# THE DETERMINATION OF POST-EXPOSURE REGROWTH EFFECTS AND THE BACTERICIDAL ACTIVITY OF SELECTED ANTIMYCOBACTERIAL AGENTS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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# DECLARATION

The experimental work described in this dissertation was carried out in the TB laboratory of the Medical Research Council, Pretoria, from March 1995 to March 1997, under the supervision of Professor Hendrik J. Koornhof.

These studies present original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

Signed R.W. Masango

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## ABSTRACT

The studies recorded in this dissertation which involved the determination of bactericidal activity and postantibiotic properties of antituberculosis drugs were performed on eight clinical isolates of *Mycobacterium tuberculosis* as well as the H37Rv strain. Their susceptibility to isoniazid (INH), rifampicin (RMP), ciprofloxacin (CIP), ethambutol (EMB) and streptomycin (SM) was determined on Lowenstein-Jensen (LJ) and Middlebrook 7H10 agar using recognized critical concentrations as breakpoints. Results obtained on the two media were similar. Two of the isolates and the H37Rv strain were susceptible to all drugs and the others had varying levels of resistance.

The minimum inhibitory concentrations (MICs) of INH, RMP, CIP and EMB for drug-resistant and drug-susceptible strains of *M. tuberculosis* determined by the Bactec and 7H10 agar methods were compared. The Bactec 7H12 broth determined MICs expressed in ug/ml ranged from 0.025 to 0.1, 0.25, 0.31 to 1.25 and 0.25 to 0.5 for INH, RMP, CIP and EM, respectively, whereas MICs on 7H10 agar ranged from 0.06 to 0.1, 0.25 to 1, 1.25 to 10 and 0.25 to 2ug/ml, respectively. The MICs were either equal in both media or slightly higher on 7H10 agar.

The minimum bactericidal concentrations (MBCs) of INH, RMP, CIP and EMB against *M tuberculosis* H37Rv were also determined using the Bactec system. INH was found to be the most bactericidal of the drugs tested (with MBC = MIC), followed by RMP (MBC = 2 X MIC), and then CIP (MBC = 4 X MIC). EMB was the least bactericidal (MBC = 8 X. MIC).

*M. tuberculosis* cells were dispersed by sonication and vortexing and the two methods of dispersion were compared. The sonication method dispersed the cells much better than the vortex by consistently breaking up clumps of bacilli to smaller units, resulting in the production of a larger number of colony forming units (CFUs) on Middlebrook 7H10 agar.

A method for determining the post-exposure regrowth effect using the BACTEC instrument was developed. The method was used to evaluate the effect of time and concentration on regrowth. The regrowth effects of RMP, CIP and EMB were dependent on both time and concentration. For INH, where only two concentrations were used, no concentration dependence was established but the duration of post-exposure regrowth of this drug was time dependent.

The Bactec method was compared with the traditional method of viable counts for the determination of postantibiotic effect (PAE). The values obtained in the Bactec system were referred to as the control-related effective regrowth time (CERT) as opposed to PAE, which is determined by the colony count method. CERT and PAE values are not directly comparable between Bactec and viable count experiments. The basic differences between PAE and CERT-type experiments is that PAE requires regrowth of post-exposure surviving bacteria to increase by $1\log_{10}$ while the CERT requires regrowth in a liquid medium to a predetermined level, usually 10-fold more than the original inoculum. Highly bactericidal drugs may reduce the original inoculum by greater than $1\log_{10}$ so that the CERT-type experiments regrowth to the original inoculum concentration, or a concentration 10-fold higher than the exposed inoculum, may result in a much longer CERT than PAE determined conventionally.

The Bactec method was also used to show whether CERTs induced by drugs in combination differed from CERTs induced by the drugs alone and in this way patterns of synergism, addition, indifference or antagonism could be determined. The CERTs of the combinations of RMP + EMB and INH + CIP were synergistic, while those of INH + RMP and INH + EMB were additive. Indifference was observed with the combination of RMP + CIP. No antagonism was demonstrated.

## ABBREVIATIONS USED IN THE TEXT

| | |
|---|---|
| ADC | - albumin, dextrose and catalase |
| CERT | - control-related effective regrowth time |
| CFU | - colony forming units |
| CIP | - ciprofloxacin |
| $C_{max}$ | - peak serum concentration achieved after a specified dose of drug |
| CSF | - cerebrospinal fluid |
| DNA | - deoxyribonucleic acid |
| DST | - drug susceptibility testing |
| EBA | - early bactericidal activity determined ex vivo by showing the extent of killing of tubercle bacilli in the sputum during the first two days of treatment |
| EMB | - ethambutol |
| ERT | - effective regrowth time |
| GI | - growth index |
| HCL | - hydrochloric acid |
| INH | - isoniazid |
| LJ | - Lowenstein-Jensen |
| MBC | - minimum bactericidal concentration |
| MIC | - minimum inhibitory concentration |
| ml | - millilitre |
| NAD | - nicotinamide adenine dinucleotide |
| NCCLS | - National Committee for Clinical Laboratory Standards |
| OFL | - ofloxacin |
| PAE | - postantibiotic effect |
| PBS | - phosphate buffered saline |
| RMP | - rifampicin |
| RNA | - ribonucleic acid |

| | |
|---|---|
| SEM | - standard error of the mean |
| SM | - streptomycin |
| SPA | - sparfloxacin |
| ug | - micrograms |

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# CHAPTER 1

## INTRODUCTION

Tuberculosis is characteristically a chronic infectious disease affecting any tissue or organ but mainly the lungs. The first signs and symptoms of the disease are weight loss, persistent cough, night sweats, dyspnea and chest pain. The causative agent is called *Mycobacterium tuberculosis*. The organism is acid fast, meaning it resists decolorisation by acid or alcohol when stained with carbol fuschin. In culture it grows slowly and produces rough colonies with bacilli compacted into dense aggregates. When cultured on favourable solid media, colonies become visible in 2 weeks but some strains require 4 to 6 weeks or even longer. Typical microscopic morphology of *M. tuberculosis* as seen in acid-fast stains exhibit thin, slightly curved bacillus measuring 0.3 to 0.6 by 1 to 4 um, deeply red staining with a distinct beaded appearance. In the preparation of smears from cultures the individual cells are often difficult to disperse, appearing as irregular aggregates in parallel strands (Roberts et.al).

The disease has been a tremendous problem for many years to humans and still continues to be a major hazard especially with the advent of infection with human immunodeficiency virus (HIV) and the associated acquired immunodeficiency syndrome (AIDS) (Sepkowitz et al., 1995). The already grave situation was further aggravated by the emergence of multiple-drug-resistant (MDR) tubercle bacilli causing several well documented outbreaks (Hobby et al., 1970; Nivin et al., 1998; CDC, 1990; Edlin et al., 1992). The World health Organisation (WHO) estimates that there are more than 8 million new cases of tuberculosis per year and that the 2.9 million people killed each year by *M. tuberculosis* are more than those caused by any other single infectious pathogen. In 1990 at least 10 million people were infected globally while 70 000 cases and 3 000 deaths were notified in South Africa alone (SA Department of Health, 1996). This global emergency underscores

the need for rapid methods of identification of the organism and for the evaluation of drugs active against the disease.

## 1.1. Chemotherapy of tuberculosis

### 1.1.1. Historical perspective

The chemotherapy of tuberculosis started in 1944 when streptomycin (SM) was introduced as the first antimycobacterial agent (Schatz and Waksman, 1944). Decline in the incidence of the disease was observed but soon after, resistance to the drug developed. Para-aminosalicylic acid (PAS) became available and thereafter isoniazid (INH), ethionamide and pyrazinamide (PZA) and rifampicin (RMP) as well as several other minor drugs were introduced.

The duration of treatment has been progressively reduced from 18-24 months, with INH, SM and PAS, to 9 months and now by a more effective short course chemotherapy (SCC) of 6 months with two or three drugs including INH and RMP supplemented with PZA during the first two months followed by four months treatment with INH and RMP (East African/ British MRC, 1974). The SCC is not only cost effective but it also has two advantages: the completion rate is higher and drug toxicity is much less (Davidson &Le, 1992).

### 1.1.2. Antituberculosis drugs

Antituberculosis drugs are divided into 3 groups:

(i) The first-line drugs used in the regimens recommended by WHO for the treatment of drug-susceptible tubercle bacilli. At present the first-line drugs in use are INH, RMP, PZA, EMB and SM.

(ii) Second-line drugs used in the treatment of tuberculosis due to resistance to one or more of the first line drugs. These include ethionamide, thiacetazone, PAS, cycloserine, kanamycin, amikacin (AMK), and capreomycin.

(iii) Drugs not used for treatment of tuberculosis but which are included in regimens of multiple drug-resistance (MDR) tuberculosis. These include quinolones [ciprofloxacin (CIP), ofloxacin (OFL) and sparfloxacin (SPA)] and the new macrolides, e.g clarithromycin because of their high concentration in macrophages and tissues (Grage et.al 1994.). The latter agents are appropriately used for the treatment of some of the infections caused by mycobacteria other than *M. tuberculosis*.

### 1.1.3. Antimycobacterial action of selected drugs used in the treatment of tuberculosis

(i) Isoniazid

Isonicotinic acid hydrazid or isoniazid (INH) was first described in 1912 but its antituberculosis activity was only discovered in the early 1950s (McClatchy, 1991). INH, a remarkably effective and nontoxic compound, used in combination with one or two other antituberculosis drugs, has made it possible to treat large numbers of patients effectively and economically. It achieves its bactericidal effect on *M. tuberculosis* by interfering with the metabolism of NAD thereby inhibiting DNA synthesis. Isoniazid also inhibits the synthesis of mycolic acid leading to a defective cell wall, which permits the exit of essential structural constituents, resulting ultimately in the loss of acidfastness and cell death (McClatchy, 1991).

INH is well absorbed in the gastrointestinal tract (Davidson & Le, 1992) and is highly reactive with various antacids and food components which reduce its bioavailability. The peak plasma concentration ($C_{max}$) after oral administration of 300 mg is 3-5 ug/ml. It is well distributed in the body and high quantities of this drug have been found in the lungs and skin, which suggest that these organs may serve as storage depots. From 70 to 95 % of a dose of INH is excreted in the urine within 24 hours, all in the form of metabolites (Holdiness, 1984). It is metabolised in the liver by acetylation. There is a bimodal distribution into rapid and slow

acetylators, based on the rate of acetylation of INH, amongst patients. Its half-life varies from 1 to 3 hours, depending on the rate of acetylation (Davidson &Le, 1992).

(ii) Rifampicin

Rifampicin (RMP) is a semisynthetic derivative from rifamycin B produced by *Streptomyces mediteranei*. The antituberculosis effect of rifamycin derivatives has been known since 1965 and rifampicin has been used clinically since 1965 (Davidson & Le, 1992). It is bactericidal against both intra- and extracellular bacterial populations and is a potent inhibitor of DNA-dependent RNA-polymerase enzymatic activity in many bacteria (McClatchy, 1991).

RMP has a particularly fast action against slow-growing tubercle bacilli. Following oral administration, RMP is well absorbed from the gastrointestinal tract and the rate of absorption increases with time (McClatchy, 1991; Holdiness, 1984). It is widely distributed in body fluids and tissues. Its $C_{max}$ has been found to be 4-32 ug/ml following oral administration of 600 mg. The cerebrospinal fluid (CSF) concentrations are relatively low, but still above the MIC levels, usually reaching 4 % of corresponding plasma concentrations in patients with inflamed meninges. RMP is deacetylated at the C-25 position, in the liver. This results in a more soluble water compound with an increased capacity for biliary excretion (Davidson & Le, 1992, Holdiness, 1984). RMP may induce hypersensitivity reactions which are often associated with the presence of auto-antibodies. It may also be hepatotoxic in some patients. The elimination half-life of RMP following initial doses ranges between 2.3 and 5.1 hours, but following repeated administration decreases to 2 to 3 hours due to increased hepatic metabolism (Holdiness, 1984).

(iii) Ethambutol

Ethambutol (EMB) is a synthetic agent active only against mycobacteria. Although it has considerable anti-TB activity, its main use is as a companion drug to prevent the development of resistance to more potent agents (Houston & Fanning, 1994). It acts by interfering with the synthesis and stabilization of RNA (McClatchy, 1991; Davidson & Le, 1992).

EMB is well absorbed in the gastrointestinal tract following oral administration and it is distributed rapidly and widely to most cells, body fluids and tissues including the lung and is localized within pulmonary alveolar and axillary lymph node macrophages. EMB penetrates the CSF in very few healthy individuals but levels of 1-2 ug/ml are achieved when the meninges are inflamed. It has a $C_{max}$ of 2 to 5 ug/ml after oral administration of 800 mg and a half-life of 2 to 4 hours. The drug is dialysable and is metabolised by oxidation in the liver (Davidson & Le 1992, Holdiness, 1984).

Hepatic and renal dysfunction increase peak serum EMB concentrations and half-life with a corresponding increase in the risk of toxicity. EMB is generally well tolerated but its major adverse effect is optic neuritis with a decrease in visual acuity, constriction of visual fields and loss of red/green discrimination (Davidson & Le 1992).

(iv) Fluoroquinolones

The new fluoroquinolones have a broad spectrum antibacterial activity including activity against mycobacteria (Rastogi & Goh, 1991; Heifets, 1991 (b); Garcia-Rodriguez & Garcia, 1993). Among the fluoroquinolones tested against mycobacteria, CIP, OFL and SPA showed highest activities against *M.* tuberculosis. These drugs inhibit DNA gyrase and topoisomerase enzymes which is essential for bacterial replication (Houston & Fanning, 1994; Sullivan et.al, 1995 ).

Fluoroquinolones are used in the treatment of MDR tuberculosis. There is no cross-resistance between fluoroquinolones and other antituberculosis agents. Resistance to these drugs can develop rapidly and they should therefore be restricted to patients with MDR tuberculosis (Sullivan et.al, 1995).

Fluoroquinolones are well absorbed in the gastrointestinal tract after oral administration (Kennedy et.al, 1993). They are widely distributed in body fluids and tissues. The highest concentrations are usually achieved in urine and bile, while low concentrations are typically found in CSF and aqueous humor (Gerding & Hitt, 1989). In meningitis with inflamed meninges the CSF concentrations are between 40-70 % of the serum concentrations. CIP and OFL penetrate well in human macrophages killing intracellular multiplying tubercle bacilli (Rastogi & Blom-Potar). In the case of CIP, a single oral 750 mg dose was shown to produce a $C_{max}$ of 2.01 mg/ml (Kennedy et.al, 1993) and its half-life has been shown to be 4.0 hours.

### 1.1.4. The basis of treatment regimens involving antituberculosis agents

The basis of the management of antituberculosis chemotherapy can be considered under 3 headings (Mitchison, 1985; Mitchison, 1992): prevention of drug resistance, early bactericidal activity (EBA), and sterilizing activity.

#### 1.1.4.1. Prevention of drug resistance

Drugs have been graded according to their activity in preventing the emergence of drug resistance to a second drug, usually INH, as follows: high - INH and RMP, intermediate - EMB and SM, low - PZA and thiacetazone. The activity of the drugs is assessed from the results of clinical studies of the treatment of smear-positive pulmonary tuberculosis in which the drug concerned was given in a 2-drug combination with INH (Mitchison, 1985).

*In vitro* studies to assess the prevention of the emergence of drug resistance have been described in which drugs were used in combination with subinhibitory concentrations of each drug and then subculturing in a drug-containing medium (Hobby & Lenert 1972). In this study RMP subinhibitory concentrations enhanced the bactericidal effect of INH and SM and suppressed the emergence of cells resistant to these drugs but failed to prevent the emergence of EMB-resistant microbial cells.

### 1.1.4.2. Early bactericidal activity (EBA)

The study of EBA was first introduced by Jindani et.al (1980) where a group of patients were given a wide variety of different regimens of single and multiple drugs for the first 14 days of treatment. During this period serial colony counts of *M. tuberculosis* in the sputum were done to measure the EBA of the regimens. EBA was defined as the ability of the drug to decrease (or kill) the number of viable bacilli in the sputum during the first 2 days of initial therapy and is expressed as $\log_{10}$ decrease in colony forming unit (CFU) per ml sputum/day.

INH was found to have the highest EBA as compared to other drugs (0.72 $\log_{10}$ CFU/ml sputum/day) when given alone. RMP and EMB had moderate EBA (0.19 $\log_{10}$ CFU/ml sputum/day and 0.25 log CFU/ml sputum/day respectively). The moderate EBA of EMB has also been reported by Botha et.al (1996) who showed a decrease in viable count of 0.245 +\-0.046 $\log_{10}$ CFU\ml sputum/day. SM, thiacetazone and PZA had been found to have a very low, if any, EBA with initial rates of kill of 0.09 to 0.04 $\log_{10}$ CFU/ml sputum/day.

CIP, one of the most promising agents in the treatment of tuberculosis has been found to have a useful EBA against *M. tuberculosis* in man (Kennedy et.al 1993), which is slightly lower than that of INH. Adult patients with smear-positive pulmonary tuberculosis were treated with CIP, the mean daily fall during a 7-day

period was 0.20 $\log_{10}$ CFU/ml/day as compared with 0.25 $\log_{10}$ CFU/ml/day for INH. This means that INH still remains the drug with the highest EBA.

EBA has no bearing in the duration of treatment, however, it is a good indication of the period during which a patient may be considered to be infectious to others (Jindani et.al, 1980). In other words, it measures how effective a drug is in killing actively growing bacilli. The EBA estimates the activity of a drug against rapidly dividing extracellular bacilli and is closely related to the conventional in vitro measure of bactericidal activity, viz. the fall in CFU counts during exposure of a culture in a logarithmic phase to a drug (Botha et.al, 1996). The ability of a drug to prevent the emergence of resistance is not related to its EBA since high bactericidal activity is not essential or even desirable for preventing resistance.

### 1.1.4.3. Sterilizing activity

Sterilizing activity is the ability of the drug to kill all or virtually all the bacilli in the lesions as rapidly as possible (Mitchison 1985). It measures the speed in which the last few viable bacilli are killed. Unlike the EBA, sterilizing activity plays an important role in chemotherapy by determining how effective the drug will be in shortening the period of chemotherapy.

When drugs are graded according to their sterilizing activity, RMP and PZA have been found to have the highest sterilizing activity, followed by INH and SM with moderate activity and then thiacetazone and EMB with very low, if any, sterilizing activity (Jindani et.al, 1980). CIP possesses no useful sterilizing activity against strains of *M. tuberculosis* (Kennedy et.al, 1993).

In clinical trials, the essential measure of sterilizing activity is the relapse rate found during a follow up period of at least 30 months after the end of chemotherapy (Mitchison, 1992). Since large numbers of patients, as well as a long follow up, are needed to measure relapse rates that are usually low, sterilizing activity is measured

as the percentage of patients with negative sputum cultures at 2 months after initiation of treatment.

An *in vitro* method for assessing sterilizing activity is to measure the bactericidal activity of the drug against stationary phase tubercle bacilli, whose metabolism has been halted by prolonged incubation. This type of experiment may approximate the response of "semi-dormant" bacilli to a drug and reflects the number of the persisting bacterial populations in lesions [Mitchison, 1992; Heifets, 1991 (b)].

### 1.1.5. Interaction of antituberculosis agents with *M. tuberculosis*

In addition to the abovementioned activities of the antimycobacterial agents which are important in our understanding of antituberculosis chemotherapy, Mitchison (1985) hypothesized that there are four subpopulations in which the tubercle bacilli exist in lesions which chemotherapy should address:

(a) Rapidly-growing organisms, which occur in the lesions at the start of treatment. This population exists in extracellular sites where the bacilli are not closely surrounded by inflammatory cells. These bacilli are killed actively by INH and to some extent SM.

(b) Semi-dormant bacilli, which occur in an acidic environment in TB lesions. The interior environment of phagolysosomes inside macrophages has a normal pH but this decreases when trapped tubercle bacilli lyse within these vacuoles. PZA is bactericidal against such organisms (Crowle et al., 1991).

(c) Semi-dormant bacilli that have occasional short spurts of metabolism lasting perhaps a few hours, RMP is active against these organisms because its bactericidal action starts very quickly during the occasional spurts of metabolism.

(d) Completely dormant populations. No antimycobacterial agent has been found to be active against such populations but recently it has been shown that the anti-anaerobe drug, metronidazole, which is also used against *Trichomonas vaginalis* and *Entamoeba histolytica* is active against dormant *M. tuberculosis* (Wayne & Sramek, 1994).

According to this hypothesis, drugs with high EBA kills populations reflected under (a), while those that have high sterilizing activity are active against populations of (b) and (c).

# CHAPTER 2

## *IN VITRO* SUSCEPTIBILITY TESTING

Drug susceptibility testing (DST) of antimicrobial agents can be divided into quantitative and qualitative procedures (Heifets, 1988). The latter is designed to measure whether a strain is resistant or susceptible to the drug and does not specify the extent of resistance or susceptibility. The former gives an indication of the degree of susceptibility or resistance of a strain which can be expressed as a bacteriostatic or bactericidal concentration or expressed in terms of whether the strain is very susceptible, intermediately resistant or very resistant. Interpretation of qualitative tests is based on 3 major components (Heifets, 1991 (a)): correlation between the minimum inhibitory concentration (MIC) and the concentration of the drug attainable in vivo ($C_{max}$) or sustained concentrations greater than the MIC under the area of the pharmacokinetic curve (auc > MIC), correlation of the MIC for the particular isolate and MICs found for other strains of the same species and, finally, the clinical experience in the use of the agents under consideration.

Standardization of DST of mycobacteria was first described by a group of specialists of mycobacteriology in 1961 (Canneti et.al, 1961). In their discussion they outlined 3 main purposes of *in vitro* susceptibility testing of tubercle bacilli to chemotherapeutic drugs:

(a) To serve as a guide to the choice of the first course of chemotherapy to be given to the patient.

(b) To confirm that drug resistance has reemerged when a patient has failed to show a satisfactory bacteriological response to treatment and, at the same time, give guidance regarding the choice of drugs for further treatment.

(c) To estimate the prevalence of primary and acquired resistance in the community.

They further defined the terms "sensitive" and "resistant". Sensitive strains were defined as those that have never been exposed to the main antituberculosis drugs

("wild" strains) and that respond to these drugs generally in a remarkably uniform manner. Resistant strains on the other hand were defined as those that differ from sensitive strains in their capacity to grow in the presence of high concentrations of a drug. The term susceptible generally indicates that treatment with a standard dosage of the antibiotic will eradicate an organism from a patient, assuming accessibility of the site of infection to the agent.

### 2.1. Methods used *in vitro* for DST of mycobacteria

In the literature relating to mycobacterial testing, there are 3 commonly used methods of measuring the sensitivity of *M. tuberculosis* to antimycobacterial agents (i) resistance ratio, (ii) absolute concentration and, (iii) proportion method (Cannetti et.al, 1963). The term conventional methods is commonly used to refer to these procedures. The methods can either be direct or indirect. Direct test is only performed with smear positive specimens and the specimen are diluted according to the number of organisms seen in the microscopic examination. Indirect tests are performed using isolates of mycobacteria. All these tests are qualitative because they only report whether the drug is resistant or susceptible.

### 2.2. Absolute concentration

This method is widely used in Eastern and Middle Europe. The medium usually employed is LJ containing 0.75% glycerol. The procedure is performed by inoculating drug-free and drug-containing media with different drug concentrations, with an inoculum containing 2 000 to 10 000 organisms (NCCLS, 1990; Canneti et.al, 1961, Cannetti et.al, 1963). For each drug the lowest concentration at which the bacilli may no longer be considered susceptible but are to be regarded as resistant (critical concentration ie. showing growth). If at this concentration growth is observed to the extent of more than 20 colonies the strain is to be considered resistant (Canneti et.al, 1961; Cannetti et.al, 1963).

## 2.3. Resistant Ratio (RR)

This procedure is performed in the same way as the absolute concentration using a small series of drug concentrations, but here, the standard strain H37Rv is tested in each set of tests. For all tests growth is defined as the presence of 20 or more colonies and the resistance ratio is the minimum concentration of the series inhibiting growth of the test strain divided by equivalent concentration of the standard strain H37Rv, in the same set of tests (Cannetti et al., 1963).

