AN INVESTIGATION INTO THE MODE OF ACTION OF PYRAZINAMIDE ON *Mycobacterium tuberculosis*

by

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Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Medical Microbiology in the Faculty of Medicine at the University of Natal-Medical School

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DECLARATION

I declare that this dissertation is my own work. It is being submitted for the degree of Doctor of Philosophy to the University of Natal-Medical School, Durban. It has not been submitted before for any degree or examination to any other university.

R. M. Dwarka

Date: August 1999

This work is dedicated to my mother

Mrs K. Dwarka

for her constant support and motivation

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List of Abbreviations

ADC: albumin dextrose catalase

AIDS: acquired immune deficiency syndrome

AraLAM: arabinose lipoarabinomannan

BCG: Bacille Calmette-Guerin

bp: base pair

BSA: bovine serum albumin

CFA: complete freud's adjuvant

CFU: colony forming units

CLA: clarithromycin

5-Cl-PZA: 5-chloro pyrazinamide

CO_2: carbon dioxide

CR: complement receptor

CSF: cerebrospinal fluid

D: daltons

DMF: N,N-dimethyl formamide

DNA: deoxyribonucleic acid

DOT: directly observed therapy

DTH: delayed type hypersensitivity

dTTP: deoxythymine triphosphate

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

FDA: Food and Drug Administration

HIV: human immunodeficiency virus

HPLC: high performance liquid chromatography

IFA: incomplete freud's adjuvant

IFN-γ: interferon γ

IgG: immunoglobulin G

IL-4: interleukin

INH: isoniazid

iNOS: inducible nitric oxide synthase

IUATLD: International Union Against Tuberculosis and Lung Disease

*kat*G: catalase peroxidase gene

KLH: keyhole limpet haemocyanin

LAM: lipoarabinomannan

LCR: ligase chain reaction

LJ: Lowenstein Jensen

LPS: lipopolysaccharide

ManLAM: mannose lipoarabinomannan

MBC: minimal bactericidal concentration

MDR: multi-drug resistant

MHC: major histocompatibility complex

MICs: minimum inhibitory concentrations

MMRc: macrophage mannose receptor

nPPA: n-propyl pyrazinoate

OADC: oleic acid albumin dextrose catalase

OFL: ofloxacin

5-OH-POA: 5 hydroxy pyrazinoic acid

5-OH-PZA: 5 hydroxy pyrazinamide

PARA: paracetamol

PAS: para-aminosalicylic acid

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFGE: pulsed field gel electrophoresis

POA: pyrazinoic acid

PPD: purified protein derivative of tuberculin

PZA: pyrazinamide

Pzase: pyrazinamidase

PZU: pyrazinuric acid

RFLP: restriction fragment length polymorphism

RNA: ribonucleic acid

RNI: reactive nitrogen intermediates

ROI: reactive oxygen intermediates

SAMSA: S-acetyl mercaptosuccinic anhydride

SDA: strand displacement assay

SMCC: N-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1- carboxylate

TEM: transmission electron microscopy

TGF: transforming growth factor

TMA: transcription mediated amplification

TNF: tumor necrosis factor

TST: tris-saline buffer supplemented with tween 20

TST: tuberculin skin test

UNG: uracil-N-glycosylase

WHO: World Health Organistion

ZN: Ziehl-Neelsen

Abstract

Tuberculosis remains a major public health problem, claiming three million deaths annually in developing countries; the resurgence of the disease in industrialized countries and the emergence of multi-drug resistant mycobacteria have renewed interest in understanding the mechanisms of drug resistance in these pathogens. The acid-fast bacillus *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). This infection is the leading cause of mortality in adults due to an infectious agent and accounts for 26% of all preventable adult deaths globally. Pyrazinamide (PZA), one of the antituberculous drugs has a special place in therapy in that it appears to kill a population of semi-dormant tubercle bacilli that are not affected by other antituberculous drugs, shortening the duration of treatment from the earlier norm of 12-18 months to the current standard of 6 months. However, the exact mode of action of PZA is unknown. A commonly accepted hypothesis is that PZA susceptible *M. tuberculosis* strains produce pyrazinamidase (Pzase), which hydrolyses PZA to the antibacterial moiety, pyrazinoic acid (POA). It is not clear whether POA has specific antimicrobial activity or that the inhibition of growth caused by POA is due to its ability to lower the pH of the environment below the limits of tolerance of *M. tuberculosis* growth.

This study attempted to address the question of the mode of action of PZA in macrophages as well as in cultures free of macrophages *i.e.*, non-cell associated free *M. tuberculosis* exposed to 100 μg/ml PZA. The survival rate was assessed by counting colony forming units (CFUs). PZA had no bacteriocidal effect on susceptible and resistant strains that were macrophage bound, nor on non cell associated mycobacteria. Intracellular multiplication was observed after 72 h in macrophages infected with *M. tuberculosis*, but not subjected to any PZA. Concurrently high performance liquid chromatography (HPLC) was used to assess intracellular, extracellular and intrabacterial PZA and POA concentrations. This methodology was developed to measure POA because this was so far impossible. Fractions were separated by silicone oil density gradient. Methanol and phosphate buffer (20%:80%) was the mobile phase and the detection was done at UV of 265 nm.

Pzase positive mycobacteria, kept in RPMI 1640 without macrophages did take up PZA and there was also POA detected in these bacteria. PZA also entered Pzase negative mycobacteria but no POA was detected. No PZA or POA could be detected in mycobacteria in macrophages over the 72h period studied. While PZA did enter the cells, no POA could be detected in cells infected with Pzase positive or negative mycobacteria. The amount of intracellular PZA was lower when the cells were inoculated with Pzase positive mycobacteria as compared to uninfected macrophages and macrophages infected with Pzase negative strains.

To further quantify and resolve the location of action of PZA, immunocytochemistry was used. However, commercial PZA antibodies are not available, thus these were raised in rabbits using the carrier keyhole limpet haemocyanin (KLH). Purified antibodies were used in gold labelling experiments where macrophages were infected with *M. tuberculosis* and the non cell associated mycobacteria were exposed to 100 µg/ml PZA for 48h. The cultures were prepared for TEM and sections were stained with PZA antibodies and counter stained with gold labelled IgG. Gold label was quantified using a Zeiss Kontron 300 Image Analyser. Most gold label was located in the non cell associated mycobacteria followed by the macrophage bound mycobacteria. Within the macrophage, the membrane bound mycobacteria had the highest label density, followed by the cytoplasm and nucleus. Although no clear distinction can be made between PZA and POA, the high accumulation of PZA label in the internalised mycobacteria could suggest that PZA acts on the mycobacteria itself.

This study shows that PZA or POA concentrates in mycobacteria inside macrophages. However, the methodologies used were unable to clarify whether the drug is present as PZA or POA or both in Pzase positive strains. The drug concentrates in both Pzase producing and non Pzase producing bacteria, even in macrophages that themselves take up only a limited amount of PZA.

INTRODUCTION

This thesis is presented in 7 chapters. Chapters 2-6 represents separate aspects of the study but collectively address the mode of action of PZA. Each chapter commences with an introduction which includes the objectives, followed by the methodologies used, the results obtained and the discussion. Each of the above chapters is presented according to the format outlined by the university. Chapter 1, the literature review places the study in perspective while chapter 7, the general discussion reconciles the differences of each chapter and concludes the study. This gives a clear understanding and logical approach to dealing with all aspects of the highly complex nature of the basic science methodologies undertaken. Summarising all methodologies in one chapter, following by a summary of all results and then discussion would make understanding the thesis difficult since a wide array of aspects are dealt with in this thesis and they need to be presented separately to enable a clear understanding of the basic science research undertaken.

Tuberculosis is back on the world stage with a vengeance perhaps at least partly as a consequence of complacency about the threat it posed in the recent past. A number of historical sources have documented that much of the currently developed world was, in the past, subject to a epidemic of tuberculosis that ran its course over a period of several centuries. It seems likely that one of the main causes of this epidemic was the process of urbanisation, industralisation, the accompanying stresses and strains imposed by overcrowding, unemployment and the associated social disruption. Long before the available chemotherapy it was apparent that the mortality associated with the tuberculosis epidemic was declining. In the opinion of some researchers this decline also antedated any improvement in the precipitating poor socio-economic factors, suggesting that the elimination of susceptible individuals had created a population with increased resistance to tuberculosis. On its introduction into a susceptible population, tuberculosis spreads rapidly and have obviously evolved mechanisms for ensuring their survival in the intracellular phagocytic host cell environment (Beyers *et al*, 1996).

Globally, the World Health Organisation (WHO) estimates that about 8 million new cases of tuberculosis occur, and that there are about 3 million deaths from tuberculosis, each year (Sudre *et al*, 1992-cited by De Cock and Wilkinson, 1996). The situation is so grave that the WHO has declared tuberculosis to be a global public health emergency (WHO-cited by De Cock and Wilkinson, 1996). With the current HIV pandemic, the tuberculosis situation (in Africa in particular) is very serious; case rates have doubled in some African countries. In central Africa more than 70% of adults with tuberculosis may be HIV positive (De Cock *et al*, 1992-cited by De Cock and Wilkinson, 1996).

South Africa's tuberculosis problem reflects that of the global community. More cases of tuberculosis than any other disease are reported annually in South Africa. There were about 82 500 cases in 1992, with an overall incidence of about 250/100 000. The basic epidemiology of tuberculosis in South Africa is well described; for example, incidence rates are highest in the coloured population in the Western Cape (700/100 000). South Africa is suffering an explosive HIV epidemic, with KwaZulu-Natal hardest hit. At Hlabisa Hospital, a typical rural district hospital in the northern part of the province, 35% of adults diagnosed with tuberculosis in mid-1993 were HIV positive (The HIV seroprevelence in women attending antenatal clinics in Hlabisa district was 7.9% at that time) (Department of National Health and Population Development, 1995-cited by De Cock and Wilkinson, 1996).

The most common agent of pulmonary mycobacteriosis is the acid-fast bacillus *Mycobacterium tuberculosis* which is one of the slow growing, non-pigmented mycobacteria (Wayne and Hawkins, 1988). The development of pulmonary tuberculosis from its onset to its various clinical manifestations may be pictured as a series of battles between host and invader. The inhaled bacillus may multiply, or it may be eliminated by alveolar macrophages before any lesions are produced. Small caseous lesions may progress or may heal or stabilize before they are detectable by radiography. Larger caseous lesions may also heal or stabilize, or they may grow locally and shed bacillus into the blood and lymph. Alternatively, caseous lesions may liquefy and introduce bacilli and their products into the bronchial tree, which makes arrest of the disease more

difficult. Thus, each successive battle is won by the host with increasing difficulty. Also, within a given lesion, the battles between the host and the bacillus are fought almost independently of the battles being fought within the other lesions (Dannenberg and Tomashefski, 1988).

It is postulated that in a patient with pulmonary tuberculosis there are three different populations of mycobacteria; those located extracellularly in open cavities, those located intracellularly in macrophages and those in closed caseous lesions. Organisms in each site have different metabolic activities and rates of replication (Wayne and Hawkins, 1988). The signs and symptoms associated with pulmonary tuberculosis can be caused by a number of infectious agents in addition to *M. tuberculosis*. These include a variety of species of mycobacteria, members of the genus Nocardia and fungi such as *Histoplasma capsulatum* and *Coccidioides immitis*. A definitive diagnosis cannot be made without data from the bacteriology laboratory (Wayne and Hawkins, 1988).

Tuberculosis has been a therapeutic challenge throughout recorded history and has earned the epithet " Captain of All Men of Death" (Howard and Damato, 1987). The era of specific chemotherapy began in 1944 with the isolation of streptomycin by Selman Waksman. Streptomycin together with isoniazid, rifampin, ethambutanol and pyrazinamide are the first line antituberculous drugs used in chemotherapy (MacGreyor, 1988). Pyrazinamide (PZA) is a synthetic pyrazine analogue of nicotinamide. It exerts bactericidal activity only in an acid environment, but this enables it to kill tubercle bacilli inside monocytes and has become an important component of short-course therapy, thus listed as a first line agent (MacGreyor, 1988). Konno *et al*. (1967) proposed the following mechanism of action of PZA to account for the need for an acid environment; susceptible *M. tuberculosis* strains are known to produce the enzyme pyrazinamidase (Pzase), which converts PZA to pyrazinoic acid (POA). It was suggested that it is this enzyme generated product, POA, that has high antibacterial activity in an acid environment, whereas PZA itself has no activity at all. Strains that are resistant to PZA do not produce Pzase, thus do not convert PZA into POA and are, therefore, not vulnerable to the antibacterial activity of POA (Heifets *et al*, 1989).

The difficulty in performing PZA susceptibility tests is due to the fact that an acid pH of the medium is required to demonstrate activity of the drug and *M. tuberculosis* strains do not grow well at pH 5.5 (Butler *et al*, 1983; Konno *et al*, 1967). The incorporation of enrichment, using oleic acid-albumin-dextrose-catalase (OADC) into 7H10 agar is necessary for optimal growth of *M. tuberculosis* at acid pH (Stottmeier *et al*, 1967), however, oleic acid in an acid medium is often inhibitory to many strains of *M. tuberculosis* (Tummon *et al*, 1975). During routine determinations of PZA susceptibility, it was noted that the growth response of *M. tuberculosis* at pH 5.5 appeared better with certain lots of albumin-dextrose-catalase (ADC), even though none of these preparations contained added oleic acid (Butler and Kilburn, 1982).

Salfinger *et al.* (1990) investigated PZA and POA activity against tubercle bacilli in cultured human macrophages and in the Bactec system. The virulent Erdman strain was used with every batch of human macrophages as an internal standard. During the 7 day period, the tubercle bacilli multiplied by a factor of 37. PZA was inhibitory at the lowest concentrations tested (25 mg. ml^{-1}). However, even at 800 mg. ml^{-1} the drug only slowed intracellular bacillary growth and was not bactericidal.

In another experiment by Salfinger *et al.* (1990), it was found that the Erdman strain grew more between days 4 and 7 than between days 0 and 4. This suggested that PZA might be losing effectiveness by being metabolised. Additional drug equal to the initial concentration was added daily to the culture medium e.g. 800 mg. l^{-1} became 5 600 mg. l^{-1} by day 7. Despite the large amounts of PZA, no significant decrease in tubercle bacilli was observed during the first 4 days and only minimal decline by day 7.

In the Bactec system there was inhibition of the Erdman strain proportional to the PZA concentration up to 400 mg. l^{-1}. Activity of PZA or POA in the Bactec system is not dependent on drug penetration, as it is in the macrophage model. POA was active in the Bactec system but not in cultured macrophages, whereas PZA which can readily enter cultured macrophages, was active in both (Salfinger *et al*, 1990). These researchers also found that the cultured macrophage must contribute actively or passively to effectiveness of PZA such as through the proposed mechanism of low pH generated by

Pzase in the phagolysosomes. A purely in vitro test such as the Bactec system can serve as a convenient tool for evaluating antimycobacterial drugs, but the cultured macrophages system may more closely approximate *in vivo* reality (Salfinger *et al*, 1990).

Although there are numerous studies on the effect of PZA on mycobacteria, the exact mode of action has not been elucidated, although the mechanism of action proposed by Konno *et al.* (1967) is widely accepted. This study was undertaken to provide further insight into the mode of action of PZA. The objectives of this study were to:

(1) assess the effect of PZA/POA on macrophage bound and non cell associated mycobacteria by colony counts

(2) measure PZA/POA concentrations in lysed macrophages (infected with Pzase positive and negative strains) and extrabacterially in non cell associated mycobacteria

(3) to assess whether the disruption of the mycobacteria contributes towards a significant increase in the concentrations of PZA and POA detected prior to their disruption

(4) identify site of location of PZA in macrophages and in non cell associated mycobacteria

Chapter 1 is the extensive literature review which sets the 'scene' for the other chapters. Chapter 2 is an introductory chapter to the experiments undertaken to understand the mode of action of PZA. It addresses objective 1 and comparison is made to other similar studies. Prior to addressing objective 2, the HPLC methodology had to be developed for POA. Although published reports show good resolution for PZA, POA was not well resolved into a clear chromatograph. These events are extensively discussed in chapter 3. Chapter 4 gives an account of all HPLC results obtained by measuring PZA and POA concentrations using different mycobacterial strains. Immunocytochemistry was then used in an attempt to further quantify PZA and POA in both systems. Since gold labelled PZA antibodies are not commercially available, chapter 5 discusses the production of these antibodies and chapter 6 gives an extensive account of all the gold labelling experiments with the various strains. Finally chapter 7, the general discussion sums up the thesis in its entirety.

CHAPTER ONE

LITERATURE REVIEW

1.1 MEDICAL IMPORTANCE OF TUBERCULOSIS

Adult tuberculosis is a disease of two worlds: no longer is it strictly divisible into developed and developing countries but rather by ethnic differences (Davies, 1996). The white population of western Europe, America and Australia have very low incidences of disease, between 5 and $15/10^5$/annum (Nisar *et al*, 1992).

Among ethnic minority groups in developed countries, rates are nearer $100/10^5$/annum and the disease is as common in young adults as it was in the white population of Europe 50 years ago. In developing countries where the incidence of infection is already high, in young adults the advent of human immunodeficiency virus (HIV) has resulted in rates of disease exceeding $1000/10^5$/annum (Nisar *et al*, 1992, Hass *et al*, 1994). The greatest impact of the interaction between HIV infection and tuberculosis has been in Sub-Saharan Africa where HIV infection has penetrated widely and tuberculosis is highly prevalent. As the HIV epidemic spreads rapidly in Asia it is likely that an even greater problem will occur in the Indian Sub-continent in the next decade (De Cock *et al*., 1992).

The prevalence of tuberculous infection in children is related to the number of smear positive adults in the community. Development of tuberculosis disease depends on age, nutrition, socio-economic circumstances, poverty, intercurrent infection, host's immune response doses of organisms (Coulter, 1996). The incidence of tuberculosis in children in developing countries is difficult to estimate because of problems in making diagnosis. Often the only guide is the number of children referred to tuberculosis units for treatment. A recent estimate of the incidence of tuberculosis in 0-14 years age group from 1990-2000 showed that the rate per 10^5 would increase from 14 to 142 in Africa. Although, in the other areas the total number of cases would increase due to increasing population, the rate per 10^5 would be static or fall. In

Sub-Saharan Africa, one major cause of this increase is the co-infection of HIV and tuberculosis in the adult population (Dolin *et al*, 1994).

1.1.1 HISTORICAL PERSPECTIVE OF THE DISEASE

The history of mycobacterial disease is divisible into three eras: those of the ignorance, hope and enlightenment. The era of ignorance lasted from the dawn of recorded history until the discovery of the causative organisms in the latter part of the nineteenth century. The era of hope lasted from then until the introduction of effective therapy around 1950. In the present era, that of enlightenment, we are acquiring detailed knowledge of the mycobacteria and the disease they cause. Can we hope that this will enable us to enter the fourth and final era?-that of conquest (Grange, 1988).

Tuberculosis is an ancient disease, the presence has been referred from early as 4000 BC from Egyptian tomb paintings and by examination of mummies for spinal tuberculosis (Cave, 1939-cited by Hart *et al*, 1996). Tuberculosis may have emerged during neolithic times when human populations increased and aggregated (Manchester, 1984-cited by Hart *et al*., 1996.) Tuberculosis was present in America prior to the arrival of Columbus (Ritchie, 1952 and Pfeiffer, 1984 - cited by Hart *et al*, 1996). The earliest evidence of tuberculosis in Britian comes from Roman times. Tuberculosis was well described by the time of Hippocrates and Aristotle in fifth century before the birth of Christ (BC) when it was described as phthisis, which was translated into English as consumption (Stirland and Waldron, 1990 - cited by Hart *et al*, 1996).

The turning point in the history of tuberculosis occurred at a meeting of the Berlin Physiological Society on the evening of 24 March 1882, when Robert Koch described the isolation of the causative organism of the disease. This was eight years after Armauer Hansen published his observation of the leprosy bacillus in lepromatous nodules, however, Hansen's work did not receive the adulation accorded to Koch as

he was unable to isolate the organism in pure culture. The germ theory of communicable disease by Louis Pasteur was clearly established and in particular by the experimental demonstration of the transmissibility of tuberculosis in rabbits by Jean Antoine Villemin, a French military surgeon in 1868. Koch's discovery heralded the era of hope. Much work was required before practical benefits were to be reaped from the discoveries of Hansen and Koch. Such work started rapidly in three main directions: isolation and culture of the bacillus for diagnostic purposes, the search for an effective cure, and the development of a vaccine (Grange, 1988).

Koch's discovery coincided with the birth of the discipline of immunology, however, he did not regard himself as an immunologist. When Metchnikoff demonstrated the phenomenon of phagocytosis, Koch remarked " I am a hygienist and it is of no interest to me where the microbes are, whether inside or outside of the cells. He attempted to develop an agglutination test for tuberculosis using the whole bacillus as antigen and also tried to attenuate a human strain for use as a vaccine for tuberculosis in cattle. However, his main interest was the development of a cure for tuberculosis which led to the development of old Tuberculin which was a filtrate of a broth culture which was concentrated by evaporation in a heated waterbath. A series of experiments in guinea pigs led to the description of the necrotic hypersensitivity reaction termed the Koch Phenomenon, which was caused by impurities derived from the medium (Grange, 1988).

Siebert (1934 - cited by Grange, 1988) tried to overcome this problem by isolating tuberculoproteins by precipitation with acetone and ammonium sulphate which resulted in purified protein derivative of tuberculin (PPD). However, PPD, despite the name is not a pure protein and suffers from several disadvantages. Its mode of preparation involves prolonged cultivation of the tubercule bacillus to enable antigens to be released by autolysis; heating to 100°C to kill the bacillus and then precipitation of the protein , which caused denaturation of protein. Stanford and his colleagues (1975), produced the " New Tuberculins", the first been Burulin, prepared for studies on Buruli ulcer in Africa. These reagents were prepared by harvesting mycobacteria during the growing phase from non-antigenic media, washing thoroughly, disrupting

the cell mass in an ultrasonicator, sterilizing by repeated membrane filtration and diluting to a suitable protein concentration.

A vaccine was eventually produced by Calmette and Guerin after passaging a supposedly bovine tubercle bacillus 230 times on potato slices soaked in bile and glycerol over a period of 13 years. This vaccine Bacille Calmette-Guerin (BCG) was first used in 1921 as oral vaccine for infants. The next milestone in the history of mycobacterial disease occurred in the mid-twentieth century with the first effective drugs (Grange, 1988).

1.1.2 *M. tuberculosis*: THE CAUSATIVE AGENT OF TB

The generic name *Mycobacterium* was introduced by Lehman and Neuman in the first edition of their Atlas of Bacteriology published in German in 1896. At that time the genus contained only two species, *Mycobacterium tuberculosis* and *Mycobacterium leprae*. The name *Mycobacterium* meaning fungus-bacterium was derived from the mould-like pellicular growth of the tubercle bacillus on liquid media (Grange, 1988). The mycobacteria are acid fast, aerobic, non-spore forming, non-motile bacteria, slightly curved or straight bacilli, 0.2 to 0.6 by 1.0 to 10 μm in size. They have cell walls rich in lipid content, which is responsible for the ability of the organism to withstand staining by usual aniline dyes (Roberts *et al*, 1991).

The growth rate for the mycobacteria is slow and requires 2 to 8 weeks or longer for detection by use of traditional mycobacteriology media. They can be detected much earlier with the use of a radiometric system that detects the metabolism of a ^{14}C labelled substrate and detection of the evolution of ^{14}C labelled CO_2 as a metabolic by-product of the organism (Middlebrook *et al*., 1977).

1.1.2.1 Ultrastructure of the cell

The mycobacteria consist of cytoplasm bounded by a plasma membrane and enclosed by a complex lipid rich wall. The single chromosome is tightly wrapped into a

nuclear body but is not bounded by a nuclear membrane. Some mycobacteria contain additional small circles of deoxyribonucleic acid (DNA) termed plasmids or episomes. The cell membrane consists of a bilayer of polar phospholipids with their hydrophobic ends facing inwards and their hydrophilic ends facing outwards (Grange, 1988). *M. tuberculosis* was an early object of study by electron microscopy because of its importance as a human pathogen (Rosenblatt *et al*, 1942). This pathogen differs from the majority of other bacteria in that it can multiply within the phagocytic cells of the host.

1.1.2.1.1 Envelope of *M. tuberculosis*

Bacterial envelopes provide protection and support for the bacterial cell and also contain the mechanisms that allow passage of substances between bacterial cell and the environment (Brennan and Draper, 1994). *M. tuberculosis* and other mycobacteria are biologically part of the gram-positive group, but they have some distinctive features in that these bacteria possess lipids rather than proteins and polysaccharides found in other bacteria (Lederer *et al*, 1975 - cited by Brennan and Draper, 1994). However, mycobacteria possess a wall polysaccharide that resembles that of other gram-positive bacteria in having a disaccharide phosphoryl linker between the peptidoglycan and the polysaccharide (McNeil *et al*, 1990 - cited by Brennan and Draper, 1994).

Since *M. tuberculosis* is one of a small group of species able to survive inside the phagocytic cells of a animal host, it is likely that its envelope has special properties defending the bacterium against host microbiocidal processes. There have been attempts to associate ultrastructural features with resistance to phagocytes, however, no special feature has yet been conclusively identified; the envelopes of *M. tuberculosis* do not differ visibly from those of non pathogenic mycobacteria. The envelope consists of two distinct parts: the plasma membrane and around it the wall. These parts provide osmotic protection, transport of ions and molecules, mechanical support and protection (Brennan and Draper, 1994).

1.1.2.1.2 Cell Wall of *M. tuberculosis*

The mycobacterial cell wall is the most complex in all of nature. Lipids consist of 60 % of the cell wall mass and they consist of a wide range of compounds, some being similar to those found in other organisms and others being unique to mycobacteria. Freeze-fracturing techniques show that the cell wall has several distinct layers (Barksdale and Kim, 1977). The inner layer overlying the cell membrane is composed of peptidoglycan (murein) which consists of long polysaccharide chains cross-linked by short peptide chains, forming a net-like macromolecule that gives the cell its shape and rigidity. The polysaccharide chains contain N-glycolyl muramic acid and N-acetyl glucosamine in alternating positions and the cross-linking peptide chains consist of the four amino acids L-alanine, D-isoglutamine, mesodiamino-pimelic acid and D-alanine. The mycobacterial murein is very similar to that in other genera except that it contains N-glycolyl muramic acid instead of the more usual N-acetyl analogue (Grange, 1988).

The most characteristic feature of the mycobacterial cell wall is the chemotype IV peptidoglycan, which is composed of substantial quantities of meso-diaminopimelic acid. The tripartite structure of the mycobacterial cell wall was labeled L1, L2 and L3 by Barksdale and Kim (1977). It was later concluded that the L3 layer corresponded to a true cell wall. The two outermost layers, L1 and L2, corresponded to capsule-like structures that were not covalently linked to the cell wall. Electron microscopy studies showed that the envelope possesses an electron dense layer of peptidoglycan that is surrounded by an electron transparent layer of arabinogalactan and mycolic acids (Besra and Chatterjee, 1994). A schematic representation of the cell wall of *M. tuberculosis* is illustrated in FIG. 1.1.

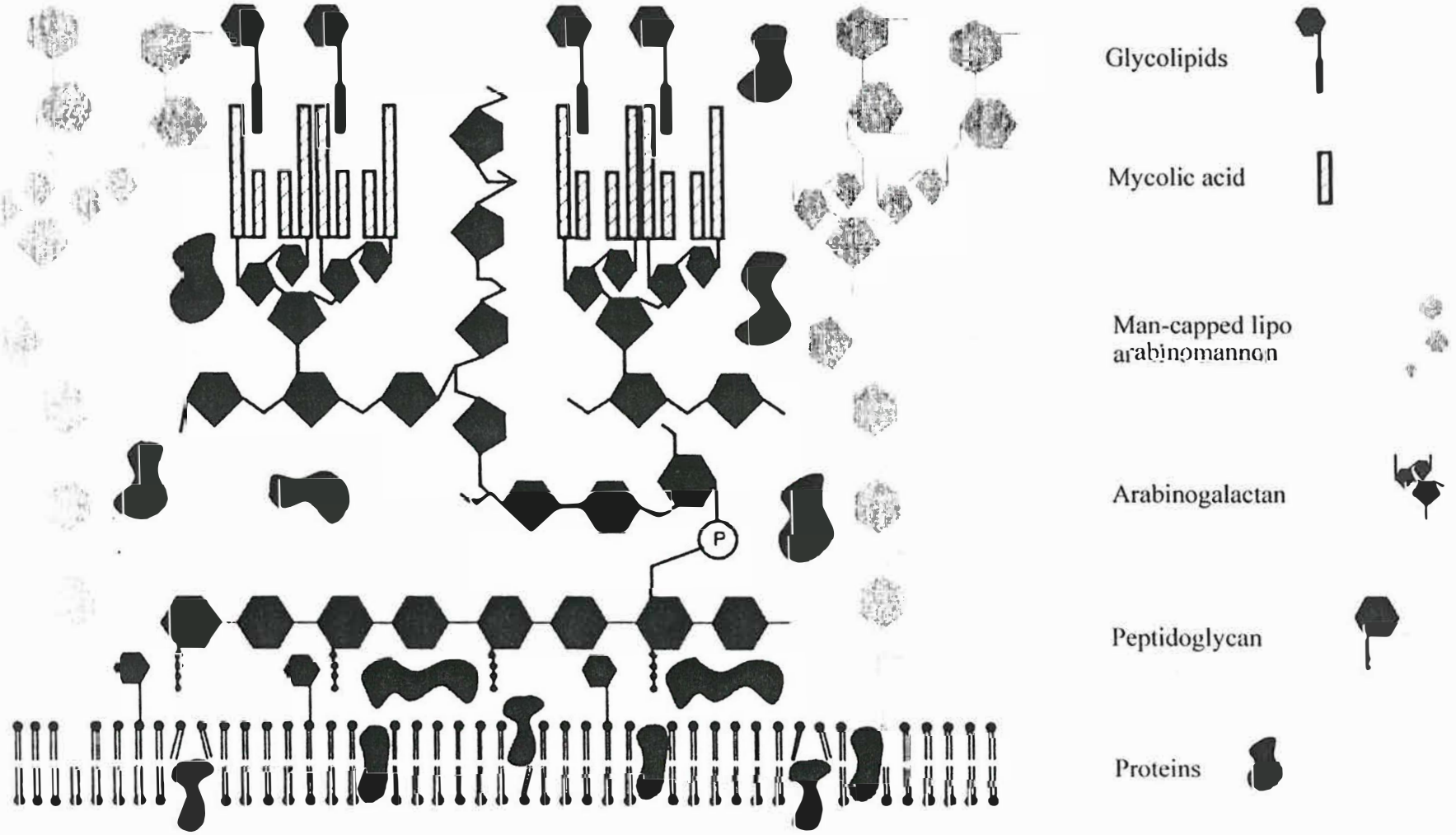


FIG. 1.1 Schematic representation of the cell wall of *M. tuberculosis* (Besra and Chatterjee, 1994).

1.2 INTERACTION OF *M. tuberculosis* WITH MACROPHAGES

The mononuclear phagocyte system is a complex system of cells widely distributed throughout the body. The earliest members of the cell line are found in the bone marrow and include monoblasts and promonocytes. These mature within the bone marrow to monocytes which leave the marrow and briefly enter the blood. After circulating for only a few hours, monocytes leave the bloodstream and enter the tissues, where they differentiate into macrophages. A schematic representation of the development of the mononuclear phagocyte system is shown in FIG.1.2.

With maturation from the monocyte to the macrophage, the cell greatly enlarges and may become multinucleate. it acquires a large number of granules (lysosomes) which are membrane bound packets containing acid activated hydrolytic enzymes. as the cell matures from monocyte to macrophage, its ability to ingest (phagocytise) particles also increases. these cells are avidly phagocytic and can engulf a wide range of organic and inorganic particles (Cline, 1981). the ultimate end stage of development of this cell is the multinucleate giant cell seen in granulomatous diseases such as tuberculosis, leprosy or sarcoidosis. these cells are very large and contain multiple nuclei, abundant cytoplasm and a large store of hydrolytic enzymes packaged in lysosomes (Cline 1981).

1.2.1 MORPHOLOGY OF MONOCYTES AND MACROPHAGES

Monocytes are found both in the blood and in tissues and body cavities in which they have morphologic variation. Study of monocytes by light microscopy showed that its nucleus occupies about half the area of the cell and is usually eccentrically placed. Under the phase contrast microscope the monocyte nucleus shows a distinct chromatin pattern. Mitochrondria are extremely fine and on occasion form a small juxtanuclear rosette surrounding the centrosome. A striking feature on the phase

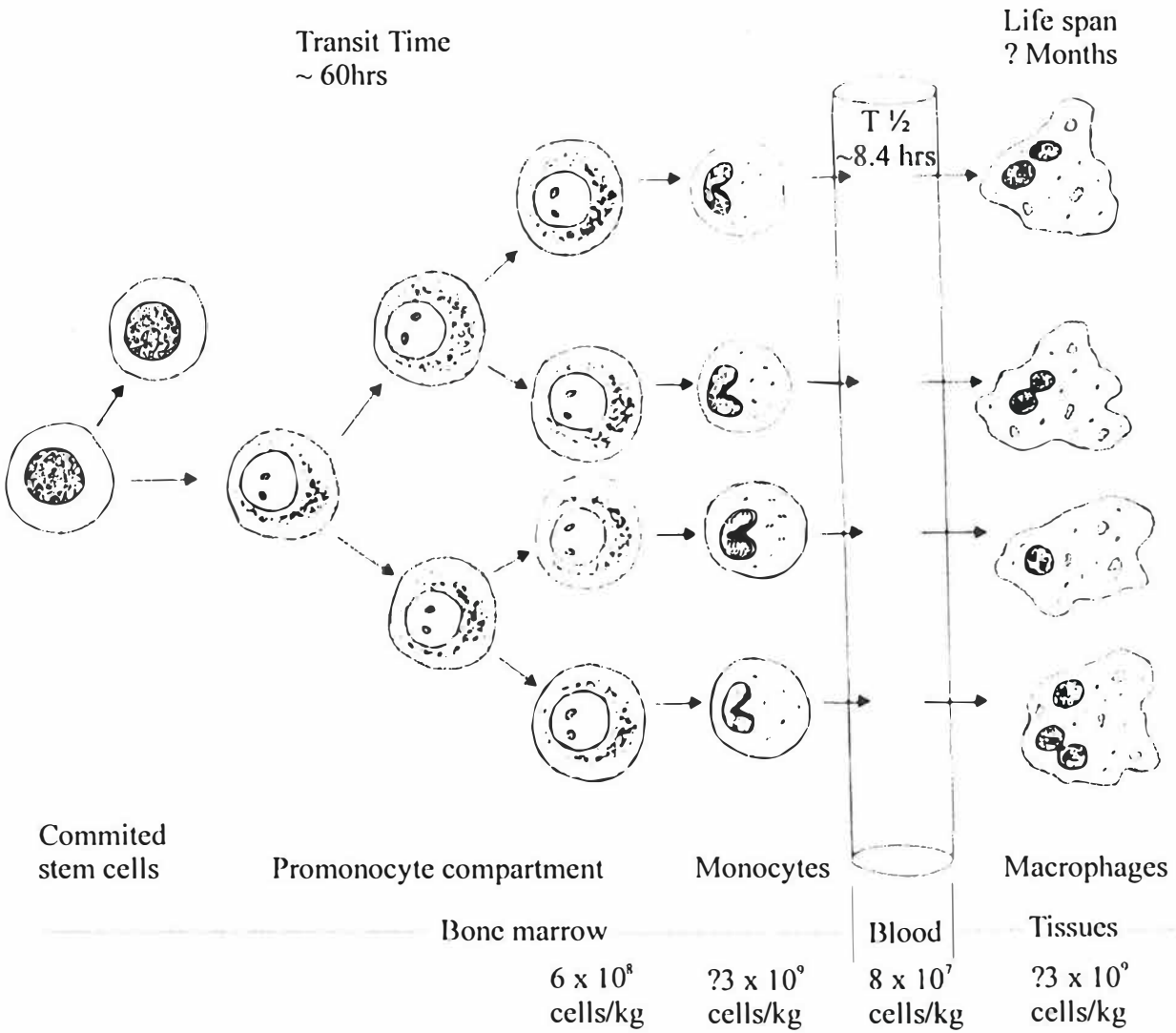


FIG. 1.2 Kinetics of the monocyte macrophage system in man. Promonocytes undergo two divisions before differentiating into monocytes. Monocytes leave the marrow, pass through the blood, and enter the tissue where they mature to macrophages (Cline, 1981).

contrast microscope is the ruffled plasma membranes which is visible as prominent phase dense folds at the cell surfaces and edges. Some cells have a dense thickening at the edge of the cytoplasm with microextensions on the thickened edge (Ackerman and Douglas, 1983; Beiss, 1973; Cline, 1981).

Scanning electron microscopy showed that very prominent ruffles and small surface blebs are apparent in monocytes. Extensive ruffling of the monocyte plasma membrane is of functional significance. The monocyte is both motile and phagocytic and those functions require physical contact with fibers or cell surfaces (Ackerman and Douglas, 1983).

Study of monocyte morphology by transmission electron microscope showed that nucleus of the monocyte contains one or two small nucleoli surrounded by nucleolar associated chromatin. The mitochondria are usually numerous, small and elongated. Golgi complex is well developed. Microtubules are numerous and microfibrils are found in bundles surrounding the nucleus (Ackerman and Douglas, 1983; Beiss, 1973 and Cline 1981).

Macrophages display attributes of morphologic specialisation specific to their location and function. Light microscopy showed that they have an eccentrically placed fusiform nucleus with one or two distinct nucleoli and finely dispersed loosely stranded nuclear chromatin which tends to clump in the nuclear interior and along the internal aspect of the nuclear membrane. On phase contrast microscopy living macrophages are large cells with a propensity to adhere and spread on glass surfaces. Vesicles and contractile vacuoles are seen about the cell periphery and in the cell interior (Ackerman and Douglas, 1983).

Transmission microscopy showed that macrophages show a variable degree of differentiation, nuclear " maturity" ribosomes, mitochrondria and lysosome content. In thin sections, the nucleus varies from horse shoe shaped to fusiform. The heterochromatin is disposed in fine clumps in the interior of the nucleus and along the internal aspect of the nuclear membrane. Polyribosomes and scant smooth and

rough endoplasmic reticulum are seen about the cell periphery. A well developed Golgi complex is in a juxtanuclear location which is often multicentric and contains a concatenation of vesicles, some with dense inclusions which mark them as early lysosome (Ackerman and Douglas, 1983).

1.2.2 UPTAKE OF *M. tuberculosis* BY MACROPHAGES

It is thought that the *M. tuberculosis* bacilli enter the macrophage via specific binding to several distinct cell surface molecules. FIG. 1.3 shows a TEM study of the ingestion of *M. tuberculosis* by macrophages. *M. tuberculosis* can bind directly via complement receptors and the macrophage mannose receptor (MMRc). The MMRc participates in nonopsonin mediated phagocytosis by recognition of terminal mannose residues on targeted particles (Fenton and Vermeulen, 1996). Macrophage phagocytosis of virulent strains of *M. tuberculosis* (e.g., Erdman and H37Rv) in the absence of serum can be substantially inhibited by soluble mannan, mannose-albumin and anti-MMRc antibodies (Schlesinger, 1993). Binding of the attenuated *M. tuberculosis* strain H37Ra to macrophages is not blocked by these agents. Expression of MMRc on the cell surface is regulated by a variety of mediators that play a role in the pathogenesis of tuberculosis. Gamma interferon (IFN-γ) down regulates MMRc expression while concomitantly increasing the capacity of the cells to kill microorganisms, suggesting enhanced coupling of the MMRc to microbicidal functions. In contrast, interleukin-4 (IL-4) is a potent enhancer of MMRc expression (Fenton and Vermeulen, 1996).

The binding of virulent *M. tuberculosis* to the MMRc may be mediated by Mannose Lipoarabinomannan (ManLAM). This was suggested by the finding that anti-ManLAM monoclonal antibodies reduced *M. tuberculosis* binding to macrophages by as much as 49% (Schlesinger *et al*, 1994).

