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Research Article

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Pyocyanin and 1-Hydroxyphenazine Produced by *Pseudomonas aeruginosa* Inhibit the Beating of Human Respiratory Cilia In Vitro

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Abstract

Pseudomonas aeruginosa culture filtrates varied in their ability to slow human ciliary beat frequency (7–71%). This activity did not correlate with known virulence factors. However, a close correlation ($r = 0.97$) existed between ciliary slowing and pigment content. In a prolonged culture, the increase in activity correlated ($r = 0.94$) with pigment accumulation. Gel filtration of lyophilized filtrate yielded a single peak of activity corresponding to the pigment fraction. Pyocyanin extracted from an active strain, and 1-hydroxyphenazine were purified by high performance liquid chromatography, and characterized by ultraviolet absorbance spectra and mass spectrometry. Both slowed cilia in a dose-dependent manner, and were synthesized and shown to be indistinguishable from the biological compounds. Pyocyanin caused gradual onset of slowing and ultimate widespread ciliostasis with epithelial disruption. 1-hydroxyphenazine caused rapid onset of ciliary slowing associated with dyskinesia and ciliostasis. Pyocyanin assayed within filtrates accounted for a significant proportion of the bioactivity present.

Introduction

The lungs of patients with cystic fibrosis and other causes of severe bronchiectasis are frequently colonized by *Pseudomonas aeruginosa*. This leads to clinical deterioration, worsening lung function, and frequently to death (1). Mucociliary clearance is the first-line defense mechanism of the human respiratory tract against inhaled particles including bacteria. Defective mucociliary activity is associated with recurrent chest infection and finally bronchiectasis (2). We have previously reported the phenomenon of slowing of human nasal ciliary beat frequency in vitro by *P. aeruginosa* culture filtrates (3). We report here the extraction, purification, and characterization of two pseudomonas factors associated with this activity.

Methods

Preparation and assay of bacterial culture filtrates

(a) Overnight aerobic incubation of sputum at 37°C on plates containing cetrimide nalidixic acid agar (Oxoid Ltd., Basingstoke, England) yielded 17 isolates of *P. aeruginosa* from patients with bronchiectasis (six with cystic fibrosis: isolates 3, 4, 7, 12, 14, and 16). The purity of isolates was

confirmed by subculture and overnight incubation. All experiments were performed with fresh isolates. After incubation in 20 ml of medium 199 (with Earle's salts and Hepes; Flow Laboratories, Inc., McLean, VA) at 37°C for 18 h and a viable count, the cultures were centrifuged at 4,656 g for 30 min at 4°C. The supernatant fluids were filtered (0.2 μm Acrodisc; Gelman Sciences, Inc., Ann Arbor, MI) to yield bacteria-free filtrates (pH 7.0–7.3; sterility of aliquots tested overnight at 37°C).

(b) Strips of normal human nasal ciliated epithelium obtained from the inferior turbinate using a cytology brush (4) were dispersed in medium 199, divided into two equal samples, and centrifuged at 186 g for 10 min. The supernatant medium 199 was aspirated and replaced by an equal volume of fresh medium 199 in one sample (control) and by the pseudomonas filtrate in the other (test). A sealed microscope coverslip-slide was prepared for measurement of ciliary beat frequency (CBF)¹ by a photometric technique (3, 4). The microscope coverslip-slide preparation was placed on an electronically controlled warmstage (Microtec, Oxford, England) at 37°C and mounted on a Leitz Dialux 20 phase-contrast microscope. A Leitz MPV compact microscope photometer transduced light intensity into an electrical signal. Strips of epithelium with beating cilia were viewed directly at a magnification of 320 by bright field illumination. The cilia were positioned to interrupt the passage of light through a small diaphragm into the photometer, and the electrical signal generated was converted into a reading of CBF (in hertz) (5). Direct viewing of the cilia allowed an assessment of their beating pattern. Ciliary dyskinesia was defined as absence of the usual coordinated ciliary movement, i.e., cilia may be seen to perform vibration about a vertical axis and those on the same strip may beat in opposite directions. Ciliostasis was defined as complete absence of ciliary movement.

CBF was measured at 30-min intervals for 4 h after initial equilibration at 37°C over 10 min. Six or more strips of ciliated epithelium were identified on the slide and the latter marked for reidentification at subsequent time points. These six strips were all used as sites of 10 subsequent CBF readings at each time point. The mean of these 10 readings at each time point was calculated for both control and test slides. Ciliary dyskinesia or ciliostasis was noted. Because static cilia become progressively more difficult to distinguish from unciliated epithelium, the CBF of static cilia was not counted unless there were insufficient groups of beating cilia to allow 10 separate readings of CBF to be made. This methodology underestimates, therefore, the overall ciliary-slowness activity of the bacterial preparations.

(c) The colony type of each isolate was noted after overnight culture on blood agar. Protease activity of the filtrate was estimated spectrophotometrically on two separate days by gentle rotation of 5 ml of filtrate with 10 mg of azocasein (Sigma Chemical Co., St. Louis, MO) at 37°C for 60 min, then precipitation of excess azocasein with 250 μl 100% TCA. After separation from the precipitate, the absorbance of the now orange filtrate was read on an ultraviolet (UV) spectrophotometer at 340 nm, the result adjusted to a standard viable count of 5×10^8 organisms and compared with results obtained after similar incubation with porcine trypsin (Sigma Chemical Co.). The result was presented as trypsin equivalent per 5×10^8 organisms. Specific elastase activity of the filtrate was estimated spectrophotometrically on two occasions after gentle rotation

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1. Abbreviations used in this paper: 1-hp, 1-hydroxyphenazine; 1-mp, 1-methoxyphenazine; CBF, ciliary beat frequency; d.e.i., desorption electron impact; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry; TFA, trifluoroacetic acid.

of 5 ml of filtrate with 20 mg of congo-red elastin (Sigma Chemical Co.) for 18 h at 37°C. Haemolytic activity of the filtrate was estimated by incubation with 5% washed sheep red blood cells for 3 h at 37°C. Production of other enzymes by the isolates was estimated by agar plate techniques in which substrate was incorporated into an agar base with appropriate nutrient; lipase (6), DNase (7), or lecithinase (8). The absorbance of each filtrate was measured at 400 nm to determine pigment content.

(d) Lipopolysaccharide was prepared by the method of Chester, Meadow, and Pitt (9). Alginate from a mucoid strain of *P. aeruginosa* was prepared by the method of Pitt and Raisbeck (10), then dried in vacuo after deproteinization with butanol and chloroform. Each was dissolved in medium 199 (1 mg/ml) and tested for inhibition of CBF. The activity of filtrates of two strains, PA 103 (high exotoxin A, non-protease producer) and PA 8505 (nonproducer of exotoxin A and protease), was also assayed for inhibition of CBF. The activity of five filtrates from nonmucoid strains was compared with the activity of five filtrates from mucoid strains.

