An investigation of the antimycobacterial mechanisms of macrophages and potential virulence factors of mycobacteria.

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ABSTRACT

Polyamine oxidation and tryptophan degradation were assessed as potential oxygen-independent antimycobacterial mechanisms of guinea-pig alveolar macrophages. Products of polyamine oxidation other than hydrogen peroxide had bactericidal effects on all the tested strains of *Mycobacterium tuberculosis*, with the most virulent strains showing the greatest degrees of resistance to products of polyamine oxidation. However, no significant correlation was found between the susceptibility of strains to products of polyamine oxidation and their virulence in the guinea-pig. Levels of polyamine oxidase were found to be significantly higher in the lung tissue from guinea-pigs vaccinated with *Mycobacterium bovis* BCG compared with lung tissue from non-vaccinated animals. This indicates that polyamine oxidation may play a part in the pathogenesis of tuberculosis in the guinea-pig. Alveolar macrophages from vaccinated and non-vaccinated guinea-pigs were found to degrade tryptophan to a similar extent, which suggests that tryptophan degradation is not involved in the antimycobacterial activity of these macrophages. Investigation of the hydrophobicity of different strains of *M. tuberculosis* found no evidence that the hydrophobicity of a strain contributed towards its virulence in the guinea-pig. Initial analysis of culture supernatants from *M. tuberculosis* 12646 suggested that this organism produced a substance similar to the homoserine lactone family of autoinducers found in Gram-negative bacteria. Two fragments of DNA from *Mycobacterium smegmatis* were isolated which appeared to code for a mycobacterial autoinducer synthase. However, after further investigation both fragments were discounted as containing a mycobacterial autoinducer synthase. Further analysis of mycobacterial supernatants found no evidence for the presence of homoserine lactone based autoinducer.
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ABBREVIATIONS

ADC = Albumin dextrose catalase complex
BCG = Bacillus Calmette-Guérin
BSA = Bovine serum albumin, plasma fraction V
CPM = Counts per minute
EDTA = Ethylene diamine tetraacetic acid
HBSS = Hank's balanced salt solution
IDO = Indoleamine 2,3-dioxygenase
LB = Luria-Bertani medium
PAO = Polyamine oxidase
PBS = Phosphate buffered saline
RIV = Root index of virulence
TE = Tris-EDTA
TSB = Tryptose soya broth
VBC = Vaccinated, boosted and challenged
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1. Introduction
1.1 History of Tuberculosis.

The mycobacterial disease, tuberculosis, is thought to be one of the oldest diseases to afflict man. Acid-fast bacilli have been found in remains dating from 5000 BC (Sager et al, 1972) and skeletons from two thousand years previous to this, also show signs of tuberculosis (Manchester, 1984). Manchester has speculated that *Mycobacterium tuberculosis* may have arisen as a mutant of *Mycobacterium bovis*, a pathogen of cattle, as the earliest recordings of tuberculous remains date from the time of domestication of cattle. At this time there was an increase in the size of the population with people living in larger communities, rather than small groups. This was important for the development of tuberculosis, which is dependent on increased, aggregated populations (Manchester, 1984). Studies in the Americas, show a much higher incidence of tuberculosis in larger, sedentary, agriculturally based communities than smaller groups of hunter-gatherers (Buickstra, 1981; Perzigian and Widmer, 1979). This appears to support the hypothesis of Manchester.

The importance of the disease to ancient cultures is evident as tuberculosis has been well documented in history and art (Evans, 1994). The term *phthisis* was introduced by Hippocrates, who also gave an excellent description of the disease (Hippocrates, 1939). Chinese documents from 2700 BC recount of a disease with symptoms very similar to tuberculosis, such as lung fever, expectoration of blood and sputum, and wasting (Keers, 1981). However, disease resulting from infection with *M.tuberculosis* can take many forms, and the various symptoms described in the historical literature may be due to other diseases.

Early treatment varied, Plato advocated no treatment at all as there was no advantage to the state or the victim (Meachen, 1978). However, the benefits of
fresh, dry air and good food were recognised by early Greek and Arab physicians. During the Middle Ages treatment took a backward step. The healing touch of the monarch was applied, with Phillip of Valois touching 1,500 sufferers in one ceremony, and Charles II touching 92,102 in his reign (Evans, 1994). Aristotle's insight into the contagious nature of the disease was also lost, until the middle of the nineteenth century, with the eminent physician Virchow claiming that tuberculosis was hereditary (Evans, 1994).

The first evidence for the contagious nature of tuberculosis came in 1865, when Villemin demonstrated that tuberculosis could be transferred to rabbits by infection with human or bovine tuberculous material (Piery and Roshem, 1931). Proof that the disease was caused by a bacterium came with a series of ground breaking experiments by Robert Koch; the results of which were presented to a meeting of the Berlin Physiological Society in 1882. Within two years, Koch had devised a method for staining tubercle bacilli in infected material, isolated the organism, and grown it in culture for several generations (Koch, 1882). He used this culture to infect animals, and was able to re-isolate tubercle bacilli from them. This work laid the foundations for the establishment of Koch's postulates; a milestone in medical microbiology.

Koch then concentrated his efforts on the immunology of the disease, and attempted to develop a vaccine based on culture filtrates of M. tuberculosis. Unfortunately, the presence of impurities in his preparations led to the deaths of several of those receiving the vaccine. A vaccine was developed in 1920 (Guérin, 1957), using the attenuated Bacille Calmette-Guérin (BCG), which is still used widely today. Antibiotics were also developed around this time; the first of which was streptomycin (Schatz and Waksman, 1944). Unfortunately, the tubercle bacillus
quickly developed resistance to this drug necessitating the development and use of other drugs in combination therapy (Snider, 1994).

The incidence of tuberculosis reached its peak in western Europe in the late eighteenth century, leading John Bunyan to name it the "Captain of all these Men of Death" (Bunyan, 1905). A general improvement in the living conditions of the lowest socio-economic groups and increased resistance to the disease appears to have been largely responsible for the decline in tuberculosis that followed (Bates and Stead, 1993). However, tuberculosis still appears to be at the epidemic stage in many parts of the world, such as sub-Saharan Africa, which has only recently been exposed to *M.tuberculosis* (Bates and Stead, 1993).

Treatment of tuberculosis in the west is largely dependent on chemotherapy and the BCG vaccine. A recent failure of the BCG vaccine to provide any protection against tuberculosis in India (Tuberculosis Prevention Trial, Madras, 1980), and the increasing prevalence of other mycobacterial species as opportunistic pathogens are other worrying trends. It has been proposed that the failure of the BCG vaccine to provide protection is because of the epidemic nature of the disease in Asia and Africa (Bates and Stead, 1993). This theory proposes that natural selection of a resistant population will lead to a decline in tuberculosis similar to that seen in Europe in the late nineteenth and early twentieth centuries.

However, there is cause for great concern, as the incidence of tuberculosis in the USA, under decline for most of this century, has shown an increase in recent years (Reider *et al*, 1989). One factor blamed for the increased incidence of tuberculosis in the USA is infection with the human immunodeficiency virus [HIV] (Rieder *et al*, 1989).
Persons infected with HIV have deficiencies in cell-mediated immunity [CMI] (Barre-Sinoussi et al, 1983; Gallo et al, 1984), including depletion and dysfunction of CD4+ cells and defective mononuclear cells (Bender et al, 1988). Those infected with HIV also have a higher incidence of tuberculosis than those not infected with HIV (Pitchenik et al, 1984; Sunderam et al, 1986; Chaisson et al, 1987). Tuberculosis in patients also infected with HIV is often extrapulmonary, suggesting that the immune system is unable to contain the mycobacteria (Pitchenik et al, 1984; Sunderam et al, 1986; Chaisson et al, 1987). The virulent nature of M. tuberculosis often means that tuberculosis occurs earlier after HIV infection than other opportunistic pathogens, leading to the suggestion that tuberculosis be used as an indicator of HIV infection (Barnes et al, 1991). Infection with Mycobacterium avium-intracellularae complex is also relatively common among patients with AIDS (Greene et al, 1982; Pitchenik et al, 1984), but only rarely reported in immunocompetent persons (Wolinsky, 1979).

Another worrying recent trend has been the emergence of strains of M. tuberculosis resistant to a variety of antibiotics (Edlin et al, 1992; Frieden et al, 1993; Iseman, 1994). Originally resistance was limited to rifampin and isoniazid (Steiner et al, 1986). However, strains have now been encountered which are resistant to all of the front-line drugs used in the treatment of tuberculosis (rifampin, isoniazid, streptomycin, ethambutol and ethionamide) and several of the second-line drugs (Heym et al, 1996). This raises the possibility of an untreatable disease (Collins, 1991). The emergence of drug-resistant strains of M. tuberculosis has been blamed on a number of factors including poor patient compliance and inadequate treatment regimes (Iseman, 1994; Yew and Chau, 1995).
However, a decrease in the number of cases of tuberculosis has been observed in New York City as a result of directly observed therapy and increased rates of completion of therapy (Frieden et al., 1995). The latest statistics show that the incidence of tuberculosis is declining in Europe and North America; unfortunately the incidence of tuberculosis is increasing in all other parts of the world (Raviglione et al., 1995).

1.2 The Mycobacteria.

The mycobacteria are typically rod shaped, aerobic, non-spore forming bacteria. Mycobacteria can be separated from most other bacteria on the basis that they are resistant to decolourisation with acid-alcohol, after staining with fuschin dyes. For this reason they are often termed acid fast. The acid fastness of mycobacteria is due to the presence of mycolic acids (Allen, 1992), which are lipid components of the cell wall unique to the mycobacteria. The mycobacterial cell wall also contains a number of other unusual components, such as lipoarabinomannan and cord factor, and a structure quite unlike that of any other bacterium (McNeil and Brennan, 1991). This thick, complex cell wall has long been associated with several other features of mycobacteria; their resistance to anti-bacterial agents, a tendency to form clumps, and their relatively slow growth rate (Wheeler and Ratledge, 1988). Pathogenic mycobacteria are also surrounded by an electron-transparent capsule (Draper and Rees, 1970; Ortalo-Mangé et al., 1995).

Species of mycobacteria fall into one of two groups depending on their growth rate. *Mycobacterium tuberculosis*, *M. avium* and *M. bovis* are all members of the slow growing mycobacteria (Smith, 1981). On the other hand *Mycobacterium smegmatis*, *Mycobacterium phlei* and *Mycobacterium chelonae* are fast growing
mycobacteria (Smith, 1981). The fast growing mycobacteria typically take two days to grow on solid media, as opposed to slow growing organisms which typically take from two to three weeks.

The slow growing mycobacteria are often pathogens, growing inside phagocytic cells and causing a chronic disease. In human tuberculosis, the infecting bacteria are most often *M. tuberculosis*, although occasionally *M. bovis* is the cause of infection (Bass *et al.*, 1990). Another mycobacterium, *Mycobacterium leprae*, is the causative agent of leprosy and several species are opportunistic pathogens of immunocompromised humans, including *M. avium-intracellulare* complex (section 1.1).

### 1.3 Pathogenesis of Tuberculosis.

Although tuberculosis is responsible for nearly three million deaths every year (Murray *et al.*, 1990; Kochi, 1991), in the majority of cases, infection with *M. tuberculosis* passes unnoticed (Grange, 1994). Many of the clinical features of the disease are due to the host's immune response, and not *M. tuberculosis* which is non-toxic. An understanding of the pathology of tuberculosis has come about through the study of the progression of the disease in rabbits and guinea-pigs, reviewed in McMurray *et al.*, 1996.

In pulmonary tuberculosis, which is by far the most common type, there are two distinct forms of the disease. Primary tuberculosis occurs with the first infection in a previously unsensitized host, whereas secondary tuberculosis is due to a re-infection or the reactivation of a previous infection. These two forms of the disease differ mainly in the level of dissemination and severity, secondary tuberculosis being the more severe.
In primary tuberculosis, infection starts with the inhalation of particles of 1-5 μm in diameter, containing 1-3 bacilli (Lurie, 1964). These particles are able to penetrate to the deepest parts of the lung (Lurie, 1964), where they encounter, and are phagocytosed by, alveolar macrophages. The low growth rate and the small number of infecting bacilli means that it take several weeks for any symptoms, as well as an immune response, to develop. The progression of the disease depends upon the activation state of the macrophage, and the virulence of the bacilli (Dannenberg, 1991). Activated macrophages are capable of inhibiting the growth of, or destroying the tubercle bacilli, whereas non-activated macrophages are incapable of controlling the growth of the mycobacteria. Failure to control the intracellular multiplication of the mycobacteria eventually leads to the destruction of the macrophages and the release of mycobacteria (Dannenberg, 1991).

Chemotactic factors such as tumour necrosis factor-α (TNF-α) and complement component C5a released from the lysed macrophages attract monocytes from the blood, which then develop into immature macrophages (Dannenberg, 1991). These unactivated macrophages phagocytose the bacilli, but again are unable to control their growth, resulting in exponential growth of the mycobacteria. A lesion known as a granuloma or tubercle forms, and grows as both the numbers of macrophages and bacilli increase (Dannenberg, 1991). Exponential growth continues inside the cells of the tubercle for two to three weeks (Lurie, 1964), and is only controlled with the development in the host of lymphocytes capable of mediating a delayed type hypersensitivity (DTH) response (Dannenberg, 1991). In pathological terms a DTH response is defined as a cytotoxic process which results in the death of the mycobacterially infected immature macrophages (Dannenberg,
This results in the formation of a caseous centre in the granuloma, in which the mycobacteria can remain dormant for years but not replicate (Hemsworth and Kochan, 1978). Thus mycobacterial growth is controlled by elimination of their favourable intracellular environment (Kaufmann, 1989), at the expense of tissue necrosis.

In resistant hosts the DTH response allows time for the development of protective cell-mediated immunity (CMI). CMI involves the activation of macrophages by CD4+ T cells reactive to mycobacterial antigens. These macrophages form a layer around the developing granuloma, and are capable of phagocytosing and destroying mycobacteria released by the caseous process, thus arresting further development of the disease (Lurie, 1964; Dannenberg, 1989). Any mycobacteria escaping from the granuloma are ingested by activated macrophages and are prevented from multiplying (Dannenberg, 1991). Thus further development of the granuloma (Ando et al, 1977) and progression of the disease is prevented (Dannenberg and Tomashefski, 1988).

Susceptible hosts, incapable of mounting an effective CMI response, attempt to control the growth of the mycobacteria by a further DTH response, causing additional tissue necrosis (Dannenberg, 1991). The mycobacteria are thus released into the blood and lymphatic systems, from where they can disseminate throughout the body, forming tubercles wherever they become lodged, often leading to the death of the host (Lurie, 1964). Patients who have also developed AIDS are very susceptible, as they lack effective macrophages and CD4+ T cells (Bender et al, 1988), which are the effector cells of the CMI response. In these patients, the disease takes on a rapidly disseminating form, similar to miliary tuberculosis seen in the very young (McMurray et al, 1996).
The generation of a well developed CMI response in resistant individuals does not necessarily prevent the further progression of the disease. Lurie noticed that if allowed to live for long enough, all of his resistant rabbits infected with virulent strains of either the human or bovine tubercle bacillus developed liquefaction of the granuloma and cavities (Lurie, 1964). This was not the case for susceptible individuals, which never reached this stage of disease (Lurie, 1964). The formation of cavities provides the mycobacteria with an environment in which they can multiply rapidly outside the cell. As macrophages cannot survive in the liquefied medium, huge numbers of bacteria can accumulate, overwhelming the normally protective CMI, and the bacilli are able to spread to other parts of the body, or other individuals. Cavity formation has been shown to be due to the DTH response (Yamamura, 1958), and desensitisation of the DTH response shown to prevent cavity formation (Yamamura, 1958; Yamamura et al, 1974). Why the resistant and susceptible rabbits differ in this process is not clear, but it has been suggested that it may be due to the increased levels of hydrolytic enzymes in activated macrophages (Dannenberg, 1994). Lysis of activated macrophages by the DTH response may release these hydrolytic enzymes into the surrounding tissues causing more extensive damage.

Secondary tuberculosis occurs as a result of reinfection or reactivation of a previous infection (Dunlap and Briles, 1993). The presence of T cells sensitised to mycobacterial antigens, and increased bacillary load in the case of reinfection, leads to a stronger DTH reaction. Persons vaccinated with M. bovis BCG also respond more quickly to infection with mycobacteria (McMurray et al, 1996). Thus BCG vaccination does not prevent reinfection, but stops the development of
tubercles beyond microscopic size (Lurie et al., 1952) thus preventing extensive damage to the lungs (McMurray et al., 1996).

1.4 Immunology of Tuberculosis.

1.4.1 Natural Resistance.

It has long been known that the genetic make-up of the host is an important factor in susceptibility and resistance to tuberculosis. Lurie showed that one week after infection, susceptible rabbits had 20-30 times the pulmonary bacillary load of resistant rabbits (Lurie, 1964). Genetic factors have also been shown in man, most notably in a study by Comstock (Comstock, 1978), who reported that among monozygotic twins there was found to be a 70-80% incidence compared with approximately 30% incidence among dizygotic twins. Whole populations also appear to vary in their resistance/susceptibility to tuberculosis. In populations which have not previously been exposed to *M. tuberculosis*, there is a very high death rate on first exposure to the disease, and the symptoms are similar to typhoid fever (Bates and Stead, 1993). It is thought that in these populations, natural selection has not had enough time to select for a resistant population.

One of the genetic factors so far identified is the expression of class II proteins of the major histocompatibility complex (MHC). The MHC is a family of polymorphic proteins involved in the presentation of antigens to lymphocytes. Processing of protein antigens involves them being broken down into peptides, which bind to a groove in the MHC molecule. The binding of peptides differs between the various MHC molecules. Thus MHC polymorphism is thought to have arisen to enable the immune system to deal with a large variety of peptides. In
human monocytes, HLA-DR expression giving rise to resistance to tuberculosis is twice as common in white as in African Americans (McPeek et al, 1992). Macrophages from resistant individuals could be induced to express these proteins constitutively, whereas they could only be induced to be transiently expressed in susceptible individuals (McPeek et al, 1992). In another study, using macrophages derived from the blood of white and African American donors, the macrophages from African American donors were found to be more permissive for the growth of \textit{M. tuberculosis} (Crowle and Elkins, 1990).

In mice, resistance to \textit{M. tuberculosis} and other intracellular pathogens has been found to be under the control of a gene named \textit{Bcg} (Forget et al, 1981; Gros et al, 1981). This gene has been mapped onto chromosome 1 of the mouse, and is found to correspond exactly with a gene coding for resistance/susceptibility to \textit{Leishmania donovani} (Lsh) and \textit{Salmonella typhimurium} (Ity). The level of control is at the innate phase of resistance in reticuloendothelial tissues, and not during acquired immunity (Skamene, 1994), the resistant phenotype being prominent in activated and not unactivated macrophages (Dannenberg, 1991). Macrophages with the resistant phenotype (Bcg') have increased respiratory burst activity and increased interleukin-2 production compared with those macrophages of a susceptible phenotype (Bcg^a). A candidate for the \textit{Bcg} gene, termed \textit{Nramp}, has been isolated recently (Vidal et al, 1993; Vidal et al, 1995). There is debate about whether or not it functions as a nitrite transport protein (Vidal et al, 1993; Formica et al, 1994).
1.4.2 Mononuclear Phagocytes.

Mononuclear phagocytes are relatively long lived cells, derived from endothelial cells within the bone marrow (Langevoort et al., 1970). Macrophages develop from circulating blood monocytes, and the populations of macrophages in different tissues differ as a result of their local environment (Lowrie, 1983). On reaching the tissue they may, or may not, be activated by cytokines released from lymphocytes. Activated macrophages have a higher metabolic rate, and are more effective at microbial killing (Cohn, 1978; Edwards and Kirkpatrick, 1986).

Mononuclear phagocytes, and in particular the alveolar macrophages, play a central role in the immune response to infection with *M. tuberculosis*. Most alveolar macrophages are highly activated cells which usually phagocytose and destroy any invading myconacteria (McMurray et al., 1996). However, if the tubercle bacilli are ingested by a poorly activated macrophage, then the mycobacteria are not destroyed but multiply within the macrophage (section 1.3). Thus the state of activation of the macrophage ingesting the mycobacteria determines whether infection with *M. tuberculosis* results in tuberculosis.

Defence against *M. tuberculosis* is as a result of CMI involving macrophages and T cells (Mackaness, 1968; Mackaness, 1969). After phagocytosis of the mycobacteria by alveolar macrophages, mycobacterial antigens are processed and presented to T cells. The T cells become activated and secrete a range of cytokines which in turn activate the macrophage (Edwards and Kirkpatrick, 1986). Macrophages are also able to produce immunomodulators, such as tumour necrosis factor-α [TNF-α] in response to mycobacteria (Valone et al., 1988). These immunomodulators may play a role in the pathogenesis of tuberculosis (Filley and Rook, 1991; Filley et al., 1992).
The work of Mackaness (Mackaness, 1968; Mackaness, 1969) showed that lymphocytes reactive to pathogenic intracellular bacteria could recruit mononuclear phagocytes to the site of infection, and activate them for antimicrobial activity. Following release of chemoattractants, monocytes migrate to the site of infection, where they differentiate into macrophages. However these immature macrophages have not been activated by mycobacterial reactive lymphocytes, and are unable to control the growth of the mycobacteria (Dannenberg, 1991). After the development of a protective CMI, it is thought that macrophages can be activated by lymphocytes resulting in them being able to destroy or control the growth of the mycobacteria (Dannenberg, 1991).

1.4.3 T Lymphocytes.

Although macrophages are the effector cells in antituberculous immunity, it is widely held that in order to destroy intracellular mycobacteria, they must first be activated by T lymphocytes. T lymphocytes recognise antigen through a heterodimeric T cell receptor (TCR). In over 90% of peripheral T cells, the TCR is composed of an \( \alpha \) and a \( \beta \) chain, the remaining cells have a TCR composed of a \( \gamma \) chain and a \( \delta \) chain. T cells expressing \( \alpha \beta \) and \( \gamma \delta \) TCRs have been shown to be of different lineages (Winoto and Baltimore, 1989).

\( \alpha \beta \) T cells can be further divided into two groups depending on the presence or absence of the surface proteins CD4 and CD8. Cells carrying the CD4 protein (CD4+) but not the CD8 protein are often termed T helper cells (Th). CD4+ cells are of particular interest in mycobacterial infections as they are thought to be the group responsible for activating cellular immunity to intracellular pathogens (Kaufmann,
1988). These cells are able to recognise antigen in association with MHC class II molecules on antigen presenting cells (APC). Binding of the CD4+ cell causes the APC to release cytokines including interleukin-1 (IL-1). These cytokines stimulate the CD4+ cell to produce IL-2 receptors and IL-2. Secreted IL-2 causes clonal expansion of the CD4+ cell in an autocrine manner. As a result of this process, there is selective expansion of only those CD4+ cells reactive to antigens presented by the APC. CD4+ cells secrete a variety of cytokines, and can be sub-divided on the basis of their cytokine secretion pattern. Although first observed in the murine system (Mosmann et al, 1986), it now appears that a similar pattern exists in man (Paronchi et al, 1991; Romagnani, 1991). The Th1 subset characteristically releases IFN-γ and IL-2 which are thought to result in macrophage activation, whereas cells of the Th2 subset release IL-4, IL-5 and IL-10 which are associated with activation of humoral immunity (Mosmann and Coffman, 1989). Cells expressing the CD8 molecule but not CD4 (CD8+) are of the described as being of the suppressor/cytotoxic phenotype, and recognise antigen in association with MHC class I molecules.

It has become apparent that γδ T cells may also play a role in immunity to tuberculosis. The majority of these cells are CD4-, CD8- and are MHC unrestricted, but are dependent on APCs for activation (Orme et al, 1993). γδ T cells appear to have both cytotoxic and helper functions (Munk et al, 1990).

Evidence that immunity to mycobacteria required the presence of T lymphocytes was based upon adoptive transfer experiments (North, 1974; Lefford, 1975). If CD4+ cells from a line capable of activating macrophages infected with M. tuberculosis, were used for adoptive transfer to irradiated mice, survival of the mice
following infection with *M. tuberculosis* was significantly prolonged (Leveton, 1989). However, a CD8+ cell line has also been shown to be able to passively transfer resistance to *M. tuberculosis* in mice with T cell deficiency (Orme and Collins, 1984).

Using antibodies, CD4+ or CD8+ populations have been specifically deleted. Deletion of either subset brought about a significant increase in the bacterial load (Müller, 1987). This study suggests that the major effector cells are CD4+ T cells as doubly deleted mice fared no worse than CD4+ deleted mice. However, the nature of the role of the CD8+ T cell in immunity to *M. tuberculosis* remains unclear. Two separate studies have found that mice depleted of CD4+ T cells had decreased survival times after infection with *M. tuberculosis*, but depletion of CD8+ T cells failed to have an effect on the survival of the mice (Leveton *et al*, 1989; Pedrazzini *et al*, 1987). Mice depleted of CD8+ T cells did however show a histopathologically distinct form of the disease, with decreased necrosis and bacterial growth (Leveton *et al*, 1989). In more recent work, mice with a targeted disruption of the β2-microglobulin gene which renders them unable to generate CD8+ T cells, were shown to develop a progressive, disseminated form of disease after infection with a normally sub-lethal dose of *M. tuberculosis* (Flynn *et al*, 1993). Further evidence for the roles of CD4+ and CD8+ T cells has come from a model based on mice vaccinated with the non-pathogen *Mycobacterium* *w*. Vaccination with killed *M. w* has been shown to induce protection against tuberculosis in mice (Singh *et al*, 1991). T cell deletion experiments, using anti-CD4+ or anti-CD8+ monoclonal antibodies, showed that both T cell subsets were involved in protection (Guleria *et al*, 1993).

CD8+ T cells have been shown to produce small amounts of the Th1 type cytokine IFN-γ (de Libero *et al*, 1988; Guleria *et al*, 1993), which corresponds with a
potential role in macrophage activation. The consensus opinion however, is that the protective effect of CD8+ cells is mediated by lysis of macrophages unable to kill phagocytosed mycobacteria (Kaufmann, 1988). It is proposed that lysis of such macrophages enables the bacteria to be taken up by macrophages able to kill them, and thus intracellular growth of the mycobacteria is controlled. Lysis of mycobacterium infected macrophages is also a property of CD4+ T cells (Mustafa and Godal, 1987; Ottenhoff et al, 1987; Kumararatne et al, 1990; Mutis et al, 1993).

The majority of isolated CD4+ T cells appear to show this function, and show a Th1 cytokine secretion profile (Mutis et al, 1993). These cells may be involved in the DTH reaction shown to be transferable with a population of Th1 CD4+ T cells (Boom et al, 1987).

A role for γδ T cells in murine immunity to M. tuberculosis was first proposed with the discovery that a large proportion of these cells were reactive to mycobacterial antigens (O'Brien et al, 1989). γδ T cells have also been found to accumulate in the lesions of leprosy patients (Modlin et al, 1989). The specificity of the γδ T cell response is also of interest. Protective vaccination against tuberculosis is only effective when live organisms are used, not heat killed organisms or antigens (Mackaness, 1962; Orme, 1988). Comparison of the populations of T cells expanded by APCs pulsed with live organisms or soluble protein antigens found that CD4+ T cells were expanded by both stimuli, whereas γδ T cells could only be expanded by APCs pulsed with live organisms (Havlir et al, 1991; Boom et al, 1992). Differences have also been found in the ability of M. tuberculosis to stimulate γδ T cell expansion in the blood of patients with mycobacterial disease compared with healthy contacts (Barnes et al, 1992; Ueta et al, 1994). Those tuberculosis patients
with protective immunity (tuberculin reactors and patients with tuberculous pleuritis) show significantly greater expansion of \( \gamma \delta \) T cells than those with ineffective immunity (patients with advanced pulmonary or miliary tuberculosis) (Barnes et al., 1992). The same study showed a similar picture in patients with leprosy, where tuberculoid patients showed significantly greater expansion of \( \gamma \delta \) T cells than patients with the lepromatous form of the disease (Barnes et al., 1992).

The role of \( \gamma \delta \) T cells in immunity to the intracellular pathogen *Listeria* appears to be in the early response to infection (Hiromatsu et al., 1992; Mombaerts et al., 1993). Either \( \alpha \beta \) or \( \gamma \delta \) cells were found to be able to mediate early protection, and although \( \alpha \beta \) T cells were mainly responsible for protection in secondary infection, \( \gamma \delta \) T cells were also shown to be involved. This also appears to be the case for tuberculosis (Inoue et al., 1991).

T cells also show patterns of emergence in the mouse model which may correlate with protective and suppressive responses. The first population to emerge are CD4+ IFN-\( \gamma \) secreting cells, recognising mycobacterial culture filtrate proteins (Orme et al., 1992; Orme et al., 1993). The emergence of this population of cells coincides with the onset of protective immunity (Orme et al., 1992; Orme et al., 1993). Later in the infection, CD4+ IL-4 secreting cells appear, which are cytolytic, and recognise the mycobacterial 65 kDa heat shock protein (Orme et al., 1992; Orme et al., 1993).

### 1.4.4 Cytokines in Tuberculosis.

The activation of macrophages for antimiicrobial activity by lymphocytes is thought to occur via the production of cytokines. These cytokines can both activate
and inhibit mononuclear phagocyte activity. However, different T cell clones produce different combination of cytokines (Paronchi et al., 1991; Romagnani, 1991). Differences in the pattern of cytokine production are apparent in patients with different forms of tuberculosis and leprosy (Sieling and Modlin, 1994).

The range of pathologies witnessed in leprosy are closely linked to the immune response of the patient. In tuberculoid leprosy, there are few lesions and the disease is contained by a strong, protective CMI response. Patients with the most severe form of the disease, lepromatous leprosy, show disseminated disease, and a weak or absent CMI response (Sieling and Modlin, 1994). Between these two extreme forms of the disease, there is a whole spectrum of pathologies. Tuberculosis does not show such a broad range of disease, but pathology and immune response are linked. Tuberculous pleurisy is regarded as showing a protective form of immunity, while active pulmonary tuberculosis correlates with a non-protective response (Barnes et al., 1992).

The different patterns of cytokine secretion by T cell clones shown by Mosmann and co-workers (Mosmann et al., 1986), led to the classification of cytokines as either type 1 or type 2. Type 1 cytokines are produced by Th1 T cells, whereas type 2 cytokines are produced by Th2 T cells. As described earlier (section 1.4.3), these cytokines activate separate sections of the immune system, and so control the type of immune response.

The patterns of cytokine production in different forms of mycobacterial diseases indicate that, while certain groups of cytokines elicit a protective response, others bring about a non-protective response. Patients with tuberculoid leprosy show a predominance of CD4+ Th1 T cells, producing type 1 cytokines such as IFN-γ and IL-2. On the other hand lesions from patients with lepromatous leprosy, show
CD8+ T cells producing the type 2 cytokines IL-4 and IL-5 (Modlin et al., 1984; Yamamura et al., 1991).

A similar dichotomy has been found in tuberculosis, where tuberculous pleuritis is regarded as being a manifestation of a protective response (Orme et al., 1993; Wallis and Ellner, 1994). Levels of IFN-γ, TNF-α and calcitriol have been shown to be elevated in pleural fluid from patients with tuberculous pleuritis (Barnes et al., 1989; Barnes et al., 1990) suggesting a role for these cytokines in protective immunity. Patients with a non-protective response, such as active pulmonary tuberculosis, have been shown to have defective production of IFN-γ and IL-2 (Vilcek et al., 1986; Toosi et al., 1986; Sánchez et al., 1994).

Elucidation of the cytokines responsible for activation of the antimycobacterial activity of macrophages has long been a goal of workers in the field. There have been many attempts at stimulating macrophages in vitro with cytokines, either individually or in combination. These difficulties have been compounded, as data comes from two very different models: murine and human macrophages.

Using the murine model, several groups have shown that IFN-γ alone is sufficient to activate macrophages to cause inhibition of growth (Rook et al., 1986; Flesch and Kaufmann, 1987; Denis, 1991) or even complete stasis of \textit{M. tuberculosis} (Rook et al., 1985). Murine macrophages activated by IFN-γ are also able to kill the vole tubercle bacillus \textit{M. microti} (Khor et al., 1986). When IFN-γ was used in conjunction with TNF-α, the macrophages were able to kill a virulent strain of \textit{M. tuberculosis} through an L-arginine dependent mechanism (Chan et al., 1992). The timing of addition of IFN-γ is also important, as macrophages activated prior to
infection with mycobacteria show increased antimycobacterial activity (Flesch and Kaufmann, 1990; Sypek et al, 1993). The importance of IFN-γ in vivo has been demonstrated using mice which have a targeted disruption of the gene for IFN-γ (Cooper et al, 1993; Flynn et al, 1993) or the IFN-γ receptor (Kamijo et al, 1993). Both groups of animals were unable to control a normally non-fatal infection with either M. tuberculosis (Cooper et al, 1993; Flynn et al, 1993) or M. bovis BCG (Kamijo et al, 1993).

The role of TNF-α in the murine model is less clear than that of IFN-γ. Sypek and co-workers found that treatment of macrophages with TNF-α before or after infection with M. bovis BCG, did not effect antimycobacterial activity (Sypek et al, 1993). However the same study found that anti-TNF-α antibodies reduced the ability of sensitised lymphocytes to activate these macrophages, suggesting that TNF-α may be more effective as a membrane-bound form. Granuloma formation and prevention of disseminated disease are also dependent on TNF-α in both M. bovis BCG (Kindler et al, 1989) and Listeria monocytogenes (Rothe et al, 1993) infection. However, these responses may not be solely dependent on macrophage activation.

The state of knowledge of how human macrophages are activated for antimycobacterial defence contrasts with that of the murine system. Where IFN-γ has been used alone, the results have varied, showing weak inhibition (Rook et al, 1986a), no effect (Denis et al, 1990; Denis, 1991; Robertson and Andrew, 1991) or even enhancement of growth (Douvas et al, 1985; Rook et al, 1986a).

