

**CELLULAR RESPONSES OF *MYCOBACTERIUM*
TUBERCULOSIS TO ANTIMYCOBACTERIAL
AGENTS**

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ABSTRACT

The effects of clinically important antimycobacterial drugs on *Mycobacterium tuberculosis* at the single cell level using cytochemical indicators of cellular activity were studied. Procedures based on rhodamine 123 (R123) uptake as an indicator of cytoplasmic membrane energisation, propidium iodide (PI) exclusion and iodonitrophenyltetrazolium chloride (INT) reduction were established. Some cells in every preparation were found to resist labelling by all of the procedures applied. This proportion was highest in broth culture (up to 70%) and lowest in cell suspensions prepared from agar spread plates. R123 uptake in growing cells was reversibly sensitive to carbonylcyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation. Several tuberculocidal treatments (70°C/30 min, 70% ethanol and 4% formaldehyde) lead to development of uncoupler insensitive R123 labelling of dead cells, demonstrating the requirement for a physiologically validated procedure where labelling was unambiguously attributed to membrane energisation. All antimycobacterial drug treatments (isoniazid, rifampicin, ethambutol, streptomycin and capreomycin) produced an excess of between 1 and 4 orders of magnitude of uncoupler sensitive R123 labelling cells over culturable units. Thus, large populations of active but nonculturable (ABNC) cells were produced by antimycobacterial drugs commonly used in the treatment of tuberculosis. Non-labelling and ABNC cell populations were further investigated using a GFP reporter strain and by exposure to the lytic mycobacteriophage D29. In addition to demonstrating many of the potential pitfalls that may be encountered when the results of cellular activity/integrity assays are equated with viability/nonviability, these studies illustrate the heterogeneous nature of *M. tuberculosis* cultures and the extent to which bulk analysis may give a misleading picture of cellular composition and physiology. Although the significance of the non-labelling and ABNC cells observed remains to be established, we speculate that these populations may have implications for the chemotherapy of tuberculosis.

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ABBREVIATIONS

ABNC	active but nonculturable
ADC	albumin-dextrose-catalase
AIDS	acquired immunodeficiency syndrome
APS	3-aminopropyltriethoxysilane
CAP	capreomycin
CCD	charge-coupled device
CCCP	carbonylcyanide <i>m</i> -chlorophenylhydrazone
CFU	colony forming units
CTC	cyanoditoly tetrazolium chloride
DAPI	4',6-diamindino-2-phenylindole
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ETH	ethambutol
GFP	green fluorescent protein
HIV	human immunodeficiency virus
INH	isoniazid
IGL	integrated grey level
INT	iodonitrophenyltetrazolium chloride
IOD	integrated optical density
LAM	lipoarabinomamman
MDR-TB	multi-drug resistant tuberculosis
MGL	mean grey level
MOD	mean optical density
MPN	most probable number
OD	optical density
OADC	oleic acid-albumin-dextrose-catalase
PBS	phosphate buffered saline
PI	propidium iodide

PFU	plaque forming unit
R123	rhodamine 123
RIF	rifampicin
RPM	revolutions per minute
SD	standard deviation
SDW	sterile distilled water
SEM	standard error of the mean
STREP	streptomycin
TB	tuberculosis
TCC	total cell counts
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organisation

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

In spite of forty years of effective chemotherapy for tuberculosis, the disease has never ceased to be a global health problem. Tuberculosis remains the major causative factor in 3 million deaths worldwide each year. Much of the recent attention that this disease has received can be attributed to the resurgence of tuberculosis in developed countries and the emergence of multi-drug resistant strains of the causative agent *Mycobacterium tuberculosis*. However, tuberculosis remains the foremost cause of death from a single infectious agent in developing countries where 95% of all cases occur. A particularly worrying aspect of the current re-emergence of tuberculosis is the association with the Acquired Immunodeficiency Syndrome (AIDS) pandemic since Human Immunodeficiency Virus (HIV) infection has emerged as the strongest known risk factor for the development of tuberculosis and has produced an epidemic within an epidemic.

The demanding, expensive and lengthy regimens required to cure tuberculosis are recognised as a significant obstacle to the control of this resurgent disease. However, the biological basis for the slow response to chemotherapy is not clearly established. While host factors may contribute to the problem by sequestering the organism at sites where the effects of antimicrobial agents are marginal, little is known about the physiological properties of the disease which could render it refractory to eradication.

1.2 THE GENUS *MYCOBACTERIUM*

Members of the genus *Mycobacterium* are considered to be aerobic, non-motile, non-sporeforming, rod-shaped bacteria, 0.2-0.7 x 1.0-10.0 µm in size (Murray *et al.*, 1990). They are included in the aggregate group of *Actinomycetales* called the nocardioform actinomycetes which includes organisms classified in the genera *Caseobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Rhodococcus* and the "*aurantiaca*" (Goodfellow *et al.*, 1984). Mycobacteria are weakly Gram-positive and are characterised by their staining property of acid-fastness. When stained with one of the basic dyes such as carbol fuchsin, they resist decolourisation with mineral acids or acidified organic solvents.

When the generic name *Mycobacterium* was introduced in 1896 (Lehmann and Neuman, 1896), the genus only contained two species, *M. tuberculosis* and *Mycobacterium leprae*, respectively the causative agents of tuberculosis and leprosy. It became apparent that other types of acid-fast bacilli were present in the environment and new mycobacterial species were placed into groups according to their speed of growth and pigmentation (Runyon, 1959). Group I contained slowly growing photochromogenic species (yellow pigment produced by culture after exposure to light), Group II was made up of the scotochromogenic slow-growers (yellow pigment produced without exposure to light) and Group III the non-chromogenic slow growers which included *M. tuberculosis*. Group IV consisted of the rapidly growing mycobacteria. Slow-growers require over 7 days at optimal temperature on nutritionally rich medium to form easily visible single colonies, whereas fast-growers achieve this in 7 days or less. Although several recognised mycobacterial species are cultivated only with great difficulty, *M. leprae* is unique amongst them, as it is not cultivable *in vitro*. The taxonomy of mycobacteria is now sufficiently complete to attach species names and Runyon group classification is rarely used. Currently, there are 78 recognised or proposed species of *Mycobacterium* (Wayne and Kubica, 1986; Shinnick and Good, 1994; Koukilakakola *et al.*, 1995; Kleespies *et*

al., 1996; Domenech *et al.*, 1997; Schroder *et al.*, 1997; Shojaei *et al.*, 1997; Reischl *et al.*, 1998; Vuorio *et al.*, 1999).

More than 25 mycobacterial species other than *M. tuberculosis* have been found in human clinical specimens, although the true pathogenicity of some is questionable (Table 1.1) (Heifets and Good, 1994). Non-tuberculous mycobacterial disease is more common in patients with pre-disposing conditions such as previously damaged lungs and immunosuppression. The *Mycobacterium avium* complex has become the second most important group of organisms among the mycobacterial species causing either disseminated infection in AIDS patients and localised pulmonary disease in non-AIDS patients (Heifets, 1994).

Table 1.1: Mycobacteria-other-than-tubercle bacilli which have been associated with human disease (Shinnick, 1994)

Slow-growers		Rapid-growers
<i>Mycobacterium asiaticum</i>	<i>Mycobacterium malmoeense</i>	<i>Mycobacterium flavescens</i>
<i>Mycobacterium avium/intracellulare</i>	<i>Mycobacterium marinum</i>	<i>Mycobacterium fortuitum</i> complex
<i>Mycobacterium celatum</i>	<i>Mycobacterium scrofulaceum</i>	<i>Mycobacterium gordonae</i>
<i>Mycobacterium genavense</i>	<i>Mycobacterium shimoidei</i>	
<i>Mycobacterium haemophilum</i>	<i>Mycobacterium simiae</i>	
<i>Mycobacterium interjectum</i>	<i>Mycobacterium szulgai</i>	
<i>Mycobacterium intermedium</i>	<i>Mycobacterium ulcerans</i>	
<i>Mycobacterium kansasii</i>	<i>Mycobacterium xenopi</i>	
<i>Mycobacterium leprae</i>		

Mycobacteria have unusually high lipid content, greater than 25% (in dry weight) in contrast to 0.5% in Gram-positive and 3% in Gram-negative bacteria (Smith, 1982). The lipids form an integral part of the distinct mycobacterial cell envelope, where they account for about 60% of the dry weight. The model of the mycobacterial cell wall, proposed by Minnikin (1982) and subsequently elaborated by McNeil and Brennan (1991), shows it to be among the most complex found in the microbial world. A schematic representation of the cell wall of *M. tuberculosis* is shown in Figure 1.1.

The innermost layer of the envelope is the cytoplasmic membrane, which is similar to that found in other bacteria. External to this is a layer of conventional peptidoglycan that is linked to arabinogalactan (a branched polysaccharide composed of arabinose and galactose). The terminal arabinose units on the sidechains are covalently bound by esterification to a group of long chain fatty acids, termed mycolic acids. Mycolic acids are high molecular weight 3-hydroxy-2-alkyl branched fatty acids and mycobacteria differ from other mycolate-containing actinomycetes in the complexity of the types of mycolic acids present. The mycolic acids of mycobacteria have between 60 and 90 carbon atoms whereas those of the other members of nocardioform actinomycetes are smaller in size, ranging from 30 to 60 carbon atoms. The outer layer is composed of a heterogeneous group of complex free lipids with medium-chain fatty acids (e.g. the mycocerosates of the phenolic glycolipids) and short-chain fatty acids (e.g. phospholipids).

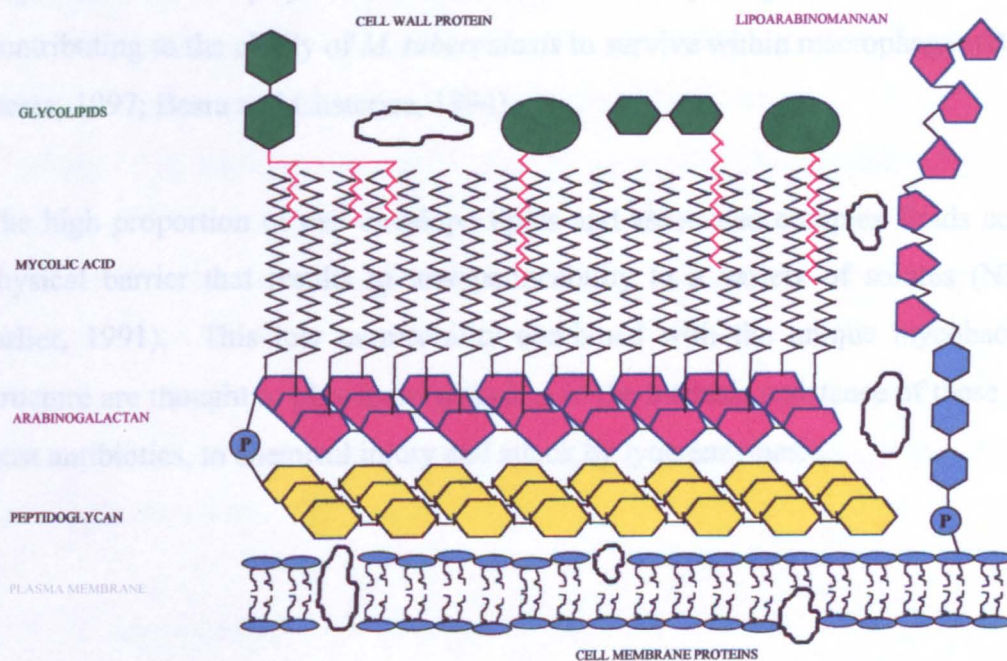


Figure 1.1: A schematic representation of the cell wall of *M. tuberculosis* (not to scale) (after Minnikin, 1982; McNeil *et al.*, 1991).

Minnikin (1982) proposed that the mycolic acids bound to arabinogalactan are arranged with their main and branched chains parallel, producing a parallel binding region which offers hydrophobic anchoring sites for the long acyl components of the complex free lipids. Essentially this produces a double membrane system analogous to the outer membrane of Gram-negative bacteria. Indeed, electron-microscopic studies of the mycobacterial cell wall show alternating zones of electron density and transparency similar to that of the Gram-negative cell wall (Brennan and Draper, 1994; Paul and Beveridge, 1994).

McNeil and Brennan (1991) adapted Minnikin's earlier model to include lipoarabinomannan (LAM), which protrudes through the cell envelope from a covalent phospholipid anchor in the cytoplasmic membrane bilayer. It is thought that LAM has an important role to play in the interaction of the pathogen with the host, possibly contributing to the ability of *M. tuberculosis* to survive within macrophages (Brennan and Besra, 1997; Besra and Chatterjee, 1994).

The high proportion of cell envelope lipids and associated complex lipids constitutes a physical barrier that results in low permeability to a variety of solutes (Nikaido and Jarlier, 1991). This low permeability combined with the unique mycobacterial wall structure are thought to play important roles in the intrinsic resistance of these bacteria to most antibiotics, to chemical injury and attack by lytic enzymes.

1.3 TUBERCULOSIS

1.3.1 *Mycobacterium tuberculosis*

Tuberculosis in humans is caused by members of the *M. tuberculosis* complex, which includes the subspecies *M. tuberculosis*, *M. tuberculosis* Asian variant, *M. tuberculosis* African variant I, *M. tuberculosis* African variant II and *Mycobacterium bovis*. *M. bovis* rarely causes disease in areas of the world where animal husbandry includes TB screening and milk pasteurisation. The *M. tuberculosis* complex also includes *Mycobacterium microtti*, a rarely encountered pathogen of voles and other small mammals which produces tubercles but is not naturally virulent for man.

Although the *M. tuberculosis* complex constitutes a relatively homogeneous group of bacteria, members of the group can be separated by several phenotypic and epidemiological characteristics (Shinnick and Good, 1994). In addition, various repetitive DNA elements that contribute to strain variation have been discovered. Two of these are insertion sequences and the remainder are short repetitive DNA sequences with no known function or phenotype (Small and van Embden, 1994). IS6110 is found in a variable number of copies and at different loci in the genomes of *M. tuberculosis* strains. Locations of these copies in the genome are different in epidemiologically unrelated isolates, and this characteristic is utilised for the typing of *M. tuberculosis* strains and in studies of tuberculosis transmission.

1.3.2 Transmission and pathogenesis of disease

Most cases of human tuberculosis result from inhalation of infected airborne droplets expectorated from a person with infectious pulmonary or laryngeal disease (Smith and Moss, 1994). These droplets are created following sneezing or coughing, dry to form droplet nuclei and can remain suspended in air currents for hours. Following the

inhalation of virulent microorganisms, the bacilli enter and proliferate intracellularly within alveolar macrophages (Fenton and Vermeulen, 1996).

Primary infection occurs when phagocytic defences fail locally and bacilli proliferate in macrophages, usually in discrete alveolar foci. Bacilli are transported to regional lymph nodes, where additional foci of disease develop. Lesions developing at the implantation site and in the lymph nodes constitute the primary complex. Bacilli spread beyond this complex by the lymphatics and bloodstream, and lodge in many organs of the body. Primary infection may give rise to mild fever and malaise, particularly when the regional lymph nodes are involved, but most cases are asymptomatic.

The possible course of events following primary infection by *M. tuberculosis* largely reflects the host's immune response and is summarised in Figure 1.2 (Grange, 1998). Where the immune system is competent, cell mediated immunity develops, macrophages infiltrate the lesion, which becomes surrounded by epithelioid cells, and granuloma develop. Subsequent caseation of dead macrophages and tubercle bacilli in the centre and the deposition of collagen in the outer layers make up the characteristic tuberculous granuloma or 'tubercle' which contains the infection. *M. tuberculosis* bacilli persist to an unknown extent, quiescent but viable in the tubercles.

In about 95% of infected individuals the primary complex resolves spontaneously and therefore usually passes unnoticed. In the remainder, particularly in some children, the primary complex causes serious complications. These include pulmonary disease, nonpulmonary disease (meningeal, bone and joint or renal tuberculosis) and miliary tuberculosis (when foci of infection erode into major blood vessels, resulting in dissemination of bacteria and the production of numerous small lesions throughout the body).

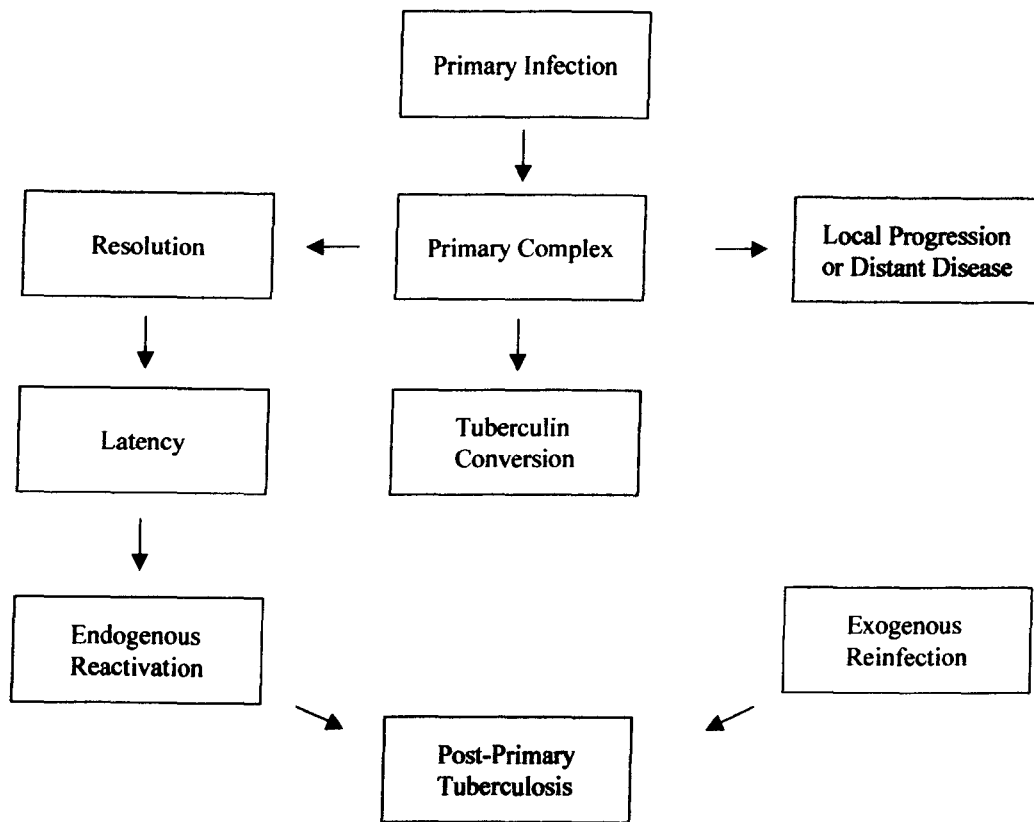


Figure 1.2: The possible course of events following primary infection by *M. tuberculosis* (Grange, 1998).

In the absence of these complications the infection becomes quiescent as stated and usually remains latent for the rest of the person's life. However, infected individuals have a 10% lifetime risk of developing post-primary tuberculosis (Hopewell, 1994). Conditions predisposing to reactivation include old age, malnutrition, malignant disease, corticosteroid therapy, immunosuppressive drugs and disease, including HIV infection. Post-primary tuberculosis may also occur from exogenous infection in communities where the disease is highly prevalent.

Post-primary tuberculosis tends to occur in the apices of the lungs irrespective of the site of the primary complex. Post-primary pulmonary lesions differ from primary lesions in that the necrotic element is much more evident, very large lesions occur and the surrounding tissues often liquefy. Local progression is much more marked and the

development of secondary lesions is common. Lesions may rupture into the bronchi and discharge their necrotic contents, thereby forming pulmonary cavities. Such patients are infectious and are said to have open tuberculosis. Bleeding can be serious when a bronchial artery is eroded. In contrast to primary tuberculosis, post-primary tuberculosis is more localised and contained as lymphatic or haematogenous spread of disease is rare. The pathologic and inflammatory processes produce the characteristic weakness, chest pain, fever, cough and bloody sputum of TB.

1.3.3 Dormancy of *M. tuberculosis*

There is currently renewed interest in the concept that *M. tuberculosis* is capable of adapting to prolonged periods of dormancy during infection (Parrish *et al.*, 1998; Gangadharam, 1995; Wayne, 1994; Grange, 1992). The existence of dormant tubercle bacilli (persistors) for long periods after chemotherapy was shown by McCune and his colleagues in 1966 (McCune *et al.*, 1966). The physiological state and nature of these persistors is unknown but it is widely thought that these bacteria are in a state of dormancy which renders them functionally resistant to many of the anti-tuberculosis drugs. Persistors may be responsible for the endogenous reactivation of tuberculosis years or even decades after the primary infection in so-called latently infected individuals.

Reports of non-acid-fast forms of *M. tuberculosis* in persistent human infection, which are suggestive of a spore-like resting state, have remained controversial for nearly a century (Stanford, 1987). In 1907 and 1908 Hans Much described spore-like granules of *M. tuberculosis* present in tuberculous tissues and abscesses that were not properly acid fast, not easily cultured, were just large enough to be resolved by light microscopy but small enough through porcelain filters then used (Much, 1907; 1908). Ultra-fine forms of *M. tuberculosis* were again described by Khomenko (1987) who observed that the proportion of ultra-fine forms increased during anti-tuberculosis chemotherapy, by the third month of treatment he detected them in 82% of patients with open cavities. These ultra-fine forms were 20 times smaller than the typical acid-fast bacteria, passed through

fine-porous filters, were spherical with dense electron opaque shells when examined by electron microscopy and reverted to typical forms of *M. tuberculosis* by passage in liquid culture medium.

The presence of homologs of *Streptomyces coelicolor* and *Bacillus subtilis* sporulation genes in *M. tuberculosis* provides further evidence that the organism may have a spore-like metabolically inactive form that may respond to activating signals (De Maio *et al.*, 1996). Although transcription of these genes is induced in stationary phase and is virtually undetected in exponential phase cultures, further molecular genetic studies are necessary to determine their true function in mycobacteria.

Recent work suggests that dormancy may be the result of metabolic adaptation to anaerobiosis, in response to conditions that the bacteria are exposed to in the human host such as in sealed-off tubercles. Wayne and Lin (1982) have shown that although very abrupt exposure to anaerobic conditions has a lethal effect on actively-growing cultures of *M. tuberculosis*, incubation under conditions in which oxygen is depleted gradually leads to a shift down from active replication to dormancy. This orderly shutdown is essential to continued survival of *M. tuberculosis* in a quiescent form. These workers found that *M. tuberculosis* was resistant to the major antituberculosis drugs whilst in this dormant phase but were susceptible to metronidazole, a drug that acts almost exclusively on anaerobes (Wayne and Sramek, 1994). An understanding of the nature and physiological properties of persisters would be of great value as it could provide the key to the development of better preventive therapy and much shorter courses of chemotherapy (Grange, 1992).

Cunningham and Spreadbury (1998) reported that there is a marked thickening of the cell wall outer electron-opaque layer in *M. tuberculosis* and *M. bovis* BCG when the bacteria were cultured under either microaerobic or anaerobic conditions. They speculated that the thickened walls act as a protective coat or mantle and help the bacilli to survive in oxygen deficient conditions *in vivo* and may offer protection against hostile environments such as the toxic conditions associated with granulomas. They found no evidence for a

spore-like or coccoid morphology and speculated that the thickened cell wall might alleviate the necessity for these bacteria to sporulate. They also observed the upregulation of an α -crystallin homolog (16 kDa small heat protein) during adaptation to low-oxygen conditions which may play a role in stabilising cell structures during long-term survival.

1.3.4 Prevention of disease by vaccination

The Bacille Calmette-Guérin (BCG) vaccine was developed by Calmette and Guérin in 1921 from an attenuated strain of *M. bovis* that had been through 231 serial cultures over 13 years and that had lost its pathogenicity in animals (Calmette, 1927; Guerin, 1957). BCG is globally the most widely used vaccine with over 3 billion doses having been administered in the past four decades and more than 70% of the world's children now receiving BCG (Fine and Rodrigues, 1990). However, the protective efficacy of the BCG vaccine has always been the subject of controversy (Bloom and Fine, 1994). A critical review of eight controlled BCG trials concluded that trials had a range of observed efficacy and protection varied from – 57% to over 75% (Clemens *et al.*, 1983). Although several explanations for BCG's variable efficacy have been proposed (different vaccine strains and doses; influence of exposure to environmental mycobacteria; differences in virulence between *M. tuberculosis* strains; and genetic differences within and between human populations), there is still no general agreement on the basis for the variation. There is no evidence that BCG vaccination can prevent the establishment of infection but it appears to limit the multiplication and dissemination of the bacilli and the development of lesions. Efficacy is greatest against disseminated disease in young children, reducing death rates from progressive pneumonic primary infection, miliary and meningeal tuberculosis.

There is much research into improved vaccines for tuberculosis. Alternative vaccines include: recombinant BCG vaccines expressing protective antigens of *M. tuberculosis* (Stover *et al.*, 1991), genetically attenuated *M. tuberculosis* vaccines (Ganjam *et al.*,

1991), atypical mycobacterial vaccines (Palmer and Long, 1966), auxotrophic or killed vaccines for use in immunocompromised recipients, and DNA vaccines (Ulmer *et al.*, 1998).

1.3.5 The return of an old nemesis

The 20th century saw a gradual decline in the incidence of tuberculosis in industrialised countries (Snider *et al.*, 1994). This decline was associated with increases in living standards and accelerated by the introduction of effective chemotherapy in the 1950s. Statistics from the United States of America (USA) compiled between 1953 and 1984 show an average decline of about 5.3% per year in the incidence of disease, and infection was typically associated with 100% cures (Centers for Disease Control and Prevention, 1993). This significant reduction in tuberculosis was observed in other developed countries. Incidence rates reached a low plateau level of 10-12 per 100,000 in the USA, however, from 1985 an increase of 20% in the number of reported cases of tuberculosis was observed.

In contrast to industrialised countries, tuberculosis remains the foremost cause of death from a single infectious agent in developing countries where 95% of all cases occur, accounting for more than 25% of avoidable adult deaths (Snider, 1994). The World Health Organisation (WHO) estimates that about one third (1.7 billion) of the world's population is infected with, or has been infected by *M. tuberculosis* (WHO, 1998). This leads to about 9 million new cases of tuberculosis and 3 million deaths from the disease annually. A particularly worrying aspect of the current almost-global re-emergence of tuberculosis is the occurrence of a series of outbreaks caused by drug resistant strains.

The re-emergence of tuberculosis as a significant public health problem in the developed world is particularly associated with immigrant groups from countries where tuberculosis is endemic, alcoholics and drug addicts, homelessness, malnutrition, and more recently with HIV infection and AIDS (Bloom and Murray, 1992). Because of the virus's ability

to disrupt the immune system, HIV has emerged as the most significant risk factor for progression of latent tuberculosis infection to clinical disease (Selwyn *et al.*, 1989). In general, individuals who are infected by *M. tuberculosis* have a 10% lifetime risk of developing clinically significant disease. For individuals coinfecting with HIV, this risk rises to 5-10% per annum, along with greatly increased case fatality, even when the disease is properly treated.

The impact of HIV on tuberculosis has been greatest in developing countries where 85% of the estimated 13 million adults and 1 million children infected with HIV live (WHO, 1998). In many of these countries, tuberculosis has been reported in 20-40% of AIDS patients. Although HIV-associated disease accounts for a major part of the recent increase in tuberculosis incidence in the USA, HIV and tuberculosis are largely restricted to distinct populations in the United Kingdom (Rieder, 1998).

1.3.6 The genome project

Since its isolation from a human patient at New York's Trudeau Sanatorium in 1905, *M. tuberculosis* strain H₃₇R_v has been used extensively, in biomedical research in laboratories all over the world (Kubica *et al.*, 1972). It has retained full virulence in animal models of tuberculosis, is susceptible to drugs used in chemotherapy and is amenable to genetic manipulation (Cole *et al.*, 1998).

The complete genomic sequence of *M. tuberculosis* H₃₇R_v has recently been determined and analysed (Cole *et al.*, 1998). The genome consists of a single circular chromosome containing 4,411,529 base pairs, encodes approximately 4,000 genes, and has a very high guanine and cytosine content (65.6%) that is reflected in the biased amino acid content of the proteins. A large proportion of its coding capacity is devoted to the production of enzymes involved in lipogenesis and lipolysis. The genome is rich in repetitive deoxyribonucleic acid (DNA), particularly insertion sequences and in new multigene families and duplicated housekeeping genes. By using database comparisons, precise

functions have been attributed to about 40% of the predicted proteins and some information or similarity for another 44%. The remaining 16% resembled no known proteins and may account for specific mycobacterial functions.

The wealth of information revealed from the systematic sequence analysis of the genome will undoubtedly fuel the next generation of research. The genome project will be used for a diverse range of purposes: deducing gene function by bioinformatics, reverse genetics and pathogenicity, new leads for chemotherapy, and immunoprophylaxis (Cole, 1996).

1.4 CHEMOTHERAPY OF TUBERCULOSIS

1.4.1 General principles

Mitchison (1985) has proposed that the *M. tuberculosis* population in an infected individual can be divided into four sub-populations distinguished by their location and metabolic activity. According to this hypothesis bacteria are either: (1) actively metabolising and relatively rapid growing, (2) semidormant in an acidic environment (early acute inflammation sites or within the phagolysosomes of the macrophages), (3) semidormant in nonacidic environments with occasional spurts of metabolism or (4) in a dormant state. It is thought that tubercle bacilli are only vulnerable to bactericidal drugs when they are metabolically active and replicating. Therefore the putative sub-populations of bacilli that are in groups 3 and 4 are not amenable to cidal action unless they become metabolically active.

Five drugs are currently regarded as essential in the management of tuberculosis - rifampicin, isoniazid, pyrazinamide, ethambutol and streptomycin (WHO, 1991). According to Mitchison, these drugs vary in their bactericidal action (ability to kill large numbers of actively metabolising bacilli rapidly), sterilising action (capacity to kill slowly or intermittently metabolising semi-dormant and persisting bacilli) and ability to prevent the emergence of acquired resistance by suppressing drug-resistant mutants in large bacterial populations (Mitchison, 1985). The relative activities of the main anti-tuberculosis drugs in each of these functions are summarised in Table 1.2.

Table 1.2: Grading of activities of anti-tuberculosis drugs (Mitchison, 1985)

Drug	Sterilising action	Early bactericidal	Prevention of drug resistance
Rifampicin	Good	Fair	Good
Pyrazinamide	Good	Poor	Poor
Isoniazid	Fair	Good	Good
Ethambutol	Poor	Fair	Fair
Streptomycin	Poor	Poor	Fair
Thiacetazone	Poor	Poor	Poor

1.4.2 Drug resistance

Drug resistance in mycobacteria results from selective growth of spontaneous resistant mutants in a wild population when exposed to a drug that can inhibit growth of sensitive organisms (Grange, 1988). Spontaneous mutations leading to drug resistance occurs in *M. tuberculosis* with a frequency of approximately 1×10^{-6} to 1×10^{-8} depending on the drug (Table 1.3) (David, 1970). There is a high probability of resistant mutants emerging and becoming predominant in single drug regimens as bacterial populations in tuberculous cavities may be as high as 10^9 (Johnson and Sepkowitz, 1995). Therefore, it is considered essential during treatment to include two or more drugs to which the strain is sensitive so that the probability that one organism will simultaneously develop resistance to the drugs becomes negligible.

Table 1.3: Mutant frequencies in unselected populations of *M. tuberculosis* (David, 1970)

Drug ($\mu\text{g ml}^{-1}$)	Average mutant frequency ^a
Rifampicin (1.0)	1.20×10^{-8}
Isoniazid (0.2)	3.50×10^{-6}
Ethambutol (5.0)	3.10×10^{-5}
Streptomycin (2.0)	3.80×10^{-6}

^a Proportion of mutants observed in an unselected population.

Although adequate regimens of anti-tuberculosis drugs are available, the incidence of drug-resistant and multi-drug resistant tuberculosis (MDR-TB) continues to increase. MDR-TB is usually defined as disease caused by bacteria that are resistant to two or more anti-tuberculosis agents including both isoniazid and rifampicin (WHO, 1997; Cohn *et al.*, 1997). Disease produced by MDR-TB is expensive and difficult to cure with cure rates less than 50% (Houston and Fanning, 1994).

Since the early 1990s, several outbreaks of MDR-TB have been reported in different regions of the world (Cohn *et al.*, 1997). In many of these outbreaks, the emergence of new strains of *M. tuberculosis* that are resistant to all current anti-tuberculosis agents has

raised the possibility of untreatable disease. This problem was documented in the New York City outbreak, where the evolution and dissemination of strain W, an extremely drug-resistant strain caused at least 22% of MDR-TB during the peak tuberculosis year of 1992 (Agerton *et al.*, 1999). Almost all the isolates of strain W were resistant to four first-line drugs and kanamycin. Most of the cases were associated with HIV-infected individuals and there was citywide nosocomial spread of W-strain TB among AIDS patients because the recommended four-drug therapy was not effective.

1.4.3 Drug regimens

Modern tuberculosis chemotherapy has had a profound effect on the outcome of tuberculosis in the individual (Houston and Fanning, 1994). The long-term mortality of active tuberculosis is well over 50% without treatment, while the cure rate of drug sensitive disease approaches 100% with optimal therapy.

Several regimens of short-course chemotherapy of tuberculosis are recommended by WHO on the basis of extensive field trials (WHO, 1991). The 6-month regimen of daily isoniazid and rifampicin, supplemented by pyrazinamide for the first 2 months, is regarded as the most effective and consistently results in a combined rate of treatment failure and relapse of less than 5% in drug-sensitive tuberculosis (Horne, 1990). The addition of a fourth drug to the initial phase is recommended in populations with a high incidence of primary resistance.

The combination of these drugs during treatment produces an early bactericidal effect and most of the freely multiplying extracellular organisms are thought to be destroyed during the first few days of therapy (Jindani *et al.*, 1980; Mitchison, 1985). The bactericidal effect is highest for isoniazid and is important in shortening the period during which the patient is most infectious to others. During the next two months, pyrazinamide and rifampicin, by virtue of their high “sterilising” activity, kill the bulk of the remaining bacteria. This powerful sterilising effect has apparently enabled shortening of the overall

period of tuberculosis chemotherapy to 6 months. Continued treatment with rifampicin is thought to ensure that dormant and near-dormant bacteria are eventually killed. The inclusion of two or more drugs throughout the treatment prevents the emergence of drug resistance. This regimen has become the therapy of choice for pulmonary tuberculosis in most countries in the developed world.

In spite of this powerful regimen, treatment failure remains a problem. The most common causes are patients who discontinue their treatment too early or who take their drugs irregularly. Directly observed therapy (DOT) is used in many countries in order to control this problem, especially amongst “problem patients” who cannot be relied upon to self-administer therapy (WHO, 1997). Patients with relapsing or unresponsive disease are a priority for obtaining culture and drug sensitivities. Single drugs are never added to a failing regimen as this may in effect, result in single-drug treatment creating ideal conditions for the development of resistance to the new medication. In the absence of reliable sensitivity tests the WHO recommend an 8-month retreatment regimen. This consists of 3 drugs throughout (isoniazid, rifampicin and ethambutol) supplemented by pyrazinamide during the first 3 months and streptomycin during the first 2 months.

Individuals who have failed the standard WHO retreatment regimen, patients with resistance to at least isoniazid and rifampicin, and other patients who have received a variety of sub-optimal regimens outside national programmes require the use of second-line drugs. These drugs are less effective and have more side effects. The initial regimen should consist of at least three drugs, preferably four or five, to which the bacilli are likely to be fully sensitive.

1.4.4 Antimycobacterial drug activity

The characteristics of the existing first and second line antimycobacterial drugs are summarised in Table 1.4 along with current understanding of their modes of action and

the genes responsible for the development of resistance (Chopra and Brennan, 1998; Evans, 1998).

Table 1.4: Characteristics of the main anti-tuberculosis drugs (adapted from Evans, 1998)

	Year of introduction	Molecular target ^a	Genes involved in drug resistance ^b	Mutation rate ^c
<u>First-line drugs</u>				
Isoniazid	1952	Mycolic acid synthesis	<i>inhA, kasA, katG</i>	10 ⁻⁸
Rifampicin	1965	RNA polymerase (β-subunit)	<i>rpoB</i>	10 ⁻¹⁰
Pyrazinamide	1970	?	<i>pncA</i>	10 ⁻³
Streptomycin	1944	30S ribosomal subunit	<i>rpoL, rrs, strA, S12</i>	10 ⁻⁸
Ethambutol	1968	Cell wall polysaccharides	<i>embA, B, C</i>	10 ⁻⁷
<u>Second-line drugs</u>				
Ethionamide	1966	Mycolic acid synthesis (?)	?	10 ⁻³
Kanamycin/ Amikacin	1957	30S ribosomal subunit	?	10 ⁻⁶
Cycloserine	1955	Cell wall synthesis	<i>AlrA</i>	10 ⁻¹⁰
Capreomycin	1967	70S ribosomal subunit (?)	?	10 ⁻³
Thioacetazone	1950	Mycolic acid synthesis (?)	?	10 ⁻³
p-Aminosalicylic acid (PAS)	1946	Folate biosynthesis / iron-chelating mycobactin synthesis (?)	?	10 ⁻⁸
Ofloxacin	1987	DNA gyrase	<i>gyrA, B</i>	?

^a (?) indicates suspected molecular targets (Chopra and Brennan, 1998); ^b ? indicates where genes involved in drug resistance are unknown; ^c mutation rate is defined as the rate of mutation per cell division at the gene(s) responsible for drug resistance.

Isoniazid is highly active against *M. tuberculosis* and is central to the treatment of disease. Although the drug was synthesised in 1912, it was not recognised as an anti-tuberculosis drug for 40 years (Heifets, 1994). Its use is estimated to kill approximately 90% of the bacterial population in tuberculous lesions during the first few days of chemotherapy (Mitchison, 1985). Efficacy of isoniazid is limited to *M. bovis* and *M. tuberculosis*, other pathogenic mycobacterial species are not susceptible to the drug (Mdluli *et al.*, 1998). Isoniazid enters the mycobacterial cell as an inactive pro-drug and is not bactericidal until oxidised by catalase-peroxidase to a still unknown active form (Zhang *et al.*, 1992; Zhang, 1993). The mode of action of isoniazid is usually associated with inhibition of mycolic acids biosynthesis (Winder, 1982). *M. tuberculosis* bacilli

treated with isoniazid show evidence of cellular deformities and loss of acid-fast staining coincident with the shut-down of mycolic acid biosynthesis (Takayama *et al.*, 1973). Recent research by Jacobs and colleagues (Banerjee *et al.*, 1994; Quémard *et al.*, 1995) identified the *inhA* gene product (InhA) as the primary target. This enzyme catalyses the NADH-specific reduction of long chain substrates (12-24 carbons) during the early stages of mycolic acid biosynthesis. More recently, Mdluli *et al.*, (1998) have reported that the *kasA* gene product (KasA) may be the target for isoniazid. They have shown that inhibition of this ketoacyl synthase results in the accumulation of long chain saturated fatty acids needed for mycolic acid biosynthesis. The evidence for several isoniazid targets has lead some researchers to speculate that the activated form of isoniazid may have multiple targets, each of which could possibly be lethal to the cell (Barry *et al.*, 1998).

Rifampicin was introduced into anti-tuberculosis drug regimens in 1966 and is an essential element of modern short course (< 1 year) regimens (WHO, 1997). It proves very effective in the inhibition of mycobacterial DNA-dependent RNA polymerase which in cell free systems is 1000 times more sensitive than the equivalent enzyme isolated from *Escherichia coli* (Nikaido and Jarlier, 1991). Rifampicin is an important bactericidal drug but is also an important sterilising drug because of its ability to kill semi-dormant bacilli capable of surviving the bactericidal effect of isoniazid (Mitchison, 1985). Resistance to rifampicin occurs by mutations leading to an altered β -subunit of the polymerase (Russell and Chopra, 1996).

Pyrazinamide is considered the third most important drug in modern therapy of tuberculosis (WHO, 1997). Its unique role for accelerating the sterilisation effect in combination with rifampicin and isoniazid is a key element that has enabled shortening of therapy from 9 to 6 months (Heifets, 1994). Pyrazinamide is only active in acidic environments (pH 5.8 optimum) and it is postulated that the drug is effective against bacterial subpopulations persisting in the low pH environments associated with acute inflammation sites and possibly within the phagolysosomes of macrophages (Mackanness,

1956; McDermott and Tompsett, 1954). Susceptible mycobacterial strains convert pyrazinamide to pyrazinoic acid and it has been suggested that this enzyme-generated product is responsible for the antibacterial activity of the drug (Chopra and Brennan, 1998). Strains resistant to pyrazinamide do not produce pyrazinamidase and therefore are not vulnerable to this agent (Scorpio and Zhang, 1996). The bactericidal activity of pyrazinamide is said to be low (33-57% killing at twofold above the minimum inhibitory concentration (MIC)). Pyrazinamide is not very active during the later stages of treatment and consequently is only included in the first two months of drug regimens (Horne, 1990).

Ethambutol is a synthetic compound and appears unrelated to the other anti-tuberculosis drugs. It produces a bacteriostatic effect and it is thought to be important in preventing the emergence of resistance to the principle antituberculosis drugs (Horne, 1990). The effect of ethambutol is primarily on polymerisation steps in the biosynthesis of the arabinan component of the mycobacterial cell wall (Takayama and Kilburn, 1989; Brennan and Besra, 1997). The absence of the arabinan receptor, which acts as the mycolic acid anchor in the cell wall prevents the transfer of mycolic acids into the cell wall and explains the ethambutol-induced accumulation of trehalose monomycolate, trehalose dimycolate, and free mycolic acids in the culture medium. The arabinosyltransferase enzymes EmbA and EmbB appear to be the primary targets. Deng *et al.*, (1995) report that ethambutol also inhibits biosynthesis of the arabinan of lipoarabinomannan and that there is also evidence that the synthesis of galactan in the cell wall core is also strongly inhibited. More recently, Brennan and Besra (1997) suggest that the primary effect of ethambutol is on arabinogalactan, not lipoarabinomannan because of the different susceptibilities of the arabinosyltransferase enzymes involved in the different biosynthetic pathways.

Streptomycin was the first drug shown to be effective against tuberculosis. It is an aminoglycoside antibiotic and inhibits prokaryotic protein synthesis by binding to the 30S ribosomal subunit (Russell and Chopra, 1996). It demonstrates potent anti-tuberculosis

activity against bacterial populations in cavities, but not intracellularly where the pH is low (Houston and Fanning, 1994). It is usually ranked third after isoniazid and rifampicin with regard to its early bactericidal activity but demonstrates poor sterilising activity on persisting bacteria (Mitchison, 1985). The other aminoglycosides have activity against *M. tuberculosis* but often show cross-resistance with streptomycin. Capreomycin has many similarities to the aminoglycoside antibiotics but has a chemically distinct structure (Russell and Chopra, 1996). There is no cross-resistance between capreomycin and streptomycin, and occasionally is used in the treatment of tuberculosis especially where streptomycin-resistant strains are encountered (Horne, 1990). There is no direct information on the mode of action of capreomycin (Chopra and Brennan, 1998), but it is likely that the drug inhibits protein synthesis (Winder, 1982).

Thiacetazone is an inexpensive antimycobacterial drug widely used in combined chemotherapy for tuberculosis in developing countries where the cost of treatment is an important factor (Horne, 1990). It has low efficacy but is effective in preventing the development of isoniazid resistance when given in combination (WHO, 1991). The mode of action of the drug is unknown. However, since many thiacetazone-resistant strains of *M. tuberculosis* exhibit cross-resistance to ethionamide (see below) it has been suggested that thiacetazone, like ethionamide might inhibit mycolic acid biosynthesis (Chopra and Brennan, 1998).

Many of the new fluoroquinolones have good antimycobacterial activity with low MIC values (Houston and Fanning, 1994). The fluoroquinolones are synthetic derivatives of nalidixic acid and include ofloxacin, ciprofloxacin and sparfloxacin. They act by inhibiting DNA gyrase which catalyses the negative supercoiling of DNA necessary for processes dependent on DNA topology such as replication and transcription (Russell and Chopra, 1996). Ofloxacin is most active against *M. tuberculosis* and is often used where the organism is resistant to one or more first-line drugs (Yew *et al.*, 1990). However, quinolone-containing regimens have demonstrated inferior sterilising ability (67% compared with 100% at 2 months) compared with the combination of pyrazinamide and

ethambutol (Kennedy *et al.*, 1993). Results obtained to date are based on limited clinical data, and the role of fluoroquinolones in antimycobacterial chemotherapy has yet to be fully established (Chopra and Brennan, 1998).

Ethionamide is structurally similar to isoniazid, and it is now believed that its mechanism of action, inhibition of mycolic acid synthesis, is also similar (Blanchard, 1996). However, ethionamide is much less active against *M. tuberculosis* than isoniazid (Heifets, 1994). Cross-resistance between the two drugs does not appear to be common.

D-cycloserine is a structural analogue of the amino acid D-alanine and inhibits cell wall synthesis in mycobacteria by competing with the amino acid during the biosynthesis of the mycolylarabinogalactan-peptidoglycan complex (David, 1970). Resistance is probably as a result of point mutations in the D-alanine racemase gene (Cáceres, 1997). D-cycloserine is used in the combination therapy of disease caused by organisms resistant to one or more of the first line drugs principally to prevent the emergence of resistance to the other drugs in the regimen (WHO, 1997).

The mode of action of para-aminosalicylic acid (PAS) is unclear but is likely that it competes with p-benzoic acid for mycobacterial dihydropteroate synthetase during folate biosynthesis (Winder, 1982; Chopra and Brennan, 1998). However, there is also evidence that PAS interferes with the salicylate-dependent biosynthesis of the iron chelating mycobactins involved in iron assimilation (Brown and Ratledge, 1975). PAS exhibits a high level of activity against *M. tuberculosis* and was widely used in combination with isoniazid to prevent the emergence of isoniazid-resistant organisms (Horne, 1990). Following the introduction of modern short-course regimens, PAS is rarely used nowadays as a chemotherapeutic drug in industrialised countries but because it is inexpensive it remains an important drug in some developing countries.

1.5 DRUG SUSCEPTIBILITY TESTING

1.5.1 Culture based phenotypic methods

Drug susceptibility testing is essential for effective management of tuberculosis. There are three widely used methods for drug sensitivity testing of *M. tuberculosis* on solid medium: the absolute concentration method, the resistance-ratio method and the proportion method (Inderlied, 1991).

