CHARACTERISATION OF THE *PSEUDOMONAS AERUGINOSA* FACTOR THAT INHIBITS MOUSE-LIVER MITOCHONDRIAL RESPIRATION

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THE extracellular products of *Pseudomonas aeruginosa* have been studied by many workers (Schoental, 1941; Liu, 1957, 1966*a* and *b*; Liu, Abe and Bates, 1961; Rugstad, 1966, 1967*a* and *b*; Kučera and Lysenko, 1968), but their mode of action in infection was not completely elucidated.

Earlier workers (Pons, 1927; Lilley and Bearup, 1928; Salvin and Lewis, 1946) isolated the organism from a variety of sites in the human body, e.g., ear, eye, skin, lung, urinary tract, meninges and joints, but gave little indication about the mode of action. It has become increasingly apparent that infections caused by *Ps. aeruginosa* are more common when other predisposing conditions prevail in the human body. Children, especially premature babies, appear to be highly susceptible, the organism entering the body via the umbilicus, gastro-intestinal tract or respiratory tract (Schaffer and Oppenheimer, 1948; Hoffman and Finberg, 1955; Neter and Weintraub, 1955; Asay and Koch, 1960; Rogers, 1960). Secondary invasion by *Ps. aeruginosa* as a complication of extensive burns is well known (Colebrook and Francis, 1941; Lowbury, 1954; Lowbury and Fox, 1954; Rabin *et al.*, 1961; Jones, Jackson and Lowbury, 1966). Jackson, Lowbury and Topley (1951) found that the colonisation of burns by *Ps. aeruginosa* led to rejection of skin grafts and prevented the normal healing process of the skin.

In a series of investigations carried out in this laboratory the modes of action of extracellular products of pathogenic bacteria on cells and subcellular components, especially mitochondria, have been studied. Since Jackson *et al.* reported that *Ps. aeruginosa* caused cellulitis our approach was extended to include this organism. It soon became apparent that pigment-containing preparations of *Ps. aeruginosa* impaired cellular respiration. The blue pigment, pyocyanin, is known to be a redox dye (Hewitt, 1950), so it was decided to explore the effect of pyocyanin and its derivatives on the mitochondrial electron-transport chain.

MATERIALS AND METHODS

Strains of organisms

Pseudomonas aeruginosa NCTC6750 and *Ps. diminuta* NCTC8545 were obtained from the National Collection of Type Cultures (Colindale, London, N.W.9). The pigmented strains of *Ps. aeruginosa* MSU 14388 and 5306 were isolated from patients at Stobhill Hospital and Western Infirmary, Glasgow, respectively.

Media

The sloppy agar medium A of King, Ward and Raney (1954) was used for pigment production: Oxoid peptone 20 g, glycerol 10 g, K₂SO₄ anhydrous 10 g, MgCl₂ anhydrous 1.4 g, Difco Bactoagar 5 g, distilled water to 1 litre. The pH of the medium was adjusted

Received 30 June 1970; accepted 16 Dec. 1970.

to 7.2 after the addition of the agar. The medium was dispensed in 200-ml amounts and autoclaved at 121°C for 15 min.

Pigment production

The organism was plated out on nutrient agar and incubated overnight at 37°C, and the growth was harvested in 5 ml of liquid King, Ward and Raney A medium. 1 ml of the suspension was pipetted on to the sloppy medium A of King *et al.* The plates were placed in sealed tins packed with tissue paper to prevent them drying. After incubation at 37°C for variable periods of time, the contents of each plate were frozen at -20°C and subsequently allowed to thaw at room temperature. The extruded fluid was collected and centrifuged at 10,000 r.p.m. for 10 min. at 4°C. The supernatant fluids containing pigment (PF2) were retained and stored at -20°C.

Chloroform extraction of the supernatant fluids

Equal volumes of supernatant fluid (PF2) and dry chloroform were mixed and shaken vigorously. The mixture was allowed to settle out in a separating funnel. The chloroform layer was collected and after evaporating off the chloroform *in vacuo* the sediment (PF30) was retained and stored under vacuum.

