Impaired respiration and mucus production by *P. aeruginosa* as a possible model for mucus production by those with cystic fibrosis*

by Ian MacL. Campbell (B.Sc., Ph.D.)

Preface

This investigation ended in 1989 and was never completed because of lack of funding. At that time, out of frustration, I promised myself I would never again think about cystic fibrosis (CF) or *Pseudomonas aeruginosa* (*P. aeruginosa*). It has taken me more than twenty years to lose that mind-set. In November of 2010 it was reported that a young woman with CF was about to have a double-lung transplant. It was the same person who, as a young child, had been selected (in the late 1980's) as the poster child by the then Canadian Cystic Fibrosis Foundation. Her image was used as part of the campaign to advertise how the discovery of the CF gene would, within a few years, lead to *the* cure. The report of this woman's transplant bothered me so much that I was compelled to review the results of our incomplete study, thinking that we must be able to do better. In what follows my goal is to present information, which I have never seen reported, that may help someone learn how to protect her new lungs from *P. aeruginosa*. Having recently developed a terminal affliction, old age, I wish to distribute this information, with some suggestions, without further delay.

Introduction

The initial bacterial invader of the pulmonary tract of those with CF is usually *Staphylococcus aureus.* Prolonged infection by this organism causes air-way obstruction. Eventually this organism is replaced by *P. aeruginosa* which invades as a non-mucoid, transforms to the mucoid state and frequently causes death. Isolates of the mucoid form are unstable and revert to the non-mucoid state when cultured *in vitro.* For a study and discussion on this instability see J. Govan (*J. Med. Microbiol*. 8 [1975]: 513-522). Our initial plan was to compare the possible effect of various fatty acids on mucus production. However, what was observed during our first experiment resulted in a change of plans.

Materials and Methods

Two strains of *P. aeruginosa* were studied: PAO-381 (non-mucoid) and PAO-579 (mucoid). These isolates were obtained from the Dept. of Bacteriology, Faculty of Medicine, of Toronto. I was told that they had been isolated by Prof. J. Govan, University of Edinburgh. For these experiments, the nutrient broth was 15 gr. of powdered Tryptic Soy medium (Difco U.S.A.) per 100 ml. of distilled water.

The regimen was as follows: At 2 PM, two 500 ml flasks containing 100 ml of sterile broth were inoculated from the stock plates of both strains and then incubated at 37 C in a shaking water-bath. This provided broth inoculum for the experimental cultures.

For the experiments, 250 ml of nutrient broth was put in 1 L flasks. After sterilization, the flasks were closed tightly with rubber stoppers. The rubber stoppers were fitted with a septum (to allow inoculation, gas purging and sampling) and passages for an oxygen- and a pH-probe. The flasks were sealed to allow assessment of oxygen consumption and the possible effect of decreasing oxygen supply on the cell cultures. At 10 PM, each flask was injected with 10 ml of the appropriate broth inoculum, purged to equilibrium with air and the probes positioned in the broth. They were then incubated in the shaking water-bath at 37 C. The oxygen content and the pH of the cultures were continuously recorded. The flasks containing the remaining inoculum were left on the bench over-night to be autoclaved in the morning.

---

* The larger research project of which this was a part was undertaken between 1974 and 1989 with Douglas N. Crozier†, C.M., M.D., F.R.C.P.C..

Results and Discussion

The initial run was to check our procedure and familiarize ourselves with the organism. By 8 AM the oxygen pressure in the sealed cultures had dropped by about one-half and the broths were opaque. There was no change in pH or a visible sign of mucus in these cultures. However, the appearance of the two inoculum cultures that had been left undisturbed over-night had changed drastically. What was observed caused a complete change in research plans. The broths were now completely clear and were covered with a layer of dense highly viscous orange-yellow mucus. Microscopic examination of the mucus and clear broth showed that all of the cells were contained in the mucus. When these flasks were swirled, which re-oxygenated the cultures, the mucus separated into a few large clumps and turned green assuming the usual appearance of mucoid cultures of this bacterium. Within 30 seconds of being placed back on the bench, the mucus rose to the surface and the original yellowish color returned. This treatment was repeated several times and always gave the same result. The color change in response to the absence or presence of oxygen suggested that an oxidation-reduction reaction was occurring in the mucus complex. (From previous experience working with insect blood, I suspect that the yellow-green color change occurred because the respiratory pigment biliverdin was present in the mucus complex.) The cause of the oxidation was apparent but the cause of the rapid reduction was not. Could there be an elevated supply of hydrogen-ion stored in the system?