## 2.4. Proportion method

The proportion method was mostly used in the US and Western Europe but now it has become the most widely used method worldwide. It consists of calculating the proportion of resistant bacilli present in a strain and below a certain proportion the strain is classified as sensitive and above resistant. The critical proportion of survivors of an inoculum to describe the drug as resistant or sensitive was suggested as 1% for INH, PAS and RMP and 10% for the remaining antituberculosis agents. Different critical concentrations of the drugs were defined. The critical concentration of a drug is the amount that inhibits the growth of most cells in wild strains of tubercle bacilli without appreciably affecting the growth of all mutants present (NCCLS, 1990; Inderlied, 1991).

Briefly, the method used LJ as a medium. Appropriate drug concentrations are incorporated into the medium before coagulation. The drug-containing and drug-free media are distributed into tubes, coagulated and kept overnight at room temperature. They are either used immediately or stored at -4$^{0}$C for up to 2 months. The inocula are prepared by adjusting the culture to the optical density which equals to that of a McFarland no1 standard. Cultures are diluted to a higher and a lower dilution. The lower dilution is the control which represents 1% of the population and it is inoculated onto the drug-free medium while the higher dilution is inoculated onto the drug containing medium. The tubes are left for 24 to 48 h

in a position that allows the inoculum to be adsorbed on to the surface of the medium, after which they are incubated at 37$^{0}$C for 4-8 weeks. The strains are considered susceptible if growth on the test culture is less than the growth in the control.

The period of incubation was shortened by the discovery of the Middlebrook 7H10 agar (Middlebrook & Cohn, 1958) and the modification of the original version of the method by the Centres for Disease Control (Vestal, 1972). They recommended the use of 7H10 agar as the medium for DST and the critical proportion of 1 % rather than 10 % was accepted for all antituberculosis drugs as a proportion of resistance to *M tuberculosis* strains. The incubation period was shortened to 3 weeks. Some laboratories including those of Heifets' group adopted 7H11 agar as the medium for some drugs while the critical inoculum concentration of 1 % for survivors was also used for all drugs. All the procedures are the same but differ in the media recommended, the incubation period, critical concentrations and the critical proportion of *M. tuberculosis* inocula inhibited.

### 2.5. Bactec radiometric system

One of the major problems in the treatment of tuberculosis remains the long period of waiting for the results of drug susceptibility testing after the diagnosis of the disease. In most instances patients are treated with the standard tuberculosis regimen and those with resistant organisms might receive inappropriate treatment that could result in the infecting organism becoming resistant to additional drugs. Therefore, any method which can reduce this period will be of advantage to the patient and the prevention of drug-resistant strains. The Bactec radiometric method was developed by Middlebrook et.al (1977) and was recommended by others (Siddiqui et.al,1985; Heifets et.al,1986; Inderlied & Young, 1981; Laszo, 1985; Rastogi et.al,1989) as a rapid method of choice for drug susceptibility testing of mycobacteria.

### 2.5.1. General principle of the Bactec technology

The delay between the clinical diagnosis of the disease and its bacteriological confirmation is a major problem in tuberculosis management. Most of the techniques used have been long and could not detect growth if the bacterial count was very low. The need for the development of rapid methods was therefore highly justified. In their study, Middlebrook et.al. (1977) used a radiometric system to detect the growth of *M. tuberculosis*. Palmitic acid and formic acid both labelled with $^{14}C$ were compared as substrate sources of $^{14}CO_2$, in 7H12A medium which is deficient in carbohydrates and contains appropriate antimicrobial agents that are not active against tubercle bacilli. The medium containing palmitic acid-1-$^{14}C$ provided the basis for practical use of this method. BACTEC 301 instrument was used to detect isotope-labelled $CO_2$ production. With the advancement of technology the BACTEC 301 instrument and 7H12A medium have been replaced by BACTEC 460-TB instrument and Bactec 12B medium, respectively. The method was found to detect *M. tuberculosis* earlier than the conventional methods. Studies have also shown that the use of Bactec significantly improved the recovery rates and times of mycobacteria from respiratory secretions and other specimens (Kirihara et al.,1985)

The method is based on the principle that organisms utilize $^{14}C$-labelled palmitic acid in Middlebrook 7H12 broth and the bacterial growth is measured by the BACTEC 460-TB as a function of the release of $^{14}C$-labelled $CO_2$ which is captured by a detector and expressed as a numerical value called the growth index (GI) which ranges between 0 to 999 (Siddiqi, 1989). The radiometric GI curves agreed closely with the growth curves plotted from CFU/ml for *M. tuberculosis* (Heifets & Lindholm-Levy, 1987). Important in the context of the present studies, is the practice in Bactec technology to use the surface-active agent for the disposal of *M. tuberculosis* cultures before they are inoculated into the drug-containing Bactec 12B medium (Siddiqi et al., 1981; Siddiqi et al., 1985).

### 2.5.2. The use of Bactec as a DST method

The Bactec radiometric system for DST is a modified version of proportion method. It was developed by Snider et al. (1981) using 7H12 medium and adjusting the inoculum size in such a way that semi-quantitative results could be achieved with the 1% threshold as determinant of resistance. Thornsberry et al. (1975) states that for newly developed procedures to be accepted they should be compared with standard reference methods and correlation values should be over 90 % with major and very major discrepancies of less than 5 % when combined. The radiometric system correlated well with conventional methods and was more reliable for the determination of resistance to INH and RMP than to SM and EMB (Siddiqi et.al, 1981; Siddiqi et al., 1985; Slazo, 1985). Woodley (1986) further tested the method to compare the results of several concentrations of SM and EMB and found, 99% agreement with susceptible strains and a 97% agreement with resistance strains, 100% agreement with both susceptible and resistant strains, respectively. Thereafter the method was evaluated with different antituberculosis agents (Rastogi et al., 1989) and the technique is now widely accepted as a DST procedure and has also been used in the evaluation of new drugs (Inderlied, 1988; Heifets, 1988).

The method is rapid, results are obtained within 4-7 days (Siddiqi et al., 1981; Rastogi et al., 1989; Siddiqi, 1989) a net gain of 3 and 2 weeks as compared to LJ and 7H10\7H11 media respectively . It is also more sensitive because in liquid medium there is more cell-to-drug contact and due to shorter incubation time, there is less loss of potency of the test drug in the medium (Siddiqi et.al, 1981). Sensitivities of 100 % to INH, SM, RMP and EMB and specificities of 95.8, 97.6, 100 and 100%, respectively, have been shown (Rastogi et.al, 1989).

The Bactec radiometric procedure is performed by adding different drug concentrations into 7H12 vials and inoculating a standardized inoculum prepared in a fatty acid-poor albumin plus Tween 80 diluent. The control is prepared by

diluting the standardized inoculum 1:100 using the Bactec diluting fluid and inoculating it into the drug free vial. The vials are read daily on the BACTEC 460 instrument. Results are obtained by comparing the GI reading of the control to that of the test. When the GI reading of the control reaches 30 or more results are interpreted by calculating the increase in GI from the previous day. If the daily GI increase of the control is greater than the daily increase in GI of the drug containing vial the bacilli are reported to be susceptible to the drug and if it is less the organism is reported as resistant (Siddiqi et al., 1981, Siddiqi, 1989). The inhibition of growth detected by the radiometric method has been found to be a reflection of true growth inhibition (Heifets et al., 1986).

# CHAPTER 3

## *IN VITRO* METHODS USED IN THE EVALUATION OF NEW AND CONVENTIONAL ANTITUBERCULOSIS DRUGS

The emergence of MDR *M. tuberculosis* strains has led to the development of new drugs for the treatment of tuberculosis. *In vitro* quantitative methods that are used extensively for the evaluation of conventional and new antituberculosis drugs include the determination of the inhibitory activity of the drug expressed as an MIC, its killing (bactericidal) activity, the minimum bactericidal activity (MBC) and the ability of the drug to affect growth by a timed pulse exposure, the so-called postantibiotic effect (PAE) (Heifets, 1991 (a)) or control-related effective regrowth time (CERT) (Hanberger et al., 1995). These factors relate to the pharmacodynamics of the antimicrobials. The term pharmacodynamics refers to the relationship of drug concentrations in serum or tissues to effects on biological systems (DiPiro et al,1996).

### 3.1. Minimum Inhibitory Concentration (MIC) determination

The minimum inhibitory concentration (MIC), necessary to inhibit bacterial growth, is an accepted standard to quantitate the relative degree of susceptibility of different bacterial species (Heifets, 1988). It is generally defined as the lowest drug concentration that inhibits more than 99% of a mycobacterial population (Heifets, et al., 1986,, Lindholm-Levy & Heifets, 1988). The procedure is the same as that described for methods of DST but differs in a sense that a range of concentrations is used.

In clinical situations, the patient whose pretreatment isolates are more susceptible, ie. the MICs of the administered drugs for the isolates are relatively low, responds to therapy more favourably than those whose isolates are considered more resistant. Heifets (1996) suggested the following categories for the tentative MIC

interpretation: (i) Susceptible indicates that the isolate is inhibited by a concentration equivalent to the highest MIC for wild *M. tuberculosis* strains (ii) Moderately susceptible or Intermediate resistance indicates that an isolate is not inhibited by a drug concentration which is at least two fold below the $C_{max}$ but is inhibited at other concentrations up to $C_{max}$ and (iii) Resistant indicates that an isolate is inhibited only by a concentration equivalent to or greater than the $C_{max}$.

The MIC of the drug is usually determined by either the agar dilution or the broth dilution method. The agar dilution procedure is performed by inoculating a standardized inoculum size of the bacteria onto drug-containing agar plates and determining the number of CFU/ml as compared to the drug-free control. The broth dilution is basically the same but the endpoint is determined by sampling of the culture from the broth tubes, plating and counting the number of CFU/ml (Heifets, 1988; Rastogi & Goh, 1991), or by turbidometry (Yajko et.al, 1987), radiometry (Siddiqi,1989; Heifets, 1991 (a), Rastogi,et al., 1989) or bioluminescence (Limb et.al. 1993) to determine growth in liquid medium. The MIC broth dilution method can also be determined by looking at tubes with inoculated medium containing serially diluted antituberculosis drugs which show no visual growth after a specified period and comparing them to that of the control. Most investigators agree that the determination of MICs or any DST in broth is superior and more rewarding than determination of the MICs by agar dilution, the reason being the longer periods of incubation of the agar plates and the fact that during this period some drugs are inactivated (Heifets, 1996; Rastogi & Goh, 1991).

The value of the MIC depends on many factors, for example, the contents of the medium and its pH, inoculum size, incubation time, techniques of measurement of growth and its inhibition, etc. (Heifets, 1989). In a medium with a high content of protein (eg. albumin) drugs that have a high affinity to the protein will bind to the protein molecules and are usually released slowly by gradient force into cells and such binding may impede penetration thereby affect antimicrobial activity (Heifets, 1991 (b). Some drugs (eg. PZA) are active only at acidic pH such that it is

important to adjust the pH of the medium. It has been reported that the inoculum size greatly affects the MIC of some antimicrobial agents, the larger the inoculum size the higher the MIC. With some techniques (eg. Bactec) when the inoculum size is low the experiment takes longer and it should be repeated. Therefore, it is important that when reporting the MIC results, conditions under which it was performed be specified.

The MICs of several drugs may also differ when performed in different media due to the differences in time required for sufficient growth in different types of media. Prolonged incubation may lead to varying degrees of drug degradation resulting in higher MIC values. Therefore, it is important to state the conditions under which the agent was evaluated (Heifets, 1991 (b)). Drugs that may be affected by media used for MIC determination include RMP (breakpoints for resistance are 40ug/ml in Lowenstein Jensen, 1ug/ml in 7H10 agar and 0.25ug/ml in Bactec 12B broth) and ethambutol (4.0 ug/ml in 7H10 agar and 2.0 ug/ml in Bactec 12B broth (Heifets 1991 (a); Luna-Herrera et al., 1995).

### 3.1.1. Limitations of the MIC

Antimicrobials often affect bacteria at concentrations below the MIC. Determination of postantibiotic effects (PAEs) at sub-MICs is one way of studying such activity. Another limitation of the MIC test is that the effect of subpopulations of resistant bacteria allowed in MIC methodology is unpredictable and may be important. (DiPiro et al., 1996). The most important and obvious limitation of MIC determination is that the method does not indicate bactericidal activity, which may be important in clinical situations eg. in infective endocarditis and in immunodeficient patients including those with neutropenia or cell-mediated defects in HIV-positive and TB patients (Odenholt-Tornquist et al., 1991 (b)).

### 3.2. The minimum bactericidal concentration (MBC)

In most infections, once the microorganism has been sufficiently inhibited (bacteriostatic activity), the host's cellular and humoral defences can complete the eradication of the offending microorganism. However, in immunocompromised patients it may be necessary to measure the bactericidal activity of the antibiotic. The patient whose immune system is impaired is more likely to benefit from the drug which has a high bactericidal activity. It can be assumed that it is most likely that the drug whose minimum bactericidal concentration (MBC) is within the concentration achievable in blood or tissue will produce a killing effect against the bacteria actively multiplying *in vivo*. Still, such a drug may not be able to kill all cells of the bacterial population *in vivo*, particularly bacteria in a semi-dormant or dormant state or multiplying within macrophages (Heifets, 1991 (a)).

Unlike the MIC determination which measures the extent of inhibition of the microorganism by the drug the MBC measures the extent of killing. *In vitro* MBC determination is based on determination of the number of surviving colony forming units per millilitre of antibiotic-containing dilutions of liquid medium after a specified period of incubation of subcultures on a solid medium. It is defined as the minimum concentration that kills more than 99% of the bacterial population in the case of slow-growing mycobacteria (Heifets et al., 1992; Inderlied, 1991). In the literature on bacteria other than mycobacteria the 99.9% criterion is used (Hallender et al., 1982; Kumar et al., 1992; Chin & Neu, 1987).

The MBC is not usually performed routinely in mycobacteriology laboratories because it is expensive, labour intensive and time consuming. In the case of rapidly growing bacteria, most authors perform MBCs simultaneously with the MICs by the broth dilution method (Heifets, 1991 (a)). After the MIC from the broth is determined by visually examining tubes for turbidity, samples are taken from tubes which show no visual growth. These are plated onto drug-free agar plates, incubated and the development of CFUs observed and counted.

A Bactec radiometric method for the determination of MBC consists of growing *M. tuberculosis* cultures in Bactec vials until a GI of 500 is reached (Heifets et al., 1986; Heifets et al., 1987, Heifets et al., 1992). At this stage different drug concentrations are added to the vials and samples are taken from alternate vials at different time intervals, diluted and plated onto 7H10 plates without drugs. The plates are read after 21 days of incubation. The MBC is defined as the lowest drug concentration that kills more than 99 % of the population. From this experiment time-kill curves may be plotted.

Most *in vitro* methods measure the ability of antimicrobial agents to kill extracellular microorganisms using broth and agar techniques. Intracellular killing of mycobacteria has also been measured in vitro using macrophages derived from human monocytes (Mor et al., 1994) and experimental animals (Yajko et al.,1989; Rastogi et al.,1987). This was determined by comparing the number of CFUs of the microorganism inside untreated macrophages at the time of addition of the drug with the number of CFUs present in macrophages after treatment with drugs. A reduction in intracellular survival below the time before the addition of drugs indicated killing in macrophages.

In the treatment of tuberculosis INH, is the most bactericidal drug (Dickinson & Mitchison, 1976; Dickinson & Mitchison, 1977 ) with its MBC against many strains being equal to the MIC . RMP is also highly bactericidal and the MBC against many tubercle bacilli is two- to three-times the MIC (Heifets, 1991 (b)) and many dilutions lower than the achievable serum concentration. Ethambutol has been described as a predominantly bacteriostatic drug (MBC:MIC ration = 1:8) and its MBC has been found to exceed the $C_{max}$. The fluoroquinolones are bactericidal against *M. tuberculosis*, MBCs are normally between two- and four-fold greater than the MICs (Garcia-Rodriguez & Gomez-Garcia, 1993). Rastogi & Goh (1991) studied the activity of 3 quinolones SPA, OFL and CIP and their bactericidal activity was found to be far below their Cmax (3ug/ml).

# CHAPTER 4

## THE POST-EXPOSURE EFFECTS OF ANTIMYCOBACTERIAL AGENTS

### 4.1. Clinical perspective

The postantibiotic effect (PAE) and the control-related effective regrowth (CERT) are two experimental approaches to measure the recovery period or persistent suppression of bacterial growth after a short exposure to an antibiotic (Craig & Gudmundsson, 1991). The effect is induced by prior antibiotic exposure rather than persisting subinhibitory concentration of the drug. As the term PAE is often used widely to cover post-exposure regrowth time irrespective of methodology and specific definitions of regrowth time (Hanberger et al., 1995; Mackenzie & Gould, 1993) the term postantibiotic regrowth time (PART) to include both the PAE and CERT will be used in this chapter when appropriate. Knowledge about the PART of an antimicrobial agent is important for deciding the frequency of administration and dosage, especially if an intermittent therapy regimen is to be considered. A prolonged PART increases the probability of a favourable clinical outcome when the concentration in the blood or tissue fall below the MIC for considerable intervals of time without allowing bacterial regrowth and loss of drug efficacy. On the other hand antimicrobial-organism combinations that do not exhibit significant PART may require dosage regimens that continuously maintain levels above the MIC (Vogelman & Craig, 1885).

For the treatment of tuberculosis, the short duration schedule of 6 months is now standard therapy worldwide. The amount of drugs to be consumed by patients in a single dose is large. This can affect the adherence of the patient to therapy (compliance) which can lead to treatment failure. Therefore, the feasibility of administering drugs intermittently is a very important consideration in the management of tuberculosis. The spacing of doses at intervals greater than one day makes it easier to administer drugs under full supervision and should therefore

improve the regularity of drug-taking (Dickinson and Mitchison,1966). Studies in the past have provided evidence that in many situations pulse dosing works as well as or better than continuous administration of antibiotics, suggesting a PART (Armstrong, 1960; Beggs & Jenne, 1969). The overall effect of a dose given in a widely intermittent regimen depends upon the balance between the bactericidal effect of a pulse in the lesion, itself dependent on the half life of the drug and the period of regrowth of bacilli after recovery from the lag period, before arrival of drug at the site of infection from the next dose (Dickinson & Mitchison, 1987 (a)).

The exact mechanism by which antimicrobials induce PAE/CERT is not known. Limited persistence of drug at a bacterial binding site inside the microorganism leading to drug-induced nonlethal damage may be part of the explanation (Vogelman & Craig, 1985). Gottfredson et al. (1995) investigated the metabolic events associated with PAE. The rate of DNA synthesis in *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas. aeruginosa* during the PAE period after a 1-h exposure of organisms to vancomycin, dicloxacillin, rifampin and ciprofloxacin was evaluated. DNA synthesis was determined by measuring the rate of [$^3$H] thymidine incorporation in *S. aureus* and *E. coli* and [$^3$H] adenine incorporation in *P. aeruginosa*. DNA synthesis was suppressed in *S. aureus* during exposure to vancomycin, dicloxacillin and rifampin, in *E. coli* with rifampin and in *P. aeruginosa* with tobramycin. Conversely, the synthesis was relatively enhanced in the Gram-negative bacilli after exposure to imipenem and in all three species after exposure to ciprofloxacin. These differences suggest multiple mechanisms of PAE.

### *4.2. In vitro* determination of PART

In vitro PART is determined by exposure of the organisms to the antimicrobial agent in broth for a certain period followed by removal of the drug, resuspension into fresh medium and quantitation of regrowth. An initial decrease in bacterial concentration may be observed during exposure followed by a lag phase after the

removal of the drug and then a period of bacterial growth in which the organisms multiply exponentially (Dickinson and Mitchison,1966).

### 4.2.1. Exposure to the antimicrobial agent

Most investigators use single exposure of the organism to the antimicrobial agent (Bermudez et al., 1992; Ellis et al., 1995; ). This is accomplished by exposing the organism in broth for a limited time, removing the antibiotic and then monitoring regrowth. However, others use multiple exposures (Armstrong, 1960; Dickinson & Mitchison, 1966). In the latter case drug-containing medium is removed at certain intervals and the same culture is re-exposed to the drug.

### 4.2.2. Methods of drug removal after exposure

Different techniques of drug removal have been used by investigators. Standard methods utilize repeated washing (filtration or centrifugation), but removal of a drug by means of a 100- to 1000-fold dilution into fresh medium can achieve the same effect (Volgeman & Craig, 1985). Ravizzola et al. (1983) used ion exchange resin mixture to remove antibiotics and the efficiency of the removal of the antibiotic was monitored by the agar-diffusion assay using a strain of high sensitivity to the drug. In the case of beta-lactam antibiotics the antibiotic can be inactivated by a beta-lactamase enzyme, usually in combination with dilution (Hanberger et al., 1995).

### 4.2.3. Quantitation of PART

To quantitate PART in vitro, samples are taken at different time intervals and regrowth is monitored. A graph of CFU/ml vs. time is then plotted. Early investigators quantitated PART by estimating the period of time of stationery growth (Dickinson & Mitchison, 1966; Amstrong, 1960; Barclay & Winberg, 1964; Beggs & Jenne, 1969). Regrowth curves do not always show a lag phase followed by a sharp log phase; sometimes a gradual increase is observed until normal growth

is reached or there can be a further decrease in the number of organisms before growth become stationery (Vogelman & Craig, 1985; Craig & Gudmundsson, 1991; Craig & Ebert, 1991).

To circumvent all these problems and to provide a standardized method for comparing different methodologies and similar experiments on different days the equation PAE = T - C was developed where, T is the time required for the count of CFUs in a test culture to increase by 1 $\log_{10}$ above the count observed immediately after drug removal and C is the time required for the count of CFU in an untreated control culture to increase by 1 $\log_{10}$ above the count observed immediately after completion of the same procedure (Craig & Gudmundsson,1991; McDonald et al., 1977). The 1 $\log_{10}$ increase was chosen because by the time the number of organisms has reached a ten-fold increase, their growth rate is identical to that of untreated control. More direct methods of measuring regrowth directly in liquid media without resolving to colony counting on solid media have subsequently been developed (Rescott et al., 1988; Hanberger et al., 1993). The term control related effective regrowth time (CERT) was coined by Hanberger (1995) to define regrowth in such models.

#### 4.2.3.1. Methods used to quantitate PART

The viable count (CFU/ml) is generally used to follow microbial growth kinetics following drug removal and this procedure alone is labour intensive, time consuming and very tedious. A study conducted by Rescott et al. (1988) involving the determination of PAE of 3 antibiotics against *E. coli* and sampling at 15-30 minutes interval showed that to perform this method on one organism exposed to one antibiotic in duplicate with a control, the procedure required approximately 140 plates, 1.7L of media, 50 serial dilutions, 6 hrs to perform and an additional 2 hrs to read the plates after overnight incubation. They proposed that more rapid methods were needed to quantitate PART.

Other methods such as spectrophotometric procedures (Jenne & Beggs, 1969; Rescott et al., 1988) measurement of intracellular ATP (Hanberger et.al, 1993; Isaksson et al., 1988), microscopy etc. have subsequently been used. Minor discrepancies between these methods and the conventional viable count method have been found. Gottfredson et al. (1991) demonstrated an excellent correlation of the Bactec NR 730 blood culture system and the standard viability counting method against strains of *Staphylococcus aureus* for the quantitation of PAE.

Hanberger et al. (1993) used bioluminescence assay of bacterial ATP, viable count and microscopy to assess PART against *E. coli.* Long PARTs were seen when growth was measured by bioluminescence and microscopy and there was almost no or only a small decrease in intracellular ATP when compared with the number of cells determined by CFU/ml. No or short PAE together with very rapid initial killing was obtained with the viable count approach. They concluded that viable counts failed to detect the cells with minor damage which could be repaired in broth as these cells have difficulty forming colonies on agar plates.

Another disadvantage of using viable count for measuring regrowth is that antibiotics with high bactericidal activity tend to decrease the number of viable bacteria such that meaningful viable counts cannot be performed (Isaksson et.al, 1988). Therefore, it is likely that viable counts will exaggerate the bactericidal activity of antibiotics, since viability depends not only on the numbers of surviving bacteria in the broth culture, but also on the ability of these survivors to form colonies on the agar plates. The growth of bacteria may be suppressed by the drug carried over with the diluent to the agar plates. It is possible that bacteria that contain a bactericidal drug intracellularly and on their cell surfaces may die later on the agar plates or may need a period of recovery before they can form colonies.

Differences between PAE and CERT and the effect of bactericidal activity of drugs are discussed in detail in Chapter 8.