Preparation of pyocyanin and 1-hydroxyphenazine from cultures of Ps. aeruginosa

Crystalline pyocyanin was prepared by a modification of the method of Wrede and Strack (1924). The supernatant fluid (PF2) containing pigment (100 ml) was extracted overnight at 4°C with 50 ml of dry chloroform. The chloroform layer was separated from the mixture and evaporated to dryness. The dried sediment was resuspended in 5 ml dry chloroform and 10–15 ml dried petroleum spirit was added dropwise until dark blue, needle-like crystals appeared. When crystallisation was complete, the apple-green solvent layer was gently decanted. The dark blue crystalline mass was dissolved in water and freeze-dried; the mass of fine crystalline pyocyanin (PF42) obtained was stored under vacuum in the dark.

An alkaline solution of this pyocyanin was left at room temperature for 18 hr, during which time the colour changed from blue to red-violet. Acidification with *N*-HCl yielded a floccular yellow precipitate, which was filtered off and freeze-dried. The resulting product was a pinkish-brown substance, 1-hydroxyphenazine (PF43).

Chemical synthesis of pyocyanin and its derivatives

The synthesis of these compounds (fig. 1) was carried out by a modification of the method of Surrey (1946*a* and *b*).

Pyrogallol 1-monomethyl ether. The apparatus consisted of a 1-litre three-necked flask fitted with a gas inlet tube extending about 3 cm into the flask and connected to the flask through a bubbler, a thermometer extending to the bottom, and a reflux condenser connected at the upper end with an exit tube leading to the hood. The reaction was carried on in an atmosphere of nitrogen. 200 ml 2*N*-NaOH was placed in the flask; 60.8 g of crushed 2-hydroxy-3-methoxy-benzaldehyde (*o*-vanillin) was slowly added to the alkali in the flask. The mixture was stirred with a magnetic stirrer until almost all the solid had dissolved. 284 ml of 6 per cent. hydrogen peroxide was added in 25-ml amounts over a period of 1 hr; the temperature was held between 40° and 50°C. The temperature increased to about 45°C and a dark solution was formed after the addition of the first portion of hydrogen peroxide; the temperature was allowed to fall to 40°C before the addition of the next portion of hydrogen peroxide.

After the addition of all the hydrogen peroxide, the reaction mixture was cooled to 20°C (room temperature) and saturated with sodium chloride. The reaction mixture was extracted with 100-ml portions of ether; a total volume of 700 ml ether was used. The combined ether extracts were dried over sodium sulphate for 48 hr. Ether was removed *in vacuo* and pyrogallol monomethyl ether was collected by heating the residue vigorously

under reduced pressure. According to Surrey (1946*b*) the pyrogallol monomethyl ether distilled over at 136°–138°C under 22 mm Hg, but in practice this did not occur. A light yellow oil was obtained that solidified on standing at room temperature for 24 hr; approximately 40 g of pyrogallol monomethyl ether were obtained.

1-Methoxyphenazine. Pyrogallol monomethyl ether (10 g) was dissolved in 3 litres of dry benzene in a flask and 200 g powdered lead dioxide was added. It was imperative that the lead dioxide was a fresh, black preparation. The mixture was shaken by hand for 20 min. and filtered through an 11-cm Buchner funnel to remove a reddish-brown solid. The filter cake was washed with 400 ml benzene. A solution of 9 g *o*-phenylenediamine in 80 ml glacial acetic acid and 200 ml benzene was immediately added to the filtrate; a mechanical stirrer was used. The dark-brown solution was left at room temperature for

FIG. 1.—The chemical synthesis of pyocyanin and its derivatives.

1½ hr and subsequently divided into two portions. Each portion was washed three times with water, twice with 5 per cent. NaOH solution and finally twice with water, 100-ml portions being used each time. The washed benzene solutions were shaken with 50 g anhydrous sodium carbonate and 5 g Norit and filtered through an 11-cm Büchner funnel. The filtrate was mixed with 60 g activated alumina; the mixture was shaken until a filtered sample was light yellow in colour. The alumina was removed by filtration through a coarse, folded filter paper and the cake of alumina was washed until the filtrate was colourless. The benzene was removed from the combined filtrates and washings by vacuum distillation. The residual yellow solid was dissolved in 10 ml hot pyridine. Distilled water was added to the point of incipient precipitation and the mixture was left at 4°C. Light yellow crystals were filtered on a 7-cm Büchner funnel, washed with water, and air-dried. The yield after 24 hr was 1.69 g, but this amount progressively increased with time so that after 3 days the total yield was 3.0 g.

