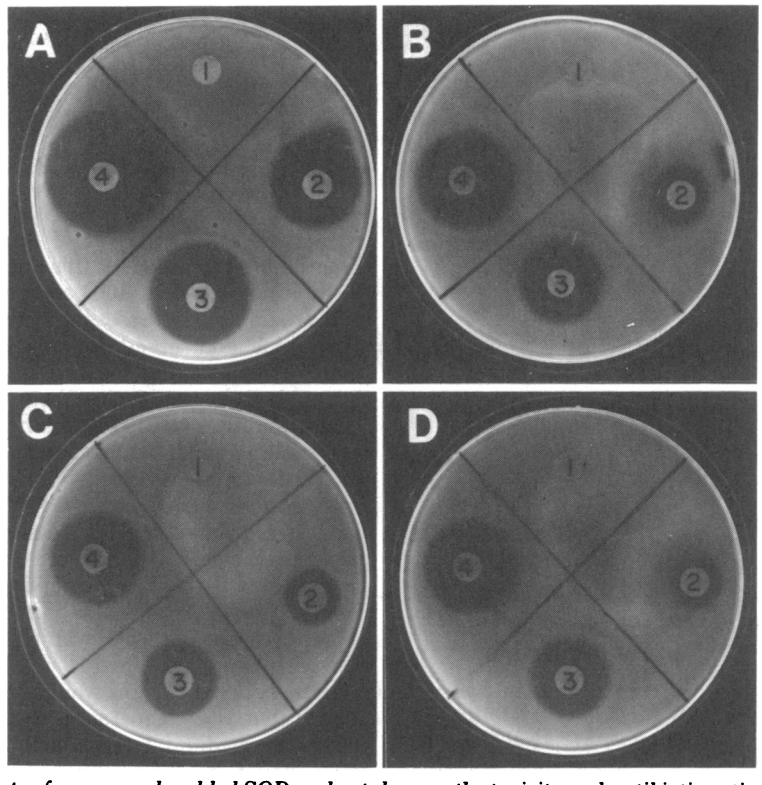

FIG. 7. *Effects of exogenously added SOD and catalase on the toxicity and antibiotic action of pyocyanine. E. coli B cells from 24-h culture (stationary-phase cells) growing in glucose-minimal medium were used to seed the glucose-minimal agar plates. Catalase (0.21 mg per plate) and copper-zinc SOD (0.5 mg per plate) were incorporated with E. coli cells into the soft agar overlay. Sterile filter-paper disks containing different concentrations of pyocyanine were placed on the top of the solidified soft agar. Plates were incubated in air at 37°C for 24 to 48 h before recording the results. (Disk 1) 0, (disk 2) 8.4, (disk 3) 21, and (disk 4) 42 μg of pyocyanine. (A) Control; (B) SOD; (C) catalase; (D) SOD plus catalase.*

present to support the antibiotic action of this dye.

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