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Effect of Respiratory Inhibitors on the Motility of *Pseudomonas fluorescens*

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The translational motility of *Pseudomonas fluorescens* was weakly inhibited by oligomycin, Dicumarol, 2,4-dinitrophenol, 2*n*-heptyl-4-hydroxyquinoline *N*-oxide, and potassium cyanide. Atabrine and antimycin A together with potassium cyanide immediately immobilized this bacterium, but antimycin A alone was without effect. Gramicidin D also immobilized *P. fluorescens*, but its action was inhibited by K^+ and NH_4^+ ions. In like manner, the effect of *p*-chloromercuribenzoate could be counteracted with cysteine, thereby suggesting the involvement of —SH groups in flagellar motility processes. It appears that the energy required for motility of *P. fluorescens* is generated by oxidative phosphorylation mediated by the cytochrome system.

There is some experimental evidence suggesting that generation of high-energy phosphate compounds, mainly adenosine triphosphate (ATP), is necessary for bacterial motility. Sherris et al. (24) reported that a strain of *Pseudomonas* actively motile under aerobic conditions ceased moving upon oxygen exhaustion, but motility could be resumed by initiating anaerobic metabolic pathways which generated ATP. Inhibitors and uncouplers affecting phosphorylation may also affect motility, but reported results are few and somewhat contradictory. Thiol inhibitors and 2,4-dinitrophenol (DNP), for example, were found to decrease motility of *Proteus vulgaris* and the motile spores of *Actinoplanes* (13, 23). Azide and cyanide, on the other hand, were without effect on the movement of *Salmonella typhimurium* (18), yet the former also immobilized *Actinoplanes* spores (13).

Considering the general role of ATP in biological motion, it has been speculated that bacterial motility also is dependent upon this compound (8, 9, 17, 29). The amount of energy required for bacterial movement is unknown, but it has been calculated that theoretically only a small fraction of the total generated by an organism ought to be needed (12, 22).

In view of the paucity of experimental evidence linking energy production and bacterial movement, a detailed study was undertaken to determine the effect of respiratory inhibitors on the motility of *Pseudomonas fluorescens*.

The cytochrome system of this bacterium is relatively well known, and the presence of cytochromes *b*, *c*, and $a + a_3$ in the respiratory chain has been established (28). Most workers (3-6, 10, 16, 28) who have studied the cytochrome system of bacteria used cell fractions, but in our studies of oxidative phosphorylation, in connection with movement, intact bacteria were used, since disruption of the integrity of the bacterial cell would, of course, cause movement to cease immediately.

MATERIALS AND METHODS

Bacteria studied. *P. fluorescens* (University of Maryland culture collection) was grown in a chemically defined medium with succinate as carbon source. Cultures were incubated at 30 C for 18 to 20 hr on an oscillating shaker operated at 60 strokes per min. The bacteria were actively motile under these conditions, but, in addition, a nonmotile (fla$^-$) mutant (27) was included as a control.

The medium contained (per liter): succinate, 5 g; $MgSO_4 \cdot H_2O$, 0.5 g; Na_2HPO_4, 1.0 g; KH_2PO_4, 0.5 g; $(NH_4)_2NO_3$, 2.5 g; $CaCl_2 \cdot 2H_2O$, 0.003 g; $FeCl_2 \cdot 6H_2O$, 0.003 g; $MnSO_4 \cdot H_2O$, 0.0001 g; and $Na_6MO_7O_{24} \cdot 4H_2O$, 0.001 g. The *p*H was adjusted to 7.2 with 0.1 N NaOH. Stock and working cultures were maintained on Cysteine Trypticase Agar (BBL). Cultures were transferred at monthly intervals, and were kept at room temperature (~20 C) between transfers.