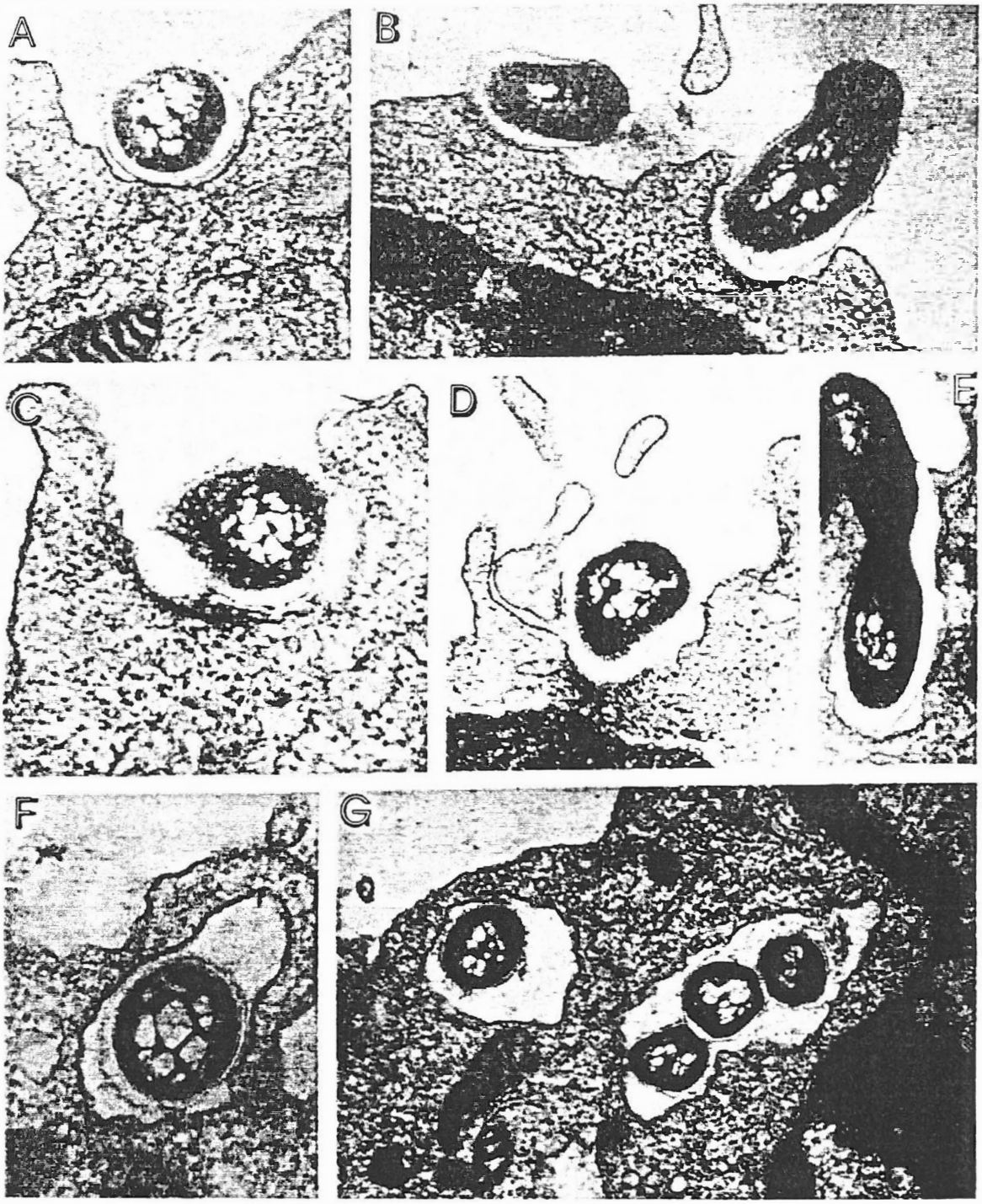


FIG. 1.3 Monocytes ingest *M. tuberculosis* by conventional phagocytosis. Micrographs A to G show sequential stages in monocyte phagocytosis of *M. tuberculosis*. Micrographs A and B show early stages of ingestion. Bacteria are tightly adherent to the monocyte surface. Micrographs C to E show more advanced stages of ingestion. The bacteria are in the process of being internalised. Micrographs F and G show the final stages of ingestion. The bacteria are completely enclosed within the membrane bound vacuoles or phagosomes. Phagocytosis appears to involve two simultaneous processes - the extension of monocyte pseudopods circumferentially about the organism until they meet at the distal side (B to D, F) and the displacement of the cytoplasm at the zone of contact between the monocyte and the bacterium such that the bacterium appears to sink into the phagocyte (A, E). (A and B) x 32 000; (C) x 40 000; (D) x 32 000; (E) x 24 000; (F) x 48 000; (G) x 24 000, (Schlesinger *et al.*, 1990).

It was reported that a mannose-dependent pathway for particle uptake by macrophages, distinct from the MMRc is mediated by lung surfactant protein A. It remains to be tested whether the surfactant protein A may serve as an opsonin for inhaled organisms and may provide an alternative means for mycobacteria to enter the macrophage. Mannose-dependent binding is likely to account for only a portion of total phagocytosed *M. tuberculosis* (Fenton and Vermeulen, 1996).

Peterson *et al.* (1995) implicated CD14, a signalling receptor for gram-negative bacterial lipopolysaccharide (LPS), in the uptake of nonopsonised *M. tuberculosis*. Treatment of human microglial cells with anti-CD14 antibodies or soluble CD14 was found to significantly block the infection of these cells by *M. tuberculosis*.

An important role for heat-labile serum components and the complement receptors CR1 (CD35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in the binding of *M. tuberculosis* to macrophages has also been well established (Schlesinger *et al*, 1990; Schlesinger and Horowitz, 1991 and Schlesinger, 1993). The adherence and ingestion of *M. tuberculosis* can be markedly inhibited (up to 84%) by antibodies against CR1, CR3 and CR4 in the presence or absence of fresh serum. Uptake of *M. tuberculosis* is also reduced in serum depleted of the complement component 3 (C3). Anti-C3 monoclonal antibodies inhibited monocyte adherence of preopsonized *M. tuberculosis* by 71%. Schlesinger *et al*, (1990) showed that a monoclonal antibody against the type 1 CR (CR1) inhibits adherence of *M. tuberculosis* by 40 ± 5% and three different monoclonal antibodies against CR3 each inhibit adherence by 39% ± 5% to 47% ± 5%. A monoclonal antibody against CR1 used in combination with one of the three monoclonal antibodies against CR3 inhibits adherence by up to 64 ± 7%. FIG. 1.4 shows a schematic representation of the antituberculous macrophages activities and evasion mechanisms.

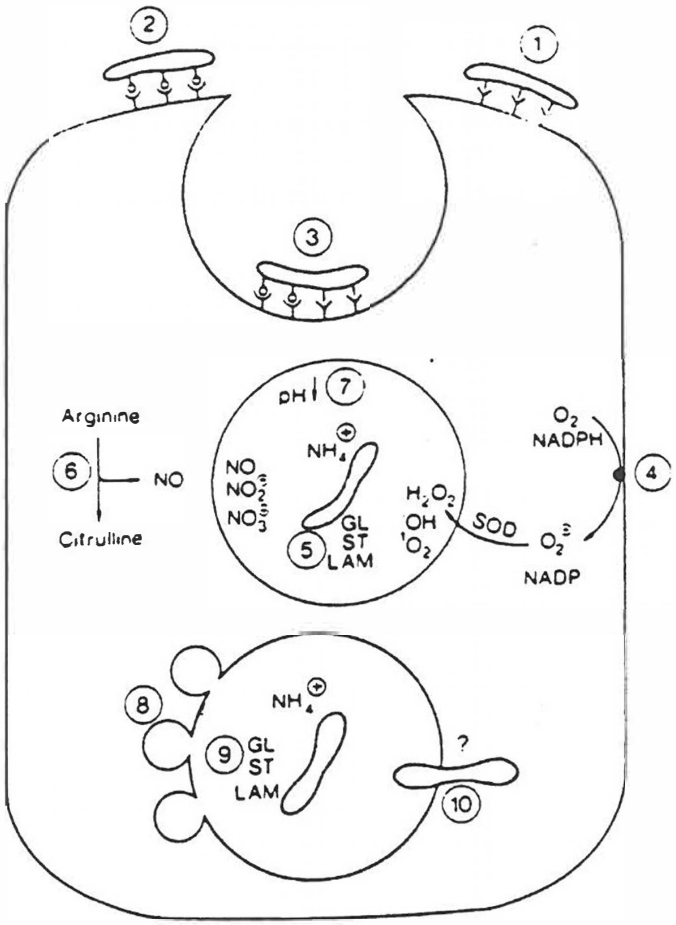


FIG. 1.4. Antituberculosis macrophage activities and evasion mechanisms. Accumulating evidence suggests that M. tuberculosis enters macrophages is specific binding to cell surface molecules of phagocytes. It has been reported that the tubercle bacillus can bind directly to the mannoase receptor via the cell wall associated mannosylated glycolipid LAM (1) or indirectly via complement receptors of the integrin family (CR1, CR3) or Fc receptors (2). Phagocytosis (3) triggered by engaging certain cell surface molecules such as the Fc receptor stimulates the production of ROI via activation of the oxidative burst (4). Experimental data indicate that *M. tuberculosis* can interfere with the toxic effect of ROI by various mechanisms. First, various mycobacterial compounds including glycolipids (GL), sulfatides (ST) and LAM can downregulate the oxidative cytotoxic mechanism. Second uptake via CR1 bypasses activation of the respiratory burst. Cytokine activated macrophages produce RNI that at least in the mouse system mediate potent antimycobacterial activity (6). The acidic condition of the phagolysosomal vacuole can be conducive to the toxic effect of RNI (7). However, NH_4^+ production by *M. tuberculosis* may attenuate the potency of the L-arginine dependent antimycobacterial mechanism and that of lysosomal enzymes (8) which operate best at an acidic pH. In addition mycobacterial products such as sulfatides and NH_4^+ may interfere with phagolysosomal fusion (9). Finally the tubercle bacillus may evade the highly toxic environment by escaping into the cytoplasm via the production of hemolysin (10) (Chan and Kaufmann, 1994).

1.2.3 THE FATE OF INTRACELLULAR *M. tuberculosis*

After attachment and subsequent phagocytosis of *M. tuberculosis*, sustained intracellular bacterial growth depends on the ability to avoid destruction by lysosomal enzymes, reactive oxygen intermediates (ROI) and the reactive nitrogen intermediates (RNI). A capacity to block the fusion of mycobacterium containing phagosomes with lysosomes could be critical for this survival; a key question is whether *M. tuberculosis* bacilli possess one. In 1975 Armstrong and Hart used ferritin-prelabelled secondary lysosomes to study the fusion of phagosomes with lysosomes. Their study showed that the virulent H37Rv strain prevented lysosome-phagosome fusion in normal mouse peritoneal macrophages.

McDonough *et al.* (1993) investigated the effect that *M. tuberculosis* has on phagolysosome fusion using thoria labelled lysosomes with virulent H37Rv, avirulent H37Ra *M. tuberculosis* strains and *Mycobacterium bovis* BCG at an electron microscopy level. At 2 h postinfection approximately 85% of the bacteria clearly resided in fused vacuoles, however, at 4 days postinfection, fusion levels for viable H37Rv and H37Ra were reduced by half, whereas, the fusion profiles of BCG and heat-killed H37Rv and H37Ra were unchanged. The results suggest a net transfer of bacteria out of the fused vacuoles and preferential bacterial multiplication in nonfused vacuoles. H37Rv and H37Ra appeared to bud from the phagolysosome into tight membrane vesicles that did not fuse with secondary lysosomes.

M. tuberculosis appears to have the ability too disrupt the normal functioning of phagosomes, preventing them from developing into acidic hydrolase-rich compartments. The restricted capacity of mycobacterial phagosomes to fuse with other vesicles suggested that their biochemical composition is altered, either preventing delivery of the mycobacteria into the lysosomal compartment or blocking association of phagosomes with host molecules that are harmful to the bacilli. This was confirmed by studies that revealed that vacuolar membranes surrounding the bacilli lacked a proton-ATPase, which may be responsible for phagosomal acidification (Xu *et al*, 1994).

1.2.4 MICROBICIDAL ACTION OF MACROPHAGES

Historically, researchers have assumed that activated macrophages can kill *M. tuberculosis*. However, this assumption has been difficult to prove unequivocally *in vitro*, especially with human monocytes and macrophages (Fenton and Vermeulen, 1996). Douvas *et al* reported in 1986 that human monocytes cultured for 3 days were measurably better at suppressing the growth of virulent *M. tuberculosis* than were either fresh monocytes or those cultured for 7 days. Also, in 1991a, Denis reported that human monocytes could be activated to high microbicidal activity following treatment with cytokines such as, interferon, IFN-γ and tumor necrosis factor TNF. However, a more recent report by Warwick-Davies (1994) suggested that this apparent killing of *M. tuberculosis* could be an artifact of the experiment and that this cytokine treatment regimen actually renders macrophages more sensitive to the toxic effects of the mycobacteria.

If the killing of ingested mycobacteria did occur then the most likely place will be within the macrophage phagolysosome. Toxic constituents found within this acidic vesicle include lysosomal hydrolases, ROI such as H_2O_2 and O_2^-, and RNI such as NO and NO_2^-. The resistance of several strains of *M. tuberculosis* to RNI *in vitro*, generated at an acidic pH, was found to correlate significantly with the virulence of the strain tested. RNI production by murine macrophages is an important effector mechanism against a variety of pathogens. In macrophages, NO and other RNI are derived from L-arginine via an enzymatic pathway controlled by an inducible nitric oxide synthase (iNOS). Cytokines are powerful modulators of murine macrophage RNI synthesis, where TNF and IFN-γ are potent activators of iNOS, while interleukin, IL-4 and IL-10 suppress it (Gazzinelli *et al*, 1992 and Ozwald *et al*, 1992). Transforming growth factor β1 (TGF-β1) has also been reported to attenuate NO production and RNI-mediated antimicrobial functions (Nelson *et al*, 1991 and Ozwald *et al*, 1992). Growth inhibition of mycobacteria by cytokine-stimulated murine macrophages strongly correlates with the generation of RNI. IFN-γ-deficient mice infected with *M. tuberculosis* are unable to restrict the growth of the organisms.

These mice develop granulomas but fail to produce RNI (Flynn *et al*, 1993 - cited by Fenton and Vermeulen, 1996).

There is much debate about the role of RNI in infected human macrophages (Denis, 1991a - cited by Fenton and Vermeulen, 1996). Although the production of iNOS mRNA and protein by primary human macrophages has been reported, efforts to demonstrate an L-arginine-dependent pathway for RNI production in human macrophages have generally produced inconsistent results. These observations may reflect intrinsic differences between human and murine cells. One possible difference may be the requirement for the cofactor tetrahydrobiopterin, which may not be present in sufficient quantities in resting human macrophages (Schoendon *et al*, 1987 and Werner *et al*, 1989). Alternatively, human macrophages may require additional induction signals for RNI production.

Nussler *et al*. (1992) showed that a combination of lipopolysaccharide (LPS) and the cytokines IL-1, TNF and IFN-γ was required in order for human hepatocytes to produce RNI via an L-arginine dependent pathway. The iNOS activity was dependent on the coinduction of tetrahydrobiopterin synthesis. Another possibility is that human macrophages may produce higher amounts of cytokines that suppress RNI production (*e.g.*, IL-4, IL-10 and TGF-β1) compared to murine cells. ROI alone may be insufficient to destroy *M. tuberculosis* but combined with RNI can significantly enhance mycobacterial killing (Stamler *et al*, 1992). Several mycobacterial products, including sulfatides and lipoarabinomannan (LAM) can scavenge ROI or inhibit the respiratory burst that generate them (Chan *et al*, 1991).

The capacities of resident alveolar macrophages and recruited monocytes to destroy mycobacteria differ significantly and progressively throughout the course of an infection. Initial infection by *M. tuberculosis* rapidly leads to the activation of alveolar macrophages, the induction of cytokines which serve to limit the growth of the ingested organisms, and the recruitment of additional leukocytes from the peripheral circulation. Enhancement of macrophage microbicidal function can be exerted by T cells, especially $CD4^+$ $\alpha\beta^+$ T cells that secrete IFN-γ and IL-2. IFN-γ

alone cannot activate macrophage sufficiently to inhibit *M. tuberculosis* replication, although combined exposure to IFN-γ and TNF was reported to be sufficient for effective killing (Denis, 1991a). The immature monocytes recruited from the periphery are thought to be less effective in killing mycobacteria and are the preferred host. The local production of IFN-γ and TNF by leukocytes is critical for the differentiation and activation of these recruited monocytes. Treatment of mice infected with BCG or *M. tuberculosis* with a neutralizing antibody directed against TNF was originally shown to block granuloma formation and to enhance bacterial growth , Denis, 1991b and Kindler *et al*, 1989). A recent study with transgenic mice (Flynn *et al*, 1995), lacking the 55 kDa THF receptor demonstrated that these animals cannot clear an *M. tuberculosis* infection. These mice and others treated *in vivo* with anti-TNF neutralizing antibody formed granulomas but in delayed manner. The granulomas observed in mice lacking normal TNF function were qualitatively distinct, lacking epitheloid cells and containing larger numbers of *M. tuberculosis*.

1.2.5 EFFECT OF LAM ON MACROPHAGES

Studies by Sturgill-Koszycki *et al.* (1994) and Xu *et al.* (1994) suggests that *M. tuberculosis* bacilli that have evaded the destruction by the macrophage not only multiply but release mycobacterial products that can affect local immune responses. Vesicles containing lipoarabinomannan (LAM) (and possibly other mycobacterial products) were released by phagosomes containing ingested mycobacteria, suggesting the active transport of mycobacterial products out of infected cells. LAM released by infected macrophages may act in a paracrine manner to modulate the function of surrounding leukocytes. The biological activities of LAMs from different mycobacteria have been studied and their chemical derivatives defined.

AraLAM (the arabinose termini are capped with additional inositol phosphates) is 100-fold more potent at inducing TNF production by macrophages than is ManLAM (the arabinose termini are capped with a few additional mannose residues). Similar results have been observed for IL-1, IL-10, NO and chemokine induction (Roach *et al*,

1993 and Roach *et al*, 1994). It has also been shown that AraLAM rapidly activates the critical transcription factor NF-κβ in murine macrophages to a much greater extent than does Man LAM. Both AraLAM and ManLAM induce sustained accumulation of KBF1, which may inhibit NF-κβ-dependent gene transcription (Brown and Taffet, 1995). While studies differ with respect to the observed biological importance of polysaccharide domains at the nonreducing termini of LAM, there was general agreement that acyl groups associated with the phosphatidylinositol end of the molecule are essential for the biological activities of LAM on monocytic cells (Barnes *et al*, 1992 and Chatterjee *et al*, 1992).

1.2.6 INTERACTIONS BETWEEN T-CELLS AND MACROPHAGES

The collaboration between macrophages and T-cells is essential for eradication of *M. tuberculosis* via antigen specific delayed-type hypersensitivity (DTH) responses. Initially, activated macrophages present mycobacterial antigens to T-cells in association with major histocompatibility complex (MHC) class 1 or class 11 proteins or with nonpolymorphic MHC-like proteins. Macrophage derived cytokines play critical roles as costimulatory molecules for antigen specific T-cells. Th1 type cytokines (*e.g.* IL-2 and IFN-γ) may enhance antigen presentation and stimulate antimicrobial responses within the infected macrophage itself. The presence of both antigen-specific T-cells and activated macrophages within the granuloma provides long-term surveillance and containment of infected macrophages, although immune responses may shift over time to favour the suppression of DTH reactions (Fenton and Vermeulen, 1996).

In murine models of tuberculosis, the initial Th 1 type response needed for effective DTH development is followed by a Th 2 type response which probably serves to limit inflammation and minimize tissue injury at the site of infection.(Orme *et al.*, 1993). The macrophage cytokine IL-2 may be important in favouring the developmemt of the DTH response by enhancing production of IFN-γ which facilitates the development of Th 1 cells and augments the cytotoxicity of antigen specific T-cells and natural killer

cells. Th 2 type responses may contribute to the immunosuppression often observed in advanced disease. Tuberculosis patients coinfected with HIV have diminished Th 1 type responses compared with HIV-seronegative tuberculosis patients (Zhang *et al*, 1994). Macrophages are likely to contribute further to these responses through the production of the immunosuppressive cytokines Il-10 and TGF-β1. The cytokine network controls a complex set of responses that may be growth modulatory to *M. tuberculosis*, proinflammatory or immunosuppressive (Toossi *et al*, 1995).

1.3 PZA: ANTIMYCOBACTERIAL AGENT UNDER STUDY

PZA, a white, odourless, crystalline powder; is a synthetic pyrazine analogue of nicotinamide (MacGregor, 1988). PZA was first shown to be an effective antitubercular drug in the year 1952 (Kushner *et al*, 1952 - cited by Lacroix *et al*, 1989). However, it fell into disrepute subsequently due to the high incidence of hepatotoxicity associated with its use in doses as high as 3g/day (Singh *et al*, 1993). PZA has regained its place in the front-line with reduced dosages and incidental minimal side effects (Singh *et al*, 1993). PZA has become an essential component of current six month regimens for therapy of tuberculosis (Salfinger *et al*, 1990). Inclusion of PZA for 2 months in regimens containing isoniazid and rifampicin throughout with ethambutol or streptomycin in the initial phase has produced highly effective short-term regimens which prevent failure associated with the emergence of resistance during chemotherapy and which are followed by negligible relapse rates (Girling, 1984).

1.3.1 PROPOSED MODE OF ACTION OF PZA

The exact mode of action of PZA is not known (Singh *et al*, 1993). A commonly accepted hypothesis explaining the mechanisms of action of PZA is based on the assumption that PZA susceptible *M. tuberculosis* strains produce pyrazinamidase, which hydrolyses PZA to the antibacterial moiety, POA. The proposed mode of

action is shown in FIG. 1.5. It is not clear whether POA has specific antimicrobial activity or the inhibition of growth caused by POA is due to its ability to lower the pH of the environment below the limits of tolerance of *M. tuberculosis* growth (Heifets *et al*, 1989; Konno *et al*, 1967 and Singh *et al*, 1993). Strains resistant to PZA do not produce pyrazinamidase, thus do not convert PZA to POA and therefore are not vulnerable to PZA.

1.3.2 PHARMACOLOGY AND PHARMACOKINETICS OF PZA

1.3.2.1 Absorption and fate

PZA is readily absorbed from the gastro-intestinal tract (Reynolds, 1989). It is widely distributed throughout the body fluids and tissues, attaining concentrations above that needed to inhibit tubercle bacilli. Peak plasma concentrations are approximately 50 $\mu g.\ ml^{-1}$, with a half life of 12-24 hours, making once daily or less frequent dosing practical (Alford, 1979 and Alford and Wallace, Jr, 1995). PZA crosses inflammed meninges and has been recommended in combination regimens for tuberculous meningitis (Donald and Seifart, 1988). Its absorption is not significantly hindered by food. The excellent penetration of PZA across the blood brain barrier is due to its moderately lipophilic nature, unchanged at body pH and not bound to serum proteins (Ellard *et al*, 1987 - cited by Singh *et al*, 1993). It is metabolised by the liver and metabolic products including principally pyrazinoic acid (POA), are excreted mainly by the kidneys. Thus, dosage modification is required in renal failure. PZA is dialyzable so that supplement dosage may be advisable after dialysis sessions (Alford and Wallace, Jr, *et al*, 1995). It is excreted through the kidney mainly by glomerular filtration. About 70% of a dose appears in the urine within 24 hours mainly as metabolites and 4 to 14% as unchanged drug. PZA is excreted in breast milk (Reynolds, 1989 and Singh *et al*, 1993).

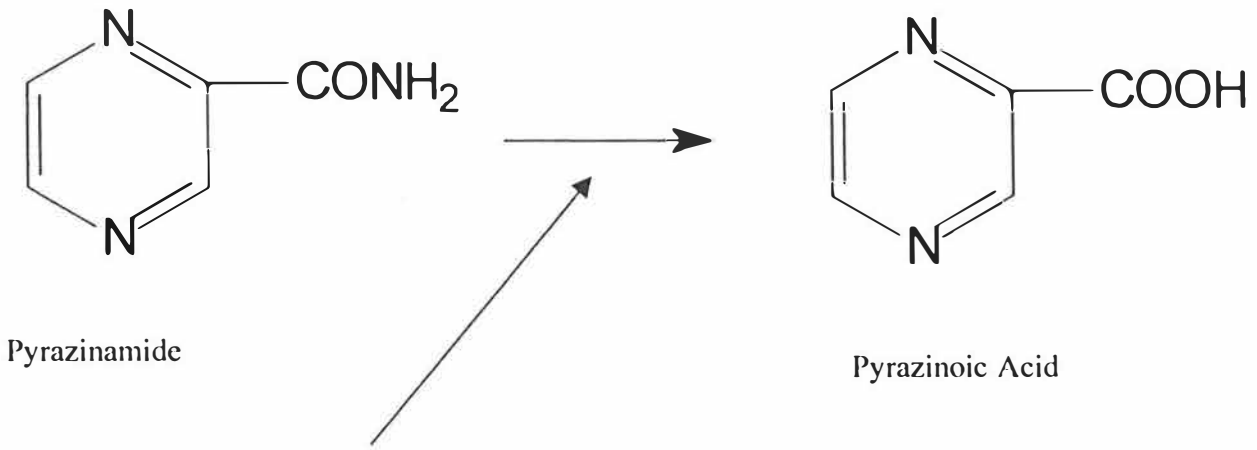


FIG. 1.5 The proposed mode of action of PZA. Susceptible *M. tuberculosis* strains are known to produce the enzyme pyrazinamidase, which converts PZA to POA. It is suggested that it is this enzyme generated product, POA, that has high antibacterial activity in an acid environment, whereas PZA itself has no activity.

1.3.2.2 Administration and dosage

The high incidence of hepatotoxicity associated with early regimens which used large doses of PZA given for prolonged periods led to PZA being considered unsuitable for use in the primary treatment of tuberculosis. Its uses was largely reserved for re-treatment regimens for drug resistant tuberculosis and for initial therapy in developing countries where the incidence of primary resistance is high. However, the currently recommended doses are considerably safer and because of its unique activity against tubercle bacilli in an acid environment, PZA is now an important first line drug in intensive phase of short-term antituberculous regimens (Reynolds, 1989 and Singh *et al*, 1993). Transplacental passage of PZA has been shown but its use in pregnancy is not contra-indicated. No embryo fetotoxity or teratogenicity has been observed. PZA, though detected in human milk is far below the therapeutic range and is considered safe for use in nursing mothers (Singh *et al*, 1993).

PZA is available in 500 mg tablets. Dosage is 20-35 mg/kg/day (often 1.5-2.0g) orally once a day or in two divided doses. PZA has been well tolerated in a twice weekly dosage of 50 mg/kg (not to exceed 3g/day) for short course regimens. It has been administered safely once weekly in a dose of 90 mg/kg (Alford, 1979 and Alford and Wallace, 1995).

1.3.2.3 Metabolism

Lacroix *et al.* (1989) studied the plasma and urine pharmacokinetic parameters of PZA and its metabolites, pyrazinoic acid (POA), 5-hydroxy-pyrazinamide (5-OH-PZA), 5-hydroxy-pyrazinoic acid (5-OH-POA) and pyrazinuric acid (PZU). These researchers found that limiting factor was the activity of a microsomal deamidase (POA formation from PZA and 5-OH-POA formation from 5-OH-PZA). In contrast, oxidation by xanthine oxidase occurred very rapidly (5-OH-PZA formation and POA catabolism to 5-OH-POA).

Weiner and Tinker (1972) described the main stages of PZA metabolism. The first stage involves microsomal deamidase which leads to formation of POA and second xanthine oxidase which produces 5-OH-POA. These researchers also showed the existence of another metabolite with polarographic characteristics similar to those of 5-OH-POA but different electrophoretic mobility. A component with a similar extinction coefficient to 5-OH-POA was isolated by Auscher *et al.* (1978 - cited by Lacroix *et al*, 1989). The nature of this component was examined by Pitre *et al.* (1981 - cited by Lacroix, 1989) using rat liver cells and by Yamamoto *et al.* (1987a) and Bereta *et al.* (1987) in man, all of whom considered it to be 5-OH-PZA, produced by the direct action of xanthine oxidase on PZA. Yamamoto *et al.* (1987b), then shown a second oxidizing enzyme which operates jointly with xanthine oxidase in the sequence PZA⇒ 5-OH-PZA. Through a minor route, POA combines with glycine to form pyrazinuric acid. Thus, a metabolic scheme was proposed as illustrated in FIG 1.6.

1.3.2.4 Adverse effects of PZA

Hepatotoxicity is the most serious side effect of PZA therapy and its frequency appears to be related to dose and duration of treatment (Reynolds, 1989). Hepatotoxicity occurred in nearly 15% of PZA recipients in early trials that employed dosages of 40-50 mg/kg/day for prolonged periods. Current regimens are much safer. Patients with pre-existing liver disease who receive PZA should have symptoms and hepatic function tests monitored closely (Zierski and Bek, 1980). Hepatomegaly, splenomegaly and jaundice may develop and in rare cases fulminating acute yellow atrophy and death have occurred. Other side effects are anorexia, nausea, vomiting, arthralgia, malaise, fever, sideroblastic anaemia and dysuria. Photosensitivity and skin rashes have also been reported. Hyperuricaemia commonly occurs and may lead to attacks of gout (Dannenberg and Tomashefski, 1988; Alford, *et al*, 1979 and Alford and Wallace, Jr, 1995; Reynolds, 1989 and Singh *et al*, 1993). Treatment of overdosage with PZA consists of gastric lavage and supportive therapy. If hyperuricaemia with acute gout arthritis occurs PZA should generally be withdrawn

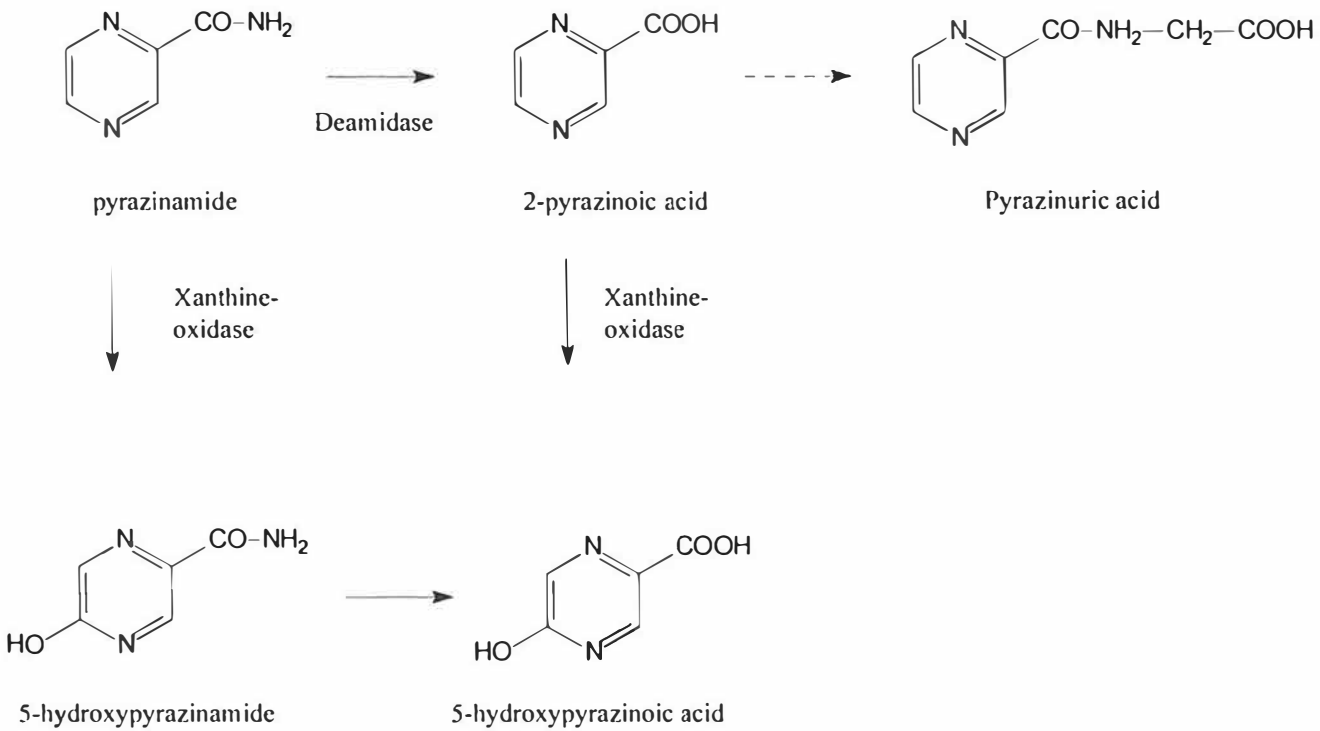


FIG. 1.6 The principal metabolic pathways of pyrazinamide (Lacroix *et al.*, 1989)

although a uricosuric agent such as probenecid has been given. PZA is contra-indicated in patients with liver damage. Liver function should be assessed before and regularly during treatment. Caution should be observed in patients with impaired renal function or a history of gout. It has also been reported that there is increased difficulty in controlling diabetes mellitus when diabetics are given PZA (Reynolds, 1989).

1.3.3 PZA ACTIVITY IN MACROPHAGES

The persistence of certain pathogenic bacteria within phagocytes, most often macrophages, may lead to progressive or chronic clinical infections (Hand *et al*, 1984). Viable intraphagocytic organisms are protected from the action of certain antimicrobial agents (Mackaness, 1952 and Suter, 1952). The efficacy of antibiotics in the treatment of these infections depends upon both the extracellular drug-bacterial interaction and the effect of intracellular drug on the phagocyte and the organism. Since chronic infections due to facultative intracellular organisms frequently occur in the lung, the interactions of antibiotics, bacteria and alveolar macrophages are of interest (Hand *et al*, 1983 and 1984; Johnson *et al*, 1980).

Carlone *et al.* (1985) investigated the killing capacity of rifampicin, PZA and POA on macrophage ingested live *M. tuberculosis*. The three drugs were used at concentrations corresponding to the average peak levels observed in humans after administration of therapeutic doses *viz.*, 30 μg/ml PZA, 10 μg/ml rifampicin and 8 μg/ml POA as determined by Acocella *et al.* (1985). Comparing the degree of killing in the control drug free cultures with that observed in the drug containing systems over a period of 3 to 24h indicated that a greater, more rapidly, although not statistically significant killing of intracellular mycobacteria took place. At 48h the degree of killing was similar in the control and the drug containing cell cultures. There was a marked growth of intracellular mycobacteria observed between 48 and 72h in the control cultures which was less evident in the drug containing cultures. There was no major increase in the killing effect with respect to that observed with the

individual drugs after exposure of the macrophages to all possible combinations of the three drugs. This finding could be explained on the basis of the hypothesis that each drug achieved a maximal (or near maximal) killing effect when used alone, so that no excess killing could be expected to occur adding one or two drugs. Another possible interpretation is the one referring to a process of competition between drugs for either entry into the macrophage or for some binding relevant to antimicrobial activity within the macrophages (Carlone *et al*, 1985).

Acocella and co-workers (1985) investigated the degree of penetration of rifampicin, PZA and POA in mouse macrophages. Unlike the study by Carlone *et al.* (1985) which investigated killing of ingested mycobacteria over 72h this study only evaluated penetration of the drugs for 24h. Also, drugs at concentrations corresponding to peak, trough and intermediate serum concentrations observed in humans after administration of therapeutic doses were used which differed from the studies by Carlone *et al.* (1985) where peak serum concentrations were used. Another difference was the use of macrophages in different metabolic stages *viz.*, resident, stimulated and dead. The drugs were radiolabelled with ^{14}C which enabled intracellular penetration to be assessed by a scintillation spectrophotometry. The results obtained in this study indicated that rifampicin, PZA and POA penetrate rapidly into macrophages, irrespective of their metabolic state. The penetration is almost complete at the lower concentrations. Increasing the concentration of exposure leads to increased penetration into the macrophage, but not proportionally with the concentration. While stimulation of macrophages had a marked effect on the degree of penetration of rifampicin at the higher concentrations, it affected PZA penetration only at the lower concentrations and seemed not to affect penetration of POA. This difference in behaviour may reflect different mechanisms of transfer through the macrophage membrane. Transfer across the macrophage membrane of the three drugs seems to be by simple diffusion. At the single concentration of each drug, the degree of penetration was similar in dead and live macrophages, strongly suggesting the absence of an energy requiring process. Rifampicin, PZA and POA appear to have different capacities to bind to intracellular proteins, where analysis of the free/bound ratios

indicates that more "binding sites" are available for rifampicin than for the PZA and POA (Acocella *et al*, 1985).

At neutral pH PZA is almost devoid of antibacterial activity against tubercle bacilli growing in commonly used bacteriologic culture mediums (Dickinson and Mitchison, 1970 and Butler and Kilburn, 1982). Its effectiveness in laboratory animals is inconsistent, thus PZA is bactericidal in mice but it seems to be inactive in the guinea pig (McCune *et al*, 1966a and b). Crowle *et al.* (1986) investigated the effect of PZA alone on tubercle bacilli within cultured human macrophages. In this study the effect of PZA on intracellular bacilli were assessed by counts of acid fast bacilli and colony forming units (CFU). PZA was active in the macrophage model as it was clinically inhibiting virulent tubercle bacilli at concentrations of 20 μg/ml or higher, however, it was ineffective in 7H9 bacteriologic culture medium. It could either be bacteriostatic or bactericidal against intramacrophage tubercle bacilli, depending on its concentration, the donor of the macrophage and the length of exposure of the infected macrophages to the drug. The results obtained by Crowle *et al.* (1986) with human blood monocyte derived macrophages agree well with the findings of Mackaness (1956) with similarly cultured rabbit peritoneal exudate macrophages.

Crowle *et al.* (1986) observed that the inhibitory effect of PZA in their macrophage cultures diminished between the fourth and seventh days after infection and that adding PZA a second time at 4 days did not change this. The following rationale could explain this paradox. Active tubercle bacilli produce amidase which converts PZA to bacillus inhibiting POA (Butler and Kilburn, 1982 and 1983; Ellard, 1969; Konno *et al*, 1967). They also produce ammonia (Gordon *et al*, 1980) which increases phagolysosome pH. Increased pH diminishes the effectiveness of POA which is almost ineffective at pH 6 or higher, as well as the effectiveness of acid dependent, potentially antimicrobial lysosomal enzymes. Thus, active bacilli will be converting PZA to POA and inhibiting themselves, but also be raising phagolysosomal pH and protecting themselves. Ammonia escapes rapidly from phagolysosomes. It would not be replenished by POA inhibited bacilli and the net early effect on active bacilli of PZA will be conversion to POA without much pH

change and therefore bacillary inhibition. Later, as the bacilli become inhibited they should make less amidase and, therefore, less POA and effectiveness of PZA should decrease. Consequently, the effects of PZA on tubercle bacilli appear to be self modulating and both bacillus interdependent (*e.g.* production of protective ammonia) and host interdependent (acid pH in phagolysosomes, lysosomal enzymes with acid pH optima, xanthine oxidase converting active POA to inactive 5-hydropyrazinoic acid (Crowle *et al*, 1986).

In this study it was also found that tubercle bacilli may replicate within human macrophage in non-acid form, thus indicating that colony forming units are inherently more accurate than acid fast bacilli counts. This also suggests that important changes in the bacillary cell wall composition may occur among tubercle bacilli within infected human macrophages after exposure to PZA (Crowle *et al*, 1986). It may also account for the undetectability of "persisting" tubercle bacilli in PZA treated mice as described by McCune *et al*. (1966a,b).

Salfinger *et al*. (1989) reported a simple radiometric method for susceptibility testing of *M. tuberculosis* against PZA at pH 6.0 *viz*., the BACTEC method. In 1990 Salfinger *et al*. reported on a comparison of PZA and POA activity in cultured human macrophages and in the BACTEC system. PZA susceptibility results obtained in cultured human macrophages were compared with those in the broth BACTEC system with 7H12 mediun at pH 6.0 for strains known to be pyrazinamidase positive or negative. Pyrazinamidase negative tubercle bacilli were resistant to PZA in both infected macrophages and the BACTEC system. However, POA was unable to inhibit tubercle bacilli in cultured macrophages, but it was able to inhibit them at high concentrations in the BACTEC system. POA's ability to inhibit tubercle bacilli in the BACTEC system was proportional to its concentration and to the decrease in pH that in itself may be inhibitory. It is possible, therefore, that PZA inhibits tubercle bacilli in cultured macrophages physically rather than biochemically (Salfinger *et al*, 1990).

The comparative activity of PZA and POA against tubercle bacilli in the infected macrophage and broth BACTEC systems provides further insights into the possible

mechanism of PZA's clinical effectiveness versus *M. tuberculosis*. Activity of PZA or POA in the BACTEC system is not dependent on drug penetration, as it is in the macrophage model. The results of Salfinger *et al.* (1990) suggest that the cultured macrophage must contribute actively or passively to effectiveness of PZA, such as through the proposed mechanism of low pH generated by pyrazinamidase in the phagolysosomes. A purely *in vitro* test such as the BACTEC system can serve as a convenient tool for evaluating antimycobacterial drugs, but the cultured macrophage system may more closely approximate *in vivo* reality (Salfinger *et al*, 1990).

The outbreaks of multi drug resistant (MDR) tuberculosis have raised questions as to the most appropriate therapeutic response for those exposed to such organisms. Sbarbaro *et al.* (1996) investigated the combined effect of PZA and a quinolone (ofloxacin) as a potential preventive therapy regimen. Three different concentrations of ofloxacin (OFL) (0.625μg, 1.25μg, 2.5μg/ml) were tested in combination with 40 μg/ml dosage of PZA, which demonstrated an inhibitory effect upon intracellular tubercle bacillary growth. PZA enhanced the effectiveness of both bacteriostatic concentrations of OFL, converting the bacteriostatic impact of 1.25 μg/ml to a bactericidal effect. The addition of PZA did not diminish the killing effect of the 2.5 μg/ml OFL. This differs from a previous study by Sbarbaro *et al.* (1992) where the bactericidal effectiveness of rifampin was reduced when administered simultaneously with PZA. The additive or interactive actions appear to be equally present even when the drugs are administered sequentially two days apart, however the killing impact of rifampin was clearly diminished when PZA was administered two days in advance to rifampin (Sbarbaro *et al*, 1992).