In the first method, resistance is defined as growth from a carefully controlled inoculum of the isolate ($\sim 2 \times 10^3$ CFU per ml) that is greater than a certain number of colonies (usually 20) at a particular drug concentration. In the resistance-ratio method the susceptibility test results are expressed in terms of the ratio of the minimal inhibitory concentration (MIC) of drug necessary to inhibit the growth of the test isolate of *M. tuberculosis* to that of the standard *M. tuberculosis* H₃₇R_v strain. A resistance ratio of 2 or less is defined as sensitive, while 8 or more is resistant. In the proportion method the bacilli are cultured on two media, one with and one without drug; the ratio of the number of colonies obtained on the drug-containing medium to the number of colonies obtained on the drug-free medium indicates the proportion of resistant bacteria. Drug resistance by this method is defined as more than 1% of a population of bacilli being resistant to a given drug.

There is a clinical need for rapid tests of drug susceptibility to disease caused by *M. tuberculosis* because of the extremely slow growth of the organism; even when cultured under optimum conditions the tubercle bacilli has a doubling time of 20-24 hours (Hatfull and Jacobs, 1994). It takes between 2 and 4 weeks to obtain bacterial colonies from a clinical sample that are large enough for further analysis and determination of whether the organism is present or not. It then requires at least a further 2 to 4 weeks to determine drug susceptibilities due principally to the slowness of colony development. By this time, the initial strain may well have acquired new resistance properties, especially in the face

of inadequate treatment or poor compliance (Heifets and Good, 1994). This problem is becoming more critical due to the emergence of drug-resistant strains of *M. tuberculosis* (Cohn *et al.*, 1997).

Techniques developed to shorten the turnaround time in mycobacterial susceptibility testing include a variety of culture-based phenotypic methods, culture-independent genotypic methods (Section 1.5.2) and a variety of colorimetric and bioluminescent cytological indicators of cellular viability and proliferation (Section 1.5.3) (Drobniewski and Wilson, 1998). The results of any new method for the assessment of drug activity should show good correlation with those from conventional methods (Gordon *et al.*, 1996).

A major improvement in the rapidity of testing was the development of the radiometric BACTEC system (Becton-Dickinson). The basis of this system is the evolution of $^{14}\text{CO}_2$ from the metabolism of ^{14}C -palmitic acid by mycobacterial clinical isolates. Growth is defined in terms of the growth index (GI) and resistance or susceptibility is determined by comparison of the GI values of control bottles relative to values from bottles containing the drug of interest. Heifets (1991) reports that it is possible to determine *M. tuberculosis* drug susceptibilities in less than two weeks using the BACTEC system and results show good agreement with the standard methods (Lee and Heifets, 1987). However, its widespread use is restricted because of cost and the generation of radioactive wastes.

Further work by Becton Dickinson lead to the development of the non-radiometric Mycobacterium Growth Indicator Tube (MGIT) fluorometric assay which utilises an oxygen-sensitive fluorescent compound to detect growth. Mycobacterial growth removes dissolved oxygen from the medium and allows the compound to fluoresce, and this provides a means of growth detection. Although the system offers a rapid, non-invasive, non-radioactive method of detection and susceptibility testing of *M. tuberculosis* it does not offer a significant saving in time over the BACTEC system, and some reports indicate that it may actually be less rapid (Pfuffer, 1995).

Recently, a novel phenotypic culture-based drug susceptibility assay that uses mycobacteriophage to identify viable *M. tuberculosis* cells has been developed (Wilson *et al.*, 1997). The PhaB assay (phage amplified biologically) is based on the ability of live tubercle bacilli to protect infecting mycobacteriophage from extracellular chemical inactivation. The protected mycobacteriophage then replicate within viable bacteria and, eventually cause the host mycobacteria to lyse and release a new generation of phage. The released mycobacteriophage are plated on to a lawn of the rapidly growing species *M. smegmatis* and after overnight incubation are detected as plaques on the test plate. Hence, a viable bacterium in the original sample will lead to a plaque on the test plate. Drug susceptibility results of cultured MDR-TB isolates were obtained within 3-4 days and the assay is being further developed for direct testing on patients samples.

1.5.2 Culture independent genotypic methods

Genetic methods for assessing drug resistance in mycobacteria have been developed and offer potential advantages over conventional methods of susceptibility testing of *M. tuberculosis* (Table 1.5) (Cockerill, 1999). Rapid detection of resistance would be produced as clinical samples could be directly tested thus avoiding the need for isolation of the organism by culture and the long wait required by conventional methods due to the slow growth of the organism. In addition, since the organism does not have to be propagated, associated biohazard risks are kept to a minimum.

However, there are disadvantages particular to genetic methods: different assays are required for each antimycobacterial drug tested; the genetic mechanism for resistance needs to be defined; and resistance to a specific antimicrobial agent may occur via different mechanisms associated with different resistance genes. Consequently, new forms of resistance produced by new mutations would not be detected.

Table 1.5: Application of genetic methods for assessing antimicrobial resistance in *M. tuberculosis* (Cockerill, 1999)

Antimicrobial agent	Genetic changes associated with resistance	Examples of reported methods
Isoniazid	Mutations in the catalase-peroxidase <i>katG</i> gene account for 50-70% of resistant strains (30-65% due to a single mutation [S315T])	PCR-SSCP ¹ , PCR-RFLP ² , PCR-CFLP ³ , PCR-DNA sequencing
	Mutations in the <i>inhA</i> locus account for 5-10% of resistant strains	PCR-SSCP, PCR-DNA sequencing
	Mutations in promoter region of <i>ahpC</i> encoding the alkylhydroperoxide reductase gene account for 6-13% of resistant strains	PCR-SSCP, PCR-DNA sequencing
Rifampicin	Mutations in <i>rpoB</i> gene, which encodes the β -subunit of RNA polymerase	PCR-SSCP, PCR-ddF, PCR-HDP analysis, PCR-CFLP, PCR-LiPA, PCR-molecular beacon sequence analysis, PCR-DNA sequencing
Ethambutol	Mutations in <i>embB</i> gene, which encodes polymerization of arabinose into arabinogalactan, account for ~70% of resistant strains	PCR-SSCP, PCR-DNA sequencing
Streptomycin	Mutations in genes encoding 16S rRNA (<i>rrs</i>) and ribosomal protein S12 (<i>rpsL</i>) account for 64-68% of resistant strains	PCR-RFLP, PCR-DNA sequencing
Pyrazinamide	Mutations in the <i>pncA</i> gene, which encodes pyrazinamidase, accounts for ~70% of resistance strains	PCR-DNA sequencing
Fluoroquinolones	Mutations in <i>gyrA</i> gene, which encodes the A subunit of DNA gyrase, accounts for ~100% of resistant strains	PCR-SSCP, PCR-DNA sequencing

¹ single-strand conformation polymorphism; ² restriction fragment length polymorphism analysis; ³ cleavage fragment polymorphism.

1.5.3 Colorimetric and bioluminescent cytological indicators of cellular viability

In the past 20 years a variety of colorimetric and bioluminescent cytological indicators of cellular viability and proliferation have been developed as rapid tests for drug susceptibility of mycobacterial clinical isolates or as high-throughput screens for detecting novel antimycobacterial agents (Table 1.6). Many rely on a metabolic process such as enzymatic activity (esterase, peroxidase) or membrane potential to identify living cells. Viable bacteria contain ion pumps and channels which serve to maintain both the appropriate levels of internal ions and the potential difference across the cell's membranes. Functioning ion pumps are necessary for cell viability.

Fluorescein diacetate (FDA) was first described as a fluorescent staining reagent for determining cytological activity of mycobacterial cells in 1980 (Jarnagin and Luchsinger, 1980). The relatively membrane-permeant FDA enters live and dead cells and is hydrolysed by various esterases to free fluorescein. Fluorescein is retained by cells with intact membranes to yield cytoplasmic green fluorescence. In a series of studies around this time, FDA was used to demonstrate activity in *M. smegmatis* and *M. phlei* (Kvach and Veras, 1982), *M. lepraemurium* (Tsukiyama *et al.*, 1985), *M. leprae* (Mor *et al.*, 1988), *M. w* and *M. tuberculosis* H₃₇R_a (Chitambar *et al.*, 1988). The emphasis of much of this work was development of a drug susceptibility assay for the non-culturable *M. leprae*. Although fluorescence after staining corresponded well with the ability of mycobacteria to multiply in culture, no correspondence was detected when death of the organisms occurred *in vivo*. Recently, there has been renewed interest in the development of a rapid FDA drug susceptibility assay for pathogenic mycobacteria using flow cytometry. Organisms investigated in these studies include *M. tuberculosis* H₃₇R_a (Norden *et al.*, 1995; Kirk *et al.*, 1998) *M. avium*, *M. fortuitum*, *M. gordonae* and *M. marinum* (Bownds *et al.*, 1996).

Table 1.6: Cytological Methods that have been used to estimate mycobacterial “viability” or activity

Method	Assay	Assay Principle	Reference
Enzyme substrates	Fluorescein diacetate	Membrane-permeant fluorescein diacetate is de-esterfied by esterases in live mycobacteria to yield cytoplasmic green fluorescence.	Kvach and Veras, 1982; Tsukiyama <i>et al.</i> , 1985; Chitambar <i>et al.</i> , 1988; Mor <i>et al.</i> , 1988; Jayapal <i>et al.</i> , 1991; Norden <i>et al.</i> , 1995; Bownds <i>et al.</i> , 1996; Kirk <i>et al.</i> , 1998.
Tetrazolium salt reduction	MTT ¹ , NBT ² , NT ³ , INT ⁴	Reduction of MTT by live <i>M. tuberculosis</i> and <i>M. avium-intracellulare</i> to formazan deposits in rapid assay for rifampicin resistance. Cytochemical indicator of activity in <i>M. smegmatis</i> .	Thom <i>et al.</i> , 1993; Gomez-Fores <i>et al.</i> , 1995; Mshana <i>et al.</i> , 1998.
Tellurite reduction	Potassium tellurite	Reduction of potassium tellurite by (non-culturable) <i>M. leprae</i> .	David <i>et al.</i> , 1982; Mor <i>et al.</i> , 1988.
Energisation-sensitive probes	R123	Accumulation of R123 by pathogenic and non-pathogenic mycobacteria with energised cytoplasmic membranes.	Matsuyama, 1984; Bercovier <i>et al.</i> , 1987; Sayles, 1994; Ibrahim, 1996.
Reporter systems	Luciferase	Luciferase reporter mycobacteriophages and luciferase producing strains of <i>M. tuberculosis</i> for rapid testing of tuberculosis drug susceptibility.	Andrew <i>et al.</i> , 1993; Cooksey <i>et al.</i> , 1993; Jacobs <i>et al.</i> , 1993; Gordon <i>et al.</i> , 1996; Cooksey <i>et al.</i> , 1995; Arain <i>et al.</i> , 1996; Shawar <i>et al.</i> , 1997; Carrière <i>et al.</i> , 1997.
	GFP	GFP reporter assays for high-throughput screening of compounds against <i>M. tuberculosis</i>	Dhandayuthapani <i>et al.</i> , 1995; Kremer <i>et al.</i> , 1995; Collins <i>et al.</i> , 1998.
	β-Galactosidase	β-Galactosidase reporter systems in <i>M. smegmatis</i> and <i>M. aurum</i> prepared for rapid screening of antimycobacterial compounds	Barletta <i>et al.</i> , 1992; Murray <i>et al.</i> , 1992

¹ methylthiazolyldiphenyl tetrazolium bromide; ² nitroblue tetrazolium chloride; ³ neotetrazolium chloride; ⁴ iodonitrophenyltetrazolium

Table 1.6 continued over

Table 1.6: Cytological Methods that have been used to estimate mycobacterial “viability” or activity

Method	Assay	Assay Principle	Reference
Passive dye exclusion	Ethidium bromide	Exclusion of nucleic acid labelling ethidium bromide by intact permeability barrier in live bacteria	Kvach and Veras, 1982; Tsukiyama <i>et al.</i> , 1985; Chitambar <i>et al.</i> , 1988; Jayapal <i>et al.</i> , 1991.
Nucleic acid staining	Molecular Probes Inc. SYTO stains	Live-cell permeant nucleic acid stain	Ibrahim, 1996; Ibrahim <i>et al.</i> , 1997.
Ratio of intrabacterial Na ⁺ to K ⁺		Mass spectrometric determination of the ratio of the intrabacterial concentrations of Na ⁺ and K ⁺ of individual bacterial organisms	
Radiolabelling	H ³ -Thymidine, Uracil	Radiometric assay for determination of <i>M. leprae</i> and <i>M. aurum</i> viability and drug susceptibility	
Adenylate energy charges		Monitoring of drug effects of cultivable mycobacteria and <i>M. leprae</i> by determination of AEC	Prioli <i>et al.</i> , 1985
RNA amplification		16S ribosomal RNA detection in <i>M. smegmatis</i> as a rapid assessment of viability	Van der Vliet <i>et al.</i> , 1994.
mRNA PCR		<i>M. leprae</i> 71kD heat shock protein	Patel <i>et al.</i> , 1993.

Rhodamine 123 (R123) is a water soluble, cationic, fluorescent dye, originally used for selectively labelling mitochondria in living cells but has also proved an indicator of cytological activity in a wide range of bacteria including *M. smegmatis*, *M. bovis* BCG and *M. tuberculosis* (Matsuyama, 1984; Resnick *et al.*, 1985; Bercovier *et al.*, 1987). Bacterial accumulation of R123 appears to be non-toxic and does not effect the viability of treated bacteria (Matsuyama, 1984). R123 accumulates down the electrochemical gradient (100-200 mV potential) of live bacteria in an energy dependent fashion. Kaprelyants and Kell (1992) demonstrated that R123 is concentrated in an uncoupler-sensitive fashion, *i.e.* fluorescence is greatly diminished in bacteria that are exposed to uncoupler agents that dissipate the proton gradient across the bacterial cytoplasmic membrane. Bercovier *et al.*, (1987) demonstrated that R123 could be used to measure the susceptibility of *M. tuberculosis* H₃₇R_a and *M. bovis* BCG to rifampicin in as short a time as 24 hours. Work in this laboratory has shown that R123 can be used as an indicator of activity in a variety of live pathogenic mycobacteria including clinical isolates of *M. tuberculosis* and *M. avium* (D.R. Walker and M.R. Barer, unpublished data; Sayles, 1994; Ibrahim, 1996).

Double staining methods may be used to assess the viability of cells by staining dead cells with one dye and live cells with another dye of different emission (López-Amorós *et al.*, 1995). Dyes that are highly impermeant to membranes will stain only cells that are dead or have compromised membranes. The most commonly used stains for identifying dead cells are cell-impermeant DNA stains, although any cell impermeant dye can potentially be used. Ethidium bromide was used in many of the mycobacterial FDA studies to identify dead cells (Kvach and Veras, 1982; Matsuo *et al.*, 1984; and Chitambar *et al.*, 1988). Propidium iodide was used to identify dead mycobacteria by D.R. Walker and M.R. Barer (unpublished data), Sayles, (1994) and Ibrahim, (1996).

Tetrazolium salts have been used extensively as cytochemical indicators of oxidative metabolism in a variety of bacteria including mycobacteria (Thom *et al.*, 1993). These agents capture electrons from oxidative metabolism and produce a localised formazan

deposit that can be detected by light microscopy. The relationship between capacity for tetrazolium salt reduction and cell viability is not clear but offers a rapid, culture-independent means of detecting microbial activity and relative rate of reducing equivalent production in micro-organisms. Gomez-Flores *et al.*, (1995) developed a colorimetric assay for drug susceptibility testing of *M. avium* complex based on the ability of live bacilli to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan. More recently, Mshana *et al.*, (1998) described a rapid test that used the ability of live *M. tuberculosis* to reduce MTT to detect resistance to rifampicin. Both studies concluded that the inhibition of MTT reduction paralleled the reduction in the number of colony forming units.

The reduction of potassium tellurite was used to investigate the effects of several substrates on the respiratory activity of *M. aurum*, *M. smegmatis* and *M. avium* (David *et al.*, 1982) and to detect activity in *M. lepraemurium* and *M. leprae* (Mor *et al.*, 1988). However, although there appeared to be correspondence between tellurite reduction for *M. lepraemurium in vitro*, correspondence with tellurite-reduction was not observed when death of *M. lepraemurium* occurred *in vivo*.

Jacobs and colleagues have demonstrated that drug susceptibility of tubercle bacilli in clinical samples could be measured with genetically modified mycobacteriophages containing the firefly *lux* or luciferase gene (Jacobs *et al.*, 1993; Cooksey, 1993, 1995). Drug-resistant mycobacteria growing in the presence of these drugs are able to express luciferase, which catalyses the reaction of luciferin with ATP to generate photons of light that can be detected in a luminometer, while mycobacteria sensitive to the drug die, no ATP is produced, and there is a cessation of light production. To date, this method has not proved sensitive and requires an expensive luminometer (Wilson *et al.*, 1997). Luciferase plasmid-based systems have also been developed as high-throughput screens for antimycobacterial drugs. Mycobacteria investigated include *M. smegmatis*, *M. avium*, *M. intracellulare*, *M. bovis* BCG and *M. tuberculosis* (Cooksey *et al.*, 1993; Andrew and Roberts, 1993; Cooksey *et al.*, 1995; Hickey *et al.*, 1996; Shawar *et al.*, 1997).

Systems for high-throughput screening of antimycobacterial drugs using *Aequorea victoria* (jellyfish) green fluorescent protein (GFP) as reporter molecule have also been developed. *Aequorea* GFP fluorescence does not require substrates for fluorescence as the active chromophore is generated by the spontaneous cyclisation and oxidation of a serine-glycine trimer within a defined hexapeptide sequence of the protein. Kremer *et al.*, (1995) demonstrated that exposure of *M. bovis* BCG to antimycobacterial drugs produced a decrease in fluorescence, while fluorescence increased in drug-free controls. More recently, a GFP reporter microplate assay for screening compounds against *M. tuberculosis* was described (Collins *et al.*, 1998).

1.6 INTRODUCTION TO LIGHT MICROSCOPIC CYTOLOGICAL ANALYSES

A central feature of this study has been the application of light microscope based techniques established in this laboratory to the investigation of mycobacterial physiology at the cellular level. Single cell cytological investigations of the types described in Section 1.5.3 can readily identify metabolically active but non-culturable cells in populations without significant cell division and thus reveal a great deal about their phenotypes. In addition, cytological analyses provide the opportunity to determine whether such phenotypes are heterogeneously distributed within a cell population and whether expression of the specific property is dependent on a spatial relationship. Investigations of the slowly growing pathogenic *M. tuberculosis* using light microscopy also offers the advantage that small quantities of culture are sufficient for most investigations (*e.g.* a few milliliters of cell suspension containing 10^7 cells ml⁻¹).

1.7 AIMS AND OBJECTIVES

The overall aim of this study was to investigate the effects of antimycobacterial drugs on *M. tuberculosis* at the single cell level using cytochemical indicators of cellular activity. The purpose was to learn more about the physiological response of *M. tuberculosis* to antimycobacterial drugs and therefore to understand better the properties which render the organism refractory to eradication and produce the characteristic slow response to chemotherapy (6-9 months).

The specific objectives were:

1. To establish a culture method for *M. tuberculosis* that would be suitable for both cytochemical labelling and antimycobacterial drug studies.
2. To evaluate and develop methods to study cytological activity in *M. tuberculosis* suitable for light microscopy and quantitative image analysis.
3. To investigate the effects of antimycobacterial drugs on *M. tuberculosis* using the techniques developed and to compare the results to those from conventional culture techniques.
4. To assess what relevance the results of these experiments would have on the chemotherapy of tuberculosis.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS, PLASMIDS AND MYCOBACTERIOPHAGE

The bacterial strains, plasmids and mycobacteriophage used in this study are summarised in Table 2.1.

Table 2.1: Bacterial strains, plasmids, and mycobacteriophages used in this study

	Description	Reference/source
<u>Bacterial strains</u>		
<i>Mycobacterium tuberculosis</i> H ₃₇ R _v	Type strain	NCTC ¹ 10942
<i>Mycobacterium smegmatis</i> mc ² 155	<i>M. smegmatis</i> strain with high transformation phenotype	Snapper <i>et al.</i> , 1990
<i>Escherichia coli</i> DH5α	<i>SupE44 hsdR17 recA1 thi-1 ΔlacU169(ϕ80/lacZΔM15) endA1 gyrA96 relA1</i>	Hanahan <i>et al.</i> , 1983
<u>Plasmids</u>		
pMV261	Mycobacterial cloning vector, Kan ^R	Stover <i>et al.</i> , 1991
pGFP	GFP cDNA source on pUC19 derivative	Clontech Laboratories ²
PGFM11	GFP-expressing mycobacterial cloning vector, Kan ^R Strep ^R	Kremer <i>et al.</i> , 1995
<u>Mycobacteriophage</u>		
D29	Lytic mycobacteriophage, infects both fast- and slow-growing mycobacteria	Kindly provided by Dr. N.G. Stoker ³

¹ National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London.

² Clontech Laboratories Inc., 4030 Fabian Way, Palo Alto, CA 94303-4607, USA; <http://GFP.CLONTECH.COM>.

³ Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London.

SAFETY

M. tuberculosis is a hazardous pathogen, therefore all the work described here was done in a containment level 3 laboratory in compliance with established advice (Advisory Committee on Dangerous Pathogens, 1995).

2.2 GENERAL LABORATORY REAGENTS AND CULTURE MEDIA

Unless otherwise stated, chemicals were obtained from Sigma-Aldrich Company Limited (Poole, Dorset, UK).

Mycobacterial strains were grown on Middlebrook 7H10 agar (Difco Laboratories, Detroit, USA) containing 0.5% (v/v) glycerol and 10% oleic acid-albumin-dextrose-catalase supplement (OADC), or in Middlebrook 7H9 broth (Difco) containing 0.2% (v/v) glycerol, 0.05% (w/v) Tween 80 and 10% albumin-dextrose-catalase (ADC) supplement. Mycobacteria transformed with *E. coli*-mycobacteria shuttle vectors were grown in the presence of kanamycin at 25 µg ml⁻¹. Mycobacteria for mycobacteriophage studies were grown in Tween 80-free media.

Middlebrook culture media were prepared according to the manufacturer's instructions and sterilised by autoclaving at 121°C at 15 psi for 15 min. The final pH of media was 6.6 ± 0.2 at 25°C. Prepared media were kept in the dark and used within a week.

Supplements were bought directly from Difco or prepared fortnightly in the laboratory. ADC supplement was prepared from 12.5 g bovine albumin fraction V, 5.0 g glucose, and 10.0 mg catalase (from bovine liver, thymol-free) made up to 250 ml in distilled water. OADC supplement was prepared from 12.5 g bovine albumin fraction V, 5.0 g glucose, 0.85 g NaCl, 0.05 g oleic acid (1% solution prepared in 0.2 N NaOH), and 10.0 mg catalase made up to 250 ml in distilled water. When dissolved, supplements were filter-sterilised, incubated overnight to check for contaminants and stored at 4°C for up to 2 weeks. Supplements were aseptically added to media after autoclaving.

2.3 STOCK CULTURES

M. tuberculosis H₃₇R_v was obtained directly as freeze dried culture from the National Collection of Type Cultures, Colindale, London, UK. A large seed pool of inoculum was prepared by subculturing freeze-dried culture through two passages of Middlebrook 7H9 broth. The second culture was divided into 1 ml aliquots and stored at -70°C in clearly labelled double sealed storage containers. *M. smegmatis* and *E. coli* were stored at -70°C in media containing 10% (v/v) glycerol as a cryoprotectant.

The Bacto TB Fluorescent Stain Set T (Difco) was used to confirm the purity of *M. tuberculosis* cultures during this study. Bacterial suspensions for acid-fast staining were smeared onto glass microscope slides, allowed to air dry, and heat fixed by passing the slide through the blue cone of a Bunsen flame three or four times. Heat fixed samples were stained with Bacto TB Auramine-Rhodamine T for 20 min at room temperature, washed with Bacto TB Decolouriser for 2 min and counterstained with Bacto TB Potassium Permanganate for 4 min. Slides were rinsed thoroughly with water after each treatment. After staining, slides were air dried and mounted in Citifluor immersion oil (Agar Scientific Limited, Essex, UK). Acid-fast bacteria were brightly fluorescent on a dark background when examined by epifluorescence microscopy.

2.4 GROWTH STUDIES

2.4.1 Growth in liquid medium

A 250 ml baffled polycarbonate conical flask (Nalgene, Fisher, Loughborough, UK) containing 50 ml of Middlebrook 7H9 broth was inoculated with 1 ml of thawed *M. tuberculosis* stock and incubated for one week at 37°C in a rotary shaker-incubator at 180 rpm. A sufficient amount of this first passage culture was added to 100 ml of Middlebrook 7H9 broth to yield a calculated optical density at 580 nm of 0.002. This culture was incubated at 37°C in a rotary shaker-incubator at 180 rpm, from which samples were taken daily for colony counts and optical density measurement.

In one experiment, growth in liquid medium was assessed for comparison using the methods of Wayne (1994) and Mitchison and colleagues (Herbert *et al*, 1996). With the first method, *M. tuberculosis* culture was prepared as described but aerated with 50-mm Teflon-coated magnetic stirring bars in flasks (70 rpm) on magnetic stirrers (Heidolph MR 1000, Kelheim, Germany). With the second method, *M. tuberculosis* was grown in 10 ml of Middlebrook 7H9 broth in unshaken tubes for 7 days at 37°C. The total number of bacilli ml⁻¹ in this culture was determined in a Thoma chamber (see Section 2.7), and appropriate amounts were inoculated into 250 ml flasks containing 100 ml of Middlebrook 7H9 broth to give 10⁵ bacilli ml⁻¹. Cultures were incubated unshaken at 37°C and sampled after 3 days growth.

2.4.2 Enumeration of colony forming units

Enumeration of colony forming units (CFU) was by a drop-plate modification of the Miles and Misra procedure (Hoben and Somasegaran, 1982). Serial tenfold dilutions of broth culture were prepared in Middlebrook 7H9 broth (containing Tween 80 but not supplement). Three 20 µl samples from each dilution were plated out on duplicate pre-dried Middlebrook 7H10 agar plates. Agar plates for colony counts were poured thickly

(25 ml) in 9 mm petri dishes to prevent desiccation during lengthy incubations. When the drops had dried, the plates were sealed with laboratory sealing film (Whatman, Maidstone, UK) and incubated, inverted in sealed plastic bags at 37°C. Colonies were counted weekly using a dissecting microscope (x 40 magnification) until counts had stabilised. Dilutions that yielded final counts of between 10 and 100 colonies were used to calculate the original number of CFUs.

2.4.3 Optical density measurements

Turbidity was determined by measuring the optical density (OD) of culture in a colorimeter (Jenway PCO1 – Jenway Ltd., Dunmow, Essex, UK) at a wavelength of 580 nm. Each measurement was made by transferring the growth culture into a 1.5 ml cuvette, which was sealed with laboratory sealing film and secured with tape. Where necessary, cultures were diluted to ODs in the range of 0.05-0.5. The OD measurement of the diluted culture was then multiplied by the dilution factor to give the actual OD.

2.4.4 Preparation of inocula from agar

A 50 ml culture of *M. tuberculosis* in Middlebrook 7H9 broth was prepared from frozen stock as described in Section 2.4.1. Pre-dried Middlebrook 7H10 agar plates were inoculated with 100 µl of subculture using the spread plate method. The plates were allowed to stand at room temperature for the inoculum to dry, sealed with laboratory sealing film and incubated in closed plastic bags at 37°C for 6-8 days. By this time the culture had grown to a sub-confluent lawn. One loopful of mycobacterial growth was transferred to a sterile glass tube and gently homogenised with the rounded end of a sterile glass rod until a smooth paste was obtained. Two 50 µl portions of Middlebrook 7H9 broth were added during homogenisation so that an even suspension was produced; the volume was made up to 1 ml and thoroughly mixed. Mycobacterial suspension prepared in this way, and adjusted to OD_{580nm} of 0.2 yielded between 5.0×10^7 and 1.0×10^8 CFU ml⁻¹.

2.5 MICROSCOPY

Bacteria were immobilised for cell counting and to study their cytochemical properties at the single cell level. Previous work in this laboratory has employed a procedure involving 3-aminopropyltriethoxysilane (APS) coated coverslips (Barer and Entwistle, 1991). The combination of APS-coated glass and centrifugation results in immobilised live cells to which analytical procedures may be applied.

2.5.1 Aminopropyltriethoxysilane (APS) coating of coverslips

APS-coated coverslips were prepared by leaving acetone-washed 19 mm circular coverslips (BDH, Poole, Dorset, UK) in a 2-3% (v/v) solution of APS in acetone overnight at room temperature. The following day the coverslips were washed twice in acetone, rinsed thoroughly in distilled water, gently blotted with tissue paper and dried at 37°C. The APS-coated face was marked with permanent pen. Coverslips prepared in this way from fresh APS were stored in clean petri dishes and used for up to one month.

2.5.2 Preparation of bacterial monolayers

Cells were immobilised as a 'monolayer' on APS-coated coverslips by centrifugation using the microchamber system developed by Walker *et al.*, (1994) (Figure 2.1). The silicone blocks with four sample wells and the teflon packing unit were made to specification for this laboratory by RAPRA Technology Limited (Billingham, Cleveland, UK; <http://www.rapra.co.uk>) and designed to fit in 30 ml disposable universals (Bibby Sterilin Limited, UK).

Thirty microliters of cell suspension were added to each well and immobilised onto APS-coated coverslips by centrifugation for 5 min at 1,000 x g. For pathogenic live mycobacteria the complete microchamber was contained in sealed centrifuge buckets. The supernatant was gently removed and the chamber assembly dismantled, taking care

not to interfere with the deposited monolayers. Cytological labelling procedures were done before or after cell deposition as indicated. Coverslips were mounted, cell deposits face down, on microscope slides in an appropriate mountant (*e.g.* 20 μ l silicone fluid, BDH) and sealed with clear nail varnish. The location of monolayers was marked with permanent pen so that they were easily located during microscopy.

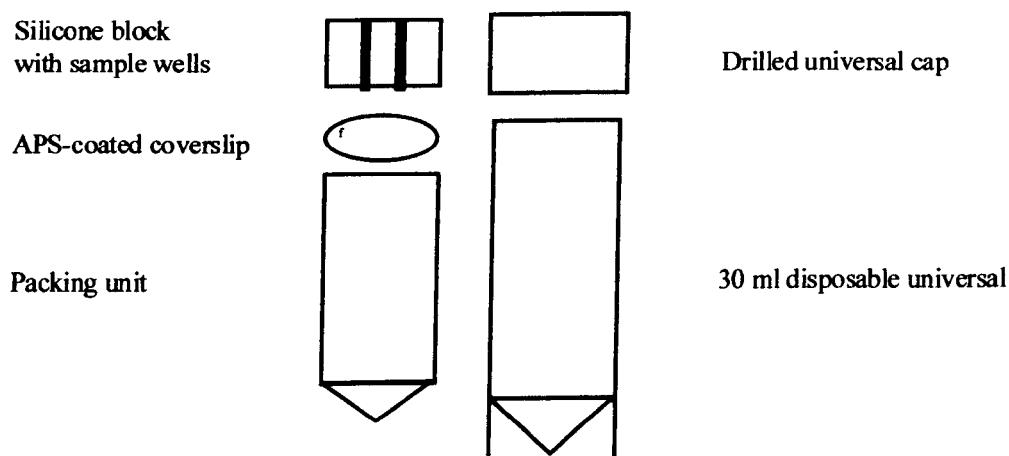


Figure 2.1: Microchamber system used for the production of bacterial monolayers

The monolayers were observed using phase contrast, brightfield and/or epifluorescence microscopy as appropriate. Silicone blocks and packing units were autoclaved for reuse, drilled universal caps and centrifuge pots were left overnight in 70% ethanol, and the 30 ml disposable universals were discarded.

2.5.3 Microscopy and recording of images

Mounted cell preparations were viewed using a Nikon DIAPHOT-300 inverted microscope (Nikon UK Limited, Surrey, UK) equipped with standard epifluorescence attachments and a 100 W high-pressure mercury-vapour light source. Images were recorded using colour charge-coupled device (CCD) cameras (Sony DXC 390, Optivision Limited, York, UK or JVC KY-S55B, Foster Findlay and Associates, Newcastle upon Tyne, UK) and stored at 24-bit resolution in standard tagged image file format (*.TIF). Camera fields of view were calibrated using a stage micrometer (Graticules Limited, Tonbridge, Kent, UK). The complete system as installed is displayed in Figure 2.2.

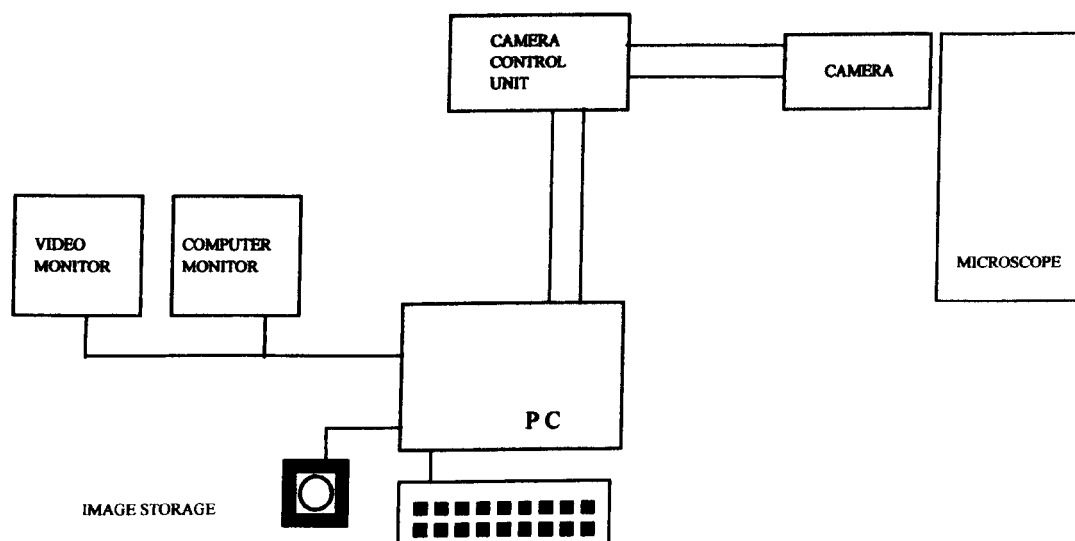


Figure 2.2: Arrangement of equipment used for the microscopy of samples and recording of images.

The spectral characteristics of the fluorophores used in the study and the appropriate filter sets used to examine the fluorescence of each are shown in Table 2.2. Citifluor immersion oil was used with oil-immersion lenses to keep background fluorescence a minimum.

Table 2.2: Characteristics of fluorescent compounds and filter sets used for viewing fluorescence

	Fluorophore characteristics ¹		Filter set used ²
	Excitation maximum (nm)	Emission maximum (nm)	
Rhodamine 123 (R123)	507	529	FITC/B-2A
Propidium iodide (PI)	535	617	B-2A
Ethidium homodimer	528	617	B-2A
Auramine O-Rhodamine B	460/540	550/625	B-2A
Green fluorescent protein (GFP)	395	509	FITC
Cyanoditotyl tetrazolium chloride (CTC)	450	630	B-2A
4',6-diamindino-2-phenylindole (DAPI)	358	461	UV-2A
Bis-(1,3-dibutylbarbituric acid) trimethine oxonal	493	516	B-2A
Live/Dead <i>Bac</i> LIGHT stain ³ , Reagent A	NA	NA	B-2A
Live/Dead <i>Bac</i> LIGHT stain, Reagent B	535	617	B-2A

¹ Spectral characteristics obtained from Molecular Probes Inc., Eugene, Oregon, USA (<http://www.probes.com>) and Polysciences Inc., Warrington, PA 18976, USA (<http://www.polysciences.com>).

² FITC specific filter block (excitation filter 420-490, barrier filter 530 ± 10, dichroic mirror 510); Nikon B-2A filter block (excitation filter 450-490, barrier filter 520-560, dichroic mirror 510); Nikon UV-2A filter block (excitation filter 330-380, barrier filter 420, dichroic mirror 400).

³ LIVE/DEAD *Bac*Light bacterial viability consists of two nucleic acid stains. Reagent A is SYTO9 whose spectral characteristics are not available (NA), Reagent B is propidium iodide.

2.6 IMAGE ANALYSIS

Images acquired by microscopy were analysed using an in house developed, image analysis programme, 'Alpha' (Adaptrix Limited, Gateshead, Tyne and Wear, UK). The protocol for the analysis of paired mask and data images adopted during the present study is outlined below. The mask image was usually a phase contrast image from which binary masks of the profiles of cells for analysis were created. The binary mask provided the morphometric data. The data image was the corresponding fluorescence or brightfield-illuminated image from which densitometric or fluorometric measurements were made. A more detailed description of image analysis using an earlier version of this software is given by Whiteley *et al.*, (1998).

Step 1 Pre-processing mask image. Mask and data image pairs were loaded together (Figure 2.3a). High quality mask images were created for accurate thresholding by applying contrast enhancement and smoothing functions (*e.g.* mean, median and gaussian filters) (Figure 2.3b).

Step 2 Thresholding. Binary images that closely represented the underlying image were created by thresholding (Figure 2.3c). Subsequent binary processing was usually necessary to create binary images that described the cells in the underlying image more precisely. These binary operators included opening, closing, erosion, dilation, thickening and thinning (Figure 2.3d).

Step 3 Cell browsing and classification. Objects were then either accepted (valid cell), rejected (classified as not being a cell), and if clumped/clustered split as precisely as possible. If necessary, binary alignment for each object was performed to ensure that the origin of the object in the mask image corresponded to the origin of the object in the data image (Figure 2.3e).

(a)



(b)



Figure 2.3: Single cell image analysis using 'Alpha' software.

Windows show (a) loaded mask and data paired images, (b) mask image processing, (c) binary creation, (d) binary processing, (e) cell browsing and detection. Figure 2.3 continued over page.

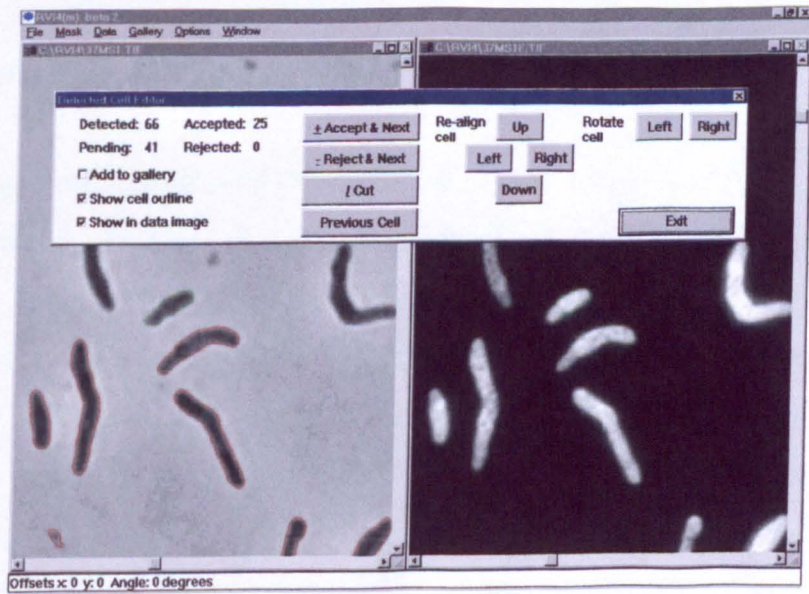
(c)



(d)



(e)



Step 4 Cell measurement. Once the browsing classification step was complete the morphometric and intensity data were saved to disc as comma-separated text files (*.prn files).

Step 5 Data export. Comma-separated text files were analysed using spreadsheet, statistical and graph-plotting computer programmes (*e.g.* Quattro Pro, Borland International, Inc.).

Morphometric measurements obtained by image analysis included length, breath, area and perimeter of cells. Densitometric measurements included integrated optical density (IOD) (sum of the optical weights of all pixels in an object) and mean optical density (MOD) (IOD divided by the number of pixels in object). Fluorometric measurements included integrated grey level (IGL) (sum of grey level values of all pixels in an object) and mean grey level (MGL) (IGL divided by the number of pixels in object).

2.7 TOTAL CELL COUNTS (TCC)

Three methods of microscopic enumeration of cells were compared, a Thoma chamber, membrane filtration and staining, and phase contrast microscopy of bacteria immobilised on APS-coated coverslips. Samples for cell counts were mixed with equal volumes of 8% formaldehyde to inactivate the mycobacteria.

Counting chamber method (Koch, 1994)

A Thoma ruled counting chamber (Hawksley Glass Limited, Lansing, UK) was filled with approximately 5 µl of bacterial suspension. The slide was viewed by phase contrast microscopy (x 40 magnification) and sufficient squares were examined to ensure that more than 300 individual cells were counted. The total number of cells ml⁻¹ of undiluted culture was calculated by use of the following formula:

$$\frac{\text{total bacteria counted} \times \text{dilution factor} \times 4 \times 10^8}{\text{number of small squares counted} \times \text{filling depth (in micrometers)}}$$

4',6-diamindino-2-phenylindole (DAPI) membrane filter method (Kepner and Pratt, 1994)

A stock solution of 33 µg ml⁻¹ DAPI was prepared in SDW and stored at 4°C in the dark.

One hundred microliters of DAPI staining solution, 100 µl of mycobacterial suspension and 800 µl SDW were mixed in a 1.5 ml centrifuge tube and incubated for 12 min in the dark at room temperature. Thirty microliters of well-mixed labelled cells were filtered onto 13 mm Nucleopore Polycarbonate filters (Costar Scientific Corporation, Bucks., UK) using a Millipore Swinney Stainless 13 mm filter holder (Millipore Corp., Massachusetts, USA). Filters were briefly dried, mounted on microscope slides between drops of citifluor immersion oil and covered with coverslips. Cells were viewed by

epifluorescence microscopy and sufficient fields were examined to ensure more than 200 bacteria were counted. The total number of cells ml⁻¹ of undiluted culture was calculated by use of the following formula:

$$\frac{(\text{total bacteria counted/number of fields}) \times (\text{area of filter/area of field}) \times \text{dilution factor}}{3 \times 10^{-2}}$$

Direct counting of immobilised bacteria on APS-coated coverslips (Gibbon, 1996)

Thirty microliters of well-mixed bacterial suspension were immobilised onto APS-coated coverslips by centrifugation and mounted on microscope slides as described in Section 2.5.2. Monolayers were viewed by phase contrast microscopy and after confirming an even distribution of cells; sufficient fields were counted to ensure that at least 500 cells were counted. The total number of cells ml⁻¹ of undiluted culture was calculated by use of the following formula:

$$\frac{(\text{total bacteria counted/number of fields}) \times (\text{area of monolayer/area of field}) \times \text{dilution factor}}{3 \times 10^{-2}}$$

2.8 METHODS USED TO ASSESS CELLULAR ACTIVITY

2.8.1 Rhodamine 123 - Propidium Iodide labelling

A stock solution of 6.5 mM rhodamine 123 (R123) was prepared in dimethyl sulphoxide (DMSO) and stored at -20°C. A working solution of 50 µM was prepared in phosphate buffered saline (PBS) from completely thawed stock on each day of use.

PBS was prepared by dissolving NaCl 8.0 g, KCl 0.2 g, Na₂HPO₄ 1.15 g, KH₂PO₄ 0.2 g in distilled water and making up to 1 L. The pH was adjusted to 7.3 prior to autoclaving.

A stock solution of 7.5 mM propidium iodide (PI) was prepared in DMSO and stored at -20°C. A working solution of 150 µM was prepared in PBS from completely thawed stock on each day of use.

Forty eight microliters of cell suspension was placed in a screw-topped 1.5 ml centrifuge tube. One microliter of R123 and PI working solutions were added to give final concentrations of 1 µM and 3 µM respectively. Suspensions were thoroughly mixed and incubated in a 37°C water-bath for 15 min. Bacteria were harvested by centrifugation (10,000 x g for 2 min), washed twice with PBS and resuspended in SDW. Bacterial monolayers were prepared and mounted on silicone oil as described in Section 2.5.2.

When large numbers of samples were being processed it was more convenient to immobilise the bacteria prior to labelling. Thirty microliters of cell suspension was immobilised onto APS-coated coverslips by centrifugation. The coverslips were placed in 5 cm petri dishes (Bibby Sterilin Limited, UK) and covered with 5 ml of PBS containing the appropriate final concentrations of R123 and PI. After 15 min, the labelling solution was removed and the coverslips gently washed twice with PBS and once with SDW. Coverslips were mounted on silicone oil.

The cells were viewed by phase contrast and epifluorescence microscopy.

2.8.2 Uncoupler sensitivity studies

A fresh stock solution of 7.5 mM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was prepared in ethanol when required.

One microliter of stock solution was added to 49 μ l of bacterial suspension in a 1.5 ml screw-topped centrifuge tube to give a final CCCP concentration of 150 μ M. Suspensions were thoroughly mixed and incubated in a 37°C water-bath for 15 min. CCCP-treated bacteria were labelled with R123/PI as described. In experiments to show the uncoupler effect was reversible, CCCP-treated bacteria were harvested by centrifugation (10,000 x g for 2 min), washed twice with uncoupler-free Middlebrook 7H9 broth and allowed to recover in a 37°C water-bath for 15 min before labelling with R123/PI.

2.8.3 Valinomycin studies

A stock solution of 1 mM valinomycin was prepared in DMSO and stored at -20°C.

Bacterial cultures were treated with valinomycin and labelled with R123 using an adaptation of the method by Porter *et al.*, (1995). Cultures were washed twice in PBS before labelling, valinomycin was added at a final concentration of 5 μ M and bacteria were labelled with R123/PI as described in Section 2.8.1.

2.8.4 Tetrazolium salt reactions

Iodonitrophenyl tetrazolium chloride (INT)

A fresh stock solution of 10 mM INT was prepared in SDW. INT was completely dissolved if the suspension was heated to 55°C and briefly sonicated (IKASONIC U 50, IKA Labortechnik, Staufen, Germany). A 2 mM working solution was prepared by dilution in PBS and sterilised by filtration through a 0.22 µm pore membrane.

Mycobacterial suspensions for INT labelling were prepared in sterile PBS and adjusted to an OD_{580nm} of 0.4. Equal volumes of bacterial suspension and INT working solution were mixed and incubated at 37°C for one hour. Cells were harvested by centrifugation (10,000 x g for 2 min), washed twice with PBS and immobilised onto APS-coated coverslips. Coverslips were mounted in PBS and the edges sealed with clear nail varnish. Formazan deposits were observed using brightfield microscopy.