Inhibitor preparation. Solutions of 1 mg of 2*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) per ml, $1.5 \times 10^{-}$ M Dicumarol, 10^{-3} M pentachlorophenol (PCP), and 1.5×10^{-3} M sodium amytal were prepared in 8 ml of distilled water, and 3 N KOH or 1 N HCl was added dropwise with vigorous stirring until clear solutions were obtained. The *p*H of each was adjusted to 7.2, and the volume was brought to 10 ml. The HQNO was adjusted to *p*H 8.5 before dilution. Other rela-

tively insoluble inhibitors, 1.5×10^{-3} M antimycin A, 1.5×10^{-3} oligomycin, and 1.5×10^{-3} M gramicidin D (GMCD), were dissolved in small quantities of acetone or ethyl alcohol (0.1 to 1.0 ml), and then brought to 10 ml with distilled water. The concentrations of inhibitors employed (Table 1) were arrived at after preliminary experiments had indicated that these levels generally lay between a null effect and complete inhibition of motility. Oligomycin, HQNO, GMCD, Atabrine, and Dicumarol were purchased from Sigma Chemical Co., St. Louis, Mo.; antimycin A and DNP were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and PCP was obtained from Eastman Organic Chemicals, Rochester, N.Y. All other compounds used were commercially available reagent grade.

Motility estimation. Motility determinations were made in modified Adler's buffer (2). Its composition was (per liter): K_2HPO_4, 2.28 g; KH_2PO_4, 1.36 g; ethylenediaminetetraacetic acid, 0.029 g; and $MgSO_4 \cdot H_2O$, 0.12 g (*p*H 7.0). Observations were made with a Zeiss photomicroscope, a bright-phase 63 × neofluar phase objective, and 12.5 × oculars. The counting procedure was that described by Shoesmith (25). One ocular was covered with an eyepiece having a pinhole aperture approximately 1 mm in diameter, and organisms in a wet mount suspension which moved past this field during 1 min were counted, the control suspension being considered "100% motile."

Inhibitor experiments. To determine the effect of inhibitors, oxygen uptake by *P. fluorescens* in their presence was determined. Bacteria were harvested from 100 ml of culture medium by centrifugation at 3,000 × *g* and washed twice in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 7.2; 14 to 16 mg (dry weight) of cells per ml was then suspended in Tris buffer. Oxygen uptake was measured with a Clark electrode equipped with a Teflon membrane-covered gold-silver electrode (model 777, Beckman Instrument Co., Fullerton, Calif.). To a jacketed glass reaction vessel were added 1 ml of Tris buffer (*p*H 7.2), 0.5 ml of inhibitor and 7 to 8 mg (dry weight) of cell suspension in 0.5 ml, giving a total volume of 2 ml. Inhibitors were added to the reaction vessel prior to introduction of the cell suspension. In control determinations, 1.5 ml of buffer was used together with 0.5 ml of cell suspension. Oxygen uptake was expressed as microatoms of O_2 per milligram (dry weight) per hour.

The uptake of inhibitors by the bacteria was ascertained by growth studies. *P. fluorescens* was cultivated in 3 ml of chemically defined medium containing one of the various compounds. Each tube was inoculated with 0.1 ml of actively growing culture, and the amount of inhibition was determined by measuring absorbance (450 nm) with a Spectronic-20 colorimeter (Bausch & Lomb, Inc., Rochester, N.Y.) after 18 hr of incubation at 30 C.

RESULTS

Effect of Atabrine. Atabrine, at the highest concentration tested (1.5×10^{-3} M), completely immobilized all bacteria within 2 min, but in a more dilute solution (1.5×10^{-4} M) it only partially inhibited movement (Fig. 1). The effect of Atabrine was nullified by the addition of 1.5×10^{-3} M riboflavine-5′-phosphate (FMN), which suggests that the inhibited site may be a flavoprotein. Although Atabrine dramatically inhibited motility, O_2 uptake was only partially inhibited. The inhibition of O_2 uptake ranged from 40 to 50%, depending upon the Atabrine concentration (Table 1).