The elevated levels of TNF-α and calcitriol found in tuberculous pleuritis (Barnes et al, 1990; Barnes et al, 1991) suggest a role for these factors in protective
immunity against *M. tuberculosis*. TNF-α has been shown to enable human macrophages to cause stasis of tubercle bacilli (Flesch and Kaufmann, 1990).

Activation of macrophage mycobacteriostasis by calcitriol has been shown (Rook *et al*, 1986b; Crowle *et al*, 1987). Metabolites of calcitriol cause maturation of the human monocytic cell line U937 (Amento *et al*, 1984) and maturation of the promyelocytic cell line HL60 into macrophage like cells (Mangelsdorf *et al*, 1984). When TNF-α and calcitriol were used in combination with IFN-γ, they were found to activate human monocytes to kill *M. tuberculosis*, regardless of whether the cytokines were added before or after infection (Denis, 1991). These findings have been disputed by Warwick-Davies and co-workers who were unable to confirm activation of human monocytes for mycobactericidal defence by this cocktail of cytokines (Warwick-Davies *et al*, 1994).

Other cytokines have been reported as having both activating and inhibitory effects on macrophages. Interleukin-12 (IL-12) can activate Natural Killer (NK) cells to produce IFN-γ, which in turn specifically directs T cells towards the Th1 phenotype (Kobayashi *et al*, 1989; Manetti *et al*, 1993). Naive T cells stimulated with antigen and antigen presenting cells, in the presence of IL-12, produce type 1 cytokines (Hsieh *et al*, 1993). Studies of IL-12 in tuberculous pleuritis (Zhang *et al*, 1993) and tuberculoid leprosy (Sieling *et al*, 1994) show very high levels of this cytokine, compared with samples from serum or tuberculoid patients respectively.

The type 2 cytokines IL-4 and IL-10 (Fiorentino *et al*, 1991a; Fiorentino *et al*, 1991b; de Waal Malefyt *et al*, 1991; Oswald *et al*, 1992; Nabioullin *et al*, 1994) along with IL-13 (Minty *et al*, 1993; Doherty *et al*, 1993) and transforming growth factor-β (TGF-β) (Oswald *et al*, 1992) have been shown to down-regulate
macrophage function. However, the picture is not totally clear as there is a report of IL-4 inducing mycobacteriostasis in murine macrophages (Flesch and Kaufmann, 1990). The immunosuppressive properties of type 2 cytokines may be involved in controlling cell-mediated immunity (Modlin and Nutman, 1993).

Cytokines may also be involved in the pathology of tuberculosis, as well as protective immunity; particularly TNF-α. Infection with live or ultrasonically disrupted *M. tuberculosis*, increases the sensitivity of cell lines to the cytotoxic effects of TNF-α (Filley and Rook, 1991; Filley et al., 1992). It is hypothesised that fever, cachexia and caseous necrosis may be as a result of a local or systemic overproduction of TNF-α (Grau et al., 1992). The destruction of nerve cells observed in leprosy may also be mediated by TNF-α (Selmaj and Raine, 1988).

### 1.4.5 Natural Killer Cells.

Natural Killer (NK) cells, also known as third population lymphocytes, form a separate lineage from B and T cells (Bancroft, 1993). Although the traditional view of the role of NK cells is the lysis of tumour or virally-infected cells (Herberman and Holden, 1978), they have also been shown to have a cytotoxic effect on cells infected with intracellular bacterial pathogens (Carl and Dash, 1986; Blanchard et al., 1987) and a direct toxic effect on bacteria themselves (Garcia-Peñarrubia et al., 1989). NK cells are also able to secrete cytokines which result in the activation of macrophages to kill intracellular bacteria, including *M. avium* (Bermudez and Young, 1991), and bias the T cell response towards a Th1 type (Kobayashi et al., 1989; Manetti et al., 1993). The beige mouse strain, which has no NK cell activity (Roder et al., 1979) also shows increased susceptibility to *M. intracellulare* (Gangadharam et
al, 1983). It is therefore conceivable that NK cells play a role in the defence against pathogenic mycobacteria.

1.4.6 Polymorphonuclear Leukocytes.

Although the macrophage-T cell relationship is central to protective immunity to *M. tuberculosis*, it is possible that polymorphonuclear leukocytes (PMNLs) are important. The majority of cells accumulating in the initial inflammatory response are PMNLs (Grange, 1984), which release chemotactic factors to attract monocytes (Anthony et al, 1983). PMNLs isolated from persons who show a positive reaction to mycobacterial purified protein derivative [PPD] (Mandell and Fuller, 1972). This, along with data showing greater adherence of PMNLs from patients with tuberculosis (Bass et al, 1981), suggests that these cells are in a greater state of activation.

Neutrophils have also been reported to kill *M. tuberculosis in vitro* (Brown et al, 1987). This killing appears to be independent of the respiratory burst, as neutrophils from patients with chronic granulomatous disease, which are unable to mount a respiratory burst, are equally microbicidal (Jones et al, 1990). The enzyme myeloperoxidase, which is found in neutrophils, but not macrophages (Beelen et al, 1979), catalyses the iodination of hydrogen peroxide, thus increasing it's toxicity (Klebanoff, 1968). Macrophages have been reported to achieve significant iodination of hydrogen peroxide after phagocytosis of material from dead neutrophils (Heifets et al, 1980) possibly augmenting their antimycobacterial ability (Edwards and Kirkpatrick, 1986). However, Brown and co-workers showed that hydrogen peroxide was equally toxic to *M. tuberculosis* in the presence or absence of myeloperoxidase and chloride ions (Brown et al, 1987).
Macrophages have been shown *in vitro* to have increased antimycobacterial ability after ingestion of granulocyte material (Silva *et al.*, 1989). The same study demonstrated that macrophages from mice infected with mycobacteria were found to contain a protein of granulocytes, lactoferrin, which is normally lacking in mature macrophages (Beelen *et al.*, 1979). Lactoferrin enhances hydroxyl radical production in neutrophils (Ambruso and Johnston, 1981) as well as having a direct antibacterial effect (Arnold *et al.*, 1977; Bortner *et al.*, 1986).

1.4.7 Humoral Immunity.

Immunity to *M. tuberculosis* is widely held to be due to T cell activation of macrophages (Mackaness, 1968; Mackaness, 1969), with the role of the humoral response unclear (Edwards and Kirkpatrick, 1986). Experiments involving the transfer of sera from animals resistant to *M. tuberculosis* infection, as a result of vaccination with *M. bovis* BCG, to non-immune animals, failed to give any protective effect (Raffel, 1955; Reggiardo and Middlebrook, 1974). There is however an antibody response to tuberculosis similar to that seen in other infections with an initial appearance of IgM followed by the appearance and persistence of IgG (Grange, 1984). Patients with tuberculosis have also been reported with elevated levels of IgA (Skvor *et al.*, 1979) and IgE (Anders *et al.*, 1989; Yong *et al.*, 1989). Antibodies can give an indication of the severity of the disease, as high concentrations of IgA correlate with increased severity (Skvor *et al.*, 1979), and an examination of patients showing a whole spectrum of cell-mediated responses showed that antibody levels were inversely related to protective cell-mediated immunity (Lenzini *et al.*, 1977). Antibodies may have a direct adverse effect on the patient as a result of the formation of circulating immune complexes of antibody and
mycobacterial antigens (Johnson et al, 1981; May et al, 1983). Formation of these complexes indicates a poor prognosis (Brostoff et al, 1981) and may impair kidney function (Shribman et al, 1983). Increased antibody responses in severe tuberculosis may be due to a type 2 cytokine response, which results in enhanced antibody production (Stevens et al, 1988) and also correlates with increased severity of disease in leprosy (Yamamura et al, 1991).

Opsonisation by complement may also affect immunity to M. tuberculosis. Complement is fixed to the mycobacteria by the alternative pathway, with the C3b component thought to be the most important opsonin (Schlesinger et al, 1990). Binding of mycobacteria coated with complement component 3 enables human macrophages to phagocytose the mycobacteria via complement receptor types 1, 3 and 4 (Schlesinger et al, 1990; Hirsch et al, 1994). Murine macrophages have also shown uptake of non-opsonised mycobacteria via the C3 receptor (Stokes et al, 1993).

1.4.8 Vaccination Against Tuberculosis.

A effective vaccine against tuberculosis has been one of the foremost topics of research into mycobacteria since the discovery of the tubercle bacillus by Koch (Koch, 1882). The first vaccine was made by Koch himself using an autoclaved culture filtrate of M. tuberculosis, which he called 'Old Tuberculin' (Koch, 1890). However, the presence of carbohydrate impurities in this extract led to the deaths of several patients, and withdrawal of the vaccine. Since this early failure, the story of the search for a protective vaccine against tuberculosis has shown an oscillating pattern of hope and disappointment.
The bacille Calmete-Guérin (BCG) vaccine is the oldest and most widespread vaccine in use, and is still the only antituberculosis vaccine available for human use (Collins, 1991). It was developed by passage of an isolate of *M. bovis* through a medium containing ox bile two hundred and thirty times. The resulting avirulent strain was shown to be a safe and effective vaccine as a result of controlled clinical trials (Stein and Aronson, 1953). The safety of the BCG vaccine for immunocompetent individuals has remained unquestioned, but its protective efficacy has been questioned, as the level of protection shown in clinically controlled trials ranges between 0% and 80% (Fine, 1994). The most recent large scale trial, which took place around the Indian village of Chingleput, found that patients receiving the vaccine were more likely to develop tuberculosis than those receiving a placebo (Tuberculosis Prevention Trial, Madras, 1980). Studies also show that protection is high against tuberculous meningitis, but lower against the more common pulmonary tuberculosis (Fine, 1994). A recent meta-analysis of data from BCG vaccinated individuals showed a 50% reduction in the risk of contracting tuberculosis in this group (Colditz *et al.*, 1994). The BCG vaccine has also shown variability in protection within the same trial. A trial in Malawi showed that BCG was not protective against tuberculosis, but was protective against leprosy (Ponnighaus *et al.*, 1992).

Several hypotheses have been put forward to explain the discrepancies of the clinical trials, the most convincing of which is the influence of atypical environmental mycobacteria. It has been suggested that environmental mycobacteria mask the protective effect of BCG vaccination (Palmer and Long, 1966), or act antagonistically to BCG (Stanford *et al.*, 1981). The finding that British children of Indian subcontinent ethnic origin were protected by vaccination with BCG (Rodrigues *et al.*, 1991), is evidence against genetic susceptibility being the
determining factor in effective BCG vaccination. Neither did the strain of BCG explain the failure of the Chingleput trial, as a strain showing a high level of protection in Britain (Medical Research Council, 1972) was ineffective in Chingleput (Tuberculosis Prevention trial, Madras, 1980).

Development of several improved antituberculosis vaccines has been instigated. The so-called rational approach, involves the evaluation of protective mycobacterial antigens. So far more than fifty mycobacterial proteins have been screened for their immunogenicity (Kaufmann and Young, 1992). One of the most interesting groups of antigens examined so far are mycobacterial proteins secreted during growth (Abou-Zeid et al, 1988; Andersen et al, 1991a). These proteins are major antigens for both human and murine T cells (Andersen et al, 1991b), and are capable of inducing memory T cells (Andersen and Heron, 1993), which could adoptively transfer resistance to T cell deficient recipients (Andersen, 1994). A mixture of proteins from a short-term culture filtrate of M. tuberculosis in combination with adjuvant, has recently been shown to engender similar protection to that given by BCG (Andersen, 1994). Andersen has proposed that a protective T cell response is dependent on the production of secreted proteins by mycobacteria (Andersen, 1994). This would explain the superior protection provided by live vaccine strains (Mackaness, 1968; Orme, 1988). The variation in BCG efficacy shown in several trials may be as a result of environmental mycobacteria sensitising the immune system to inhibit the growth of a subsequent dose of BCG vaccine. Inhibition of growth of the BCG vaccine prevents production of protective secreted protein antigens (Andersen, 1994). Andersen has suggested that use of short-term culture filtrates may overcome the variation in efficacy of BCG vaccination, as protective antigens are present regardless of their immune status.
Special Note

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to challenge with realistic numbers of *M. tuberculosis* through the usual routes of infection (Rook, 1985), and murine macrophages show only tuberculostatic and not tuberculocidal activity. Most experiments use macrophages derived from the peritoneal cavity or bone marrow precursor cells and not those from the lungs, which may show distinct properties. Further, the tuberculostatic properties of murine macrophages have been shown to be dependent on the generation of reactive nitrogen intermediates (RNI) from L-arginine (Chan *et al*, 1992). There is still a great deal of doubt over whether this pathway exists in human macrophages (section 1.5.2).

Attempts to activate human macrophages for tuberculocidal activity have, with the exception of one report (Denis, 1991b), failed (Section 1.4.4). Even reports of tuberculostasis have been inconsistent (Crowle and May, 1981). It has been necessary therefore, to use a model system, which shows similarities to that of humans.

Vaccination of guinea-pigs with *M. bovis* BCG under a protocol shown to result in killing of *M. tuberculosis* in vivo has been developed (Jackett *et al*, 1981a). Alveolar macrophages isolated from guinea-pigs vaccinated under this protocol have been shown to kill *M. tuberculosis* in vitro (O'Brien *et al*, 1991). The susceptibility to strains of mycobacteria and the pathology of the disease is similar in guinea-pigs compared with that observed in humans (Brown, 1983), establishing the suitability for this animal as a model for tuberculosis in humans.

**1.5.1 Generation of Reactive Oxygen Intermediates.**

Macrophages are able to reduce molecular oxygen to superoxide (O$_2^-$) by the addition of an electron from NADPH oxidation. During phagocytosis, there is an
increase in oxygen consumption by macrophages which is independent of cytochromes (Sbarra and Karnovsky, 1959), accompanied by increased superoxide and hydrogen peroxide production (Iyer et al, 1961), termed the respiratory burst. O$_2^-$ generated by this system is converted to hydrogen peroxide (H$_2$O$_2$), by the enzyme superoxide dismutase (SOD) (Beaman and Beaman, 1984). O$_2^-$ and H$_2$O$_2$ can also combine to form other toxic products such as singlet oxygen (O$_2$) and hydroxyl radical (OH) (Andrew et al, 1985). These products, termed reactive oxygen intermediates (ROI) have been shown to be toxic to intracellular pathogens including *M. tuberculosis* (Murray and Cohn, 1979; Walker and Lowrie, 1981).

Although ROI are toxic for mycobacteria, there is contradictory evidence for their involvement in the defence of macrophages against *M. tuberculosis*. A study of isoniazid sensitive strains of *M. tuberculosis* isolated from British and south Indian patients, found that there was a correlation between their sensitivity to hydrogen peroxide and their virulence in the guinea-pig (Mitchison et al, 1963). The differences in susceptibility were not due to differences in the amount of catalase produced, which was not significantly different between strains. It has also been demonstrated that there is an association between loss of virulence and decreased catalase activity (Middlebrook, 1954). There is no correlation between virulence of strains of *M. tuberculosis* and susceptibility to superoxide or levels of superoxide dismutase (Jackett et al, 1978).

Macrophages from animals shown to kill *M. tuberculosis in vivo* (Jackett et al, 1981a) show higher production of superoxide and hydrogen peroxide than macrophages from unvaccinated, control animals (Jackett et al, 1981b). Resistance to isoniazid is associated with an increase in susceptibility to hydrogen peroxide (Cohn et al, 1954; Knox et al, 1956). The survival of two strains of *M. tuberculosis*,
one of low virulence and one of high virulence, and the corresponding isoniazid
resistant mutants was compared in guinea-pigs by Jackett and co-workers (Jackett
et al., 1981a). The peroxide resistant, isoniazid sensitive strains showed greater
survival than their peroxide sensitive, isoniazid resistant counterparts.

The killing of the vole tubercle bacillus, *M. microti* by murine
macrophages, could be reversed by the addition of catalase (Walker and Lowrie,
1981) was claimed as evidence that hydrogen peroxide was responsible for
macrophage killing of mycobacteria. However, this view could be challenged as
catalase can act as a scavenger of reactive nitrogen intermediates [RNI] (Li et al,

However, sufficient evidence has now accumulated that demonstrates that
ROI alone are not responsible for the killing of *M. tuberculosis* by activated
macrophages. A model using macrophages from guinea-pigs vaccinated under the
same protocol as Jackett (Jackett et al., 1981a; Jackett et al., 1981b) has been used
to investigate the role of ROI in guinea-pig alveolar macrophage killing of *M.
tuberculosis* (O'Brien et al., 1991; O'Brien and Andrew, 1991). There was found to
be no correlation between the sensitivity of the strain of *M. tuberculosis* and the
degree to which it was killed by macrophages from vaccinated animals. All of the
strains tested were killed to a similar extent. Strains of differing virulence also
showed no difference in their ability to avoid triggering the respiratory burst of the
macrophages (O'Brien et al., 1991). Guinea-pig alveolar macrophages showed a
similar level of mycobactericidal activity in the presence of the scavengers of
hydroxyl radical and hydrogen peroxide, manitol and catalase respectively (O'Brien
and Andrew, 1991). Therefore, although Jackett and co-workers found decreased
survival of peroxide sensitive strains *in vivo*, and increased production of hydrogen

Murine macrophage killing of M. tuberculosis in vitro was not affected by addition of catalase (Chan et al, 1992). In the same study, a mutant of the murine macrophage cell line J774, which was unable to produce a respiratory burst, was able to kill M. tuberculosis to the same extent as the parental strain. This appears to be in contradiction of the work of Walker and Lowrie (Walker and Lowrie, 1981).

1.5.2 Generation of Reactive Nitrogen Intermediates.

Reactive nitrogen intermediates (RNI) are a group of nitrogen oxides which include nitric oxide (NO), a by product of the production of citrulline from L-arginine (Marletta et al, 1988). Nitric oxide is a relatively short lived molecule, with a half life of approximately 6 seconds (Geller and Billiar, 1993), and reacts readily with oxygen to produce other RNI species (Marletta, 1989). The function of NO is dependent upon its concentration. At low concentrations, NO acts as a messenger molecule, by activating guanylate cyclase (Bredt and Snyder, 1989). At higher concentrations it has been found to have potent antitumour effects (Hibbs et al, 1987) and is toxic to a wide range of micro-organisms (Alspaugh and Granger, 1991; Mauèl et al, 1991; Vincendeau et al, 1992; Assreuy et al, 1994) including mycobacteria (Chan et al, 1992; O’Brien et al, 1994). The toxicity of NO is increased in combination with superoxide, when peroxynitrite is formed (Beckman et al, 1990; Zhu et al, 1992; Denicola et al, 1993). A correlation has been found between the root index of virulence of M. tuberculosis strains and their susceptibility to nitric oxide (O’Brien et al, 1994) in vitro.
The enzyme nitric oxide synthase (NOS), which is responsible for the production of NO from L-arginine, occurs in two forms in mammalian cells and shows differing patterns of expression (Geller and Billiar, 1993). The constitutive form (cNOS), is expressed continuously, producing small amounts of NO upon activation. The inducible form (iNOS) is expressed only after activation of the cell, and produces large amounts of NO. The iNOS from murine macrophages has been cloned and characterised (Lyons et al, 1992; Xie et al, 1992), and is structurally different from cNOS (Lowenstein et al, 1992).

The importance of generation of RNI in murine macrophages has been shown by inhibition of NOS activity using the competitive inhibitor N\textsuperscript{6}-monomethyl-L-arginine (N\textsuperscript{6}MMA). This reduces the antimicrobial activity of murine macrophages against a number of pathogens (Granger et al, 1988; James and Glaven, 1989; Green et al, 1990; Feng and Walker, 1993), including M. leprae (Adams et al, 1991), M. bovis BCG (Flesch and Kaufmann, 1991) and M. tuberculosis (Denis, 1991). Mice administered with NOS inhibitors showed increased mortality, tissue damage and bacterial numbers, after infection with virulent M. tuberculosis (Chan et al, 1995).

The importance of generation of RNI to the antimicrobial activity of human macrophages is still unknown. There is still a great deal of debate as to whether NOS is a component of human mononuclear cells. Nitric oxide synthase activity, or an inhibitory effect of N\textsuperscript{6}MMA on antimicrobial activity, has not been demonstrated in several studies where mononuclear cells were activated by cytokines or lipopolysaccharide (Murray and Teitelbaum, 1992; Sakai and Milstein, 1993; Schneemann et al, 1993; Zembala et al, 1994). However, others claim to have shown an inhibitory effect of N\textsuperscript{6}MMA on human macrophage antimycobacterial
activity (Denis, 1991c), or production of NO in human mononuclear cells stimulated with cytokines (Muñoz-Fernández et al., 1992), *M. avium* (Dumarey et al., 1994) or tumour cells (Zembala et al., 1994). Others have found iNOS in human alveolar macrophages by immunohistochemistry, especially in chronically inflamed areas (Kobzik et al., 1993). This is with regard to the protective function of the granuloma in human tuberculosis. Human macrophages also appear to lack tetrahydrobiopterin (Stuehr et al., 1991), which is required as a co-factor by iNOS (Sakai et al., 1992).

### 1.5.3 Lysosomal Enzymes.

It is commonly held that material phagocytosed by mononuclear phagocytes is held in a membrane-bound structure termed the phagosome. Lysosomes containing degradative enzymes fuse with the phagosome to form a phagolysosome, releasing enzymes which degrade the ingested material.

Lysosomes contain a wide range of hydrolytic enzymes, some of which are toxic to micro-organisms (Andrew et al., 1985). Guinea-pig alveolar macrophages capable of killing *M. tuberculosis in vitro* were found to possess an acid soluble fraction which is toxic to *M. tuberculosis* at pH 5.5 but not pH 7.0. A similar fraction was not found in non-immune animals (O'Brien, 1995).

A pioneering study by Armstrong and D'Arcy Hart (Armstrong and D'Arcy Hart, 1971) found that virulent mycobacteria phagocytosed by murine macrophages could inhibit phagosome-lysosome fusion (P-LF). This could be reversed by coating the mycobacteria with antibody prior to infection, but this did not affect the survival rate of the mycobacteria (Armstrong and D'Arcy Hart, 1975). The vole pathogen *M. microti* also possesses the ability to avoid P-LF (D'Arcy Hart et al., 1972), possibly mediated by release of cAMP (Lowrie et al., 1975). Dead mycobacteria were not able...
to avoid P-LF (Armstrong and D'Arcy Hart, 1971; Lowrie et al, 1975). A correlation has also been found between inhibition of P-LF and intracellular survival of *M. avium* (de Chastellier et al, 1993). It appears then, that mycobacteria can avoid lysosomal contents by inhibiting P-LF, and may even be resistant to the contents of lysosomes. Mycobacteria are known to be very resistant to the digestive properties of phagocytes (Stähelin et al, 1956)

This view has recently been challenged however by a study which followed the location of various mycobacteria within macrophages over a period of several days (McDonough et al, 1993). Twenty four hours after infection, 85% of the mycobacteria were found in fused phagolysosomes, but four days after infection, 50% of *M. tuberculosis* strains H37Rv and H37Ra were found in non-fused vesicles with tightly opposed membranes. *Mycobacterium bovis* BCG and heat killed *M. tuberculosis* showed no change in their intracellular location. Some of the phagocytosed *M. tuberculosis* appeared to escape into the cytoplasm, a feature also of *M. leprae* (Evans et al, 1973), however there is conflicting evidence that *M. tuberculosis* can escape into the cytoplasm (Myrvik et al, 1984; Douvas et al, 1986).

The findings of McDonough and co-workers have also been challenged (Clemens and Horwitz, 1995). This study found the same pattern of inhibition of P-LF demonstrated by Armstrong and D'Arcy Hart (Armstrong and D'Arcy Hart, 1971). The discrepancy has been explained by differences in the viability and preparation of the mycobacteria (Clemens and Horwitz, 1995).

Another mycobacterial survival mechanism may be prevention of acidification of the phagosome. Phagosomes containing mycobacteria have been shown not to be acidified (Gordon et al, 1980; Crowle et al, 1991). Vacuoles containing *M. avium* have been found to lack the vesicular proton ATPase, which is responsible for
phagosome acidification (Sturgill-Koszycki et al, 1994). This may explain the survival of mycobacteria in fused vacuoles, as the neutral pH would restrict the activity of lysosomal hydrolases. High pH has been associated with reduced efficacy of cytokine-induced bacteriostasis (Appelberg and Orme, 1993).

1.5.4 Cationic Proteins.

Phagocytes possess a range of antibacterial proteins with relatively high proportions of the amino acids lysine, arginine or proline (Patterson-Delafield et al, 1980; Frank et al, 1990; Spitznagel, 1990; Lehrer et al, 1991; Lehrer et al, 1993). The antibacterial properties of these proteins is not due to enzymic activity, but rather as a result of their cationic nature (Elsbach and Weiss, 1983). The defensin proteins of human neutrophils are toxic to M. avium (Ogata et al, 1992) and M. fortuitum (Lehrer et al, 1993), but it is unknown whether they are toxic to M. tuberculosis (Lehrer et al, 1993). A further three proteins have been isolated from murine macrophages, and found to be toxic to M. fortuitum (Hiemstra et al, 1993).

Other investigations have tested the crude extracts of macrophages against bacteria. Antibacterial activity was found to be higher in homogenates from activated macrophages than unactivated macrophages (Sharma and Middlebrook, 1976). The tuberculostatic activity of guinea-pig macrophages was also higher in homogenates from vaccinated animals (Ramsuer and Suter, 1964).

1.5.5 Iron Sequestration.

Iron is essential for bacterial growth as it is a component of many enzymes and other proteins, such as cytochromes. Micro-organisms deprived of iron grow
poorly and eventually undergo complete stasis or death (Wheeler and Ratledge, 1985). It has been estimated that iron is required by aerobic bacteria at a concentration of $10^{-6}$M (Weinberg, 1978). Although the concentration of iron in human serum is $10^{-5}$M, most is bound to the iron binding proteins lactoferrin and transferrin, so that the concentration of free iron is approximately $10^{-18}$M (Barclay and Ratledge, 1983). Like most other organisms, mycobacteria produce high-affinity iron-binding proteins, to sequester iron in environments where it is at a low concentration (Barclay and Ratledge, 1983). Two groups of iron-binding proteins are known to be produced by mycobacteria: exochelins, which solublise iron from ferric hydroxide or ferric phosphate, and mycobactins, which store iron intracellularly (Wheeler and Ratledge, 1985). Addition of transferrin or lactoferrin to cultures of *M. tuberculosis* or *M. smegmatis* resulted in approximately 50% reduction of growth as measured by cell mass (Raghu et al, 1993). This study also showed that mycobacteria grown in the presence of lactoferrin or transferrin had increased concentrations of both exochelins and mycobactins.

Human serum has been shown to have an inhibitory action on both *M. tuberculosis* and *M. avium* and that for both organisms, apotransferrin mediates the bacteriostatic effect, addition of iron was shown to reverse the bacteriostasis (Kochan et al, 1963; Kochan, 1973; Douvas et al, 1993). Macrophages do not normally possess lactoferrin, but they may acquire it by ingestion of granulocyte material (Silva et al, 1989). Lactoferrin and transferrin inhibit growth of *Legionella pneumophila* (Byrd and Horwitz, 1989; Byrd and Horwitz, 1991; Byrd and Horwitz, 1993), whereas high concentrations of iron promote intracellular growth (Byrd and Horwitz, 1989). Down regulation of human monocyte transferrin receptors by IFN-$\gamma$
suggests that this may be an important defence against *L. pneumophila* (Byrd and Horwitz, 1989; Byrd and Horwitz, 1993).

**1.5.6 Degradation of Tryptophan.**

Degradation of tryptophan is a mechanism by which macrophages and other cells can deprive intracellular parasites of a vital nutrient. Tryptophan is required for protein synthesis and is necessary for synthesis of nicotinamide adenine dinucleotide (Taylor and Feng, 1991). Murine lung tissue simulated with a crude supernatant from fibroblasts, which contained interferon, was found to enhance levels of the enzyme indoleamine 2,3-dioxygenase [IDO] (Yoshida et al., 1981). IDO is responsible for the first step in the degradation of tryptophan to kynurenine (Shimizu et al., 1978). Interferons have also been found to induce IDO activity in human cells. Recombinant human IFN-γ but not IFN-β was found to induce tryptophan degradation in human lung fibroblasts (Byrne et al., 1986a). The same study also showed decreased levels of tryptophan in the plasma of patients receiving intravenous IFN-γ but not IFN-α in a clinical trial. Both IFN-γ and IFN-β have been shown to stimulate IDO activity in human mononuclear cells, but the effect of IFN-β was greatest in the presence of lipopolysaccharide (Carlin et al., 1989).

Increased IDO activity in cells stimulated with interferon-γ has been found to be a cause of their antiproliferative abilities. Human fibroblasts treated with IFN-γ inhibit the intracellular growth of *Toxoplasma gondii* (Pfefferkorn, 1984). The mechanism by which cells achieved this antiproliferative activity was found to be due to starvation of tryptophan. The concentration of IFN-γ required, increased with
increased levels of tryptophan in the growth medium (Pfefferkorn et al, 1984). Addition of exogenous tryptophan to the growth medium reversed the inhibition of growth (Pfefferkorn, 1984; Scmitz et al, 1989). Degradation of tryptophan does not appear to be a universal antimicrobial mechanism however, as the antitoxoplasma effects of human umbilical vein endothelial cells could not be reversed by addition of exogenous tryptophan (Woodman et al, 1991). Similarly degradation of tryptophan is not the mechanism by which IFN-γ-activated fibroblasts restrict the growth of Rickettsia prowazekii (Turco and Winkler, 1986). Murray compared the role of tryptophan degradation in both human and murine macrophages against T. gondii, Chlamydia psittaci and Leishmania donovani (Murray et al, 1989). Addition of exogenous tryptophan only partly reversed the antimicrobial effect of human macrophages against all three organisms. In contrast murine macrophages showed no evidence of IDO activity (Murray et al, 1989).

The findings with T. gondii have been repeated with C. psittaci (Byrne et al, 1986b) and C. trachomatis (Shemer-Avni et al, 1989). IDO activity appears to be a crucial factor in the IFN-γ stimulated antimicrobial response of human fibroblast cell lines. Mutant cell lines with reduced levels of this enzyme could not inhibit the growth of C. psittaci, C. trachomatis or T. gondii to the same extent as the parental cell line lacking the mutation (Thomas et al, 1993). Treatment of human epithelial cells with levels of IFN-γ below that required to activate inhibition of growth of C. trachomatis, results in the generation of persistent form of the organism (Beatty et al, 1993). Tryptophan depletion was found to be involved, as the cells showed substantial IDO activity, and persistent forms did not develop in IDO-deficient cell lines treated with IFN-γ (Beatty et al, 1994). The authors also reported that addition
of exogenous tryptophan resulted in the development of more typical chlamydial forms. One of the differences between persistent and normal forms of *C. trachomatis* is the presence of the 60 kDa major outer membrane protein (MOMP) (Beatty et al., 1993). MOMP contains seven tryptophan residues (Baehr et al., 1988; Hamilton and Malinowski, 1989; Peterson et al., 1990) and therefore starvation of tryptophan may prevent *C. trachomatis* expressing this protein.

Starvation of tryptophan may also be involved in the *in vitro* antitumour activity of IFN-γ (de la Maza and Peterson, 1988; Ozaki et al., 1988; Takikawa et al., 1988). Transplanted murine tumour cells undergoing rejection have also been shown to have elevated IDO activity, which was mediated by IFN-γ (Takikawa et al., 1990).

Although tryptophan is not essential for growth of mycobacteria (Grange, 1976), mycobacteria have been shown to grow better when supplemented with a number of amino acids which included tryptophan (Sundaram and Venkitasubramanian, 1978). Mycobacteria grown *in vivo* show a greater affinity for tryptophan compared with those grown *in vitro* (Sundaram and Venkitasubramanian, 1978), which may be an adaptation to compete with the host cell for this amino acid (Wheeler and Ratledge, 1988). Using the vaccinated, boosted and challenged guinea-pig model of Jackett and co-workers (Jackett et al., 1981), it has been found that addition of exogenous tryptophan did not reverse the antimicrobial activity of guinea-pig alveolar macrophages against *M. tuberculosis* or *T. gondii* (O'Brien, 1992). However, tryptophan degradation may be one of several mechanisms acting in concert to produce an antimicrobial effect as suggested recently (Gebran et al., 1994). It is possible that by depriving intracellular pathogens of favorable conditions
for growth, these pathogens will be less able to proliferate and resist antimicrobial
defences such as toxic oxygen and nitrogen species or degradative enzymes.

1.5.7 Polyamines and Polyamine Oxidation.

Polyamines are a group of low molecular weight aliphatic bases with two or
more amino groups. The importance of polyamines can be inferred from their
presence in the cells of all living organisms (Morgan, 1987a). Under normal
conditions, the function of polyamines appears to be in the control of cellular
division and differentiation (Heby, 1981; Pegg, 1986). Intracellular levels of
polyamines vary with the stage of the cell cycle (Scalabrino and Ferioli, 1981;
Scalabrino and Ferioli, 1982), as increases in polyamine concentrations often
precede synthesis of DNA, RNA and proteins (Morgan, 1987a). Elevated levels of
polyamines are found in regenerating liver (Hölttä et al, 1973). Studies using
mutants of *Escherichia coli* with reduced polyamine synthetic capacity has shown
that there is a reduction in cell size, protein content and growth rate (Morris and
Jorstad, 1973). It has been suggested that these changes are a result of reduced
DNA gyrase activity (Morris and Harada, 1980). DNA gyrase is dependent on the
presence of spermidine for activity (Gellert et al, 1976), and partial inhibition of DNA
gyrase shows the similar changes to a polyamine depleted state (Morris and
Harada, 1980).

