KILLING OF MICROBES BY NEUTROPHILS, WITH PARTICULAR REFERENCE TO THE ROLE OF PROTEOLYTIC ENZYMES.

By

Emer Patricia Reeves

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To

Richard Reeves and Christopher O’Dwyer.
ABSTRACT

According to the hitherto accepted view, neutrophils kill ingested microorganisms by subjecting them to highly toxic reactive oxygen species (ROS) and myeloperoxidase catalysed halogenation. Work presented in this project shows that this simple scheme, which for many years has served as a satisfactory working hypothesis, is inadequate. It was found that mice deficient in neutrophil granule proteases but normal in respect of superoxide production and iodination, are unable to resist staphylococcal and candidal infections.

It was further shown that accumulative ROS concentrations could reach some 4 mols/l within the phagocytic vacuole. To compensate for the movement of charge incurred in making superoxide, a large concentration of potassium ions ($K^+$) cross the membrane. The rate of superoxide generation stimulated by phorbol 12-myristate 13-acetate (PMA) is accelerated in the presence of valinomycin, a specific $K^+$ ionophore, and reduced by 4-aminopyridine, a $K^+$ channel inhibitor. Diphenylene iodonium (DPI), an inhibitor of the oxidase blocks the release of $K^+$ and the activation of the $K^+$ channel is pH-dependent, being inhibited at pHs above 8.0.

The massive rise in ionic strength within the vacuole engenders the release of cationic granule proteins, including elastase and cathepsin G, from the anionic sulphated proteoglycan matrix. It is these proteases that destroy the bacterium. The inferred killing mechanism is confirmed by the lack of solubilized granule proteases in CGD neutrophils or those treated with DPI. In addition the
bactericidal properties of hydrogen peroxide and hypochlorous acid were re-examined, illustrating, that neither oxidants possessed adequate effects against staphylococci and E. coli *in vitro*. Killing that is promoted by the addition of potassium to the granules is eliminated by protease inhibitors.

**PUBLICATION**


Killing activity of neutrophils is mediated through activation of proteases by K⁺ flux.

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ABBREVIATIONS

aa amino acid
4-AP 4-aminopyridine
BPI bactericidal/permeability-increasing protein
BSA bovine serum albumin
°C degrees Celsius
C. albicans Candida albicans
CARS coherent anti-Stokes Raman scattering microscopy
CETAB Cetyltrimethylammonium bromide
CGD chronic granulomatous disease
Ci Curie
cpm counts per minute
C-terminal carboxy terminal
2D two dimensional
Da Daltons
DIFP diisopropylfluorophosphate
DMSO dimethyl sulphoxide
DPI diphenylene iodonium, inhibitor
DTT dithiothreitol
ECL enhanced chemiluminescence
E. coli Escherichia coli
FAD flavin adenine dinucleotide
FMLP N-formyl-methionyl-leucyl-phenylalanine
FPLC fast protein liquid chromatography
g relative centrifugal force
GAP GTPase activating protein
GDI GDP-dissociation inhibitor
GDP guanosine 5’-diphosphate
HEPES N-(2-hydroxylethyl)piperazine-N’-(2-ethane-sulphonic acid)
H₂O₂ hydrogen peroxide
OH hydroxyl radical
HOCl hypochlorous acid
hr hours
IgG immunoglobulin G
IPG immobilized pH gradient
K⁺ potassium ion
l litre
LB Luria-Bertani media
LLO long lived oxidants
m milli
mA milliamp
M molar
MALDI-TOF matrix-assisted laser-desorption ionisation-time of flight
<table>
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<tr>
<td>MAP kinase</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>Mol</td>
<td>mole</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>mV</td>
<td>millivolt</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
</tr>
<tr>
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<td>amino terminal</td>
</tr>
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<td>superoxide</td>
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<td>oxygen</td>
</tr>
<tr>
<td>O2•-</td>
<td>superoxide</td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAK</td>
<td>p21-activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>phox</td>
<td>phagocytic oxidase</td>
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<tr>
<td>PI</td>
<td>protease inhibitors</td>
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<td>protein kinase A</td>
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</tr>
<tr>
<td>PMA</td>
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</tr>
<tr>
<td>PNS</td>
<td>post-nuclear supernatant</td>
</tr>
<tr>
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<tr>
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<td>Staphylococcus aureus</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
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</tr>
<tr>
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<td>Src-homology 3 domain</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloro-acetic acid</td>
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<tr>
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<td>threonine</td>
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<tr>
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<td>N’-p-tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
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<td>tetratricopeptide repeat</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>Val</td>
<td>valinomycin</td>
</tr>
<tr>
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CHAPTER 1
GENERAL INTRODUCTION

1.1 THE NEUTROPHIL AND ACTIVATION OF THE OXIDASE.

Phagocytosis is a critical event in immune defence and is essential for survival of all multicellular organisms. Phagocytosis is the process of recognition and engulfment of particles such as pathogens or tissue debris that accumulate during infection, inflammation and wound repair. The phagocytic cells of the immune system (neutrophils, eosinophils, monocytes and macrophages) play a vital role in the body’s ongoing battle against disease. The neutrophil granulocyte is the most numerous of the circulating phagocytes in humans, accounting for approximately 60% of total blood phagocytes in adulthood.

Phagocytosis requires sequential signal transduction events that lead to, (i) the recruitment of the phagocytes at the site of infection, (ii) The recognition of the particles to be ingested which is initiated by the interaction between receptors on the neutrophil surface and the microbe, (iii) The movement of the cytoskeleton of the phagocyte that forms pseudopodia around the particle to be ingested. (iv) The formation and maturation of a phagosome, and (v) degranulation of cytoplasmic granules by fusion of their membrane with that of the formed phagosomal membrane (Figure 1.0).

The phagosome is where degradation of the ingested particle occurs by a combination of oxidative and non-oxidative mechanisms. The antimicrobial systems which can operate in the absence of oxygen are due to neutral serine proteases, hydrolytic enzymes and cationic proteins. Oxidative mechanisms
FIGURE 1.0. Phagocytosis by neutrophils. Electron micrographs of neutrophils during the process of phagocytosis of *Staphylococcus aureus* (approximate diameter of bacterium, 0.8μM).

(a) A bacterium is recognised by receptors on the plasma membrane of a neutrophil. This interaction triggers the formation of pseudopodia around the bacterium so that the bacterium eventually becomes fully enclosed within a phagocytic vacuole. The membrane of the vacuole is derived from the plasma membrane.

(b) Cytoplasmic granules migrate to and fuse with the phagosomal membrane, releasing their microbicidal contents into the vacuole.
refer to bacterial killing that is dependent on the production of oxidants in the phagocytic vacuole. The chain of reactions that produce oxidants is triggered by an enzyme called the NADPH oxidase, which causes the single electron reduction of oxygen to superoxide\textsuperscript{9,10}.

Although the purpose of the phagocytic oxidase is to remove infectious agents, foreign particles and damaged tissue from the body, reactive oxygen species and granule enzymes can also damage host tissue in the vicinity of an inflammatory site\textsuperscript{11}. Neutrophil activation could be an important pathogenic mechanism underlying several medical disorders including autoimmune arthritis and inflammatory bowel diseases\textsuperscript{12,13}. Hence the NADPH oxidase of phagocytes is a powerful immune defence mechanism which, requires tight regulation.

1.2 THE NADPH OXIDASE.

The role of phagocytes in host defence was discovered late in the nineteenth century by Elie Metchnikoff\textsuperscript{14}, who noticed that these cells crowed around an inflicted site of infection in transparent starfish larvae. The first inkling that oxygen might have something to do with the function of the phagocytes was the discovery in 1935, of the "respiratory burst". Baldridge and Gerard measured a small but significant increase in the oxygen consumption of canine neutrophils during phagocytosis of bacteria\textsuperscript{15}. This was the level of understanding of the respiratory burst until 1959, when Sbarra and Karnovsky made the important observation that this specific increase in oxygen consumption was resistant to conventional inhibitors of mitochondrial respiration\textsuperscript{16}. This finding ruled out the possibility that mitochondrial function had anything to do with the respiratory
burst and raised the question as to the purpose of this increase in oxygen uptake when phagocytes were exposed to their targets.

Several reports appeared in the paediatric literature between 1954 and 1960 which described a clinical syndrome characterised by recurrent life threatening sepsis and widespread granulomatous infiltration\textsuperscript{17}. In 1967, Quie demonstrated that neutrophils collected from a male patient with this disease were unable to kill \textit{Staphylococcus aureus in vitro}, and that the primary abnormality was of neutrophil function\textsuperscript{18}. In the same year it was discovered that neutrophils from patients with this chronic granulomatous disease (CGD) failed to exhibit the "respiratory burst" during phagocytosis\textsuperscript{19}. Together, these observations indicated a crucial function for this system in host defence.

Following discovery of the respiratory burst, the identity of the substrate was the subject of considerable disagreement\textsuperscript{20-22}. The activity of the hexose monophosphate shunt is considerably enhanced\textsuperscript{23} and coincident with activation of the respiratory burst. Together with enzyme kinetics in reactions containing purified components of this oxidase supported the identification of NADPH as the natural substrate. As a consequence, this enzyme system became known as the "NADPH-oxidase"\textsuperscript{24}.

The NADPH oxidase remains inactive until exposed to appropriate stimuli, after which there is a short lag phase before oxygen consumption increases by up to one hundred fold, depending on the stimulus used\textsuperscript{25,26}. The classical stimulus of the respiratory burst \textit{in vivo} is a particle opsonized with immunoglobulin and/or complement, which bind to receptors on the plasma membrane. This triggers phagocytosis and vacuolar closure before the localized
activation of the electron transport, across the wall of the vacuole and the formation of superoxide \((O_2^-)\) \(^9\) (Figure 1.1).

1.3 THE STRUCTURE OF THE NADPH OXIDASE.

The active oxidase is a multi-component system, the molecular basis of which remained obscure until 1978 when Segal and Jones discovered cytochrome \(b_{558}\), showing that it was missing in some patients with CGD, and identified it as the terminal electron transporting component of the NADPH oxidase\(^{27}\). \(b_{558}\) based on its absorption properties (or \(b_{245}\)), is a membrane-bound flavocytochrome, which carries a non-covalently bound flavin adenine dinucleotide (FAD) cofactor and two heme prosthetic groups. The consensus is that the cytochrome directly binds NADPH for oxidation, which is supported by the identification of NADPH binding motifs\(^{28}\). The cytochrome takes electrons from NADPH and passes them, via FAD and haem, to \(O_2\), with kinetics of cytochrome reduction correlating with the observed rate of superoxide generation\(^{29}\). Thus the cytochrome oxidase catalyses the following reaction:

\[
\text{NADPH} + 2O_2 \rightarrow \text{NADP}^+ + H^+ + 2O_2^-
\]

1.3.1 Cytochrome \(b_{558}\).

Flavocytochrome \(b_{558}\) comprises two protein subunits with a stoichiometry of 1:1\(^{30}\). The larger of the two subunits, known as gp91\(^{\text{phox}}\) (according to standard nomenclature the superscript suffix phox represents “phagocyte oxidase-associated”) or the \(\beta\) subunit, is 569 amino acids in length, heavily glycosylated\(^3\), and runs as a broad band with an approximate molecular weight of 91 kDa on SDS–PAGE gels\(^{32}\). The NADPH analogue 2-azido-NADPH has
**FIGURE 1.1.** Diagrammatic representation of activation of the respiratory burst.

Bacteria (dark circle) is phagocytosed and enclosed within a phagocytic vacuole. The hexose monophosphate shunt is activated, and this generates NADPH, which is the substrate for the oxidase. The oxidase itself is then activated via translocation and assembly of individual component parts, and accepts electrons from NADPH to reduce $O_2$ to $O_2^-$. 

\[
NADPH \rightarrow NADP^+ + H^+ + O_2^- + O_2
\]
been used to affinity label the β subunit, thus confirming that the β subunit of the cytochrome, directly binds to NADPH.

The second smaller subunit, referred to as p21phox, p22phox or the α subunit, comprises 194 amino acids, is non-glycosylated and has a derived molecular weight of 21-22 kDa. Phosphorylation of both subunits of the flavocytochrome occurs upon stimulation of neutrophils.

The most common and often the most severe form of CGD is X-linked CGD which accounts for roughly two-thirds of all cases. Here the mutation lies within the gene for the gp91phox, which is located on the X-chromosome. The result in the majority of cases is the total loss of the membrane-bound flavocytochrome, with both the β and α subunits missing, despite normal mRNA encoding the α subunit. This suggests that protein stability of the cytochrome depends on the interaction of the two subunits.

The connection between the X-linked CGD gene and flavocytochrome b558 was established on obtaining N-terminal sequence from pure gp91phox and demonstrating that it was in fact coded, from what had been designated as non-coding DNA, 5' to the X-linked CGD protein cDNA sequence. The amino acid sequencing data showed that errors had been made in the cDNA sequencing data, one of them altering the true initiating codon. Dinaeur et al., (1987), raised antibodies to synthetic peptides corresponding to the C-terminus of the predicted protein. These cross-reacted with gp91phox, confirming that gp91phox was coded for by the X-linked CGD locus.

In cases of variant CGD, reduced or even normal amounts of a defective protein are present and the patient may demonstrate some residual oxidase activity.
The cytochrome b is found in the plasma membrane of the phagocytic membrane anchored by a series of hydrophobic transmembrane segments. The location of extracellular glycosylation sites, by site directed mutagenesis has aided in the understanding of the topology of this membrane-spanning protein. The cytochrome b is also found in the membranes of the secondary granules which fuse with the vacuole during phagocytosis, and replenish stores of this and other specialized plasma membrane proteins.

The carboxy terminal half of the large subunit is relatively hydrophilic and is believed to form a soluble globular domain which houses the NADPH and FAD binding sites and which must be located on the cytoplasmic side of the membrane to allow access to the NADPH substrate.

1.3.2 Association with heme.

The location of the hemes is uncertain but they probably lie within the hydrophobic N-terminal intra-membranous region of the protein. The midpoint potentials for the heme centres have been determined from redox titrations and are $-225$ and $-265$ mV. These unusually low values are crucial to the functioning of the protein since they facilitate the transfer of electrons directly to $O_2$ to form $O_2^-$, the midpoint potential of which is $-160$ mV.

The number of heme groups involved has been thoroughly investigated, with groups providing evidence that there must be more than one. Lizuka et al., (1985), showed that the heme redox spectrum at low temperature had a shoulder on the $\alpha$-band at 558nm and Yamaguchi et al., (1985) employed circular dichroism spectroscopy and found a bilobed pattern. In addition Hurst et al., (1991), used electron paramagnetic resonance spectroscopy and were unable to
detect a heme signal\(^4\). In this case, signals were believed to be cancelling each other out completely.

Conclusive evidence for two heme was found by Cross et al., (1995), who showed that the midpoint potential titration for an X91 CGD variant patient (Arg 54-Ser) showed two, non-equivalent heme groups, with mid-point potentials of \(-220\) and \(-300\) mV \(^4\). This case was unable to transfer electrons from FAD to heme. Reassessment of normal flavocytochrome \(b_{558}\) mid point potential titration showed that there could be two components making up the curve, with values for \(E_{m,7.0}\) of \(-225\) and \(-265\) mV.

The precise location of the hemes is uncertain, but recent evidence suggests that they are both located on the \(\beta\) subunit. Expression of the \(\beta\) subunit in cultured COS cells in the absence of the \(\alpha\) subunit has shown this molecule to house the hemes\(^4\).

1.3.3 Association with FAD.

The nucleotide binding protein in an electron transport chain is usually a flavoprotein\(^4\), so the presence of a flavin-binding protein linking NADPH and heme had been speculated about. FAD was found to enhance activity of the oxidase\(^9\), whereas FAD analogues were inhibitory\(^9\). Diphenylene iodonium (DPI), an inhibitor of electron transport in flavocytochrome \(b_{558}\), is thought to act at the flavoprotein site\(^9\).

Isolation of the flavoprotein was difficult because the FAD was invariably lost during detergent solubilization\(^3\). Segal et al., (1992), showed that the flavoprotein was not cytosolic\(^3\), and found a degree of homology with
the flavin binding domains of the ferredoxin-NADP⁺ reductase flavoenzyme family, a putative FAD binding site.

The FAD: heme ratio in resting and activated membrane was found to remain constant, and since cytochrome $b_{558}$ was recruited from specific granules, FAD was thought to accompany, or possibly be associated with the cytochrome $b_{558}$. gp91phox⁻/⁻ CGD patient membrane invariably contained less FAD than control, and undifferentiated HL60 cells with low or no oxidase components, showed similar levels of FAD as the X-linked patient cells. An estimation of FAD: heme was made, deducting the level of FAD in HL60 cells as 1:2. Using the photoaffinity ligand $[^3H]NAP_4$-FAD, Doussiere et al., showed direct binding of FAD to the β subunit.

1.4 CYTOSOLIC COMPONENTS OF THE NADPH OXIDASE.

The onset of oxidase activity is initiated by the binding of chemotactic molecules such as the N-formylmethmethionyl oligopeptides (fMLP), which are produced by bacteria and damaged tissues and bind to receptors at the neutrophil cell surface. Thereafter, a complex series of signal transduction pathways leads to the formation of the cytosolic NADPH oxidase activation complex.

The first indication that cytosolic factors were necessary for activation of the NADPH oxidase came from cell-free studies on whole cell homogenates. The particulate fraction of the homogenate, which was believed to contain the membrane bound NADPH oxidase, could not be activated unless cytosol was also present. Furthermore, some patients with autosomally inherited
CGD were deficient in this cytosolic activity\(^6\). Cells from these patients have a normal flavocytochrome\(^6\) but are unable to pass electrons on to the haem which suggested an abnormality of the activation.

1.4.1 \(p47^{phox}\).

Following a series of phosphorylation studies the first biochemical abnormality noted in autosomal recessive CGD was the failure to phosphorylate a 47 kDa protein\(^6\). Extensive studies revealed the absence of this 47 kDa protein in most patients with autosomal recessive CGD. Phosphorylation of \(p47^{phox}\)\(^9,10,63\) correlated with activation of the oxidase\(^64\) and occurs at its C-terminal end in which 9 or 10 serine phosphorylation sites have been identified by phosphopeptide sequencing and site directed mutagenesis\(^65\). Several kinases are known to phosphorylate \(p47^{phox}\) \textit{in vitro}, among them protein kinase C\(^66,67\), cAMP-dependent protein kinase A (PKA), p21-activated kinase (PAK)\(^68\) and a number of kinases activated by phosphatidylinositol-3-kinase (PI-3 kinase)\(^69\).

1.4.2 \(p67^{phox}\).

\(p67^{phox}\) was the second cytosolic component discovered to be required for a functional NADPH oxidase\(^70\). Its absence accounts for the defective microbicidal activity of neutrophils from autosomal recessive CGD patients which are both \(p47^{phox}\) and cytochrome-positive\(^71\) and accounts for just 5% of all cases. \(p67^{phox}\) phosphorylation occurs upon stimulation of neutrophils with fMLP and PMA\(^72\) and it has been suggested that PKC is the likely kinase\(^72\).
1.4.3 p21\textsuperscript{rac}.

Fractionation of cytosol from guinea pig macrophages in the cell free assay led to the isolation of a third cytosolic component that was required to complement p47\textsuperscript{phox} and p67\textsuperscript{phox} and membranes in the cell free assay. This cytosolic component comprised a heterodimeric complex of p21\textsuperscript{rac}, a small GTP binding protein, and rho GDI (GDP-dissociation inhibitor). In the inactive state p21\textsuperscript{rac} exists in a complex with GDI but when neutrophils are stimulated the complex dissociates and p21\textsuperscript{phox}, in the GTP bound form binds to p67\textsuperscript{phox} in the activation complex and then translocates to the membrane. However it is most likely that p21\textsuperscript{rac2} which shares 92% amino acid identity with p21\textsuperscript{rac} and expressed predominantly in myeloid cells is the physiologically active molecule in neutrophils.

The minimal requirements for the \textit{in vitro} cell-free activity have now been recognised as lipid-reconstituted cytochrome (neutrophil-extracted or recombinant), p47\textsuperscript{phox} and p67\textsuperscript{phox}, and p21\textsuperscript{rac} (in the GTP-bound state) FAD, NADPH and an amphiphile such as sodium dodecyl sulphate (SDS).

1.4.4 p40\textsuperscript{phox}.

The final addition to the cytosolic oxidase factors is a 40kDa protein, p40\textsuperscript{phox}. It was observed that p47\textsuperscript{phox} and p67\textsuperscript{phox} exist in the cytosol of resting neutrophils in a large complex of approximately 240kDa. By immunoprecipitation studies it was found that p40\textsuperscript{phox} formed a complex with p67\textsuperscript{phox} and greatly diminished levels of p40\textsuperscript{phox} were found in p67\textsuperscript{phox} deficient patients. p40\textsuperscript{phox} becomes phosphorylated when the NADPH oxidase is activated however the role of this phox protein is controversial.
Oxidase activity can be established in K562 cells by transfecting them with plasmids that express recombinant p47phox, p67phox and gp91phox. Cells that express p40phox along with other recombinant oxidase components produced only half the amount of superoxide production generated by cells not expressing p40phox. Similarly, adding p40phox to a cell-free system inhibits superoxide production, suggesting p40phox is an inhibitory oxidase component.

On the other hand interfering with the binding of p40phox to p67phox reduced the superoxide production within a cell free system by 50% \(^8^3\), a result implying that p40phox is a stimulatory component of the oxidase. CGD due to a mutation affecting p40phox has not yet been found, possibly it could be a lethal mutation as it has been suggested that p40phox is also expressed in the brain.

1.5 INTERACTIONS BETWEEN THE CYTOSOLIC FACTORS.

Characterization of p47phox, p67phox and p40phox revealed that they contain specific domains important for protein-protein interactions and NADPH oxidase activation. Four classes of domain have been characterized including the Src-homology 3 (SH3) domain, PC motif, tetratrico-peptide repeats and the phox domain (Figure 1.2).

1.5.1 SH3 domains.

The presence of SH3 domains within the sequence of a number of the cytosolic proteins is evident. These 50-60 amino acid domains, mostly in \(\beta\)-sheet configuration, have been implicated in mediating protein-protein interactions in receptor signalling processes \(^8^4\) and have been found to bind proline-rich motifs.
FIGURE 1.2. The domain structure of the cytosolic phox proteins.

Schematic showing the known domains as elucidated by sequence homology and expression and functional studies of truncated proteins.
Two such SH3 domains are found in both p47phox and p67phox, and one within the p40phox sequence.

1.5.2 Tetratricopeptide repeat domains.
The tetratricopeptide repeat, (TPR) is a 34-residue repeat, identified in a wide variety of proteins and implicated in protein-protein interactions. It has been postulated that p67phox contains four TPR domains which are found in the N-terminal region. A single site mutation within TPR3 (Gly 78 to Glu substitution) leading to disruption of TPR folding and potential TPR mediated interactions, results in non-functional p67phox, thus implicating a role for this region for successful oxidase activation.

1.5.3 Phox domain.
The N-terminal parts of p40phox and p47phox show strong sequence homology and contain a phox domain (PX domain). A PX domain is of approximately 100 residues and may contain an SH3 binding poly-proline motif. The first function assigned to this domain was the interaction of the PX domain of p47phox and p40phox with the N-terminal part of moesin, a member of the ezrin-radixin-moesin family of cytoskeletal proteins.

1.5.4 PC Motif.
Multiple sequence alignment revealed a 28 residue repeat at the C-terminus of p40phox. This motif is thought to be important for protein-protein interactions and biochemical studies and site-directed mutational analysis have highlighted the importance of this region in the binding to p67phox.
1.5.5 Established interactions.

Studies have revealed that in the NADPH oxidase system, there are no simple one-domain-one-domain protein interactions but that activation/deactivation of the oxidase is a dynamic multiple-site interaction process. A number of investigators, using various techniques such as blot overlays, binding to proteins on beads, and the yeast two hybrid system, have found that the C-terminal SH3 domain of \( p67^{\text{phox}} \) binds to the C-terminal proline rich motif of \( p47^{\text{phox}} \) [89-91].

Binding partners to the two SH3 domains of \( p47^{\text{phox}} \) have been clarified, the first (most N-terminal) binds to \( p22^{\text{phox}} \) [92], whereas the second binds to \( p67^{\text{phox}} \) [93].

\( p40^{\text{phox}} \) binds to both \( p67^{\text{phox}} \) and \( p47^{\text{phox}} \) although the \( p40^{\text{phox}}-p67^{\text{phox}} \) interaction is of significantly greater affinity [94]. The \( p40^{\text{phox}}-p47^{\text{phox}} \) interaction is between the SH3 domain of \( p40^{\text{phox}} \) and the C-terminus of \( p47^{\text{phox}} \) [94,95]. The interaction on \( p67^{\text{phox}} \) for \( p40^{\text{phox}} \) was shown to be the \( p67^{\text{phox}} \) inter-SH3 region [88,90]. The \( p67^{\text{phox}} \) binding region on \( p40^{\text{phox}} \) in turn was shown to bind the C-terminal half of the protein [88,90,94,95], with the PC motif being especially important [88].

*In vitro* binding studies indicated that \( p47^{\text{phox}}, p67^{\text{phox}} \) and \( p40^{\text{phox}} \) bind to one another and once at the membrane, \( p67^{\text{phox}}, p47^{\text{phox}} \) and rac appear to be present in equimolar amounts [96].

The entire complex docks at the membrane via an association between \( p47^{\text{phox}} \) and the proline rich region at the carboxy-terminus of the small subunit [93,95&97]. Evidence for this interaction came from studies of a \( p22^{\text{phox}} \) CGD patient with normal expression of an inactive flavocytochrome \( b_{558} \). The patient
had a Pro 156-Gln substitution, disrupting one of the p22phox proline rich sequences, resulting in no translocation of the cytosolic factors.

Further indications of a multiple-site interaction between p47phox and the flavocytochrome came from experiments with random-sequence peptide display libraries. In these studies gp91phox sequences Ser86-Lys93 and Phe450-Lys457 were identified as binding regions for p47phox and the corresponding synthetic peptides inhibited translocation of the cytosolic factors and activation of the oxidase. In addition, a point mutation in the gp91phox has been described, substituting Asp500 for Glu, that resulted in defective translocation of p47phox and p67phox although normal amounts of the flavocytochrome were present in the membrane.

Another binding partner for a cytosolic phox protein is rac. In vitro binding studies showed it to bind to the N-terminal 199 amino acids of p67phox in a GTP-dependent manner and more recently to interact directly with cytochrome.

1.6 INTERACTION WITH THE CYTOSKELETON.

There is some evidence for the interaction of components of the NADPH-oxidase with the cytoskeleton, although the nature of this interaction is not totally understood. Experimentally p67phox and p47phox are associated with the detergent-insoluble fraction when neutrophils are permeabilised with Triton X-100, a result taken as evidence for the interaction of the phox proteins with the cytoskeleton.

Cytoskeletal involvement is also implicated by experimental observations that rac, when injected into the cytosol of fibroblasts in the active GTP-bound
form, may control cytoskeletal events such as membrane ruffling and the formation of actin stress fibres. Evidence for modulation of the oxidase by membrane alterations and the amount and localization of filamentous actin has also been presented.

Within the laboratory of A.W. Segal a number of cytoskeletal interactions have been identified by in vitro binding assays. As already mentioned an interaction of p47phox and p40phox with the cytoskeletal actin-binding protein moesin, in a phosphoinositide-dependent manner, was identified and a clear interaction between the C-terminal of p40phox and the actin binding protein coronin was observed. Interestingly oxidase activity was essential for cytoskeletal rearrangements of actin and coronin. Rearrangements did not occur in cells deficient in p47phox or p67phox suggesting a role for these proteins in cytoskeletal rearrangements which accompany neutrophil activation.

1.7 CHRONIC GRANULOMATOUS DISEASE (CGD).
The syndrome of CGD provides the most definitive evidence for the physiological and clinical importance of the respiratory burst. CGD is a syndrome the unifying features of which are a predisposition to infection with bacteria and fungi and the inability of their cells to kill bacteria in vitro and is coupled to the absence of NADPH oxidase activity. The infecting organisms are commonly Staphylococcus aureus, Klebsiella, E. coli, Pseudomonas, Serratia marcescens and also fungi especially Aspergillus fumigatus and therefore similar to those that normal neutrophils fail to kill under anaerobic conditions. The diagnostic tests for CGD include measurements of oxygen
consumption\textsuperscript{112}, superoxide dismutase (SOD) inhibitable reduction of cytochrome C\textsuperscript{113} and nitroblue tetrazolium\textsuperscript{114}.

CGD patients have played and continue to play an invaluable role in elucidating the structure and mechanism of the oxidase. Characterisation of the wide range of genetic lesions underlying CGD has enabled the identification of domains of proteins likely to be of functional importance\textsuperscript{38}.

The accepted dogma on explaining why CGD patients are prone to infection was initiated in 1964, when two laboratories provided experimental evidence that the neutrophil oxidase mediated the increase in oxygen consumption\textsuperscript{112,115}. The burst of oxidative metabolism associated with phagocytosis and the combination in CGD of the absence of this process together with a defective bacterial killing system, was interpreted as indicating that the oxygen that is consumed becomes converted into microbicidal products, thus explaining the pathogenesis of granulomatous disease.

In the early 1960's, Iyer and co-workers\textsuperscript{116} observed that phagocytosis was associated with increased production of hydrogen peroxide and the role of hydrogen peroxide in the killing system was supported by observations on neutrophils from patients with CGD. It was also believed that these cells kill bacteria that themselves produce hydrogen peroxide, but not those bacteria that contain catalase, which catabolises the hydrogen peroxide\textsuperscript{117}. This point is expanded in section 1.15.

Whilst studying the microbial killing in phagocytes, Klebanoff and McRipley and Sbarra\textsuperscript{118,119} independently showed the tripartite interaction of the neutrophil primary granule enzyme myeloperoxidase, a halide such as chloride
and hydrogen peroxide, resulting in the formation of hypohalous acid, the most microbicidal oxidant known to be produced by neutrophils.

Using human neutrophils triggered by an opsonized strain of *Staphylococcus aureus*, Cohen and co-workers recovered 99% of oxygen consumed as superoxide and 40% as hydrogen peroxide\(^{120}\). The failure of phagocytes from patients with CGD to generate superoxide during phagocytosis was shown\(^ {113}\) and the requirement of superoxide as a precursor to hydrogen peroxide formation in the phagocyte was confirmed\(^ {121}\).

1.8 ADVANCES IN THERAPY.

As already discussed, all cases of CGD so far have been found to result from a deficiency of one of four oxidase proteins. Autosomal recessive CGD arising as a result of a deficiency in p47\(^{phox}\), p67\(^{phox}\) or p22\(^{phox}\) and X-linked CGD as a result of the loss of gp91\(^{phox}\). Large studies from the United States and Europe have identified the distribution of genetic lesions within CGD patients\(^ {122,123}\). Overall, two thirds of families had an X-linked recessive pattern of inheritance with a defect of the β-subunit of the cytochrome. Of the remaining patients with an autosomal recessive pattern of inheritance, the majority, (25% of the total) were deficient in p47\(^{phox}\). Defects in the small α-subunit of the cytochrome and in p67\(^{phox}\) made up the other 10% of the cases.

Genetically engineered mice, close replicas of the X chromosome-linked (gp91\(^{phox}\)) or the autosomally inherited (p47\(^{phox}\)) types of the disease, were developed for research on phagocyte biology and treatment\(^ {124,125}\). Affected mice lack phagocyte superoxide production and are susceptible to infection with
*Staphylococcus aureus* and *Aspergillus fumigatus*, reflecting a similar phenotype to that seen in human CGD.

The discovery that interferon gamma could enhance superoxide production of normal phagocytes was followed by studies in patients with CGD. Interferon gamma increased cytochrome *b* activity from nil to as much as half normal, superoxide production by as much as tenfold, and restored the capability of killing *Staphylococcus aureus* by patients' phagocytes\textsuperscript{126,127}. These findings led to administrations of the cytokine to patients in a clinical trial resulting in significantly decreasing the frequency of severe infections.

Much of the recent work on the oxidase in CGD has been directed towards developing a genetic cure of the disease. B lymphocytes from patients with various forms of CGD have been cured by transfection with vectors expressing the oxidase component missing from the patients' cells. Various retroviral vectors have been used to correct CGD cells from patients with the X-linked form of the disease, who are missing gp91\textsuperscript{phox}, with the active cells producing superoxide at about 60% of the normal rate\textsuperscript{128}. CD34\textsuperscript{+} cells from a p67\textsuperscript{phox} deficient patient were transfected with a retrovirus that expressed p67\textsuperscript{phox}. Superoxide production was restored in neutrophils arising from the transfected cells\textsuperscript{129}.

Investigators have cured CGD *in vivo* by genetic methods, but only in mice. Knockout mice lacking gp91\textsuperscript{phox} were transplanted with their own marrow cells after transfection with a murine stem cell virus vector that expressed the missing protein\textsuperscript{130}. Superoxide was expressed by neutrophils from these transplanted mice, though at levels lower than seen in wild-type mice but they
did withstand a challenge with *Aspergillus fumigatus* that produced pneumonia in all the untransplanted CGD mice.

1.9 PRIMING.

Sub-stimulatory levels of a wide variety of agonists can induce an accelerated and often exaggerated response to subsequent stimuli\(^{131,132}\). Hence the primed neutrophil represents a state distinct from the resting and activated states\(^{133}\). It is not known if it is a physiological phenomenon, although it has been demonstrated that subpopulations of neutrophils from subjects with acute bacterial infection have increased oxidant production upon PMA stimulation\(^{134}\). The mechanism underlying the "priming" response are unknown but are associated with increases in levels of cytosolic calcium\(^{135}\), upregulated tyrosine phosphorylation\(^{136}\), cytoskeletal rearrangements\(^{137}\) and probably operate at an early stage in the activation process.

1.10 CELLULAR ACTIVATION PATHWAYS.

Activation of the respiratory burst is characteristically associated with a delay or lag phase, between the application of the stimulus and onset of the respiratory burst\(^{138}\). This lag reflects the complex series of integrated signal transduction events that take place during activation. The duration of the lag phase preceding onset of the respiratory burst, and the duration and magnitude of activation are determined by the particular activating stimulus, or combinations of stimuli and the pre-activation or primed status of the cell. Most obviously different are responses that follow stimulation with a receptor ligand such as fMLP and a direct agonist of PKC such as PMA, which activates the respiratory burst
independently of surface receptors. In response to fMLP the lag phase is short and onset of the respiratory burst is relatively rapid, within 5-10 seconds, but the reaction is brief tailing off over about 1 minute. In contrast the burst induced by PMA takes about 25 seconds to be initiated but lasts many minutes.

Phosphorylation and dephosphorylation are dynamic processes and continued phosphorylation is probably required for activity \textit{in vivo}. This is supported by the massive prolongation of the otherwise rather transient response to fMLP, with okadaic acid, an inhibitor of phosphatase 1 and 2A\textsuperscript{139}. Pre-treatment of cells produces a greatly prolonged response to fMLP\textsuperscript{139}.