Effect of uncouplers. GMCD was also an effective inhibitor of motility; a concentration of 1.5×10^{-3} M in Adler's buffer gave 100% inhibition, whereas a concentration of 1.5×10^{-5} M gave 50%. GMCD at a concentration of 1.4×10^{-4} M was more effective in distilled water than in Adler's buffer, but the inhibition caused by GMCD could be reversed by an equal amount of $(NH_4)_2NO_3$ (Fig. 2). Oxygen uptake was increased in the presence of GMCD (Table 1), whereas Dicumarol, DNP, and PCP all inhibited movement and O_2 uptake, to various limited degrees (Fig. 3 and Table 1).

Effect of antimycin A and potassium cyanide. KCN (2.5×10^{-2} M) reduced motility to 50% of the control (Fig. 4), whereas antimycin A had little effect. When both compounds were added together, motility completely ceased within 2 min (Fig. 5). KCN (2.5×10^{-2} M) alone reduced O_2 uptake by 93% and antimycin A increased it by 48% (Table 1). It should be pointed out that, whereas the combined effect of antimycin A and KCN on motility was almost immediate, the effect of KCN alone was much slower.

Effect of other inhibitors. Oligomycin (1.5×10^{-3} M) inhibited motility to 52% after 1 min, affecting O_2 uptake and growth to a lesser degree (Table 1). Azide did not affect movement or O_2 uptake, but its inhibition of multiplication indicated penetration into the bacteria.

The effect of *p*-chloromercuribenzoate (CMB)

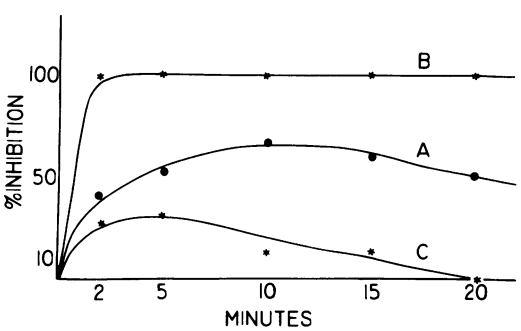

FIG. 1. *Effect of Atabrine on the motility of P. fluorescens. A, 1.5×10^{-4} M; B, 1.5×10^{-3} M; C, Atabrine (1.5×10^{-3} M) + FMN (1.5×10^{-3} M).*

on motility was similar to that of GMCD. At a concentration of 1.5×10^{-3} M in distilled water, the inhibition of movement was rapid and complete (Fig. 6), but in Adler's buffer the rate of inhibition was much slower; complete inhibition occurred after 20 min. The action of CMB could be largely negated by including an equivalent amount of cysteine in the medium. Oxygen uptake was partially inhibited, and growth was almost completely halted by the same concentration of CMB that affected motility.

HQNO inhibited motility, but at the lowest concentration (10 μg/ml) this effect was only transitory. At higher concentrations, HQNO was more effective, giving almost complete inhibition in 15 min (Fig. 7). It inhibited O_2 uptake to only a limited degree (Table 1) and had almost no effect on growth. All compounds, with the exception of antimycin A, GMCD, and HQNO, when incorporated into the growth medium in the concentrations shown in Table 1, caused significant decreases in absorbance after 18 hr of incubation as compared with controls. We consider this additional evidence of their uptake by *P. fluorescens*.

