THE EFFECT OF 1-HYDROXYPHENAZINE AND PYOCYANIN FROM *PSEUDOMONAS AERUGINOSA* ON MAMMALIAN CELL RESPIRATION

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IN 1953 Cruickshank and Lowbury found that pyocyanin was toxic to human skin cells and leucocytes *in vitro*, and suggested that this might explain the local pathogenic effects caused by *Pseudomonas aeruginosa* in burns.

Previously, Jackson, Lowbury and Topley (1951) had found that pyocyanin was non-toxic when injected into the skin of animals. Berk, Nelson and Pickett (1960*a* and *b*) studied the interaction between *Ps. aeruginosa* and mouse peritoneal monocytes. The oxygen uptake by the mixture was greater than that attributable to an additive effect of bacterium and monocyte alone. Similar results were obtained with heat-killed monocytes, kidney homogenates or HeLa cells; it was concluded that the organism metabolised cellular materials. It was also shown that the addition of heat-killed cells of *Ps. aeruginosa* inhibited the endogenous and succinoxidase activities of mouse monocytes, but did not affect their cytochrome oxidase activity. Nelson and Berk (1960) extended this work and found that not only the whole organism but also its component parts inhibited the respiration of mouse monocytes, spleen and HeLa cells.

Armstrong, Stewart-Tull and Roberts (1971) examined the effect on liver mitochondrial respiration of pyocyanin and its derivatives prepared either from *Ps. aeruginosa* culture fluids or by chemical synthesis. It was shown that 1-hydroxyphenazine completely inhibited the uptake of oxygen by suspensions of mouse liver mitochondria. Later, it was shown that 1-hydroxyphenazine acted at the site of ubiquinone-cytochrome *b* within the electron transport chain (Armstrong and Stewart-Tull, 1971). However, these investigations did not reveal whether 1-hydroxyphenazine would inhibit the respiration of intact cells, and it was decided to explore the effect of 1-hydroxyphenazine and pyocyanin on the respiration of intact mammalian cells.

MATERIALS AND METHODS

Preparation of pseudomonas fractions and chemical synthesis of pyocyanin and 1-hydroxyphenazine

The methods used were identical with those described by Armstrong *et al.*

Preparation of suspensions of mouse liver and hamster kidney cells

Two cell-lines were used in this investigation: (a) L“S”, derived from mouse liver and (b) BHK strain C13, derived from hamster kidney. The cells were grown in Eagle's complete medium (Eagle, 1955*a*, *b* and *c*) as modified by Paul (1959). Burler bottles containing 200 ml

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Eagle's medium were seeded with approximately 20×10^6 cells and gassed with CO_2 . After 3 days at 37°C the cells were harvested from the tissue cultures.

(a) The contents of burler bottles containing L"S" cells in suspension were removed aseptically and centrifuged at 1000 r.p.m. for 5 min. The pellet was resuspended in Eagle's medium minus glucose; substrate was added during the measurement of respiratory activity.

(b) After removal of the medium the monolayer of BHK strain C13 cells was washed with 25 ml trypsin-Versene solution. The washed monolayer was suspended in 25 ml trypsin-versene solution and centrifuged at 1000 r.p.m. for 5 min. The pellet was resuspended in Eagle's medium minus glucose.

Preparation of a suspension of macrophage cells from the peritoneal exudate of a guinea-pig

Sterile paraffin oil (20 ml) was injected intraperitoneally into an albino guinea-pig. After 4 days the animal was killed and 200 ml Hanks' fluid containing 20 IU non-phenolised heparin per ml was injected into the peritoneal cavity. The peritoneal washings were aspirated, pooled and centrifuged at 1000 r.p.m. for 10 min. at 4°C . The pellet was resuspended in Hendry's sucrose phosphate buffer, pH 7.3.

Warburg manometry and oxygen polarography

The procedures for measuring cell respiration were those described by Armstrong *et al.*

Determination of the optimal number of tissue culture cells for manometric measurements of respiration. Variable numbers of cells, from 17×10^6 to 80×10^6 , were suspended in 2.5 ml of Eagle's medium minus glucose. Glucose (0.3 ml containing 5.0 mg) was added from the side-arm; 0.2 ml of 0.1M-NaOH was contained in the centre pot of the Warburg flask.