Polyamine oxidases have also, like polyamines, been found in the tissues of
most higher organisms (Morgan, 1985). However their role is even less clear that
that of the polyamines. Among the polyamine oxidases isolated from mammalian
tissues, the rat liver polyamine oxidase (RLPAO) (Höltta, 1977) and the bovine
serum polyamine oxidase (BSPAO) (Tabor et al, 1954) are best characterised. The
action of these enzymes upon spermine and spermidine results in the production of different products (Fig. 1.1).

Notably both enzymes require oxygen and produce aminoaldehydes and hydrogen peroxide. Human retroplacental serum (RPS) also contains high levels of polyamine oxidase activity (RPSPAO), which increases during the course of the pregnancy (Illei and Morgan, 1979a; Illei and Morgan, 1979b).

The toxic properties of products of polyamine oxidation were first noted when a substance extracted from bovine kidney, subsequently shown to be identical to spermine, was added to media containing tubercle bacilli (Hirsch and Dubos, 1952). The antimycobacterial effect occurred only in the presence of bovine serum albumin (BSA) Cohn fraction V. It has since been shown that BSA contains BSPAO as a contaminant (Livingstone et al., 1977). Addition of spermine or spermidine, but not other related compounds, to cultures of *M. tuberculosis* caused inhibition of growth (Hirsch, 1953a). These studies were extended to show that spermine and spermidine were acted upon by an enzyme which was termed spermine oxidase (Hirsch, 1953b). Spermine oxidase was found in both bovine and ovine sera but not in others tested, including human serum. Crude extracts of guinea-pig kidney and heart were also shown to contain the enzyme, whereas rabbit organs did not (Hirsch, 1953b). Interestingly, fast-growing saprophytic mycobacteria were not killed, nor were passaged mycobacteria more resistant to these toxic effects (Hirsch and Dubos, 1952).

A wide range of bacteria have since been shown to be susceptible to products of polyamine oxidation (Bachrach and Persky, 1964). Other organisms affected include the parasites *Plasmodium* (Rzepczyk et al., 1984; Morgan et al., 1986), *Schistosoma* (Ferrante et al., 1986) and *Trypanosoma* (Ferrante et al., 1982;
Fig. 1.1 Oxidation of spermine and spermidine by bovine serum polyamine oxidase (BSPAO).

\[
\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2 \rightarrow \text{OCH(}\text{CH}_2)_2\text{NH}(\text{CH}_2)_4(\text{CH}_2)_2\text{CHO}
\]

Spermine \hspace{1cm} \text{N,N'-Bis(3-aminopropanal)-1,4-diaminobutane (Oxidised spermine)}

\[
+ 2\text{O}_2 + 2\text{H}_2\text{O} \rightarrow + 2\text{NH}_3 + 2\text{H}_2\text{O}_2
\]

\[
\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{OCH(}\text{CH}_2)_2\text{NH}(\text{CH}_2)_4\text{NH}_2 + \text{NH}_3 + \text{H}_2\text{O}_2
\]

Spermidine \hspace{1cm} \text{N-(4-aminobutyl)-3-aminopropanal (Oxidised spermidine)}

Oxidation of Spermine and spermidine by Rat Liver Polyamine Oxidase (RLPAO).

\[
\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2 \rightarrow \text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2 + \text{NH}_2(\text{CH}_2)_2\text{CHO}
\]

Spermine \hspace{1cm} \text{Spermidine} \hspace{1cm} \text{3-Aminopropanal}

\[
+ \text{O}_2 + \text{H}_2\text{O} \rightarrow + \text{H}_2\text{O}_2
\]

\[
\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{NH}_2(\text{CH}_2)_4\text{NH}_2 + \text{NH}_2(\text{CH}_2)_2\text{CHO}
\]

Spermidine \hspace{1cm} \text{Putrescine} \hspace{1cm} \text{3-Aminopropanal}

\[
+ \text{H}_2\text{O}_2
\]
Storer et al., 1988), fungi (Levitz et al., 1990), viruses (Fukami et al., 1967; Bachrach et al., 1971) and eukaryotic cells (Pulkkinen et al., 1978; Morgan, 1987b; Storer et al., 1988; Averill-Bates et al., 1993). Both BSPAO and RPSPAO produce toxic aminoaldehydes (Morgan et al., 1986b; Morgan, 1987). The products of polyamine oxidation can cause the death of intraerythrocytic parasites without lysing the cell (Rzepczyk et al., 1984).

In order to identify the toxic agent, the oxidised polyamines have been separated from hydrogen peroxide and other potentially toxic substances. When organisms are incubated in the presence of purified aminoaldehydes, similar toxic effects are demonstrated (Bachrach et al., 1967; Ferrante et al., 1986; Morgan et al., 1986). A synthetic compound, POX-3, which shows structural similarities with oxidised spermine, shares a similar inhibition of DNA, RNA, and protein synthesis (Bachrach and Rosenkranz, 1969). When acetylated polyamines are used as the substrate for PAO, they were found to be non-toxic, although the reaction produces hydrogen peroxide (Morgan, 1987b). In all but one case (Averill-Bates et al., 1993), addition of catalase has ruled out hydrogen peroxide as the toxic species (Rzepczyk et al., 1984; Ferrante et al., 1986). Ammonium ions were also found not to be mediating the toxic effect (Ferrante et al., 1986). It appears therefore, that the toxic effects of polyamine oxidation are as a result of the production of aminoaldehydes and not hydrogen peroxide or ammonium ions.

The aminoaldehyde derivatives are not very stable, with half lives of 42, 137 and 137 minutes for dioxidised spermine, monooxidised spermine and monooxidised spermidine respectively (Kimes and Morris, 1971a). Oxidised polyamines can undergo β-elimination to form acrolein (Alarcon, 1964; Alarcon, 1970), or condense to form oligoamines (Kimes and Morris, 1971a). In a further
communication, Kimes and Morris (1971b) showed that pre-incubation of oxidised spermine or oxidised spermidine increased their inhibitory action on *E. coli* macromolecular biosynthesis. The authors demonstrated similar results to those obtained by pre-incubating oxidised polyamines, by including acrolein at concentrations found after pre-incubation (Kimes and Morris, 1971b). Condensation products were ruled out as they are only produced at much higher concentrations (Kimes and Morris, 1971a). Other workers have found that five times the molar amount of acrolein was required to show the same damage as spermine (Ferrante et al, 1986a). The same report also suggested that the rapid reaction between the oxidised polyamines and the cells with which they are incubated would limit the amount of acrolein formed. When a range of viruses were tested for susceptibility to acrolein or oxidised polyamines, different patterns of susceptibility were found (Bachrach et al, 1971).

Oxidised spermine binds to the amino groups of DNA bases (Eilon and Bachrach, 1969) and DNA itself (Bachrach and Leibovici, 1966), via covalent bonds (Bachrach and Eilon, 1967). This binding is irreversible (Bachrach and Eilon, 1969), and prevents strand separation (Bachrach and Leibovici, 1966). Guanine and cytosine residues have a higher affinity for oxidised polyamines than adenine or thymine residues (Eilon and Bachrach, 1969).

Oxidation of polyamines has however yet to be demonstrated as an *in vivo* antimicrobial defence. There is evidence that peritoneal macrophages from mice vaccinated with *M. bovis BCG* or *Corynebacterium parvum* have higher levels of polyamine oxidase than macrophages from unvaccinated animals (Morgan et al, 1980). The first enzyme in the pathway for the production of polyamines is ornithine decarboxylase (ODC), which converts ornithine to putrescine, from which the
polyamines spermine and spermidine are produced. Elevated levels of ODC and ODC mRNA have been shown in human monocytes treated with IFN-γ (Messina et al., 1990; O'Brien, 1995). Treatment of macrophages or macrophage cell lines with mycobacterial cell walls has also been shown to raise levels of ODC activity (Nichols and Prosser, 1980; Taffet and Haddox, 1985). Inhibition of ODC in macrophages results in reduced uptake of *Trypanosoma cruzi*, which is reversible by the addition of exogenous putrescine, suggesting the involvement of polyamines (Kierszenbaum et al., 1987). Polyamine levels have been reported to be several hundred times higher in leukocytes compared with plasma and erythrocytes (Cohen et al., 1976; Cooper et al., 1976). However this may due to the role of polyamines in control of cell division and differentiation (Heby, 1981).
1.6 Factors Affecting Mycobacterial Virulence.

It has been known for some time that strains of *M. tuberculosis* isolated from different patients show differing patterns of disease in experimental animals (Mitchison *et al*, 1960; Mitchison *et al*, 1963). This work investigated the ability of isolates of *M. tuberculosis* to cause gross disease in guinea-pigs, with isolates being assigned a root index of virulence (RIV). Strains with an RIV of one or more were termed virulent, those with an RIV of less than one were termed avirulent (Mitchison *et al*, 1963; Jacket *et al*, 1978). A linkage between virulence and the ability to cause progressive disease has also been found in a murine model of tuberculosis (North and Izzo, 1993). Several phenotypes have been reported to be linked to mycobacterial virulence (Mitchison *et al*, 1963; Goren *et al*, 1974; O'Brien *et al*, 1994), but a perfect correlation with virulence has not been found for any of these phenotypes. Furthermore, no single genetic determinant of virulence has yet been identified (Bloom and Murray, 1992). Also considered here are autoinducers, which control the expression of virulence determinants in several Gram-negative bacteria (Salmond *et al*, 1995).

1.6.1 Components of the Mycobacterial Cell Wall.

The mycobacterial cell wall contains a number of molecules which have been implicated in pathogenicity. Several lipids are among those molecules which have been postulated to be involved in mycobacterial pathogenicity. The presence of high levels of sulpholipids was found to be associated with virulence (Goren *et al*, 1974). Sulpholipid can act as a stimulus for TNF-α release (Bronza *et al*, 1991) and so may indirectly contribute towards some of the pathogenic features of the disease (Filley
and Rook, 1991; Filley et al, 1992; Grau et al, 1992). Inhibition of phagosome-lysosome fusion by M. tuberculosis has also been claimed to be due to the presence of sulpholipids (Goren et al, 1976).

The phenolic glycolipids of M. leprae have been reported to inhibit T-cell activity (Mehra et al, 1984) and macrophage oxidative activity (Vachula et al, 1989), as well as scavenging ROI (Chan et al, 1989).

*Mycobacterium tuberculosis* and M. leprae also contain a highly antigenic lipopolysaccharide, lipoarabinomannan [LAM] (Hunter et al, 1986). Several immunosuppressive abilities have been attributed to LAM, including inhibition of macrophage antimicrobial ability (Sibley et al, 1988), inhibition of protein kinase C activity (Chan et al, 1991), suppression of T-cell activation (Kaplan et al, 1987; Moreno et al, 1988), and scavenging of ROI (Chan et al, 1991). LAM is also able to stimulate the release of high levels of TNF-α (Moreno et al, 1989), and so may contribute towards the pathology of tuberculosis (Filley and Rook, 1991; Filley et al, 1992; Grau et al, 1992). However, LAM can act as a macrophage activating factor, either as an inducer of early response genes (Roach et al, 1994), or as a second signal for macrophages activation (Adams et al, 1993). LAM has also been demonstrated to stimulate production of RNI (Adams et al, 1993).

Comparison of LAM from virulent and avirulent strains of *M. tuberculosis* and M. leprae has found differences in the structure of the molecule itself and its properties. In LAM isolated from avirulent strains of *M. tuberculosis*, the terminal sugars are predominantly arabinose residues, whereas the LAM of virulent strains has extensive capping of the arabinose residues with mannose residues (Chatterjee et al, 1991; Chatterjee et al, 1992a). Molecules of LAM capped by mannose residues have greatly reduced ability to stimulate TNF-α production (Chatterjee et
al., 1992b; Adams et al., 1993), and show ability to induce early response genes in macrophages (Roach et al., 1994). Despite this evidence, the relationship between the structure of a strain's LAM and its virulence is not clear, as the avirulent vaccine strain *M. bovis* BCG was found to have no major difference in the structure of its LAM compared with that of the virulent *M. tuberculosis* Erdman strain (Prinzis et al., 1993). However, it has since been disclosed that the *M. tuberculosis* H$_3$7Ra strain used was found to be a non-pathogenic mycobacterium in comparison to other strains of *M. tuberculosis* (Barnes and Modlin, 1996).

1.6.2 Resistance to Oxidising Agents.

Oxidising agents such as ROI and RNI are thought to be partly responsible for macrophage antimicrobial activity (sections 1.5.1 and 1.5.2). The resistance of a mycobacterial strain to these oxidising agents can affect its virulence in the guinea-pig as discussed previously in sections 1.5.1 and 1.5.2.

1.6.3 Interaction of Mycobacteria with Macrophages.

The route by which macrophages bind and phagocytose mycobacteria is unclear. Mycobacteria have been shown to be taken up by macrophages after binding to fibronectin receptors (Abou-Zied et al., 1988), complement receptors CR1, CR3 and CR4 (Schlesinger et al., 1990; Schlesinger, 1993) and mannose receptors (Schlesinger, 1993). Uptake via the complement receptors avoids triggering of the macrophage's respiratory burst activity (Schlesinger et al., 1990), and so could affect the intracellular fate of the mycobacteria. Mannose receptors on the macrophage are only effective in taking up virulent strains of *M. tuberculosis* (Schlesinger, 1993). As noted previously (section 1.6.1) LAM of virulent strains of mycobacteria is
capped with mannose residues, and may therefore be involved in uptake by the macrophage. In a separate experiment particles coated with mannose capped LAM were found to bind more readily to macrophages than those coated with LAM in which arabinose is the terminal sugar (Schlesinger et al, 1994).

It is possible however, that mycobacteria are not dependent upon macrophages for entry into the cell. Mycobacteria have been shown to enter non-phagocytic cells (Shepard, 1957; Arruda et al, 1993). Moreover, a fragment of DNA from the avirulent strain *M. tuberculosis* H37Ra has been isolated which confers a similar invasive character on non-pathogenic strains of *E. coli* (Arruda et al, 1993).

Another fragment of mycobacterial DNA which may be associated with virulence has been reported. A fragment of DNA from the virulent strain *M. tuberculosis* H37Rv was found to increase the in vivo growth rate of the avirulent strain H37Ra (Pascopella et al, 1994).

1.6.4 Autoregulatory factors.

The bioluminescent marine bacteria *Vibrio harveyi* and *Photobacterium fischeri* (formerly known as *Vibrio fischeri*) occur both as free-living organisms and as symbionts of fish and squid (Hastings and Nealson, 1977; Ruby and McFall-Ngai, 1992). However, expression of a bioluminescent phenotype is cell density-dependent (Nealson, 1970). The linking of expression of particular physiological traits to cell density has been termed 'quorum sensing' (Fuqua et al, 1994). Both *V. harveyi* and *P. fischeri* produce small diffusible molecules termed autoinducers, which have similar structures but are species specific in their actions (Greenberg et al, 1979). In the case of *P. fischeri* grown in batch culture, autoinducer gradually accumulates in the medium over a period of time (Kaplan and
Greenberg, 1985). Upon reaching a threshold concentration, the autoinducer

The portion of the \( \textit{P. fischeri} \) genome which contains the \textit{lux} genes has been cloned into \( \textit{E. coli} \) (Engebrecht \textit{et al}, 1983) and used to study the autoinduction system. The complete nucleotide sequence of this 9 kilobase fragment has since been determined (Devine \textit{et al}, 1988; Baldwin \textit{et al}, 1989; Swartzmann \textit{et al}, 1990) and shown to contain seven genes, which are organised into two separate transcriptional units \( \textit{luxR} \) and \( \textit{luxICDABE} \) (Engebrecht \textit{et al}, 1983; Engebrecht and Silverman, 1987; Devine \textit{et al}, 1988). The arrangement of these genes is shown in Fig 1.2. \textit{LuxA} and \textit{luxB} code for the two subunits of the enzyme luciferase (Ziegler and Baldwin, 1981). The \textit{luxC}, \textit{luxD} and \textit{luxE} genes code for proteins involved in the biosynthesis of the aldehyde substrate of luciferase (Engebrecht and Silverman, 1984). Both the \textit{luxl} and \textit{luxR} genes are involved in regulation of the \textit{lux} operon. LuxR, the \textit{luxR} gene product, acts as a transcriptional activator in the presence of autoinducer, increasing the transcription of \textit{luxR} itself and \textit{luxICDABE} (Nealson \textit{et al}, 1970; Engebrecht \textit{et al}, 1983). \textit{Luxl} is thought to code for a polypeptide responsible for the synthesis of autoinducer (Engebrecht and Silverman, 1984).

As noted previously, expression of a bioluminescent phenotype is cell density-dependent (Nealson \textit{et al}, 1970). At low cell density, there is only a low level of expression of \textit{luxl} and also therefore low levels of autoinducer (Meighen, 1991).
Fig. 1.3 The Organisation of the *lux* Genes in *Photobacterium fischeri*.

The heavy arrows denote luxR and the luxICDABE operon.

However, as the cell density rises, so autoinducer accumulates until its concentration reaches a threshold level. At this concentration, autoinducer is thought to bind to luxR, which binds to the luxR-binding site and increases transcription of luxICDABE (Kaplan and Greenberg, 1985, Shadel and Baldwin, 1991). Increased transcription of the lux operon leads to increased levels of luxI, which in turn leads to elevated levels of autoinducer. Thus a positive feedback loop is generated, leading to increased expression of the whole of the lux operon (Salmond et al, 1995). A second feedback loop acting via an autoregulatory mechanism also exists in P. fischeri (Shadel and Baldwin, 1992). In this system, a region of the luxD gene acts as a low affinity binding site for luxR, and results in negative autoregulation of the luxR gene (Shadel and Baldwin, 1992).

Recently, several bacteria have been shown to produce autoinducers which are structurally similar to those of P. fischeri and V. harveyi. The first of these to be discovered was the plant pathogen Erwinia carotovora, where autoinducer controls the production of the antibiotic 1-carbapen-2-em-3 carboxylic acid [carbapenem] (Bainton et al, 1992a, b). The autoinducer was found to be identical to that found in P. fischeri (Eberhard et al, 1981; Bainton et al, 1992b). It has since been discovered that as well as carbapenem, synthesis of several extracellular enzymes are controlled in E. carotovora by this autoinducer (Jones et al, 1993; Pirhonen et al, 1993).

A recombinant strain of E. coli was developed for use as a bioluminescent sensor for autoinducer molecules analogous to those produced by P. fischeri and E. carotovora (Bainton et al, 1992a; Swift et al, 1993). Spent culture supernatants from a range of Gram-negative organisms [Citrobacter freundii, Enterobacter agglomerans, Erwinia herbicola, Hafnia alvei, Proteus mirabilis, Pseudomonas 54
Ps. aeruginosa, Rahnella aquatilis and Serratia marcesens] were found to give positive results for the presence of autoinducer (Bainton et al, 1992; Swift et al, 1993). It was found that autoinducer tended to accumulate in the supernatants of stationary-phase cultures of these bacteria (Swift et al, 1993). An autoinducer which is highly similar in structure to that of P. fischeri has also been isolated from culture supernatants of Agrobacterium tumefaciens (Zhang and Kerr, 1991).

Further research has found two component regulatory systems homologous to the luxl-luxR system in several bacteria (Latifi et al, 1995; Salmond et al, 1995; Throup et al, 1995). The two best described examples are those found in Ps. aeruginosa, an opportunistic pathogen of man (Nicas and Iglewski, 1986) and the plant pathogen A. tumefaciens (Nester et al, 1984). In Ps. aeruginosa the luxR homologue, lasR, controls production of several exoenzymes which act as virulence factors including elastase, alkaline protease and exotoxin A (Gambello and Iglewski, 1991; Toder et al, 1991; Gambello et al, 1993).

The identity of the Ps. aeruginosa autoinducer, however, is debatable. The homologue of luxl isolated from Ps. aeruginosa, lasl, when cloned into E. coli, effects the production of N-(3-oxododecanoyl)-L-homoserine lactone suggesting that this is the Ps. aeruginosa autoinducer (Passador et al, 1993; Pearson et al, 1994). The P. fischeri autoinducer was found not to activate lasR in E. coli (Gray et al, 1994). However, a second report in which mass spectrometry and high-performance liquid chromatography were used to identify the structure of the autoinducer, found the Ps. aeruginosa autoinducer to be identical to that of P. fischeri (Jones et al, 1993). The small differences in the structures of the autoinducers of different bacteria have been claimed to prevent these autoinducers from cross-reacting with luxR homologues from other bacteria (Gray et al, 1994). However, others have
found that there is a degree of cross-reaction between autoinducers and luxR homologues from different bacterial species (Swift et al., 1993).

The various luxR homologues do show some degree of conservation of function in the presence of the appropriate autoinducer. In E. coli, luxR in the presence of the P. fischeri autoinducer was able to activate the elastase gene (lasB) from Ps. aeruginosa (Gray et al., 1994). Under similar conditions, lasR in the presence of the Ps. aeruginosa autoinducer was able to activate the P. fischeri lux operon (Gray et al., 1994). However, the luxR homologues in the presence of heterologous autoinducer (that is LuxR with Ps. aeruginosa autoinducer and LasR with P. fischeri autoinducer) showed no significant activity, leading to the suggestion that autoinducer and transcriptional activator pairs are species specific (Gray et al., 1994). There are suggestions, however, that other bacteria do produce the same autoinducer as P. fischeri (Salmond et al., 1995; Throup et al., 1995).

A similar situation to that in Ps. aeruginosa exists in A. tumefaciens, where the homologues of luxl and luxR (tral and traR respectively) along with the autoinducer N-(3-octoctanoyl)-L-homoserine lactone control genes carried on the Ti plasmid (Piper et al., 1993; Zhang et al., 1993). The Ti plasmid is involved in bacterial conjugation and production of opines, which act as bacterial nutrients (Nester et al., 1984).

Autoregulatory systems other than those based on the lul-luxR family also exist, the best known of which is that found in the Actinomycetes (Horinuchi and Beppu, 1992). A diffusible factor, termed A-factor, has been isolated from culture supernatants of Streptomyces griseus and found to induce streptomycin production and spore formation in mutant strains (Khokhlov et al., 1967; Hara and Beppu, 1982). Several other A-factor homologues have since been isolated from species of
Streptomyces (Yamada et al, 1987; Horinuchi and Beppu, 1992). A-factor from S. griseus shows some structural similarity with the autoinducer found in P. fischeri, but does not cause autoinduction in P. fischeri (Horinuchi and Beppu, 1992). So far no autoregulatory systems based on HSL have been found in Gram-positive bacteria (Salmond et al, 1995).

1.6.5 Autoinduction as a potential factor in bacterial pathogenicity.

Control of metabolic processes by cell density-dependent autoinduction allows bacteria to assess their population size before switching on certain genes or groups of genes (Salmond et al, 1995). How could this be advantageous to the bacteria?

In the symbiotic relationship between P. fischeri and its host, the host through the luminescence of bacteria in the light organs has gained a mechanism for attracting prey and communicating with other members of its species (Engebrecht et al, 1983). How the bacteria benefit from the association is unknown. Bioluminescence appears to have no beneficial effects for individual bacteria, as autoinduction and therefore bioluminescence does not occur in P. fischeri in seawater where the concentration of these bacteria does not exceed 100 cells per millilitre (Ruby et al, 1980). Generation of bioluminescence can account for up to 10% of the bacterium's energy usage (Karl and Nealson, 1980) and so by regulating expression of bioluminescence, bacteria can make a major energy saving.

Many of the other bacteria which have been found to have autoregulatory systems analogous to that found in P. fischeri are pathogens of animals or plants. Extracellular enzymes such as pectinases and cellulase are major virulence determinants of E. carotovora (Barras et al, 1994). The parasitised plant can mount
a defensive action against attack by such enzymes (Dixon et al, 1994). It has been hypothesised that bacteria which reach a high density before releasing exoenzymes could overwhelm the host before it has time to initiate a defensive reaction (Salmond et al, 1995). A similar situation may also be the case for the opportunistic human pathogen Ps. aeruginosa. Density of the infecting bacterial population has been reported to be a major contributor towards the ability to colonise infected tissues (Passador et al, 1993), so autoinduction may play a key role in the pathogenicity of this organism.

1.7 Aims of the study.

The primary aim of this research was to investigate potential antimycobacterial mechanisms of macrophages.

The toxicity of products of polyamine oxidation has been shown previously (Hirsch and Dubos, 1952; Hirsch, 1953b). In this investigation it was intended to verify the work of Hirsch and examine the toxicity of products of polyamine oxidation on a wider range of mycobacterial strains. It was also intended that the product(s) mediating the toxic effect be identified.

Comparison of the susceptibilities of mycobacterial strains of different RIV to the toxic product(s) would allow determination of whether there was a correlation between strain virulence in the guinea-pig and susceptibility to products of polyamine oxidation.

The levels of PAO in tissues and macrophages from vaccinated and control guinea-pigs would also be investigated. It could then be determined whether vaccination has an effect on PAO levels, and if activation of antimycobacterial activity was accompanied by an increase in PAO.
The role of tryptophan degradation in the antimycobacterial activity of guinea-pig macrophages would be assessed. Alveolar macrophages from vaccinated and control guinea-pigs would be compared for their ability to catabolise tryptophan to kynurenine.

As well as looking at macrophage antimycobacterial mechanisms, two features of mycobacteria that could play a role in vivo would also be investigated. The hydrophobicity of a range of strains of *M. tuberculosis* would be assessed by determining their adherence to hexadecane.

Several species of bacteria produce autoregulatory molecules which may be virulence factors (section 1.6.5). Culture supernatants from mycobacteria would be analysed for the presence of these autoregulatory factors, and libraries of mycobacterial DNA would be screened for the presence of genes for such factors.
2. Materials and Methods
2.1 Chemicals.

Except where stated otherwise, the chemicals used were either from Sigma Chemical Company Ltd. or BDH.

2.2 Bacteria.

The Mycobacterium tuberculosis strains, 79499, 79500, 79112, I2646, B1453, H₃₇Rv and H₃₇RaHR, and Mycobacterium bovis 81470, were obtained from the University of Leicester Culture Collection. Mycobacterium tuberculosis H₃₇Ra was obtained from the National Collection of Type Cultures (Porton Down, Salisbury). Mycobacterium tuberculosis strains 24, 24 (p16R1) and 24 (pYZ66) were gifts from Dr Ying Zhang, Department of Medical Microbiology, St. Mary's Hospital Medical School, London. Mycobacterium bovis BCG was obtained as a freeze dried culture from Glaxo Laboratories U.K. Ltd. The characteristics of the mycobacterial strains used are detailed in Table 2.1.

Eschericia coli K12 strain LE392 and Listeria monocytogenes C52 were obtained from the University of Leicester Culture Collection. Escherichia coli JM101 (pSB237) and E. coli JM101 (pSB315) were kindly donated by Professor G.S.A.B. Stewart, University of Nottingham.
Table 2.1 Mycobacterial Strain Characteristics.

<table>
<thead>
<tr>
<th>Mycobacterial Strain</th>
<th>RIV\textsuperscript{a}</th>
<th>Catalase\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> 79499</td>
<td>1.29</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> I2646</td>
<td>1.17</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H\textsubscript{37}Rv</td>
<td>1.01</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79500</td>
<td>0.98</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79112</td>
<td>0.92</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> B1453</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H\textsubscript{37}Ra</td>
<td>0.52</td>
<td>+</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H\textsubscript{37}RaHR</td>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 24</td>
<td>NA</td>
<td>\textsuperscript{b}</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 24 (p16R1)</td>
<td>NA</td>
<td>\textsuperscript{b}</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 24 (YZ66)</td>
<td>NA</td>
<td>\textsuperscript{b}</td>
</tr>
<tr>
<td><em>M. bovis</em> 81470</td>
<td>&gt;1.29</td>
<td>+</td>
</tr>
</tbody>
</table>

NA = Not available

\textsuperscript{a} Strains with a root-index of virulence (RIV) in the guinea-pig of \(\geq 1.0\) or \(\leq 1.0\) were termed high and low virulence strains respectively. The RIV values and data on catalase were from studies of the pathology of tuberculosis \textit{in vivo} (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison \textit{et al}, 1963; Jackett \textit{et al}, 1978).

\textsuperscript{b} Data from Zhang \textit{et al}, 1992.
2.2.1 Growth and Maintenance of Bacteria.

Mycobacteria were grown at 37°C as static cultures in 10 ml volumes of Middlebrook 7H9 medium (Difco) supplemented with 0.05% (v/v) Tween 80 (Sigma) and 10% (v/v) albumin dextrose catalase complex (ADC, Difco). After seven days growth, mycobacteria were passaged at a ratio of 1:10 into fresh 7H9 medium plus supplements.

Mycobacterial strains were maintained by growth on slopes of Lowenstein-Jensen medium (Difco) followed by storage at -20°C. Alternatively, 4.5 ml of 7H9 medium containing 50% glycerol (v/v) were added to a 10 ml seven-day old culture, and 1 ml aliquots stored at -70°C.

*Escherichia coli* was grown at 37°C in 10 ml nutrient broth (Oxoid) as an overnight culture in a shaking incubator. Stocks of *E. coli* were maintained at 4°C on nutrient agar (Oxoid) and subcultured every seven days.

*Listeria monocytogenes* was grown at 37°C in 10 ml tryptose soya broth [TSB] (Oxoid) as an overnight culture in a shaking incubator. Stocks were maintained at 4°C on blood agar base (Oxoid) and subcultured every seven days.

Stocks of *E. coli* and *L. monocytogenes* were also kept at -70°C. To 8.5 ml of an overnight culture of *E. coli* or *L. monocytogenes* was added 1.5 ml of glycerol. The culture was vortexed gently for 5 s to disperse the glycerol evenly. 1 ml aliquots of the glycerol cultures were stored in Eppendorf vials at -70°C.

To recover bacteria from glycerol cultures, the cultures were allowed to thaw at room temperature and 500μl used to inoculate 10 ml of LB (*E. coli*) or TSB (*L. monocytogenes*). The cultures were grown overnight and sub-cultured before being used in experimental work.
2.3 Tissue Culture.

2.3.1 Tissue Culture Plasticware.

All tissue culture plasticware was from Nunc UK Ltd., unless otherwise stated. Syringes were from Beckton Dickinson Ltd. and needles from Terumo Europe Ltd.

2.3.2 Tissue Culture Reagents.

Unless otherwise stated, tissue culture reagents and media were from Gibco UK Ltd.

2.3.3 Tissue Culture Cells.

The murine fibroblast cell line L929 was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury).

2.3.4 Growth and Maintenance of Tissue Culture Cells.

All cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated foetal calf serum and 1% (w/v) L-glutamine at 37°C with 5% (v/v) CO₂.

L929 cells were kept sub-confluent, as a monolayer in 250 ml flasks in 10 ml of growth medium, and typically subcultured every three days. For subculture, the medium was removed and replaced with 0.05% (w/v) trypsin and 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA) in calcium- and magnesium-free Hank's Balanced Salt Solution. The cells were incubated for 5-10 min at 37°C and 5% (v/v) CO₂, after which time the trypsin-EDTA solution was removed and the flasks incubated for a further 10 min or until the cells were observed to detach from the
surface of the flask. 10 ml of fresh growth medium plus supplements was added and the cells removed from the flask by gentle pipetting with a Pasteur pipette. The cell suspension was inoculated into flasks at a ratio of 1:5 in fresh growth medium.

2.3.5 Preservation of Cells in Liquid Nitrogen.

L929 cells were detached from flasks as described above, and harvested by centrifugation at 600g for 10 min. The cell pellet was washed by resuspending in Hank's Balanced Salt Solution buffered with 25mM hepes pH 7.2 (HBSS-hepes) and centrifuged at 600g for 10 min. This was repeated to give a total of two washes. The cell pellet was resuspended in RPMI 1640 containing 20% (v/v) heat-inactivated new-born calf serum. The cell concentration was estimated using a haemocytometer, and adjusted to 1 x 10^7 ml^-1. Aliquots of 1 ml were dispensed into sterile cryotubes, and placed in racks in an isopropanol-containing cryopreservation unit (Nalgene) at -70°C overnight. The vials were then transferred to a liquid nitrogen storage container.

2.3.6 Recovery of Cells from Liquid Nitrogen.

Cells were thawed rapidly at 37°C and added gently to 10 ml of RPMI 1640 warmed to 37°C. The cells were collected by centrifugation at 600g for 10 min, resuspended in RPMI plus supplements and grown at 37°C with 5% CO₂ in culture flasks.

2.4 Animals.

Female Dunkin-Hartley guinea-pigs were obtained at a weight of 300-400g from David Hall Ltd, and housed and maintained by staff of the Biomedical Services Unit, University of Leicester.
2.4.1 Vaccination of Guinea-pigs.

For use as a vaccine, *Mycobacterium bovis* BCG was grown for seven days in Middlebrook 7H9 broth plus supplements. The bacilli were harvested by centrifugation at 2,000g for 10 min, and washed by resuspending in 10 ml 0.01% (v/v) Tween 80, followed by centrifugation at 2,000g for 10 min. This was repeated twice to give a total of three washes. The final pellet was resuspended in 2 ml nanopure water, and clumps disrupted by ultrasonication at 40W (Ultrasonic Engineering, U.K.) for 3 periods of 5 seconds (O'Brien, 1992). The bacterial concentration was assessed using a Thoma counting chamber, and adjusted to an appropriate concentration.

Guinea-pigs were vaccinated with three doses of *M.bovis* BCG over a period of 5 weeks to produce animals termed vaccinated, boosted and challenged (VBC). Five weeks before collection of the macrophages, the guinea-pigs were vaccinated with $2 \times 10^7$ *M.bovis* BCG intraperitoneally. Two weeks before collection, $1 \times 10^7$ *M.bovis* BCG were given intramuscularly. Six days before collection, guinea-pigs were anaesthetised, and challenged with $1 \times 10^7$ *M.bovis* BCG via an ear vein. This procedure has been shown to produce alveolar macrophages capable of killing *M. tuberculosis* both *in vivo* (Jackett et al, 1981a), and *in vitro* (O'Brien et al, 1991).