Meanwhile, the cultures that were being incubated in the shaking water bath were monitored. Every hour samples of both cultures were prepared for both light and electron microscopy. By 18 hours, when the oxygen saturation became nearly stable at about one-third saturation, some of the bacteria had an altered appearance. Instead of these cells having a sharply defined margin they appeared fuzzy with strands of fibrous material extending outward. By 19 hours many of these “fuzzy cells” had aggregated to form mucoid clumps and shortly thereafter these clumps had enlarged enough to become visible as typical green aggregates of mucoid *P. aeruginosa.* These aggregates also underwent the green to yellow change in response to the presence or absence of oxygen.

Surprisingly, the pH of the cultures remained unchanged at about pH 7.0. It then occurred to us that something in the cultures may be “storing” hydrogen-ion (i.e. a strong buffer). To check this possibility, small samples of mucus and mucus-free material, taken from each culture, were shaken into 10 ml of water (double-distilled and de-ionized) and changes in pH were recorded. Whereas the pH of the non-mucus samples came into equilibrium at between 5 and 6, that of the mucus samples stabilized at between 1.5 and 3. Evidently there was a sizable store of hydrogen-ion within the mucus complex. Several repeats of the experiment gave us similar results. Because funds and equipment were limited, we decided to restrict the remainder of the study to the mucoid strain PAO-579.

Since an Aminco dual wave-length spectrophotometer was available, the oxidation-reduction response of the cytochromes to these conditions was investigated. This spectrophotometer was designed specifically for the study of opaque material such as mucus and bacterial suspensions. The wavelength settings were at 410 nm., the isosbestic point, and 430 nm., the maximal light-absorbing wavelength of reduced cytochrome-C. A 1.0 cm. cuvette was three-quarters filled with a sample of mucus- or non-mucus containing culture diluted (3:1) in 10% glucose solution. The sample was then aerated by vigorous shaking and immediately placed in the spectrophotometer. Once the optical-density reading became minimal, indicating that cytochrome oxidation was complete, the subsequent increase in optical density as the cytochrome underwent reduction, as the dissolved oxygen became depleted, was continuously recorded.

Fig. 1 is a diagrammatic presentation of the results of a study on the mucus from a 24 hr. culture of the mucoid strain, PAO-579. Line 1 shows the increase in optical density during reduction in a sample that had been aerated. The maximum reading was interpreted as complete reduction of the cytochrome (O.D. = 1). The procedure was then repeated on the same sample

after the addition of a few grains of succinate. In the Krebs cycle, surplus succinate restricts the generation of hydrogen-ion by severely limiting the rate of its own conversion to fumarate. Line 2 shows that with restricted hydrogen-ion generation, a large additional portion of cytochrome was oxidized by aeration. The addition of more succinate did not reveal more cytochrome. Moreover, the addition of hydrogen-peroxide to a matched sample from the same culture gave the same result, Line 3. This minimal reading was interpreted as complete oxidation of the cytochrome (i.e. O.D.=0). The minimal density readings (Lines 2 and 3, compared to that for Line 1) indicate that approximately only 35% of the total cytochrome was oxidized and 65% remained reduced with aeration alone. When the same treatments were applied to samples of the same culture that contained no mucus, only about 25% of the contained cytochrome remained reduced when aerated. Apparently, the rate of reduction in the mucoid cells was much greater than in those which had not yet become mucoid.