***α*-Hydroxyphenazine.** A solution of 2 g *α*-methoxyphenazine in 125 ml of 55 per cent. hydrobromic acid was placed in a 250-ml round-bottomed flask fitted with a reflux condenser. The flask was immersed in an oil-bath and the solution was heated at 110°–120°C for 5 hr; any gases evolved were absorbed in a water trap. After 5 hr the dark-brown solution was cooled to room temperature, diluted with 125 ml distilled water and almost neutralised with 100 ml of 35 per cent. NaOH followed by an additional volume of 10 per cent. NaOH. The reaction mixture was faintly acid to litmus; at this point there was also a change in colour from dark brown to bright yellow. The mixture was extracted with 40-ml portions of ether until the ether extracts were colourless; a total volume of 600 ml ether was used. The combined ether extracts were extracted with 25-ml portions of 10 per cent. NaOH solution. The purple sodium salt, which separated during this extraction and adhered to the wall of the separating funnel, was dissolved in water and added to the NaOH extracts. This extraction was continued until no more purple sodium salt was removed from the ether

extracts. Water was added to the NaOH extracts until all salts were in solution. The extracts were made acid to litmus with acetic acid; this process was accompanied by a colour change from purple to bright yellow. The acidified extracts were re-extracted five times with 50-ml portions of ether. These extracts were dried over anhydrous sodium sulphate and the ether was removed by vacuum distillation. The residue was dissolved in 10 ml hot ethanol, and water was added to the point of incipient precipitation. Norit (0.5 g) was added and the hot solution was filtered. The filtrate was cooled in ice-water and the bright yellow solid was filtered on a 7-cm Büchner funnel, washed with water and dried at 100°C. The yield of α -hydroxyphenazine was 1.0 g.

Pyocyanin. α -Hydroxyphenazine (0.5 g) was dissolved in 10 ml methyl sulphate (0.1 mole) and placed in a 250-ml flask fitted with a calcium chloride drying tube and heated at 100°C in an oil-bath for 10 min. The solution was cooled to room temperature and 75 ml dry ether was added. The dark-brown solid that formed was filtered on a 7-cm Büchner funnel and washed with 150 ml of dry ether. The dry methosulphate was dissolved in 30 ml water and made alkaline by the addition of 10 per cent. NaOH. The dark-ruby-coloured solution was extracted exhaustively with successive 15-ml portions of chloroform until no more blue substance was removed in the extract; this procedure was continued for 36 hr and 5 litres of chloroform were used. The combined chloroform solutions were extracted three times with 20-ml portions of 5 per cent. HCl. The combined red-coloured, acid extracts were made alkaline with 10 per cent. NaOH, during which a colour change to blue occurred. The alkaline solution was extracted exhaustively with 25 ml amounts of chloroform until no more blue substance was removed; 2 litres of chloroform were used. The combined chloroform solutions were dried over anhydrous sodium sulphate. The dried extracts were decanted and the chloroform was removed by vacuum distillation. The blue crystalline residue was dissolved in 5 ml water at 60°C and cooled in an ice-bath, when a mass of dark blue crystals were formed. This product was filtered on a 7-cm Büchner funnel and dried in the dark in a vacuum desiccator over calcium chloride. The yield was 386 mg of dark-blue needles.

Amino acid chromatography

The two-dimensional detection of amino acids was carried out as described by Stewart-Tull and White (1967).

Preparation of mouse-liver mitochondrial suspensions

A fresh suspension of mitochondria was prepared for each experiment by a modification of Schneider's method (1948). Three Porton white mice (35–45 g) were killed by cervical dislocation and exsanguinated. The liver was removed from each mouse and placed in 10 ml Hendry's sucrose phosphate buffer (0.25M sucrose in 0.023M phosphate buffer, pH 7.3; Hendry, 1948). Connective tissue was gently removed and the liver was minced; the minced tissue was carefully homogenised in a tissue grinder. The homogenate in 50 ml of Hendry's sucrose phosphate buffer was centrifuged at 2000 r.p.m. for 10 min. at 4°C; the supernatant fluid was retained and the sediment was discarded. The supernatant fluid was centrifuged at 8500 r.p.m. for 10 min. at 4°C; the sediment was washed and re-centrifuged under the same conditions. Finally the sediment was resuspended in 4 ml buffer and stored at 4°C for 1 hr.

Absorption of pseudomonas fractions with mouse-liver mitochondria

Mouse liver mitochondria (1 ml) were incubated at 37°C for 1 hr with 1 ml of the supernatant fluid (PF2). Subsequently, the mitochondria were removed by centrifugation at 8500 r.p.m. for 10 min. at 4°C, and the supernatant fluid (PF2A) was retained for use in manometric experiments.