The effect of pseudomonas fractions on mouse liver and hamster kidney cells. The cell suspension (1.8 ml) and 0.5 ml oxidisable carbohydrate (either 5 mg glucose or 0.2M sodium succinate) were placed in the main well of the Warburg flask. The pseudomonas fraction (0.5 ml) was placed in the side-arm, and 0.2 ml of 0.1M-NaOH in the centre pot. The oxidation of the substrates was measured at 37°C .

The effect of pseudomonas fractions on the respiration of guinea-pig macrophages. The respiration of peritoneal macrophages was measured in the oxygen polarograph with either 0.2M sodium succinate or 0.2M glucose as substrate. Pyocyanin or 1-hydroxyphenazine was added to respiring macrophages or was pre-incubated with the macrophages for periods up to 2 hr before measurement of respiratory activity.

The in-vivo effect of Pseudomonas aeruginosa NCTC6750, 1-hydroxyphenazine and pyocyanin on mammalian cell respiration

Groups of six mice each received by intraperitoneal injection 1×10^8 *Ps. aeruginosa* NCTC6750 organisms. The test and control mice were killed by cervical dislocation 17 hr after the injection. The livers were removed, mitochondrial suspensions were prepared and respiratory activity was measured.

Pyocyanin (PF 49) or 1-hydroxyphenazine (PF 48) was injected intravenously or intraperitoneally into groups of six mice. Mitochondrial suspensions were prepared from animals that died or were killed, and their respiration was measured in the oxygen polarograph. The total volume in each test was 3 ml, consisting of 2.5 ml Hendry's sucrose phosphate buffer, 0.3 ml of 0.2M sodium succinate and 0.2 ml mitochondrial suspension.

RESULTS

The inhibition of respiration in mouse liver and hamster kidney tissue-culture cells by pseudomonas fractions

Preliminary experiments were done to determine the optimal number of mouse liver (L"S") and hamster kidney (BHK strain C13) cells that gave a

measurable rate of respiration per Warburg flask; optimal conditions were obtained with 47×10^6 or more cells per flask.

The uptake of oxygen by L" S " cells utilising sodium succinate was reduced by the pigment-containing supernatant culture fluid of *Ps. aeruginosa* (PF 2) and also by the sediment of a chloroform extract (PF 30) to 18 μl in 90 min., whereas in control flasks it was 240 μl in 90 min. However, the uptake of oxygen by the same tissue-culture cells utilising glucose was not inhibited by these pseudomonas fractions (fig. 1).

The BHK cells were found to utilise glucose but not succinate. Both PF 2 and PF 30 reduced the uptake of oxygen by these cells when they were utilising glucose to 160 μl in 100 min., whereas in the control flask it was 220 μl (fig. 1).

FIG. 1.—The effect of pseudomonas fractions on the respiration of mouse liver, L" S ", and baby hamster kidney BHK strain C13 tissue-culture cells. A: Control Warburg flask containing L" S " cells utilising sodium succinate or glucose; B: flask containing L" S " cells utilising glucose plus PF 2 or PF 30; C: flask containing L" S " cells utilising succinate plus PF 2 or PF 30; D: control flask containing BHK cells utilising glucose; E: flask containing BHK cells utilising glucose plus PF 2 or PF 30. PF 2, a crude fraction containing 1-hydroxyphenazine prepared from *Ps. aeruginosa* culture fluid. PF 30, the sediment of a chloroform extract of *Ps. aeruginosa* culture fluid.

The action of 1-hydroxyphenazine and pyocyanin on macrophage respiration

The effect of pseudomonas fractions on the uptake of oxygen per minute by peritoneal macrophages was measured in the polarograph. When 75 μg of 1-hydroxyphenazine (PF 43 or PF 48) or pyocyanin (PF 42 or PF 49) was added to macrophages utilising 0.2M sodium succinate respiratory activity was not reduced. However, the addition of four times the amount of 1-hydroxyphenazine to respiring macrophages utilising 0.2M glucose caused a 77 per cent. reduction in respiratory activity (fig. 2).