(e) Sequential samples of supernatant fluid taken during a continuous 4-d culture of isolate 4 in medium 199 at 37°C were centrifuged (4,656 g; 30 min) then filtered (0.2 µm Acrodisc; Gelman Sciences, Inc.). The spectrophotometric absorbance of each filtrate was measured at 400 nm and its activity assayed for inhibition of CBF. The filtrate of a 4-d culture of *Staphylococcus aureus* in medium 199 at 37°C was also assayed for ciliary-slowng activity.

Gel filtration

Three isolates (Nos. 2, 5, and 8) were separately cultured in 100 ml of medium 199 for 18 h at 37°C, and the cultures centrifuged and filtered (0.2 µm Acrodisc; Gelman Sciences, Inc.) to yield bacteria-free preparations, which were immediately frozen and then lyophilized overnight. 100 ml of medium 199 were treated similarly to act as control. Each solid was reconstituted in 5 ml distilled water, 2 ml of this being passed down a Sephacryl-200 gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C.

Phosphate-buffered saline (PBS) was used to elute, and 4-ml aliquots were collected and stored at -30°C until their cilioinhibitory activity was tested. The column void volume was 60 ml, and absorbance at 280 nm indicated that pseudomonas products appeared between elution volumes of 92 and 148 ml. Cilia beat normally in PBS and aliquots from the column were tested for their cilioinhibitory activity against a control of PBS alone. Osmolality of aliquots was checked by absence of crenation of human red blood cells and corrected with deionized water if required. The percent ciliary slowing produced by each aliquot was calculated as 100 minus (minimum CBF during experiment/control CBF at same experimental time-point × 100). The absorbance of each aliquot was measured at 400 nm.

Factor purification, synthesis, and assay

Pyocyanin and 1-hydroxyphenazine (1-hp) were prepared by methods adapted from those described by Armstrong et al. (11).

PREPARATION OF PYOCYANIN

10 King's A Agar (12) plates were cultured aerobically at 37°C for 18 h, left at room temperature for a further 24 h, the organisms washed from the surface of the agar using sterile distilled water, and the agar cut aseptically into 1-cm squares that were placed in a sterile glass bottle. A 20-ml aliquot of dry chloroform was added, the mixture shaken vigorously and the chloroform (colored blue) removed by pipette and filtered (No. 1, double; Whatman Chemical Separation, Inc., Clinton, NJ). The procedure was repeated to extract the remaining pigment. The chloroform was evaporated in an air stream and the blue sediment redissolved in 5 ml of dry chloroform. Dry petroleum ether was added dropwise until blue crystals of pyocyanin formed (~15 ml). The apple-green supernatant was decanted and the blue crystals recrystallized from chloroform. The crystals were dissolved in distilled water, filtered (0.2 µm Acrodisc; Gelman Sciences, Inc.), and freeze-dried for estimation of weight. 2 mg of crude crystalline pyocyanin was obtained.

PREPARATION OF 1-HP BY BASE HYDROLYSIS

An alkaline solution (0.2 N NaOH) of pyocyanin was left at room temperature for 18 h, during which time the color changed from blue to red-violet. Acidification with 1 N HCl yielded a yellow precipitate of crude 1-hp, which was dissolved in distilled water and freeze-dried for weight estimation. 200 µg of crude 1-hp was obtained from 4 mg of pyocyanin.

SYNTHESIS OF 1-HP AND PYOCYANIN

All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) unless otherwise stated. HPLC (high pressure liquid chromatography) solvents were purchased from Rathburn, Walkerburn, England; water was of Milli Q quality.

1-hp and pyocyanin were synthesized following the method of Armstrong et al. (11). Essentially, 1-methoxy catechol (2 g) was oxidized with lead dioxide (40 g) in dry benzene, and the resultant quinone coupled with o-phenylenediamine (1.8 g) forming 1-methoxyphenazine (1-mp). 1-mp was crystallized from pyridine/water to give red-orange crystals in 12.5% yield. 1-mp was hydrolyzed in 60% HBr (5 h, reflux), neutralized, and extracted into ether to form 1-hp in good yield. 1-hp was purified by exhaustive ether extraction, first from base (10% NaOH) and then from acid (5% acetic acid, pH 3).

1-hp was converted into pyocyanin by heating with methyl sulphate (100°C, 10 min). Crude pyocyanin was precipitated by addition of dry ether. The solid was dissolved in water (pH 11) and extracted into chloroform. Pyocyanin was further extracted into 5% HCl, and back into chloroform after neutralization. Excess solvent was removed under nitrogen to yield a dark blue solid.

PURIFICATION AND CHARACTERIZATION

Biological and synthetic 1-hp and pyocyanin were purified by HPLC before biological or structural studies.

HPLC. HPLC was undertaken on a Waters gradient elution system, monitoring at 280 and 254 nm in the UV. Elution was carried out either:

(i) at 2 ml/min on a Nova Pak octadecylsilyl column (Waters Associates, Millipore Corp., Milford, MA) with acetonitrile/water/trifluoroacetic acid (TFA) (100:0:0.04 vol/vol/vol) isocratically for 5 min followed by a 10-min linear gradient to acetonitrile/water/TFA (60:40:0.04 vol/vol/vol).

(ii) For thermospray liquid chromatography/mass spectrometry (LC/MS), at 1.8 ml/min on a Nova Pak octadecylsilyl column (Waters Associates, Millipore Corp.) eluting with a 10-min linear gradient from aqueous ammonium acetate (0.05 M) to 70% acetonitrile/water-containing 0.05 M ammonium acetate.

(iii) For assay of pyocyanin in culture filtrates, at 2 ml/min on a µBondapak C18 column (30 × 0.8 cm; Waters Associates, Millipore Corp.) with a 20-min linear gradient from 0 to 40% propan-2-ol in aqueous acetic acid (5%).

MS. Mass spectra were recorded in the electron impact (e.i.), desorption e.i. (d.e.i.), or thermospray ion modes (where applicable) on a mass spectrometer (model 4,500; Finnigan, Sunnyvale, CA). Samples were introduced either directly, or via gas chromatography (GC)/HPLC. The HPLC was coupled to the mass spectrometer via a "Vestal" thermospray interface (Finnigan, Sunnyvale, CA). The source block was kept at 200°C and the interface jet was operated at 140°C. HPLC/MS was undertaken with solvent system ii, although for loop injection of pyocyanin, the salt concentration was varied.