The observations of Sbarbaro *et al.* (1996) suggest that the combination of a fluoroquinolone and PZA will provide an effective alternative regimen for prophylactic or preventive intervention. However, both OFL and PZA are eliminated from the kidneys. When used together clinically, competitive inhibition of drug elimination might be expected, and probably underlies the unexpectedly high rate (44%) of arthralgias observed by 7 of 16 health workers treated with a preventive regimen of 800 mg/day of OFL and 1500 μg/day PZA (Horn *et al*, 1994). Arthralgias

are a well documented side effect of PZA and can be minimized with a reduction in daily dosage or by limiting treatment with PZA to three times per week (Sbarbaro *et al*, 1996).

Mor and Esfandiari (1997) investigated the synergistic activities of clarithromycin (CLA) and pyrazinamide against *M. tuberculosis*. CLA is an acid stable macrolide antibiotic with good bioavailability after oral administration, however, its activity against *M. tuberculosis* has been relatively poor (Gorzynski *et al*, 1989 and Traffert-Pernot, 1995). Since PZA works most effectively on bacteria in an acid environment and within macrophages, any drug that could enhance its activity should therefore be invaluable in the treatment of tuberculosis. The combination of CLA and PZA exerted a synergistic effect in preventing intracellular bacterial growth, however the combination of rifampin and PZA produced only an additive effect. CLA, even at subinhibitory concentrations significantly enhanced the effect of PZA. This indicates that CLA tends to enhance the effectiveness of PZA against tubercle bacilli within cultured human macrophages, however this effect was not demonstrated to be bactericidal during the eight days of this study (Mor and Esfandiari, 1997).

1.3.4 ANTIBACTERIAL ACTIVITY OF PZA

The commonly accepted hypothesis regarding the mechanism of action of PZA is the production by PZA susceptible *M. tuberculosis* strains of pyrazinamidase, which hydrolyses PZA to the antibacterial moiety POA. It is not clear whether POA has specific antimicrobial activity or that the inhibition of growth caused by POA is due to its ability to lower the pH of the environment below the limits of tolerance of *M. tuberculosis* growth (Konno *et al*,1967). Pavlov *et al*. (1974 - cited by Heifets *et al*, 1989) showed that exposure to PZA increased the acidity in small areas surrounding phagocytosed *M. tuberculosis* organisms, lowering the usual intracellular pH range from 5.0 to 5.3 to 4.5 to 4.7 which could be due to the transformation of PZA to POA. Heifets *et al*. (1989) then addressed the question whether POA has any specific antimicrobial activity or whether it affects the growth of *M. tuberculosis* simply by

lowering the pH below the limits of tolerance. There was a clear dose-effect correlation when the inhibitory activities of different concentrations of POA under identical pH conditions (5.6) in 7H12 liquid medium was evaluated. This showed that POA does have specific activity against *M. tuberculosis*. The minimum inhibitory concentrations (MICs) of POA were determined using the radiometric BACTEC system (Heifets *et al*, 1985; Salfinger and Heifets, 1988) and it was found that under these conditions the MICs were 240 to 480 μg/ml. This is 8 to16 times higher than the MICs of PZA. There were previous suggestions that POA is less active *in vitro* than PZA (Dickinson and Mitchison, 1970; Ellard, 1969), with only a two fold difference between the MICs of POA and PZA (Ellard, 1969). However, the ultimate question concerns the validity of the theory that PZA acts by its conversion to POA *in vivo*, since the concentration of POA in human serum does not exceed 10 μg/ml (Ellard, 1969). This study supports that part of the hypothesis that states that POA is an antimicrobial agent, but seems to contradict (owing to the high MICs) the suggestion that it is the only antimicrobial moiety of PZA. A possible explanation is the assumption that high concentrations of POA, close to the MIC and much higher than the concentrations of both PZA and POA found in serum, might be achieved in the immediate surroundings of mycobacterial cells, which is supported by studies by Pavlov *et al*.(1974 - cited by Heifets *et al*, 1989). An assumption made is that mycobacterial cells act like pumps consuming PZA from the surrounding environment and transforming it into POA, which accumulates at the surface of each cell and is delayed in diffusing into the medium. Another possibility is that the MIC for POA decreases with a drop in pH. The action of POA in an acid environment could be a combined effect of its specific activity and its ability to lower the pH below the limits of tolerance of the target organism (Heifets *et al*, 1989).

The activity of PZA can be demonstrated only in an acid medium *in vitro* (McDermott and Tompsett, 1954), which is not favourable to the growth of *M. tuberculosis*. Thus, this poses a problem when testing the susceptibilities of *M. tuberculosis* strains to PZA as shown by Butler and Kilburn (1982). The incorporation of enrichment, usually oleic acid-albumin-dextrose-catalase (OADC) into the 7H10 agar is necessary for optimal growth of *M. tuberculosis* at acid pH (Stottmeier *et al*, 1967), however,

oleic acid in an acid environment is inhibitory to many strains. (Tummon, 1975 - cited by Butler and Kilburn, 1982). Butler and Kilburn (1982) reported that growth of 25% of *M. tuberculosis* strains were inhibited by the presence of oleic acid at pH 5.5. Growth appeared better with certain lots of albumin-dextrose-catalase (ADC) at pH 5.5 but some lots did not support growth at low pH. The growth supporting ability of different lots of ADC were assayed for by observing the turbidimetric measurements of *Bacillus subtilis* in an ADC-heart infusion broth test which provided a rapid 24 hour preselection method for ADC. It was found that lots of ADC that gives a change in OD of 0.145 or below with *B. subtilis* were able to support growth of 37% of *M. tuberculosis* strains, whereas those that showed a change in OD of 0.165 or greater were able to support 90% of the strains of *M. tuberculosis* (Butler and Kilburn, 1982).

In the same study Butler and Kilburn (1982) investigated the effect of carbon dioxide (CO_2) and inoculum size on PZA inhibition. Strains of *M. tuberculosis* incubated in the presence of 7% CO_2 in air at pH 5.5 for three weeks yielded larger colonies than the same strains incubated without CO_2. Inoculum size was also important, when drug-containing medium was inoculated with greater than 300 CFU of PZA susceptible strains. Colonies developed in 3 weeks in the absence of added CO_2 in the atmosphere, but growth was stimulated (greater number of colonies and larger colony size) by added CO_2 during incubation (Butler and Kilburn, 1982).

In vitro experiments only approximate *in vivo* conditions. Important cofactors for certain biologic effects may be lacking in *in vitro* models because they are unknown. Crowle *et al.* (1989) showed the synergistic effect of 1,25 $(OH)_2$-vitamin D_3 ($1,25D_3$) with PZA to kill intracellular bacilli. The two used together synergises to decrease concentrations of PZA which were inhibitory and to switch the action of PZA from weakly inhibitory to bacteriostatic or mildly bactericidal, however, $1,25D_3$ has no anti-tuberculosis effect in the absence of macrophages as determined in acidified bacteriologic culture medium (Crowle *et al*, 1989).

The difficulty in performing the PZA susceptibility tests because an acid of the medium is required to demonstrate activity of the drug (McDermott and Tompert, 1954) and *M. tuberculosis* strains do not grow well at pH 5.5 (Stottmeier *et al*, 1967) lead to alternatives been investigated. Butler and Kilburn (1983) reported on the detection of pyrazinamidase activity as an alternative as PZA susceptible strains have the pyrazinamidase enzyme that hydrolyses PZA to POA. McClatchy *et al.* (1981) also recommended the detection of pyrazinamidase activity as a useful indicator for screening *M. tuberculosis* for PZA susceptibility, as this would overcome the problem of acid medium. The standard Wayne's test was used to investigate the enzyme activity (Wayne, 1974), where a postive is indicative of a pink to red colour development upon addition of freshly prepared ferrous ammonium sulfate solution. However, Butler and Kilburn (1983) found that the detection of pyrazinamidase activity by Wayne's (1974) method was of limited value when compared to the standard PZA susceptibility tests, especially when high levels of PZA resistance was found. When resistance to PZA reached a level of 150 to 200 μg/ml, there was too much variability in Wayne's tests results to accurately define PZA susceptibility (Butler and Kilburn, 1983).

The MICs of PZA at pH 5.5 or 5.6 has been reported (Dickinson and Mitchison, 1970; Salfinger and Heifets, 1988 and Heifets *et al*, 1989), however, the MBC *i.e.*, the lowest concentration killing more than 99% of the bacterial population *in vitro* was not reported. In 1990 (Heifets and Lindholm-Levy) reported on the MBC using PZA susceptible strains cultivated in 7H12 broth at pH 5.6, the lowest pH favourable for active bacterial growth. 33 to 57% of the bacterial population was killed during the 15 day period of cultivation in the presence of PZA concentrations two fold greater than the MIC. Increasing the PZA concentration as high as 500 and 1000 μg/ml did not substantially increase the proportion of bacterial population killed, which was not greater than 74%. Thus, the MBC could not be determined and they concluded that the MBC was greater than 1000 μg/ml. It is a paradox that one of the most clinically efficient antituberculosis drugs has such poor bactericidal activity *in vitro* against actively multiplying bacteria (Heifets and Lindholm-Levy, 1990).

Heifets and Lindholm-Levy (1992) then tried to find some explanations to this paradox by determining the *in vitro* activity of PZA against tubercle bacilli at a lower pH, closer to that in which the part of the bacterial population presumably affected by PZA *in vivo*. This approach required cultivation at pH 5 and lower, instead of pH 5.5 to 5.6 used in their previous study (Heifets and Lindholm-Levy, 1990). The number of viable tubercle bacilli remained almost unchanged within a period of 3 to 4 weeks of cultivation in a liquid medium with a pH of 4.8 to 5.0. They referred to this state as semidormant rather than dormant, assuming that the relatively stable number of viable cells was a reflection of the simultaneous existence of cells in various states of dormancy, multiplication and dying (Heifets and Lindholm-Levy, 1992).

The CFU/ml declined 1000 fold in the presence of 50 μg/ml PZA over the same period. The high "bactericidal" effect of PZA at pH 4.8 to 5.0 occurs because the tubercle bacilli are probably somehow damaged by this acidic environment but continue to be metabolically active. It is also possible that this increased vulnerability of tubercle bacilli to PZA is a result of some changes of the metabolic pathways at pH 4.8 to 5.0 Therefore, the sterilising activity of PZA found under these conditions is most likely a result of the combined effect of the unfavourable pH environment and PZA (Heifets and Lindholm-Levy, 1992).

The ability of tubercle bacilli to grow at low pH had not been questioned because one of the accepted sites of multiplication *in vivo* was the phagolysosomes of macrophages. However, in recent times this theory has being challenged by Crowle *et al.* (1991), who proposed that vesicles containing living, virulent *M. tuberculosis* or *M. avium* in cultured macrophages are not acidic. It was confirmed by immunocytochemistry that both chloroquine and NH_4Cl raise the pH of acidic vesicles in the infected macrophage. However, neither caused any pH related change in the antimycobacterial activities of PZA, streptomycin or isoniazid. Therefore, the suggestions by Heifets and Lindholm-Levy (1992) that PZA may accelerate the toxic effect of low pH *in vitro* supports the same events taking place *in vivo*, especially considering that most of the bacterial population persisting there in an acid environment is extracellular rather than intracellular (Jindani *et al*, 1980).

The sterilising effect is quite different from the actual bactericidal activity of other drugs against actively growing cultures, which usually can be expressed in MBC values. The importance of the distinction between early bactericidal and sterilizing activity of the antituberculous drugs in patients was suggested by Mitchinson (1985). A special clinical observation on 14 days of monotherapy has shown that PZA, along with thiacetazone and streptomycin, had the poorest early bactericidal activity among all the antituberculous drugs, especially during the first two days of treatment when the bacteria are actively multiplying (Jindani *et al*, 1980). However, Mitchinson (1985) found that PZA was one of the most potent drugs when judged by its activity in patients. According to Mitchinson's hypothesis (1985) the early bactericidal activity of a drug reflects its interaction with the actively multiplying part of the bacterial population, whereas the sterilising activity of PZA is a result of its ability to affect the semidormant subpopulation at low pH, mostly extracellular in sites of acute inflammation (Jindani *et al*, 1980).

The *in vitro* observations on differences in the activity of PZA against actively growing (Heifets and Lindholm-Levy, 1990) and the semidormant bacterial populations (Heifets and Lindholm-Levy, 1992) can be considered a confirmation of Mitchinson's explanation of the activity of PZA in patients: low bactericidal activity of PZA against bacteria actively growing at pH 5.6 correlates with the poor early bactericidal activity in patients and the high sterilising effect *in vitro* at pH 4.8 to 5.0 against non-multiplying bacterial population correlates with the high sterilising activity *in vivo*.

Susceptibilities to PZA correlates with amidase activity of *M. tuberculosis*. Spiers *et al.* (1995) studied the *in vitro* activity of n-propyl pyrazinoate (nPPA) against a series of PZA-susceptible and PZA-resistant isolates of *M. tuberculosis*. In a previous study by Cynamon *et al.* (1992) a series of synthesised esters of pyrazinoic acid demonstrated enhanced *in vitro* activity against both PZA susceptible and PZA resistant *M. tuberculosis* isolates. Spiers *et al.* (1995) hypothesised that POA esters would not require activation by amidase but would be converted to POA by mycobacterial esterase. The MICs of POA and nPPA for *M. tuberculosis* were found

to be more favourable than those of PZA. PZA resistant isolates retain susceptibility *in vitro* to POA and nPPA. This supports the hypothesis that esters of POA can circumvent the requirement for activation by mycobacterial amidase. The MICs of nPPA for *M. tuberculosis* were found to be the same or one to fivefold lower than those of POA which suggests that the biochemical basis for antituberculosis activity of POA is not simply the reduction in pH caused by the POA moiety. An alternative explanation could be that POA and nPPA are transported more efficiently than PZA into the mycobacterial cell (Spiers *et al*, 1995). These researchers are performing further studies which investigate the effects of modifications of the alcohol and pyrazine moieties of pyrazinoate esters on *in vitro* and *in vivo* antituberculosis activity, which may lead to a candidate compound with enhanced activity against both PZA susceptible and PZA resistant *M. tuberculosis* isolates (Spiers *et al*, 1995).

Cynamon *et al.* (1998) evaluated the *in vitro* activity of 5-chloro-PZA (5-Cl PZA) and 5-Cl POA against various mycobacterial isolates, including resistant *M. tuberculosis* cultures using the broth dilution method. 5-Cl PZA was more active than PZA against all organisms tested. The assumption based upon the effect of POA increasing the intracellular pH was confounded by the observation that 5-Cl POA is significantly less effective than POA against *M. tuberculosis*. The largest difference, an eightfold increase in the MIC of 5-Cl POA relative to that of POA was found in a strain ATCC 35828, which was resistant to PZA and deficient in amidase (Cynamon *et al*, 1998).

1.3.5 A MOLECULAR PERSPECTIVE

Pyrazinamidase is the same as nicotinamidase in *M. tuberculosis*. Nicotinamidase is involved in the degradation of nicotinamide to nicotinic acid (Konno *et al*, 1967). *M. tuberculosis* strains that are resistant to PZA (an analog of nicotinamide) lose both pyrazinamidase and nicotinamidase activity. It has been suggested that the role of pyrazinamidase/nicotinamidase is to convert PZA to bactericidal POA inside the bacterial cell and that the absence of pyrazinamidase may be responsible for PZA resistance in both acquired PZA-resistant *M. tuberculosis* and naturally resistant *M.*

bovis strains. To provide insights into why *M. bovis* are naturally resistant to PZA and to define the molecular mechanism of PZA resistance in *M. tuberculosis*, Scorpio and Zhang (1996) cloned and characterised the pyrazinamidase gene (*pncA*) and demonstrated that mutations identified in the *pncA* gene are the cause for PZA resistance in both types of resistant bacilli.

1.3.5.1 Cloning and characterisation of the *M. tuberculosis pncA* gene

Since the pyrazinamidase enzyme is the same as the nicotinamidase in *M. tuberculosis* Scorpio and Zhang (1996) cloned the *M. tuberculosis pncA* gene by the polymerase chain reaction (PCR) using degenerate primers based on the amino acid sequence derived from *Escherichia coli* nicotinamidase gene (*pncA*) (Jerlstrom *et al*, 1989). The *M. tuberculosis pncA* gene was initially cloned on a 500 base pair PCR product from a PZA susceptible *M. tuberculosis* strain and subjected to sequence analysis, which showed that the PCR product contained a partial open reading frame with homology to the *E. coli pncA* sequence (Jerlstrom *et al*, 1989). The *M. tuberculosis* integrating cosmid DNA library was screened using the 500 bp *pncA* containing PCR product as a probe to obtain the complete *M. tuberculosis pncA* gene. A *pncA* hybridising cosmid clone was isolated and used to transform bacillus Calmette-Guerin (BCG), an attenuated vaccine strain derived from *M. bovis*, in order to confirm the identity of the putative *M. tuberculosis pncA*. The cosmid DNA containing the putative *M. tuberculosis pncA* conferred pyrazinamidase activity to BCG, a natural mutant defective in pyrazinamidase. Southern analysis conferred the integration of the *pncA* cosmid DNA into *M. bovis* BCG genome (Scorpio and Zhang, 1996).

The functional *M. tuberculosis pncA* gene was localised on a 3.2 kilobase *Eco*R1-*Pst*1 fragment by restriction mapping of the cosmid DNA in combination with BCG transformation studies using DNA constructs derived from the cosmid DNA insert. To confirm that pyrazinamidase activity is due to the *pncA* gene, but not to other DNA in the *pncA* upstream region on the 3.2 kb *Eco*R1-*Pst*1 fragment, BCG was

transformed with the 2.3 kb *Eco*R1-*Sma*1 DNA construct that contains the upstream region and partial *pncA* gene, however no pyrazinamidase activity was detectable in the BCG transformant. Sequence analysis of the 2.3 kb *Eco*R1-*Sma*1 fragment does not show significant open reading frames with homology to other known proteins in the database. Transformation of BCG with a construct that contains the *pncA* gene alone with its 120-bp upstream sequence as a PCR fragment gave functional expression of pyrazinamidase activity, indicating that the pyrazinamidase activity was indeed conferred by the *pncA* gene (Scorpio and Zhang, 1996).

Sequence analysis showed that the *M. tuberculosis pncA* gene (558 bp) encoded a protein of 186 amino acids with 35.5% overall amino acid identity to the *E. coli* nicotinamidase (Jerlstrom,1989). The predicted molecular mass of the *M. tuberculosis* pyrazinamidse is about 20 kDa and is smaller than the *E. coli* homolog, which consists of 213 amino acids with a size of 23 kDa (Scorpio and Zhang, 1996).

1.3.5.2 Identification of mutations in the *pncA* gene of resistant strains

Scorpio and Zhang (1996) wanted to determine the genetic basis of the defective pyrazinamidase and PZA resistance in tubercle bacilli. Southern analysis was performed using the 3.2 kb EcoR1-Pst1 *pncA* containing DNA fragment as a probe on a panel of PZA resistant strains. There was no gross deletions of the *pncA* gene or restriction fragment length polymorphism. The *M. bovis* species, which is well known to lack pyrazinamidase activity had the *pncA* gene as that in *M. tuberculosis* which was showed by the same size hybridisation fragments from the two species. This indicated that these PZA resistant strains probably had point mutations in the *pncA* gene. Sequence analysis was carried out on the *pncA* gene to determine if point mutations did exist. Point mutations were found and it was concluded that a particular substitution caused the defective pyrazinamidase in these resistant strains of *M. tuberculosis* and *M. bovis* (Scorpio and Zhang, 1996).

1.3.5.3 Transformation of PZA resistant strains with *pncA* gene

To determine whether PZA resistance is due to point mutations identified in the *pncA* gene of resistant strains, the susceptibility of PZA of naturally PZA resistant BCG and the acquired PZA resistant *M. tuberculosis* strain PZA-R derived from H37Rv was tested. These strains were transformed with the 3.2 kb *Eco*R1-*Pst*1 plasmid construct that contains the functional *pncA* gene. The recombinant BCG and the PZA-R *pncA* transformants expressed functional pyrazinamidase activity and became susceptible to PZA (MIC = 50 μg/ml) under acid conditions (pH 5.5) *in vitro*, whereas the BCG and PZA-R vector control strains remained pyrazinamidase negative and PZA resistant (MIC>1000 μg/ml). The susceptibility of recombinant BCG to PZA inside macrophages were also tested. It was shown that the PZA susceptibility of the recombinant BCG is due to the expression of the pyrazinamidase activity conferred by the *M. tuberculosis pncA* gene (Scorpio and Zhang, 1996).

1.3.5.4 *pncA* mutations in PZA resistant *M. tuberculosis*

A study of 38 PZA resistant clinical isolates of *M. tuberculosis* was carried out by Scorpio *et al.* (1997). Various mutations were found *viz.*, nucleotide substitution, insertions or deletions in the *pncA* gene. The identified mutations were dispersed along the *pncA* gene but some degree of clustering of mutations was found at the following regions: Gly132-Thr142, Pro69-Leu85, and Ile5-Asp12. PCR-single strand conformation polymorphism (SSCP) analysis was useful for the rapid detection of *pncA* mutations in the PZA resistant strains (Scorpio *et al*, 1997). A similar study carried out by Hirano *et al.* (1998) on 168 Asian isolates of *M. tuberculosis*, where 135 isolates were Pzase positive and 33 were negative. Among the 33 Pzase negative isolates 32 (97%) had mutations within the *pncA* gene. Three strains that were PZA resistant mutants were Pzase positive and showed no change in the *pncA* gene, suggesting an additional mechanism may be involved in PZA resistant. No mutations were observed in all of the 135 Pzase positive isolates tested, indicating that mutations in the *pncA* gene could be involved in the loss of Pzase activity (Hirano *et al*, 1998, and Ramaswamy and Musser, 1998).

1.4 TREATMENT OF TUBERCULOSIS

A century of treating tuberculosis is well described by Bignall (1982), where he starts with one of the earliest documented treatment regimens of Professor Jaccoud of Paris who published "The Curability and Treatment of Pulmonary Phthisis" The treatment consisted of 'hygienic measures'. The patient should reside in a dry dwelling in rooms facing south and the temperature of the bedroom should not exceed 15^0C. The windows should be closed even in summer. In addition several drugs were advised, including salts of iron, cod liver oil, preparations of arsenic and antimony, quinine salts, salicylates. Bignall (1982) comments: "It is tempting to look at condescension and faint amusement at the efforts of physicians to treat tuberculosis a century ago. The temptation should be firmly resisted. Is our present treatment of some diseases more rational or effective? And do we not, in our efforts to be scientific, sometimes forget Jaccoud's concept of 'our duty' that the treatment should benefit the patient and not cause distress".

Sanatorium treatment was another alternative, which was first used in 1859 at Gorbersdorf in Germany. Although sanatorium treatment remained one of the main weapons for over 50 years, there is no scientifically acceptable evidence that it did reduce the toll of the disease. Nevertheless, sanatoriun treatment remained popular until effective chemotherapy arrived in the 1950's. In 1882, the year of Koch's discovery, Carlo Forlanani of Milan speculated on the feasibility and efficacy of collapsing the lung by introducing air into the pleural space, however, like the sanatorium treatment, it was difficult to estimate its value in reducing mortality and transmission of the disease. There was also the curious episode of the "gold decade", which began in 1925 by the publication of Mollgaard who published with enthusiasm the benefits of gold salt, sodium aurothiosulphate. However, just as fast as it had spread it began to fade and by 1935 the fashion was over.

Then came the modern chemotherapy which began with the discovery of the first effective drug, streptomycin in 1944.

1.4.1 ANTIMYCOBACTERIAL AGENTS

1.4.1.1 Streptomycin

Streptomycin was the first of the aminoglycoside antibiotics to be developed and the first drug to be effective in treating pulmonary tuberculosis (MacGregor, 1988 and Alford and Wallace Jr, 1995). Streptomycin is bactericidal against *M. tuberculosis in vitro*, but is inactive against intracellular tubercle bacilli because of its failure to penetrate into phagocytes and its lack of activity within the acid environment of caseous necrosis (Chambers and Jawetz, 1998). Sensitive organisms are inhibited by concentrations of less than 10 μg/ml, sometimes as low as 0.4 μg/ml (MacGregor, 1988 and Alford and Wallace Jr, 1995).

Streptomycin binds to bacterial ribosomes, causing misreading of the mRNA codons and subsequent production of nonfunctional proteins. It also inhibits bacterial cell respiration and damages cellular membranes. Approximately 1 in every 10^8 *M. tuberculosis* bacteria are resistant. When used as a single agent to treat cavitary pulmonary tuberculosis in the late 1940's, up to 75% of patients were found to have resistant organisms after four months of treatment, thus streptomycin must be used in combination with other drugs. Streptomycin is not absorbed from the gastrointestinal tract and therefore requires parenteral administration. This is a disadvantage when compared to other first line drugs that can be administered orally. It is widely distributed into extracellular fluid but does not cross the uninflamed meninges or enter intracellular space (MacGregor, 1988). Streptomycin toxicity is like that of other aminoglycoside antibiotics but with less renal and acoustic toxicity and greater vestibular toxicity than more commonly used aminoglycosides (Alford and Wallace Jr, 1995).

1.4.1.2 Isoniazid

Isoniazid (INH), the hydrazide of isonicotinic acid was shown to be effective against human tuberculosis in 1952 (Chambers and Jawetz, 1998). INH is bactericidal against actively growing *M. tuberculosis* (Alford and Wallace Jr, 1995). It acts by inhibition

of oxygen-dependent synthetic pathways of mycolic acid, an important constituent of mycobacterial cell walls (Herman and Weber, 1980).

INH concentrations of 0.025-0.05 μg/ml is inhibitory to *M. tuberculosis*, and higher concentrations are bactericidal against replicating organisms. When INH is administered alone for three months, resistance tends to emerge in over 70 % of cases. Resistance results from selection under antimicrobic pressure of incompletely susceptible variants of *M. tuberculosis* that number 1 in 10^6 among untreated bacillary populations. Surviving tubercle bacilli exhibit decreased INH uptake. The large populations (10^9-10^{10}) bacilli in pulmonary cavities are especially likely to contain significant numbers of inherently resistant tubercle bacilli that regrow because of inadequate therapy to express secondary resistance (Alford and Wallace, 1995).

INH is well absorbed orally or intramuscularly and is distributed throughout the body, with therapeutic levels detectable in pleural, ascitic and cerebrospinal fluids as well as in tissue including caseous abscess material (Alford and Wallace, 1995; Chambers and Jawetz, 1998, and MacGregor, 1988). Metabolism of INH occurs initially by liver *N*-acetyltransferase. Diminished acetylation capacity is inherited as an autosomal recessive trait, which varies from a 5 % prevalence rate in Canadian Eskimos to 83 % in Egyptians. Ten to fifteen % of Orientals are "slow" acetylators as are 58 % of American Whites. The striking bimodal distribution of plasma half lives of INH depending on acetylator status does not affect outcome with daily therapy, because plasma levels are maintained well above inhibitory concentrations (MacGregor, 1988, and Alford and Wallace, 1995).

INH has infrequent major toxicities, most notably hepatitis. Approximately 15% of INH recipients have minor asymptomatic elevations in serum aspartate aminotransferase levels that usually resolve with continued therapy. Patients should be advised to discontinue INH therapy at the onset of symptoms consistent with incipient hepatitis, such as nausea, loss of appetite and dull midabdominal pain. Peripheral neuropathy developed in 17% of recipients taking 6mg/kg/day of INH but

is less frequent when adults receive the standard dose of 300 mg/day. INH is available as tablets, syrup and injectable solutions (Alford and Wallace, 1995).

1.4.1.3 Rifampin

Rifampin (termed rifampicin in the United Kingdom) is a semisynthetic derivative of a complex macrocyclic antibiotic, rifamycin B, produced by *Streptomyces mediterranei* (Chambers and Jawetz, 1998). It was introduced into clinical trails in 1967. Its mode of action involves the inhibition of mycobacterial DNA-dependent RNA polymerase; human RNA polymerase is insensitive. Rifampicin binds to the β-subunit of the polymerase enzyme which acts as the catalytic centre of the enzyme, thus inhibits the initiation of RNA synthesis (Tillotson *et al*, 1996). It is bactericidal against susceptible *M. tuberculosis* strains at 0.005-0.2 μg/ml (Alford and Wallace, Jr,. 1995). Organisms can develop one-step resistance by modifying the target polymerase and mutants occur with a frequency of 1 in 10^7 to 10^8 organisms. To avoid selection of resistant strains, rifampin should always be used in combination with other antituberculous drugs (MacGregor, 1988)

Rifampin is well absorbed orally with peak plasma concentration yields of 7-8 μg/ml after dose of 600 mg. It is widely distributed throughout the body, including CSF. Its high lipid solubility enhances phagosomal penetration. Rifampin is bactericidal against actively replicating *M. tuberculosis* to a degree comparable to INH. It is also active against intracellular, slowly replicating bacilli. Its efficacy in treatment is indicated by sputum conversion two weeks earlier with rifampin containing regimens than those without it. Due to rapid resistance emerging so frequently, precludes it from being used alone. Resistance results from a mutation causing specific amino acid substitution in RNA polymerase. Its major adverse effect is hepatotoxicity, which caused 16 deaths in 500 000 recipients (Alford and Wallace Jr, 1995).

1.4.1.4 Ethambutol

Ethambutol was discovered in 1961 among synthetic compounds screened for antituberculous activity. When administered orally 75-80% is absorbed, yielding peak plasma concentrations of 5 μg/ml after dose of 25 mg/kg. It is distributed throughout the body including the CSF. Ethambutol is bacteriostatic *in vitro* or within macrophages at concentrations of 1 μg/ml against susceptible strains of *M. tuberculosis*. (Alford and Wallace Jr, 1995 and MacGregor, 1988). Two target sites for ethambutol have been elucidated, polyamine function and cell wall synthesis, where ethambutol was a specific inhibitor of spermidine synthase (Poso *et al.*, 1983) and inhibited arabinogalactan synthesis which is critical in the development of cell wall synthesis (Takayama and Kilburn, 1989). Stepwise resistance occurs whenever ethambutol is administered without companion drugs, thus the principal role of ethambutol has been as "companion" drug to curtail resistance (Alford and Wallace, Jr, 1995, and Chambers and Jawetz, 1998). The major toxicity of ethambutol is neuropathy. Peripheral neuropathy is infrequent, however retrobulbar neuritis is more common (Alford and Wallace Jr, 1995).

1.4.1.5 Rifapentine

The Food and Drug Administration (FDA), US Department of Health and Human Services granted approval of rifapentine as a new drug for pulmonary tuberculosis, making it the first drug approval in 25 years (FDA talk paper-www.FDA.com, 1998). Clinical trials compared to standard therapy, evaluating the drug were carried out predominantly in patients in South Africa. Rifapentine was studied with three other drugs: isoniazid, PZA and ethambutol. In follow ups there was a 10 % rate of relapse in rifapentine patients compared to 5 % in rifampin patients, however the FDA's Antiviral Drugs Advisory Committee recommended the approval of the drug due to the following reasons:

i) Patients are more likely to comply with the obligations of the therapy because the drug regimen is easier to follow,

ii) In the second course of therapy, patients are dosed weekly (rifapentine plus isoniazid) rather than twice weekly (rifampin plus isoniazid). For compliance reasons patients must undergo "directly observed therapy",
iii) Better compliance not only improves patient outcomes, but should deter drug resistance,
iv) While the relapse rate with rifapentine was higher than that of rifampin, neither was alarmingly high (10% to 5%).

Its safety profile is similar to rifampin. Rifapentine could cause increased liver enzyme levels which can be a precursor to liver damage and increased uric acid levels, which can be a precursor to gout. One of the most common side effects is discoloured. orange-reddish urine (FDA talk paper-www.FDA.com, 1998).

1.4.1.6 Second-line Antituberculous Drugs

Para-Aminosalicylic Acid (PAS) is a synthetic compound that inhibits growth of tubercle bacilli by impairment of folate synthesis. It is incompletely absorbed orally and its chief side effect is gastrointestinal intolerance. It has limited use in modern chemotherapy because of its poor compliance and primary resistance (Alford and Wallace Jr, 1995 and MacGregor, 1988).

Cycloserine is a broad spectrum antibiotic which acts as an antimetabolite of D-alanine, a major component of bacterial cell walls. It is readily absorbed orally, producing peak plasma concentrations of 20-50 μg/ml. Cycloserines can cause peripheral neuropathy or central nervous system dysfunction, including confusion, irritability, somnolence, headache, nervousness, vertigo, dysarthria and seizures (Alford and Wallace Jr, 1995).

Ethionamide is a derivative of isonicotinic acid and was first synthesised in 1956. It is tuberculostatic at 0.6-2.5 μg/ml against susceptible strains presumably by inhibition of oxygen-dependent mycolic acid synthesis. It is well absorbed orally, yielding peal plasma concentrations of 20 μg/ml. Gastrointestinal distress with nausea and

vomiting frequently leads to poor compliance and drug discontinuance, although ethionamide can be chosen for treatment of resistant tuberculosis (Alford and Wallace Jr, 1995, and MacGregor, 1988).

Capreomycin, amikacin, kanamycin and viomycin are considered a group of antimycobacterial drugs as they are all administered by intramuscular injection, have similar pharmacokinetics and toxicities and are excreted by the renal route. Capreomycin is a polypeptide antibiotic obtained from *Streptomyces capreolus* which is active against *M. tuberculosis*, including MDR tuberculosis strains. It is emerging as a first line injectable agent in multidrug regimens for treatment of drug resistant tuberculosis, especially when there is resistance to streptomycin as there is no cross resistance between them. Amikacin is among the most active aminoglycosides against *M. tuberculosis*, however there is limited experience with it in human tuberculosis. Kanamycin is an aminoglycoside that has activity against most strains of streptomycin resistant tubercle bacilli. Except for its lower cost, kanamycin has no advantage over amikacin in combination therapy. Viomycin is a complex basic polypeptide antibiotic for injection and many MDR tuberculosis strains are susceptible to it. Cross resistance to viomycin and capreomycin occurs frequently but less between viomycin and kanamycin (Alford and Wallace Jr, 1995).

Amithiozone, a thiosemicarbazole is active against many strains of *M. tuberculosis*, where susceptible strains are inhibited by 1 μg/ml. Due to the development of resistance when used in single drug therapy, amithiozone is used in combination regimens (Alford and Wallace Jr, 1995).

The emergence of MDR tuberculosis has stimulated investigation of new fluorinated quinolone antibiotics with activity against mycobacteria. These are bactericidal against *M. tuberculosis* by inhibiting its DNA gyrase at concentrations well within achievable plasma levels. Ciprofloxacin and ofloxacin inhibit 90 % of strains of susceptible tubercle bacilli at concentrations of 0.5 and 1.0 μg/ml, respectively (Caekenberghe, 1990; Chen *et al*, 1989; and Gay *et al*, 1984). Usage as a single agent in animal models has led to resistance, similarly human trials with fluoroquinolone

alone or with inactive drugs has led to the rapid emergence of resistance. Fluoroquinolones, particularly sparfloxacin have produced additive effects with other antituberculous drugs *in vitro* and in animals. Initial clinical trials of ofloxacin in combination with INH and rifampin indicate activity comparable to ethambutol. Ofloxacin used alone in a dose of 300 mg/day in patients having MDR tuberculosis has produced decreases in sputum colony counts, with sputum conversion in 26 %, in non converters resistance emerged (Alford and Wallace Jr, 1995).

Several β-lactamase resistant β-lactam antibiotics or combinations with β-lactamase inhibitors such as clavulanic acid are active *in vitro* against *M. tuberculosis* and other nontuberculous mycobacteria (Alford and Wallace Jr, 1995). Voladri *et al.* (1998) reported that the major β-lactamase of *M. tuberculosis* is a class A β-lactamase with predominant penicillinase activity and that a minor β-lactamase with greater cephalosporinase activity is also present. A better understanding of the mechanisms by which *M. tuberculosis* expresses resistance to β-lactams might ultimately lead to strategies in which these agents could be used in the treatment of tuberculosis as they are extensively developed, highly effective, widely used and relatively non toxic antimicrobial agents (Voladri *et al*, 1998).

1.4.2 TUBERCULOSIS CHEMOTHERAPY

There are numerous articles and books that discuss the recommended tuberculosis chemotherapy *e.g.*, Merck Manual of Diagnosis and Therapy (Berkow, 1992). Weyer and Kleeberg (1992) discuss the recommended therapy in South Africa in their article on primary and acquired resistance in South Africa. I have chosen to use the South African Medicines Formulary produced by the Department of Pharmacology, University of Cape Town, South Africa to discuss the therapy of tuberculosis mainly taking into account the South African scenerio (Gibbon and Swanepoel, 1997).

Standardised short-course chemotherapy in developing countries has been successful when combined with a control programme in which drug administration is supervised

and facilities for sputum examination are available. Treatment regimens are divided into intensive and follow-up phases. During the intensive phase the bactericidal effect treatment leads to rapid bacteriological sputum conversion and culture negativity and an improvement of the illness. During the follow-up phase, when fewer drugs are given the sterilising effect of the treatment eliminates the remaining viable bacilli and prevents subsequent relapse (Gibbon and Swanepoel, 1997).

For standardised chemotherapy it is necessary to categorise tuberculosis cases and to determine treatment regimens for each category:

1.4.2.1 Regimen 1: Newly diagnosed adults

Cases of smear or culture positive pulmonary tuberculosis and other newly diagnosed seriously ill patients with tuberculosis (including tuberculosis meningitis, pericarditis, peritonitis, disseminated tuberculosis, bilateral or extensive pleurisy, spinal disease with complications, extensive pulmonary parenchyma involvement and gastrointestinal or genitourinary tuberculosis) who have never been treated before, or who have been previously treated for less than four weeks (Gibbon and Swanepoel, 1997). This regimen is very similar to that recommended by Weyer and Kleeberg (1992).

Table 1.1: Regimen 1- Newly diagnosed adults

Intensive Phase: 2 months	under 50 kg	over 50 kg
Rifampicin/INH/PZA combination tablet: 120/80/250 mg	4 tablets	5 tablets
Ethambutanol 400 mg	2 tablets	3 tablets
Continuation Phase 4 months		
Rifampicin/INH Combination tablet 150/100 mg Combination tablet 300/150 mg	 3 tablets --	 -- 2 tablets

1.4.2.2 Regimen 2: Retreatment adults

Relapse or treatment failure (smear or culture positive). Anyone who has been treated at any time before for more than four weeks. These patients should have drug sensitivity tested at the start of treatment (Gibbon and Swanepoel, 1997).

Table 1.2: Re-treatment of Adults

Intensive Phase: 2 months	Under 50 kg	Over 50 kg
Rifampicin/INH/Ethambutanol Combination tablet 150/75/300 mg	3 tablets	4 tablets
PZA 500 mg	2 tablets	3 tablets
Streptomycin*	750 mg	1000 mg

* Streptomycin dose must be reduced to 750 mg in patients older than 45 years and must not be given to those over 65 years or during pregnancy. The dose in renal patients should be reduced or the dosing interval increased.

Table 1.3: Treatment at 3 months

Third month	under 50 kg	over 50 kg
Rifampicin/INH/ethambutol combination tablet: 150/75/300 mg	3 tablets	4 tablets
PZA 500 mg	2 tablets	3 tablets
Continuation Phase 5 months		
Rifampicin/INH/ethambutol Combination tablet 150/75/300 mg	3 tablets	4 tablets

1.4.2.3 Regimen 3: Newly diagnosed children

Children with newly diagnosed primary tuberculosis and/or pleural effusions should be treated with regimen 3 drugs (Gibbon and Swanepoel, 1997).

Table 1.4: Regimen 3- Newly diagnosed children

Intensive Phase 2 months	5-10 kg	11-20 kg	21-30 kg
Rifampicin/INH combination tablet: 150/100 mg	1/2 tablet	1 tablet	2 tablets
PZA 500 mg	1/2 tablet	1 tablet	2 tablets
Continuation Phase 2 months			
Rifampicin/INH Combination tablet 150/100 mg	1/2 tablet	1 tablet	2 tablets

Regimen 4 combination should be used for newly diagnosed tuberculosis in children with progressive primary or cavitating tuberculosis or non-pulmonary tuberculosis. This regimen is the same as regimen 3 with the exception that the continuation phase is longer *i.e.*, 4 months (Gibbon and Swanepoel, 1997).

1.4.2.4 Regimen 5: Chemoprophylaxis in Children < 2 years

Children under 2 years of age who have been in close household contact with a smear positive case of tuberculosis are at considerably increased risk of infection and disseminated disease. Regimen 5 is the recommended combination of therapy.

Table 1.5: Chemoprophylaxis in Children

3 months	under 5 kg	5-10 kg	11-20 kg
Rifampicin/INH combination tablet: 150/100 mg	1/4 tablet	1/2 tablet	1 tablet

1.4.2.5 Regimen 6: Chemoprophylaxis for healthy 2-5 year old contacts

Children 2-5 years of age should receive 6 months of INH prophylaxis (5-10 mg/kg/day). Regimen 6 is shown in the table below.

Table 1.6: Chemoprophylaxis for healthy 2-5 year old contacts

6 months	10-20 kg	21-30 kg
INH	100 mg	200 mg

1.4.2.6 Treatment of multi-drug resistant tuberculosis

MDR tuberculosis is diagnosed when sensitivity tests show resistance to both isoniazid and rifampicin at least. Choice of appropriate regimen should be made individually, based on evaluation of previous treatment episodes or, preferably on sensitivity testing. A single drug should not be added to a failing regimen. Treatment should not be adjusted while awaiting bacteriological results, except in rapidly deteriorating patients. Treatment of drug resistant tuberculosis should not be attempted at general tuberculosis clinics; these patients should be referred to a hospital (Gibbon and Swanepoel, 1997).