Various physiological stimuli have been used in experimental systems to study the oxidase. These include phagocytosable particles (bacteria, viruses, aggregated material, cell debris, etc.) and a number of soluble factors, (chemotactic peptides-lectins, complement factors - C5a, Ca\textsuperscript{+} ionophores, leukotrine B\textsubscript{4}\textsuperscript{108}, cytochalasins, phorbol esters\textsuperscript{25}, diacylglycerol, platelet activating factor and endotoxin\textsuperscript{10,24,140}). Most of the stimulatory agents are of biological importance, since they frequently come in contact with the phagocytic cells at the inflammatory sites or in the blood stream. Many of the stimuli induce more than one response at different concentrations. For example the optimal chemotactic response to fMLP takes place at 10\textsuperscript{-11}M and the respiratory one at 10\textsuperscript{-7}-10\textsuperscript{-6}M.

Interaction of the neutrophil receptor with specific ligands, results in the activation of a large number of signalling pathways. Three classes of receptors are described. The first is the transmembrane or serpentine receptor, for example the receptor to fMLP, inducing the activation of the heterotrimeric G-proteins Gi and Gq\textsuperscript{141,142}. The second class of receptors are described by the \(\beta\)-
adrenergic receptor resulting in the activation of the heterotrimeric G-protein Gs
Finally the Fcγ receptors employ tyrosine kinases and tyrosine phosphorylation as part of their signalling response.

One of the first responses to receptor-ligand interaction is the activation of PI-3 kinase. Neutrophils contain several PI-3 kinase isoforms and their importance for the respiratory burst is highlighted by the fact that a highly specific PI-3 kinase inhibitor, wortmannin, is one of the most potent inhibitors of fMLP and opsonised zymosan induced respiratory burst. PI-3 kinase regulates a number of signalling processes including the activation of kinases such as protein kinase D and small GTP-binding proteins such as ras and rac. PI-3 kinase also regulates phospholipase D.

Serpentine receptors as well as Fcγ receptors regulate phospholipase C isoforms. Activation leads to hydrolysis of the lipid precursor phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG activates protein kinase C, itself an important activator of the NADPH oxidase. Soluble IP3 diffuses into the cytosol and mediates a biphasic rise in intracellular Ca²⁺ that results, initially from release of Ca²⁺ by intracellular stores and later from sustained entry across the plasma membrane. The respiratory burst is preceded by the rise in Ca²⁺, suggesting an important function for this divalent cation in the signalling pathway. Furthermore, depletion of intracellular Ca²⁺ stores results in failure of the respiratory burst when stimulated by receptor agonists such as fMLP.

Under some circumstances the oxidase maybe activated independently of the Ca²⁺ signalling pathway, by direct stimulation of PKC. The membrane-associated activity of DAG is raised transiently when the cell is stimulated
resulting in PKC activation\textsuperscript{147}. The contribution of PKC to oxidase activation has long been recognised. PKC is able to phosphorylate components of the oxidase and PKC stimulation by PMA leads to respiratory burst\textsuperscript{65,148}. The use of staurosporine a PKC inhibitor invariably inhibited the PMA stimulated respiratory burst\textsuperscript{149}. Recently more selective PKC inhibitors have been developed from staurosporine. These include indocarbazoles and bisindolylmaleimides, yielding results indicating a role for PKC in fMLP mediated oxidase activation\textsuperscript{150}.

Various studies show the presence of PKC isotypes in neutrophils\textsuperscript{151}. The use of inhibitors indicates that many isotypes are involved in activation of the oxidase and that different stimuli use different subsets of isotypes to activate the NADPH oxidase. For example neutrophils from PKC-\(\beta\) deficient mice showed about 50\% oxidase activity in response to PMA or Fc\(\gamma\) receptor stimulation\textsuperscript{152}, confirming the role of PKC-\(\beta\) and indicating the involvement of other kinases. Using neutrophils of PKC-\(\delta\) deficient mice, this isotype was found to be important for activation by PMA or non-opsonised zymosan. Additionally employing an oligonucleotide interference strategy, it was confirmed that PKC-\(\mu\)/PKD mediates the Fc\(\gamma\) receptor response but not that to PMA\textsuperscript{153}.

1.11 THE NADPH OXIDASE IS AN ELECTROGENIC PROCESS.

1.11.1 Plasma membrane depolarization reaches a steady state.

Through the use of cytoplasts and an optical membrane-potential indicator, Henderson (1987), showed that the generation of superoxide anions at the exterior of the cytoplast involves the uncompensated charge transfer, by the
oxidase, of an electron across the plasma membrane from the cytosolic NADPH to external $O_2$. Thus the oxidase is electrogenic$^{154}$. Stimulation by PMA causes a depolarization across the plasma membrane from $-60$ to $-20$ mV. The depolarization is complete within 1 minute, and can persist for 10 minutes. With the use of indirect probes of membrane potential$^{155}$, the fluorescent cyanine dye 3,3'-dipentyl-oxacarbocyanine and the radiolabeled ion TPMP$,^+$, depolarization was shown to be due to activity of the oxidase rather than a signal transduction mechanism. This was confirmed by membrane potential changes elicited by fMLP and PMA, which were reduced or entirely absent in neutrophils obtained from patients with CGD$^{155}$. In addition, inclusion of the oxidase inhibitor, DPI, to cytoplasts after PMA stimulation, lead to rapid membrane repolarisation.

As the depolarization reaches a steady state after one minute, while superoxide generation continues, the positive charge left in the cytosol must be dissipated, either by efflux of positive ion or influx of negative ion. One suggestion is that a $Na^+/H^+$ exchange protein stabilizes the system. An alternative suggestion is that flavocytochrome $b_{558}$ itself transports protons$^{156}$.

1.11.2 $Na^+/H^+$ exchange restores pH.

pH of neutrophils and cytoplasts can be monitored with a pH sensitive fluorescent dye. Neutrophils maintain a resting pH of 7.3 when in a pH 7.4 medium. Stimulation brings on an initial fall in pH to 7.2 in 1min, followed by a gradual alkalinisation to pH 7.5 over 5 min. The generation of $O_2^-$ imposes a metabolic acid load ($CO_2$) on the cell. When $Na^+$ transport is blocked (with amiloride), the initial fall is greater, and the alkalinisation does not take place, suggesting that $Na^+/H^+$ exchange takes part in recovery from the acid load$^1$. 
1.11.3 Preventing $H^+$ flux leads to a greater fall in pH and superoxide generation is prevented.

$Zn^{2+}$ and $Cd^{2+}$ are commonly used to block $H^+$ channels. When these were applied to stimulated cytoplasts, the fall in pH was more rapid, and to a greater extent (by 0.25 pH unit). Inhibition of the channel led to inhibition of superoxide generation. When valinomycin and $K^+$ were supplied as an alternative charge compensator, activity was temporarily restored.$^{157}$

1.11.4 Evidence that the $H^+$ channel is gp91$^{phox}$.

The identity and nature of the $H^+$ channel involved in compensation of charge, induced by the NADPH oxidase has been well studied and it has been suggested that gp91$^{phox}$, the flavocytochrome $b$ of the NADPH oxidase is itself the channel$^{157-159}$. In support of this theory Henderson and colleagues (1995)$^{159}$ identified the fully functional arachidonate, activated $H^+$ channel in EBV-transformed B lymphocyte cell lines, derived from normal and CGD patients, lacking the expression of p47$^{phox}$ and p67$^{phox}$. This channel was not identified in those cells lacking gp91$^{phox}$. A later study employing a patch clamp technique, on Chinese hamster ovary cells examined whole cell currents from cells expressing mutated versions of gp91$^{phox}$. Their findings located the voltage sensitive residues of gp91$^{phox}$ in the NH$_2$-terminal 230 amino acids and that histidine residues at positions 111, 115, and 119 on the membrane-spanning helical region of the protein contributed to $H^+$ permeation$^{160}$.

The theory that the gp91$^{phox}$ subunit of the NADPH oxidase contained proton channels was disputed by DeCoursey (2001), who found no difference in $H^+$ currents in cells of the human myelocytic PLP-985 cell line$^{423}$, in which
gp91\textsuperscript{phox} was knocked out and those that were re-transfected with the gp91\textsuperscript{phox}. Similarly, monocytes from CGD patients lacking gp91\textsuperscript{phox} exhibit normal levels of H\textsuperscript{+} currents\textsuperscript{156}.

1.12 VACUOLAR pH.

In 1981, Segal and co-workers employed pH indicator fluorescein, conjugated to \textit{Staphylococcus aureus} and following phagocytosis, measured early pH changes within the phagocytic vacuole\textsuperscript{161}. Results indicated a transient increase in pH to 7.8-8.0 within the first two minutes, which was followed by a slow fall to 6.0-6.5 after two hours. Jiang (1997), using similar fluorometric techniques later confirmed these observations\textsuperscript{162}. The pattern of pH with phagocytosis was clearly different in CGD neutrophils or with normal neutrophils in anaerobic conditions, where the pH fell rapidly from 7.4 to 6.7 within the first two minutes\textsuperscript{161} and carried on falling to 5.5 after two hours.

Because of deficient pH regulation in CGD phagocytic vacuoles, it has been suggested that the products from superoxide are generated to consume protons, thereby regulating the pH of the vacuole\textsuperscript{163}. The rise in pH achieved within the vacuole facilitates killing and bacteriolysis by granule proteins\textsuperscript{7,164}, as the pH in the vacuoles of CGD patient cells is below the optimum for both elastase and cathepsin G\textsuperscript{165}. Indeed it has been demonstrated that cells treated with chloroquine or ammonium chloride, had markedly increased antifungal activity against the AIDS-related pathogen \textit{Cryptococcus neoformans}, which resides in acidic phagosomes. Both of these agents raise the lysosomal pH, suggesting that the increased antifungal activity was a function of alkalinizing the phagosome\textsuperscript{166}. 

The subsequent drop in pH of the vacuole, observed by Segal et al., is optimal for the activity of hydrolases and other proteins with acid pH optima.

1.13 BACTERICIDAL MECHANISMS.

Once the microbe has been contained within a phagocytic vacuole it must be actively killed or else it multiplies, destroys the cell and propagates the infection. As Klebanoff suggested, the mechanisms by which phagocytic cells kill, can be classified according to whether or not molecular oxygen or its reduction products are participants in the process. Therefore, bacterial clearance can be divided into two sets of processes, those that are oxygen independent, and those that are oxygen dependent. It is probable that the total microbicidal potential of the neutrophil is in excess of its needs under most circumstances. Particular organisms are susceptible to more than one antimicrobial system and thus may be effectively handled by back-up systems when one is absent.

1.14 BIOLOGICAL FUNCTION OF THE NADPH OXIDASE: OXYGEN DEPENDENT MICROBIAL KILLING.

The burst of oxidative metabolism associated with phagocytosis and the combination in CGD of the absence of this process together with a defective bacterial killing system has been interpreted as indicating that the oxygen that is consumed becomes converted into microbicidal products and indeed phagocytes manufacture a battery of reactive oxidants that in vitro have been shown to successfully kill microorganisms, as illustrated in Figure 1.3.
**FIGURE 1.3.** Possible oxidant generation reactions within stimulated neutrophils.

$O_2^-$, superoxide, mild oxidant and reductant with limited biological activity.  
$H_2O_2$, hydrogen peroxide, slow acting oxidizing agent.  
$OH^-$, hydroxyl radical, extremely reactive with most biological molecules.  
$^1O_2$, singlet oxygen, electronically excited state of oxygen.  
HOCl; hypochlorous acid, strong non-radical oxidant.  
R-NHCl; chloramines, milder and longer lived oxidants than HOCl.
1.14.1 Superoxide: \( O_2^- \)

The importance of \( O_2^- \) as a harmful biological oxidant is emphasised by the ubiquitous presence of superoxide dismutase (SOD) in all cells (eukaryotic as well as prokaryotic) that contain a cytochrome system (i.e., depend on aerobic metabolism for energy)\(^{170}\). Cytochemical techniques based on a manganese dependent diaminobenzidine oxidation, demonstrated the generation of reduced oxygen within the phagocytic vacuole\(^{171}\).

The first observation of \( O_2^- \) production was by Babior and his group in 1973 as stimulated neutrophils were seen to reduced cytochrome C in the surrounding medium, and this was inhibited by the enzyme, superoxide dismutase (SOD)\(^9\). As a result of these experiments it was thought that stimulated neutrophils generated \( O_2^- \), and that \( H_2O_2 \) is formed as a result of the spontaneous dismutation of the \( O_2^- \) according to the following equations:

\[
O_2 + 1 \text{ electron} \rightarrow O_2^- (\text{superoxide})
\]

\[
O_2^- + O_2^- \rightarrow O_2^{2-} + O_2 (\text{peroxide and oxygen})
\]

\[
O_2^{2-} + 2H^+ \rightarrow H_2O_2 (\text{protonated peroxide})
\]

\[
2H_2O_2 \rightarrow 2H_2O + O_2 (\text{breakdown of hydrogen peroxide to water and oxygen})
\]

Experiments were also conducted in which the artificial \( O_2^- \) generating system of xanthine oxidase and purine were employed. Results were interpreted as showing that \( O_2^- \) kills bacteria\(^{172}\), however this has been disputed as \( O_2^- \) does not appear to be directly microbicidal\(^{173,174,371}\).
1.14.2 Hydrogen peroxide: $H_2O_2$

$H_2O_2$ is formed by the two-electron reduction of molecular oxygen or by the dismutation of $O_2^-$. In 1961, Iyer, Islam & Quastel demonstrated that formate was effectively converted to $^{14}CO_2$ by phagocytosing neutrophils. They ascribed this to a catalytic oxidation of formate due to the release of substantial amounts of peroxide during phagocytosis\textsuperscript{116}.

This theory was later confirmed by the more sensitive fluorometric assay for the detection of $H_2O_2$\textsuperscript{175}. This assay depends upon the utilization of $H_2O_2$ by exogenous horseradish peroxidase for the oxidation of scopoletin. Specificity was conferred by the evolution of $O_2$\textsuperscript{176} or the inhibition of the decreased scopoletin fluorescence\textsuperscript{177} by the addition of catalase.

Cytochemical studies also supported the production and localization of $H_2O_2$ to zones in the phagosome between the ingested particles and the inner membrane. In this study reaction of peroxide with $Ce^{3+}$ produced precipitates of cesium perhydroxy compounds, which were detectable by electron microscopy\textsuperscript{178}.

The role of $H_2O_2$ in the killing system was confirmed by the introduction of an artificial $H_2O_2$ generating system in the form of glucose oxidase coated latex particles, some of which entered the same phagosome as the bacteria, resulting in enhanced bacterial killing\textsuperscript{179}.

Further evidence for the role of $H_2O_2$ in bacterial killing was the fact that catalase negative organisms rarely infect CGD patients\textsuperscript{180}. The explanation proposed was that these bacteria generated enough $H_2O_2$ to mediate their own destruction within the phagocytic vacuole of CGD cells\textsuperscript{117}.
It is not known what concentration of H$_2$O$_2$ is attained within the vacuole. Measurement from 0.01 μM to 100 mM have been estimated, depending on the amount of phagocytosis and the intracellular pH. It seems unlikely however that they could exceed 100 mM because at such high levels the catalase activity of MPO supervenes. H$_2$O$_2$ generated during the respiratory burst has limited bactericidal properties and the best-defined function of H$_2$O$_2$ in the antimicrobial activities of granulocytes comes from the function of H$_2$O$_2$ as a substrate for myeloperoxidase (MPO) in the presence of halides.

1.14.3 Myeloperoxidase and the products of chloride oxidation:

HOCl

1.14.3a Myeloperoxidase.

The neutrophil peroxidase was first purified by Agner in 1941 and because of its intense colour, was called verdoperoxidase, its name subsequently changed to myeloperoxidase (MPO). It is present in exceptionally high concentrations in neutrophils, with levels estimated to be no less than 5% of the dry weight of the cell. MPO is synthesised and packaged into azurophilic or primary granules of neutrophils during the promyelocyte stage of granulocyte development and is present in mature resting granulocytes. Mature MPO is a 150 kDa tetramer composed of two glycosylated 59-64 kDa heavy subunits and two unglycosylated 14 kDa light subunits as a pair of protomers linked together by a disulphide bond. Each heavy subunit carries a covalently bound heme group.

Monocytes also contain MPO-positive cytoplasmic granules although they are fewer in number than in neutrophils and are generally lost as monocytes.
mature into macrophages. Eosinophils also contain a peroxidase in their cytoplasmic granules\textsuperscript{188}, however it differs from MPO both structurally and functionally.

When the phagosome containing microorganisms fuses with cytoplasmic granules, MPO, along with the other components of the granules, is released into the vacuole\textsuperscript{169}. A role for MPO as a component of the antimicrobial armamentarium of neutrophils was proposed in 1967 with the finding that MPO was strongly microbicidal when combined with $\text{H}_2\text{O}_2$ and a halide\textsuperscript{118,189}. MPO and $\text{H}_2\text{O}_2$ form an enzyme-substrate complex, which oxidises ions to the toxic agent, hypohalous acid. Any of the halide ions ($\Gamma^-$, $\text{Br}^-$, $\text{Cl}^-$) can be oxidized with iodide and bromide being more effective than chloride on a molar basis\textsuperscript{189}. It is more likely however that the phagocyte uses $\text{Cl}^-$ because it is present in high concentration in biological fluids, resulting in the formation of hypochlorous acid (HOCl).

The following paragraph outlines the reactions of MPO. At relatively low (equimolar) concentrations, $\text{H}_2\text{O}_2$ formed by the NADPH oxidase reacts with the heme iron of MPO to form a complex referred to as Compound I (Figure 1.4). Compound I, is the primary catalytic complex of MPO and $\text{H}_2\text{O}_2$ and it can oxidize substances with the regeneration of the native MPO and production of HOCl. When there is excess of $\text{H}_2\text{O}_2$, Compound I is converted to an inactive complex, Compound II. Compound II can be reduced to the active MPO by a number of reducing agents with restoration of catalytic activity. Among the reducing agents that can act in this way is $\text{O}_2^-$\textsuperscript{190,191}. A third MPO complex, Compound III, is an oxyperoxidase that can be formed by the reaction of $\text{O}_2^-$ with MPO. Compound III can be catalytically active, however its formation by
FIGURE 1.4. Reactions of MPO.

Ferric MPO (MP$^{3+}$) reacts with H$_2$O$_2$ to form the redox intermediate Compound I, which oxidizes Cl$^-$ by a single 2-electron transfer to produce the respective HOCl. When there is an excess of H$_2$O$_2$, Compound I is converted to an inactive complex, Compound II. Compound II can be reduced to the active MPO by O$_2^-$, which is released into the phagosome as the primary product of the respiratory burst oxidase. Thus one of the functions of O$_2^-$ may be to maintain MPO in a catalytically active form. A third complex Compound III can be formed by reaction of O$_2^-$ with MPO. Compound III is unstable, decaying to native MPO.
the reaction of MPO with $O_2^-$ is an order of magnitude slower than the reaction of MPO with $H_2O_2$, and Compound III is unstable, decaying to native MPO.

1.14.3b Hypochlorous acid: $HOCl$

The microbicidal potency of $HOCl$ is convincingly demonstrated by its pervasive use in treatment of municipal sewage and other waste-waters. $HOCl$, as Dakin’s solution was extensively used in medicine in the treatment of topical wounds until antibiotics became available.

It is generally believed that $HOCl$ is the most bactericidal oxidant known to be produced by the neutrophil. Levels of $HOCl$ produced upon activation of the NADPH oxidase are based on calculations made after stimulation with PMA, which induces the oxidase to secrete $O_2^-$ across the membrane to the extracellular space. In this case $HOCl$ is produced in the surrounding supernatant and levels achieved are estimated at 80μM. More definite calculations have come from recent studies using chlorinated fluorescein as a specific marker for $HOCl$ production. It has been calculated that 11% of the oxygen consumed was converted to $HOCl$, resulting in concentrations of 28μM within the phagosome.

$HOCl$ is an extremely strong non-radical oxidant of a wide range of biological compounds. Bacterial targets include adenosine triphosphate (ATP)-generating systems, the origin of replication site for DNA synthesis, activation or inactivation of enzymes and electron transport systems, disruption of basement membranes or cell membranes, and fragmentation of proteins.

Chloramines are generated indirectly through the reaction of $HOCl$ with the many amines that are found in biological systems. These compounds are
less reactive than HOCl but much more stable, and are therefore called “long lived oxidants” (LLO). Chloramines concentrations of 30-100μM have been detected in the supernatants of 0.25x10^7 to 1.0x10^7 PMA stimulated neutrophils. It has been reported that they are capable of maintaining oxidizing capacity for many hours during inflammation and have been detected in bronchial mucus of patients suffering from cystic fibrosis.

LLO consist of a mixture of chlorinated proteins and amino acids. Because of the high intracellular concentration of the β-amino acid taurine (50mM), N-chlorotaurine (NCT) is the major compound of low molecular weight LLO produced by neutrophils and is thought to maintain oxidizing capacity that could be used in vivo for transhalogenation reactions (transfer of the active chlorine) to lipophilic oxidants, especially monochloramine, which has strong microbicidal properties (Figure 1.5).

NCT, the most stable N-chloro amino acid exerts broad-spectrum activity against bacteria, fungi, viruses and helminths down to physiological concentrations in vitro.

Finally the reaction of HOCl with amino acids leads through chloramines to aldehydes, another class of very reactive molecules.

1.14.4 Hydroxyl radical: \( \cdot \text{OH} \)

The hydroxyl radical (\( \cdot \text{OH} \)) is one of the most reactive oxidant known and its formation within the phagocytic vacuole has been controversial. Johnston and co-workers provided the first evidence that human neutrophils produce \( \cdot \text{OH} \) during phagocytosis in 1975. SOD, catalase, and scavengers of \( \cdot \text{OH} \), (mannitol and benzoate) inhibited bactericidal activity of neutrophils.
FIGURE 1.5. Transhalogenation.

N-chlorotaurine maintains oxidation capacity for transhalogenation reactions (transfer of the active chlorine).
The first potential mechanism in which \( \cdot \text{OH} \) can be produced is through the interaction of \( \text{O}_2^- \) with \( \text{H}_2\text{O}_2 \). The interaction of these reactants to form \( \cdot \text{OH} \) was proposed by Haber and Weiss\(^{208}\) as follows:

\[
\text{H}_2\text{O}_2 + \text{O}_2^- \rightarrow \text{O}_2 + \text{OH}^- + \cdot \text{OH}
\]

However studies indicated that the direct interaction of \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) is slow\(^{173}\) making it unlikely that \( \cdot \text{OH} \) is generated in biological systems by this mechanism. Also release of MPO may inhibit neutrophil associated Haber-Weiss-mediated \( \cdot \text{OH} \) formation by decreasing the availability of \( \text{H}_2\text{O}_2 \) for this reaction\(^{209}\).

An alternative mechanism of \( \cdot \text{OH} \) production is via the superoxide-driven Fenton reaction\(^{210,211}\) between hydrogen peroxide and an appropriate transition metal catalyst, measured by electron spin resonance spectroscopy\(^{212}\):

\[
\text{Fe}^{3+} + \text{O}_2^- \rightarrow \text{Fe}^{2+} + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot \text{OH}
\]

However while investigating this mechanism, Winterbourn (1983), established that neutrophils do not have an endogenous transition metal catalyst and that release of lactoferrin inhibits the reaction by complexing iron\(^{213}\).

A potential third source of \( \cdot \text{OH} \) is the MPO system. HOCI reacts with \( \text{O}_2^- \) to form \( \cdot \text{OH} \)\(^{214}\) leading to the proposal that \( \cdot \text{OH} \) may be generated in phagocytes by the following mechanism:

\[
\text{HOCl} + \text{O}_2^- \rightarrow \cdot \text{OH} + \text{O}_2 + \text{Cl}^-
\]

Electron paramagnetic resonance spectroscopy, using spin trap procedures indicated the formation of \( \cdot \text{OH} \) by PMA stimulated neutrophils, this production was inhibited in the presence of SOD and azide, implicating \( \text{O}_2^- \) and MPO respectively\(^{215}\). However the fact that less than 1\% of the \( \text{O}_2^- \) formed was
accounted for in the formation of OH and no products of fluorescein oxidation by OH were detected\textsuperscript{162}, raises questions on the biological importance of the neutrophil-derived OH.

1.14.5 Singlet oxygen: $^1O_2$

Oxygen is a diradical with two unpaired electrons and the pairing of these two electrons leads to a highly reactive form of oxygen, known as singlet oxygen. In 1972, Allen and working colleagues observed that neutrophils displayed a chemiluminescence during phagocytosis and suggested that this phenomenon reflected the generation of $^1O_2$\textsuperscript{216}. They proposed that the radical might function as a bactericidal agent. Although it is now accepted that the chemiluminescence results from another series of interactions, this paper was of special interest as it suggested that cidal agents (active oxygen species) other than $H_2O_2$ might be produced by the respiratory burst.

Two laboratories reported\textsuperscript{217,218} that $^1O_2$ is produced \textit{in vitro} by the MPO/\textsubscript{H}_2O\textsubscript{2}/halide antimicrobial system described by Klebanoff\textsuperscript{167,219}. An established mechanism for the formation of $^1O_2$ is the reaction of hypochlorite and $H_2O_2$:

$$HOCl \rightarrow H^+ + OCl^-$$

$$OCl^- + H_2O_2 \rightarrow Cl^- + H_2O + ^1O_2$$

Because $OCl^-$ is a product of the MPO-$H_2O_2$-chloride system and $H_2O_2$ is a product of the respiratory burst, their interaction in neutrophils to form $^1O_2$ has been sought with conflicting results. Early studies by Krinsky (1974), reported that neutrophil were incapable of killing wild-type \textit{S. lutea in vitro} over a 90min incubation, in the presence of serum\textsuperscript{220}. A mutant strain lacking carotenoid pigments was rapidly killed. Since carotenoids function as efficient quenchers of
$^{1}\text{O}_2$\textsuperscript{221}, these data suggested the production of $^{1}\text{O}_2$ by these cells and of a role for the latter as a bactericidal agent. Toxicity by inactivation of membrane respiratory chain enzymes has been reported.

However the most convincing evidence for $^{1}\text{O}_2$ formation is detection of near infrared light emission at 1268nm. With the near infrared spectrometry, Kanofsky \textit{et al.}, (1984), demonstrated that $^{1}\text{O}_2$ generation mediated by MPO occurred only at very acidic pH with a high H$_2$O$_2$ concentration or at a high bromide ion (Br$^-$) concentration\textsuperscript{222}. Based on these results, they concluded that physiological production of $^{1}\text{O}_2$ by an MPO-mediated reaction appeared unlikely\textsuperscript{223}.

Contrarily, Steinbeck and colleagues (1992), have used a singlet oxygen trap and reported a high 19\% conversion of available oxygen to the singlet form\textsuperscript{224}. A result suggesting that $^{1}\text{O}_2$ is likely to be responsible for some of the damage inflicted by phagocytes on their targets.

1.14.6 Reactive nitrogen species.

Reactive nitrogen species are produced by the reaction of NO with O$_2^-$ resulting in the formation of peroxynitrite\textsuperscript{225} (ONOO'). ONOO$^-$ then undergoes a secondary reaction to produce an agent that is able to nitrate tyrosine. These include ONOOH that breaks down to NO$_2^-$\textsuperscript{225}, O$_2$N-O-CO$_2^-$ produced by reaction of ONOO$^-$ with carbon dioxide\textsuperscript{226} and NO$_2$Cl, formed when HOCl reacts with nitrite\textsuperscript{227}, a degradation product of ONOO$^-$. The large amount of NO produced by macrophages may contribute to their microbicidal activity, but work by Klebanoff and Nathan (1993) concluded that
human neutrophils appear to generate so little\(^{228}\), that its microbicidal role remains controversial.

However in urinary tract infection, neutrophils isolated from urine showed a dramatic increase in nitric oxide synthase activity\(^{229}\). In addition nitric oxide synthase knockout mice have demonstrated the enzyme's essential contribution to host defence against a restricted set of pathogens, including *Mycobacterium tuberculosis* and *Leishmania major*. Also mice doubly deficient in both NO and NADPH oxidase (gp91\(^{phox}\) knockout) formed massive abscesses even when reared under specific pathogen-free conditions with antibiotics, whereas neither parental strain showed such infection\(^{230}\).

1.15 VIRULENCE OF CATALASE MICROBES IN CGD QUESTIONED.

Catalase is a heme protein enzyme that decomposes \(H_2O_2\) to water and oxygen. CGD patients lacking a functional NADPH oxidase are highly susceptible to catalase-producing bacteria and fungi but do not appear to be at increased risk of infection with catalase negative organisms\(^{180}\). The lack of virulence was explained by the pathogen producing appreciable amounts of \(H_2O_2\), which can substitute for the absence of reactive oxidant formation within the phagosome of CGD neutrophils, leading to MPO mediated halogenation\(^{19,231}\). This theory was supported by work carried out by Mandel (1975), in which *in vitro* mutagenesis was used to generate strains of *Staphylococcus aureus* containing varying levels of catalase. Their virulence in mice was found to be inversely proportional to their catalase content\(^{232}\).
More recently, Chang et al., (1998) cast doubts upon this theory. They generated genetically engineered catalase-deficient strains of *Aspergillus nidulans* and carried out infection studies in CGD mice. Results indicated that catalases do not play a significant role in pathogenicity of *Aspergillus nidulans* in CGD mice and questioned the central role of catalases as a fungal virulence factor in CGD.

The theory was further challenged by Messina et al., (2002), using *Staphylococcus aureus*, as this is the commonest cause of infection in patients with CGD. A mouse model of CGD was infected with two naturally occurring clinical isolates of staphylococci and results showed these organisms to be equally virulent. In the same study it was reported that the bacteria produced about 170pmols/10^8 organisms/min of H_2O_2, whereas when the same number of particles are phagocytosed by neutrophils approximately 100nmols are produced. Variant CGD neutrophils posses approximately 10% of normal oxidase and produce approximately 2 orders of magnitude more H_2O_2 than would be generated by the bacteria, but these levels are insufficient to cause efficient microbial killing. Thus the possibility that CGD phagocytes could utilize microbial H_2O_2 to generate significant microbial activity, by MPO mediated conversion into HOCl seems remote.

1.16 A DEBATE ON THE “ROLE OF MPO”

Experimental data suggesting an important role for MPO in the microbicidal activity was derived from studies employing heme enzyme inhibitors such as NaN_3 and KNC and attributed the decrease in bacterial killing to the depression of peroxidase activity of MPO. The MPO-dependent process has been
suggested to be strongly favored by human neutrophils as their prime mechanism of oxidative killing.

A summary of the outstanding evidence supporting the main role of MPO to be that of a peroxidase, resulting in the formation of HOCl, the principal oxidative toxin produced by neutrophils is outlined. This is followed by a number of contradictory facts, which dispute this role and further propose that MPO serves to protect the microbicidal degradative enzymes against oxidative damage, by breaking down hydrogen peroxide through its catalase action.

1.16.1 Evidence for the primary role of MPO, as a peroxidase.

- MPO, H_2O_2 and chloride can form a powerful antimicrobial system effective against bacteria, fungi, and viruses.
- Where oxidative killing is important, evidence with *Staphylococcus aureus* points to the process being overwhelmingly dependent on MPO. Adding the NADPH oxidase inhibitor DPI, to prevent O_2^- production, decreased the killing rate to 25%, indicating that oxidative killing mechanisms predominate in this system.
- MPO and H_2O_2 are released or formed by neutrophils at a time appropriate to the microbicidal act. MPO is a strongly basic protein, which binds avidly to negatively, charged surfaces and can be seen coating microorganisms.
- HOCl is produced in the phagosome and up to 12% of O_2 consumed is accounted for by HOCl production.
- H_2O_2 as a substrate for MPO mediated halogenation is formed by the respiratory burst of neutrophils and is required for optimum antimicrobial
activity. Thus, neutrophils of CGD patients lacking a respiratory burst, in turn lack H$_2$O$_2$ production. The microbicidal defect observed in these cells \textit{in vitro} can be reversed in part by the introduction of H$_2$O$_2$ into the system by latex particles coated with glucose oxidase$^{179,242}$.

- Studies of inhibitors of peroxidase activity, or of neutrophils from patients with hereditary MPO deficiency$^{243}$, or knockout mice deficient in MPO$^{244}$, suggest that MPO is required for optimum activity, against infections of \textit{Candida albicans}.

- Aerobic killing of the bacterium \textit{Actinobacillus actinomycetemcomitans} was nearly completely inhibited by addition of CN$^-$ and comprised the predominant mechanism based upon reaction half-times determined from initial killing rates under aerobic and anaerobic conditions$^{245}$.

\textit{1.16.2 The halogenation reaction of MPO is not necessarily the major or natural function of MPO.}

- MPO deficiency occurs with a prevalence of 1 in 2000 in the population and patients are not clinically afflicted by serious bacterial infections$^{246}$. Infections by \textit{Candida} species being only of importance$^{247}$. Although more slowly than normal, neutrophils deficient in MPO can kill bacteria \textit{in vitro},$^{243,248}$ a result incompatible with suggestions that MPO is the major mediator of neutrophil bactericidal function in man. Cells illustrate a prolonged respiratory burst resulting in increased levels of H$_2$O$_2$$^{176,249}$ and this coupled with non-oxidative methods of bacterial killing, has been proposed to compensate for MPO deficiency. Furthermore, chicken neutrophils lack this enzyme$^{250}$. 
❖ The second query on the importance of the oxidative, MPO/ H$_2$O$_2$/Cl$^-$ killing is evident in-patients with CGD. Despite the fact that neutrophils of these patients do not produce O$_2^-$ and H$_2$O$_2$, studies by Qui, Segal, and Lehrer, showed that neutrophils from CGD patients were capable of killing significant numbers of phagocytosed bacteria and yeast $^{18,161,251}$. Mandell showed that many organisms including *S. epidermidis, enterococci, Viridans streptococci, Pseudomonas aeruginosa, Streptococcus anaerobius, B. fragilis, C. perfringens and Peptococcus magnus* are killed normally in the absence of oxygen$^{111}$.

❖ When experiments were conducted with radioactive iodide to demonstrate that bacteria were iodinated via MPO mediated halogenation; no evidence was found that they were. Instead it was found that the proteins that were iodinated came from the neutrophils rather than the bacteria$^{252}$.

❖ MPO is a heme enzyme that has several different activities $^{253}$. The environmental condition in which the enzyme functions will determine the substrates used and the activity exhibited. Its main physiological activity is generally accepted to be the production of HOCl, however activity is decreased at high pH and at high peroxide and chloride concentrations$^{181,190}$. In 1963 Agner$^{254}$ found that MPO displays a catalase like activity in which it degrades H$_2$O$_2$ to oxygen and water. A much more recent study by Kettle *et al.*, concluded that H$_2$O$_2$ reacts with Compound I and catalase activity is efficiently maintained by O$_2^-$ $^{255}$. This third point raises justified questions about the true function of MPO within the phagocytic vacuole.

❖ The physiological environment created within the vacuole is not consistent with conditions favourable for MPO peroxidase activity. Following phagocytosis there is a transient rise in vacuolar pH, increasing as high as 7.8 to 8.0$^{161,162,256}$. 
Studies by Klebanoff (1975) found maximal MPO activity at pH 5.0\textsuperscript{167}. Whilst Zgliczynski and colleagues showed the process of \textsuperscript{36}Cl incorporation to an insoluble fraction decreased as the pH was elevated from 4 to 7.4\textsuperscript{257,258}. Following the initial rise, the pH within the vacuole falls to about 6.0 after one hour. This pH is more suitable for MPO peroxidase activity, however within 4 min of phagocytosis 90\% of bacteria are killed and oxidase activity is complete\textsuperscript{161}.