The pattern of the reduction of the cytochrome, following its' complete oxidation in response to aeration following the addition succinate (Line 2) or hydrogen peroxide (Line 3),was biphasic. The cytochrome that had been revealed by these additives underwent reduction first and, after a delay, that which was oxidized by aeration alone underwent reduction. This indicates that the mucus contained two different types of cells, one type generating hydrogen-ion at a considerably higher rate than the other.

When these experiments were repeated with cells from young (6 to 8 hr.) cultures, which contained no "fuzzy cells" or mucus, it was found that with aeration still about 10% of the cytochrome remained reduced. When samples of these cultures were aerated with either succinate or hydrogen-peroxide present, so that the cytochrome was completely oxidized, the pattern of the of the subsequent reduction was again biphasic indicating the presence of the two different cell types. In this instance those with the lower hydrogen-ion generation predominated. (NOTE: Increasing the quantity of succinate did not reveal more cytochrome but prolonged the subsequent reduction period. This was overcome by the addition of malate which allows the hydrogen-ion generating cycle to bypass the succinate blockage. See Fig.1 Line 4.)

With prolonged incubation at low oxygen pressure, the mucus appeared to reach maximal density and viscosity at between 45 and 50 hrs. Within the "splash region" of the flask walls, mucus strongly adhered and contained hard pellets of material that required a steel spatula to remove them from the glass. Some of these pellets were sectioned and observed under an electron microscope. The pellets consisted of dehydrated mucus, surrounding masses of cells which were so tightly packed that they resembled multi-cellular tissue, i.e. encapsulated *P. aeruginos*a.

After between 85 and 90 hours of incubation approximately 90% of the cytochrome contained in the free-floating mucus remained reduced upon aeration. We could not measure the oxygen content because, as mucus adhered to the nylon guard surrounding the membrane of the oxygen sensor, the reading dropped from about 25 to 0% saturation. Evidently, the mucus severely restricts oxygen diffusion. The pH of the culture remained at about 7.0. All of the foregoing experiments were repeated more than 10 times and gave very similar results.

In a few instances the duration of the incubation period was extended beyond 50 hours. By about 65 hours, the quantity, viscosity and adhesiveness of the mucus appeared to decrease. By 75 hours there was no visible mucus, only the pellets on the wall of the flasks remained. When samples of these cultures were added to de-ionized water the pH *increased* to between 11 and12. This suggested that the loss of mucoidy coincidental with high pH may result from de-animation of protein. In older cultures carbohydrate and lipid could be depleted so that protein became the sole source of carbon for the cells. The consequent de-animation would result in the release of basic ammonia. Testing showed that the addition of carbohydrate to older cultures *did* restore the formation of mucus. Unfortunately, spectral measurements were not taken on the cytochromes.

Knowing that, with impaired oxygen supply, surplus hydrogen-ion accrues and mucus production is initiated, we planned to investigate treatments that could reverse its production.

However, it was now 1989 and funding was exhausted. It is now 2011 and, prompted by the report of the lung-transplant of the former CF poster child, I have turned back to my research and asked, how could the excess production of hydrogen-ion relate to mucus production? After studying various sources, especially Chapter 2 *of Algal Physiology and Biochemistry* (W.D.P. Stewart ed., Botanical Monographs, Vol. 10, 1974, Univ. of California Press), my understanding of the formation of mucus is as follows. The primary structure of the mucus of P. *aeruginosa* is alginic acid, a linear polymer of acid sugar units (usually mannuronic but sometimes guluronic) connected through an oxygen atom that links carbons 1 and 4 of the linear units. (Figure 2 is an illustration of a section of polymanuronic alginic acid.) The synthesis of the alginic acid is genetically regulated. However, the formation of mucus requires the formation of weak hydrogen-bonds between adjacent polymeric chains. When the carboxyl groups are not protonated they carry a strong negative charge and accordingly are hydrophilic and repel each other so that hydrogen-bonding cannot occur. However, protonation of a carboxyl group with a hydrogen-ion neutralizes the charge and it becomes hydrophobic. As more of these charges along the alginic acid chains are neutralized, more sugar-acid units will aggregate in the hydrophobic domain. This allows inter-chain linkage by hydrogen-bonds forming between a carboxyl group in one chain and a hydroxyl-group in an adjacent chain. With manuronic units, the remaining hydroxyl-group, within a unit, hydrogen-bonds with the ring oxygen in the next unit and thereby further increases the rigidity and hydrophobicity of the chain. Thus, the more excess hydrogen-ion generated the more carboxyl groups will be neutralized, the more hydrophobic the alginate will become and the greater the opportunity for hydrogen-bonding between chains which results in more and more viscous mucus being formed.