Contacts of sputum smear-negative drug resistant tuberculosis patients are managed according to the standard recommendations for contacts of drug-sensitive tuberculosis. The treatment of household contacts of smear-positive drug resistant tuberculosis patients can be carried in two ways: children younger than 2 years and all HIV postive contacts should be given preventive therapy, irrespective of their state of health and tuberculin response, where preventive therapy should include at least two drug to which the source strain is susceptible. The decision to treat depends on clinical assessment in the case of children older than two years and adult contacts, however, routine preventive chemotherapy is not recommended (Gibbon and Swanepoel, 1997).

Cohn (1995) in his retrospective study on the treatment of MDR tuberculosis showed that in immunocompetent patients with secondary MDR tuberculosis, only 56% responded to prolonged courses of multiple drug regimens and 22% died of tuberculosis. In patients with AIDS even fewer patients respond, with median survivals of 2-4 months. Cohn (1995) comments that MDR tuberculosis is best

prevented by directly observed therapy of patients with susceptible organisms and rigorous infection control practices in areas of high incidence of MDR tuberculosis.

1.4.2.7 Supervision of therapy

The taking of every capsule and tablet should be ideally supervised (directly observed therapy-DOT). Non-compliance with self-medication can result in drug resistance. Supervisors of treatment do not necessarily need to be health workers (Gibbon and Swanepoel, 1997). Wilkinson and De Cock (1996) showed that non-health workers *e.g.*, store keepers were just as good as clinic sisters where 89% of cases reached completion of treatment. They should keep the drugs and the treatment card, supply the medication, watch the patient taking the treatment and sign according (Gibbon and Swanepoel, 1997).

Wilkinson and De Cock (1996) reported on the treatment of tuberculosis in a rural South African community, Hlabisa. World Health Organisation (WHO) and International Union against Tuberculosis and Lung Disease (IUATLD) recommend passive case finding and provision of short course (6 months) chemotherapy which should cure the vast majority of cases and if coverage is high enough, which will lead to a sustained reduction in case rates. Wilkinson and De Cock (1996) reported success achieved in Hlabisa with an alternative approach that being: all patients to be treated with an intermittent rather than a daily drug regimen, there should be only one regimen, all patients to receive directly observed therapy for full duration of their treatment, patients need not have sputum taken after diagnosis *i.e.*, no smears at 2 months and 6 months, the tuberculosis control programme must be structured and resourced in a way that is fully cognisant of the needs of a comprehensive district health system that provides a wide range of essential services, of which tuberculosis treatment is only one.

Bacille Calmette-Guerin (BCG) is a freezed dried live vaccine prepared from an attenuated strain of bovine tubercle bacillus. BCG should not be administered concurrently with antituberculous therapy or chemoprophylaxis. Vaccination with

BCG is required in South Africa. Immunisation is commenced at birth, and repeated at 3 months if no scar is visible. Efficacy is controversial (Gibbon and Swanepoel, 1997).

1.4.3 MOLECULAR MECHANISMS OF RESISTANCE

Until recently the molecular mechanisms of resistance in *M. tuberculosis* remained largely unknown. The recent resurgence of tuberculosis, along with the outbreaks of MDR tuberculosis, has stimulated a great deal of interest in understanding the molecular basis of drug resistance in *M. tuberculosis* (Bloom and Murray, 1992). There has been tremendous progress in this field due to the application of mycobacterial molecular genetics (Zhang, 1996). Molecular analysis of some MDR tuberculosis strains suggest that the MDR phenotype is caused by sequential accumulation of individual mutations in separate genes, not by novel mechanisms due to single mutagenic event (Heym *et al*, 1994).

Zhang *et al*. (1992) reported on the catalase-peroxidase gene, *kat*G gene, which was responsible for isoniazid resistance. The *kat*G gene was cloned and used as a probe, a *kat*G deletion was identified in some highly INH-resistant clinical isolates of *M. tuberculosis*. To determine whether the loss of catalase activity (either due to *kat*G gene deletion or point mutations) is the cause of INH resistance, INH resistant *M. tuberculosis* strains that were defective in catalase activity were transformed with a functional *kat*G gene, which resulted in complete restoration of INH sensitivity to these strains resistant to different concentrations of INH (Zhang *et al*, 1993). Among INH resistant *M. tuberculosis* strains, *kat*G deletion seems to be a relatively rare event, accounting for 5-10% of INH resistant *M. tuberculosis* strains, most have point mutation in the *kat*G gene (Heym *et al*, 1994) and a few have point mutations in a second gene, *inh*A, which has being implicated in INH resistance (Banerjee *et al*, 1994). Point mutations in the *inh*A gene (Ser to Ala change at amino acid residue 94) was found to confer a low level of INH resistance as well as ethionamide (a structural

analogue of INH) resistance in *M. smegmatis* by allelic exchange experiments. Mutations in the *inh*A gene appear to be associated with low level INH resistance, in contrast to the high level of resistance caused by inactivation of catalase-peroxidase due to *kat*G deletion or point mutations (Zhang *et al*, 1992).

Resistance to rifampin in *M. tuberculosis* has been associated with alterations in the segment of the *rpo*B gene encoding amino acids 430-456 of the RNA polymerase β subunit (Telenti *et al*, 1993 and Miller *et al*, 1994). Morris *et al*. (1995) analysed the mutations in rifampin resistant strains by PCR and nucleotide sequencing. Six different *rpo* B mutations were identified, with the most frequent gene alteration associated with rifampin resistant phenotype occurred at position 456. Sixteen of the rifampin resistant strains had a C to T transition in codon 456, which converted serine (TCG) to leucine (TTG). Five rifampin resistant isolates had mutations at position 451: three C to T transition that changed histidine (CAC) to tyrosine (TAC) and two C to G transversions that substituted aspartic acid (GAC) at this position. Five A to T transversions that convert aspartic acid at position 441 to valine (GTC) and a single G to T transversion that substitutes a tyrosine at codon 451 were detected. A unique G to A transition converted the codon 440 methionine (ATG) to isoleucine (ATA) (Morris *et al*, 1995).

Mutations in genes encoding the ribosomal S12 protein (*rps*L) and 16s rRNA (*rrs*) have been detected in streptomycin resistant strains of *M. tuberculosis* (Finken *et al*, 1993). Morris *et al*. (1995) studied the genetic mutations in 11 singly streptomycin resistant clinical isolates and 33 streptomycin resistant MDR tuberculosis strains. Seven of the 11 strains resistant to only streptomycin had *rps*L mutations. Six of these seven *rps*L genes exhibited an A to G transition at codon 43, which converts lysine to arginine; the other was an A to G transition in codon 88, which also converts lysine to arginine. No *rrs* mutations were identified in singly streptomycin resistant strains.

Eighteen of the 33 streptomycin resistant MDR tuberculosis isolates also had *rps*L mutations. Sixteen had the A to G transition at codon 43 and 2 had the same codon 88

mutation described for singly streptomycin resistant strains. Also, A to C transversions were identified at position 513 of the *rrs* genes in 5 of 33 MDR tuberculosis strains. A, C to T transition was also detected at position 491 in 2 MDR tuberculosis strains. No alterations were detected at other positions of the *rrs* gene (512, 516 and 904) that have been associated with streptomycin resistance (Morris *et al*, 1995).

1.4.4 INFLUENCE OF THE AIDS-HIV PANDEMIC ON TUBERCULOSIS CHEMOTHERAPY

Human immunodeficiency virus (HIV) infection affects up to 10 million persons worldwide. It exerts a pronounced influence on the natural history of tuberculosis in several ways; in addition, infection with *M. tuberculosis* appears to affect the course of HIV disease as follows:

i) Among persons latently infected with tuberculosis who become HIV infected, active tuberculosis develops at a rate of 7 to 10% per year rather than 8 % per lifetime.

ii) Persons with HIV (regardless of $CD4^+$ cell counts) who are newly infected with *M. tuberculosis* progress to active tuberculosis at a rate as high as 37 % in the first 6 months rather than 2 to 5 % in the first 2 years.

iii) HIV confers anergy upon a large number of persons with HIV infection, thus confounding tuberculin skin test (TST) interpretation, the prevalence of anergy increases as the CD4 cell counts decreases. In many areas where tuberculosis is endemic, prophylactic INH therapy is recommended for anergic persons with HIV infection.

iv) HIV-infected persons may malabsorb drugs perhaps because of HIV related enteropathy, which may further complicate the treatment of tuberculosis. In addition, immunocompetent persons who have undergone gastrectomy may, in the course of treatment for drug susceptible tuberculosis, progress to drug resistant tuberculosis because of malabsorption of drugs (Sepkowitz *et al*, 1995).

Tuberculosis may influence the natural history of HIV infection by activating macrophages that harbor HIV. The result of activation is expression of HIV, rather than prolonged latency without expression of HIV. In a study carried out by Pape *et al.* (1993) progression to AIDS occurred sooner among TST-positive persons not treated with INH than those treated with INH, even when tuberculosis was excluded as AIDS indicator disease, which suggests that treatment of latent tuberculosis can help preserve the immune function of HIV-infected individuals. These influences have conspired to create an epidemic of tuberculosis within the HIV epidemic that is particularly threatening to the public health in areas such as Africa, where large numbers of individuals are dually infected with *M. tuberculosis* and HIV (Sepkowitz *et al*, 1995).

Wilkinson and Moore (1996) assessed the difference between HIV infected and non-infected tuberculosis patients in the Hlabisa hospital, Kwazulu-Natal, South Africa. They found that HIV-positive patients with tuberculosis were three times more likely to fail to complete treatment than HIV-negative patients. HIV infection is clearly altering the epidemiological profile of tuberculosis in rural South Africa and poses an additional challenge to tuberculosis control programmes to maintain high case-holding rates among HIV-infected tuberculosis patients.

M. tuberculosis can cause disease at nearly all stages of HIV infection. There is a wide spectrum of clinical disease in adults which ranges from typical pulmonary presentation, in those with relatively well preserved immunity and CD4 counts in excess of 200 cells/mm^3, to atypical disseminated disease in markedly immunosuppressed individuals (Gilks, 1996).

In adults with pulmonary disease, cavity formation occurs less frequently and there is much less predilection for the apical regions. Unusual lower lobe infiltrates which may be bilateral and are smear negative but culture positive are the commonest diagnostic challenge. Without bronchoscopy or induced sputum, many diagnoses are established by a 1-month therapeutic trial. Pleural effusions occur more frequently with HIV infection and can be sometimes be bilateral (Gilks, 1996).

The response to standard treatment appears adequate, at least in the earlier stages of HIV infection. The high mortality seen in endemic areas during therapy is usually the result of other severe bacterial infections such as pneumococcal pneumonia or disseminated non-typhi salmonellosis rather than a failure to respond to appropriate therapy. A difficulty in treating HIV-tuberculosis in poor resource countries is the widespread routine use of thiacetazone as a first line anti-tuberculous drug. This drug is toxic in individuals with underlying HIV infection and can cause hypersensitivity reactions and severe Stevens-Johnson syndrome in up to 20% of cases (Drobniewski *et al*, 1995 and Gilks, 1996). The cost implications of replacing thiacetazone with expensive short-course regimens containing rifampin are significant and many African countries cannot afford to switch even though this is a much safer policy (Gilks, 1996). In Kenya the recurrence rate of tuberculosis in HIV-infected patients was 34-fold greater than in HIV negative patients treated with thiacetazone containing regimens (Hawken *et al*., 1993). In a study in Zambia, 22 of 237 children developed cutaneous hypersensitivity reactions whilst on a regimen that included thiacetazone; 12 of the 22 children who developed Stevens-Johnson syndrome were HIV-positive (Chintu *et al*, 1993).

There is little to be complacent or optimistic about when considering the impact that HIV has had and increasingly will have on tuberculosis. The impact is already devastating and in some areas totally disrupting existing tuberculosis programmes. The burden of the disease is increasing everywhere. The HIV epidemic has cruelly exposed weaknesses in tuberculosis control worldwide and is undoubtly making them worse. Tuberculosis remains a global priority even without the interaction with HIV (Gilks, 1996).

1.5 *M. tuberculosis*: THE MOLECULAR STORY

The diagnosis of *M. tuberculosis* infections are often delayed due to slow generation time, about 15-20 hours which means that visible cultures are only apparent after several weeks of incubation. Direct microscopy on specimens by auramine-rhodamine or Ziehl-Neelsen staining is rapid but has a detection limit of 10 000

bacilli/ml and sensitivity could range from 22 to 78% of culture proven cases. Culture is recognised as the "gold standard" for the diagnosis of tuberculosis and has a detection limit of 100 bacilli/ml. Although the BACTEC, a culture based method has been introduced, the procedures are still considerably slower than most conventional bacteriology (Butcher *et al*, 1996).

The explosive development of the technology of molecular biology has provided a host of potential applications for diagnostic microbiology. Initially nucleic acid probes were applied to both cultured bacteria and clinical specimens, subsequently various radioactive and non-radioactive probes have been found to be most useful for the rapid identification of cultured mycobacteria. Nucleic acid amplification techniques, polymerase chain reaction (PCR), can be applied to the detection of specific and non-specific mycobacterial gene sequences in clinical specimens, cultures and environmental samples to provide an extremely sensitive and rapid diagnostic test. PCR may also be used to produce probes for the confirmation of amplimer identity, restriction fragment length polymorphism (RFLP) to amplify species specific rRNA sequences for species identification and direct PCR typing (Hawkey *et al*, 1996).

1.5.1 MOLECULAR TECHNIQUES FOR DIAGNOSIS

Nucleic acid amplification-based methods for the detection and identification of mycobacteria can be divided into three types:

a) amplification of genus specific sequences;

b) amplification of genus specific sequences followed by oligonucleotide probing to give a species specific test;

c) amplification of species specific sequences

A wide range of target sequences has been used, although in the case of genus specific primer pairs, the genes encoding the 65 kDa antigen are popular and for the species specific primers those based on IS6110/IS986 (Hawkey *et al*, 1996). The choice of target gene is an important determinant of sensitivity and specificity (Butcher *et al*,

1996). The method of extracting DNA from specimens has a great influence on the sensitivity and specificity of PCR protocols (Hawkey *et al*, 1996). The main objectives are the efficient release of mycobacterial DNA and the removal of any material in the sample that may be inhibitory to the PCR; at the same time, it is desirable to avoid introducing chemicals that may themselves inhibit the PCR. A method that is suitable in a research laboratory with a handful of samples may not be workable in a diagnostic laboratory. Lysis of mycobacteria can be difficult because of the thick lipid-rich cell wall and the cell wall components can also contaminate the DNA preparation (Butcher *et al*, 1996). Good results can be obtained with simple methods, such as boiling, mechanical disruption and sonication (Hawkey *et al*, 1996).

It is important to detect inhibition in order to identify false negative reactions. A simple method is the division of the extracted specimen into two and spiking one portion with a low level *M. tuberculosis* DNA, however, this involves doubling the number of PCR tests. This could be avoided by using an internal control, which yields a product that can be distinguished from the test product. Use of a different DNA template is not ideal, as different primers may vary in their sensitivity to inhibition or amplification efficiencies. It is preferable to use an internal control that has the same primer binding sites as the natural target, but the product has to be distinguishable. Therefore, the control can be cloned, modified template yielding a different size of fragment, or with part of the target replaced by another sequence so that they can be distinguishable by specific probes (Butcher *et al*, 1996).

Contamination occurs in all laboratories, but can be minimised. The first step is to design the laboratory and develop protocols to minimise contamination. Common procedures include physical separation of different stages of the process, and the use of dedicated equipment for clean processes. A widely used method involves the use of uracil-N-glycosylase (UNG) which breaks down amplicons containing deoxyuracil, resulting in the use of dUTP instead of dTTP in the PCR mix. The UNG destroys any amplicons present initially and is then inactivated at 94^0C at the start of the PCR reaction. The efficiency of the PCR is not affected, however, this does not protect the PCR against contamination from other sources, including other uses of the target

DNA in the same laboratory or pre-existing contamination. Procedure such as the use of UNG must be used to supplement and not replace basic procedures against contamination. Also, a negative control should be included in all batches of a samples (Butcher *et al*, 1996).

PCR in theory can detect the presence of a single bacillus within a few hours. In reality, the adaptation of PCR for the detection of mycobacterial disease is problematic. The problems include the difficulty and unreliability of extracting DNA from specimens; presence of inhibitors in the specimens; problem of contamination with amplicons giving false positives results; non-robust methods for the detection of the amplified product; poor sensitivity when there are few organisms present; the difficulty in interpreting the results in respect to active disease and PCR positive, culture negative samples. In a blind folded comparative study carried out by Noordhoek *et al.* (1994) in seven laboratories experienced in PCR for *M. tuberculosis*, they reported false positive rates ranging from 3-20% up to 77%. Reliable detection of *M. tuberculosis* is dependent on conventional methods, however this situation can change as automated techniques become available, thus reducing labour costs and increasing reliability. Until then the main value of PCR will be for specific situations where conventional methods are especially inadequate; limiting examination to selected cases makes it possible to carry out the PCR more thoroughly, using different sets of primers, hybridization to confirm specificity, checking for inhibition and repeating positive tests to exclude contamination (Butcher *et al*, 1996).

Non-PCR based amplification techniques have been applied to the diagnosis of mycobacterial infections, these include: strand displacement assay (SDA) developed by Becton Dickinson, transcription mediated amplification (TMA) developed by Gen-Probe and the reporter phage systems. The principle of the commercially available TMA is that specific rRNA is amplified isothermally by an autocatalytic process, the products being detected with an acridium-labelled DNA probe in a homogeneous solution hybridisation following a reverse transcriptase step (Hawkey *et al*, 1996).

The detection limit of specific labelled gene probes that can detect mycobacteria by hybridisation is of the order of 10^5 bacteria, whereas the number of bacilli in many clinical samples is much lower than this. Gene probes are not sufficiently sensitive for the direct detection of mycobacteria in clinical specimens, but have become the method of choice for speciation of mycobacteria after isolation and growth in culture. Probes available (Gen-Probe) have >95% specificity for several mycobacterial species. The target sequences are often short. The occurrence of a mutation or natural variation may lead to failure to identify that organism (Butcher *et al*, 1996).

A further system is the Ligase Chain Reaction (LCR). This involves a thermostable ligase joining two oligonucleotides that are immediately adjacent to each other when they hybridise to target DNA. The products of a cycle of denaturation, annealing and ligation steps serve as templates for the next cycle, resulting in exponential amplification. The LCR is the basis of the Abbott Corporation mycobacterial detection test; it can detect down to five mycobacteria and show a sensitivity and specificity similar to PCR and other amplification systems (Butcher *et al*, 1996).

Cole (1996) reported on the need to study the nerve centre of the tubercle bacillus, its genome, by means of systematic deoxyribonucleic acid sequences analysis. He wrote this "will provide a wealth of information about *M. tuberculosis* that will undoubtly fuel the next generation of research". Cole *et al.* (1998) reported the complete genome sequence of the best characterised strain of *M. tuberculosis*, H37Rv. This will improve our understanding of the biology of this slow growing pathogen and help the conception of new prophylactic and therapeutic interventions. The genome comprises 4 411 529 base pairs and contains around 4 000 genes and has high guanine + cytosine content (Cole *et al*, 1998).

1.5.2 MOLECULAR EPIDEMIOLOGY

Identification of subtypes of *M. tuberculosis* has been a long-standing problem that has dogged epidemiologists and microbiologists. The development of molecular

fingerprinting techniques that identify subtypes that are related to the apparent epidemiology of cases promises to be one of the most exciting developments in the study of tuberculosis. The standard technique (restriction fragment length polymorphism) is based on the insertion sequence IS*6110*. Insertion elements are sequences of DNA bounded by short inverted repeated DNA sequences together with a direct repeat of the target sequence which are capable of movement within a replicon by a process analogous to transposition (Hawkey *et al.* 1996).

In the standardised technique genomic DNA is cut with the restriction endonuclease *Pvu* II and the fragments are separated on an agarose gel and transferred to a nylon membrane. The restriction endonuclease *Pvu* II has one asymmetric site within IS6110 and the genomic DNA is probed with IS*6110* DNA generated from DNA to the right hand side of this site. The resulting fingerprint will contain a number of bands which correspond to the number of copies of IS*6110* in an individual strain. This technique has been found to produce a level of typability and strain discrimination that is appropriate for the investigation of cross-infection by *M. tuberculosis* (Hawkey *et al*, 1996).

Some strains of *M. tuberculosis* have only a few copies of IS*6110* which greatly limits strain differentiation. Such strains have been distinguished by hybridisation of genomic DNA with probes to a polymorphic GC-rich repetitive sequence (PGRS), $(GTG)^5$ or a 36 base pair DR (Direct repeat) sequence. PGRS offers a somewhat better discriminatory power over IS6110 (Butcher *et al.*, 1996 and Hawkey *et al*, 1996).

Restriction endonuclease analysis types are stable and provide a valuable tool for studying mycobacterial transmission, however a very large number of fragments are produced which makes the electrophoretic conditions critical for good results and subsequent analysis is difficult. Some of the disadvantages of conventional restriction endonuclease analysis can be overcome by using restriction enzymes that cut less frequently and these can be separated by pulsed field gel electrophoresis (PFGE).

RFLP typing with specific probes is much easier to perform and is therefore preferred for the analysis of large numbers of isolates (Butcher *et al*, 1996).

With the current increases in the incidence of tuberculosis and emerging drug resistance molecular techniques will be implemented as routine diagnostic and epidemiological tests. They will make substantial contributions to disease management and in community based infection control measures (Butcher *et al*, 1996 and Hawkey *et al*, 1996).

Summary:

The literature was reviewed with a view to emphasise the major health care problem that tuberculosis presents to the world, to draw attention to the place that PZA has in modern chemotherapy of tuberculosis. Also to emphasise the importance of understanding the mode of action of a drug used in tuberculosis chemotherapy. The disease is first placed in its historical perspective followed by commenting on the interaction of tuberculosis with macrophages, since researchers have shown that the macrophage plays an important role in the mode of action of PZA. The proposed mode of action of PZA as outlined by Konno *et al.* (1967) and the present molecular characterisation of the *pnc*A gene coding for the enzyme Pzase is discussed. Chemotherapy of the entire millieu of drugs available for tuberculosis chemotherapy with their advantages and disadvantages are discussed. Finally, the influence of the HIV-AIDS pandemic on tuberculosis and the molecular studies undertaken for diagnosis and epidemiology are reviewed. The later two aspects emphasise the need for continued research in the tuberculosis arena as the eradication of the disease is far from over. With the following points in mind the experimental component of the study was commenced and are discussed in individual chapters.

CHAPTER 2

SURVIVAL OF *M. tuberculosis* FOLLOWING EXPOSURE TO PZA

2.1 INTRODUCTION

This chapter serves as a introduction to the other experimental procedures undertaken to study the mode of action of PZA on *M. tuberculosis, viz.*, HPLC to measure the concentrations of PZA and POA in non cell associated mycobacteria as well as in the extracellular, intracellular and intra-bacterial compartments of infected macrophages, production of polyclonal antibodies to PZA and immunogold labelling experiments to locate the site of action of PZA. Thus, the objective of this chapter is to assess the survival or death of non cell associated mycobacteria and mycobacteria bound within macrophages following PZA treatment.

The activity of PZA can be demonstrated only in an acid medium *in vitro* (McDermott and Tompset, 1954), which is not favourable to the growth of *M. tuberculosis*. There have been studies which investigated the effect of PZA on *M. tuberculosis* at acid pH 5.5. Butler and Kilburn (1982) investigated the effect of carbon dioxide and inoculum size on PZA inhibition where *M. tuberculosis* was incubated in the presence of 7% CO_2 in air at pH 5.5. Heifets and Lindholm-Levy (1990) reported on the minimal bactericidal concentration using PZA susceptible strains cultivated in 7H12 broth at pH 5.6. In 1992, Heifets and Lindholm-Levy investigated the *in vitro* activity of PZA at a lower pH of 5. Also, in our department, the *in vitro* activity of PZA and POA at various pH values has been extensively studied. Thus, the aim of this study was to evaluate the effect of the tissue culture medium, RPMI 1640 on non cell associated mycobacteria and their potential to convert PZA to POA in this medium. No manipulations of the pH of the medium were performed.

There are also numerous reports on the activity of PZA in macrophages with many studies investigating the effect of combinations of drugs with PZA. Mor and Estandiari (1997) investigated the synergistic activities of clarithromycin (CLA) and PZA against *M. tuberculosis*. Sbarbaro *et al.* (1992) investigated the effect of rifampin and PZA on mycobacteria and in 1996 investigated the combined effect of PZA and a quinolone (ofloxacin) as a potential preventive therapy regimen. Salfinger *et al.* (1990) reported on a comparison of PZA and POA activity in cultured human macrophages and in the BACTEC system. Crowle *et al.* (1986) investigated the effect of PZA alone on tubercle bacilli with cultured human macrophages, whereas Carlone *et al.* (1985) investigated the killing capacity of PZA, POA and rifampin on macrophages ingested live *M. tuberculosis*.

In this study the goal was to investigate the mode of action of PZA, thus combinations of drugs were not assessed for antibacterial activity. In experiments with non cell associated and macrophage bound mycobacteria exposed to PZA; the HPLC experiments were carried out concurrently with the growth assessment experiments. Although presented separately in the thesis for purposes of clarity we were able to co-operatively assess PZA activity on mycobacteria in relationship to the PZA and POA concentrations detected in the non cell associated mycobacteria as well as in the different compartments *viz.*, intracellular, extracellular and intra-bacterial, of the infected macrophages. We were also able to assess at which time interval PZA was converted to POA and the effect of this conversion on the growth of non cell associated mycobacteria in RPMI 1640.

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL ISOLATES

Mycobacterium tuberculosis strains resistant and susceptible to PZA as obtained from the Department of Medical Microbiology, University of Natal-Medical School are listed in appendix 1. Cultures were maintained on antibiotic free Middlebrook 7H10

agar slants (Appendix II), Lowenstein Jensen (LJ) slants and Middlebrook 7H9 broth (Appendix II). Ziehl-Neelsen (ZN) staining was performed regularly on all cultures to confirm purity. The Wayne's test (Appendix II) was performed on all strains to determine the presence of the pyrazinamidase (Pzase) enzyme, thus indicating whether the strain was susceptible or resistant to PZA. Stock cultures were frozen in freezing medium containing proteose peptone broth supplemented with glycerol.

One week old broth cultures were used in invasion assays with macrophages as well as in experiments with non cell associated mycobacteria in RPMI 1640. For the invasion assays with the macrophages, the mycobacterial cultures were centrifuged and the pellet resuspended in phosphate buffered saline (PBS). This suspension was vortexed for 1 min with 5 mm glass beads and allowed to settle for 15 mins at room temperature. The supernatant containing single mycobacteria and small clumps was adjusted to a McFarland No 1 standard (equivalent to 10^7 cells/ml- Hindler, 1992) before use in the macrophage assays.

2.2.2 SURVIVAL OF NON CELL ASSOCIATED MYCOBACTERIA FOLLOWING EXPOSURE TO PZA

A McFarland No 1 standard of all clinical isolates and the reference strain, H37Rv was exposed to 100 µg/ml PZA in RPMI 1640 tissue culture medium. At time intervals of 4, 8, 16, 24, 48, 72, 144, 240 and 336 h the mycobacteria were plated out onto Middlebrook 7H10 agar plates. The colony forming units enabled us to assess survival or death of mycobacteria following exposure to PZA.

2.2.3 ISOLATION OF MONOCYTES

Peripheral blood monocytes were obtained by overlaying blood from healthy volunteers onto Ficoll-Histopaque 1077 (Sigma) in the ratio 5:4, followed by centrifugation at room temperature at 400 x g for 30 mins (Hirsch *et al*, 1994 and

Johnson *et al*, 1977). The cells were washed twice with RPMI 1640 tissue culture medium and resuspended in RPMI 1640 supplemented with 10 % fetal calf serum that was inactivated at 56°C for 30 min. The monocyte suspension was aliquoted in 5 ml volumes into 65 mm diameter petri dishes at a concentration of approximately 10^5 cells/ml to obtain maximum yield for HPLC analysis. Monocytes were grown for 6 days with changes of RPMI 1640 supplemented with 10 % fetal calf serum every 2 days. On the 6th day the monocyte derived macrophages were used in invasion assays. The trypan blue exclusion assay confirmed viability of the macrophages, while the esterase test confirmed maturation to the macrophage stage.

2.2.4 SURVIVAL OF MACROPHAGE-BOUND MYCOBACTERIA FOLLOWING EXPOSURE TO PZA

Macrophages were exposed to the bacteria for 1 hour in the ratio 1: 10, after which all non cell associated bacteria were washed off. Five ml of fresh RPMI 1640 supplemented with 100 µg/ml PZA was added to the petri dishes. At time intervals of 4, 8, 16, 24, 48 and 72 h, the cells from 3 petri dishes were harvested and pooled to separate extracellular, intracellular and intra-bacterial fractions.

2.2.5 SEPARATION OF EXTRACELLULAR, INTRACELLULAR AND INTRA-BACTERIAL FRACTIONS

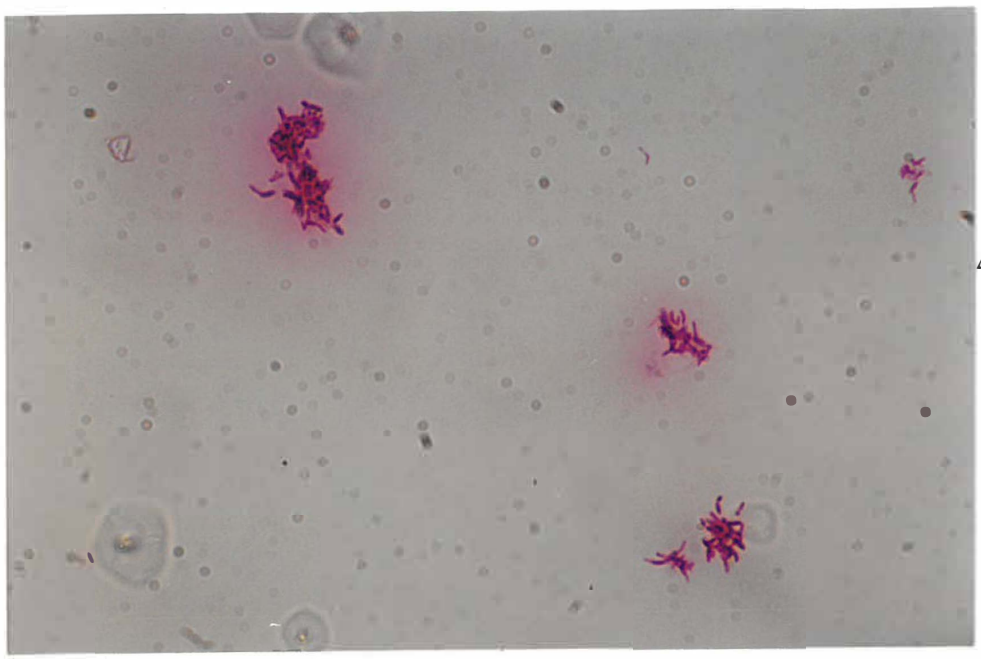
The infected macrophages were gently scraped off the petri dishes using a Sterilin quadloop. The suspension obtained was centrifuged at 250 x g for 10 mins. The supernatant was discarded except for 500 µl which was used to resuspend the pellet. To separate the extracellular from the intracellular fraction, silicone oil velocity gradient centrifugation was used (Johnson *et al*, 1980, Koga, 1987, Pascual *et al*, 1994, Klemper and Stryt, 1981). A 6:5 mixture of silicone oils (500 µl) of densities 1.07 and 0.98, respectively, was placed in an eppendorf tube and 500 µl of macrophage suspension was overlaid on the oil. This was then centrifuged for 3 mins

at 12 000 x g which resulted in a pellet of macrophages at the bottom of the tube. The extracellular fluid above the oil layer was removed. The pellet was resuspended in 500 µl of distilled water, vortexed and incubated at 37°C for 10 mins to completely disrupt the macrophages. This fraction contained the intracellular mycobacteria. Serial dilutions of this fraction was made in distilled water and plated out onto Middlebrook 7H10 agar plates to assess survival or death of macrophage bound mycobacteria following exposure to PZA.

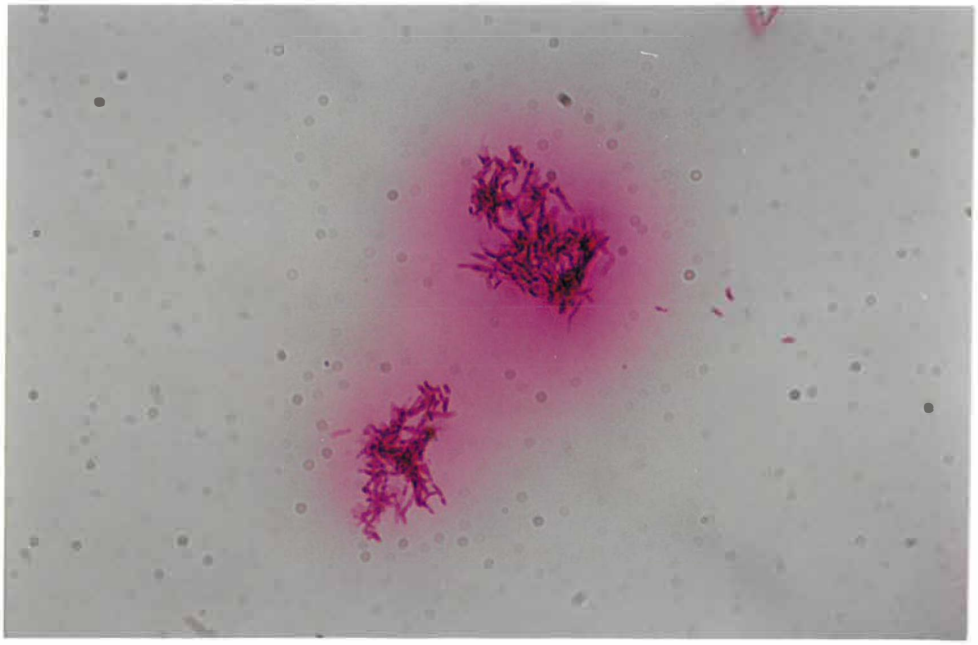
2.3 RESULTS

Prior to assessing the effect of PZA on non cell associated and macrophage bound mycobacteria, purity of the strains and maturation of the macrophages had to be assessed. ZN staining of *M. tuberculosis* was performed regularly to confirm purity of the culture as shown in FIG. 2.1. The esterase test, FIG. 2.2., was performed on macrophages at 6 days of incubation in RPMI 1640 supplemented with 10% fetal calf serum. A positive esterase test is indicated by black granulation over the macrophages, this indicates mature macrophages. Subsequent to infecting the macrophages with *M. tuberculosis*, ZN staining was performed to assess the degree of infection as shown in FIG. 2.3.

The reference strain, H37Rv, as well as 10 other clinical *M. tuberculosis* strains that are Pzase positive were assessed for their ability to survive or die in non cell associated cultures as well as in macrophage co-cultures following PZA exposure. These susceptible strains were able to survive in both systems *i. e.*, over a 336 h period in non cell associated cultures and over 72 h in macrophage co-cultures (FIG. 2.4-2.14). The same pattern of survival was observed in *M. bovis* (naturally Pzase negative) as well as in clinical isolates that were Pzase negative (FIG. 2.15-2.20). These results are presented graphically. Composite graphs show the mean CFU/ml for susceptible and resistant strains as well as strains incubated without antibiotics (FIG. 2.21-2.22).



A



B

FIG. 2.1. ZN staining of *M. tuberculosis* cultures. (A) single mycobacteria and (B) characteristic clumps of mycobacteria

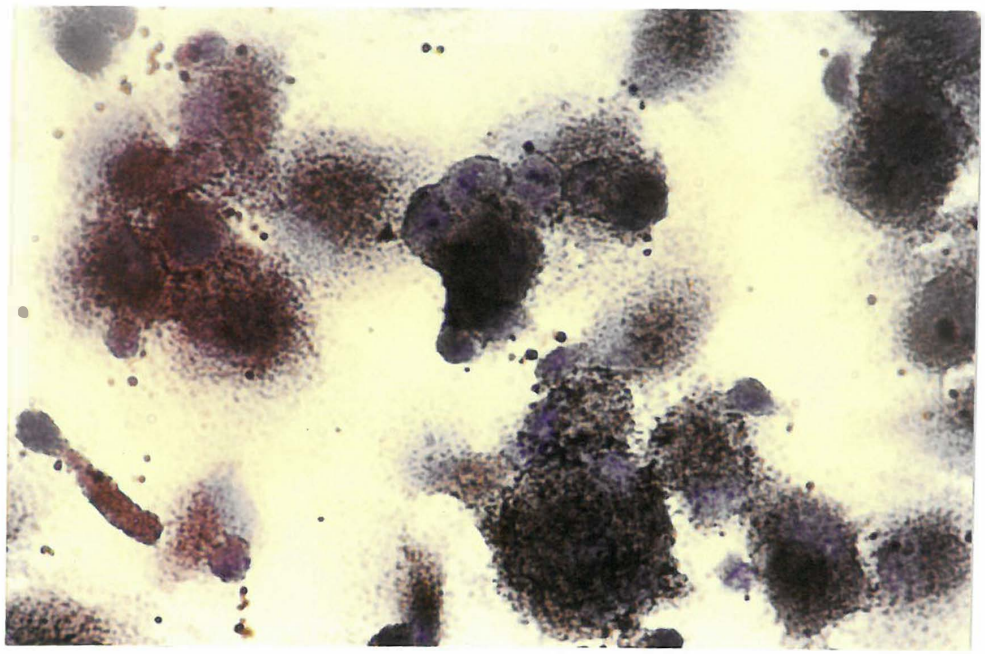
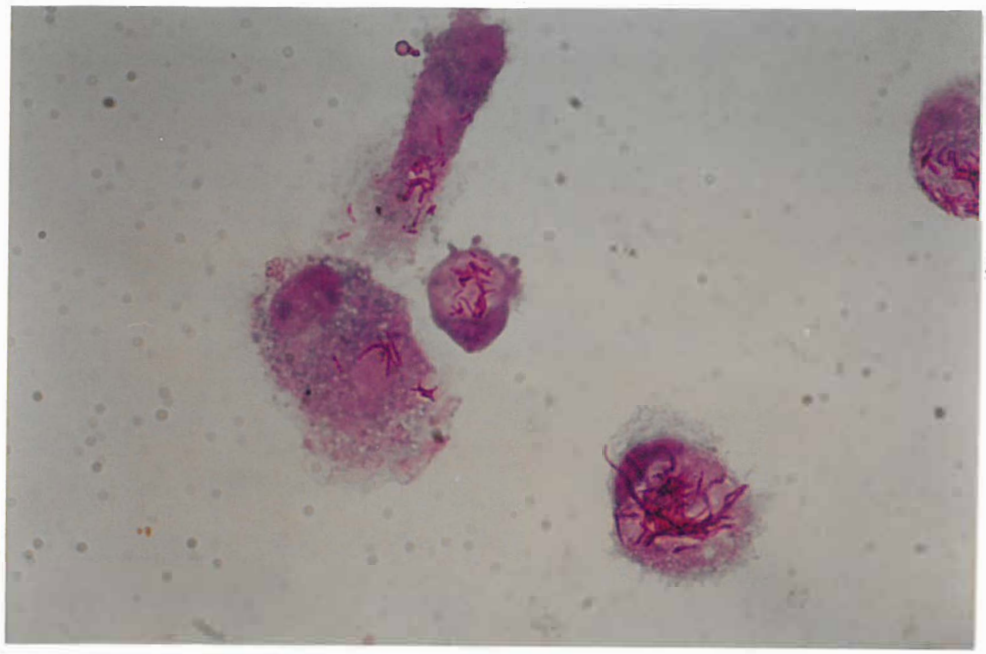
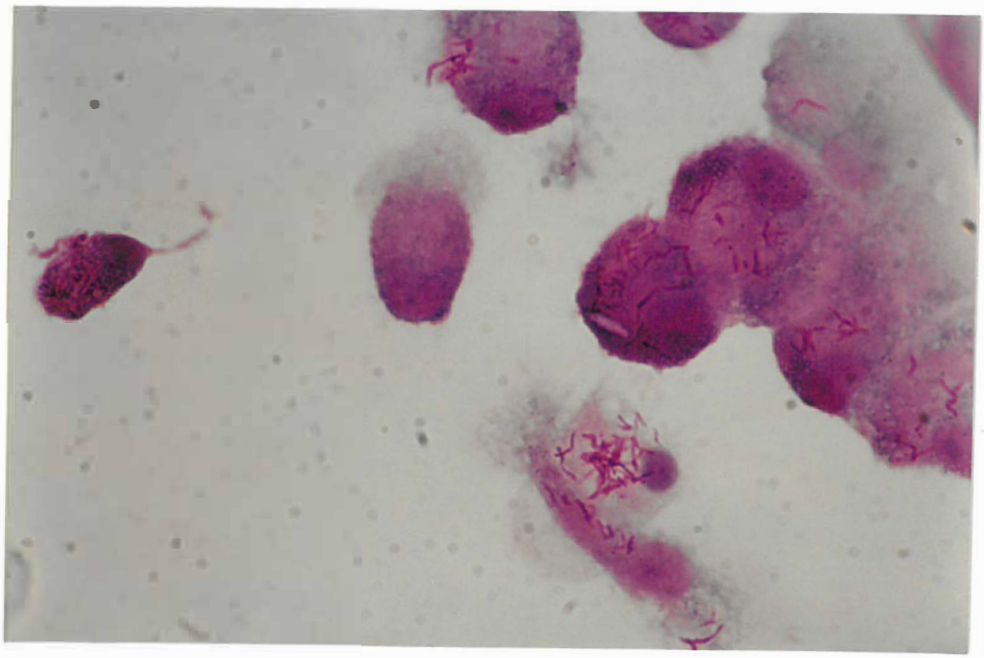


FIG. 2.2. Positive esterase test indicating mature macrophages



A



B

FIG. 2.3. (A) and (B) ZN staining on macrophage bound mycobacteria.