- The major contributors to the chemical composition of the phagosome are the contents of the cytoplasmic granules that empty into it. Granule protein contents are released within seconds of ingestion and constitute a significant proportion of the phagosomal volume\textsuperscript{169,259}. In 1982 Nathan and Klebanoff found that increasing concentrations of protein inhibited the toxicity of the MPO-H\textsubscript{2}O\textsubscript{2}-halide system\textsuperscript{260}, as HOCl is consumed immediately by reaction with all organic material\textsuperscript{198}. This inhibition could only be overcome if the bacterium was coated with MPO prior to the addition of free protein\textsuperscript{260}. Furthermore MPO has been shown to reconstitute microbial activity of cytoplasts (neutrophils from which the granules and nuclei are removed, but which are able to mount a respiratory burst) when introduced into the phagocytic vacuole\textsuperscript{261}. But the use of cytoplasts excludes all other granule protein, which may effect the overall toxicity of the MPO/ H\textsubscript{2}O\textsubscript{2}/Cl\textsuperscript{-} system.

Thus it is not entirely clear that microbial killing via MPO mediated halogenation reaction is necessarily the major or natural function of MPO within the phagocytic vacuole. Certainly, HOCl has been demonstrated to be produced within the vacuole and indeed the activation of gelatinase and collagenase was
shown to be under oxidative regulation and coupled to the generation of HOCl\textsuperscript{262}. However alternative roles for MPO have been suggested. As MPO has the ability to react directly with O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2}, it may be more important for the detoxification and removal of superoxide and peroxide\textsuperscript{181} and to protect microbicidal enzymes against oxidative damage, as the enzymatic activity of proteases is diminished by HOCl\textsuperscript{263}.

Studies that will be described in this thesis have been conducted in an attempt to further understand how effective the O\textsubscript{2} metabolites (O\textsubscript{2}\textsuperscript{-} and H\textsubscript{2}O\textsubscript{2}) and products of chloride oxidation (HOCl) are, in killing bacteria under conditions prevailing within the vacuole. Particular attention will be devoted to pH and granule protein concentrations.

1.17 OXYGEN INDEPENDENT MICROBIAL KILLING.

The presence of an oxygen-independent microbial mechanism in neutrophils is demonstrated by the ability of these cells to kill certain bacteria under anaerobic conditions\textsuperscript{111}. In 1956 Hirsch\textsuperscript{264} observed that neutrophil lysates killed bacteria and that this effect could be attributed to material that he called phagocytin. The component responsible for bacterial killing was localized to the cytoplasmic granules which are released into the phagocytic vacuole\textsuperscript{239}. Neutrophil-derived microbicidal molecules are packaged within the granules that are released upon cell activation\textsuperscript{265}(Figure 1.6).
Figure 1.6. Electron micrograph of a neutrophil vacuole following phagocytosis of *Staphylococcus aureus* (approximate diameter of the bacterium 0.8μM). The image captures the moment of degranulation. During this process the granule membranes incorporate into the membrane of the phagosome, and the granules are discharged into the vacuole.
1.17.1 Four granule populations.

Granule biogenesis follows the granulocyte differentiation pathway. The azurophilic granules first emerge at the stage of promyelocytes\(^\text{266}\) and contain MPO, serine proteases, and antibiotic proteins, (Table 1.0). Azurophilic granules are thus considered as the true microbicidal compartment mobilized upon phagocytosis.

Later in differentiation, at the metamyelocyte stage, specific granules containing lactoferrin and collagenase emerge, followed by a third population termed the gelatinase granules. A forth type of granule, called the secretory vesicles, appears at the stage of mature neutrophil. Their origin might be endocytic, because they contain plasma proteins such as albumin. However, recent findings have shown that proteinase 3, a serine proteinase described in azurophilic granules is also present in secretory vesicles\(^\text{267}\).

1.17.2 Degranulation.

Disappearance of the cytoplasmic granules following ingestion of bacteria was first observed by Robineaux and Frederic in 1955\(^\text{268}\), by phase contrast motion pictures of guinea pig leukocytes. With the use of chicken leukocytes, with their large-dense granules, Hirsch observed by phase contrast microscopy, degranulation and release of the granule contents directly into the phagocytic vacuole, by fusion of the granule membrane with the invaginated cell membrane\(^\text{269}\).

The mechanism underlying the degranulation of the four morphologically distinct populations of granules maybe under separate control. The order of exocytosis observed after ionophore-induced progressive elevation of cytosolic
### TABLE 1.0. Contents of neutrophil granules.
The most abundant organelles within the cytoplasm are the granules, which are membrane-bound organelles containing an array of antimicrobial proteins. Three major types have been identified, azurophilic, specific and gelatinase-containing granules. When granules fuse with the invaginating membrane, or the formed vacuole, they deliver a potent mixture of enzymes, including neutral degradative proteases into the vacuolar space around the microbe. In resting cells, approximately 20% flavocytochrome b\textsubscript{558} is found in the plasma membrane pool, and 80% in the specific granules.

<table>
<thead>
<tr>
<th>Azurophilic granules</th>
<th>Specific granules</th>
<th>Gelatinase-granules</th>
<th>Secretory vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>(primary)</td>
<td>(secondary)</td>
<td>(peroxidase positive)</td>
<td>(peroxidase negative)</td>
</tr>
<tr>
<td>Elastase</td>
<td>Lactoferrin</td>
<td>Gelatinase</td>
<td>Proteinase 3</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>Collagenase</td>
<td>Collagenase</td>
<td></td>
</tr>
<tr>
<td>Proteinase 3</td>
<td>Lysozyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azurocidin</td>
<td>Flavocytochrome b\textsubscript{558}</td>
<td></td>
<td>(membrane spanning)</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Neutral proteases**

**Acid hydrolases**
- β-glucuronidase
- α-mannosidase
- α-fucosidase

**Cationic proteins**
- Defensins
- BPI
- Lysozyme
- MPO
calcium was secretory vesicles, gelatinase granules, specific granules, and lastly azurophilic granules.\textsuperscript{270}

1.17.3 Granule defects.

Two inherited defects affect neutrophil granule structure\textsuperscript{271}. The first is the specific granule deficiency, which is marked by frequent and severe bacterial infections. Neutrophils are characterised by a lack of specific granules and defensins, and impaired bactericidal activity. The second inherited granule deficiency is Chediak-Higashi syndrome, which is a rare autosomal recessive disorder associated with a prominent defect in cellular function and formation of neutrophil granules, resulting in increased susceptibility to infection\textsuperscript{271,272}.

1.18 GRANULE STRUCTURE.

The structure of the neutrophil granule is pivotal in its function of packaging and storage of microbicidal molecules. The composition of granules has been biochemically and cytochemically identified to contain sulphated glycosaminoglycans (GAGs), including chondroitin (Figure 1.7) and heparin sulphate\textsuperscript{273-275}. Ultrastructurally, granule sulphate and anionic complex carbohydrates can be stained in immature granules\textsuperscript{274,275} and radiosulphate is incorporated into immature azurophilic granules and appears to persist to maturity of the cell.

In light microscope studies promyelocyte azurophilic granules lose affinity for cationic dyes with cytoplasmic maturation, which could result from the complexing of GAGs with other cationic granule components. Parmley, (1986) showed that the sulphate groups can be re-exposed for binding to cationic dyes.
FIGURE 1.7. Structural formula of the repeating disaccharide unit of the glycosaminoglycan chondroitin 6-sulphate, with illustrated negative charged groups. An interaction between chondroitin and the cationic granule enzymes, regulates their activity and packages them in an inactive form. (Biochemistry, 3rd ed. By L. Stryer. W.H. Freeman & Company/New York. 1975)
in degranulating primary granules\textsuperscript{276}, following release of their microbicidal proteins. Finally the use of cationic colloidal gold staining, revealed the presence of GAGs in both azurophil and specific granules\textsuperscript{277}.

It was proposed that GAGs may interact with granule enzymes and regulate their activities, packaging them in an inactive form adsorbed to acidic residues\textsuperscript{278}. Of interest is the virulence mechanism described by Schmidtchen (2001), whereby \textit{Pseudomonas aeruginosa}, \textit{Enterococcus faecalis} and \textit{Streptococcus pyogenes} released host dermatan sulphate, which bound neutrophil-derived $\alpha$-defensin neutralizing its bactericidal activity\textsuperscript{279}.

\section*{1.19 GRANULE MICROBIAL MOLECULES.}

What follows, is a description of the antimicrobial granule proteins including some proteases, hydrolytic enzymes and cationic proteins (Table 1.0). More detail is given to those, which relate directly to the work outlined within this thesis.

\subsection*{1.19.1 Serine proteases.}

Serine proteinases have a catalytically essential serine residue at their active site. Their activity depends on a catalytic triad consisting of residues Asp\textsuperscript{102}, His\textsuperscript{57}, and Ser\textsuperscript{195}, which are widely separated in the primary sequence but are brought together at the active site of the enzyme in their tertiary structure\textsuperscript{280}. Neutrophil-derived proteases are packed in azurophilic granules and include \textit{cathepsin G}, \textit{elastase}, \textit{proteinase 3} (PR3) and \textit{azurocidin}. Mg\textsuperscript{2+} and Ca\textsuperscript{2+} display a protective effect against the microbicidal activity of the proteases, indicating the operation of charge interactions between cationic protein and bacterial surface\textsuperscript{7}.  
Elastase and PR3 display very similar patterns of proteolytic activities\textsuperscript{281}. They are both capable of cleaving insoluble elastin and a variety of matrix proteins, including fibronectin, laminin and collagen\textsuperscript{282}, within a pH optimum of about 8.0\textsuperscript{283}.

In spite of their original role in host defence, elastase and PR3 have been implicated in pulmonary pathology including rheumatoid arthritis\textsuperscript{12}, cystic fibrosis\textsuperscript{284}, and emphysema\textsuperscript{285}, due to unrestrained proteolytic activity in the lung extracellular matrix. The main physiological defence against elastase and PR3 is plasma $\alpha_1$-antitrypsin. Emphysema may result from inactive $\alpha_1$-antitrypsin unable to inhibit neutrophil elastase in the lung\textsuperscript{286}, (Figure 1.8).

Elastase is a basic glycoprotein of molecular weight 29-31 kDa with at least three elastase isoenzymes differing in their carbohydrate content\textsuperscript{287}. It is highly cationic, with a strongly basic isoelectric point (pH 10-11). Elastase has long been regarded as an antibacterial protein and mice made homozygous for a disrupted neutrophil elastase gene have demonstrated that elastase is required for host defence against Gram-negative, but not Gram-positive, bacteria, as mice have diminished resistance to *Klebsiella pneumoniae* and *Escherichia coli* sepsis\textsuperscript{288}. In contrast, mice that were made deficient in the neutrophil protease cathepsin G were phenotypically normal when challenged with the same bacteria. In addition elastase was identified as the only granule component responsible for the killing of *Borrelia burgdorferi*\textsuperscript{289} in vitro.

One possible target for elastase is a bacterial outer-membrane protein OmpA\textsuperscript{290}. Elastase degradation of OmpA results in cell death and it has been postulated that death arises as a result of loss of bacterial integrity, or by localized weakening of the cell wall followed by osmotic lysis\textsuperscript{290}.
Release of proteases into the extracellular environment could give rise to considerable damage as the proteases digest the tissues into which they are released. This damage is prevented by \(\alpha_1\)-antiproteinase, a powerful protease inhibitor that is normally present in the plasma and extracellular fluids. As rapidly as the neutral proteases are released from the cells, they are neutralized by \(\alpha_1\)-antiproteinase, saving the tissues from damage.

\(\alpha_1\)-Antiproteinase deficiency is an inherited disorder caused by the abnormal processing of the \(\alpha_1\)-antiproteinase precursor as it is synthesized by the liver. Emphysema is a characteristic complication of \(\alpha_1\)-antiproteinase deficiency.

An acquired \(\alpha_1\)-antiproteinase deficiency appears to be induced in alveolar fluids by tobacco smoke. Alveolar phagocytes from smokers were found to manufacture oxidants at several times the rate seen with similar cells from non-smokers. In turn, the rate at which neutrophil elastase was inhibited by alveolar fluid was lower in fluids from smokers than in fluids from non-smokers.

Oxidants produced by activated neutrophils, oxidize a methionine on \(\alpha_1\)-antiproteinase, blocking its activity to neutralize elastase. The free elastase then over time destroys the tissue of the lung, leading to emphysema.
The amino acid composition and crystal structure of cathepsin G are known\(^{291,292}\) and it shares 37% sequence homology with elastase. The azurophil granules of human neutrophils contain several isozymes of cathepsin G, all of which have basic isoelectric points, and have a molecular weight of 25 to 29 kDa. Cathepsin G has been proposed to play a role in neutrophil responses against a variety of bacteria. Purified cathepsin G has been shown to inhibit the growth of several organisms, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Neisseria gonorrhoeae*.\(^{7,164,293}\) It also displays toxic properties against *Eimeria tenella* sporozoites\(^{294}\), *Capnocytophaga*\(^{295}\), and *Listeria monocytogenes*\(^{296}\). The enzyme activity of cathepsin G is not required for its antibacterial activities, in fact, 3 peptides derived from cathepsin G (aa 1-5, aa 77-83, and aa 117-136) have direct antimicrobial properties.

Contrary to *in vitro* results, it was surprising that neutrophils of cathepsin G deficient mice were able to clear *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* infections, as efficiently as wild-type animals\(^{297}\). This suggests that this enzyme is not necessary for the normal clearance of any of these bacterial species. Tkalecovic (2000) carried out further *in vivo* studies of mice deficient in cathepsin G, elastase or both. These experiments illustrated the importance of cathepsin G in the successful clearance of *Aspergillus fumigatus*. Wild type mice almost completely cleared the fungal pathogen, while the single mutants showed an “intermediate” phenotype between the wild type and double mutants\(^{298}\), thus establishing a critical role of both elastase and cathepsin G in the control of *Aspergillus fumigatus* infection *in vivo*. 
1.19.2 Metalloproteinases.

Neutrophils contain metalloproteinases such as, *collagenase* which is stored in the specific granules and specifically cleaves type I, II, and III collagen, whereas the 92kDa-*gelatinase*, degrades type IV and V collagens. Metalloproteinases are stored in a latent form within granules\(^2\). When neutrophils are stimulated, these latent enzymes must be activated before they can attack their substrate. The activation of collagenase was shown to be under oxidative regulation and coupled to the generation of HOCl\(^2\) and enhanced by cathepsin G activity\(^3\). Similarly, using gelatin as a substrate, gelatinase was shown to be activated by HOCl (0.5nmol), but the fact that CGD neutrophils were capable of activating greater than 30% of the total gelatinase of the cell, suggested a second regulatory factor\(^2\). This was confirmed by Shamamian (2001), by *in vitro* experiments which showed the involvement of serine proteases, cathepsin G, elastase and PR3\(^3\).

1.19.3 Granule proteins.

One of the most active participants in host defence against Gram-negative bacterial infections is *bactericidal/permeability-increasing protein* (BPI), a 50 kDa protein stored in azurophil granules\(^3\). The selective toxicity of BPI for Gram-negative bacteria relies on the binding capacity of its 21-25 kDa aminoterminal fragment to LPS\(^3\). Changes in BPI sensitivity have been shown to parallel differences in binding affinity of *Escherichia coli* for BPI, which closely correlate with changes in the chain length of LPS, as assessed by SDS-PAGE\(^3\). BPI is potently bactericidal at low nM concentrations both in artificial media and in biological fluids\(^3\).
Another important group of antimicrobial peptides is the group of beta-sheet defensins that comprise four members: HNP1-HNP4. Defensins are small (4 kDa) cationic, antibiotic peptides. The widespread occurrence of defensins in higher animals and more distant defensin relatives in plants and insects, is consistent with an early evolutionary origin.

*In vitro* studies reveal the microbicidal activity of defensins against a variety of bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, many fungi, and some viruses, by inducing microbial membrane permeabilization and lysis of the target cells. However, in physiologic and biologic fluids their activity towards most bacterial species is markedly reduced or even eliminated, thus questioning their effect within the phagosome. Various other extracellular activities of defensins have been described, including chemotactic activity for monocytes, lymphocytes and leukocytes.

Human lactoferrin is a major component of the specific granules and is active against a variety of pathogens. This protein binds to bacteria through its highly positively charged N-terminus and displays antimicrobial properties against Gram-positive and Gram-negative bacteria by limiting the availability of environmental iron. However, since iron-saturated lactoferrin is also able to kill certain bacteria, mechanisms other than iron depletion apparently are involved. Recent studies have indicated that peptides obtained after enzymatic hydrolysis of lactoferrin and bovine lactoferrin are much more effective in killing bacteria than is the intact protein. It is likely that the N-terminal cationic domain of human lactoferrin plays an essential role in the bactericidal activity.
and has been shown to be highly effective against infections with antibiotic-resistant Staphylococcus aureus, in mice with experimental infection\textsuperscript{317}.

The concept of degradative enzymes acting as independent antimicrobial agents was based on the discovery of lysozyme by Fleming in 1922\textsuperscript{318}. Lysozyme, a constituent of both azurophilic and specific granules, is a 14.4 kDa cationic protein with the ability to kill a wide range of Gram-positive bacterial species, by virtue of its ability to hydrolyze cell wall components. Bacterial cell wall consist in general of linear polysaccharide chains containing repeating units of N-acetylglucosamine and N-acetylmuramic acid residues in $\beta$-1-4 linkage\textsuperscript{319}. Lysozyme exerts its effects by cleavage of the $\beta$-1-4 glycoside bond between the two acid residues. Access to its substrate contributes to the toxicity of lysozyme, and because an outer membrane protects this peptidoglycan structure in Gram-negative bacteria, these organisms are not very susceptible to the lytic effects. Furthermore lysozyme is absent from bovine leukocytes\textsuperscript{320}.

\subsection*{1.20 AN OUTLINE OF IDEAS AND RESULTS ACHIEVED IN THIS THESIS.}

\textit{In vivo} murine infection studies were carried out on CGD mice, deficient in the p47$^{\text{phox}}$ cytosolic component of the NADPH oxidase and mice deficient in the two serine proteases, elastase and cathepsin G. Results revealed that both the generation of reactive oxygen species and activation of granule proteases are essential for bacterial killing, whereas neither is sufficient on its own. Therefore experiments were set out to determine the mechanism of their interaction.

Examination of electron micrographs showed differences in the morphology of the phagocytic vacuoles in the presence and absence of the
respiratory burst. In normal cells the granule contents were generally dispersed throughout the cavity of the vacuole, and that the vacuole subsequently swelled to 2-3 times its original size. In the absence of the oxidase the granule contents remain in discrete aggregates and the vacuoles do not swell.

Investigation into these differences discovered it to be due to the association of the oxidase activity with the pumping of large amounts of K⁺ into the vacuole (Figure 1.9). An estimate of approximately 4 moles/l of superoxide is generated within the vacuole. Compensation by H⁺ alone would not alter the pH in the vacuole, which has been shown to be significantly elevated by the oxidase, whereas K⁺ allows the O₂⁻ and O₂⁻ to be protonated by H⁺ from within the vacuolar cavity, causing the pH to rise.

A further study made a correlation between oxidase activity and activity of the K⁺ channel. This was confirmed using valinomycin, a specific K⁺ ionophore and 4-aminopyridine, a non specific K⁺ channel blocker. Measurements of K⁺ were made using a K⁺ sensitive electrode, more quantitatively using ⁸⁶⁶Rb⁺ and directly estimated using X-ray microanalysis. Results showed that approximately 5% of the charge is compensated by K⁺.

Charge compensation must be synchronous with O₂⁻ generation and therefore the K⁺ must enter the vacuole early after phagocytosis. There is considerably delay between the end of the respiratory burst and vacuolar swelling. This delayed swelling was found to be due to cytoskeletal constraints, as determined by confocal microscopy. Consequently there is a stage at which the K⁺ is hypertonic. Measurements of 270mM K⁺ were made using X-ray microanalysis and added to the 150mM NaCl of biological fluids, a hypertonic vacuolar environment is established.
FIGURE 1.9. The primary role of $O_2^-$ appears to be indirect, by displacing the proteolytic enzymes released into the vacuole from the cytoplasmic granules. To compensate for the movement of charge incurred in making $O_2^-$, $H^+$ and $K^+$ cross the membrane into the vacuole. The massive rise in ionic strength within the vacuole engenders the release of cationic granule proteins, including elastase and cathepsin G, from the anionic sulphated proteoglycan matrix and allows them to participate in the killing process.
The cationic enzymes are tightly bound to strongly negatively charged sulphated proteoglycans within the granules\(^{321}\). In order to activate the enzymes they must be released from the matrix, and this is achieved by the ionic strength generated by the K\(^+\) that is driven into the vacuole to compensate the charge. Killing of *Staphylococcus aureus* proved the solubilized granule proteins to be microbicidal.

Another consequence of partial compensation of the charge by K\(^+\) is that the pH is elevated, sufficient to overcome the considerable buffering capacity of the granules, to the optimal pH for the neutral granule proteases.

Having defined conditions within the vacuole, including concentrations of protein, experiments were designed to test the effects of reactive oxygen species, and granule proteins separately and together on microbial viability. Studies showed that high concentrations of H\(_2\)O\(_2\) or HOCl kill bacteria when incubated on their own but that the addition of granule proteins completely abrogated this microbicidal activity. The possibility that MPO might protect the microbicidal degradative enzymes against oxidative damage by breaking H\(_2\)O\(_2\) down through its catalase activity\(^{255}\) is proposed.

1.21 TESTS OF NEUTROPHIL FUNCTION.

Whilst studying neutrophil function a number of techniques are routinely used and are summarized in this short section. Tests of neutrophil function are generally within the field of research interest of the individual investigator. Most techniques require the purification of neutrophils from whole blood and take into account that they have a limited *in vitro* life.
Both light and electron microscopy can study neutrophil morphology. The movement of these cells on glass slides can be directly observed by placing a preparation of adherent cells on a warm stage of the microscope. They will be seen to orientate along and crawl in the direction of a concentration gradient of a chemotactic stimulus\(^{322}\). In assays of phagocytosis\(^{323}\), the number of cell-associated larger particles can be measured by direct microscopic counting or by the use of radiolabelled bacteria\(^{161}\) or those that contain a dye that can be measured spectrophotometrically. The difficulty with these techniques is in distinguishing phagocytosed from adherent particles. This can be done directly by electron microscopy or by using a particle, which can be differently manipulated if it is exposed outside the cell or protected within it. For example, the enzyme lyststaphin\(^{161}\), when added to a mixture of cells and staphylococci, will lyse those bacteria outside the cell, similarly the dye trypan blue will differentially quench fluorescence of external bacteria but not those that are protected within the cell\(^{234}\).

The granule contents can be measured by assaying the activity of the individual proteins in an homogenate of the cells, or in the granules which have been separated by centrifugation techniques\(^{324}\). Degranulation can be assayed by the technique of "frustrated phagocytosis"\(^{325}\) whereby the cells are exposed to objects that are too large or numerous to be engulfed, under which circumstances the vacuole is unable to close and the degranulated material is released into the medium. A more direct method of examining degranulation is to isolate the phagocytic vacuoles and measure their contents\(^{326}\). The cells are disrupted after they have been allowed to phagocytose, the phagocytic vacuoles can then be
separated from the rest of the cellular components by flotation and the granule contents within the phagocytic vacuoles can then be separately assayed.

Microbial **killing** is determined by mixing isolated leukocytes with live microbes and then determining the rate at which the microbes are killed^{18}. Killing is generally assayed by culturing the organisms. In these experiments it is best to select particular organisms to which the immunodeficient patients are unusually susceptible^{161,327}. **Digestion** is best assayed by presenting the cell with a radiolabelled particle and measuring the subsequent release of soluble radioactivity^{161,327}. 
CHAPTER 2.
MATERIALS AND METHODS.

2.1 CHEMICALS.

All chemicals and reagents were of the highest purity and were purchased from Sigma Aldrich, unless stated otherwise. Radiochemicals, chromatography resins and molecular weight markers were from Amersham Pharmacia Biotech.

2.1a Concentrations and storage conditions of frequently used chemicals.

Protease Inhibitors, (PI)

♦ 1 mM di-isopropyl fluorophosphate (DIFP)*, (stock: -20°C at 1M in isopropanol). Molecular formula C₆H₁₄FOP.
♦ 10μg/ml N-alpha-p-tosyl-L-lysine chloro methyl ketone (TLCK), (stock: -20°C at 10mg/ml in DMSO). Molecular formula C₁₄H₂₁ClN₂O₅S.HCl
♦ 10μg/ml pepstatin A, (stock: -20°C at 10mg/ml in ethanol).
Molecular formula C₃₄H₆₃N₅O₉.
♦ 10μg/ml leupeptin, (stock: -20°C at 10mg/ml in H₂O₂).
Molecular formula C₂₀H₃₈N₆O₄.HCl
♦ 10μg/ml aprotinin, (stock: -20°C at 10mg/ml in H₂O₂).

* DIFP, was added to concentrated cell pellets or slurries, as it is subject to hydrolysis in aqueous solutions.

K⁺ ionophore and channel blocker.

♦ 3μM valinomycin (Val), (stock: -20°C at 3mM in DMSO).
Molecular formula C₅₄H₉₀N₆O₁₈.
♦ 4mM 4-aminopyridine (4-AP), (stock: -20°C at 1M in DMSO).
Molecular formula C₅H₆N₂.
NADPH oxidase activator and inhibitor.
♦ 1µg or 10ng/ml 12-phorbol myristate 13-acetate (PMA), (stock: -20°C at 1M in DMSO). Molecular formula C_{36}H_{56}O_{8}.
♦ 5mM diphenylene iodonium (DPI), (freshly prepared at 5mM in DMSO). Molecular formula C_{6}H_{14}FOP.

2.2 PROTEIN ELECTROPHORESIS AND MASS SPECTROMETRY.

2.2a Protein gel electrophoresis and western blotting.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by standard Laemmli methods\(^\text{328}\), using 30% (w/v) acrylamide/ 0.8% (w/v) bisacrylamide solution (Protogel, National Diagnostics) for a final concentration of 10.0-12.5% acrylamide, depending on protein size. In all cases, a stacking gel containing 2.5% polyacrylamide and running buffer (200mM glycine, 0.1% SDS, 25mM Tris/HCL; pH 8.9) were used. Samples were prepared for electrophoresis by adding 1/10 volume 10x SDS-PAGE sample buffer (20% SDS, 1M DL-Dithiothreitol (DTT), 10mM ethylenediaminetetraacetic acid (EDTA), 0.04% bromophenyl blue, 10% (w/v) sucrose, 625mM Tris/HCL; pH 6.7) and boiled for three minutes.

Hoefer Mighty Small II SE 250 (for mini gels 10x8 cm), or SE 600 (midi gels 18x16 cm) apparatus was used for electrophoresis, operated at 30mA per gel. Gels were stained with Coomassie R-250 stain. Gels were either dried onto chromatography paper (Whatman) or cellophane (Biorad) using a Biorad 543 gel dryer.

For western blotting, samples were first run on 12.5% SDS-PAGE minigels and transferred to nitrocellulose membrane (Anderman) with a semidry
blotter (Pharmacia LKB 2117 Multiphor 11) in transfer buffer (200mM glycine, 0.1% SDS (w/v), 10% methanol (v/v), 25mM Tris/HCL, pH 8.9) for 1 hour at 1.4mA/cm². Membrane blots were rinsed with distilled water and protein molecular weight markers visualized by reversible staining with Ponceau Red. The nitrocellulose membranes were blocked with 3% fat free milk powder (w/v) and 1% bovine serum albumin (w/v) in Tris buffered saline (TBS, 200mM NaCl, 50mM Tris/HCL; pH 7.4) with 0.05% Tween 20 (w/v), for 1 hour. Blots were probed with primary antibodies overnight. And then rinsed with TBS/0.05% (v/v) Tween 20. After washing horse radish peroxidase (HRP)- conjugated species-specific antibodies against IgG were incubated as per manufacturer's instructions (Amersham Pharmacia Biotech). Blots were developed using an enhanced chemiluminescence method (ECL, Amersham Pharmacia Biotech), and the autoradiograph processed by a Velopex MD2000 automated developer (Medivance).

2.2b Antibodies.

The antibodies used for western blotting were raised against purified proteins of neutrophil elastase, cathepsin G, myeloperoxidase, lactoferrin and lysozyme using standard protocols329. Briefly purified protein antigens (100µg) were mixed to form an emulsion, with an equal volume of Freund's complete adjuvant oil and injected subcutaneously at four separate sites in New Zealand white rabbits. Four weeks later the animals were injected in a similar fashion, replacing the Freund's complete adjuvant with incomplete adjuvant. Every two weeks the animals were reimmunized and a 10ml bleed obtained. The clotted blood was centrifuged (200g/20min) and the serum removed. Stocks of serum
were stored at -80°C. Antibodies were affinity purified on the purified proteins coupled to N-hydroxysuccinimide-activated Sepharose (Hitrap column, Amersham Pharmacia Biotech)\(^7\).

All purified antiserum recognized the appropriate protein in whole cell lysate, but were used at various dilutions for western blotting. Antiserum to myeloperoxidase, lysozyme and lactoferrin was used at 1/2000 and antiserum to elastase and cathepsin G at 1/500.

Antibodies to paxillin and vinculin were purchased from Santa Cruz. Polyclonal antiserum specific for \textit{S. aureus} catalase was supplied by De La Fuente\(^3\).

\subsection{2.2c Two-dimensional electrophoresis of proteins using immobilized pH gradients.}

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), is a method in which proteins are separated according to charge (pI) by isoelectric focusing (IEF) in the first dimension and according to size (Mr) by SDS-PAGE in the second dimension. Immobilized pH gradient (IPGs) strips (Amersham Pharmacia Biotech), 3mm wide and cast on GelBond PAG film, having a pH gradient from 3-10 were stored at -20°C, dehydrated.

Samples were prepared by precipitating the protein with 10% Trichloroacetic acid (TCA) and centrifuged (15,000g, 10min, 4°C). The pellet was washed four times with ice cold 80% acetone (readjustment of pH) and air-dried. The pellet was then resuspended in 250μl of IEF sample buffer (8M Urea, 2M Thiourea, 4% CHAPS, 1% Triton X-100, 65 mM DTT, 10mM Tris base, 0.8% Ampholyte), sonicated briefly (10 pulses, 10 sec) using a MSE Soniprep
150 sonicator and centrifuged (10,000g/10min/4°C). IEF was preformed in an IPGphor within which in-gel rehydration and IEF were performed in one step overnight. The IPGphor included a temperature control (samples run at 16°C) and an inbuilt programmable power supply. Prepared samples (0.5mg/250μl) were pipetted on top of IPG strips, within aluminum oxide ceramic strip holder and overlaid with mineral oil (500μl). The IPGphor running conditions are outlined below:

<table>
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<tr>
<th>Step</th>
<th>Voltage</th>
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<tbody>
<tr>
<td>Step 1</td>
<td>30</td>
<td>360</td>
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<tr>
<td>Step 2</td>
<td>300</td>
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<td>Step 3</td>
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<td>Step 4</td>
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<tr>
<td>Step 5</td>
<td>8000</td>
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<tr>
<td>Step 6</td>
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After IEF, those IPG gel strips, which were not used immediately for second dimension, were stored between two sheets of plastic film at −80°C. Those strips intended for second dimension were equilibrated twice, each 15 min in equilibration buffer (30% v/v glycerol, 2% SDS w/v, 6M Urea, 50mM Tris-HCl; pH 6.8, stored in 10ml aliquot’s at −20°C). The first equilibration step was carried out, in 10ml equilibration buffer containing 2% DTT and the second, containing 4.5% iodoacetamide. The equilibrated IPG gel strips were blotted to remove excess equilibration buffer and then applied onto the second dimension 12.4 % SDS gel.
2.2d Mass spectrometry – sample preparation.

The protein bands/spots of interest were excised from the SDS gel and exposed to in-gel digestion according to the protocol described by Rosenfeld et al., 1992. In brief, the gel piece was destained overnight with continuous shaking in 40% ethanol, freshly prepared in 50mM NH$_4$HCO$_3$. The pH of the gel piece was re-equilibrated with 25mM NH$_4$HCO$_3$ and then washed three times with 25µl acetonitrile and dried. 5µl of freshly prepared trypsin (75ng/µl in 25mM NH$_4$HCO$_3$) was added to the gel piece and incubated overnight at 30°C. For acquisition of mass-spectrometric peptide maps of the proteins, 0.5µl aliquots of the generated trypsin cleavage products were dispensed onto a sample support and overlaid with 0.5µl of matrix (α-cyano-4-hydroxy-cinnamic acid dissolved in 35% acetonitrile, 0.1% trifluoroacetic acid).

2.2e MALDI-TOF mass spectrometry.

The following peptides were used as external standards for MALDI spectra calibration: human angiotensin I and II, ACTH (clip 18-39), [glu]-fibrinopeptide B, renine substrate tetradecapeptide and insulin B chain. The amount of each peptide was 25 pmol per spot.

MALDI-TOF mass spectra of the peptides were obtained using a Biflex III mass spectrometer (Bruker, Breman, Germany). All spectra were acquired in a positive-ion reflector. Typically 200 shots were recorded. Proteins were identified by comparing mass fingerprints to NCBI's database using Matrix Science searching machine (http://www.matrixscience.com/).
2.3 PREPARATION OF NEUTROPHILS.

2.3a Purification from fresh blood, buffy coat or buffy coat residues.

Venous blood was obtained from healthy volunteers and collected in heparin (5 units/ml) containing tubes. Neutrophils were isolated as described by Segal and Jones, (1980). In short, 10% dextran (mean MW, 260 kDa) dissolved in single strength saline (0.9% NaCl, 5 units/ml heparin) was added to the blood at a final concentration of 1%. The mixture was mixed and allowed to settle at room temperature to sediment erythrocytes. The top layer containing both leukocytes and red blood cells was taken, underlayed with Lymphoprep (Nycomed), and centrifuged (200g/10min/RT) in a MSE Mistral 3000i to remove lymphocytes and monocytes. The pellet was resuspended in distilled water for 10-20 seconds for hypotonic red cell lysis, followed by an equal volume of double strength saline (1.8% NaCl, 10 units heparin/ml), restoring osmolarity. This was then centrifuged (150g/5min/RT) to pellet neutrophils.

When working with buffy coats or buffy coat residues, the blood was first diluted with 1/3 volume, single strength saline.

2.3b Purification from chronic myeloid leukemia source.

Chronic myeloid leukemia (CML) is a condition in which there is a massive proliferation of myeloid cells. Patients can be temporarily treated by leukopheresis of the buffy coat layer of their blood. When available, the leukopheresed cells are a rich source of neutrophils. CML buffy coats were diluted with ammonium chloride (0.15M NH₄Cl with 5 units heparin/ml) to lyse red blood cells. Neutrophils were collected by centrifugation (200g/15min/RT). The procedure was repeated to lyse residual red cells.
2.3c Temporary storage of neutrophils.

If not used immediately, neutrophils were resuspended in phosphate buffered saline (PBS; 140mM NaCl, 10mM KCl, 10mM NaH₂PO₄; pH 7.2, with NaOH) with 5mM glucose and stored for no longer than 12 hr at 4°C. Before use, neutrophils were centrifuged (200g/15min/RT) and resuspended in the appropriate buffer.

2.3d Neutrophil function - measurement of oxygen consumption and superoxide generation.