GC. GC was undertaken on an SE54 capillary column with helium carrier gas. Samples in octane were injected in the splitless mode at 250°C injector temperature. The injector temperature was also varied between 200°-300°C for pyocyanin. Elution was carried out for 1 min isothermally (100°C) followed by a linear gradient of 20°C/min to 300°C. The GC was monitored directly in the mass spectrometer in the e.i. mode.

UV spectrophotometry. Full UV spectra were obtained in methanol, 0.1 M HCl, or 0.1 M NaOH on a spectrophotometer (model 555; Perkin-Elmer Corp., Norwalk, CT). Quantitation for bioassay was based on the

absorbance in 0.1 M HCl/pyocyanin: λ max 278 nm, ϵ max 50,000; and 1-hp: λ max 273 nm, ϵ max 30,000.

ASSAY OF CRUDE AND PURIFIED FACTORS ON CBF

The crude and purified (HPLC) biological and the synthetic pyocyanin and 1-hp were dissolved in PBS for assay of their cilioinhibitory activity.

EFFECT OF ADJUSTING pH

In preliminary experiments, the effect of pH on CBF was tested in PBS for the range 6.5 to 8.0. The CBF assay was then performed after adjusting the pH (with 1% HCl or NaOH) of the bacterial filtrates, and of the purified synthetic 1-hp and pyocyanin. In each experiment the pH of the control slide was identical to that of the test slide.

Assay of cilioinhibitory factors within culture filtrates

After characterization and synthesis of pyocyanin and 1-hp, methodology was developed to assay for the presence or absence of these compounds in culture filtrates without prior extraction. Nine new isolates (Nos. 9–17) were used to produce 18-h culture filtrates for assay of ciliary-slowness activity. Two longer cultures (a 48-h culture of one of the new isolates [No. 13] and a sample from the 4-d culture of isolate No. 4 described earlier) were also similarly assayed. Pyocyanin and 1-hp were chromatographed on a μ Bondapak C18 HPLC column in system iii (13). The HPLC eluate was monitored at 280 and 254 nm. Pyocyanin eluted as an A_{280} UV-absorbing peak at 25.4 ml (and 1-hp at 50 ml). The peak height (A_{280}) was proportional to the amount of pyocyanin loaded, allowing a standard curve to be constructed for synthetic pyocyanin across the range 50–500 ng. Culture filtrates (250 μ l in medium 199) were similarly chromatographed, and A_{280} peak height for pyocyanin and 1-hp measured.

The remaining filtrate was made basic (pH 12) with 5% NaOH and extracted twice with two volumes of chloroform. Chloroform was removed under a stream of nitrogen and the full UV spectrum obtained in 0.1 M HCl.

Statistical analysis

Each experiment yielded a series of 10 CBF readings at each timepoint, and the mean CBF at each time point was calculated for test and control preparations. The slowest mean CBF of the test preparation was identified and compared with the control mean at the same timepoint by the unpaired *t* test (20 readings, 18 d.f.).

Results

Culture filtrates. The slowing of human nasal ciliary beating by 17 filtrates of medium from 18-h cultures of separate isolates of *P. aeruginosa* was measured. Three patterns of ciliary inhibition were observed: inhibition with no recovery during the 4-h experiment—by the filtrates from seven isolates (Nos. 1, 2, and 9–13); inhibition with recovery—by the filtrates from six isolates (Nos. 3–7, 14); and no inhibition at all—by the filtrates from four isolates (8, 15–17). For clarity these patterns are illustrated in Fig. 1 by 10 of these 17 isolates. The CBF of control specimens did not vary significantly over 4 h (Fig. 1). Marked slowing was associated with dyskinesia, which usually preceded ciliary stasis in some areas of the epithelium and disruption of the epithelium itself. Complete reversal of the filtrate effect could be demonstrated by washing the cilia with fresh culture medium before ciliostasis and disruption were observed.

This activity of the filtrates neither correlated with their protease, specific elastase or haemolytic activity, nor with production of other pseudomonas enzymes as detected by plate techniques (Table I), nor with other virulence factors as demonstrated in experiments using lipopolysaccharide, alginate, and specific strains (Table II). Five nonmucoid strains produced more active filtrates than five mucoid strains (mean 46% ciliary slowing compared with 12%). UV absorbance at 400 nm indicated a

close correlation ($r = 0.97$) between percent ciliary slowing and pigment contained in the filtrate (Table I).

Sequential samples of supernatant fluid taken during a continuous 4-d culture of isolate 4 in medium 199 at 37°C increased progressively in their capacity to slow ciliary beating when compared with control medium alone (Table III). There was close correlation ($r = 0.94$) between this activity and the gradually increasing pigment accumulation in the filtrate. After a 4-d culture of *S. aureus* in medium 199 at 37°C to control for medium exhaustion, a filtrate of the culture medium did not slow ciliary beating. *S. aureus* has previously been shown not to produce factors slowing ciliary beating in vitro (3). Therefore, it is unlikely that the activity of the filtrates of *P. aeruginosa* was due to exhaustion of the medium 199 of factors necessary for normal ciliary beating.

Gel filtration. Gel filtration of the filtrates (lyophilized and later reconstituted in water) obtained from strongly cilioinhibitory strain 2 and protease-producing strain 5 yielded fractions with a single peak of activity (Fig. 2) corresponding to the fractions containing pigment. Fractions from similarly treated control medium and from noncilioinhibitory strain 8 were without activity.

Characterization of 1-hp and pyocyanin. Authentic 1-hp and pyocyanin were synthesized. GC/MS and thermospray LC/MS methodologies were developed and applied both to define the molecular weight and structure of the synthetic materials, and also to facilitate gas chromatographic/mass spectrometric comparison of the synthetic and natural species. The natural and synthetic compounds were indistinguishable by all spectroscopic, mass spectrometric, and chromatographic tests.

Synthetic 1-hp was characterized in terms of its UV absorbance spectrum and e.i. mass spectrum. The full UV spectrum of 1-hp in 0.1 M HCl showed an intense absorbance at λ max = 273 nm ($\epsilon = 30,000$) and a series of weaker peaks at $\lambda = 363, 370, \text{ and } 383$ nm ($\epsilon = 5,500, 6,000, \text{ and } 8,500$, respectively). In 0.1 M NaOH, the λ max shifted from 273 to 291 nm (14). 1-hp chromatographed on GC with a retention time of 7.2 min, and on HPLC at 12.6 or 10.6 min (systems i and ii). The HPLC/MS thermospray mass spectrum showed an intense molecular ion species ($M + H$)⁺ at m/z 197 defining the molecular weight as 196. The e.i. mass spectrum of 1-hp (post-GC) showed two intense ions at m/z 196 [M^+] and 168 [$M^+ - CO$] together with minor ions at m/z 154, 157, 142, 140, 129, 122, 115, 114, 102, 98, and 77. The spectrum was in good agreement with that reported for the isomer 2-hp (15).