Warburg manometry

In the standard Warburg assay four flasks were used, each containing 1.7 ml Hendry's sucrose phosphate buffer, 0.5 ml mitochondrial suspension and 0.3 ml of 0.2M succinate in the main well. Two of these flasks contained a pseudomonas fraction in the side arm

and two contained a control fluid (0.5 ml). The fifth flask was a thermobarometer. The total volume was always 3 ml in each flask. Readings were taken over a period of 90 min.

The crude supernatant fluid (PF2), the chloroform extract (PF30), the biological preparation of pyocyanin (PF42) and 1-hydroxyphenazine prepared from biological pyocyanin (PF43), the chemical preparations of pyrogallol monomethyl ether (PF46), 1-methoxyphenazine (PF47), 1-hydroxyphenazine (PF48) and pyocyanin (PF49) and absorbed supernatant fluid (PF2A) were tested for their activity on mouse-liver mitochondria utilising sodium succinate.

Polarographic measurement of mouse-liver mitochondrial respiration

The Y.S.I. Model 53 biological oxygen monitor (Shandon Scientific Co., London) provides a means of measuring oxygen uptake by biological systems. In all experiments 0.3 ml 0.2M sodium succinate and 0.1 ml mouse-liver mitochondrial suspension were placed in the sample chamber. Different concentrations of the pseudomonas fractions were tested for their activity on the mitochondria; the total volume in each test was adjusted to 3 ml with Hendry's sucrose phosphate buffer pH 7.3. The culture supernatant fluid (PF2) was diluted 1 in 6 before use, and all other fractions (PF42, 43 and 46-49) were used as stock solutions containing 0.5 mg per ml.

In order to determine the activity of these fractions mitochondria were allowed to utilise the sodium succinate for 90 s after which the fractions were injected into the sample chamber through narrow catheter tubing. The uptake of oxygen was determined before and after the addition of the fractions.

*Pigment production by *Pseudomonas aeruginosa**

Maximum pigment production by *Ps. aeruginosa* NCTC6750, and by MSU 14388 and 5306 was evident in King, Ward and Raney A sloppy agar; after 24 hr the culture supernatant fluid (PF2) obtained by centrifugation, was a deep blue-green in colour. The organisms produced negligible amounts of blue pigment either in 1 per cent. nutrient sloppy agar, or in static or shaking cultures in King, Ward and Raney A liquid medium.

Various peptones (Oxoid peptone, Evans peptone, Eupiptone, Difco Neopeptone and BDH peptone) were separately incorporated into different batches of King, Ward and Raney A sloppy agar and it was found that the pigmentation varied from a pale yellow-green with B.D.H. peptone to the typical deep blue-green with Oxoid peptone. Consequently, the latter was used in order to maintain maximum pigment production.

Chloroform extraction of the culture supernatant fluid PF2. Extraction of the crude pseudomonas fraction with chloroform gave a yield of 1 mg of a deep-blue substance (PF30) per 1 ml supernatant fluid.

The biological and chemical synthesis of pyocyanin and its derivatives

Paper chromatographic analysis of the fractions indicated that they were non-protein in nature; no trace of amino acids was found in the biological preparation of pyocyanin (PF42) or in the sample of 1-hydroxyphenazine (PF43) chemically prepared from PF42.

The ultraviolet spectra of pyocyanin (PF42) and 1-hydroxyphenazine (PF43) were obtained in the Unicam SP 800 spectrophotometer, with methanol as the reference solvent. The maximum ultraviolet absorption was found at 320 nm for pyocyanin and at 297 nm for 1-hydroxyphenazine.

Maximum absorption for the chemically synthesised compounds was 320 nm for pyocyanin, 263 nm, for 1-hydroxyphenazine, 260 nm for 1-methoxyphenazine and 268 nm for pyrogallol monomethyl ether; these figures compared favourably with those of Corbett (1964). The absorbance of pyocyanin (PF49) was also examined in 0.1M-HCl in 50 per cent. methanol; a peak was obtained at 388 nm, which agreed with the published figure of 387 nm (Corbett).