Production of superoxide by 1x10⁶ neutrophils after stimulation with PMA (10ng/ml or 1μg/ml) was measured by superoxide dismutase (SOD) inhibitable reduction of cytochrome C⁹. Experiments were repeated in the presence of valinomycin (3μM) or 4-Aminopyridine (4mM).

Oxygen consumption was measured in a Clark-type oxygen electrode (Rank Brothers, Bottisham). The cells (1x10⁷) were incubated in a rapidly stirred, thermostatically controlled (37°C) chamber above the electrode and were stimulated by the addition of *S. aureus* opsonized with IgG³²³.

2.3e Iodination studies.

Iodination studies were performed using the method described by Klebanoff & Clark, (1976)²¹⁹, with a number of small changes. Neutrophils (1x10⁶) were resuspended in Hepes Buffer (Gibco) supplemented with 50nM KI, 10μCi ¹²⁵I and 5% human serum. In experiments carried out on neutrophils isolated from mice, serum was obtained from mice preimmunized with *S. aureus* (100μg
protein) three weeks before. The cell mixture was placed in a magnetically stirring oxygenated, 37°C chamber and *S. aureus* added at a ratio of 10:1, 50:1 or *E. coli* at 100:1. Aliquots were removed periodically and placed in ice cold PBS plus 10% trichloroacetic acid (TCA). Pellets were washed (x6) in the same buffer and counted for radioactivity in a liquid-scintillation gamma counter. Experiments were repeated in the presence of valinomycin (3μM) or the absence of serum as a control.

2.3f Preparation of neutrophil lysates.

The method of cell lysis was either by sonication or nitrogen cavitation. To the pellet of neutrophils, 1mM DIFP, was added, whirly mixed and left on ice for 10min. Cells were then resuspended in Break Buffer (10mM KCl, 3mM NaCl, 4mM MgCl₂, 10mM Pipes; pH 7.2), containing protease inhibitors (10μg/ml of the following, leupeptin, TLCK, pepstatin A and aprotinin). Lysis by sonication, (3x5 second bursts), was performed on ice. Alternatively the cells were transferred to a cavitation chamber and brought up to 400 psi with nitrogen gas for 20min, allowing neutrophils to equilibrate with this pressure. When the outlet was opened, a slow stream of neutrophils, with a high internal pressure, is exposed to atmospheric pressure (approximately 15 psi) and rupture. Phagocytic vacuoles and nuclei are left intact and cytoskeletal components are not subjected to shearing. A post nuclear supernatant (PNS) was prepared by centrifugation (400g/15min/4°C), and the pellet (unbroken cells and nuclei) discarded. The PNS was stored by freezing at -80°C.
2.4 MYELOPEROXIDASE.

2.4a Purification of myeloperoxidase from azurophilic granules.

Human neutrophil lysates were obtained from $10^{10}$ neutrophils. The PNS was layered on top of a discontinuous sucrose gradient of 30%, 43% and 55% (w/w) and centrifuged (1 hr/100,000g/4°C) in a Beckman SW41 head. The primary azurophilic granules passed through the 43% sucrose and were isolated from the top of the 55% sucrose. The secondary, specific granules were collected from the surface of the 43% and any residual cytosolic and membrane proteins floated on top of the 30% layer. The azurophilic granules were removed from the interface and homogenized in 120mls of Break Buffer (2.3f) containing 0.75% Cetyltrimethylammonium bromide (CETAB; BDH) and centrifuged (30,000g/10min/4°C). To the supernatant, 311mg/ml or 50% final, ammonium sulphate was added. The resulting precipitate was removed after centrifugation (30,000g/10min/4°C) and the pellet discarded. To the supernatant, 100mg/ml ammonium sulphate was added, at which concentration the MPO came out of solution and was pelleted by centrifugation (30,000g/10min/4°C). The pellet was resolubilized in 50mM Phosphate buffer pH 7.2.

The MPO was purified by ion exchange chromatography, at 4°C. The protein was loaded on Fast Flow S-Sepharose (1.5cm x 10cm column, flow rate of 1ml/min, 1ml fractions collected, eluted with a 100ml linear gradient of 50-500mM Phosphate Buffer; pH 7.2). Peak fractions were pooled together, the ionic concentration diluted to 50mM and the pH adjusted to pH6.5. The sample was then reloaded onto a Mono S column, (1ml column, flow rate of 0.5ml/min, eluted with a linear gradient of 50 to 500mM phosphate buffer; pH 6.5 and 1ml
fractions collected). The peak fractions of MPO were pooled and stored at 
80°C.

2.4b Myeloperoxidase Assay.

The assay employed for the detection of MPO is as described in Immunochemical techniques, (1986). Briefly increasing concentrations of sample containing MPO were incubated at 37°C for 5min in 0.5ml substrate buffer, (100mM Citrate buffer pH 5.5, 3μM O-dianisidine dissolved in distilled water, 10μM H2O2 and 0.05% Triton X-100 (w/v). To stop the reaction 0.5ml Stopping mixture was added (35% perchloric acid (w/v), 20% DMSO (w/v)) and the sample read at 560nm using a Unikon 860 spectrophotometer (Kontron Instruments). Horse radish peroxidase (0-20ng) was used as a standard.

2.4c Killing by, and oxidative capacity of, purified myeloperoxidase.

An enzyme preparation of MPO with a purity index (A430/A280) of 0.82 was used in this study. Hydrogen peroxide solutions were prepared daily by diluting a 30% stock solution and the concentration determined by measuring its absorbance at 240nm (ε240 43.6M⁻¹ cm⁻¹). Bacteria (2x10⁷) from an overnight culture, were washed twice in PBS and resuspended in PBS of pH 7.5, 6.5 or 5.5 with purified MPO (5mg/ml). Increasing concentrations of H2O2 (0.1, 1.0, 10 or 100mM) was added and incubated at 37°C, for times of 0,1,2,4,8 & 16min. Aliquots were removed at each time point and diluted 1/10 in ice cold LB. Results were calculated as the mean (±S.E.) from at least three experiments. Colony counts were performed in triplicate or quadruplicate for each sample and expressed as a percentage of the
original viability, as determined by control assays performed in buffer alone. The pH remained stable during assays to within 0.15 pH units of the starting pH.

The oxidative capacity (COX) of purified MPO was determined by the addition of potassium iodide in molar excess. The product triiodide (>N-Cl + 3I⁻ + H⁺ ↔ I₃⁻ + >N-H) was measured by its absorbance at 350nm which is the maximum wavelength (λ_max) of I₃⁻ (ε=22,900/mol/cm)^205. The concentration of oxidants was calculated by absorption / ε according to the formula A = c x ε x d (A.. absorption, c... concentration in M/l, d... thickness of the cuvette in cm)^201.

2.5 CATIONIC PROTEINS.

2.5a Purification of cationic granule proteins, lactoferrin, cathepsin G, elastase & lysozyme.

Throughout the purification process, for rapid desalting or buffer exchange, prepacked NAP-5 or PD-10 columns were used according to the manufacturers description (Amersham Pharmacia Biotech).

Granules were isolated as described above (2.4a). A 25ml mixed granule pellet was homogenized in Break Buffer (2.3f) containing protease inhibitors (1μg/ml of the following; leupeptin, TLCK, aprotinin and pepstatin A) and 1% CETAB. The homogenate was centrifuged (30,000g/10min/4°C). The remaining pellet was re-extracted and the supernatants from both extractions pooled. The resulting protein was loaded on Fast Flow S-Sepharose in 10mM Phosphate buffer pH 6.95, (1.5cm x 20cm column, 2ml/min, 4ml fractions collected, eluted with a linear gradient from 10mM-1M NaCl). For the isolation of lysozyme, fractions 16-18 were pooled and rerun on Mono S. Buffers were the same, as for the Fast Flow S-Sepharose (1ml column, 0.5ml/min, 10mM-0.5M NaCl over 50
fractions, 0.5ml fractions collected). Fractions 28-30 from the Fast Flow S-Sepharose were pooled together and rerun on Mono S for the purification of cathepsin G and lactoferrin (1ml column, 10mM Phosphate Buffer pH 7.5, eluted from 10mM-1M NaCl over 100 fractions, 1ml fractions collected). Fractions 22-24 from the Fast Flow S-Sepharose, were pooled and reloaded on Heparin Agarose (10ml column, 10mM Phosphate Buffer pH 6.9, 1ml/min, 1ml fractions collected) for the isolation of elastase. From the Heparin agarose, fractions 43-46 were pooled and loaded onto Gel Filtration Superdex 75 (3ml column, buffer as for Heparin Agarose with 500mM NaCl, 50μl/min, 80μl fractions collected). All proteins were at least 95% pure and stored at -80°C in 10% glycerol.

2.5b Biocore studies.

All proteins, lactoferrin, MPO, cathepsin G, elastase and lysozyme were confirmed by MALDI. Protein binding to heparin was measured using surface plasmon resonance (Biacore) as described by Sadir et al., 2001. 

2.5c Enzyme activity.

pH-dependence of the activities of elastase and cathepsin G (both from Sigma) were measured as described by Barrett (1981). For cathepsin G the principle substrate was nitroanilide (Suc-Ala-Ala-Pro-Phe-NPhNO2, dissolved at 20mM in DMSO and stored at 4°C) and reaction buffer 0.10M N-(2-hydroxyethyl)piperazine-N’-(2-ethane-sulphonic acid (HEPES) at the appropriate pH. The product 4-nitroaniline was quantifiable at A410. For quantification of elastase activity the stock substrate MeOSuc-Ala-Ala-Pro-Val-NMec (1mM in DMSO
and stored at 4°C) was diluted to 20μM in 0.2M Tris-HCl at the appropriate pH. The product was quantified by excitation at 370nm with monitoring of emission at 460nm.

2.6 GRANULES AND THE PHAGOCYTIC VACUOLE.

2.6a Preparation of demembraned granules.

Granules were purified as described (2.4a) and the method of Percoll granule disruption is as published by Vita et al., (1997){superscript}335. In brief, the azurophilic and secretory granules were pooled together. The sucrose surrounding the granules was diluted to 9% (w/w) and mixed at a ratio of 6:4 with Percoll (1.130g/ml, Amersham Pharmacia Biotech). The granules were then centrifuged (20,000g/45min/4°C) in a swing out Beckman SW41 head. The demembranated granules appeared as a loose band and were withdrawn from above the Percoll pellet, whereas the granule membranes were evident as a white band, high up the Percoll gradient.

2.6b Isolation of phagocytic vacuoles.

The major steps in the experimental procedure are outlined by Segal et al., (1980){superscript}326. In short, a neutrophil suspension (2ml containing 2x10^8 cells) was rapidly stirred in an oxygen electrode (Rank Brothers LTD), thermostatically controlled chamber. Latex particles, (0.81μM in diameter, Difco laboratories) were precoated with human IgG by resuspending them in 1.0ml of 100mM Tris, pH 8.5 together with 1.0 ml of human IgG (250mg/ml, Leo Laboratories). The mixture was incubated at 37°C for 45min and then centrifuged (8000g/2min). The supernatant was discarded and the latex particles were washed by
resuspending in a similar volume of PBS, centrifuged under the same conditions and finally, resuspended in PBS at a final concentration of $1 \times 10^{11}$ particles/ml. To the neutrophils, $2 \times 10^{10}$ particles were added in a rapidly stirring $37^\circ C$, temperature controlled chamber. The experiment was also carried out in the presence of $5 \mu M$ DPI or under anaerobic conditions. In the latter experiments the oxygen was depleted from the system by bubbling a constant flow of nitrogen over the cells. Phagocytosis was stopped after 4 min, in ice cold PBS. The cells were centrifuged ($75g/10\text{min}/4^\circ C$) and the pellet resuspended in 11.2% (w/w) sucrose and recentrifuged as before. All sucrose solutions contained 1mM ethylenediamine tetra-acetic acid (EDTA), pH 7.4 and 5 units/ml heparin. The pellet was homogenized in 5ml of 11.2% sucrose in a 7ml Dounce homogenizer with 100 strokes of a tight fitting (B) pestle. This homogenate was then mixed with 12ml of 60% sucrose and placed at the base of a 50ml Sorvall centrifuge tube (DuPont instruments-Sorvall). Overlaid with 16ml of 33% sucrose and then 5ml of 11.2% sucrose and centrifuged ($20,000g/20\text{min}/4^\circ C$) in a Sorvall SS3 centrifuge with an SS-34 fixed angle rotor. Latex particles at the interface between the 11.2% and 33% sucrose were harvested and the concentration of sucrose determined with a refractometer (B & S Abbe), and diluted to a concentration of 11.2%. The suspension of latex was centrifuged ($10,000g/10\text{min}/4^\circ C$) and either placed in fixative for electron microscopy (2.7a) or processed further to investigate solubilization of granule proteases. In the case of the latter, the latex was resuspended in Break Buffer plus protease inhibitors as described (2.3f) with the addition of 50mM NaCl. The vacuoles were lysed by freezing and thawing three times and centrifuged ($250,000g/10\text{min}/4^\circ C$). The supernatant was removed and analysed by western blotting (2.2a).
2.6c Measurement of vacuolar permeability to H$_2$O.

Purified neutrophils were adhered to poly-L lysine coated slides for 15 min at 37°C. The slides were washed with PBS and then overlaid for 15 min with a suspension of *C. albicans* labelled with the fluorophore ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, Molecular Probes Inc.) and opsonised with human IgG. Unattached candida were washed away and non-linear coherent anti-Stokes Raman scattering (CARS) microscopy in combination with isotopic exchange was performed as described by Potma et al., (2001)\textsuperscript{336}.

2.6d To quantitate the amount of granule protein entering the vacuole upon phagocytosis.

To determine the quantity of granule proteins in the vacuole, *S. aureus* were radiolabelled by culturing overnight in LB in the presence of 250μCi $^{35}$S methionine, centrifuged (200g/10min/RT) and washed three times in PBS to remove free radiolabel. The bacteria were heat killed (60°C/30min), opsonised with human IgG (1hr/37°C), and mixed with purified neutrophils at a ratio of 25:1 (1ml volume) in a rapidly stirring oxygenated chamber (37°C/4min). Unopsonized bacteria were used as a control. Phagocytosis was stopped by placing the cells in ice cold PBS and washed twice (100g/3min/4°C). Cells were then resuspended in Break Buffer (2.3f) containing protease inhibitors (10μg/ml of leupeptin, TLCK, pepstatin A and aprotinin) and 11.2% (w/w) sucrose. Lysis was by nitrogen cavitation as described (2.3f). A continuous sucrose gradient (30-60% w/w in Break Buffer) was made by overlaying equal volumes of decreasing sucrose concentrations, differing by 2.5% (w/w) and warming the gradient (12ml) to 37°C for 1hr obtaining continuity, from 11.2% to 60%
sucrose. The PNS was centrifuged through the gradient (150,000g, 2hrs, 4°C in a Beckman SW41 head) and fractions (0.5ml) removed. The percentage sucrose in each fraction was measured using a refractometer. The bacteria were located by counting for radioactivity in a liquid-scintillation counter (channel fully open). Bradford/Protein Assay reagent (Biorad Laboratories) was used to assay the amount of protein in each fraction, using BSA as a standard and MPO was detected using the O-dianisidine assay (2.4b).

This experiment was also carried out on a discontinuous sucrose gradient. The PNS was prepared and loaded on top of two sucrose cushions (40% and 53% (w/w)) and centrifuged as for the continuous gradient above. Analysis of the granule content at the interface between the two sucrose layers and the pellet below the 53% sucrose was performed. Protein concentrations, bacterial counts and MPO assays were calculated as already described.

2.6e Buffering capacity of granule protein.

To measure the buffering capacity of the granule proteins, various concentrations of granules lacking membranes, suspended in saline (0.9% NaCl) were titrated against KOH and the pH measured, (pH meter; Mettler Delta, model 340).

2.7 MICROSCOPY.

2.7a Electron microscopy.

To assess sizes of phagocytic vacuoles and their contents, 5x10^7 fresh human neutrophils were mixed with 5x10^8 IgG opsonised S. aureus in a rapidly stirred chamber at 37°C. At timed intervals (1,2,4,8&16 min), aliquots were taken into
fixative of gluteraldehyde (1.5%), picric Acid (0.1%) in cacodylate (0.1M, pH 7.2). They were postfixed in osmium tetroxide, dehydrated in ethanol and embedded in araldite. Sections were collected on copper grids, contrasted with uranyl acetate and lead citrate and viewed at 16,000x. Morphometry was by projecting the photographic EM negatives through a microfilm reader, giving a further enlargement of 9x or 12x. The relevant structures (and the calibration bars) were traced on paper and the profiles were then measured with a digitizing tablet. Volumes were calculated using the formula $4/3 \pi r^3$.

To measure the effect of valinomycin (3μM), DPI (5μM) or protease inhibitors (10μg/ml of leupeptin, TLCK, pepstatin A and aprotinin and 1mM DIFP) on vacuolar volumes, chemicals were added immediately prior to the addition of S. aureus. To study the effect of 1μM jasplakinolide (1mM stock in DMSO, stored at -20°C. Molecular formula, C$_{36}$H$_{45}$BrN$_4$O$_6$) without inhibiting phagocytosis, cells were mixed with bacteria for 2min, the agent was then added and cells were incubated at 37°C for a further 14min.

For assessment of the dispersion of granule contents in the vacuole, the cells were treated as above and the incubation ended at 4 min. Granule contents in the vacuoles were empirically assessed as dispersed or not by two independent "blinded" observers.

2.7b Confocal microscopy.

Confocal immunohistochemical analysis of phagocytosing neutrophils was conducted as described$^{107}$ with slight variations. Phagocytosis of 5x10$^8$ IgG /FITC opsonised S. aureus by 5x10$^7$ fresh human neutrophils was carried out in a rapidly stirred chamber at 37°C, for up to 16min. Reactions were stopped in ice
cold PBS and cells adhered to glass slips by centrifugation (150g/3min/4°C). Cells were fixed in 4% paraformaldehyde, washed in PBS and permeabilised with 0.2% Triton X-100 in PBS for 10mins. As a blocking agent NaBH₄ (10mM) in PBS was used for 1 hr. Antibodies were incubated for 2 hr followed by rhodamine labelled goat anti-rabbit IgG antibody (Stratech Scientific Ltd). Control samples were exposed to non-specific rabbit IgG or goat anti-rabbit IgG antisera alone.

The fluorescence intensity around 50 vacuoles was measured at each time point and compared with that in the surrounding cytosol using a Leica TCS NT confocal microscope.

2.8 GRANULE PROTEINS.

2.8a Elution of proteins from granule proteoglycan matrix.

Membranes were removed from isolated granules (20mg/ml) by the method of Percoll granule disruption described (2.6a). Increasing concentrations of KCL (0, 50, 100, 200, & 400 mM) was added to equal aliquots of demembraned granules suspended in 20mM Hepes pH 7.8 and 150mM NaCl. Reactions were incubated at 37°C for 2min and centrifuged (100,000g/10min/4°C). Solubilized proteins were recovered in the supernatant.

The effect of pH on granule protein solubilization was carried out by resuspending granules in 20mM Hepes buffer pH 6.0 to 8.5 and centrifuged as above.
2.8b Solubilization of granule protein in CGD neutrophils.

Neutrophils were obtained from a normal healthy donor and a CGD patient. In the absence of available CGD cells, DPI (5μM) was used. To investigate solubilization of granule protein upon PMA (1μg/ml) stimulation, resulting in degranulation to the outside of the cell. Cells (5x10^8) in 1ml of PBS supplemented with protease inhibitors (10μg/ml of leupeptin, TLCK, pepstatin A, aprotinin and (1mM) DIFP) were placed in a stirring oxygenated chamber at 37°C and the stimulus added. Aliquotes (250μl) were removed periodically, rapidly cooled to 4°C and centrifuged (700g/5min/4°C) to pellet intact cells. The supernatant was further centrifuged (100,000g/10min/4°C) and analysed for the presence of solubilized granule protein by western blotting.

In experiments using phagocytic stimuli, cells were resuspended in PBS with protease inhibitors. Induction of frustrated phagocytosis was achieved by the addition of 100 S. aureus to a single neutrophil.

2.9 MICROBIAL STUDIES.

2.9a Culturing conditions and cell efficiency.

Overnight cultures of S. aureus (NCTC 12981) and E. Coli (ACCC 11775) were grown in Luria-Bertani media (LB; 1% Bacto-tryptone (Oxide), 0.5% Bacto-yeast extract (Oxide), 0.5mM NaCl; pH 7.0 with NaOH, and autoclaved). For the optimal growth of C. albicans, YEPD was used, containing the required standard nutrients supplemented with glucose as a carbon source (2% Bacto-peptone (Difco), 1% Bacto-yeast extract (Oxide), 2% Glucose).

To estimate the number of organisms killed under different experimental conditions known numbers were used. A reading of 0.17 and 0.365 at 600nm
resulted in $1 \times 10^8$ *S. aureus* and *E. Coli* respectively. *C. albicans* were counted using a haemacytometer. Cell densities were confirmed by plating onto LB or YEPD agar (15g/L; Gibco BRL). Plates were incubated at 37°C overnight and the following morning the number of colonies counted.

**2.9b Killing of *S. aureus* by granule proteins.**

Granules were isolated in the absence of protease inhibitors and demembraned as described (2.6a). The prepared granules (44mg/ml) were incubated (2min, 37°C) with either 200 or 400mM KCl in PBS. Exposure of *S. aureus* (1x10$^7$ CFU/ml) for 6min at 37°C assessed the bacteriocidal properties of the granules. The reaction was then plated in triplicate on LB agar plates and colonies counted.

The experiment was also performed in the presence of protease inhibitors (10μg/ml of leupeptin, TLCK, pepstatin A, aprotinin).

As controls the effect of protease inhibitors and 200 and 400mM KCl on the integrity of the bacteria was also assessed.

An additional experiment was carried out by treating granules (44mg/ml, 2min, 37°C) with 350mM KCl in PBS, followed by centrifugation (100,000g/10min/4°C). The ionic strength of the supernatant was diluted to 150mM to allow reabsorption of proteins to anionic matrix. The bactericidal properties of the supernatant containing solubilized granule proteins was assessed as described above. This was compared to killing following absorption of the proteins to heparin sepharose.

To assess the bactericidal properties of the individual solubilized granule proteins, granules were treated as above and centrifuged (100,000g/10min/4°C).
Fractionation of the supernatant by Gel filtration (Superdex 75, 3ml column, in PBS with 500mM NaCl, 50μl/min, 80μl fractions collected) was carried out.

2.9c In vitro killing of S. aureus and E. Coli by hydrogen peroxide, hypochlorous acid and superoxide (KO$_2$).

From an overnight culture, bacteria (2x10$^7$) were washed twice in PBS and resuspended in PBS at pH 7.5, 6.5 or 5.5. Increasing concentrations of H$_2$O$_2$ (1.0, 10 or 100mM) or HOCl (1 or 5μM) was added and incubated at 37°C, for times of 0,1,2,4,8,16 & 32min. Aliquots were removed at each time point and diluted 1/10 in ice cold LB. Serial dilutions of each reaction were made and plated in triplicate on LB agar plates. Results were calculated as the mean (±S.E.) from at least three experiments with colony counts performed in triplicate and expressed as a percentage of the original viability at time zero. The pH remained stable during assays to within 0.2pH units of the starting pH.

This experiment was also carried out in the presence of granules (25mg/ml), prepared using protease inhibitors (10μg/ml of the following, leupeptin, TLCK, pepstatin A, aprotinin and 1mM DIFP) and demembraned as described above (2.6a). Under these conditions 100mM H$_2$O$_2$ and up to 1mM HOCl was added.

Killing of bacteria by O$_2^-$ was performed similarly to that described for H$_2$O$_2$ and HOCl. As a source of O$_2^-$, KO$_2$ was employed and added as a powder to the reactions. Concentrations of KO$_2$ greater than 50mM elevated the pH of the reaction. To overcome this, bacteria were suspended in PBS, pH of 6.5 prior to the addition of 100mM KO$_2$, which resulted in an increase in the pH to approximately 7.5.
**2.9d In vitro killing of S. aureus by the MPO/H₂O₂/Cl⁻ system**

Bacteria (1x10⁷) from an overnight culture were washed extensively (x4) and resuspended in PBS (pH 7.5, 6.5 or 5.5) together with purified MPO (5mg/ml). Increasing concentrations of H₂O₂ (0.1, 1.0, 10, 100mM) were added and the reaction incubated at 37°C. Aliquots were removed at times 0,1,2,4 & 16mins. and diluted 1/10 in ice cold LB. Serial dilutions of each reaction were plated on LB agar plates and after an overnight incubation at 37°C the colonies were counted. The pH remained stable during assays to within 0.15 pH units of the starting pH.

**2.10 K⁺ STUDIES.**

**2.10a Movement of K⁺.**

K⁺ release from neutrophils (4x10⁷/ml) stimulated with 1μg/ml PMA was measured continuously with a K⁺ electrode (Thermo Russell, Model 93-3199) at 37°C. The cells were kept in PBS and buffer exchanged to a K⁺ free PBS (NaCl 140mM, MgSO₄ 0.5mM, CaCl₂ 1mM, Glucose 5mM, phosphate 10mM; pH 7.3) just before each measurement. The K⁺ efflux caused by superoxide production was measured as the difference obtained in the absence and in the presence of DPI (5μM). K⁺ concentration was calculated using a standard curve.

**2.10b ⁸⁶Rb⁺ as a tracer for K⁺ movement.**

For ⁸⁶Rb⁺ efflux, 1x10⁷ neutrophils were suspended in 1ml of HEPES Buffer (136mM NaCl, 1mM MgSO₄, 1mM CaCl₂, 5mM KCl, 5.6mM Glucose, 20mM Hepes; pH 7.4, 2.5μCi ⁸⁶Rb⁺ and BSA 0.1mg/ml) and incubated at 30°C for 30 min, washed x3 in the buffer without the ⁸⁶Rb⁺ and then placed in a stirred
oxygenated chamber at 37°C. Cells were stimulated either by 2x10^8 IgG opsonised *S. aureus* or 1μg/ml PMA. Stimulated cells were immediately cooled to 4°C and centrifuged through Ficoll/Hypaque (400g/10min/4°C). The supernatant above the Ficoll/Hypaque and the neutrophil pellet were removed and counted for radioactivity in a liquid-scintillation counter. Experiments were repeated in the presence of DPI (5μM) or valinomycin (3μM) added immediately prior to neutrophil activation.

^86Rb^ efflux was also measured in 20mM Hepes, buffered at pH 6, 7, 8 & 8.5, stimulated with PMA (1μg/ml final, 3min, 37°C) and centrifuged through Ficoll/Hypaque as described above. ^86Rb^ efflux is expressed as a percentage of the total.

2.10c *Electron probe X-ray microanalysis.*

K^+^ concentrations inside the phagocytic vacuole and cytoplasm were measured by X-ray microanalysis on snap frozen, freeze dried cells. Neutrophils (+5μM DPI or 3μM valinomycin) were mixed with IgG opsonised *S. aureus* at 37°C for 3 min, centrifuged at 600g for 30 seconds, drops of the thick cell pellet were collected on a piece of Millipore filter paper and cryofixed by plunging into liquefied propane. The cryofixed pellets were stored under liquid nitrogen. Cryosections, 250 nm thick, were cut at -120°C in a RMC MTXL cryoultramicrotome. The sections were supported on Pioloform covered nickel grids (100 mesh hexagonal) and freeze dried overnight under temperature controlled conditions. The sections were coated with a thin layer of carbon in the vacuum chamber and analysed using a Zeiss EM10 electron microscope fitted with an Oxford Instruments detector and PGT Avalon 8000 microanalysis
system. Areas within the vacuole space and in the adjacent cytoplasm were analysed using a reduced area raster for 60 secs of live time at 80 kV accelerating voltage and 1 nA beam current in STEM mode at ambient temperature. Spectra were processed and quantification was achieved using the peak to continuum ratio method\textsuperscript{338}.

\textbf{2.11 VACUOLAR AND CYTOSOLIC PH.}

\textit{2.11a Vacuolar pH.}

Vacuolar pH was determined from the excitation spectrum of fluorescein-labelled, IgG coated \textit{S. aureus}. The latter were produced as described by Segal \textit{et al.}, (1981)\textsuperscript{161}. In brief heat killed (60°C, 30min), IgG opsonized \textit{S. aureus} were incubated with FluorX reactive dye (1µg/ml, in 0.1M Sodium carbonate buffer pH9.3, Amersham Pharmacia Biotech) and incubated at room temperature, with mixing for 30min. The bacteria (0.5x10\textsuperscript{7}) were then washed three times in PBS and added to neutrophils (2.8x10\textsuperscript{7}/ml), in the rapidly stirred chamber of an oxygen electrode at 37°C for 2.5min. A comparison of fluorescence ratio $I_{493}/I_{433}$ was made with a calibration curve generated using free bacteria in buffers of different pH's (Jiang Q., 1997)\textsuperscript{162}.

\textit{2.11b Cytosolic pH.}

Cytosolic pH was measured in cells in suspension with the carboxyfluorescein, BCECF (Molecular Probes). The method employed is as described by Pocock & Richards, (1992)\textsuperscript{339}. The cells (2x10\textsuperscript{7}/ml) in PBS were loaded by incubation with 10µM BCECF for 15min at 37°C. Loading was terminated by washing the cells with PBS three times. For the experiment fluorescence was excited at 490
and 440nm and fluorescence emission ratios calculated (490/440). pHs were standardized with phosphate- or Hepes-buffered salt solutions, and cells equilibrated in the presence of nigericin (10μM).

2.12 **IN VITRO & IN VIVO NEUTROPHIL KILLING ASSAYS.**

2.12a **Thioglycolate-induced peritonitis.**

Mice were injected intraperitoneally with 0.7ml/20g body weight of 3% fluid thioglycolate medium (Difco). After 12hr, peritoneal exudate cells were harvested by peritoneal lavage with 10ml of RPMI 1640 (25mM Hepes buffer, Gibco BRL) containing heparin at 5 units/ml. Total cell numbers were determined with a hemocytometer.

2.12b **In vitro murine killing studies.**

Thioglycolate elicited mouse neutrophils (2.8x10⁷) in 0.5ml PBS were mixed with 0.5x10⁷ *S. aureus* or 0.25x10⁷ *C. albicans* in the rapidly stirred chamber of an oxygen electrode at 37°C. Killing was measured as described by Segal et al., (1981)¹⁶¹ omitting lysostaphin. Briefly, aliquotes of the phagocytosing cells were removed at periodic time points and lysed by placing in ice cold distilled sterile water. All *in vitro* killing studies were performed in duplicate with cells from 3-5 mice, and colonies counted on 3 aliquots taken at each time point. Bacterial killing was expressed as a percentage of time zero.
2.12c Systemic infection studies.

*S. aureus* & *C. albicans* were grown overnight at 37°C and washed in PBS. Microbial numbers were determined as described, (section 2.9a) and confirmed by plating onto LB agar plates.

In studies of systemic infections, wild type and homozygous mutant mice all of the strain 129 genetic background, deficient in cathepsin G and/or elastase or p47phox deficient CGD, were used. Mice were age and sex matched and maintained in microisolator cages. *S. aureus* (4x10^7) or *C. albicans* (1x10^4) were injected intravenously in a 100μl volume via the tail vein. Mice were monitored twice daily for 25 days. Signs of stress, weight loss and subsequent death were documented.

2.12d In vitro killing of *S. aureus* or *E. coli* by human neutrophils.

To measure the effect of valinomycin (3μM), protease inhibitors (10μg/ml of, leupeptin, TLCK, pepstatin A, aprotinin and 1mM DIFP) or DPI (5μM) on killing by human neutrophils, the chemicals were added without subsequent incubation to 1x10^8 cells in 1ml PBS containing 5mM glucose. IgG opsonised *S. aureus* (1x10^8) were added to the cells in a rapidly stirring 37°C chamber of an oxygen electrode and then treated as above (2.12b). As a control the effect of all chemicals and the solvent in which they were dissolved, on bacterial viability (2.9a) and neutrophil function (2.3d) was tested.
2.12e In vitro digestion of $^{35}$S radiolabelled *S. aureus* by human neutrophils.

Bacteria were grown in LB (2.9a) containing $^{35}$S-methionine (1μCi/ml) and then washed, killed by heating to 60°C for 30min, rewashed and opsonized with IgG $^{161}$. Radiolabelled bacteria were mixed with neutrophils (1:1) and rapidly stirred at 37°C. After 30min the cells and bacteria were precipitated by adding trichloroacetic acid (TCA, 10% final concentration) and centrifuged at 8,000g for 10min. Digestion was expressed as the % of TCA-soluble radioactivity released into the supernatant. Digestion was also carried out in the presence of valinomycin (3μM), DPI (5μM) or protease inhibitors (10μg/ml of, leupeptin, TLCK, pepstatin A, aprotinin and 1mM DIFP).

2.12f Survival analysis.

Survival of mice was analysed by Cox proportional hazards survival analysis. *In vitro* killing was analysed by determining the rate of loss of viability of the organisms by regression analysis of a logarithmic transformation of the numbers of surviving microbes in relation to time. Slopes were compared with a 2-tailed t test.
CHAPTER 3.

3.0 CONDITIONS WITHIN THE PHAGOCYTIC VACUOLE.

3.0 A BRIEF SUMMARY OF CONTENTS.

The aim of the work described in this chapter was to establish conditions prevailing within the vacuole, immediately after phagocytosis, and within the time of bacterial killing\textsuperscript{161}. Systematic experiments were carried out to calculate the vacuolar volume from cross-sectioned areas of phagocytic vacuoles. Subcellular centrifugation studies determined the concentration of granule protein entering the vacuole and calculations were made on the amount of oxygen consumed and the resulting $O_2^-$ produced within the vacuole for each bacterium engulfed.

Experiments reveal that an indirect consequence of $O_2^-$ production, is an increase in ionic strength, as $K^+$ is transported into the vacuole as a counter ion to the negative charge induced by the electrogenic NADPH oxidase. Studies on the effect of valinomycin, a specific $K^+$ ionophore and of 4-aminopyridine, a non-specific $K^+$ channel blocker were performed to correlate the activity of the oxidase with activity of the $K^+$ channel.

Following PMA stimulation the cytosolic $K^+$ is released to the extracellular medium, measured with a $K^+$ sensitive electrode and more quantitatively using $^{86}\text{Rb}^+$\textsuperscript{340}. The NADPH oxidase inhibitor DPI\textsuperscript{154}, was shown to block the release of $K^+$. 
Electron probe X-ray microanalysis was then used to obtain a direct estimate of the K⁺ content of the vacuole\textsuperscript{337,341}. Cytochrome C reduction assays\textsuperscript{9} at increasing pH, were employed to show, K⁺ channel activity and efflux is pH dependent.

3.0 INTRODUCTION

Phagosomes are pivotal organelles in the ability of neutrophils to perform its key function of killing and digestion of engulfed microbes. It was recognized at an early stage that cytoplasmic granules containing digestive and antibacterial compounds are emptied into the phagosome\textsuperscript{342} and that phagocytosing neutrophils undergo a burst of oxygen consumption that is caused by the NADPH oxidase complex that assembles at the phagosomal membrane\textsuperscript{140}. The passage of electrons across the vacuolar membrane in the production of O₂⁻ is an electrogenic process and the required charge compensation is commonly thought to be encompassed solely by the transfer of H⁺ into the vacuole\textsuperscript{154}.