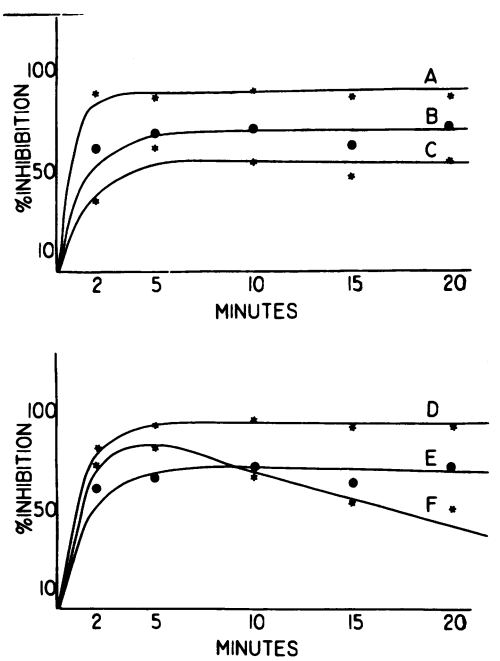

FIG. 2. *Effect of gramicidin D (GMCD) on the motility of P. fluorescens. Concentrations of GMCD in Adler's buffer: A, 1.5×10^{-3} M; B, 1.5×10^{-4} M; C, 1.5×10^{-5} M; D, 1.5×10^{-4} M GMCD in distilled water; E, 1.5×10^{-4} M GMCD in Adler's buffer; F, GMCD (1.5×10^{-4} M) + $(NH_4)_2NO_3$ (1.5×10^{-4} M) in distilled water.*

TABLE 1. *Effect of inhibitors on O_2 uptake by Pseudomonas fluorescens*

Inhibitor	O_2 uptake (% of control)[a]	Time elapsed for inhibition shown (min)
Potassium cyanide		
2.5×10^{-3} M	7	20
2.5×10^{-3} M	2	20
Antimycin A		
1.5×10^{-3} M	148	20
Sodium azide		
1.5×10^{-3} M	100	20
Atabrine		
1.5×10^{-3} M	61	30
1.5×10^{-4} M	50	30
Gramicidin D		
1.5×10^{-3} M	103	30
1.5×10^{-4} M	171	30
Oligomycin		
1.5×10^{-3} M	75	30
2,4-Dinitrophenol		
1.5×10^{-3} M	79	30
1.5×10^{-4} M	51	20
Dicumarol		
1.5×10^{-3} M	71	30
Pentachlorophenol	71	30
1.5×10^{-3} M	71	30
2*n*-Heptyl-4 hydroxyquinoline *N*-oxide		
1 mg/ml	68	10
100 μg/ml	57	10
10 μg/ml	100	10
Chloromercuribenzoate		
1.5×10^{-3} M	44	30

[a] Controls were generally around 1.40 μatoms of O_2 per mg (dry weight) per hr.

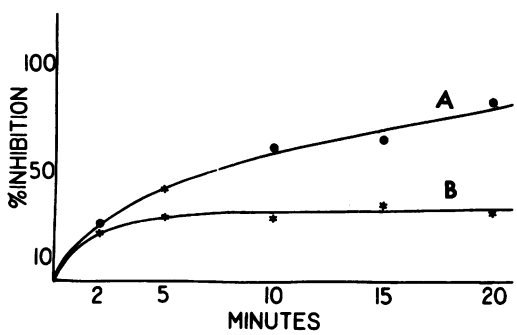

FIG. 3. *Effect of 2,4-dinitrophenol on motility of P. fluorescens. A, 1.5×10^{-3} M; B, 1.5×10^{-4} M.*

DISCUSSION

Asano and Brodie (4), using a cell-free bacterial system, reported that Atabrine inhibited electron flow through the cytochromes between succinate and cytochrome *b*. This site is a flavoprotein (20).