Analysis of the biological sample of pyocyanin (PF42) and the sample of 1-hydroxyphenazine (PF43), prepared from PF42, in the mass spectrometer, indicated that they were

not chemically pure. This was confirmed by thin-layer chromatography; the sample of pyocyanin was found to contain a small amount of 1-hydroxyphenazine. Mass spectrometry of the chemical preparations indicated that they were chemically pure. The observed molecular weights were 140 for pyrogallol monomethylether (PF46), 210 for 1-methoxyphenazine (PF47) and 196 for 1-hydroxyphenazine (PF48). The mass spectra of the latter two compounds were in accord with the results of Morita (1966) and Holliman, Johnstone and Millard (1967). An anomaly was found for pyocyanin (PF49) since the expected molecular weight was 210 and the mass spectrometric analysis indicated a molecular weight of 224. This observation of an extra 14 mass units has been found in certain alkaloids and by deuterium labelling the mechanism has been shown to involve a bimolecular transmethylation with a subsequent loss of H (Budzikiewicz, Djerassi and Williams, 1967).

RESULTS

The action of pseudomonas fractions on mouse-liver mitochondrial respiration measured by Warburg manometry

The effect of Ps. aeruginosa culture supernatant fluid PF2

Before the fractions were tipped into the main well of the Warburg flask there was no difference in the uptake of oxygen between the test and control systems. After tipping, the uptake of oxygen in the control, containing supernatant fluid from uninoculated medium, was 328 μ l after 30 min., whereas the uptake in the presence of PF2 was 96 μ l; a reduction of some 71 per cent.

The effect of chloroform extracts from Ps. aeruginosa culture

The uptake of oxygen (a) by mitochondria treated with the chloroform extract (PF30) was 107 μ l in 90 min. and (b) by mitochondria treated with the supernatant fluid (PF2) was 87 μ l in 90 min. The chloroform-insoluble residues (PF32) of the supernatant fluid caused no inhibition of the mitochondria; the oxygen uptake was 274 μ l in 90 min., which was similar to the result obtained in the control flasks.

The effect of pseudomonas fractions absorbed with mitochondria

The uptake of oxygen by mitochondria treated with the culture supernatant fraction PF2 was 117 μ l in 85 min., and by mitochondria treated with absorbed PF2, 200 μ l in 85 min. In the control flask the uptake was 367 μ l in 85 min. It was apparent that absorption of the pseudomonas fraction with mitochondria reduced its inhibitory effect on mitochondrial oxygen uptake by 22.5 per cent.

The effect of pyocyanin and 1-hydroxyphenazine prepared either directly or indirectly from culture supernatant fluids

As shown in fig. 2, both pyocyanin (PF42) and 1-hydroxyphenazine (PF43) caused an inhibition of oxygen uptake by mouse-liver mitochondria. The oxygen uptake by mitochondria treated with 1-hydroxyphenazine (PF43) was 81 μ l in 75 min., regardless of the concentration of chemical tested. The uptake by mitochondria treated with the supernatant fluid (PF2) was 104 μ l in 75 min.; mitochondria in the control flasks took up 357 μ l in 75 min. Contrariwise,

pyocyanin (PF42) caused a lesser inhibitory effect on the uptake of oxygen by mouse-liver mitochondria and this inhibition was concentration-dependent; inhibition with 0.25 mg pyocyanin per ml > that with 0.16 mg per ml > that with 0.08 mg per ml.

FIG. 2.—The effect of pyocyanin and its derivatives on the respiration of mouse-liver mitochondria measured in the Warburg apparatus. All fractions were added at the time marked with an arrow. The asterisk indicates that a similar result was obtained with 0.08, 0.16 and 0.25 mg fraction per ml.

The effect of chemically synthesised derivatives of pyocyanin

A similar result was obtained using chemically synthesised pyrogallol monomethylether (PF46), 1-methoxyphenazine (PF47) and 1-hydroxyphenazine (PF48). When these substances were tested, at concentrations of 0.25 mg per ml, 0.16 mg per ml and 0.08 mg per ml, the uptake of oxygen by mitochondria was 80 μ l in 75 min.; in the control test it was 270 μ l (see fig. 2).

The action of pseudomonas fractions on mouse-liver mitochondrial respiration measured in the oxygen polarograph

The effect of pseudomonas fractions, at various concentrations, on the uptake of oxygen per minute by mitochondria was measured in the polarograph (table). The biological preparation of 1-hydroxyphenazine (PF43) completely inhibited mitochondrial respiration at concentrations of 25 and 33 μ g per ml (fig. 3). A similar result was obtained with the chemical preparation of 1-hydroxyphenazine (PF48). However, the biological preparation of pyocyanin

(PF42) caused no inhibition at a concentration of 16 μg per ml, little inhibition at a concentration of 33 μg per ml and a more marked inhibition at a concentration of 50 μg per ml (fig. 4).