A paradox exists however, as work carried out by Segal \textit{et al.}, (1981) and later by Jiang \textit{et al.}, (1997), provided evidence that the pH in the vacuole rises from about 6.0 to 7.8-8.0, in consequence of oxidase activity\textsuperscript{161,162}, despite the entry of acid granule contents\textsuperscript{343}. Compensation of an electron by a H⁺ is pH neutral and cannot explain the observed elevation in vacuolar pH. As an alternative compensatory charge for the production of superoxide, Cl⁻ is the most obvious candidate and could migrate from the vacuole to the cytosol. However Cl⁻ has been shown to move in the opposite direction in stimulated cells\textsuperscript{344}. Outward movement of cations provides an alternative mechanism, and K⁺
seemed to be the most likely of these because of its abundance in the cytoplasm (125mM)\(^{345}\).

3.0 RESULTS AND DISCUSSION.

3.1.0 Conditions within the vacuole.

The first set of experiments was designed to establish conditions within the vacuole with respect to vacuolar volume, \(O_2^-\) production and granule protein concentrations.

3.1.1 Volume of the phagocytic vacuole.

To estimate the volume, cross-sectional areas of phagocytic vacuoles and of their internalized bacteria, that were judged to have been sectioned in the region of maximum width, were determined from electron micrographs (see methodology section 2.7a). EM negatives of vacuoles containing a single bacterium were enlarged by projection through a microfilm reader. The vacuoles and calibration bars were traced on paper and then measured with a digitizing tablet. The volume of the vacuole during the respiratory burst and up to 4min after the onset of phagocytosis was calculated to be about 0.2\(\mu\)M\(^3\).

3.1.2 Vacuolar protein concentration.

The concentration of granule protein entering the vacuole was first measured by determining the migration of protein from the granules to the phagocytic vacuoles that were separated on continuous or discontinuous sucrose gradients (Figure 3.1 & 3.2.). Employing methods of O-dianisidine detection for MPO\(^{333}\) and Bradford/Protein assay using purified MPO as a standard, it was found that
FIGURE 3.1. Movement of granular proteins to the vacuole during phagocytosis of S. aureus, part 1.

Separation of cellular components was performed on a continuous gradient, from 11.2 to 60% sucrose. (a) Distribution of MPO in phagocytosing (●) was compared with resting neutrophils (□) following phagocytosis of $^{35}$S radionlabelled S. aureus. (b) are the same fractions as in (a) where shading indicates movement of MPO to dense vacuoles containing $^{35}$S radionlabelled S. aureus.

As a control the entire experiment was performed using unopsonized bacteria with reproducible results over five separate experiments.

Methodology section 2.6d.
**FIGURE 3.2.** Movement of granular proteins to the vacuole during phagocytosis of *S. aureus*, part 2.

Separation of cellular components was performed on a discontinuous sucrose gradient of 40 and 53% sucrose. Distribution of MPO in phagocytosing was compared with resting neutrophils by analysis of the granule content at the interface between the two sucrose layers and the pellet below the 53% sucrose containing vacuoles of $^{35}$S radiolabelled *S. aureus*.

As a control the experiment was performed using unopsonized $^{35}$S radiolabelled bacteria with no radioactivity detected below the 53% sucrose.

The entire experiment was carried out five times and the value reported is the average ± S.E.

Methodology section 2.6d.
MPO comprised 26% of the total granule protein. MPO was then used as a marker for granule movement to the vacuole. The protein content of 1x10⁷ cells was 1.2 mgs, of which 9.4 ± 1.0% (Mean ± S.E.M., n=5) was in the granule fraction. Of this, 24%, or approximately 0.1 pg / vacuole migrated to the vacuoles after the phagocytosis of 25 bacteria/cell. With a vacuolar volume of 0.2 μm³, the protein concentration was estimated at 0.5 g/ml.

3.1.3 Calculations of O₂⁻ production.

The final part of this set of experiments was to calculate the concentration of O₂⁻ produced within the vacuole. The background information for this calculation is logically illustrated in Figure 3.3.

4 molecules of O₂⁻ rapidly dismutate to give rise to 2O₂ and 2H₂O₂. The 2H₂O₂, in turn break down to 2H₂O and 1O₂, thus for each molecule of O₂ consumed 4O₂⁻ ions are generated. Between 0.2³³² and 0.5 fmols (this study) of oxygen is consumed for each bacterium engulfed and with a vacuolar volume of 0.2 μM³ the concentrations of intravacuolar O₂⁻ generation would be about 4 moles/l.

Accumulation of O₂⁻ does not occur and the steady state concentrations are unknown, with dismutation rates obtained by stopped-flow kinetics, of 2.3x10⁹ M⁻¹s⁻¹ at neutral pH³⁴⁶, calculated for the reaction,

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2. \]

Vacuolar concentrations of H₂O₂ are difficult to predict because the rates of decomposition and diffusion are unknown. Measurement from 0.01 μM¹⁷⁵,³⁴⁷ to 100 mM have been estimated, depending on the amount of phagocytosis and
FIGURE 3.3. K\(^+\) charge compensation is regulated by pH.

The pH in the vacuole rises\(^{161}\), despite the influx of acidic granule contents, because of the consumption of H\(^+\) within this compartment by protonation of O\(_2^−\) to O\(_2^{2−}\) with the resulting formation of H\(_2\)O\(_2\).

The volume of the vacuole during the respiratory burst is about 0.2\(\mu\)M\(^3\). For each molecule of O\(_2\) consumed 4 O\(_2^-\) are generated. Between 0.2 \(^{332}\) and 0.5fmols (this study) of oxygen is consumed for each bacterium engulfed, resulting in an intravacuolar O\(_2^-\) generation of about 4moles/l. The steady-state concentration of O\(_2^-\) and of H\(_2\)O\(_2\) attained are uncertain, but have been surmized to be in the micromolar range\(^{347}\).

If all the charge produced by pumping electrons across the phagosomal membrane were compensated by H\(^+\)s from the cytosol, the pH in the vacuole would not rise.

Within this chapter it will become evident that part of the charge is compensated by K\(^+\), which elevates the pH in a regulated manner and causes the vacuole to become hypertonic.
the vacuolar pH\textsuperscript{116}. Also the concentration consumptioned by the peroxidase\textsuperscript{189,348} or catalase\textsuperscript{181,255} activity of myeloperoxidase, is unknown.

- \(2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2\) (catalase activity)
- \(\text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{HOCl} + \text{H}_2\text{O}\) (peroxidase activity).

3.1.4 Conclusion.

From this set of experiments it was concluded that large quantities of superoxide are produced. This suggested that its products might be exerting an electrostatic, or other physical effect, rather than simply producing substrate for MPO. This inference was strengthened by observations on patients with incomplete, or “variant CGD” \textsuperscript{37}, who still displayed impaired microbicidal killing despite generating about 0.5 moles/litre of \(\text{O}_2^-\) in the vacuole. Accordingly experiments were designed to examine phagocytic vacuoles by electron microscopy to search for evidence of changes in morphology induced by the oxidase.

3.2.0 Dispersion of granule proteins is associated with oxidase activity.

The aim of this experiment was to identify physical differences in the appearance of vacuoles induced by oxidase activity following phagocytosis of \(S. \text{aureus}\). Vacuoles of normal neutrophils were compared to those of a CGD patient. Vacuolar morphology was studied by electron microscopy.
3.2.1 *Electron microscopy of phagocytosing vacuoles.*

CGD neutrophils were donated by a previously diagnosed, X-linked CGD patient and an equal number of normal cells obtained as a control. The electron microscopy images are presented (Figure 3.4), where (a) is representative of control cells and (b) CGD cells. The appearances of the vacuoles, of normal cells were strikingly different from those of the CGD patient. The most obvious characteristic of the control cells was the homogeneous appearance or uniform dispersion of the granule material throughout the vacuole. This was in total contrast to the appearance of material in most of the vacuoles of CGD cells, where granules appeared as discrete clumps.

Phagocytosis and degranulation have previously been reported to be equal between normal and CGD neutrophils\(^9\) however by visual analysis it became apparent that degranulation is followed by granule protein dispersion throughout the vacuole as a result of oxidase activity.

3.2.2 *Dispersion of granule contents assessed.*

The granule contents in the vacuoles were empirically assessed as dispersed or not, in normal neutrophils, those from a patient with X-linked CGD with complete absence of oxidase activity and a variant patient\(^{37}\) with 12% oxidase activity. The result shows (Figure 3.5), that complete absence of oxidase activity, corresponds to a lack of granule material dispersion. Possession of 12% oxidase activity was insufficient to cause dispersion of the granule contents.
FIGURE 3.4. Superoxide generation is associated with dispersion of granule contents in the phagocytic vacuole.

Transmission electron micrographs of normal (a) and CGD (b) neutrophils, 4 min after phagocytosis of *S. aureus*. Vacuoles containing bacteria (S) are shown (➔).

Granule contents within the vacuole (a) were classified as diffuse, because of their homogenous distribution (D), or clumped (➔ in b).

Methodology section 2.7a.
FIGURE 3.5. Dispersion of granule contents in the vacuole.

Granule contents in the vacuole were empirically assessed as dispersed or not by two independent “blinded” observers. The proportion of vacuoles with a diffuse appearance in normal neutrophils (n=280) and those from patients with X-linked CGD with complete absence of oxidase activity (CGD 1, n=244) or a variant patient with 12% oxidase activity (CGD 2, n=206) is shown. (p=10^{-148} and 10^{-68} between normal and X-CGD and normal and variant X-CGD patients respectively by Chi-squared analysis).
3.2.3 Conclusion.

These conclusive results provided evidence for a role of the oxidase in dispersing the granule proteins within the vacuole. A failure of the granules to disperse was correlated with a lack of respiratory burst, as such dispersion in vacuoles of CGD neutrophils was not apparent. This observation is the basis for the next set of experiments.

3.3.0 $O_2^-$ induced charge compensation by $K^+$.

3.3.1 Introduction.

The following group of experiments was designed to show that the oxidase disperses the granule proteins by pumping large amounts of $K^+$ into the vacuole. There is no evidence that the oxidase reaction results in the efflux of $K^+$ into the vacuole and therefore the first experiment was designed to investigate whether disruption of the natural movement of $K^+$ would influence $O_2^-$ production. Stimulation of the cells was by PMA. In response to this agent the oxidase is induced to secrete $O_2^-$ across the membrane to the outside of the cell as measured by cytochrome C reduction.

3.3.2 The effect of the $K^+$ ionophore, valinomycin, on $O_2^-$ production.

Experiments studied the effect on $O_2^-$ production of valinomycin, a specific $K^+$ ionophore. Valinomycin has the chemical structure depicted in Figure 3.6a. It is readily dissolved in organic solvent and in cell membranes to which it imparts a high selective permeability for $K^+$ and $Rb^+$. Valinomycin renders membranes
**FIGURE 3.6a STRUCTURE OF VALINOMYCIN.**
Valinomycin is a repeating cyclic molecule made up of L-lactate, L-valine, D-hydroxyisovalerate and D-valine, with a central opening which can bind K⁺.


**FIGURE 3.6b.** Valinomycin makes neutrophils membranes permeable to K⁺.
Neutrophils (1x10⁷/ml) were preloaded with ⁸⁶Rb⁺ as a tracer for K⁺ movement. ⁸⁶Rb⁺ efflux was compared in the presence and absence of valinomycin (3μM).

Values are derived from three separate experiments ± S.E.
Methodology section 2.10b.
more permeable to the ion\textsuperscript{349,350} and results in the redistirbute of the cellular K\textsuperscript{+}, establishing equilibrium of the ion between the inside and outside of the cell.

Preliminary experiments were performed to confirm a positive effect of valinomycin on neutrophil cytosolic K\textsuperscript{+}. By taking advantage of the radiolabelled ion, \textsuperscript{86}Rb\textsuperscript{+}, which is a commonly used marker of K\textsuperscript{+} in transport studies\textsuperscript{340}, as all K\textsuperscript{+} channels show selectivity of K\textsuperscript{+} \approx Rb\textsuperscript{+} \textsuperscript{351}, a quantitative measure of the effect of valinomycin was made. Cells were preloaded with \textsuperscript{86}Rb\textsuperscript{+} and movement of K\textsuperscript{+}/\textsuperscript{86}Rb\textsuperscript{+} from the high cytosolic 125mM\textsuperscript{337,345} to the low 5mM in the surrounding buffer was measured. This concise experiment (Figure 3.6b) revealed the addition of valinomycin to almost double the release of \textsuperscript{86}Rb\textsuperscript{+} from unstimulated cells.

The effect of the addition of the ionophore to stimulated cells was assessed by cytochrome C reduction following PMA stimulation and oxygen consumption as a result of bacterial phagocytosis (Figure 3.7). On the addition of valinomycin, it was evident that by facilitating the movement of K\textsuperscript{+}, in the direction of superoxide production, the latter was accelerated.

Under exactly the same experimental conditions the effect of 4-aminopyridine, a non-specific K\textsuperscript{+} channel blocker was studied\textsuperscript{349}. Lower levels of cytochrome C reduction were observed, arising as a direct result of the inhibition of K\textsuperscript{+} movement.

\textbf{3.3.3 Conclusion.}

This result implicated K\textsuperscript{+} as a likely candidate for charge compensation of O\textsubscript{2}\textsuperscript{-} production. Thus, the availability and rate of movement of K\textsuperscript{+}, acting as a compensatory charge, limits the activity of the electrogenic NADPH oxidase.
FIGURE 3.7. K⁺ transport is associated with the generation of superoxide by neutrophils.

The rate of superoxide release from 1x10⁶/ml neutrophils is stimulated by valinomycin (val, Δ, 3μM, 3min) and inhibited by 4-aminopyridine (4-AP, ■, 4mM, 3min) compared with control cells (con, ▲).

Representative of 3 experiments in response to PMA (10ng/ml).

O₂ consumption by 1x10⁷ cells phagocytosing *S. aureus* in absence (●) and presence (○) of valinomycin is also shown.

Methodology section 2.3d.
3.4.0 Measurements of $K^+$ release.

3.4.1 Introduction.

The aim of this set of experiment was to measure the release of $K^+$ upon neutrophil activation.

3.4.2 A calculation of $K^+$ release following PMA activation, using a $K^+$ electrode.

As there is no known indicator of the required specificity and sensitivity to measure $K^+$, its release was determined, on the exterior of the cell into the surrounding medium, after stimulation with PMA using a $K^+$ sensitive electrode. Neutrophils were stimulated in the presence and absence of DPI. The compound DPI, in PMA-treated cytoplasts has been shown to inhibit 96% of the oxidase activity. Calculations of release following PMA activation were the difference obtained in the presence of DPI. The result in Figure 3.8 shows the release of $K^+$ into the medium and paralleled the time course of oxygen consumption.

3.4.3 Quantitation of $K^+$ movement using $^{86}\text{Rb}^+$. 

Measurements of $K^+$ movement upon neutrophil stimulation with the $K^+$ electrode were only semiquantitative so $^{86}\text{Rb}^+$, the marker of $K^+$ was used for quantitation.

The experiment was devised employing two different stimuli. PMA was used and as already discussed stimulation with this soluble stimulus results in production of $O_2^-$ outside the cell membrane. The second stimulus, IgG opsonized S. aureus, results in phagocytosis of the bacterium into an enclosed
FIGURE 3.8. Time course of K⁺ release.

K⁺ release from neutrophils (4x10⁷/ml) stimulated with PMA (1µg/ml) in a K⁺ free PBS was measured continuously with a K⁺ electrode. Efflux was measured in the presence and absence of DPI (5µM).

The result shown is representative of three separate experiments and illustrates the time course of release of K⁺ into the medium in relation to oxygen consumption.

Methodology section 2.10a.
intracellular vacuole. Superoxide production under these conditions is confined to the vacuole as illustrated, (Figure 3.9a). By association, PMA activation would result in the efflux of $K^+/86Rb^+$ to the outside of the cell and phagocytosis of $S. aureus$ would result in the channelling of $K^+/86Rb^+$ into the vacuolar space (Figure 3.9b). The experimental technique designed for this experiment is described in the methodology section (2.10b).

Results of this experiment are illustrated in Figure 3.10. PMA stimulation resulted in the release of $86Rb^+$ from the cell, representative of 4.5% $K^+$ release. Secretion of this ion was dependent upon $O_2^-$ generation as demonstrated by its abrogation by DPI. Treatment of the cells with the oxidase inhibitor prior to stimulation reduced the $86Rb^+$ efflux almost to the level of unstimulated cells. This result is supportive of an observation by Lehrer in 1977, when it was reported that neutrophils from patients with CGD had low $86Rb^+$ efflux following stimulation, which the authors suggested was closely related to the abnormality of oxidative metabolism$^{352}$.

To show that this release was spatially associated with the $O_2^-$ generating machinery, a measurement of secretion in conditions favouring the flux of $K^+$, $86Rb^+$ and $O_2^-$ into the closed phagocytic vacuole surrounding an engulfed bacterium was made. In these circumstances in which the vacuole is separated from the external medium by two membranes and the cytosol, no release to the exterior was observed, even though an equivalent amount of oxygen was consumed. Total oxygen consumed for both stimuli was 170nmoles per $10^7$ cells.
FIGURE 3.9. Schematic representation of the cellular localization of $O_2^-$ production by activated neutrophils.

(a) the NADPH oxidase molecules on the plasma membrane may be activated by a soluble agonist (i.e. PMA) and $O_2^-$ production occurs to the outside of the cell. Alternatively activation of the NADPH oxidase may occur on phagocytosis of a bacterium, resulting in the production of $O_2^-$ within the phagocytic vacuole.

(b) Production of $O_2^-$ on the outside of the cell will result in efflux of $K^+$, $H^+$ and/or $^{86}\text{Rb}^+$ to the surrounding medium. On activation of the NADPH oxidase during phagocytosis, $K^+$, $H^+$ and/or $^{86}\text{Rb}^+$ follow the production of $O_2^-$ and are channelled into the vacuole.
**FIGURE 3.10.** K⁺ transport is associated with the generation of O₂⁻ by neutrophils.

⁸⁶Rb⁺ was used as a tracer for K⁺ movement and its release before (Cells) or after stimulation with PMA (1µg/ml, 3.5min) or phagocytosis of *S. aureus* was measured.

⁸⁶Rb⁺ efflux following PMA activation was compared to cells in which the NADPH oxidase was inhibited by DPI (5µM), added prior to activation.

Oxygen consumption was equivalent for the two stimuli (170nmoles/10⁷cells) and was inhibited by DPI.

All results are the mean ± S.E. of three separate measurements.

Methodology section 2.10b.
3.4.4 Conclusion.

To conclude, the concept of $K^+$ as a compensating charge for superoxide production is supported by the following observations. The rate of $O_2^-$ generation stimulated by PMA is accelerated in the presence of valinomycin, a specific $K^+$ ionophore, and reduced by 4-aminopyridine, a $K^+$ channel inhibitor. After PMA stimulation the cytosolic $K^+$ is released to the extracellular medium, measured directly with a $K^+$ electrode and with radioactive $^{86}\text{Rb}^+$. Furthermore, $^{86}\text{Rb}^+$ release is not seen when an equal amount of oxygen consumption is induced by phagocytosis of bacteria, or in the presence of the oxidase inhibitor, DPI.

3.5.0 A direct estimate of the $K^+$ content of the phagosome.

3.5.1 Introduction.

Electron probe X-ray microanalysis (EPXMA) of thin biological specimens has the capability of providing information at high resolution about the spatial distribution of elements/ions within cells and was used to directly estimate the $K^+$ content within the phagosome.

3.5.2 Measurement of vacuolar $K^+$ concentrations using X-ray microanalysis.

Vacuolar $K^+$ measurements were calculated in the presence or absence of oxidase activity, inhibited by DPI and in the presence of the $K^+$ ionophore valinomycin. Results are presented in Figure 3.11. In DPI-treated cells the vacuolar potassium concentration was greatly reduced compared to that in
**FIGURE 3.11.** $K^+$ concentrations within the phagocytic vacuole.

$K^+$ concentrations in phagocytic vacuole of neutrophils after phagocytosis of *S. aureus*, and the effects on this of the addition of valinomycin (3μM) and DPI (5μM), as determined by electron probe X-ray microanalysis.

Horizontal line depicts mean concentration (280 mMol/g) in control cytosol.

Median, mean, n and S.E. of values in vacuoles were: 236, 322, 39 and 50; 152, 213, 39 and 34; and 100, 121, 39 and 13 mMol/g for control, valinomycin and DPI treated cells respectively.

Corresponding values in cytosols were: 291, 280, 41 and 22; 211, 269, 46 and 278; and 278, 345, 37 and 46 mMol/g for control, valinomycin and DPI treated cells respectively.

Methodology section 2.10c
untreated control cells (122±15 mean±S.E., n=35 and 314±22, n=40 mMol/g respectively. p=<0.001 by Wilcoxon rank-sum), clearly implicating the oxidase in the accumulation of K⁺ in this compartment.

A wide range of values is measured in normal vacuoles in consequence of the asynchronous nature of phagocytosis. To take into account the wide spread of results obtained with the microprobe technique, and to detect a skewing of the distribution towards higher concentrations, a comparison of the upper quartiles of K⁺ measurements in the vacuoles with those in the corresponding cytosol was made. Vacuolar K⁺ concentrations were found to be significantly higher in the control cells (p=<0.03 by Wilcoxon rank-sum), no different in the valinomycin treated cells (p=0.16) and much lower in cells exposed to DPI (p=0.0003).

K⁺ channels contribute to the control of cell volume and membrane potentials. In this study valinomycin had a profound effect on the vacuolar K⁺ concentration. Therefore a further control study was undertaken to look for any adverse effects of valinomycin on neutrophils over the short time that it was used in these experiments.

Results showed there were no major differences in cell volume, as measured from cross sectioned areas on electron micrographs, between control (310±14 μ³, n=43) and in valinomycin-treated (327±18μ³, n=42) cells.

In addition K⁺ levels were slightly lower in the cytosol of valinomycin treated (254±37.5, n=46 mMol/g) than in control cells (280±22,n=41 mMol/g) (p=0.011) by microprobe analysis, consistent with our observation that this agent doubled the rate of leakage of ⁸⁶Rb⁺ in resting and cells (Figure 3.6b).
3.5.3 Conclusion.

Microprobe analysis gave a median concentration of $\text{K}^+$ of 290mMol/g in the cytosol of normal cells, which was taken to correspond to the experimentally measured concentration of 125mM$^{345}$. This ratio leads to a value of 270mM for the upper quartile in normal vacuoles, corresponding to the microprobe measurement of 631mMol/g. Thus it was concluded that $\text{K}^+$ concentrations in the range of 200-300mM were attained in the vacuole.

3.6.0 Two separate factors set the level of charge compensation by $\text{K}^+$.

3.6.1 Introduction.

The passage of electrons across the vacuolar membrane in the production of superoxide is electrogentic and full charge compensation by $\text{K}^+$ would result in an excessively elevated pH. However the pH in the vacuole is elevated from pH 6.0 to 7.8-8.0, which would not occur if compensation were due entirely to $\text{H}^+$. Therefore what sets the level of charge compensation by $\text{K}^+$? Did the vacuolar granules themselves provide a buffered environment such that higher levels of charge compensating $\text{K}^+$ can enter the vacuole?

3.6.2 The superoxide enters a vacuole containing the microbe and granule proteins, which serve as an internal pH buffer.

The buffering capacity of granule proteins was determined by measuring the amount of KOH required to raise the pH from 5.5, at which the granules are maintained in the intact cell$^{343}$, to a final value of pH 8.0$^{161,162}$. The result is
illustrated in Figure 3.12. The titre proved to be about 400μmols of KOH per gram of granule protein, so the amount of protein predicted to be present within the vacuole (0.5g/ml) would buffer approximately 200μmoles of OH⁻. In this way the amount of K⁺ entering the vacuole is linked to degranulation. The more granule contents released into the vacuole, the more the pH is depressed and the greater the buffering capacity.

3.6.3 The transport of K⁺ is pH dependent.

The regulation of the proportion of compensating charge made up by K⁺ appears to be governed by the pH itself. In an experiment employing PBS of increasing pH, and ⁸⁶Rb⁺ as a marker for K⁺, the efflux of ⁸⁶Rb⁺ to the exterior following PMA stimulation was measured.

The efflux of ⁸⁶Rb⁺ to the exterior following PMA stimulation was reduced when the pH of the medium was elevated to 8.0, and abolished at 8.5, even though superoxide production was almost normal at the higher pHs, as measured by cytochrome C reduction (Figure 3.13). At high pH the charge is likely to be compensated almost entirely by H⁺.

To determine the effect of changes in the external pH on that of the pH of the cytosol, the latter was measured using BCECF (2',7'-bis(2-carboxyethyl)-5-(and-6-)carboxyfluorescein). The cells were preloaded with this pH-sensitive fluorescent dye, that obtains access into the cytosol by means of its lipid-soluble acetoxyster. Cleavage by intracellular esterases exposes four negative charges, which trap the dye in the cell. The response of the dye was calibrated by alterations in the external pH of the cells in the presence of the electroneutral H⁺:K⁺ exchanger, nigericin. Neutrophils maintain a resting pH of 7.3 and
**FIGURE 3.12.** Buffering capacity of the granule proteins.

Buffering capacity of granule proteins (1ml in 0.9% NaCl) at various concentrations titrated against KOH.

(a) The amount of KOH required to elevate pH from 5.5 to 8.0 for different amounts of protein (b).

Methodology section 2.6e.
**FIGURE 3.13.** $K^+$ charge compensation is regulated by pH.

The efflux of $^{86}\text{Rb}^+$ to the exterior of the cell following PMA stimulation was assessed over a pH range of 6-8. $^{86}\text{Rb}^+$ release (■) but not superoxide generation (□) was inhibited by elevating the pH to 8 and above.

Buffer pH and cytosolic pH (in brackets) are shown.

All results are the mean ± S.E. of at least three experiments.

Methodology section 2.10b and 2.11b.
therefore, as illustrated in Figure 3.13, the external pH had little effect on the cytosolic pH.

3.6.4 Conclusion.

In conclusion, the amount of K⁺ entering the vacuole is linked to granule contents released into the vacuole. The more granule contents released, the more the pH is depressed and the greater the buffering capacity. A parallel increase in the proportion of the charge compensated for by K⁺ would be required before transport of this ion would be blocked by an elevation in pH.

3.7.0 K⁺ influx renders the vacuole hypertonic.

The following sets of calculations were carried out to quantify the concentration of K⁺ within the vacuole; this was then crosschecked against the concentration detected by X-ray microanalysis.

Upon stimulation, 10⁷ neutrophils produce about 300 nmols of O₂⁻ within 3 min. (Figure 3.7 & 3.8). With a cellular volume of 345 μm³ and a K⁺ concentration of 125 mM, 2.9x10⁹ cells occupy 1 ml, and contain 125 μmols of K⁺, of which, judging from the ⁸⁶Rb⁺ measurement, they would release approximately 4.5%, or 5.6 μmols. Over the same time they would produce about 87 μmols of O₂⁻, a molar ratio to K⁺ secretion of approximately 15:1. Extrapolation from the generation of 4 mols/l of O₂⁻ in the vacuole would predict levels of K⁺ in the vacuole of about 270 mM, very similar to those measured by X-ray microanalysis (200-300 mM). The charge of each electron that is compensated by K⁺ generates one OH⁻, so the levels of OH⁻ that would be
produced roughly accord with those found to elevate the pH in the presence of
the predicted concentration of granule contents (0.5g/ml).

3.8.0 Time scale of hypertonicity.

3.8.1 Introduction

Tonicity in the vacuole must initially be high. The K$^+$ (270mM) driven into the
vacuole by the oxidative burst is added to the predominantly 150mM NaCl,
incorporated from the extracellular medium when the vacuole forms. This would
be expected to cause osmotic swelling of the vacuole. Vacuolar swelling coupled
with a failure of enlargement of CGD vacuoles was observed, but only briefly
discussed by Nathan et al.$^{353}$ in 1969 and addressed later by Segal et al. in
1981$^{161}$. Therefore an experiment was designed to compare oxidase activity with
the volume of the vacuole.

3.8.2 Vacuolar volumes.

To estimate the volume, cross-sectional areas of phagocytic vacuoles were
determined from electron micrographs. Phagocytosis was performed over a time
course of up to 16min. The result is illustrated in Figure 3.14a. The volume of
the vacuole, and the vacuolar volume minus that of the bacteria are plotted. The
respiratory burst was essentially complete by 4 min, as measured by oxygen
consumption and the volume of the vacuole during this time was maintained at
approximately 0.2$\mu$m$^3$. Tonicity in the vacuole during this time must initially be
high and dilution of these salts only occurs later, as swelling of the vacuoles is
seen well after the respiratory burst has ended. Swelling to more than double
FIGURE 3.14. Superoxide generation is associated with vacuolar swelling.

Electron micrographs were examined at different times after phagocytosis of *S. aureus*. Result (a) shows the time course of changes in the volume of the entire phagocytic vacuole (■) and the intervening space, (vacuolar volume minus bacterial volume), (□).

(b) illustrates the changes in the bacterial volume.

n=24, 43, 65, 45 and 24 vacuoles at 1, 2, 4, 8 and 16min respectively.

The bacteria swelled significantly between times 8 and 16min, p<0.05 by Student t test.

The time course of oxygen consumption is shown (- -o- -) and the respiratory burst was essentially complete by 4min.

Methodology section 2.3d and 2.7a.
their original size indicates that the salt concentration in the vacuoles is likely to have reached at least twice normal, or 300mM.

An independent indication of elevated vacuolar tonicity in normal vacuoles is provided by the observation that the ingested bacteria act as osmometers, shrinking to about half their original volume (Figure 3.14b), possibly after enzyme induced permeabilisation of their capsules. After 16 min, when the vacuoles are swollen by the influx of water, and tonicity is diminished, the bacteria partially regain their original size.

To show that this high ionic strength was partly due to K⁺ efflux into the vacuole, acting as a compensating charge for oxidase activity, the same experiment was repeated in the presence of DPI, the oxidase inhibitor or valinomycin, the K⁺ ionophore. The result of this experiment is illustrated in Figure 3.15. Both vacuolar swelling and bacterial shrinkage were inhibited by DPI, and by valinomycin, confirming their dependence upon the oxidase and a K⁺ gradient.

3.8.3 Delayed vacuolar swelling resolved.

To understand why swelling of the vacuole did not occur immediately it was first proposed that movement of water into the vacuole was restricted by the vacuolar membrane, as the vacuolar and plasma membranes are known to have distinct protein compositions. Membranes are permeable to H₂O which passes through aquaporins. The delayed swelling was first thought to be due to inactivation or the exclusion of aquaporins from the vacuolar membrane as it formed. To investigate the latter possibility, quantitation of aquaporin 9 (AQP9), one of the two aquaporins
FIGURE 3.15. Vacuolar swelling and its control.

The effect on the size of the vacuoles, of exposing cells to valinomycin (Val, 3μM, n=106), DPI (5μM, n=44), and jasplakinolide (Jas*, 1μM, n=74) compared with untreated cells (Con, n=111) at 16min after phagocytosis of S. aureus.

Results show the volume of the entire phagocytic vacuole, S. aureus and the intervening space, (vacuolar volume minus bacterial volume).

All additions resulted in highly significant differences (p<0.001) from control cells.

* The effect of jasplakinolide will be discussed later.

Methodology section 2.7a.
found in humans using polyclonal antibodies was attempted. Unfortunately by western blotting, no signal was detected for AQP9.

A second approach was taken to determine whether the vacuolar membrane was unusually impermeable to water. This involved coherent anti-Stokes Raman scattering (CARS) laser-scanning microscopy, probing for intracellular hydro-dynamics\(^{336}\). Neutrophils were allowed to adhere to a glass slide following phagocytosis of ANTS fluorescent labelled \textit{C. albicans}. The slide was placed in a perfusion chamber, which was rapidly flushed with isotonic D\(_2\)O containing phosphate buffered NaCl. During the process of H\(_2\)O/D\(_2\)O exchange, the interior and cytoplasm of a single cell vacuole, was imaged by repetitive scanning. The results illustrated in Figure 3.16, showed the membrane surrounding the vacuole to be permeable to H\(_2\)O, with a permeability constant of 6 \(\mu\)m/s or higher, roughly similar to that of the plasma membrane of \textit{D. discoideum} cells\(^{336}\). Thus the delayed swelling of the vacuole was clearly not a result of the impermeability of the vacuolar membrane to water. Another explanation was required.

### 3.8.4 The vacuole is constrained by a surrounding meshwork of cytoskeletal proteins.

Fluorescence microscopy was used with antibodies to neutrophil cytoskeletal proteins to follow the distribution of the cytoskeleton in phagocytosing cells, as it had previously been observed by confocal microscopy that vacuoles in macrophages were surrounded by a constraining meshwork of cytoskeleton\(^{357}\), including two cytoskeletal proteins, paxillin and vinculin. It has previously been
FIGURE 3.16. The use of Coherent anti-Stokes Raman scattering (CARS) laser scanning microscopy to determine the water permeability of the vacuolar membrane.

(a) CARS image of neutrophil with superimposed fluorescence image of ANTS-labelled phagocytosed, *C. albicans*.

(b) Real time permeability experiment showing the CARS signal from water on left and the ensuing drop in signal after rapid exchange (arrow) of aqueous medium against isotonic D₂O buffer. Solid lines mark the plasma membrane of the cell and dashed lines indicate the edges of the intracellular vacuole.

(c) Dynamics of water efflux from the vacuole upon rapid H₂O/D₂O exchange. Dots refer to experimental data points. The solid line fitted to the data reveals an efflux time of H₂O from the vacuole of about 0.1s.
demonstrated that vinculin promotes cytoskeletal alterations, associated changes in cell shape and binds directly to F-actin and paxillin\textsuperscript{358}. In this study fluorescein labelled \textit{S. aureus} were used to identify vacuoles within the cells. Antibodies to the cytoskeletal proteins paxillin and vinculin were visualized using rhodamine labelled secondary antibodies. The result illustrated in Figure 3.17, clearly shows the distribution of the two proteins to be similar with pronounced accumulation around the phagosome, strongly suggesting a restraining role preventing swelling.

Quantification of fluorescence intensity demonstrated the initial accumulation of the cytoskeletal proteins around the vacuole, with highest intensity at 4min when tonicity within the vacuole must be at its highest. At 8 and 16min the cytoskeletal proteins slowly disperse as swelling proceeds (Figure 3.18).

A further experiment employed jasplakinolide\textsuperscript{359,360}, the membrane-permeable fungal toxin and potent inducer of actin polymerization \textit{in vitro}. Upon inclusion of this toxin normal cytoskeletal rearrangements were prevented as actin filaments were stabilized and swelling of the vacuoles was prevented (Figure 3.15). This result confirmed the importance of the neutrophil cytoskeleton in creating specific phagosomal conditions. By constraining the volume and preventing movement of water into the vacuole the cytoskeleton is indirectly maintaining a hypertonic vacuolar environment, the importance of which will be addressed in the next chapter.
FIGURE 3.17. The neutrophil cytoskeleton surrounds the phagocytic vacuole.

Fluorescein-labelled S. aureus (approximate diameter of 0.8μm) in phagocytic vacuoles (green, left) of neutrophils stained for vinculin or paxillin (red, middle panels). These cytoskeletal proteins surround the vacuole (composite images, right).

Methodology section 2.7b.
**Figure 3.18.** The cytoskeleton is responsible for delayed vacuolar swelling.