Could the secretion of mucus by the bacterial cells function to excrete surplus hydrogen-ion and thereby assist in regulating the pH within the cells? Perhaps a surplus of intra-cellular hydrogen-cation indirectly activates translation of the gene for alginic acid synthesis by displacing the di-cationic iron, which is known to block its translation. This could be a feed-back mechanism for the excretion of surplus intra-cellular hydrogen-ion that is generated in hypobaric-oxygen environments and allows this obligate aerobe to survive and flourish under such conditions. Moreover, the excreted mucus surrounds the cells and affords them protection from hostile entities such phagocytes and antibiotics. This could facilitate the invasion of lungs when ventilation is severely restricted by an existing *Staphylococcus aureus* infection. Our observation on the formation of pellets containing tightly-packed cells suggests that with prolonged severe hypoxia the mucus completely dehydrates encapsulating the bacteria. Encapsulation is known to occur in many species and it provides complete isolation from hostile environments.

The accepted wisdom in the 1980's was that when the supply of oxygen to cells is decreased their cytochrome content decreases. Our research indicated that, at least in *P. aeruginosa,* this decrease is apparent rather than real. The addition of either succinate or hydrogen peroxide to the substrate either to suppress the rate of reduction or increase the rate of oxidation revealed all of the cytochrome by facilitating its' complete oxidation. Evidently, what does seem to change in response to low oxygen is increased capacity to generate hydrogen-ion.

Mucoidy in *P. aeruginosa* is known to occur in three medical conditions: cystic fibrosis, chronic bronchitis and 3rd degree burns. A standard treatment for the latter is hyperbaric oxygen. We had planned an *in vitro* study on the effect of hyperbaric oxygen in reversing mucoidy in this bacterium. Treatments that assist in oxidizing the hydrogen-ions from the mucus should increase hydration, decrease viscosity and thereby facilitate some clearance of the air-ways. However, if our observations are correct, they will not prevent the intracellular production of mucus. Only increasing the capacity of the electron-transport system to utilize the surplus hydrogen-ion being generated by the Krebs cycle will arrest mucus formation.

Our findings may relate directly to the pathology of cystic fibrosis. With CF, as with *P. aeruginosa,* when the uptake and transport of oxygen is impaired, the secretion of mucus is elevated and the mucus is unusually viscous. Are the glycoproteins of human mucus similarly

affected by an excess of hydrogen-ion being generated because of restricted oxygen supply? It seems to me that elevated hydrogen-ion in any organism could destabilize both cation and anion regulation in a way that could modify normal biochemical and physiological processes (e.g. elevation of sodium and chloride-ion in the sweat and mucus blockage of the pancreatic ducts). A study of the oxidation-reduction system in mitochondria of CF individuals might lead to a better understanding of the disease.