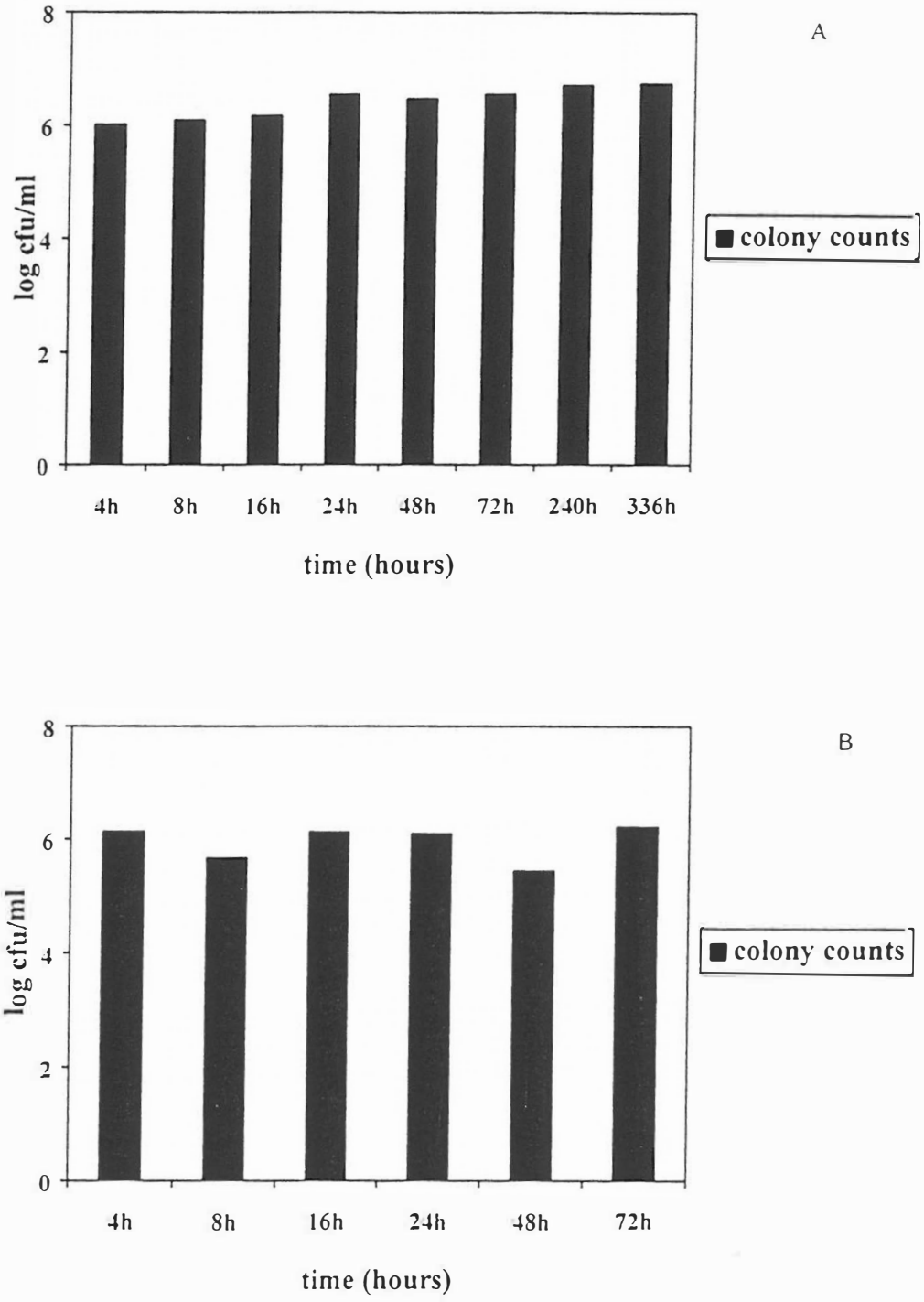


FIG. 2.4. Colony counts of H37Rv exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

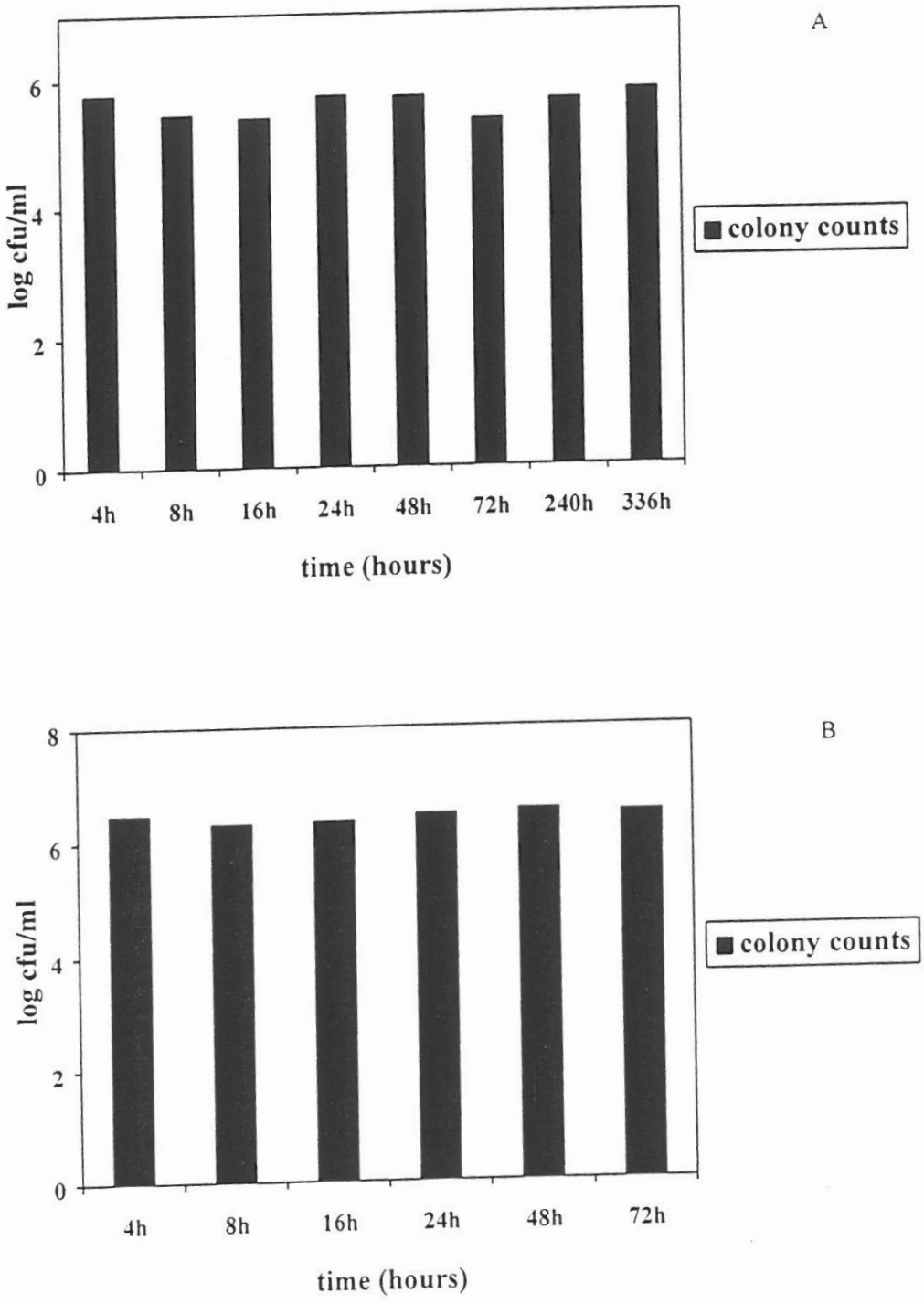


FIG. 2.5 Colony counts of a susceptible strain, 200R exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

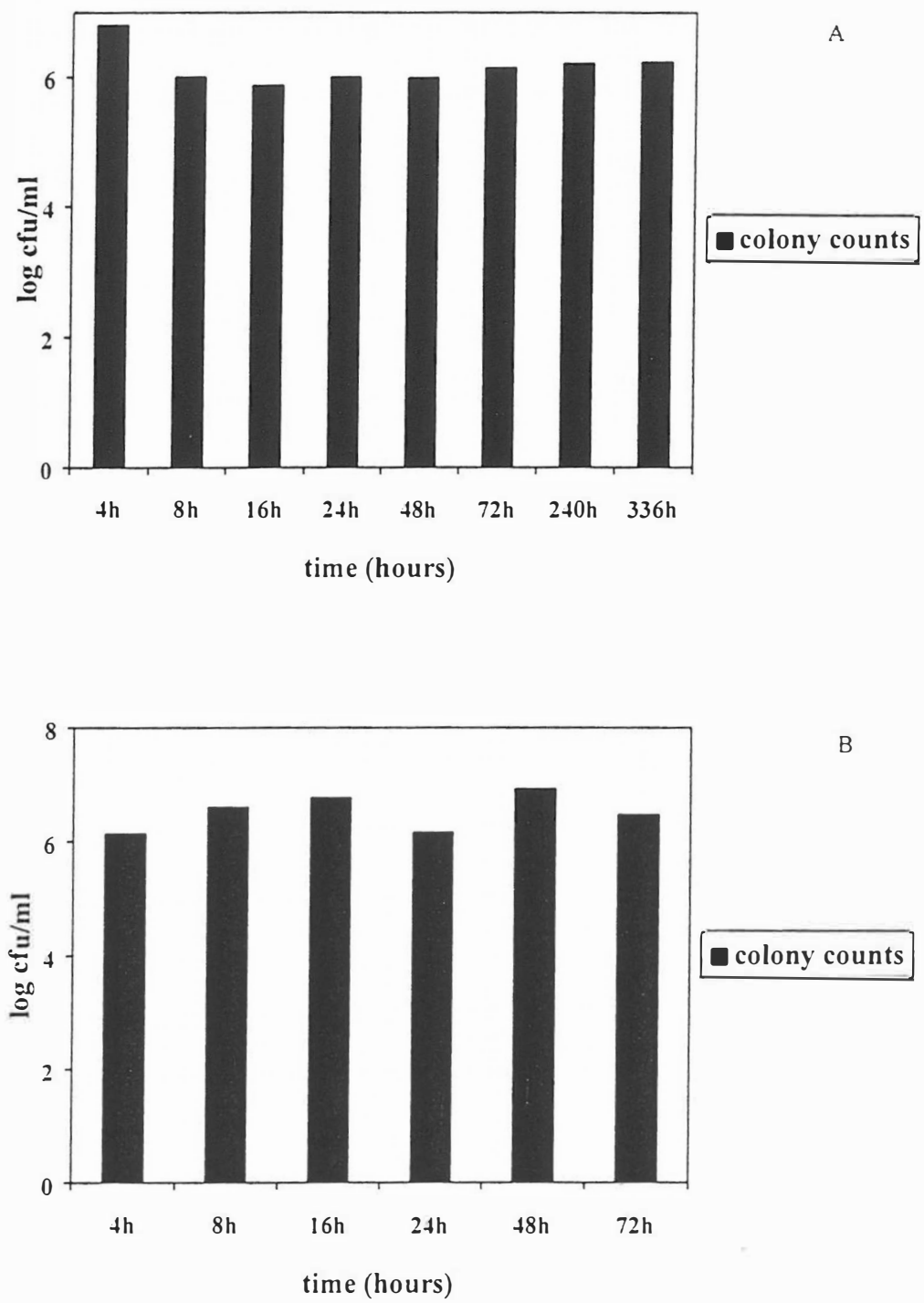


FIG. 2.6 Colony counts of a susceptible strain, 100R exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

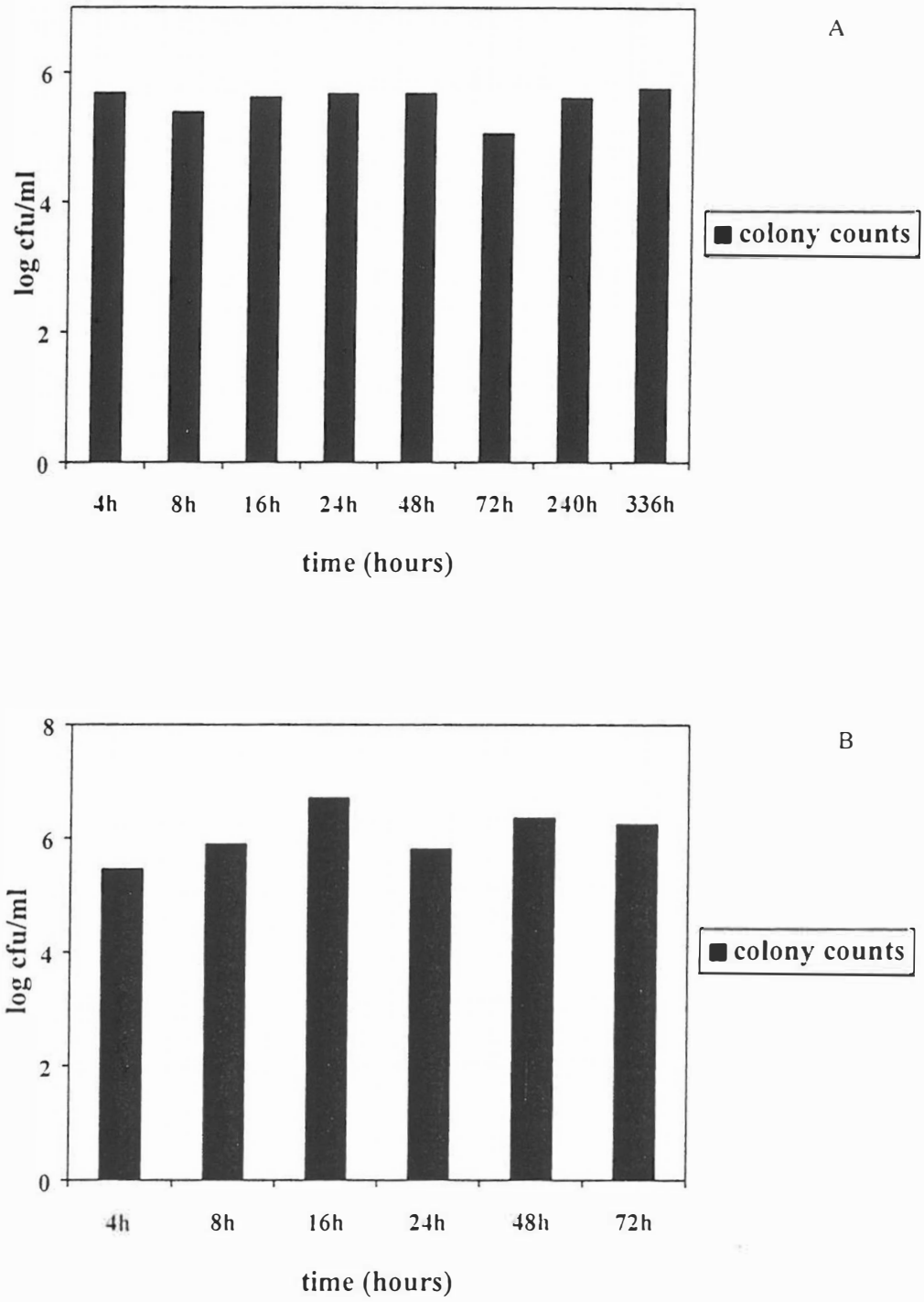


FIG. 2.7 Colony counts of a susceptible strain, 11344 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

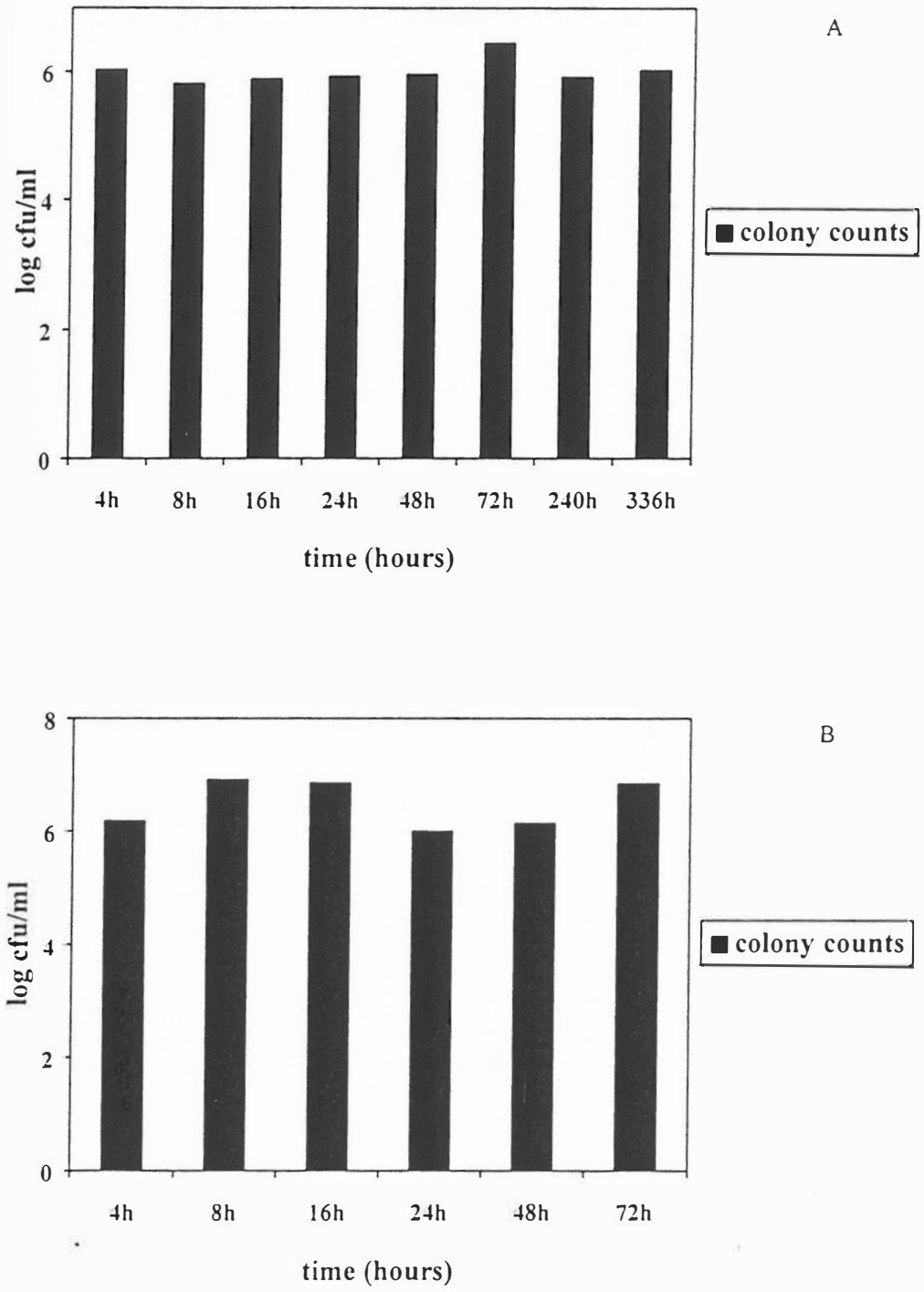
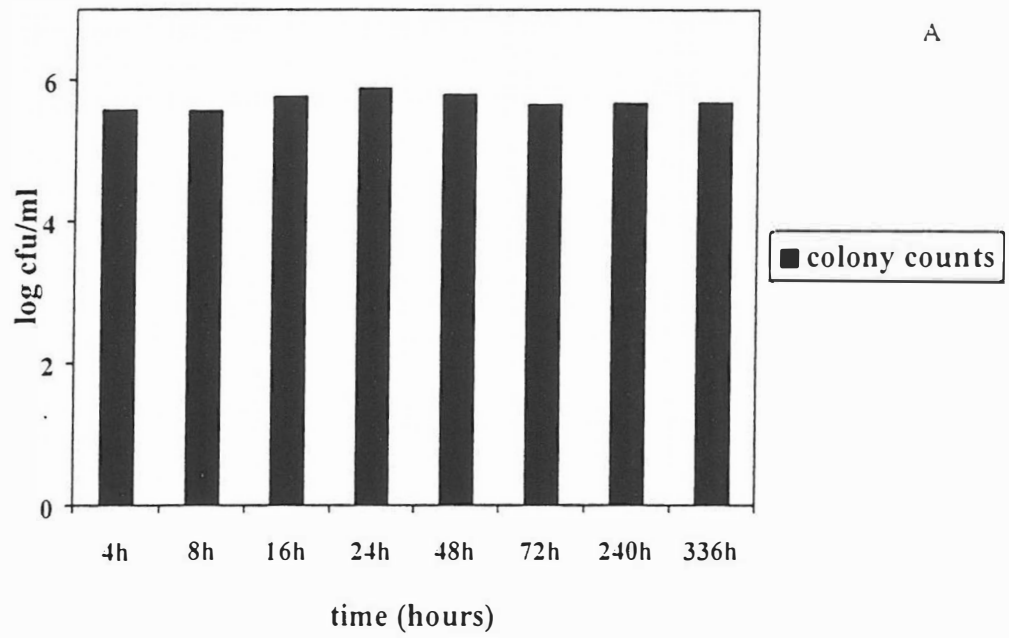


FIG. 2.8 Colony counts of a susceptible strain, 90s exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.



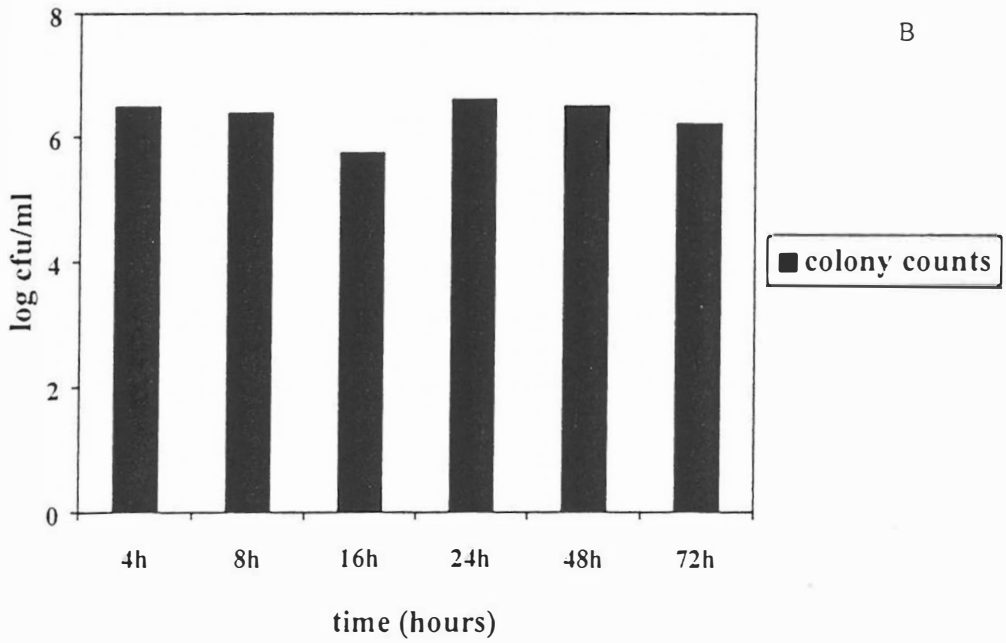


FIG. 2.9 Colony counts of a susceptible strain, 11102 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

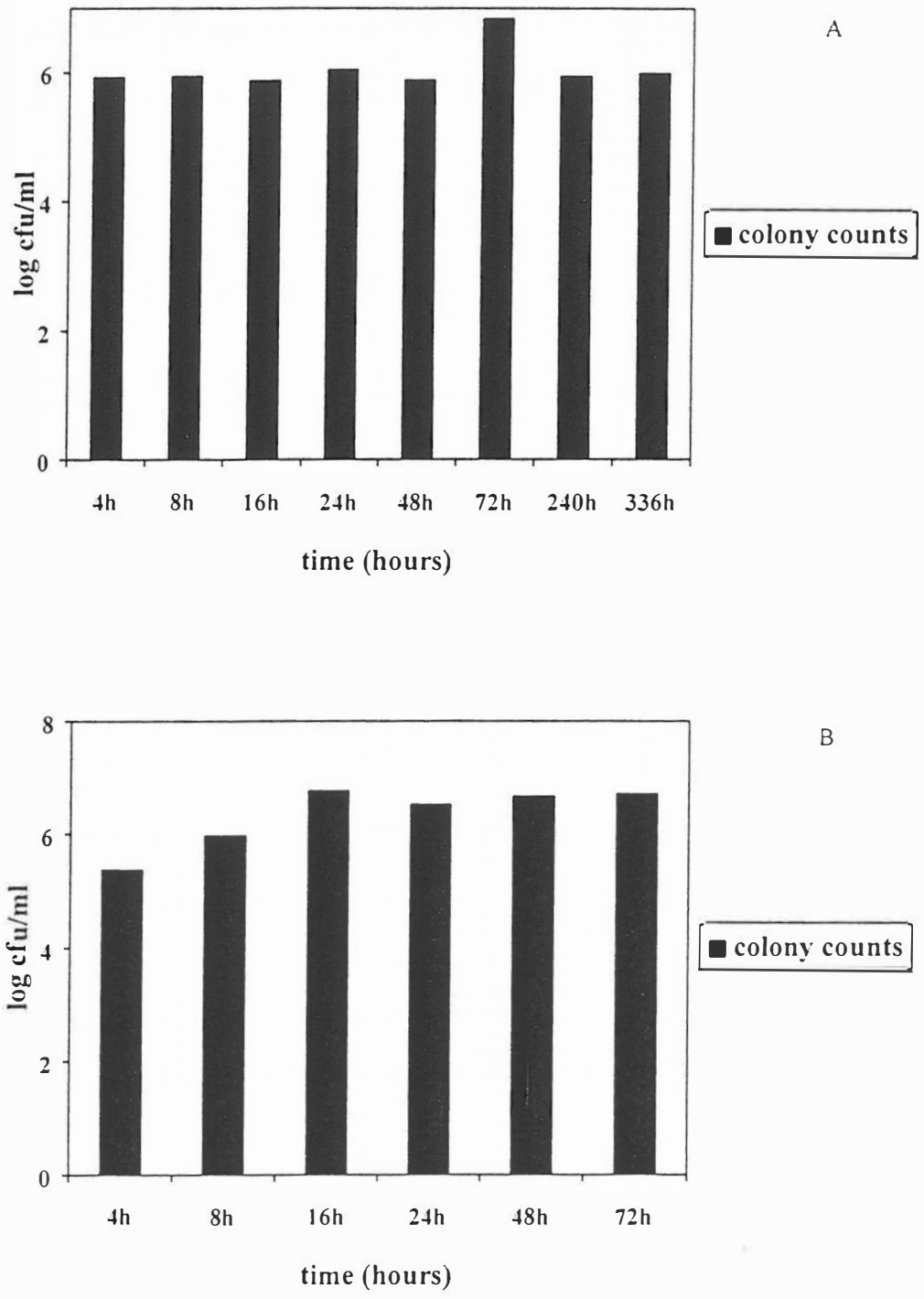


FIG. 2.10 Colony counts of a susceptible strain, 11191 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

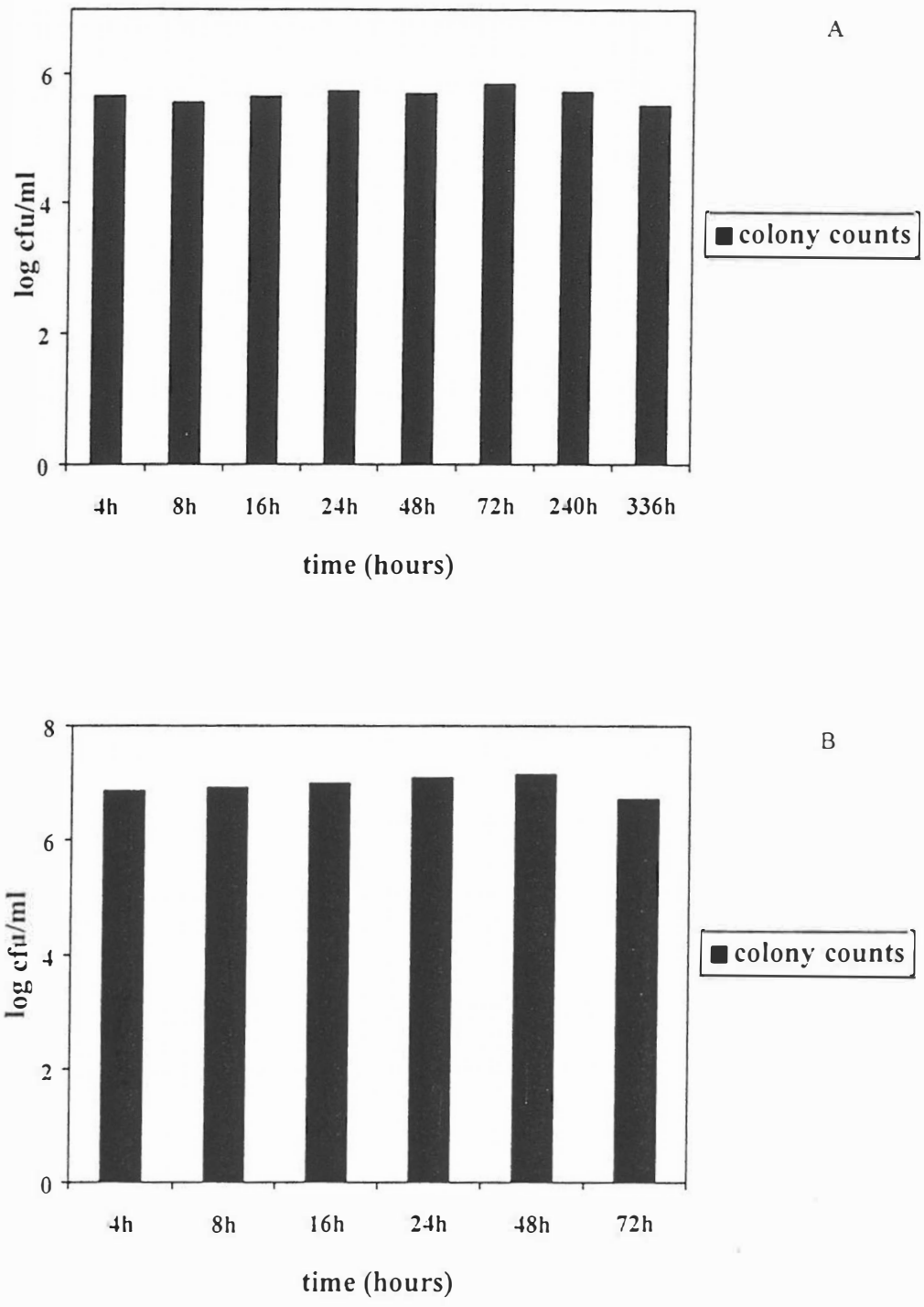


FIG. 2.11 Colony counts of a susceptible strain, 11341 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

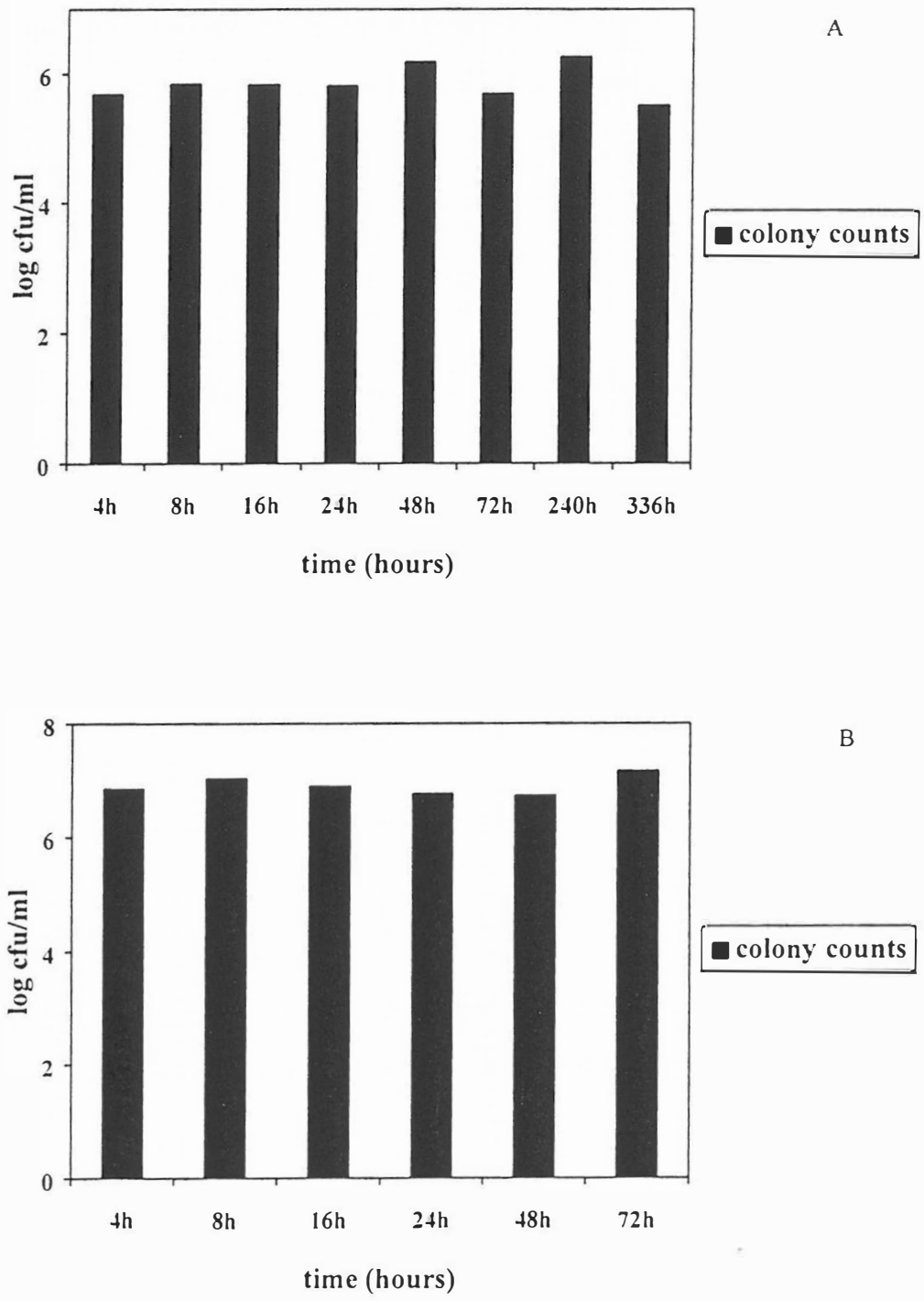


FIG. 2.12 Colony counts of a susceptible strain, 11851 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

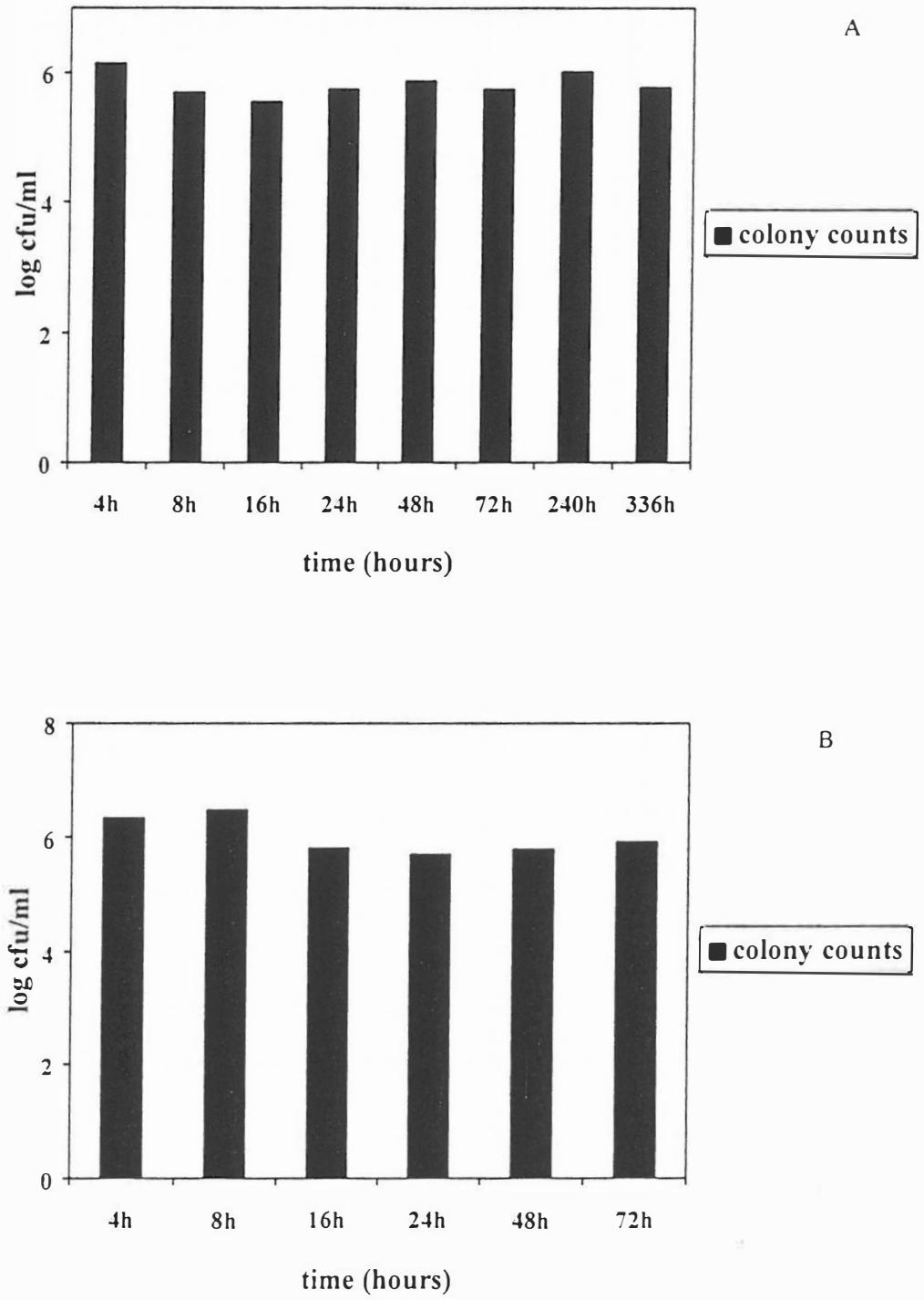


FIG. 2.13 Colony counts of a susceptible strain, 10486 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

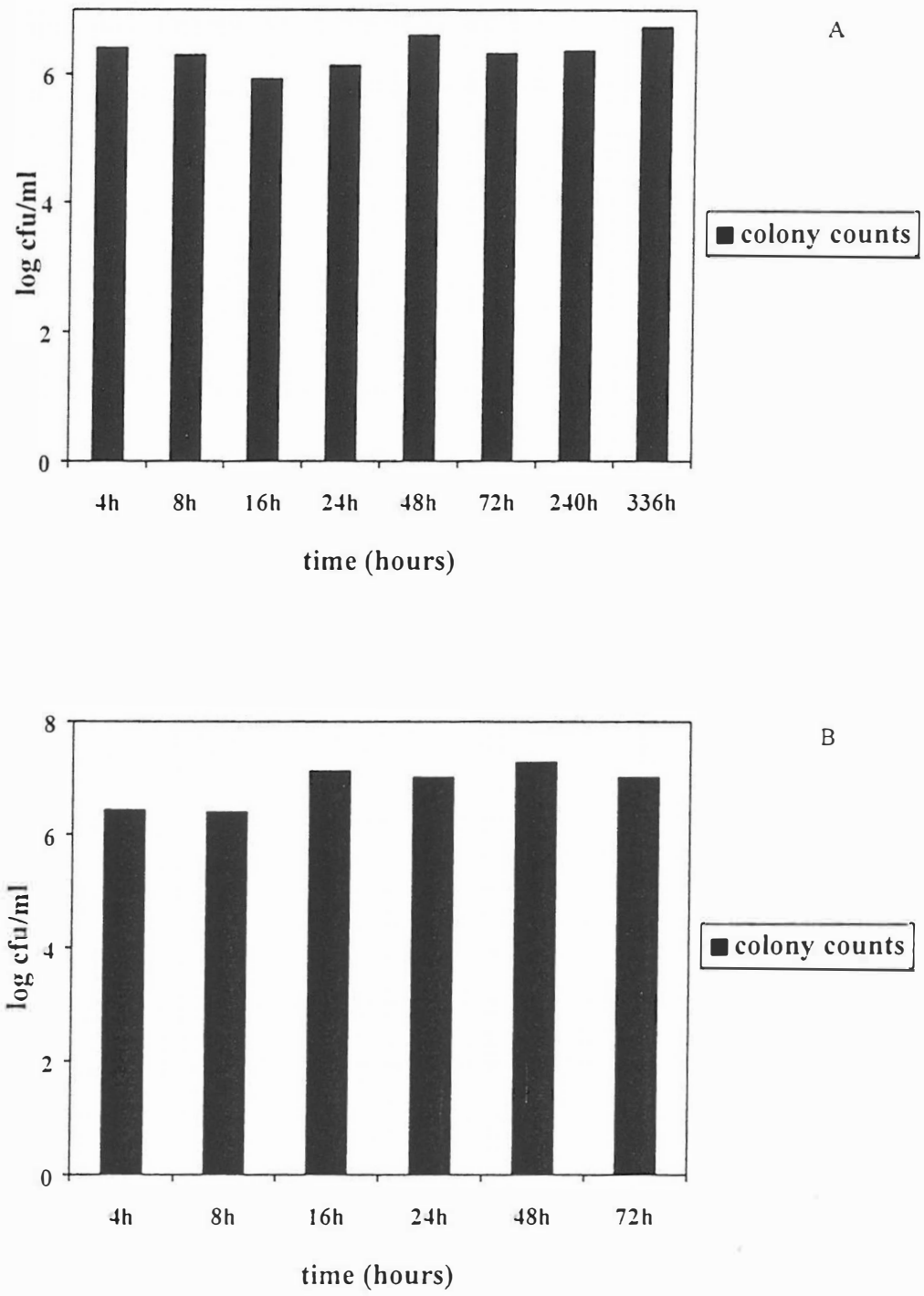


FIG. 2.14 Colony counts of a susceptible strain, 1195 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