### 4.3. Factors affecting PART *in vitro*

Although PARTs have been produced by most antimicrobials and observed with a wide range of bacteria, the presence and the duration of PART can differ significantly for specific antimicrobial-organism combinations. Like any other in vitro method used in the evaluation of drugs there are certain factors which affect the duration or even the presence of PART. These include the drug concentration, duration of exposure, organism-drug combination, the inoculum size, etc. (Vogelman & Craig, 1985; Craig & Ebert, 1991).

### 4.4. The postantibiotic sub-MIC effect (PA SME)

PART at sub-MIC levels has been determined by PAE methodology and the term PAE has traditionally been used to describe PART in this context. During the postantibiotic phase bacteria have been found to be very sensitive when re-exposed to the same drug at concentrations that are below the MIC (sub-MIC) (Lowdin et.al, 1993; Cars & Odenholt, 1993, Odenholt et al., 1992). The delay in regrowth during this phase may be longer than that produced during the postantibiotic phase. Odenholt et al. (1991), referred to this phenomenon as the postantibiotic sub-MIC effect (PA SME). This phenomenon closely reflects the clinical situations of intermittent therapy in which there is a gradual decrease in the antibiotic concentration where supra concentrations are followed by a period of subinhibitory concentrations.

*In vitro* studies of PA SME can be demonstrated by exposing the bacteria to concentrations above the MICs (supra-inhibitory concentrations) for a limited time, removing the drug and re-exposing the cultures to sub-MIC concentrations.

### 4.5. *In vivo* methods used to determine PAE

*In vivo* PAE has been demonstrated in animal models. Experimental models in which repeated sampling of tissues or body fluids for bacterial numbers and drug concentrations are performed have provided information on the in vivo determination of PAE. In this situation PAE is defined as the difference in time for the number of bacteria in treated and control tissues or body fluids to increase 1 $\log_{10}$ over values when drug concentrations in serum or the site of infection fell below the MIC (Craig, 1993). The models which have been studied include: (i) thigh infections in mice, (ii) pneumonia in mice, (iii) infected subcutaneous threads in mice, (iv) infected tissue cages in rabbits, (v) meningitis in rabbits and (vi) endocarditis in rats (Craig, 1993).

An in vitro method to determine the regrowth pattern of bacteria exposed to antibiotic concentrations simulating those observed in vivo uses a two compartment model with a dialyser interconnection (Guggenbichler et al., 1985). In this model regrowth patterns of bacteria are determined under fluctuating antibiotic concentrations.

### 4.6. PAE of selected antituberculosis drugs against *M. tuberculosis* strains

An *in vitro* method which describes this phenomenon was first observed by Bigger in 1944 who noted delayed development in turbidity after adding penicillinase to cultures of staphylococci and streptococci previously exposed to penicillin G. Since then many studies have been done with gram-positive and gram-negative bacteria (Chin & Neu, 1987; Kumar et al., 1992, Li et al., 1997) and they were extended to fungi (Neu et al., 1987; Scalarone et al., 1992). Although studies have been done with *M. tuberculosis* and other mycobacteria information is relatively sparse and inadequate.

Early *in vitro* studies of PAE in mycobacteriology were done by Amstrong (1960) who observed time-concentration relationship of INH with tubercle bacilli. He showed that nearly complete bacteriostasis could be accomplished by a daily exposure to INH at a concentration of only 2ug/ml for as short as 2 h (Dickinson & Mitchison,1966) using the same technique but with single exposure of tubercle bacilli to 1ug/ml INH found that there was no delay before growth after exposure periods of 12 hr or less. Beggs & Jenne (1969) observed that exposure to 0.5ug/ml INH for as little as 2 h produced a well marked transient growth inhibition. These data indicated that growth inhibition of tubercle bacilli following pulsed exposure to INH is a function of time-concentration product rather than a function primarily of either concentration or time and is closely correlated with the amount of drug bound by the cell. McDonald et.al. (1977) used the term postantibiotic effect (PAE) to describe the recovery period or persistent suppression of bacterial growth after short exposure to antimicrobial agents.

*In vitro* studies of *M. tuberculosis* using 1ug/ml of RMP for 6, 24 and 96 h showed that there was a period of slow growth, starting immediately after removal of the drug lasting 2 to 3 days after 6 and 24 h and up 6 days after 96 h exposure after which the culture gradually returned to its full growth rate (Dickinson & Mitchison 1987 (b)). This implies that recovery from a 24 h exposure would be partial if doses were spaced at intervals of 2-3 days but would have been complete for 2-3 days if doses were separated by 7 days. These results correlated with those in guinea pigs (Dickinson & Mitchison,1987 (a)). In studies in mice, when RMP was used alone, intermittent treatment twice a week was more effective than daily treatment when the drug was given at higher dosage. However, higher once weekly regimens were ineffective (Grosset, 1992)

EMB appears to be suitable for intermittent chemotherapy, the efficacy of the drug in this setting being dose related (Gangadharam et al., 1990). *M. tuberculosis* exposed for 24 hrs either to 1 or 10ug/ml of EMB showed only

minimal antimycobacterial activity and with 96 hrs exposure, growth resumed after 3 days at 1ug/ml and 6 days at 10ug/ml (Gangadharam et al., 1990). This suggests that EMB starts acting after longer periods of exposure and that PAE for this drug is dose dependent.

The PAE for CIP against *M. tuberculosis* has not been evaluated before but it has been demonstrated in other bacteria (Neu et al., 1987; Minguez et al., 1991).

# CHAPTER 5

## DRUG COMBINATIONS

Several highly effective short-course chemotherapy regimens of 6-8 months' duration have been evolved for the treatment of sputum-positive pulmonary tuberculosis. In most regimens four drugs, viz, RMP, INH, pyrazinamide and streptomycin or ethambutol are given together, usually for two months initially, in a single dose either daily or intermittently (Hong Kong Chest Service, 1977; O'Brien & Snider, 1985; Paramasivan et al., 1993).

The use of antimicrobial agents in combination has been practised since the discovery of the first antibiotics. Combinations of antimicrobial agents are used mainly for 3 purposes: (i) to prevent drug resistance, (ii) to increase the bactericidal activity of the agent especially in immunocompromised patients and, (iii) to cover infections of unidentified organisms (Heifets, 1991 (c)). Current use of drug combinations for the treatment of tuberculosis is aimed at achieving the most efficient short course regimen without the emergence of drug resistance. This can be achieved by demonstrating synergistic effects between the drugs. A combination is considered synergistic when its effect is greater than the sum of those produced by each agent singly (Heifets, 1991 (c)).

Other effects that can be produced by drug combinations are additive, indifference and antagonism. An additive effect is produced when the combination effect is equal to the sum of those produced by each agent singly. Indifference is when the combined effect is not different from the effect of the most active drug. An antagonistic effect is when the combination is less active than either drug alone (Heifets, 1991(d)).

There are four accepted mechanisms of antibacterial synergism: 1. serial (sequential) inhibition of a common biochemical pathway (e.g. TMP-SMZ), 2.

inhibition of protective bacterial enzymes (e.g. clavulanic acid or sulbatum + b-lactam sensitive penicillin), 3. combinations of cell wall active agents if their target are different (e.g. amdinocillin + ampicillin), and 4. use of cell wall- active agents to enhance the uptake of other antimicrobials (e.g. penicillin + streptomycin (Krogstad & Moellering, 1991). Other mechanisms are unclear (e.g. SMZ + colistin).

The two best known methods to asses the combined effects of antibiotics are the checkerboard method and the time-kill curve. PAE has also been used in combination studies to determine the combined effects of antibiotics for intermittent therapy.

### 5.1. The checkerboard method

The checkerboard titration is a broth determined method and measures only the inhibitory effect and two antibiotics are tested in serial dilutions and in all combinations of these dilutions together to find the concentrations of each antibiotic, both alone and in combination, that produce some specified, easily determined effect (Berenbaum,1978). The method is determined in broth or on agar. With the checkerboard titration interaction between two antibiotics is determined either algebraically, by expressing the effect as a fraction of the concentration that produces the same effect when the antibiotic is used alone ie. its fractional inhibitory concentration, or geometrically by plotting a graph (isobolograms) with the axes representing antibiotic concentration on linear scales (Krogstad & Moeller, 1991; Berenbaum,1978; Hsieh, 1993). Berenbaum (1978) modified the checkerboard method in an attempt to test for inhibitory or bactericidal synergy with any number of agents using the fractional indices. With this method, the concentrations of each of *n* agents producing some specified effect (such as MIC or MBC) are determined.

Results of the checkerboard are read by examining the tubes (or wells of the microtiter plate) for evidence of visible growth after 16 to 20 hrs of incubation in the case of rapidly growing bacteria. Bactericidal data may be obtained if the tubes (or wells) without visible evidence of bacterial growth are sampled to determine the concentration of the combination which produced >\= 99.9% killing with the fractional index method. Synergy is defined as an FIC or FBC index <\= 0.5, 1.0 for additivity and >\= 20 for antagonism (Krogstad & Moellering, 1991; Hallander, 1982). With isobolograms, synergy is deemed synergistic if in an isobol joining the points representing the drugs used singly and passing through the point representing the combination is concave-up, antagonistic if it is concave-down and indifferent if it is straight.

### 5.2. The kill curve method

The killing curve or time kill curve method which measures the bactericidal effect of a drug combination is more relevant for clinical situations in which bactericidal therapy is desired. The method has been done extensively in Gram-positive and Gram negative bacteria (Wiland et al., 1994; Chidiac et al., 1995, Hallander et al., 1982). The technique provides information on the interaction of an antimicrobial agent and bacteria over time (Heifets, 1991 (c)). The effect is determined on broth by sampling and plating. Aliquots from the drug-containing and drug-free tubes are diluted usually $10^0$ to $10^8$ and plated on an agar plate without antimicrobials. Initial sampling for colony counts is performed as soon as the inoculum is added (within 15 min) (Krogstad & Moellering, 1991) followed by sampling at different time intervals. Results are interpreted by plotting a graph of CFU/ml vs time. Synergism is defined as a >\= 100 fold increase in killing between the combination and its most active constituent after 24 h and antagonism is defined as a <\= 100 fold decrease in killing under the same combination. Additivity (or indifference) is defined as less than 10-fold change (increase or decrease) in killing at 24 hrs with the combination in comparison with the most active drug alone (Heifets, 1991 (c); Moellering, 1979).

If the results of a kill curve are to be useful in guiding therapy it is essential that the antimicrobial concentrations tested be chosen carefully, and that they represent concentrations which are achievable at the presumed site of the infection (Krogstad & Moellering, 1991). When choosing concentrations of the agents one of the pair must be present in a concentration that by itself does not alter the growth curve (Moellering,1979). Most authors based their concentrations on the previously determined MICs or MBCs. The concentrations which are one half to one of the MBC have been used (Hallender, 1983). Nash & Stangrube (1980) suggested a probable usefulness for multiple drug sensitivity assay in selecting drug combinations most likely to be effective as therapeutic regimens in multidrug resistant strains of *M. tuberculosis.*

The major disadvantage of the killing curve technique is that the repetitive sampling necessary from each of the flasks being tested and the multiple colony counts required limit the number of antimicrobial combinations that can be tested. Therefore, a method which is rapid and can test for the bactericidal action of a combination with a number of antimicrobials is needed.

The PAE has been described extensively above and only a few comments need to be mentioned with its use in combination studies. Paramasivan et al. (1993) evaluated the bactericidal effect of pulsed exposure to antituberculosis drugs in which *M. tuberculosis* strains were treated with pulsed exposure to drug combinations on alternate days using a filtration and resuspension procedure. This method is similar to the multiple exposure of the microorganisms used to evaluate single drugs. This study could not explain the effect of the combination of pulse exposure in terms of synergy, antagonism, additive or indifference. Gudmundsson et al. (1991) used the standard method of PAE for single agents to determine the PAE of drug combinations against fast growing bacteria. These authors defined PAE synergism as the PAE induced by an antimicrobial combination being at least one hour longer than the sum of the PAEs for individual drugs, and addition as the combination PAE being roughly similar to

the mathematical sum of the individual PAEs. Indifference was defined as the combination PAE being no different from the longest of the individual PAEs and antagonism as the combination PAE being at least one hour less than the longest of the individual PAEs. In a study on PAE in *M. avium* strains, Fuursted (1997) defined a significant PAE as a lag period lasting more than the mean generation time of the strains tested. Synergistic prolongation of PAE was defined as having occurred when the PAE form the drug combination compared with the most active single drug alone was longer than the generation time.

### 5.3. Drug combinations used in the treatment of tuberculosis

The current situation in the treatment of tuberculosis patients in South Africa is based on the six months SCC recommended by the World Health Organisation (WHO). The treatment is based on whether the patient is a new patient (has never been treated for TB before or who has only been treated for less than 4 weeks) or a retreatment patient (treated for more than 4 weeks at any time in the past)(SA Dept. Health, 1996). Combination tablets are mostly used than the single agents and the dose depends on the weight of the patient before treatment is started. There are 2 adult treatment regimens divided into phases:

Regimen 1 is for new adult patients whose strains are sensitive to all TB drugs and consists of two phases. In the intensive phase the combination tablet of RMP/INH/PZA plus an EMB tablet are given for 2 months. The continuation phase which is given for 4 months consist of the combination of INH and RMP.

In regimen 2, which is for retreatment of adults, the intensive phase consist of the combination of RMP/INH/EMB plus PZA and SM and is prescribed for the initial 2 months of retreatment. This is followed by 1 ( which is the 3$^{rd}$) month of RMP/INH/EMB plus PZA. The last 5 months of treatment fall under the continuation phase and the combination tablet of RMP/INH/EMB is given. This

regimen is based on the fact that during the period the patient was treated, resistance to one or more of the antituberculosis drugs might have developed.

The treatment of tuberculosis in immunocompromised patients ( e.g. patients receiving antineoplastic drugs) is not well established (Davidson and Le, 1992). HIV-induced immunosuppression has stimulated more comprehensive studies on the immunology of tuberculosis and its treatment in immunocompromised patients. Recent reports have indicated that a regimen of RMP, INH and EMB for 6 months supplemented with PZA during the first 2 months is quite effective, although relapse has been reported (Davidson & Le, 1992)

Children are treated in essentially the same way as adults, using appropriately adjusted doses of the drugs. Because of difficulty in assessing their visual acuity and colour vision, ethambutol is not used in children who are too young (Davidson & Le, 1992). Streptomycin is also not recommended for young children because of its toxicity.

# CHAPTER 6

## MATERIALS AND METHODS

In this chapter the materials and methods used for the determination of the bactericidal activity and PAE of anti-TB agents will be discussed.

### 6.1. Materials

#### 6.1.1. Strains

Nine strains were collected from different sources. The H37Rv strain from the American Tissue Culture Collection (ATTC) 27294, and the clinical strains number MR 83713, 249/941 and 342/941 were obtained from the MRC, Pretoria. The other clinical strains number 3883, 4491, 11270, 12107 and 11400 were obtained from the South African Institute for Medical Research (S.A.I.M.R.), Johannesburg.

To obtain log phase cultures, strains were grown by frequent stirring, using a magnetic stirrer, in 7H9 broth supplemented with Albumin Dextrose Catalase (ADC) (Difco laboratories, Detroit M148232-7058 USA) for 7 days at 37$^{0}$C. For each drug exposure experiment bacteria in the log phase were subsequently subcultured into 7H9 broth (Difco laboratories, Detroit M148232-7058 USA).

#### 6.1.2. Drugs

The following drugs were obtained from different sources as reference powders with known potencies (for potencies refer to Appendix) and were used throughout the experiments: Isoniazid (Noristan, Pretoria - RSA), rifampicin (Ciba-Geigy, Johannesburg - RSA), ethambutol (Rolab, Johannesburg - RSA),

ciprofloxacin (Bayer, Johannesburg - RSA) and streptomycin (Novo-Nodisk, Johannesburg -RSA).

Drug stock solutions to be used in the experiments were prepared and stored as described in appendix B of this dissertation.

### 6.1.3. Media

LJ slopes were prepared as described in appendix A, 7H9 broth and 7H10 agar base were purchased from Difco laboratories, Detroit M148232-7058 USA, Bactec 12B broth was kindly donated by Bactlab Pty Ltd, RSA.

## 6.2. Methods

### 6.2.1. Drug susceptibility testing (DST) on LJ medium using critical concentrations

DST was determined on LJ and 7H10 agar media using the proportion method. The sensitivity patterns determined on the two media were compared. The proportion method for drug susceptibility testing described earlier (Cannetti et al. 1963, Kleeberg et.al. 1980) was used.

#### 6.2.1.1. Strains tested

Eight strains were tested i.e. H37Rv, 342/941, 249/941, 3883, 4491, 11400, 11270, and 12107. These strains were selected as they were previously shown in routine laboratories to exhibit varying degrees of resistance. The DSTs were performed blindly by the candidate i.e. the resistance patterns were not known by the candidate at the time when the DSTs were conducted.

### 6.2.1.2. Drugs tested

Isoniazid (INH), rifampicin (RMP), streptomycin (SM), ethambutol (EMB) and ciprofloxacin (CIP) were chosen for the studies.

### 6.2.1.3. Inoculation of the slopes

For each strain, representative colonies were scraped from 14-day-old LJ cultures and transferred to tubes containing 5 - 6 ml water and 6 -8 glass beads using an inoculation loop. The glass beads were used to disperse the cells. A suspension was prepared by vortexing the tubes for 1 to 2 minutes and allowing the larger particles to settle down for 30 to 45 minutes. The supernatant was removed and added into a test tube without glass beads. The inoculum was adjusted to a turbidity which equals to that of a McFarland no 1 standard (see appendix C for the preparation) using sterile distilled water and contained about $10^7$ CFU/ml cells. The inoculum was diluted $10^{-1}$ and $10^{-3}$.

Using a sterile plastic inoculation loop with a diameter of 3 mm, one loopful of the $10^{-1}$ dilution was inoculated onto the drug-containing media and one control tube. One loopful of the $10^{-3}$ dilution was spread on the surface of the drug-free slopes. Care was taken to cover as much surface of the media as possible. The two inocula containing approximately $10^6$ and $10^4$ CFU/ml of bacteria. The former represented the whole population and the latter represented 1 % of the population (1:100 control). The test inocula were prepared by inoculating slopes containing antimicrobial agents with the $10^{-1}$ suspension.

The slopes were left at room temperature for about 2 hours until the inocula were absorbed after which they were incubated at 37°C. The slopes were checked for contamination by fast growers during the first and the second week of incubation.

### 6.2.1.4. Recording of results

The results were read for the first time after 4 weeks of incubation. During this time the growth of *M. tuberculosis* is visible and colonies have been formed. The colonies were counted on the slopes, containing antibiotics, that had produced growth and the growth was compared to that of the 1:100 control. Results were recorded as follows:

| | |
|---|---|
| Confluent growth | + + + + |
| Innumerable discrete colonies | + + + |
| 100 to 200 colonies | + + |
| 50 to 99 colonies | + |
| Less than 50 colonies | actual number of colonies |
| No growth | - |

After 4 weeks of incubation if the results of the tests indicated sensitivity, the slopes were reincubated for a further 6 weeks. If the slopes still showed no growth by this time they were recorded as sensitive. If the results indicated resistance after 4 weeks incubation, no further reading of the test was done and they were recorded as resistant.

The drug concentrations used and the criteria for critical concentration of resistance are indicated in Table 6.1. The critical concentrations used are those recommended by Cannetti et al. (1963) and Kleeberg et al.(1980).

Table 6.1. Drug concentrations used and critical proportion of resistance.

| Drug | Concentration (ug/ml) | Critical proportion for resistance (%) |
|---|---|---|
| INH | 0.2 | 1 |
| | 1 | 1 |
| RMP | 30 | 1 |
| SM | 4 | 10 [a] |
| CIP | 2 | 1 |
| EMB | 2 | 10 [a] |

[a] The NCCLS (1990) recommended 1% when Middlebrook 7H10 and 7H11 media are used.

### 6.2.2. The determination of the pattens of resistance and MICs on 7H10 agar medium

The MICs were determined as described earlier (NCCLS, 1990) using the proportion method. To compare the susceptibility patterns of the strains determined by 7H10 agar proportion method with the LJ DST method, critical concentrations of the drugs were included in the test.

#### 6.2.2.1. Strains tested

All the eight strains listed in 6.1.1. were tested.

#### 6.2.2.2. Drugs tested

INH, RMP, SM, EMB and CIP.

For susceptibility testing using breakpoints, all the drugs were tested and for MIC determinations all but SM were evaluated.

### 6.2.2.3. Preparation of drug-containing agar plates

Middlebrook 7H10 medium was prepared as described in Appendix A but was not allowed to solidify until drugs were added. To prepare drug-containing plates, drug stock solutions were thawed and 2-fold dilutions of the drugs were prepared using sterile distilled water. The concentrations of the drugs were 50 times the desired final concentration. Using a 1000 ul pipette 1 ml of each dilution was added to 49 ml of 7H10 agar supplemented with ADC in a 200ml flask to achieve the final desired concentration. After mixing the drugs with the media thoroughly, 20 to 25ml amounts of the antimicrobial agent-containing medium were dispensed aseptically and quickly, before the medium solidified, into sterile petri dishes. The plates were allowed to solidify at room temperature and divided into 9 parts with a marker. Each plate contained one drug concentration. Drug-free plates were also prepared. For each drug the critical concentrations were included in the test. MICs for SM were not determined but only the resistance pattern using critical concentrations were determined.

The plates were sealed with transparent plastic bags to avoid drying out of the medium. They were protected from light with a black plastic bag and incubated at 37$^{0}$C overnight to check for sterility. The NCCLS (1990), guidelines specify that only 10% of the plates need to be tested for sterility but in this study all the plates were incubated to ensure complete sterility. The plates were used immediately or stored at 4$^{0}$C for no more than 7 days. For use after storage, the plates were allowed to reach room temperature before use.

### 6.2.2.4. Inoculation of the plates

For each strain, an inoculum with a turbidity which equals to a McFarland no.1 standard (see Appendix C for preparation) was prepared and diluted $10^{-1}$ and $10^{-3}$, using 7H9 broth without supplement. Using a 20 ul pipette, 10 ul of the $10^{-1}$ dilution was inoculated onto one of the marked spaces on the drug-

containing media. Duplicate plates were inoculated and each plate accommodated nine strains. Two control plates were prepared using drug-free 7H10 agar plates, one was inoculated in the same way as the test inoculum (using the $10^{-1}$ suspension) and the other, the 1:100 control, was prepared by inoculating 10 ul of the $10^{-3}$ suspension onto the drug-free 7H10 agar plates. The former control was used for standardization of the inoculum and the latter represented 1% of the bacterial population.

The plates were allowed to stand at room temperature until the spots were dry, sealed with the transparent plastic bags and protected from the light with black plastic bags. They were incubated at 37°C in +\- 10% $CO_2$ for 3 weeks and checked for contamination of rapidly growing organisms during the first week. Readings were recorded after the second and third week but optimal reading was after the third week.

Table 6.2. Critical concentrations used in the sensitivity testing

| Drug | Critical concentration (ug/ml) |
|---|---|
| INH | 0.2 |
| | 1.0 |
| RMP | 1.0 |
| CIP | 2.0 |
| EMB | 5.0 |
| | 10.0 |
| SM | 2.0 |
| | 10.0 |

The critical concentrations used are in accordance with those recommended by the NCCLS. The concentrations for CIP and EMB were recommended by the Centres for Disease Control (CDC), USA (Heifets et.al,1987).

### 6.2.2.5. Recording of results

The amount of growth on each spot was recorded as follows:

- Confluent growth with too numerous to count colonies + + + +
- Discrete colonies + + +
- Approximately 100 to 200 colonies + +
- 50 to 99 colonies +
- Less than 50 colonies, the actual count was recorded.

### 6.2.2.6. Interpretation of results

For susceptibility testing, growth on the test plates was compared with that of the 1:100 control. If the growth was less than that of the control the test was regarded as sensitive and if it was greater it was considered to be resistant. Any growth equal to that of the control was considered borderline resistant. The critical proportion of resistance used was 1 % for all the drugs tested.

The MIC was defined as the lowest drug concentration that inhibited more than 99% of the population. Plates containing the lowest drug concentration and colonies exhibiting fewer than that of 1:100 control inoculum were considered to have inhibited more than 99% of the population and the drug concentrations in these plates were therefore considered to be the MICs.