The amounts of vinculin and paxillin around the vacuole diminishes with time as swelling occurs.

Each time point is the mean ±S.E. of 50 measurements.

Methodology section 2.7b.
3.9 CONCLUDING REMARKS.

The recognition of invading microorganisms by neutrophils results in the phagocytic internalization and sequestration of the pathogen into an enclosed phagocytic vacuole. Bacterial killing occurs within the first 2-4 minutes\textsuperscript{161} of ingestion and experiments were designed to evaluate the conditions prevailing within the vacuole within this time.

Groundwork established vacuolar volumes, superoxide concentrations and granule protein concentrations entering the vacuole. Individual phagocytic vacuoles were examined and volumes estimated at 0.2\(\mu\)m\(^3\), consequently an immensely high concentration of 4 moles/l of intravacuolar superoxide was calculated for each bacterium engulfed. By isolating vacuoles, it was possible to quantitate the concentration of granule protein entering the vacuole. This also proved to be enormously high at 0.5 g/ml.

It has long been known that the formation of superoxide via the NADPH oxidase is an electrogenic process, with the transfer of electrons, ultimately resulting in increased levels of OH\(^-\) and an increase in vacuolar pH from about 6.0 to 7.8-8.0 soon after phagocytosis\textsuperscript{161,162}. This change in pH occurs despite the entry of acidic granule contents into the phagocytic vacuole\textsuperscript{343}. In order for the neutrophil to compensate for the massive influx of electrons into the vacuole, a balancing charge movement must occur, and this has largely been thought to be by way of a compensatory H\(^+\) influx\textsuperscript{154}. Compensation of an electron by a proton would not alter the pH and therefore would prevent the observed increase in pH. Within the work described, it was shown that charge compensation is also due to K\(^+\) entering the vacuole. Studies on the effect of valinomycin, a specific K\(^+\) ionophore and of 4-aminopyridine, a non-specific K\(^+\) channel blocker were
performed and correlated the activity of the oxidase with activity of the K\(^+\) channel.

The proportion of K\(^+\) as opposed to H\(^+\) entering the phagocytic vacuole is determined by the vacuolar pH, as activity of the K\(^+\) channel and K\(^+\) movement is reduced as the pH rises above 8.0. In addition the entry of acidic granule protein (0.5g/ml) into the vacuole also buffers pH changes and therefore the entry of K\(^+\) also appears to be linked to the amount of degranulation within the vacuole.

Electron probe X-ray microanalysis was used to obtain a direct estimate of the K\(^+\) concentration within the vacuole, which proved to be in the range of 200-300mM. Following phagocytosis the phagocytic vacuole swells, an effect that is inhibited by DPI and valinomycin, respectively, suggesting that the swelling depends on both superoxide production and high concentration of K\(^+\) in the vacuole. Thus the vacuole creates a hypertonic environment that is maintained by a cytoskeletal meshwork. This hypertonic environment persists for the duration of oxidase activity and the time required for bacterial killing\(^{161}\).

An encouraging prospect was the possibility of an additional consequence of the elevated K\(^+\) concentration within the vacuole. The clue to this was the appearance of vacuoles of normal cells, which were impressively different to those of patients with CGD. The granule contents were uniformly dispersed throughout the normal vacuoles, but remained clumped in most of the CGD vacuoles. The next chapter provides data that it is the hypertonic environment created by the K\(^+\) entry that solubilizes the bacterial proteases and allows them to participate in the killing process within the vacuole.
CHAPTER 4.

4.0 THE IMPORTANCE OF K⁺ WITHIN THE VACUOLE.

4.0 A BRIEF SUMMARY OF CONTENTS.

The primary role of superoxide appears to be indirect, by solubilizing the proteolytic enzymes released into the vacuole from the cytoplasmic granules.

From the results obtained and described in the previous chapter, it was evident that as a consequence of superoxide production, K⁺ is channelled into the vacuole by the electrogenic NADPH oxidase. Proceeding data provided evidence, that it is the highly hypertonic environment engendered by the K⁺ entry that solubilizes the cationic granule enzymes from anionic proteoglycan matrixes and allows them to participate in the killing response.

In support of this theory treatment of neutrophils with the K⁺ ionophore, valinomycin or protease inhibitors resulted in decreased killing despite equal phagocytosis of staphylococci. A result, which equates the importance of increased K⁺ levels in the vacuole with that of granule proteases in microbial killing.

In vivo experiments illustrated the significance of serine proteases as mice lacking elastase and cathepsin G or CGD mice lacking the p47phox component of the NADPH oxidase, succumbed equally to staphylococcal and candidal infection, despite the protease deficient mice producing normal amounts of superoxide. This result demonstrated the importance of both oxidase activity with O₂⁻ production, and protease activity, in microbial killing in vivo.
Results demonstrate that a major consequence of the defective function of the NADPH oxidase in neutrophils from CGD patients is an absence of K⁺ influx into the vacuole. As a result the normal solubilization and activation of granular enzymes is not apparent.

Results also uncover a previously unsuspected mechanism of antimicrobial activity in the phagocytic vacuole associated with K⁺ influx. As previously shown measurements of pH within the vacuole illustrate an elevation to the pH optima of serine proteases elastase and cathepsin G. This rise in pH was abrogated in the presence of valinomycin and DPI, illustrating its dependence upon K⁺ influx and a functional NADPH oxidase respectively.

4.0 INTRODUCTION.

The chief view held, is that neutrophils kill ingested microorganisms by subjecting them to high concentrations of highly toxic ROS and myeloperoxidase-catalysed halogenation. The most convincing evidence was provided by CGD patients who have a profound susceptibility to bacterial and fungal infection and the inability of phagocytes from these individuals to generate ROS. Thus, according to this prevailing ROS model, lack of proteases should not affect the ability of neutrophils to kill bacteria. Earlier studies have however shown a requirement for neutrophil elastase to kill some gram-negative bacilli, and of both cathepsin G and elastase to protect against infection with Aspergillus fumigatus.

To clarify the importance of granule proteases in host defence, in vivo murine infection studies were carried out to investigate killing of microbes that
are generally accepted as requiring oxygen dependent killing mechanisms for eradication.

Experiments investigated the possibility that activation of these proteases depends on the influx of $K^+$ into the phagocytic vacuole. The granules of neutrophils can be described as regulated storage organelles of proteolytic and bactericidal proteins that are kept in store until liberated either to the outside of the cell or to the phagocytic vacuole. The structure of the neutrophil granule is pivotal in its function of packaging and storage of microbicidal proteins. The composition of granules has been identified to contain sulphated proteoglycans, including chondroitin and heparin sulphate$^{273-275}$. The interaction between the strongly anionic proteoglycan matrix and the cationic proteases renders the granule proteins in a bound state in which they cannot reach the ingested microbe and must be solubilized to become functional. Work presented shows that this solubilization and activation is brought about by the hypertonic $K^+$, driven into the vacuole by the NADPH oxidase.

In addition, the $K^+$ may enter into the killing process by regulating the phagosomal pH. The latter is under strict regulation and NADPH oxidase activity, results in the critical elevation of pH to 7.8-8.0$^{161,162}$, optimal for bacteriolysis by granule proteins. Results will be presented which clearly show, a second way in which $K^+$ enters the killing process is enabling the pH in the vacuole to rise as it substitutes for $H^+$ in compensating the potential difference across the membrane generated by the oxidase.
4.0 RESULTS AND DISCUSSION.

4.1.0 Susceptibility of cathepsin G and elastase deficient mice to microbial infection.

4.1.1 Introduction.

The aim of the first set of experiments was to investigate the importance of granule proteases in killing of microbes that are generally accepted as requiring oxygen dependent killing mechanisms for eradication. *Staphylococcus aureus* (*S. aureus*), a gram-positive bacterium, was chosen because it is the archetype organism requiring oxygen dependent killing and the most commonly cause of infection in CGD. *Candida albicans* (*C. albicans*) was selected as mice lacking myeloperoxidase and subsequently the production of HOCl, have been shown to be sensitive to this infectious microbe. The importance of serine proteases, cathepsin G and elastase, to clear systemic infections of these two organisms was investigated.

4.1.2 Murine in vivo studies.

In protease deficient mice, elastase and cathepsin G are absent. The CGD mice were deficient in the p47phox cytosolic component of the NADPH oxidase. The results of intravenous injections of *S. aureus* and *C. albicans* on the survival of protease deficient and CGD (*S. aureus* only) mice are illustrated in Figure 4.0. The mice were infected with a dose sublethal to wild-type mice and the survival was monitored. As shown wild type mice were resistant to infection with *S. aureus* (4x10^7) and *C. albicans* (10^6), whereas both organisms were much more virulent in the mice lacking both cathepsin G and elastase. In fact mice deficient in both proteases tolerated the microbial challenge as badly as CGD mice did.
**FIGURE 4.0.** Susceptibility of protease deficient mice to bacterial and fungal infection.

Survival probability plots (Kaplan-Meier) of wild type mice (WT, n=32), neutrophil elastase (E, n=12), cathepsin G (C, n=12), elastase and cathepsin G (Dual, n=31) and p47phox deficient (CGD, n=24) following intravenously injection with $4 \times 10^7$ *S. aureus* (a) or $1 \times 10^4$ *C. albicans* (b) (WT, n=9; E, n=8; C, n=7; Dual, n=8).

The rates of decreased probability of survival for all mice were significantly different (p<0.05) from WT except for elastase deficient in (a) and cathepsin G deficient in (b) where p=0.1.

Methodology section 2.12c
This result demonstrated the importance of both proteases in providing full protection against the invading microorganisms. Interestingly, there was a difference in the sensitivity to the two microbes of mice lacking one or other of these enzymes. Cathepsin G deficient mice resisted \textit{C. albicans} but not \textit{S. aureus}, whereas elastase proved important for the successful clearance of \textit{C. albicans}.

\subsection*{4.1.3 Murine in vitro studies.}

It was important to extent these studies to exclude the possibility that susceptibility to infection in mice deficient in elastase and cathepsin G \textit{in vivo}, was not a result of a defect in phagocytosis, degranulation, oxidase activity or, a result of another abnormality of the immune response. Therefore the activity of purified thioglycolate elicited neutrophils was determined \textit{in vitro}. To achieve this, an iodination test was performed as described by Klebanoff (1976)\textsuperscript{219}. The method requires normal MPO activity, the production of hydrogen peroxide by the oxidase system and an oxidizable cofactor such as Cl\textsuperscript{-} or in this case, \textsuperscript{125}I\textsuperscript{-}.

The result of this experiment, (Figure 4.1), shows that the iodination patterns of wild-type and protease deficient neutrophils are identical over time. Thus neutrophils deficient in cathepsin G and elastase have a functional NADPH oxidase and therefore possess oxidative mechanisms of killing including the full complement of ROS. Conversely, the lack of iodination detected for CGD neutrophils is characteristic of a nonfunctional NADPH oxidase\textsuperscript{363}.
FIGURE 4.1. Normal neutrophil function in mice deficient in elastase and cathepsin G.

Time course of iodination by thioglycolated elicited neutrophils from mice deficient for both elastase and cathepsin G (Dual), p47^{phox} deficient (CGD) and control wild type (WT).

Each measurement is the average of triplicate experiments and error bars shown ± S.E.
The method employed is as described by Klebanoff & Clark (1976).

Methodolgy section 2.3e.
4.1.4 Murine in vitro killing assays.

To ensure that the susceptible mice succumbed through defective intracellular killing, rather than as a result of another abnormality of the immune response, the microbicidal activity of purified neutrophils was determined in vitro. Under the same experimental conditions, used for quantification of iodination levels, killing of *S. aureus* and *C. albicans* by purified mouse neutrophils or by neutrophils deficient in both elastase and cathepsin G was determined. The results are illustrated in Figure 4.2. The pattern of killing was almost a mirror image of the in vivo susceptibility (see Figure 4.0). Elastase deficient neutrophils killed *S. aureus* well and *C. albicans* weakly, whereas the converse was true with cathepsin G deficiency. CGD cells or those lacking both enzymes killed both organisms poorly.

4.1.5 Conclusion of infection studies in protease deficient mice.

Bacterial killing was defective in cells from CGD mice and mice made deficient in elastase and cathepsin G, suggesting that both oxidase activity and protease action are necessary to destroy *S. aureus* and *C. albicans*, the two organisms which had previously been thought to be killed primarily through oxidative mechanisms. Thus, the activation of granule proteins of neutrophils is imperative for the generation of sufficient microbicidal activity to control yeast and staphylococcal infection in vivo.

4.1.6 In vitro killing by human neutrophils.

In the light of the information obtained, experiments were extended to investigate bacterial killing by human neutrophils in the presence of the NADPH
FIGURE 4.2. *In vitro* microbicidal activity of mouse neutrophils.

Killing of *S. aureus* (0.5x10^7 CFU/ml) (a) or *C. albicans* (0.25x10^7 CFU/ml) (b) by mouse neutrophils (2.8x10^7/ml) of wild type (WT), p47^phox^ deficient (CGD), elastase (E), cathepsin G (C) or both (Dual).

Killing rates for all mice were significantly different from WT (p<0.01) except for elastase deficient in (a) and cathepsin G deficient in (b) where p=0.4 and 0.9 respectively.

Killing was measured as described by Segal *et al.*, (1981). Described in methodology section 2.12b.
oxidase inhibitor, DPI or added protease inhibitors. The addition of protease inhibitors was intended to prevent microbial killing via the nonoxidative mechanism, by inhibiting the activity of the granule proteases.

Oxygen consumption following bacterial phagocytosis was determined to ensure that added protease inhibitors had no adverse effect on neutrophil function. Total oxygen consumption for control and treated cells was 165 nmoles per $10^7$ cells. The result of microbial killing is illustrated in Figure 4.3 and as expected bacterial killing was defective in cells from a CGD patient and also in cells treated with DPI. Similarly, protease inhibitors impaired the ability of human neutrophils to kill *S. aureus*. These observations confirm that both oxidase activity and protease action are necessary to destroy this organism.

4.2.0 Hypertonicity activates proteases.

4.2.1 Introduction

Results of electron probe X-ray microanalysis estimated the concentration of $K^+$ entering the vacuole at 200-300 mM (section 3.5.0 and Figure 3.11). To assess whether this influx of $K^+$ is involved in bacterial killing, we measured killing by isolated neutrophils in the presence of valinomycin. The effect of valinomycin would allow $K^+$ to leak from the vacuole and cytoplasm into the medium. Electron probe X-ray microanalysis calculated the concentration of $K^+$ entering the vacuole in the presence of valinomycin to be no higher than the cytosolic $K^+$ concentration (125 mM). Killing of *S. aureus* by normal neutrophils, in the presence or absence of valinomycin was compared to CGD cells.
FIGURE 4.3. The importance of both ROS and proteases for successful bacterial killing.

Killing of S. aureus (1x10^8 CFU/ml) by human neutrophils (1x10^8/ml) was assessed in the presence or absence (Control) of protease inhibitors (PI) and compared to p47phox deficient (CGD) or cells in which the oxidase had been inhibited by 5μM DPI.

Rates of bacterial killing by CGD, DPI and PI treated cells were not significantly different, but all differed from control cells p<0.01.

Methodology section 2.12d.
4.2.2 Effect of valinomycin on neutrophil function.

It was first important to prove that the addition of valinomycin did not impair neutrophil (human) phagocytosis, degranulation, H$_2$O$_2$ generation or MPO activity, therefore any defect in bacterial killing would be a direct result of reducing the level of K$^+$ in the vacuole.

Normal neutrophil function was confirmed using iodination quantification, employing techniques exactly as already described (see 4.1.3 and methodology 2.3e). Iodination results are illustrated in Figure 4.4, and show equal iodination levels for normal and valinomycin treated cells. In the absence of a respiratory burst, as is the case with CGD neutrophils, an increase in iodination over time is not apparent. The result clearly showed that valinomycin did not affect, uptake, degranulation or MPO activity of the neutrophil.

4.2.3 The effect of valinomycin on neutrophil bacterial killing.

The importance of elevated K$^+$ levels within the vacuole was addressed by assessing the ability of neutrophils to kill bacteria in the presence of valinomycin. The latter reduced the level of K$^+$ in the vacuole (section 3.5.2).

Killing of *S. aureus* by neutrophils was carried out as described (methodology section 2.12d) and the result is illustrated in Figure 4.5. Valinomycin on its own had no effect on the viability of the bacteria. It was found however that valinomycin suppressed neutrophil bacterial killing to the low level seen in CGD cells. Rates of bacterial killing by CGD and valinomycin treated cells were not significantly different, but both differed from control cells (p<0.01).
FIGURE 4.4. Normal neutrophil function in cells treated with valinomycin.

Time course of iodination by neutrophils of a normal control (Con) in the presence and absence of valinomycin (Val, 3μM) and compared to p47phox deficient (CGD).

Each measurement is the average of triplicate experiments and error bars shown ± S.E.

The method employed is as described by Klebanoff & Clark, (1976).

Methodology section 2.3e.
FIGURE 4.5. The importance of K⁺ for successful bacterial killing.

Killing of *S. aureus* (1x10⁸ CFU/ml) by human neutrophils (1x10⁸/ml) was assessed in the presence of valinomycin (Val, 5µM) and compared to killing by normal (Control) and p47^phox^ deficient (CGD) neutrophils.

Rates of bacterial killing by CGD and valinomycin treated cells were not significantly different, but both differed from control cells p<0.01. The effect of valinomycin on bacterial viability is also shown (◊).

Methodology section 2.12d.
The result of this experiment provided evidence for the importance of the elevated $K^+$ within the vacuole, but the question next asked, was how might the $K^+$ enter the killing process?

4.3.0 *The cationic granule proteins bind strongly to proteoglycans.*

4.3.1 *Introduction.*

The granules comprise a strongly anionic sulphated proteoglycan matrix to which the cationic proteases are tightly bound\(^3\). Release from this matrix is necessary for these enzymes to interact functionally with the ingested microorganism and the following experiments will demonstrate a functional role for $K^+$ in affecting this release.

4.3.2 *Interaction of granule proteins and proteoglycan matrix.*

Biocore studies were designed to study the interaction of the granule proteins with heparin proteoglycan matrix. Binding of purified human neutrophil granule proteins MPO, lactoferrin, elastase, cathepsin G and lysozyme was measured. Surface plasmon resonance plots are illustrated in Figure 4.6. The high rate at which the proteases bound to the immobilised heparin, and reached equilibrium, is evident. Lysozyme did not bind to the heparin proteoglycan matrix, an observation that is supported by work by Olsson & Venge (1974), who showed by electrophoretic mobility, that lysozyme is less cationic than other granule proteins\(^2\).

The work that follows proves that displacement or solubilization of the granule proteins is brought about by the hypertonic $K^+$ that is driven into the vacuole by the NADPH oxidase.
FIGURE 4.6. Binding of purified granule proteins to heparin proteoglycan matrix.

Surface plasmon resonance plots of binding of pure MPO (MPO, $K_d$ 20.4 nM), cathepsin G (C, $K_d$ 5.3nM), lactoferrin (L, $K_d$ 28.3nM), elastase (E, $K_d$ 8.9nM) to heparin proteoglycan. Lysozyme (LY) did not bind significantly. All proteins at 4μg/ml.
4.3.3 Elevated ionic strength solubilizes protease.

The aim of the next experiment was to show solubilization of the granule proteins from anionic proteoglycan matrix in the presence of increasing concentrations of $K^+\). 

To imitate physiological conditions it was necessary to use granules devoid of their membranes, because, as degranulation occurs the granular membrane fuses with the vacuolar membrane. Granule membranes were removed by a simple one step method based on the granule damaging capacity of Percoll (see methodology section 2.6a)$^{335}$. 

By subjecting isolated demembraned granules to increasing concentrations of KCl, the concept of hypertonic potassium being responsible for the solubilization and activation of granule proteins was challenged. The result is illustrated in Figure 4.7 and argues a parallel between K$^+$ concentration and protein displacement. In fact, almost all proteins are solubilized from the proteoglycan matrix at the concentration of K$^+$ predicted in the vacuole (200-300mM).

Solubilized proteins were analyzed by mass spectrometry. Figure 4.8, shows the MALDI-TOF spectrum of the tryptic digests. Database searching with these mass fingerprints showed these proteins to be lactoferrin, MPO, cathepsin G and elastase.

It has previously been shown that mouse mast cell protease 7 (mMCP-7) possess a strong positive electrostatic potential in the acidic pH of the granule which allows it to interact with the proteoglycan$^{364}$. As this binding requires positively charged His residues, native mMCP-7 is able to dissociate from the protease/proteoglycan macromolecular complex when the complex is
FIGURE 4.7. Most of the granule protein is solubilized at the K\(^+\) concentration achieved in the vacuole.

(a) is a Coomassie stained gel illustrating release of granule proteins with increasing KCl concentrations. Solubilized proteins were identified by MALDI-TOF.

(b) MPO, cathepsin G / elastase and lactoferrin were released from the granule matrix at elevated salt concentrations (granules 20mg/ml). Solubilization is expressed as a % of total granule protein.

Methodology section 2.8a.
**FIGURE 4.8.** Hypertonic K⁺ driven into the vacuole solubilizes granule proteins. Granule proteins were eluted with increasing concentrations of KCl (a). Proteins were cut from the gel, tryptic digested and subjected to MALDI-TOF mass spectrometry (b, c, d & e). Proteins were identified by comparing mass fingerprints to NCBI’s database. Methodology section 2.2d & e.
exocytosed from bone marrow-derived mast cells into a neutral pH environment. The pH in the vacuole is elevated as high as pH 7.8 to 8.0, thus the effect of pH on protease solubilization was also investigated. Under similar experimental conditions, granules were suspended in HEPES Buffer of increasing pH. Detection of displaced proteins was by western blotting and results are illustrated in Figure 4.9. It can be seen that elevated pH on its own was without effect, with no increase in the amount of soluble MPO and lactoferrin from pH 6.0 – 8.5. Undetectable amounts of elastase and cathepsin G were solubilized.

4.3.4 Solubilization by $K^+$ accompanies protease activation.

The previous experiment clearly demonstrated the elution of granule proteins from proteoglycan matrixes by $K^+$. The next experiment shows that once solubilized the granule enzymes are activated.

The bactericidal effect of purified neutrophil granules against $S.\ aureus$, in the presence and absence of $K^+$ was investigated. The experiment was also repeated, by purifying the granules in the presence of protease inhibitors.

The result presented (Figure 4.10) shows that granules alone were not bactericidal, and high concentrations of KCl and protease inhibitors had no effect on bacterial viability. However granules became bactericidal when exposed to KCl of two different concentrations (200 and 400mM). Under these conditions the proteases, cathepsin G and elastase are solubilized (see Figure 4.7&4.8). This killing effect was reversed in the presence of protease inhibitors, but only partially abrogated at the higher KCl concentration. The fact that some of the antimicrobial activity of the cationic proteins has been shown to be
FIGURE 4.9. Elevated pH had no effect on the solubilization of granule protein.

Granules (Gran) were resuspended in Hepes Buffer of indicated pH and incubated at 37°C for 2 min. Following centrifugation, the supernatant was analysed for solubilized proteins.

(a) is a Coomassie stained gel, illustrating the limited extent of granule protein solubilization and (b), western blots probed for lactoferrin, MPO, cathepsin G and elastase.

Volumes were equalized and 1μl of granules and 20μl of eluted protein loaded.

Methodology section 2.8a.
**FIGURE 4.10.** K⁺ elutes and in turn activates proteases.

The effect of purified demembranated neutrophil granules (44mg/ml), in the presence and absence of KCl and/or protease inhibitors (PI) on the viability of *S. aureus*.

All results are the mean ± S.E. of 3 different measurements.

Methodology section 2.9b.
independent of their enzymic function could account for this inhibitor resistant killing\textsuperscript{164}.

Further evidence that activation of granule proteins accompanies solubilization by K\textsuperscript{+} is seen in an experiment where granules were exposed to 350mM KCl and solubilized proteins were recovered in the supernatant following centrifugation. Exposure of \textit{S. aureus} to the solubilized granule proteins resulted in killing of over 60\% of the bacteria. The rebinding and clearance of the solubilized proteins, to heparin sepherose prior to bacterial exposure inhibited this killing (\textbf{Figure 4.11}).

In an additional experiment K\textsuperscript{+} solubilized granule proteins were separated according to size by gel filtration, as illustrated in \textbf{Figure 4.12}. The bactericidal property of each fraction was tested and results show that killing can be attributed to the combined effect of each granule protein, however high levels of activity against \textit{S. aureus} at pH 7.5 was retrieved in fraction 15 & 16. The major components of fraction 15 & 16 were the neutral proteases elastase and cathepsin G, but small amounts of BPI, the 37 kDa cationic protein and other granule proteins are probably also present. Further studies will be necessary to determine the individual components responsible for killing. This experiment was not performed with a source of H\textsubscript{2}O\textsubscript{2} for MPO peroxidase activity and therefore only addresses the non-oxidative mechanisms of bacterial killing.

\textit{4.3.5 Conclusion.}

It has previously been published that neutrophils from CGD patients are unable to damage fungal hyphae significantly \textit{in vitro}, while purified cationic neutrophil granule proteins are able to do so\textsuperscript{365}. This suggests that neutrophil serine
FIGURE 4.11. Bacterial killing is inhibited in the presence of anionic heparin sepharose.

Exposure of *S. aureus* (1×10⁷ CFU/ml) to granule eluate obtained by KCl (350mM) treatment of demembraned granules (25mg/ml) resulted in reducing the bacterial colony count. This microbicidal effect was reversed by re-absorption of eluted proteins on heparin sepharose.

KCL (control) had no effect on bacterial viability.
Each point is the mean ± S.E. of three separate measurements.

Methodology section 2.9b.
**FIGURE 4.12.** Microbicidal activity of solubilized granule proteins.

Demembraned granule proteins (44mg/ml) were exposed to 300mM KCl and the resulting solubilized proteins (St), fractionated by Gel Filtration in PBS pH 7.8 plus 500mM NaCl (Buf).

(a) the protein profile were visualized by Coomassie stained SDS-PAGE. Molecular mass markers are shown on the left.

(b) exposing *S. aureus* (1x10^7 CFU/ml) for 6min in the same buffer at 37°C assessed the bactericidal properties of each fraction. Buffer alone had no effect on bacterial viability. The entire experiment was plated on LB agar in triplicate and the value reported is the mean ± S.E.

Methodology section 2.9b.
proteases may remain latent in neutrophils until activated by products of the respiratory burst. Our studies lend further support to this theory. The granules comprise a strongly anionic sulphated proteoglycan matrix to which the cationic proteases are tightly bound, as evidenced by the high rate at which proteases bound to the heparin on the sensor chip and reached equilibrium. In this state they are inaccessible to the ingested microbe, and must be solubilized to become active.

The work presented proposes that this activation is brought about by the hypertonic K\(^+\) that is driven into the vacuole by the NADPH oxidase. Experiments successfully show that most of the granule protein is solubilized at the K\(^+\) levels predicted in the vacuole. Granules alone were not bactericidal, but became so when exposed to KCL, an effect that was partially abrogated in the presence of protease inhibitors and re-absorption of eluted granule proteins on heparin sepharose.

4.4.0 Lack of solubilization of granule proteins in CGD neutrophils.

4.4.1 Introduction.

As the mechanism of granule protease solubilization and activation described involves regulation by hypertonic K\(^+\) driven into the vacuole as a result of oxidase activity, work according examined solubilization of granule proteins in neutrophils of CGD patients or in cells treated with the NADPH oxidase inhibitor DPI.
4.4.2 Purification and polyclonal antibody production of granule proteins.

As studies in this section, required analysis of granule proteins by western blotting, polyclonal antibodies were prepared by immunizing rabbits with purified proteins.

MPO, lactoferrin, cathepsin G, elastase and lysozyme were purified from granules by conventional chromatography (methodology section 2.4a & 2.5a). The latter four proteins were retained on Fast Flow S-Sepharose with one further purification step required for the isolation of pure lactoferrin, cathepsin G and lysozyme on Mono S (Figure 4.13). Elastase required a final purification step by Gel filtration as illustrated in Figure 4.14.

MPO was fully retained on Fast Flow S-Sepharose with peak fractions further chromatographed on Mono S resulting in essentially pure protein. The ratio between the absorption at the Soret peak and that at 280nm (the RZ value) is commonly used as a criterion of purity of haem peroxidases. The final MPO preparation had an RZ value of 0.82, a value very similar to that expected for the pure enzyme. Figure 4.15 (a) is a Coomassied gel of the final purified proteins. MPO appeared as two bands, at 60 and 12 kDa, as previously described. The protein bands were analysed by mass spectrometry which identified them successfully as the individual proteins.

Polyclonal antibodies were prepared by immunising rabbits with 100μg of purified protein. Specificity of affinity purified antibodies was confirmed by western blotting of whole neutrophil cell lysate, as illustrated in Figure 4.15 (b).
Material for the first purification step was obtained from neutrophil granules. This start material (st) was loaded onto Fast Flow S-Sepharose, with almost all the material binding as evident from the small amount of protein present in unbound (un). As illustrated separate fractions were taken for further FPLC chromatography for the successful isolation of lysozyme, lactoferrin and cathepsin G. Proteins were visualized by Coomassie stained SDS-PAGE. Molecular mass markers are shown on the left.
FIGURE 4.14. Purification of cationic granule proteins, part II.

Fractions from the original Fast Flow S-Sepharose (Figure 4.13) were taken for further FPLC chromatography by heparin agarose and gel filtration for the successful isolation of neutrophil elastase.
Figure 4.15. Final purified proteins and specificity of polyclonal antibodies.

(a) Coomassie stained SDS-PAGE of purified neutrophil granule proteins. Molecular mass markers are shown on the left. Final protein concentrations were estimated; lactoferrin (0.5mg/ml), MPO (5.0mg/ml), elastase (2.0mg/ml), cathepsin G (0.5mg/ml) and lysozyme (4.0mg/ml).

(b) Polyclonal antibodies raised against the individual proteins were affinity purified and their specificity tested on neutrophil cell lysate. Blots were developed using an enhanced chemiluminescence method.

Methods and antibody dilutions are described in the methodology section 2.2a & b.
4.4.3 Solubilization of granule proteins following PMA stimulation.

This experiment is based on the fact that stimulation of neutrophils by PMA induces the oxidase to secrete $O_2^-$ across the membrane to the exterior. Results have shown that $K^+$ is released into the medium to compensate the charge induced by electrogenic oxidase activity (Figure 3.8 & 3.10). PMA also leads to degranulation to the outside of the cell and this experiment was designed to look at solubilization of degranulated material in the surrounding buffer, as a result of $K^+$ efflux.

Neutrophils were harvested into PBS at $5 \times 10^8$ cells/ml. Such a high cell number was employed to enable easy detection of degranulated and soluble proteins by western blotting. Cells were stimulated with PMA in the presence of protease inhibitors to prevent autolysis of the released proteases. In order to separate solubilized from unsolubilized granule proteins, two centrifugation spins were performed. The first was a gentle spin to sediment intact neutrophils. The supernatant of this spin was then recentrifuged at high speed and consequently only granule proteins, which were soluble, remained in the supernatant.

Solubilized proteins were identified by western blotting and results are illustrated in Figure 4.16. The Coomassie stained gel shows equal total protein loading for the three time points. Blots of the high speed supernatant displayed successful solubilization of the four granule proteins, lactoferrin, MPO, elastase and cathepsin G over time.

Under exactly the same conditions the experiment was repeated in the presence of DPI and results are shown in Figure 4.17. By inhibiting oxidase activity degranulation was not effected, as evident from the equal amount of
**FIGURE 4.16.** Solubilization of granule proteins to the outside of the cell following PMA stimulation.

Cells (2x10^7/ml) were activated by PMA (1µg/ml) in PBS plus proteases inhibitors. It was confirmed that this buffer had no effect on granule integrity and did not cause release of granule proteins.

Aliquots were removed at the indicated times. The intact cells were pelleted and the high speed supernatants of each time point subjected to SDS-PAGE.

(a) Coomassie stained 12.5% acrylamide gel of the supernatants, illustrating equal protein loading of each time point.

(b) Immunoblots of the same samples as in (a), probed for 4 different granule proteins.

Methodology section 2.8b.
**Figure 4.17.** Solubilization of serine proteases requires a functional NADPH oxidase.

Cells were stimulated with PMA (1μg/ml) in the presence or absence of DPI (5μM) and for the indicated time points. Following activation the cells were pelleted gently and the supernatant further centrifuged. Solubilization of elastase and cathepsin G was observed in the high speed supernatant by western blotting, but not in the presence of DPI.

The experiment shown is representative of three independent ones.

Methodology section 2.8b.
degranulated elastase and cathepsin G compared to the control. PMA stimulation resulted in solubilization of both cathepsin G and elastase after 1 min. However inhibition of oxidase activity with DPI, previously shown to prevent K⁺ efflux (Figure 3.10) also prevented solubilization of these two proteases.

An additional experiment was carried out, employing neutrophils donated by an X-linked CGD patient. Cells were stimulated by frustrated phagocytosis, which results in poor vacuole formation and release of granules to the outside of the cell. Results are shown in Figure 4.18. Supernatants of the high-speed centrifugation spin were subjected to SDS-PAGE and western blotting. From the Coomassie gel equal total protein loading can be seen between the unstimulated and stimulated cell types. The blots show almost no granular protein in the unstimulated supernatants. PMA activation of control cells results in solubilization of the four granule proteins lactoferrin, MPO, cathepsin G and elastase. This is in contrast to the situation seen in the supernatants of the CGD cells, where only a small amount of MPO is released, with a total absence of soluble lactoferrin and the two serine proteases.

4.4.4 Conclusion.

Although the results of these experiments appear to support the theory that a lack of oxidase activity and superoxide production, results in decreased solubilization of granule protein, it is questionable. Due to the small volume of the vacuole, high K⁺ concentrations are achievable. The use of soluble stimuli however, results in release of K⁺ to the surrounding cellular medium and though a high neutrophil number is employed, it is of concern whether K⁺ levels reach an optimum to release the latent proteases from the proteoglycan matrix. Therefore
FIGURE 4.18. Solubilization of granule proteins following stimulation of normal and CGD neutrophils.

Solubilization of granule proteins to the outside of the cell following frustrated phagocytosis of 100 S. aureus to a single target neutrophil. Cells and bacteria were placed in a rapidly stirring chamber for a period of 5 min.

(a) Coomassie stained gel of the supernatants following high speed centrifugation. The gel illustrates equal protein loading between the unstimulated (un) and stimulated (stim) supernatants of both control (con) and p47phox deficient (CGD) cells.

(b) Immunoblots of the same samples as in (a) probed for the indicated granule proteins.

Two further experiments showed similar solubility results.

Methodology section 2.8b.
it was decided to look for solubilization of granule proteins within the vacuole following phagocytosis of IgG opsonized latex particles.

4.4.5 Solubilization of granule protein within phagocytic vacuole.

A procedure for the isolation of phagocytic vacuoles following the engulfment of latex particles had been previously established[^26].

Phagocytic vacuoles were separated from cell homogenates by floatation on sucrose gradients and examined by electron microscopy to search for evidence of soluble protein. 2min after phagocytosis the appearance of the vacuoles of control cells (Figure 4.19 a) were very different from those in which oxidase activity had been inhibited by the addition of DPI (Figure 4.19 b). The most obvious characteristic of the vacuoles of control cells was the homogeneous appearance of the granule material. This was in total contrast to the appearance of material in vacuoles following DPI treatment, where granules appeared as discrete bundles of non-homogeneous material.