Natural 1-hp cochromatographed with synthetic material both on GC and HPLC. The UV and mass spectra (e.i. and thermospray) were indistinguishable from those reported above for synthetic 1-hp.

Synthetic pyocyanin chromatographed on HPLC with retention times of 10.4 or 7 min (systems i and ii). It possesses a characteristic UV spectrum in both methanol ($\lambda_1 = 238, \epsilon_1 = 26,840, \lambda_2 = 316$ nm, $\epsilon_2 = 30,000, \lambda_3 = 690$ [broad], and $\epsilon_3 = 4,210$) and in 0.1 M HCl ($\lambda_1 = 278, \epsilon_1 = 50,000, \lambda_2 = 385, \epsilon_2 = 21,840, \lambda_3 = 520$ [broad], and $\epsilon_3 = 3,160$). Under normal thermospray HPLC conditions (0.05 M ammonium acetate in the solvents), mass spectrometric ionization yielded an intense ion species at m/z 212, with a weaker ion at m/z 211 (corresponding to $M + H^+$). At low ammonium ion concentrations, the intensity of m/z 211 increased until it became the major ion. The d.e.i. probe mass spectra showed the presence of both the protonated ($M + H^+$) species (m/z 211, 196, 168, 140, 129)

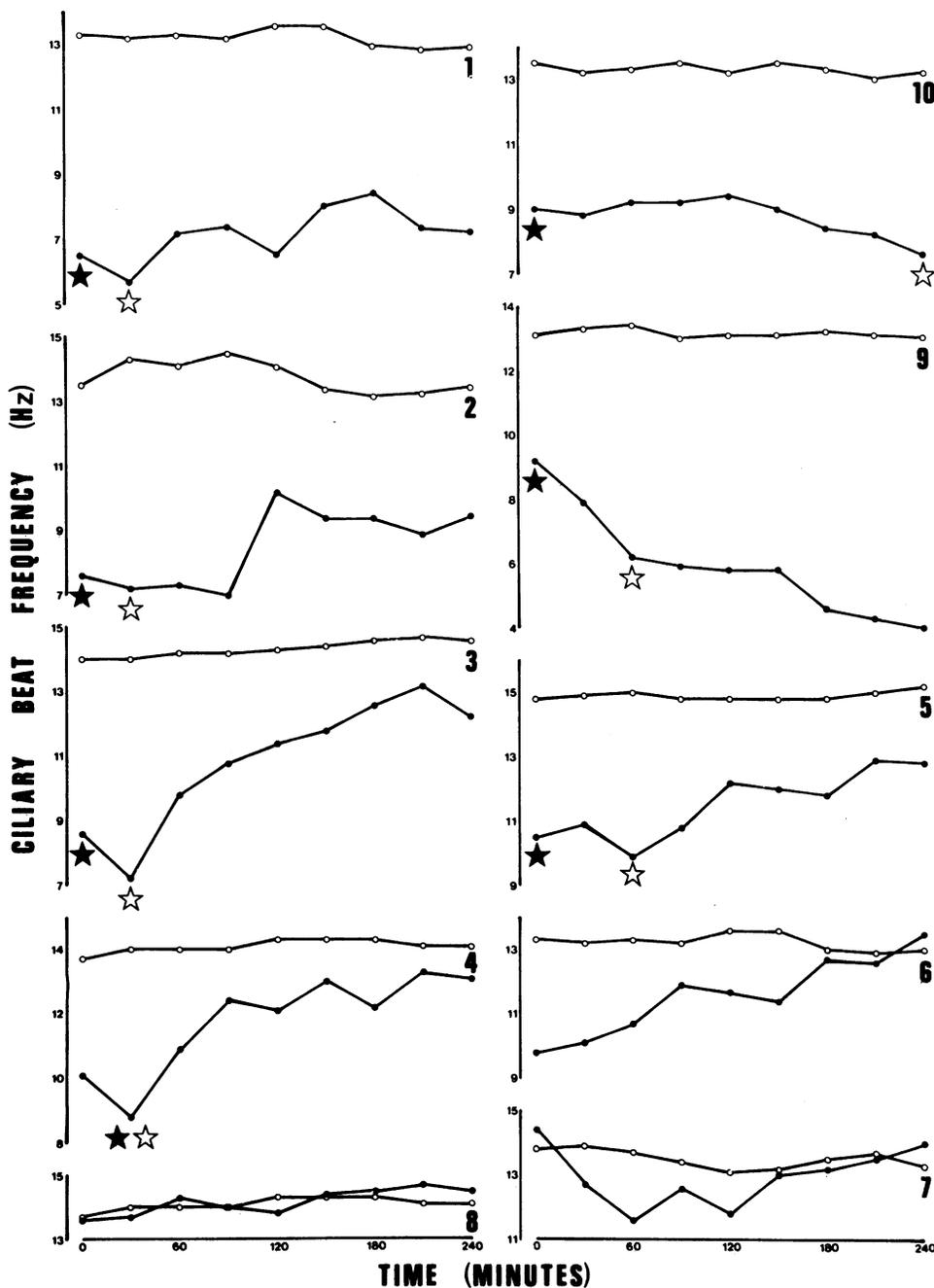


Figure 1. The effect of culture filtrates from ten *P. aeruginosa* isolates on human ciliary beat frequency (CBF) in vitro. The CBF produced by each culture filtrate (solid circles) and the time-based control CBF (open circles) are shown. The top right hand corner of each graph shows the isolate reference number. Filled stars, ciliary dyskinesia; open stars, ciliostasis.

and deprotonated (M^+) species: m/z 224 ($M^+ + CH_2$, [trans-methylation (11)], 210 (M^+), 196 ($M^+ - CH_3^+$), 181, 168, 129)—as expected, the protonated species desorbed from the probe at lower current. A sample of pyocyanin injected onto the gas chromatograph at 250°C eluted as a single peak with retention time 7.2 min. The e.i. spectrum was indistinguishable from 1-hp. At higher injector temperatures, a species cochromatographing with 1-hp was also observed (retention time 8.1 min); the e.i. MS spectrum (m/z 210 (M^+), 209, 181, 180, 179, 167, 153, 140, 127, 114, and 102) was similar, both to that obtained with our synthetic 1-hp and that reported by Holliman et al. (15).