### 6.2.3. Determination of MIC using the Bactec radiometric system

The method described earlier was used (Heifets, 1987; Siddiqi, 1989). The BACTEC 460-TB instrument measures the amount of radiolabelled $CO_2$ liberated by bacteria in a confined atmosphere. The growth in terms of the amount of $CO_2$ liberated is expressed as a numerical value called the growth index (GI) which ranges from 1 to 999.

All the vials were tested on the BACTEC 460 instrument before use (see Appendix C for the initial testing of vials). Because this method is expensive only five strains and four drugs were tested.

#### 6.2.3.1. Strains tested

The following strains were tested
(i) H37Rv ATCC 27294
(ii) 342\941
(iii) 249\941
(iv) 4591 and
(v) MR 83713

#### 6.2.3.2. Drugs tested

INH, RMP, EMB and CIP.

#### 6.2.3.3. Preparation of 12B medium containing drugs

The tubes of frozen stock solutions were thawed and serial 2-fold dilutions of the drug concentrations were prepared using sterile distilled water in 5ml tubes. The drug concentrations were 40X the desired concentrations. Using a 1ml tuberculin syringe, 0.1ml of each dilution was inoculated into the vials each containing 4ml of 12B broth. One test vial was used for each drug concentration. The drug-containing media was used immediately or stored at $4^{0}$C. All the drug-containing media were inoculated within 7 days of preparation.

#### 6.2.3.4. Preparation of inoculum

For each strain, an inoculum with a turbidity which equals that of a McFarland no.1 standard was prepared as described earlier (see Appendix C) and diluted

1:2 in 7H9 broth without antimicrobials. Using a 1ml tuberculin syringe, 0.1ml of this dilution was inoculated into drug-free 12B medium. The vials were incubated at 37°C and tested daily on the BACTEC 460 instrument until a GI reading of 400 to 500 was reached, providing a cell suspension of $10^5$ to $10^6$ CFU/ml.

### 6.2.3.5. Inoculation of the 12B medium with and without drugs

When growth on the 12B vials reached a GI reading of 400 to 500, 0.1ml of this suspension was added into each drug-containing 12B vial, yielding approximately $10^4$ to $10^5$ CFU/ml. Two controls were prepared, one was inoculated the same way as the test vials and the other, the 1:100 control, was prepared by adding 0.1ml of the bacterial suspension that had reached a GI of 500, into 9.9ml of the special Bactec diluting fluid and inoculating 0.1ml of this dilution into a drug-free 12B vial. The first control is necessary to confirm that the actual inoculum is within the desired limit and the 1:100 control represents 1% of the bacterial population.

### 6.2.3.6. Incubation and reading of the vials

All the vials including the control vials were incubated at 37°C and the GI reading was monitored daily on the BACTEC 460 instrument for a minimum of 4 and a maximum of 12 days or until the GI reading of the 1:100 control reached 30 or more for three consecutive days. If the GI reading in the 1:100 control reached 30 or more in less than 4 days it implied that the inoculum was too heavy and the experiment was repeated. If it took more than 12 days the inoculum was low but the experiment was not repeated.

#### 6.2.3.7. Interpretation of results

The lowest drug concentration producing a daily decrease or an increase and a final GI reading lower than that of the 1:100 control was considered to have inhibited more than 99% of the bacterial population and was therefore defined as the MIC.

### 6.2.4. Determination of MBC using the Bactec radiometric system

The method described previously (Heifets et.al, 1986; Heifets & Lindholm-Levy, 1987) was used. Because the method is costly and labour intensive only one strain was tested.

#### 6.2.4.1. Strains tested

Only the H37Rv ATCC 27294 strain was tested.

#### 6.2.4.2. Drugs used

INH, RMP, CIP and EMB.

#### 6.2.4.3. Preparation of Bactec 12B medium with or without antimicrobial agents

The inoculum was prepared as described for MIC determination but here duplicate vials were allowed to incubate drug-free until the GI reading reached 400 to 500, yielding about $10^5$ to $10^6$ CFU/ml. At this point stock solutions of drugs were thawed and serial 2-fold dilutions of 1, 2, 4, 8 and 16X the previously Bactec determined MICs were prepared using sterile distilled water. These were 40 times the desired final concentration. Using a 1 ml tuberculin syringe, 0.1 ml of the each serial dilution was added into the culture containing

vials. One test vial was used for each drug concentration. A drug-free control was also prepared.

#### 6.2.4.4. Incubation of vials and CFU/ml assay

7H10 agar without drugs was prepared at the beginning of the experiment and marked into 6 parts with a permanent marker. Samples of 0.1ml were taken from the control and test vials immediately after the addition of drugs using a tuberculin syringe. They were diluted $10^{-1}$ to $10^{-6}$ with the Bactec diluting fluid. Using a 20 ul pipette, 10ul of each dilution was plated in duplicate, on each marked space on 7H10 agar. The inoculum was distributed by tilting the plate slightly and gently taking care not to mix the diluted suspensions on the spaces.

The drug containing vials were incubated at 37°C and read daily on the BACTEC 460 instrument. If the vials were not read daily the $CO_2$ in the vial accumulated and resulted in a false increase in the GI reading. This would be misleading and could lead to an underestimation of killing activity. On day 3, 6, 9, 12 and 15, samples of 0.1ml were taken, using a tuberculin syringe, diluted and plated onto 7H10 agar as in day 0 after the addition of drugs. Cultures with a GI reading of >600 or which showed an increase in GI were not diluted for colony counting because they showed no killing.

All the plates were sealed in plastic bags to prevent the drying of the media and incubated at 37°C in +\- 10% $CO_2$ for 3 weeks. The plates were checked for contamination with rapid growers during the first and second week.

#### 6.2.4.5. Recording of results

After 3 weeks of incubation, the plates were examined for growth. The growth of the test culture on each day of sampling was compared with that of the control on day 0 when the drugs were added. To obtain the percentage of

killing, growth of the dilutions that resulted in colony formation was recorded and the CFU/ml calculated.

#### 6.2.4.6. Interpretation of results

The MBC was defined as the lowest drug concentration that killed more than 99% of the population after 15 days of cultivation. The percentage of killing was calculated by comparing the number of colonies in the control on day 0 (when drugs were added) with the number of colonies of the test on the 15th day of sampling. The means of the CFUs/ml of the dilutions were used in the calculations.

### 6.2.5. Determination of PAE and CERT

A procedure was developed to measure CERT using the Bactec system and it was compared with PAE using the colony count method. Two methods to disperse the cells were used, the glass beads and the sonicator. The effect of time and concentration on PAE and CERT were determined. The experiments were done in triplicate.

#### 6.2.5.1. Strain tested

Only the *M. tuberculosis* H37Rv strain was tested.

#### 6.2.5.2. Drugs used

The following drugs were used in the experiment: INH, RMP, CIP and EMB.

### 6.2.5.3. Preparation of drug-containing medium

Drug stock solutions were thawed and diluted, using sterile distilled water, to prepare different concentrations which were 10 X the desired final concentrations. Using a 100 ul pipette, 0.5 ml of the solution was added into a test tube containing 8.5 ml 7H9 broth supplemented with ADC to make up a volume of 9 ml.

### 6.2.5.4. Exposure of bacterial cells to antibiotics

Because of the simplicity and safety aspects of determining CERT in Bactec vials the effect of time and concentration on CERT was determined in this system by exposing the *M. tuberculosis* culture to different drug concentrations and different times, respectively.

One ml of the log phase culture with a turbidity adjusted to equal that of a McFarland no1 standard was added to 9 ml 7H9 broth containing the desired concentrations of the drugs tested. A control was prepared by adding 1 ml of the culture to 9 ml 7H9 broth without antibiotics. The initial bacterial concentration was approximately $10^6$ CFU/ml.

All the test tubes were incubated at $37^0$C in +/- 10 % $CO_2$.

***Effect of time on CERT*** : The culture was exposed to a fixed drug concentration of 8X the previously Bactec determined MIC. In most published studies PAEs and CERTs have been determined at drug concentrations of 8 to 10X the MIC (Craig & Ebert, 1991; Craig & Gudmundsson,1991). The culture was exposed by incubating at $37^0$C for 2, 12, 24, 48 and 96 hours.

***Effect of concentration on CERT*** : Unlike in the determination of the effect of time on CERT, cultures were exposed to different concentrations of 2, 4, and

8X the MICs of RMP, CIP and EMB. The *M. tuberculosis* strain was exposed to 8 and 40X the MIC of INH. This was based on the results obtained in previous experiments by other authors that due to regrowth in response to INH exposure is not dependent on the concentration (Dickinson & Mitchison, 1966). The exposure time was fixed at 24 hours.

#### 6.2.5.5. Removal of antibiotics

After incubating at 37$^{0}$C for a limited time antibiotics were removed by filtering through a microfil filter system (Millipore, RSA).

***The filter system*** :the Microfil millipore filter system consists of a grinded S-Pak membrane with a pore size of 0.45 um and a diameter of 4.5 cm, 6X 100 ml glass funnels, 6X filter holders, 6X filter holders support rubbers, filter holder clamps and a microfil stainless steel support (or stand or manifold). A vacuum supply and a filtrate collection flask are also essential components of the system.

The support and glassware were autoclaved prior to sample processing . The filter holders were fitted to the support using the rubbers provided. Using sterile forceps, the membranes were placed, with the grinded side up, onto the centre of the filter holders. The funnels were placed onto the filter holders and clamped firmly into position. A vacuum supply and a 1 000 ml filtrate collection flask were connected to the system.

***Filtration*** : the 7H9 broth culture-containing tubes were removed from the incubator, passed over a flame and 0.1 ml samples were taken, diluted and plated onto 7H10 agar. The cultures were poured down the funnel and filtered under vacuum pump until all the liquid passed through the membrane. To ensure complete drug removal the bacteria were washed twice by adding 100 ml PBS

and filtering as before. The membrane filters were removed with sterile forceps and placed in sterile petri dishes.

### 6.2.5.6. Resuspension and mechanical dispersion of bacterial cells into drug-free and drug-containing media

Bacteria were resuspended by adding 10 ml prewarmed 7H9 broth over the membranes in the petri dishes and mixing thoroughly with a loop, scraping all parts of the membrane and mixing with a 10 ml pipette. The unexposed control culture was treated similarly. The bacterial suspension was transferred into McCartney bottles using a 10 ml pipette.

***Bactec assay***: Regrowth in Bactec vials was performed on both sonicated cultures and cultures dispersed by vortexing. Resuspended bacteria were sonicated for 30 seconds at 20W using a Branson Sonifier (Branson Sonic Power Company, Danburg, Connecticut, USA) with a tapered micro-tip inserted into the bacterial suspension (Paul et.al., 1996; Bermudez & Goodman, 1996). In order to protect against aerosol inhalation all sonication procedures were performed with special precautions in a biosafety cabinet vertical laminar flow hood.

Vortexing was performed with glass beads for 45 seconds to disperse the cells. Triplicate samples of 0.1 ml were taken and inoculated into drug-free Bactec 12B broth vials using a tuberculin syringe . The vials were incubated at 37$^{0}$C and read daily on the BACTEC 460 instrument until the GI reading reached 999.

***CFU/ml assay***: After resuspension of the cells the McCartney bottles containing resuspended cultures were incubated at 37$^{0}$C. At each point in time before samples were taken and plated, the cells were dispersed as described for Bactec assay using a sonicator and a vortex. Samples of 0.1 ml were taken on day 0, immediately after resuspension, and on day 2, 5, 8, and 12. The samples were

diluted $10^{-1}$ to $10^{-6}$ with 7H9 broth without supplement and plated, in triplicate, onto drug-free 7H10 agar. The agar plates were incubated at 37°C in +\- 10% $CO_2$ and were read after 21 days of incubation. Graphs of time versus log CFU/ml were plotted.

### 6.2.5.7. Interpretation of results

PAE was defined as PAE = T - C, where T is the time required for the bacterial population in the test culture to increase by 10-fold after elimination of the drug and C, the corresponding time for the control culture to reach the same increase in CFU/ml after the removal of the drug.

CERT was also defined as CERT = T - C, where, T was the time required for cumulative $CO_2$ production by the exposed organisms to reach a GI reading of 500 and C was the corresponding time for the unexposed control culture to reach the same GI reading.

### 6.2.6. Drug combination studies

The CERT of drug combinations was determined using the Bactec radiometric system to follow regrowth. The same procedure used for single drugs was used but here the CERT of drug combinations including the single agent and the control was determined after 24 hours exposure. Experiments were done in duplicate. The drug combinations and the concentrations used were as follows:

| Drug combination | Concentration |
|---|---|
| INH + RMP | 8 X MIC + 2 XMIC |
| INH + EMB | 8 X MIC + 2 X MIC |
| INH + CIP | 8 X MIC +2 XMIC |
| RMP + EMB | 2 XMIC + 2 XMIC |
| RMP +CIP | 2 XMIC +2 XMIC |

For each combination single drugs were also included in a concentration used in the combination; eg in the combination of INH + RMP, 8 XMIC of INH and 2 X MIC of RMP were included as single agents. The concentrations used in the drug combination experiments were based on achievable blood levels and the MICs of the fully susceptible H37Rv strain.

The formula for the interpretation of results CERT = T - C was used, as defined in 6.2.5.7.

### 6.2.6.1. Interpretation of results

For the purpose of this study, the CERT of the combination was considered synergistic if it was greater than the sum of the CERT of the individual drugs. An additive effect was defined as the combination effect which was equal to the sum of the CERTs produced by each agent singly. The combination effect was said to be indifferent if it was not different from the effect of the longest CERT of the individual drugs and antagonism was defined as the duration of CERT which was less than the shortest PAE of the drugs used singly. These criteria were adopted from those suggested by Gudmundsson et al (1991) for PAE-based synergy studies.

### 6.2.7. Statistical analysis

The PAE of INH and RMP was measured using the colony count method and CERT in the Bactec system in three experiments. There were up to 3 samples in each experiment subjected to each treatment and samples were taken on day 0, ie. immediately after the removal of drugs, day 2, 5, 8 and 12. The mean ($\bar{x}$) and the standard error of the mean for $\log_{10}$ counts and the GI readings for all the experiments were calculated.

# CHAPTER 7

## RESULTS AND COMMENTS ON TECHNICAL ASPECTS

### 7.1. Drug susceptibility testing (DST) using breakpoints

DST of *M. tuberculosis* is determined by various methods on solid and in liquid media. Among the solid media used are the LJ, 7H10 and 7H11 agar media (Cannetti et. al, 1963, Heifets,1991 (a); Heifets, 1988, Rastogi et.al, 1989). During our experiments LJ and 7H10 agar media were compared for their efficiency in determining the susceptibility patterns of different *M. tuberculosis* strains. For both media to be comparable the resistance patterns had to be similar, taking into account that the critical concentrations that are prescribed for the media are either similar or different.

Table 7.1. DST of *M. tuberculosis* on LJ slopes and 7H10 agar

| Strain no | LJ slopes | | | | | | 7H10 agar | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | INH 0.2 | INH 1.0 | RPM 30 | CIP 2.0 | EMB 2.0 | SM 5.0 | INH 0.2 | INH 1.0 | RMP 1.0 | CIP 2.0 | EMB 5.0 | EMB 10 | SM 2.0 | SM 10 |
| H37Rv | S | S | S | S | S | S | S | S | S | S | R | S | S | S |
| 342/94 | R | R | R | S | S | S | R | R | R | S | R | S | S | S |
| 249/94 | R | S | R | S | S | S | R | S | R | S | R | S | S | S |
| 4591 | S | S | R | S | S | S | S | S | R | S | S | S | S | S |
| 3883 | R | R | R | S | S | S | R | R | R | S | S | S | S | S |
| 11270 | R | R | R | S | R | R | R | R | R | S | S | S | R | R |
| 11400 | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| 12107 | S | S | S | S | S | S | S | S | S | S | S | S | S | S |

S = sensitive, R = resistant

Table 7.1. shows the resistance patterns of strains of *M. tuberculosis* on LJ and 7H10 agar. The strains H37Rv, 342/94 and 249/94 were resistant to 5ug/ml EMB but sensitive to 10 ug/ml when tested on 7H10 agar. However, they were inhibited by 2ug/ml when tested on LJ medium. The patterns of resistance shown by strains 342/94, 3883 and 11270 are those of MDR strains. These strains were highly resistant to INH and RMP, 11270 was also highly resistant based on critical concentrations of SM used in 7H10 agar. The strain 249/94 was resistant to RMP and showed borderline resistance to INH, ie. it was resistant to 0.2ug/ml but sensitive to 1ug/ml concentrations of INH in both media. According to the context of the term "wild strains" of *M. tuberculosis* used by Cannetti et al. (1963), the two clinical strains 11400 and 12107 were classical "wild strains" of *M. tuberculosis* because they were sensitive to all the drugs used and on both media.

### 7.2. The minimum inhibitory concentrations (MICs)

The MIC is an accepted standard to quantitate the degree of resistance of different mycobacteria and other bacterial species. It measures the extent of susceptibility or resistance by employing a wide range of drug concentrations. (Heifets, 1991 (c)).

Eight strains were tested on 7H10 agar and only four were tested in 7H12 broth, the reason being that Bactec is very costly and we only wanted to compare the two methods. The guidelines for interpretive criteria of MICs determined in 7H12 broth as recommended by Heifets et al (1986) are shown in Table 7.2.. These "critical" or "breakpoint" concentrations were chosen to indicate whether the strain is very susceptible, moderately susceptible, moderately resistant or resistant to the anti-TB drug tested.

Table 7.2. Guidelines for interpretation of MICs determined in 7H12 broth radiometrically

| Drug | Very susceptible | Moderately susceptible | Moderately resistant | Resistant |
|---|---|---|---|---|
| INH | $\leq = 0.1$ | 0.2-1.0 | 2.0 | $\geq = 4.0$ |
| RMP | $\leq = 0.5$ | 1.0-4.0 | 8.0 | $\geq = 16$ |
| CIP | $\leq = 2.0$ | 4.0 | 8.0 | $\geq = 16$ |
| EMB | $\leq = 2$ | 4.0 | 8.0 | $\geq = 16$ |

The results of the MICs determined on 7H10 agar and in 7H12 broth are presented in Table 7.3.. Most strains selected for this experiment were shown to be resistant to INH. When the criteria in Table 7.1 and Table 7.2 are employed to interpret the 7H10 agar amd the Bactec results respectively, H37Rv and 4591 are fully sensitive to INH while H37Rv and MR 83173 were very susceptible to RMP. All the strains were very susceptible to CIP and EMB.

The MICs of EMB on 7H10 agar were very high (ranging from 2.5 to 10ug/ml) for all strains when compared to those determined in 7H12 Bactec broth (ranging from 0.3 to 1.25ug/ml). These differences in MICs are reflected by the higher breakpoint concentrations for EMB on 7H10 agar compared with Bactec (see Table 7.1 and Table 7.2) and can be attributed to variable rates of absorption to medium components and degradation of the drug during the long period of cultivation in 7H10 medium as compared to the Bactec 7H12 broth. In the latter the period of cultivation is short and there is a low rate of drug absorption and degradation (Heifets,1996). Some studies have shown MICs of up to 16ug/ml for EMB against the H37Rv strain (Gangadharam & Gonzales, 1970).

The MICs of RMP for the sensitive strains showed some variation. For H37Rv they were the same in both methods and for MR 83173 they were much higher

when determined in 7H10 agar (1 ug/ml as compared to 0.06 ug/ml). RMP is known to bind to components in solid media and this is especially noticeable in LJ medium where breakpoint concentrations are much higher for this reason.

For CIP the MICs determined on 7H10 agar were either equal to or slightly higher than those determined on 7H12 broth. For H37Rv and MR83173 the MICs were the same (0.5 and 0.25, respectively) in both methods and were higher for the strain 342/94 and 4591 (1 and 2$\mu$g/ml respectively) in 7H10 agar as compared to those in 7H12 Bactec broth (0.25ug/ml in both).

Table 7.3. Comparison of 7H10 agar and 7H12 Bactec broth determined MICs

| strain no | 7H10 agar | | | | Bactec broth | | | |
|---|---|---|---|---|---|---|---|---|
| | INH | RMP | CIP | EMB | INH | RMP | CIP | EMB |
| H37Rv | <0.06 | 0.25 | 0.5 | 10 | 0.025 | 0.25 | 0.5 | 1.25 |
| 342/94 | 2 | >16 | 1 | 10 | 0.8 | >64 | 0.25 | 0.6 |
| 4591 | 0.125 | >16 | 2 | 2.5 | 0.1 | 24 | 0.25 | 1.25 |
| MR 83173 | 4 | 1 | 0.25 | 5 | 0.8 | 0.06 | 0.25 | 0.31 |
| 3883 | >8 | >16 | 1 | 5 | ND | ND | ND | ND |
| 11270 | 8 | 8 | 1 | >20 | ND | ND | ND | ND |
| 11400 | <0.06 | <0.25 | 2 | <1.25 | ND | ND | ND | ND |
| 12107 | <0.06 | <0.25 | 1 | 10 | ND | ND | ND | ND |

ND = NOT DETERMINED

The overall MIC results show that the MICs in Bactec radiometric 7H12 broth were either equal or slightly lower than the 7H10 agar determined MICs.

### 7.3. The minimum bactericidal concentrations (MBCs)

The MBCs of INH, RMP, CIP and EMB were determined using the Bactec against *M. tuberculosis* H37Rv strain only, the reason being that MBC determinations in Bactec are not only costly but also labour intensive. Samples were taken from

7H12 broth at 0 (after addition of drugs), 3, 6, 9, 12, and 15 days of incubation and plated onto 7H10 agar.

Table 7.4. 7H12 Bactec broth determined MBCs of the strain H37Rv compared with MICs

| DRUG | MIC (ug/ml) | MBC (ug/ml) | MBC/MIC ratio |
|---|---|---|---|
| INH | 0.025 | 0.025 | 1 |
| RMP | 0.25 | 0.5 | 2 |
| CIP | 0.5 | 2.0 | 4 |
| EMB | 1.25 | 10 | 8 |

INH was shown to be more bactericidal as compared to the other drugs tested and its MBC was equal to that of the MIC (Table 7.3.). The bactericidal activity of INH in the treatment of tuberculosis is well documented (Heifets, 1991 (b)). RMP, whose MBC was twice the MIC was also bactericidal but less so when compared to INH. CIP was less bactericidal (MBC equals to 4X MIC). EMB is bacteriostatic and the its MBC was found to be 8X the MIC. RMP exerted its effect earlier than other drugs with colony counts dropping earlier than was the case with the other agents.

## 7.4. Comparison of the sonicator and vortex as methods of dispersing *M. tuberculosis* cells

One of the typical features of the bacilli of *M. tuberculosis* is to clump. If the clumps are not broken up, suspensions containing clumps will produce fewer colonies on solid media than when properly dispersed. This will lead to artificially low colony counts. Methods of dispersing the cells in suspension to produce single colonies have been used to simplify the counting of single colonies but have not been evaluated in detail. In this study two methods of dispersion, ie. sonication and vortexing were evaluated during the PAE experiments. Plating was done on 7H10 agar and colonies were counted by naked eye. These

experiments were repeated three times for each method. The mean log count ($\bar{x}$) and the standard error of the mean (SEM) for all the experiments were calculated for each point on the graphs.

Figures 7.1 - 7.3 show the graphs of vortex versus sonicator for experiments with the control, INH and RMP. As can be seen from the SEMs, quite large variations occurred with both methods.The overall counts when vortex dispersion was used were however consistantly lower compared to those obtained when the tubercle bacilli were sonicated. The implication of lower counts is that there was considerable of clumping even after dispersion. The higher counts obtained with the sonicator is an indication of better dispersion of bacilli before subculture on a solid medium to produce CFUs.