Vacuoles were also examined following phagocytosis under anaerobic conditions. By diminishing the available oxygen an immitation of the CGD neutrophil phenotype was achieved. Neutrophils suspensions were freed of most of their oxygen and then incubated with IgG coated latex in an atmosphere of N₂. The rate of oxygen consumption (8 nmoles per 10⁷ cells) was a little above resting cells and similar to DPI treated cells. Isolated vacuoles were then gently lysised by freezing and thawing once, a method found not to disrupt the granule integrity and cause protein displacement.

Solubilization of granule protein within the vacuole of control cells, following DPI treatment and under anaerobic conditions was examined by
FIGURE 4.19. Superoxide generation is necessary for the solubilization of granule protein in the phagocytic vacuole.

Transmission electron micrographs were examined of normal (a) and DPI treated (b) phagocytic vacuoles (v) after phagocytosis of latex particles (0.81μM) for 4min. Granule proteins in (a) were classified as soluble because of the homogenous distribution around the latex particle (←) or insoluble because of their clumped appearance (b, ←). Vacuoles were also isolated from neutrophils in which anaerobic (anaer) phagocytosis took place. Solubilization of granule protein under anaerobic conditions was compared to the control (con) and DPI vacuoles. (c)Coomassie stained gel of isolated vacuoles and soluble proteins following vacuolar lysis and centrifugation. The gel illustrates equal protein loading of isolated vacuoles. (d)Immuneblots of the same samples in (c), probed for MPO.

Methodology section 2.6b & 2.7a.
western blotting. Figure 4.19 (c) shows a Coomassie stained gel of isolated vacuoles and corresponding soluble protein following vacuole lysis. Equal degranulation into the vacuole, under the three conditions is evident, as the intensity of the signal of the antibody against MPO is similar Figure 4.19 (d). Isolation of vacuoles of aerobic phagocytosis resulted in the solubilization of relatively large levels of MPO. However by inhibiting oxidase activity with DPI or by limiting available oxygen much less MPO was solubilized.

A limitation to the technique employed in this experiment was the length of time required to isolate the phagocytic vacuole. Despite the inclusion of protease inhibitors detection of the proteases proved fruitless, possibly due to autolysis. Nevertheless this experiment satisfactorily demonstrated that a lack of oxidase activity and superoxide production results in decreased solubilization of at least one granule protein (MPO).

4.4.6 Conclusion.

The study so far reveals that the superoxide generating system of phagocytes acts through the novel mechanism, by creating a hypertonic environment within the phagocytic vacuole. This releases the latent toxicity of the proteases within the confines of the vacuole from the proteoglycan matrix to which they are bound in the granule.

Studies that follow reveal that not only does oxidase activity dissociate enzymes through the use of $\text{K}^+$ compensating electrogenic charge, but also as its transport is pH regulated, it optimizes the pH in the neutral range for the proteases.
4.5.0 Activation of the microbicidal proteases requires $K^+$ and neutral $pH$.

4.5.1 Introduction.

As valinomycin had such a profound effect on vacuolar $K^+$ concentration and in suppressing bacterial killing, experiments were designed to measure the effect of valinomycin upon vacuolar $pH$. To measure vacuolar $pH$ the reliable method used was previously described by Segal et al., (1981) using the pH indicator fluorescein conjugated to $S. aureus^{161}$. 

4.5.2 Intravacuolar $pH$.

The application of fluorescent conjugates to studies in single living cells demands the use of highly fluorescent fluorophores which absorb in the visible spectrum, so that adequate signals are obtained while minimizing interference from autofluorescence. Furthermore the conjugates must be associated by stable covalent bonds and be devoid of non-covalently bound fluorophores. The $pH$ of the bacterial environment was determined by measuring fluorescein emission at selected excitation wavelengths (493 and 433nm) at various times after the initiation of phagocytosis.

In normal neutrophils an increase in peak fluorescence corresponding to a rise of intravacuolar $pH$ from 7.3 to 7.55 was observed within the first 2 min (Figure 4.20), an increase just slightly below that previously published$^{161}$. Thereafter the $pH$ fell to approximately 6.5, after 16min.

The pattern of $pH$ changes with phagocytosis was clearly different in valinomycin treated cells as no initial fluorescence increase was observed. Valinomycin caused a large drop in vacuolar $pH$, to the low level observed in
**FIGURE 4.20.** The effect of valinomycin on vacuolar pH.

The effect of DPI (5μM) and valinomycin (3μM) on vacuolar pH was measured over time and compared to control vacuoles.

Each point is the mean of at least 3 measurements with fluorescein coated bacteria as pH indicators.

Methodology section 2.11a.
CGD cells\textsuperscript{161}, and produced by DPI. The pH fell rapidly from 7.3 to approximately 6.0. A possible explanation of this effect is that valinomycin almost doubles the oxygen consumed for each bacterium engulfed and therefore requires more cations to balance the charge. K\textsuperscript{+} ions are lost from the vacuole through the ionophore, so charge compensation by H\textsuperscript{+} dominates with consequent acidification.

4.5.3 *Vacuolar pH is below the optimum for elastase and cathepsin G.*

To determine whether the excessively acid conditions within the vacuole of valinomycin and DPI treated cells could explain the defective bacterial killing, digestion and expulsion of \textsuperscript{35}S radiolabelled heat-killed *S. aureus* was examined (Figure 4.21). The bacteria were incubated under conditions employed in the killing assay and timed samples were collected and counted for soluble \textsuperscript{35}S, a measure of bacterial digestion. It was found that control cells released three to four times more soluble \textsuperscript{35}S than cells treated with DPI or proteases inhibitors, confirming the necessity of oxidase and protease activity for microbial digestion. Control cells solubilized two to three times more bacterial \textsuperscript{35}S than cells treated with valinomycin. The reduction in solubilization of bacterial \textsuperscript{35}S, in the presence of valinomycin can be attributed to a combination of reduced solubilization of granule proteins as well as an excessively acid pH.

To confirm that the pH in the vacuole of the valinomycin treated cells was below the optimum for both elastase and cathepsin G the pH optimum for both proteases was determined according to established procedures\textsuperscript{361,362}. As
**FIGURE 4.21.** Digestion of $^{35}$S-labelled *S. aureus*.

The effect of valinomycin (3μM), DPI (5μM) and protease inhibitors (PI) on the digestion of $^{35}$S-labelled, heat killed *S. aureus*.

Each point is the mean of 3 measurements ± S.E.

Methodology section 2.12e.
illustrated in Figure 4.22. The result shows maximal enzymatic activity at pH 7.5 to 8.0 for both enzymes with little activity below pH 7.0.

4.5.4. Vacuolar swelling readdressed.

In section 3.8.2, it was concluded that swelling occurred following a delay of 4 min after phagocytosis of bacteria and was due to increased tonicity within the vacuole as a result of $K^+$ influx. However the previous result showed a lack of bacterial digestion within the valinomycin treated vacuoles possibly due to a combination of low vacuolar pH and $K^+$ concentration. In 1981 Segal et al., suggested that the abnormally small size of phagocytic vacuoles in CGD was due to defective bacterial digestion\textsuperscript{161}. To clarify this situation it was decided to calculate vacuolar volumes of normal cells following phagocytosis of \textit{S. aureus} in the presence and absence of protease inhibitors, to prevent bacterial digestion.

The result is illustrated in Figure 4.23 and shows swelling in normal vacuoles to be almost double that of vacuoles in cells treated with protease inhibitors. As previously established the presence of DPI or valinomycin prevented vacuolar swelling, illustrating its dependence on both oxidase activity and $K^+$ flux. However the result also shows that protease inhibitors which would not perturb $K^+$ fluxes also prevented swelling. Therefore the osmotic effect of the added $K^+$ is in itself probably insufficient. Swelling seems instead to be linked to the digestion of vacuolar contents and thus probably results from the osmotic pressure exerted by the products of this process.

Digestion and swelling are both impaired in the absence of the oxidase, either naturally in CGD\textsuperscript{161} or after inhibition by DPI, by valinomycin or by
FIGURE 4.22. Relation between pH and activity of cathepsin G and elastase.

The pH optimum of both elastase and cathepsin G was carried out exactly as described by Barrett (1981). For cathepsin G the product was quantifiable at $A_{410}$ and for elastase, by excitation at 370nm with monitoring of emission at 460nm.

Methodology section 2.5c.
FIGURE 4.23. Swelling is linked to digestion of vacuolar contents.

The effect on the size of the vacuoles, of exposing cells to valinomycin (Val, 3μM, n=106), DPI (5μM, n=44), and protease inhibitors (PI, n=30) compared with untreated cells (Con, n=111) at 16min after phagocytosis of S. aureus.

Results show the volume of the entire phagocytic vacuole, S. aureus and the intervening space, (vacuolar volume minus bacterial volume).

All additions resulted in highly significant differences (p<0.001) from control cells.

Methodology section 2.7a.
protease inhibitors. Also when the microbe is replaced by latex particles which are undigestable, vacuolar swelling is not seen

4.5.5 Conclusion.

What are the additional consequences of K⁺ influx?

Granule proteins including elastase and cathepsin G are bound in an inactive form to acidic proteoglycans until their latent toxicity is liberated within the confines of the phagocytic vacuole through the activity of the NADPH oxidase. The oxidase accomplishes this by pumping O₂⁻ across the membrane, which has the dual effect of creating a hypertonic environment that dissociates the sequestered enzymes through the use of K⁺ to compensate the charge, which as its transport is pH regulated, optimizes the pH in the neutral to slightly alkaline range for the proteases.

Measurements of vacuolar pH in the presence of valinomycin, showed that by preventing the natural movement of K⁺ into the phagocytic vacuole a large drop in pH occurred, to the low level seen in DPI treated cells in which the NADPH oxidase was inhibited or in CGD cells. It was further shown that pH in the vacuoles of the valinomycin treated cells was below the optimum for both elastase and cathepsin G (Figure 4.22), a contributing factor to the impaired killing (Figure 4.5) and digestion (Figure 4.21) induced by the ionophore.

Phagocytic vacuoles enlarge as a result of oxidase activity resulting in K⁺ influx and protease activation. In turn the osmotically active digestion products of the ingested bacteria attract water into the vacuole and induce swelling.
4.6.0 Concluding remarks.

Results presented suggest that the normal sequences of events following phagocytosis of microbes such as staphylococci are as follows. After entry of the bacteria, the vacuole oxidase system elevates the intravacuolar pH via a cytochrome \( b_{245} \) mediated transport of electrons from the cytosol to the inner phagosomal surface of the vacuolar membrane. The formation of \( O_2^- \) via NADPH oxidase is an electrogenic process, ultimately resulting in increased levels of OH\(^-\) and an increase in vacuolar pH from about 6.0 to 7.8-8.0 soon after phagocytosis. In order for the leukocyte to compensate for the massive influx of electrons into the phagocytic vacuole, a balancing charge movement must occur and this has largely been thought to be by way of a compensatory proton influx. Compensation of an electron by a proton would not alter the pH, and compensation by this ion alone could not explain the observed rise in vacuole pH. Results have clearly shown that a proportion of the charge is compensated by the influx of \( K^+ \) into the phagocytic vacuole, a process that accounts for the observed increase in pH. The transport of \( K^+ \) is pH dependent, providing a mechanism for regulating the pH in the vacuole and elevating it to the optimal for the activity of the neutral proteases, but not beyond. The raised pH facilitates killing and bacteriolysis by granule proteins (Figure 4.21). Following which the pH of the vacuole is reduced possibly to optimize the activities of hydrolases and other proteins with acid pH optima.

The additional consequences of the \( K^+ \) influx was observed by examining phagocytic vacuoles purified from neutrophils of a CGD patient (Figure 3.4) or from cells treated with DPI (Figure 4.19). Vacuoles from these cells appeared strikingly different from normal vacuoles. Granule contents that were uniformly
dispersed in the phagocytic vacuoles of normal cells instead remained clumped in the absence of oxidase activity. Cationic proteases such as cathepsin G and elastase within the neutrophil granules are bound to a strongly anionic sulphated proteoglycan matrix. Release from this matrix is necessary for these enzymes to interact functionally with the ingested microorganism. Electron probe X-ray microanalysis was used to directly estimate the K$^+$ content of phagosome to be 200-300 mM (Figure 3.11) and results have shown that it is the highly hypertonic environment of the phagosome that solubilizes the bacterial proteases and allows them to participate in the killing process. Cytoskeletal elements associated with the phagocytic vacuole indirectly affect the killing process by constraining vacuolar volume and maintaining the hypertonic environment. To confirm that influx of K$^+$ is involved in bacterial killing, measurements in the presence of valinomycin were made and it was found to suppress killing of *S. aureus* to the low level seen in CGD (Figure 4.5).

*In vitro* studies showed that most of the granule protein is solubilized at the concentration of KCL corresponding to the K$^+$ levels predicted to occur in the vacuole (Figure 4.7). Elevated pH on its own was without effect (Figure 4.9).

Additional studies employing neutrophils donated by a CGD patient revealed abnormally low levels of soluble protein including elastase and cathepsin G (Figure 4.18). As expected, bacterial killing was also defective in cells from CGD patients and in cells treated with the NADPH oxidase inhibitor DPI (Figure 4.5).

The importance of the serine proteases cathepsin G and elastase, for microbial killing was shown by studies on mice deficient in these granule
proteins (Figure 4.0). Neutrophils of these mice were ineffective at killing \textit{S. aureus} and \textit{C. albicans}, even though they produced normal NADPH oxidase activity. Similarly, protease inhibitors impaired the ability of human neutrophils to kill \textit{S. aureus} (Figure 4.3).

Thus, the products of the NADPH oxidase activity do not kill organisms such as \textit{S. aureus} and \textit{C. albicans} directly, but rather act through the activation of granule proteases that act as the final mediators of antimicrobial action. The profound predisposition to microbial infections in CGD patients, are not due to the lack of reactive oxygen species, H$_2$O$_2$ and HOCl, produced within the phagosome$^{235}$. Instead the lack of K$^+$ that normally compensates the passage of electrons into the vacuole resulting in defective solubilization and activation of granule proteins. The inactivity of these enzymes is responsible for the observed lack of bacterial killing.

However the importance of the MPO-H$_2$O$_2$ mechanism and HOCl production, in eliciting effective killing and degradation has been extensively investigated. Research in patients deficient in MPO$^{367}$ and the use of MPO knock-out mice$^{244,368}$ have shown the significance of this mechanism for killing \textit{Candida albicans}, however killing does occur if somewhat slower. Additional roles for MPO have been suggested, including protecting granule enzymes against oxidative damage by reactive oxygen species, due to its ability to act as a catalase at high H$_2$O$_2$ concentrations$^{181}$ and also its ability to interact with O$_2^-$ $^{255}$. Indeed the enzymatic activity of proteases has been shown to be diminished in the presence of reactive oxygen species$^{263}$.

Studies addressed in the following chapter were designed to investigate how effective the O$_2$ metabolites (O$_2^-$ and H$_2$O$_2$) and products of chloride
oxidation (HOCl) are in killing bacteria under conditions established to prevail within the vacuole.
CHAPTER 5

5.0 A REASSESSMENT OF THE MICROBICIDAL ACTIVITIES OF REACTIVE OXYGEN SPECIES AND HOCL WITH REFERENCE TO THE PHAGOCYTIC VACUOLE OF THE NEUTROPHIL GRANULOCYTE.

5.0 A BRIEF SUMMARY OF CONTENTS.

The immediate task was to develop in vitro bactericidal assay systems that mimicked the phagosomal milieu. Human neutrophils produce a number of oxidants upon phagocytosis of bacteria. Experiments were designed to evaluate the contribution of the oxidants $\text{O}_2^\cdot$, $\text{H}_2\text{O}_2$ and the MPO-formed oxidant, HOCl, to the intracellular killing of engulfed bacteria under conditions found in the vacuole at the time of bacterial killing. Observations would provide a useful framework for interpreting the physiological relevance of various in vitro studies that examine $\text{O}_2^\cdot$, $\text{H}_2\text{O}_2$ and HOCl inflicted bacterial killing.

The pH of the phagosome was the first condition taken into account when designing experiments. The pH in the vacuole rises from approximately 6.0 to 7.8-8.0$^{161}$ in consequence of oxidase activity, and therefore the effect of pH on bacterial viability was determined over the range of 5.5 to 7.5.

Vacuolar protein concentrations of 0.5g/ml have been established within the vacuole (Section 3.1.2). Killing of bacteria by $\text{H}_2\text{O}_2$ and HOCl in the presence and absence of high granule protein concentration was assessed at three different pHs.
To evaluate the contribution of the MPO-formed oxidant HOCl to the intracellular killing of engulfed micro-organisms, cellular and bacterial proteins oxidized during this process were separated by 2D gel electrophoresis. Iodinated spots were detected by autoradiography and these oxidized proteins identified by mass spectrometry.

Results indicate that oxidants produced within the phagocytic vacuoles are consumed mainly by reaction with cellular proteins and are not the main contributors to the rapid intracellular killing of bacteria.

5.0 INTRODUCTION.

The aim of this study was to evaluate how effective the O\textsubscript{2} metabolites (O\textsuperscript{2-} and H\textsubscript{2}O\textsubscript{2}) and products of chloride oxidation (HOCl) are, in killing gram positive and gram negative bacteria under conditions prevailing within the vacuole. The microbicidal activity of O\textsuperscript{2-}, H\textsubscript{2}O\textsubscript{2} and HOCl have been extensively studied. Nevertheless, this composite set of experiments was committed to investigate the microbicidal effectiveness of these ROS under conditions of various pHs and high granule protein concentration as found within the phagocytosing vacuole.

5.1.0 RESULTS AND DISCUSSION.

5.1.1 To recapitulate conditions within the vacuole.

This first set of experiments was used in combination with previously published data to establish conditions present within the vacuole, early after phagocytosis and at a time of bacterial killing. The effect of pH alone on bacterial killing was
assessed. The time scale of bacterial killing was confirmed and the concentration of protein within the vacuole discussed further.

5.1.2 The effect of pH on bacterial viability.

The first condition within the vacuole considered was pH. The pH is elevated to 7.8 soon after phagocytosis in consequence of oxidase activity despite the entry of acid granule contents, which are maintained at a pH of 5.5 within the intact cell. However, activation of H^+ and K^+ conductance mechanisms occurs so that ion pumping from the cytoplasm into the phagosome restricts this increase to pH 7.8–8.0, following which the pH decreases to approximately 6.0. Therefore, pH of buffers employed in experiments mimicked the pH range achieved within the vacuole, i.e. pH 5.5, 6.5 & 7.5.

Within this study two bacterial strains were used, the gram negative bacteria, *S. aureus* and gram positive, *Escherichia coli* (*E. coli*). To realize the effect of pH alone on bacterial survival, an experiment employing PBS of the three different pH's, 5.5, 6.5 and 7.5 was used and survival monitored over time. The result in Figure 5.0 (a) & (b) shows that pH had no effect on the survival of *E. coli*. *S. aureus* also appeared to be unaffected by the elevated pH, but over longer periods of exposure (32 min), succumbed to the low pH of 5.5 with a decrease in survival of approximately 50%.

5.1.3 Time scale of bacterial killing.

Bacterial killing by normal cells is very rapid. In an experiment carried out by Segal and co-workers (1981) employing *S. aureus*, bacterial viability was reduced by 90% after just 4 min. Therefore when investigating the bactericidal
FIGURE 5.0. The effect of pH on bacterial viability, destruction by stimulated neutrophils and bactericidal effects of O$_2^\cdot$.

a & b, S. aureus or E. coli (1x10$^7$ CFU/ml) were incubated at 37°C in 0.01M phosphate buffer pH 5.5 (□), 6.5 (○) or 7.5 (●). Reduction in survival of S. aureus at pH 5.5 compared to 6.5 was found to be significant, p< 0.033.

Methodology section 2.9c

c, IgG opsonized S. aureus (■) or E. coli (□) (1x10$^8$ CFU/ml) were mixed at a ratio of one target organism to five neutrophils in 1ml PBS pH 7.5 for the indicated periods of time.

Methodology section 2.12d

d, S. aureus (1x10$^7$ CFU) were resuspended in 0.01M phosphate buffer pH 7.5 (●) and increasing concentrations of KO$_2$ (○) added at 37°C for 6min.

Methodology section 2.9c

In all experiments aliquots were removed periodically and two serial dilutions plated in triplicate on LB agar. Each point is the average of triplicate experiments and error bars shown ± S.E. Statistical significance was determined by Student’s t test.
effects of ROS within this study, it was necessary to do so within a time scale consistent with killing by fully functional neutrophils.

In an experiment where IgG opsonized *S. aureus* and *E. coli* were exposed to isolated human neutrophils, (Figure 5.0c) bacterial killing occurred quickly, with over 50% killed after just 2 min and 20% remaining after 4 min, as described previously\(^{161}\).

### 5.1.4 Vacuolar protein concentration.

The third and final factor taken into consideration when designing a model of conditions predominant within the vacuole, was protein concentration.

In chapter three (section 3.1.2) a quantitative measure of the granule protein entering the vacuole was made upon phagocytosis of \(^{35}\)S labelled *S. aureus*. Measurements were accomplished by determining the migration of protein from the granules to the phagocytic vacuoles that were separated on sucrose gradients (Figure 3.1.2). The result showed that the protein concentration would accumulate as high as 0.5g/ml with MPO in the range of 100mg/ml.

### 5.1.5 Conclusion.

The immediate task was to develop *in vitro* bactericidal assay systems that mimicked the phagosomal milieu. To evaluate the effectiveness of \(\mathrm{O}_2^*\), \(\mathrm{H}_2\mathrm{O}_2\) & \(\mathrm{HOCl}\), on the killing of bacteria, a model of the conditions prevailing within the vacuole was made. Routinely bacteria were exposed to ROS in the presence or absence of demembraned granule protein, within PBS at pHs of 5.5-7.5 and for a time scale of up to 32 min. Due to the tremendous viscosity of the granules at
high concentrations, for technical handling purposes the granules were used at a concentration of 25mg/ml.

5.2.0 The bacteriocidal effects of ROS under established vacuolar conditions.

5.2.1 The involvement of $O_2^-$ in bacterial killing.

The bactericidal effect of increasing concentrations of $O_2^-$ was investigated at pH 7.5 and a time of 6min (methodology section 2.9c). As a source of $O_2^-$, KO$_2$ was employed. The result illustrates (Figure 5.0 d) that $O_2^-$ itself inflicted little toxicity on the bacteria, a result consistent with findings of other researchers$^{370,371}$. Superoxide produced by neutrophils acts as a precursor of H$_2$O$_2$ and the bactericidal effect seen on addition of 100mm O$_2^-$, was possibly the result of the production of 50mM H$_2$O$_2$.

5.2.2 The bactericidal effects of H$_2$O$_2$.

A systematic study of the bactericidal effects of H$_2$O$_2$ was made under the established vacuolar conditions. The results illustrated in Figure 5.1, represent the killing of $S. aureus$ and $E. coli$ when challenged with increasing concentrations of H$_2$O$_2$ within an environment of low to elevated pH.

From the result it is evident that H$_2$O$_2$ was bactericidal only at high concentrations, as already reported$^{167}$, with $E. coli$ being more susceptible than $S. aureus$. It was clear that as the pH was elevated to 7.5, a pH similar to that within the vacuole when killing occurs, an extreme concentration of 100mM H$_2$O$_2$ was required to reduce the survival of $S. aureus$ by 50%. The bactericidal effects of H$_2$O$_2$ were marginally enhanced when the pH was reduced to 5.5.
**FIGURE 5.1.** Kinetics of bactericidal activity against *S. aureus* and *E. coli* by HgOg within three different pH’s.

The reaction mixture contained $2 \times 10^7$ CFU/ml *S. aureus* (a,c,e) or *E. coli* (b,d,f) in 0.01M phosphate buffer pH 5.5, 6.5 or 7.5 and 1(●), 10(○) or 100mM (□) H$_2$O$_2$.

Incubations were carried out at 37°C and aliquots removed at the indicated times.

Two serial dilutions were made and reactions plated in triplicate on LB agar.

Mean S.E. of 4 experiments. Methodology section 2.9c.
The exact experiment was repeated, but this time purified cytoplasmic granules devoid of their membranes were included. Prior to their use, granules had previously been purified in the presence of protease inhibitors to exclude killing of bacteria by activated digestive enzymes.

From the previous experiment it was evident that 100mM H$_2$O$_2$ demonstrated greatest bactericidal activity and therefore the effect at this concentration in the presence of granules was investigated.

Results of this experiment clearly showed (Figure 5.2) the microbicidal influence of 100mM H$_2$O$_2$ on *S. aureus* and *E. coli* was totally eliminated in the presence of granule protein. In addition the concentration of granules used is only a fraction of that predicted to be present within the vacuole and yet the amount added illustrated a marked protective effect and was capable of restoring full bacterial survival.

### 5.2.3 The bactericidal effects of HOCl

Investigation of the microbicidal effectiveness of ROS under conditions existing within the phagocytosing vacuole was extended to the effects of HOCl. HOCl is a strong non-radical oxidant and is the most bactericidal agent thought to be produced by neutrophils.$^{116, 189}$ The result of incubating *S. aureus* and *E. coli* in 1µM and 5µM HOCl is illustrated in Figure 5.3. The effect was clearly lethal and immediate, as measured by inhibition of colony formation. Following incubation with as little as 5µM HOCl all bacteria were killed within one minute.
FIGURE 5.2. The effect of granule protein on H$_2$O$_2$ bacterial killing.

The reaction mixture contained 0.01M phosphate buffer, at pH 7.5 (●), 6.5 (○) or 5.5 (□), together with demembranated granule protein (25mg/ml). Bacteria (2x10$^7$ CFU/ml) were added to the granule protein prior to the addition of 100mM H$_2$O$_2$. Each value is derived from plating the reaction in triplicate. Mean ± S.E. of 3 separate experiments. Methodology section 2.9c.
**FIGURE 5.3.** Kinetics of bactericidal activity against *S. aureus* and *E. coli* by 1 & 5μM HOCl within three different pH’s.

The reaction mixture contained 0.01M phosphate buffer at pH 7.5 (●), 6.5 (○) or 5.5 (□). *S. aureus* (a,c) or *E. coli* (b,d, 2x10⁷CFU/ml) were introduced and HOCl added in the concentrations indicated. Incubation period was up to 32min at 37°C.

Each line is representative of the mean ± S.E. of 3 experiments. Methodology section 2.9c.
1 μM HOCl killed more efficiently at low pH and an explanation for this is possible because HOCl is more lipophilic at low pH and therefore can enter and damage the bacterium more easily.

The effect of HOCl on bacterial killing was next assessed in the presence of isolated demembraned granules. In this experiment bacteria were exposed to 1 and 5μM HOCl, in phosphate buffer pH 7.5 (Figure 5.4). The observed effect in the absence of granules was exactly as described (Figure 5.3). Conversely, when bacteria were added to a suspension of granules at a concentration of 25mg/ml, prior to the addition of HOCl, it was clear that the bacteria were totally protected from the oxidizing effects, as no killing was evident.

Consequently the experiment was taken further by including even higher concentrations of HOCl in the presence of granules in phosphate buffer pH 7.5 or 5.5. The result (Figure 5.5 a & b) demonstrated HOCl levels as high as 1.0mM had no bactericidal effect in the presence of high granule protein concentrations and pH 7.5, conditions similar to those found in the phagosome early after phagocytosis and when most neutrophil killing occurs. This was an unpredicted result due to the outstanding evidence supporting a bactericidal role for HOCl in the neutrophil vacuole\(^{167,370}\).

The experiment was also repeated at pH 5.5 (Figure 5.5 c&d), with no bactericidal properties observed for HOCl concentrations of 0.1, 0.25 and 0.5 mM, and only 40% bacterial killing by 1mM.

5.2.4 Conclusion.

Various publications document successful microbial killing by H\(_2\)O\(_2\), over a wide spectrum of peroxide concentrations. Data have illustrated killing of 50%
**FIGURE 5.4.** The effect of granule protein on bacterial killing by HOCl.

Reaction mixtures contained $2 \times 10^7$ CFU/ml of *S. aureus* or *E. coli* in 0.01M phosphate buffer pH 7.5. HOCl 1μM (○, ●) or 5μM (□, ■) was added, in the presence (○, □) or absence (●, ■) of 25mg/ml demembranated granule protein.

Each value is derived from plating the reaction in triplicate.

Mean ± S.E. of 3 separate experiments.

Methodology section 2.9c.
FIGURE 5.5. The bactericidal effect of high concentrations of HOCl in the presence of granule protein.

Inhibition of *S. aureus* (a,c) or *E. coli* (b,d) killing by HOCl was observed in 0.01M phosphate buffer pH 7.5 or 5.5 with added granule protein.

Bacteria (2×10⁷ CFU/ml) were exposed to 100 (●), 250 (○), 500μM (▲) or 1mM HOCl (△) in the presence of demembraned granule protein (25mg/ml). Incubation period was up to 32 min at 37°C and aliquots removed at indicated time points.

Each line is representative of the mean ± S.E. of 3 experiments.

Methodology section 2.9c.
S. aureus by 2.5μM H₂O₂, no effect of 2mM H₂O₂ on the viability of Pseudomonas aeruginosa and 300mM required to kill 50% Streptococcus faecalis. In this study 100mM H₂O₂ demonstrated microbicidal activity, an effect emphasised at lower pH. This observed killing was reversed in the presence of granule proteins, at a fraction of the protein concentration predicted within the vacuole.

Killing is more drastic with HOCl than H₂O₂. Low concentrations (1-5μM) of HOCl were found bacteriocidal but again this effect was completely reversed in the presence of granule protein. Estimates of approximately 28μM HOCl production would be ineffective against bacteria within the high protein concentration of the vacuole.

5.3.0 The bactericidal effects of the myeloperoxidase system.

5.3.1 Introduction.

Myeloperoxidase is a promiscuous heme enzyme that has several different activities. The physiological milieu in which the enzyme functions will determine the substrates it uses and the activity it exhibits. Its main physiological activity however is generally accepted to be production of HOCl. As this study is structured to address the effect of ROS under conditions existing within the vacuole at the time of bacterial killing, it was of particular interest to investigate the bactericidal effect of the MPO/H₂O₂/Cl⁻ system within the designed model.
5.3.2 The MPO/H₂O₂/Cl⁻ system.

As the pH within the phagosome increases in consequence of oxidase activity, an experiment was designed to assess how bactericidal the MPO/H₂O₂/Cl⁻ system is against *S. aureus* at elevated pH. The bactericidal activity was assessed using purified neutrophil granule MPO with an RZ value of 0.82, a value very similar to that expected for the pure enzyme. As MPO comprises approximately a quarter of the total granule protein and experiments so far have employed concentrations of granules at 25mg/ml, the relative concentration of MPO employed was 5mg/ml.

Bacteria were washed in PBS of pH 5.5, 6.5 and 7.5 to remove any proteins from the culture medium, which may have obscured true bactericidal effects. The bacteria were then resuspended in PBS of appropriate pH containing MPO. Reactions were started by the addition of 1.0, 10 or 100mM H₂O₂.

The result showed the bactericidal effect to be dependent upon the concentration of H₂O₂ and the pH. In the presence of 1mM H₂O₂ the result illustrated (Figure 5.6) the inability of the MPO/H₂O₂/Cl⁻ system to kill bacteria, despite low pH of 5.5 and the presence of Cl⁻. With the use of 10mM H₂O₂ there was a marked bactericidal effect seen at pH 5.5 and 6.5, but not at pH 7.5. Finally, the use of 100mM H₂O₂ resulted in total killing of all bacteria at pH 5.5 and 6.5, but at pH 7.5 killing via the MPO/H₂O₂/Cl⁻ system was not evident.

The result provided evidence of the bactericidal properties of MPO at low pH, which is supportive of work by Klebanoff S.J. (1976). However as the pH is raised from 5.5 to 7.5 the MPO/H₂O₂/Cl⁻ system became inefficient with regard to bacterial killing.
The bactericidal effects of the MPO/H$_2$O$_2$/Cl$^-$ system against *S. aureus* (1x10$^7$ CFU/ml) was carried out in 0.01M phosphate buffer at pH 7.5 (●), 6.5 (O) or 5.5 (□). H$_2$O$_2$, 1(a), 10(b) or 100mM (c) was added and incubated at 37°C for up to 16 min with aliquots removed at the indicated time points.

Each line is representative of the mean ± S.E. of 3 experiments. Methodology section 2.9d.
5.3.3 Catalase activity of MPO at pH 7.5.

At this point it was important to compare the level of killing seen on the addition of 100mM H$_2$O$_2$, pH 7.5 (Figure 5.1a) with killing under the same conditions including MPO (Figure 5.6c). The killing effect of 100mM H$_2$O$_2$ alone resulted in a reduction of the bacterial count by 60%, and this killing was inhibited to less than 10% in the presence of MPO. It was postulated, at pH 7.5 MPO was functioning as a catalase rather than a peroxidase, preventing oxidation of the bacteria by 100mM H$_2$O$_2$.

As it is difficult to compare between two separate experiments with total confidence, the result was confirmed on repetition including all parameters (Figure 5.7). 100mm H$_2$O$_2$ resulted in a decrease of the colony count by 60%. MPO on its own was without effect and MPO at pH 7.5 inhibited killing incurred by 100mM H$_2$O$_2$.

The number of reports providing evidence for the predominant role of MPO in converting H$_2$O$_2$ and Cl$^-$ to HOCl greatly outweigh the role of the enzyme as a catalase, degrading H$_2$O$_2$ to O$_2$ and H$_2$O$^{181,254}$. Recently however Kettle & Winterbourn, (2001) demonstrated that MPO did indeed have true catalase activity and it could efficiently be maintained by O$_2^-$ within the phagosome$^{255}$.

The result obtained indicates, that at a time of bacterial killing when the pH is elevated to 7.8 within the phagosome and MPO is present in abundant concentrations the main activity of the enzyme may possibly be that of a catalase.

It was important at this stage to prove that the catalase activity of MPO was not due to contamination by neutrophil cytosolic catalase. This is unlikely
FIGURE 5.7. MPO catalase activity as opposed to peroxidase activity at pH 7.5.

*S. aureus* (1x10^7 CFU/ml) were suspended in 0.01M phosphate buffer pH 7.5 and the effect of 5mg/ml MPO (O), 100mM H$_2$O$_2$ (●) and MPO plus H$_2$O$_2$ (□) was tested over time.

Each time point measurement is the average of triplicate samples and error bars show ± S.E.
Methodology section 2.9d.
for the following reasons. The same result was obtained with two preparations of MPO with quite different purity indexes. The purest form had a very high purity index (RZ, 0.83), and because purification involved cation exchange chromatography, contamination with catalase would be very unlikely. In addition, a western blot using antibodies against catalase, identified catalase within neutrophil whole cell lysate but not in the purified preparation of MPO (Figure 5.8).