2.4.2 Collection of Macrophages.

Alveolar macrophages were collected by bronchopulmonary lavage (Myrvik et al, 1961). Guinea-pigs were killed by a single blow to the back of the head, followed by exsanguination. To collect the macrophages, an incision was made below the ribcage and continued to the top of the jaw. The trachea was exposed and clamped below the larynx with Spencer Wells forceps, and the diaphragm removed. The
lungs were lavaged by injecting 15 ml of RPMI 1640 containing 5U ml⁻¹ heparin (Leo Laboratories Ltd., U.K.) into the trachea. The lungs were gently massaged with the syringe and needle still in place. The lavage medium was then removed using the syringe and needle originally used to inject the medium. The syringe and needle were removed and the trachea clamped below the puncture mark caused by the needle. The lavage process was repeated, after which the trachea was clamped below the puncture mark and the lavage process carried out for a third time. Fresh 15ml volumes of RPMI 1640 containing 5U ml⁻¹ heparin was used for each lavage along with a new, sterile syringe and needle. The macrophages were harvested by centrifugation at 200g for 10 min, resuspended in 1 ml of the medium and their concentration estimated using a haemocytometer. Between 1-2 x 10⁷ macrophages per animal were recovered by this method.

2.5 Bacterial Viable Count Assay.

50µl of sample to be counted was serially diluted from 10⁻¹ to 10⁻⁴ in sterile distilled water. Ten 5 µl volumes from each dilution were spotted onto nutrient agar (E. coli) or blood agar base (L. monocytogenes) and grown until distinct colonies were visible. Listeria monocytogenes was grown at 30°C for 24 hours, E. coli was grown at 37°C for 24 hours and mycobacteria were grown for 3-4 weeks at 37°C. Where possible between 100 and 400 colonies were counted.
2.6 Measurement of the Indoleamine 2,3-dioxygenase (IDO) Activity of Guinea-pig Alveolar Macrophages.

IDO activity was measured using a modification of the method of Byrne and co-workers (Byrne et al., 1986a). IDO catalyses the breakdown of tryptophan to kynurenine. By measuring the relative amounts of tryptophan and kynurenine present in the culture medium after various periods of incubation with guinea-pig alveolar macrophages, the amount of tryptophan which has been converted to kynurenine can be calculated.

Guinea-pig alveolar macrophages were collected and counted as described previously (section 2.4.2). Either 5 x 10^8 or 2 X 10^6 cells were added to the wells of 24-well multiwell plates, and the volume made up to 1 ml with fresh RPMI 1640 plus supplements. The cells were allowed to adhere for one hour at 37°C with 5% (v/v) CO_2. After this period, the supernatant was aspirated, and the cell monolayer washed six times with 1 ml volumes of HBSS-hepes, to remove non-adherent cells. The monolayer was then overlaid with 1 ml HBSS-hepes containing 1.0 mCi L-[5-3H]tryptophan [specific activity 28.0 Ci/mmol] (Amersham) with 80 μM tryptophan as carrier, and incubated at 37°C and 5% (v/v) CO_2. Supernatant samples were taken from each well at the desired times and stored at -20°C until they were processed.

To each sample was added 25 μl 10mgml⁻¹ L-tryptophan and 25 μl 10mgml⁻¹ L-kynurenine, which were to act as markers. Each sample was spotted onto Whatmann 3MM chromatography paper, 5 μl at a time, and dried under a gentle heat. Tryptophan (Rf typically 0.65) was separated from a mixture of kynurenine and N-formyl kynurenine (Rf typically 0.85) by ascending paper chromatography with 0.1M HCl as solvent (Pfefferkorn, 1984). Separation was carried out over 20cm,
after which time the chromatograms were removed and allowed to dry. The positions of the markers were visualised under ultraviolet light, and noted. The lanes were cut out and subsequently cut into 1 cm pieces, each of which was added to a scintillation vial insert containing 5 ml Optiphase 'Safe' (Pharwall) as scintillant. Radioactive counts per minute (CPM) were determined using a Minaxi TRI-CARB scintillation counter (United Technologies Packard Ltd.). Each vial was counted for 5 min, and the average CPM calculated.

2.6.1 Assessment of the Uptake of Tryptophan into Acid-soluble and Acid-insoluble Intracellular Pools.

Uptake of radiolabelled tryptophan was determined by the method of Byrne and co-workers (Byrne et al., 1986b). Guinea-pig alveolar macrophages were collected and counted as described previously (section 2.4.2). 5 x 10⁵ cells were added to the wells of 24-well multiwell plates, and the volume made up to 1 ml with fresh RPMI 1640 with supplements. The cells were allowed to adhere for 1 hour at 37°C and 5% (v/v) CO₂, after which time the medium was aspirated and the monolayer washed six times with 1 ml volumes of HBSS-hepes, to remove non-adherent cells. The cells were then overlaid with 1 ml HBSS-hepes containing 1.0mCi L-[5-³H]tryptophan [specific activity 28.0 Ci/mmol] and 80 µM tryptophan as carrier, and incubated at 37°C with 5% (v/v) CO₂. After 0, 2 and 8 hours, the medium was aspirated and the monolayer washed six times with 1 ml HBSS-hepes. After washing, 500 µl HBSS-hepes was added to each well and the cells removed by scraping with the plunger of a sterile 1 ml syringe. The cells were collected by centrifugation at 2,000g for 5 min. The supernatant was removed and the cells
resuspended in 100 μl 10% (w/v) trichloroacetic acid. Acid-soluble material was separated from acid-insoluble material by centrifugation at 5,000g for 5 min. The supernatant was removed and added to 4 ml Optiphase ‘Safe’ (Pharwall) as scintillant. The pellet of acid-insoluble material was resuspended in 100 μl distilled water and centrifuged at 5,000g for 5 min to remove any remaining acid-soluble material. The supernatant was removed, and added to the vial containing acid-soluble material. The acid-insoluble pellet was resuspended in 200 μl distilled water and added to 4 ml Optiphase ‘Safe’ as scintillant. Scintillation was above described above (section 2.6).

2.7 Collection of Serum.

Bovine and ovine blood samples were collected from freshly slaughtered animals (Parkers, Leicester) and allowed to clot at room temperature. The clotted portion of the blood was then removed by centrifugation at 1,000g for 10 min. The serum was drawn off, sterilised by filtration through 0.2μm filter (Gelman) and stored at -20°C until required.

2.8 Collection and Homogenisation of Tissue Samples.

Tissue samples and macrophages to be assayed for polyamine oxidase activity were collected from guinea-pigs culled by a single blow to the back of the head, followed by exsanguination (section 2.4.2). PAO homogenisation buffer was that used by Seiler and co-workers (Seiler et al, 1980). Tissue samples weighing approximately 5g were homogenised for 1 minute in 10 ml polyamine oxidase (PAO) homogenisation buffer. Samples weighing less than 5g were similarly homogenised,
but in 5 ml PAO homogenisation buffer. Organ homogenates were centrifuged at 2000g for 5 min to remove any cellular debris. The supernatant was removed and used in polyamine oxidase assays. Macrophages were collected and counted as described in section 2.4.2, centrifuged at 600g for 10 min and then resuspended in PAO homogenization buffer.

**PAO homogenisation buffer:**

- 1.55g Boric acid
- 5.00g Sodium borate
- 1.53mg Dithiothreitol
- 146mg Ethylene diamine tetraacetic acid (EDTA)
- 6 ml 1M Sodium hydroxide

Volume was made up to 500 ml with distilled water. The solution was pH 9.0.

### 2.9 Assays for Polyamine Oxidase.

Two methods were used to assay polyamine oxidase activity.

#### 2.9.1 Fluorimetric Polyamine Oxidase Assay.

This method was used to test whether or not samples contained any polyamine oxidase (PAO) activity. Oxidation of polyamines by PAO results in the production of hydrogen peroxide. The enzyme horse-radish peroxidase (HRP) catalyses the oxidation of p-hydroxyphenylacetic acid (HPAA) by hydrogen peroxide.
resulting in the production of a product which is fluorescent at 414nm (Guilbault et al, 1968).

100 ml of the PAO source to be tested was incubated with 0.5U of HRP, 0.7mgml⁻¹ HPAA, and 1mM spermidine and the volume was made up to 1 ml with 0.14M Tris buffer pH 7.2. The assay was carried out at 37°C for 90 min, after which time, the reaction was stopped by the addition of 1 ml of ice-cold 0.1M borate buffer pH 10.4. An zero-time point was taken by adding borate buffer to the assay mixture before the spermidine. Fluorescence was measured using a fluorescence spectrophotometer (Perkin-Elmer, U.K.) with an excitation wavelength of 310nm and an analysis wavelength of 414nm. Fluorescence increase was given by subtracting the initial zero time value from the value achieved after 90 min of incubation.

2.9.2 Radiometric Polyamine Oxidase Assay.

This was used as a quantitative assay for PAO in guinea-pig tissues, and is based on the method of Morgan and Illei (Morgan and Illei, 1981). Oxidation of polyamines by PAO results in deamination of the polyamines (section 1.5.7; Fig. 1). The loss of the positively-charged amino groups reduces the ability of the oxidised polyamines to associate with negatively-charged ion-exchange resins (Morgan and Illei, 1981). Oxidised polyamines are therefore eluted by less concentrated solutions than their non-oxidised counterparts. By using radiolabelled polyamines, the levels of oxidised and non-oxidised polyamines can be measured, and the PAO activity of the sample calculated.

The assay was carried out in 0.5M Tris buffer pH7.4 containing 0.1% (v/v) Triton X-100 and 7.6 mM benzaldehyde (incubation buffer). 5 μl ¹⁴C spermine tetrahydrochloride [specific activity 110mCi/mmol] (Amersham, U.K.) was added to
445 μl of 4mM unlabelled spermine, and the volume made up to 5 ml with incubation buffer. This was known as buffered substrate. 100 μl of the sample to be tested was added to 200 μl of buffered substrate, and incubated at 37°C for 90 min. At the end of the incubation period, the reaction was stopped by the addition of 50 μl of 50% (w/v) trichloroacetic acid. The contents are mixed, and centrifuged at 5000g for 5 min to precipitate acid-insoluble material. The supernatants are removed and stored at -20°C until required.

Oxidised spermine was separated from non-oxidised spermine by ion-exchange chromatography. Dowex 50W, mesh size 200-400 with 2% cross-linking (Sigma Chemical Company Ltd.), was resuspended in distilled water at a ratio of 15g Dowex per 10 ml. 1 ml of the suspension was aliquoted into long-form Pasteur pipettes, plugged with polymer wool. The column was washed with water until the eluate was colourless. Samples of 200 μl were applied to the columns followed by 200 μl of distilled water. Oxidised and non-oxidised spermine were separated by a discontinuous gradient of hydrochloric acid. The gradient was created by sequentially adding 9 ml of 1.25M, 1.75M and 3M hydrochloric acid. 3 ml fractions were collected and added to 10 ml of Optiphase 'Safe' in 20 ml scintillation vials. Scintillation counting was performed as described previously (section 2.6).

The radioactivity contained in those fractions eluted by 1.25M hydrochloric acid was considered to be oxidised spermine as 14C spermine which had not been incubated in the presence of PAO was eluted at concentrations of HCl of 1.75M or greater. For this reason, the radioactivity eluted by concentrations of HCl of 1.75M or greater was considered to be non-oxidised spermine. The background radioactivity was measured by performing the assay with non-radiolabelled
spermine in place of the radiolabelled spermine. The background count was subtracted from each of the counts obtained for each sample in the radiolabel assay. The percentages of the radiolabel which was eluted as oxidised spermine and non-oxidised spermine were calculated. Knowing the concentration of spermine in the reaction, it was possible to work out the amount of spermine which had been oxidised.

2.10 Toxicity of Products of Polyamine Oxidation to *Listeria monocytogenes* and *Escherichia coli*.

This was a modification of the method of Bachrach and Persky (1964). For use in toxicity assays, bacteria were grown overnight in 10 ml volumes of the appropriate broth (section 2.2.1). Cells were harvested by centrifugation at 2,000g for 10 min, and washed by resuspending in 10 ml of nanopure water, before re-centrifuging as described above. The washing procedure was repeated for a total of two washes. The concentration of bacteria was estimated using a Thoma counting chamber. Toxicity assays were carried out in a total volume of 1 ml of 0.05M phosphate buffer pH7.2. The PAO source was either 100μl adult bovine serum (section 2.7) (heat-inactivated at 56°C for 30 min) or bovine serum albumin, plasma fraction V (Advanced Protein Products) at a final concentration of 5mg/ml. Polyamine oxidase has been shown to be present as a contaminant in bovine serum albumin (BSA) (Livingstone *et al*, 1977). This was confirmed before use of BSA by the fluorimetric method described in (section 2.9.1). Bacteria were added at a final concentration of 1 x 10^7 ml^-1. At the appropriate times, samples were taken and the viable counts determined as described in section 2.5.
2.11 Toxicity of Products of Polyamine Oxidation to *Mycobacterium tuberculosis*.

The polyamine oxidase sources used was BSA, as in the experiments with *E. coli* described previously (section 2.10). For use in toxicity assays, mycobacteria were grown for seven days as described previously (section 2.2.1). The bacteria were harvested by centrifugation at 2,000g for 10 min. after which the supernatant was discarded and the bacteria washed by resuspending in 0.01% (v/v) Tween 80, followed by centrifugation at 2,000g for 10 min. This washing was repeated for a total of two washes. The bacterial pellet was resuspended in 1-2 ml nanopure water and clumps disrupted by ultrasonication at 40W for three periods of five seconds. The bacterial concentration was estimated using a Thoma counting chamber, and adjusted to 1x10⁸ ml⁻¹.

The antimycobacterial activity of polyamine oxidation was evaluated using a modification of the method of Bachrach and Persky (Bachrach and Persky, 1964). The assay was carried out in 1 ml volumes of 0.05M phosphate buffer pH7.2. Bacteria at a concentration of 1x10⁷ ml⁻¹ were incubated in the presence of 5mg ml⁻¹ BSA (Advanced Protein Products), and the appropriate concentration of spermine or spermidine. After 0 and 24 hours of incubation samples were taken and viable count assays performed as described previously (section 2.5).

To test whether hydrogen peroxide was responsible in part or in full for the toxic effect of polyamine oxidation on *M. tuberculosis*, catalase was included at a final concentration of 400U ml⁻¹. This concentration was shown to remove more than 99% of the hydrogen peroxide produced by polyamine oxidation (section 3.1.2 and Table 3.5).
2.12 The Toxicity of Ammonium Ions and Acrolein to *Mycobacterium tuberculosis*.

The toxicity of acrolein and ammonium ions to mycobacteria were assessed by incubating mycobacteria in the presence of various concentrations of acrolein or ammonium chloride. Mycobacteria were prepared as described in section 2.11. The assays were carried out in 1 ml volumes of 0.05M phosphate buffer pH7.2. 1x10^8 bacilli were incubated for 24 hours in the presence of the appropriate concentration of acrolein or ammonium chloride. Samples were taken after 0 and 24 hours and bacterial viable counts performed as described in section 2.5.

2.13 Assay of the Hydrophobicity of *Mycobacterium tuberculosis* Strains.

The assay of hydrophobicity of *M. tuberculosis* was based on that of Stormer and Falkingham (Stormer and Falkingham, 1989). Mycobacteria were vortexed with a biphasic solution of phosphate buffered saline (PBS) (hydrophilic) and hexadecane (hydrophobic). Those bacteria which have a hydrophobic surface will be partitioned into the hexadecane layer, whereas those bacteria with a hydrophilic surface will remain in the PBS layer.

Mycobacteria were grown in 50 ml volumes of 7H9 medium with supplements as described in section 2.2.1. The bacilli were collected by centrifugation at 2,000g for 10 min and washed twice with 10 ml volumes of PBS.
Phosphate buffered saline, (PBS).

4g Sodium chloride
0.2g Potassium chloride
1.44g di-Sodium hydrogen phosphate
0.24g Potassium di-hydrogen phosphate

Water added to 500ml

Adjust to pH 7.4 with HCl.

The bacteria were then resuspended in 1-2 ml of PBS, sonicated and the bacterial concentration estimated as described in section 2.11. The concentration of the bacterial suspension was adjusted to $1 \times 10^9$ bacilli ml$^{-1}$. 1 ml of this suspension was diluted with an equal volume of formol saline (8% (v/v) formaldehyde solution, 0.85% (w/v) NaCl). This was the untreated sample. To a further 1.5 ml of the bacterial suspension was added 100 µl of hexadecane, in a 15 x 50mm glass test tube. The tube was vortexed at maximum speed for 2 min and then left to stand for a further 10 min. The lower aqueous layer was gently drawn off with a long form Pasteur pipette, avoiding disturbance of the hexadecane layer. This was then diluted by adding an equal volume of formol saline, to give the treated sample.

The absorbance at 600nm of both samples was measured (P8720 UV/VIS Scanning Spectrophotometer, Phillips, U.K). By comparison of the optical density of both samples, the percentage of the bacteria partitioned into the hexadecane could be calculated.
2.14 Molecular Biology Methods.


**Luria-Bertani (LB) medium.**

Media were as described by Sambrook and co-workers (Sambrook et al, 1989).

10g Tryptone
5g Yeast extract
10g Sodium chloride

Add water to 950ml and adjust to pH 7 with sodium hydroxide. Sterilise at 15psi for 20 min.

**Terrific medium (Tartof and Hobbs, 1987).**

12g Tryptone
24g Yeast extract
6ml Glycerol

Sterilise at 15 psi for 20 min, allow to cool then add 100ml of supplement solution.

Adjust volume to 1L with sterile nanopure water.

**Supplement solution.**

0.17M Potassium di-hydrogen phosphate
0.72M di-Potassium hydrogen phosphate

Sterilise by filtration through 0.2μm filter before use.
SOC medium.

20g Tryptone
5g Yeast extract
0.5g Sodium chloride
10ml 250mM Potassium chloride

Add 950ml water and adjust to pH 7 with sodium hydroxide. Sterilise at 15 psi for 20 min. Before use add 5ml of sterile 2M MgCl₂

2.14.2 Molecular Biology Reagents.

Reagents were made up as described by Sambrook and co-workers (Sambrook et al, 1989) unless otherwise stated.

Alkaline lysis solutions.

Solution I.
50mM Glucose
25mM Tris HCl (pH 8.0)
10mM Ethylenediaminetetraacetic acid (EDTA) (pH 8.0)

Solution II.
0.2M Sodium hydroxide
1% (w/v) Sodium dodecyl sulphate (SDS)

Solution III.
60ml 5M Potassium acetate
11.5ml Glacial acetic acid
2.14.3 Small Scale Preparation of Plasmid DNA.

This was by a modification of the alkaline lysis method described by Sambrook et al. (Sambrook et al., 1989). A single bacterial colony containing the plasmid to be prepared was used to inoculate 10 ml Luria-Bertani (LB) medium, (section 2.14.1) with the appropriate antibiotic(s), and the culture grown overnight at 37°C with constant agitation. 1.5 ml of the culture was harvested in each of three 1.5 ml Eppendorf tubes by centrifugation at 10,000g for 2 min. The supernatant was discarded and each pellet was resuspended in 200 μl of Solution I containing 4 mg ml⁻¹ lysozyme, and left at room temperature for 10 min. 200 μl of Solution II was added and the contents of the tube gently mixed by inverting the tube several times. Cellular debris was precipitated by the addition of 300 μl 7.5M ammonium acetate, left on ice for 10 min and the precipitate pelleted by centrifugation at 10,000g for 10 min. 800 μl of supernatant from each tube was added to 480 μl of isopropanol in a fresh 1.5 ml Eppendorf tube, the contents were mixed gently by inversion and the tube left at room temperature for 10 min. The supernatant was poured off, and the pellet washed with 100-200 μl of 70% (v/v) ethanol. The tube was inverted and allowed to dry for 15-20 min at room temperature. The contents of the three tubes were resuspended in a single 50 μl volume of TE (section 2.14.2) or nanopure
water, and phenol:chloroform extracted as described in section 2.14.5. After the chloroform extraction step, the plasmid DNA was precipitated with ethanol as described previously and resuspended in approximately 20 μl. The DNA was visualised by running 2-5 μl on an agarose gel (section 2.14.8).

2.14.4 Large Scale Preparation of Plasmid DNA.

This was a modification of the alkaline lysis-polyethylene glycol precipitation method of Sambrook and co-workers (Sambrook et al, 1989). A single bacterial colony was used to inoculate 10 ml LB containing the appropriate antibiotic, and the culture grown for 6-8 hours at 37°C with constant agitation. This culture was then used to inoculate 500 ml of Terrific broth (section 2.14.1) in a 2 L flask, and the flask incubated at 37°C overnight with constant vigorous agitation. The bacteria were harvested by centrifugation at 5,000g for 10 min at 4°C, and the supernatant discarded, taking care to remove the last of the supernatant. The pellets were resuspended in a single 25 ml volume of Solution I containing 5mg ml⁻¹ lysozyme, and left at room temperature for 10 min. 50 ml of Solution II was added and the contents mixed gently by inversion. 37.5 ml of ice-cold Solution III was added to precipitate the cellular debris, and the contents mixed gently and left on ice for 10 min. The precipitate was pelleted by centrifugation at 5,000g for 10 min at 4°C, and the supernatant filtered through polymer wool to remove any remaining debris. 70 ml of isopropanol was added to the supernatant to precipitate the plasmid DNA, and the mixture incubated at room temperature for 10 min. The DNA was collected by centrifugation at 5,000g for 10 min at room temperature. The pellet was washed gently with 5-10 ml of 70% (v/v) ethanol, the tube was inverted and allowed to dry at room temperature for 15 min. The pellet was then resuspended in 3 ml TE and 3 ml
of 5M LiCl was added to precipitate high molecular weight RNA, and the contents of the tube mixed gently. After centrifugation at 5,000g for 10 min, the supernatant was added to 30 ml of isopropanol, and incubated at room temperature for 10 min. The precipitated plasmid DNA was collected by centrifugation at 8,000g for 10 min, washed with 70% (v/v) ethanol and allowed to dry at room temperature for 15-20 min. Once dry, the pellet was dissolved in 500 ml TE and transferred to a 1.5 ml Eppendorf vial, to which was added RNase at a final concentration of 20mg/ml⁻¹ and incubated at 37°C for 30 min. If DNA was required in a high state of purity, 500μl of 13% (w/v) polyethylene glycol (Mr 8,000) in 1.6M sodium chloride was added, and the contents of the tube mixed and left on ice for 10 min. The precipitated DNA was collected by centrifugation at 10,000g for 10 min in a microfuge, the supernatant was discarded, and the pellet redissolved in 400 μl TE. Any protein remaining was removed by phenol:chloroform extraction as described in section 2.14.5 and the nucleic acid precipitated with ethanol and redissolved in 100-200 μl of TE, as described in section 2.14.6.

2.14.5 Phenol:Chloroform Extraction of DNA.

Phenol:chloroform extraction of DNA was as described by Sambrook and co-authors (Sambrook et al, 1989). An equal volume of phenol:chloroform was added to the DNA sample and vortexed at maximum speed for 10 seconds. The tube was centrifuged at 10,000g in a bench top centrifuge for 5 min to separate the phases. The upper aqueous phase contained the DNA and between the upper phase and the lower phenolic phase was the layer of precipitated protein. The upper phase was carefully withdrawn so as not to disturb the lower phase or the layer of
protein, leaving some of the upper phase if necessary. The recovered upper phase was extracted for a second time with phenol:chloroform, and the upper phase again recovered. Any remaining phenol present was removed by extraction with chloroform:iso-amyl alcohol (ratio 25:1). An equal volume of chloroform:iso-amyl alcohol was added to the DNA sample and vortexed at maximum speed for 10 seconds. The phases were separated by centrifugation at 10,000g for 2 min in a microfuge (MSE, U.K.) and the upper aqueous layer removed carefully.

2.14.6 Ethanol Precipitation of DNA.

The method of Sambrook and co-authors (Sambrook et al, 1989) was used for the ethanol precipitation of DNA. To a tube containing a solution of DNA was added one tenth of a volume of sodium acetate pH 5.2, and two volumes of ice-cold ethanol. The contents of the tube were mixed gently and the DNA allowed to precipitate at -20°C for 30 min. The DNA was recovered by centrifugation at 10,000g for 10 min in a microfuge. The supernatant was discarded and replaced with 100-200 µl of 70% (v/v) ethanol and the sample centrifuged as previously for 2 min. The supernatant was removed carefully and the tube inverted and allowed to dry at room temperature for 15-20 min. The DNA could then be dissolved in TE or nanopure water.

2.14.7 Restriction Digests of DNA.

Restriction digests were carried out in accordance with the manufacturer's instructions. Restriction enzymes were supplied by Gibco BRL.
2.14.8 Agarose Gel Electrophoresis.

Agarose (Seakem) was added to TAE buffer at a concentration of 0.7% (w/v) and dissolved by sterilising at 15 psi for 15 min. Before the gel was poured, both ends of the plate were sealed with tape, and an appropriately sized comb inserted. Cooled molten agarose was poured onto the slide to a depth of approximately 5mm. The gel was allowed to set and the comb and tape removed. Gel-loading buffer was added to samples for electrophoresis at 10% (v/v). For each gel, one lane contained size markers in the form of 1μg of 1 Kb ladder DNA (Gibco BRL). For gels with larger wells, the amount of 1 Kb ladder DNA used was increased in proportion to the increase in volume of the well. The gel was placed in an electrophoresis tank, and TAE buffer containing 5mgml⁻¹ ethidium bromide was added to just cover the surface of the gel. Gels were electrophoresed at 100 V until adequate separation of the bands was achieved. Ultra-violet light was used to visualise the DNA.

2.14.9 Purification of Restriction Fragments from Agarose Gels.

Restriction fragments were purified from agarose gels using a Sephaglass Band Prep Kit (Pharmacia, U.K.) according to the manufacturer’s instructions.

2.14.10 Preparation of Electrocompetent *Escherichia coli*.

The method of Sambrook and co-workers was used (Sambrook *et al*, 1989). 10 ml culture of *E. coli* was grown as described in section 2.14.3, and used to inoculate 1 L of LB medium. This culture was incubated at 37°C until it had reached an optical density of 0.5-1 at 600 nm, typically 1.5-2 hours. This gave cells at early log phase. The culture was chilled on ice for 20 min, after which the cells were
harvested by centrifugation at 2000g for 15 min. The bacterial pellet was
resuspended in 1 L of sterile distilled water and recentrifuged as previously. This
pellet was then resuspended in 500 ml of sterile distilled water, and recentrifuged as
previously. The pellet was resuspended in 20 ml of sterile 10% (v/v) glycerol, and
recentrifuged as previously. This final pellet was resuspended in 3 ml of sterile 10%
(v/v) glycerol and stored at -70°C in 40 μl aliquots.

2.14.11 Transformation of *Escherichia coli* by Electroporation.

A 40 μl aliquot of electrocompetent cells was allowed to thaw on ice. 1-5 ml of
plasmid DNA at a concentration of approximately 1mgml⁻¹ was mixed with the cells
and allowed to stand for 1 min on ice. The mixture was transferred to a chilled 0.2
cm gap electroporation cuvette (Biorad), avoiding the formation of bubbles. The
cuvette was placed in a safety chamber slide, and the pulse controller (Biorad) set
at 200 W, and the gene pulser (Biorad) set at 25 μF and 2.5 kV. The sample was
pulsed once, following which the cells were immediately resuspended in 1 ml of
SOC medium (section 2.14.1), transferred to a sterile test tube and incubated at
37°C for 1 hour with agitation.

Transformants were identified by plating onto LB medium containing an
appropriate antibiotic. Competent cells without plasmid DNA were always used as a
negative control.

2.14.12 Preparation of Chromosomal DNA from *Escherichia coli*.

A 10 ml culture of *E.coli* LE392 was grown for 16 hours at 37°C with agitation.
The cells were harvested by centrifugation at 2000g for 10 min, resuspended in 5 ml
of 10mM NaCl and harvested by centrifugation as previously. The cells were resuspended in 5 ml of lysis solution, and left on ice for 30 min.

Lysis solution:
10mM EDTA
20mM Tris-HCl
2% (w/v) Glucose
5mgml^-1 Lysozyme

Sodium lauryl sulphate was added to a final concentration of 1% (w/v) along with 500 ml of 0.5M EDTA, and the contents of the tube mixed and allowed to stand at room temperature for 20 min or until the contents of the tube went clear. The contents were transferred to a glass universal vial, 5 ml of phenol:chloroform added, and the vial centrifuged at 2000g for 20 min to collect material precipitated by the phenol:chloroform. The upper phase was removed and extracted a further two times with phenol:chloroform. Any remaining phenol was removed by extracting with chloroform:iso-amyl alcohol. After this extraction, the DNA was precipitated by addition of sodium acetate to a final concentration of 500mM, and three volumes of ethanol and kept at -20°C for 16 hours. Precipitated chromosomal DNA was collected by centrifugation at 10000g for 10 min and dissolved in 2 ml of TE.


The method of Davis and co-workers (Davis et al, 1991) was used. 20 ml of a two day old culture of M. smegmatis were added to 180 ml of double-strength Dubos broth base (Difco Laboratories, MI) and incubated at 37°C. After two days 20 ml of
2M glycine was added, and the cells incubated for a further two day at 37°C. Mycobacteria were harvested by centrifugation at 3000g for 10 min and resuspended in 10 ml of wash buffer, and centrifuged as before.

**Wash Buffer:**

300mM Sucrose

50mM Tris-HCl pH 8.0

10mM EDTA

The cells were resuspended at 2mgml⁻¹ of the wet weight in wash buffer, containing 2mgml⁻¹ of lipase (Sigma) and 2mgml⁻¹ lysozyme (Sigma) and incubated at 37°C for 60 min in a water bath. Four volumes of mycobacterial lysis solution were added and the incubation continued for a further 2 hours.

**Mycobacterial Lysis Solution:**

6M Guanidium Hydrochloride

1% (v/v) Sarkosyl

20mM EDTA

The preparation was extracted once with an equal volume of chloroform:iso-amyl alcohol, after which the upper phase was removed and the DNA precipitated at -20°C for 16 hours as described in section 2.14.6. The precipitated DNA was collected by centrifugation at 8,000g for 15 min, resuspended in 400 µl of TE containing 0.5mgml⁻¹ proteinase K and 4,000 U of RNase T1. After 3 hours of
incubation at 37°C, the DNA preparation was extracted once with phenol:chloroform, and once with chloroform:iso-amyl alcohol, and stored at 4°C.
3. Results and Discussion.
3.1 Polyamine Oxidation as an Antimicrobial Defence of Macrophages.

It has long been known that polyamine oxidation can yield products which are toxic to a range of bacteria, including *M. tuberculosis* (Section 1.5.7; Hirsch and Dubos 1952; Hirsch, 1953b; Bachrach and Persky, 1964). Oxidation of polyamines has also been shown to be generate products toxic to viruses (Bachrach *et al*, 1971), fungi (Levitz *et al*, 1990), parasitic protozoa (Ferrante *et al*, 1982; Rzepczyk *et al*, 1984; Morgan *et al*, 1986) and helminths (Ferrante *et al*, 1986).

Elevated levels of polyamine oxidase (PAO) have been found in activated macrophages (Morgan *et al*, 1980). It has been suggested that the increased PAO activity found in activated macrophages may contribute towards the antimicrobial activity of activated macrophages (Levitz *et al*, 1990). Further evidence for the importance of spermine and spermidine in macrophage function has come from studies using inhibitors which block the production of these polyamines. Macrophages treated with these inhibitors show reduced tumour necrosis factor-induced respiratory burst activity (Kaczmarek *et al*, 1992) and reduced phagocytic capacity (Kierzenbaum *et al*, 1987). However, little is known about the possible role polyamine oxidation may play as a defence of macrophages against intracellular bacterial pathogens such as *M. tuberculosis*. Research has so far concentrated on their possible defensive function in ruminant sera (Ferrante *et al*, 1982).

3.1.1 Polyamine Oxidase Mediated Killing of *Listeria monocytogenes*.

The Gram-positive bacterium *Listeria monocytogenes* was used as a model organism in preliminary studies of the antimicrobial effects of polyamine oxidation. However, *L. monocytogenes* has the advantage of forming a visible colony on agar
after approximately twenty four hours. This facilitates rapid repetition of experiments, and enables the determination of optimum conditions for the antimicrobial assay.

The antilisterial effects of polyamine oxidation were determined by incubating $1 \times 10^6$ bacilli in 1 ml of phosphate buffer pH7.2, containing 10% (v/v) PAO source and 100μM spermine. Viable count assays were performed at various times, and the number of colonies formed twenty four hours later counted.