Peritoneal macrophages were incubated at 37°C in the presence of approximately half the quantity of chemical, namely, 33 μg of 1-hydroxyphenazine or 33 μg of pyocyanin. The uptake of oxygen by macrophages utilising glucose as substrate was measured in the polarograph after variable periods of pre-treatment with the chemicals. Figure 3 shows that there was no significant difference

in the percentage reduction of oxygen uptake by macrophages treated for 1, $1\frac{1}{2}$ or 2 hr with 1-hydroxyphenazine. A similar result was found with macrophages treated with pyocyanin. However, whereas 1-hydroxyphenazine caused 54–56 per cent. inhibition of macrophage respiration, pyocyanin caused only 8–31 per cent. inhibition.

FIG. 2.—The effect of 1-hydroxyphenazine (PF 48) on the respiration of guinea-pig peritoneal macrophages measured in the oxygen polarograph. A: after the addition of $300\text{ }\mu\text{g}$ 1-hydroxyphenazine; B: control without 1-hydroxyphenazine. Fractions were added at the point marked with an arrow.

FIG. 3.—The effect of pre-incubation with 1-hydroxyphenazine (PF 48) and pyocyanin (PF 49) on the respiration of guinea-pig peritoneal macrophages measured in the oxygen polarograph. A: control macrophages; B: pyocyanin-treated macrophages; C: 1-hydroxyphenazine-treated macrophages.

The respiratory activity of liver cell mitochondria from infected mice

Mice that received 28×10^8 or 5.6×10^8 *Ps. aeruginosa* NCTC6750 organisms died within 24 hr of the injection; none died after receiving 22×10^6 or 4×10^7 organisms. It was decided to inject 1×10^8 organisms, since this number was about 1 LD₅₀. The average uptake of oxygen by isolated liver mitochondria from uninfected animals was $350\text{ }\mu\text{l}$ and from infected animals $260\text{ }\mu\text{l}$ —a reduction of 25 per cent. in the respiratory activity.

The respiration of liver cell mitochondria from mice given an injection of pyocyanin (PF 49) or 1-hydroxyphenazine (PF 48)

Mice given an intravenous injection of 0.2 ml saline containing 6.6 mg pyocyanin (PF 49) or 2 mg 1-hydroxyphenazine died within 2 min. of the injection. During the preparation of mitochondrial suspensions from the liver of these animals it was found that the supernatant fluid from the disintegrated liver from pyocyanin-treated animals was pale green; from 1-hydroxyphenazine-treated animals it was brownish-yellow. The polarograph results indicated that

FIG. 4.—The effect of 1-hydroxyphenazine (PF 48) on mouse liver mitochondria *in vivo*. A: liver mitochondria from mice given an intravenous injection of 1 mg 1-hydroxyphenazine; B: liver mitochondria from mice given an intraperitoneal injection of 2 mg 1-hydroxyphenazine; C: liver mitochondria from control animals.

there was a reduction in the respiratory activity of the isolated mitochondria by 36.0 per cent. in 1-hydroxyphenazine-treated animals and by 41.0 per cent. in pyocyanin-treated animals. The uptake of oxygen by mitochondria from animals killed after 3 hr, utilising sodium succinate as substrate, is shown in fig. 4. It was apparent that the intravenous injection of 1 mg of 1-hydroxyphenazine caused a 59 per cent. reduction in mitochondrial respiratory activity, whereas the intraperitoneal injection of 2 mg of 1-hydroxyphenazine caused only an 8 per cent. reduction. On the other hand, pyocyanin (6.6 mg) caused a 19 per cent. reduction in the respiratory activity of mouse liver mitochondria obtained from animals that died 10 min. after intraperitoneal injection.

DISCUSSION

In previous communications Armstrong *et al.* (1971) showed that 1-hydroxyphenazine inhibited mitochondrial respiration. It was suggested that 1-hydroxyphenazine might act as an “electron shunt” at the site of ubiquinone-cytochrome *b*. However, no evidence was presented to show that 1-hydroxyphenazine could traverse the intact cell membrane.