Natural pyocyanin cochromatographed on HPLC with synthetic material and was indistinguishable from it in terms of its

UV and mass spectra (under both d.e.i. and thermospray conditions).

Bioassay of crude pyocyanin and 1-hp. Crude pyocyanin dissolved in PBS (16 $\mu\text{g/ml}$) caused ciliostasis and epithelial disruption during the 4-h experiment. At 4 $\mu\text{g/ml}$, gradual ciliary slowing occurred and at 4 h caused a test value of 9.3 Hz, and a control value of 14.2 Hz. Crude 1-hp dissolved in PBS caused early onset of ciliary slowing with dyskinesia and ciliostasis (32 $\mu\text{g/ml}$), and at 8 $\mu\text{g/ml}$ caused a test value of 11.4 Hz, and a control value of 13.0 Hz after 60 min.

Bioassay of purified factor. Pyocyanin extracted from an active strain and its base hydrolysis product, 1-hp, were purified by reverse-phase HPLC. HPLC of the apple-green supernatant remaining after pyocyanin crystallization demonstrated the

Table I. Correlation of Colony Type, Pigment Production, and Enzymic Properties of *P. aeruginosa* with Their Cilioinhibitory Properties

| Isolate | Colony type | Filtrate absorbance | Slowing of CBF | Filtrate protease | Filtrate elastase activity | Filtrate hemolytic activity | Lipase activity | Lecithinase activity | DNase activity |
|---------|-------------|---------------------|---------------------------|----------------------------------------------------------------------|----------------------------|-----------------------------|-----------------|----------------------|----------------|
| | | 400 nm* | % (SD in Hz) [‡] | $\mu\text{g trypsin equivalent to } 5 \times 10^8 \text{ organisms}$ | | | | | |
| 1 | Nonmucoid | 0.338 | 57 (1.8) [§] | 5.2 | 1+ | 0 | 1+ | 1+ | 3+ |
| 2 | Nonmucoid | 0.288 | 51 (2.0) [§] | 14.8 | 2+ | 0 | 3+ | 3+ | 0 |
| 3 | Nonmucoid | 0.230 | 49 (2.2) [§] | 1.1 | 0 | 0 | 3+ | 1+ | 0 |
| 4 | Nonmucoid | 0.240 | 37 (1.4) [§] | 7.7 | 0 | 0 | 2+ | 1+ | 0 |
| 5 | Nonmucoid | 0.224 | 34 (3.0) [§] | 37.5 | 3+ | 0 | 3+ | 2+ | 1+ |
| 6 | Nonmucoid | 0.156 | 26 (1.5) [§] | 1.7 | 0 | 0 | 3+ | 0 | 0 |
| 7 | Mucoid | 0.116 | 15 (1.8) [§] | 0.0 | 0 | 0 | 3+ | 3+ | 3+ |
| 8 | Mucoid | 0.014 | 3 (1.0) [§] | 0.0 | 0 | 0 | 3+ | 3+ | 0 |

* Correlation coefficient between filtrate absorbance and percent ciliary slowing = 0.97. ‡ % ciliary slowing = 100

– $\left[\frac{\text{minimum CBF during experiment}}{\text{control CBF at same experimental time point}} \times 100 \right]$. § $P < 0.001$ *t* test, test vs. control at same experimental point, 18 d.f.

presence of both pyocyanin and 1-hp. Pyocyanin and 1-hp were characterized in terms of UV absorbance spectra and MS (molecular weights 210 and 196 respectively).

Pyocyanin dissolved in PBS produced gradual slowing of CBF without recovery (Figs. 3 and 4). Ciliary dyskinesia was observed only late in the experiments when ciliostasis and epithelial disruption were also noted. In control experiments using PBS alone, CBF did not vary significantly over 4 h (Fig. 3), however over 36 h CBF was more variable (Fig. 4). Significant slowing ($P < 0.001$, *t* test) occurred at 20 μM at 30 min (Fig. 3), at 1 μM at 20 h and at 0.1 μM at 36 h (Fig. 4). In contrast, Fig. 3 shows 1-hp to produce rapid onset of ciliary slowing, dyskinesia, and some immediate ciliostasis but some recovery of beating during the course of the experiment (the pattern seen with some isolates in Fig. 1). However, significant changes ($P < 0.001$) only occurred at a concentration of 10 μM 1-hp (Fig. 3) even with more prolonged exposure (Fig. 4). The activity of pyocyanin dissolved in PBS did not change after incubation at 37°C for 4 h; similarly, its UV spectrum and HPLC retention time were unchanged after 4 h in 0.04% TFA or methanol.

Synthetic 1-hp and pyocyanin were dissolved in PBS and assayed as above. Identical patterns of ciliary slowing were produced, and equivalent concentrations gave similar quantitative CBF results compared with control: 50 μM 1-hp, a test value of 7.8 Hz, and a control value of 12.8 Hz (60 min) with widespread

dyskinesia and ciliostasis; 10 μM 1-hp, a test value of 10.2 Hz, and a control value of 12.8 Hz (60 min) with dyskinesia only. 75 μM pyocyanin, a test value of 6.8 Hz, and a control value of 11.9 Hz (240 min) with widespread epithelial disruption and ciliostasis; 20 μM , a test value of 9.1 Hz, and a control value of 11.9 Hz (240 min) with some epithelial disruption and ciliostasis.

Effect of pH. In the preliminary experiments CBF did not vary significantly (unpaired *t* test, 18 d.f.) within the pH range 7.0 to 8.0. When CBF measured within the range 6.5 to 7.0 was compared with that measured within the range 7.0 to 8.0, a reduction in CBF of <10% was noted. This is in agreement with previously published data (16). Subsequent experiments were controlled with slides at identical pH to the test slide. Increasing the pH of the bacterial filtrates from 6.5 to 8.0 markedly reduced their cilioinhibitory properties (at pH 6.5, 43% max ciliary slowing compared with control, $n = 3$; at pH 7.5, 24%, and at pH 8.0, 5%). A pH change from 7.5 (used in the above experiments with biological and synthetic 1-hp) to 6.5 profoundly increased the cilioinhibitory properties of synthetic 1-hp and synthetic pyocyanin (compared with control medium pH 6.5). At pH 6.5,

Table II. Effect on CBF of *Pseudomonas* Lipopolysaccharide, Alginate, and 18-h Culture Filtrates (0.2 μm) of Strains PA103 and PA8505

| | Minimum CBF during 4 h of exposure | | Control CBF at same experimental time point |
|------------------------------|------------------------------------|-------------|---------------------------------------------|
| | Hz (SD) | | Hz (SD) |
| Lipopolysaccharide (1 mg/ml) | 12.9 (1.2) | NS | 13.7 (1.6) |
| Alginate (1 mg/ml) | 12.8 (1.0) | NS | 13.3 (0.6) |
| PA 103 | 12.4 (1.1) | NS | 12.6 (1.4) |
| PA 8505 | 5.6 (1.3) | $P < 0.001$ | 12.8 (1.1) |

$P < 0.001$, *t* test, 18 d.f.