At first, the source of PAO used was bovine serum, and samples were taken after zero, one, two, and four hours of incubation. Strong inhibition of protein synthesis and nucleic acid synthesis by oxidised polyamines has been reported to take place within 30 min of incubation of *E. coli* with oxidised polyamines (Bachrach and Persky, 1964; Bachrach and Rosenkranz, 1969). The results of this initial experiment are shown in Table 3.1. Over the four hour incubation period, the bacteria showed greater than 90% survival in phosphate buffer, indicating its suitability as a buffer for these experiments. Addition of spermine at a concentration of 100μM led to decreased viability, but *L. monocytogenes* still showed 80% viability at the end of the four hour incubation period. It was therefore concluded that spermine alone did not have a dramatic antilisterial effect at the concentration used. The inclusion of PAO in the form of 10% (v/v) bovine serum brought about the most noticeable effect on viability of *L. monocytogenes*. Comparable rates of survival (approximately 58%) were seen regardless of whether or not spermine is included. This indicates that the toxic effect is not mediated by products of polyamine oxidation, but rather by a component of bovine serum. The component mediating the
3.1 Toxicity of Oxidised Polyamines to *L. monocytogenes* C52.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Buffer</th>
<th>PAO&lt;sup&gt;b&lt;/sup&gt; + 100μM Spermine</th>
<th>100μM Spermine</th>
<th>PAO&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>67</td>
<td>74</td>
<td>72</td>
</tr>
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<tr>
<td>4</td>
<td>93</td>
<td>58</td>
<td>80</td>
<td>59</td>
</tr>
</tbody>
</table>

* Data are the mean of one experiment performed in triplicate

* Bovine serum

**Table 3.2** Effect of Incubating *L. monocytogenes* C52 in the Presence of Spermine and Different Sources of Polyamine Oxidase.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Buffer</th>
<th>Isoniazid control</th>
<th>PAO&lt;sup&gt;b&lt;/sup&gt; + 100μM Spermine</th>
<th>PAO&lt;sup&gt;b&lt;/sup&gt; + Isoniazid + 100μM Spermine</th>
<th>PAO&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PAO&lt;sup&gt;c&lt;/sup&gt; + 100μM Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>109</td>
<td>109</td>
<td>56</td>
<td>65</td>
<td>102</td>
<td>103</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>101</td>
<td>37</td>
<td>33</td>
<td>109</td>
<td>106</td>
</tr>
</tbody>
</table>

* Data are the mean of one experiment performed in triplicate

* Bovine serum

* Bovine serum albumin
toxicity was not complement because all sera were heat-inactivated at 56°C for 30 minutes before use.

In order to confirm that PAO was not involved in toxicity, PAO was inhibited by the addition of 2mM isonicotinic acid hydrazide (isoniazid). Isoniazid is known to be a potent inhibitor of PAO (Tabor et al, 1954), and this was confirmed using the fluorimetric PAO assay. Incubation of bovine serum with 2mM isoniazid for 40 minutes at 37°C caused a decrease from 141 fluorescence units to 2 fluorescence units (section 3.2.1 and Table 3.2), that is a 99% inhibition of polyamine oxidase activity. The data shown in Table 3.2 indicate that inhibition of PAO by 2mM isoniazid failed to remove the toxic effect of bovine serum on L. monocytogenes. Similar antilisterial effects were observed with bovine serum and spermine in the presence or absence of isoniazid (approximately 33% and 37% respectively). Wells containing 2mM isoniazid showed greater than 100% viability at the end of the four hour incubation period, therefore isoniazid at this concentration was not toxic to L. monocytogenes. As isoniazid is highly inhibitory to PAO without having any toxic effect on L. monocytogenes, the similar antilisterial effects of bovine serum and spermine in the presence or absence of isoniazid must be due to some factor other than polyamine oxidation.

The toxic nature of bovine serum to L. monocytogenes meant that another source of PAO had to be found. It has been reported that PAO is present as a contaminant in bovine serum albumin (BSA) (Livingstone et al, 1977). Indeed, the toxic effects of polyamine oxidation on M. tuberculosis were first noted when polyamines were added to cultures of M. tuberculosis containing BSA (Hirsch and Dubos, 1952; Hirsch, 1953b). As BSA is a relatively pure product, it was hoped that the PAO-independent toxic nature of the serum would be removed, whilst the PAO
activity remained. The level of PAO activity present in BSA was therefore investigated (Table 3.18). BSA at a concentration of 50mgml⁻¹ was found to have 53% of the PAO activity of bovine serum. This would still, however, result in complete oxidation of the spermine within the four hours of the assay.

The effect of BSA on *L. monocytogenes* in the presence or absence of spermine was therefore examined (Table 3.3). A toxic effect was not seen with BSA either in the presence or absence of spermine over the four hour incubation period. A small increase in bacterial numbers was observed after two hours of incubation and a slightly larger increase after four hours of incubation. This contrasts with experiments where bovine serum is used as the PAO source. In these experiments, viability decreases after two hours and shows a further decrease after four hours.

These preliminary experiments with *L. monocytogenes* failed to provide an adequate model for PAO-mediated killing of bacteria. However, as a result of these experiments, bovine serum was found to be unsuitable as a source of PAO due to its' bactericidal properties. The purified product BSA at a concentration of 50mgml⁻¹ was found to have a high level of PAO activity (Table 3.19). Unfortunately, although BSA itself was non-toxic to *L. monocytogenes*, neither was it toxic in the presence of spermine over the four hour incubation period. Polyamine oxidation is known to have an antimicrobial effect on a range of Gram-positive and Gram-negative organisms (Bachrach and Persky, 1964). However, in the work of Bachrach and Persky (1964), the antimicrobial effect was assessed after 20 hours of incubation. It is possible that extension of the incubation period would have resulted in a toxic effect of PAO oxidation. The low survival rate of *L. monocytogenes* in the buffer control would make accurate quantification of any antimicrobial effect difficult. For this reason, another model organism, *Escherichia coli*, was tested.
3.1.2 Polyamine Oxidase-Mediated Killing of *Escherichia coli*.

*Escherichia coli*, is a Gram-negative bacterium which is known to be susceptible to the PAO-polyamine system (Bachrach and Persky, 1964; Kimes and Morris, 1971b). The strain used, LE 392, is widely used for genetic studies and was chosen for this study as it carries the *RecA* gene (Sambrook *et al.*, 1989), which is important in protection against hydrogen peroxide (Carlsson and Carpenter, 1980). As hydrogen peroxide is produced during polyamine oxidation, this strain may give a clearer indication of the toxic properties of oxidised polyamines as opposed to hydrogen peroxide. Having found that spermine at a concentration of 100μM in the presence of PAO, had no toxic effect upon *L. monocytogenes*, it was decided to use two higher concentrations of spermine, 500μM and 250μM to ensure that a toxic effect was seen.

The data in Table 3.3 are from an initial experiment examining the toxicity of polyamine oxidation to *E. coli* over a four hour incubation period. Bacterial numbers in the buffer-only control after 4 hours of incubation, were 131% of that at the start of the experiment, establishing its suitability for use in the experiment. The PAO source, BSA at the same concentration used previously, was non-toxic to the bacteria, and supported a greater than two-fold increase in bacterial numbers. Spermine itself was also found not to be toxic to *E. coli*, with bacterial numbers slightly increased over the four hour incubation period. Spermine at either 250μM or
3.3 Toxicity to *E. coli* of Incubation for 4 Hours in the Presence of Spermine and Polyamine Oxidase.

<table>
<thead>
<tr>
<th>Percentage Survival After 4 Hours of Incubation in the presence of:⁰</th>
<th>Buffer</th>
<th>PAO</th>
<th>500μM Spermine</th>
<th>PAO + 500μM Spermine</th>
<th>PAO + 500μM Spermine + CAT⁵</th>
<th>PAO + 250μM Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>131</td>
<td>229</td>
<td>111</td>
<td>0, (36)⁶</td>
<td>107</td>
<td>0, (69)⁶</td>
</tr>
</tbody>
</table>

a Data are the mean of one experiment performed in triplicate.

b CAT = 4000 Uml⁻¹ catalase.

c Figures in brackets indicate the levels of survival observed after the agar plates had been incubated for a further 14 hours at 37°C.
500μM in the presence of PAO appeared to exert a complete bactericidal effect on 
*E. coli* over the four hour incubation period.

In order to distinguish whether the observed antimicrobial effect was due to 
death of the bacteria or a temporary inhibition of replication, from which the bacteria 
could recover after an extended period of time, the plates were incubated for a 
further eight hour period. Towards the end of this period, very small colonies were 
observable, and therefore incubation was continued until these colonies could be 
counted easily.

A difference in survival rate was found between *E. coli* incubated at the two 
spermine concentrations. Those bacteria incubated with PAO and spermine at a 
concentration of 500μM, showed approximately 36% survival whereas those 
incubated with 250μM spermine and PAO showed approximately 69% survival. The 
bactericidal effect of 500μM oxidised spermine in the presence of PAO is 
approximately double that of 250μM oxidised spermine, toxicity is therefore 
dependent upon polyamine concentration. This linkage between polyamine 
concentration and toxicity confirms the involvement of the polyamine in toxicity. 
Neither spermine or PAO alone had any toxic effect upon the bacteria (Table 3.3).

There appear to be two types of toxic effect of polyamine oxidation on *E. coli*. 
It is apparent that oxidation of spermine generates products which are bactericidal 
to *E. coli* as bacterial numbers fall after incubation with PAO and spermine. 
However, the delay in the appearance of colonies suggests that oxidation of 
spermine can also result in products which inhibit the replication of *E. coli*. Products 
of polyamine oxidation have previously been reported to inhibit DNA, RNA and 
protein synthesis in *E. coli* (Bachrach and Persky, 1964; Bachrach and Persky ,

97
1966; Bachrach and Rosenkranz, 1969; Kimes and Morris, 1971b). The inhibition or replication of *E. coli* shown in Table 3.4 could therefore be attributed to inhibition of macromolecular synthesis by products of polyamine oxidation. It is feasible that the inhibitory effect could become bactericidal if inhibition of bacterial macromolecular synthesis was continued for a long enough period of time. The inhibitory and bactericidal effects may therefore be mediated by the same toxic species.

Polyamine oxidation produces a number of products which are potentially toxic to bacteria (Section 1.5.7 and Fig 1.1), including aminoaldehydes, hydrogen peroxide and ammonium ions. The role of hydrogen peroxide can be established by the addition of catalase at a concentration sufficient to remove all of the hydrogen peroxide produced. Catalase at a concentration of 400Uml\(^{-1}\) was found to remove more than 99% of the hydrogen peroxide produced by oxidation of 500\(\mu\)M spermine (Table 3.4). When this concentration of catalase was included in the antibacterial assay, the toxic effect of polyamine oxidation was removed. Numbers of *E. coli* after four hours of incubation with PAO, 250\(\mu\)M spermine and 400Uml\(^{-1}\) catalase were 104% of those at zero-time (Table 3.3). This suggests that over the four hour incubation period, the toxicity of polyamine oxidation to *E. coli* was mediated solely by hydrogen peroxide and not by aminoaldehydes or ammonium ions.

Although hydrogen peroxide appears to be the sole mediator of toxicity, other products of polyamine oxidation may prove to be toxic if the incubation period is extended. Therefore, the incubation period of the assay was extended to twenty four hours. Cultures of *E. coli* were incubated with either spermine or spermidine in the presence or absence of PAO. Agar plates used to determine bacterial viability were incubated for an extra 12 hours in order to discriminate between inhibitory and
Table 3.4 Removal by Catalase of Hydrogen Peroxide Generated as a Result of Polyamine Oxidation.

<table>
<thead>
<tr>
<th>Catalase Concentration</th>
<th>Fluorescence Increase Over 120 min</th>
<th>Percentage of Hydrogen Peroxide Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>58</td>
<td>0</td>
</tr>
<tr>
<td>Catalase (400 Uml⁻¹)</td>
<td>19.2</td>
<td>33</td>
</tr>
<tr>
<td>Catalase (4000 Uml⁻¹)</td>
<td>0.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>
bactericidal effects of products of polyamine oxidation. Over the twenty four hour incubation period, the bacteria increased their numbers approximately two and a half fold in buffer alone (Tables 3.5 and 3.6). Similar levels of multiplication were observed in the presence of both concentrations of spermine in the absence of PAO. The inclusion of PAO however resulted in the death of all the bacteria at both concentrations of spermine. In this case the failure of the bacteria to form colonies was not as a result of the inhibitory effect observed previously (Table 3.3), as plates kept for several days showed no signs of the formation of colonies.

The antimicrobial effects of spermidine were also investigated after both four and twenty four hours (Table 3.6). A slight decrease in viability was noted in the buffer alone control after four hours of incubation, but this was found not to be significant. *Escherichia coli* showed a slight increase in number after four hours of incubation with either 500µM or 250µM spermidine in the absence of PAO. In contrast to the findings with oxidation of spermine (Table 3.3), incubation for four hours with spermidine and PAO had no antibacterial effect on *E. coli* (Table 3.6). However, a significant (P<0.1) bactericidal effect was observed when *E. coli* was incubated in the presence of 500µM spermidine and PAO for four hours. The bactericidal effect observed after four hours of incubation with PAO and 500µM spermidine is lower than that with PAO and 500µM spermine, but similar to that observed with 250µM spermine (Table 3.3). This was as expected, as complete oxidation of spermine produces twice the molar amount of hydrogen peroxide and aldehyde groups as oxidation of spermidine (Fig. 1.1). As catalase was not included in the experiments shown in Table 3.6, it could not be determined whether the
3.5 Effect on *E. coli* of Incubation for 24 Hours in the Presence of Spermine and Polyamine Oxidase.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Percentage survival after incubation with:* $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>265.51±</td>
</tr>
<tr>
<td></td>
<td>106.92</td>
</tr>
</tbody>
</table>

*a Data are the mean ± standard deviation from three experiments*

3.6 Effect on *E. coli* of Incubation for 24 Hours in the Presence of Spermidine and Polyamine Oxidase.

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Percentage survival after incubation with:* $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91.72±</td>
</tr>
<tr>
<td></td>
<td>18.72</td>
</tr>
<tr>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>286.43±</td>
</tr>
<tr>
<td></td>
<td>42.57</td>
</tr>
</tbody>
</table>

*a Data are the mean ± standard deviation from 3 experiments*
observed antibacterial effects were due to hydrogen peroxide or other products of polyamine oxidation.

After twenty four hours of incubation, the buffer control and both concentrations of spermidine in the absence of PAO showed an approximately three fold increase in bacterial numbers. However, bacteria incubated with either 500\(\mu\)M or 250\(\mu\)M spermidine in the presence of PAO showed no survival after twenty four hours of incubation. The failure to observe colonies after incubating the plates for several days, illustrates that this is a bactericidal effect as seen previously with spermine (Table 3.5).

Therefore, it has been demonstrated that oxidation of either spermine or spermidine by bovine serum polyamine oxidase, can result in the generation of products which kill \textit{E. coli}. Investigations could therefore begin into the antimycobacterial effects of polyamine oxidation.

3.1.3 The Antimycobacterial Effects of Polyamine Oxidation.

The experiments performed with \textit{E. coli} (section 3.1.2) were repeated with two strains of \textit{M. tuberculosis}; strain B1453 and strain 79500. The characteristics of both strains are summarised in Table 2.1. Both strains are classed as avirulent in the guinea-pig and susceptible to 0.02\% (v/v) hydrogen peroxide. Strain 79500 is positive for the enzyme catalase, whereas strain B1453 is catalase-negative. By choosing a catalase-positive strain and a catalase-negative strain, it was hoped to assess whether possession of the enzyme catalase increased the resistance of \textit{M. tuberculosis} to products of polyamine oxidation.

The effects of PAO and spermine on the survival of both strains are shown in Table 3.7. Both strains showed a slight increase in bacterial numbers over the
twenty four hour period of incubation in the buffer control. The presence of spermine at both concentrations (250μM and 500μM) had different effects upon the two strains. A decrease in number was found after incubating strain B1453 for twenty four hours at either concentration. The observed decrease was higher at the higher concentration (500μM) of spermine. On the other hand, strain 79500 showed a slight increase in number at both concentrations of spermine. The magnitude of these increases was similar to that observed in the buffer control. Where PAO was added in the presence of either of the two concentrations of spermine, no survival was observed with strain B1453 or strain 79500. The antimicrobial effect of polyamine oxidation observed was a bactericidal effect, because no colonies were observed on the agar plates after five or six weeks of incubation.

When spermidine was used in place of spermine as the polyamine source, similar results were obtained (Table 3.8). After twenty four hours of incubation in the buffer control, a decrease in number was observed with strain B1453, whereas a slight increase in number was observed with strain 79500. Both strains did however show an approximately 30% decrease in number when incubated with 500μM spermidine alone. At the lower concentration of 250μM spermidine alone, a slight decrease in number was observed with strain B1453, and a slight increase observed with strain 79500. Therefore spermidine itself appears to have some antibacterial effect, especially at a concentration of 500μM. When spermidine was added in the presence of PAO, a similar bactericidal effect to that described above with spermine was observed (Table 3.7). Both concentrations of spermidine in the presence of
3.7 Effect on Strains of *M. tuberculosis* of Incubation for 24 Hours in the Presence of Spermine and Polyamine Oxidase.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> Strain</th>
<th>Percentage survival after 24 hours of incubation in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>B1453</td>
<td>123</td>
</tr>
<tr>
<td>79500</td>
<td>108</td>
</tr>
</tbody>
</table>

a Data represent the mean of two or three replicates of a single experiment.

3.8 Effect on Strains of *M. tuberculosis* of Incubation for 24 Hours in the Presence of Spermidine and Polyamine Oxidase.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> Strain</th>
<th>Percentage survival after 24 hours of incubation in the presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>B1453</td>
<td>84</td>
</tr>
<tr>
<td>79500</td>
<td>109</td>
</tr>
</tbody>
</table>

a Data represent the mean of two or three replicates of a single experiment.
PAO resulted in the death of 100% of both strains of *M. tuberculosis*. As described previously, colonies were not observed at any of the dilutions plated after four to five weeks of incubation. It appears therefore that oxidation of this concentration of spermidine results in the generation of products as mycobactericidal as those produced by oxidation of spermine.

Having found that polyamine oxidation had a bactericidal effect on two strains of *M. tuberculosis*, it was decided to look at a wider range of strains. The results described above show that both concentrations of spermidine and spermine were bactericidal in the presence of PAO. It was decided to investigate whether there was a link between strain virulence and susceptibility to products of polyamine oxidation. Incubation of both strain 79500 and strain B1453 for twenty-four hours in the presence of PAO and polyamine at a concentration of 250μM resulted in the death of 100% of bacteria of both strains. This does not allow a comparison of the susceptibilities of the two strains to products of polyamine oxidation to be made. By using lower concentrations of polyamine, it was hoped to demonstrate differences in the susceptibilities of different strains of *M. tuberculosis* to products of polyamine oxidation. Eight strains of *M. tuberculosis* were tested for their susceptibility to three concentrations of spermine in the presence of PAO. The concentrations of spermine used were 250μM, 25μM and 2.5μM. The results of these experiments are detailed in Table 3.9.

Apart from strain 79499, none of the strains tested showed a significant decline in number after incubation for twenty four hours in the buffer control. Spermine at a concentration of 250μM in the presence of PAO was significantly bactericidal to all strains (P<0.05). The highest level of survival at this concentration
3.9 Toxicity to *M. tuberculosis* of Various Concentrations of Spermine in the Presence of Polyamine Oxidase.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>RIV</th>
<th>Percentage Survival After 24 Hours of Incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buffer only</td>
</tr>
<tr>
<td>79499</td>
<td>1.29</td>
<td>57.73 ± 17.51*</td>
</tr>
<tr>
<td>I2646</td>
<td>1.17</td>
<td>82.94 ± 18.27</td>
</tr>
<tr>
<td>H37Rv</td>
<td>1.01</td>
<td>105.73 ± 11.73</td>
</tr>
<tr>
<td>79500</td>
<td>0.98</td>
<td>101.13 ± 15.38</td>
</tr>
<tr>
<td>79112</td>
<td>0.92</td>
<td>98.80 ± 16.80</td>
</tr>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>87.84 ± 12.90</td>
</tr>
<tr>
<td>H37Ra</td>
<td>0.5</td>
<td>84.94 ± 16.57</td>
</tr>
<tr>
<td>H37RaHR</td>
<td>0.12</td>
<td>109.84 ± 25.56</td>
</tr>
</tbody>
</table>

a Data are the mean ± standard deviation of three or four experiments

* Denotes a value significantly (P<0.05) different from that at zero-time.
of spermine was shown by strain 79499 (23.75%), all other strains showed less than 3% survival. However, it is possible that the true level of toxicity of polyamine oxidation to strain 79499 is partially masked by the toxic effect of the buffer. That is, if the apparent toxic effects of the buffer could be removed, survival of strain 79499 may be greater than the value observed. Despite the uncertainty of the true level of toxicity to strain 79499, it is worth noting that as well as being the strain with the greatest resistance to PAO and 250μM spermine, strain 79499 is also the strain with the highest RIV in the guinea-pig (1.29).

Spermine at a concentration of 25μM was not uniformly bactericidal to all of the tested strains. Three strains (H37Rv, 79500 and 79112) were not significantly affected by oxidation of this concentration of spermine. The other tested strains showed a significant (p<0.05) decline in number after incubation with this concentration of polyamine and polyamine oxidase. For three of these strains (79499, I2646 and H37Ra) percentage survival after twenty four hours of incubation was greater than 50%. Strain 79499 again shows a significant decline in number at this concentration of spermine. This, however, may be attributable solely to the toxic effects of the buffer, as there is no significant difference between the survival rates of this strain in buffer or 25μM spermine and PAO. Similarly for strain H37Ra, the number of viable bacteria present after twenty four hours of incubation is significantly different from the initial number. As in the case of strain 79499, there is not a significant difference between the survival rates of this strain in buffer or 25μM spermine and PAO. Strain I2646 shows the greatest decrease of these three strains. Incubation of this strain in the presence of 25μM oxidised spermine results in significantly lower survival (p<0.05) when compared with the buffer control.
The two strains showing greatest sensitivity to 25μM spermine in the presence of PAO are strain B1453 and strain H₃₇RaHR. Both strains are catalase-negative, and thus would be more susceptible to hydrogen peroxide than the other strains tested, which are catalase-positive. When compared with it's catalase-positive parent H₃₇Ra, H₃₇RaHR shows significantly reduced survival (P<0.02), in the presence of PAO and 25μM spermine. As oxidation of polyamines results in the production of hydrogen peroxide this may be a contributing factor in the susceptibility of these strains to oxidation of low polyamine concentrations.

At the lowest concentration of spermine used, greater than 70% survival was shown by all strains. Strains B1453 and H₃₇RaHR were significantly affected by oxidation of 2.5μM spermine. As described above, this may be due to hydrogen peroxide generated as a result of polyamine oxidation. The decreased survival of strain 79499 whilst significant, is not significantly different from its survival in the buffer control. The decline in number cannot, therefore, be attributed to polyamine oxidation. It is also difficult to ascribe the decrease in viability of strain 79500 to polyamine oxidation, as this strain is not significantly affected by a ten fold higher concentration of spermine in the presence of PAO. However, none of the strains tested showed significantly different rates of survival in the presence of 2.5μM spermine and PAO compared with the buffer control.

When three concentrations of spermine were tested against a panel of mycobacterial strains, a pattern of susceptibility was observable. Those strains which are catalase-positive, were generally less susceptible to oxidation of lower concentrations of spermine than the catalase-negative strains. It would therefore
appear that hydrogen peroxide produced by polyamine oxidation was a major contributor to the observed bactericidal effects.

The borderline virulent/avirulent strains H37Rv, 79500 and 79112 were not significantly affected by oxidation of 25µM spermine. In contrast, the highly virulent strains 79499 and I2646 showed significant loss of viability after incubation with 25µM spermine and PAO. In the case of strain 79499, it is doubtful whether the decline in numbers over the incubation period of the assay is due to toxicity of 25µM oxidised spermine. This is concluded because there is no difference in the rates of survival of this strain in the presence of 25µM oxidised spermine compared with the buffer control. The decline in numbers of this strain may be due in part to the observed toxic effects of the buffer. Similarly the survival of the avirulent strain H37Rv, has significantly reduced after twenty four hours of incubation with PAO and 25µM spermine. However, as is the case with strain 79499, the fact that there is no significant difference between levels of survival of this strain in 25µM oxidised spermine or the buffer control suggests that products of polyamine oxidation are not responsible for the decline in number.

In contrast, strain I2646 shows significantly lower survival in the presence of 25µM oxidised spermine compared with the buffer control. As strain I2646 is classed as catalase-positive and hydrogen peroxide resistant, it is difficult in this case to ascribe this toxic effect of PAO and 25µM spermine to hydrogen peroxide. This would not be expected if susceptibility to polyamine oxidation is linked to virulence, as this strain has a high RIV (1.17). Strain I2646 has also, however, been found to have unexpectedly low resistance to reactive nitrogen intermediates (O’Brien et al, 1994).
The findings of this series of experiments have confirmed and enlarged upon the work of Hirsch and Dubos (Hirsch and Dubos, 1952; Hirsch, 1953a). Oxidation of spermine by PAO results in the generation of products which have antimycobacterial properties. This antimycobacterial effect was described by Hirsch (1953a) as an inhibition of replication as no growth was observed in cultures of mycobacteria incubated for 14 days with PAO and spermine. Hirsch and Dubos had previous shown, using a viable count assay, that significant numbers of viable mycobacteria were present after incubation for up to 24 hours in the presence of PAO and spermine. However, no mycobacterial colonies were observed after incubation for 4 days in the presence of PAO and spermine (Hirsch and Dubos, 1952).

In the present study the antimycobacterial effect was measured by determining the number of viable bacteria present after 0 and 24 hours of incubation. In contrast to the findings of Hirsch and Dubos, the antimycobacterial effect of polyamine oxidation observed in the present study was deemed to be as a result of the death of the mycobacteria as no colonies were observable on the agar plates, even after 6 weeks of incubation at 37°C.

One possible explanation for this discrepancy is that different concentrations of PAO and polyamine were used. The concentration of spermine needed to bring about the almost complete bactericidal effect observed in the present study was approximately ten-fold greater (250μM) than that used to cause bacteriostasis in the work of Hirsch (17.5μM-70μM) (1953a). However, this does not completely explain the differences in the findings of the two studies as there was also a difference in the number of bacteria used, (1x10^7 in the present study and 5x10^4 in the work of Hirsch).
The present study has also expanded upon the work of Hirsch and Dubos (1952) by looking at the effects of products of polyamine oxidation on avirulent and catalase-negative strains of *M. tuberculosis*. The strains used by Hirsch and Dubos were virulent strains of *M. tuberculosis* and *M. bovis*, various strains of *M. bovis* BCG and the avirulent *M. tuberculosis* strain H$_{37}$Ra.

3.1.4 The Relationship Between the Virulence of Mycobacterial Strains and Their Susceptibility to Polyamine Oxidation.

The experiments described above demonstrated that products of polyamine oxidation are bactericidal to *M. tuberculosis*. Catalase-negative strains show the greatest sensitivity to these products, which may be as a result of the hydrogen peroxide generated during polyamine oxidation. A correlation has been found between sensitivity to hydrogen peroxide and virulence in the guinea-pig of strains of *M. tuberculosis* (Mitchison et al., 1963). If oxidation of the polyamines spermine and spermidine is an antimycobacterial defence of macrophages, then it can be hypothesised that virulent strains of *M. tuberculosis* are more resistant than avirulent strains. In order to clarify whether this is due to products specifically generated by polyamine oxidation, hydrogen peroxide must be eliminated from the assay. Oxidised polyamines are known to have short half lives, and therefore it was decided to generate these products *in situ*. Hydrogen peroxide was removed by adding catalase at an appropriate concentration, as described previously (section 3.1.2).

The mycobacterial strains tested were incubated for twenty four hours in the presence of PAO, catalase and a range of concentrations of spermine, from 250μM to 25μM. The results of these experiments are shown in Table 3.10. Concentrations
of spermine up to and including 100μM in the presence of PAO and catalase, were found to be highly toxic to six of the tested strains, including the five avirulent strains (79500, 79112, B1453, H$_{37}$Ra, and H$_{37}$RaHR) and the virulent strain with the lowest RIV (H$_{37}$Rv), as defined previously (Table 2.1; Mitchison et al, 1963). Oxidation of spermine at concentrations of 50μM or 25μM had a reduced bactericidal effect on several of these strains compared with that of concentrations of spermine of 100μM or more. Comparison of the data in Table 3.9 with that in Table 3.10 shows that while strains 79500 and H$_{37}$Rv were not significantly affected by oxidation of 25μM spermine in the absence of catalase, they were affected by oxidation of 25μM spermine in the presence of catalase, when PAO was present.

Of the six strains described above, three (B1453, H$_{37}$Ra and H$_{37}$RaHR) are significantly affected by 250μM spermine in the presence of catalase, but the absence of PAO. The toxic effects of 25μM and 50μM spermine in the presence of PAO and catalase may therefore partly be due to catalase or unoxidised spermine rather than products produced as a result of polyamine oxidation. However, catalase or unoxidised spermine cannot account for the highly bactericidal effect observed at concentrations of 100μM spermine and above when PAO is present.

The three strains with the highest RIVs (M. bovis 81470, 79499 and I2646), were the most resistant to polyamine oxidation. However, the low degree of survival of M. bovis 81470 in the buffer control and the low degree of survival of strain 79499 in the 250μM spermine plus catalase control make it difficult to draw conclusions on
3.10 Toxicity to *M. tuberculosis* of Various Concentrations of Spermine in the Presence of Polyamine Oxidase and Catalase.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>Buffer</th>
<th>250µM Spermine + PAO + CAT</th>
<th>200µM Spermine + PAO + CAT</th>
<th>150µM Spermine + PAO + CAT</th>
<th>100µM Spermine + PAO + CAT</th>
<th>50µM Spermine + PAO + CAT</th>
<th>25µM Spermine + PAO + CAT</th>
<th>250µM Spermine + CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em> 81470</td>
<td>55.87 ± 16.90 *</td>
<td>50.78 ± 17.8 *</td>
<td>71.63 ± 32.00</td>
<td>52.12 ± 16.76 *</td>
<td>79.76 ± 22.7</td>
<td>64.99 ± 28.75</td>
<td>83.42 ± 17.06</td>
<td>102.73 ± 2.53</td>
</tr>
<tr>
<td>79499</td>
<td>110.65 ± 31.46 *</td>
<td>30.46 ± 10.46 *</td>
<td>30.16 ± 3.97 *</td>
<td>55.95 ± 27.44 *</td>
<td>58.41 ± 27.91 *</td>
<td>65.60 ± 23.02 *</td>
<td>32.71 ± 9.67 *</td>
<td>49.44 ± 11.48 *</td>
</tr>
<tr>
<td>l2646</td>
<td>93.87 ± 15.01 *</td>
<td>1.06 ± 1.26 *</td>
<td>11.64 ± 12.03</td>
<td>23.43 ± 13.46 *</td>
<td>74.32 ± 18.55</td>
<td>97.95 ± 29.84</td>
<td>81.75 ± 29.48</td>
<td>105.68 ± 27.38</td>
</tr>
<tr>
<td>H37Rv</td>
<td>105.62 ± 4.11 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>70.85 ± 9.26</td>
<td>66.76 ± 8.17</td>
<td>91.50 ± 21.05</td>
</tr>
<tr>
<td>79500</td>
<td>ND</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>41.43 ± 30.92</td>
<td>61.55 ± 165.75</td>
<td>165.75 ± 35.69</td>
<td>ND</td>
</tr>
<tr>
<td>79112</td>
<td>103.61 ± 26.10</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>43.60 ± 26.10</td>
<td>118.60 ± 51.17</td>
<td>64.28 ± 21.23</td>
<td>ND</td>
</tr>
<tr>
<td>B1453</td>
<td>76.62 ± 8.48 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 0.00 *</td>
<td>6.30 ± 4.10 *</td>
<td>70.58 ± 18.67</td>
<td>57.18 ± 3.27</td>
<td>69.82 ± 10.42 *</td>
</tr>
<tr>
<td>H37Ra</td>
<td>84.09 ± 25.12</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 2.41 *</td>
<td>12.93 ± 2.79</td>
<td>85.00 ± 6.66</td>
<td>106.87 ± 23.10</td>
<td>67.40 ± 20.63</td>
<td>ND</td>
</tr>
<tr>
<td>H37RaHR</td>
<td>ND</td>
<td>0.00 ± 0.00 *</td>
<td>0.00 ± 13.78</td>
<td>38.71 ± 9.08</td>
<td>38.71 ± 8.54</td>
<td>59.82 ± 18.79</td>
<td>69.50 ± 3.96</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Data are the mean ± standard deviation of three or four experiments ND = Not determined
* Denotes a value significantly (P<0.05) different from that at zero-time. CAT = 4000 Uml⁻¹ catalase
the effects of polyamine oxidation on these two strains. It is clear, however, that both strains are partially resistant to the toxic effects of 250\( \mu \)M spermine in the presence of PAO and catalase. At this concentration of spermine the only other strain to show any degree of survival was the virulent strain I2646 (1.06% survival).

Strain I2646 was not significantly affected by either the buffer control or the 250\( \mu \)M spermine and catalase control. The lowest concentration of spermine in the presence of PAO which was significantly bactericidal to this strain was 150\( \mu \)M. This contrasts with the experiments in which hydrogen peroxide was not removed (Table 3.9), where 25\( \mu \)M spermine in the presence of PAO was significantly bactericidal to strain I2646. Hydrogen peroxide would therefore appear to be an important contributor towards the toxicity of polyamine oxidation to strain I2646. The survival of strain I2646 in the presence of concentrations of spermine of 150\( \mu \)M or greater with catalase and PAO is less than that shown by \textit{M. bovis} 81470 and strain 79499 but greater than that shown by the avirulent strains.

All of the mycobacterial strains tested can be placed into either one of two groups depending upon their resistance to concentrations of spermine of 150\( \mu \)M or greater in the presence of PAO and catalase. The virulent strains with the highest RIV scores (\textit{M. bovis} 81470, 79499, I2646) show a greater degree of resistance to these high concentrations of spermine in the presence of PAO and catalase when compared with the other strains tested. With one exception (strain H\textsubscript{37}Ra at 150\( \mu \)M spermine), the less virulent strains (H\textsubscript{37}Rv, 79500, 79112, B1453, H\textsubscript{37}Ra, H\textsubscript{37}RaHR) showed a complete loss of viability at concentrations of spermine of 150\( \mu \)M or greater in the presence of PAO and catalase. Therefore, there is some evidence of a link between strain virulence in the guinea-pig and resistance to products of
polyamine oxidation, with the highly virulent strains being more resistant to products of polyamine oxidation.