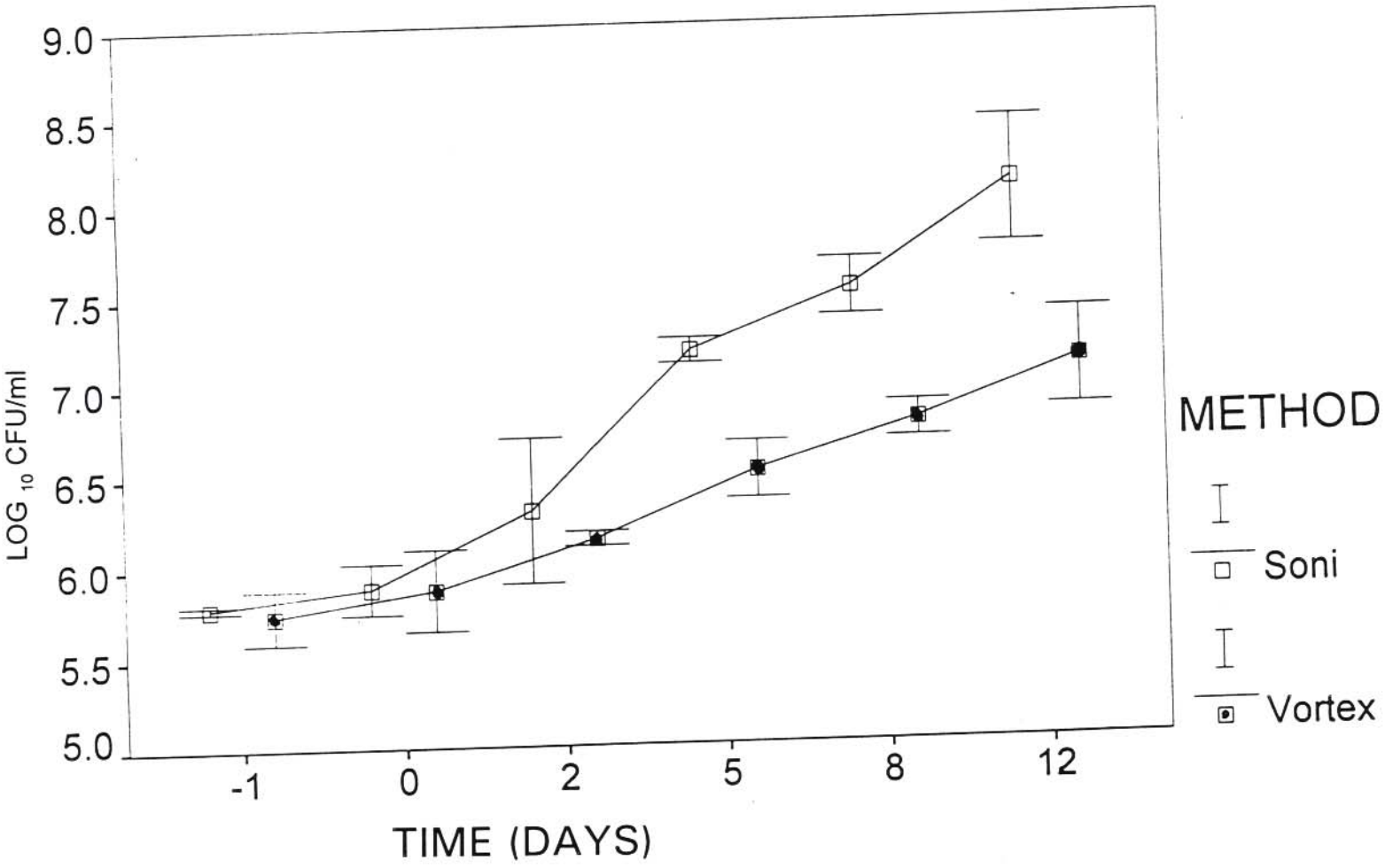


Fig 7.1. Growth curve of the dispersion of *M. tuberculosis* cells using vortex and sonicator for the unexposed control. The bars indicate means +\- 2 SEM as determined by AMLOG.

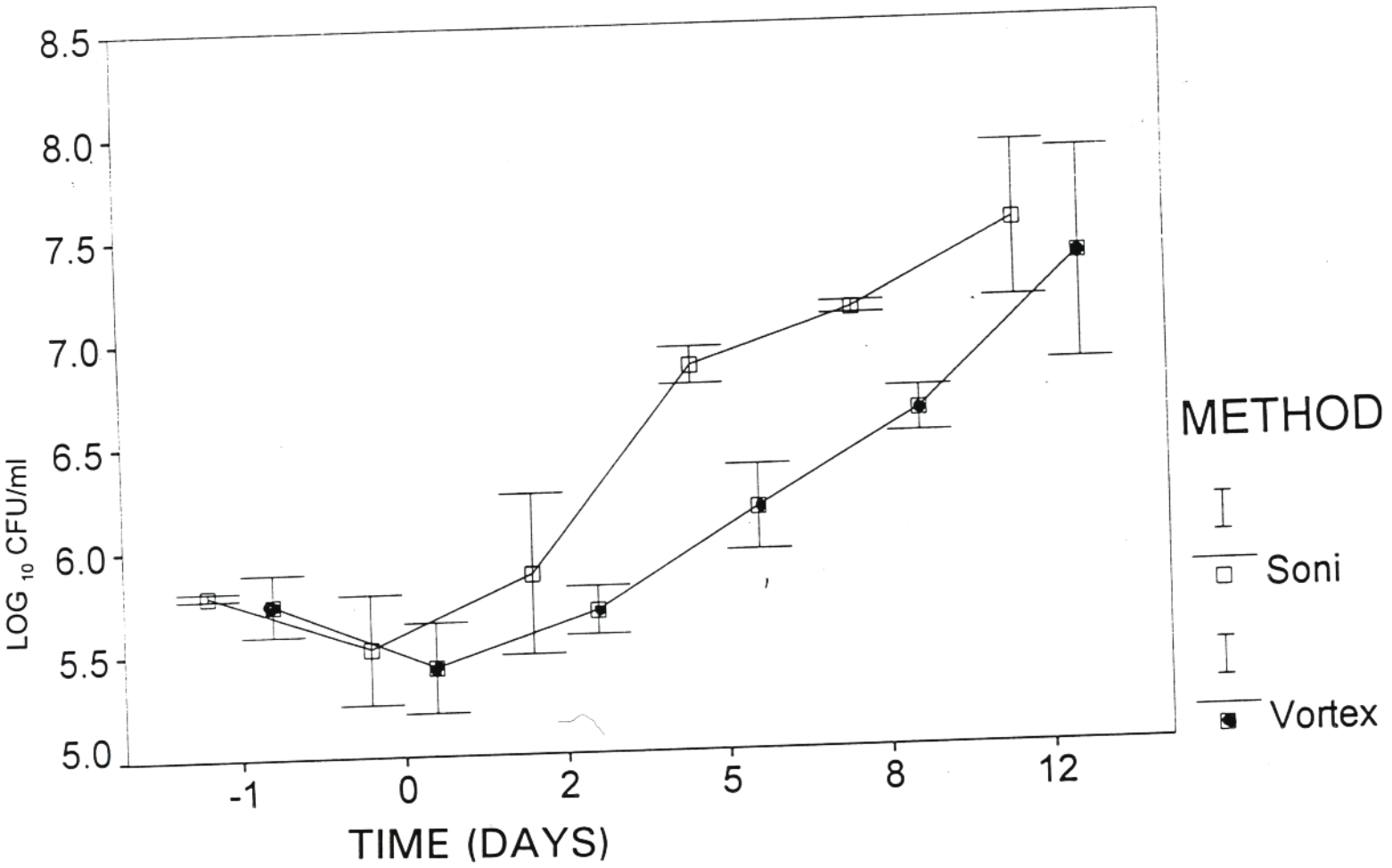


Fig 7.2. Growth curve of the dispersion of *M. tuberculosis* cells using vortex and sonicator after exposure to INH. The bars indicate means +\- 2 SEM as determined by AMLOG.

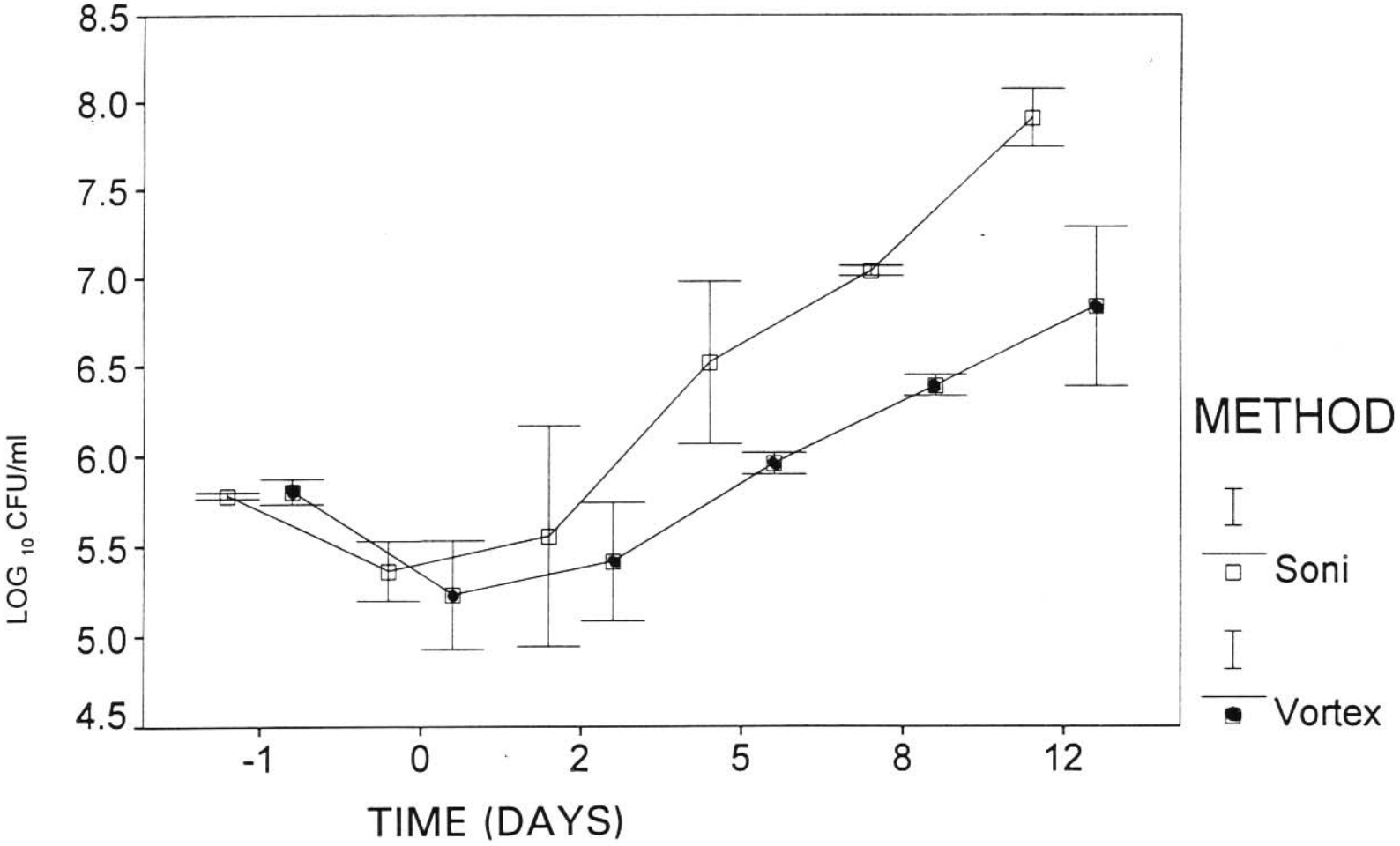


Fig 7.3. Growth curve of the dispersion of *M. tuberculosis* cells using vortex and sonicator after exposure to RMP. The bars indicate means +\- 2 SEM as determined by AMLOG.

## 7.5. PAE AND CERT

### 7.5.1. Comparison of the duration of PAE determined by the viable count (CFU/ml) and CERT by the Bactec radiometric method.

A Bactec radiometric method for the determination of CERT was developed and compared with the traditional CFU/ml method. A set of experiments were conducted to determine the post-exposure regrowth using the two methods of dispersion. PAE and CERT determinations were treated separately for the each dispersing method.

*M. tuberculosis* H37Rv strain was exposed to 0.2ug/ml INH ( 8 X MIC) and 0.5ug/ml RMP (2 X MIC) for 24 hrs, and regrowth was monitored either by Bactec or by viable counts for a maximum of 12 days after washing.

#### 7.5.1.1. Sonication experiments

***CERT determined by Bactec***. Fig 7.6. is a graph of time vs GI for INH, RMP and the control culture using the Bactec system. The duration of CERT was 1.7 and 2.4 days for INH and RMP, respectively.

***PAE determined by viable count***. The PAE of INH determined by this method was -0.1 days and that of RMP was 0.4 days (Fig 7.7.). The PAEs detemined by the viable count method were shorter compared with CERTs determined by the radiometric (Bactec) technique. In both methods the PAE/CERT for RMP was longer than that of INH. The marginally negative PAE (-0.1 days) for INH shows that regrowth after exposure to INH was slightly faster than the growth rate of the control culture.

Fig 7.7. also shows that the colony count in the RMP exposed cells increases rapidly after the fifth day of regrowth.

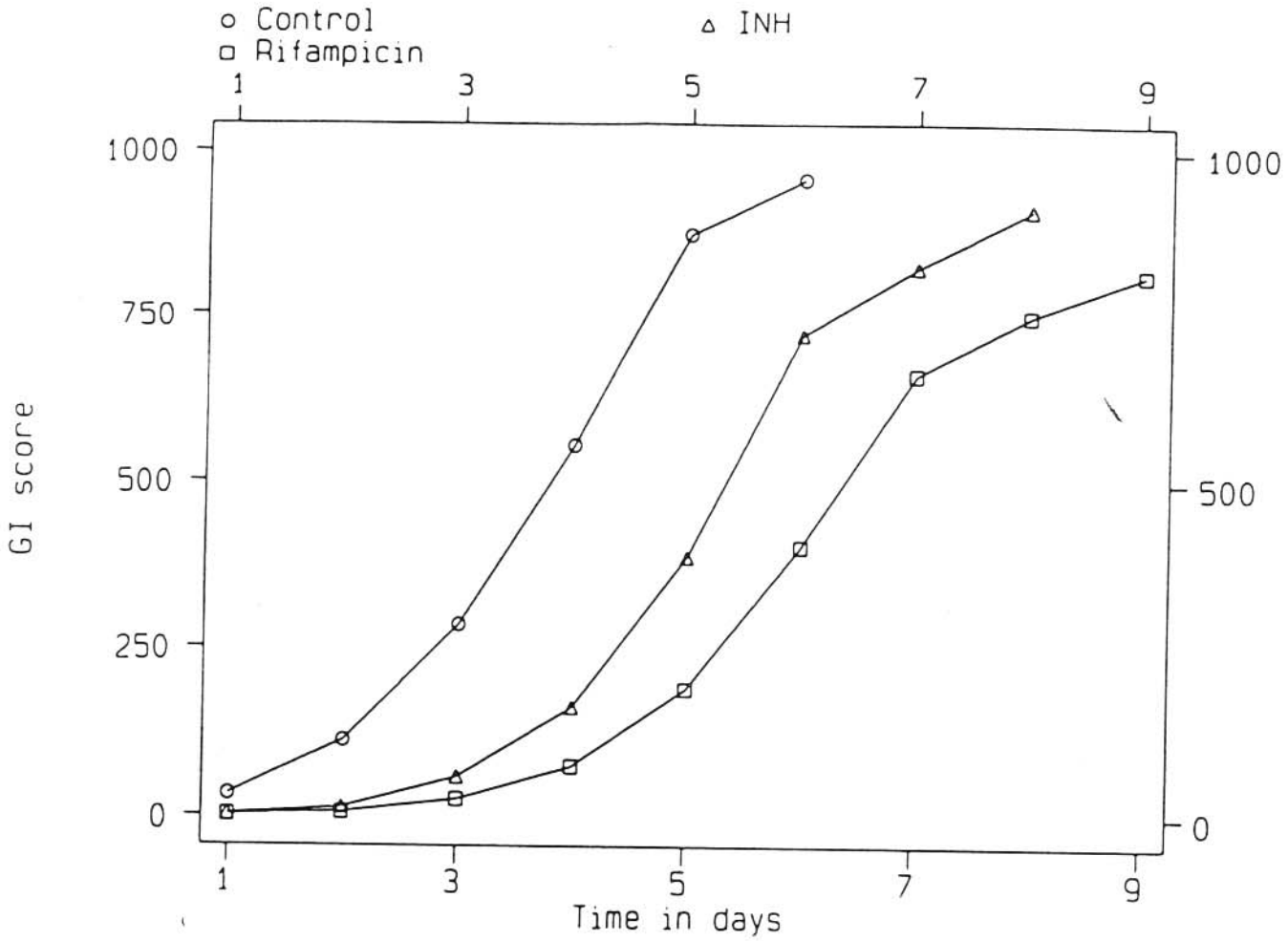


Fig 7.4. Regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to INH (0.2 ug/ml) and RMP (0.5 ug/ml) determined by Bactec (sonicator experiments). The points in graph represent the mean of the GI readings.

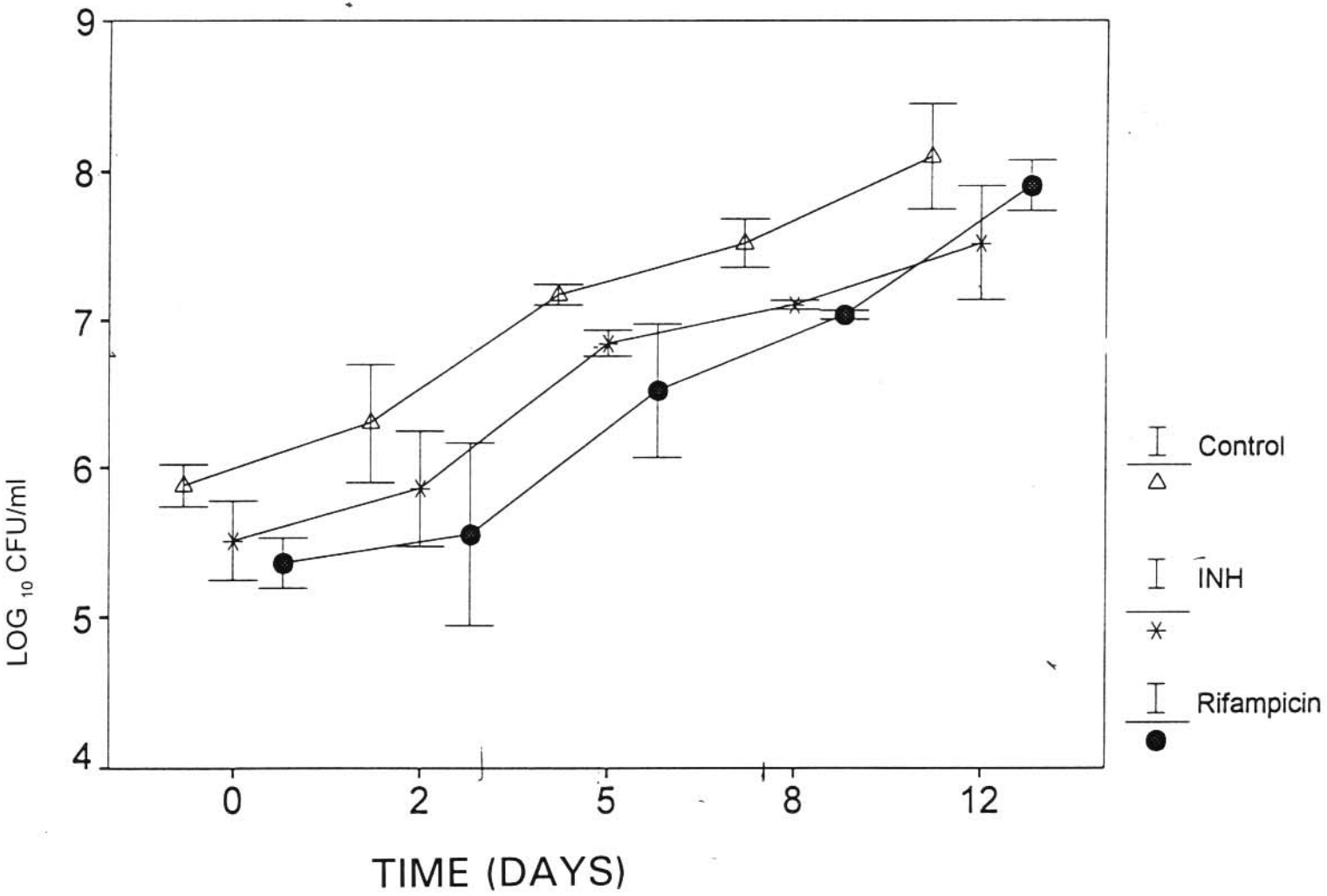


Fig 7.5. Regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to INH (0.2 ug/ml) and RMP (0.5 ug/ml) determined by viable counts (sonicator experiments). The bars indicate the means +\- 2SEM.

### 7.5.1.2. Vortexing experiments

***CERT determined by Bactec.*** Figure 7.6 presents graphs of GI readings vs time for INH and RMP exposed cells. It took the control 3.8 days, 5.4 days for INH and 6.5 days for RMP to reach a GI of 500, resulting in CERTs of 1.5 and 2.7 days for INH and RMP, respectively.

**PAE determined by colony counts method after vortexing**

The time taken by the control culture to reach a $1\log_{10}$ increase was 9.3 days while the same increase in CFU/ml took 6.6 days for INH and 6.8 days for RMP after the removal of the drugs. Therefore, the PAE for INH was -2.7 days and for RMP it was -2.5 days (Fig 7.9).