TABLE

The effect of biological and chemical preparations of pyocyanin and its derivatives on liver mitochondrial respiration as measured in the oxygen polarograph

Substance added	Amount added (μg)	μl O ₂ consumed per min. by 0.5 ml mitochondrial suspension	
		before addition of substance	after addition of substance
PF2—crude fraction from culture supernatant fluid .	16	5.85	6.75
	33	4.80	0.45
	50	6.45	0.45
PF42—biological preparation of pyocyanin . . .	16	5.4	5.85
	33	5.1	4.70
	50	5.1	2.25
PF43—1-hydroxyphenazine prepared from biological pyocyanin (PF 42)	16	5.17	2.40
	25	5.50	0
	33	6.50	0
PF46—pyrogallol monomethyl ether	16	4.95	5.6
	33	3.60	3.15
	50	4.05	5.85
PF47—1-methoxyphenazine	16	5.1	4.65
	33	5.6	4.95
	50	5.4	4.35
PF48—1-hydroxyphenazine	16	5.6	3.15
	25	4.5	0
	33	4.75	0
PF49—Pyocyanin	16	4.05	4.35
	33	4.5	4.05
	50	4.7	5.70
None (controls)	...	5.4	5.70
	...	4.27	4.05
	...	4.35	4.20

In contrast with the results obtained by Warburg manometry no significant inhibition of oxygen uptake was found with pyrogallol monomethyl ether (PF46), 1-methoxyphenazine (PF47) or pyocyanin (PF49) at concentrations of 16, 33 or 50 μg per ml.

DISCUSSION

The culture supernatant fluids of *Pseudomonas aeruginosa* NCTC6750 and MSU 14388 and 5306 caused a 90–100 per cent. inhibition of oxygen uptake by mouse-liver mitochondria. This effect appeared to be related to pigment

production in these strains, since *Ps. diminuta* NCTC8545 did not produce pigment and caused no inhibition of mitochondrial respiration. In addition,

FIG. 3.—The effect of varying concentrations of preparations of 1-hydroxyphenazine (PF43 or PF48) on mouse liver mitochondrial respiration, as measured in the oxygen polarograph. A—after the addition of 25 μ g or 33 μ g 1-hydroxyphenazine per ml; B—after the addition of 16 μ g 1-hydroxyphenazine per ml; and C—control without 1-hydroxyphenazine. Fractions were added at the point marked with an arrow.

under certain growth conditions *Ps. aeruginosa* is non-pigmented and the supernatant fluid from cultures in this state did not cause any respiratory inhibition. Maximum pigment production was obtained in King, Ward and

Raney A medium containing Oxoid peptone. *Ps. aeruginosa* NCTC6750 constantly produced a deep blue-green pigment in this medium. When a

FIG. 4.—The effect of varying concentrations of pyocyanin (PF42) on mouse liver mitochondrial respiration, as measured in the oxygen polarograph. A, B and C—after the addition of 50, 33 and 16 µg pyocyanin per ml respectively; D—control without pyocyanin. Fractions were added at the point marked with an arrow.

variety of peptones were incorporated into this medium it was found that the inhibitory activity of supernatant fluids, from cultures grown in media containing different peptones, was related to the degree of pigment formation.

However, *Ps. aeruginosa* MSU5306, a variant of *Ps. aeruginosa*, produced a red-brown pigment that was shown to contain 1-hydroxyphenazine. Acidification of the red-brown pigment caused the deposition of yellow needles of 1-hydroxyphenazine.

The pseudomonas culture supernatant fluid (PF2) and the chloroform extract (PF30) of PF2 retained their inhibitory effect after heating at 100°C for 30 min. Schoental (1941) showed that pyocyanin and 1-hydroxyphenazine were resistant to boiling; Wrede and Strack (1924) had already found that heating pyocyanin led to the production of 1-hydroxyphenazine. The heat resistance provided evidence for the non-enzymic nature of the factor responsible for mitochondrial inhibition. This was confirmed by experiments that showed that the active fractions contained no amino acids, and were dialysable.