In order to determine whether there is a correlation between strain virulence and resistance to products of polyamine oxidation, the survival rates of strains in the presence of PAO and catalase and either 50µM or 25µM spermine were compared using linear regression. Concentrations of spermine of 100µM and greater were deemed unsuitable, as several of the strains showed complete loss of viability under these conditions. No correlation was found between strain virulence and susceptibility to either 50µM \( (r = 0.3186) \) or 25µM \( (r = -0.1295) \). From the results of this experiment it was not possible to determine whether there was a link between strain virulence and susceptibility to products of polyamine oxidation.

The catalase-negative strains (H37RaHR, B1453) had similar levels of susceptibility to products of polyamine oxidation in the presence of catalase as the avirulent catalase positive strains. This would appear to confirm that the greater susceptibility of catalase-negative strains to products of polyamine oxidation (Table 3.9) is due to a greater susceptibility to hydrogen peroxide. In the absence of hydrogen peroxide (Table 3.10) catalase-negative strains show a similar level of susceptibility to products of polyamine oxidation. Potentially toxic species produced by polyamine oxidation other than hydrogen peroxide (amminoaldehydes, ammonium ions) appear to be equally toxic to the avirulent strains regardless of whether these strains possess catalase.

To summarise, mycobacteria were found to be susceptible to polyamine oxidation in the presence of catalase. Therefore products of spermine oxidation other than hydrogen peroxide (for example amminoaldehydes, ammonium ions) have
mycobactericidal properties. Under these conditions, the most resistant strains were those with the highest virulence in the guinea-pig. Oxidation of spermine by PAO in the presence of catalase was equally toxic to all avirulent strains regardless of the possession of catalase.

Having found no correlation between the virulence of mycobacterial strains and their susceptibility to products of spermine oxidation other than hydrogen peroxide, it was decided to investigate whether there was a linkage to products of spermidine oxidation. Oxidation of spermidine by bovine serum PAO (BSPA0) results in the production of a monoaldehyde, as opposed to the dialdehyde produced by oxidation of spermine.

Spermidine at concentrations between 250μM and 25μM was used to determine whether there was a link between mycobacterial strain virulence and susceptibility to oxidation of this polyamine (Table 3.11). Of the eight strains tested, only *M. bovis* 81470, the most virulent strain, was found to be resistant to all concentrations of spermidine in the presence of PAO and catalase. However, this strain showed a small decrease in number in the buffer control which was significant (*P* < 0.05). At a concentration of 100μM spermidine, a significantly bactericidal effect was observed with all of the strains tested except *M. bovis* 81470, I2646 and 79500. All of the strains tested, with the exception of strain 79112, were resistant to oxidation of 50μM spermidine. The apparent susceptibility of this strain to low concentrations of spermidine in the presence of PAO and catalase may partly be due to a toxic effect of either the buffer or catalase, as both the buffer control and the 250μM spermidine plus catalase control, show significantly reduced numbers of
3.11 Toxicity to *M. tuberculosis* of Various Concentrations of Spermidine in the Presence of Polyamine Oxidase and Catalase.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> Strains</th>
<th>Percentage Survival After 24 Hours of Incubationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td><em>M. bovis</em> 81470</td>
<td>84.25 ± 5.53 *</td>
</tr>
<tr>
<td>79499</td>
<td>229.35 ± 106.82</td>
</tr>
<tr>
<td>I2646</td>
<td>116.65 ± 55.77</td>
</tr>
<tr>
<td>H37Rv</td>
<td>109.94 ± 26.64</td>
</tr>
<tr>
<td>79500</td>
<td>112.12 ± 29.22</td>
</tr>
<tr>
<td>79112</td>
<td>66.99 ± 9.45 *</td>
</tr>
<tr>
<td>B1453</td>
<td>117.95 ± 10.46</td>
</tr>
<tr>
<td>H37RaHR</td>
<td>94.37 ± 30.67</td>
</tr>
</tbody>
</table>

* Data are the mean ± standard deviation of three or four experiments. * Denotes a value significantly (P<0.05) different from that at zero-time. ND = Not determined

CAT = 4000 Uml⁻¹ catalase
bacteria ($P<0.05$ in both cases). However, strain 79112 was also found to be highly susceptible to oxidation of spermine in the presence of catalase.

The possibility of a correlation between strain virulence and resistance to oxidised spermidine was again investigated. The strongest correlations were found at $200\mu$M ($r = 0.5358$) and $100\mu$M ($r = 0.5102$). However, these were found not to be significant.

Comparing the results in Tables 3.10 and 3.11, it is seen that oxidation of both spermine and spermidine generates products which are toxic to mycobacteria. The degree of toxicity varies between the two polyamines. For example, at concentrations between $250\mu$M and $100\mu$M spermine, there is a complete loss of viability with strains $H_37$Rv, 79500 and 79112 (Table 3.10). In contrast, at the same concentration of spermidine, no strains show a complete loss of viability. Indeed, at $100\mu$M spermidine no bactericidal effect is observed upon strain 79500, whereas strains $H_37$Rv and 79112 show only partial loss of viability (62.07% and 27.97% survival respectively). Similar effects can be seen at polyamine concentrations of $250\mu$M, $200\mu$M and $150\mu$M.

In contrast, the virulent strains $M. bovis$ 81470, 79499 and I2646 show similar degrees of susceptibility to oxidation of either spermine or spermidine. This is demonstrated by strain I2646, which has similar rates of survival when incubated with PAO in the presence of $200\mu$M spermine or $200\mu$M spermidine (Tables 3.10, 3.11).

The pattern of susceptibility of the mycobacterial strains to oxidation of spermidine in the presence of catalase is different to that observed with spermine. $Mycobacterium bovis$ 81470 was resistant to all of the tested concentrations of
oxidised spermidine. This strain was previously found to be highly resistant to oxidised spermine (Table 3.10).

Unlike *M. bovis* 81470, the highly virulent strains 79499 and I2646 were significantly affected by concentrations of oxidised spermidine up to and including 150µM. The less virulent strains on the other hand do not show complete loss of viability at any of the concentrations of oxidised spermidine. At a concentration of 150µM, oxidised spermidine has a similar level of toxicity for a number of strains of widely differing virulence such as I2646 and H₃₇RaHR. This is in contrast to the findings with 150µM oxidised spermine, where the less virulent strains (with the exception of strain H₃₇Ra) show complete loss of viability.

The differences between the susceptibilities of mycobacterial strains to the two oxidised polyamines suggest that these oxidised polyamines achieve their toxic effects by different mechanisms.

Analysis of the data pertinent revealed that there was no correlation between strain virulence and resistance to any of the tested concentrations of oxidised spermidine. This is a similar situation to that seen with spermine, as virulence is found to be independent of resistance to oxidised spermidine. Oxidation of spermidine would therefore not appear to be the sole mechanism by which guinea-pig macrophages kill *M. tuberculosis*.

### 3.1.5 The Effect of Transformation with *katG* on the Susceptibility of Catalase-negative strains of Mycobacteria to Polyamine Oxidation.

It was observed earlier (section 3.1.3 and Table 3.9) that strains of *M. tuberculosis* which lack catalase (catalase-negative), show greater susceptibility to
products of polyamine oxidation than a similar strain possessing catalase (catalase-positive). This is shown by the catalase-positive strain H₃⁷Ra, and the catalase-negative mutant of this strain, H₃⁷RaHR. At 25µM spermine, strain H₃⁷Ra showed significantly greater survival (p<0.02) than H₃⁷RaHR (73.74% compared with 27.13%, Table 3.9). This suggests that the increased susceptibility of strain H₃⁷RaHR to 25µM spermine in the presence of PAO could be due to the loss of the enzyme, catalase. In the same series of experiments, a second catalase-negative strain, B1453, was also found to be highly susceptible to 25µM spermine in the presence of PAO (Table 3.9).

In order to confirm that possession of catalase increased the resistance of mycobacteria to products of polyamine oxidation, another catalase-negative strain was investigated. *Mycobacterium tuberculosis* strain 24 has been transformed with a plasmid (pYZ66) bearing the *M. tuberculosis* catalase gene katG, to obtain a catalase-positive phenotype, 24(pYZ66) (Zhang et al, 1993). For these experiments, strain 24 and a recombinant strain 24 containing the parental plasmid (p16R1), were also included. The susceptibility of the three strains to 250µM and 25µM spermine, in the presence of PAO, was tested. The effect of catalase on the toxicity of 25µM spermine in the presence of PAO to the three strains was also tested. The results are shown in Table 3.12.

Of the three strains tested, all were highly susceptible to 250µM spermine in the presence of PAO (P<0.05). While both of the catalase-negative strains showed a total loss of viability under these conditions, strain 24 (pYZ66) which is catalase-positive showed a small degree of survival (6.3%). The susceptibility of
3.12 Effect of katG on the Susceptibility of *M. tuberculosis* Strain 24 to Products of Polyamine Oxidation.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>Percentage Survival After 24 Hours of Incubation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>24</td>
<td>104.31 ± 7.48</td>
</tr>
<tr>
<td>24 (p16R1)</td>
<td>78.35 ± 15.04</td>
</tr>
<tr>
<td>24 (pYZ66)</td>
<td>83.60 ± 7.36</td>
</tr>
</tbody>
</table>

* Results are the mean ± standard deviation of three or four experiments

* Denotes a value significantly (P<0.05) different from that at zero-time.

CAT = 4000 Uml⁻¹ catalase
the three strains tested is similar to that found previously (Table 3.9) with both catalase-positive and catalase-negative strains.

However, the differences between the strains in their susceptibility to products of polyamine oxidation was demonstrated at a concentration of 25μM spermine in the presence of PAO (Table 3.12). The catalase-negative strains, 24 and 24 (p16R1) show 0.09% and 0.43% survival respectively under these conditions, whereas the catalase-positive strain 24 (pYZ66) shows 69.66% survival. Analysis of these results showed that the catalase-positive strain 24 (pYZ66) showed significantly (P<0.01) greater survival than the two catalase-negative strains. Transformation of the catalase-negative strain 24 with a plasmid containing the *M. tuberculosis* catalase gene increased the resistance of this strain to products of the oxidation of spermine.

Addition of catalase led to significantly (P<0.05) increased survival of both catalase-negative strains. Indeed, incubation of strain 24 with 25μM spermine, PAO and catalase resulted in no significant loss in viability of the bacteria. Therefore, it can be concluded that hydrogen peroxide was responsible for the mycobactericidal effects of 25μM spermine in the presence of PAO. Strain 24(YZ66) was significantly affected by 25μM spermine in the presence, but not the absence, of catalase. It would appear that inclusion of catalase has a toxic effect on this stain. The toxicity of similar concentrations of catalase to mycobacteria has been observed previously (section 3.1.4; O'Brien, 1995).
3.1.6 The Antimycobacterial properties of Ammonium Ions and Acrolein.

Polyamine oxidation results in the generation of several toxic products (section 1.5.7). While hydrogen peroxide appears to play a part in the toxicity of polyamine oxidation to mycobacteria (section 3.1.5), other bactericidal products are generated (section 3.1.4) which possibly include aminoaldehydes, acrolein and ammonium ions. As a result of the instability of the aminoaldehydes, it was decided to investigate the toxicity of acrolein and ammonium ions, and from these results infer the role of the aminoaldehydes.

Ammonium ions and hydrogen peroxide are formed in equimolar quantities during polyamine oxidation. For each mole of dioxidised spermine produced, two moles of ammonium ions are produced, and for each mole of monooxidised spermine or monooxidised spermidine produced, one mole of ammonium ions is produced (Fig 1.1). It was therefore necessary to investigate the toxicity of concentrations of ammonium ions up to 500μM, in order to account for that which could result from dioxidised spermine. The three strains of M. tuberculosis used were the virulent strain I2646, the avirulent strain B1453, and strain 79500 which is near the borderline between virulent and avirulent strains. The results are displayed in Table 3.13.

The highest concentration of ammonium ions tested (500μM) did not show a bactericidal effect on any of the three strains. Nevertheless, significant effects (P<0.05) were observed on strain I2646 at 250μM ammonium, strain B1453 at 100μM ammonium and strain 79500 at 50μM ammonium. However, the survival of B1453 in the presence of 100μM ammonium was not found to be significantly different from its survival in the buffer control. The degree of the toxic effects was,
Table 3.13 Toxicity of Ammonium Ions to *M. tuberculosis*.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>Percentage Survival After 24 Hours of Incubation in the Presence of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
<td>500μM NH₄Cl</td>
</tr>
<tr>
<td>I2646</td>
<td>98.35 ± 14.50</td>
<td>88.35 ± 8.76</td>
</tr>
<tr>
<td>79500</td>
<td>143.53 ± 12.79</td>
<td>92.32 ± 23.07</td>
</tr>
<tr>
<td>B1453</td>
<td>91.18 ± 9.28</td>
<td>110.45 ± 11.37</td>
</tr>
</tbody>
</table>

a Data are the mean ± standard deviation of three or four experiments

* Denotes a value significantly (P<0.05) different from that at zero-time.
however, small in comparison with that observed in previous experiments with polyamine and PAO, with all of the strains showing greater than 70% survival. The evidence that ammonium ions are mycobactericidal is inconsistent. It is likely that the loss of viability of strains 79500 and 12646 at 50µM and 100µM ammonium was due to an effect of the buffer as described previously (section 3.1.4). Previous work has found that the inclusion of ammonium ions at concentrations up to 1000µM had no effect on the survival of *Plasmodium falciparum* (Rzepczyck *et al.,* 1984) or *Cryptococcus neoformans* (Levitz *et al.,* 1990). Complete oxidation of 250µM spermine and 250µM spermidine would produce concentrations of 500µM and 250µM ammonium respectively. As ammonium concentrations of 500µM and 250µM had no significant antimycobacterial effect, it is concluded that ammonium ions are not responsible for the antimycobacterial effects of polyamine oxidation.

Oxidation of the polyamines spermine and spermidine results in the production of relatively unstable aminoaldehydes (Kimes and Morris, 1971a), which have been shown to breakdown by the process of β-elimination, with the resultant formation of acrolein. Acrolein has been shown to inhibit nucleic acid synthesis in *E. coli* (Kimes and Morris, 1971b) and to cause inactivation of phages (Bachrach *et al.,* 1971). The relatively short half-lives of dioxidised spermine and oxidised spermidine [42 minutes and 137 minutes respectively, (Kimes and Morris, 1971a)] would theoretically allow more than 90% of the oxidised spermine to undergo β-elimination to form acrolein. However, a yield of more than 30% acrolein from oxidised spermine has not been achieved even in the presence of trapping agents (Alarcon, 1970).

Concentrations of acrolein, from 250µM to 5µM were therefore investigated for their bactericidal effects on two strains of *M. tuberculosis*: 12646 and B1453.
Both strains were susceptible to acrolein, up to and including concentrations of 25μM. The degree of toxicity varied between the strains. For example, at a concentration of 50μM acrolein, strain I2646 showed significantly higher (P<0.05) survival (14.95%), compared with 4.22% survival for strain B1453. Comparing these results with those in Table 3.10, it is seen that 50μM acrolein has similar toxic properties as 200μM spermine for strain I2646, and 100μM spermine for strain B1453. This is in contrast to previous reports which have found that on a molar basis, acrolein was only as toxic as oxidised spermine to mammalian cells (Alarcon, 1964).

It has been suggested (Ferrante et al, 1986a) that as the aminoaldehydes produced by polyamine oxidation are highly reactive, inhibiting bacterial macromolecular synthesis within 20 minutes of their production (Bachrach and Persky, 1964), they would react before they degraded to form acrolein. The aminoaldehydes form electrostatic and covalent linkages with DNA (Bachrach and Eilon, 1967) and would therefore be unable to undergo β-elimination to form acrolein. Whether a concentration of 50μM acrolein could accumulate in the experiments carried out in this study (Tables 3.10 and 3.11) is therefore debatable.
### Table 3.14 Toxicity of Acrolein to Strains of *M. tuberculosis*.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>Buffer</th>
<th>250μM Acrolein</th>
<th>100μM Acrolein</th>
<th>75μM Acrolein</th>
<th>50μM Acrolein</th>
<th>25μM Acrolein</th>
<th>10μM Acrolein</th>
<th>5μM Acrolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2646</td>
<td>98.35 ± 14.50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00 ± 0.00 *</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.74 ± 6.21 *</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>2.86 ± 1.16 *</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14.92 ± 5.16 *</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>50.21 ± 20.84 *</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90.72 ± 16.47</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>74.31 ± 11.75 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1453</td>
<td>91.18 ± 9.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.00 ± 0.00 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.51 ± 0.26 *</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75 ± 0.11 *</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.22 ± 1.14 *</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>31.10 ± 3.16 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>85.78 ± 9.81 *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86.52 ± 15.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Data are the mean ± standard deviation of three or four experiments.

* Denotes a value significantly (P<0.05) different from that at zero-time.
3.1.7 Antimycobacterial Activity of Rat Tissue Polyamine Oxidase.

Oxidation products of the polyamines spermine and spermidine produced by bovine serum PAO (BSPAO) were found to be toxic to mycobacteria (section 3.1.3). However, the form of PAO found in macrophages more closely resembles the mammalian tissue-type PAOs (Morgan et al., 1980). The aminoaldehyde produced when polyamines are oxidised by this group of enzymes is 3-aminopropanal (Fig 1.1). Homogenates of rat tissues found to contain high levels of PAO (Table 3.19) were investigated for their antimycobacterial activity in the presence of spermine (Table 3.15). Rat tissues were used in preference to guinea-pig tissues as PAO level in rat tissues were higher than those in the corresponding guinea-pig tissues (Tables 3.19, 3.20 and 3.21).

Preparations of spleen and kidney were found to have no antimycobacterial activity either in the presence or absence of spermine. Liver homogenate on the other hand, had a significant (P<0.02) toxic effect upon strain I2646 but this occurred both in the presence and absence of spermine. The observed toxic effects of liver homogenate were of a similar magnitude regardless of the presence or absence of spermine. Polyamine oxidation cannot therefore be claimed to be mediating the mycobactericidal effects of the liver homogenate. It is possible that the toxicity of the liver extract may be masking an antimycobacterial effect of liver PAO and spermine. Bile salts are known to have antimycobacterial effects. It was hoped to avoid contamination of the liver extract with bile salts by careful removal of the gall bladder and by using sections of the liver distant from the site of the gall bladder. However, it is still possible that small amounts of bile salts present in the liver could be responsible for the observed antimycobacterial effects of the liver extract.
3.15 Toxicity to *M. tuberculosis* Strain I2646 of Extracts from Rat Tissues in the Presence and Absence of Spermine.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> Strain</th>
<th>Buffer</th>
<th>250µM Spermine + spleen extract</th>
<th>Spleen extract</th>
<th>250µM Spermine + Kidney extract</th>
<th>Kidney extract</th>
<th>250µM Spermine + liver extract</th>
<th>Liver extract</th>
<th>250µM Spermine + PAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2646</td>
<td>82.38 ± 19.93</td>
<td>112.65 ± 36.55</td>
<td>122.15</td>
<td>93.74 ± 7.83</td>
<td>107.45 ± 10.25</td>
<td>63.05 ± 13.53*</td>
<td>56.08 ± 17.49*</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

*a* Data are the mean ± standard deviation of three or four experiments.

* Denotes a value significantly (P<0.05) different from that at zero-time.
Other tissues (kidney, spleen) had no antimycobacterial effects in the presence of spermine despite having higher levels of PAO than the liver extract (Table 3.19). Extracts from guinea-pig kidneys have been shown previously (Hirsch, 1953a) to have antimycobacterial properties in the presence of polyamines. This was not found to be the case in the present study.

It is possible that 3-aminopropanal is not toxic for mycobacteria to the same extent as the products produced through oxidation of polyamines by BSPAO. Previous research has found that for some organisms, oxidation of polyamines by human retroplacental serum PAO (RPSPAO), which produces the same oxidation products as rat tissue PAO (3-aminopropanal) had less of an antimicrobial effect than oxidation of polyamines by BSPAO (Ferrante et al., 1986a; Morgan, 1987b). When RPSPAO was used as PAO source, higher concentrations of spermine were required to result in the same level of damage to *Dirofilaria immitis* microfilariae (Ferrante et al., 1986a). This contrasted with the situation for *Schistosoma mansoni* schistosomula, where both PAO sources were equally effective (Ferrante et al., 1986a).

Another possible explanation for the failure to observe a toxic effect with rat tissue extracts as the PAO source is that the oxidised polyamines produced reacted with components present in the tissue extracts. Oxidised polyamines bind irreversibly to DNA (Bachrach and Leibovici, 1966), therefore the DNA released from cells disrupted during the homogenesis of the tissue may have acted as a scavenger, removing all of the aminoaldehydes generated by PAO.

In conclusion, no evidence was found for oxidation of spermine by rat tissue PAO having an antimycobacterial effect. However, it is possible that the toxic products of polyamine oxidation are reacting with components such as DNA in the
tissue extracts and therefore are not able to react with mycobacteria. PAO from human retroplacental serum produces the same products as rat tissue PAO, but the serum does not contain DNA. An effort was made to collect retroplacental serum, however, sufficient serum could not be collected to carry out toxicity assays.

3.2 Polyamine Oxidase Levels in Vaccinated and Control Guinea-pigs.

The aims of this experiment were to look at whether vaccination of guinea-pigs to produce macrophages which are capable of killing *M. tuberculosis* in *vitro*, resulted in elevated levels of PAO in tissues or macrophages. Intra-peritoneal vaccination of rabbits with *M. bovis* BCG has been shown previously to result in elevated levels of PAO in the alveolar macrophages from these animals (Morgan *et al*, 1980). If oxidation of polyamines by PAO is part of the antimycobacterial defence of guinea-pig alveolar macrophages, then it is possible that levels of this enzyme will be greater in immunised animals than susceptible control animals. Comparison of the VBC guinea-pig model with non-vaccinated guinea-pigs would indicate whether the antimycobacterial effects of alveolar macrophages could be mediated by polyamine oxidation.

Three of the tissues studied (spleen, liver and kidney) have been reported to show high levels of PAO activity (Seiler *et al*, 1980; Suzuki *et al*, 1984), making detection of PAO easier. As the spleen is a lymphoid tissue, and the immune response to tuberculosis is a lymphoid response, any effect of vaccination may be expected to be most prominent in this tissue. The liver was studied because vaccination of guinea-pigs with *M. bovis* BCG under the VBC schedule results in the formation of observable granulomas in the liver. Tuberculosis is primarily a disease of the lungs, therefore levels of PAO in the lungs were measured.
3.2.1. Fluorimetric Polyamine Oxidase Assay.

The fluorimetric PAO assay is based upon the fact that polyamine oxidation results in the generation of hydrogen peroxide. Horseradish peroxidase catalyses the oxidation of the non-fluorescent substrate (p-hydroxyphenylacetic acid) by hydrogen peroxide, to give a fluorescent product. As oxidation of each terminal amino group of the polyamine produces a molecule of hydrogen peroxide, fluorescence is directly linked to the number of aldehyde groups produced. This assay also has the advantages that it is quick to perform and does not involve the use of radioactive substances.

The fluorimetric PAO assay was used to assess PAO activity in several serum and tissue samples (Table 3.16). Over the 150 minute incubation period of the assay, only bovine serum demonstrated activity. Neither of the guinea-pig tissue samples or the human serum showed any activity using this assay. Previous reports (Seiler et al, 1980; Suzuki et al, 1984) have found high levels of PAO in rat tissues, and detectable levels of PAO in human pregnancy serum (Morgan and Illei, 1980 and 1981). Therefore, it was concluded that the failure to detect PAO activity in guinea-pig tissues and human serum was as a result of the fluorimetric PAO assay being unsuitable for use with these samples.

In order to confirm that the fluorescence produced after incubation of bovine serum with spermidine was due to PAO activity, the serum was incubated with 4mM isoniazid at 37°C prior to use in the fluorimetric PAO assay (Table 3.17). The
Table 3.16 Polyamine Oxidase Activity in Sera and Guinea-pig Tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fluorescence Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum</td>
<td>296.7</td>
</tr>
<tr>
<td>Guinea-pig kidney</td>
<td>0.9</td>
</tr>
<tr>
<td>Guinea-pig spleen</td>
<td>0</td>
</tr>
<tr>
<td>Human serum</td>
<td>0</td>
</tr>
<tr>
<td>Human pregnancy serum</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.17 Effect of Isoniazid on Polyamine Oxidase Activity of Bovine Serum.

<table>
<thead>
<tr>
<th>PAO source</th>
<th>Fluorescence Increase</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum</td>
<td>141.05</td>
<td>NA</td>
</tr>
<tr>
<td>Bovine serum + isoniazid</td>
<td>2.1</td>
<td>98.51</td>
</tr>
</tbody>
</table>

NA = Not applicable
increase in fluorescence observed previously (Table 3.17) was inhibited by 99% in the presence of this concentration of isoniazid, an inhibitor of PAO (Tabor et al., 1954). It can therefore be assumed that the increased fluorescence was due to the production of hydrogen peroxide by polyamine oxidation.

It was thought possible that the hydrogen peroxide was interacting with cell components present in the tissue homogenates, thus masking any PAO activity. Whether this would be sufficient to account for all of the hydrogen peroxide produced is not known. Fluorimetric PAO assays based upon the production of hydrogen peroxide have been suggested as not being as reliable for use with crude cell extracts as radiometric assays (Höltta, 1983). It was therefore decided to use a radiometric assay, which does not depend upon the generation of hydrogen peroxide.

3.2.2 Radiometric Polyamine Oxidase Assay.

The radiometric PAO assay used was based upon that developed by Morgan and Illei (Morgan and Illei, 1981). This assay has been extensively used by Morgan and co-workers to determine PAO activity in a variety of samples (Morgan et al., 1983; Morgan, 1985b, 1985c; Ferrante et al., 1986a; Morgan, 1987; Ferrante et al., 1990). In this assay, samples are incubated with a known amount of $^{14}$C-labelled spermine. At the end of the incubation period, the reaction is stopped by the addition of trichloroacetic acid. The products of the reaction are separated from the unreacted substrate by ion-exchange chromatography, with a stepwise gradient of hydrochloric acid.

A range of samples were tested using the radiometric PAO assay, and the results are shown in Table 3.18. The highest levels of activity were found in bovine
Table 3.18 Polyamine Oxidase Activity in Sera and Tissues as Determined by the Radiometric PAO Assay.

<table>
<thead>
<tr>
<th>PAO Source</th>
<th>Polyamine Oxidase Activity&lt;sup&gt;a&lt;/sup&gt; (nmol substrate converted/min/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum</td>
<td>2571</td>
</tr>
<tr>
<td>Bovine serum albumin (50mg/ml&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>1328</td>
</tr>
<tr>
<td>Ovine serum</td>
<td>2006</td>
</tr>
<tr>
<td>Non-pregnant human serum</td>
<td>2</td>
</tr>
<tr>
<td>Pregnant human serum A</td>
<td>23</td>
</tr>
<tr>
<td>Pregnant human serum B</td>
<td>45</td>
</tr>
<tr>
<td>Guinea-pig Kidney</td>
<td>33</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are the mean of two or three replicates.
and ovine serum (2571 and 2006 nmol substrate converted/minute/l respectively). The source of PAO used in most of the toxicity assays, BSA at a concentration of 50mgml⁻¹, was found to have approximately half that of whole bovine serum (1328 nmol substrate converted/minute/l).

The human sera tested showed very low levels of PAO in comparison to the ruminant sera. The non-pregnant female serum showed the lowest level of PAO of all the tested samples (2 nmol substrate converted/minute/l). This is a similar level to that found by Morgan and Illei [1.7 ± 6.53 nmol substrate converted/minute/l (Morgan and Illei, 1981)].

In comparison, the sera of females during the advanced stages of pregnancy (30-35 weeks) was found to have approximately ten to twenty times the PAO activity as that of the non-pregnant female (23 and 45 nmol substrate converted/minute/l). The demonstration of elevated levels of PAO in the serum of pregnant human females in this experiment is in agreement with the findings of Morgan and Illei (Morgan and Illei, 1981). At a similar stage of pregnancy (30-32 weeks) Morgan and Illei found PAO levels of 128.08 ± 97.19 nmol/minute/l. Therefore the PAO levels detected in Morgan and Illei's study appear to be a little higher than those found in the present study. However, the large standard deviation shown in Morgan and Illei's work indicates that PAO levels vary greatly between individuals.

Apart from the natural variability between subjects, one factor that may be responsible for the lower level observed in the present study is a difference in the pattern of elution of the polyamines. In the study of Morgan and Illei, spermidine was eluted by 1.75M hydrochloric acid and spermine was eluted by 3M hydrochloric acid. In the present study spermine was eluted by 1.75M hydrochloric acid and both spermidine and putrescine were eluted by 1.25M hydrochloric acid. As spermidine is
produced by oxidation of spermine and putrescine by oxidation of spermidine, there may be some underestimate of PAO activity in the present study.

The level of PAO in guinea-pig kidney homogenates (33 nmol substrate converted/minute/l) was found to be similar to that in the human pregnancy sera (Table 3.18). Therefore both guinea-pig kidney and human pregnancy sera have PAO activity which could not be detected using the fluorimetric PAO assay but which was detectable using the radiometric PAO assay. It is possible that this is as a result of the inclusion of benzaldehyde in the incubation buffer of the radiometric PAO assay. Addition of benzaldehyde has been shown to result in increased activity of PAO in human pregnancy sera (Morgan and Illei, 1981). It has been proposed that benzaldehyde forms a Schiff base with the polyamine (Morgan, 1985a).

The radiometric PAO assay was therefore selected for use in determining the PAO levels in tissues of vaccinated and non-vaccinated guinea-pigs. However, the fluorimetric assay was still used to test whether or not batches of BSA had similar levels of activity to previous batches.

3.2.2.1 Polyamine Oxidase Activity in Rat Tissues.

Before using the radiometric PAO assay to compare levels of PAO in control and VBC guinea-pigs, it was decided to look at the PAO levels in rat tissues. PAO levels in rat tissues have been assessed using thin layer chromatography (Seiler et al, 1980) and measurement of oxygen consumption (Pavlov et al, 1991). For these experiments the tissues were homogenised in the buffer previously used by Seiler and co-workers (Seiler et al, 1980), which contained dithiothreitol (DTT), as did the buffer of Pavlov and co-workers (Pavlov et al, 1991). Addition of DTT has been
shown to result in elevated PAO activity in rat liver homogenates (Höltta, 1977). The results of this experiment are shown in Table 3.19.

The highest levels of PAO activity were found in spleen and kidney preparations (10.65 and 9.78 nmol substrate converted/minute/l respectively). Liver had approximately half the PAO activity of these tissues (4.75 nmol substrate converted/minute/l). Levels in the heart and lung samples were approximately one tenth and one twentieth those in liver and kidney (0.80 and 0.39 nmol substrate converted/minute/l respectively). The findings of this study show a similar pattern of PAO activity to that found previously with an assay based upon thin layer chromatography of dansylated polyamines (Seiler et al, 1980). However, the levels of PAO reported by Seiler and co-workers are higher than those reported here. Liver was reported by Seiler and co-workers to have fifty times the PAO activity of that demonstrated in this study (Seiler et al, 1980). Furthermore, in the study of Seiler and co-workers, levels of PAO in spleen and kidney were found to be fifteen times higher. Lung and heart homogenates had one hundred, and twenty five times the PAO activity demonstrated in this study. A separate study using an assay based upon oxygen consumption, has found even higher levels of PAO than those found by Seiler and co-workers (Pavlov et al, 1991). Acetylated polyamines were used as the substrate for PAO by the groups of Seiler, Pavlov and Kumazawa in the experiments described above. It would also appear that acetylated polyamine are the natural substrates for mammalian tissue type PAO, as they are more readily oxidised than non-acetylated polyamines (Bolkenius and Seiler, 1981; Seiler, 1989) which may explain the differences in PAO activity between these reports and the present study.
Table 3.19 The Polyamine Oxidase Activity in Rat Tissues.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Polyamine Oxidase Activitya (nmol substrate converted/min/l)</th>
<th>Polyamine Oxidase Activitya (nmol substrate converted/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>1065</td>
<td>10.65</td>
</tr>
<tr>
<td>Liver</td>
<td>475</td>
<td>4.75</td>
</tr>
<tr>
<td>Kidney</td>
<td>978</td>
<td>9.78</td>
</tr>
<tr>
<td>Heart</td>
<td>80</td>
<td>0.8</td>
</tr>
<tr>
<td>Lung</td>
<td>39</td>
<td>0.39</td>
</tr>
</tbody>
</table>

aData are the mean of two or three replicates.
A more recently published radiometric assay (Kumazawa et al, 1990) has found PAO levels in rat tissue to be closer to those in this report. However, the two studies are not comparable as Kumazawa and co-workers did not include DTT in the incubation mixture, which may lead to an underestimation of the PAO activity.

Although the levels of PAO found in rat tissue were less than those previously described, it was decided to use the radiometric PAO assay to evaluate differences in PAO activity between vaccinated and control guinea-pigs. This was because the pattern of distribution of PAO was similar to that found in other assays (Seiler et al, 1980; Kumazawa et al, 1990; Pavlov et al, 1991). The assay was also sensitive enough to detect PAO in all of the tissues tested.

One improvement upon the radiometric PAO assay used in this study would be the use of acetylated spermine instead of spermine. However, radiolabelled acetylated polyamines were not used as they are not yet commercially available. The method of synthesising N1-acetylspermine described by Kumazawa and co-workers (Kumazawa et al, 1990) was deemed too time consuming for use in the present study.