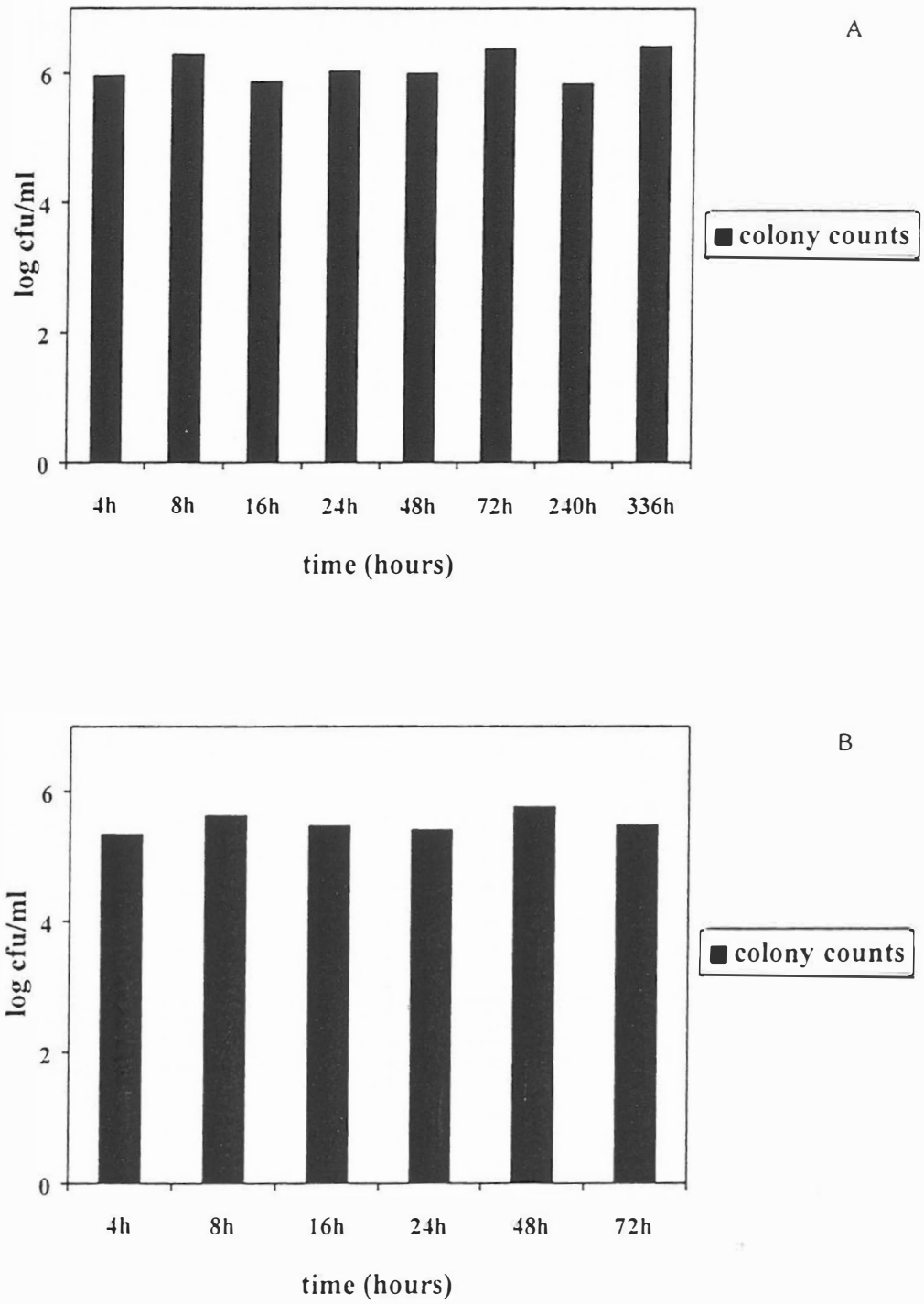


FIG. 2.15 Colony counts of *M. bovis*, a mycobacterial strain in which the Pzase enzyme is not present, exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

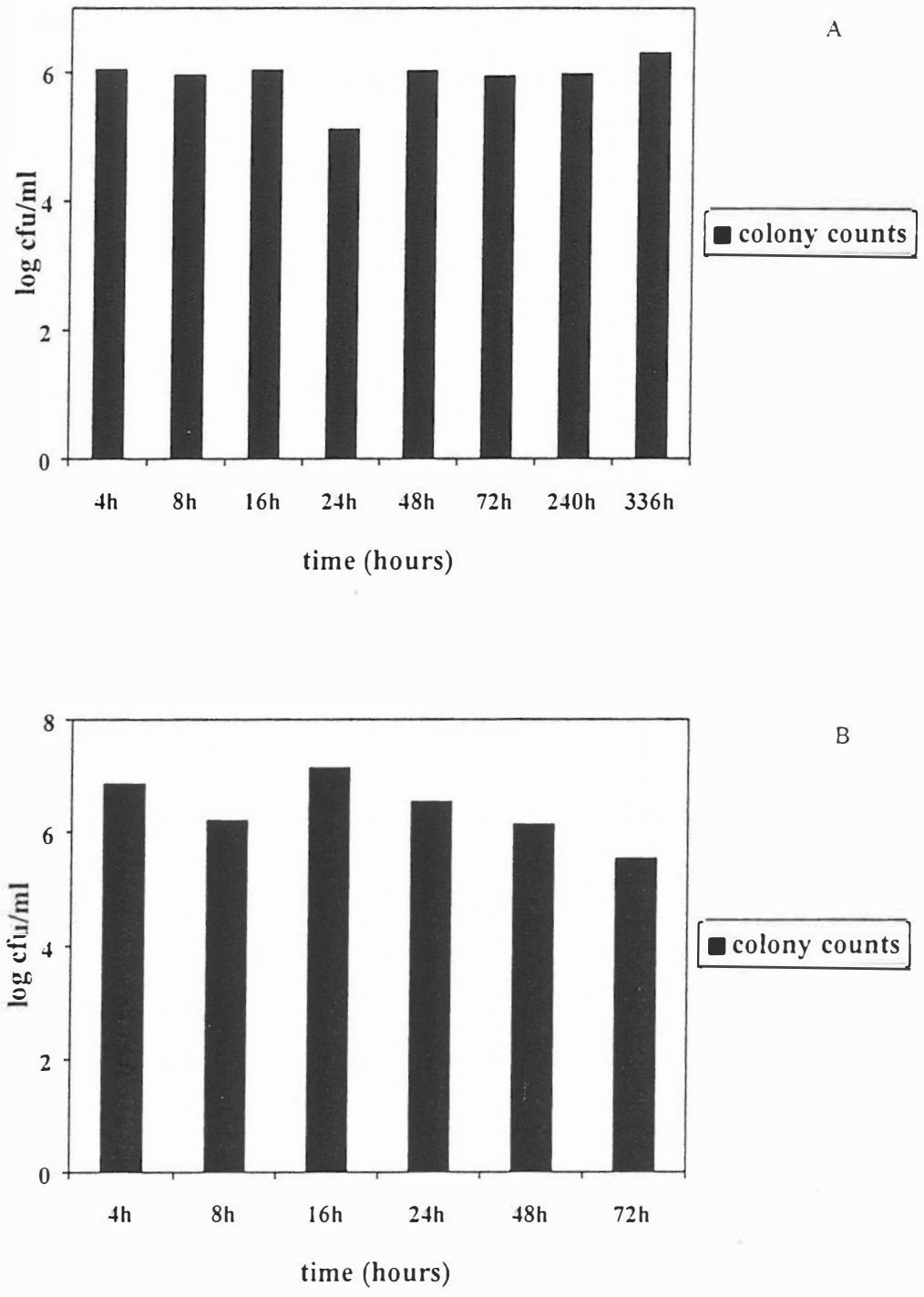


FIG. 2.16 Colony counts of a resistant strain, 3886 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

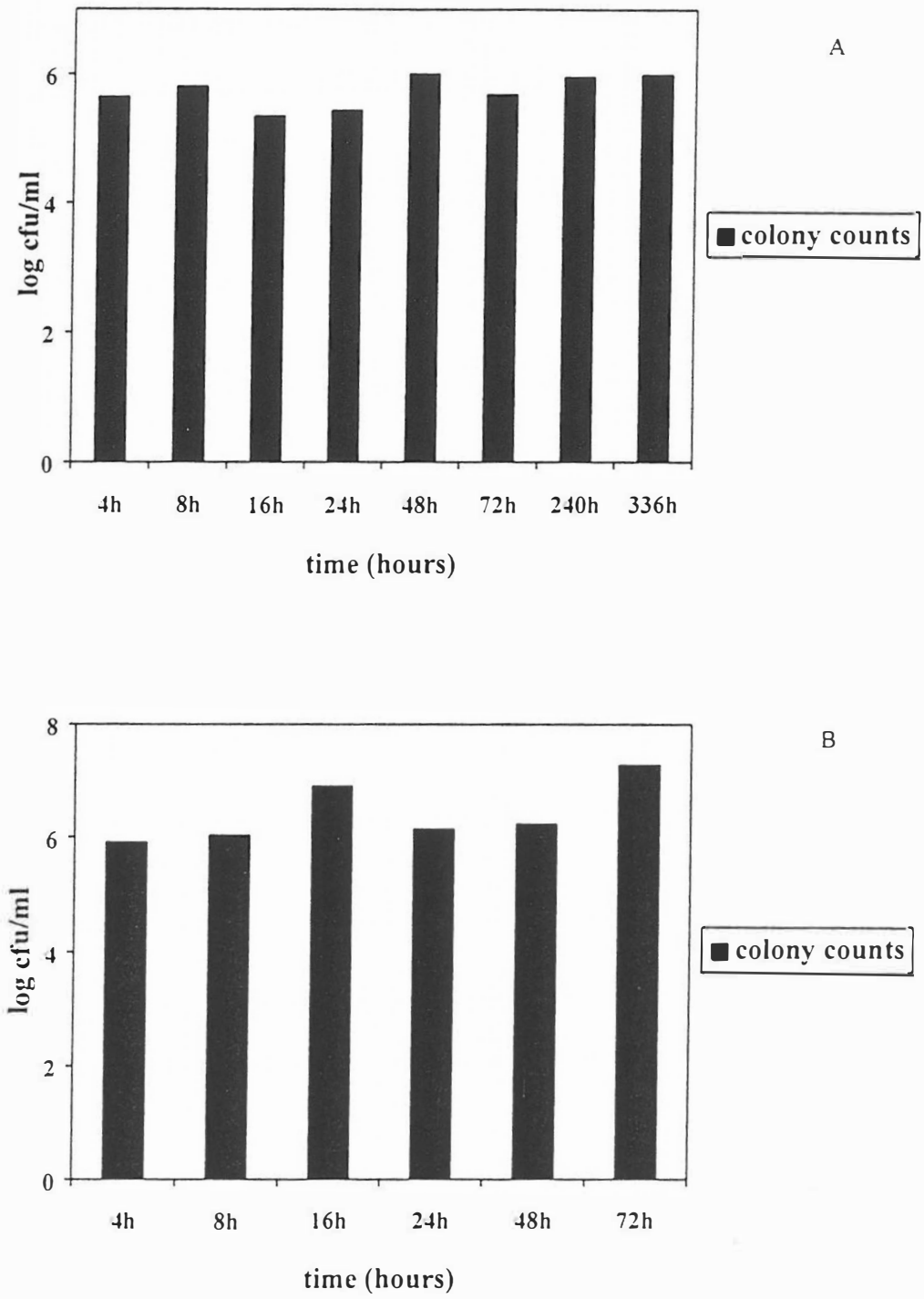


FIG. 2.17 Colony counts of a resistant strain, 3732 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

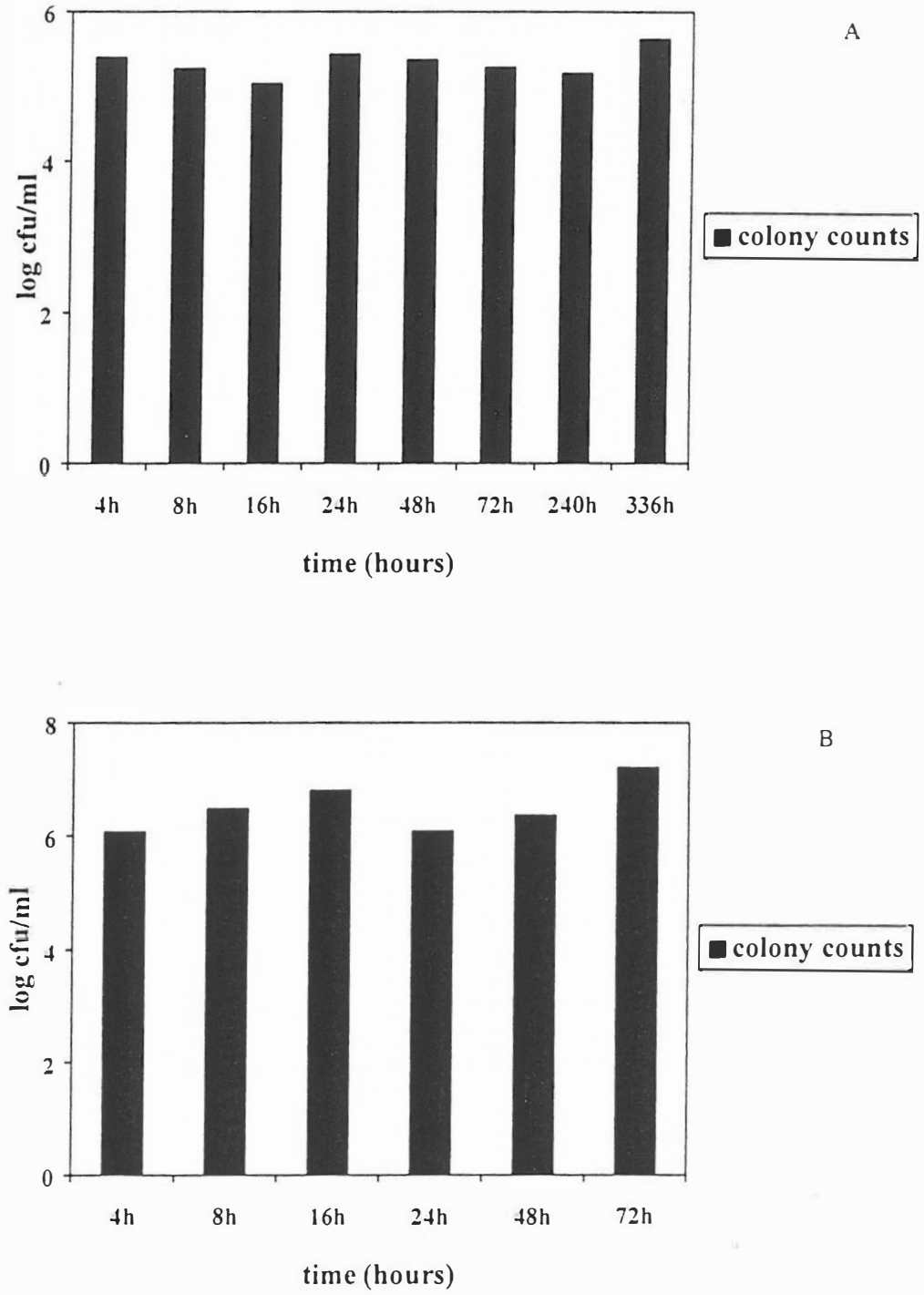


FIG. 2.18 Colony counts of a resistant strain, 60650 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

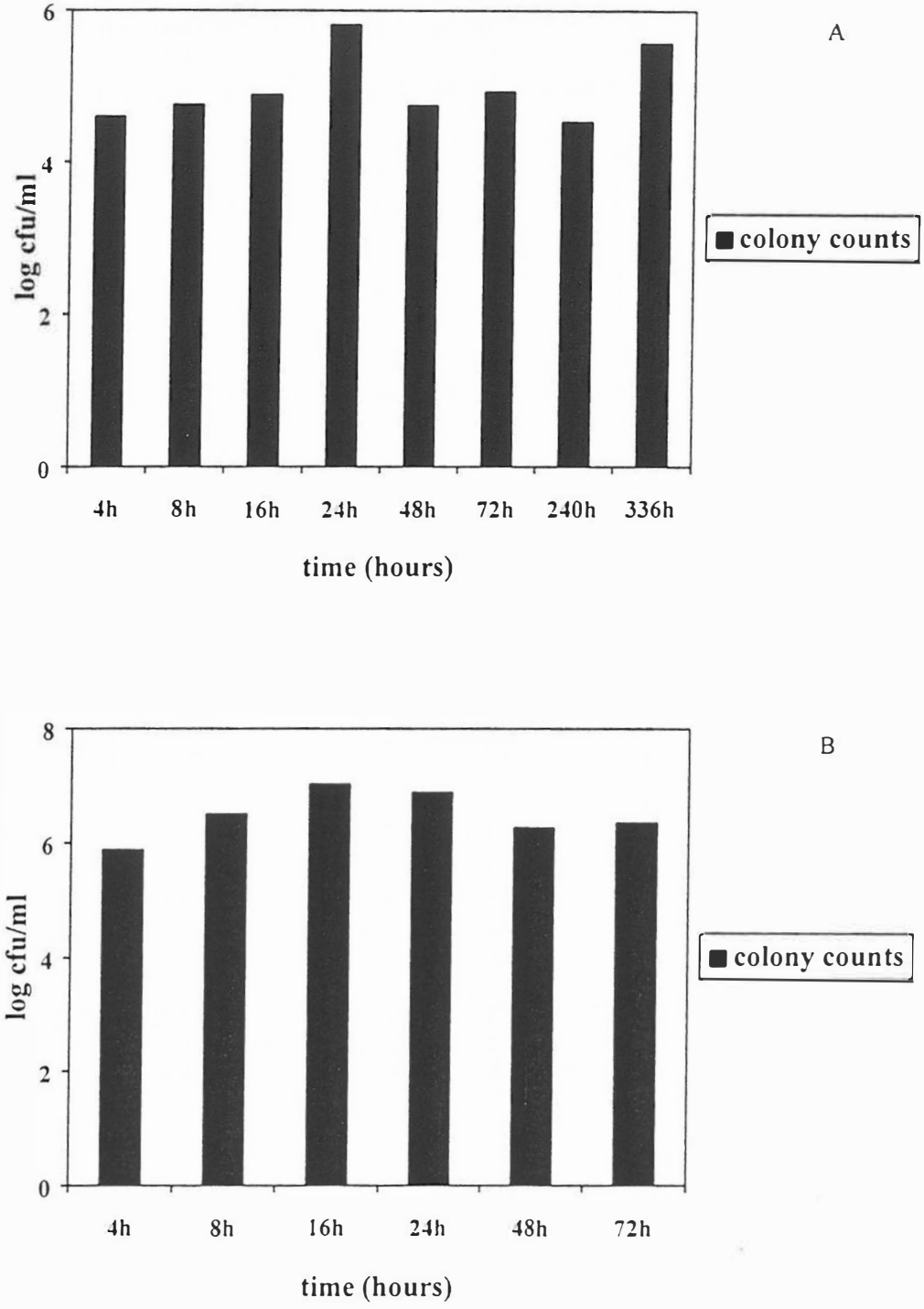


FIG. 2.19 Colony counts of a resistant strain, 79386 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

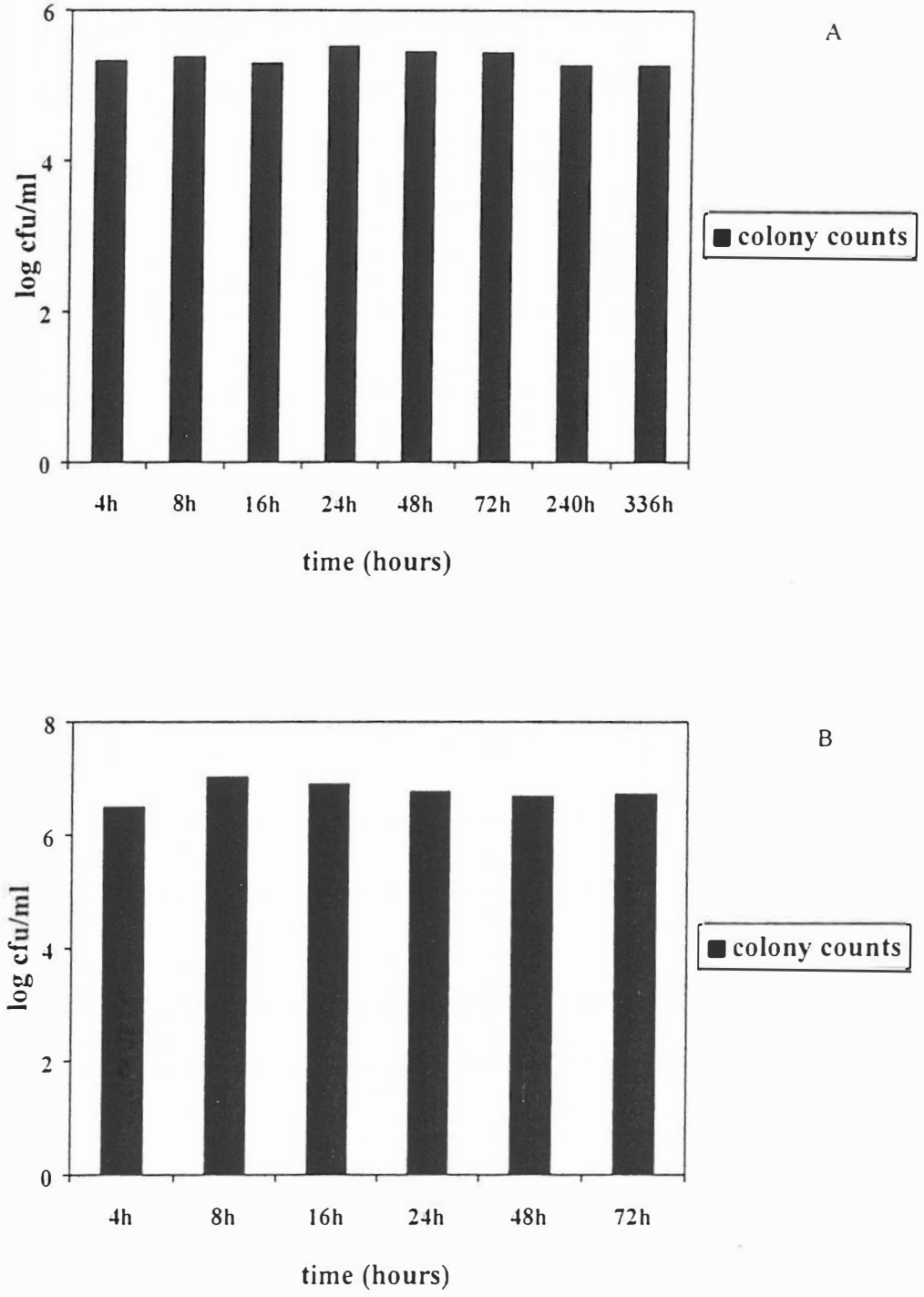


FIG. 2.20 Colony counts of a resistant strain, 6114 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

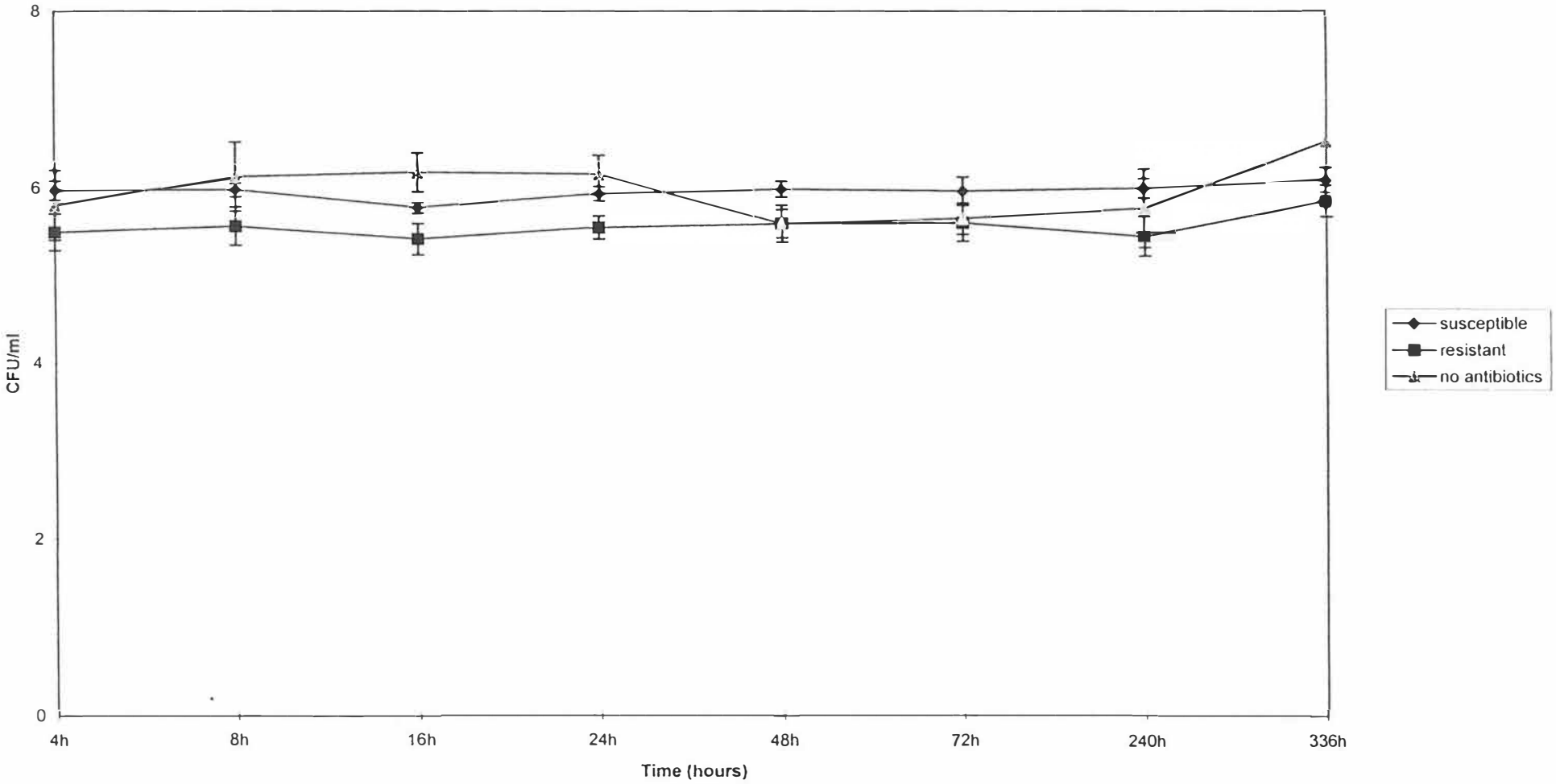


FIG. 2.21 Mean CFU/ml for susceptible and resistant *M. tuberculosis* strains grown in RPMI 1640 supplemented with PZA as well as antibiotic free medium. Irrespective of the presence or absence of PZA in RPMI 1640, no growth or death of susceptible and resistant *M. tuberculosis* strains were observed when assessed by colony counts.

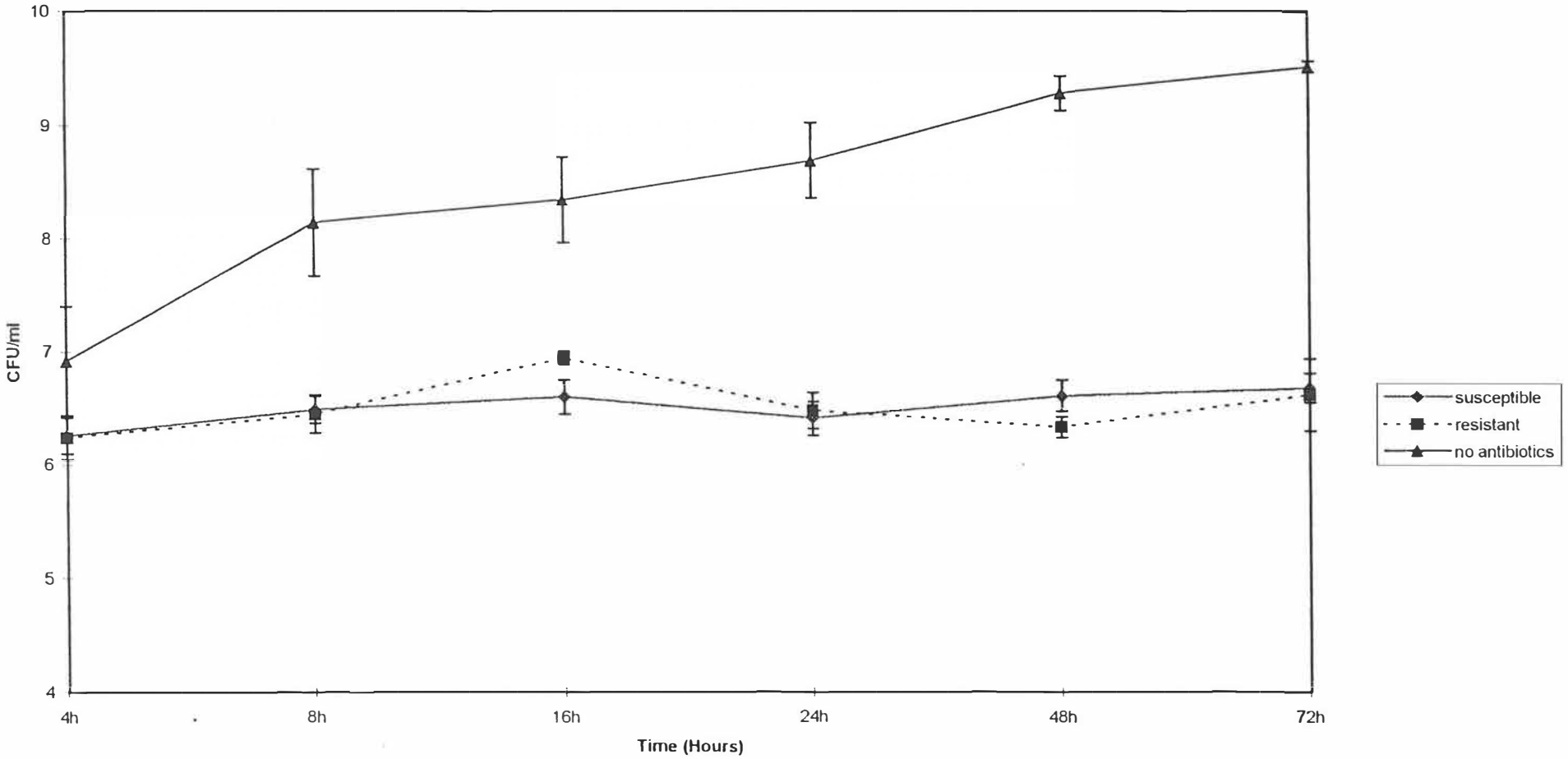


FIG. 2.22 Colony counts of *M. tuberculosis* strains located intracellularly in macrophages. No growth or death of resistant and susceptible strains were observed when macrophages were exposed to PZA, however, intracellular growth was observed in PZA free macrophages.

2.4 DISCUSSION

The goal of this chapter was to assess the growth capacity of PZA susceptible and resistant clinical isolates of *M. tuberculosis* on exposure to PZA, as non cell associated cultures as well as in macrophages. Prior to these experiments purity of the *M. tuberculosis* cultures were confirmed by ZN staining indicated in FIG 2.1 which shows single mycobacteria stained with ZN staining (FIG 2.1A) as well as the characteristic clumping of mycobacteria (FIG 2.1B). All cultures were subject to ZN staining to confirm purity prior to be used in experiments. Any cultures that were contaminated were subject to the decontamination procedure used for sputum and cultured on LJ slants and in Middlebrook 7H9 broth. After incubation at 37^0C, cultures were stained again to confirm purity.

The macrophages were subject to the esterase test at 6 days of incubation in RPMI 1640 supplemented with 10 % fetal calf serum. This confirmed maturation to the macrophage stage. A positive esterase test was indicated by black granulation on the macrophages. Thereafter, the macrophages were infected with the different strains of *M. tuberculosis*. To confirm that the macrophages were indeed infected with *M. tuberculosis*, ZN staining was repeated (FIG 2.3). Thereafter, PZA assays were carried out.

The non cell associated PZA assays were carried out by incubating strains at a final concentration of 10^7 CFU/ml. Also, the CO_2 concentration was maintained at 5% throughout the experiments. The main goal was to investigate the mode of action of PZA on intracellular *M. tuberculosis*. The experiments with non cell associated bacteria were performed as controls for the experiments in macrophages. Thus, standard growth conditions were maintained throughout the experiments with emphasis being placed on the effect of the conversion or non-conversion of PZA to POA on *M. tuberculosis* strains. This differed from the studies by Butler and Kilburn (1982) who investigated the effect of CO_2 and inoculum size on PZA inhibition. Strains of *M. tuberculosis* incubated in the presence of 7% CO_2 in air at pH 5.5 for 3 weeks yielded larger colonies than the same strains incubated without CO_2. They also

found that when medium was inoculated with greater than 300 CFU/ml of PZA susceptible strains, colonies developed in 3 weeks in the absence of added CO_2 in the atmosphere, but growth was stimulated by addition of CO_2 during incubation. In this study 7H10 agar plates were incubated with 5% CO_2 and the initial inoculum used at the start of the experiments was 10^7 CFU/ml. Both conditions served adequately to evaluate growth on plates after 3 weeks of incubation. There was no difficulty in counting of colonies formed and no further manipulations were carried out.

The colony forming units observed in the experiments with non cell associated mycobacteria as well as with mycobacteria in macrophages have being plotted as individual graphs. PZA has no bactericidal effect on susceptible and resistant strains that were macrophage associated (FIG 2.4B-2.20B). This is further emphasised by analysing the composite graph, FIG. 2.22, where the mean CFU/ml are plotted for PZA susceptible and resistant strains. Intracellular growth was observed in macrophages infected with *M. tuberculosis*, but not subject to any PZA treatment. Thus, intracellular *M. tuberculosis* has the ability to survive and multiply within untreated macrophages.

This study differed from the other studies on PZA where the effect of combination of drugs on macrophages infected with *M. tuberculosis* strains were investigated (Acocella *et al*, 1985, Carlone *et al*, 1985, Mor and Esfandiari, 1997 and Sbarbaro *et al*, 1996). Carlone *et al.* (1985) investigated the killing capacity of rifampicin, PZA and POA on macrophages ingested live *M. tuberculosis*. They found over a 3 to 24 h period greater, more rapid, although not statistically significant killing of intracellular bacteria. There was a marked growth of intracellular mycobacteria observed between 48 and 72 h in the control cultures which was less evident in the drug containing cultures. A similar trend was observed with the control experiments set up in this PhD study. No combinations of drugs were studied in antibiotic assays, thus there was no competition among the drugs for either entry into the macrophage or for binding to targets relevant to antimicrobial activity within the macrophage. It was, therefore, possible to assess the nett effect of PZA as an antimycobacterial agent. Carlone *et al.* (1985) found that there was no major increase in the killing effect with

respect to that observed with the individual drugs after exposure of the macrophage to all possible combinations of the 3 drugs. Another study that differed from this study was the study by Acocella *et al.* (1985) who investigated the degree of penetration of rifampicin, PZA and POA in mouse macrophages, where PZA was only evaluated for 24 h. Macrophages in different metabolic phases were used *viz.*, resident, stimulated and dead, which differed from this study where only actively growing peripheral blood derived macrophages were used. Also, Acocella *et al.* (1985) radiolabelled the antibiotics used and measured their penetration into macrophages by scintillation spectrophotometry. This study did not involve the addition of POA into the system. Thus, conversion from PZA to POA was of importance to us and HPLC was used as it could quantify PZA as well as the PZA converted to POA.

Crowle *et al.* (1986) investigated the effect of PZA alone on tubercle bacilli within cultured human macrophages, similar to the culture aspect of this study. This studied the effect of PZA on intracellular bacilli by counts of acid fast bacilli and colony forming units. They found that PZA was active in the macrophage model as it was inhibiting virulent tubercle bacilli at concentrations of 20 μg/ml or higher, however, it was ineffective in 7H9 bacteriologic culture medium. Crowle *et al.* (1986) comments that PZA could either be bacteriostatic or bactericidal against intramacrophage tubercle bacilli depending on its concentration, the donor of the macrophages and length of exposure of the infected macrophage to the drug. In this study, irrespective of the donor of the macrophages, PZA had no bactericidal effect over the 72 h period studied. A fixed concentration of 100 μg/ml was used throughout the experiments as high antibiotic concentrations were required to enable efficient quantification of PZA by HPLC in *in vitro* experiments as shown in later chapters. This has no bactericidal effect over the 72 h period studied.

Combinations of drugs have also been studied. Sbarbaro *et al.* (1996) investigated the effect of PZA and a quinolone (ofloxacin) as a potential preventive therapy regimen. Three different concentrations of ofloxacin (OFL) (0.625, 1.25 and 2.5 μg/ml) were tested in combination with 40 μg/ml dosage of PZA. This demonstrated an inhibitory

effect upon intracellular tubercle bacillary growth. PZA enhanced the effectiveness of both bacteriostatic concentrations of OFL, converting the bacteriostatic impact of 1.25 μg/ml to a bactericidal effect. The addition of PZA did not diminish the killing effect of the 2.5 μg/ml OFL.

Mor and Esfandiari (1997) investigated the synergistic effect of clarithromycin (CLA) and PZA against *M. tuberculosis*. This combination exerted a synergistic effect in preventing intracellular bacterial growth. CLA tends to enhance the effectiveness of PZA against tubercle bacilli within cultured human macrophages, however, this effect was not demonstrated to be bactericidal during the 8 days of this study. In this PhD study no combinations of drugs were studied as this had no purpose in the main aims of the project.

The effect of pH on *M. tuberculosis* has been studied extensively in our department (Matai, 1997) as well as internationally. Heifets *et al.* (1989) investigated whether POA has any specific antimicrobial activity or whether it affects the growth of *M. tuberculosis* simply by lowering the pH below the limits of tolerance. They found that there was a clear dose effect correlation when inhibitory activities of different concentrations of POA under identical pH conditions (5.6) in 7H12 liquid medium was evaluated.

Matai (M. Sc thesis) investigated the MICs for PZA and POA against 17 *M. tuberculosis* strains at each of the following pH values: 5.3, 5.8, 6.3, 6.8. She found that as the pH increased, the MICs of both drugs increased. At pH 6.3 the highest concentration of PZA of 512 mg/l had no activity on any of the organisms whereas POA at that concentration still retained some activity. She also found that all 17 *M. tuberculosis* strains used in these experiments were able to grow at all of the 4 pH values tested, however, a reduction in colony size was observed at the lower pH values of pH 5.3 and pH 5.8. Since the effect of pH was extensively studied in our department the purpose of my experiments with non cell associated *M. tuberculosis* was to evaluate the effect of the tissue culture medium on the mycobacteria Also, to assess if the amount of PZA converted to POA by Pzase positive cultures has the

ability to inhibit the growth of *M. tuberculosis*. FIG. 2.21 shows the mean values of 10 susceptible and 5 resistant strains that were exposed to 100 μg/ml PZA in RPMI 1640. The control involved incubating 3 cultures in RPMI 1640 with no antibiotics. The graphs show that the cultures remain in a dormant state when incubated in antibiotic free RPMI 1640. Addition of PZA to the system does not change the overall effect *i.e.*, the cells remain in a dormant state. Thus, PZA or the PZA converted to POA (see chapter 4 for HPLC results) in RPMI 1640 has no effect on the non-cell associated mycobacteria. This results differs from the macrophage bound mycobacteria where there is an increase in CFU/ml of intracellular mycobacteria exposed to antibiotic free RPMI 1640. Addition of PZA to the co-culture system shows that the mycobacteria are maintained in a bacteriostatic state throughout the experiment. Thus, there seems to be a clear distinction between the effect of PZA on intracellular mycobacteria as compared to non cell associated mycobacteria.