Table III. Effect of Centrifuged, Filtered (0.2 μm) Aliquots Removed from a 4-d Culture of *P. aeruginosa* (Strain 4) in Medium 199 on the Beat Frequency of Human Nasal Cilia in Vitro

| Culture duration when aliquot removed | Spectrophotometric absorbance | Minimum CBF during 4 h of exposure | |
|---------------------------------------|-------------------------------|------------------------------------|---------|
| | | Test | Control |
| <i>h</i> | 400 nm | Hz | Hz |
| 6 | 0.017 | 12.4 | (13.8) |
| 18 | 0.244 | 8.1 | (13.5) |
| 24 | 0.314 | 8.3 | (13.8) |
| 36 | 0.412 | 8.7 | (13.7) |
| 48 | 0.648 | 4.5 | (15.2) |
| 72 | 0.676 | 4.1 | (15.2) |
| 96 | 0.704 | Stasis at 15 min | (13.2) |

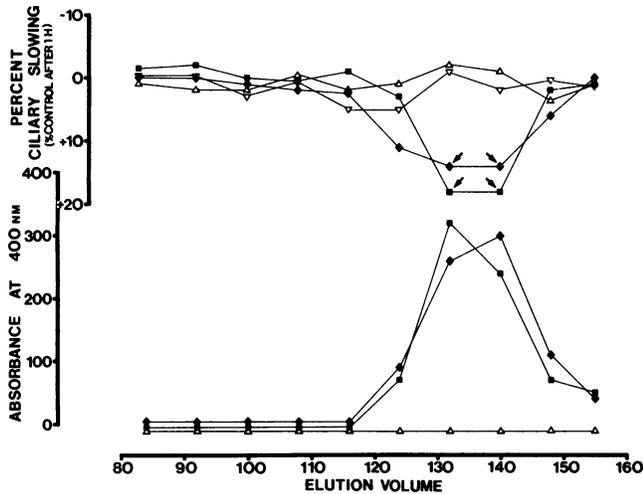


Figure 2. Inhibition of human nasal CBF by eluates of gel-filtered, freeze-dried filtrate; medium 199 (∇), isolate 2 (\blacksquare), isolate 5 (\blacklozenge), isolate 8 (\triangle). Absorbance (400 nm) of filtrates from isolates 2, 5, and 8. \blacktriangle , significant $P < 0.001$ t test.

10 μ M 1-hp caused complete ciliostasis at 240 min; 2 μ M caused a test value of 7.8 Hz, and a control value of 11.7 Hz with some dyskinesia and ciliostasis at 210 min; 1 μ M caused a test value of 8.6 Hz, and a control value of 11.4 Hz at 150 min; and 0.3 μ M caused a test value of 9.6 Hz, and a control value of 11.4 Hz at 60 min. At this lower pH, 1-hp still caused no epithelial

disruption and its ciliary-slowing properties were again immediate but tended to progress rather than plateau.

At pH 6.5, 5 μ M pyocyanin caused a test value of 8.1 Hz, and a control value of 12.2 Hz with epithelial disruption and ciliostasis at 240 min; and 1 μ M caused a test value of 8.2 Hz, and a control value of 11.9 Hz with some epithelial disruption at 240 min.

Measurement of cilioinhibitory factors within culture filtrates.

Nine filtrates were assayed for the presence of pyocyanin and 1-hp by HPLC, five of these caused substantial ciliary slowing, two caused minor changes in CBF, and two produced no change in CBF (Table IV). The filtrates from two longer cultures (48 and 96 h) were similarly assayed.

The conditions used for HPLC allowed direct analysis of high salt samples without the need for prior extraction (and attendant losses). Pyocyanin eluting at 25.4 ml in the propanol/acetic acid system was quantitated against an HPLC/UV standard curve. In all the 18-h culture filtrates the bioactivity of the filtrate correlated (Kendall's rank correlation coefficient $\tau = 0.72$, $P < 0.01$) with the amount of pyocyanin present, as determined by A_{280} peak height (Table IV). Representative HPLC/UV profiles are shown in Fig. 6.

In each sample in which pyocyanin was observed on HPLC, the characteristic UV spectrum for pyocyanin could be obtained after base extraction of larger quantities of the parent filtrate into chloroform.

1-hp was below the limit of detection in each of the 18-h filtrates examined, although it was detected in the chloroform extract from agar plate cultures.