3.2.2.2 Comparison Between the Polyamine Oxidase Activity of Tissues from Vaccinated and Control Guinea-pigs.

The levels of PAO in five tissues (spleen, kidney, liver, lung and heart) from non-vaccinated guinea-pigs were investigated (Table 3.20). The highest levels of activity were found in spleen samples. Lower levels of activity were found in kidney and liver homogenates, with lung and heart showing the lowest levels of activity. This is a similar distribution to that found in rat tissue (section 3.2.2.1; Seiler et al,
Table 3.20 Polyamine Oxidase Levels in Tissues From Non-Vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Polyamine Oxidase Activity$^a$ (nmol substrate converted/min/g tissue)</th>
<th>Guinea-pig 1</th>
<th>Guinea-pig 2</th>
<th>Guinea-pig 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>6.64, 6.33, 6.31</td>
<td>8.01, 11.08, 7.76</td>
<td>9.17, 12.49, 7.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\mu = 6.43$</td>
<td>$\mu = 8.95$</td>
<td>$\mu = 9.71$</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>3.01, 4.59</td>
<td>1.77, 1.29, 2.01</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\mu = 3.80$</td>
<td>$\mu = 1.69$</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>2.47, 4.96, 2.79</td>
<td>2.80, 2.61, 3.49</td>
<td>1.86, 2.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\mu = 3.41$</td>
<td>$\mu = 2.97$</td>
<td>$\mu = 2.08$</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>1.79, 0.76, 0.81</td>
<td>0.64, 0.45</td>
<td>0.32, 0.21, 0.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\mu = 1.12$</td>
<td>$\mu = 0.55$</td>
<td>$\mu = 0.49$</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>0.95, 1.66, 0.66</td>
<td>0.36, 0.05</td>
<td>0.06, 0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\mu = 1.09$</td>
<td>$\mu = 0.21$</td>
<td>$\mu = 0.08$</td>
</tr>
</tbody>
</table>

ND = Not done

$^a$ Data shown are two or three replicates of the same experiment and the mean ($\mu$) of those replicates.
The levels of PAO detected in non-vaccinated guinea-pig tissue were also comparable to that found in normal rats using the same assay (Table 3.19).

The tissues of animals vaccinated under the VBC schedule described previously (section 2.4.1) were also investigated (Table 3.21). Again the highest levels of PAO were found in spleen homogenates, with slightly less activity found in liver and kidney homogenates. Heart and lung samples had the lowest levels of activity detected. This is a similar pattern to that described above for non-vaccinated guinea-pigs, and that reported previously for rat tissue (section 3.2.2.1; Seiler et al, 1980).

In order to determine whether there was a difference in the PAO activity of tissues of non-vaccinated and vaccinated guinea-pigs, the mean of the two or three replicates was calculated. Using the Student's t test, the mean PAO activities of the two populations of animals (non-vaccinated and vaccinated) were compared for each tissue.

No significant difference was found between the levels of PAO in either the liver or spleen samples of the two populations. This would seem to suggest that PAO is not elevated in macrophages found in these tissues. However, macrophages make up a very small percentage of the cells present in these tissues, making detection of any elevation of PAO in these macrophages less likely. This could be improved by attempting to purify these macrophages, before measuring their PAO activity. The granulomas observed in the livers of vaccinated guinea-pigs also make up a very small percentage of the total liver tissue. Therefore, elevated levels of PAO activity in the granulomas will be difficult to detect for a similar reason. The use of immunohistochemical techniques could provide an answer to this problem.
Table 3.21 Polyamine Oxidase Levels in Tissues From Vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Polyamine Oxidase Activity&lt;sup&gt;a&lt;/sup&gt; (nmol substrate converted/min/g tissue)</th>
<th>Guinea-pig 1</th>
<th>Guinea-pig 2</th>
<th>Guinea-pig 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td>7.56, 9.68</td>
<td>5.04, 4.91</td>
<td>9.53, 9.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\mu = 8.62)</td>
<td>(\mu = 4.98)</td>
<td>(\mu = 9.68)</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td>4.89, 5.30, 4.24</td>
<td>4.45, 2.26, 2.22</td>
<td>2.53, 2.65, 2.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\mu = 4.81)</td>
<td>(\mu = 2.98)</td>
<td>(\mu = 2.66)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td>3.53, 3.81</td>
<td>4.41, 4.21, 6.34</td>
<td>3.16, 2.82, 2.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\mu = 3.67)</td>
<td>(\mu = 4.99)</td>
<td>(\mu = 2.87)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td>2.28, 1.95, 1.60</td>
<td>1.57, 1.50, 1.61</td>
<td>2.32, 0.99, 1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\mu = 1.94)</td>
<td>(\mu = 1.56)</td>
<td>(\mu = 1.48)</td>
</tr>
<tr>
<td>Heart</td>
<td></td>
<td>1.36, 1.32, 1.50</td>
<td>2.14, 1.54, 1.20</td>
<td>2.24, 1.55, 1.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\mu = 1.39)</td>
<td>(\mu = 1.63)</td>
<td>(\mu = 1.75)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data shown are two or three replicates of the same experiment and the mean (\(\mu\)) of those replicates.
As data for only two non-vaccinated guinea-pigs was available for kidney, a significance test could not be performed. However it can be seen from the data available for the kidney samples, that there is a degree of overlap between both populations. However, a significant difference between the two groups of animals might have been found if a greater number of animals had been used.

Vaccinated guinea-pigs were however found to have significantly higher levels of PAO in lung tissue compared with non-vaccinated animals. Alveolar macrophages were removed from the lungs of the animals tested by bronchopulmonary lavage. However, this technique does not result in the removal of all of the macrophages present in the lungs. Therefore, the elevated levels in the lung could be due to alveolar macrophages not removed by the lavage process. Again, immunohistochemical techniques could be used to confirm the findings of this study.

The lung is a major site of tuberculous infection in the guinea-pig (Brown, 1983). A previous report has shown that alveolar macrophages from guinea-pigs vaccinated under the same schedule used in the present study were mycobactericidal (O'Brien et al, 1991). Therefore, the vaccination schedule used in the present study is known to result in the generation of antitubercular immunity in the lung. The finding that PAO levels are elevated in the lungs of vaccinated guinea-pigs supports the view that polyamine oxidation is involved in the antimycobacterial defence of guinea-pig lungs. However, an increased level of PAO in the lungs of vaccinated animals would not necessarily mean a role for polyamine oxidation as an antimycobacterial defence. Elevated levels of polyamine oxidase are found in the synovial fluid of patients with rheumatoid arthritis (Ferrante et al, 1990) and the plasma of pregnant women (Morgan et al, 1983). Oxidised polyamines have
been shown to inhibit lymphocyte proliferation (Byrd et al., 1977; Allen et al., 1979) and neutrophil chemotaxis (Ferrante, 1985) and respiratory burst activity (Ferrante et al., 1986b). An anti-inflammatory role has been proposed for PAO in rheumatoid arthritis synovial fluid (Ferrante et al., 1990). Polyamine oxidase in human retroplacental serum has similarly been proposed to be involved in protection of the foetus from the maternal immune system (Morgan and Illei, 1980). It is conceivable that elevated PAO levels may function in an immunoregulatory role as well as an antitmycobacterial mechanism of macrophages.

3.2.2.3 Polyamine Oxidase Levels in Guinea-pig Alveolar Macrophages.

Elevated PAO levels have been reported in peritoneal macrophages from mice vaccinated intra-peritoneally with proteose-peptone (Morgan et al., 1980). Similarly, alveolar macrophages of rabbits vaccinated with *M. bovis* BCG had elevated levels of PAO (Morgan et al., 1980). In both of these cases, however, PAO does not appear to be specifically synthesised, as the specific activity of PAO was the same in both unactivated and activated cells of rabbits and mice.

To test the effect of vaccination on PAO activity of guinea-pig alveolar macrophages, macrophages were collected from the lungs of two non-vaccinated and one vaccinated guinea-pig, and assayed for PAO activity (Table 3.22). Macrophages from both populations appear to have similar levels of PAO activity. The small number of experiments performed was due to problems experienced in recovering sufficient macrophages to be able to detect any PAO activity. However, the possible roles of polyamine oxidation as an antitmycobacterial or immunoregulatory mechanism would appear to merit further investigation.
Table 3.22 Polyamine Oxidase Levels in Alveolar Macrophages from Vaccinated and Non-vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Guinea-pig Population</th>
<th>Polyamine Oxidase Activity (nmol converted/min/10⁷ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-vaccinated</td>
<td>0.07, 0.05</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>0.08, 0.09, 0.07</td>
</tr>
<tr>
<td></td>
<td>µ = 0.08</td>
</tr>
</tbody>
</table>

a Data are the results of a single replicate from each of two experiments
b Data are the results and mean (µ) of a single experiment performed in triplicate.
3.3 Degradation of the Amino Acid Tryptophan as an Antimicrobial Defence of Guinea-pig Macrophages.

The enzyme indoleamine 2,3-dioxygenase (IDO) is responsible for the breakdown of tryptophan to kynurenine in a wide range of tissues (Yamazaki et al, 1985). A second enzyme, tryptophan 2,3-dioxygenase is responsible for the breakdown of tryptophan to kynurenine in the liver (Taylor and Feng, 1991). Interferon-γ-induced tryptophan degradation has been shown to have an antimicrobial effect on several intracellular pathogens including Toxoplasma gondii (Pfefferkorn, 1984) and Chlamydia psittaci (Byrne et al, 1986b). Levels of IDO have also been shown to be raised by bacterial lipopolysaccharide [LPS] (Yoshida and Hayaishi, 1978) and during infection with influenza virus (Yoshida et al, 1979). It has been suggested that tryptophan degradation may be a component of the antimicrobial defences of human macrophages (Murray et al, 1989).

Previously, alveolar macrophages from BCG-vaccinated guinea-pigs have been shown to kill M. tuberculosis in an oxygen-independent manner (O'Brien and Andrew, 1991; O'Brien et al, 1991). Addition of exogenous tryptophan had no effect on the antimycobacterial or antitoxoplasmal effects of these macrophages (O'Brien, 1992). The levels of IDO in macrophages from control and vaccinated guinea-pigs were investigated in order to confirm that tryptophan degradation is not an antimycobacterial defence of macrophages from BCG-vaccinated guinea-pigs.
3.3.1 Measurement of IDO Activity in Alveolar Macrophages from Control and BCG-vaccinated Guinea-pigs.

Estimation of the IDO activity of guinea-pig macrophages was by a modification of the method of Byrne and co-workers (Byrne et al., 1986a). As described above, (section 3.2), the action of IDO on tryptophan results in the production of kynurenine (Taylor and Feng, 1991). Therefore, by measuring the degree of conversion of tryptophan to kynurenine, the IDO activity in cells can be estimated.

Samples taken from the supernatants from cultures of alveolar macrophages from vaccinated guinea-pigs show that there is a change in the distribution of the radiolabel (Fig 3.1). The figure shows the results of a single chromatogram at each of two timepoints. The initial sample displays only one peak at 15 cm from the origin, which corresponds with the position of tryptophan. However, the sample taken after eight hours of incubation has two major peaks at 15 cm and 17 cm from the origin, which correspond with the positions of tryptophan and kynurenine. This would appear to indicate that tryptophan has been converted to kynurenine, and hence that alveolar macrophages from vaccinated guinea-pigs possess IDO. A peak is also visible near the origin of the eight hour chromatogram. This peak could be due to incorporation of 14C-tryptophan into proteins which then have been secreted into the supernatant. However, this was not investigated further.
Fig 3.1 Action of Macrophages on Tryptophan.

Alveolar macrophages from vaccinated guinea-pigs were incubated with $^{14}$C-labelled tryptophan. Paper chromatography was used to separate $^{14}$C-tryptophan from its metabolites $^{14}$C-kynurenine and $^{14}$C-N-formylkynurenine. Radioactive counts per minute were determined for 1cm strips of the chromatograms by scintillation counting. A similar pattern is shown in results from control animals.
Fig 3.1 Action of Macrophages on Tryptophan

Radioactive Counts per Minute (CPM)

Fraction Number

0 Hours

8 Hours
The positions of known samples of tryptophan and kynurenine on the chromatogram were determined by looking at the chromatograms under ultra-violet light. By comparison, this allowed the position of $^{14}$C-tryptophan and $^{14}$C-kynurenine in culture supernatants to be determined. The distribution of the radiolabel after incubation for various periods of time with alveolar macrophages from two vaccinated guinea-pigs is shown in Table 3.23.

For animal 1, there were found to be significant reductions in the amount of $^{14}$C-tryptophan present in the supernatant at the two (P < 0.001), three (P < 0.01), four (P < 0.001), six (P < 0.002) and eight (P < 0.01) hour timepoints, compared with the initial timepoint. However, this was not accompanied by a concomitant increase in the levels of $^{14}$C-kynurenine present in the supernatant at any of the sampled timepoints. Indeed, the level of kynurenine was found to be significantly (P < 0.02) reduced at the six hour timepoint. It would therefore appear that in the case of macrophages from animal 1, tryptophan is being taken up by the macrophages, but kynurenine is not released into the supernatant. In fact, the results of the six hour timepoint suggest that kynurenine is also being taken up by the macrophages.

The results from an identical experiment with a second animal (animal 2) contrast with those found with animal 1. In these experiments, a significant decrease in the level of $^{14}$C-tryptophan in the supernatants of alveolar macrophages from vaccinated guinea-pigs was not observed at any of the timepoints (Table 3.23). This contrasts with the findings with animal 1, where a significant decrease in the level of tryptophan present in the supernatant was found at each of the sampled timepoints. For the experiment with animal 2, the level of kynurenine in the supernatant was found to be significantly increased after eight hours of incubation (P < 0.01). This again contrasts with the findings of the experiment with animal 1, where no such
Table 3.23 Distribution of Radiolabel in Supernatants of Alveolar Macrophages from Vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Radiolabel Present as Tryptophan and Kynurenine (CPM)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal 1</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
</tr>
<tr>
<td>0</td>
<td>1814 ± 202</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>994 ± 109</td>
</tr>
<tr>
<td>3</td>
<td>1209 ± 81</td>
</tr>
<tr>
<td>4</td>
<td>900 ± 119</td>
</tr>
<tr>
<td>6</td>
<td>875 ± 37</td>
</tr>
<tr>
<td>8</td>
<td>1232 ± 78</td>
</tr>
</tbody>
</table>

ᵃ = Data are the mean ± standard deviation of three or four replicates.

ND = Not done.
increase was observed. The increased level of $^{14}$C-kynurenine in the supernatants of alveolar macrophages from vaccinated guinea-pig 2, suggest that $^{14}$C-tryptophan has been converted to $^{14}$C-kynurenine by the action of IDO.

A more accurate method would be to compare the proportions of the total radioactivity in the supernatants due to $^{14}$C-tryptophan and $^{14}$C-kynurenine rather than the actual counts per minute. This is similar to the measurement of tryptophan degradation used by Paguirigan and co-workers (Paguirigan et al, 1994) Therefore, the proportions of the total radioactivity due to $^{14}$C-tryptophan and $^{14}$C-kynurenine were calculated. These results are shown in Table 3.24.

For animal 1, there were found to be a significant decrease in the proportions of the total radioactivity due to $^{14}$C-tryptophan after four (P < 0.01) and eight (P < 0.01) hours. There is, however, no significant change in the proportion of the total radioactivity in the supernatant which is present as kynurenine, at any of the timepoints. Despite there being a decrease in the proportion of the radiolabel present as $^{14}$C-tryptophan, there is no evidence for the formation of $^{14}$C-kynurenine. Reduction in the proportion of the radiolabel present as $^{14}$C-tryptophan alone, does not constitute evidence for IDO activity. An increase in the proportion of the radiolabel due to $^{14}$C-kynurenine can only be due to IDO activity. Therefore, for this animal, there is no evidence for any IDO activity. This suggests that the decrease in the proportion of radioactivity due to $^{14}$C-tryptophan after 4 and 8 hours is due to uptake of $^{14}$C-tryptophan by the macrophages.

A slightly different picture was found with macrophages from animal 2. After eight hours of incubation, there was a significant (P < 0.001) decrease in the percentage of $^{14}$C-tryptophan in the supernatants from cultures of macrophages from animal 2. There was also a significant (P < 0.002) increase in the percentage of the
Table 3.24 Degradation of Tryptophan by Alveolar Macrophages from Vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Percentage of Radiolabel Due to Tryptophan and Kynurenine*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Animal 1</td>
</tr>
<tr>
<td></td>
<td>Tryptophan</td>
</tr>
<tr>
<td>0</td>
<td>69.7 ± 2.6</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>57.7 ± 6.3</td>
</tr>
<tr>
<td>3</td>
<td>66.4 ± 2.1</td>
</tr>
<tr>
<td>4</td>
<td>61.25 ± 2.2</td>
</tr>
<tr>
<td>6</td>
<td>64.9 ± 5.2</td>
</tr>
<tr>
<td>8</td>
<td>56.7 ± 4.3</td>
</tr>
</tbody>
</table>

a = Data are the mean ± standard deviation of three or four replicates.

ND = Not done.
radiolabel present as $^{14}$C-kynurenine in the supernatants of these macrophages, at the eight hour timepoint. This decrease in the level of $^{14}$C-tryptophan accompanied by an increase in the level of $^{14}$C-kynurenine constitutes evidence for IDO activity in the macrophages of this animal. Neither a significant increase in the percentage of the radiolabel present as $^{14}$C-kynurenine or a significant decrease in the percentage radiolabel present as $^{14}$C-tryptophan was observed after one or two hours of incubation.

Alveolar macrophages from guinea-pigs which had not been vaccinated were also assayed for IDO activity using the same method. The results of these experiments are shown in Table 3.25.

For animal 1, the levels of $^{14}$C-tryptophan present at each of the timepoints sampled were not found to be significantly different to those present at the start of the experiment. However, the level of $^{14}$C-kynurenine was found to be significantly higher ($P < 0.01$) in the sample taken after eight hours when compared with the initial sample. The sample taken after one hour of incubation was also found to have a significantly higher ($P < 0.02$) level of $^{14}$C-kynurenine in comparison with the initial sample. However, the levels of $^{14}$C-kynurenine in the two and three hour samples were not significantly different to that in the initial sample. The increased level of $^{14}$C-kynurenine in the supernatant, despite the lack of an observable decrease in the level of $^{14}$C-tryptophan, suggests that these macrophages possess IDO activity.

For the second control animal, no data were available for the eight hour timepoint. Also, only two replicates were available for the initial timepoint. There was no significant increase in level of $^{14}$C-tryptophan in the supernatant of these macrophages at any of the three tested timepoints. Similarly, no significant increase in the level of $^{14}$C-kynurenine was observed at any of the three sampled timepoints.
Table 3.25 Distribution of Radiolabel in Supernatants of Alveolar Macrophages from Non-vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Radiolabel Present as Tryptophan and Kynurenine (CPM)*</th>
<th>Animal 1</th>
<th>Animal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tryptophan</td>
<td>Kynurenine</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>0</td>
<td>1900 ± 252</td>
<td>402 ± 37</td>
<td>1883.9b</td>
</tr>
<tr>
<td>1</td>
<td>1580 ± 127</td>
<td>661 ± 91</td>
<td>1557 ± 92</td>
</tr>
<tr>
<td>2</td>
<td>1692 ± 145</td>
<td>384 ± 60</td>
<td>1396 ± 193</td>
</tr>
<tr>
<td>3</td>
<td>1529 ± 150</td>
<td>425 ± 63</td>
<td>1360 ± 220</td>
</tr>
<tr>
<td>8</td>
<td>1816 ± 290</td>
<td>1027 ± 229</td>
<td>ND</td>
</tr>
</tbody>
</table>

*a = Data are the mean ± standard deviation of three or four replicates except where stated.

b = Data are the mean of two replicates.

ND = Not done.
The proportions of $^{14}$C-tryptophan and $^{14}$C-kynurenine in the supernatants of macrophages from control guinea-pigs at the various timepoints are shown in Table 3.26. Analysis of the data from animal 1 found that the proportion of tryptophan in the supernatant was significantly reduced after one ($P < 0.05$), three ($P < 0.05$) and eight hours ($P < 0.01$), compared with the initial sample. The sample taken after two hours of incubation was not found to have a significantly different proportion of the radiolabel present as tryptophan. Therefore, it would appear that tryptophan has been preferentially removed from the supernatant by the macrophages. It is possible that the tryptophan has either been broken down to kynurenine by the enzyme IDO, or it has been stored within the macrophages.

Comparison of the proportions of $^{14}$C-kynurenine in the supernatants of macrophages from animal 1 at the various timepoints found that the proportion of kynurenine in the supernatant was significantly increased after one ($P < 0.02$) and eight ($P < 0.001$) hours of incubation. The increased levels of $^{14}$C-kynurenine in the supernatants suggest that $^{14}$C-tryptophan has been broken down by IDO to form $^{14}$C-kynurenine. Analysis of the data for animal 2 showed that the proportion of the radioactivity due to $^{14}$C-tryptophan was significantly decreased after one ($P < 0.02$), two ($P < 0.05$) and three ($P < 0.01$) hours, compared with the initial sample. The proportion of the total radioactivity accounted for by $^{14}$C-kynurenine was found to be significantly increased after one ($P < 0.01$), two ($P < 0.05$) and three ($P < 0.05$) hours, compared with the initial sample. Taken together, the results from the experiments with animal 2 suggest that tryptophan has been degraded by the enzyme IDO to form kynurenine. Thus it would appear that IDO activity can be demonstrated in non-vaccinated guinea-pigs as well as vaccinated guinea-pigs.
Table 3.26 Degradation of Tryptophan by Alveolar Macrophages from Non-vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Percentage of Radiolabel Due to Tryptophan and Kynurenine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Animal 1</th>
<th>Animal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tryptophan</td>
<td>Kynurenine</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>0</td>
<td>71.9 ± 1.3</td>
<td>15.5 ± 2.4</td>
<td>76.93&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>62.2 ± 3.6</td>
<td>26.1 ± 3.5</td>
<td>69.7 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>70.3 ± 1.9</td>
<td>15.9 ± 1.1</td>
<td>61.8 ± 5.5</td>
</tr>
<tr>
<td>3</td>
<td>65.9 ± 3.0</td>
<td>18.4 ± 4.3</td>
<td>61.4 ± 3.0</td>
</tr>
<tr>
<td>8</td>
<td>57.2 ± 5.2</td>
<td>30.0 ± 5.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are the mean ± standard deviation of three or four replicates except where stated.

<sup>b</sup> Data are the mean of two replicates.

ND = Not done.
If degradation of tryptophan is an antimycobacterial defence of alveolar macrophages from VBC guinea-pigs, then it would be expected that levels of IDO would be higher in these macrophages than in similar macrophages from control animals. Reduction in the level of \(^{14}\)C-tryptophan in the supernatant is not an accurate measure of IDO activity as uptake of tryptophan into acid-soluble or acid-insoluble intracellular pools within the macrophage may also account for the disappearance of \(^{14}\)C-tryptophan from the medium. However, measurement of the proportion of the radiolabel present as kynurenine, which is formed from tryptophan by IDO, will infer the level of IDO in the macrophages. No significant increase in the level of \(^{14}\)C-kynurenine was observed in the supernatant of macrophages from vaccinated animal 1. However, the proportion of the radioactivity in the supernatant of the macrophages from vaccinated animal 2 was found to increase by 100% over the eight hour incubation period. Over the eight hour incubation period, the proportion of the radiolabel present as kynurenine in the supernatant of macrophages from control animal 1 was found to be increased by 95%. No data was available for the eight hour timepoint of control animal 2.

The uptake of \(^{14}\)C-labelled tryptophan by macrophages from VBC guinea-pigs into acid-soluble and acid-insoluble intracellular pools was also investigated (Table 3.27). Radiolabelled tryptophan was found to accumulate in both acid-soluble and acid-insoluble intracellular pools. The amount of radiolabel found to accumulate inside acid-soluble and acid-insoluble pools of macrophages after eight hours was found to be up to 20% of that found in the supernatant at the initial timepoint. Therefore accumulation of tryptophan in acid-soluble and acid-insoluble intracellular pools could account for a substantial proportion of the \(^{14}\)C-tryptophan which is observed to be removed from the supernatant during the course of the experiment.
Table 3.27 Uptake of Radiolabelled Tryptophan Into Acid-soluble and Acid-insoluble Intracellular Pools by Alveolar Macrophages from Vaccinated Guinea-pigs.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>Radioactive counts per minutea</th>
<th>Animal 1</th>
<th>Animal 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acid-soluble</td>
<td>Acid-insoluble</td>
<td>Acid-soluble</td>
</tr>
<tr>
<td>0</td>
<td>42 ± 13</td>
<td>21 ± 6</td>
<td>55 ± 22</td>
</tr>
<tr>
<td>2</td>
<td>140 ± 50</td>
<td>78 ± 7</td>
<td>239b</td>
</tr>
<tr>
<td>8</td>
<td>200 ± 21</td>
<td>254 ± 91</td>
<td>190 ± 15</td>
</tr>
</tbody>
</table>

a = Data are the mean ± the standard deviation of three or four replicates except where noted

b = Data are the mean of two replicates
(Table 3.23 and Table 3.25). Thus the decrease in $^{14}$C-tryptophan in the supernatant may, in part, be due to incorporation into acid-soluble and acid insoluble material rather than degradation of tryptophan to kynurenine. However, this does not explain the increase in kynurenine observed. The results in Table 3.23 show that macrophages from VBC guinea-pigs take up more than 50% of the radiolabel present in the supernatant. However, no evidence was found for this level of radiolabel in the acid-soluble and acid-insoluble intracellular pools of the macrophages. In summary, uptake of tryptophan into acid-soluble and acid-insoluble intracellular pools occurs in macrophages from VBC guinea-pigs. However, the extent and rate of uptake into these pools is not sufficient to account for the decrease in tryptophan found previously (Table 3.23 and Table 3.25).

Previous research has found transport of tryptophan into acid-soluble intracellular pools was greatly increased in cells treated with IFN-$\gamma$ (Byrne et al., 1986b). The ability of macrophages from control guinea-pigs to transport $^{14}$C-tryptophan into acid-soluble and acid-insoluble intracellular pools was not determined.

In this study, no evidence was found for increased levels of IDO activity in macrophages from VBC guinea-pigs compared with non-vaccinated animals. However, the number of animals studied was very small and macrophages from only one of the vaccinated guinea-pigs showing any measurable IDO activity at all. Previously, addition of exogenous tryptophan to the supernatants of macrophages, failed to reverse the antimycobacterial or the antitoxoplasmal effects of these macrophages (O'Brien, 1992). Starvation of the amino acid tryptophan had previously been shown not to be involved in the antimicrobial response of murine macrophages (Murray et al., 1989; Gebran et al., 1994). This study therefore confirms
the view that degradation of the amino acid tryptophan is not involved in the antimycobacterial defence of alveolar macrophages from VBC guinea-pigs. Considering that *M. tuberculosis* is not auxotrophic for tryptophan, (Grange, 1976) this conclusion is not surprising.

3.4 Hydrophobicity as a Potential Virulence Factor of Mycobacteria.

Tuberculosis starts with the phagocytosis of *M. tuberculosis* by alveolar macrophages. The precise mechanism by which the mycobacteria are taken up by human macrophages remains unclear (section 1.6.3). It is known that uptake via complement receptors avoids the triggering of the respiratory burst activity of the macrophage (Schlesinger *et al*, 1990), thus possibly affecting the intracellular fate of the mycobacteria.

It has previously been found that there is an inverse correlation between the virulence of strains of *M. tuberculosis*, and their binding to the surface of alveolar macrophages from vaccinated guinea-pigs (O'Brien *et al*, 1991). That is, strains with a high root index of virulence (RIV) were taken up to a lesser extent than strains with a lower RIV. No difference was found in the uptake of the strains by alveolar macrophages from non-vaccinated guinea-pigs, (O'Brien *et al*, 1991). One possible explanation is that virulent strains have different surface characteristics, which affects their uptake by activated macrophages.

A study of pigmented and non-pigmented strains of *M. avium* has found that the pigmented (more virulent) strains are more hydrophobic than the non-pigmented (less virulent) strains (Stormer and Falkinham, 1989). It was therefore decided to investigate the hydrophobicity of mycobacterial strains of different RIV. Where
possible, the strains investigated were the same as those used by O'Brien and co-workers (O'Brien et al, 1991).

3.4.1 Measurement of the Hydrophobicity of Mycobacterial Strains.

The hydrophobicity of eight strains of *M. tuberculosis* and one strain of *M. bovis* was determined by a modification of the method of Stormer and Falkinham (Stormer and Falkinham, 1989). This method is based on measurement of relative partition of mycobacteria into hexadecane and aqueous phases. The results of these experiments are shown in Table 3.28.

If the hydrophobicity of mycobacterial strains significantly contributes to their virulence in the guinea-pig, there would be a correlation between the virulence of the strains and their hydrophobicity. Comparing the results obtained from the four experiments it can be seen that the hydrophobicity of each strain, expressed as the percentage of the cells partitioning into the hexadecane, varies between the four experiments. For example, strains 79112 and B1453 were found to have low hydrophobicity in experiment 2 (33% and 43% partitioning into hexadecane respectively). However, both strains were found to have very high hydrophobicity in experiment 4 (100% adherence to hexadecane).

Comparison of the strains I2646 and 79500 shows that any difference in hydrophobicity between two strains was not constant throughout the four experiments. For example, in experiment 2 both strains showed similar degrees of hydrophobicity; 46% adherence for strain I2646 and 50% for strain 79500. Strain 79500 was found to have a similar degree of hydrophobicity in experiment 3 (48%
Table 3.28 Measurement of the Hydrophobicity of Mycobacterial Strains.

<table>
<thead>
<tr>
<th>Mycobacterial Strain</th>
<th>RIV</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. bovis</em> 81470</td>
<td>1.29</td>
<td>64</td>
<td>ND</td>
<td>80</td>
<td>75</td>
</tr>
<tr>
<td>79499</td>
<td>1.29</td>
<td>42</td>
<td>28</td>
<td>22</td>
<td>66</td>
</tr>
<tr>
<td>I2646</td>
<td>1.17</td>
<td>45</td>
<td>46</td>
<td>93</td>
<td>72</td>
</tr>
<tr>
<td>H37Rv</td>
<td>1.01</td>
<td>34</td>
<td>62</td>
<td>72</td>
<td>91</td>
</tr>
<tr>
<td>79500</td>
<td>0.98</td>
<td>64</td>
<td>50</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>79112</td>
<td>0.92</td>
<td>75</td>
<td>33</td>
<td>46</td>
<td>100</td>
</tr>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>64</td>
<td>43</td>
<td>51</td>
<td>100</td>
</tr>
<tr>
<td>H37Ra</td>
<td>0.52</td>
<td>30</td>
<td>60</td>
<td>35</td>
<td>98</td>
</tr>
<tr>
<td>H37RaHR</td>
<td>0.12</td>
<td>71</td>
<td>54</td>
<td>90</td>
<td>95</td>
</tr>
</tbody>
</table>

ND = Not Done
adherence), whereas strain 12646 was found to have a much higher adherence to hexadecane (93%). Similarly, strain H$_{37}$Ra has a very low degree of hydrophobicity in experiments 1 and 3 (30% adherence and 35% adherence respectively), but a very high degree of hydrophobicity in experiment 4.

From Table 3.28 it can be seen that each of the strains tested showed a high degree of variation in hydrophobicity within the four experiments. For this reason, a mean of the four experiments was not calculated. Regression analysis of the four experiments individually, found no significant correlation (P>0.05) between virulence and adherence to hexadecane in any of the four experiments. Therefore, there is no evidence from this study that hydrophobicity is a factor in determining the extent of the uptake by macrophages. Although no correlation between RIV and hydrophobicity was found and a high degree of variability was observed in the experiments, the highly virulent strain 79499 was found to be consistently hydrophilic while the strain with the lowest RIV (H$_{37}$RaHR) was consistently among the most hydrophobic strains. This is the opposite of that found by Stormer and Falkingham (Stormer and Falkingham, 1989) where virulent strains of M. avium were found to have a greater degree of hydrophobicity than avirulent strains.

The variation in percentage adherence to hexadecane shown by each of the strains indicates that hydrophobicity is not constant when mycobacteria are grown in liquid media. Different liquid media were not used as it was intended to compare the findings of the current experiment with previous work showing a link between RIV and adherence of mycobacteria to activated macrophages (O'Brien et al, 1991). Attempts were made to compare the results obtained after growth in liquid media with hydrophobicity measurements of the same strains grown on solid media.
However, sufficient mycobacteria could not be recovered after growth on solid media for four weeks.

It is also possible that the variability in hydrophobicity is due to the relatively crude nature of the assay. However, the assay has been used previously to show differences in hydrophobicity between two strains of *M. avium* (Stormer and Falkingham, 1989).

### 3.5 Investigation of the Production of Autoregulatory Factors by Mycobacteria.

It is known that many bacteria produce small, diffusible molecules which accumulate in culture supernatants and activate expression of specific genes on reaching a certain concentration (section 1.6.1). In several if these cases, the genes which are activated by this means are involved in controlling the synthesis of virulence factors (Gambello and Iglewski, 1991; Toder *et al.*, 1991; Gambello *et al.*, 1993; Jones *et al.*, 1993; Pirhonen *et al.*, 1993). Furthermore, autoinducers are also found in the genus *Streptomyces* (Miyake *et al.*, 1989; Miyake *et al.*, 1990), a close relative of the mycobacteria. However, the autoinducers found in the genus *Streptomyces* differ structurally from those found in Gram-negative bacteria (Horinuchi and Beppu, 1992). It was decided, therefore, to look for evidence of autoinducible factors in mycobacterial culture supernatants.

#### 3.5.1 Analysis of Mycobacterial Culture Supernatants for the Presence of Autoinducers.

Autoinducers produced by bacteria are secreted into the culture medium (Swift *et al.*, 1993). Bacteria have been analysed for production of autoinducers by adding the culture supernatants to a recombinant strain of *E. coli* which contains the
lux RAB genes and acts as a reporter system for homoserine lactone-like molecules in the presence of long-chain aldehyde (Bainton et al, 1992a, 1992b; Swift et al, 1993). It was decided to investigate whether there was evidence of autoinducers in mycobacterial culture supernatants.

Spent culture supernatants from *Pseudomonas aeruginosa*, which are known to produce autoinducers detectable by this method (Bainton et al, 1992a), were investigated for the presence of autoinducer. Culture supernatant from *M. tuberculosis* was similarly investigated. The results of these experiments are shown in Table 3.29.