Substrate-enhanced tetrazolium reduction (Gribbon and Barer, 1995)

Working solutions of 1.0 M aspartate, α-ketoglutarate, pyruvate, cellobiose, malate, serine, fumarate, citrate, succinate, glucose and glutamate were prepared in distilled water. The solutions were adjusted to pH 7.0, sterilised by filtration through 0.22 µm pore membranes and stored at 4°C.

Mycobacterial suspensions for substrate-enhanced INT reduction were prepared in PBS as described. Equal volumes of bacterial suspension and appropriate INT solution supplemented with 100 mM substrate were mixed to give a final reaction concentration of 50 mM substrate. Reaction tubes were mixed thoroughly and incubated at 37°C for 60 min. Bacteria were harvested, washed, immobilised onto APS-coated coverslips and mounted in PBS. Formazan deposits were observed using brightfield microscopy.

Cyanoditoly tetrazolium chloride (CTC)

A stock solution of 20 mM CTC (Polysciences, Inc.) was prepared in sterile PBS. The solution was dispensed into 50 µl aliquots and stored at -20°C. A working solution of 2 mM was prepared in sterile PBS when required.

Bacteria for labelling were harvested by centrifugation (10,000 x g for 2 min), resuspended in 2 mM CTC working solution and incubated at 37°C for 30 min. After incubation, cells were washed with PBS, resuspended in SDW and immobilised onto APS-coated coverslips. When the monolayers were dry, the coverslips were mounted in silicone fluid and sealed with clear nail varnish. CTC deposits were observed by epifluorescence microscopy.

2.8.5 Combined R123/PI labelling and INT reduction assays

The INT reduction assay was combined with R123/PI labelling to further investigate the cytological activity of *M. tuberculosis* at the single cell level. In these experiments, R123 and PI were added for the last 15 min of the INT step incubation at final concentrations of 1 µM and 3 µM respectively. Bacteria were harvested by centrifugation (10,000 x g, 2 min), washed in PBS and resuspended in SDW. Bacterial monolayers were prepared as described and mounted in silicone fluid on a microscope slide. Cells were observed by epifluorescence and brightfield microscopy.

2.8.6 Live/Dead BacLIGHT viability stains

M. tuberculosis was labelled with the Live/Dead BacLight viability stains (Molecular Probes, Inc.) according to the manufacturer's protocol. In summary, mycobacterial suspensions were washed with PBS and resuspended in SDW to an OD_{580nm} of 0.2. Equal volumes of reagents A and B were combined and mixed thoroughly. Three microliters of this solution were added to 1 ml of bacterial suspension in 1.5 ml

centrifuge tubes and incubated for 15 min, at room temperature in the dark. Bacteria were immobilised onto APS-coated coverslips as described, gently washed with SDW and mounted in silicone fluid. Labelled cells were observed by epifluorescence microscopy.

2.8.7 Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄) labelling

M. tuberculosis was labelled with DiBAC₄ using an adaptation of the methods described by Mason *et al.*, (1995) and Jepras *et al.*, (1997). A stock solution of 5 mM DiBAC₄ (Molecular Probes, Inc.) was prepared in DMSO and stored at -20°C. Fresh working stocks of 50 µM were prepared in PBS when required.

Mycobacterial suspensions for labelling were prepared in PBS and adjusted to an OD_{580nm} of 0.2. One microliter of oxonol working stock was added to 49 µl of bacterial suspension in 1.5 ml centrifuge tubes to give a final concentration of 1 µM. Reaction tubes were thoroughly mixed and incubated at 37°C for 15 min in the dark. Bacteria were immobilised onto APS-coated cover slips by centrifugation as described, washed gently with PBS and water and mounted in silicone fluid. Cells were viewed by phase contrast and epifluorescence microscopy.

2.8.8 SYTO16 live cell nucleic acid stain

A working solution of 250 µM SYTO16 (Molecular Probes, Inc.) was prepared in SDW from the 1 mM solution supplied and stored at -20°C.

M. tuberculosis cell suspensions were labelled using the method of Ibrahim *et al.*, 1997. One microliter of SYTO16 working solution was added to 50 µL of cell suspension in SDW in 1.5 mL centrifuge tubes to give a final concentration of 5 µM. The cell mixture was briefly vortexed and incubated for 10 min at 37°C. Cells were harvested by centrifugation at 10,000 x g for 2 min, washed once with SDW and resuspended in SDW.

Cells were immobilised onto APS-coated coverslips by centrifugation and mounted in silicone fluid as described previously. Cells were viewed by phase contrast and epifluorescence microscopy.

2.8.9 Effect of cytological assays on colony counts

The effect of routinely used cytological assays on mycobacterial colony counts was investigated. Mycobacterial suspensions were treated with R123, PI, CCCP and INT using conditions described in their staining procedures. Samples were taken for enumeration of colony forming units as described in Section 2.4.2 and compared to those from untreated controls.

2.8.10 Heat, formaldehyde and ethanol treatments

Cytochemical labelling was used to study the effect of heat, formaldehyde and ethanol on cultures of *M. tuberculosis*.

Heat treatment: Five milliliters of mycobacterial suspension in screw-capped pyrex test tubes (20 x 125 mm) were heat treated for 30 min in a 70°C water bath. The tubes were mixed thoroughly once or twice during heat treatment and afterwards were cooled to 37°C for at least 2 hours before being utilised for staining.

Formaldehyde treatment: Fresh 8% para-formaldehyde was prepared by dissolving 8 g para-formaldehyde in 100 ml PBS heated to 60°C on a heated magnetic stirrer and cooled to room temperature before use. Equal volumes of mycobacterial suspension and 8% formaldehyde solution were mixed thoroughly and incubated for 3 h or overnight.

Ethanol treatment: Mycobacteria were harvested by centrifugation (4,000 x g for 5 min) and resuspended in an equal volume of 70% ethanol for 5 min.

Formaldehyde and ethanol treated bacteria were harvested by centrifugation (4,000 x g for 5 min), washed twice with fresh broth and resuspended in an equal volume of broth before being utilised for staining. Samples of mycobacteria after each of the bactericidal treatments were taken for colony counts and cytochemical labelling.

2.9 TREATMENT WITH ANTIMYCOBACTERIAL DRUGS

2.9.1 Antimycobacterial drugs

The antimycobacterial drugs tested in the study were isoniazid, rifampicin, ethambutol HCl, streptomycin sulphate and capreomycin. Antibiotics were purchased from Sigma Chemical Company and stored as recommended by the manufacturer. Stock solutions were prepared according to Inderlied, (1991), sterilised by filtration through 0.22 μm pore membranes and stored at -70°C for up to 12 months. The diluents used and the stock concentrations are summarised in Table 2.3.

Table 2.3: Preparation of stock, working stock and final concentrations of antimycobacterial drugs

Drug	Solvent ^a	Diluent	Stock Conc ($\mu\text{g ml}^{-1}$)	Working Conc ^b ($\mu\text{g ml}^{-1}$)	Final Conc ^c ($\mu\text{g ml}^{-1}$)
Rifampicin	DMSO	SDW	10,000	2.0	1.0
Isoniazid	SDW	SDW	10,000	0.4	0.2
Streptomycin	SDW	SDW	10,000	4.0	2.0
Ethambutol	SDW	SDW	10,000	12.0	6.0
Capreomycin	SDW	SDW	10,000	20.0	10.0

^a Rifampicin was dissolved in DMSO and decimally diluted in SDW before sterilisation by filtration.

^b "Working Concentration" was prepared by dilution in Middlebrook 7H9 broth.

^c "Final Concentration" was obtained by mixing equal volumes of "Working Concentration" and mycobacterial suspension.

2.9.2 Drug exposure protocol

Inocula for assessment of antimycobacterial activity were prepared from sub-confluent lawns of *M. tuberculosis* cultured on Middlebrook 7H10 agar as described in Section 2.4.4, adjusted to an $\text{OD}_{580\text{nm}}$ of 0.4 in Middlebrook 7H9 broth and distributed into 5 ml aliquots in sterile screw-capped tubes (20 x 125 mm). Equal volumes of mycobacterial suspension and broth containing the 'Working Concentration(s)' of the drug(s) to be tested were mixed to yield the final drug concentration(s) noted in Table 2.3. Drug-free control cultures were included in each assay.

Cultures were incubated without shaking at 37°C for 7 days and were thoroughly mixed before sampling. Daily samples were taken for colony counts and cytochemical labelling. Samples were taken at the end of the assay for TCC enumeration and Most Probable Number (MPN) estimation.

2.9.3 Most Probable Number (MPN) estimates

The MPN and colony count methods for estimating the number of propagules of *M. tuberculosis* in antimycobacterial drug treated cultures were compared. A five-tube, ten-dilution MPN protocol was used. Diluent tubes were prepared aseptically by aliquoting 1.8 ml of Middlebrook 7H9 broth into 5 ml plastic disposable bijoux (Bibby Sterilin Ltd., UK) and incubated overnight at 37°C to check for contaminants. Hence, fifty tubes were prepared for each estimate.

Two millilitres of mycobacterial culture were sampled at day 7 of each drug assay and harvested by centrifugation (3000 x g for 5 min). The supernatant was carefully discarded; the bacteria were washed once in Middlebrook 7H9 broth and thoroughly resuspended in 2 ml of Middlebrook 7H9 broth. Five replicas of serial tenfold dilutions (10^{-1} - 10^{-10}) were prepared for each MPN estimate. The bijoux were sealed with parafilm to prevent evaporation and incubated at 37°C for 2 months. Turbidity was scored as a positive result and the number of positive tubes at each dilution was recorded. The combination of positive and negative tubes was used to calculate the MPN from standard MPN tables as described in Reports on Public Health and Medical Subjects, No. 71 (1969). Random samples of turbid tubes were taken for acid-fast staining to check for contaminants.

2.10 MYCOBACTERIAL GREEN FLUORESCENT PROTEIN STUDIES

GFP-expressing *M. tuberculosis* were produced to further investigate the non-labelled sub-population observed in broth culture and as a reporter assay to follow the effect of drugs during the antimycobacterial drug assays.

2.10.1 Construction of shuttle plasmid expressing the *gfp* gene

The strategy was to clone the *Aequorea victoria gfp* gene into the *E. coli*-mycobacterial shuttle vector pMV261 downstream of the *hsp60* promoter using the unique *Bam*HI and *Eco*RI sites as shown schematically in Figure 2.4a. *M. tuberculosis* was also transformed with the *gfp* expressing vector pGFM-11 which was kindly supplied by Kremer *et al.*, (1995). The structure of pGFM-11 is shown in Figure 2.4b.

Plasmids and DNA manipulation

All DNA manipulations were performed under standard conditions, as described by Sambrook *et al.*, (1989). Restriction enzymes, T4 DNA polymerase and T4 DNA ligase were purchased from Boehringer Mannheim.

All cloning steps were performed in *E. coli* DH5 α . Preparation of electro-competent cells and electro-transformation with plasmid DNA of *E. coli* DH5 α was by the method of Dower *et al.*, (1988).

Preparation of electro-competent cells of *E. coli* DH5 α

One litre of Luria-Bertani broth* was inoculated with 1/100 volume of a fresh overnight culture and incubated with vigorous shaking to OD_{600nm} of 0.5-1.0. The culture was chilled on ice for 15-30 min and harvested by centrifugation at 4000 x g for 15 min at 4°C. The pellet was resuspended in 1 L of chilled SDW and harvested by centrifugation.

The pellet was resuspended in 500 ml of chilled SDW and harvested by centrifugation. The pellet was resuspended in 20 ml of sterile chilled 10% (w/v) glycerol and harvested by centrifugation. The bacteria were carefully resuspended to a final volume of 2-3 ml in 10% glycerol, divided into 50 μ l aliquots and snap-frozen in ethanol cooled to -70°C. The aliquots were stored at -70°C and used for up to 6 months.

*Luria-Bertani broth contained: 10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl made up to 1 L in SDW and was sterilised by autoclaving (15 psi for 30 min). Luria-Bertani agar was solidified with 1.5% (w/v) agar.

Procedure for high efficiency electro-transformation of *E. coli* DH5 α

The cells were thawed at room temperature and placed on ice. Resuspended cells (40 μ L) were mixed with 1-2 μ l of DNA, vortexed and placed on ice for 1 min. The mixture was transferred to a cold 0.2 cm gap electroporation cuvette (Biorad) and electroporated (2.5kV, 25 μ F, 200 Ω) in a Gene Pulser (Biorad). Cells were immediately diluted with 1 ml of SOC* medium at room temperature, incubated at 37°C for 1 h and plated onto solid medium containing 25 μ g ml⁻¹ kanamycin.

*SOC medium contained: 2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose in SDW. SOC medium was sterilised by filtration.

Small scale extraction of plasmid DNA was by the STET buffer* method. Bacteria in 1.5 ml of overnight culture were harvested by centrifugation (13,000 x g for 1 min), resuspended in 200 ml STET buffer and boiled for 1 min. Cell debris was pelleted by centrifugation (13,000 x g for 10 min) and removed with a tooth pick. The supernatant and 150 μ l isopropanol were thoroughly mixed and centrifuged for 15 min at 13,000 x g. The supernatant was aspirated and the pellet dried under vacuum for 30 min. The pellet was resuspended in 50 μ l SDW and 1 μ l RNase.

*STET buffer contained: 40 g sucrose, 25 mL 10% (w/v) Triton X, 50 ml 0.5 M EDTA (pH 8.0), 25 ml Tris HCl (pH 8.0) in 500 ml SDW.

The Qiagen Midi protocol (Qiagen Ltd., UK) was used for large scale extraction of plasmid DNA. Cloned genes were sequenced at the Facility for Molecular Biology at the University of Newcastle upon Tyne, UK.

Expression studies were performed in *M. tuberculosis* H₃₇R_v. Preparation of electro-competent cells and electro-transformation with plasmid DNA of *M. tuberculosis* H₃₇R_v was carried out as described by Wards and Collins, (1996).

Preparation of electro-competent cells of *M. tuberculosis* H₃₇R_v

A 250 ml conical flask containing 100 µL of Middlebrook 7H9 broth was inoculated with 1 ml of *M. tuberculosis* stock and incubated at 37°C in a rotary shaker-incubator at 180 rpm for 7 days. Cells were harvested by centrifugation, washed twice with 10% glycerol and resuspended in approximately 1 ml 10% (w/v) glycerol (at room temperature).

Procedure for high efficiency electro-transformation of *M. tuberculosis* H₃₇R_v

Aliquots of cells (200 µL) were mixed with 1 µg DNA in a 0.2 cm gap electroporation cuvette (Biorad) and electroporated (2.5 kV, 25 µF, 1000 Ω) in a Gene Pulser (Biorad). Cells were immediately diluted with 1 ml fresh Middlebrook 7H9 broth at room temperature, incubated for 4-8 h at 37°C, and plated onto solid medium containing 25 µg ml⁻¹ kanamycin. Once dried, the plates were sealed with parafilm, inverted and incubated at 37°C in sealed bags.

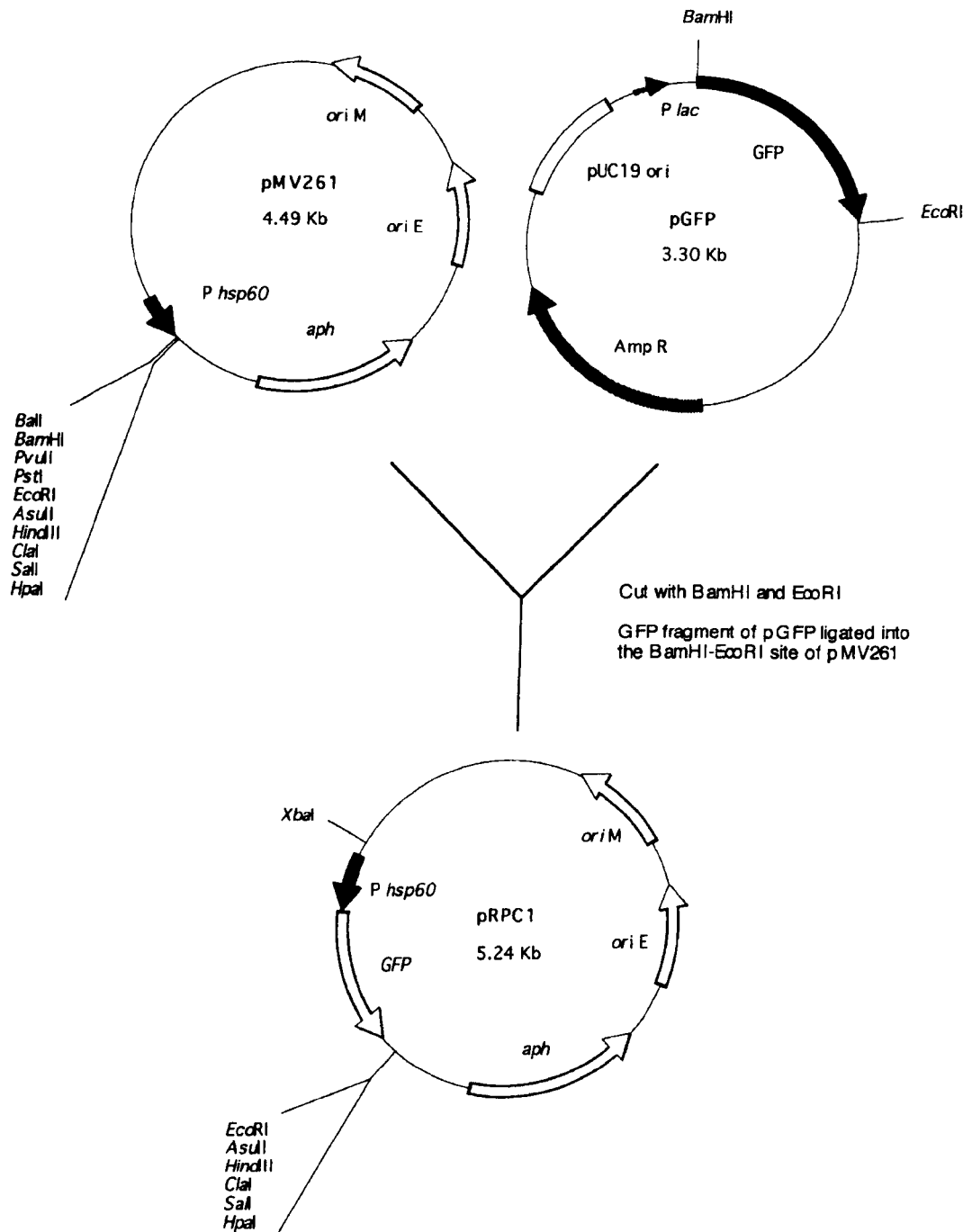


Figure 2.4a: Construction of the *gfp* expression vector pRPC1.

The *Aequorea victoria gfp* gene was cloned into the *E. coli*-mycobacterial shuttle vector pMV261 downstream of the *hsp60* promoter using the unique *Bam*HI and *Eco*RI sites.

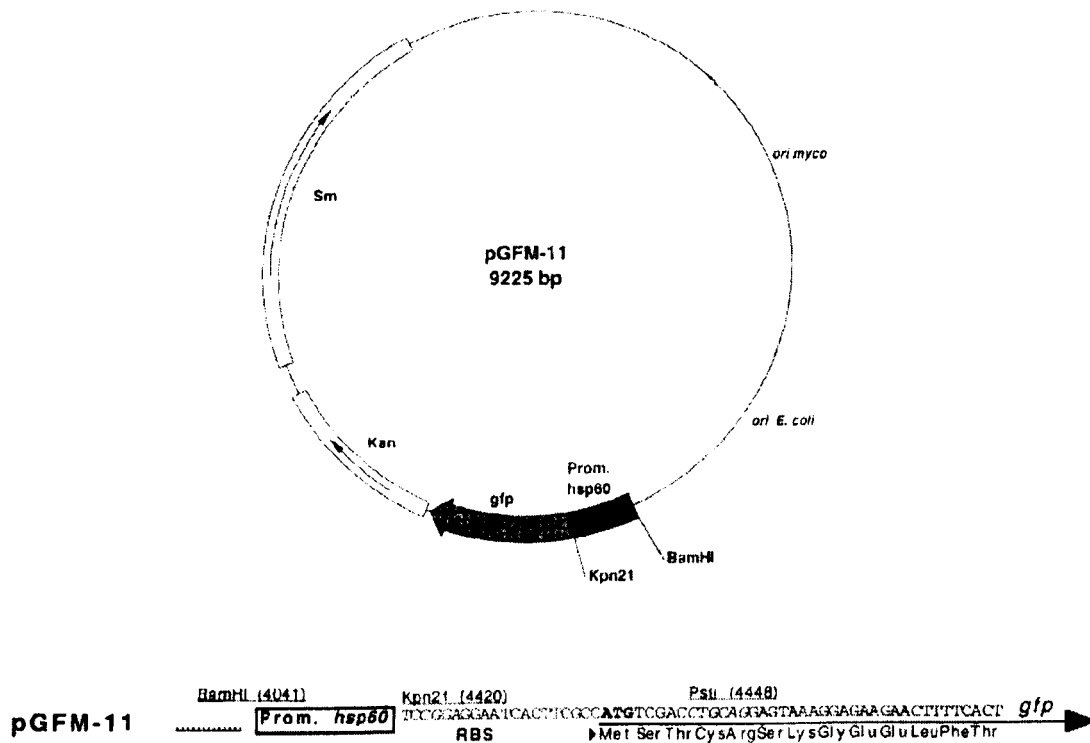


Figure 2.4b: Structure of the *gfp* expression vector pGFM-11 (reproduced from Kremer *et al.*, 1995).

The vector contains 0.4 kb of the 5' end of the BCG *hsp60* gene, including the promoter region, ribosome-binding site and the initiation codon, upstream of the *gfp* coding sequence. The vector also contains a kanamycin and streptomycin resistance cassette as well as a mycobacterial and an *E. coli* origin of replication.

2.10.2 Method for GFP detection

Direct detection of *gfp* expression was by irradiation of colonies at 366 nm with a long-wave UV light source. For microscopical studies, bacteria were harvested from agar or broth culture and immobilised on APS-coated coverslips by centrifugation as described in Section 2.5.2. Coverslips were rinsed with PBS, mounted in PBS on microscope slides and sealed with clear nail varnish. Cells were viewed by phase contrast and epifluorescence microscopy.

Colonies with the most intense GFP fluorescence were isolated and selected for study. Mycobacterial *gfp* transformants were cultured in Middlebrook 7H9 broth or on Middlebrook 7H10 agar containing 25 $\mu\text{g ml}^{-1}$ kanamycin.

2.10.3 Treatment with antimycobacterial drugs

The effect of the antimycobacterial drugs on *gfp* expression by *M. tuberculosis* transformants was investigated. Drug assays were carried out as described in Section 2.9.2, with the exception that each was supplemented with 25 $\mu\text{g ml}^{-1}$ kanamycin. Cultures were incubated without shaking at 37°C and sampled at day 7 for CFU enumeration and microscopic assessment of GFP expression.

2.11 MYCOBACTERIOPHAGE D29 STUDIES

2.11.1 Preparation of high-titre lysates of mycobacteriophage

High-titre lysates of mycobacteriophage D29 were prepared using the method described by Jacobs *et al.*, (1991). *M. smegmatis* for phage infection were prepared by inoculating 25 ml of Middlebrook 7H9 broth with 100 µl of starter culture in a 250 ml flask and incubated with shaking overnight at 37°C. Phage was decimally diluted in Phage Buffer* (PB) to obtain approximately 5×10^4 plaque forming units ml⁻¹. Two millilitres of *M. smegmatis* cells were mixed with 1.0 ml of phage solution in 30 ml disposable universals and incubated for 30 min at 37°C to allow adsorption to occur. 0.3 ml of the phage-cell mixture was added to 3.0 ml of overlay agar (Tryptone Soya Broth (Difco) containing 0.75% agar), mixed by gentle rotation and aseptically poured on top of prepoured agar plates (Tryptone Soya Broth containing 1.2% agar). The agar was allowed to solidify, inverted and incubated at 37°C for 24-36 h. Five plates were prepared for each lysate. After incubation, 5 ml of PB was pipetted onto each plate and the plates were set upright on a rocking table at 4°C overnight. As much liquid as possible was pipetted from each plate and combined. The lysates were centrifuged at 4°C at 3000 x g for 10 min, the supernatant was sterilised by filtration through 0.2 µm membranes and stored at 4°C.

*Phage Buffer contained: 10 mM Tris (pH 7.6), 100 mM NaCl, 10 mM MgSO₄, and 2 mM CaCl₂ in SDW and was sterilised by autoclaving (15 psi for 30 min).

2.11.2 Plaque forming unit (PFU) enumeration / phage titres

Enumeration of PFU was by the method described by Fullner and Hatfull, (1997). Bacterial lawns were prepared by addition of 3.0 ml of softened overlay agar to 0.3 ml of overnight culture of *M. smegmatis* (OD_{600nm} ~ 0.2) and the mixture poured onto prepoured agar plates and allowed to solidify. Mycobacteriophage D29 was decimally diluted in PB (to 10⁻¹⁰) and triplicate 10 µl drops of each dilution were spotted, allowed to

dry and incubated inverted at 37°C for 1-2 days. The dilution that produced discrete countable plaques was used for the enumeration of PFU ml⁻¹.

2.11.3 Mycobacteriophage infectivity studies

The effect of mycobacteriophage D29 infection on broth culture and antimycobacterial drug treated cultures of *M. tuberculosis* was investigated using R123/PI cytochemical labelling. Mycobacterial cultures were infected with D29 as described by Fullner and Hatful (1997). Cultures were incubated for 24 h in Tween 80-free medium prior to D29 infection because Tween 80 was shown to inhibit phage adsorption.

Broth culture of *M. tuberculosis* was prepared as described in Section 2.4.1. Bacteria were harvested by centrifugation (3000 x g for 10 min), resuspended in Tween 80-free Middlebrook 7H9 broth and incubated for with shaking for 24 h at 37°C. Cultures were then either infected with D29 (1 ml of high titre phage solution to 9 ml culture) or PB control and incubated overnight. Samples were taken for CFU enumeration and R123/PI labelling.

M. tuberculosis culture was treated with antimycobacterial drugs as described in Section 2.9.2. Bacteria were harvested by centrifugation (3000 x g for 10 min), resuspended in Tween 80-free Middlebrook 7H9 broth containing the appropriate antimycobacterial drug and incubated for 24 h. Cultures were then either infected with D29 (1 ml D29 to 9 ml culture) or PB control and incubated overnight. Samples were taken for CFU enumeration and R123/PI labelling.

2.12 ADAPTATION OF *M. TUBERCULOSIS* TO ANAEROBIOSIS

Anaerobiosis was induced in *M. tuberculosis* cultures using two different methods developed by Wayne and colleagues (Wayne and Lin, 1982; Wayne and Hayes, 1996). Cells were cultured in Dubos Tween-albumin broth prepared according to the manufacturer's instructions from Dubos broth base (Difco) and Dubos medium albumin (Difco) at a final pH of 6.6 ± 0.2 . Inocula were prepared in 250 ml baffled polycarbonate conical flask containing 50 ml of Dubos Tween-albumin broth and 1 ml of thawed *M. tuberculosis* stock, which were incubated for one week at 37°C in a rotary shaker-incubator at 180 rpm. By this time the culture contained approximately 1×10^7 CFU ml⁻¹.

In the first method, cells were adapted by the slow settling of bacilli through a self-generated oxygen gradient in unshaken tubes. Aliquots (10 ml) of Dubos Tween-albumin broth in 20-by-125-mm tubes fitted with screw-cap caps with latex liners were inoculated with 100 µl of culture. The caps were tightly screwed down and the tubes incubated in an upright position without agitation, with care not to disturb the sediment that formed or to aerate the medium. Cells were sampled from the anoxic region in the sediment at the bottom of the tube and labelled with R123/PI as described in Section 2.8.1.

In the second method, adaptation to anaerobiosis was achieved in slowly stirred sealed liquid cultures exposed to limited headspace volumes of air (one-half volume of initial headspace air per volume of media). Conical flasks with total fluid capacity of 600 ml containing 400 ml of Dubos Tween-albumin broth and 1 cm Teflon-coated magnetic stirrers were inoculated with 1 ml of culture. The flasks were tightly sealed with sterile rubber septa (Sigma) and incubated on magnetic stirrers (Heidolph MR 100, Germany) at 37°C at the slowest magnetic stir. Samples of culture for cytochemical staining were taken using sterile syringes and needles.

Control cultures containing sterile methylene blue at a final concentration of $1.5 \mu\text{g ml}^{-1}$ were included in each experiment. Reduction and decolourisation of this dye served as a visual indication of oxygen depletion.

3. RESULTS

3.1 METHODOLOGICAL STUDIES

A reliable method for obtaining consistent defined populations of *M. tuberculosis* for use in cytochemical labelling and antimycobacterial drug assays was required early in this study. Considerable effort was invested in establishing growth in broth under defined conditions as the basis for these experiments. However, as will be seen, inocula produced from broth culture were unsuitable, as many cells proved recalcitrant to labelling. Therefore, cells from agar culture, although more difficult to describe in terms of recognised growth phases were used for most of the work.

3.1.1 Growth characteristics in broth

Representative growth characteristics of *M. tuberculosis* in broth, incubated in a rotary shaker-incubator for two weeks are shown in Figure 3.1. An exponential increase in OD was observed for the first seven days after which little change occurred. Towards the end of the 2 week culture period a decrease in OD was observed. Colony counts were shown to increase by between 3 and 4 log cycles during the culture period. Cultures sampled at day 7 routinely yielded between 5.0×10^7 and 1.0×10^8 CFU ml⁻¹ and were used as a source of inocula in the early studies. It was noted that continued incubation (> 2 weeks) in a rotary shaker-incubator produced clumping of mycobacteria and deposition of biomass at the liquid/air interface on the flask wall.

3.1.2 R123/PI labelling of cells cultured in broth

Samples of *M. tuberculosis* were taken from broth culture over the first week of growth and labelled with R123/PI. Due to the low cell density, samples taken early in broth culture were concentrated by centrifugation prior to staining. It was repeatedly observed that only between 20 and 40% of broth-cultured bacteria labelled with R123. No PI or R123 label was observed in the remaining (60-80%) cells. An example of *M. tuberculosis* culture sampled on day 7 and labelled with R123/PI is shown in Figure 3.2.

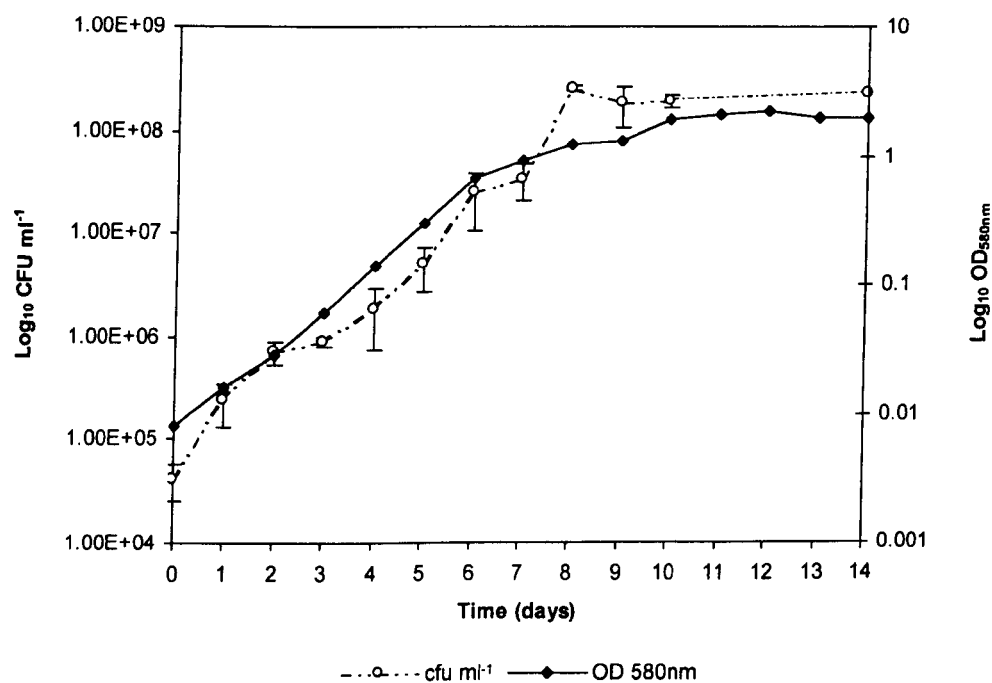


Figure 3.1: Growth curve of *M. tuberculosis* in Middlebrook 7H9 broth.

The inoculum was prepared from a 7 day broth culture as described in Section 2.4.1. Cultures were incubated with constant agitation (180 rpm) in a rotary shaker-incubator at 37°C. Growth was measured as CFU ml⁻¹ (± 2 S.D.) and OD_{580nm}.

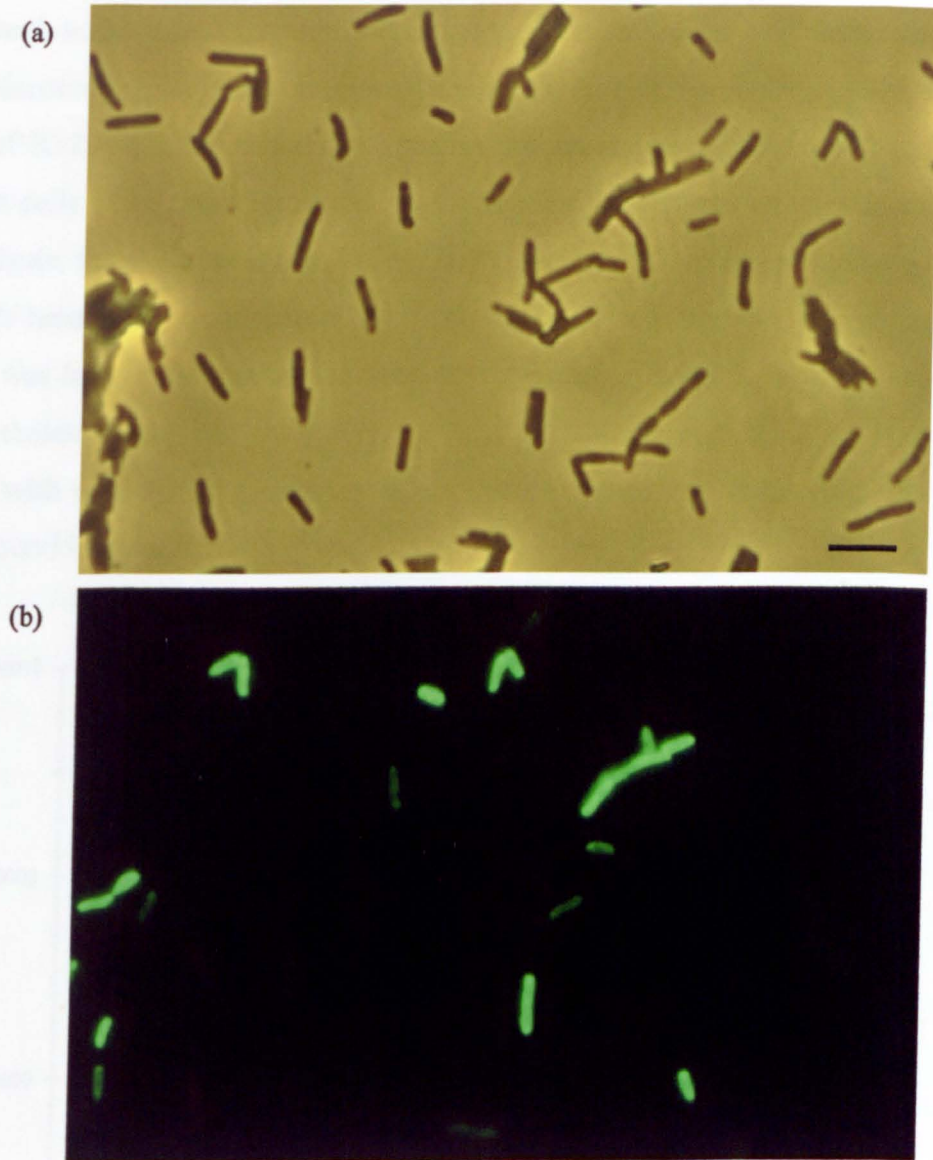


Figure 3.2: Broth-cultured *M. tuberculosis* dual labelled with R123 and PI.

Phase-contrast and fluorescence images of the same field of view of broth-cultured *M. tuberculosis* dual labelled with R123 (1 μ M) and PI (3 μ M). The organism was cultured in Middlebrook 7H9 broth with constant agitation and sampled after 7 days growth. Cells were viewed at 1000 x magnification. Scale bar = 3 μ m.

Labelled and non-labelled bacteria were indistinguishable from each other by phase-contrast microscopy (*i.e.* based on opacity, refractivity or cell morphology). Fluorescence intensity of R123-labelled bacteria was heterogeneous and ranged from dull to brightly fluorescent cells. The total fluorescence associated with each cell may be quantified by image analysis; the example shown in Figure 3.3 displays this data in a scatter plot from which both heterogeneity of fluorescence intensity and cell size is evident. Although Tween 80 was included in the broth to prevent clumping of bacteria, many small clumps were nonetheless observed. It should be noted, that clumping did not appear to be associated with non-labelling, as cells found in clumps were as likely to be labelled as those not found in clumps.

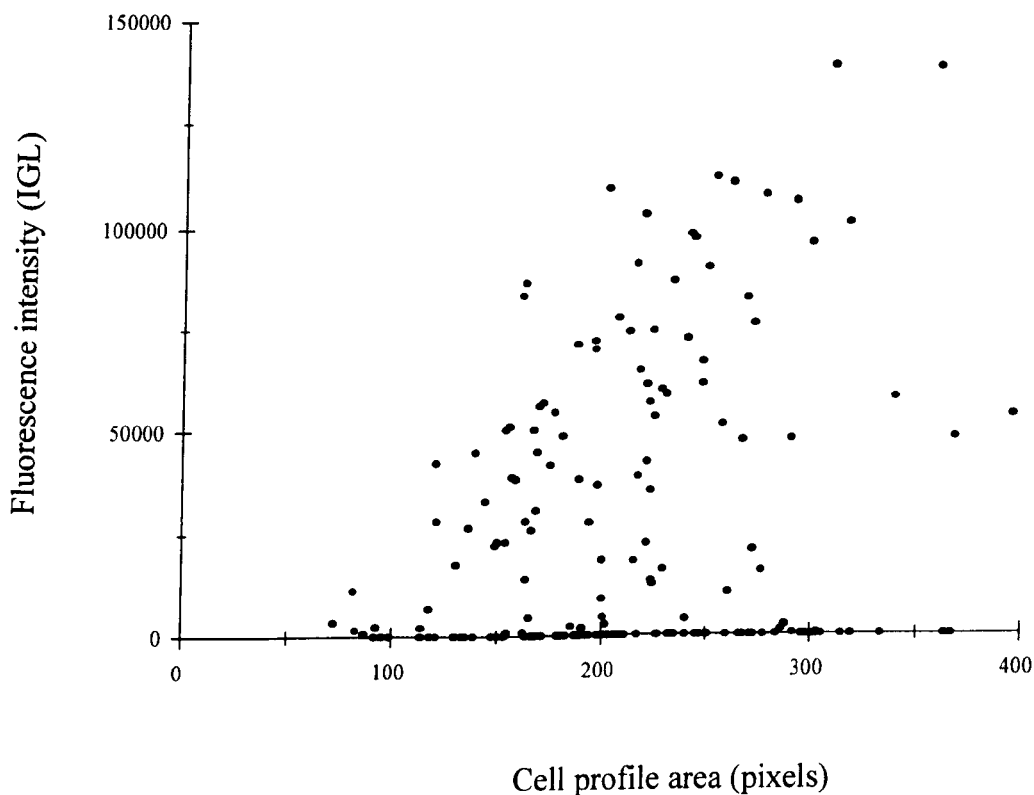


Figure 3.3: Scatter plot demonstrating heterogeneity of fluorescence intensity in broth-cultured *M. tuberculosis* labelled with R123.

Fluorescence is expressed as the integrated grey level (IGL) attributable to each cell. IGL is expressed in arbitrary units.

Cytochemical labelling of mycobacterial cells from shaken broth cultures was compared to that of cells produced by other methods of broth culture (Section 2.4.1). These methods included agitation and aeration of culture by magnetic stirrers (Wayne, 1994) and incubation of *M. tuberculosis* without shaking (Herbert and Mitchison, 1996). Regardless of how broth culture was produced, there were more unlabelled than labelled cells when stained with R123/PI.

Increased incubation times and concentrations of dye were investigated during optimisation of the mycobacterial R123/PI cytochemical reaction. Figure 3.4 shows that longer incubation periods (> 15 min) had no effect on R123 labelling. Indeed, overnight incubations produced neither an increase in fluorescence intensity of individual labelled cells nor an increase in the proportion of labelled bacteria (results not shown). Similarly, increasing the concentration of R123 did not alter the observed labelling pattern with *M. tuberculosis*.

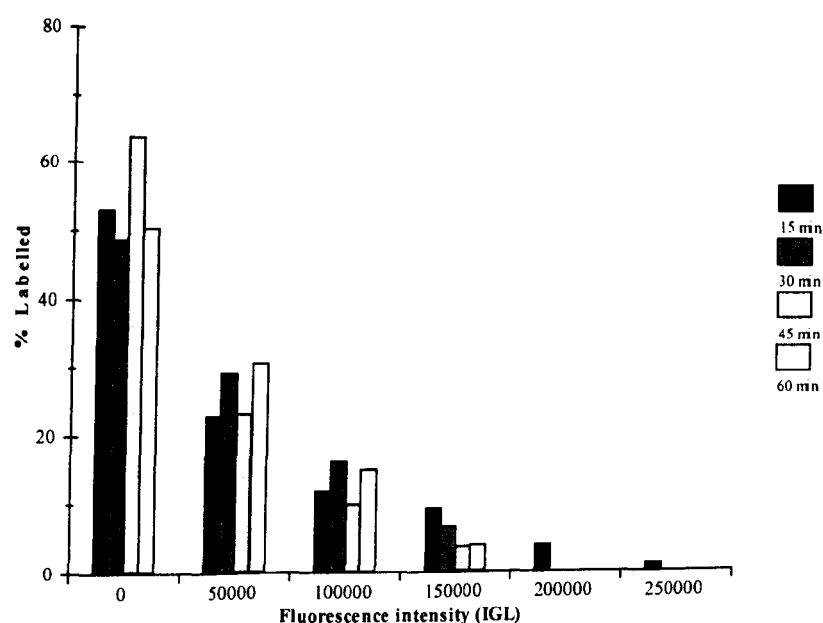


Figure 3.4: Effect of incubation time on the R123 accumulation by *M. tuberculosis*.

Accumulation is expressed as the percentage of bacteria in each of the fluorescence intensity ranges. The number of cells analysed for each incubation time was in the range of 100-150.

The non-labelled cells observed in the R123/PI assay were investigated for low-level fluorescence using prolonged integration times and increased electronic gain (analogous to longer exposure time and enhanced camera chip sensitivity respectively). However, under these conditions our general-purpose colour CCD camera produced high levels of background noise making it difficult to differentiate non-labelled cells. When a Peltier-cooled CCD camera (Type KAF-0400, Wright Instruments Ltd., Enfield, England; array 768H x 512V, pixel size 9 μm square, operating temperature 200 K) with 40% photon detection efficiency at 500 nm was used to examine these cells, no fluorescence signal above background was detected.

Experiments designed to improve R123 labelling of potential low-activity non-labelled cells in *M. tuberculosis* broth culture by valinomycin cytoplasmic membrane hyperpolarisation (Section 2.8.3) met with little success. In fact, rather than increasing dye uptake through the generation of a potassium ion gradient, treatment with valinomycin inhibited R123 accumulation and reduced fluorescence even in the proportion of cells that were originally labelled. Valinomycin-treated bacteria did not stain with PI.

In summary, between 20 and 40% of broth cultured *M. tuberculosis* cells labelled with R123. The remainder did not label with either R123 or PI. It should be noted, that the presence of non-labelling bacteria would have been difficult to detect and quantify if brightfield illumination (the standard form of illumination on most microscopes) rather than phase contrast illumination had been used.

3.1.3 Growth on agar

Provided that the initial inoculum was well dispersed (*e.g.* 100 μl of day 7 broth culture) and thoroughly spread on pre-dried agar plates, sub-confluent lawns of *M. tuberculosis* were produced within a week. Bacteria were easily harvested and dispersed as single cells with a minimum of clumping using the glass rod homogenisation method described

in Section 2.4.4. When adjusted to an OD_{580nm} of 0.2, bacterial suspensions prepared by this method yielded between 5.0×10^7 and 1.0×10^8 CFU ml^{-1} . However, with continued incubation (> 1 week), lawn cultures became confluent, dry and wrinkled. It was more difficult to prepare evenly dispersed suspensions from older cultures and they were not used in the cytochemical or antimycobacterial drug studies.

3.1.4 R123/PI labelling of cells cultured on agar

In contrast to broth culture, most *M. tuberculosis* cells cultured on agar labelled with R123 (typically, 85-90%). The remainder were non-labelled and few bacteria in these preparations stained with PI. An example of R123/PI labelled *M. tuberculosis* prepared from sub-confluent lawn culture as described in Section 2.4.4 is shown in Figure 3.5. Heterogeneity of fluorescence intensity in R123-labelled cells was also observed; an example of data from image analysis is displayed in scatter plot form in Figure 3.6.

Apart from the difference in labelling with R123/PI, the most obvious difference in growth produced in broth and on agar was cell size. Broth cultured *M. tuberculosis* bacilli were consistently larger than those grown on agar. Comparison of the length, breadth and area obtained by image analysis is shown below in Table 3.1. These differences were shown to be statistically significant ($p < 0.05$, Student's t-test).

Table 3.1: Morphological properties of agar and broth cultured *M. tuberculosis**

	length (μm)	breadth (μm)	area (μm^2)
Agar	2.85 ± 0.01	1.39 ± 0.01	2.63 ± 0.01
Broth	3.71 ± 0.02	1.67 ± 0.01	4.02 ± 0.03

*Data based on 13 independent experiments with analysis of 5364 bacteria and are expressed as means \pm standard error of the means.

Since the majority of agar cultured bacteria labelled, bacterial suspensions prepared from sub-confluent lawns were used as the standard inoculum for the remainder of the study.