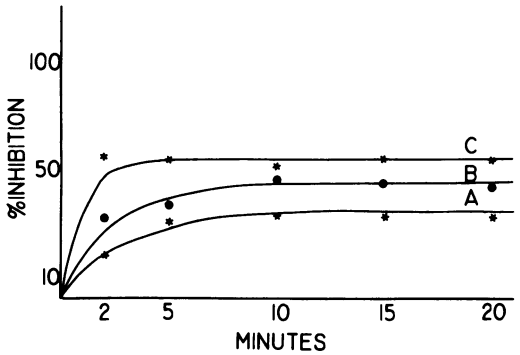

FIG. 4. *Effect of potassium cyanide on motility of P. fluorescens. A, 2.5 × 10⁻⁴ M; B, 2.5 × 10⁻³ M; C, 2.5 × 10⁻² M.*

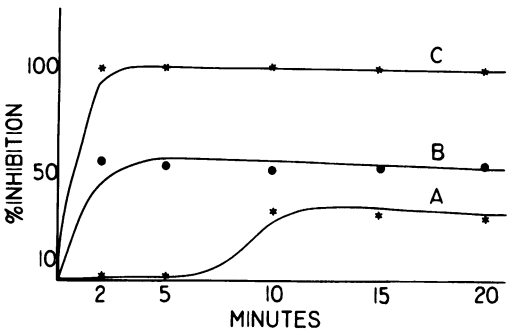

FIG. 5. *Effect of antimycin A and potassium cyanide on motility of P. fluorescens. A, antimycin A (1.5 × 10⁻⁴ M); B, KCN (1.5 × 10⁻³ M); C, antimycin A (1.5 × 10⁻⁴ M) + KCN (1.5 × 10⁻³ M).*

In our experiments, the effect of Atabrine was countered by addition of riboflavine to the solution, and this suggests that the inhibited site may also be a flavoprotein. One should remember, however, that, because intact organisms were employed, determination of the precise site affected was not possible. Since Atabrine inhibited motility completely, whereas growth was inhibited only partially, other possible effects of Atabrine cannot be discounted.

GMCD was also an effective motility inhibitor, although oxygen uptake was increased in its presence. The primary effect of this antibiotic is thought to be uncoupling of oxidative phosphorylation (6, 7, 15), but Harold and Baarda (11) noted that it affected membranes of *Streptococcus faecalis* with resultant K^+ ion leakage. Similar leakage of K^+ ions from mitochondria, together with increased O_2 consumption, also has been reported (7, 15). Motility is a property of the functional organism, and is obviously lost when the cells are disrupted, so that in all likelihood the integrity of the membrane systems must be maintained for functional motility (8). It is possible that the prime effect of GMCD on bacterial movement is not an uncoupling, but rather an alteration of membrane function. The effect of Adler's buffer on GMCD could be explained by assuming that it decreased K^+ leakage. When the medium supplied NH_4^+ ions, the effect of GMCD on motility was not pronounced, indicating again that the cation concentration of the milieu influences its effectiveness. These observations are similar to those of Chappel and Crofts (7), namely, that NH_4^+ ions reversed the GMCD-induced swelling of mitochondria.

Dicumarol, DNP, and PCP inhibited movement and O_2 uptake, but the latter effect was expected. These compounds act differently in bacterial systems than in mitochondria, and in the former they inhibit, rather than increase, O_2 uptake (4, 16). Imai et al. (16) noted that for bacterial systems

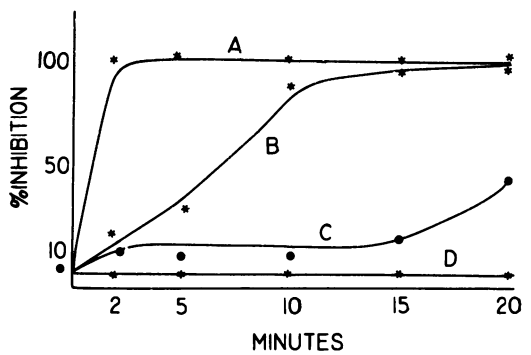

FIG. 6. *Effect of chloromercuribenzoate (CMB) on the motility of P. fluorescens. A, CMB (1.5 × 10⁻³ M) in distilled water; B, CMB (1.5 × 10⁻³ M) in Adler's buffer; C, CMB (1.5 × 10⁻³ M) + cysteine (10⁻³ M) in distilled water; D, cysteine (10⁻³ M in distilled water)*