5.3.4 Quantitation of HOCl produced within the designed model.

Results showed total killing of bacteria by the MPO/H$_2$O$_2$/Cl$^-$ system at low pH, an effect, which was reversed by increasing the pH to 7.5. It was therefore necessary to quantitate the production of HOCl by MPO under the same experimental conditions. The measurement of HOCl generated by MPO (5mg/ml) in the presence of 1, 10, and 100mM H$_2$O$_2$ was performed at pH 5.5-7.5 using the method of taurine trapping. By addition of 10mM taurine to the MPO/H$_2$O$_2$/Cl$^-$ system, most of the produced HOCl chlorinates taurine to N-chlorotaurine, the most stable chloramine compound. Subsequent to incubation times of 0.3, 2.0, and 16.0 min, sodium iodide was added in molar excess to aliquots, and the absorption at 350 nm (triiodide peak, $\varepsilon = 22900$/mol/cm) was measured spectrophotometrically. Control values measured from 1, 10, and 100mM H$_2$O$_2$ in buffer solution at pH 5.5, 6.5, and 7.5 in the absence of MPO were subtracted from these values.

Results clearly showed the activity of MPO to be pH and H$_2$O$_2$ concentration dependent. As shown, the production of HOCl increased with decreasing pH and increasing H$_2$O$_2$ concentration, (Table 5.0).
FIGURE 5.8. Western blot analysis of purified MPO.

Purified neutrophil MPO was electrophoresed and the resulting Comassie Blue stained gel and corresponding western blot is shown. Bovine catalase was used as a control for the antibody. A positive signal although weak, was detected for catalase in neutrophil whole cell lysate but not in the purified MPO preparation.

Methodology section 2.4a, 2.2a & b.
| Methodology section 2.4c. |
|-------------------------|----------------|----------------|----------------|
|                         | Minutes | pH 7.5 | pH 6.5 | pH 5.5 |
| 1mM H₂O₂ | 0.3     | 0.09 ± 0.16 | 0.35 ± 0.12 | 0.19 ± 0.11 |
|            | 2.0     | 0.02 ± 0.16 | 0.23 ± 0.12 | 0.24 ± 0.19 |
|            | 16.0    | n.d.      | 0.28 ± 0.12 | 0.22 ± 0.08 |
| 10mM H₂O₂ | 0.3     | 1.14 ± 0.22 | 2.02 ± 0.79 | 4.84 ± 0.09 |
|            | 2.0     | 0.99 ± 0.10 | 1.86 ± 0.57 | 4.44 ± 0.09 |
|            | 16.0    | 1.30 ± 0.05 | 1.57 ± 0.16 | 3.49 ± 0.09 |
| 100mM H₂O₂| 0.3     | 1.29 ± 0.40 | 14.80 ± 1.13 | 13.26 ± 1.58 |
|            | 2.0     | 0.18 ± 0.37 | 13.64 ± 0.59 | 12.61 ± 1.32 |
|            | 16.0    | 0.82 ± 0.26 | 11.80 ± 0.55 | 12.16 ± 0.37 |

**TABLE 5.0.** Quantification of hypochlorite production.

Hypochlorite (mM) produced by purified MPO (5mg/ml) was carried out in PBS of different pH's and in the presence of 1-100mM H₂O₂.

The taurine method was employed and measurements made at three different time points at 37°C.

Mean values (± S.E.) of three independent experiments. (nd; none detected) Methodology section 2.4c.
The addition of 1mM H$_2$O$_2$, produced low levels of HOCl which coincided with the low level of bacterial killing observed for the same parameter (Figure 5.6a). As the concentration of H$_2$O$_2$ increased to 10mM there was a corresponding increase in the production of HOCl, most obvious at low pH 5.5. Finally as the H$_2$O$_2$ concentration is elevated to 100mM there is no increase in HOCl production at pH 7.5, a result in total contrast to that observed at pH 6.5. At this low pH, the production of HOCl increases approximately ten times, from 1.0mM to 10.0mM, an increase paralleled with the total killing of all bacteria under this condition (Figure 5.6c).

5.3.5 Conclusion.

Most of the HOCl, *in vitro* was produced within the first few seconds after the addition of H$_2$O$_2$, with no significant further production. This might indicate that MPO is no longer active in the phagosome when the pH becomes acidic. On the other hand, it is not known how long MPO is active *in vivo*. Accumulation of long-lived oxidants (i.e. chloramines) in the supernatant of neutrophils, stimulated by PMA for longer periods of time (30-60min), indicated activity of MPO lasting more than a few minutes$^{205}$. However it has not been clarified whether this activity takes place intra- or extracellularly.

5.4.0 A proteomics approach to the identification of iodinated proteins.

5.4.1 Introduction.

According to previous studies the phagocytosing granulocyte possesses all factors needed for the production of HOCl and the chlorination process, i.e.
MPO coating the bacteria in the phagosome\textsuperscript{348}, chloride ions, and pH 6.6 within the vacuole\textsuperscript{161}. Chlorination studies are largely an extrapolation from that of iodination and a wide body of evidence has been collected which appears to support the chlorination/iodination theory. Autoradiographic evidence indicated that iodination occurred in close proximity to phagocytosed bacteria\textsuperscript{348}, and the specific radioactivity of phagocytic vacuoles isolated from neutrophils that had phagocytosed droplets of paraffin oil was greater than that of the cells as a whole\textsuperscript{375}. Iodination is decreased or absent in patients with congenital absence of MPO\textsuperscript{251,376}, and in normal cells treated with haem ligands azide and cyanide\textsuperscript{376}.

The accuracy of the iodination theory however was queried\textsuperscript{252} on the presentation of results illustrating autoradiographs after SDS-PAGE of cells stimulated with either opsonized bacteria or PMA. Both stimuli resulted in a similar distribution of iodinated components regardless of the obvious absence of bacteria upon PMA activation. Thus positive evidence for the iodination of bacteria had not been produced and the role of iodination/chlorination remained to be established.

5.4.2 Identification of iodinated proteins.

This set of experimental studies was undertaken to investigate targets of the iodination reaction and thus identify exactly which proteins within the phagosome were becoming iodinated. A proteomics approach was taken whereby, iodinated proteins were cut from 2-D PAGE, digested with trypsin and subjected to MALDI-TOF mass spectrometry. Proteins were identified by comparing mass fingerprints with protein sequence in NCBI's database. A variety of iodinated spots became apparent in the stimulated phagocytosing cells...
when compared with resting cells (Figure 5.9). The autoradiographs of solubilized iodinated trichloroacetic acid precipitates showed at least forty spots, varying from very high molecular weights to about 8 kDa. Most of these spots could be identified by mass spectrometry. A list of these iodinated proteins is provided in Table 5.1. On identification of these proteins it was found that they appeared to belong mainly to the azurophilic and specific granules (mainly lactoferrin, MPO 14kDa light subunit, lysozyme, gelatinase-associated lipocalin and lysozyme). Also other intracellular cytoskeletal proteins (profilin, annexins) and plasma proteins (hemoglobin, fibrinogen, fibrin) of the neutrophil. In contrast, the main bacterial protein identified was the opsonin, IgG. The clear iodination of granular proteins demonstrates at least a certain topical specificity of iodination, since these proteins are colocalized with MPO upon phagocytosis. Iodination of non-neutrophilic proteins like hemoglobin and fibrinogen can be explained either by their uptake with phagocytosed particles or by extracellular iodination during the oxidative burst.

Iodination of granular and cytosolic proteins as well as extracellular human proteins was more abundant than that of bacterial proteins. Only if $10^9$ colony forming units of bacteria/ml were used, iodinated outer membrane proteins (OMP-A, OMP-NMPC) and some enzymes of *E. coli* were observed (Table 5.2). However at such high numbers of bacteria to neutrophils (100:1), frustrated phagocytosis takes place with degranulation and $O_2^-$ production released to the outside of the cell, thus iodination is probably occurring in the surrounding media.$^{377}$

Thus the object of enhanced iodination that occurred when bacteria were phagocytosed by neutrophils appeared mainly to be components of the ingesting
FIGURE 5.9. Electrophoretic patterns of components from cells after phagocytosis of opsonized *S. aureus*.

Neutrophils (1x10^6/ml) were suspended in HEPES Buffer supplemented with 10μCi ^125^I and placed in a rapidly stirring oxygenated chamber at 37°C. *S. aureus* (1x10^7 CFU/ml) were added for 4 min and the reaction stopped by precipitation of all proteins in 10% TCA.

Coomassie Blue stained gel is shown (a&b) with corresponding autoradiographs (c&d), 216 hr exposure to film.

Methodology section 2.2 c,d,e & 2.3e.
<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Neutrophilic</th>
<th>Other human</th>
<th>Bacterial (S. aureus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>lactoferrin</td>
<td>19 hemoglobin beta</td>
<td>27 Ornithine-transcarbamoylase</td>
</tr>
<tr>
<td>2</td>
<td>gelatinase-associated lipocalin</td>
<td>20 hemoglobin alpha 2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>cathepsin G</td>
<td>21 albumin</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>lysozyme</td>
<td>22 fibrin</td>
<td></td>
</tr>
<tr>
<td>5&amp;6</td>
<td>Calgranulin A</td>
<td>23 fibrinogen</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>elastase fragment</td>
<td>24&amp;25 IgG</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>myeloperoxidase</td>
<td>26 fructose-bis-phosphate-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>grancalcin</td>
<td>adolase A</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>profilin</td>
<td>13 actin</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>annexin III</td>
<td>14 GDP-dissociation inhibitor</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>annexin V</td>
<td>16 glucose-6P-dehydrogenase</td>
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<tr>
<td>13</td>
<td>actin</td>
<td>17 glutathione-S-transferase P</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>GDP-dissociation inhibitor</td>
<td>18 esterase D</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>profilin</td>
<td>19 phosphoglycerate mutase 1</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>myeloperoxidase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>grancalcin</td>
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<tr>
<td>18</td>
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<tr>
<td>19</td>
<td>phosphoglycerate mutase 1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 5.1.** List of iodinated proteins following phagocytosis of *S. aureus* by isolated neutrophils.

Proteins of interest were excised from the SDS gel and exposed to in-gel digestion with trypsin. Mass-spectromatric peptide maps were acquired and proteins identified by comparing mass fingerprints to NCBI’s database.

Methodology section 2.2d & e.
**Bacterial (E. coli)**

- outer membrane protein A
- OMP-NMPC
- OMP W precursor
- superoxide dismutase
- malate dehydrogenase
- asparaginase
- hydroperoxide reductase
- fructose-bis-P-aldolase

**TABLE 5.2.** List of iodinated bacterial proteins following stimulation of neutrophils by *E. coli*.

Neutrophils (1x10⁶/ml) were suspended in HEPES Buffer supplemented with 10μCi ¹²⁵I.

*E. coli* were added at a ratio of 100 bacteria to one neutrophil for 4 min and the reaction stopped by precipitation of all proteins in 10% TCA.

Proteins of interest were excised from the SDS gel and treated as described in legend of Table 5.1.
Methodology section 2.2d & e.
cell rather than bacterial proteins. This indicates that oxidants produced within
the neutrophil vacuole are consumed mainly by reaction with proteins of the
phagocyte and provides further evidence that they are unlikely to contribute to
the rapid intracellular killing of bacteria.

This result is supportive of the lack of bacterial killing by HOCl, observed
in vitro in the presence of high granule protein concentrations (Figure 5.4 &
5.5). HOCl does not distinguish between bacterial and proteins of the
phagocytosing cell, and it is consumed rapidly by reaction with the organic
material present within the phagocytic vacuole\textsuperscript{198}. Long-lived oxidants
(chloramines) generated by chlorine transfer from HOCl to amino groups
(transhalogenation) do not kill bacteria immediately at micromolar
concentrations\textsuperscript{205}. Thus HOCl concentrations which have been shown to be
produced by phagocytosing cells\textsuperscript{162} would have little if any bactericidal effect in
the high protein environment of the phagosome, and within the time scale of
neutrophil bacterial killing.

5.5.0 Concluding remarks.

There are many reports supporting a role for H\textsubscript{2}O\textsubscript{2} and to a greater extent HOCl
in the successful killing of invading organisms\textsuperscript{116,119,168}. However some of the
early studies should perhaps be reassessed.

In initial experiments to demonstrate the participation and toxicity of the
MPO/H\textsubscript{2}O\textsubscript{2}/Cl\textsuperscript{-} system, in vitro experiments with MPO were performed with
very low concentrations of enzyme, with MPO concentrations in the range of
50\textmu g/ml\textsuperscript{190,214,255,369,378}, rather than the tens of mgs/ml found in this study to be
present within the vacuole. Also, studies have been done at pHs of 5.0-
5.5^{118,189,348,379,380} or less^{238}, rather than the alkaline to neutral conditions^{161,162,256} that pertain in the vacuole at a time when almost all bacteria are killed^{161}. Thus previous studies investigating killing by MPO may have been performed under unphysiologically low concentrations of reactants and too low a pH. Indeed, this study has shown that killing by H$_2$O$_2$ and HOCl can be completely reversed by the presence of granule protein, or pure MPO despite the presence of Cl$^-$, which could provide substrate for chlorination reactions in the medium. Therefore the cell-free MPO/H$_2$O$_2$/halide system which has been shown to kill a wide range of microbes in vitro may not be the primary defence mechanism in vivo.

How does this new data fit in current dogma about the role of ROS in microbial killing? Certainly this work does not dispute the production of HOCl within the vacuole but readdress its role. Confirmation of the involvement of MPO in the killing process was observed in patients deficient in MPO and through the use of MPO knock-out mice which provide data implicating the importance of this mechanism for the killing of Candida albicans^{244}. However studies showing the relevance of the MPO mechanism against infections of Aspergillus fumigatus and Trichosporon asahii were less convincing and carried out for a maximum of only 48hr^{368}. As CGD neutrophils failed to activate their released collagenase^{262}, it is tempting to suggest that the MPO/H$_2$O$_2$/Cl$^-$ system resulting in low concentrations of HOCl production is necessary for the activation of metalloproteinases such as collagenase and gelatinase within the vacuole^{262,299}.

The role of MPO may not be direct but may act by protecting granule proteins and enzymes against oxidative damage by ROS. It acts as a catalase at high H$_2$O$_2$ concentrations and also interacts with O$_2^-$, an interaction which has
been suggested maintains the catalase activity of MPO\textsuperscript{181,255}. One final experiment illustrated in Figure 5.10 shows the oxidative damage increasing concentrations of HOCl inflicted on granule protein. Whilst neither O\textsubscript{2}\textsuperscript{-} or H\textsubscript{2}O\textsubscript{2} affected the integrity of the granule protein, the addition of HOCl resulted in total degradation. This result should have included a measurement of enzyme activity, however it is supported by the observation that HOCl decreased markedly the activity of proteolytic enzymes\textsuperscript{263}, as assessed by NMR spectroscopy and MALDI-TOF mass spectrometry.

One last consideration, why do neutrophils produce superoxide? The dogma is that superoxide quickly dismutates to form the necessary substrate, H\textsubscript{2}O\textsubscript{2}, for MPO mediated halogenation. But most enzymes that produce H\textsubscript{2}O\textsubscript{2} (e.g. all the flavin oxidases) do so without making O\textsubscript{2}\textsuperscript{-}. The answer to this maybe the discovery that K\textsuperscript{+} is one of the ions compensating the charge induced by O\textsubscript{2}\textsuperscript{-} generation. K\textsuperscript{+} influx into the vacuole has the dual effect of creating a hypertonic environment that dissociates the sequestered granule enzymes such as cathepsin G and elastase and as its transport is pH regulated, optimizes the pH in the neutral to alkaline range for the proteases. Thus the role of O\textsubscript{2}\textsuperscript{-} is not simply that of a precursor for degradative oxygen free radical production, but rather a mechanism for promoting protease activation.
**FIGURE 5.10.** The effect of HOCl, $O_2^-$ and $H_2O_2$ on granule protein.

Purified demembranated neutrophil granules (25mg/ml) were exposed to 5, 10 & 50μM HOCl or 5, 10 & 50mM $O_2^-$ or $H_2O_2$. Following a 6min, 37°C incubation an aliquot of the reaction was removed and electrophoresised.

A Coomassie Blue-stained dried gel and corresponding western blots are shown.
CHAPTER 6.

6.0. CONCLUSION.

6.1. Combining existing theories with new discoveries.

This study describes a novel model for the molecular mechanisms utilized by phagocytic neutrophils to kill their target organisms. Results clearly show a link between the formation of superoxide anion and the activation of granule microbicidal enzymes. It has been shown that the formation of superoxide has the dual effect of creating a hypertonic environment that dissociates sequestered enzymes through the use of $K^+$ to compensate the charge, which, as its transport is pH regulated, optimizes the pH in the neutral range for the proteases\(^{327}\).

The sequence of events following phagocytosis of staphylococci is as follows (Figure 6.0). After the bacteria is phagocytosed by normal cells the vacuole closes and the components of the electron transport chain are activated and pump electrons across the membrane of the phagocytic vacuole to form $O_2^-$ and $H_2O_2$ in the lumen. $H^+$ and $K^+$ ions accompany the movement of electrons, and are channeled into the vacuole in a coordinated manner resulting in the elevation of vacuolar pH. The fusion of the cytoplasmic granules with the phagocytic vacuole releases their contents from an environment in which the enzymes are maintained inactive at low pH and bound to proteoglycan matrixes. $K^+$ influx creates a hypertonic environment that dissociates the sequestered enzymes and optimizes the pH for their activity\(^{327}\). These enzymes proceed to kill and digest the bacteria or other ingested particle. After a short time in normal cells the pH within this compartment falls\(^{161}\) and at this abnormally low pH the activity of hydrolases and other proteins with acid pH may take over the killing.
FIGURE 6.0. Proposed sequence of events in the phagocytic vacuole. The insert shows a cytoplasmic granule which has fused with the phagocytic vacuole. After the microbe is phagocytosed by normal cells the vacuole closes and the components of the electron transport chain are activated and pump electrons across the wall of the phagocytic vacuole to form $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ in the lumen. The movement of electrons is accompanied by $\text{K}^+$ and $\text{H}^+$ resulting in creating a hypertonic environment of elevated pH, optimal for granule protein activation. These enzymes kill and digest the bacterium. Products of digestion are osmotically active and attract water producing considerable osmotic swelling.
process. Bacterial digestion products are sufficient to cause osmotic swelling of
the vacuole, until it comes into contact and fuses with the plasma membrane,
releasing the digested bacterial particles, into the extracellular environment. In
CGD, or under anaerobic conditions, the environment within the vacuole rapidly
becomes acidic\textsuperscript{161}, and would be predicated to result in unfavorable conditions
for the killing and digestion of the microbe. Absence of oxidase activity
certainly results in a lack of hypertonicity and granule protein activation,
consistent with impaired microbial killing observed in these cells. However
CGD patients suffer less infections, compared to individuals without neutrophils,
who rapidly succumb. The bacterial killing by CGD cells or cells under
anaerobic conditions may possibly be via enzymes like lysozyme, which are not
bound to proteoglycan matrices and possess optimal activity at low pH.

6.2. The bactericidal effects of $O_2^-$ and $H_2O_2$.

The long standing dogma, that microbial killing is brought about by reactive
oxygen species, developed from the discovery of non-mitochondrial respiration
by phagocytic cells\textsuperscript{16}, diminished killing in the absence of oxygen\textsuperscript{111,381}, and the
discovery of CGD\textsuperscript{18}. The killing of microbes was directly attributed to the direct
toxic effect of $O_2^-$\textsuperscript{172} and $H_2O_2$\textsuperscript{179}.

The production of $O_2^-$ by the one-electron reduction of oxygen in
biological systems, together with its reactivity, suggested it as a possible killing
agent in neutrophils. Babior and colleagues first reported the production of $O_2^-$
by phagocytosing cells in 1973\textsuperscript{9}. Experiments demonstrated the reduction of
cytochrome C following phagocytosis of latex particles, an effect inhibited by
the addition of SOD. It is likely, that the rate of $O_2^-$ recorded refered to a fraction
of the $O_2^-$ produced (1.03 nmol/10^7 neutrophils per 15 min) because of the use of phagocytic stimuli and the production of $O_2^-$ within the confines of the phagocytic vacuole, rather than to the surrounding medium.

Evidence for the participation of $O_2^-$ in bacterial killing was reported by Babior in 1974\textsuperscript{47}. Experiments were conducted using high concentrations of xanthine oxidase, an enzyme capable of reducing oxygen to $O_2^-$. However some of the results and reasonings are questionable. For example, in these experiments buffers contained 2.3M $(NH_4)_2SO_4$ and killing of *S. epidermidis* occurred in the presence of the xanthine oxidase system but also in the absence of the substrate purine. Dismutation of $O_2^-$ in the presence of SOD was predicted to protect killing by xanthine oxidase and though some protection of *S. epidermidis* was observed, protection of *E. coli* was minimal or absent.

To further evaluate the microbicidal activity of $O_2^-$, Klebanoff (1974), employed the xanthine oxidase system using xanthine as substrate\textsuperscript{370}. In accordance with results presented within this study, the microbicidal activity of the anion was shown to be weak when compared to the $H_2O_2$ formed from it, and when combined with other components of the MPO-mediated antimicrobial system. *E. coli*, *S. aureus* and *C. albicans* were unaffected by the $O_2^-$ generating system, under conditions in which they were rapidly killed by the xanthine oxidase system supplemented with chloride and MPO\textsuperscript{370}.

In 1979, Rosen and Klebanoff, carried out a more thorough investigation into the bactericidal activity of $O_2^-$.\textsuperscript{382} The acetaldehyde-xanthine oxidase system in the presence and absence of MPO and chloride was employed as a model of the oxygen-dependent antimicrobial system. The bactericidal activity in the absence of MPO was inhibited by SOD and catalase, implicating $O_2^-$ and
respectively. The 'OH scavengers, mannitol and benzoate also inhibited activity and the authors concluded that the bactericidal activity of the xanthine oxidase system was not by O$_2^-$ or H$_2$O$_2$ but mediated through the reduction of H$_2$O$_2$ by O$_2^-$ with the resulting formation of 'OH (Haber-Weiss reaction). Thus the idea of O$_2^-$ as a microbicidal agent fell into disfavor.

The consideration that H$_2$O$_2$ formed by phagocytosing neutrophils may be microbicidal in situ was suggested in 1961 by Iyer et al. The idea was supported by a correlation between H$_2$O$_2$ production and microbicidal activity in neutrophils treated in a number of ways. Thus, anaerobiosis$^{373}$, catalase$^{373}$, and phenylbutazone$^{383}$ decreased the H$_2$O$_2$ production and microbicidal activity of isolated neutrophils.

Concentrations of H$_2$O$_2$ production within the vacuole have been estimated between 0.01$\mu$M$^{176}$ and 100mM depending on the amount of phagocytosis and intracellular pH$^{116}$. Exact concentrations of H$_2$O$_2$ are difficult to predict as H$_2$O$_2$ is uncharged and can penetrate cellular and organelle membranes. Evidence indicates that several metabolic pathways exist within neutrophils which catabolize free H$_2$O$_2$ in different subcellular locations, including catalase and glutathione peroxidase within the cytosol and MPO$^{384}$ within phagocytic vacuoles.

In vitro studies$^{374}$ indicate that if high levels of H$_2$O$_2$ (10mM) can be substained for long periods of time, H$_2$O$_2$ may be an effective bactericidal agent. A possible adaptation of this activity has been proposed in MPO deficient cells which have increased respiratory burst and greater than normal amounts of H$_2$O$_2$.$^{249,385}$ It has been suggested that in these cells the increased H$_2$O$_2$ concentrations are microbicidal in the absence of MPO, thus providing an explaination as to
why these patients are not unduly effected\textsuperscript{243}. However, results described in this study have shown that concentrations of \( \text{H}_2\text{O}_2 \) as high as 100mM are not bactericidal under physiological concentrations of granule protein and pH, thus questioning this theory and implicating nonoxidative methods of bacterial killing.

6.3. \textit{MPO-derived oxidants in inflammation.}

Since Klebanoff (1967) showed that a myeloperoxidase system is strongly bactericidal\textsuperscript{118}, the enzyme has been considered as the most important component of the neutrophil’s antimicrobial armory. Conveniently, however it has been maintained that the main activity of myeloperoxidase is converting chloride to hypochlorous acid, the major oxidant produced by neutrophils. Indeed, from the foregoing, reactive oxidizing species produced from phagocytes have been implicated in the pathogenesis of many conditions. As a result an entire industry has developed aimed at the production of drugs to antagonize these oxidizing species. It is well established that myeloperoxidase-derived oxidants damage cells and tissue \textit{in vitro}\textsuperscript{386,387}. The oxidants produced by phagocytes are also implicated in many inflammatory diseases including respiratory distress, pulmonary injury\textsuperscript{388}, peptic ulcer formation, and gastric cancer\textsuperscript{389}. Early studies were based on tissue damage induced by infusion of MPO and glucose oxidase (as a source of \( \text{H}_2\text{O}_2 \)) into the rat lung. Similarly, the infusion of \( \text{H}_2\text{O}_2 \) and MPO into the renal artery induced proteinuria, combined with morphological evidence of glomerular cell damage\textsuperscript{390,391}. However such evidence that MPO derived oxidants contribute to the pathology is largely indirect.
The application of assays for specific markers has enabled a more direct assessment of the role of oxidants in disease. Potential biomarkers of HOCl include chlorinated tyrosines, chlorohydrins, 5-chlorocytosinen and protein carbonyls\[^{392}\], successfully detected by gas chromatography and mass spectrometry. In addition antibodies that recognized HOCl-treated proteins (HOP1)\[^{393}\] gave a positive response to inflammatory lesions in the kidney. This antibody enabled analytic techniques to be complemented by immunocytochemical investigation of the localization of modified protein.

The presence of neutrophils in the inflamed joint raised the possibility that reactive oxygen species may be partly responsible for the damage sustained in rheumatoid arthritis. Joint fluid from patients with rheumatoid arthritis have an increased content of carbonyl groups, indicating sustained damage by reactive oxidants\[^{394}\]. Also, in two mouse models of autoimmune arthritis, superoxide dismutase has been shown to ameliorate the disease\[^{395,396}\].

Within the neutrophil vacuole the generation of ROS is essential for efficient killing of bacteria\[^{118,189}\] and fungi\[^{237}\] by neutrophils. The question is how these ROS accomplish this. There is extensive doubt on the role or even the existence of some oxygen radicals including, the hydroxyl radical\[^{162}\], singlet oxygen\[^{223}\], and reactive nitrogen species\[^{228}\] within the confines of the vacuole. However the same cannot be said for killing via the MPO/H\(_2\)O\(_2\)/Cl\(^-\) mechanism. Confirmation of the involvement of MPO in the killing process was made through the use of MPO knock-out mice\[^{244}\], in which killing of Candida albicans was defective. However, as already mentioned, deficiency of MPO is a common condition in humans and does not lead to obvious susceptibility to bacterial infection\[^{243}\]. Therefore an alternative system must dominate to compensate for
halogenation reactions are largely those of the engulfing neutrophil rather than the microbial prey. This was demonstrated by results of this, and a previous study^252. Regarding chlorination, in a recent publication Chapman and co-workers established that 94% of the total chlorinated tyrosine residues formed during phagocytosis were those of neutrophil proteins^397, indicating that oxidants produced within the neutrophil vacuole are consumed mainly by reaction with proteins of the phagocyte.

Alternatively, MPO may protect microbicidal enzymes against oxidative damage^327 by ROS. In addition to its peroxidase activity, MPO can also act as a catalase and this latter role may dominate under conditions in the vacuole in which the concentration of H_2O_2 is high and where the catalase activity of MPO can be constantly regenerated through the reduction by O_2^-^255. Preliminary studies carried out within Prof. Segal’s lab, have found cathepsin G to be very sensitive to oxidation by H_2O_2 and to be inactivated at a greatly increased rate in phagocytosing neutrophils treated with azide to inhibit MPO. This theory is supported by the observation that HOCl decreased markedly the activity of proteolytic enzymes^263.

A similar, mechanisms indicating a protective effect for MPO was described in mice, deficient in MPO and exhibiting increased susceptibility to atherosclerosis^398. Evidence placing MPO at the site of atherosclerosis, the major cause of heart disease and stroke, arises from immunological studies^399.
this deficiency. With the use of elastase and cathepsin G deficient mice this study showed that killing of *C. albicans* was grossly defective despite perfectly normal iodination\textsuperscript{327}, implicating granule proteases and questioning the conventional theory of MPO action. Furthermore the target proteins of
Oxidative modification to low-density lipoprotein (LDL) is thought to contribute to the accumulation of cholesterol-loaded macrophages, termed “foam cells” that are a hallmark of the disease\textsuperscript{400}. When LDL receptor-deficient mice were lethally irradiated and their bone marrow repopulated with MPO deficient or wild type cells, lesions in MPO-deficient mice were about 50% larger than control\textsuperscript{398}. This result was contrary to predictions. It may be possible that neutrophil enzymes are involved in clearance of atheromatous material and that the catalase activity of MPO protects the enzymes against oxidation by H\textsubscript{2}O\textsubscript{2}. Increased oxidative damage was coupled with impaired ability to clear infections of \textit{C. albicans}, which is not so surprising as cathepsin G, which is necessary to kill \textit{C. albicans}\textsuperscript{327}, is sensitive to oxidation\textsuperscript{263}.

In a similar fashion, a role for MPO in mopping up oxidizing species was observed in EAE, a demyelinating disease that serves as an animal model for multiple sclerosis (MS). MPO has been implicated in MS through the release of reactive oxidants from phagocytes and cytotoxic damage to the oligodendrocytic myelin sheath is thought to occur. In the murine model this process is associated with nerve dysfunction, accompanied by a progressive ascending paralysis, beginning in the tail and progressing through the limbs. Unexpectedly MPO knockout mice had significantly increased incidence of EAE\textsuperscript{401}.

Doubt has also been cast on another aspect of oxidative killing. It was thought that patients with CGD were more susceptible to catalase positive microbes because the catalase negative organisms generated H\textsubscript{2}O\textsubscript{2} as substrate for MPO mediated halogenation, thereby providing the substrate for their own destruction\textsuperscript{117}. However catalase deficient \textit{Staphylococcus aureus}\textsuperscript{234} and
Aspergillus nidulans were shown to be at least as virulent as the catalase positive variety in a mouse model of CGD.

Still on the subject of ROS but changing the line of thought, early studies looking at the possible signal-transduction molecules that might be influenced by the presence of ROS identified the NADPH oxidase complex and a H$_2$O$_2$ sensitive potassium channel within the same cells. Exposure of NEB cells to H$_2$O$_2$ resulted in the outward movement of K$^+$ (Figure 6.1), indicating that H$_2$O$_2$ could be the transmitter modulating the O$_2^-$ sensitive K$^+$ channel. Similar K$^+$ fluxes were observed on treatment of erythrocyte membranes to 5mM hydrogen peroxide. It is therefore intriguing to speculate that within the phagosome, H$_2$O$_2$ may not only be acting as substrate for MPO but is also necessary to modulate the K$^+$ channel and influence the influx of K$^+$ leading to granule protein activation.

6.4. The Importance of the neutrophil cytoskeleton.

Neutrophils constantly rearrange their actin cytoskeleton to perform chemotaxis and phagocytosis. Rho-GTPase regulate actin rearrangement by signalling to multiple downstream effector complexes such as the Wiskott-Aldrich syndrome protein (WASp) and the Arp2/3 complex.

As the novel mechanism described involves regulation by hypertonicity, the control of the phagocytic vacuole volume by associated cytoskeletal elements plays an important role in modulating the killing process. Vinculin and paxilin were found to initially accumulate around the vacuole and then slowly disperse as swelling proceeded. Similar observations were made in
It has been postulated that $H_2O_2$ is involved in the modulation of potassium-channel function.

**FIGURE 6.1.** Role of $H_2O_2$ in signalling.
A distinct ring of F-actin accumulated at the vacuole membrane and included an enrichment of vinculin and paxillin.

Earlier studies employing rhodamine phalloidin staining combined with quantitative fluorescence and confocal microscopy have measured local F-actin changes in neutrophils phagocytosing yeast particles. Results showed a distinct ring of F-actin around the phagosome, which was calcium regulated and decreased shortly after particle ingestion. Such rearrangements depend on actin severing and capping proteins, of which gelsolin is the best characterized and has been shown to translocate to the actin ring.

It is essential that the volume of the vacuole is restricted for the required hypertonicity to develop. It is intriguing to speculate that disruption of the cytoskeleton around the vacuole by microbial products could offer a mechanism of virulence by inhibiting the activation of granule proteins. Interestingly, it has been shown that phagosomes containing live *Mycobacterium avium* are unable to mature normally, with marked disorganization of the phagosomal F-actin network, the progressive disappearance of the small filaments and the appearance of large numbers of tiny punctuate structures.

6.5. Defective proteolytic power.

The cationic enzymes are tightly bound to strongly negatively charged proteoglycans within the granules. Results have shown that in order to activate the enzymes they must be released from this matrix, and this is achieved by the ionic strength generated by the K⁺ that is driven into the vacuole. One might question the need for such an elaborate activation system, but a possible explanation lies in the very large numbers of neutrophils that infiltrate sites of
acute inflammation. As opposed to strong oxidants which are short lived, react once and are incapable of fastidiously selecting targets, an enzyme can remain active for exceptionally long periods, can repetitively catalyze a given reaction, and will ignore all targets save those dictated by the range of its substrate spectrum. The potential of their enzymes to damage autologous tissues if released from cells in a freely soluble active form is indisputable.

Thus while playing a protective role in host immunity, neutrophil proteolytic enzymes, including elastase and cathepsin G, have also been implicated in numerous inflammatory diseases. These serine proteinases have the distinct ability to attack key components of the extracellular matrix, composed of a complex mix of collagens, elastin, proteoglycans and glycoproteins that lie under epithelia and surround connective tissue cells. Proteinases have been implicated in a number of inflammatory diseases such as adult respiratory distress syndrome, ischemia reperfusion injury, emphysema, rheumatoid, arthritis and endotoxic shock. The hazards of injurious effects from these enzymes in normal tissues is reduced by packaging them in an inactive form adsorbed to acidic proteoglycans, from which they are released and activated only by a combination of the unusual conditions of hypertonicity and alkalinity that prevails in the phagocytic vacuole.

In 1998, an interesting article was published by Kainulainen et al who showed that syndecans, which are a major cellular source of heparin-like glycosaminoglycan are shed into acute human dermal wound fluids and bind tightly proteases such as cathepsin G and elastase. Therefore a corollary of the mechanism of enzyme packaging within the granules exists. The reabsorption of
these proteases onto heparin is an ideal mechanism of reducing their proteolytic activity at sites of inflammation.

However not all enzymes need to be released from proteoglycan matrices for their activation. Indeed the reverse is true for rat mast cell protease 1 (RMCP-1) and tryptase. RMCP-1:heparin complexes are stored in the secretory granules of the cells and are released following mast cell activation. Dissociation of RCMP-1 from heparin resulted in loss of proteolytic activity, as measured by its ability to inactivate thrombin. Within our study almost all granule enzymes were eluted from the proteoglycan matrix by about 300mM KCl, an ionic strength that is achieved within the vacuole. However, in vitro studies showed that 1.2M NaCl was required for elution of RMCP-1 from heparin affinity matrix, an ionic strength unlikely to occur within a cellular system, thus securing the activity of these proteinases.

The fate of the proteoglycan matrix, devoid of granule proteins within the vacuole is of interest. With the use of specific synthetic inhibitors, there are many publications indicating a role of elastase and cathepsin G in proteoglycan matrix degradation, with the release of single chain chondroitin sulphate-peptides. Therefore it is possible that protease subjacent activity may play an important role in the degradation and clearance of the granule proteoglycan within the vacuole. Neutrophils of CGD patients have diminished heparin degrading activity which the authors concluded was a result of lack of oxidant generation, but may possibly be due to lack of soluble active proteases.


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