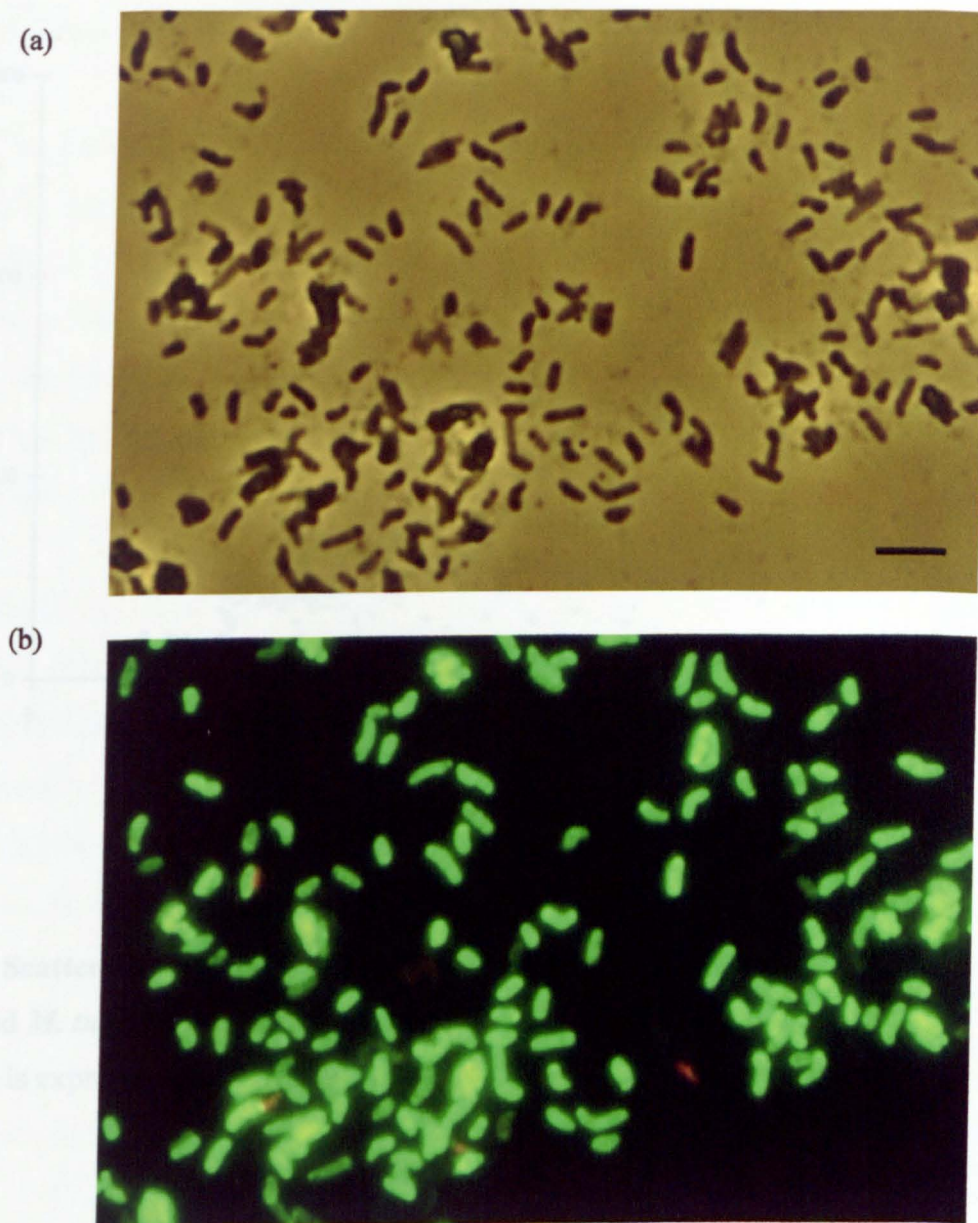


Figure 3.5: Agar-cultured *M. tuberculosis* dual labelled with R123 and PI.

Phase-contrast and fluorescence images of the same field of view of agar-cultured *M. tuberculosis* labelled with R123 (1 μM) and PI (3 μM). The organism was cultured on Middlebrook 7H10 agar and sampled after one weeks growth. Cells were viewed at 1000 x magnification. Scale bar = 3 μm .

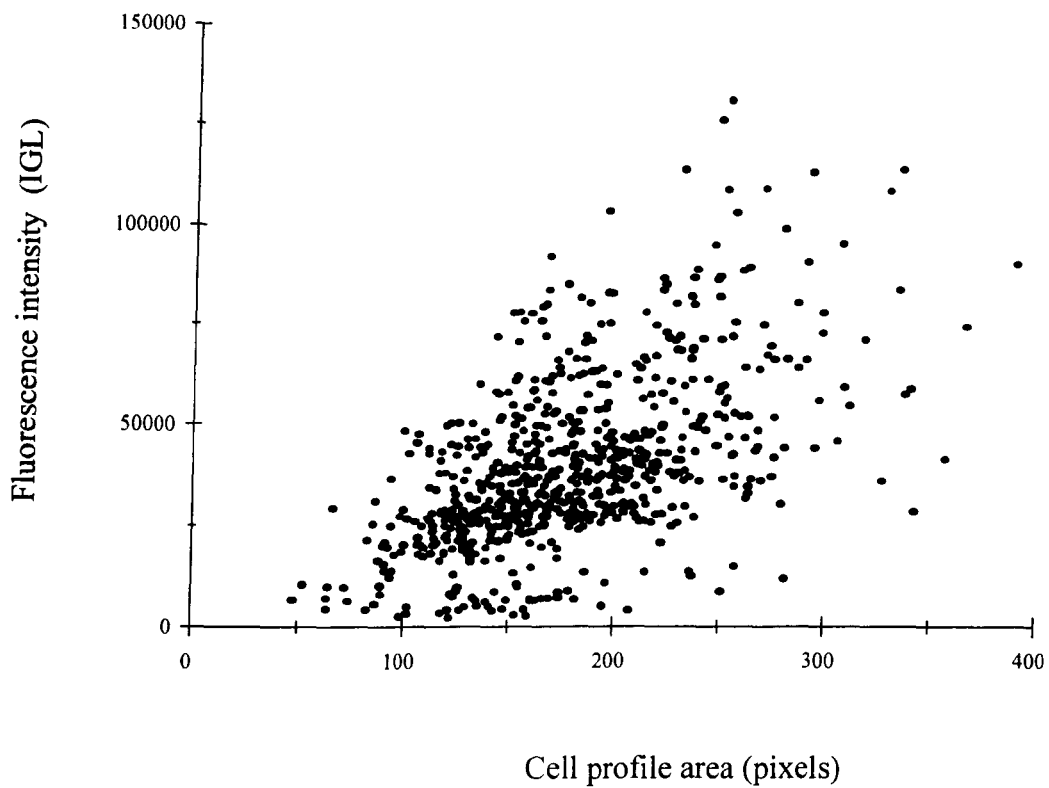


Figure 3.6: Scatter plot demonstrating heterogeneity of fluorescence intensity in agar-cultured *M. tuberculosis* labelled with R123.

Fluorescence is expressed as the integrated grey level (IGL) attributable to each cell.

3.1.5 R123 Uncoupler sensitivity studies

Labelling of *M. tuberculosis* with R123 in the presence of the uncoupler agent CCCP was investigated, as described in Section 2.8.2, to demonstrate that the fluorescent dye was concentrated by the bacteria in an uncoupler-sensitive fashion. In the initial experiments, bacteria were treated for 15 min with 15 μM CCCP and labelled with 13 μM R123. Under these conditions no detectable effect on the accumulation of R123 was produced. Consequently, a series of experiments were designed where different concentrations of CCCP and R123 were tested.

CCCP concentration: The effect of CCCP on R123 accumulation by *M. tuberculosis* was negligible until concentrations greater than 15 μM were used. Thereafter, each increase in CCCP concentration produced a concomitant decrease in bacterial fluorescence (Figure 3.7). Cytochemical labelling of bacteria treated with 150 μM CCCP produced almost complete inhibition of accumulation of R123 and this concentration was used in all further experiments to demonstrate uncoupler-sensitive accumulation of dye. CCCP does not itself quench the fluorescence of R123.

Since CCCP stocks were prepared in ethanol, the impact of ethanol on accumulation was assessed. The addition of 2% ethanol alone in labelling experiments had no effect on the accumulation of R123.

It was noted that, even at optimal concentrations of CCCP, complete inhibition of accumulation of R123 by all bacteria was not observed. However, only low-level fluorescence was observed in these residual labelled cells.

R123 concentration: The accumulation of R123 by *M. tuberculosis* was uncoupler sensitive only when lower concentrations of dye ($\leq 1 \mu\text{M}$) were used. Labelling with 13 μM R123 was not sensitive to CCCP, even when high concentration of the uncoupler agent was used. Results from *M. tuberculosis* cell suspensions, labelled with 1 μM and

13 μM R123 in the presence and absence of CCCP are shown in Figure 3.8. In all further experiments R123 was added at a final concentration of 1 μM .

The uncoupler effect of CCCP on R123 accumulation was not permanent, as CCCP-treated bacteria were able to accumulate the dye once the uncoupler was removed. It is shown in Figure 3.9 that an *M. tuberculosis* cell suspension treated for 15 min with CCCP, washed and left to recover in fresh Middlebrook 7H9 broth for 15 min before labelling, demonstrated a return of fluorescence to a level similar to that detected in untreated bacteria.

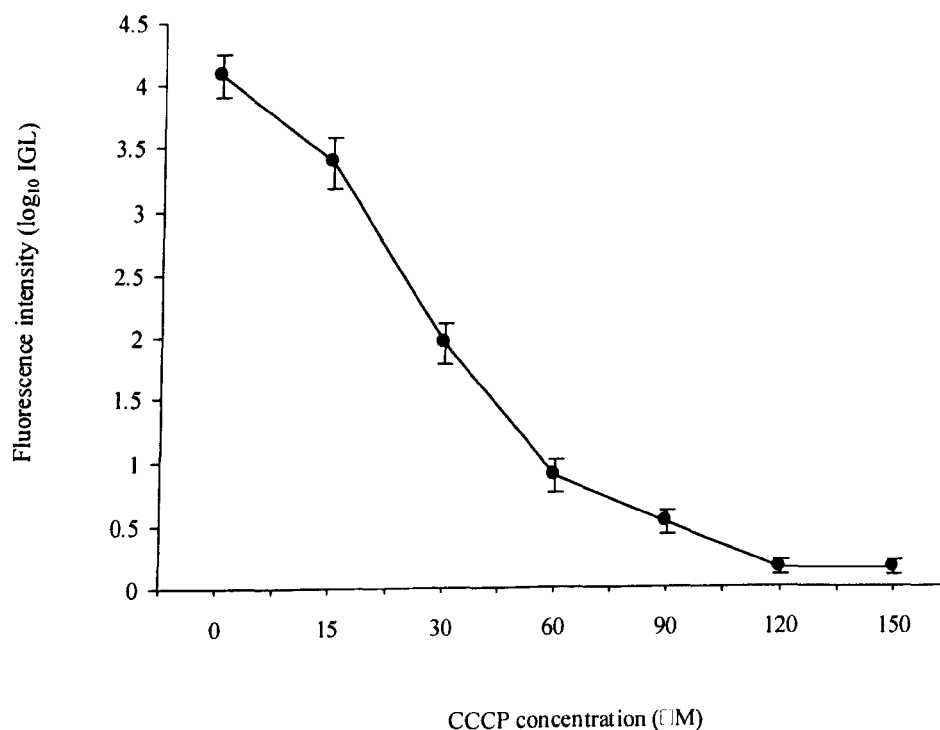


Figure 3.7: Effect of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) concentration on the accumulation of R123 by *M. tuberculosis*.

Fluorescence is expressed as \log_{10} of the average integrated grey level (IGL) produced by bacteria at each of concentrations tested. The number of cells analysed for each concentration was in the range of 70-120; symbols represent mean \pm S.E.M.

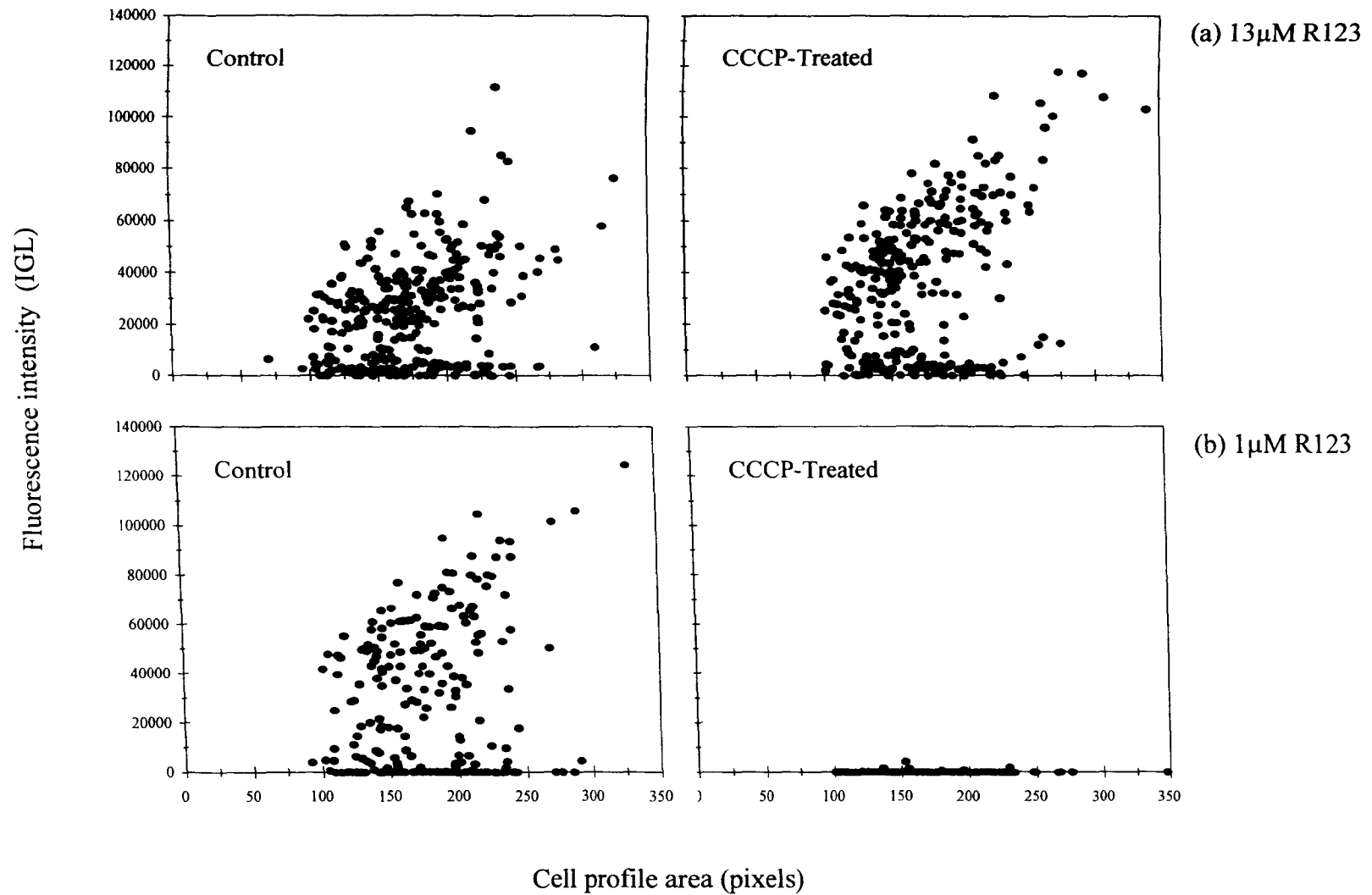


Figure 3.8: Effect of R123 concentration on the uncoupler-sensitive accumulation of the dye by *M. tuberculosis*.

Scatter plots of the fluorescence produced by bacteria labelled with (a) 13 μ M and (b) 1 μ M R123 with and without 150 μ M CCCP. Fluorescence is expressed as integrated grey level (IGL).

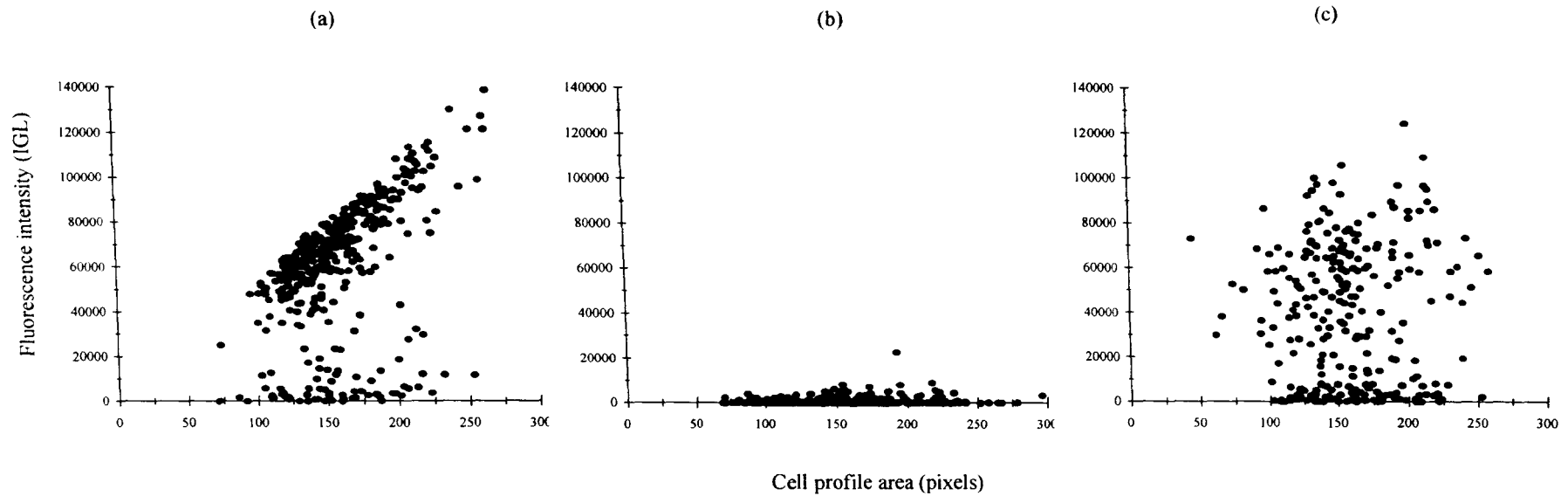


Figure 3.9: Effect of CCCP on R123 accumulation by *M. tuberculosis*.

(a) Untreated, (b) CCCP-treated and (c) washed and resuspended CCCP-treated populations of R123 labelled bacteria. Fluorescence intensity was measured by integrated grey level (IGL) and plotted against the cell profile area of each bacterium analysed.

3.1.6 Heat, ethanol and formaldehyde treatments

The effects of bactericidal treatments (defined as treatments shown to be lethal to *M. tuberculosis* as assessed by colony forming unit potential on Middlebrook 7H10 agar) on cytochemical labelling were assessed as described in Section 2.8.10. Each of the treatments was shown to significantly alter the accumulation of R123 and PI by *M. tuberculosis*.

Formaldehyde treatment produced uniform homogenous low-level labelling with R123 that was not sensitive to uncoupler agent (Figure 3.10a & b). PI staining was not observed. Treatment with formaldehyde did not produce fluorescence without staining (Figure 3.10c).

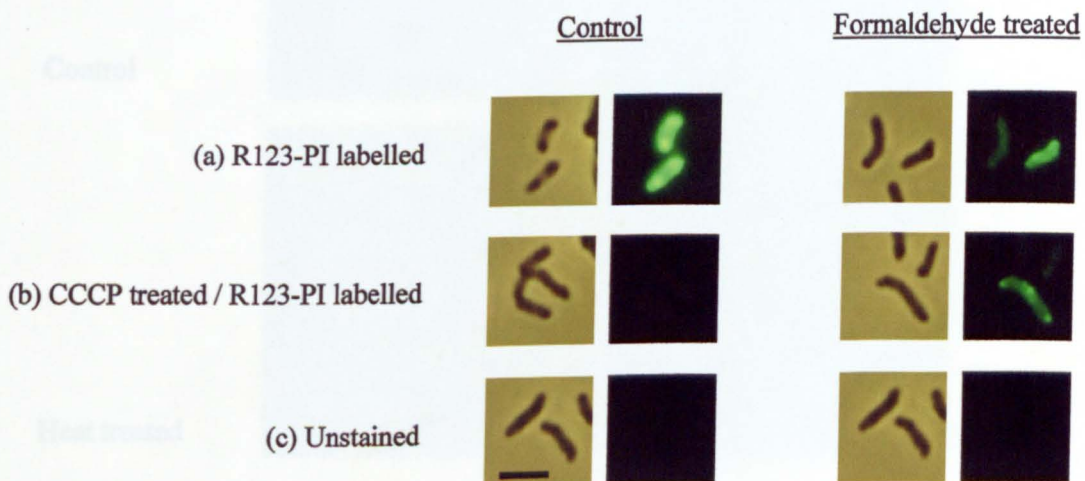


Figure 3.10: Effect of formaldehyde treatment on R123 accumulation by *M. tuberculosis*.

Phase-contrast and corresponding fluorescence microscopic images of (a) R123 labelled, (b) R123 labelled CCCP-treated and, (c) unstained populations are shown. Cells were viewed at 1000 x magnification. Scale bar = 2 μ m.

Approximately 90% of heat-treated bacteria labelled with PI and fluoresced red. The remaining 10% retained the ability to accumulate R123 and fluoresced with similar intensity to bacteria in untreated populations. However, the accumulation of R123 by the residual labellers in heat-treated populations was not sensitive to CCCP. An example of R123/PI labelled heat-treated *M. tuberculosis* is shown in Figure 3.11. Heat-treated bacteria were not fluorescent without R123 labelling.

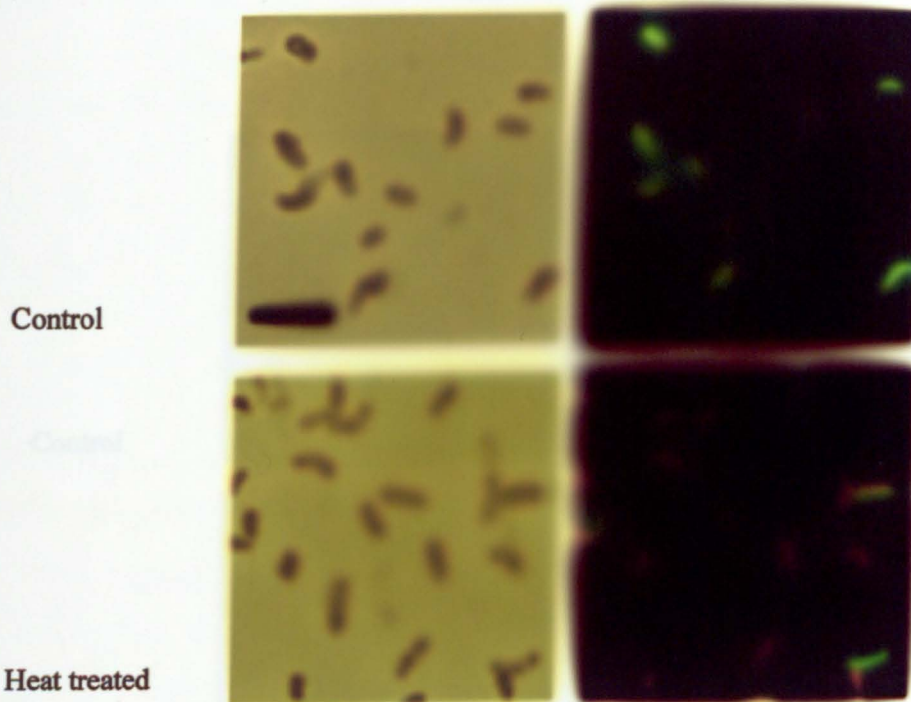


Figure 3.11: Effect of heat treatment (70°C/30 min) on R123 accumulation by *M. tuberculosis*.

Phase-contrast and fluorescence images of the same microscopic fields of view of treated and control populations are shown. Cells were viewed at 1000 x magnification. Scale bar = 3 μm .

The R123-labelled heat-treated cells were unexpected as previous work in this laboratory established that heat-treated mycobacteria labelled exclusively with PI (D.R. Walker and M.R. Barer, unpublished data). However, closer inspection of this work revealed that *M. tuberculosis* was not among the mycobacterial species investigated. Heat treatment of *M. smegmatis* and *M. aurum* (organisms included in the previous study) produced results that agreed with the earlier findings, *i.e.* all bacteria identified by phase-contrast microscopy were PI stained and no R123 fluorescence was observed. An example of R123/PI labelled heat-treated *M. smegmatis* is shown in Figure 3.12. Prior to heat treatment, almost all of *M. smegmatis* cells accumulated R123 and fluoresced green.

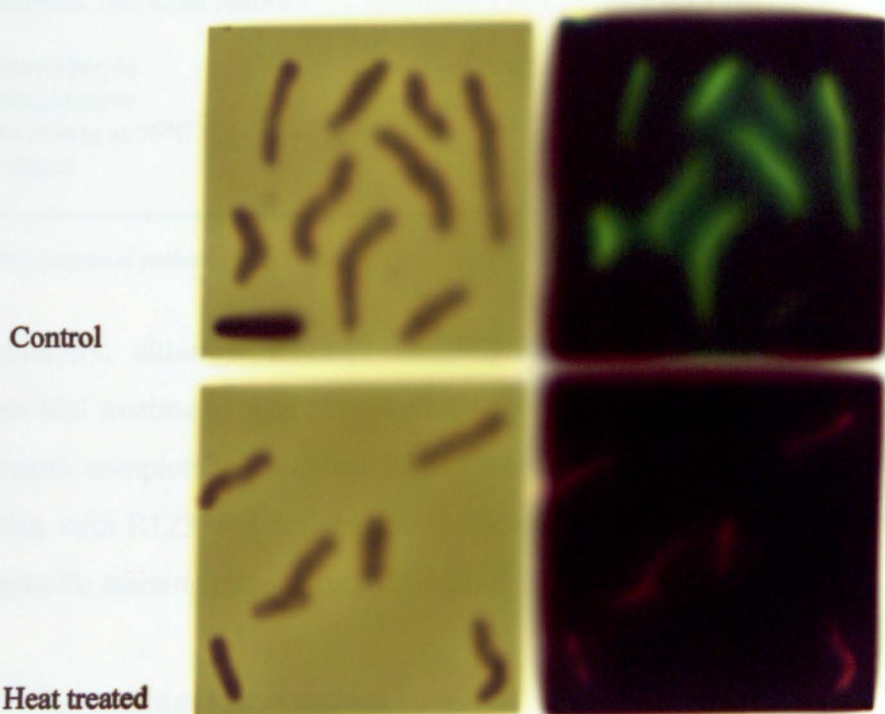


Figure 3.12: Effect of heat treatment (70°C/30 min) on R123 accumulation by *M. smegmatis*.

Phase-contrast and fluorescence images of the same microscopic fields of view of treated and control populations are shown. Cells were viewed at 1000 x magnification. Scale bar = 3 μm .

Ethanol treatment produced the same pattern of R123/PI labelling as heat treatment in *M. tuberculosis* (results not shown). In like manner, residual R123 accumulation was not uncoupler sensitive and ethanol-treated bacteria did not fluoresce without R123 labelling.

Effects of the bactericidal treatments on colony counts are shown in Table 3.2. Each of the treatments produced a reduction in counts to levels below that detectable by the drop plate method used ($< 50 \text{ CFU ml}^{-1}$).

Table 3.2: Effect of bactericidal treatments on colony counts of *M. tuberculosis*.

Treatment	Time	CFU ml ^{-1a}
Middlebrook 7H9 broth control		$1.8 \pm 0.4 \times 10^7$
4% formaldehyde	2 h or o/n	< 50
1% formaldehyde	2h or o/n	< 50
Heat treatment at 70°C	30 min	< 50
70% ethanol	5 min	< 50

^a Limit of detection of method is 50 CFU ml⁻¹.

In summary, although R123/PI labelling of *M. tuberculosis* cell suspensions after bactericidal treatments was phenotypically different from untreated controls, none of the treatments completely abolished R123 fluorescence. However, in each case, residual labelling with R123 was not sensitive to uncoupler agent and, was therefore regarded as non-specific staining rather than evidence of physiological activity.

3.1.7 Tetrazolium salt reactions

Tetrazolium salt reduction was evaluated as an alternative cytochemical indicator of physiological activity in *M. tuberculosis* (Section 2.8.4). Preliminary studies showed that both INT and CTC were reduced intracellularly by physiologically active cells to insoluble formazan and were detectable by microscopy. However, more cells remained non-labelled than labelled and deposits detected in many cells were small. Consequently, various reaction conditions were tested in order to improve tetrazolium reduction.

INT

Initial experiments used short incubation times (5-10 min), high concentrations of INT (5 mM), and test cells prepared in Middlebrook 7H9 broth. Although this resulted in some reduction of INT, formazan deposits were small and often difficult to detect.

Deposits were found to increase in size with longer incubation times and lower concentrations of INT. The results of these experiments were quantified by image analysis and are shown in Figures 3.13 and 3.14 respectively. The most substantial deposits in *M. tuberculosis* were observed with 1 mM INT incubated for 1 h. Examples of brightfield microscopy images of formazan deposits at each of the concentrations tested are included in Figure 3.14. With longer incubation times (> 1 h), deposits produced swollen cells with distorted morphology. Larger formazan deposits were observed in test cells prepared in PBS (pH 7.3) rather than Middlebrook 7H9 broth (results not shown).

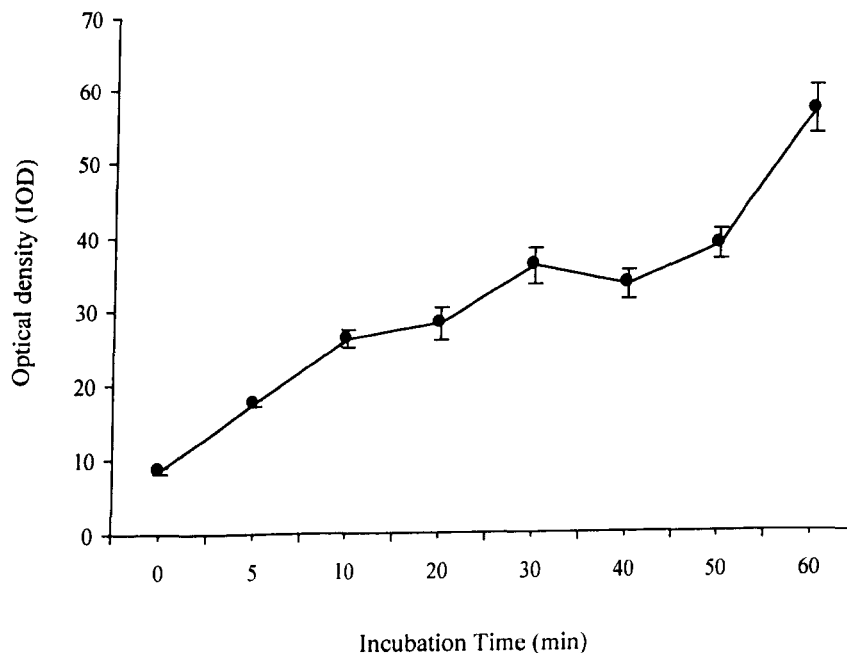


Figure 3.13: Effect of incubation time on INT reduction by *M. tuberculosis*.

Formazan deposits are expressed as the average integrated optical density (IOD) produced by bacteria at each of incubation times tested. INT was added at a final concentration of 5 mM. The number of cells analysed for each time point was in the range of 80-302; symbols represent mean \pm S.E.M.

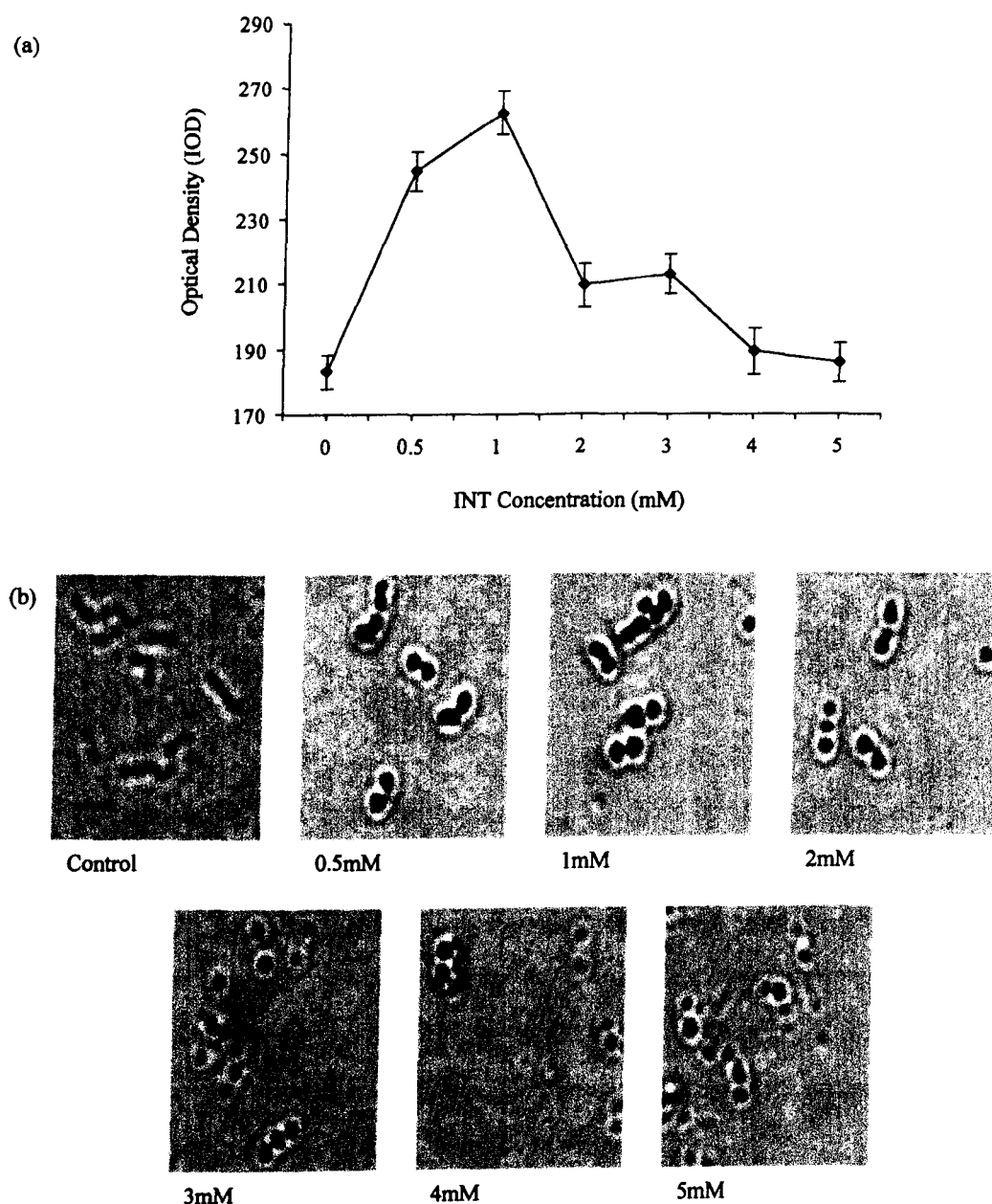


Figure 3.14: Effect of INT concentration on the production of formazan deposits by *M. tuberculosis*.

(a) Formazan deposits produced at each of the concentrations tested expressed as the average integrated optical density (IOD). The number of cells analysed for each concentration was in the range of 50-100; symbols represent the mean \pm S.E.M. Examples of brightfield images of bacteria at each of the concentrations are shown in (b). Cells were viewed at 1000 x magnification. Scale bar = 3 μ m.

Although, the addition of exogenous oxidisable substrates (aspartate, α -ketoglutarate, pyruvate, cellobiose, malate, serine, fumarate, citrate, succinate, glucose and glutamate) was shown to stimulate tetrazolium salt reduction with other bacterial species (Gribbon, 1995; Whiteley, 1996), neither an increase in the number of *M. tuberculosis* cells that produced formazan deposits or an increase in deposit size was observed in the present study.

In summary, although optimisation of INT reduction in *M. tuberculosis* produced larger formazan deposits, increase in the proportion of labelling to non-labelling bacteria was not observed. The number of cells that demonstrated cytological activity with the INT reduction assay was approximately the same as the number of cells that labelled with R123.

CTC

Cells that reduced CTC were easily identified by their bright red fluorescent deposits. However, non-labelling and inconsistent results were the norm. Some bacteria contained what appeared to be intracellular formazan deposits when observed by phase contrast microscopy, which, were not fluorescent when examined by fluorescence microscopy. Extracellular fluorescent formazan deposits were also observed. Extended incubation times and varying the concentration of CTC produced little improvement in labelling and consequently experiments with CTC were discontinued.

3.1.8 Combined R123/PI labelling and INT reduction assays

Both the R123-labelling and INT-reduction assays identified non-labelled cytologically inactive bacteria in *M. tuberculosis* cell suspensions. In order to assess whether these populations were the same a combined assay was developed.

Results of the combined assay indicated that more bacteria accumulated R123 than produced measurable formazan deposits. Analysis of R123-labelled cells from broth culture revealed that approximately 10% of the fluorescent cells did not contain detectable formazan deposits (Figure 3.15a). This analysis was often difficult as R123 fluorescence was obscured in bacteria that were filled with large formazan deposits (Figure 3.15b). It should be noted that INT deposits themselves are not fluorescent.

In summary, although the non-labelling proportion was more or less the same, there were a higher number of cytologically inactive cells in the INT assay than the R123 assay. Bacteria that contained formazan deposits always accumulated R123 but cells that accumulated R123 did not always contain detectable deposits.

The combined assay was also used to further investigate the residual R123-labelled cells produced by the bactericidal treatments described in Section 3.1.6. Examples of heat-treated and control populations of *M. tuberculosis* labelled using the combined assay are shown in Figure 3.16. Whereas formazan deposits were clearly observed in most R123 labelled cells in control populations, none of the R123 labelled cells in heat-treated samples contained deposits.

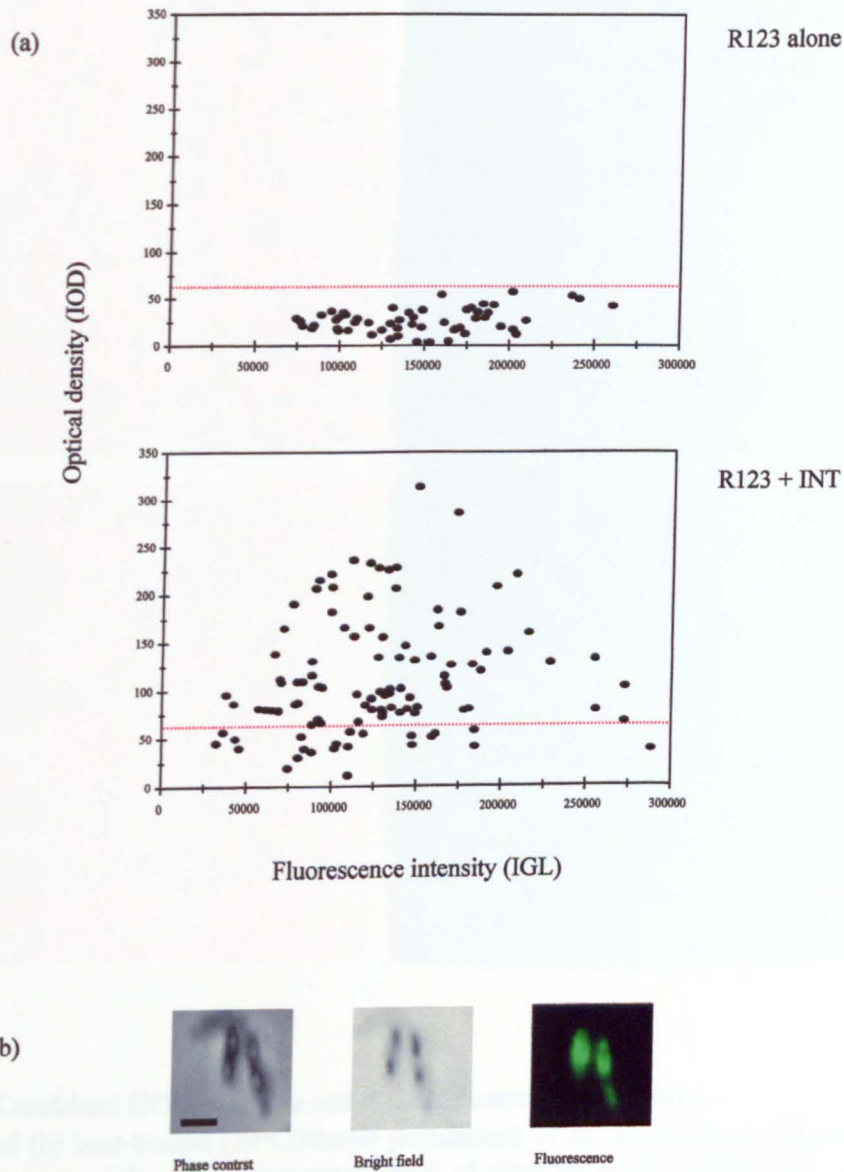


Figure 3.15: INT reduction in R123-labelled *M. tuberculosis*.

(a) Formazan deposits were quantified by integrated optical densities (IOD) and fluorescence intensities by integrated grey levels (IGL). Cells with IOD < 60 did not contain detectable formazan deposits. (b) Phase-contrast, brightfield and fluorescence images of R123-labelled bacteria with large formazan deposits demonstrating the quenching of fluorescence. Scale bar = 2 μ m.

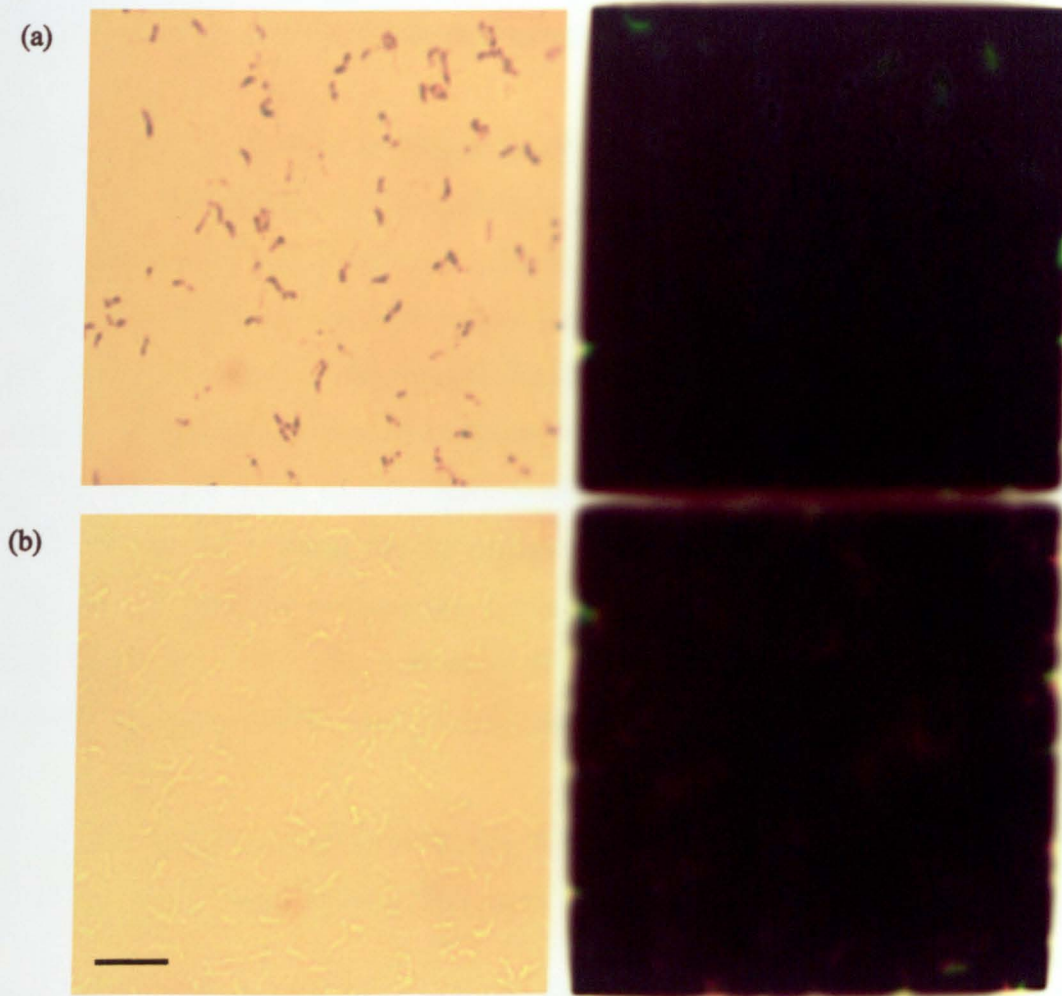


Figure 3.16: Combined INT reduction and R123/PI accumulation assay.

(a) Control and (b) heat-treated (70°C/30min) populations of *M. tuberculosis*. Brightfield and fluorescence images of the same microscopic field of view are shown. Cells were viewed at 1000 x magnification. Scale bar = 5 μm .

3.1.9 Results of other cytological assays

The other assays used to investigate cytological activity in *M. tuberculosis* met with little success. Whereas the control organism *Staphylococcus aureus* labelled as expected in the oxonol labelling experiments (*i.e.* cells inactivated by heat-treatment were readily stained while untreated cells excluded oxonol and did not fluoresce), no difference in labelling between heat-treated *M. tuberculosis* and untreated controls was observed. Mycobacteria inactivated by heat or ethanol treatment were expected to stain fluorescent red using the Live/Dead BacLIGHT kit but a mixture of fluorescent red, green and orange bacteria was observed instead. Substantial background fluorescence was observed with both of these assays. Although, *M. avium* stains successfully with the live cell nucleic acid SYTO16 (Ibrahim, 1996; 1997), the number of *M. tuberculosis* cells stained with this dye in the present study was found to be less than the number labelled with R123 or INT.

Since cytochemical labelling of *M. tuberculosis* with oxonol, Live/Dead stains, SYTO16 and CTC proved unsatisfactory, they were not included in further work.

3.1.10 Effect of labelling on colony counts

The effects of treatment with R123, PI, CCCP and INT on colony counts of *M. tuberculosis* are shown in Table 3.3. Treated bacteria produced approximately the same colony-forming ability as the controls. One notable observation was that sufficient INT was present in the undiluted and 10^{-1} diluted samples used to enumerate colony counts to produce dark red/purple coloured colonies.