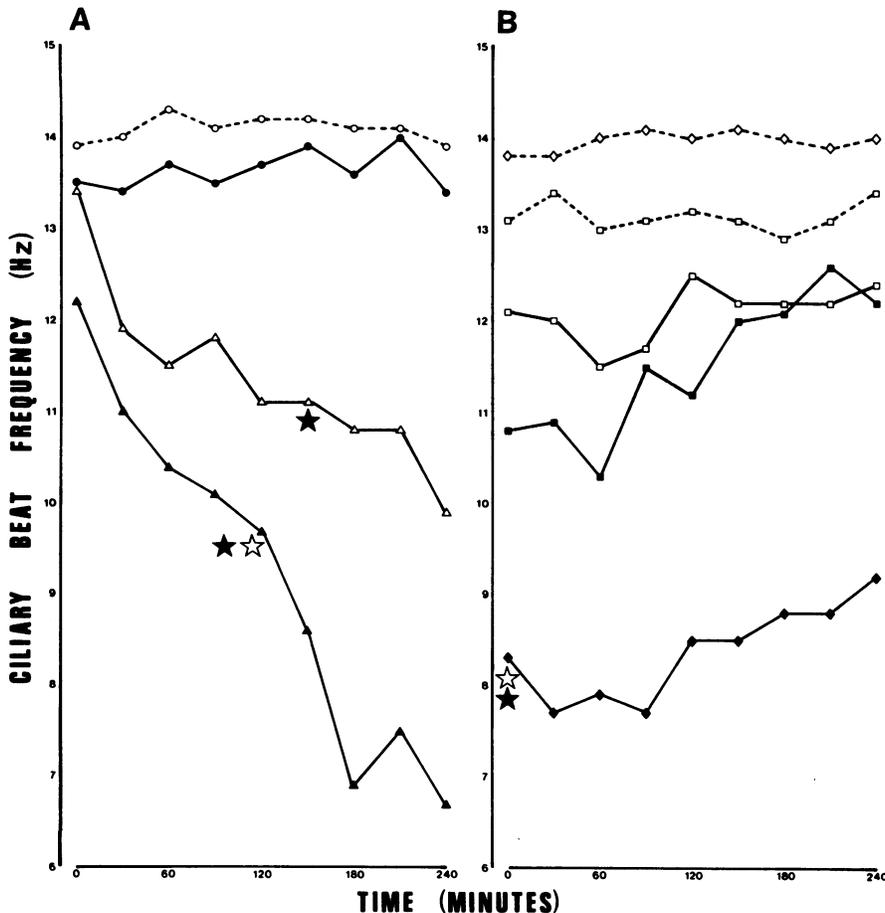


Figure 3. The dose-response effect of purified pyocyanin and purified 1-hp on human nasal CBF at pH 7.4. In A, the CBF produced by pyocyanin 10 μ M (\bullet), 20 μ M (\triangle), 100 μ M (\blacktriangle), and the time-based control CBF (\circ) are shown. In B, the CBF produced by 1-hp 10 μ M (\square), 20 μ M (\blacksquare), and the time-based control CBF (\square - \square) are shown. Also in B is the CBF produced by 50 μ M 1-hp (\blacklozenge) and the time-based control CBF (\diamond). \star = ciliary dyskinesia; \star = ciliostasis.

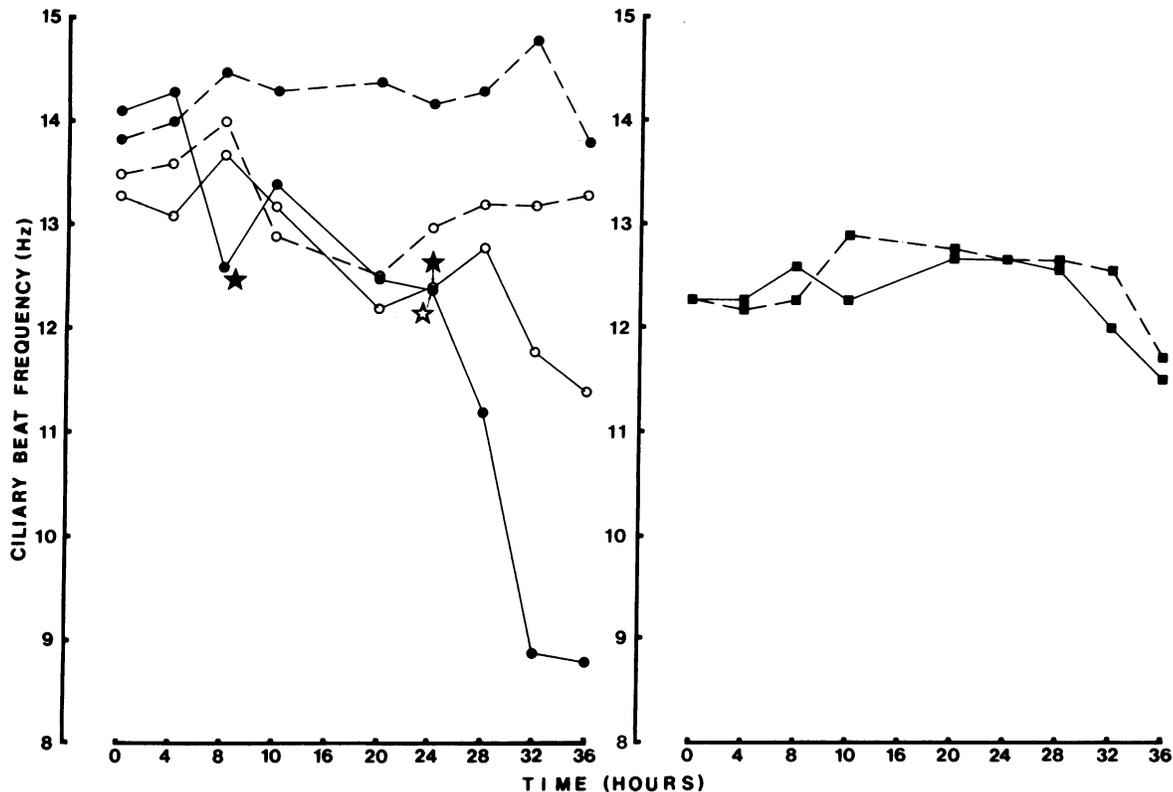


Figure 4. The effect of purified pyocyanin (solid circles, 1 μ M; open circles, 0.1 μ M) and 1-hp (solid boxes, 1 μ M) on human nasal CBF at pH 7.5 over 36 h; solid lines, test; dashed lines, control. Solid stars, first observation of epithelial disruption; open stars, ciliostasis first observed.

In the filtrates from longer cultures, the pyocyanin content was reduced (Table IV) and other UV-absorbing species became apparent (at 4.3 and 48.5 ml).

Discussion

Mucociliary clearance is delayed in patients with chronic bronchial sepsis (17). There are likely to be several reasons for this,

Table IV. Ciliary Slowing Properties and Pyocyanin Concentrations in Active and Inactive Culture Filtrates

| Isolate | Duration of culture | Ciliary slowing | | Pyocyanin |
|---------|---------------------|-----------------|-------|-----------|
| | h | % control | ng/ml | μ M |
| 9 | 18 | 71* | 4,745 | (24) |
| 10 | 18 | 40* | 3,297 | (16) |
| 11 | 18 | 42* | 2,950 | (15) |
| 12 | 18 | 31* | 1,250 | (6) |
| 13 | 18 | 29* | 800 | (4) |
| 14 | 18 | 15* | 0 | |
| 15 | 18 | 0 | 0 | |
| 16 | 18 | 12 | 0 | |
| 17 | 18 | 3 | 0 | |
| 13 | 48 | 37* | 440 | (2) |
| 18 | 96 | 77* | 0 | |

* $P < 0.001$ t test, test vs. control at the same experimental time point, 18 d.f.

including change in mucus properties and damage to respiratory ciliated epithelium by leucocyte proteolytic enzymes released during the inflammatory response. Human leucocyte elastase has been shown to arrest rabbit ciliary beating in vitro (18), and bronchiectatic sputum sol with elastase activity has been shown to slow the beating of normal human nasal cilia in vitro (19).