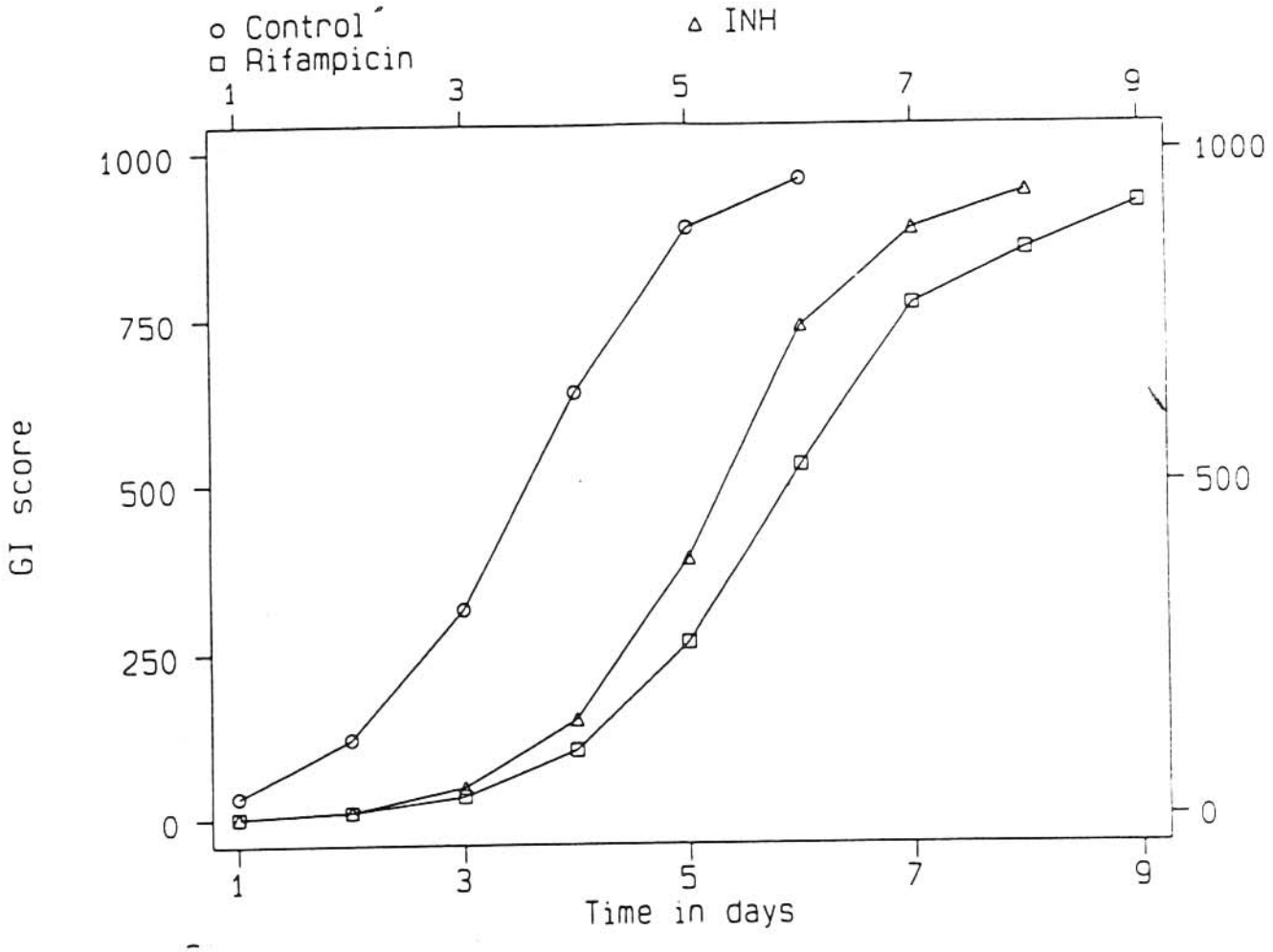


Fig 7.6. Regrowth curve of *M. tuberculosis* H37Rv exposed for 24 hours to INH (0.2 ug/ml) and RMP (0.5 ug/ml) determined by Bactec (vortex experiments)

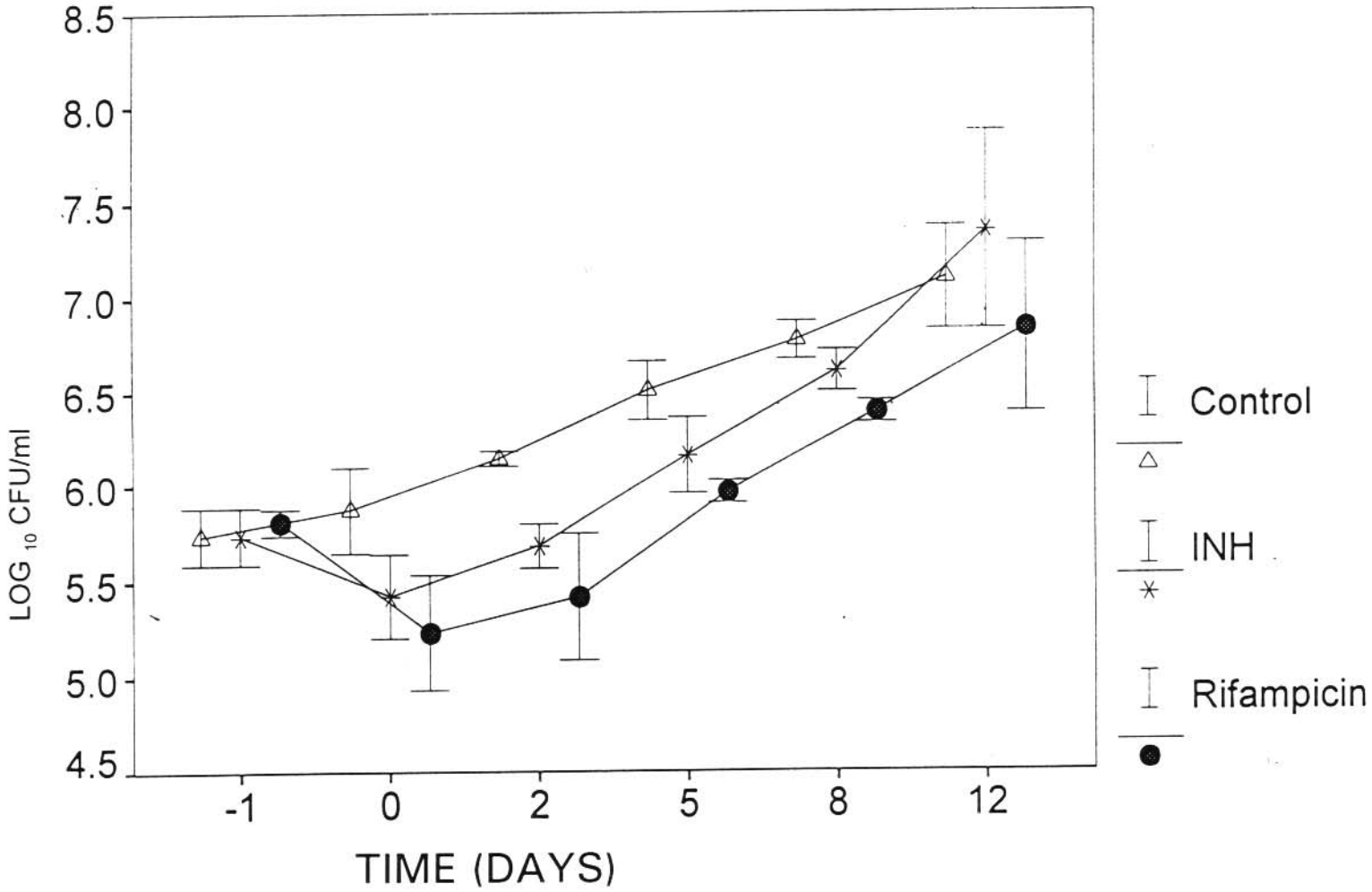


Fig 7.7. Regrowth curve of *M. tuberculosis* H37Rv exposed for 24 hours to INH (0.2 ug/ml) and RMP (0.5 ug/ml) determined by viable counts (vortex experiments). The bars indicate the means +\- 2SEM.

### 7.5.2. Effect of exposure time on CERT

Table 7.5. CERT determined at 8X MICS of each drug at different exposure times

| Drug (ug/ml) | Duration of exposure (h) | Time (d) to reach GI 500 | Duration of CERT (d) |
|---|---|---|---|
| CONTROL | | 5 | 0 |
| INH .02 | 2 | 5 | 0 |
| | 12 | 5 | 0 |
| | 24 | 6 | 1 |
| | 48 | 7 | 2 |
| | 96 | 8 | 3 |
| RMP 2.0 | 2 | 8 | 3 |
| | 12 | 12 | 7 |
| | 24 | 13 | 8 |
| | 48 | 18 | 13 |
| | 96 | 19 | 14 |
| CIP 4.0 | 2 | 5 | 0 |
| | 12 | 5 | 0 |
| | 24 | 6 | 1 |
| | 48 | 7 | 2 |
| EMB 10 | 2 | 5 | 0 |
| | 12 | 5 | 0 |
| | 24 | 6 | 1 |
| | 48 | 8 | 3 |

d = days, h = hours

To determine the effect of duration of exposure on CERT the organisms were exposed to a fixed concentration of 8X the previously determined MICs of INH, RMP, CIP and EMB. This was based on the fact that most organisms exhibit a maximum PAE at concentrations of approximately 10X the MIC (Volgeman & Craig, 1985). Organisms were exposed to the drugs for 2, 12, 24, 48 and 96 hours.

Table 7.5 demonstrates that INH, CIP and EMB showed no CERT after exposure for 2 and 12 hours but started showing CERT at 24 hours (CERT of 1 day for each). RMP started showing a long CERT of 3 days even when exposed for as little as 2 hours. With all the drugs CERT increases with an increase in time. The CERT of RMP was even longer at 96 hours (14 days). Compared with RMP, INH had a CERT of only 3 days at 96 hours. The values given were rounded to the nearest number of days to reach GI 500.

### 7.5.3. Effect of drug concentration on CERT

To study the effect of concentration on CERT the organism was exposed for 24 hours to different drug concentrations of 2, 4 and 8X the MIC. INH was tested at 8 and 40X the MIC.

Table 7.6. shows that the increase in concentration of RMP prolonged the duration of CERT. There was no increase in the duration of CERT when the concentration of INH and EMB was increased. The persistent suppression of growth with CIP showed an increase in CERT at 2 and 4X the MIC but there was no increase at 8X the MIC. Also in these experiments the trend of an increasing concentration was looked at and the values given were rounded off to the nearest number of days.

Table 7.6. CERT of H37Rv strain after 24 hrs exposure at different concentrations

| Drug | Concentration (ug/ml) | Time (d) to reach GI 500 | Duration of PAE (d) |
|---|---|---|---|
| Control | - | 5 | 0 |
| INH | 0.2 | 6 | 1 |
| | 1.0 | 6 | 1 |
| RMP | 0.5 | 7 | 3 |
| | 1.0 | 8 | 4 |
| | 2.0 | 12 | 7 |
| CIP | 1.0 | 6 | 1 |
| | 2.0 | 7 | 2 |
| | 4.0 | 7 | 2 |
| EMB | 2.5 | 6 | 1 |
| | 5 | 6 | 1 |
| | 10 | 6 | 1 |

### 7.7. Drug combinations

Figure 7.8. to Figure 7.12 are graphs of CERTs of different drug combinations. The CERT of the combination could be defined using the Bactec and the interpretation of results was discussed in Chapter 6. The combination of INH-CIP (Figure 7.8) had a CERT of 2.5 d and that of RMP-EMB was 3.5 d (Figure 7.9). Both combinations showed a synergistic effect which was more than the sum of the drugs used singly, ie. the sum of 1.6 and 2.1, respectively. An additive effect was demonstrated in the combination of INH-RMP; the CERT of the combination was 2.9 days (Figure 7.10). The combination INH-EMB had a CERT of 1.75 days (figure 7.11) which was also an additive effect. The CERT for the combination of RMP-CIP was indifferent and the duration was 1.7 days, ie. it was less than the sum of the combination effect which was 2.1 days (Figure 7.12).

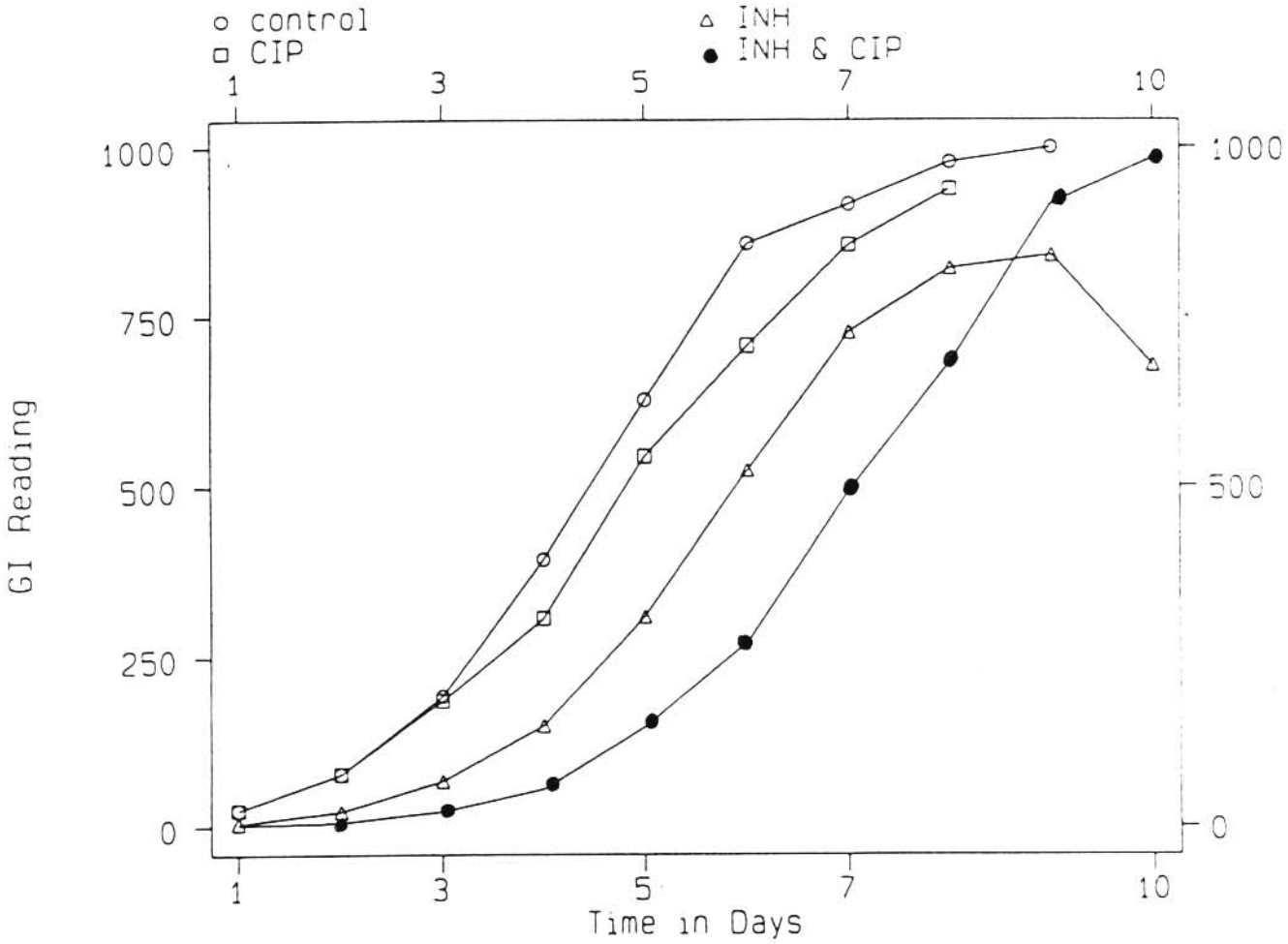


Fig 7.8. Typical regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to INH (0.2 ug/ml) and CIP (1ug/ml) both alone and in combination using the Bactec system.

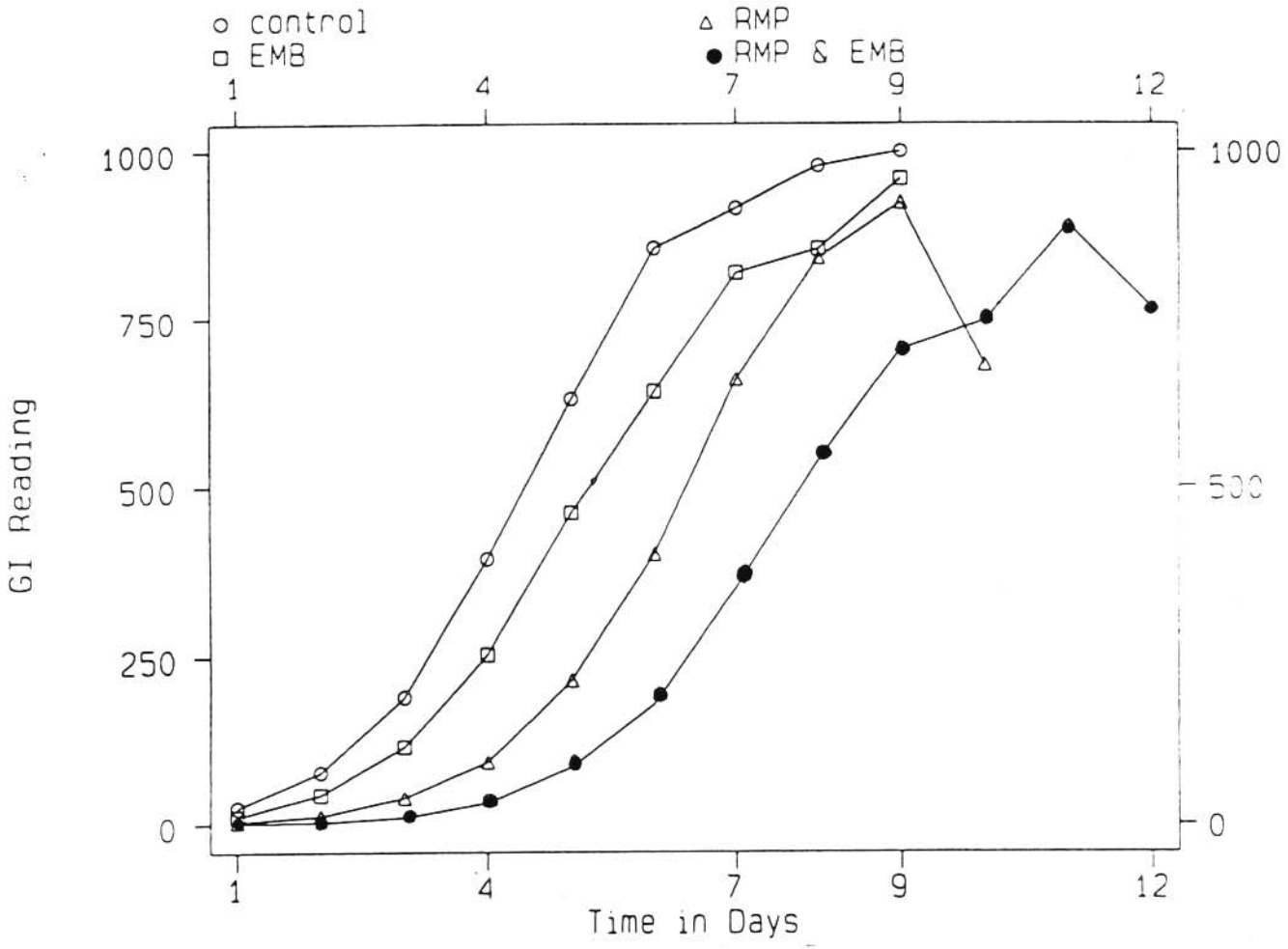


Fig 7.9. Typical regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to RMP (0.5 ug/ml) and EMB (2.5 ug/ml) both alone and in combination using the Bactec system.

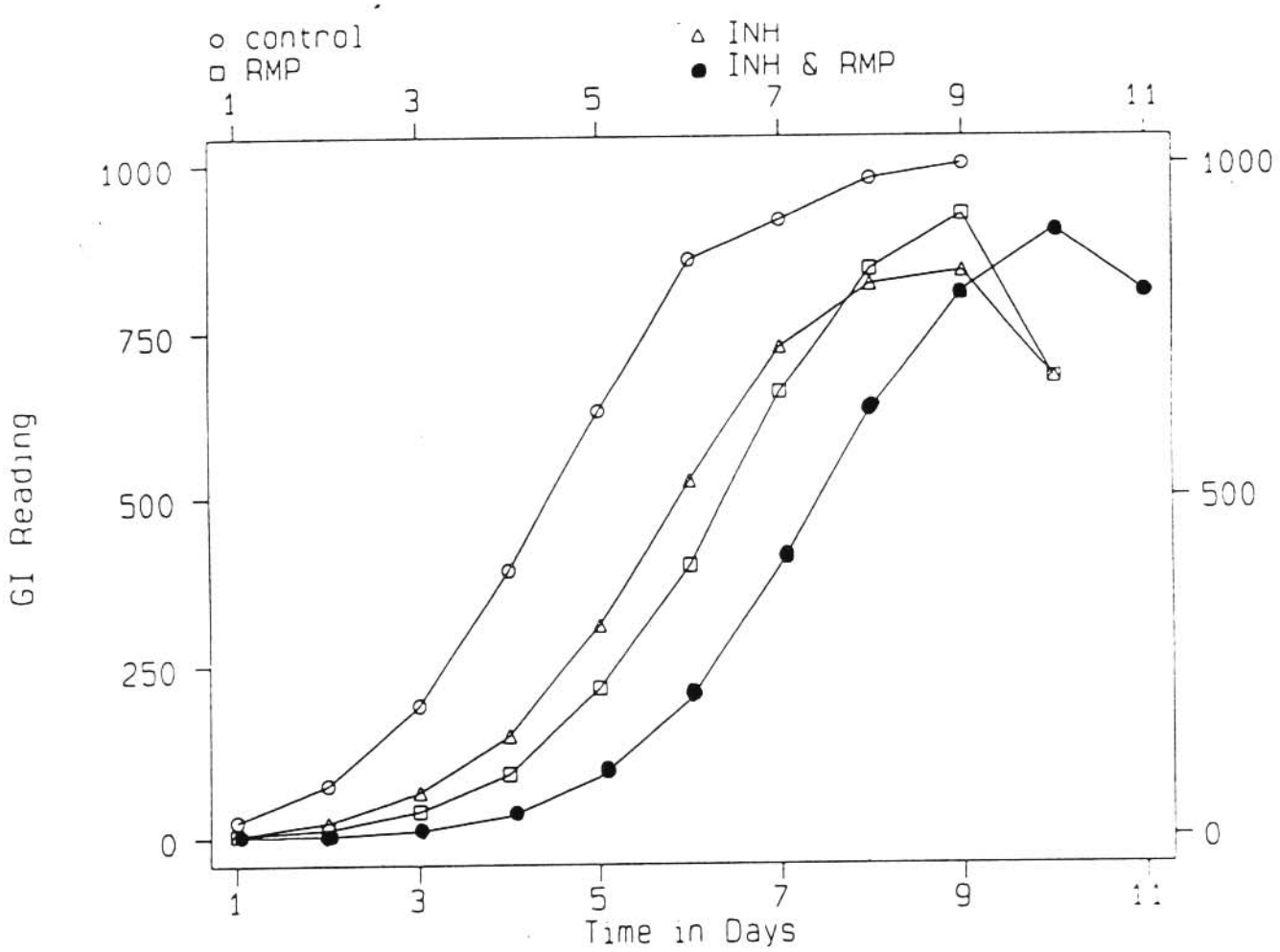


Fig 7.10. Typical regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to INH (0.2 ug/ml) and RMP (0.5 ug/ml) both alone and in combination using the Bactec system.

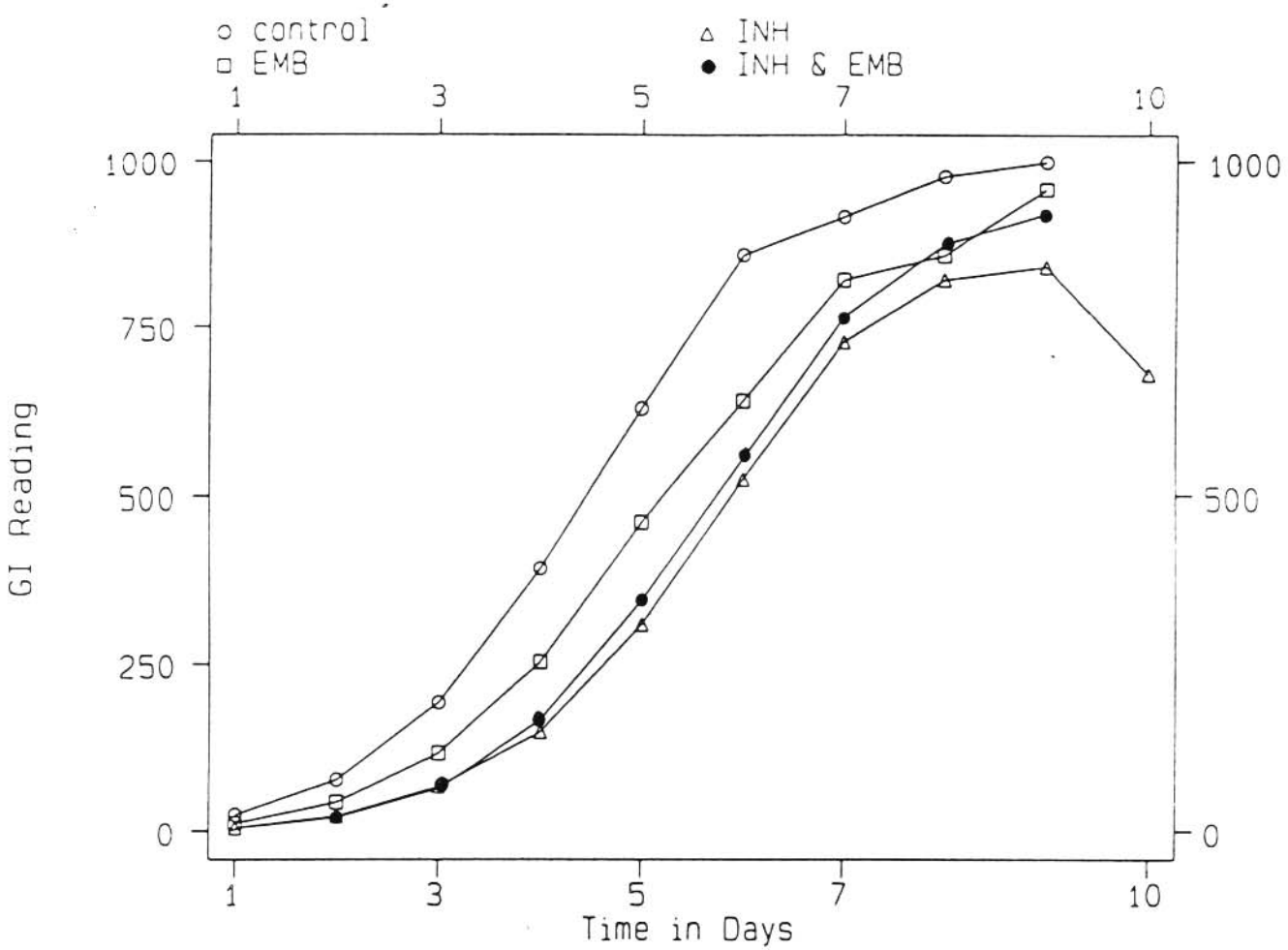


Fig 7.11. Typical regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to INH (0.2 ug/ml) and EMB (2.5 ug/ml) both alone and in combination using the Bactec system.