Table 3.3: Effect of cytochemical assays on culture of *M. tuberculosis*

Treatment	CFU ml ⁻¹ ^a
Control	1.8 ± 0.4 × 10 ⁷
1 µM R123/ 3 µM PI 15 min	1.2 ± 0.2 × 10 ⁷
13 µM R123/ 3 µM PI 15 min	1.7 ± 0.4 × 10 ⁷
15 µM CCCP 15 min	1.8 ± 0.2 × 10 ⁷
150 µM CCCP 15 min	1.6 ± 0.4 × 10 ⁷
5 mM INT 15 min	1.5 ± 0.5 × 10 ⁷

^a Colony counts are expressed as means ± standard deviation of the means.

3.1.11 Development of GFP reporter strain

The *Aequorea victoria gfp* gene was cloned into the *E. coli*-mycobacterium shuttle vector pMV261 using the unique *Bam*HI and *Eco*RI sites downstream of the *hsp60* promoter, and transformed into *M. tuberculosis* as described in Section 2.10. However, none of the transformants selected by high-level kanamycin resistance produced fluorescence when examined at the single cell or colony level. Restriction digests of the *gfp* constructs (Figure 3.17) revealed that a DNA fragment of the expected size was inserted into pMV261 and analysis of the DNA sequence demonstrated that the *gfp* gene was successfully cloned downstream of the promoter. Problems with this cloning strategy had apparently also occurred in other laboratories (Locht, personal communication¹). At this time, Kremer *et al.*, (1995) and Dhandayuthapani *et al.*, (1995), described *gfp* expression in mycobacteria and the former group kindly provided their construct (pGFM11) for use in this study. *M. tuberculosis* was transformed with this construct as described.

Gfp-expressing *M. tuberculosis* produced fluorescent colonies when illuminated by long-wave UV light. At the single-cell level, GFP was brightly fluorescent and evenly distributed throughout the cell. Fluorescence was stable and did not fade quickly when illuminated. Apart from high-level kanamycin and streptomycin resistance, no obvious

difference in culture characteristics between transformants and the wild type strain was observed.

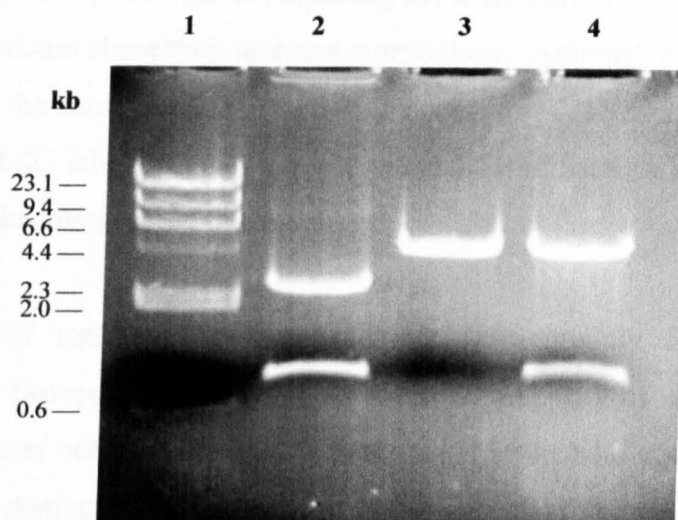


Figure 3.17: Restriction endonuclease digest of plasmids pGFM (lane 2), pMV261 (lane 3) and pRPC1 (lane 4).

Plasmid maps are shown in Figure 2.4. Plasmids were digested with *Eco*RI and *Bam*HI and analysed on 0.8% agarose gel. DNA size markers (*Hind* III digested lambda DNA) are shown in lane 1.

3.1.12 Total cell counts (TCC)

Three different methods of direct microscopic enumeration of mycobacteria were assessed as described in Section 2.7. Although the specifications of the Thoma counting chamber are such that its optical path with an ordinary coverslip is short enough so the chamber can be used under an oil-immersion objective at high power, it was both difficult and time consuming to use. Consequently, differentiating bacteria from debris or marks on the slide or coverslip and determining whether an object was a single large cell or a group of small cells proved difficult.

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Direct counting of bacteria immobilised on APS-coated coverslips was more successful, as cells were clearly identified using phase-contrast microscopy and readily distinguished from debris present. As well as estimating the total number of bacteria it was possible to record information about their size and morphology. Although there was no need to stain the bacteria, the same preparation could be used for TCC enumeration and cytological assays (e.g. R123 labelling). As a result, the number of bacteria demonstrating activity or a particular phenotype could be determined.

With the third method, DAPI-stained cells were brightly fluorescent on a black background. However, there was variation in the intensity of staining and background fluorescence was occasionally noted. Staining of amorphous shapes occurred but these were usually distinguishable from bacteria. Individual fluorescent cells were clearly labelled in small clumps.

The three methods were used to calculate the number of bacteria in suspensions of *M. tuberculosis* prepared as described in Section 2.4.4. Table 3.4 shows that there was little difference in the counts obtained. However, because of the straightforwardness of the techniques, direct counting of DAPI-stained bacteria on filters and bacteria immobilised on APS-coated coverslips were used for the remainder of the study.

Table 3.4: Comparison of direct microscopic methods used in total cell counts of *M. tuberculosis* ^a

Counting Method	TCC ml ⁻¹ ^b
Counting chamber	$1.27 \pm 0.34 \times 10^8$
Membrane filter sampled DAPI-stained	$1.14 \pm 0.52 \times 10^8$
Immobilised on APS-coated cover slip	$8.49 \pm 0.13 \times 10^7$

^a Bacterial suspensions were prepared from agar culture as described in Section 2.4.4 and diluted to an OD_{580nm} of 0.2.

^b Data are expressed as means \pm standard error of the mean.

3.1.13 Development of mycobacteriophage infectivity studies

High titre lysates of mycobacteriophage D29 were obtained using the *M. smegmatis* infection method described in Section 2.11.1. These lysates produced approximately 2 x

10^{10} PFU ml⁻¹ on *M. smegmatis* after 1-2 days incubation using the phage titres enumeration method described by Fullner and Hatfull (1997) (Section 2.11.2). Infection of mycobacterial cultures prepared in Tween-80 free media was essential for high titre lysates. Although 2 mM CaCl₂ was reported to enhance productive infection by D29 (David *et al.*, 1980), phage titres were not substantially reduced by its absence. All plaques observed were discreet and clear, surrounded by confluent bacterial growth. Phage titres prepared from mycobacteriophage lysates stored aseptically either at 4°C or at -20°C for several months remained the same.

M. tuberculosis cultures were prepared for phage infectivity studies as described in Section 2.11.3. Overnight culture in Tween 80-free broth before incubation with phage was essential for successful infection. Cultures treated with a multiplicity of infection of between 10 and 100 did not produce colonies when subcultured.

Effects of mycobacteriophage D29 infection on antimycobacterial drug treated *M. tuberculosis* cultures and *M. tuberculosis* broth culture were assessed using R123/PI cytochemical labelling and are presented in Section 3.2.10 and Section 3.3.2 respectively.

3.2 EFFECTS OF ANTIMYCOBACTERIAL DRUGS

The suitability of *M. tuberculosis* cell suspensions prepared from agar culture for cytochemical labelling and the development of R123/PI labelling, INT reduction, and *gfp* reporter strains as useful indicators of cytological activity in these populations were reported in Section 3.1. Here, the effects of clinically important antimycobacterial drugs on *M. tuberculosis* cultures are assessed using these techniques and compared to those obtained with conventional culture methods.

The experiments were done on many occasions during development and optimisation of the cytochemical assays using different inocula preparations, incubation conditions, sampling regimes, labelling procedures, recording and image analysis protocols. Bacteria in the early drug experiments were taken from the exponential growth phase of broth culture and consequently contained many unlabelled cells. These early experiments also used concentrations of R123 that were later shown to produce uncoupler-insensitive accumulation of dye, therefore it not known whether fluorescence reflected membrane potential or non-specific labelling. The results presented here are the culmination of this work and characterise the effects of the drugs with protocols that gave low numbers of non-labelling or ambiguous cells.

The effect of antimycobacterial drugs on colony counts, total cell counts, cytochemical labelling and most probable number estimates are presented individually in this section. The results are not presented together because of the complexity of the data.

3.2.1 Colony counts

The colony counts of *M. tuberculosis* cultures treated with single antimycobacterial drugs over 7-day assay periods as described in Section 2.9.2 are shown in Figure 3.18a. Isoniazid and rifampicin produced between two and three log reductions in CFU ml⁻¹. The effect of ethambutol was considerably less, and at most, a decline of one log cycle

was recorded. Although streptomycin initially produced reduction in colony counts comparable to isoniazid and rifampicin, the effect was transient as counts increased after day 4. Similar results were obtained with capreomycin (results not shown). Colony counts in drug-free controls increased by about one log over the assay period.

The colony counts of *M. tuberculosis* treated with isoniazid combinations and rifampicin combinations are shown in Figures 3.18b and 3.18c respectively. In each case, the result produced when these drugs were used alone is included for comparison. The antimicrobial activity of drug combinations was almost always greater than that of single drugs, with greatest reduction in colony counts produced by isoniazid-rifampicin, isoniazid-streptomycin and rifampicin-streptomycin combinations (reductions of between five and six log CFU ml⁻¹). Colony counts continued to decline after the 7-day assay period, and few colonies were detected when cultures were sampled at 14 and 21 days (results not shown). Regrowth, such as that observed when streptomycin and capreomycin when used individually, was not observed when combinations of antimycobacterial drugs were used.

It was noted that colonies of *M. tuberculosis* treated with antimycobacterial drugs occasionally took longer to grow to visible size than those of drug-free controls. Consequently, plates for CFU enumeration were incubated for six weeks before being discarded to ensure detection of any slow-growing colonies.

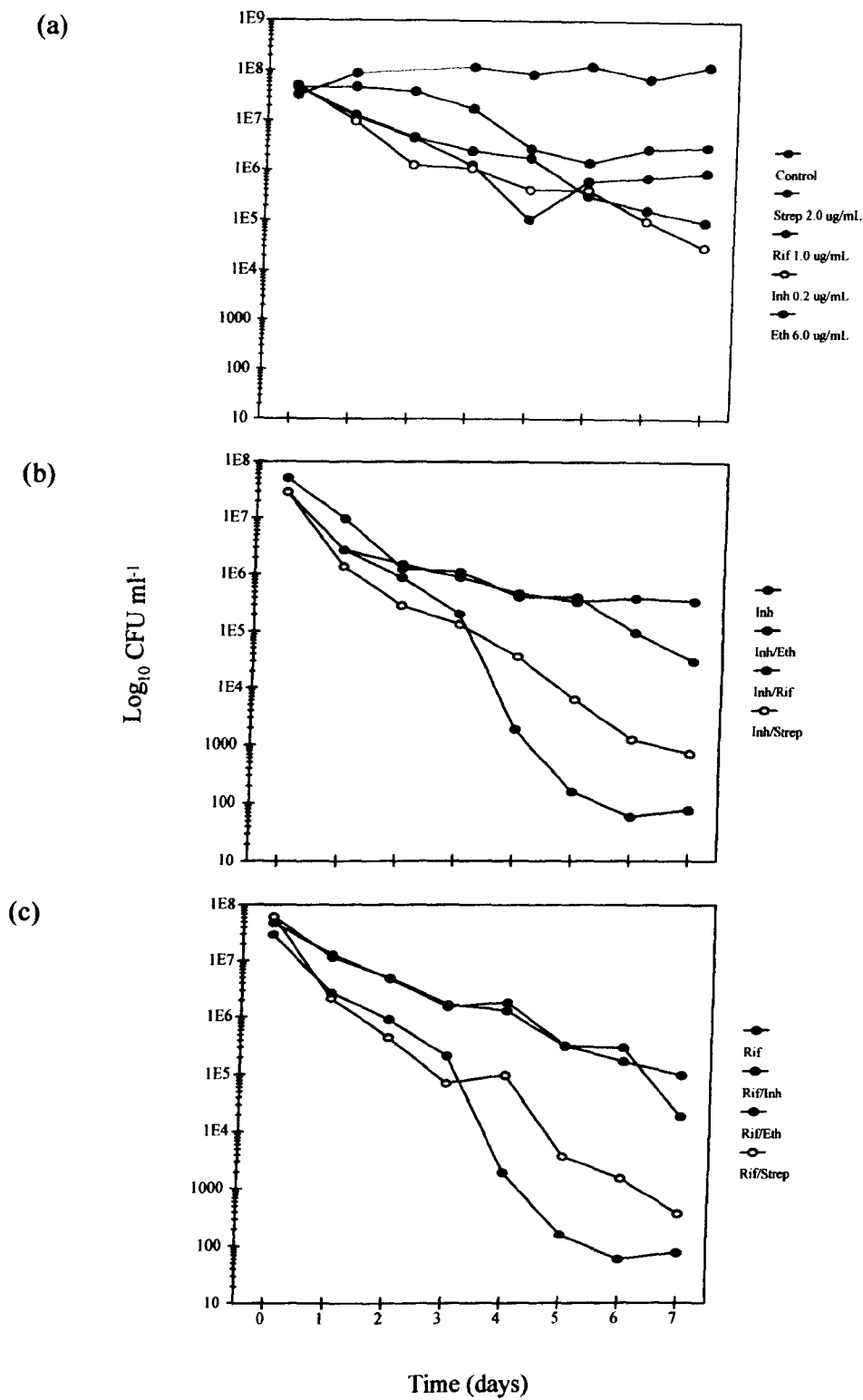


Figure 3.18: Effect of antimycobacterial drugs on colony counts of *M. tuberculosis*.

(a) single anti-mycobacterial drugs, (b) isoniazid combinations and, (c) rifampicin combinations. Colony counts are expressed as log₁₀ CFU ml⁻¹.

3.2.2 Total cell counts

None of the antimycobacterial drugs tested, either singularly or in combination, produced more than a half log change in the total cell count of *M. tuberculosis* cultures during the 7-day assay periods. Total cell counts and colony counts before and after 7 days treatment are compared below in Figure 3.19.

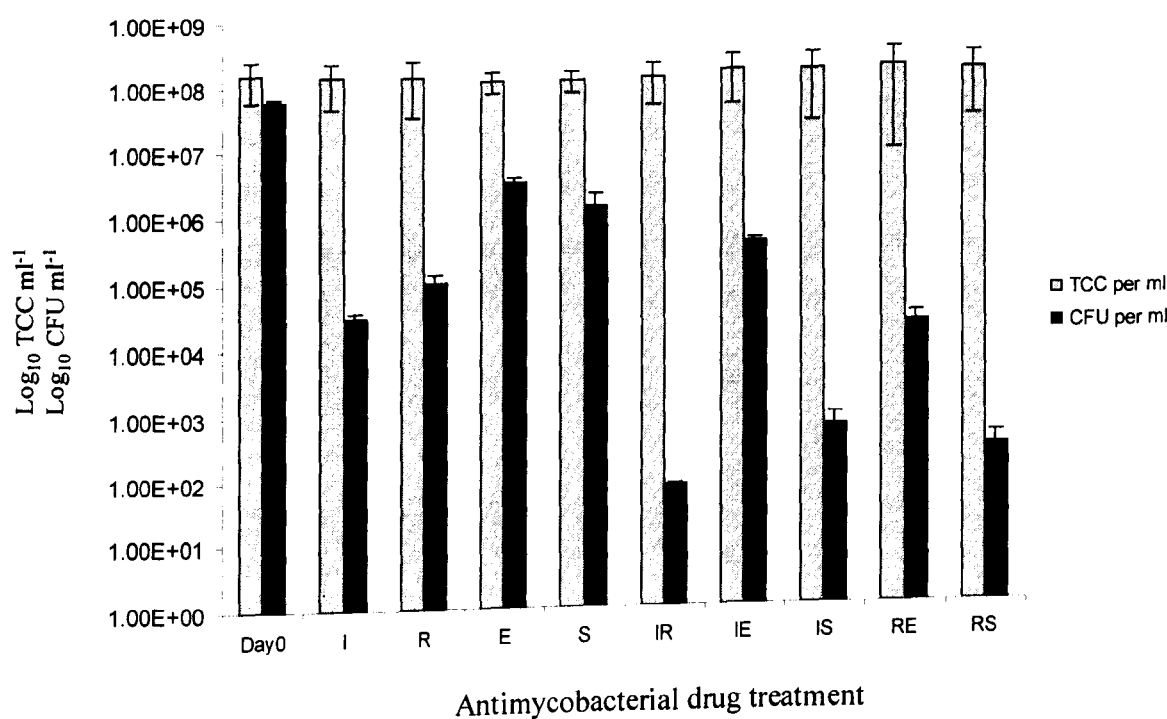


Figure 3.19: Total cell counts and colony forming unit counts of *M. tuberculosis* after 7 days treatment with antimycobacterial drugs.

Cultures were treated for 7 days with antimycobacterial drugs (S, streptomycin; I, isoniazid; R, rifampicin; E, ethambutol) and sampled for TCC and colony counts. Day 0 represents the counts before drug treatment. Counts are expressed as means \pm standard deviation of the means of data from independent experiments. TCC were by direct microscopic enumeration of DAPI-stained bacteria.

3.2.3 R123/PI labelling

Prior to treatment with antimycobacterial drugs, approximately 90% of bacteria labelled with R123, 1% stained with PI and the remainder were non-labelled. During the 7-day drug assays, there was a reduction in the proportion of R123-labelled bacteria and a concomitant increase in the number of PI-stained cells. The total number of R123-labelled bacteria ml^{-1} was calculated from the number of green fluorescent cells in monolayers of bacteria immobilised on APS-coated coverslips, as described in Section 2.7, and compared to the colony counts for each of the drugs tested (since it was necessary to process large number of images for these calculations, R123-labelled cell counts were evaluated from fluorescent cells visible on recorded fields of view rather than from quantitative data obtained using image analysis). To simplify interpretation, the results of exposure to individual drugs are shown in Figure 3.20, isoniazid in combination with other drugs in Figure 3.21 and rifampicin in combination with other drugs in Figure 3.22.

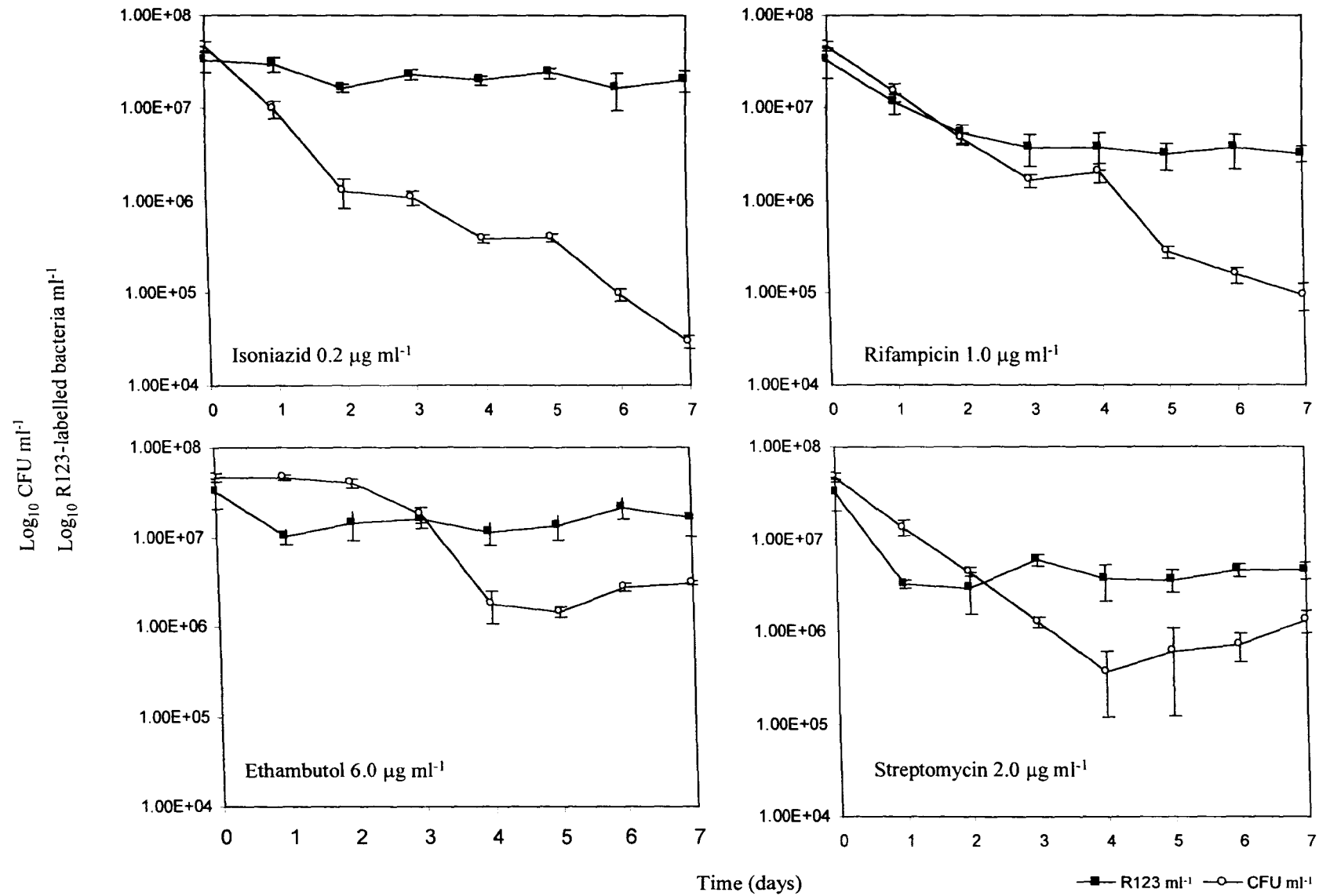


Figure 3.20: Comparison of colony counts and the number of R123-labelled bacteria in cultures of *M. tuberculosis* treated with single anti-mycobacterial agents.

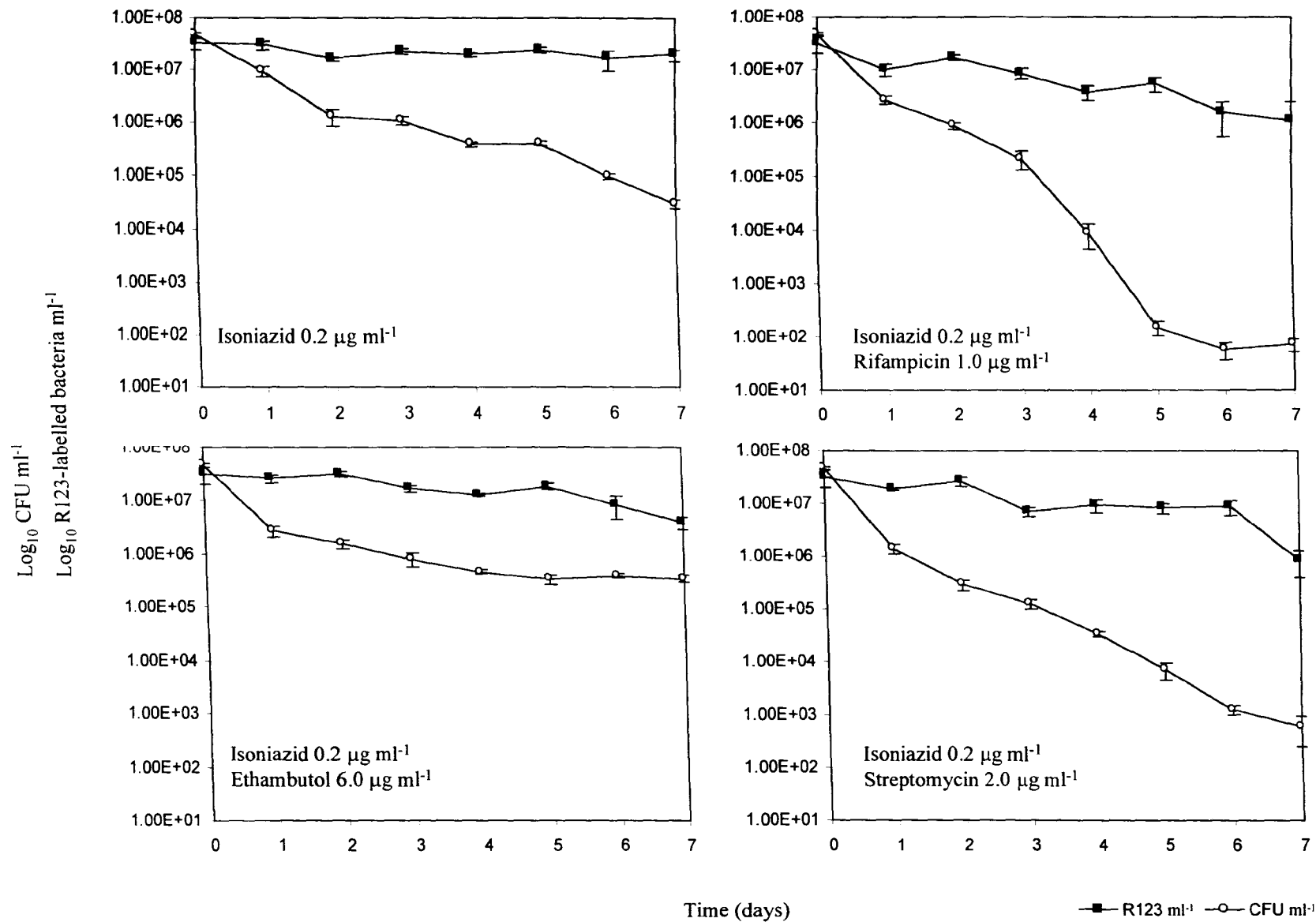


Figure 3.21: Comparison of colony counts and the number of R123-labelled bacteria in cultures of *M. tuberculosis* treated with isoniazid combinations.

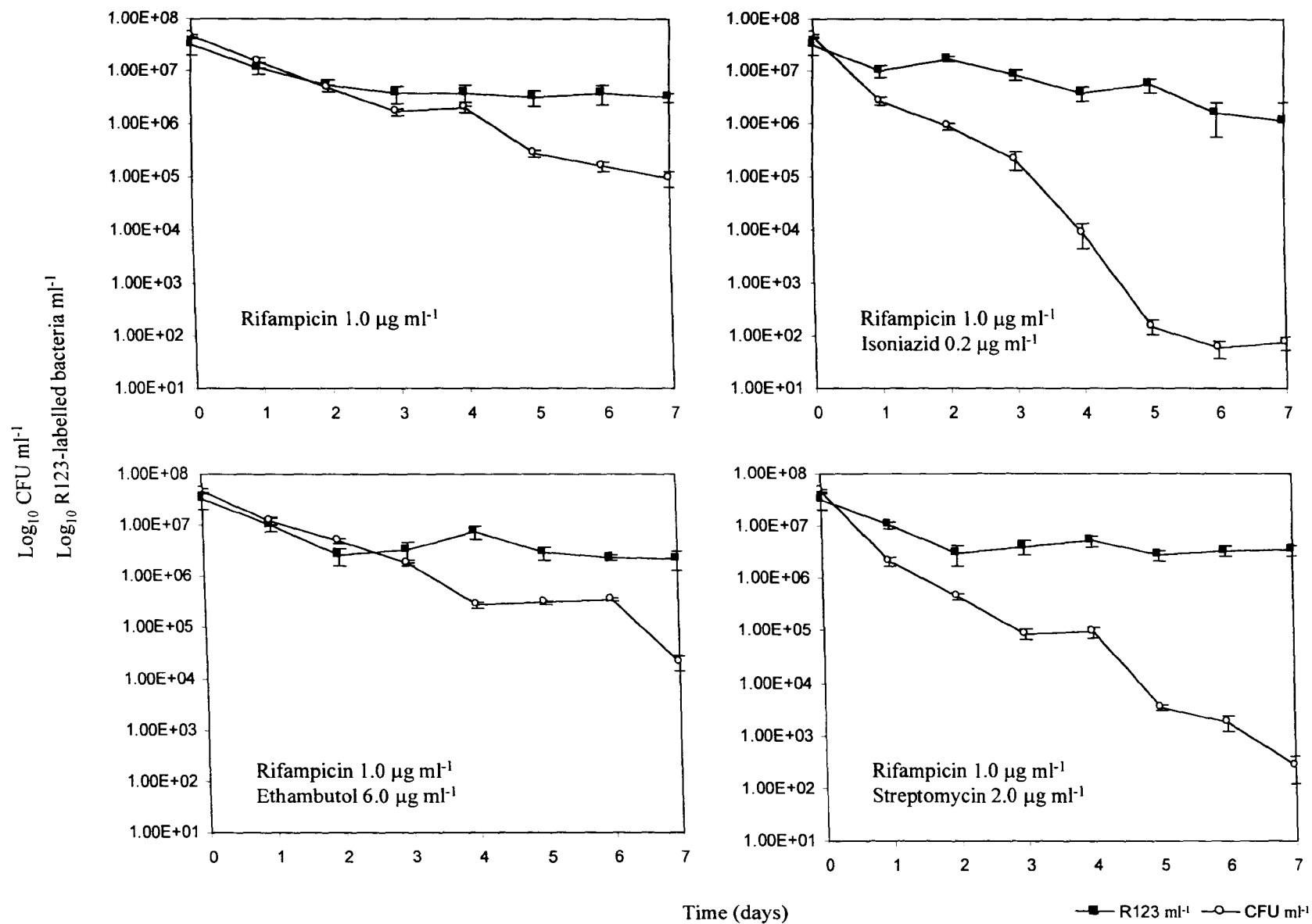


Figure 3.22: Comparison of colony counts and the number of R123-labelled bacteria in cultures of *M. tuberculosis* treated with rifampicin combinations.

It is evident from these graphs that treatment with antimycobacterial drugs produced substantial dissociation between the number of R123-labelled bacteria and the number of colonies counted. In all cases, the number of R123-labelled cells exceeded the number of cells that produced colonies. Exposure to single drugs resulted in between half and two logs more R123-labelled bacteria than colonies. Treatment with drug combinations produced greater dissociation, in the cases of isoniazid-rifampicin, isoniazid-streptomycin and rifampicin-streptomycin combinations differences greater than three and four logs were observed. The discrepancies between the number of R123-labelled bacteria and colony forming units after 7 days treatment with antimycobacterial drugs are summarised in Table 3.5.

Table 3.5: Comparison of R123-labelled cell counts (R123 ml⁻¹) and colony forming unit counts (CFU ml⁻¹) of *M. tuberculosis* culture after 7 days treatment with antimycobacterial drugs^a

Antimycobacterial drug(s)	R123 ml ⁻¹	CFU ml ⁻¹	Log ₁₀ difference
Isoniazid	1.4±0.5 x 10 ⁷	3.0±0.5 x 10 ⁴	2.8
Rifampicin	2.2±0.4 x 10 ⁶	9.6±3.2 x 10 ⁴	1.5
Ethambutol	1.1±0.4 x 10 ⁷	3.1±0.6 x 10 ⁶	0.7
Streptomycin	3.3±0.7 x 10 ⁶	1.3±0.7 x 10 ⁶	0.6
Isoniazid & Rifampicin	7.6±0.9 x 10 ⁵	7.6±0.5 x 10 ¹	4.2
Isoniazid & Ethambutol	2.7±0.6 x 10 ⁶	3.5±0.4 x 10 ⁵	1.1
Isoniazid & Streptomycin	5.7±2.8 x 10 ⁵	5.9±3.3 x 10 ²	3.1
Rifampicin & Ethambutol	1.5±0.6 x 10 ⁶	2.1±0.7 x 10 ⁴	2.0
Rifampicin & Streptomycin	2.7±0.6 x 10 ⁶	2.8±1.5 x 10 ²	4.1

^a Counts are expressed as means ± standard deviation of the mean of data from replica experiments.

The fluorescence intensity of R123-labelled cells after drug treatment was heterogeneous and mixtures of bright, dull and non-fluorescent bacteria were seen. Image analysis was used to measure and quantify fluorescence intensities throughout the 7-day drug assays. Scatter plots of the integrated grey level and mean grey level of green fluorescence of individual bacteria from selected analyses are shown in Figures 3.23-3.29. From such data, the average daily fluorescence intensities for each of these drug assays were calculated and are shown in Figure 3.30.

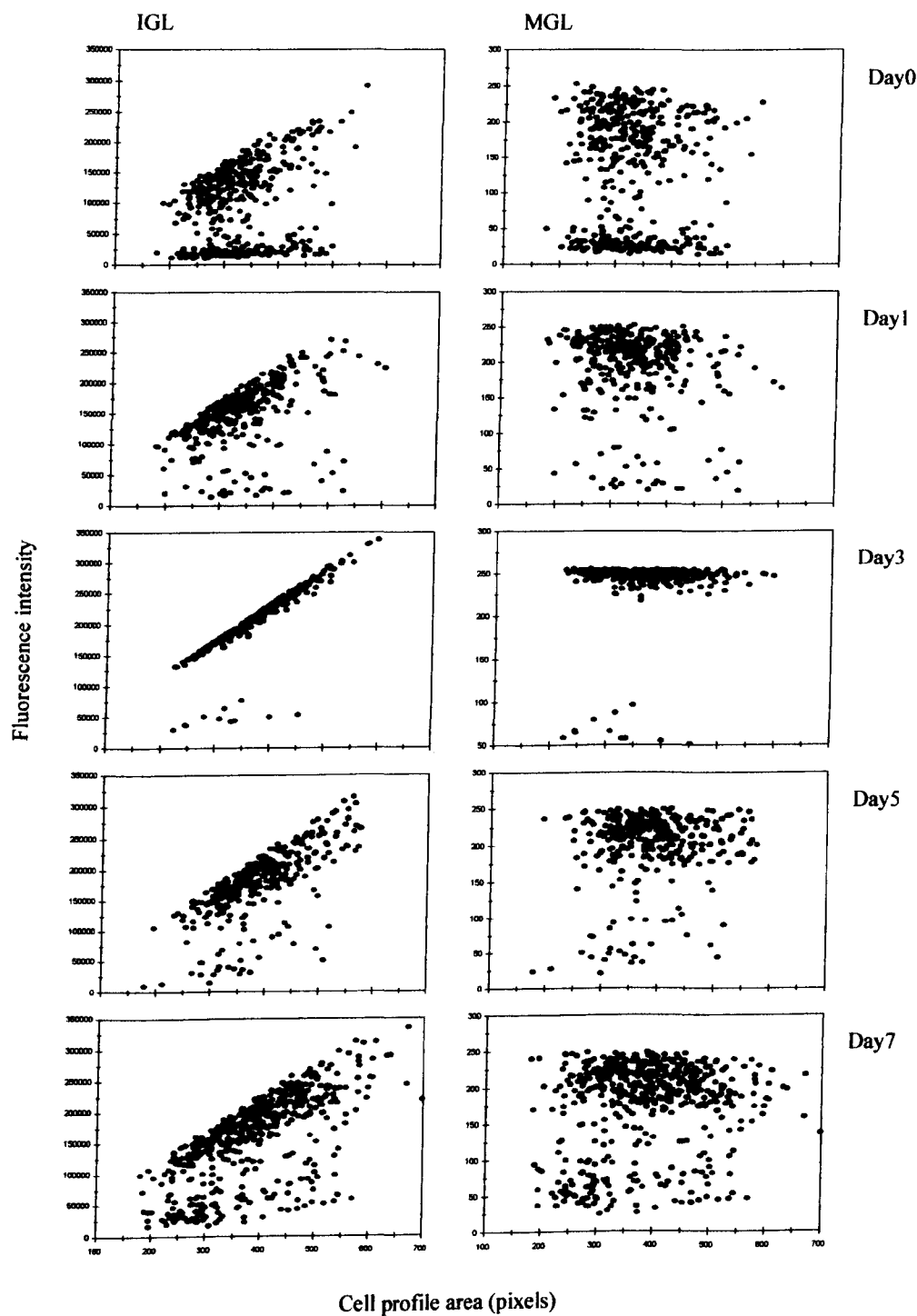


Figure 3.23: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with isoniazid.

Fluorescence is expressed both by integrated grey level and mean grey level.

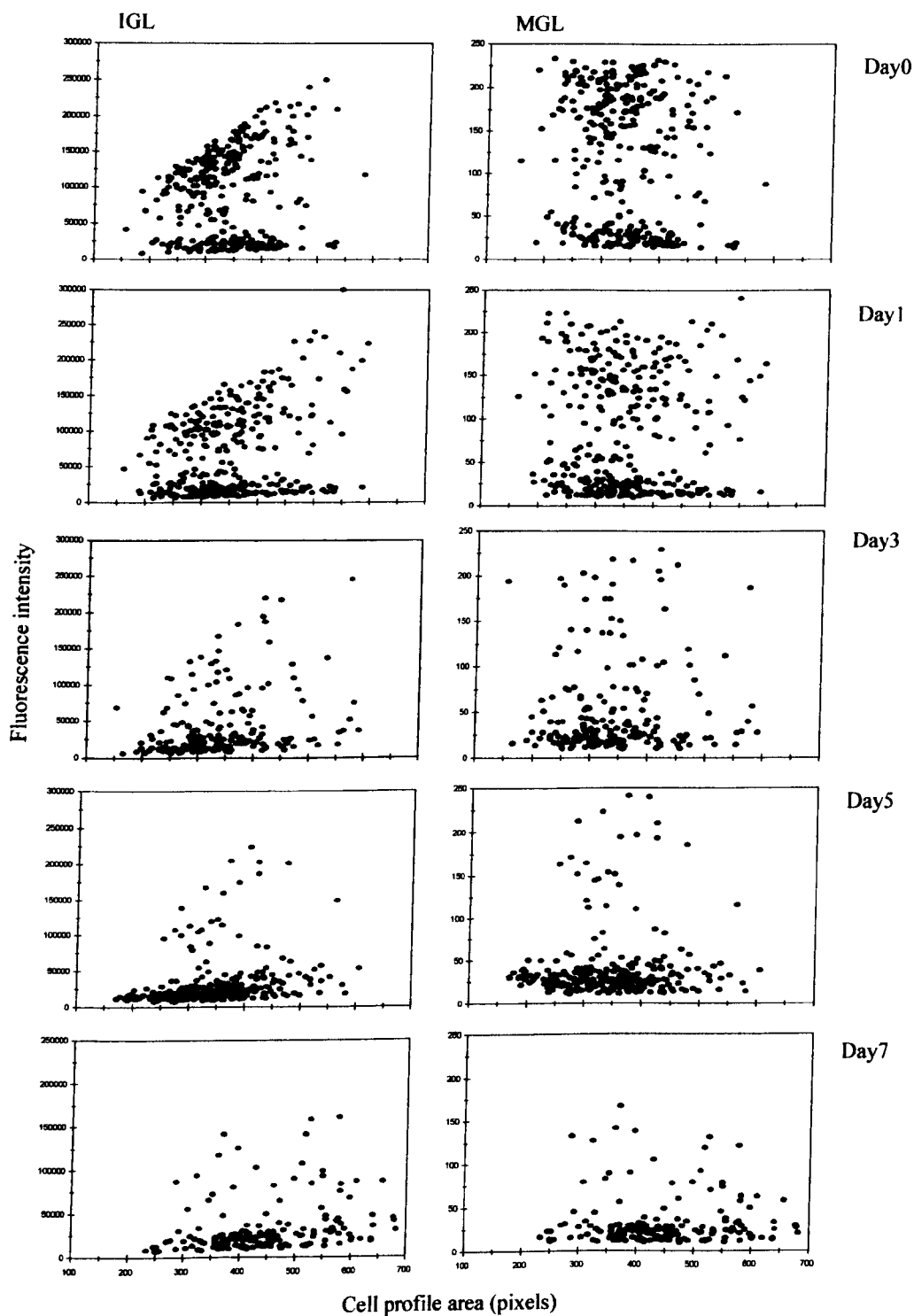


Figure 3.24: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with rifampicin.

Fluorescence is expressed both by integrated grey level and mean grey level.

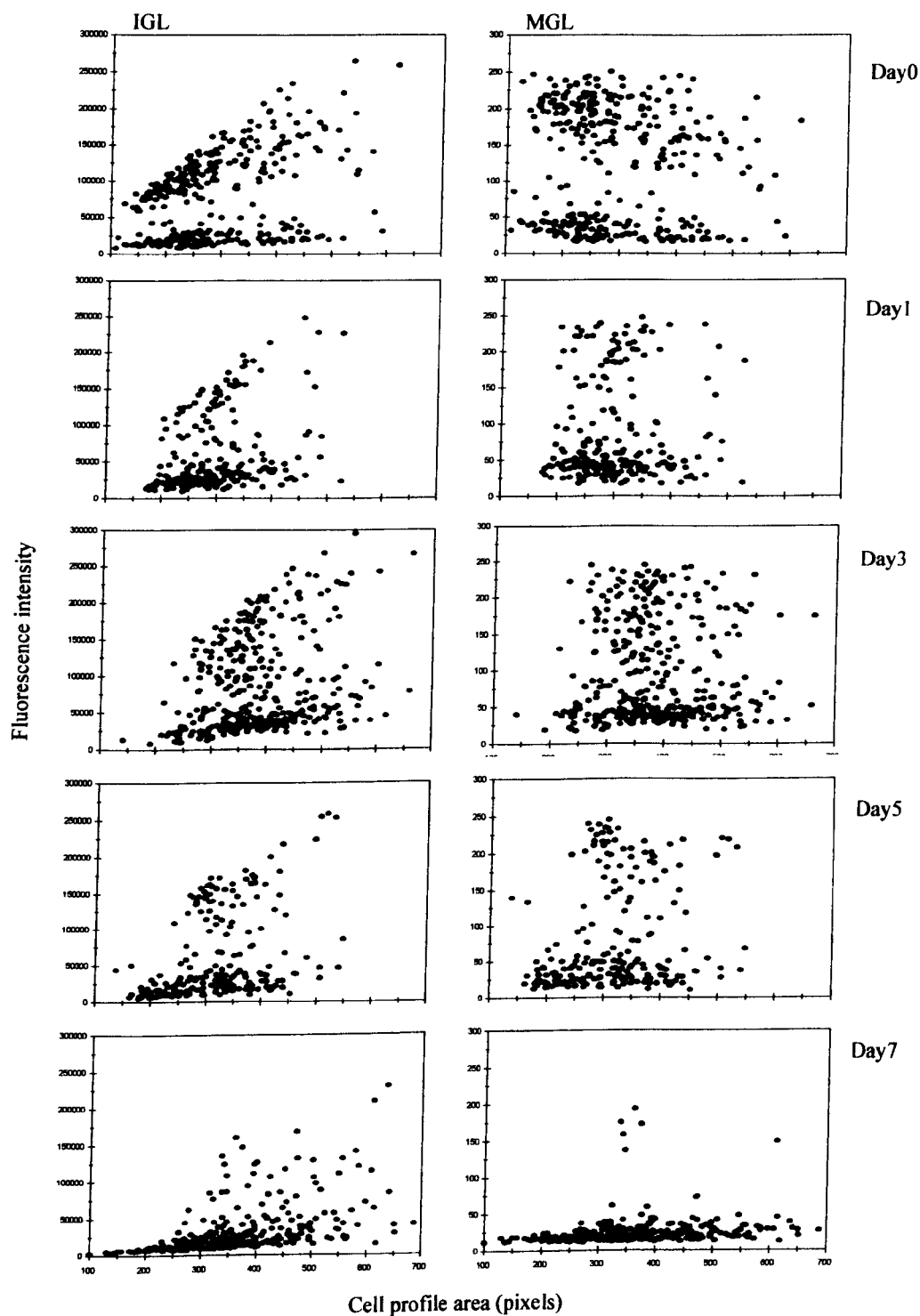


Figure 3.25: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with a combination of isoniazid and rifampicin.

Fluorescence is expressed both by integrated grey level and mean grey level.

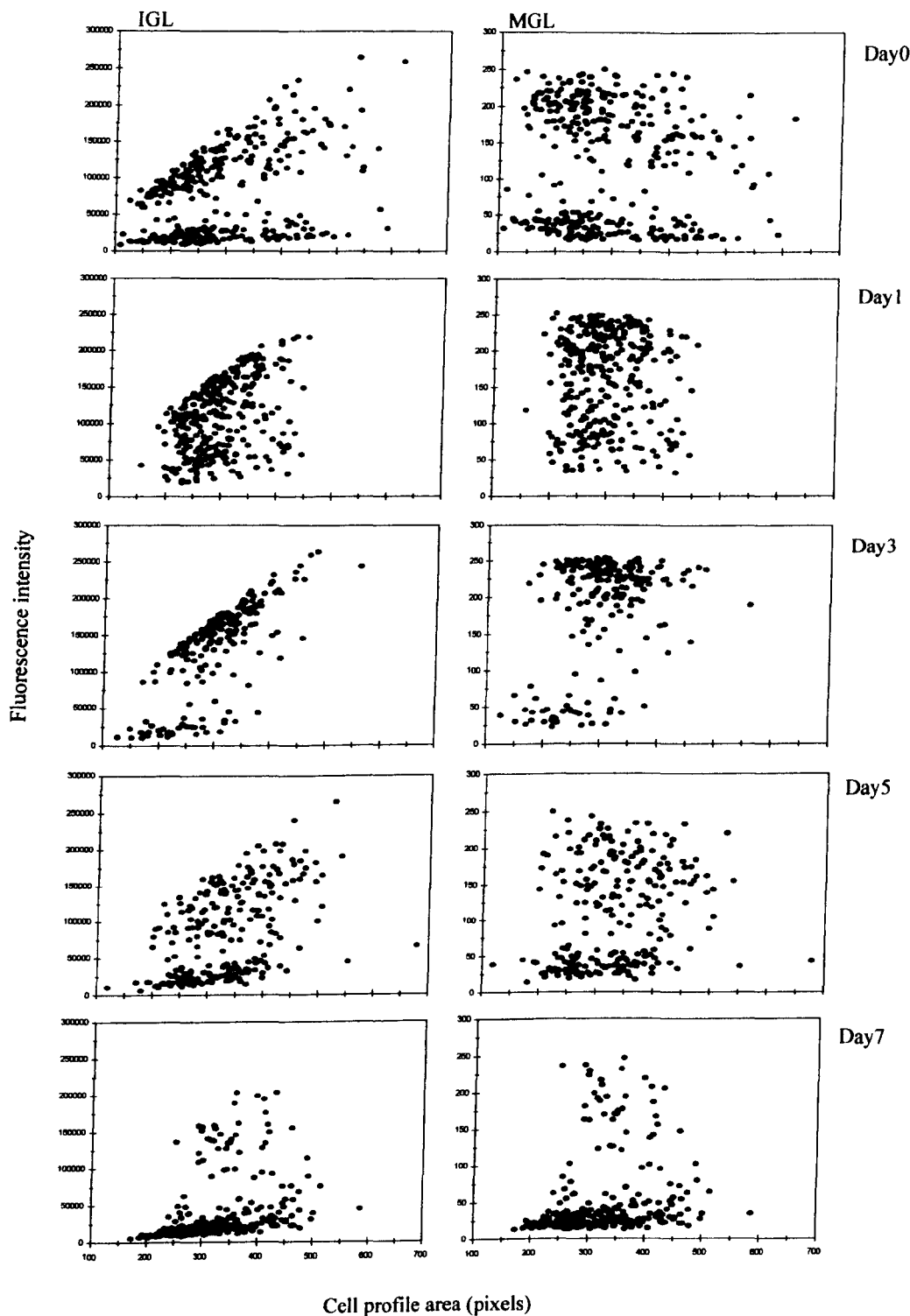


Figure 3.26: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with a combination of isoniazid and ethambutol.
Fluorescence is expressed both by integrated grey level and mean grey level.