Bacterial products may delay clearance by their effect on human cilia. Culture filtrates of *P. aeruginosa* and *Haemophilus influenzae* slow human nasal ciliary beating in vitro (3). Infection with *Mycoplasma pneumoniae* destroys human ciliated epithelium (20), and *H. influenzae* causes ciliostasis in rat and chick tracheal organ cultures (21). Southern et al. (22) demonstrated delayed clearance from the lungs of normal mice infected with some strains of *P. aeruginosa*, and this phenomenon appeared to correlate with lecithinase production by the strains. Reimer et al. (23) used rabbit cilia in an in vitro model to investigate filtrates from three strains of *P. aeruginosa*. A concentration-dependent slowing of ciliary beating was demonstrated, and this was reproduced by pyocyanin partially purified by the method of Armstrong et al. (11) (therefore containing 1-hp) and by haemolysin extracted from the organism. Caution must be exercised in extrapolating such results in the rabbit to man, because considerable interspecies variation in susceptibility of cilia to slowing has been demonstrated (24).

P. aeruginosa synthesizes numerous products that have been tested in vitro and in vivo (25, 26) for possible pathogenic significance. Most clinical isolates of this organism produce a pigment, pyocyanin. Mucociliary clearance is the first-line defense mechanism of the human respiratory tract against inhaled par-

ticles. Our results show that purified 1-hp, a degradation product of pyocyanin, rapidly slows and disorganizes the beating of cilia and stops a proportion completely, whereas pyocyanin itself slows cilia gradually but finally disrupts ciliary epithelium completely. We have confirmed the potency of these two molecules by synthesis. At lower pH, the bioactivity of both 1-hp and pyocyanin markedly increases. As pH is reduced, there will be increasing protonation of the phenolic hydroxyl group (Fig. 5) which suggests that this may be important for bioactivity—either through enhanced entry into the cell or possibly through more favorable interaction with receptors.

The ciliary-slowng activity of 18-h culture filtrates correlated ($r = 0.97$) only with pigment content as measured by spectrophotometric absorbance. Subsequent development of methodology to assay pyocyanin within filtrates showed a similar good correlation ($r = 0.72$) between the amount of pyocyanin present and biological activity.

The filtrates of two longer cultures contained less pyocyanin but maintained biological activity. The reduction in amount of pyocyanin is likely to be due to degradation to other phenazine compounds—which may contribute to the ciliary slowing properties of these filtrates from prolonged cultures. Indeed, other less polar UV-absorbing species were observed on HPLC of these filtrates and these may possess cilioinhibitory activity.

We have shown 1-hp to be active and to reproduce the rapid onset of ciliary slowing with subsequent recovery, observed in experiments using some filtrates (Fig. 1). However, there was no evidence of 1-hp in the HPLC profile of culture filtrates and therefore, if present, it is below the limit of detection of this system. It is possible that, if formed from pyocyanin, 1-hp is further metabolized to other (possibly cilioinhibitory) species. 1-hp was however observed in chloroform extracts of agar plate cultures (as noted by Armstrong et al. [11]), perhaps reflecting diffusion of 1-hp away from the immobilized organism. We have recently examined the pulmonary secretions obtained from the lung of a patient suffering from cystic fibrosis removed before heart-lung transplantation and found both pyocyanin and 1-hp to be present. The concentration of each of these pigments was measured using HPLC and found in each case to be in excess of that slowing cilia in vitro.

At the beginning of this century a pigment-containing preparation "pyocyanase", produced from old cultures of *P. pyocyanea*, was shown to have antibacterial properties and was used in the treatment of patients (27). During the first World War, physicians noted the improvement in erysipelas when *P. pyocyanea* infection supervened (27). In 1947, Young (28) described a number of pigments produced by *P. aeruginosa*, including

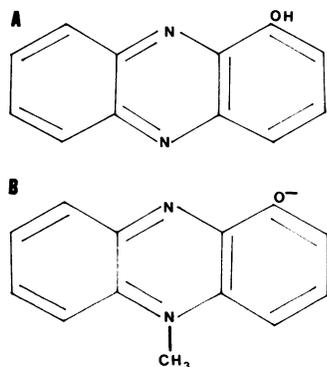


Figure 5. The molecular structure of 1-hp (A) and pyocyanin (B).

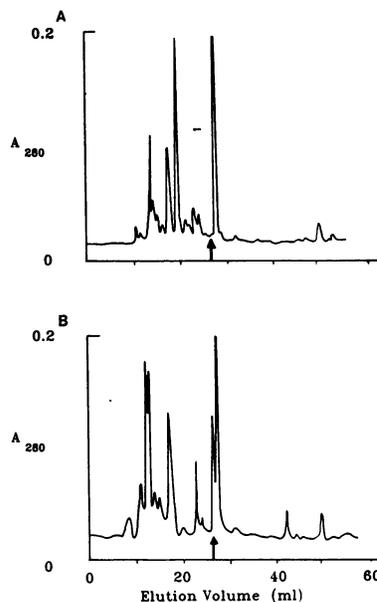


Figure 6. Representative HPLC UV absorbance profiles from the purification of culture filtrates. The samples were chromatographed directly (system iii, propan-2-ol/acetic acid) without prior purification. The elution position of pyocyanin is marked with an arrow. (A) A_{280} UV profile of an inactive filtrate. A number of UV-absorbing peaks are present but there is no evidence of pyocyanin or 1-hp. (B) A_{280} UV profile of an active filtrate showing the presence of pyocyanin.

pyocyanin, which is blue and its yellow breakdown product 1-hp. Schoental (29) isolated three antibacterial substances from chloroform extracts of *P. pyocyanea*, two of which were pyocyanin and 1-hp.

Pyocyanin is a redox dye (30), and its complicated mass spectral behaviour (notably on thermospray LC/MS but also under d.e.i. ionization) arises from its unusual structural properties (i.e., its zwitterionic nature, which confers solubility in both water and chloroform). Pigment-containing preparations from *P. aeruginosa* have been shown to impair cellular respiration (11). Oxygen consumption by mouse liver mitochondria was measured for 8 min after addition of the potential inhibitor. The active fraction that caused inhibition of cellular respiration was 1-hp while pyocyanin was inactive (11). The apparent discrepancy between our results and those of Armstrong et al. (11) may arise from the slower onset of pyocyanin action.

Early in an infection it would be a critical advantage for pseudomonas to produce a virulence factor that compromises ciliary function and enables the organism to establish itself within the respiratory tract. We propose that, in the microenvironment of the ciliated epithelium, pseudomonas produces pyocyanin and 1-hp, which paralyze mucociliary clearance. Both are active in vitro in nanomolar concentrations. Pyocyanin slows ciliary beating and ultimately disrupts the epithelium completely. 1-hp, as well as slowing ciliary beating, also disorganizes the beating pattern. Once established, subsequent spread of the organism within the lung would be facilitated by these properties, leading to considerable morbidity and mortality in patients with cystic fibrosis and other forms of severe bronchiectasis.

Acknowledgments

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