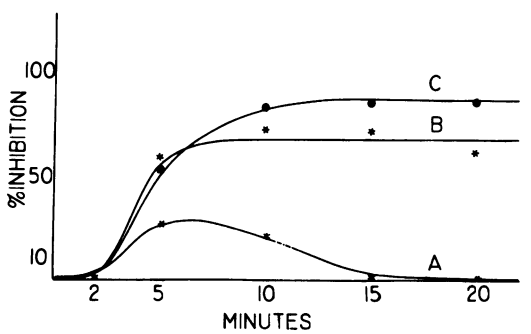

FIG. 7. *Effect of 2n-heptyl-4-hydroxyquinoline N-oxide on motility of P. fluorescens. A, 10 μg/ml; B, 100 μg/ml; C, 1 mg/ml.*

they should be classified as energy-transfer inhibitors rather than uncouplers. This view seems consistent with our results, and satisfactorily explains the effect of these compounds on growth, O_2 uptake, and motility.

The observation that KCN had only a slight effect on motility confirms the results of Kerridge (18), who reported that cyanide and azide were not effective in this regard against *S. typhimurium*. Antimycin A also had little effect on motility. However, when both compounds were added together, motility completely ceased. Antimycin A increased O_2 uptake, and this was possible only because of branching of the cytochrome chain, thereby permitting a bypass of the inhibited site(s). The existence of a cytochrome side chain has been reported in bacteria (5), and it is possible that in our experiments the presence of antimycin A "forced" the electron flow in such a way that it was rendered sensitive to cyanide inhibition. Inhibition of O_2 uptake by KCN in the presence of antimycin A in *Bacillus megaterium* was observed by Marquis (21) and can be interpreted similarly. The slight inhibitory effect of antimycin A on motility may be explained by the relative ineffectiveness of the branched cytochrome components in generating high-energy phosphate compounds. In mitochondria, antimycin A and HQNO both inhibited the same site of the respiratory chain (14, 19, 29), but this may not be true for bacteria. Bongers (5) suggested that the HQNO-sensitive site in the respiratory chain lies before that inhibited by antimycin A in *Hydrogenomonas*. HQNO was a more effective inhibitor of movement than antimycin A, and the possibility exists that the sites of inhibition are not the same.

The inhibitory effect of oligomycin in bacteria, unlike that in mitochondria, lies in blocking hydrolysis of ATP rather than affecting the high-energy intermediates (10, 16). In either case, it may act as an electron-chain inhibitor and its effect on motility may thereby be explained. The ineffectiveness of azide has been noted previously for motility (18) and for O_2 uptake (5, 6); however, it immobilized the motile spores of *Actinoplanes* (13). The effect of CMB on motility and its reversal by the inclusion of an equivalent amount of cysteine in the medium suggest that —SH groups are involved in the energetics of bacteria (1, 13, 23, 30).

The effect of inhibitors and their possible site(s) of action as postulated here must be regarded as tentative, because we have used intact functional bacteria. Several inhibitors, namely, Atabrine and antimycin A plus KCN, immediately immobilized the bacteria; others, such as oligomycin, HQNO, PCP, Dicumarol, and DNP, decreased movement but were not able to halt it completely. From the patterns of O_2 uptake, as influenced by the inhibitors, we are led to believe that they involved the cytochrome system, and, because of consequent effects on energy generation, thereby affected motility. These data, however, do not show what might happen if the bacteria cannot get energy for motility via the cytochromes over an extended period of time. Here we have to accept the possibility shown by Sherris et al. (24) that bacteria may generate energy for movement anaerobically through substrate-level phosphorylation.

The possible effect of GMCD on membrane leakage, and inhibition of its activity by the presence of cations, may be an important facet of the "bacterial motility problem" and ought to be further investigated. Under aerobic conditions, we have not found evidence for the existence of a specific energy-generation mechanism for motility of *P. fluorescens*.

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