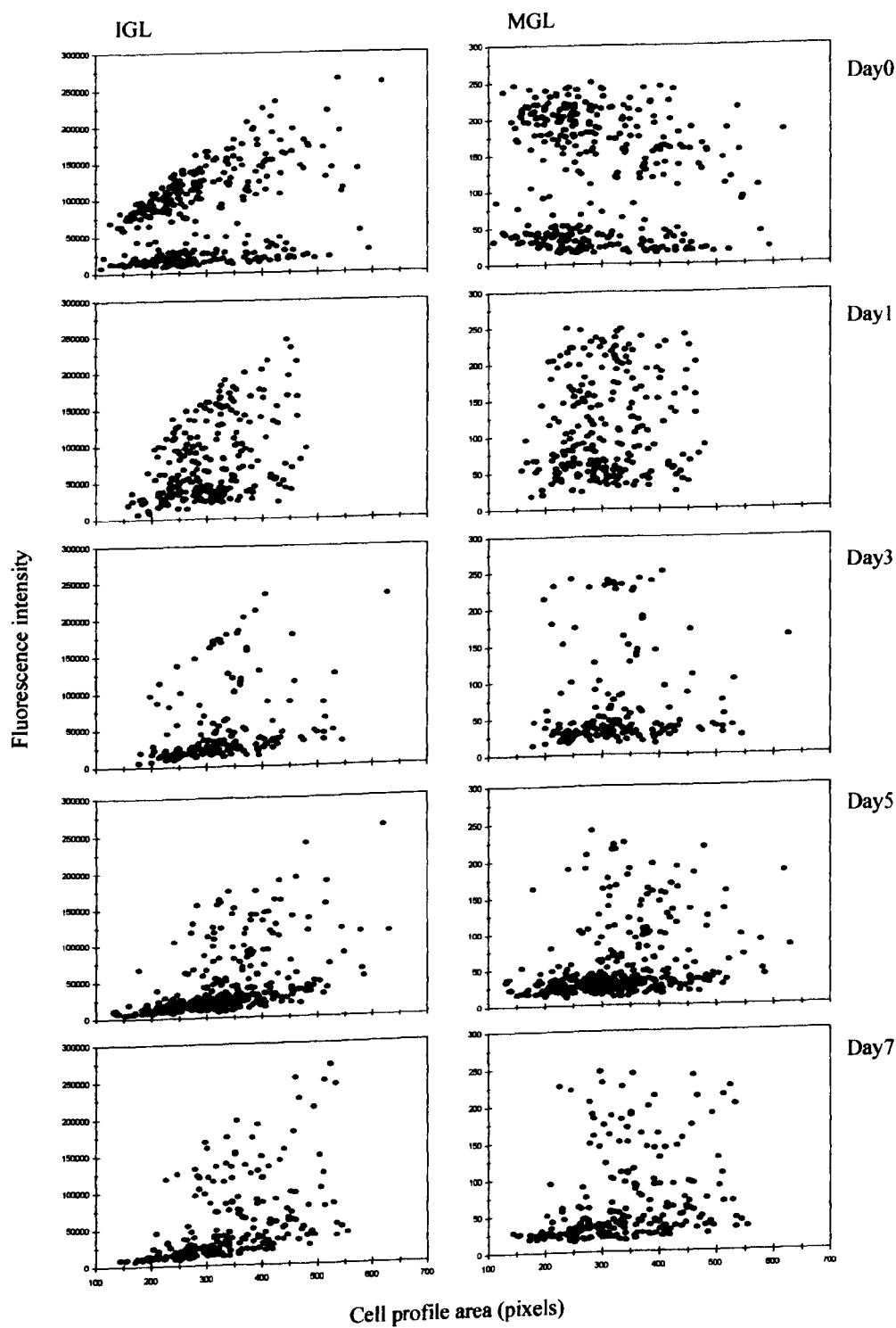


Figure 3.27: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with a combination of isoniazid and streptomycin.

Fluorescence is expressed both by integrated grey level and mean grey level.

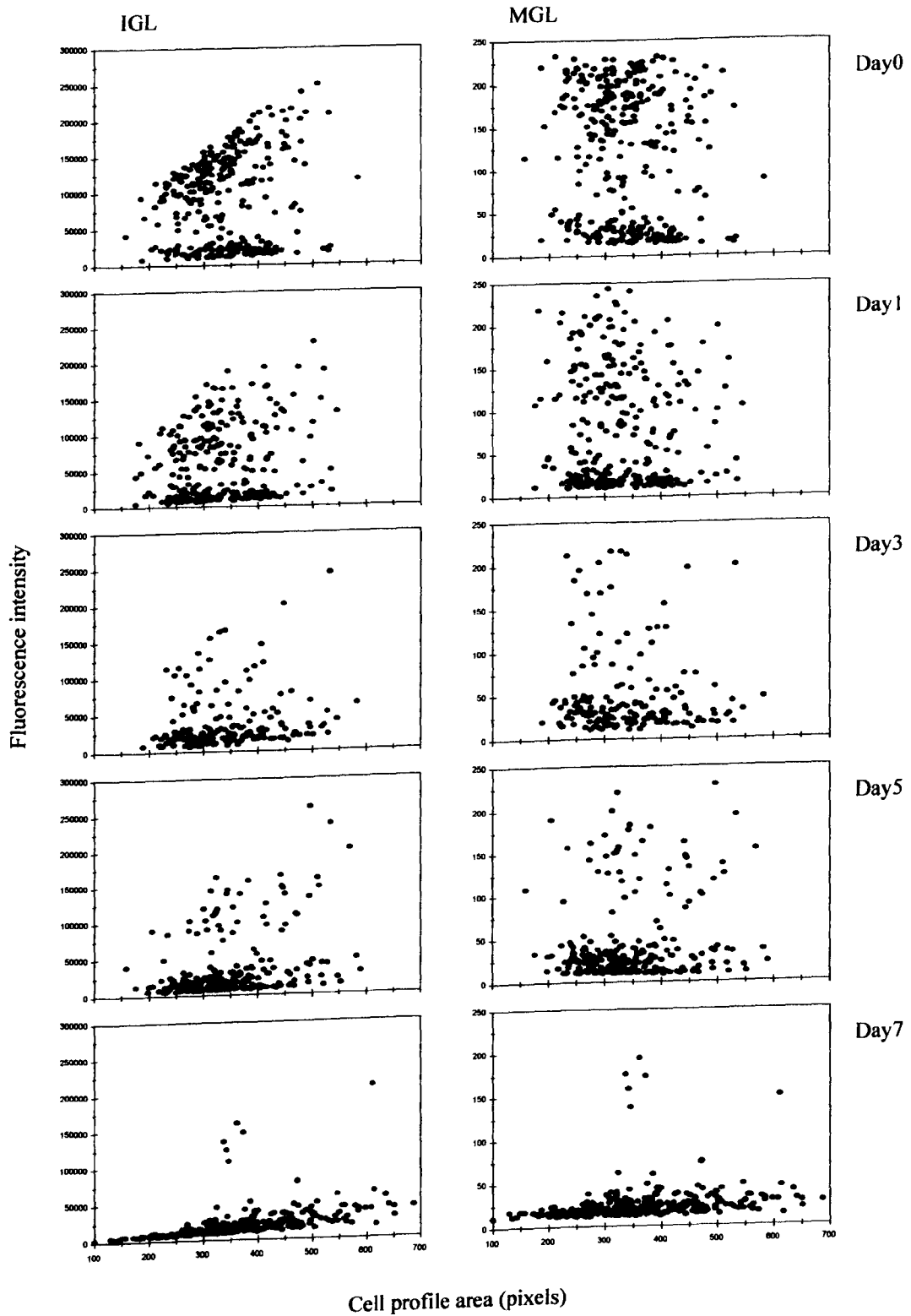


Figure 3.28: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with a combination of rifampicin and ethambutol. Fluorescence is expressed both by integrated grey level and mean grey level.

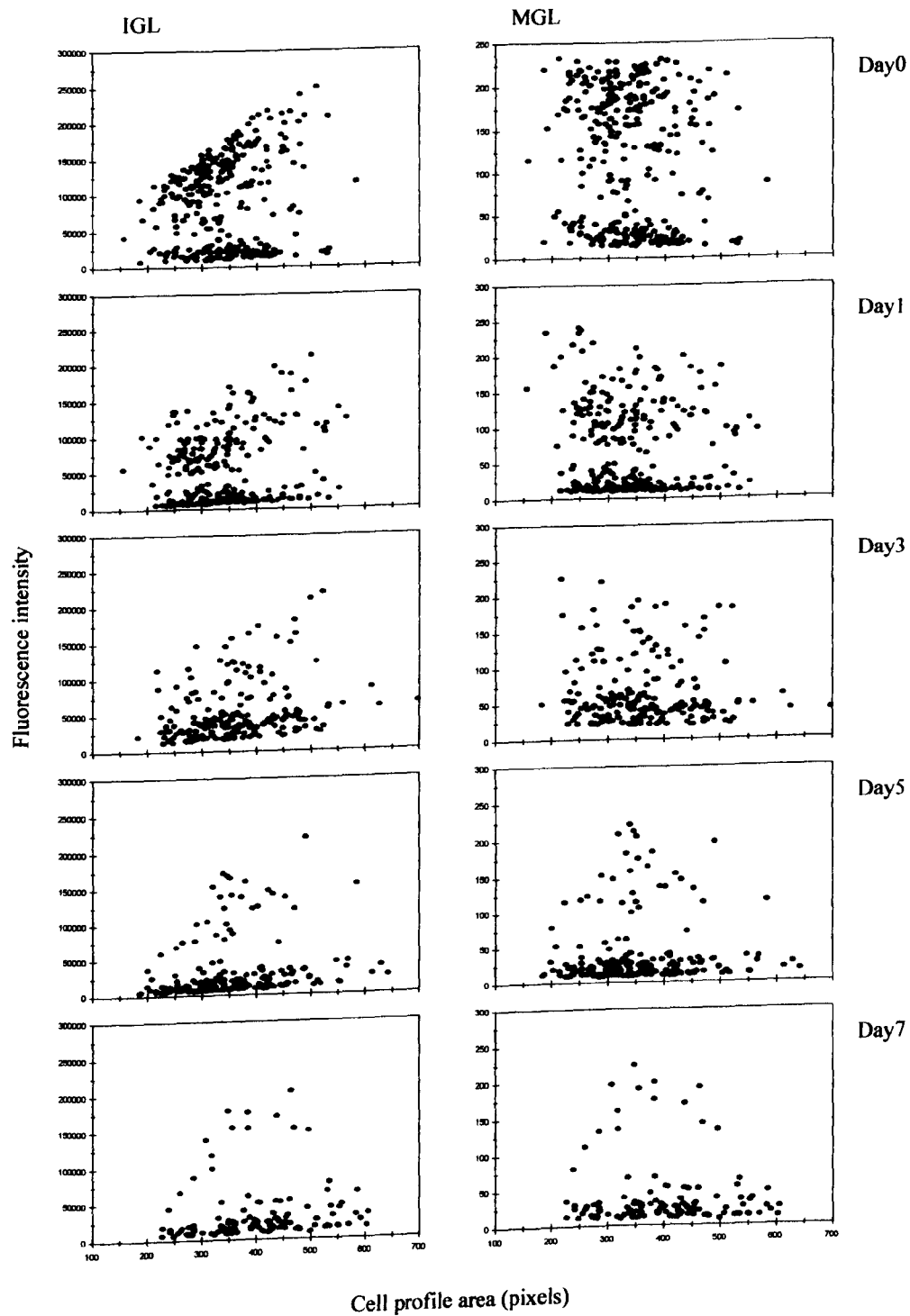


Figure 3.29: Scatter plots of the fluorescence intensities of R123-labelled *M. tuberculosis* during treatment with a combination of rifampicin and streptomycin.
 Fluorescence is expressed both by integrated grey level and mean grey level.

Two labelling trends with R123 were observed from the quantitative image analysis results. With the exception of treatments with isoniazid, and isoniazid-ethambutol combination, the scatter plots demonstrate the progressive reduction in the number of green fluorescent cells during the course of the 7-day treatments with antimycobacterial drugs already described. This reduction is reflected in the decrease of the average daily fluorescence intensities shown in Figure 3.30. Rifampicin treatment, for example, reduced the average green fluorescence intensity by a minimum of 30% within 24 hours and by at least 65% over the 7 days.

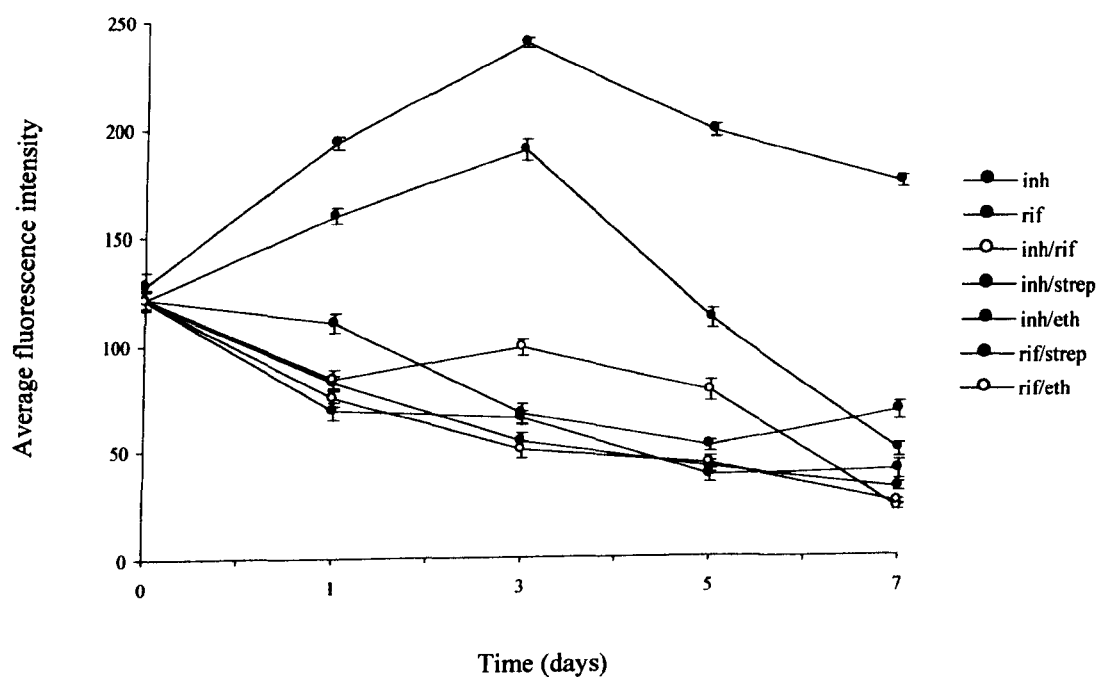


Figure 3.30: Effect of antimycobacterial drugs on the average fluorescence intensity of *M. tuberculosis*.

The average fluorescence intensity was calculated from the green fluorescence intensity attributable to each cell (MGL), including R123-labelled and non-labelled cells. Symbols represent mean \pm S.E.M of these values.

However, exposure of *M. tuberculosis* culture to isoniazid or to a combination of isoniazid and ethambutol produced enhanced uptake of R123 resulting in both increased fluorescence intensity per cell and an increase in the proportion of cells labelled. Increased average fluorescence intensities were observed within 24 hours of treatment (increases of 50% and 30% respectively) and were greatest after 3 days (increases of 85% and 55% respectively) (Figure 3.30). By this time, bacteria were almost exclusively labelled with R123 and few of the non-labelled proportion present before treatment were observed. By day 7, the average fluorescence intensity of isoniazid-treated cells remained higher than that measured in bacteria prior to treatment at day 0, whereas, the average fluorescence intensity of bacteria treated with isoniazid and ethambutol was reduced to less than 50% of that measured at day 0. Although increased R123 fluorescence intensities were not observed when *M. tuberculosis* cells were treated with isoniazid-streptomycin or isoniazid-rifampicin combinations, declines in average fluorescence intensities were not as gradual as those produced by rifampicin and the other drug combinations.

A noteworthy effect arising from the standardisation of image acquisition during experiments (*i.e.* using the same camera integration times and electronic gain for each image so that fluorescence intensities could be compared in meaningful way) can be observed in the scatter plots in Figure 3.23. The enhanced R123 fluorescence observed following isoniazid treatment meant that the brightest cells produced pixel saturation with the CCD camera. In such instance, quantitative fluorescence intensity data obtained using image analysis undervalues the total fluorescence produced by these cells and gives the inaccurate impression that they belong to distinct homogenous groups.

In addition to increased fluorescence, many bacteria treated with isoniazid and combinations of isoniazid-ethambutol produced intense polar labelling when stained with R123 (Figure 3.31). The majority of cells in these bacterial populations were characterised by increased refractility when viewed by phase-contrast microscopy and many oddly shaped, small cells were observed. Although ethambutol-treated cells also

became more refractile and stunted, increased fluorescence intensity and polar labelling were not observed. The morphological changes produced by the cell wall active antimycobacterial drugs were associated with a lower frequency of clumps in the monolayer preparations. Enhanced R123 uptake and polar labelling was not produced by the other antimycobacterial drugs tested and treated cells gradually became less refractile when viewed using phase contrast microscopy.

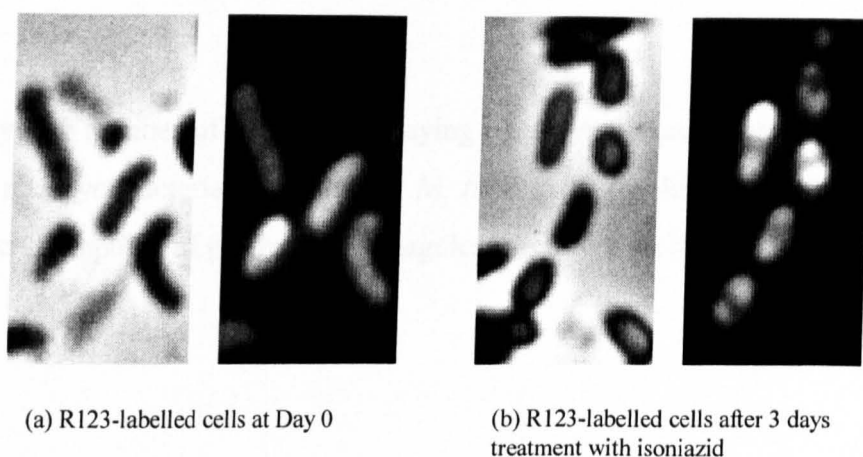


Figure 3.31: R123 polar labelling of isoniazid-treated *M. tuberculosis*.

Phase-contrast and fluorescence images of the same field of view of (a) bacteria before treatment and (b) bacteria treated with $0.2 \mu\text{g ml}^{-1}$ isoniazid for 3 days.

When viewed using phase contrast microscopy, R123-labelled and PI-labelled cells from *M. tuberculosis* cultures treated with antimycobacterial drugs were indistinguishable from each other. In other words, groups of morphologically similar bacteria could contain R123-labelled, PI-labelled and non-labelled cells. It was noted that a small number of bacteria labelled simultaneously with R123 and PI. In such cases, R123 fluorescence was low-level and localised in one part of the cell.

3.2.4 R123 uncoupler sensitivity studies

Cultures of *M. tuberculosis* treated with antimycobacterial drugs were labelled with R123 in the presence of CCCP (150 μ M) at intervals during the assays to demonstrate that accumulation of dye was uncoupler sensitive. Results of labelling experiments on cultures treated for 7 days with isoniazid, rifampicin and a combination of the two are shown in Figures 3.32-3.34 and demonstrate that fluorescence of cells was sensitive to uncoupler agent. Similar results were obtained with the other antimycobacterial drugs tested.

In summary, the number of bacteria displaying phenotypes consistent with physiological activity in antimycobacterial drug-treated *M. tuberculosis* cultures greatly exceeded the number of cells capable of producing propagules by culture on Middlebrook 7H10 agar.

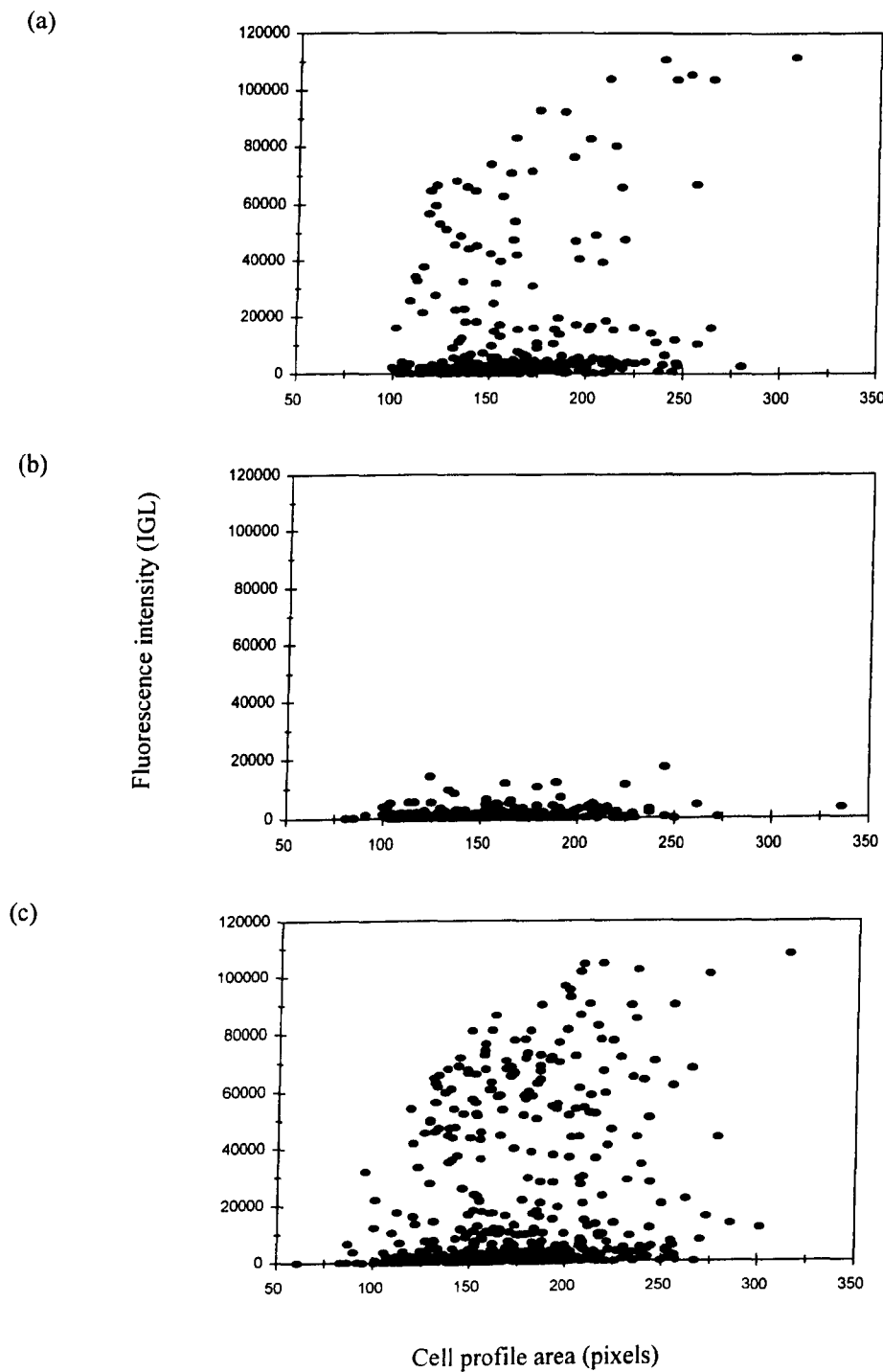


Figure 3.32: Uncoupler-sensitive accumulation of R123 by isoniazid-treated *M. tuberculosis*.

Bacteria treated for 7 days with $0.2 \mu\text{g ml}^{-1}$ isoniazid were (a) labelled with $1 \mu\text{M}$ R123 (b) treated with $150 \mu\text{M}$ CCCP and labelled with R123 (c) treated with CCCP, washed in fresh broth and labelled with R123.

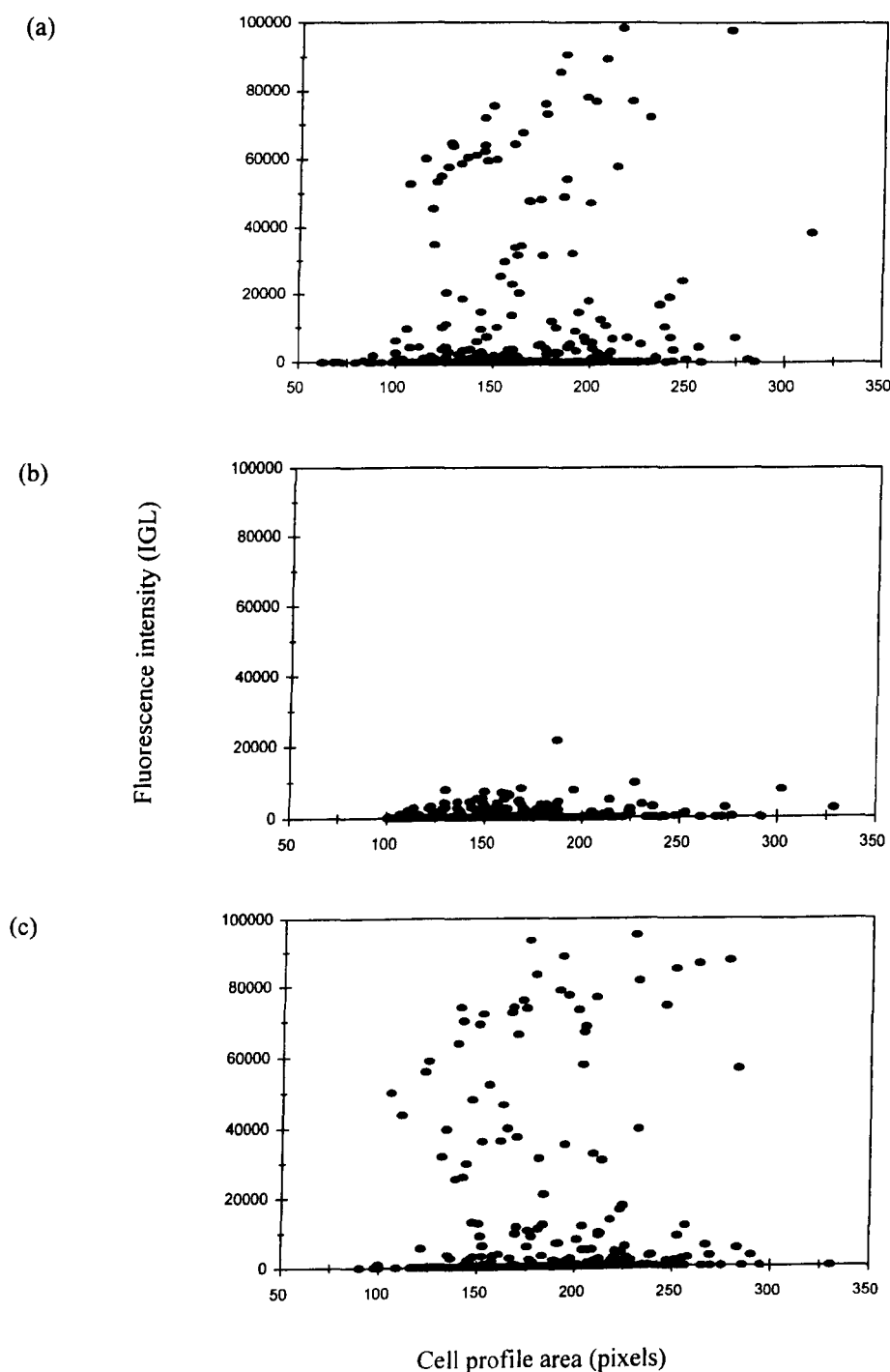


Figure 3.33: Uncoupler-sensitive accumulation of R123 by rifampicin-treated *M. tuberculosis*.

Bacteria treated for 7 days with $2.0 \mu\text{g ml}^{-1}$ rifampicin were (a) labelled with $1 \mu\text{M}$ R123 (b) treated with $150 \mu\text{M}$ CCCP and labelled with R123 (c) treated with CCCP, washed in fresh broth and labelled with R123.

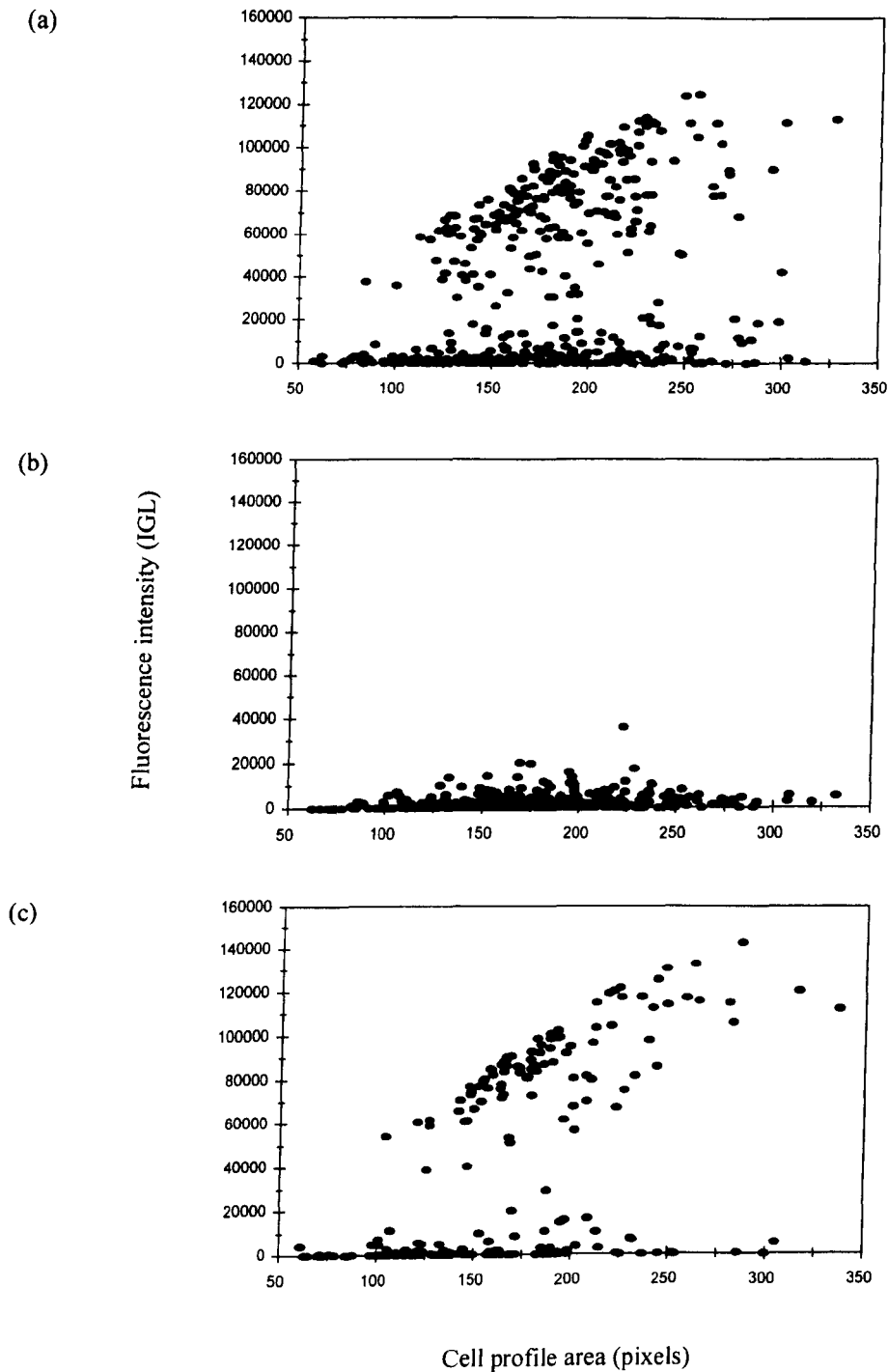


Figure 3.34: Uncoupler-sensitive accumulation of R123 by *M. tuberculosis* treated with a combination of isoniazid and rifampicin.

Bacteria treated for 7 days with a combination of $0.2 \mu\text{g ml}^{-1}$ isoniazid and $2.0 \mu\text{g ml}^{-1}$ rifampicin were (a) labelled with $1 \mu\text{M}$ R123 (b) treated with $150 \mu\text{M}$ CCCP and labelled with R123 (c) treated with CCCP, washed in fresh broth and labelled with R123.

3.2.5 MPN enumeration

In light of the discrepancies between the number of cells with physiological activity and colony counts on Middlebrook 7H10 agar, propagules of *M. tuberculosis* in drug-treated cultures were also enumerated in Middlebrook 7H9 broth using the MPN method described in Section 2.9.3. The results from preliminary experiments revealed that sufficient active drug was present in the cultures at day 7 to prevent growth in the undiluted and least diluted tubes of the MPN assay. Consequently, bacteria were harvested at day 7 by centrifugation and washed in drug-free Middlebrook 7H9 broth for MPN enumeration.

The transition from 5 positive to 5 negative tubes for all samples took place within two or three dilutions steps, thus indicating Poisson distribution of cells (Niemelä, 1983). MPN estimates and colony counts of *M. tuberculosis* at day 7 of the drug assays are compared in Figure 3.35. Although the MPN estimates for each of the antimycobacterial drugs and drug combinations tested were almost always greater than the colony counts, difference were usually about a half log and never more than one log.

Whereas, results of colony counts changed little after 4 weeks incubation, MPN tubes were not discarded until after 6 weeks of continued incubation as turbidity in some of the dilutions was not observed until then. All randomly sampled positive MPN tubes contained acid-fast bacteria.

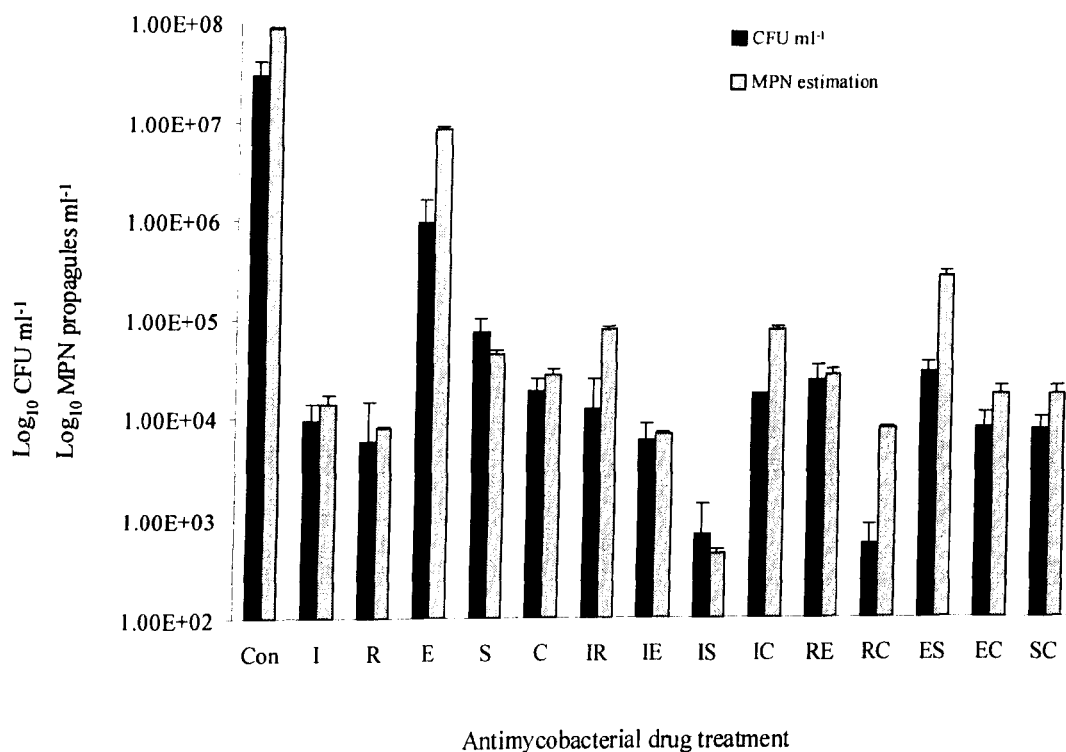


Figure 3.35: Comparison of colony forming unit (CFU) and most probable number (MPN) estimates of propagules of *M. tuberculosis* after 7 days treatment with antimycobacterial drugs.

Cultures were treated for 7 days with antimycobacterial drugs (I - isoniazid, 0.2 µg ml⁻¹; R - rifampicin, 2.0 µg ml⁻¹; E - ethambutol, 6.0 µg ml⁻¹; S - streptomycin, 2.0 µg ml⁻¹ and C - capreomycin, 10 µg ml⁻¹), washed in drug-free Middlebrook 7H9 broth and sampled for MPN and CFU counts. Con represents the counts before drug treatment. Colony counts are presented as CFU ml⁻¹ ± S.D. and most probable numbers as MPN ml⁻¹ ± S.D. of log₁₀MPN as described by Niemelä (1983).

3.2.6 Cell elongation and loss of R123 labelling in drug-free controls

The effect of antimycobacterial drugs on R123/PI labelling of *M. tuberculosis* was discussed in Section 3.2.3 (*i.e.* a decrease in the number of R123-labelled bacteria and a concomitant increase in the proportion of PI-labelled cells). In these experiments, changes in labelling were compared to labelling before drug treatment (*i.e.* at day 0) rather than labelling in drug-free controls. In the drug-free controls, morphological and labelling changes occurred which were later shown to be characteristic of *M. tuberculosis* when transferred from agar to broth culture. These changes, summarised in Figure 3.36, were most obvious over the first three days and did not occur in the presence of any of the antimycobacterial drugs tested. In the absence of drugs, individual cells elongated and increased in length from an average of 3.0 to 4.1 μm within 72 h. During this time bacteria became recalcitrant to labelling with R123 and the proportion of labelled cells decreased from approximately 80-90% to as low as 10%. The non-labelled cells did not stain with PI and were indistinguishable from labelled cells when viewed using phase-contrast microscopy. Over the same period a four-fold increase in colony counts was observed (1.7×10^7 to 7.2×10^7 CFU ml^{-1}).

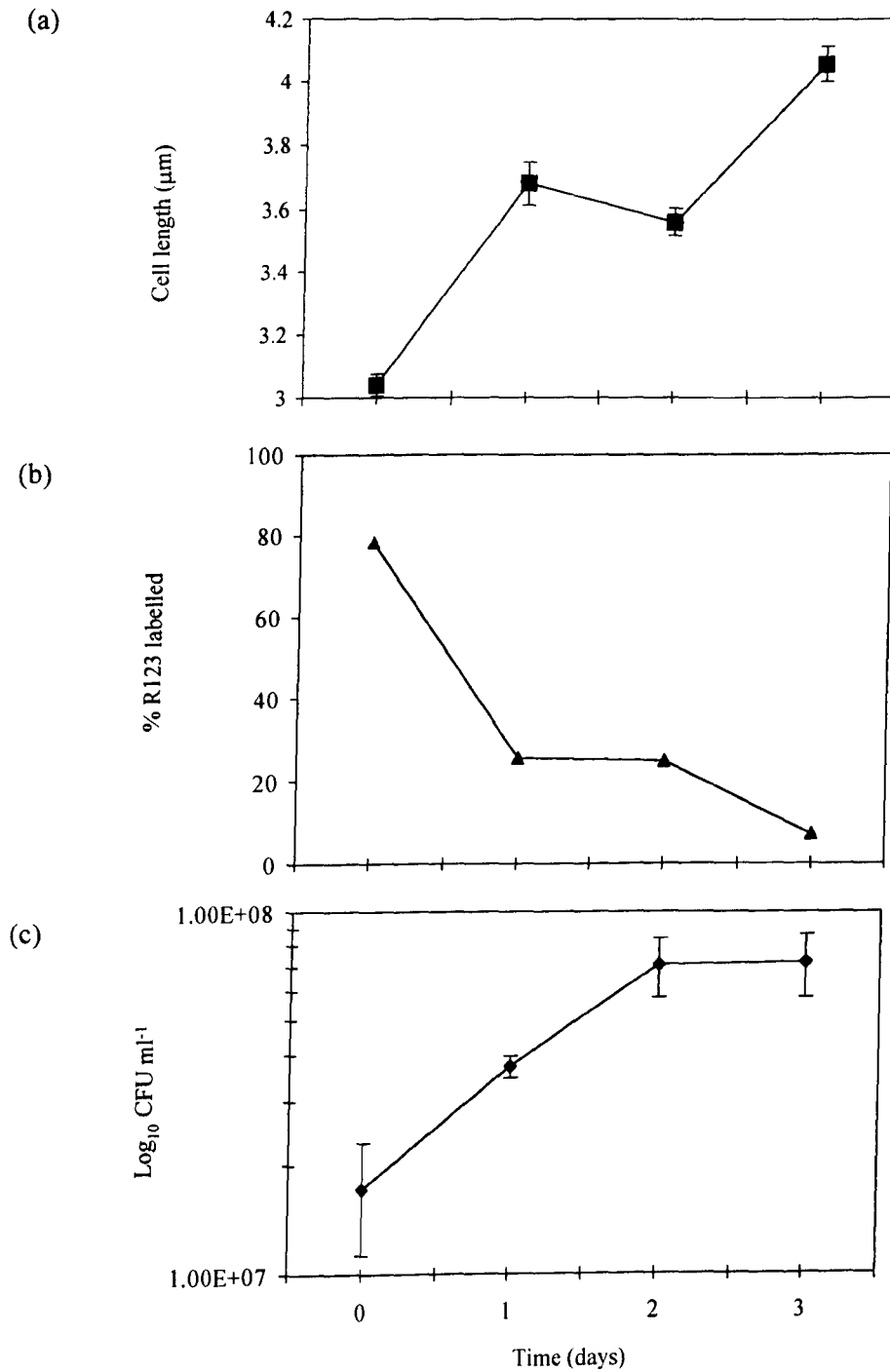


Figure 3.36: Changes in cell length, R123 labelling and colony counts of *M. tuberculosis* following transfer from Middlebrook 7H10 agar to Middlebrook 7H9 broth.

(a) Cell lengths are expressed as means \pm S.E.M., (b) R123 labelling as percentage labelled and (c) colony counts as means \pm S.D.

3.2.7 INT reduction

The effects of isoniazid and rifampicin on cultures of *M. tuberculosis* were also assessed using INT reduction as an indicator of oxidative metabolism. Two trends were produced, comparable to the distinct trends produced with R123 accumulation.

Enhanced reduction of INT was observed in isoniazid-treated cultures resulting both in larger formazan deposits and an increased proportion of labelled cells (Figures 3.37 and 3.38). Analysis of these cells revealed that increased formazan deposits were produced within 24 hours of treatment and were greatest after 3 days (Figure 3.37a). Examples of formazan deposits in cells before treatment and at day 3 of treatment are shown in Figure 3.37b. Although the deposits filled the cells at day 3, they appeared more concentrated at the bacterial poles. By day 7, the optical density measurements of isoniazid-treated cells had returned to a level close to that measured in untreated bacteria at day 0. The proportion of cells labelled with INT increased from approximately 65% to 95% after 24 hours treatment with isoniazid (Figure 3.38). This increase was followed by a gradual reduction in the proportion of labelled cells and by day 7 the number of cells containing formazan deposits was approximately the same as that observed at day 0 before drug treatment. Isoniazid treatment produced a 2.5 log reduction in colony counts over the assay period.

Although a small increase in the proportion of labelled cells was produced in cultures treated with a combination of isoniazid and rifampicin after 24 hours (Figure 3.38), an increase in formazan deposit size was not observed (results not shown). This was followed by a rapid decrease in the number of labelled bacteria and by day 7 less than 20% of the cells identified by phase contrast microscopy contained detectable deposits.