There have been many manipulations of the media used to study the activity of PZA. Incorporation of enrichment usually oleic acid-albumin-dextrose-catalase (OADC) into 7H10 agar is necessary for optimal growth of *M. tuberculosis* at acid pH, however, oleic acid in an acid environment is inhibitory to many strains (Tummons, 1975-cited by Butler and Kilburn , 1982). They also reported that growth of 25 % of *M. tuberculosis* strains were inhibited by presence of oleic acid at pH 5.5. Growth appeared better with certain lots of albumin-dextrose catalase (ADC) at pH 5.5 but some lots did not support growth at low pH. My study did not involve the evaluation of the OADC used in the *M. tuberculosis* growth medium. All bottles of OADC used promoted optimum growth of *M. tuberculosis* strains, thus it did not warrant further evaluation.

Butler and Kilburn (1983) reported on the detection of Pzase activity which hydrolyses PZA to POA as an alternative to PZA susceptibility tests. The standard Wayne's test (Wayne, 1974) was recommended, where a positive is indicative of pink to red colour development upon addition of freshly prepared ferrous ammonium sulfate solution. The Wayne's test was used initially on all the *M. tuberculosis* strains used in my study to establish their Pzase status. There was good correlation with all

the strains, where all PZA resistant strains were Pzase negative and PZA susceptible strains were Pzase positive.

Heifets and Lindholm-Levy (1990) reported on the MBC *i.e.*, the lowest concentration killing more than 99 % of the bacterial population *in vitro*, using PZA susceptible strains cultivated in 7H12 broth at pH 5.6, the lowest pH favourable for active bacterial population growth. 33 to 57 % of the bacterial population was killed during the 15 day period of cultivation in the presence of PZA concentration two fold greater than the MIC. Increasing the PZA concentration as high as 500 and 1000 μg/ml did not substantially increase the proportion of bacterial population killed. They concluded that PZA has no bactericidal activity *in vitro* against actively multiplying bacteria. This is similar to this study where actively multiplying bacteria were incubated in RPMI 1640. PZA displayed poor bactericidal activiy under these conditions. The POA formed by PZA susceptible strains was not sufficient to lower the pH to levels intolerable to *M. tuberculosis* strains.

Heifets and Lindholm-Levy (1992) tried to determine *in vitro* activity of PZA against tubercle bacilli at a lower pH *i.e.*, pH 5 or lower. The number of viable tubercle bacilli remained almost unchanged within a period of 3 to 4 weeks of cultivation in liquid medium with a pH of 4.8 to 5.0. This was referred to as the semi-dormant rather than dormant state, assuming that the relatively stable number of viable cells was a reflection of the simultaneous existence of cells in various states of the dormancy, multiplication and dying. The POA formed by Pzase positive strains, incubated in RPMI 1640 supplemented with PZA, was not able to decrease the pH to less than 5.0 to have a bactericidal effect on the cultures, thus the cultures remained in a (semi) dormant state.

CHAPTER 3

DEVELOPMENT OF THE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODOLOGY FOR SIMULTANEOUS DETECTION OF PZA AND POA

3.1 MANUSCRIPT

This chapter is presented as a manuscript being submitted for consideration for publication. It discusses the high performance liquid chromatography (HPLC) methodology that was developed for the simultaneous detection of PZA and POA. It also shows the initial results obtained with the reference strain H37Rv.

Aspects not discussed extensively in the manuscript are presented as additional information.

ABSTRACT

Pyrazinamide (PZA) is known to be converted to pyrazinoic acid (POA) by the enzyme pyrazinamidase (Pzase). It is generally accepted that this conversion plays a role in the mechanism through which PZA exerts its antimycobacterial activity. This activity is thought to take place intracellularly in infected macrophages. High performance liquid chromatography (HPLC) methodology was developed to measure the concentration of PZA and POA in biological systems. A mobile phase of methanol and 10 mM phosphate buffer (20%:80%, v/v), pH 3.5 most effectively separated both compounds. The wavelength of 265 nm was most efficient to resolve PZA and POA peaks. This methodology was used to quantify PZA and POA in the different compartments of a macrophage-mycobacterium co-culture system as well as in extracellularly growing mycobacteria, using strain H37Rv. Macrophages were separated from extracellular fluid by means of silicone oil velocity gradient centrifugation. The macrophages were lysed by means of distilled water and the bacterial cell walls disrupted by means of silica/zircona beads in a mini-bead beater. There was minimal entry of PZA into the macrophage, with most of the PZA remaining in the extracellular fraction. Also, no intracellular or extracellular POA could be detected.

INTRODUCTION

The *Mycobacterium tuberculosis* population present in a patient with active tuberculosis is divided into four subpopulations: actively multiplying extracellular organisms, actively multiplying intracellular organisms, dormant extracellular organisms and dormant intracellular organisms (Mitchison, 1985). Each of the different drugs used in treatment of tuberculosis is thought to play a specific role in eradication of these subpopulations.

It has been postulated that the role of pyrazinamide (PZA) is to eradicate dormant and actively multiplying organisms from the intracellular compartment. This thought is supported by the observation that the optimal pH at which this drug is active is between 5.0 and 5.5. This is the pH in the phagolysosomes of activated macrophages. *M. tuberculosis* is a facultative intracellular pathogen of these cells.

To be able to test this hypothesis it must be possible to measure drug penetration in macrophages as well as the effect of the drug on intracellular mycobacteria. Circumstantial evidence suggests that the metabolic product, pyrazinoic acid (POA), produced on hydrolysis of PZA by the bacterial enzyme pyrazinamidase (Pzase), is the active compound (Heifets *et al*, 1989, Konno *et al*, 1967, Singh *et al*, 1993). Thus, there is a need for a test which is able to measure PZA as well as POA concentrations intracellularly. High performance liquid chromatography (HPLC) with acetonitrile and phosphate buffer pH 3.5 as mobile phase has been used to measure PZA in human plasma (Walubo *et al*, 1994) and cerebrospinal fluid (CSF) as well as in plasma of rabbits (Chan *et al*, 1986). This method does not resolve POA. So far, tests that

measure POA are based on spectrophotometry (Wayne, 1974). This technique is not sensitive enough to detect POA at concentrations expected to be present intracellularly in macrophages.

We report on the development of a HPLC based method to measure both PZA and POA simultaneously in macrophages as well as in bacteria.

MATERIALS AND METHODS

Bacteria. Pyrazinamide susceptible *M. tuberculosis*, reference strain H37Rv (ATCC 37294) was grown in antibiotic free Middlebrook 7H9 broth. One week old cultures were used in invasion assays with macrophages. The cultures were centrifuged at 12 000 x g at room temperature and the pellet resuspended in phosphate buffered saline. This suspension was vortexed for 1 min with 5 mm glass beads and allowed to settle for 15 mins at room temperature. The supernatant containing single bacteria and small clumps adjusted to a McFarland No 1 standard (10^7cells/ml) was used in the macrophage assays.

PZA uptake by mycobacteria . A McFarland No 1 standard of H37Rv was exposed to 100 μg/ml PZA in RPMI 1640 tissue culture medium. At time intervals of 4, 8,16, 24, 48 and 72h the mycobacteria were disrupted with 0.1-0.15 mm diameter zirconia/silica beads using a mini bead beater set at homogenise for 3 mins. Mycobacteria were centrifuged at 12 000 x g for 10 mins (Biospec Products). The supernatant was used to assess PZA and POA by HPLC.

Isolation of monocytes. Peripheral blood monocytes were obtained by overlaying blood from healthy volunteers onto Ficoll-Histopaque 1077 (Sigma) in a ratio of 5:4, followed by centrifugation at room temperature at 400 x g for 30 mins. The cells were washed twice with RPMI 1640 tissue culture medium and resuspended in RPMI 1640 supplemented with 10 % fetal calf serum that was inactivated at 65°C for 30 min. The monocyte suspension was aliquoted in 5 ml volumes into 65 mm diameter petri dishes at a concentration of approximately 10^5 cells/ml to obtain maximum yield for HPLC

analysis. Monocytes were grown for 6 days with changes of RPMI 1640 supplemented with 10 % fetal calf serum every 2 days. On the 6th day the monocyte derived macrophages were used in invasion assays. The trypan blue exclusion assay confirmed viability of the macrophages. The monocyte suspension was stained with 0.4 % trypan blue made in phosphate buffered saline. Viable cells have intact cell walls and does not allow for the uptake of the stain. Thus clear uncoloured cells are viable while blue cells are dead. Percentage viability of the cells were assessed. The esterase test confirmed maturation to the macrophage stage. The α-naphthyl-acetate esterase kit (Sigma) was used where black granulation over the macrophage cultures indicate mature macrophages.

PZA uptake by infected macrophages. Macrophages were exposed to the bacteria for 1 hour, after which all extracellular bacteria were washed off by gently pipetting the RPMI and allowing the medium to flow over the petri dish. This washing procedure was carried out thrice before the medium was discarded. The infected macrophages were stained with ZN stain to determine if washing was effective and all free bacteria removed. Also, transmission electron microscopy was used to assess mycobacteria bound within macrophages (data not shown). Fresh RPMI 1640 supplemented with 100 μg/ml PZA was added to the petri dishes. To quantify extracellular, intracellular and intra-bacterial PZA/POA by HPLC, cells from 3 petri dishes were harvested and pooled at intervals of 4, 8, 16, 24, 48, and 72 hours.

Separation of extracellular, intracellular and intra-bacterial fractions. The infected macrophages were gently scraped off the petri dishes using a 10 μl disposable

loop (Sterilin). The suspension obtained was centrifuged at 250 x g for 10 mins. The supernatant was discarded except for 500 µl which was used to resuspend the pellet. To separate the extracellular from the intracellular fraction, silicone oil velocity gradient centrifugation was used (Johnson *et al*, 1980, Klempner and Styrt, 1981, Koga, 1987, Pascual *et al*, 1994). A 6:5 mixture of silicone oils (500 µl) of densities 1.07 and 0.98, respectively, was placed in an eppendorf tube and 500 µl of macrophage suspension was overlayed on the oil. This was then centrifuged for 3 mins at 12 000 x g which resulted in a pellet of macrophages at the bottom of the tube. The extracellular fluid above the oil layer was removed. The pellet was resuspended in 500 µl of distilled water, vortexed and incubated at 37°C for 10 mins to completely disrupt the macrophages. This constituted the intracellular extra-bacterial fraction. The mycobacteria in this fraction were disrupted with 0.1-0.15 mm diameter zirconia/ silica beads using a mini bead beater (Biospec Products).

HPLC. HPLC was performed using a Hewlett Packard 1090 HPLC with a Merck reverse phase C8 column (250 mm) fitted with a C8 guard column and a column oven temperature of 40 °C. Mobile phases used to separate PZA and POA were a mixture of acetonitrile and 10mM phosphate buffer pH 3.5 (20%:80%, v/v) (Chan *et al*, 1986, Woo, 1987); phosphate buffer pH 2.56 alone (Yamamoto *et al*, 1987); and a mixture of methanol and 10 mM phosphate buffer pH 3.5 (20%:80%, v/v) (Ono *et al*, 1996). The flow rate of the mobile phase was 1ml/min with a sample injection volume of 25 ul in all experiments. Product analysis was done using a UV detector, at wavelengths of 215 nm (Chan *et al*, 1986), 254 nm and 265 nm (Ono *et al*, 1996). PZA, POA as well as paracetamol (PARA) were dissolved in warm distilled water (HPLC pure) to

obtain the following: standard solutions: 5, 10, 25, 50, 100 μg/ml of PZA; 5, 10, 25, 50 μg/ml of POA, with the internal standard PARA at a final concentration of 100 μg/ml. RPMI 1640 tissue culture medium without any of the drugs was used as a blank and the HPLC results obtained were subtracted from all measured extracellular concentrations.

RESULTS

The combination of methanol and 10 mM phosphate buffer, pH 3.5 as the mobile phase was able to separate POA, PZA as well as PARA (FIG. 1). The retention times were 2.7, 4.1 and 5.5 mins respectively. The mobile phases of acetonitrile with 10 mM phosphate buffer, pH 3.5 (Chan *et al*, 1986 and Woo, 1987) and 0.02 M phosphate buffer, pH 2.56 (Yamamoto *et al*, 1987) were able to resolve PZA only. With these mobile phases tailing of the POA peak occurred. Hence, acceptable correlation factors could not be obtained. Optimal resolution was achieved at wavelength 265 nm. At wavelength 215 nm (Chan *et al*, 1986) interference peaks were obtained. Wavelength of 254 nm was optimal for PARA but not for PZA and POA. RPMI 1640 gave rise to HPLC peaks at wavelength 265 nm that interfered with the POA peak. Subtraction of results obtained with RPMI 1640 only was used to resolve this. With this method the minimal concentration of PZA and POA that gave a detectable peak was 0.1 and 0.5 μg/ml respectively.

Results of the application of this HPLC methodology on H37Rv only and on macrophages infected with H37Rv and exposed to PZA are shown in FIG. 2 and FIG. 3, respectively. The concentration of PZA extracellularly decreased gradually over the first 24 h without the appearance of detectable amounts of POA with free mycobacteria in RPMI 1640. POA was first detected at 48 h. At that point there were also measurable concentrations of PZA and POA in the bacteria. The POA concentration increased in the next 24 h in the extra-bacterial fraction but not in the intra-bacterial fraction (FIG. 2). Intra-bacterial concentration of PZA and POA at 72 h were 4.58 and 0.650 μg/ml, respectively. In macrophages infected with H37Rv, no

POA was detected in the extracellular, intracellular or the intra-bacterial fractions. The PZA concentration was higher extracellularly than intracellularly. No PZA could be detected in the intra-bacterial fraction (FIG. 3).

DISCUSSION

The combination of PZA with isoniazid and rifampicin is one of the most effective antituberculous regimens (Walubo *et al*, 1994). However, the exact mode and site of action of PZA are unknown. One of the hypothesis is that in macrophages infected with PZA susceptible strains, PZA is converted to POA by Pzase. This causes a lowering of pH in the macrophage environment. Either the decrease in pH alone or this enzyme generated product, POA, is responsible for the high antibacterial activity of PZA. We developed HPLC based methodology that can quantify PZA and POA simultaneously at concentrations as low as 0.1 and 0.5 μg/ml, respectively. This allows detection of PZA and POA extracellularly, intracellularly as well as in the intra-bacterial compartment of infected macrophages.

We choose HPLC as a tool since it has the potential to quantify PZA and POA simultaneously in a single assay. Although, there have been numerous reports on the use of HPLC to quantify PZA in plasma, serum and CSF, the methodolgy used only yields a well resolved chromatograph for PZA (Chan *et al*, 1986, Walubo *et al*, 1994 and Yamamoto *et al*, 1987). When the same mobile phase is used for POA, a peak with distinct tailing is observed, which resulted in poor correlation factors when standard curves were plotted. Thus, published mobile phases were unable to resolve POA. We successfully attempted to develop a mobile phase that would separate POA, PZA and the internal standard PARA into well resolved and distinctly separate peaks.

Methanol and phosphate buffer proved to be the mobile phase that separates PZA, POA and PARA efficiently. Reproducibility was optimal at pH 3.5. Prolonged usage

of the column (over a 2 year period) as well as pH of the buffer affects the POA peak drastically as compared to the PZA peak where no change in retention time was observed. The tissue culture medium RPMI 1640 gave rise to intereference peaks that affected the POA peak, thus RPMI 1640 had to be run as a blank with the results subtracted from measured concentrations. A UV wavelength of 265 nm was the optimum wavelength for the analysis of extracellular and intracellular PZA and POA as compared to 215 nm as previously reported (Chan *et al*, 1986). The UV wavelength of 265 nm is also used for the separation of isoniazid and isonicotinic acid (Ono *et al*, 1996) which is similar in structure to PZA.

We also report on the results obtained with bacteria (H37Rv) exposed to PZA in tissue culture medium as well as in macrophages infected with H37Rv. With mycobacteria only, POA was detected at 48 h in tissue culture medium, where the quantity of detectable POA formed increased over time with the PZA concentration decreasing. This suggests that PZA is converted to POA.

There are numerous studies that have investigated the effect of PZA in macrophage models infected with *M. tuberculosis* and challenged with PZA (Acocella *et al*, 1985, Carlone *et al*, 1985, Salfinger *et al*, 1989 and Sbarbaro *et al*, 1996). However, the main methodology applied was assessement of intracellular kill by bacterial counts. Carlone *et al* (1985) investigated the killing capacity of rifampicin, PZA and POA on macrophages infected with live *M. tuberculosis* with drug concentrations corresponding to the average peak levels observed in human after administration of therapeutic doses *viz*., 30 μg/ml PZA, 10 μg/ml rifampicin and 8 μg/ml POA. There

was a marked growth of intracellular mycobacteria observed between 48 and 72 h in the control cultures which was less evident in the drug containing cultures. The studies of Acocella *et al* (1985) differed from the studies by Carlone *et al* (1985) in that the concentration of rifampicin, PZA and POA corresponded to the peak, trough and intermediate serum concentrations observed in human. Also, macrophages were in different metabolic stages. The results obtained in that study indicated that rifampicin, PZA and POA penetrate rapidly into macrophages, irrespective of their metabolic state. The penetration is almost complete at lower concentrations. Sbarbaro *et al* (1996) investigated combined effect of PZA and ofloxacin as a potential preventive therapy regimen. Three different concentrations of ofloxacin (0.625, 1.25, 2.5 μg/ml) were tested in combination with 40 μg/ml of PZA. This demonstrated an inhibitory effect upon intracellular bacillary growth. However, there are no reports on the quantification of intracellular and extracellular PZA and POA. This is the key to understanding the location of action of PZA.

To do so, cells had to be concentrated to enable intracellular PZA or POA to be quantified. Silicone oil velocity gradient separation using a mixture of silicone oil of two densities as described by Klempner and Stryt (1981) was used to separate the extracellular from the intracellular fractions. This resulted in efficient recovery of the antibiotic. Even with the high concentration of 100 ug/ml, there was minimal detectable PZA in the infected macrophages. Most of it remained in the extracellular tissue culture medium. No POA was detected in the extracellular or intracellular compartment of the infected macrophage model nor was any PZA or POA detected after the bacteria were lysed. However, addition of the extracellular and intracellular

drug concentrations at each time interval equals approximately 80 ug/ml (FIG. 3). Thus, 20 % of the initial amount of 100 μg/ml could have been converted to POA, that was used up by the intracellular mycobacteria resulting in antimycobacterial activity. This is supported by our findings with bacteria only (FIG.2). In this system, using the same medium and organism, we found over the first 24 h a gradual decrease of PZA before POA became detectable extra- or intracellularly. In contrast to our observations with free bacteria, we did not find any PZA or POA in the intracellular bacteria. This might reflect a concentration dependent effect. Although exposed to a concentration 3 to 4 times higher than that achievable in blood, the intra-macrophage concentrations of PZA did not exceed 8 μg/ml. There was no detectable free drug intra-bacterially when exposed to this low concentration. In patients, with serum peak levels of PZA of approximately 30 μg/ml, one expects this to be even lower. MIC for PZA amongst *M. tuberculosis* are in the order of 20 μg/ml at pH 5.5. If PZA has any anti-mycobacterial effect intracellularly it is most likely because of trapping of the drug (or its metabolite) in a intra-bacterial structure. Such an event could result in a constant influx even at low extra-bacterial concentrations and it explains why PZA disappears in the absence of detectable POA.

With the establishment of an effective mobile phase as well as a method to separate test system fractions we are now able to carry out further experiments with clinical isolates of *M. tuberculosis* susceptible and resistant to PZA.

In conclusion, we developed HPLC methodology that allows simultaneous quantification of PZA and POA with minimal detection levels of 0.1 and 0.5 μg/ml.

Application of this methodology in macrophages infected with H37Rv suggest that, if the site of activity of PZA is in the macrophages, minimal amounts of the drug are needed. This is in contrast to the minimal inhibitory concentrations (MICs) obtained with free mycobacteria.

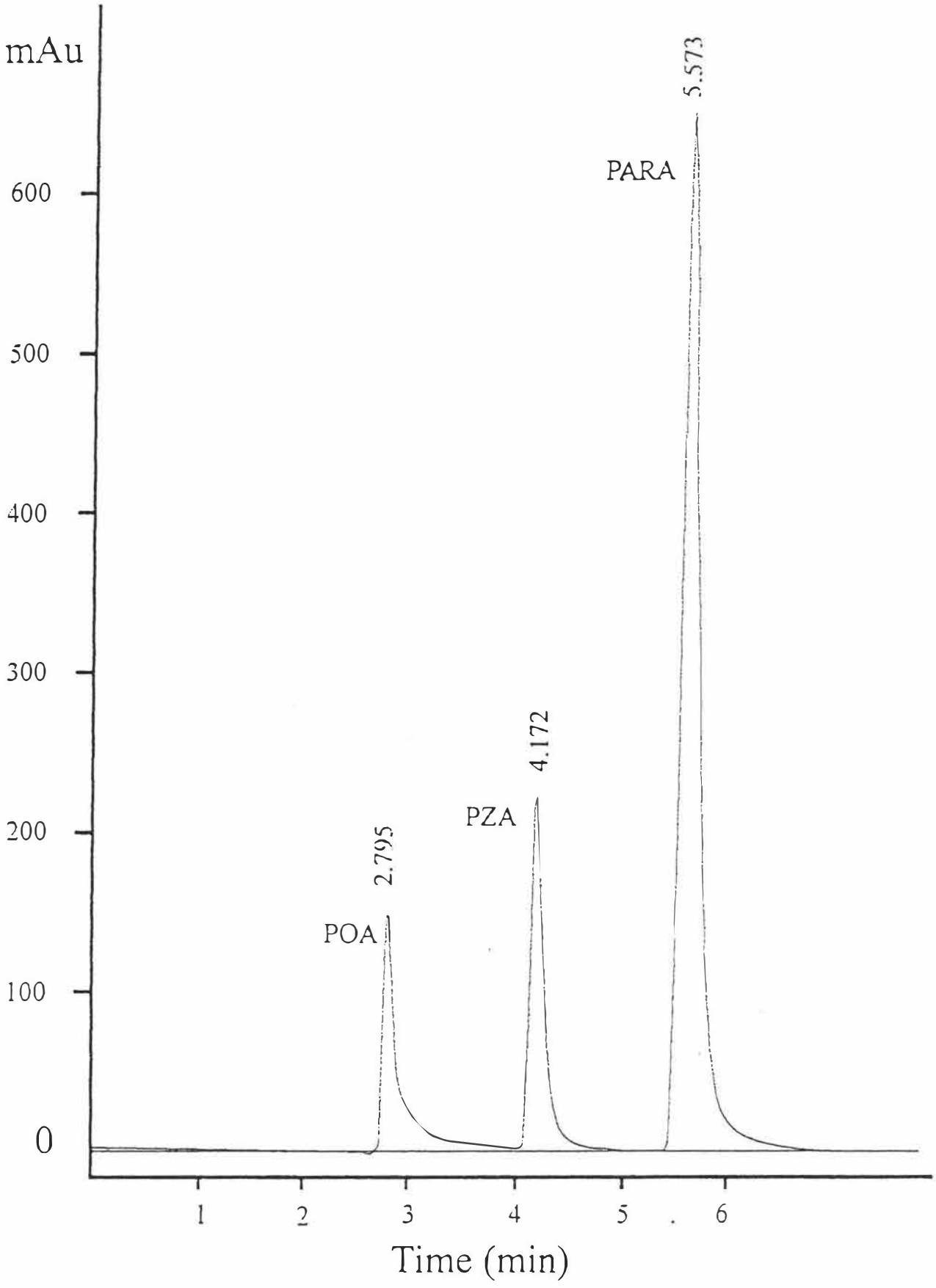


Fig 1: Separation of PZA, POA and PARA simultaneously using methanol and 10mm Phosphate buffer pH 3.5 (20%:80% v/v) as mobile phase on a C8 RP-Merck column.

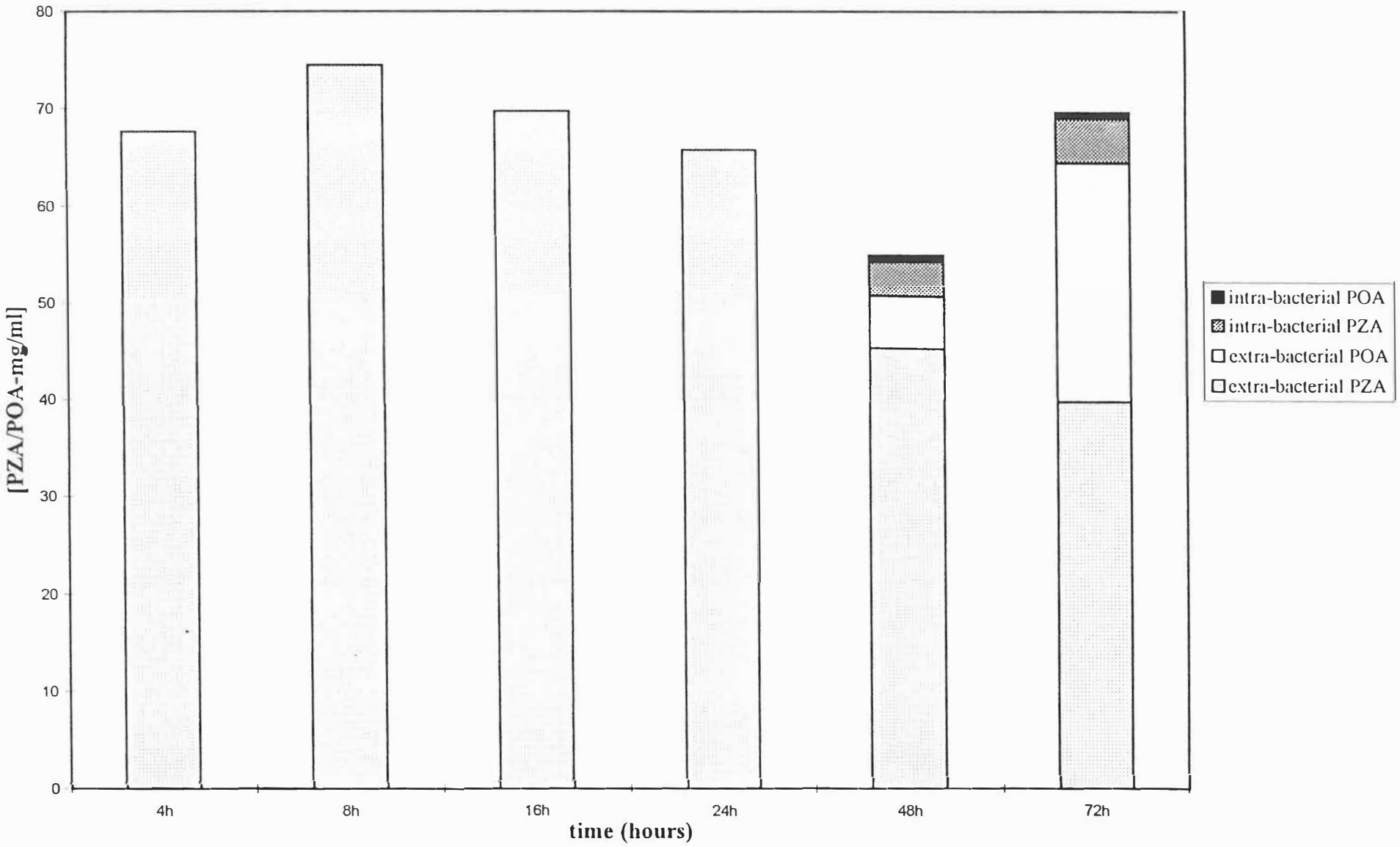


FIG. 2. Pyrazinamide and pyrazinoic acid concentrations in H37Rv grown in RPMI 1640.

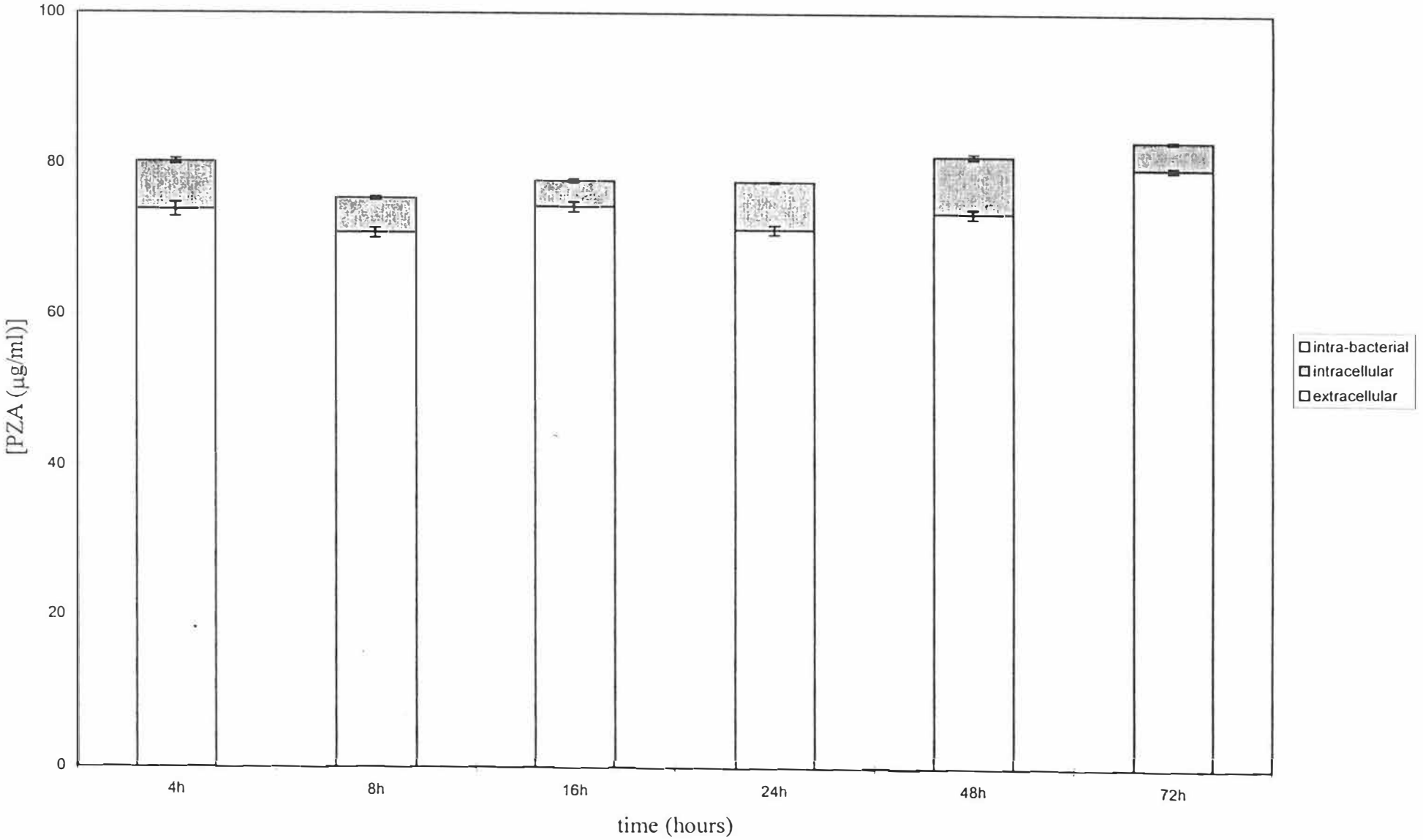


FIG.3. Extracellular, intracellular and intra-bacterial concentrations of PZA in macrophages infected with H37Rv (n=3).

3.2 ADDITIONAL INFORMATION

3.2.1 HPLC INSTRUMENTATION

The following figure indicates the functions of the different components of the HPLC.

COMPONENT	FUNCTION
Automatic injector	injects samples automatically into the column
Diode array UV-vis detector	measures absorbance at specific wavelengths and/or UV-vis spectrum of mobile phase and analytes
C8 guard column	to filter solid impurities that may be in sample
C8 analytical column	separates the components of samples injected
Autosampler	allows for the series of samples to be queued for automatic injection
Column oven	keeps column at optimum temperature for separation of samples
Mobile phase solvents	Carries samples across analytical column and allows optimum separation of sample
Control panels	instrument operation panel
Chemstation and computerised control	software that runs the HPLC as well as analysis of results can be done
Low and high pressure pumps	pumps mobile phase across the column
Electronic panels	electronics of instruments

FIG. 3.1 Functions of the different components of the HPLC.

3.3 ADDITIONAL RESULTS

PZA, POA and the internal standard was separated by the reported mobile phase of acetonitrile and phosphate buffer, pH 3.5. FIG. 3.2A, B, C and FIG 3.3A, B, C shows the chromatographs for PZA and POA, respectively. This mobile was not able to resolve POA satisfactory to obtain good correlation factors for the standard curves, thus other mobile phases were tried. Methanol and phosphate buffer, pH 3.5 was able to resolve all three antibiotics and was therefore the mobile phase of choice (FIG. 3.4). Also, three different wavelengths were used to obtain the best separation, *viz.*, 215, 254 and 265 nm. FIG. 3.5A, B, C shows the separation of the standard solutions of PZA while FIG. 3.6A, B, C shows the separation of POA at wavelengths 215, 254 and 265nm, respectively. The tissue culture medium, RPMI caused interference peaks, therefore the chromatographs obtained for RPMI was substracted from all the chromatographs as showed in FIG. 3.7. With prolonged use of the column, deterioration of the packing material occurred which interfered with the retention time of POA only, causing the peak to coincide with PZA (FIG. 3.8). Different pH values was used in an attempt to correct this as shown in FIG. 3.9. pH values above and below pH 3.5 was used in an attempt to resolve the peaks. This was corrected by changing the pH of the buffer to 4.0 as shown in FIG. 3.10. The standard curves are shown in appendix II.

FIG. 3.2A. Chromatographs of PZA (50 μg/ml) at wavelengths of (A) 215, (B) 254 and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.2B. Chromatographs of PZA (25 μg/ml) at wavelengths of (A) 215, (B) 254, and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.2C. Chromatographs of PZA (10 μg/ml) at wavelengths of (A) 215, (B) 254 and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.3A. Chromatographs of POA (100 μg/ml) at wavelengths of (A) 215, (B) 254 and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.3B. Chromatographs of POA (50 μg/ml) at wavelengths of (A) 215, (B) 254 and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.3C. Chromatographs of POA (25 μg/ml) at wavelengths of (A) 215, (B) 254, and (C) 265 nm using mobile phase of acetonitrile and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.4. Chromatographs of POA, PZA and internal PARA with retention times of 2.795, 4.172 and 5.573 mins respectively as separated by methanol and phosphate buffer, pH 3.5 (20%:80%).

FIG. 3.5A. Chromatographs of PZA standard solutions (A) 100, (B) 50, (C) 25, (D) 10, (E) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 215 nm.

FIG. 3.5B. Chromatographs of PZA standard solutions (A) 100, (B) 50, (C) 25, (D) 10, (E) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 254 nm.

FIG. 3.5C. Chromatographs of PZA standard solutions (A) 100, (B) 50, (C) 25, (D) 10, (E) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 265 nm.

FIG. 3.6A. Chromatographs of POA standard solutions (A) 50, (B) 25, (C) 10, (D) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 215 nm.

FIG. 3.6B. Chromatographs of POA standard solutions (A) 50, (B) 25, (C) 10, (D) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 254 nm.

FIG. 3.6C. Chromatographs of POA standard solutions (A) 50, (B) 25, (C) 10, (D) 5 μg/ml separated with methanol and phosphate buffer, pH 3.5 (20%:80%) at 265 nm.

FIG. 3.7. Subtraction of RPMI 1640 from all HPLC calculations to eliminate the interference peaks caused by RPMI 1640 (A) subtraction result-all interference peaks eliminated except for internal standard, (B) chromatograph of RPMI 1640, (C) RPMI 1640 with internal standard.

FIG. 3.8. Chromatograph of POA (3.638 mins) that coincides with PZA (4.100 mins) as the column deteriorates with prolonged use.

FIG. 3.9. pH values above and below pH 3.5 was used in an attempt to separate the PZA and POA chromatographs after deterioration of the column (A) pH 5.2, (B) pH 3.5, (C) pH 3.1.

FIG. 3.10. Separation of POA (3.119 min), PZA (4.091 min) and PARA (5.442 min) at pH 4 after deterioration of the column.

3.4 ADDITIONAL DISCUSSION

The chromatographs obtained for the PZA and POA standards with a mobile phase of acetonitrile and phosphate buffer pH 3.5 is shown in FIG 3.2A, B, C and FIG 3.3A, B, C. Although many trials were carried out with this mobile phase the POA peak was not satisfactory resolved. New stock powder was used to eliminate contamination. Also, different solvents were used to dissolve POA, but all solutions of POA resulted in poor chromatographs. Thus, an attempt was made to change the mobile phase to methanol and phosphate buffer which resolved POA peaks. The best chromatographs for PZA, POA and the internal standard PARA were obtained at 265 nm.

CHAPTER 4

DISTRIBUTION OF PZA AND POA IN NON CELL ASSOCIATED AND MACROPHAGE BOUND MYCOBACTERIA: A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSESSMENT

4.1 INTRODUCTION

The objectives of the high performance liquid chromatography (HPLC) experiments presented in this chapter were:

a) to measure PZA concentrations as well as the amount of PZA converted to POA upon exposure of mycobacteria in RPMI 1640 supplemented with 100 μg/ml PZA over time,

b) to measure PZA concentration and the amount of PZA converted to POA in macrophages infected with mycobacteria and exposed to 100 μg/ml PZA, *i.e.*, in the various fractions of the mycobacterial-macrophage co-cultures, *viz.*, the extracellular, intracellular and intra-bacterial fractions.

There are several methods available to quantify pharmaceutical compounds. Spectrophotometric methods (UV/visible, fluorescence) are fairly non-specific and usually do not discriminate between closely related metabolites or degradation products. Thin-layer chromatographic methods are widely used and inexpensive. However, quantitative analysis is difficult. Gas chromatography shows good specificity and sensitivity. However, the elevated operating temperatures used may occasionally cause thermal degradation of compounds, and it is necessary to derivatise samples to increase their volatility and improve their chromatographic behaviour. HPLC offers many advantages for quantitative analysis. Extraction procedures and

sample clean-up prior to injection are much simpler than for most other methods. In the analysis of pharmaceutical products it is frequently sufficient to crush or mix the product with a suitable solvent, followed by filtration and injection into the analyser (Knox, 1978).

HPLC has been used to measure PZA in human plasma (Walubo *et al*, 1994) and cerebrospinal fluid (CSF) as well as in plasma in rabbits (Chan *et al*, 1986). These HPLC studies were only able to quantify PZA. With acetonitrile and phosphate buffer, pH 3.5 as mobile phase, it was not possible to resolve POA. We developed a mobile phase to resolve PZA and POA simultaneously. Also, there have been no HPLC studies that have investigated the effect of PZA on macrophages infected with mycobacteria as well as non cell associated mycobacteria.

By means of this method, we were able to measure PZA/POA in the various fractions of the macrophage. This enabled us to establish how these compounds were distributed in a mycobacterial macrophage co-culture system. We were also able to compare the degree of conversion of PZA to POA in non cell associated mycobacteria in RPMI 1640 as compared to macrophage bound mycobacteria.

4.2 MATERIALS AND METHODS

4.2.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) CONDITIONS

HPLC was performed using a Hewlett Packard 1090 HPLC with a Merck reverse phase C8 column fitted with a C8 guard column. The column oven temperature was 40 °C. Mobile phases used to separate PZA and POA were combinations of acetonitrile and 10mM phosphate buffer pH 3.5 (20%:80%, v/v) (Appendix II) (Woo, 1987 and Chan, 1986); phosphate buffer pH 2.56 (Yamamoto *et al*, 1987a); and a combination of methanol and 10 mM phosphate buffer pH 3.5 (Ono *et al*, 1996) and

with prolonged use of the column had to be changed to pH 4. The flow rate of the mobile phase was 1ml/min with a sample injection volume of 25 µl in all experiments. Product analysis was done using a UV detector, at wavelengths of 215 nm (Chan *et al*, 1986), 254 nm and 265 nm (Ono *et al*, 1996). PZA and POA as well as paracetamol (PARA) were dissolved in warm distilled water to obtain the following standard solutions: 5, 10, 25, 50, 100 µg/ml of PZA and 5, 10, 25, 50 µg/ml of POA, with the internal standard PARA at a final concentration of 100 µg/ml. RPMI 1640 tissue culture medium without any of the drugs was used as a blank and the HPLC results were subtracted from all extracellular calculations as the extracellular fraction only was in RPMI 1640.

4.2.2 PZA UPTAKE BY MYCOBACTERIA ONLY

A McFarland No 1 standard of the reference strain, H37Rv, as well as all Pzase positive and negative clinical mycobacterial isolates were exposed to 100 µg/ml PZA in RPMI 1640 tissue culture medium. At time intervals of 4, 8, 16, 24, 48, 72, 144, 240, 336 h the mycobacteria were disrupted with 0.1-0.15 mm diameter zirconia/silica beads using a mini bead beater (Biospec Products) and PZA and POA concentrations were measured by HPLC.

4.2.3 PZA UPTAKE BY INFECTED MACROPHAGES

Macrophages were exposed to the mycobacteria in the ratio 1:10 for 1 hour, after which all unbound mycobacteria were washed off. Fresh RPMI 1640 supplemented with 100 µg/ml PZA was added to the petri dishes. To quantify extracellular, intracellular and intrabacterial PZA/POA by HPLC, cells from 3 petri dishes were harvested and pooled at intervals of 4, 8, 16, 24, 48, and 72 h.