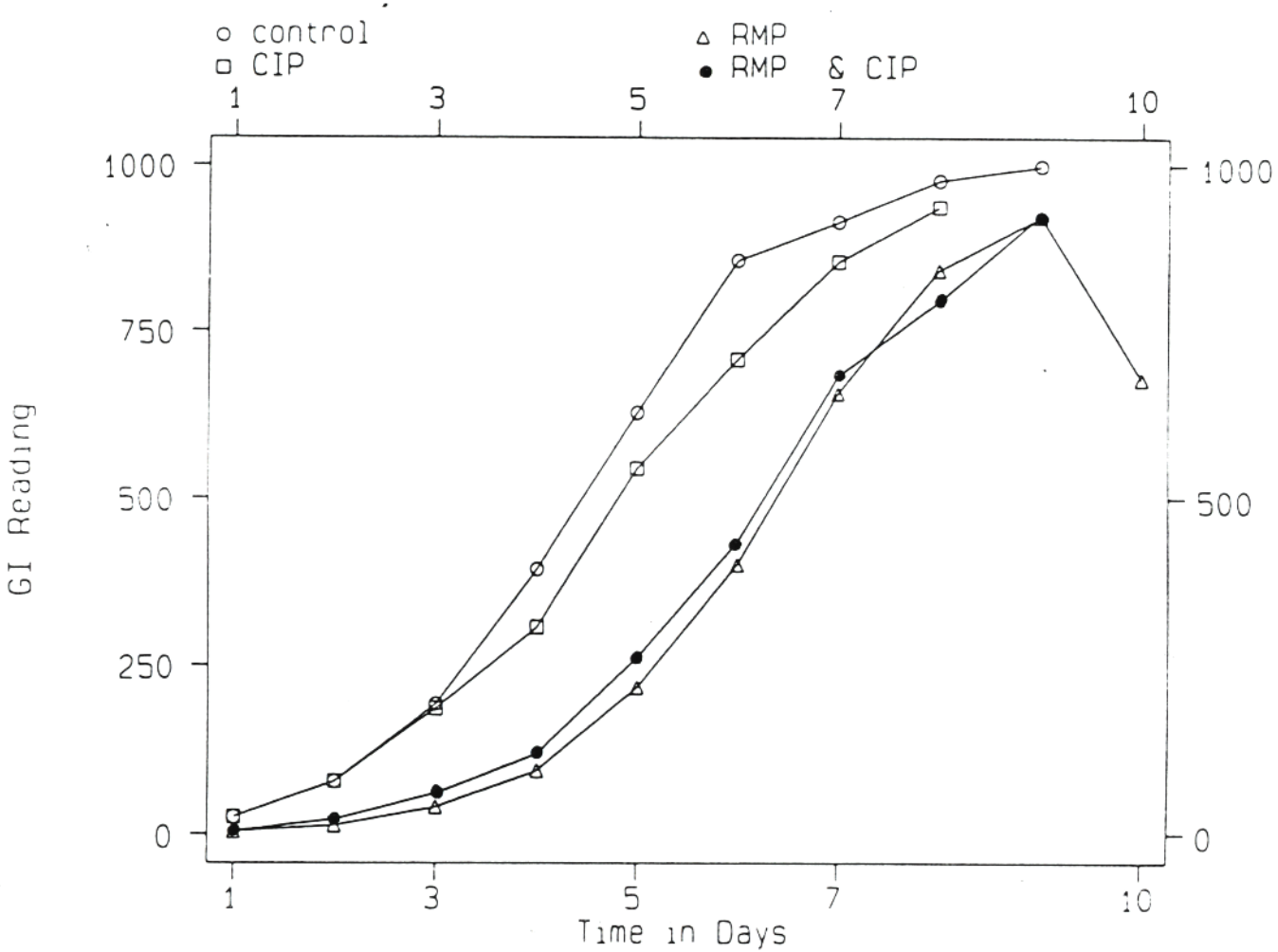


Fig 7.12. Typical regrowth curve of *M. tuberculosis* H37Rv strain exposed for 24 hours to RMP (0.5 ug/ml) and CIP (1.0 ug/ml) both alone and in combination using the Bactec system.

# CHAPTER 8

## DISCUSSION AND CONCLUSIONS

### 8.1. Drug susceptibility testing including MICs and MBCs

#### 8.1.1. Susceptible and resistant strains used in the present study

As this dissertation concerns mainly technical aspects of DST, the vast questions surrounding drug resistance, including mechanisms of resistance and how it arises during treatment and the tremendous importance of MDR strains, will not be discussed in detail.

As mentioned in Chapter 7, the two clinical strains 11400 and 12107 are "wild strains" of *M. tuberculosis* which were sensitive to all the drugs using both solid (LJ and 7H10 agar) and liquid (Bactec) media. This suggests that the patients from whom these strains were isolated either never received treatment involving the drugs which were tested or if having had exposure to these drugs, used them under conditions which would not tend to select for resistant mutants such as being subjected to monotherapy through poor compliance, wrong dosage, drug incompatibility or drug absorption problems.

One problem in the treatment of tuberculosis remains the development of drug resistance. Multidrug resistant (MDR) strains are those that are resistant to 2 or more antituberculosis drugs especially INH and RMP(SA TB control programme, 1996). MDR strains (342/94, 3883 and 11270) were isolated and they were highly resistant to INH and RMP. The strain no 11270 was also resistant to SM. Conditions under which resistance may develop during antituberculosis treatment have recently been discussed by Mitchison (1998).

### 8.1.2. Comparison of methods

The patterns of resistance of the *M. tuberculosis* strains tested in this study were similar on 7H10 agar and LJ medium using the proportion method. The two media gave comparable results. Based on these findings and experience elsewhere (Heifets, 1991 (a)), the use of 7H10 agar is preferred to LJ because it gives results more quickly (within 2 to 3 weeks) as compared to the long period of incubation of LJ slopes (4 to 8 weeks). The 7H10 agar is also easy to prepare because it comes as a 7H10 agar base and one only needs to add water, autoclave, and then add the desired drug concentrations whereas with the LJ medium the preparation is tedious and the drugs are added before inspissation. Those drugs that are not stable can be degraded during inspissation (see appendix A for the preparation of both media). LJ is however much cheaper than 7H10 agar.

Generally, the 7H10 agar determined MICs were higher than the Bactec determined MICs but in some cases they were similar. These differences in MICs can be attributed to variable rates of binding of drugs to media components and degradation of the drug during the long period of cultivation in 7H10 medium as compared to the Bactec 7H12 broth in which the period of cultivation is short and there is a low rate of drug absorption and degradation (Heifets, 1996). Such differences are particularly evident in the case of EMB, one of the antituberculosis agents which has been found to have different MICs in different media. *M. tuberculosis* strains were inhibited at higher concentrations of EMB in 7H10 agar than in LJ medium and Bactec. The MIC of EMB against H37Rv and other clinical strains was 10 ug/ml when tested on 7H10 medium as compared to 2ug/ml on LJ medium and 0.31 to 1.25 in Bactec 12B broth. Gangadharam and Gonzales (1970), found that the standard H37Rv strain was inhibited by 8 or 16ug/ml in 7H10 agar medium as compared to 1 or 2g/ml in LJ medium. The finding that 7H10 agar gave higher MICs for EMB is not suprising. Heifets states that susceptible "wild strains" will grow in 7.5ug/ml EMB and this concentration

has been recommended as the critical concentration. In our experiments H37Rv and strain 342/94 had EMB MICs of 10ug/ml on 7H10 agar suggesting that in the case of EMB the 7H10 agar method on this occasion slightly overestimated the MIC of EMB. This may have been due to excessive binding of EMB to the 7H10 agar batch used by us or other unknown technical factors.

The Bactec method, though it is costly, is easier to work with and faster (results are obtained within 5 to 12 days of incubation) than the 7H10 agar method for MIC determination. The medium is commercially prepared, which makes it easier to use but it involves the use of needles which can be dangerous if care is not taken. The counting of colonies in 7H10 agar is laborious and time consuming. Our findings relating to the bactericidal activity of antituberculosis agents are also in agreement with those of most investigators in that INH together with RMP are the most bactericidal antimycobacterial agents (Heifets, 1985 (b)), with their MBCs close to their MICs and the concentrations required for bactericidal activity are far less than their $C_{max}$. The MBC of INH was equal to its MIC. RMP was also highly bactericidal with MBCs equal to twice the MIC and its action was more rapid than that of INH. There was a drop in colony count within the first 3 days of sampling which was not seen with INH when the MBCs were performed (data not shown). CIP can also be regarded as bactericidal against *M. tuberculosis* with an MBC of 4X the MIC. The MBC is less than the $C_{max}$ of this drug which is 2.01 mg/ml after oral administration. The predominantly bacteriostatic activity of EMB was confirmed in this study. The MBC of this drug was 8XMIC, a concentration which is much higher than the $C_{max}$.

### 8.2. Post-exposure regrowth experiments

#### 8.2.1. The problem of clumping of tubercle bacilli: use of mechanical dispersion

When the methods of dispersing tubercle bacilli were compared, sonication proved to perform better than the vortex procedure (Figure 7.1 - Figure 7.3).

Variation in counts encountered by both methods was however considerable, illustrating the problem of clumping. The colonies of cultures dispersed by the sonicator could be counted easily because they were well separated into single colonies. The machine is easy to use although it has some disadvantages. Among these are its size and the necessity to use it in a safety cabinet and it is also more expensive than the vortex equipment. The tubes which contain the culture to be dispersed by the sonicator have to be opened during use and this may produce aerosols which may pose a health hazard or it may lead to the introduction of foreign organisms into the culture. The advantage of using the vortex is that the apparatus is available in most laboratories and is relatively cheap. Also, the culture containing medium is maintained in a closed container during vortexing thus avoiding contamination and aerosol discharge into the air. The disadvantage of using the vortex is that the problem of clumping of the tubercle bacilli is not adequetely solved. This can be seen during the counting of colonies on the agar which is difficult especially for someone who is not used to the technique. In the present study different methods of dispering cells were studied, avoiding the use of surface active agents such as Tween 80, which may interfere with the action of antituberculosis drugs in our studies. Dispersal methods need to be studied further and standardized to reduce the problem of clumping of cells and produce reliable and reproducible results.

### 8.2.2. Post-exposure regrowth indices

During our experiments a method for determining CERT using Bactec was developed. This was based on the fact that Bactec measures the growth of microorganisms by detecting the amount of radiollabled $CO_2$ which is proportional to the growth of microorganisms. A 1 in 40 dilution (0.1 ml into 4 ml in a Bactec 12B vial) of the exposed and control culture was used for regrowth. The equation CERT = T - C was used, where T, is the time taken by the drug-exposed culture to reach a GI reading of 500 and C, is the corresponding time for the unexposed control culture to reach the same reading.

This reading was chosen because earlier studies of growth curves in 7H12 broth showed that with rising GI readings, bacteria are actively multiplying at a GI of 500 and the number of CFU/ml at this reading is approximately $10^5$ CFU/ml (Heifets et al. 1985 (a), Heifets et al 1985 (b), Heifets et al. 1986). In order to calculate a regrowth concentration of $+\backslash - 1\log_{10}$ greater than the original inoculum regrowth to a GI Of 600 was determined for $ERT_{10}$ and $CERT_{10}$ estimation. The 7H9 broth was used during the drug exposure stage of the experiments as it forms the base of 12B broth but lacks ADC which, because of its high protein content (albumin and the enzyme catalase), could have resulted in protein binding of the drugs. It is also easier to remove the antibiotics from 7H9 broth in wide mouth containers than from Bactec 12B broth vials.

The terminology of the effects of antibiotic exposure on regrowth is confusing, also with regard to the term PAE which is usually used in a specific context comparing regrowth of viable bacteria in terms of cfus to a $1\log_{10}$ increase in bacterial numbers post-exposure, with a similar increase with an unexposed control culture. The term PAE is however, also used in models where post-exposure regrowth in a liquid medium to a prescribed concentration of organisms before exposure is measured in hours or days and compared with an unexposed control culture growing up to the same predetermined level (Rescott et al, 1988; Gould et al. 1989; Gottfredsson et al, 1991; Zodivin et al, 1993; Hanberger et al, 1993). The latter type of PAE determination has been redefined more specifically by Hanberger et al (1995) as control-related effective regrowth time (CERT).

### 8.2.3. Post-exposure regrowth models

The post-exposure regrowth model and the specific PAE definition related to the counting of viable bacteria based on cfus on solid media is the traditional and "gold standard" approach used in this field.

Various models in which regrowth is determined in liquid medium utilise a variety of systems/indicators capable of measuring growth quantitatively. These include bioluminiscence based on ATP activation of luciferin, obtained from fire flies, to luminesce (Hanberger et al, 1993) and radiometrically by determining the release of $^{14}CO_2$ from an isotope-labelled substrate as used in the commercially available Bactec system. Other principles used to measure regrowth involve impedance technology (Gould et al, 1989) and continous vertical photometry (Zodivin et al, 1993). The basic differences between PAE as measured by viable counting and regrowth in liquid media were emphasized by Hanberger et al (1995) who proposed the term control-related effective regrowth time (CERT) for the latter-type experiments to distinguish between regrowth time in such systems and conventional PAEs based on cfus on solid media. This difference is related to the fact that in conventional PAE determination the regrowth of surviving bacteria to increase by $1\log_{10}$ immediately after exposure and removal of the drug is compared with the time taken by the control culture to increase by the same margin while the methodologies involving liquid media measure the time taken, after exposure, to reach a predetermined growth level (usually equivalent to a 10-fold increase of that of the original pre-exposed culture), compared with the $1\log_{10}$ (10-fold) increase in concentration of the control culture. In PAE experiments involving highly bactericidal antibiotics, it is therefore possible that a small number of surviving bacteria may show a 10-fold increase in number fairly quickly giving a low PAE result, as opposed to a much higher PAE or, more specifically, CERT value in the liquid medium regrowth model where the surviving orgainsms will have to increase to a much higher cell concentration (10 times that of original inoculum).

Usually the two approaches give comparable results when the exposure times are sufficiently short to exert a PAE with minimal loss of bacteria due to bactericidal activity (Gottfredsson et al, 1991; Gould et al. 1989; Rescott et al, 1988). An explantion for this good correlation was provided by Hanberger et al (1991) who showed that the growth curves of control and exposed bacterial

cells are parallel once regrowth starts. There was therefore, no difference in PAEs whether a 10-, 100-, 1 000- or 10 000-fold increase in bacterial growth was used to measure PAE.

MacKenzie et al. (1994) and Hanberger et al. (1995) however, showed much shorter PAEs against gram-negative bacilli when measured by viable counting compared with bioluminiscence for imipenem and meropenem, respectively. These two carbapenem antibiotics produce spheroplasts and filamentous forms of gram-negative bacteria which will tend to give low counts as determined by cfus. These cell wall-deficient forms, however, recover quickly after removal of the antibiotic and the filamentous forms may produce more than one colony when the previously affected PBP2 recovers its function to form transverse cell wall partitions. This process results in an increase in cell numbers in terms of the CFUs and a shortened PAE compared with PAE measured as regrowth in liquid media by bioluminescence. In the latter situation the time taken for the damaged and reduced number of surviving organisms to grow up to their original pre-exposed numbers is longer. Furthermore, PAE measured by bioluminiscence shows a linear relationship with increasing concentrations of the carbapenem agent, while PAEs measured by CFU technology did not (Mackenzie et al, 1994). Examining the same phenomenon, Hanberger et al (1995) showed that in the case of meropenem the CERT with an endpoint of $1\log_{10}$ above the pre-exposure inoculum size for both test and control cultures when measured by viable counting or bioluminescence was virtually identical. In contrast, the PAEs determined simultaneously in the same system for *Enterobacter cloacae*, *Escherichia coli*, *Klebsiela pneumoniae* and *Pseudomonas aeruginosa* were -0.4, -0.5, -0.1 and 0.7h, respectively, as opposed to corresponding CERT findings of 3.9, 4.8, 4.7 and 3.5h. These findings are very similar to ours, which showed negative or very low PAEs of INH and RMP. As pointed out earlier, the reduction in the number of surviving bacteria due to the bactericidal activity of the antibiotic tested, requiring extra time to grow to levels 10-fold or higher than the

original inoculum, the lag period and subsequent exponential regrowth phase are all part of the CERT regrowth assessment model.

A modified CERT approach has also been applied to a mouse thigh infection model in which effective regrowth time (ERT) was determined in neutropenic mice infected *E. coli* and exposed to different quinolones (Shibata et al., 1991). In such models the effect of sub-MICs have been studied by several researchers (Odenholt et al., 1990; Gudmundsson et al., 1994; Odenholt et al., 1997) and could be applied to the CERT in vitro model in order to simulate in vivo conditions (Hanberger et al., 1995).

From the literature discussed above, it is clear that in the case of rapidly growing bacteria two important factors may account for differences in PAE determned by viable counting as opposed to methods based on measuring metabolic activity including bioluminiscence, Bactec, impedence or bacterial cell density (Lodwin et al, 1993). These are (a) the bactericidal effect of the antibiotic tested and (b) problems which may affect viable counting on solid media (cfu determinations) such as spheroplasts and filamentous forms resulting from cell wall active agents on gram-negative bacteria.

### 8.2.4. Comparison of different dispersal methods according to PAE and CERT

In the present study *M. tuberculosis* cells were exposed to 0.2 ug/ml INH and 0.5 ug/ml RMP for 24 hrs and the two methods of quantitation of CERT and PAE were compared, ie Bactec and viable count. The post-antibiotic regrowth time (PART) determined in Bactec was redefined after considering what was discussed ealier and expressed in terms of CERT because it was determined in liquid medium and corresponded to CERT as defined by Hanberger et al. (1995). The actual PAE findings obtained in the present study determined by the viable count method as well as calculated regrowth indices extrapolated from

experimental data referred to by Mitchison (1998) are presented in Table 8.1 and will be discussed in the light of experience reported in the literature.

Table 8.1. Comparison of PAEs between three dispersion methods

| | | PAE in days following exposre to | | | |
|---|---|---|---|---|---|
| Dispersion method | Inoculum concentration | INH 0.2ug/ml | INH 1ug/ml | RMP 0.5ug/ml | RMP 0.2ug/ml |
| Sonication | ~ $10^6$/ml | -0.1 | - | 0.4 | - |
| Vortexing | ~ $10^6$/ml | -2.7 | - | -2.5 | - |
| Tween 80* | ~ $10^5$/ml | - | 4.6 | - | - |
| Tween 80* | ~ $10^7$/ml | - | - | - | 2.4 |

* Experiments by Mitchison and Dickinson (1971) performed in 7H9 broth with Tween 80 for dispersal of organisms and viable counting in terms of CFU/ml in a solid medium.

In both the mechanical dispersion methods regrowth appeared to be faster during the logarithmic growth phase in the exposed cultures compared with the control cultures accounting for the negative PAE findings (Table 8.1). The apparently slow growth of the vortexed control culture, 9.3 days (see Figure 7.7) vs 3.0 - 4.4 for the Tween 80 and sonication experiments to achieve a 10-fold increase in bacterial count (see Figure 8.1) contributed to a large extent to negative PAEs for both INH and RMP in the vortex experiments. It is clear from Figure 7.1 to Figure 7.3 that sonication gives a better dispersal of organisms than vortexing as evidenced by the consistently higher cfu counts obtained by the former method. The variation in count encountered by both methods was however considerable, illustrating the problem of clumping as a result of cord formation, which was not satisfactorily resolved with either method. Dickinson and Mitchison (1966) used a 7H10 Tween-albumin medium during exposure while following washing by filtration in a closed flask system, their viable counts were performed in medical flat-bottles containing silica gelatinized medium forming a 3mm deep layer on the floor of the bottles. The extent to which Tween 80 may interfere with various antimycobacterial agents is uncertain.

As the exposure concentrations of INH (0.2 ug/ml) and RMP (0.5 ug/ml) used in our experiments differ from those used by Mitchison and Dickinson (1971), the latter being 5 times higher in the case of INH (1ug/ml) and 2.5 times lower in the case of RMP (0.2 ug/ml), the PAEs obtained in these sets of experiments are not directly comparable (Table 8.1). Although other workers have found that, at least in the case of INH, the duration of pulsed growth inhibition and regrowth is both concentration- and time- dependent and is a function of the time-concentration product (Armstrong, 1960; Beggs & Jenne, 1969), these researchers also showed that in the case of INH, regrowth time was less affected by concentration than duration of exposure. Like them, we also found no increase in regrowth time when INH concentrations were increased from 0.2ug/ml to 1.0 ug/ml (see Table 7.6). Even in the case of RMP where regrowth time is clearly concentration dependent it is unlikely that differences in PAE between our studies and those of Mitchison & Dickinson (1971) can be explained entirely on methodological aspects such as different concentrations used and the presence of Tween 80 in the Mitchison & Dickinson's experiment, which may have affected dispersal of bacilli and drug penetration into bacterial cells. The most plausible explaination of the differences between the two sets of experiments is provided by the work of Hanberger et al. (1995) who, as discussed in section 8.2, showed that the effect of bactericidal activity, leading to a substantial reduction in bacterial counts during the drug-exposure period, resulted in disproportionately low PAEs compared with CERTs. In Mitchison & Dickinson's experiments there was virtually no bactericidal activity of INH during the 24h exposure period but a long lag period before regrowth started (See Figure 8.1), while in our viable count-based experiments, a definite reduction in CFU/ml was seen with a shorter lag period (See Figure 7.5 and Figure 7.7). Once regrowth starts, a small cohort of surviving bacteria can rapidly increase in number by $1\log_{10}$ CFU/ml. Similar trends were seen with RMP (See Figure 8.2, Figure 7.5 and Figure 7.7) except that a definite post-exposure reduction in CFU/ml was also evident in Mitchison & Dickinson's experiments.

The reasons for the longer lag periods in the latter experiments are unclear. One may speculate that the lag period may be enhanced by the presence of Tween 80 while failure of drugs to penetrate bacilli in clumps in our studies, where dispersal of clumps was suboptimal, may also have played a role. It is also possible that the anti-tuberculosis drugs in our studies may have altered cell wall surfaces of bacteria, without killing them but affecting their adherence properties as was demostrated in experimental endocarditis by Scheld et al. (1981). This could lead to better dispersal of tubercle bacilli in the drug-exposed cultures compared with the control cultures, resulting in artificially short PAEs and CERTs. At a more basic level, however, it is accepted that sublethal damage to bacterial cells is responsible for the lag period. It is possible that at higher concentrations and/or longer exposure times, irreversible damage with cell death is caused to the majority of bacterial cells and that the survivors are refractory to cell processes associated with delayed regrowth and therefore have the ability to immediately resume growth.

When growth rates of control cultures are compared with those of post-exposure cultures, and the lag periods are excluded (Table 8.2), it is clear that regrowth, once it starts, is approximately equal to that of the control culture (See RMP experiments by Mitchison & Dickinson, 1971) or slightly more rapid in the case of INH and RMP in our sonication experiments (See Table 8.2 and Figure 7.5, Figure 7.7, Figure 8.1 and Figure 8.2). In our vortexing experiments, however, growth appeared to be much slower, especially that of the control culture, again illustrating the poor dispersal of bacilli in clumps following vortexing.

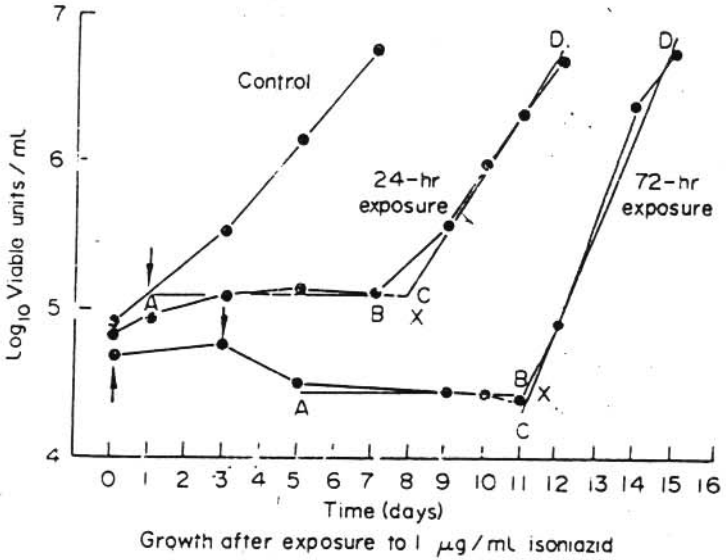


FIG. 1. Growth during and after exposure to 1 μg/ml isoniazid. ↑, Addition of isoniazid; ↓, removal of isoniazid by filtration.