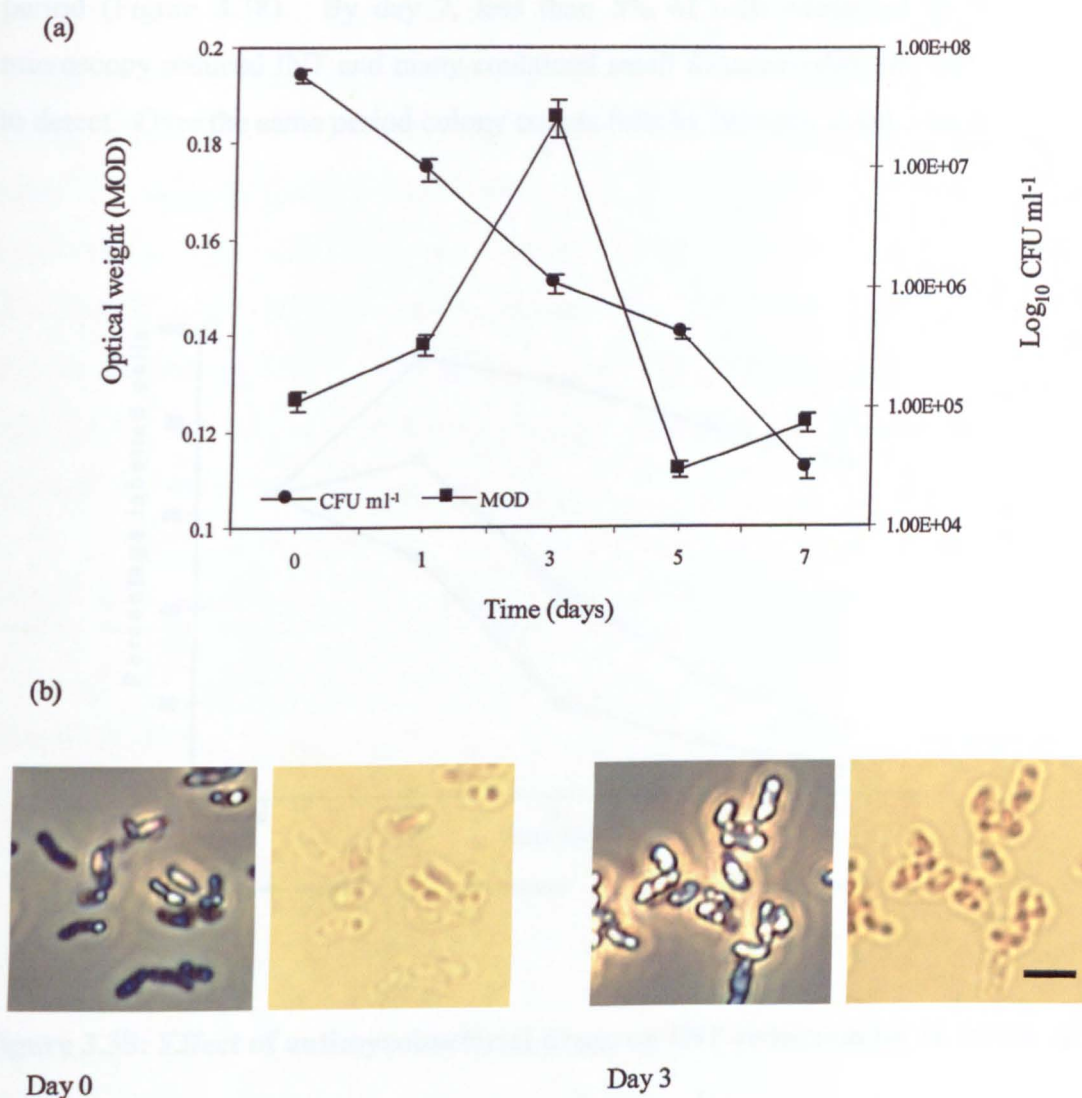


Figure 3.37: Effect of isoniazid treatment on colony counts and INT reduction by *M. tuberculosis*.

Bacteria were treated with $0.2 \mu\text{g ml}^{-1}$ isoniazid. (a) INT reduction is expressed as the average mean optical density (M.O.D. \pm S.E.M.) of bacteria with formazan deposits, colony counts are expressed as $\log_{10} \text{CFU ml}^{-1}$ (\pm S.D.). (b) Phase-contrast and bright-field microscopic images comparing formazan deposits in cells before treatment with those in cells after 3 days isoniazid treatment. Scale bar = $2 \mu\text{m}$.

In contrast, cultures of rifampicin-treated *M. tuberculosis* demonstrated a gradual and progressive reduction in both the number and size of formazan deposits over the assay period (Figure 3.38). By day 7, less than 5% of cells identified by phase contrast microscopy reduced INT and many contained small formazan deposits that were difficult to detect. Over the same period colony counts fell by between 2 and 3 log units.

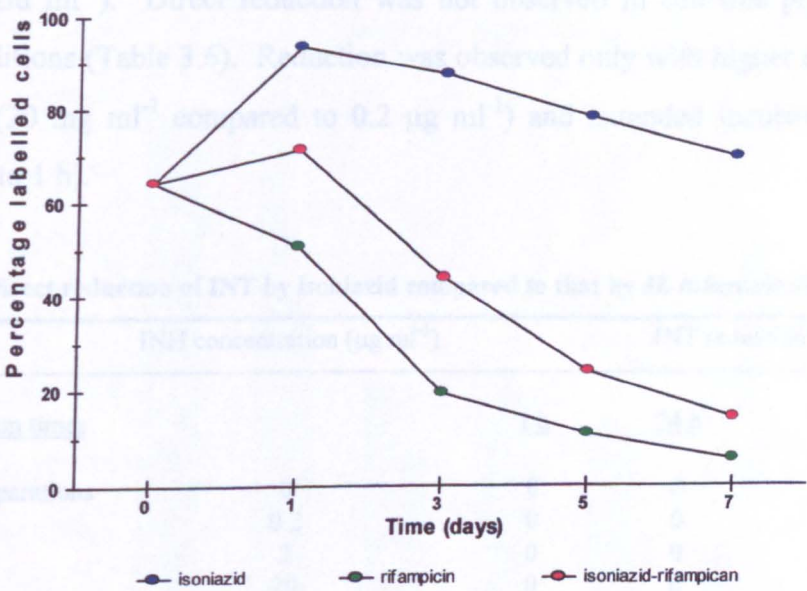


Figure 3.38: Effect of antimycobacterial drugs on INT reduction by *M. tuberculosis*

Cell suspensions were treated with 0.2 $\mu\text{g ml}^{-1}$ isoniazid and 1.0 $\mu\text{g ml}^{-1}$ rifampicin. The percentages of INT labelled bacteria were calculated from the number of cells containing detectable formazan deposits counted directly from recorded phase contrast and brightfield microscopy images. The number of cells counted for each point was in the range of 450-1248.

3.2.8 Direct reduction of INT by isoniazid

In light of the results observed in Section 3.2.7, investigations were undertaken to determine whether or not isoniazid had sufficient intrinsic reducing power to directly reduce INT under the test conditions used to observe physiological activity in drug treated mycobacterial cell suspensions (*i.e.* 1 mM INT for 60 min at 37°C in the presence of 0.2 µg isoniazid ml⁻¹). Direct reduction was not observed in cell-free preparations under these conditions (Table 3.6). Reduction was observed only with higher concentrations of isoniazid (20 mg ml⁻¹ compared to 0.2 µg ml⁻¹) and extended incubation times (24 h compared to 1 h).

Table 3.6: Direct reduction of INT by isoniazid compared to that by *M. tuberculosis* cell suspension

INH concentration (µg ml ⁻¹)		INT reduction ^a		
<u>INT incubation times</u>		<u>1 h</u>	<u>24 h</u>	<u>36 h</u>
Cell-free preparations	0	0	0	0
	0.2	0	0	0
	2	0	0	0
	20	0	0	0
	200	0	0	0
	2,000	0	(+)	(+)
	20,000	(+)	++	++
<i>M. tuberculosis</i> cell suspension control ^b	0.2	++++	+++++	+++++

^a Reactions were carried out in PBS (pH 7.3) at 37°C with 1.0 mM INT for 1, 24 and 36 h. INT reduction was assessed visually and scored out of 5 depending on colour change. No colour change was recorded as 0; minimum colour change was recorded as (+), whereas maximum colour change was recorded as +++++.

^b *M. tuberculosis* bacterial suspension (OD_{580nm} of 0.2) was prepared in PBS (pH 7.3) containing 0.2 µg ml⁻¹ INH.

3.2.9 Effect of antimycobacterial drugs on GFP production

The effects of antimycobacterial drugs on GFP production and colony counts of the *M. tuberculosis* [pGFM11] reporter strain are shown in Figure 3.39. Reductions in colony counts over the 7-day assay period were similar to those already described for the wild type strain; greatest reductions were observed with isoniazid, rifampicin and a

combination of the two, whereas treatment with ethambutol produced the least reduction. Since *M. tuberculosis* [pGFM11] contained a streptomycin-resistance cassette, cultures were not affected by streptomycin treatment and an increase in colony count over the 7 days was observed.

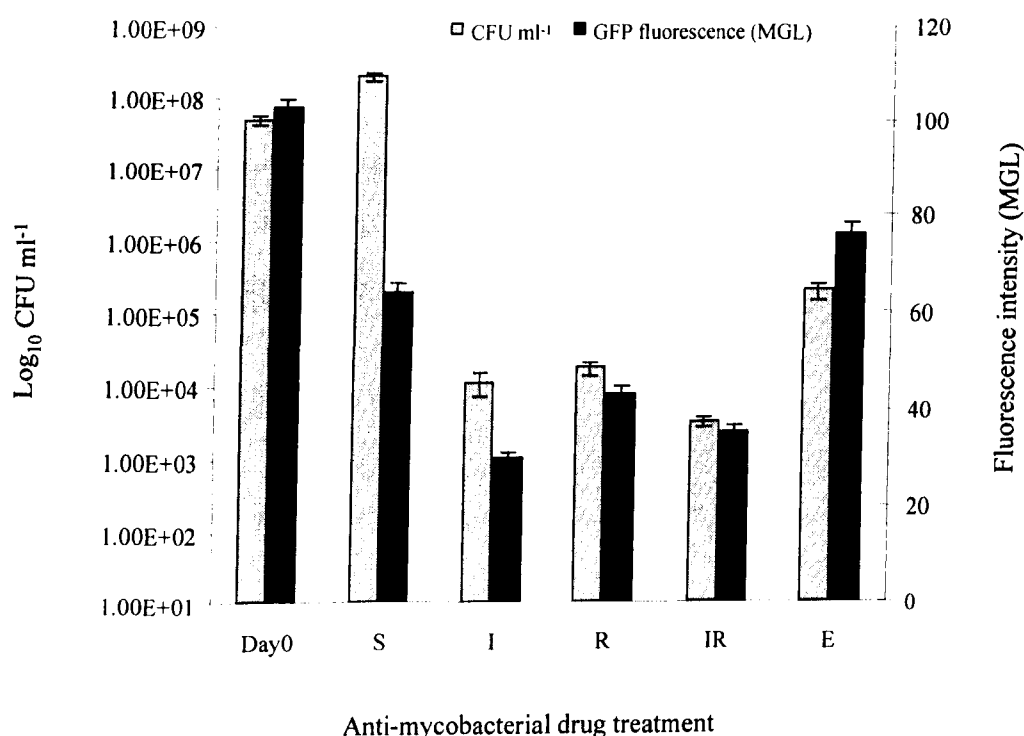


Figure 3.39: Effect of antimycobacterial drugs on colony counts and GFP fluorescence in *gfp*-expressing *M. tuberculosis*.

Cultures were treated for 7 days with antimycobacterial drugs (S, streptomycin; I, isoniazid; R, rifampicin; E, ethambutol) and sampled for fluorescence and colony counts. Day 0 represents fluorescence and colony counts before drug treatments. Fluorescence intensity is expressed as the average of the mean grey levels (MGL \pm S.E.M.) of analysed bacteria, colony counts are expressed as log₁₀ CFU ml⁻¹ (\pm S.D.).

GFP fluorescence was expressed as mean grey levels per cell (*i.e.* the average fluorescence produced by each pixel of a cell) so that changes in morphology produced by different antimycobacterial drugs would not interfere with comparisons. Treatment with isoniazid, rifampicin and a combination of the two produced the greatest decrease in GFP

fluorescence. Less than 3% of cells treated with these drugs had MGL values greater than the average MGL value (102) measured at day 0. Ethambutol-treated cells produced least decrease in fluorescence. Curiously, even though GFP clones were resistant to the antibiotic, fluorescence in streptomycin-treated bacteria decreased to lower levels than those found in ethambutol-treated bacteria.

Unfortunately, identification of membrane energisation and oxidative metabolism in cells with high GFP fluorescence intensity following antimycobacterial drug treatment using R123 and INT was hampered (results not shown). In the first instance, little information could be taken from cells co-labelled with R123 and GFP because of the similar spectral characteristics of these fluorophores (Table 2.2). In the second instance, GFP fluorescence was obscured in cells containing formazan deposits.

3.2.10 Mycobacteriophage infectivity studies

Overnight incubation of antimycobacterial drug treated *M. tuberculosis* cultures with mycobacteriophage D29 produced no detectable effect on cytochemical labelling with R123/PI; *i.e.* drug-treated cell suspensions inoculated with D29 contained the same proportion of fluorescent green, fluorescent red and non-labelled cells as those incubated in phage buffer without D29 (results not shown). Although reductions in colony counts were noted in drug-treated cultures after incubation with D29, corresponding reductions were also observed in the phage buffer controls (Table 3.7) indicating that the decrease in culturability was not due to phage infection.

It should be noted that the *M. tuberculosis* cell suspension used in the drug studies (*i.e.* prepared from barely confluent agar culture) was less susceptible to D29 infection than the exponential phase broth culture described in Section 3.1.13, and at most a reduction of half a log in colony counts was observed. D29 infection of these cell suspensions also produced little detectable effect on R123/PI labelling, unlike the changes observed in exponential phase broth culture cells reported in Section 3.3.2 below. Overnight

incubation in phage buffer was shown to have no effect on colony counts in control cell suspensions.

Table 3.7: Effect of mycobacteriophage D29 infection on colony counts of antimycobacterial drug treated *M. tuberculosis*¹

Antimycobacterial drug	Day 7 (CFU ml ⁻¹)	O/N Phage Buffer (CFU ml ⁻¹)	O/N D29 (CFU ml ⁻¹)
Control cell suspension	5.58×10^7	7.83×10^7	1.18×10^7
Isoniazid	2.05×10^5	1.53×10^4	2.98×10^4
Rifampicin	4.67×10^3	1.80×10^3	0.58×10^3
Isoniazid-rifampicin	8.50×10^3	1.17×10^2	0.58×10^2
Isoniazid-streptomycin	1.48×10^4	3.67×10^3	4.33×10^3
Rifampicin-streptomycin	2.33×10^3	1.25×10^2	0.50×10^2

¹ *M. tuberculosis* cell suspension was prepared from barely confluent agar culture as described in Section 2.4.4 and treated with antimycobacterial drugs for 7 days as described in Section 2.9.2. Isoniazid was used at a final concentration of $0.2 \mu\text{g ml}^{-1}$, rifampicin at $1.0 \mu\text{g ml}^{-1}$ and streptomycin at $2.0 \mu\text{g ml}^{-1}$. After 7 days treatment, cultures were washed for 24 h in Tween 80 free broth (containing the appropriate antimycobacterial drugs) and infected with mycobacteriophage D29 as described in Section 2.11.3.

3.3 FURTHER STUDIES ON NON-LABELLING POPULATIONS AND DORMANCY MODELS

The recalcitrance of cell suspensions prepared from *M. tuberculosis* broth culture to cytochemical labelling was highlighted in Sections 3.1.2. The physiological status of these non-labelling cells was unknown with possible explanations including: physiologically active bacteria with low permeability properties to the cytochemical reagents; metabolically inactive or dormant sub-populations; empty cell husks devoid of nucleic acid, membrane potential and oxidative metabolism. Here, the results of experiments designed further investigate non-labelling populations are presented. These include observations from *gfp* reporter strains, mycobacteriophage D29 infection studies and the anaerobically induced dormancy model.

3.3.1 Comparison of GFP production by bacteria cultured in broth and on agar

Expression of *gfp* in *M. tuberculosis* [pGFM11] cells cultured in Middlebrook 7H9 broth was compared to that produced by cells grown on Middlebrook 7H10 agar. Scatter plots of fluorescence intensities obtained by image analysis and examples of microscopy images are shown in Figure 3.40. With each of the culture methods, most cells (> 90%) identified by microscopy produced sufficient GFP to be brightly fluorescent. Evidence of a predominantly cytologically inactive population in broth culture was not observed.

Although detailed growth studies were not undertaken, expression of *gfp* by *M. tuberculosis* did not appear to affect growth (assessed by OD and colony counts in broth culture and the time taken for growth to produce a barely confluent lawn on agar). Like the wild type strain, cells taken from *M. tuberculosis* [pGFM11] broth culture were greater in length than cells in suspensions prepared from agar (average cell lengths of 4.0 and 3.5 μm respectively).

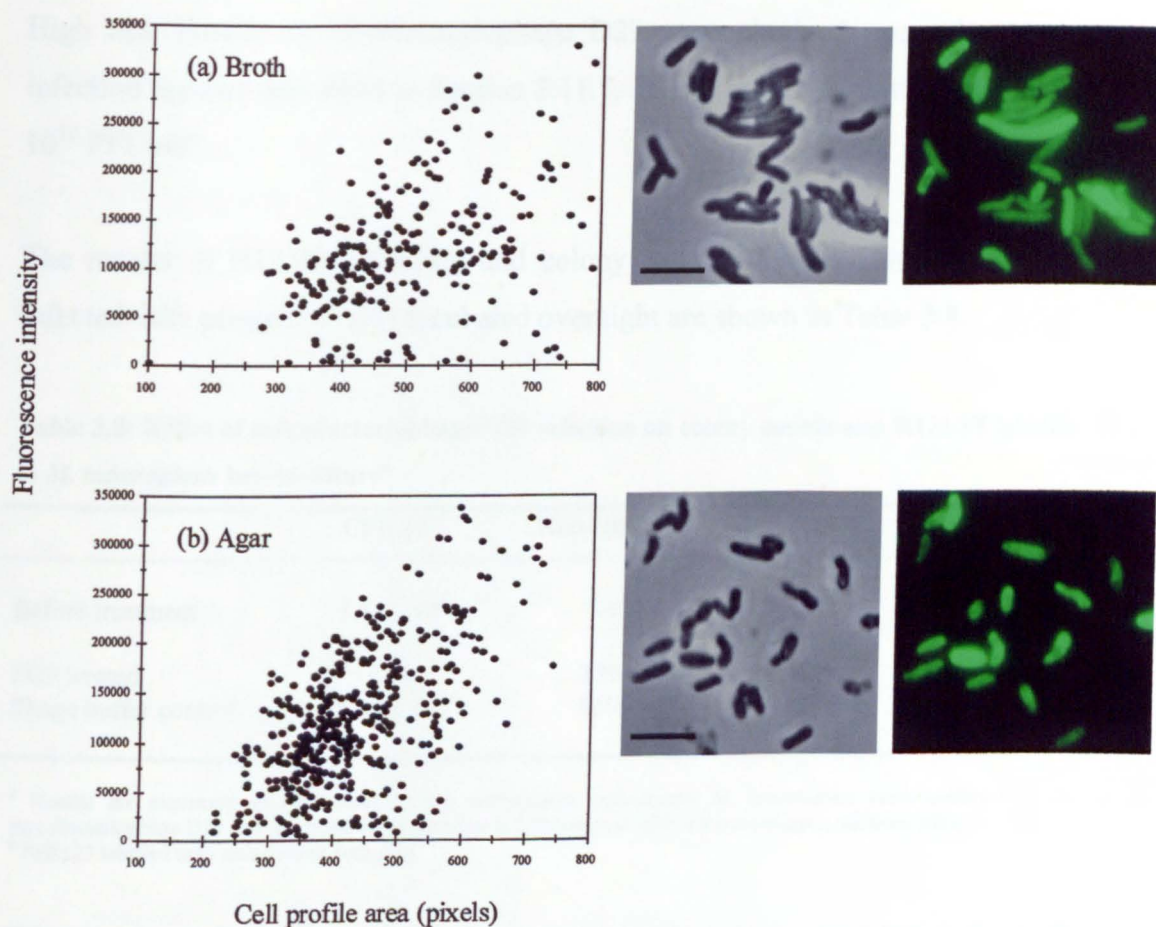


Figure 3.40: Expression of GFP in *M. tuberculosis*.

Scatter plots of fluorescence intensities, phase-contrast and corresponding fluorescence micrographs of *gfp*-expressing *M. tuberculosis* cultured (a) in Middlebrook 7H9 broth and (b) on Middlebrook 7H10 agar. Cells were viewed at 1000 x magnification. Scale bar = 3 μ m.

3.3.2 Mycobacteriophage infectivity studies

High titre lysates of mycobacteriophage D29 were obtained using the *M. smegmatis* infection method described in Section 2.11.1. These lysates produced approximately 2×10^{10} PFU ml⁻¹.

The results of R123/PI labelling and colony counts of broth-cultured *M. tuberculosis* infected with phage D29 and incubated overnight are shown in Table 3.8.

Table 3.8: Effect of mycobacteriophage D29 infection on colony counts and R123/PI labelling of cells in *M. tuberculosis* broth culture^a

	CFU ml ⁻¹	Non-labelled	R123	PI & PI/R123 ^b
Before treatment	3.52×10^7	73%	27%	< 1%
D29 treated	< 50	37%	12%	51%
Phage buffer control	3.05×10^7	83%	17%	< 1%

^a Results are expressed as the means of two independent experiments. *M. tuberculosis* broth culture was infected with mycobacteriophage D29 and incubated overnight prior to cytochemical labelling and colony count enumeration.

^b PI/R123 labelled cells stained with both dyes.

The number of R123-labelled and non-labelled cells was reduced in D29-treated populations (from 27 to 12% and 73 to 37% respectively). The remaining 50% of bacteria were dual-stained cells, characterised by a green fluorescent periphery of variable thickness and a diffuse to well-defined red-orange fluorescent nuclear area. These cells were phase-light with dark regions at the poles when viewed using phase contrast microscopy. Examples of the different labelling patterns observed in D29-treated *M. tuberculosis* are shown in Figure 3.41. No growth (< 50 CFU ml⁻¹) was recorded in D29-infected samples.

Overnight incubation of broth-cultured *M. tuberculosis* in phage buffer also produced a decrease in the number of R123-labelled bacteria (from 27 to 17%). However, unlike infected culture, the remaining bacteria were predominantly non-labelled and

indistinguishable by phase contrast microscopy. Incubation in phage buffer produced a small reduction in colony counts.

In summary, the percentage of non-labelled cells in R123/PI stained *M. tuberculosis* cell suspensions prepared from broth culture was reduced by infection with mycobacteriophage D29.

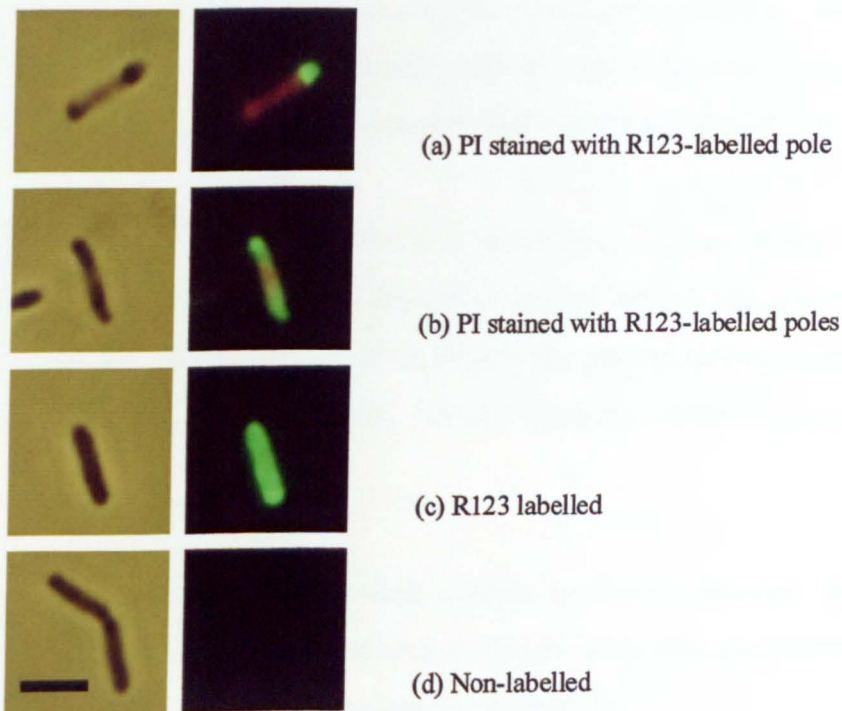


Figure 3.41: Effect of mycobacteriophage D29 treatment on R123/PI labelling of *M. tuberculosis*.

Phase-contrast and fluorescence images of the same fields of view of D29-treated *M. tuberculosis* broth culture demonstrating the different R123/PI labelling patterns observed: (a & b) PI stained cells with R123 labelled pole(s), (c) R123 labelled cells and, (d) non-labelled cells. Scale bar = 2 μm .

3.3.3 R123 labelling of dormant bacteria

Two different methods were used to generate *M. tuberculosis* cultures in a dormant phase by adaptation to anaerobiosis. Decolourisation of methylene blue in control tubes indicated when oxygen was used up and cells were sampled for labelling one week later. In the first method, unagitated broth culture was adapted to anaerobiosis by settling through a self-generated oxygen depleted gradient. Most of the cells found in the anoxic sediment had compromised cytoplasmic membranes and stained with PI. However, many cells labelled with R123 even with extended culture. R123 labelled cells present in the sediment 20 weeks after the oxygen was depleted are shown in Figure 3.42a.

In the second method, cells were adapted to anaerobiosis in slowly stirred flask cultures by gradual depletion of oxygen. Cytochemically labelled cells in the anoxic stirred flask culture were indistinguishable from cells in aerobically aerated culture; between 20-40% accumulated R123, few stained with PI, and the remainder were non-labelled (Figure 3.42b).

In summary, cells in *M. tuberculosis* culture adapted to a non-replicating dormant phase by anaerobiosis using the methods developed by Wayne and colleagues (Wayne and Lin, 1982; Wayne and Hayes, 1996) could be labelled with R123.

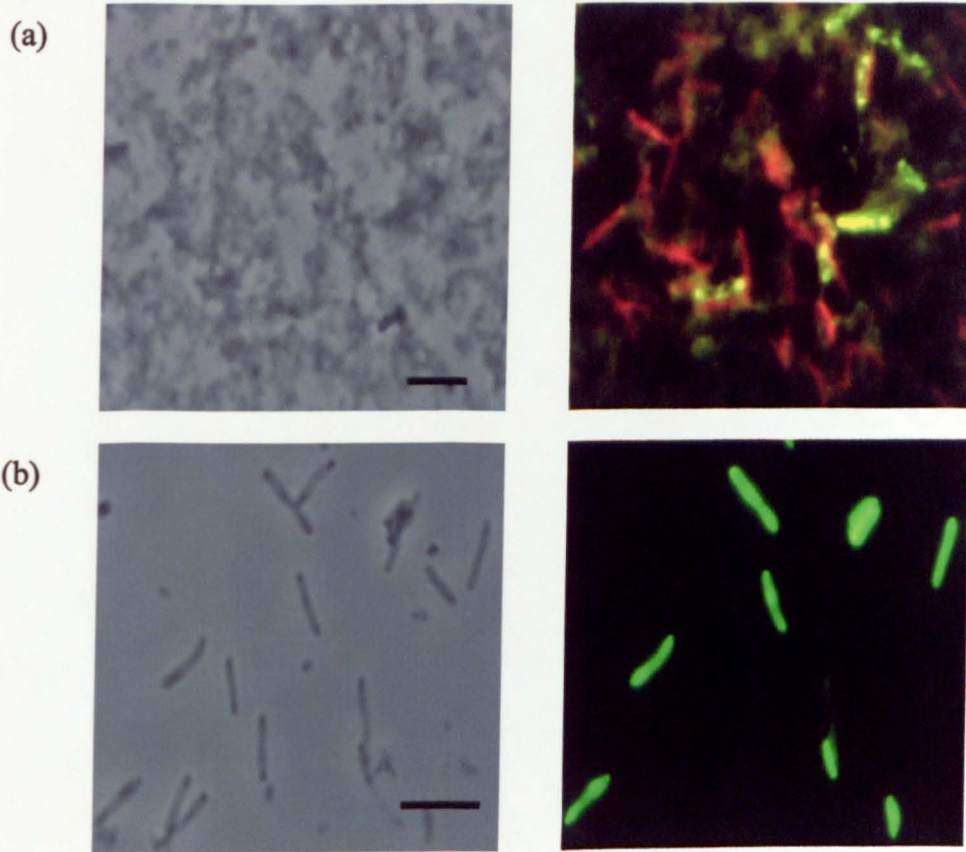


Figure 3.42: R123/PI labelled *M. tuberculosis* cells adapted to anaerobiosis

Paired phase contrast and fluorescence microscopic images of:

(a) Cells in the sediment of unagitated broth culture adapted to anaerobiosis by settling through a self-generated oxygen depleted gradient as described by Wayne and Sramek (1994). Cells were sampled and labelled after 20 weeks of culture.

(b) Cells adapted to anaerobiosis in slowly stirred flask cultures by gradual depletion of oxygen as described by Wayne and Hayes (1996). Cells were sampled 7 days after complete decolourisation of methylene blue ($0.5 \mu\text{g ml}^{-1}$) indicated depletion of oxygen.

Cells were viewed at 1000 x magnification. Scale bar = $3 \mu\text{m}$.

4. DISCUSSION

4.1 METHODOLOGICAL STUDIES

Different methods of cultivating *M. tuberculosis* are available; the choice of medium and conditions of incubation used in investigations has generally depended on the aspect of the organism to be studied. In the present study, cytological assays were being used to assess the physiology of bacteria at the single cell level and therefore, considerable effort was invested in establishing a culture method to produce consistent defined populations of *M. tuberculosis* suitable for these assays.

4.1.1 Growth characteristics in broth

Culture in liquid media was originally investigated as it is generally considered to produce bacterial cells in a defined state of growth with minimum heterogeneity of physiology. As stated in Section 3.1.1, continuously agitated culture exhibited logarithmic growth over the first week with daily increases in colony counts and OD consistent with expected generation times of *M. tuberculosis* (16 to 18 h). During the second week growth slowed, cultures entered the stationary phase, small clumps appeared and biomass was deposited at the liquid/air interface on the flask wall. Mycobacteria, by virtue of their hydrophobicity, tend to accumulate at the triphasic interface of air, glass and culture medium where, the wave motion produced from centrifugal forces deposit cells on the flask wall out of the reach of fresh medium. This effect may be exaggerated by the cord factor (6,6'-dimycolate) produced which tends to aggregate in veils that naturally "climb" container walls (Wayne, 1994). It has been suggested that deposition on the flask wall may be the cause of inhibition of growth reported by many workers when *M. tuberculosis* is cultured in flasks aerated on rotary shaker incubators. However, inhibition of growth was not observed in the present study, possibly because polycarbonate baffled flasks were used, to which cells adsorb less than to glass.

4.1.2 R123/PI labelling of cells cultured in broth

It soon became evident that although broth culture was considered appropriate for producing defined populations of *M. tuberculosis*, it was unsuitable as a starting point for the cytochemical labelling reactions. Regardless of when bacteria were sampled during the growth curve, more cells were non-labelled than labelled (Section 3.1.2). From the results obtained with R123/PI dual labelling, few PI stained cells were observed indicating that although the majority of the cells did not fluoresce with R123, they had intact cytoplasmic membranes. The lack of staining in cells sampled during the exponential phase of growth is difficult to explain since it seems unlikely that actively growing bacteria exist without a detectable membrane potential. It is more likely that the dye cannot penetrate the mycobacterial cell wall and in such cases, membrane potential cannot be measured using R123. A more detailed discussion of the non-labelled cells found in broth culture is given in Section 4.3 including observations from other experiments used to investigate this sub-population. In addition, although the detergent Tween 80 was included in media to disperse the hydrophobic mycobacteria during growth, much clumping was observed on the bacterial monolayers making image analysis and interpretation difficult.

4.1.3 Growth on agar

Some of the problems faced when cells from broth culture of *M. tuberculosis* were used for cytochemical labelling were highlighted in the previous section. Mycobacterial culture on agar media was investigated as an alternative source of cell suspension for these studies. Mature colonial growth was not used because this can be expected to include a wide range of environments, from an abundance of oxygen and nutrients at the edge, to almost no oxygen or nutrients available to those cells in the centre. All phases of growth are probably represented, and therefore cellular heterogeneity is likely. Consequently, bacterial suspensions prepared from growth on agar were from barely confluent spread plate cultures as described in Section 2.4.4.

4.1.4 R123/PI labelling of cells cultured on agar

The results obtained with these cell suspensions demonstrated that approximately 90% of individual bacteria accumulated R123. In addition, bacterial monolayers prepared from culture on agar showed little evidence of clumping on slide preparations and more uniform cell morphology was observed. This made microscopy, image recording and subsequent image analysis more straightforward than with cell suspensions from broth culture. Therefore, it was decided to use bacterial suspensions prepared from culture on solid medium for the remainder of the study. The inherent morphological differences between *M. tuberculosis* cells prepared from culture in broth and on agar presented in Table 3.1 will be discussed in more detail in Section 4.2.6.

Interestingly, apart from a report by Ibrahim (1996), other workers who used R123 to label mycobacteria (Matsuyama, 1984; Bercovier, 1987; Walker (D.R. Walker and M.R. Barer, unpublished observations); and Sayles, 1994) did not comment on non-labelled cells. Ibrahim observed substantial non-labelling in *M. avium* cultured in liquid media. As in the present study, this proportion was reduced in suspensions prepared from culture grown on solid medium. Matsuyama, Walker and Sayles prepared cell suspensions from culture grown on solid medium in their studies and therefore probably did not observe large unlabelled sub-populations. Although, Bercovier and colleagues prepared their cultures in liquid media, fluorescence was measured using spectrophotofluorometry and therefore non-labellers in their experiments would have been overlooked.

4.1.5 R123 Uncoupler sensitivity studies

The accumulation of R123 across the energised mycobacterial cytoplasmic membrane was demonstrated by showing that uptake of dye was sensitive to dissipation of the membrane potential by the uncoupler agent CCCP (Section 3.1.5). The mechanism of uncoupling by CCCP is demonstrated schematically below in Figure 4.1 (Mitchell, 1966; Lewis *et al.*, 1994).

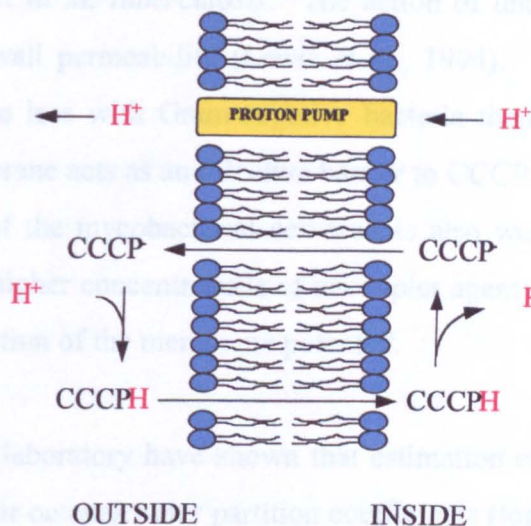


Figure 4.1: Dissipation of the cytoplasmic membrane potential by the uncoupler agent carbonyl cyanide m-chlorophenyl hydrazone (Lewis *et al.*, 1994).

It was established during this study that both the concentration of R123 used to label *M. tuberculosis* cell suspensions and the concentration of CCCP used to dissipate the membrane potential were decisive when demonstrating uncoupler-sensitive accumulation. Whereas other workers successfully labelled mycobacteria with 13 μM R123 (Matsuyama, 1984; Bercovier, 1987; D.R. Walker and M.R. Barer, unpublished observations; Sayles, 1994; and Ibrahim, 1996), the evidence presented here shows that uncoupler-sensitive accumulation of R123 can only be demonstrated at lower dye concentrations (*e.g.* 1 μM). Kaprelyants and Kell (1992) made similar observations with their studies on *Micrococcus luteus* and also concluded that uptake was fully uncoupler sensitive only at low R123 concentrations. At higher concentrations R123 may bind non-specifically and produce fluorescence independent of membrane energisation.

Whereas, uncoupler-sensitive accumulation of R123 was reported with 5 μM CCCP for *S. aureus* (Matsuyama, 1984) and 15 μM CCCP for *M. luteus* (Kaprelyants and Kell, 1992), almost ten-fold higher concentrations were required in the present study to

produce the same effect in *M. tuberculosis*. The action of uncoupler agents on whole cells depends on cell wall permeability (Lewis *et al.*, 1994). The effect of uncoupler agents is reported to be less with Gram-negative bacteria than Gram-positive bacteria because the outer membrane acts as an effective barrier to CCCP uptake (Nikaido, 1994). The low permeability of the mycobacterial cell wall is also well documented (Nikaido and Jarlier, 1991), and higher concentrations of uncoupler agent are probably required to achieve effective dissipation of the membrane potential.

Previous studies in this laboratory have shown that estimation of the hydrophobicity of compounds based on their octanol:water partition coefficients ($\log P_{\text{oct}}$) may be calculated using the semi-empirical algorithms of Hansch and Leo (1979) and used to predict the likelihood of a compound crossing the mycobacterial cell membranes (Christensen *et al.*, 1999). Extremely hydrophobic compounds ($\log P_{\text{oct}} > 8$) are likely to localise in the mycobacterial cell wall lipids where they become trapped, weakly hydrophobic compounds ($8 > \log P_{\text{oct}} > 0$) are likely to be membrane permeant and diffuse into the cytoplasm, while hydrophilic compounds ($\log P_{\text{oct}} < 0$) do not pass through membranes by passive diffusion. Calculation of the $\log P_{\text{oct}}$ values of non-ionised and ionised CCCP (+ 0.7 and < 0 respectively) suggest that although the non-ionised compound is readily membrane permeant, once ionised it is much less so (R.W. Horobin, personal communication¹). The rate of uncoupling may therefore be limited by the rate of diffusion of the anionic species across the cytoplasmic membrane.

4.1.6 Heat, ethanol and formaldehyde treatments

Interestingly, none of the bactericidal methods used to produce defined cytologically inactive populations of *M. tuberculosis*, produced populations that exclusively stained with PI. As stated in Section 3.1.6, heat, ethanol and formaldehyde treated cultures contained cells that labelled with R123. However, in each case, this residual labelling

¹ R.W. Horobin, Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN, UK.

was shown to be uncoupler-insensitive and was therefore not specifically attributable to membrane energisation.

Several workers have commented on non-specific R123 fluorescence produced by bactericidal treatments. Mason *et al.*, (1995) reported that heat-treated *E. coli*, *Salmonella typhimurium* and *S. aureus* showed increased fluorescence compared to controls when labelled with R123 whereas low-level R123 fluorescence was observed in formaldehyde-fixed cells of *E. coli* and *S. aureus* by Diaper *et al.*, (1992) and Comas and Vives-Rego (1997). Suggested mechanisms for non-specific binding include cell surface changes produced by increases in surface hydrophobicity or exposure of negatively charged components resulting in increased R123 adsorption and binding of the dye to alternative cellular binding sites, possibly within the hydrophobic regions of the membrane.

However, it remains unclear why small sub-populations of *M. tuberculosis* cells consistently demonstrated uncoupler-insensitive R123 fluorescence after heat or ethanol treatment while the majority labelled intensely with PI. Much variability has been reported in the outermost layer of the *M. tuberculosis* cell wall. These include thickness, electron density and appearance (fibrillar, granular or homogeneous) when viewed using electron microscopy (Rastogi *et al.*, 1986). Whereas, the outer layer tends to be thin for the majority of the population, a small proportion of *M. tuberculosis* cells have a wide electron transparent zone around individual cells (Frehel *et al.*, 1986; 1988). Cell wall variation may account for residual uncoupler-insensitive labelling observed after heat or ethanol treatment should binding of dye to non-specific binding sites found only in a small proportion of the population occur.

Curiously, R123 labelling was not observed in heat or ethanol treated *M. smegmatis* or *M. aurum*, or in previous studies with *M. goodii* (D.R. Walker and M.R. Barer, unpublished results). Brennan and Draper (1994) report that although *M. tuberculosis* shares many ultrastructural features with other mycobacteria, the various species differ

considerably in their complement of lipids, glycolipids and proteins resulting in important differences in wall structure and properties. Disruption of the *M. tuberculosis* cell wall might well expose non-specific binding sites for the cationic R123, e.g. LAM, that are absent in the rapidly growing species.

Thus while there were significant differences between *M. tuberculosis* cells treated with formaldehyde, heat and ethanol and untreated controls, presence of R123 labelling was not consistently correlated with the ability of cells to form colonies. However, loss of CCCP suppressible R123 labelling was strongly correlated with the bactericidal (CFU reducing) activity of these treatments.

4.1.7 Tetrazolium salt reactions

INT

Initially, formazan deposits were small and difficult to detect in cells but optimisation of reactions, increased incubation time and decreased tetrazolium salt concentration resulted in greater deposition (Section 3.1.7). The effect of INT concentration was not expected as previous work in this laboratory using a variety of bacteria suggested that higher concentrations of tetrazolium salt resulted in optimal reduction. Concentrations of 10 mM were routinely used with the environmental bacteria *Vibrio vulnificus* and *Pseudomonas putida* (Whiteley *et al.*, 1996), and 5 mM with *M. avium* (Ibrahim, 1996) and the enteric pathogen *Helicobacter pylori* (Gribbon and Barer, 1995). However, at these concentrations, tetrazolium salt reduction was inhibited in *M. tuberculosis*. Exposure to INT was shown not to be tuberculocidal (Table 3.3) and therefore some form of inhibitory effect must be attributed to INT.

Larger deposits occurred with longer incubation times in *M. tuberculosis* and continued to grow intracellularly as more INT diffused into bacteria. Indeed, formazan deposits often completely filled and distorted cells, raising the possibility of cell lysis. However, phase

contrast microscopy showed that although cells tended to swell in association with large formazan deposits, there was no evidence of lysis and cell envelopes appeared morphologically intact with continuous smooth surfaces around the swollen deposits.

Although reaction optimisation improved labelling, marked cell-to-cell variation in staining remained, ranging from some cells that were filled with beads of deposits to others with no detectable formazan. As reported with R123/PI labelling, this variation was more obvious with *M. tuberculosis* cells cultured in liquid media. Other workers have shown that addition of oxidisable substrates to the incubation medium enhanced both the intensity and proportion of cells yielding positive INT reactions in a variety of microbial species (Gribbon and Barer, 1995; Whiteley *et al.*, 1996). These included *E. coli*, *H. pylori*, *V. vulnificus* and *P. putida*. However, attempts at substrate enhanced tetrazolium salt reduction in the present study with *M. tuberculosis*, and previously by Ibrahim (1996) with *M. avium*, indicated that none of the substrates tested (aspartate, α -ketoglutarate, pyruvate, cellobiose, malate, serine, fumarate, citrate, succinate, glucose and glutamate) had any effect on formazan deposition. It is possible that the exogenous substrates selected in both these studies were inappropriate for demonstrating activity in mycobacterial species. In addition, there is substantial evidence in the literature to show that mycobacteria produce intracellular storage bodies (Garton, 1998) and therefore the addition of exogenous substrates would not be expected to show any effect until these endogenous pools of substrates were exhausted.

Since INT detects the overall rate of production of reducing equivalents within cells, one interpretation of the results would be that many cells demonstrate little evidence of oxidative metabolism even when exogenous energy sources are supplied. This seems unlikely especially in cells sampled during the exponential phase of growth. It seems more probable that the cell-to-cell variation in INT reduction, like the previously described variation in R123 accumulation, reflects heterogeneity of cell envelope permeability in *M. tuberculosis* cells.

CTC

As stated in Section 3.1.7, the fluorogenic tetrazolium salt CTC was investigated as an alternative determination of oxidative metabolism in *M. tuberculosis* since this would facilitate rapid analysis by fluorescence microscopy. However, in these studies CTC was found to be less sensitive to reduction than INT. There are several explanations for this observation. Firstly, mycobacterial cells may be more permeable to INT than CTC resulting in greater diffusion and/or transport to the site of reduction. Secondly, mycobacterial cells may be more permeable to CTC formazan deposit than INT formazan deposit resulting in the former leaching out of the cell. This possibility could explain the presence of the extracellular fluorescent deposits seen in *M. tuberculosis* cell suspensions. Thirdly, INT may be reduced more readily than CTC and hence produce more detectable amounts of formazan within the reaction period. Regardless, reaction with CTC did not produce useful results with *M. tuberculosis* and consequently only INT was used in the remaining experiments involving tetrazolium salt reduction.

4.1.8 Combined R123/PI labelling and INT reduction assays

The development of a combined INT reduction and R123 accumulation assay was pursued to provide simultaneous determination of oxidative potential and membrane potential in individual cells. One application of this approach was to investigate whether the same cytologically active population was being identified by the separate assays. The general observation was that most R123 labelled cells also contained detectable formazan deposits (Section 3.1.8). Unfortunately, simultaneous measurement of R123 fluorescence intensity and formazan optical weight was difficult because cells that contained large deposits obscured or quenched fluorescence. Therefore, it wasn't possible to establish a quantitative relationship between R123 accumulation and INT reduction in individual cells. However, approximately 10% of R123 labelled cells did not contain detectable deposits. Whether this reflects differences in the physiological status or the permeability of those cells compared to the dual-labelled population remains uncertain.

None of the cells that demonstrated uncoupler-insensitive R123 labelling after bactericidal treatment with heat, ethanol or formaldehyde reduced INT. The absence of oxidative metabolism in these cells was not unexpected, since it was speculated in Section 4.1.6 that this fluorescence was not evidence of membrane energisation but of non-specific staining produced by the treatments.

4.1.9 Results of other cytological assays

Although there are many reports of oxonol and the Live/Dead *Bac*LIGHT stains being used to differentiate physiologically active and inactive cells, neither was shown to label *M. tuberculosis* in a satisfactory manner (Section 3.1.9). Oxonol labelled cell preparations were always poorly stained, most likely because of dye exclusion by the impermeant mycobacterial cell wall. The myriad of labelling obtained with the Live/Dead *Bac*LIGHT stain made interpretation difficult and suggested that much of the staining was non-specific. Although the 'live cell' nucleic acid stain SYTO16 was shown to label *M. tuberculosis* cell preparations, the low number stained in control populations precluded useful application.

4.1.10 Effect of labelling on colony counts

Previous workers have reported that some of the cytochemical reagents used to demonstrate bacterial physiological activity were toxic and inhibited the cell functions they were supposed to measure (Nebe-Von Caron and Badley, 1995; Diaper *et al.*, 1992; Mason *et al.*, 1993). In the present study, none of the cytochemical procedures used were shown to be bactericidal (as assessed by colony forming unit counts) with *M. tuberculosis*.

4.1.11 Development of GFP reporter strain

A major drawback in monitoring the cytological activity of intact live bacteria using cytochemical reagents is cell wall permeability. For example, it was suggested in Section 4.1.9 that oxonol was excluded from labelling *M. tuberculosis* cells because of poor permeability. Similarly, it was highlighted in Sections 4.1.2 and 4.1.7 that as few as 20% of *M. tuberculosis* cells found in the exponential phase of growth in broth labelled with R123 and INT respectively. The absence of membrane potential and oxidative metabolism seems unlikely in rapidly dividing cells; it seems more likely that the reagents cannot enter the cell and therefore activity cannot be assessed. A GFP reporter strain of *M. tuberculosis* was developed to look for cytological activity in such populations recalcitrant to labelling since GFP is produced intracellularly and does not require external substrates or cofactors due to the intrinsically fluorescent nature of the protein.