Addition of supernatant from any of the three bacteria used to the sensor bacteria resulted in increased bioluminescence within 30 minutes of incubation. There appears to be a great deal of variation between each of these experiments. Therefore, the first value was used to calculate the increase in bioluminescence.

Addition of supernatant from *M. tuberculosis* had the greatest effect upon the production of bioluminescence, bringing about an approximately twenty seven-fold increase. This was surprising as the detection system was designed to detect HSL-like molecules produced by Gram-negative bacteria. Bioluminescence was found to be increased approximately three-fold by the addition of supernatants from *Ps. aeruginosa*. A previous study which used a similar reporter system to identify autoinducer in the supernatant of Gram-negative bacteria (Swift et al, 1993) found that there was an approximately two-log increase in bioluminescence in the presence of culture supernatants.

To determine whether the induction of bioluminescence was due to a product of *M. tuberculosis* or whether it was due to a normal component of 7H9 medium in which the *M. tuberculosis* was grown, supernatant samples were taken from
Table 3.29 Induction of Bioluminescence by Bacterial Culture Supernatants.

<table>
<thead>
<tr>
<th>Bacterial Culture Supernatant</th>
<th>Bioluminescence* after incubation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Minutes</td>
</tr>
<tr>
<td><strong>M. tuberculosis</strong></td>
<td>0.45, 0.59, 0.55</td>
</tr>
<tr>
<td><strong>Ps. aeruginosa</strong></td>
<td>1.78, 0.72, 0.76</td>
</tr>
</tbody>
</table>

* Bioluminescence was measured in relative light units (RLU)
mycobacterial cultures over a period of seven days. Bacterial production of autoinducer has been found to be greatest during the late log and early stationary phases of growth (Swift et al, 1993). The mycobacterial supernatant samples were compared with 7H9 medium for their ability to induce bioluminescence in the sensor bacteria (Table 3.30 and Fig. 3.2).

No induction of bioluminescence was observed with any of the culture supernatants or the medium control after 0 minutes of incubation. However, all of the tested samples, including the medium control, were able to induce bioluminescence to some degree after 30 and 60 minutes of incubation. The data displayed in Table 3.30 are from three separate experiments. Again, the first value was considered to be the truest representation of the level of bioluminescence. Despite some variation, a pattern is still observable with the values for the three experiments. The highest levels of bioluminescence were produced by the addition of supernatants from 2 and 3 day old cultures of M. tuberculosis. Comparing these data with estimates of the viable cell count shows that the supernatants which induced the greatest bioluminescence came from cultures in late log phase (Fig 3.1). The level of bioluminescence induced by supernatants from cultures in late log phase was five to six times that induced by medium alone. Cultures taken later, during stationary phase show decreased induction of bioluminescence.

The pattern of induction of bioluminescence by supernatants from M. tuberculosis appears to be cell density-dependent. The cell density-dependent induction of bioluminescence is similar to that observed in P. fischeri, which is mediated by the autoinducer N-(3-oxohexanoyl)-L-homoserine lactone (Eberhard et al, 1981). It is possible, therefore, that the induction of bioluminescence by mycobacterial supernatant is due to an autoinducer similar to that found in P.
Table 3.30  Induction of Bioluminescence by Mycobacterial Culture

Supernatants from *M. tuberculosis* I2646.

<table>
<thead>
<tr>
<th>Day Number</th>
<th>Bioluminescence* after incubation for:</th>
<th>Cell Density (cells/ml⁻¹ x 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Minutes</td>
<td>30 Minutes</td>
</tr>
<tr>
<td>0</td>
<td>0.35, 0.35, 0.31</td>
<td>4.49, 3.25, 3.01</td>
</tr>
<tr>
<td>1</td>
<td>0.40, 0.33, 0.15</td>
<td>5.49, 4.79, 3.03</td>
</tr>
<tr>
<td>2</td>
<td>0.28, 0.19, 0.25</td>
<td>7.96, 5.07, 5.40</td>
</tr>
<tr>
<td>3</td>
<td>0.63, 0.33, 0.40</td>
<td>7.65, 3.95, 5.05</td>
</tr>
<tr>
<td>4</td>
<td>0.58, 0.19, 0.40</td>
<td>5.73, ND, 4.16</td>
</tr>
<tr>
<td>5</td>
<td>0.45, 0.41, 0.29</td>
<td>4.45, 4.02, 4.48</td>
</tr>
<tr>
<td>6</td>
<td>0.49, 0.26, 0.31</td>
<td>3.34, 3.06, 3.20</td>
</tr>
<tr>
<td>7</td>
<td>0.25, 0.56, 0.31</td>
<td>3.06, 3.28, 2.75</td>
</tr>
<tr>
<td>Medium only</td>
<td>0.38, 0.45, 0.46</td>
<td>2.89, 4.08, 4.85</td>
</tr>
</tbody>
</table>

ND = Not determined

* Bioluminescence was measured in relative light units (RLU)
Fig 3.2 Induction of Bioluminescence by Mycobacterial Culture Supernatants
Cell density-dependent patterns of induction similar to that observed in *P. fischeri* have also been observed in several species of Gram-negative enteric bacteria (Swift *et al.*, 1993). The finding that induction of bioluminescence by culture supernatants from *M. tuberculosis* corresponded with increased cell density was surprising as this pattern has only previously been observed in Gram-negative bacteria.

The increase in bioluminescence induced by supernatants from cultures in late log phase is still low in comparison to that observed previously with other bacteria (Swift *et al.*, 1993). However, studies using another reporter system and the *P. fischeri* autoinducer found only a three to five-fold increase in bioluminescence in the presence of autoinducer (Shadel and Baldwin, 1992a, 1992b). The studies of Shadel and Baldwin used the *P. fischeri* autoinducer (N-(3-oxohexanoyl)-L-homoserine lactone) and the *P. fischeri lux* operon (Shadel and Baldwin, 1992a, 1992b). This is the same system used by *P. fischeri* to regulate bioluminescence *in vivo*.

Culture supernatants from a range of strains of *M. tuberculosis* and other slow-growing mycobacterial species were compared for autoinducer activity (Table 3.31). The supernatants were obtained from three day old cultures as this represented the peak of autoinducer activity measured previously (Table 3.30).

Incubation of the sensor bacteria with mycobacterial culture supernatants resulted in increased bioluminescence. However, the increase in bioluminescence was similar in all cases with that observed when sensor bacteria were incubated in the presence of 7H9 medium in which mycobacteria had not been grown. In the case of *M. tuberculosis* strain I2646, the increase in bioluminescence was much lower than that observed in the previous experiment (Table 3.30). The fact that the
Table 3.31 Induction of Bioluminescence by Supernatants from Cultures of Various Mycobacteria.

<table>
<thead>
<tr>
<th>Bacterial Culture Supernatant</th>
<th>Bioluminescence* after incubation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 minutes</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79499</td>
<td>1.54, 1.37, 0.93</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> I2646</td>
<td>1.41, 1.01, 1.74</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>1.04, 0.89, 2.25</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79500</td>
<td>1.74, 1.57, 1.46</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79112</td>
<td>1.91, 2.13, 1.88</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> B1453</td>
<td>1.27, 1.21, 1.64</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Ra</td>
<td>0.90, 1.43, 0.99</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37RaHR</td>
<td>0.78, 1.35, 1.40</td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td>1.70, 1.44, 1.31</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>0.84, 1.57, 0.72</td>
</tr>
<tr>
<td><em>Mycobacterium microti</em></td>
<td>0.19, 1.45, 1.43</td>
</tr>
<tr>
<td>Medium control</td>
<td>0.96, 0.81, 1.00</td>
</tr>
</tbody>
</table>

* Bioluminescence was measured in relative light units (RLU)
increase in bioluminescence after incubation with mycobacterial culture supernatant was of a similar magnitude to that observed with 7H9 medium alone would suggest that no autoinducer was present in any of the mycobacterial culture supernatants. Previously a difference had been found between bioluminescence induced by culture supernatant from \textit{M. tuberculosis} 12646 and that induced by 7H9 medium (Table 3.30).

3.5.2 Attempts to Isolate and Characterise a \textit{lux I} Homologue from \textit{Mycobacterium smegmatis}.

In order to clarify whether mycobacteria produce an autoinducer similar to that found in several species of Gram-negative bacteria, it was decided to screen mycobacterial DNA for genes homologous in function to \textit{lux I}. A library of \textit{M. tuberculosis} DNA was not available, therefore a library of \textit{M. smegmatis} DNA was screened for the presence of \textit{lux I} homologues.

The method of Swift and co-workers (Swift \textit{et al}, 1993) was used to identify potential \textit{lux I} homologues from a library of \textit{M. smegmatis} DNA constructed in pTTQ18 (Gordon, 1995). The library was screened for homologues of \textit{lux I} using \textit{E. coli} strain JM 109, containing the plasmid pSB315. This plasmid was constructed by Swift and co-workers (Swift \textit{et al}, 1993) by cloning the \textit{P. fischeri lux RAB} genes into the \textit{EcoRV} site of plasmid pACYC184, which confers chloramphenicol resistance. Electrocompetent \textit{E. coli} (pSB315) were electroporated with the \textit{M. smegmatis} library, which confers ampicillin resistance (Stark, 1977). Therefore, transformants resistant to both chloramphenicol and ampicillin were screened for bioluminescence in the presence of decanal, the assumption being that bioluminescent colonies contained potential \textit{lux I} homologues from the \textit{M. smegmatis} library.
Two transformants out of five thousand screened resistant to both chloramphenicol and ampicillin were identified as bioluminescent (Fig. 3.3). The bioluminescent transformants contained a sensor plasmid as well as the plasmid from the mycobacterial library. Therefore, before the plasmid containing the mycobacterial DNA could be purified, it was necessary to select for bacteria which contained the plasmid from the library of M. smegmatis DNA but not the sensor plasmid. The bacteria were grown in five changes of LB medium containing ampicillin, with daily sub-culture, over 5 days. For each transformant, colonies which were resistant to ampicillin, but not resistant to chloramphenicol, were selected. Thus, the sensor plasmid was removed whilst the plasmids containing M. smegmatis DNA were retained. The plasmids containing fragments of M. smegmatis DNA were purified and named pBJR1 and pBJR2. Both inserts were found to be approximately four kbp in size. This was expected as the library was made from DNA approximately three to four kbp in size. Each plasmid was then used to re-transform the sensor strain E. coli (pSB315). Transformation with either pBJR1 or pBJR2 produced transformants which were all ampicillin resistant, chloramphenicol resistant and bioluminescent in the presence of decanal.

In order to determine whether the two cloned fragments had any homology to each other, the fragments were compared by DNA hybridisation.

The restriction enzymes Xba I and Sst I cut the plasmid pBJR1 on either side of the insert, but did not cut within the insert. Therefore, the insert was removed by digestion of plasmid pBJR1 with Xba I and Sst I, and purified as described previously (section 2.14.7). This fragment was labelled with [α\(^{32}\)P] dCTP and used to probe the plasmids pBJR1, pBJR2 and pTTQ18 (Fig. 3.4). The fragment was found to bind only to the positive control, pBJR1 and not pBJR2 or pTTQ18. The
Fig. 3.3 Identification of Bioluminescent Clones from a Library of *M. smegmatis* mc²155 in *E. coli* (pSB315).

Bioluminescent transformants were identified by placing plates of media containing colonies derived from individual transformants onto undeveloped photographic film. The colonies were supplied with decanal and left on the photographic film for 15 minutes after which time the film was developed.

Of 8 colonies tested, two were found to be bioluminescent.
Fig 3.4 Southern Hybridisation of \textit{Xba I/}Sst I Fragment of pBJR1 to pBJR2 and pTTQ18.
mycobacterial DNA inserts from plasmids pBJR1 and pBJR2 do not appear to be related to each other. It was decided to investigate the insert from pBJR1 before looking at the insert in pBJR2.

In order to confirm that the cloned fragment of DNA contained in pBJR1 had originated from *M. smegmatis*, the Xba I/Sst I fragment from pBJR1 was used to probe genomic DNA from *M. smegmatis* and *E. coli* (Fig. 3.5). This probe bound to the *M. smegmatis* DNA, but did not bind to the *E. coli* DNA.

Having found that the insert in plasmid pBJR1 originated from *M. smegmatis*, it was decided to investigate whether the insert bound to genomic DNA from *M. tuberculosis* 12646 (Fig 3.5). The radiolabelled Xba I/Sst I fragment from plasmid pBJR1 was found to bind to a fragment of genomic DNA from *M. tuberculosis* 12646 cut with BamHI of approximately 3 kbp in size. Therefore, the fragment of DNA contained in the Xba I/Sst I insert appears to be conserved to some degree between species of mycobacteria. If the Xba I/Sst I fragment from pBJR1 codes for an autoinducer synthase, then both fast and slow-growing mycobacteria would appear to possess this gene.

In order to further study the insert contained in pBJR1, the plasmid was digested with restriction enzymes known to have a single site within the parental plasmid pTTQ18. By analysing fragments of the mycobacterial insert it was hoped to identify more specifically that region of the insert responsible for inducing bioluminescence when cloned into *E. coli* (pSB315). Digestion of pBJR1 with the restriction endonuclease Pst I produced three fragments of approximately 5.5, 1.8 and 0.8 kbp (Fig. 3.6). The 1.8 and 0.8 kbp fragments were purified and each was cloned into the Pst I site of plasmid pTTQ18. These plasmids were named pBJR3 (1.8 kbp insert) and pBJR4 (0.8 kbp insert).
Fig. 3.5 Southern Hybridisation of Xba I/Sst I Fragment of pBJR1 to Chromosomal DNA from *E. coli* and *M. smegmatis*.

Lane 1 Blank
Lane 2 Kb ladder
Lane 3 *E. coli* genomic DNA digested with Bam HI
Lane 4 *M. smegmatis* genomic DNA digested with Bam HI
Lane 5 pBJR1 (positive control)
Fig 3.6 Digestion of Plasmid pBJR1 with the Restriction Endonuclease Pst I.

- 1.8 kb → fragment
- 0.8 kb → fragment
Each of these plasmids was used to transform *E. coli* (pSB315). For each plasmid, transformants resistant to both chloramphenicol and ampicillin were selected by growth on medium containing both antibiotics. These transformants were then screened for bioluminescence in the presence of long-chain aldehyde. Transformation of *E. coli* (pSB315) with either pBJR3 or pBJR4 resulted in colonies which were resistant to both ampicillin and chloramphenicol, and bioluminescent in the presence of decanal (Fig 3.7). The level of bioluminescence was similar with *E. coli* (pSB315) transformed with either of the plasmids.

The 1.8 kbp fragment from pBJR3 was resistant to further digestion by the restriction enzyme *Pst* I. Therefore the 0.8 kbp fragment could not have been derived from the 1.8 kbp fragment. The 1.8 kbp fragment and the 0.8 kbp fragment must have been derived from separate parts of the mycobacterial insert of pBJR1. When *E. coli* (pSB315) is transformed with either pBJR3 or pBJR4, the resulting transformants are all bioluminescent in the presence of long-chain aldehyde. By digesting the insert in pBJR1 it was hoped to identify that region of the insert which induces a bioluminescent phenotype in the sensor strain. However, as both restriction fragments induce bioluminescence in the sensor strain it would appear that both contain potential *lux* I homologues.

The marine bacterium *V. harveyi* has been shown to possess more than one gene responsible for the production of an autoinducer (Bassler *et al*, 1994). However, these genes code for different autoinducers, which control separate sensory pathways (Bassler *et al*, 1994). It therefore seems unlikely that both the 1.8 and the 0.8 kbp *Pst* I fragments from pBJR1 could contain a gene responsible for the synthesis of autoinducer. It is probable that one or both of the plasmids, pBJR3 and pBJR4, has given a false positive result. The technique of screening a DNA
Fig 3.7 Assay of Plasmids pBJR3 and pBJR4 for the Presence of a *lux* I Homologue.
library with a sensor strain has given false positive results with other bacteria (personal communication from J. Ketley, Department of Genetics, University of Leicester).

The gene lasl is a homologue of luxl, which regulates expression of several virulence determinants of Ps. aeruginosa (Gambello and Iglewski, 1991: Toder et al, 1991: Gambello et al, 1993). The autoinducer molecule produced by Ps. aeruginosa has been identified from supernatants of E. coli transformed with a plasmid containing the lasl gene (Pearson et al, 1994). It may be possible to detect mycobacterial autoinducer in supernatants of E. coli transformed with either pBJR1 or pBJR2, if either of the plasmids pBJR1 or pBJR2 contain a gene responsible for the production of such an autoregulatory factor.

Culture supernatants from E. coli JM 109 transformed with either pBJR1 or pBJR2 were assayed for the presence of autoinducer as previously (Table 3.32). Transformed E. coli were grown both in the presence and absence of isopropyl-1-thio-β-D-galactopyranoside (IPTG), which regulates the tac promoter of pBJR1 and pBJR2. An increase in bioluminescence of approximately three logs was observed after incubation with supernatants from transformed E. coli, M. tuberculosis I2646 or LB medium. The increase in bioluminescence cannot therefore be due to the presence of autoinducer in the supernatant of E. coli transformed with either pBJR1 or pBJR2. Autoinducer could not therefore be detected in the supernatants of E. coli transformed with either pBJR1 or pBJR2, either in the presence or absence of IPTG. Neither was there any evidence for the presence of autoinducer in the culture supernatant of M. tuberculosis I2646. The relatively high levels of bioluminescence are due to a change in the sensitivity of the luminometer.
Fig 3.32 Induction of Bioluminescence by Culture Supernatants Bacteria Containing Potential lux I Homologues.

<table>
<thead>
<tr>
<th>Bacterial Culture Supernatant</th>
<th>Bioluminescence* after incubation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 Minutes</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> I2646</td>
<td>0.90, 1.07, 1.32</td>
</tr>
<tr>
<td>pBJR1 + IPTG</td>
<td>2.01, 2.67, 2.85</td>
</tr>
<tr>
<td>pBJR1 - IPTG</td>
<td>2.56, 2.96, 2.93</td>
</tr>
<tr>
<td>pBJR2 + IPTG</td>
<td>1.84, 2.31, 2.45</td>
</tr>
<tr>
<td>pBJR2 - IPTG</td>
<td>1.84, 2.41, 2.44</td>
</tr>
<tr>
<td>Medium control</td>
<td>1.18, 1.31, 1.41</td>
</tr>
</tbody>
</table>

* Bioluminescence was measured in relative light units (RLU)
Initial experiments suggested that a component of the culture supernatants from *M. tuberculosis* 12646 and *M. smegmatis* had activity analogous to that of the *P. fischeri* autoinducer. In *M. tuberculosis* 12646, this activity was found to be maximal at three days growth, and was found to be related to cell density as seen in other bacteria. However, this activity was not demonstrable in later experiments.

Attempts to clone a *luxI* homologue from a library of *M. smegmatis* identified two clones which were bioluminescent in the presence of long-chain aldehyde. The DNA insert from the first clone, pBJR1, hybridised to DNA from *M. smegmatis* and *M. tuberculosis*, but not *E. coli*. However, after digestion of the insert with the restriction endonuclease *Pst* I, two separate fragments were bioluminescent in the sensor bacteria in the presence of long-chain aldehyde. This suggested that pBJR1 did not contain a mycobacterial *luxI* homologue. Partial sequencing of the second clone, pBJR2, revealed that this was not of mycobacterial origin, but contained the *P. fischeri* *luxR* gene. Culture supernatants from *E. coli* transformed with either pBJR1 or BJR2 were found to be negative for the presence of autoinducer.

From the analysis of the culture supernatants of mycobacteria, it would appear that mycobacteria do not produce an autoinducer which cross-reacts with the *luxR* gene of *P. fischeri*. Components of similar autoregulatory systems from different bacteria have been found not to complement each other (Gray *et al.*, 1994). If mycobacteria do possess an autoregulatory system, then it may be more similar to that of the actinomycetes (Horinouchi and Beppu, 1992) than the *P. fischeri* system.
4. Conclusions
The main aim of this research was to investigate possible antimycobacterial mechanisms of guinea-pig alveolar macrophages. The pathology of tuberculosis in the guinea-pig is similar to that observed in humans (Brown, 1983; Smith and Weigeshaus, 1989). Therefore, from the observations made with the guinea-pig model, inferences could be made about the situation with human alveolar macrophages.

The oxidation of spermine and spermidine by PAO generates products which are inhibitory to a number of micro-organisms including *E. coli* (Bachrach and Persky, 1964). In this study, it was found that over a 4 hour incubation period, products of polyamine oxidation inhibited the growth of *E. coli* (section 3.1.2). The bacteria were able to recover fully from this inhibitory effect, but when the period of incubation with PAO and polyamines was extended to 24 hours, the products of polyamine oxidation were bactericidal rather than inhibitory. These effects were seen when either spermine or spermidine was used as the polyamine source.

Oxidation of polyamines has also been claimed to inhibit the replication of mycobacteria (Hirsch and Dubos, 1952; Fletcher *et al*, 1953; Hirsch, 1953b; Bachrach and Persky). However, the present study reveals that oxidation of polyamines by PAO generated products which were mycobactericidal (section 3.1.3). Mycobacteria incubated with PAO and polyamines for 24 hours did not form colonies on solid media after 6 weeks of incubation. The technique used by Hirsch to estimate the antimycobacterial activity of oxidised polyamines involved measuring the turbidity of liquid cultures of mycobacteria (Hirsch, 1953b). This technique would not distinguish an inhibitory effect from a mycobactericidal effect. Therefore, the antimycobacterial activity of polyamine oxidation described by Hirsch (1953b) may have been incorrectly described as an inhibition of replication.
Other workers (Bachrach and Persky, 1964) have measured the growth of mycobacteria in Dubos medium to quantify the antmycobacterial effects of polyamine oxidation. Again, this technique will not distinguish between an inhibitory effect or a mycobactericidal effect as growth could either be due to mycobacteria recovering from an inhibitory effect or growth of a small population of mycobacteria resistant to the mycobactericidal effects of oxidised polyamines.

The work of Hirsch (1953b) was expanded by comparing a greater range of strains of *M. tuberculosis*, including both catalase-positive and catalase-negative strains. The catalase-negative strains 24, B1453 and H$_{37}$RaHR were susceptible to 25µM spermine in the presence of PAO to which catalase-positive strains were resistant. Addition of catalase increased the survival of these strains (section 3.1.4). Strain 24 transformed with a functional mycobacterial catalase gene expressed a catalase-positive phenotype and was resistant to 25µM spermine in the presence of PAO. It was concluded that the catalase-negative strains were susceptible to the low concentrations of hydrogen peroxide generated by oxidation of 25µM spermine.

Products of polyamine oxidation were mycobactericidal when the hydrogen peroxide generated was removed by catalase. Therefore, products of polyamine oxidation other than hydrogen peroxide, such as aminoaldhydes, have mycobactericidal properties. This is in agreement with what has been found with other micro-organisms (section 1.5.7).

Comparison of the toxicity to mycobacteria of spermine and spermidine in the presence of PAO and catalase showed that oxidation of spermine had a greater mycobactericidal effect than oxidation of spermidine. This has been found previously with fungi (Levitz *et al*, 1990) and helminths (Ferrante *et al*, 1986a). The
aminoaldehyde produced by oxidation of spermine has two aldehyde groups, whereas the aminoaldehyde produced by oxidation of spermidine has a single amino group (Fig 1.1). The increased number of reactive aldehyde groups would account for the increased reactivity of oxidised spermine compared with oxidised spermidine.

The resistance of a range of mycobacterial strains to oxidation of either spermine or spermidine was assessed. There was found to be no correlation between strain virulence in the guinea-pig and resistance to oxidation of either spermine or spermidine. However, the highly virulent strains I2646, 79499 and M. bovis 81470 also showed the greatest resistance to products of polyamine oxidation. Therefore, there is conflicting evidence for polyamine oxidation as an antymycobacterial defence of guinea-pigs. While there is no doubt that products of polyamine oxidation are mycobactericidal, it has yet to be demonstrated that polyamine oxidation exists as an antimicrobial defence of macrophages.

Ornithine decarboxylase (ODC) catalyses the rate-limiting step in the pathway that leads to the formation of spermine and spermidine (Messina et al, 1990). The activity of ODC and inducible nitric oxide synthase, which produces another potential antimicrobial agent - nitric oxide, are up-regulated on stimulation with mycobacterial cell wall material (Nichols and Prosser, 1980; Stuehr and Marletta, 1987). This raises the possibility that polyamine oxidation may be part of the antimicrobial armature of macrophages in conjunction with other systems such as production of nitric oxide.

Hydrogen peroxide is known to have antymycobacterial effects (Murray and Cohn, 1979; Walker and Lowrie, 1981), however, polyamine oxidation was bactericidal to mycobacteria after removal of hydrogen peroxide by catalase. There
are three products which could mediate the peroxide-independent antimycobacterial effects of polyamine oxidation: ammonium ions, acrolein and aminoaldehydes.

There was no conclusive evidence that ammonium ions are mycobactericidal, even at the theoretical maximum concentration generated by oxidation of 250μM spermine. Therefore ammonium ions can be ruled out as mediating the peroxide-independent antimycobacterial effects of polyamine oxidation. This is in agreement with previous reports on the antimicrobial effects of ammonium ions (Rzepczyk et al, 1984; Levitz et al, 1990)

Acrolein was mycobactericidal to *M. tuberculosis*, with the virulent strain 12646 being more resistant than the avirulent strain B1453. On a molar basis acrolein was more toxic than oxidised spermine. Therefore, it is possible that acrolein mediates the peroxide-independent antimycobacterial effects of polyamine oxidation. It is debatable, however, whether the aminoaldehydes produced in the assay would break down to form acrolein, or whether they would react with mycobacteria before breaking down. The highly reactive nature of the aminoaldehydes suggests the latter. The concentrations of acrolein generated during the antimycobacterial assay needs to be measured in order to clarify whether concentrations of acrolein generated are sufficient to account for the mycobactericidal effects of polyamine oxidation. If concentrations of acrolein generated during the assay are not sufficient to account for the antimycobacterial effects of polyamine oxidation, then the toxic species must be the aminoaldehydes.

Oxidation of polyamines by mammalian tissue polyamine oxidase generates products with antimicrobial activity (Egan et al, 1986; Ferrante et al, 1986a; Morgan et al, 1986; Storer et al, 1988). Hirsch (1953a) also found that extracts from guinea-pig kidneys were inhibitory to mycobacteria in the presence of spermine. In
the present study homogenates of rat spleen and kidney with high levels of PAO
activity had no antimycobacterial effect in the presence of spermine. Homogenates
of rat liver were mycobactericidal, but the toxicity could not be ascribed to polyamine
oxidation as it occurred in both the presence and absence of spermine. It is possible
that toxic species produced by PAO in the rat tissue homogenates reacted with
other components of those homogenates such as DNA from lysed cells. This would
prevent the toxic species from reacting with the mycobacteria, and so a
mycobactericidal effect would not be observed.

Elevated levels of PAO in the tissues of immunised guinea-pig compared with
tissues from control guinea-pigs would suggest the involvement of polyamine
oxidation in the response to infection with mycobacteria. A radiometric assay for
PAO was used. This was a very accurate assay, able to detect the very low levels of
PAO in human serum. PAO levels in rat tissues measured using this assay were
lower than those found by other workers (Seiler et al, 1980; Pavlov et al, 1991).
However, this is probably due to differences in the substrate used for measuring
PAO activity.

The distribution of PAO in the rat followed a similar pattern to that shown
previously (Seiler et al, 1980; Pavlov et al, 1991), being highest in the spleen and
kidney and lowest in the lung and heart. A similar distribution of PAO activity was
found in the guinea-pig. Comparison of the PAO levels in vaccinated and control
guinea-pigs found that PAO levels were elevated in the lungs of vaccinated
guinea-pigs. This suggests that polyamine oxidation is somehow involved in the
response to infection with mycobacteria, but only in lung tissue.

No difference was found in the PAO activity of alveolar macrophages from
vaccinated and control guinea-pigs. However, the low numbers of macrophages
recovered made measurement of PAO levels difficult. It is possible that with larger numbers of macrophages and more replicates for each group a difference may be seen between the PAO levels of macrophages from vaccinated and control guinea-pigs. Previously it had been found that intra-peritoneal infection of mice with *M. bovis* BCG resulted in elevated PAO activity in peritoneal macrophages (Morgan *et al*, 1980). There is evidence from this study that the response of guinea-pigs to infection involves polyamine oxidation. However, more work is needed to determine whether polyamine oxidation is involved as an antimycobacterial mechanism of macrophages or as a response to inflammation caused by mycobacterial infection.

PAO levels in human macrophages could be studied by stimulating human macrophages *in vitro* with immunomodulators or *M. bovis* BCG. Treatment of the human monocyte cell line U937 with interferon-γ leads to an elevation in ODC (O’Brien, 1995) the first enzyme in the pathway for the formation of spermine and spermidine (Messina *et al*, 1990).

Degradation of the amino acid tryptophan has been shown to be responsible for the inhibition of growth of *T. gondii* by human macrophages (Pfefferkorn, 1984). Cytokine-stimulated human monocytes have increased levels of indoleamine 2,3-dioxygenase [IDO] (Carlin *et al*, 1987), which degrades tryptophan to kynurenine (Taylor and Feng, 1991). If degradation of tryptophan is an antimycobacterial mechanism of alveolar macrophages from vaccinated guinea-pigs, then levels of IDO would be expected to be higher in alveolar macrophages from vaccinated guinea-pigs compared with similar cells from control guinea-pigs.

This study found that levels of IDO were similar in macrophages from both control and vaccinated guinea-pigs. Therefore there is no evidence that degradation of tryptophan is an antimycobacterial mechanism of alveolar macrophages from
vaccinated guinea-pigs. This is in agreement with a previous finding that addition of exogenous tryptophan did not reverse the antimycobacterial or antitoxoplasmal effects of alveolar macrophages from vaccinated guinea-pigs (O'Brien, 1992). A similar situation has been found in the mouse, as murine macrophages do not show IDO activity (Murray et al, 1989) and addition of exogenous tryptophan did not reverse the inhibition of growth of Legionella pneumophila by murine macrophages (Gebran et al, 1994).

The relationship between the hydrophobicity and virulence of mycobacterial strains was investigated as a virulent strain of M. avium has been shown to be more hydrophobic than a similar avirulent strain (Stormer and Falkingham III, 1989). A correlation between virulence and hydrophobicity was not found, with the individual strains showing a great deal variability in their hydrophobicity within the four experiments. However, the highly virulent strain 79499 was consistently found to be one of the most hydrophobic strains and the least virulent strain H37RaHR was consistently one of the least hydrophobic strains. This appears to fit the pattern found by Stormer and Falkingham III (1989), however, the other 7 strains tested did not fit this pattern. Therefore, there is no clear evidence that the degree of hydrophobicity of mycobacterial strains affects their virulence in the guinea-pig.

The relatively crude nature of the assay used to determine hydrophobicity in the present study may have contributed towards the high degree of variability. It is possible that with the use of a more accurate assay a relationship between virulence and hydrophobicity may be found.

The expression of virulence factors in several species of Gram-negative bacteria is under the control of a family of small, diffusible molecules termed autoinducers (section 1.6.4). A second family of autoinducers are found in the genus
**Streptomyces**, a close relative of the mycobacteria (section 1.6.4). Using a recombinant strain of E. coli, which is bioluminescent in the presence of autoinducer, supernatants from *Pseudomonas aeruginosa* and *M. tuberculosis* l2646 were found to contain autoinducer. This system has been successfully used to detect autoinducer in *Ps. aeruginosa* (Swift *et al*, 1993), however, it was exciting to find an autoinducer in mycobacterial culture supernatant as this system specifically detects autoinducers belonging to the homoserine lactone (HSL) family, which have previously been found only in Gram-negative organisms (section 1.6.4). Autoinducers produced by the Gram-positive *Streptomyces* do not cross-react with the targets of the HSL-based autoindecers of Gram-negative bacteria (Horinouchi and Beppu, 1992). The production of autoinducer appeared to be cell density-dependent, with the greatest concentration of autoinducer being present in three day old cultures, which corresponds to log phase. Cell density-dependent production of autoinducer has been found previously with Gram-negative bacteria, however this tended to appear during the transition from log to stationary phase (Swift *et al*, 1993).

Further experiments with supernatants from a range of mycobacterial strains found no evidence for the presence of autoinducer, including supernatant from *M. tuberculosis* l2646. Therefore, there is conflicting evidence as to whether mycobacteria possess autoinducers belonging to the homoserine lactone family.

For this reason a second approach was used, in which mycobacterial DNA was screened for the presence of genes homologous to those which synthesise homoserine lactone autoinducers in Gram-negative bacteria, termed *lux I* homologues. Two clones containing potential *lux I* homologues were identified from
a library of *M. smegmatis* mc²155 DNA. The plasmids containing the inserts from the library of *M. smegmatis* were named pBJR1 and pBJR2.

The insert contained in pBJR1 was found to hybridise to DNA from *M. smegmatis* mc²155, confirming that it was mycobacterial in origin. However, pBJR1 did not hybridise to pBJR2, indicating that different fragments of DNA were contained within the two clones. Digestion of the mycobacterial insert contained in pBJR1 gave two fragments each of which appeared to have *lux I* activity. This suggests that pBJR1 does not contain a mycobacterial *lux I* homologue, rather, it would appear to be a false positive. Further investigations were not carried out with pBJR2.

Overall there is no consistent evidence that mycobacteria possess an autoregulatory system homologous to that found in Gram-negative bacteria. Previous work has found that the autoregulatory systems of different Gram-negative bacteria require very specific autoinducer molecules in order to function (Gray *et al.*, 1994). Therefore it is unlikely that a system homologous to that found in Gram-negative bacteria would be also be found in mycobacteria. However, it is still possible that mycobacteria possess an autoregulatory system similar to that found in the closely related *Streptomyces*.


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