Figure 8.1. Reproduced from Mitchison and Dickinson, 1971

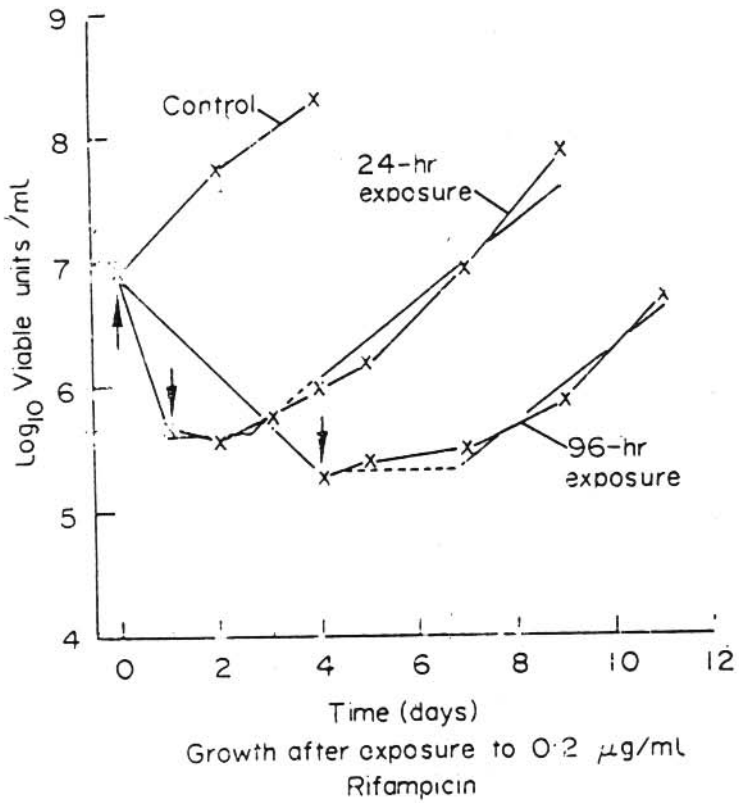


FIG. 2. Growth during and after exposure to 0·2 μg/ml rifampicin. ↑, Addition of rifampicin; ↓, removal of rifampicin by filtration.

Figure 8.2. Reproduced from Mitchison and Dickinson, 1971

Table 8.2. Time in days required for a $1\log_{10}$ increase in CFUs following sonication, vortexing and Tween 80 dispersal

| Type of exposure | Time taken (days) for $1\log_{10}$ increase in cultures from | | | |
|---|---|---|---|---|
| | Present study[1] | | Mitchison's studies[2] | |
| | Sonication | Vortexing | Tween 80 medium in | |
| | | | INH experiment | RMP experiment |
| Control cultures | 4.2 | 9.3 | 4.4 | 3.0 |
| Post-INH cultures | 3.7 (4.1)[3] | 5.0 (6.6) | 3.1 (9.0) | NA[4] |
| Post-RMP cultures | 3.4 (4.6) | 6.0 (6.8) | NA | 3.2 (8.2) |

[1] See Figure 7.5 and Figure 7.7

[2] See Figure 8.1 and Figure 8.2.

[3] Numbers in brackets denote regrowth times in days, including lag periods.

[4] NA = Not applicable

## 8.3. Comparison of effective regrowth time (ERT) and CERT findings in the three dispersal models

The ERT and CERT findings of the dispersal models used in the present study and that by Mitchison and Dickinson (1971) are given Table 8.3. ERTs and CERTs to reach the original inoculum size are given, $ERT_0$ and $CERT_0$, while those following regrowth to a level $1\log_{10}$ greater than the initial bacterial cell concentration of the control inoculum as tested as $ERT_{10}$ and $CERT_{10}$.

ERT determined by Bactec was not directly comparable with ERT obtained with other models. This was the case because the initial bacterial cell concentrations inoculated into Bactec 12B vials for regrowth were 40 times smaller than those of the exposed cultures ($2.5 \times 10^4$ CFU/ml vs $10^5$ CFU/ml). It is to be expected that regrowth to GI 500, which represents the bacterial cell concentration of the

drug-exposed cultures, will take longer than the regrowth of survivors, which are reconstituted in the same volume of regrowth medium than the original exposed culture, after filtration and washing, to reach a concentration which is the same or only 10-fold higher than the pre-exposed culture.

The data in Table 8.3 demonstrates that, with the exception of the $CERT_{10}$ findings which have already been discussed, the experiments involving mechanical dispersion (ours) and those of Mitchison & Dickinson (1971) involving Tween 80 are comparable, while the trends shown by these experiments were reflected in the Bactec model.

Table 8.3. Comparison between different inocula. Dispersal methods as assessed by control-related effective regrowth time (CERT) and effective regrowth time (ERT) indeces after 24h drug exposure.

| | Regrowth times in days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $ERT_0$[1] | | $ERT_{10}$[2] | | $CERT_0$[3] | | $CERT_{10}$[4] | |
| | INH ug/ml[5] 0.2 (1.0) | RMP ug/ml[5] 0.5 (0.2) | INH ug/ml 0.2 (1.0) | RMP ug/ml 0.5 (0.2) | INH ug/ml 0.2 (1.0) | RMP ug/ml 0.5 (0.2) | INH ug/ml 0.2 (1.0) | RMP ug/ml 0.5 (0.2) |
| Viable count, sonication ($5.3 \log_{10}$) | 2.2[6] | 3.0[6] | 5.8 | 7.1 | 2.2[6] | 3.0[6] | 1.8 | 3.1 |
| Viable count, vortex ($5.7 \log_{10}$) | 3.1 | 4.4 | 8.5 | 11.1 | 3.1 | 4.4 | 0.2 | 2.4 |
| Viable count, Tween 80 ($5 \log_{10}$, $7 \log_{10}$)[7] | $<2.0$[8] | 5.8 | 8.5 | 8.2 | $<2.0$[8] | 5.8 | 4.6 | 5.8 |
| Bactec sonication ($5 \log_{10}$)[9] | 5.4 | 5.7 | 6.4 | 6.8 | 1.7 | 2.0 | 2.2 | 2.6 |
| Bactec, vortex ($5 \log_{10}$)[9] | 5.2 | 5.6 | 5.9 | 6.4 | 1.6 | 2.0 | 2.0 | 2.5 |

1. $ERT_0$ = regrowth time to original inoculum concentration.
2. $ERT_{10}$ = regrowth time to $1 \log_{10}$ greater than original concentration.
3. $CERT_0$ = control-related $ERT_0$.
4. $CERT_{10}$ = control-related $ERT_{10}$.
5. INH and RMP exposure concentrations of 0.2ug/ml and 0.5 ug/ml, respectively except forTween 80 experiments by Mitchison & Dickinson (1971) when concentrations of 1.0 ug/ml for INH and 0.2 ug/ml for RMP were used.
6. $ERT_0$ and $CERT_0$ values are the same as regrowth end point concentrations which is the same as that of the original inoculum, ie. control = 0 days.
7. Inocula of 5 $\log_{10}$ and 7 $\log_{10}$ were used respectively for INH and RMP experiments.
8. Two days after exposure the viable count of the exposed culture was already higher than that of the original inoculum.
9. Exposure inoculum was 5 $\log_{10}$ but initial regrowth bacterial concentration was 1 in 40 dilution, resulting in 3.3979 $\log_{10}$ (2500) CFU/ml.

Our findings of the Bactec experiments suggest that regrowth to GI 500 will take slightly longer than regrowth in the viable count experiments. In the latter the post-exposure cultures were, after filtration and washing, reconstituted to the same volume of regrowth medium as the original exposed culture and were observed until the same or a 10-fold higher concentration than the pre-exposed culture was reached.

The findings in Table 8.3. again demonstrate, as was the case with PAE (Table 8.1), how differences in design and disposal procedures could lead to differences in ERT and CERT. These latter indices, however, exhibited less variation between the models than PAE and, in the case of $ERT_{10}$ the findings of the three models were roughly comparable. The ERT findings are also more in keeping than PAE with the way Mitchison & Dickinson (1971) reported their findings.

It is noteworthy that the ERT and CERT findings of our own viable count and Bactec experiments were comparable. Significantly, however, very little variation was found between CERTs obtained with the sonication and vortex dispersal experiments in the Bactec model.

CERT, as was the case with PAE, was longer in Tween 80 experiments of Mitchison & Dickinson (1971) compared with those of the present study, even if one allows for the different exposure concentration of INH and RMP. This can be directly linked to the pronounced lag periods before regrowth started in the Tween 80 studies. Similarly long lag periods were observed when the surface active agent Triton R WR1339, was used by Beggs and Jenne (1969). We are not aware of any similar studies in which either of these chemical dispersal agents was not used.

What was also noticed in the viable count method (Figure 7.5 and Figure 7.7) is that the RMP exposed cells of *M. tuberculosis* showed a rapid initial killing

compared with INH. The rapid initial killing can be attributed to the fact that RMP kills *M. tuberculosis* cells faster than INH. Although INH is more bactericidal than RMP when exposure is longer than 24h this is not shown in Figure 7.7 as the exposure time in those experiments was 24h. This also supports the concept on the sterilizing activity of RMP (Dickinson & Mitchison, 1977, Mitchison, 1992) in which it is assumed that there may be organisms lying for most of the time dormant within lesions which periodically metabolize for short periods, long enough for RMP but not INH to start killing.

Based on the abovementioned analysis there was significantly strong evidence to suggest that the Bactec model should be useful and give comparable results with previously described models while it is much simpler and safer to perform. It was therefore decided to study this model in greater detail and to use it in drug combination experiments.

### 8.4. Effect of drug concentration and exposure time

With regard to the effect of exposure time and drug concentration on regrowth in our Bactec model (Table 7.5 and 7.6), CERT was time dependent in the case of all four agents tested (INH, RMP, CIP and EMB). Previous studies have shown that lengthening the exposure time prolongs the duration of PAE in rapidly growing bacteria (Vogelman & Craig 1985). RMP in a concentration of 2.0 ug/ml had a PAE of approximately 3 days even when exposed for a short period of 2h and the duration of PAE increased with increasing time of exposure becoming about 14 days after a 96h exposure. Mitchison & Dickinson (1971) evaluated post-exposure regrowth involving RMP and other rifamycins in which RMP at a concentration of 0.2 ug/ml, pulsed for 2h, produced a delay in regrowth of 1.8 days after removal of the drug. After exposure times of 6, 10, 24, or 96h the periods of delay were 2 to 3 days long. Other *in vitro* studies of *M. tuberculosis* using 1ug/ml of RMP for 6, 24 and 96h showed that there was a period of depressed growth, starting immediately after removal of the drug lasting 2 to 3

days after 6 and 24h and up to 6 days after 96h exposure, after which the culture gradually returned to its full growth rate (Dickinson & Mitchison, 1987 (b)). These studies and the present study confirm that the postantibiotic regrowth activity of RMP is dependent on exposure time.

At a concentration of 0.2 ug/ml INH had a CERT of 1 day after exposure time of 24h but there was no delayed regrowth following exposure at 12h or less. The CERT increased with increasing time of exposure and it was 3 days after 96h of exposure (Table 7.5). This has also been demonstrated by Mitchison and Dickinson (1987 (b)). In their experiments the drug was removed by filtration after a pulse exposure, 2 ug/ml INH produced a 3- to 13-day delay in regrowth only after exposure to 24h but no delay in regrowth was observed at shorter exposure times. Our results are in accordance with these findings.

Both CIP and EMB demonstrated a CERT of 1 day at 24h of exposure and no CERT was found at 12h of exposure or less. The CERT of EMB at 10 ug/ml increased with time and was 3 days after 48h exposure. In experiments with *M. tuberculosis,* Pattisuta et.al. (1980) exposed the organisms to the same concentration of EMB and found minimal PAE after 24h of exposure but PAE was 6 days after 96h. The results of the present study therefore clearely correlate with those of Pattisupa et.al (1980).

Previous studies have demonstrated that the effect of INH in delaying regrowth is a time-concentration product (Amstrong, 1960; Beggs & Jenne, 1969). At the low concentrations of 0.2 ug/ml and 1.0 ug/ml used in our studies as opposed to 0.25 ug/ml to 16.0 ug/ml in the Amstrong (1960) experiments no concentration dependence of INH was demonstrated. If higher concentrations were used, concentration dependence could possibly have been established. Although we used 1.0 ug/ml of INH which is more than 40X the INH MIC in Bactec, other studies have been conducted at concentrations of 1 ug/ml and 2ug/ml and even as high as 32 ug/ml (Mitchison & Dickinson, (1971);

Amstrong, 1960; Amstrong,1965). RMP was concentration dependent with 0.5 ug/ml and 2.0 ug/ml giving a CERT of 3 and 7 days, respectively. In our experiments, post-exposure regrowth of EMB is not dose dependent and this was also demonstrated by Pattisapu et.al (1990). *M. tuberculosis* exposed for 24h either to 1 or 10ug/ml of EMB showed only minimal antimycobacterial activity. In our study EMB at the concentrations of 2.5, 5.0 or 10 ug/ml had a CERT of 1 day after 24h of exposure, which is also minimal and does not show any concentration dependence. There are no reports in the literature of PAE studies on fluoroquinones against *M. tuberculosis*. Our study showed some evidence of concentration dependence in the case of CIP as concentrations of 1, 2, and 4 ug/ml, exhibited CERTs of 1, 2, 2, days, respectively.

When the results of time and concentration dependence are combined, RMP appears to be ideally suited for intermittent therapy because it is dependent on both time and concentration such that the right doses and the right time can be selected based on this data. These results correlate with those obtained in experiments with guinea pigs (Dickinson & Mitchison 1987) and mice (Grosset et. al, 1992). When RMP was used alone, intermittent treatment twice a week was more effective than daily treatment when the drug was given at higher dosage. However, higher once weekly regimens were ineffective. Because of the concentrations achieved and maintained in patients INH is also ideally suited to intermittent therapy (Mitchison & Dickinson, 1971).

### 8.5. Drug combination studies

The Bactec method was used successfully to determine CERTs of drug combinations and to compare these with CERTs of single drugs to demonstrate synergy, addition, indifference and antagonism. These terms have previously been used by Gudmundsson et al. (1991) to determine PAE induced by antimicrobial combinations against strains of *E. coli*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa*. In vitro studies of the PAE/CERT of drug combinations have

the potential of being a useful tool in the spacing of doses of drugs in combination used in the treatment of tuberculosis. This can improve patients' compliance which is in many instances affected by the bulk of drugs taken daily. Ideally the relevance and validity of the in vitro drug combination studies should be confirmed in animal studies. A neutropenic mouse thigh-infection model has been devised (Volgeman et al., 1988) and could possibly be used with *M. tuberculosis* and antituberculosis drugs.

Previous experiments on short course preventive chemotherapy for tuberculosis in mice have found antagonism between INH and the combination RMP-PZA during both the initial and continuation phase of antituberculosis treatment . However, pharmacokinetic analysis of these drugs suggested that the pharmacological interaction between INH and RMP was very likely to be involved in the mechanism of antagonism (Grosset et.al, 1992; Dickinson & Mitchison, 1977). Our in vitro CERT findings involving the INH-RMP combination suggested an additive effect and Dickinson & Mitchison (1977) reported an indifference effect of this combination on *M. tuberculosis* cultures and antagonism between the combination RMP-EMB. In our study the combination RMP-EMB was synergistic, an effect which was also demonstrated against *M. avium* complex strains in a Bactec growth inhibition model by Hoffner et al. (1981). The combination of INH-EMB was also additive but it was reported to be synergestic earlier (Dickinson & Mitchison, 1977). Our results indicate an indifferent CERT effect for the RMP-CIP combination while the INH-CIP combination proved to be synergistic. These findings suggest that further experiments including in vivo studies, are required to evaluate combinations involving CIP.

### 8.6. Concluding remarks

The susceptibility tests, including MICs and MBCs recorded in this dissertation were performed to introduce the author into the technological field of

mycobacteriology presently used world-wide. The findings correlated well with those reported in the literature.

In the post-exposure regrowth experiments, attempts were made to clarify differences between published models designed to study the post-exposure regrowth phenomenon. A Bactec-based model was then used to study ERT and CERT and the findings were compared with those of earlier studies. To achieve this it was necessary to reanalyse and interpret the findings of other workers in the field of tuberculosis in terms of ERT and CERT. It was concluded that our model demonstrated similar trends to those published before. As the Bactec regrowth model was shown to be less affected by problems related to mechanical dispersion of tubercle bacilli, and was safer, it was used in further experiments to study dose and exposure time effects and drug combinations.

# APPENDIX

In this section the preparation of media, working solutions and the inocula will be outlined. The procedures performed on the BACTEC-460 machine before the use of this instrument will described.

## APPENDIX A

### GROWTH MEDIA

***LJ slopes***

The LJ slopes were prepared as described earlier (Kleeberg et al. 1987).

Ingredients

Mineral salt solution
2 % malachite green solution (aqueous)
Beaten, whole hen's eggs

Preparation of mineral salt solution

| | |
|---|---|
| Potassium dihydrogen phosphate, $KH_2PO_4$ | 2.4g |
| Magnesium sulphate, $MgSO_4.7H_2O$ | 0.24g |
| Magnesium citrate | 0.6g |
| Asparagine | 3.6g |
| Glycerol | 12ml |
| Distilled water | 600ml |

The ingredients were dissolved by heating and autoclaved at 121°C for 25 minutes. This solution was stored at room temperature.

Malachite green solution

| | |
|---|---|
| Malachite dye | 2.0g |
| Sterile distilled water | 100ml |

Using aseptic techniques, the dye was dissolved in sterile distilled water by placing the solution in the incubator for 1 - 2 hours and it was stored at room temperature.

Beaten whole eggs

The eggs were washed thoroughly in warm water with a brush and a plain alkaline soap. After draining off the water, the eggs were allowed to dry. The eggs were cracked with a sterile knife into a sterile beaker and beaten with a sterile electric waring blender.

Preparation of complete medium

The following ingredients were aseptically added in a large sterile flask and mixed well:

| | |
|---|---|
| Mineral salt solution | 600ml |
| Malachite green solution | 20ml |
| beaten whole egg | 1000ml |

Using a sterile 5ml dispenser, the medium was distributed in 5ml amounts in sterile McCartney bottles. The bottles were closed tightly, placed in horizontal racks and put in the inspissator. The medium were inspissated at 80°C for 45

minutes and allowed to cool at room temperature. The media were stored at 4°C or used immediately.

***Middlebrok 7h9 broth***

Materials

| | |
|---|---|
| 7H9 broth base | 0.94g |
| Sterile distilled water | 180ml |
| ADC | 20ml |

The 7H9 broth was prepared according to the manufacturer's instructions but in final volumes of 200ml instead of 1000ml. Using a 250ml measuring cylinder, the 7H9 broth base was dissolve in distilled in a 250ml conical flask. This was sterilised by autoclaving at 121°C for 10 minutes and cooled to about 45°C. The broth was supplemented with ADC to make a final volume of 200ml.

Sterility was checked by incubating the medium at 37°C overnight. The medium was allowed to cool to room temperature and used immediately or stored at 4°C for not more than 7 days.

***Middlebrook 7H10 agar***

Material

| | |
|---|---|
| 7H10 agar base | 3.8g |
| Sterile distilled water | 180ml |
| ADC | 20ml |
| Glycerol | 1ml |

The 7H10 agar was also prepared according to the manufacturer's instructions also in the final volume of 200ml. The 7H10 agar base was dissolved in sterile distilled water containing glycerol and autoclaved at 121°C for 10 minutes. It was allowed to cool to about 55°C in a water bath and ADC was added to make a final volume of 200ml.

The media were allowed to solidify and incubated at 37°C overnight to check for sterility. The media were used immedietely or stored at 4°C for not more than 7 days.

## APPENDIX B

### WORKING SOLUTIONS

***Stock solutions of drugs***

The stock solutions of drugs were prepared in volumes of 100ml. The final drug concentrations were calculated by adjusting the potency and calculating using the formula :

$$\text{Weight} = \frac{\text{volume X concentration}}{\text{potency}}$$

Drugs were weighed and diluted (using the specified diluents) as follows:

| Drug | Potency | Amount (mg) | Diluent (ml) |
|---|---|---|---|
| INH | 100 | 100 | 100ml $dH_2O$ |
| RMP | 100 | 100 | 75ml $dH_2O$ + 25ml ethanol |
| CIP | 100 | 100 | 100ml $dH_2O$ |
| EMB | | 140 | 100ml $dH_2O$ |
| SM | | 130 | 100ml $dH_2O$ |

The final concentration of each stock solution was 1000 ug/ml.

Using a filter with a pore diameter of 0.22um, the drugs were filtered into small aliquots and stored at -70$^{0}$C.

***Barium sulphate suspension***

This suspension is used to facilitate the standardization of the inoculum and corresponds to the McFarland No. 1 standard which is equivalent to about $10^{7}$ cfu/ml.

Ingredients

| | |
|---|---|
| 1 % barium chloride ($BaCl_2$) | 0.1ml |
| 1 % sulphuric acid ($H_2SO_4$) | 9.9ml |

The two solutions were combined, mixed well and distributed into a test tube. The tube was tightly sealed with a rubber stopper and kept at room temperature.

***Phosphate buffered saline (PBS)***

PBS was prepared as described early (Sambrook et al., 1989)

Ingredients

| | |
|---|---|
| NaCl | 8g |
| KCl | 0.24g |
| $Na_2HPO_2$ | 1.44g |
| $KH_2PO_4$ | 0.23g |
| HCl | |

The ingredients were dissolved in 800ml water and the pH was adjusted to 7.4 using HCl. Water was added to make a volume of 1 litre. The solution was dispensed into 200ml aliquots and sterilized by autoclaving at 121$^{0}$C for 20 minutes. It was stored at room tempeture.

## APPENDIX C

### INOCULA

**Inoculum with a turbidity which equals to McFarland No.1 standard**

The inoculum was prepared according to the NCCLS standards.

Material

Test tubes containing about 8 to 10 glass beads
Test tubes without glass beads
Vortex mixer
Biosafety cabinet
McFarland No. 1 standard
White paper with black contrasting lines
Bunsen burner

Method of preparation

Bacteria were grown on LJ slopes for 14 days to obtain log phase cultures. Representative colonies were scraped from LJ and transferred to 4 to 5 ml 7H9 broth containing glass beads. The culture was vortexed for about 1-2 minutes and the larger particles were allowed to settle for 30 - 45 minutes. The supernatant was removed and added to a test tube without beads. The turbidity

was adjusted, using fresh 7H9 broth, under the white background with contrasting black lines, to equal to that of a McFarland No 1 standard.

## APPENDIX D

### BACTEC 460 machine procedures performed during or before the use of the instrument

#### *Performance test*

This test was done daily before testing the Bactec vials to ensure that the Bactec 460 machine is working well. A performance kit is supplied which consist of one vial of 11A $^{14}C$ solution containing 1 uCi of $^{14}C$ radioactive material in 20ml of bicarbonate solution and three vials of 11B test acid each containing 10ml of 2N hydrochloric acid solution.

Using a 1ml tuberculine syringe, 0.2ml of the standard $^{14}C$ solution was inoculated into one of the three test acid vials. The vial was shaken vigorously for about 10 seconds to liberate known amount of $^{14}CO_2$. The vial was tested on the Bactec 460 instrument and a GI reading of 40 to 65 was said to be normal and if outside this range, the procedure was repeated using the same vial. If it was still outside the range this was an indication that something was wrong with the machine and that was taken care of.

#### Initial testing of the 12B medium

The Bactec 12B vials were tested on the Bactec 460 instrument before use to eliminate contaminated vials. The vials were said to be contaminated when the GI reading was 10 or more. The testing of vials was also performed to establish a $CO_2$ enriched atmosphere in the vial.

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