It is difficult to explain why attempts to clone the GFP gene downstream of the HSP60 promoter in the *E. coli*-mycobacterial shuttle vector pMV261 failed since there are many reports of this exact cloning strategy being used for recombinant protein expression in a variety of mycobacterial species (Stover *et al.*, 1991; Andrew and Roberts, 1993; Cooksey *et al.*, 1993). Regardless, transformation of *M. tuberculosis* with the GFP construct pGFM-11 (Kremer *et al.*, 1995) produced expression that was readily detected both at colony and single cell level. Comparison of GFP production by *M. tuberculosis* cells cultured in broth and on agar is discussed in Section 3.3.1. The effects of antimycobacterial drugs on GFP production are discussed in Section 3.2.9.

4.1.12 Total cell counts (TCC)

The results of the three direct microscopic methods of enumeration used to obtain total cell counts of mycobacterial cell suspensions (immobilisation of bacteria on APS-coated coverslips, membrane filtration of DAPI stained cells and the Thoma counting chamber method) were compared in Section 3.1.12. Little difference was found between the

counts obtained by the different methods. Direct counting of cells immobilised on APS-coated coverslips by phase contrast microscopy had a number of advantages over the other methods. Firstly, cell suspensions did not have to be formalin fixed prior to counting since the microchamber system is suitable for pathogens requiring level 3 containment. Secondly, the same preparation used for counting could be used for cytochemical labelling and thus the number of cells displaying certain phenotypes could be calculated. Thirdly, the size and morphology of the cells being counted were readily recorded for analysis. Fourthly, where clumping did occur it was usually possible to identify individual bacteria. Consequently, most of the total cell counts were evaluated using this method.

4.1.13 Development of mycobacteriophage infectivity studies

Results of the development of mycobacteriophage infectivity studies for investigation of effects on cytochemical labelling of *M. tuberculosis* were presented in Section 3.1.13. The results corroborate previous reports that although Ca^{2+} may enhance productive infection by D29 it is not essential for productive infection (Sellers *et al.*, 1962; David *et al.*, 1980). The inhibitory effect of Tween 80 on phage infection was highlighted. Although this effect has been known for some time (White and Knight, 1958), culture of *M. tuberculosis* in liquid media necessitates the use of the detergent to prevent clumping. To prevent inhibition, cultures were washed and incubated for 24 h in detergent-free broth before infection. However, it is known that Tween 80 extracts lipids, glycolipids and carbohydrates from the mycobacterial cell wall (Daffé and Draper, 1998) and consequently phage binding sites may have been removed from many of the cells. Nonetheless, high titre lysates of mycobacteriophage D29 were obtained and infection of *M. tuberculosis* culture was shown to be cidal.

The effects of mycobacteriophage D29 infection on antimycobacterial drug treated *M. tuberculosis* cultures and *M. tuberculosis* broth culture assessed using R123/PI cytochemical labelling are described in Section 4.2.10 and Section 4.3.2 respectively.

4.2 EFFECTS OF ANTIMYCOBACTERIAL DRUGS

The resurgence of tuberculosis has caused considerable effort to be focused on the development of rapid methods for determining the susceptibility of *M. tuberculosis* to antimycobacterial agents. Firstly, there is a clinical need for a quicker turnaround time in susceptibility testing because of the slow growth of the organism, especially with the diagnosis and management of MDR-TB. Secondly, little is known about the physiological properties of *M. tuberculosis* which render it refractory to eradication and produce the characteristic slow response to chemotherapy. In Section 4.1 the development of physiologically validated procedures for determining cytological activity in *M. tuberculosis* was discussed. The results demonstrated that cytological activity could be detected in *M. tuberculosis* at the single cell level using a variety of cytochemical assays. Uncoupler sensitive accumulation of R123 and reduction of the tetrazolium salt INT readily demonstrated membrane energisation and oxidative metabolism in cytologically active cells. The effects of antimycobacterial drugs on *M. tuberculosis* at the single cell level using these cytochemical assays are discussed here.

4.2.1 Colony counts

The effects of antimycobacterial drugs are most often assessed using colony counts. In Section 3.2.1, the individual antimycobacterial drugs tested were shown to produce different bactericidal activity *in vitro*. The reduction in colony counts following treatment with rifampicin or isoniazid was greatest and demonstrated the high level of bactericidal activity observed by other workers with these drugs (Herbert *et al.*, 1996). There was much less reduction with ethambutol, even though this agent is reported to show substantial early bactericidal activity *in vivo* (lower than isoniazid but higher than rifampicin) (Jindani *et al.*, 1980). However, ethambutol was also reported to be less bactericidal *in vitro* than isoniazid and rifampicin (Dickinson *et al.*, 1977).

After initial declines in culturability, cultures treated with streptomycin or capreomycin demonstrated increases in colony counts. It seems unlikely that resistance in the previously sensitive *M. tuberculosis* H₃₇R_v strain would arise so quickly since wild-type resistance is reported at 1 in 10⁷ and the rate of mutation per cell division leading to drug resistance is 10⁻⁸ (Evans, 1998; WHO, 1997). It seems more likely that loss of drug activity occurred through deterioration which allowed cell division to resume, yet reports show that deterioration of streptomycin and capreomycin in Middlebrook media at 37°C was minimal and indeed less than that of isoniazid, rifampicin and ethambutol (Griffith and Bodily, 1992). Mitchison and colleagues reported that many of the antimycobacterial drugs they investigated (*e.g.* novel rifamycins) were unstable in Middlebrook 7H9 media and to compensate for potential loss of activity routinely added additional drug during susceptibility assays so that inhibitory concentrations were constantly maintained (Dickinson and Mitchison, 1990; Herbert *et al.*, 1996). However, since treatments with combinations of antimycobacterial drugs were shown to produce enhanced and permanent reduction in colony counts few further studies were designed where streptomycin or capreomycin were used in isolation.

The antimicrobial activities of isoniazid-rifampicin, isoniazid-streptomycin, isoniazid-capreomycin, rifampicin-streptomycin and rifampicin-capreomycin combinations were greater than those observed with individual drugs. Although there is well-documented synergy between ethambutol and other antimycobacterial drugs in the treatment of disease caused by mycobacteria-other-than-tuberculosis (*i.e.* where the antimicrobial effect produced by the drug combinations is significantly greater than the expected result based on their independent effects, probably as a result of increased permeability of the ethambutol-induced damage to the mycobacterial cell wall) (Heifets, 1994), synergy was absent with *M. tuberculosis* in the present study. It should be noted that, even though drug combinations produced increased bactericidal activity, with reductions of up to 5 log₁₀ in colony counts over the 7-day assay periods, none of the treatments resulted in complete bactericidal activity.

4.2.2 Total cell counts

No substantial change in TCC over the 7-day assays was observed with any of the drugs or drug combinations used in this study (Section 3.2.2). This was interesting because lysis and an associated reduction in TCC were expected, especially following treatment with the cell wall active drugs isoniazid and ethambutol. There is surprisingly little specific information in the literature regarding what eventually happens to mycobacterial cells following treatment with antimycobacterial drugs. Scanning electron microscopy studies on isoniazid-treated *M. tuberculosis* suggest that extensive fragmentation of the mycobacterial cell wall should occur within 24 h (Takayama *et al.*, 1973). However, the results presented here show that although treatment was bactericidal (assessed by reduction in colony counts), exposure to isoniazid does not immediately lead to cell lysis since the layers of peptidoglycan, arabinogalactan, mycolic acids and associated components in the sturdy mycobacterial cell envelope retains structural integrity for some time after wall synthesis is halted.

Although it was highlighted in Section 3.1.3 that preparation of *M. tuberculosis* cell suspensions from barely confluent lawn produced monolayers of bacteria largely free from clumping, some clumping was nonetheless observed. Treatment of culture with isoniazid or ethambutol resulted in noticeably lower levels of clumping and development of bacillus-shaped cells to shorter and broader forms with increased refractility when viewed by phase contrast microscopy (Section 3.2.3). Loss of clumping, however, did not result in greater total cell counts since individual bacteria in small clumps observed prior to drug treatment were easily identified using phase contrast microscopy and accurately enumerated. Other workers have commented on loss of clumping of mycobacteria after treatment with ethambutol (Kilburn and Greenberg, 1977). This was associated with an initial rapid rise in colony-forming units following exposure of *M. smegmatis* to an inhibitory concentration of ethambutol along with a change in cell shape to shorter and broader forms. Early increases in colony counts of *M. tuberculosis* were not observed following treatment with any of the drugs in the present study.

4.2.3 R123/PI labelling

It was reported in Section 3.2.3, that treatment of *M. tuberculosis* culture with antimycobacterial drugs produced two patterns of R123/PI labelling. The first trend was produced by treatment with isoniazid and was shown to be unique to this drug, while, the second trend was characterised by treatment with rifampicin and was produced by the other drugs tested.

Treatment of *M. tuberculosis* culture with isoniazid produced enhanced accumulation of R123 in cells, which was observed within 24 hours and peaked at day 3. This was unexpected since the bactericidal effect of isoniazid was shown to be rapid; on day 3 colony counts were already reduced by 2 log cycles indicating that approximately 99% of the cells identified by microscopy were unable to divide and produce detectable colonies. These cells were expected to show reduced cytological activity and accumulate less R123. As cell death ensued it was expected that isoniazid treated cells would lose the integrity of their cytoplasmic membranes and stain with PI.

In addition to increased fluorescence intensity many of these R123 labelled cells displayed intense staining at their poles. It has been firmly established that isoniazid inhibits mycolic acid synthesis at a very early stage of the action and produces profound changes in the mycobacterial cell envelope (Bardou *et al.*, 1996). One of the earliest effects of subinhibitory concentrations of isoniazid on the ultrastructure of mycobacteria, produced within one generation time after the addition of the drug, was alteration of the bacterial poles accompanied by release of intracellular material. These workers suggest that as well as morphological changes induced by the inhibition of mycolic acids, the drug produced change in the permeability of the mycobacterial cell envelope, affecting both the influx and efflux of compounds. Based on these observations, it is likely that isoniazid treatment increases the permeability of the mycobacterial cell wall and allows greater influx of R123, especially at the poles where earliest weakening was shown to occur. Similarly, many workers have reported increased R123 labelling of many Gram-

negative organisms following permeabilization of their outer membrane with ethylenediaminetetraacetic acid (EDTA) (Kaprelyants and Kell, 1992; Diaper *et al.*, 1992; Mason *et al.*, 1995). However, interpretation of the increase in fluorescence in *M. tuberculosis* cells based on the physiological understanding of R123 uptake would suggest that greater influx of dye implies greater electrochemical gradient rather than greater cell wall permeability. Indeed, one of the early changes in mycobacteria following treatment with isoniazid is an increase in respiration associated with a change in cell envelope permeability (Schaefer, 1960). An increase in cellular respiration may produce an increase in the electrochemical gradient across the mycobacterial cytoplasmic membrane resulting in a greater influx of R123. This increase in respiration may also be responsible for the unexpected increase in INT reduction produced by isoniazid (Section 3.2.7) and discussed in further detail below in Section 4.2.7.

Although leakage of cell material undoubtedly occurs following treatment with isoniazid, most cells actually became more refractile when viewed using phase contrast microscopy (Section 3.2.3). Increased refractility was also observed with ethambutol treatment but not with the other antimycobacterial drugs tested and could be produced by accumulation of polysaccharides and other cell wall precursors in the cytoplasm that would ordinarily be incorporated into the cell wall. Increases in protoplasmic viscosity in isoniazid-treated cells were also observed by Takayama *et al.*, (1975).

In contrast, the average fluorescence intensity produced by R123 accumulation gradually declined following treatment with rifampicin so that by day 7 fluorescence was approximately one third of that measured at day 0. Similarly, Bercovier *et al.*, (1987) observed that cultures of *M. tuberculosis* H₃₇R_a treated with rifampicin for 6 days yielded between 20-30% of total fluorescence measured at day 0. However, it was unknown from their study whether 70-80% of individual cells no longer accumulated R123 following treatment or if the average fluorescence per cell was reduced by 70-80% in all the cells since fluorescence was measured at the population level using spectrofluorometry. The results in the present study clearly show that as many as one third of cells in rifampicin-

treated *M. tuberculosis* culture could be labelled with R123. The fluorescence intensities of these cells were mostly indistinguishable from those measured before treatment. However, the vast majority of these cells were not expected to label with R123 since less than 1 in 100 of them were able to produce detectable colonies.

Cells treated with rifampicin gradually became less refractile when viewed using phase contrast microscopy suggesting a decrease of intracellular contents. According to Konno *et al.* (1973), exposure of *M. bovis* to rifampicin for as little as 12 hours produced extensive changes in cell ultrastructure including fragmented mesosomes, less defined ribosomes, loosening of the cytoplasmic structure and shrinking of the cytoplasm. It seems that lack of freshly synthesised RNA eventually initiates secondary changes in cell structure as the cell dies.

Although combinations of isoniazid-rifampicin, isoniazid-streptomycin and rifampicin-streptomycin produced synergistic reduction in colony counts, a corresponding synergistic reduction in the number of R123 labelled cells was not observed. Rather, the discrepancy between the number of cells demonstrating cytological activity and the number of these cells that produced colonies increased (Table 3.6). For example, approximately 1 in 10 cells treated with rifampicin-streptomycin labelled with R123 whereas less than 1 in 10,000 produced colonies.

The central observation in the present study, regardless of which antimycobacterial drug was used, was that cytochemical phenotypes consistent with viability far exceed the number of cells capable of forming colonies. Retained cytological activity in mycobacteria treated with antimycobacterial drugs is by no means restricted to the present study. The application of various cytochemical assays as rapid assessment of viability in mycobacteria indicate that the total number of cytologically active cells demonstrable by microscopy greatly exceeds the number capable of growth by several orders of magnitude. Retained activity in non-culturable drug-treated mycobacterial cell suspensions was reported in studies with FDA (Norden *et al.*, 1995; Bownds *et al.*, 1996;

Tsukiyama *et al.*, 1985), MTT (Mshana *et al.*, 1998) and as already discussed with R123 (Bercovier *et al.*, 1987). Indeed the general subject of bacterial viability, cytological activity and culturability has recently received renewed attention (Kell *et al.*, 1998). These authors reject the view advanced in many publications that demonstrable cellular activity can be used as an operational definition of viability (*i.e.* activity = viability). They propose that these assays are indicators of (metabolic) activity since they demonstrate important aspects of cellular physiology and that a positive result should lead to classification of the cell as active rather than viable.

Evidence of cytological activity in *M. tuberculosis* cells unable or electing not to divide and produce colonies provides a substantial opportunity for speculation. This may be no more than residual activity in cells without the potential to divide and therefore these cells should only be considered as slowly degrading packages of pre-existing enzymes. For instance, it is not known how long enzymes involved in oxidative metabolism, esterases or cytoplasmic membrane energisation persist for after the death of the cell (*i.e.* after it loses division potential). With the exception of nonculturable cells of pathogens capable of expressing virulence factors such as toxins and invasins in response to exogenous stimuli, activity in such bags of enzymes is not important so long as the cells cannot divide and grow.

However, evidence of cytological activity in nonculturable cells of *M. tuberculosis* following treatment with antimycobacterial drugs could be important in elucidating many unanswered problems in the clinical treatment of tuberculosis. As stated already, little is known about the physiological properties of *M. tuberculosis* which render it refractory to eradication and produce the characteristic slow response to chemotherapy (6-9 months). It is generally considered that the tubercle bacillus is susceptible to the bactericidal effects of antimycobacterial drugs only when it is actively dividing and growing (Mitchison, 1985). The results presented here show that there are many individual cells in *M. tuberculosis* cultures which cannot be grown but remain cytologically active either

because non-replicating sub-populations always exist in large populations or sub-populations convert to a non-growing state after exposure to antimycobacterial drugs.

Phenotypic adaptations that enable pathogenic bacteria to resist the action of drugs in response to prolonged exposure have been reported (Handwerger and Tomasz, 1985). *S. aureus* and *Salmonella enteritidis* can enter physiological states in which normally bactericidal antibiotics produce only a bacteriostatic effect, thus enabling the microorganisms to outdistance the course of antibiotic therapy and produce recurrent infections. Parrish *et al.*, (1998) suggest that phenotypic adaptation might explain the unusually long treatment duration required for successful chemotherapy of active tuberculosis. Whereas bacteriological cultures of *M. tuberculosis* are sterilised in a matter of days with antimycobacterial drug treatment, it is essential to treat patients for at least 6 months with multiple drugs to eradicate the infection. It is possible that actively growing *M. tuberculosis* cells adapt to the administration of antimycobacterial drugs by moving into a physiological state that is associated with increased drug resistance. Identifying the means to rapidly eliminate this population *in vitro* with novel combinations of known drugs or novel drugs could lead to development of regimens that could accelerate chemotherapy *in vivo*.

Indeed, the endogenous reactivation of tuberculosis in humans is generally considered to be the result of reactivation of dormant tubercle bacilli that persisted, sequestered in tubercles after the initial infection. The putative mycobacterial dormancy phase adopted to survive this lengthy persistence may well be responsible for the recalcitrance of *M. tuberculosis* cells to killing by antimycobacterial drugs.

4.2.4 R123 uncoupler sensitivity studies

The inhibitory effect of the uncoupling agent CCCP was used to show that R123 accumulation in antimycobacterial drug treated cell suspensions was attributable to membrane energisation and to discount the possibility that labelling unrelated to

membrane energisation might be concealing genuine inhibitory and bactericidal effects (Section 3.2.4). The results of quantitative image analysis show clearly that R123 labelling was uncoupler sensitive with all the antimycobacterial agents tested. In accordance with previous observations CCCP-inhibited cells were capable of regenerating membrane potential when washed and re-labelled with R123.

In previous work where R123 was used to monitor mycobacterial cytological activity, decisive uncoupler sensitivity studies were not included (D.R. Walker and M.R. Barer, unpublished data; Bercovier, 1987; Sayles, 1994; Ibrahim, 1996). Indeed the results presented in Section 3.1.5 clearly show that inhibition would not have been observed in these studies, since the high concentration of R123 (13 μ M) used would have produced non-specific staining. Consequently, there is considerable doubt concerning the significance of R123 labelling observed in these studies.

4.2.5 MPN enumeration

The numbers of culturable cells following treatment with antimycobacterial drugs were assessed using the MPN method and compared to the colony counts obtained by conventional agar plate dilution (Section 3.2.5). Improved recovery and enumeration of surviving bacteria using the MPN assay could help explain the significant difference observed between the number of cytochemically labelled cells detected by microscopy and the number which can form colonies highlighted in Section 4.2.3. Although greater numbers of propagules were detected with the MPN method, differences of at least 1.5-2 orders of magnitude are needed before considering significant improvement over conventional plate counts (Kell *et al.*, 1998). It is worth noting that a major disadvantage to enumeration of culturable cells using the MPN approach is the inherent low precision of the procedure; the coefficient of variation is about 40% for 10 parallel tubes (Koch, 1994).

Recently, Sun and Zhang (1999) have shown that early-stationary-phase culture supernatant improved the viability of aged cultures of *M. tuberculosis* H₃₇R_a by allowing the formation of 20-fold more colonies on plates and allowed small inocula to initiate growth in liquid culture. They speculate that an acid labile and heat stable resuscitation factor with a mass of less than 1,375 Da was probably responsible for this effect. It would be interesting to assess the effect of such a resuscitation factor in the recovery of antimycobacterial drug treated *M. tuberculosis* cells in the model established in the present study.

4.2.6 Cell elongation and loss of R123 labelling in drug-free controls

It was reported in Section 3.2.6 that cells in the drug-free controls quickly elongated and became recalcitrant to labelling. In contrast, cells exposed to drugs did not grow and the number of cells stained with PI gradually increased concomitantly with a reciprocal decrease in the number of R123 labelled cells. This was interesting because it was observed that the most obvious morphological difference between *M. tuberculosis* cells cultured in liquid and on solid media was cell size, the former being approximately 25% longer and 20% wider (Section 3.1.4). Further studies revealed that this appears to be a general phenomenon with *M. tuberculosis* and that cell suspensions prepared in broth from agar cultures quickly elongate and by day 3 resemble cells maintained in broth culture.

References to morphological differences between *M. tuberculosis* cells cultured in broth and on agar are scanty. Wayne and Hayes (1996) commented on cell elongation in their anaerobic adaptation model for dormancy. They observed that the length of bacilli increased by 32%, from a median length of 2.23 μm during active aerobic growth to 2.94 μm during the period of O₂ depletion, which lasted over 150 hours. However, inocula for their experiments were prepared in broth culture. Ingraham *et al.*, (1983) comment that stationary-phase cells are always smaller than those in the exponential phase as cell division continues after the synthesis of most macromolecules has slowed. These

observations provide little basis for interpreting the morphological changes reported here. Nonetheless, they identify trends that may be relevant. Most importantly, however, the changes clearly signify shifts in the physiological status of the cells, the basis of which has yet to be established.

4.2.7 INT reduction

The effects of isoniazid and rifampicin on *M. tuberculosis* culture were also assessed using INT reduction as an indicator of oxidative metabolism (Section 3.2.7). The results were comparable to those reported with R123 in that two trends were observed. Isoniazid treatment produced enhanced reduction of INT in almost every cell, which was apparent after 24 hours and greatest after 3 days, whereas rifampicin treatment produced a gradual and progressive reduction in both the number and size of formazan deposits over the assay period.

The simplest interpretation of the increase in INT reduction in isoniazid-treated cells is that isoniazid treatment increased the permeability of the mycobacterial cell wall resulting in a greater influx of tetrazolium salt where it was reduced to INT formazan. This would explain both the increase in deposit size and the increase in the proportion of labelled cells. However, it is also likely that increased INT reduction reflects an increase in the oxidative metabolism of isoniazid-treated *M. tuberculosis* cells and is associated with the increase in respiration reported by Schaefer (1960) as one of the early changes in mycobacteria following treatment. Indeed, activation of isoniazid by crude extracts of *M. tuberculosis* and purified hydrogen peroxidase is known to be accompanied by a burst of toxic oxygen species (including superoxide and hydroxy radicals) (Shoeb et al., 1985a; 1985b) leading to speculation that oxidative stress contributes to the bactericidal action of isoniazid (Barry *et al.*, 1998).

Other workers have also observed enhanced tetrazolium reduction with isoniazid. Ibrahim (1996) reported enhanced reduction of INT by isoniazid-treated *M. avium*, larger

formazan deposits were produced and the proportion of cells that reduced INT increased to almost 100%. More recently, Mshana *et al.*, (1998) reported difficulties detecting isoniazid resistance in *M. tuberculosis* using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay because formazan production remained high despite inhibition of mycobacterial proliferation.

Curiously, the enhanced reduction of INT (or increased R123 fluorescence intensity) produced by isoniazid was minimal in cells treated with combinations of isoniazid and rifampicin. In addition, the distinctive cellular morphological characteristics (stunted cells, bulging poles and increased refractility) produced by isoniazid treatment were absent. These observations suggest that the inhibitory effects of rifampicin start very quickly, before isoniazid produces gross cell wall changes and/or an increase in oxidative metabolism. Indeed, the progressive reduction in both the number and size of formazan deposits in rifampicin-treated cultures reflects the expected reduction of oxidative metabolism in cells treated with bactericidal concentrations of the antibiotic and mirrors the reduction in colony counts.

4.2.8 Direct reduction of INT by isoniazid

An alternative explanation for the enhanced reduction of INT during treatment with isoniazid is that the antimycobacterial drug itself has sufficient intrinsic reducing power to reduce tetrazolium salts. Indeed, Issopoulos and Economou (1992) reported that both neotetrazolium chloride and tetrazolium violet chloride could be used in the spectrophotometric determination of microquantities of isoniazid as demonstrated by the redox reaction in Figure 4.2.

The results presented in Section 3.2.8 clearly demonstrate that direct reduction of INT by isoniazid *in vitro* only occurs at levels well above those used in the biological experiments (20 mg ml⁻¹ vs. 0.2 µg ml⁻¹) and after much longer exposure times (overnight vs. 10-60 min). Thus, it is unlikely that the direct reducing capacity of isoniazid reported

Along with detecting the decline in average intensity fluorescence, image analysis of cells treated with antimycobacterial drugs identified sub-populations with GFP fluorescence intensity comparable to that measured before treatment (approximately 3% of drug-treated bacteria had fluorescence intensities greater than the average fluorescence intensity measured before treatment). However, these results should be interpreted with caution as once formed GFP seems to be very stable (Tombolini *et al.*, 1997; Andersen *et al.*, 1998). Therefore, fluorescence may be detected in cells that are no longer capable of protein production. Recently, new unstable variants of GFP with reduced half-life (40 min to a few hours) compared to that of the wild-type protein have been produced (Andersen *et al.*, 1998). The effect of antimycobacterial drugs on reporter strains of *M. tuberculosis* expressing such variants might be more revealing concerning real-time gene expression.

Unfortunately, as stated in Section 3.2.9, identification of membrane energisation and oxidative metabolism in cells with high GFP fluorescence intensity following antimycobacterial drug treatment using R123 and INT was hampered due to the similar spectral characteristics R123 and GFP and the obscuring of GFP fluorescence in cells containing formazan deposits. However, spectrally shifted GFP variants have been produced recently (*e.g.* Clontech's enhanced blue fluorescent protein which has an emission maximum of 440 nm) which could be used in conjunction with R123 labelling and provide information on the energy status of any brightly fluorescing GFP cells.

4.2.10 Mycobacteriophage infectivity studies

Mycobacteriophage D29 was also used to investigate whether the R123 labelled proportion of cells observed in cultures of *M. tuberculosis* treated with antimycobacterial drugs could be infected and support viral replication. However, as stated in Section 3.2.10, treatment with D29 did not reduce the number of R123 labelled bacteria and unlike D29-treated broth culture (Section 3.3.3), cells partially labelled with R123 and PI

were not observed suggesting that the R123 labelled cells in the drug-treated cultures did not support lytic development.

It should be noted that, although the antimycobacterial drug treated cultures were washed in Tween 80-free media and incubated overnight before infection, these media were prepared with antimycobacterial drugs that may have affected the outcome of these experiments. Firstly, although the effect of addition of fresh drug to drug-treated cell suspensions was not studied, it is possible that this contributed to the reduction in colony counts observed following incubation in D29-free phage buffer controls (Table 3.7). Secondly, in the case of rifampicin it has been reported that development of D29 can be inhibited in *M. smegmatis* at concentrations below the MIC (Jones and David, 1971) suggesting that phage transcription is more sensitive to the drug than the host. In hindsight, phage development in cell suspensions containing rifampicin would probably not have been produced. Unfortunately, time constraints precluded the repetition of these experiments such that the effects of D29 on drug-treated cells washed in drug-free media prior to infection could be investigated.

However, under the experimental conditions established in this study, little effect was observed in cells in drug-treated *M. tuberculosis* cultures suggesting that although membrane energisation may be demonstrated in many of these cells, they do not seem to support phage development.

4.3 FURTHER STUDIES ON NON-LABELLING POPULATIONS AND DORMANCY MODELS

It was highlighted in Section 4.1.2 that most *M. tuberculosis* cells cultured in broth did not label with R123 or the other cytochemical stains used in the study (*e.g.* INT, PI or SYTO16). The physiological nature of these cells, therefore, remained undefined. Non-labellers could be: (1) active bacteria that were impermeable to cytochemical stains; (2) active bacteria with a low energy status; or (3) empty bacterial remnants devoid of intracellular material.

Heterogeneity of R123 labelling in other bacterial species has been reported and even in species that stained well, R123 did not label 100% of the cells detected, *e.g.* *S. aureus* (96%), *E. coli* (91%), *Salmonella pullorum* (89%) and *Aeromonas hydrophilia* (77%) (Diaper *et al.*, 1992). Most reports agree that the uptake of R123 and other fluorogenic probes that measure membrane potential is dependent on the age of culture, with accumulation greatest during the exponential phase and decreasing during the stationary phase. They suggest that variations in uptake reflect differences in the cell surface physiology and/or permeability of the bacteria. (Jepras *et al.*, 1995; Monfort and Baleux, 1996 and Comas and Vives-Rego, 1997). Indeed, it has long been reported that the concentration of lipids in mycobacteria increases with age and bacteria in older cultures often have two or three layered cell walls when observed using electron microscopy (Imaeda *et al.*, 1969). On this basis older cultures with thickened cell walls and lower permeability properties might be expected to show recalcitrance to cytochemical labelling. However, poor labelling with R123 was observed regardless of when cells were sampled from *M. tuberculosis* broth culture; there was little difference between bacteria stained early in the exponential phase or late in the stationary phase of growth.

According to Kaprelyants and Kell (1992, 1993), the extent by which individual cells accumulate R123 depends on their energetic status and physiological state. They suggest that fluorescent cells have high metabolic activity, whereas non-labelled cells are

metabolically inert or dormant. For example, starvation of *Micrococcus luteus* culture produces a gradual decrease in the number of cells exhibiting R123 fluorescence until few can be detected, whereas resuscitation in fresh liquid medium results in most of these cells reverting to accumulating R123. Similarly, Porter *et al.*, (1995) observed non-staining sub-populations in *Pseudomonas fluorescens* cultures stressed by starvation and suggested that they were low-activity bacteria with insufficient membrane potential to accumulate sufficient R123 for detection. The addition of the ionophore valinomycin to these cells was shown to increase the uptake of R123. Valinomycin is a potassium-selective ionopore and under conditions of high concentrations of sodium ions, but low concentrations of potassium ions produces hyperpolarisation by selective transport of potassium until the membrane potential is almost entirely due to the potassium ion gradient. Using this interpretative framework, the non-labelled cells observed in *M. tuberculosis* broth culture could be metabolically inert or dormant. However, attempts to hyperpolarise the plasma membrane in these cells with valinomycin produced inhibition of fluorescence even in the originally labelled portion. Explanation of this inhibition by valinomycin in *M. tuberculosis* under conditions where the cell membrane should be hyperpolarised remains unclear.

Heterogeneity of labelling with R123 was also reported with other mycobacteria. A recent investigation in this laboratory which characterised the morphological changes during the life cycle of *M. avium*, demonstrated that culture in liquid media produced an abundance of cell-like particles that did not label with either R123 or PI (Ibrahim, 1996). Ibrahim postulated that this non-staining proportion consisted of lipid bodies rather than bacteria, which were made from sloughed off mycobacterial lipids produced from culturing these extremely hydrophobic organisms in broth. However, the particles observed with *M. avium* were generally small and spherical, and should not be confused with the non-staining cells observed with *M. tuberculosis* which were bacillus shaped and morphologically indistinguishable from their staining counterparts.

Other workers have commented on persistent low-level lysis of *M. tuberculosis* in broth culture (Wayne, 1994; Cole *et al.*, 1998). Non-labelled cells could well be bacteria that had undergone disintegration/lysis leaving behind ghost cells or empty mycobacterial husks devoid of cellular contents. Such remnants would not accumulate R123 since they lack membrane potential, would not reduce INT due to the absence of oxidative metabolism and would not contain nucleic acid to stain with PI or SYTO16. However, labelled and non-labelled cells had similar morphological and refractile properties when viewed using phase contrast microscopy, evidence that lysis had not occurred and that husks devoid of cellular contents were not present.

The results of further investigations into the cytochemically non-labelled cells found principally in broth culture were presented in Section 3.3 and are discussed below.

4.3.1 Comparison of GFP production by bacteria cultured in broth and on agar

The growth and morphological characteristics of wild type H₃₇R_v differed little from *gfp*-expressing strains of *M. tuberculosis* (Section 3.1.1), suggesting that, although *gfp* was being expressed constitutively by cells and presumably reaching substantial concentrations, it was not toxic.

Regardless of culture method, most *M. tuberculosis* cells transformed with the *gfp*-expression cassette p-GFM11 produced GFP and were brightly fluorescent green. Therefore, the majority of bacteria had at some stage been able to produce intact GFP. This evidence that the majority of cells supported protein synthesis favours the view that the high numbers of cytochemically non-labelled cells observed in broth culture were cytologically active but had decreased cell wall permeability properties.

As stated in Section 4.1.11, this highlights one advantage of using GFP as a reporter molecule to demonstrate cellular activity in *M. tuberculosis* since it requires neither substrate nor cofactor due to the intrinsically fluorescent nature of the protein. However,

as discussed in Section 4.2.9 a major drawback of GFP is that once formed it is very stable and the half-life in *M. tuberculosis* is not really known. Hence, while fluorescence may be detected in cytologically inactive cells in broth culture that are no longer capable of protein synthesis or division, these cells may not label with cytochemical reagents such as R123 and INT. Real-time gene expression studies using unstable variants of GFP with reduced half-lives would help solve this problem.

4.3.2 Mycobacteriophage infectivity studies

Mycobacteriophage D29 was used to determine whether the non-labelling subpopulations observed in *M. tuberculosis* broth culture could be infected and support viral replication. D29 is a lytic phage and is reported to cause rapid bacterial cell lysis (David *et al.*, 1980; Donnelly-Wu *et al.*, 1993; Hatfull and Jacobs, 1994; Carrière *et al.*, 1997). It was hypothesised that R123-labelled cells being cytologically active would be infected, support phage replication and lyse during lytic development whereas the cytologically inactive non-labelled cells would not be infected and/or support replication and would not lyse. If this had been the case, the proportion of R123-labelled cells should have decreased following phage infection and the proportion of non-labelled cells should have increased.

In contrast, the results presented in Section 3.3.2 show that the proportion of non-labelled cells was greatly reduced after overnight incubation with D29, suggesting that these cells had become infected, supported viral replication and during phage lytic development had lost their intact cell membrane and become permeable to PI or lysed completely. Many of the PI-stained cells showed evidence of shrunken cytoplasm when viewed by phase contrast microscopy (*e.g.* Figure 3.39a) suggestive of plasmolysis. It appears that although most *M. tuberculosis* cells in broth culture are recalcitrant to cytochemical labelling, many of these cells can be infected by mycobacteriophage and support replication.

Although the proportion of cells that accumulated R123 was reduced after infection with phage, many still accumulated the dye. There are several possible reasons for this. (1) David *et al.*, (1980) demonstrated that the timing of D29 replication was related to the average division time of the host, and therefore, one would expect that only cells which underwent replication during the time frame of the experiment would show evidence of lytic development. (2) Longer phage development may take place in slowly dividing cells and probably does not occur in non-dividing or resting cells. (3) Although Tween 80 is known to inhibit D29 infection (White and Knight, 1958), culture of *M. tuberculosis* in liquid media necessitates the use of detergent to prevent clumping. Consequently, cultures were washed and incubated for 24 h in detergent-free broth before infection. However, Tween 80 extracts lipids, glycolipids and carbohydrates from the mycobacterial cell wall (Daffé and Draper, 1998) and may have removed phage binding sites from many cells. Another important effect resulting from the addition of Tween 80 to mycobacterial cells is insertion of the detergent into the outer lipid layer of the wall (Christensen *et al.*, 1999). Such accumulation might well mask the D29 specific receptors in many of these cells.

4.3.3 R123 labelling of dormant bacteria

There has been renewed interest in mycobacterial dormancy to explain both the capacity of *M. tuberculosis* cells in tissues to survive in the presence of antimycobacterial drugs and the endogenous reactivation of tuberculosis years or even decades after primary infection (Gangadharam, 1995; Grange, 1992; Wayne, 1994). Wayne and colleagues have investigated nonreplicating persistence produced by adaptation to anaerobiosis as an *in vitro* model to study this phenomenon (Wayne and Lin, 1982; Wayne and Hayes, 1996). Cells adapted to anaerobiosis in liquid culture remain viable for long periods but do not replicate. Adaptation involved changes in oxygen-dependent metabolic pathways, which may facilitate the phenomenon of dormancy *in vivo*. Recently Cunningham and Spreadbury (1998) observed that under these low-oxygen conditions *M. tuberculosis*

develops a thickened cell wall possibly for protection and produces an α -crystallin homolog that may play a role in stabilising cell structures during long-term survival.

It was reported in Section 3.3.3 that *M. tuberculosis* cells adapted to anaerobiosis using either of Wayne's models could be labelled using the R123/PI assay. In the first model, the slow settling of bacilli through a self-generated oxygen gradient, most of the cells found in the anoxic region at the bottom of the tube had not adapted to anaerobiosis as they were stained with PI. The PI stained cells must have succumbed to the lethal shock of oxygen depletion. Wayne (1996) found that this model yielded erratic results and that slight changes in the growth or settling rate was sufficient to upset the equilibrium between replication and settling that is required to maintain the oxygen gradient and support the orderly shift down to dormancy. Nonetheless, many R123 labelled cells were found in the anoxic sediment as long as 6 months after oxygen was depleted.

In the second model, which was based on controlled agitation of sealed liquid cultures exposed to limited headspace volumes of air, few PI labelled cells were found. Here, it appears that the transition to anaerobiosis was more gradual, allowing the bacilli to adapt to the gradual depletion of oxygen producing an orderly shift down to dormancy.

In summary, cytological activity in *M. tuberculosis* cells adapted to anaerobiosis using Wayne's *in vitro* model could be monitored using R123/PI labelling. Unfortunately, time constraints precluded assessment of the effects of antimycobacterial drugs on these cells.

4.4 GENERAL DISCUSSION

The central aim of this project was to develop cytochemical indicators of cell activity to investigate the effect of antimycobacterial drugs on *M. tuberculosis*. It was hoped that these techniques would contribute to our understanding of the physiological properties of *M. tuberculosis* that render it refractory to eradication and produce the characteristic slow response to chemotherapy (6-9 months).

A principal feature of the early work was to establish a culture method that would produce consistent defined populations of *M. tuberculosis* that was suitable for both the cytochemical labelling and *in vitro* antimycobacterial drugs studies. It was clear from these results that there were substantial differences between cells grown in broth and those grown on agar. While culture in liquid media was expected to produce bacterial cells in a defined state of growth with minimum of heterogeneity, it proved unsuitable for cytochemical labelling due to the large number of cells (up to 70%) at any time of observation that were recalcitrant to staining. These cells were unlabelled and therefore undefined. In contrast, most bacteria (approximately 90%) in cell suspensions prepared from growth on agar were readily labelled with the cytochemical stains evaluated and consequently this source was used as the standard *M. tuberculosis* cell suspension for the study.

Not all the cytochemical methods evaluated demonstrated activity in *M. tuberculosis*, e.g. poor labelling was observed with the membrane potential indicator oxonal (DiBAC₄), extracellular formazan with the tetrazolium salt CTC and non-specific labelling with the Molecular Probes Live/Dead BacLIGHT reagents. The most consistent results were obtained with R123/PI dual labelling and INT reduction, which demonstrate transmembrane electrochemical potential in cells with intact plasma membranes and oxidative metabolism respectively.

The accumulation of R123 was shown to be energy dependent as dissipation of the electrochemical gradient with the uncoupler agent CCCP abolished uptake. However, this was only demonstrable in *M. tuberculosis* with low concentrations of dye and high concentrations of uncoupler reagent. CCCP-inhibited cells were capable of regenerating membrane potential when washed and re-labelled. The requirement for a physiologically validated procedure where cellular labelling could be unambiguously attributed to membrane energisation was highlighted when the effects of a range of tuberculocidal treatments on *M. tuberculosis* cell suspensions were assessed. Several of the treatments (70°C for 30 min, 70% ethanol and 4% formaldehyde) lead to development of uncoupler-insensitive R123 labelling of ‘dead’ cells. This identified non-specific labelling of dye that was unrelated to membrane energisation, which would have concealed the genuine bactericidal effects of the treatments at the single cell level.

All of the cytochemical stains evaluated identified heterogeneity in labelling among the *M. tuberculosis* cell population irrespective of growth stage and growth conditions. It is clear that all the cells in a mycobacterial culture are not identical and that all stages of the growth and division cycle are probably represented. With each labelling reaction, heterogeneity may reflect either the level of the physical or chemical activity available to be demonstrated in each cell (*e.g.* high versus low oxidative metabolism, high versus low electrochemical gradient) or the access of the label to that activity (*e.g.* variation in cell permeability). The heterogeneity of physiological phenotypes in *M. tuberculosis* cell populations no doubt produces cell-to-cell variation in antimycobacterial drug susceptibility.

Once the suitability of *M. tuberculosis* cell suspensions prepared from agar culture for cytochemical labelling with R123/PI and INT was established, the effects of clinically important antimycobacterial drugs on cytological activity were assessed in parallel with conventional growth based techniques. Prior to drug treatment approximately 90% of cells labelled with R123, 1% stained with PI and the remainder were non-labelled. The general observation during the 7-day drug treatments was, with the exception of isoniazid

(and isoniazid-ethambutol combination), that there was a reduction in the proportion of R123-labelled bacteria and a concomitant increase in the number of PI-stained cells. Although substantial bactericidal effects were achieved with single drug treatments (1 to 3 log reductions in CFU ml⁻¹), drug combinations (*e.g.* isoniazid-rifampicin, isoniazid-streptomycin and rifampicin-streptomycin) produced the greatest reduction in colony counts (3 to 5 log reductions in CFU ml⁻¹). However, the central feature of the antimycobacterial drug treatments was that the number of cells with cytochemical phenotypes consistent with viability (*i.e.* energy-dependent uptake of R123 or INT reduction) far exceeded the number of cells capable of forming colonies. Typically, an excess of between 1 and 4 orders of magnitude of uncoupler sensitive R123 labelling cells over culturable units was observed. Increased recovery of *M. tuberculosis* propagules in drug-treated cultures was not observed with the MPN enumeration method. Thus, large populations of active but nonculturable (ABNC) cells were produced by antimycobacterial drugs commonly used in the treatment of tuberculosis.

Isoniazid (and isoniazid-ethambutol combinations) caused enhancement in the labelling properties of cells during the first 3 days of treatment, producing both an increase in the proportion of labelled cells and an increase in the intensity of fluorescence and size of formazan deposit. This was associated with gross morphological changes characterised by a transformation from bacillus-shaped cells to smaller stunted cells with intense polar labelling. The simplest interpretation of these observations is that isoniazid treatment increased the permeability of the mycobacterial cell walls to the cytochemical stains resulting in greater influx of R123 and INT. However, it is possible that isoniazid causes an increase in cell respiration, thus producing greater activity in the electron transport system, an increased rate of production of reducing equivalents and therefore greater labelling with INT and R123.

Non-labelling cells and ABNC cells were further investigated using a GFP reporter strain and by exposure to the lytic mycobacteriophage D29. Regardless of culture method, the majority of bacteria (> 90%) in cell suspensions prepared using the reporter strain

produced GFP and were brightly fluorescent. Evidence of the predominant non-labelling population identified by cytochemical labelling in broth culture was not observed. In addition, the results of studies with D29 revealed that some non-labelled cells in broth culture were readily infected with D29, supported viral replication and during lytic development were permeabilised to PI or lysed completely. It seems likely that the non-labelled cells identified in *M. tuberculosis* broth culture are cytologically active cells with low permeability properties to the cytochemical reagents rather than empty cell husks. The identification of large numbers of cells with low permeability properties in *M. tuberculosis* cell populations has significance with regard to antimycobacterial drug susceptibility and needs to be investigated further.

Treatment with antimycobacterial drugs caused a reduction in the expression of GFP in the reporter strain; the greatest reductions were seen with drugs that produced the greatest reduction in colony counts. It was disappointing that the membrane potential and oxidative metabolism in drug-treated GFP expressing cells could not be assessed using the cytochemical assays developed during the study since the spectral characteristics of R123 and GFP overlapped and formazan deposits obscured GFP fluorescence. Although the results made clear that many drug-treated cells contained sufficient GFP to brightly fluoresce, they should be taken with caution as GFP seems to be very stable once formed and its half-life in *M. tuberculosis* is not known. Exposure of antimycobacterial drug treated cultures to D29 did not appear to have any effect on the ABNC cells identified by R123 labelling. It was hoped that the mycobacteriophage would infect these cells and lyse them during lytic development demonstrating that these cells contained the necessary machinery for replication.

In addition to demonstrating ABNC in antimycobacterial drug treated *M. tuberculosis* culture, cytological activity was demonstrated in non-replicating cells adapted to anaerobiosis using Wayne's *in vitro* model of dormancy. Cells in the anoxic sediment generated accumulated R123 months after the oxygen was depleted. Unfortunately, time constraints precluded assessment of the effects of antimycobacterial drugs on these cells

since it is known that *M. tuberculosis* derived from the Wayne model reveals a different drug susceptibility pattern.

The presence of large numbers of ABNC bacteria in antimycobacterial drug treated cultures offers much opportunity for speculation. Since antimycobacterial agents are generally considered to affect *M. tuberculosis* only when the bacilli are actively growing, phenotypic adaptation to ABNC forms in response to chemotherapy may produce drug-resistant phenotypes in which normally bactericidal drugs produce only a bacteriostatic effect. Such phenotypes would have the capacity to outdistance the course of chemotherapy, which might explain both the unusually long treatment required for successful chemotherapy of tuberculosis and reactivation tuberculosis. Indeed, by adopting the rapid elimination of ABNC cells as a determinant for the outcome of chemotherapy, novel drugs or novel combinations of known drugs might be discovered that could produce more rapid treatment of disease.

Although many publications implicitly or explicitly state that viability can be assayed at the cellular level by a variety of cytological methods, it should be stressed that viability implies the capacity for replication and growth and that methods that can predict these properties have not been found. Direct demonstration of growth and replication by culture remains the only unambiguous operational definition of culturability applicable to bacteria. Although we have demonstrated R123 accumulation, INT reduction and *gfp* expression in nonculturable *M. tuberculosis* cells on many occasions, the viability of these cells is indeterminate at present. The significance of the non-labelling and ABNC cells observed remains to be established, but it is speculated that these populations may have implications for the chemotherapy of tuberculosis.

4.5 CONCLUSIONS

The following conclusions can be drawn from this study

1. There are substantial differences between cells grown in broth and those grown on agar with regard to morphology and physiology. The majority of cells grown in broth are recalcitrant to labelling with cytochemical stains.
2. Of the cytochemical indicators of cell activity evaluated, R123/PI dual labelling and INT reduction give the most consistent results with *M. tuberculosis*.
3. All of the cytochemical stains evaluated identified heterogeneity in *M. tuberculosis* cell population irrespective of growth stage and growth conditions. It is clear that all the cells in a mycobacterial culture are not identical.
4. All antimycobacterial drug treatments produce an excess of between 1 and 4 orders of magnitude of uncoupler sensitive R123 labelling cells over culturable units after 7 days exposure. Activity in ABNC cells can also be detected using INT reduction and GFP reporter strains.
5. Isoniazid produces enhanced cytochemical labelling with R123 and INT early in treatment that is associated with gross morphological cell changes.
6. Non-replicating cells adapted to anaerobiosis using Wayne's *in vitro* model of dormancy can be labelled with R123.
7. Non-labelled cells identified by cytochemical staining techniques seem to be cytologically active cells with low permeability properties. Some of them can be infected and lysed by the lytic mycobacteriophage D29. All cells in GFP reporter strains of *M. tuberculosis* produce GFP.

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