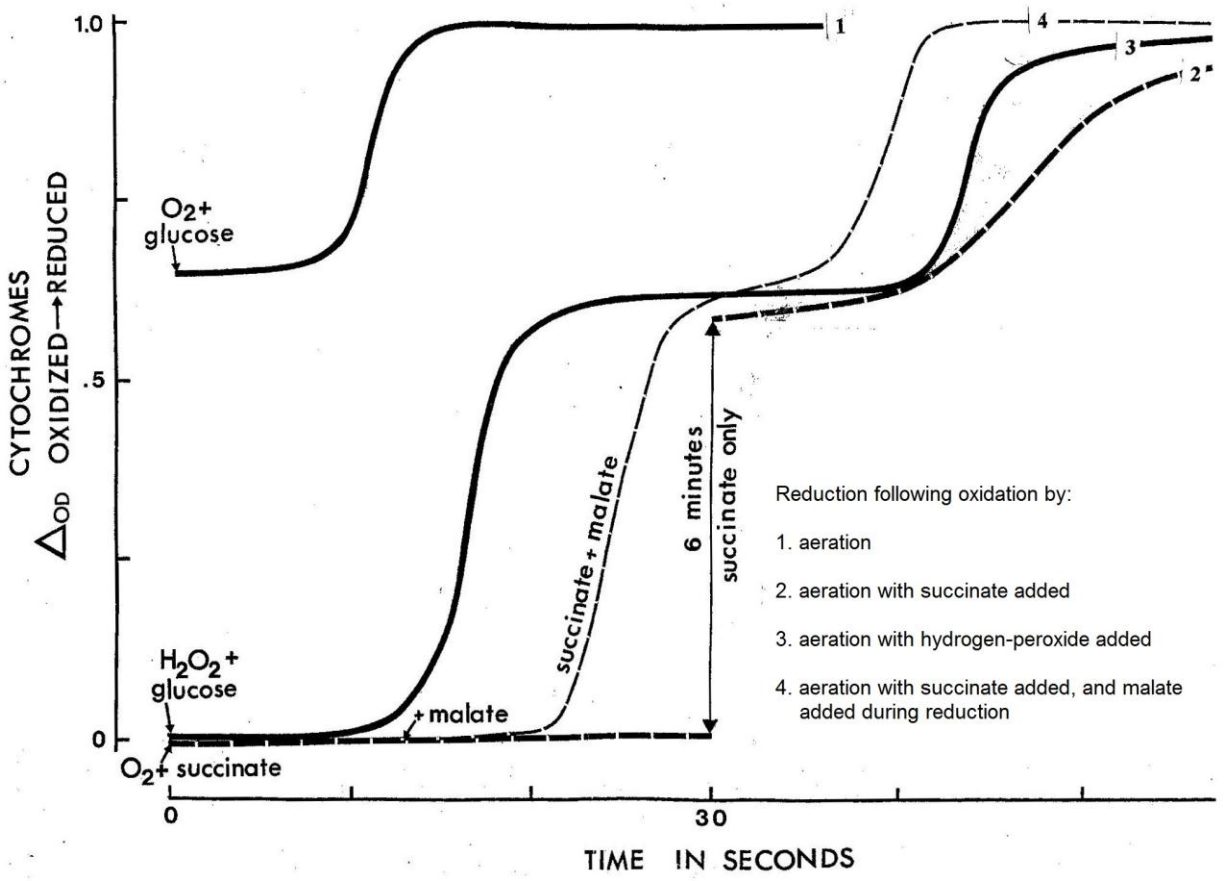

Figure 1. Reduction of cytochrome-C in mucoid cells following oxidation of Pseudomonas aeruginosa (PAO-579).

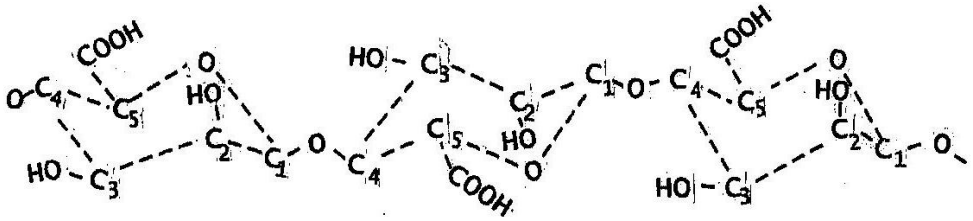

Figure 2. Three oxygen-linked units of polymanuronic alginic acid. (Adapted from W.D.P. Stewart, 1974. See text.)