A biological preparation of 1-hydroxyphenazine (PF43) completely inhibited the uptake of oxygen regardless of the concentration tested (fig. 2). However, the inhibitory activity of the biological preparation of pyocyanin (PF42) was concentration-dependent. Since the biological preparations were found to be mixtures of pyocyanin and hydroxyphenazine it was decided to synthesise pure chemical preparations of pyocyanin and its derivatives in order to ensure a complete characterisation of the inhibitory factor. When the activity of these chemical preparations (PF46–49) on mouse-liver mitochondrial respiration was tested by Warburg manometry they caused an inhibition of oxygen uptake (fig. 2—expt 2). However, when more sensitive measurements were made in the oxygen polarograph it was found that the inhibitory activity was confined to both preparations of 1-hydroxyphenazine (PF43 and PF48); the inhibition was insignificant with pyrogallol monomethyl ether (PF46), 1-methoxyphenazine (PF47), or pyocyanin (PF49) (table). It is evident that investigations into the inhibitory activity of microbial substances on mitochondrial respiration must be carried out with the more sensitive oxygen polarographic technique since Warburg manometry did not reveal fine differences in inhibition. These results show that the factor produced by *Ps. aeruginosa* that inhibits mouse-liver mitochondrial respiration is not pyocyanin but 1-hydroxyphenazine (α -oxyphenazine, α -hydroxyphenazine or 1-phenazinol).

Current theories of *Ps. aeruginosa* pathogenicity do not take into account the precise in-vivo biochemical action of pseudomonas products. Chemical constituents have been isolated from the cells of this organism, but their interactions in *Ps. aeruginosa* pathogenicity still remain inconclusive. These constituents include an endotoxin moiety (Liu *et al.*, 1961); an exotoxin (Liu, 1966*a*); haemolysins (Liu, 1957); extracellular enzymes, namely, lecithinases, lipases, and proteases (Liu *et al.*; Liu, 1966*b*), kininases (Rugstad, 1966, 1967*a* and *b*) and proteinases (Kučera and Lysenko, 1968). In addition, abnormal physiological conditions in the host, such as those caused by either shock or the presence of a previous infection or antibiotic therapy, may play an important role in the over-all mechanism of *Ps. aeruginosa* pathogenicity.

Earlier workers (Bouchard, 1889; Hettche, 1933; Kramer, 1935; Schoental)

concentrated on the antibacterial action of products of this organism, but the importance of these with respect to pathogenicity was not considered. It is possible that in a mixed infection competing bacteria would be eliminated through the action of these products, thus allowing the unimpeded growth of *Ps. aeruginosa*. Jones *et al.* (1966) believed that the failure of antibiotic therapy in the treatment of *Ps. aeruginosa* burn infections was due to the irreparable damage (cellulitis, invasion of the walls of small blood vessels and septicaemia) that had occurred by the time that the infection was diagnosed. Nelson and Berk (1960) suggested that the development of lesions involved an alternation between toxic destruction of cells and bacterial multiplication. This could explain why lesions persist even after antibiotic treatment, since a toxic substance would be unaffected by the antibiotics and could continue to cause cytolysis.

It is possible to postulate from the results presented in this paper that in a superficial wound or burn infected with *Ps. aeruginosa* first 1-hydroxyphenazine would be produced which might cause an impairment of efficiency and ultimately death of macrophage cells; thus removing the first line of defence of the body. This would be followed by the destruction of tissue cells and finally 1-hydroxyphenazine would be released into the bloodstream. The 1-hydroxyphenazine produced at the site of infection would reach the liver and accumulate in liver cells including liver macrophages. Eventually, the liver cell mitochondria would be totally inhibited with subsequent cytolysis and death of the animal. Although this may not account for the total pathogenic effect, it would explain, in part, the complex mechanism of *Ps. aeruginosa* pathogenicity.

SUMMARY

A chloroform-soluble, extracellular product of *Pseudomonas aeruginosa* was obtained from sloppy-agar cultures. Pyrogallol 1-monomethyl ether, 1-methoxyphenazine, 1-hydroxyphenazine and pyocyanin were chemically synthesised and also tested for an inhibitory effect on mitochondrial respiration. It was found that the *Ps. aeruginosa* substance and 1-hydroxyphenazine inhibited the uptake of oxygen by mouse liver mitochondria utilising sodium succinate.

In a *Ps. aeruginosa* infection this substance would be released into the bloodstream, accumulate in the liver and cause cytolysis, thus contributing to the complex mechanism of *Ps. aeruginosa* pathogenicity.

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