The use of mouse liver (L“S”) and baby hamster kidney (BHK strain C13) tissue cultures simulated to some extent the permeability barrier of the animal cells. The results indicated that the pigment-containing fraction (PF 2) obtained

from *Ps. aeruginosa* culture supernatant fluid and also the sediment of a chloroform extract (PF 30) inhibited the uptake of oxygen by L“S” cells respiring on sodium succinate. The BHK strain C13 cells were similarly inhibited when respiring on glucose. The variable response of the tissue culture to utilisation of sodium succinate or glucose has also been observed by Dr C. G. Gemmell (personal communication). It would seem that the age of the cells, method of suspension preparation, and pretreatment with the chemicals may alter the response to a particular substrate.

It was noticed that the addition of 75 μ g of pseudomonas fractions (PF 42, PF 43, PF 48 or PF 49) to respiring guinea-pig peritoneal macrophages did not cause any inhibition of respiration. However, this situation was reversed when much larger amounts of 1-hydroxyphenazine (300 μ g) were added or the cells were pre-incubated for 1–2 hr with 33 μ g of 1-hydroxyphenazine or pyocyanin. It was interesting that pyocyanin was found to have an inhibitory effect on the peritoneal macrophages. These results might indicate that pyocyanin was altering the cell membrane and impeding the permeation of the substrate into the cell. Armstrong *et al.* showed that pyocyanin did not possess any inhibitory activity against liver cell mitochondria utilising sodium succinate. To determine whether pyocyanin affected the permeability of the cell membrane and not the mitochondrial membrane, in-vivo experiments were done. Mice were given an injection of 1-hydroxyphenazine and pyocyanin, and subsequently the respiratory activity of the liver cell mitochondria was determined. It was significant that the intravenous injection of pyocyanin caused a 41 per cent. reduction in respiratory activity—a similar effect to that of 1-hydroxyphenazine. It was also shown (fig. 4) that the maximum reduction was obtained with liver cell mitochondria from mice given 1-hydroxyphenazine by intravenous injection. These results indicate that pyocyanin does not have a selective effect against the cell membrane affecting substrate permeability. However, Armstrong and Stewart-Tull (1971) suggested that pyocyanin was a protoxic substance activated by a demethylase to yield the inhibitory 1-hydroxyphenazine. The results obtained with mouse liver L“S”, hamster kidney (BHK strain C13) and guinea-pig peritoneal macrophages, together with the liver cell mitochondria prepared from mice after injection of pseudomonas fractions, support this hypothesis. In addition, a similar effect was observed when mice were infected with *Ps. aeruginosa* NCTC6750. Conclusive evidence to prove this hypothesis would be obtained by observing the intracellular metabolism of pyocyanin.

The mechanism of *Ps. aeruginosa* pathogenicity is complex and, although it is apparent that 1-hydroxyphenazine causes inhibition of respiration in an experimental model, the control of this process may not be simple in an infection. The results indicate that there is an inhibitory effect on both cytoplasmic and mitochondrial respiration, and the extent of such inhibition and cytolysis is controlled by the amount of chemical absorbed by the cells and the duration of exposure. This could explain the persistence of *Ps. aeruginosa* in burns since lysis and regeneration of macrophages would occur continuously. On the other hand, it could also explain why *Ps. aeruginosa* infections do not, in general, have a high mortality rate, but are nevertheless persistent.

SUMMARY

Since 1-hydroxyphenazine was found to inhibit mitochondrial respiration, attempts were made to determine whether this material would affect whole cells. Oxygen uptake by mouse liver L“S” cells, hamster kidney cells (BHK strain C13) and guinea-pig peritoneal macrophages was inhibited by 1-hydroxyphenazine and pyocyanin. In-vivo experiments confirmed that these compounds permeated the cell membrane. Pyocyanin did not inhibit mitochondrial respiration, and the hypothesis was put forward that this compound is acted on by a demethylase to produce the inhibitory 1-hydroxyphenazine and thus cause inhibition of cell respiration.

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