4.2.4 SEPARATION OF EXTRACELLULAR, INTRACELLULAR AND INTRA-BACTERIAL FRACTIONS

The infected macrophages were gently scraped off the petri dishes using a Sterilin quadloop. The suspension obtained was centrifuged at 250 x g for 10 mins. The supernatant was discarded except for 500 µl which was used to resuspend the pellet. To separate the extracellular from the intracellular fraction, silicone oil velocity gradient centrifugation was used (Johnson *et al*, 1980, Koga, 1987, Pascual *et al,* 1994, Klemper and Stryt, 1981). A 6:5 mixture of silicone oils (500 µl) of densities 1.07 and 0.98, respectively, was placed in an eppendorf tube and 500 µl of macrophage suspension was overlayed on the oil. This was then centrifuged for 3 mins at 12 000 x g which resulted in a pellet of macrophages at the bottom of the tube. The extracellular fluid above the oil layer was removed. The pellet was resuspended in 500 µl of distilled water, vortexed and incubated at 37°C for 10 mins to completely disrupt the macrophages. This constituted the intracellular extra-bacterial fraction. The intra-bacterial antibiotic concentration was calculated after PZA and POA concentrations were measured in the intracellular fraction, in which the mycobacteria were disrupted by means of 0.1-0.15 mm diameter zirconia/ silica beads using a mini bead beater (Biospec Products).

4.3 RESULTS

Pzase positive mycobacteria, grown in RPMI 1640 without macrophages did take up PZA and there was also POA detected in these bacteria. FIG. 4.2A-FIG. 4.18A shows the variation in concentration of PZA and POA over a 336h period. PZA also entered Pzase negative bacteria but no POA was detected (FIG. 4.13A-FIG. 4.18A). No PZA or POA could be detected in bacteria in macrophages over the 72h period studied (FIG. 4.2B-FIG. 4.12B). While PZA did enter the cells, no POA could be detected in infected cells or in cells infected with Pzase positive and negative mycobacteria. The differences in intracellular and extracellular concentrations of the drug for the 3 different test systems, *viz*., uninfected macrophages, infected macrophages and free

mycobacteria are shown in figures FIG. 4.1, 4.2B-4.18B and FIG. 4.2A-4.18A, respectively. The amount of intracellular PZA was lower when the cells were inoculated with Pzase positive mycobacteria as compared to uninfected macrophages and macrophages infected with Pzase negative strains. This difference was statistically significant ($p=0.001$). There was no difference in extracellular concentration over time in the test system with Pzase positive and negative bacteria ($p=0.07$). However, the extracellular concentration in the test system without bacteria were significantly lower over the 72 h studied when compared to infection with susceptible strains ($p=0.0008$) and resistant strains ($p<0.0001$).

All results are presented graphically for non cell associated cultures as well as macrophage bound mycobacteria. Composite graphs show the mean values for PZA and POA of all susceptible and resistant strains as macrophage bound mycobacteria (FIG. 4.19-FIG. 4.20) and non cell associated cultures (FIG. 4.21-FIG. 4.22). Graphs for statistically analysis are shown in FIG. 4.23 and FIG. 4.24.

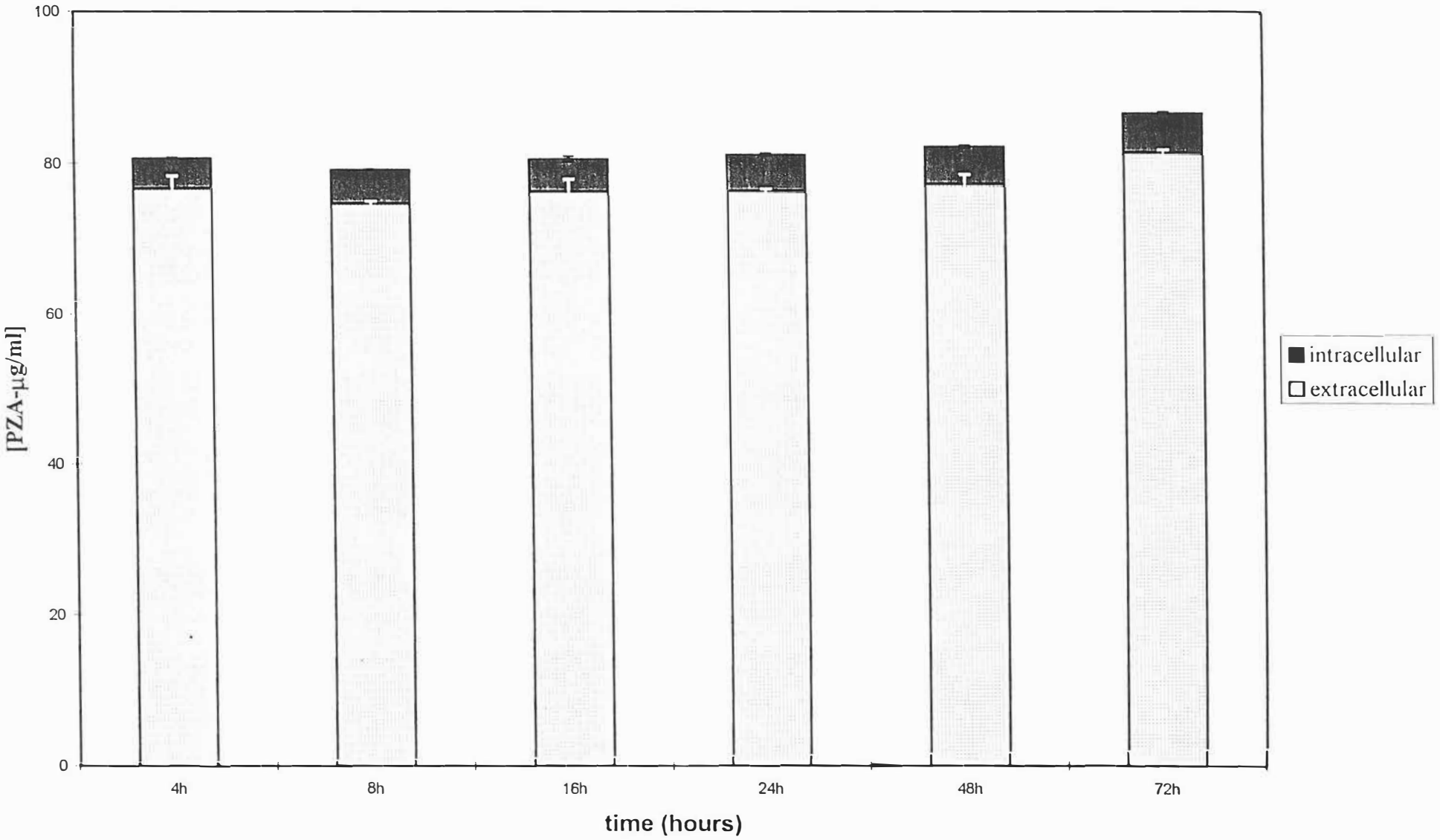


FIG. 4.1 Mean values of extracellular and intracellular PZA concentrations in uninfected macrophages (n=5).

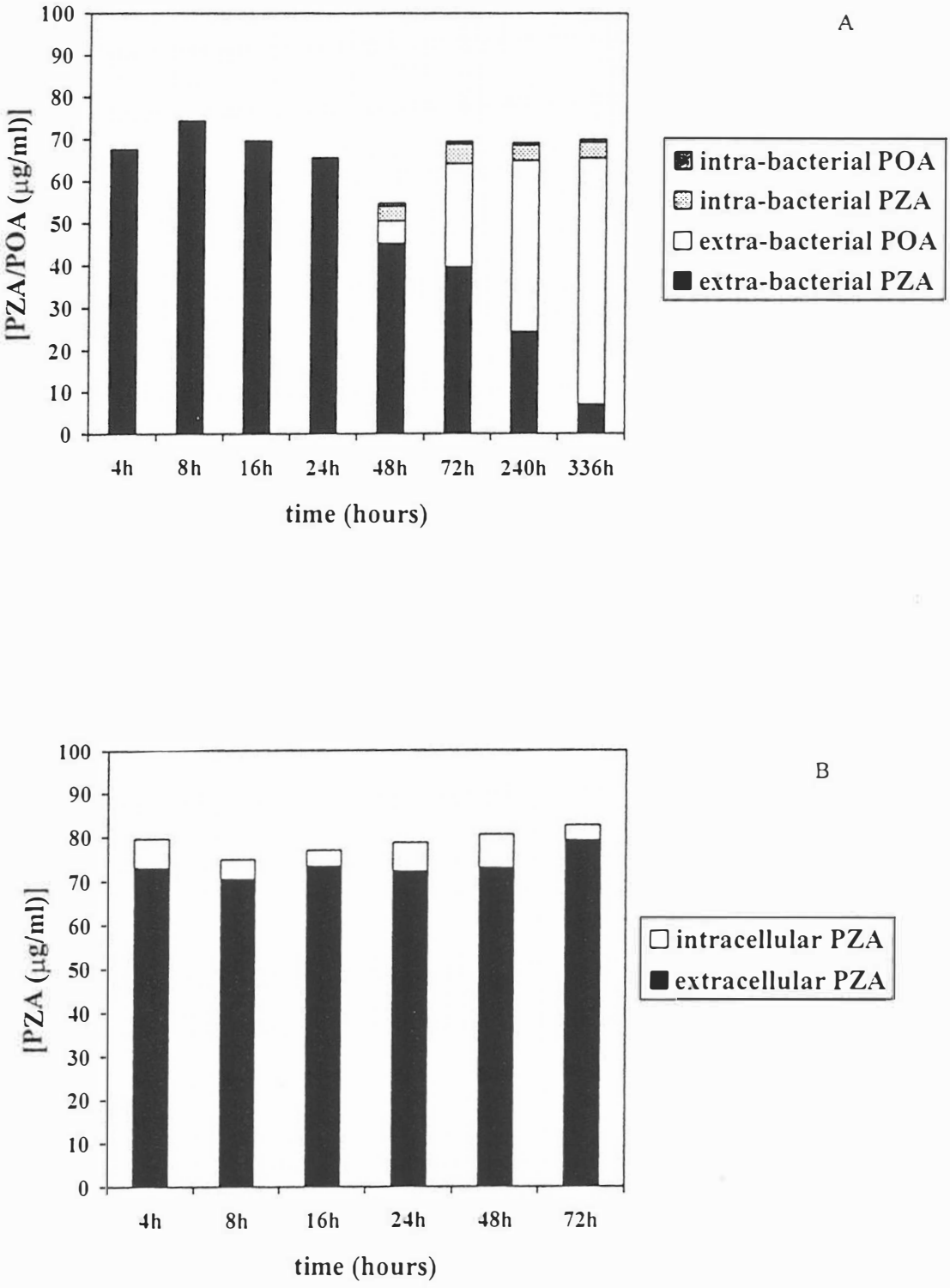


FIG. 4.2. PZA and POA concentrations in reference strain, H37Rv exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

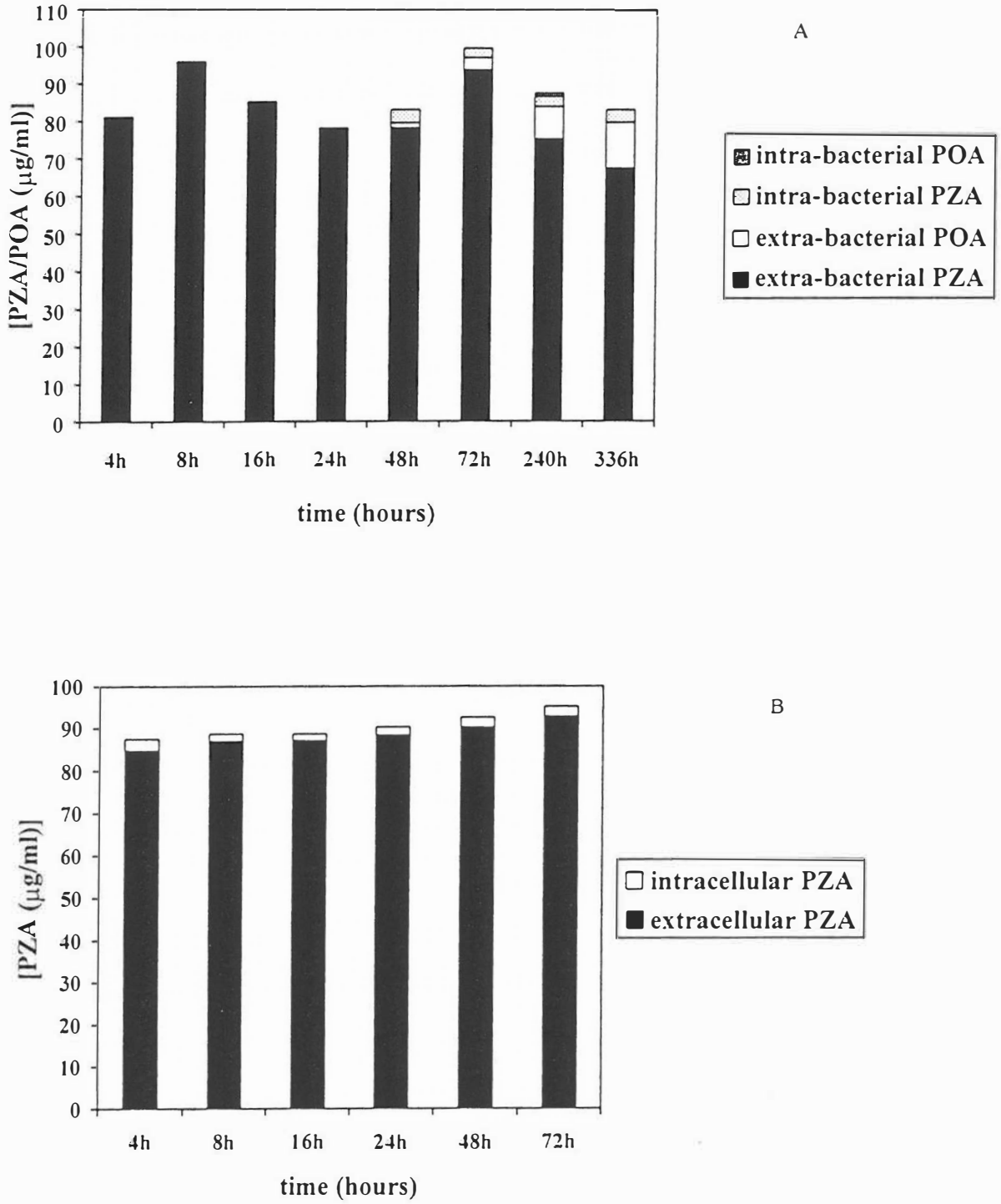


FIG. 4.3. PZA and POA concentrations in a susceptible strain, 200R exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

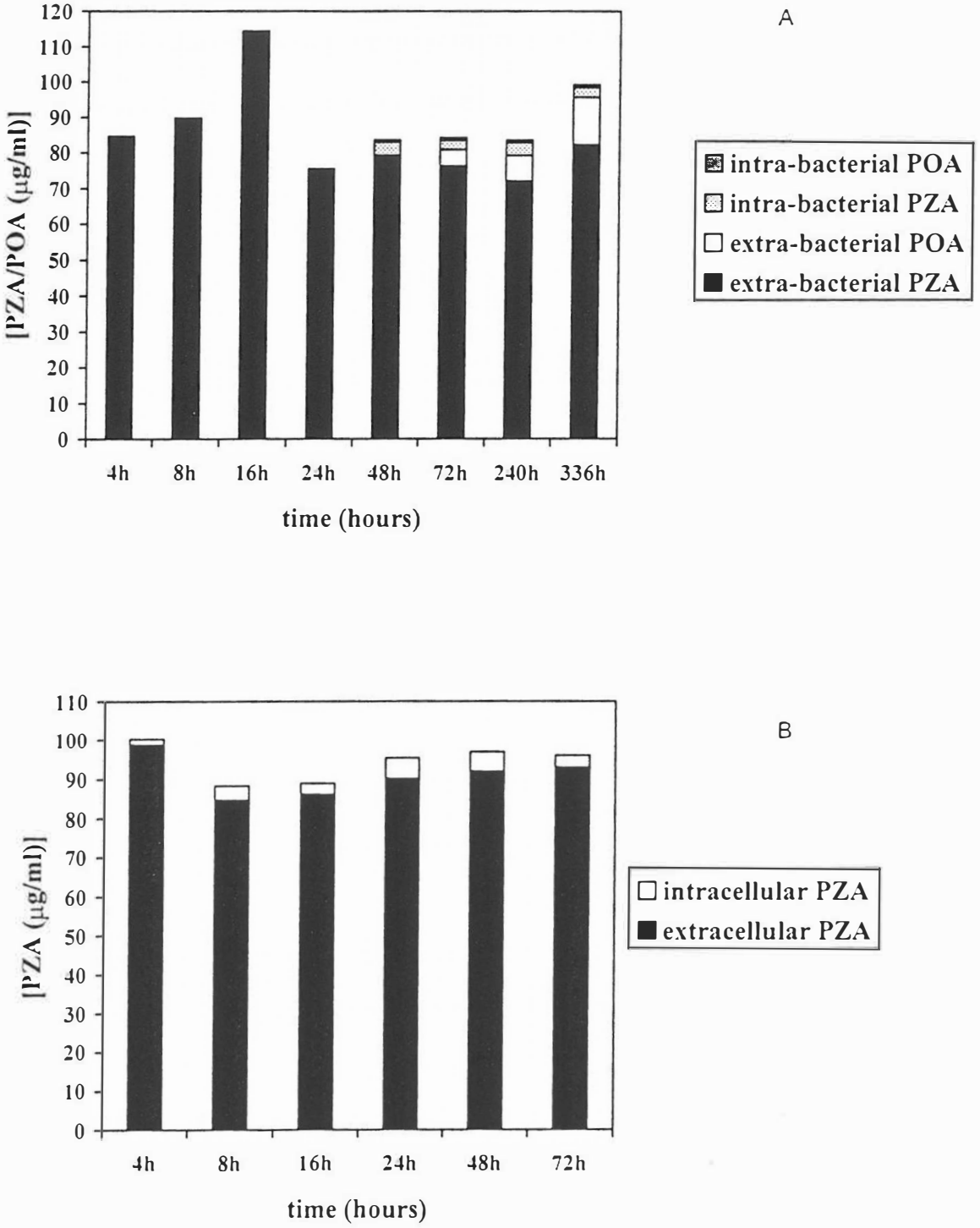


FIG. 4.4. PZA and POA concentrations in a susceptible strain, 100R exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

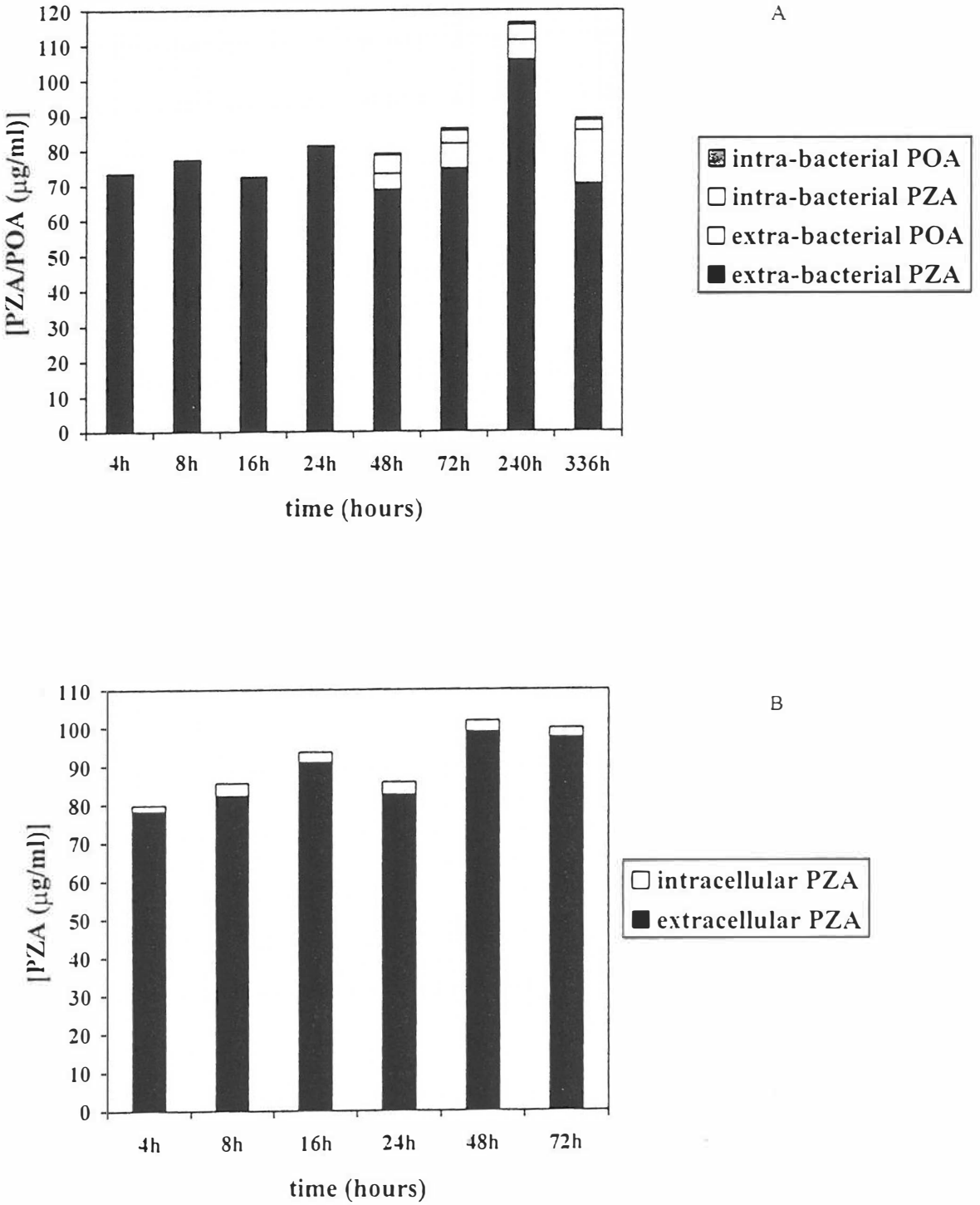


FIG. 4.5. PZA and POA concentrations in a susceptible strain, 11344 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

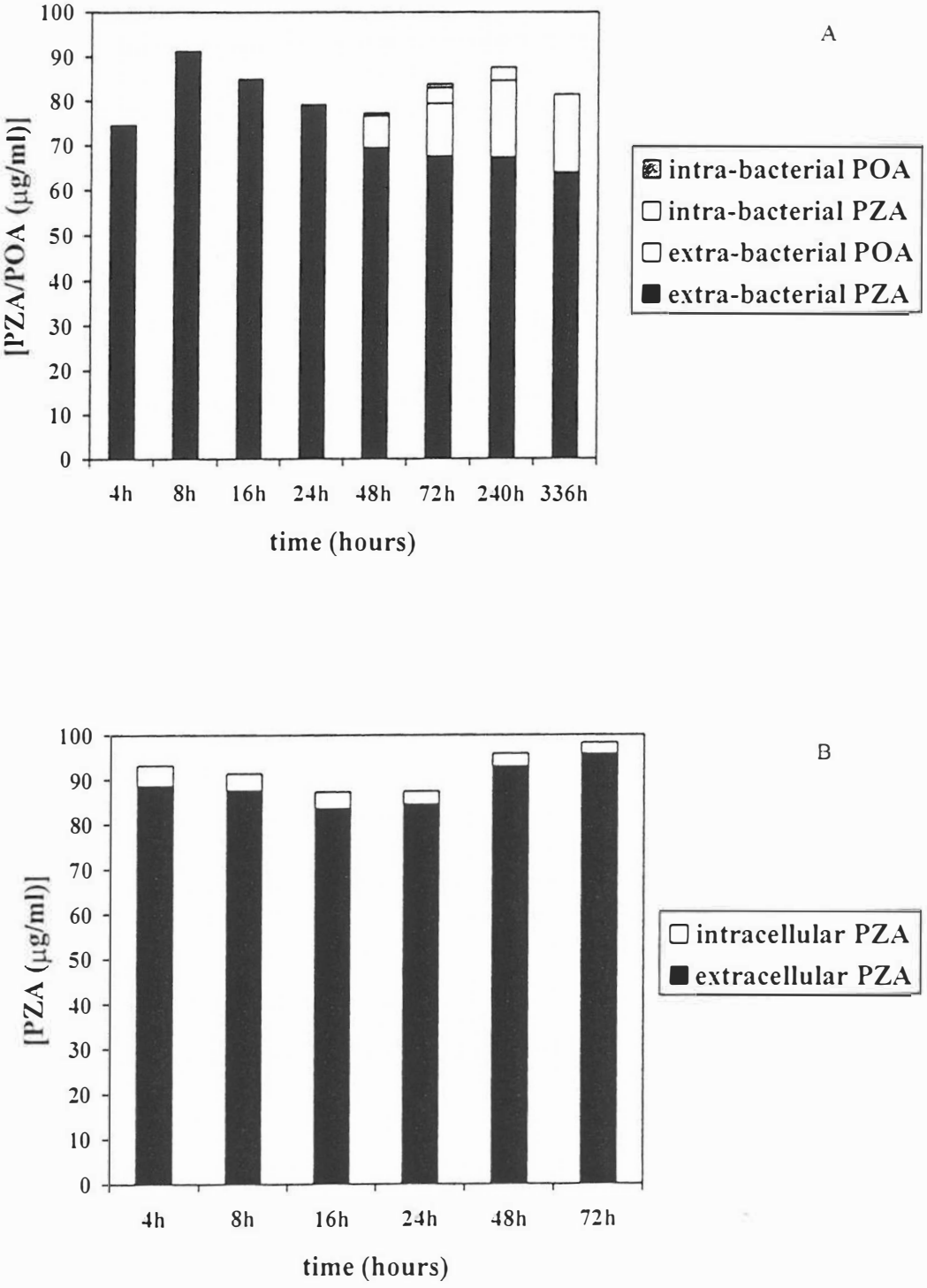


FIG. 4.6. PZA and POA concentrations in a susceptible strain, 90s exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

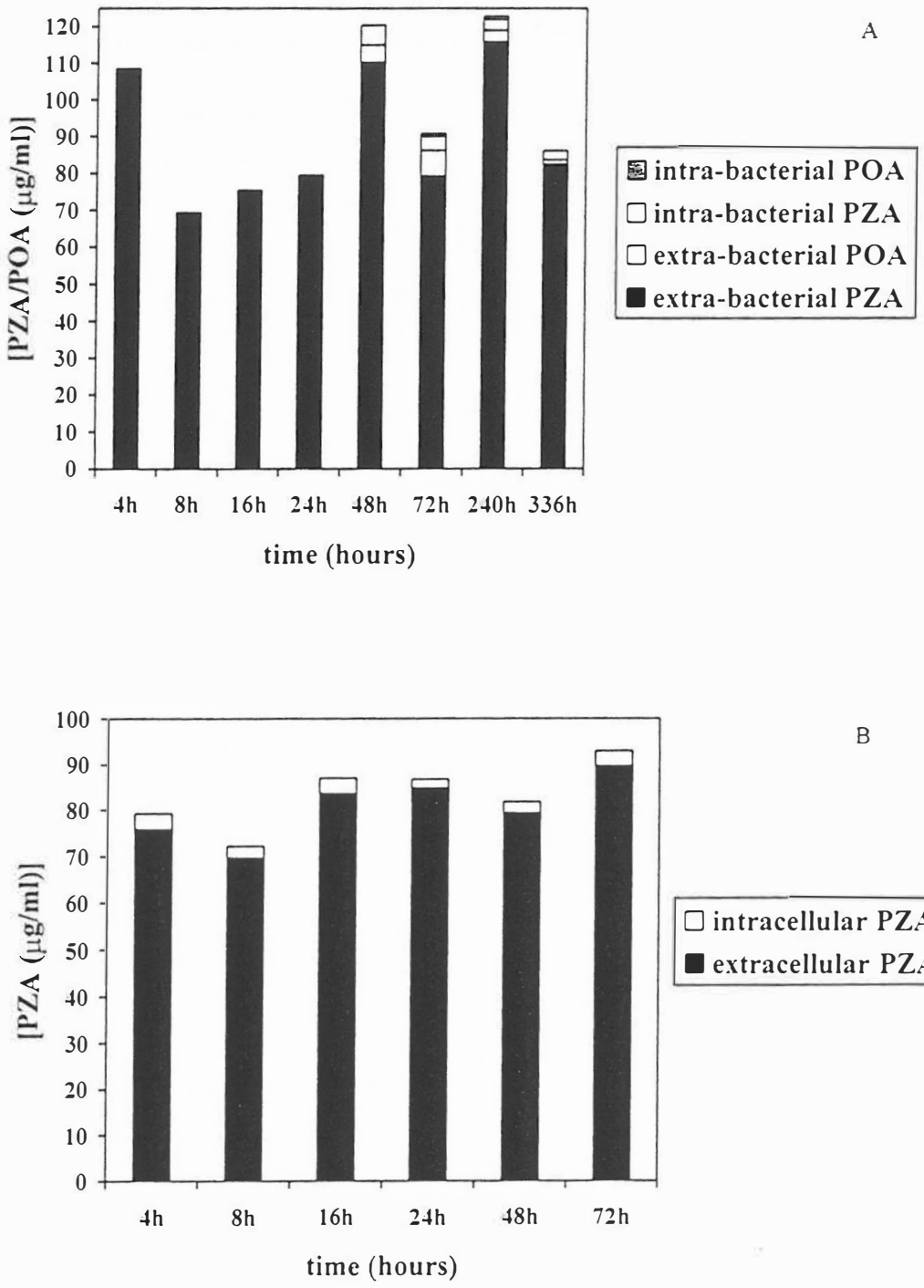


FIG. 4.7. PZA and POA concentrations in a susceptible strain, 11102 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages

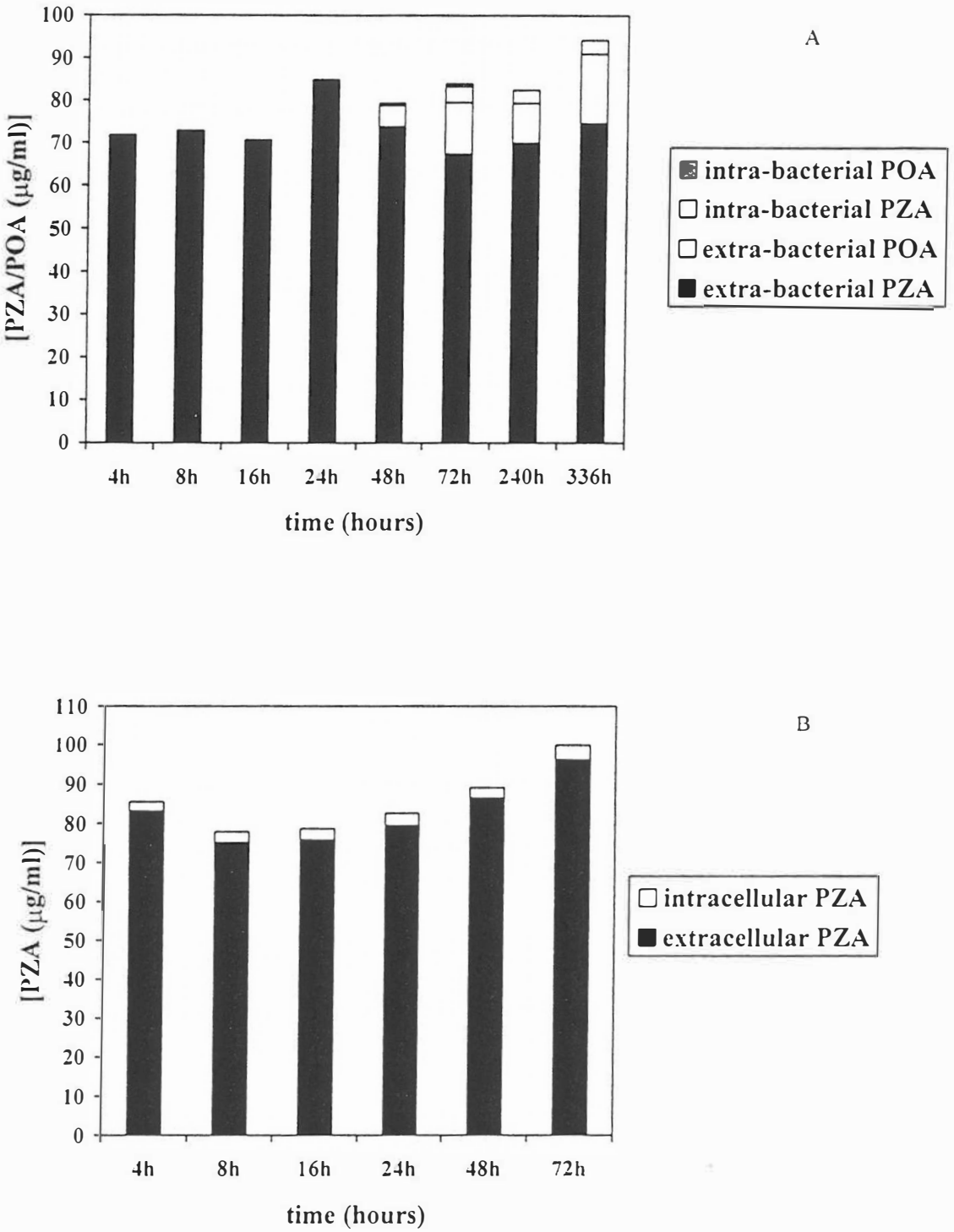


FIG. 4.8. PZA and POA concentrations in a susceptible strain, 11191 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

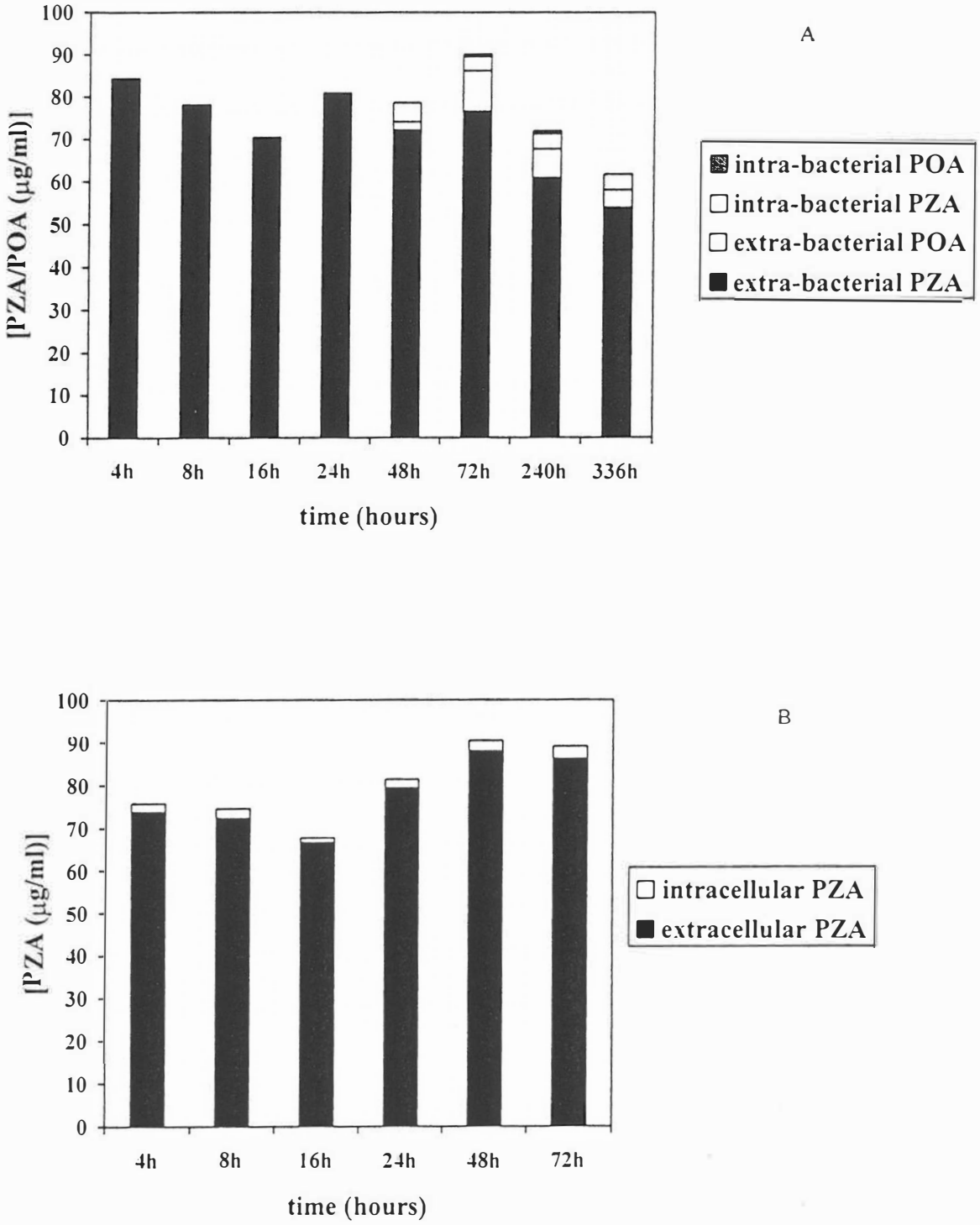


FIG. 4.9. PZA and POA concentrations in a susceptible strain, 11341 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

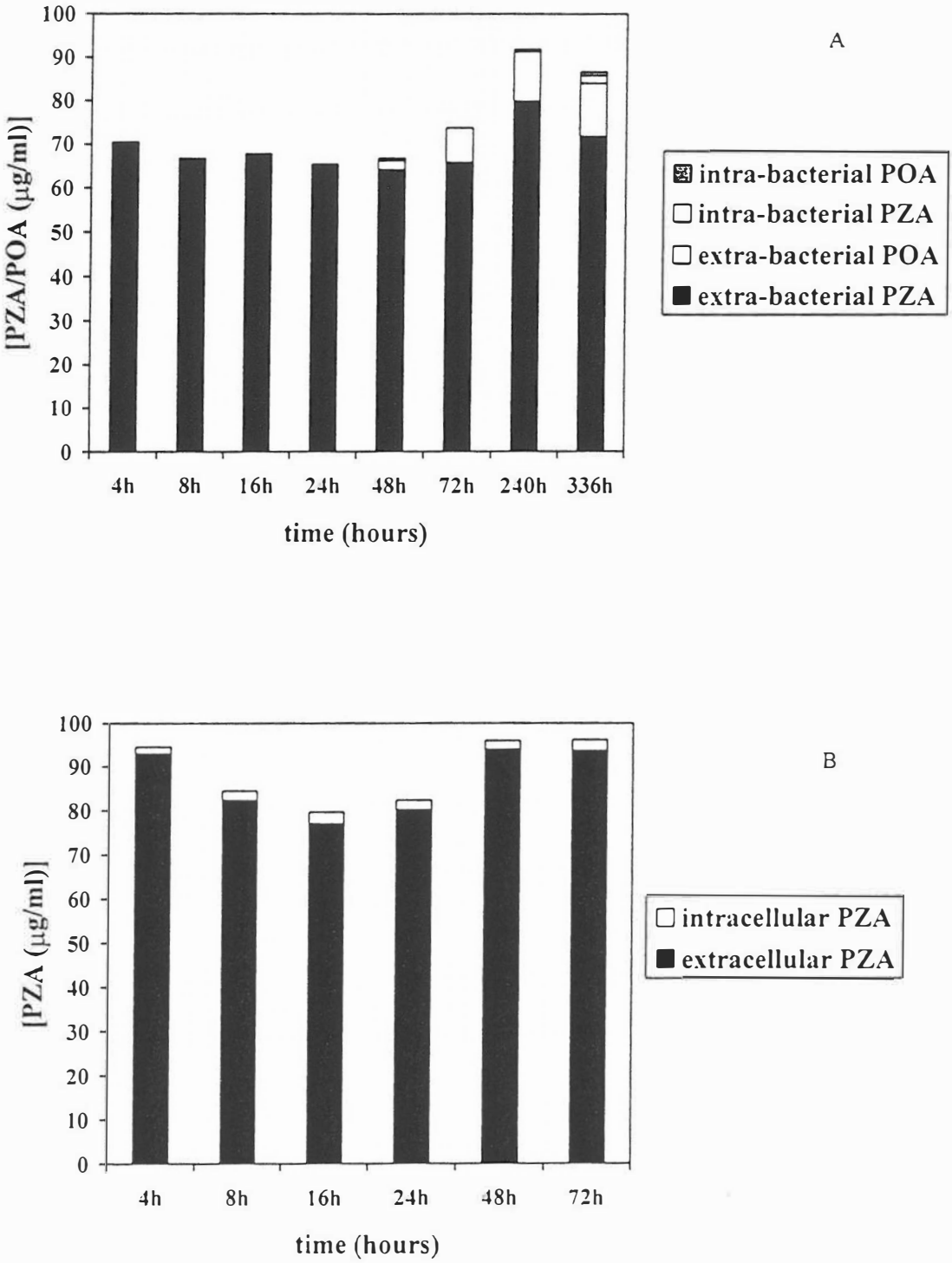


FIG. 4.10. PZA and POA concentrations in a susceptible strain, 11851 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

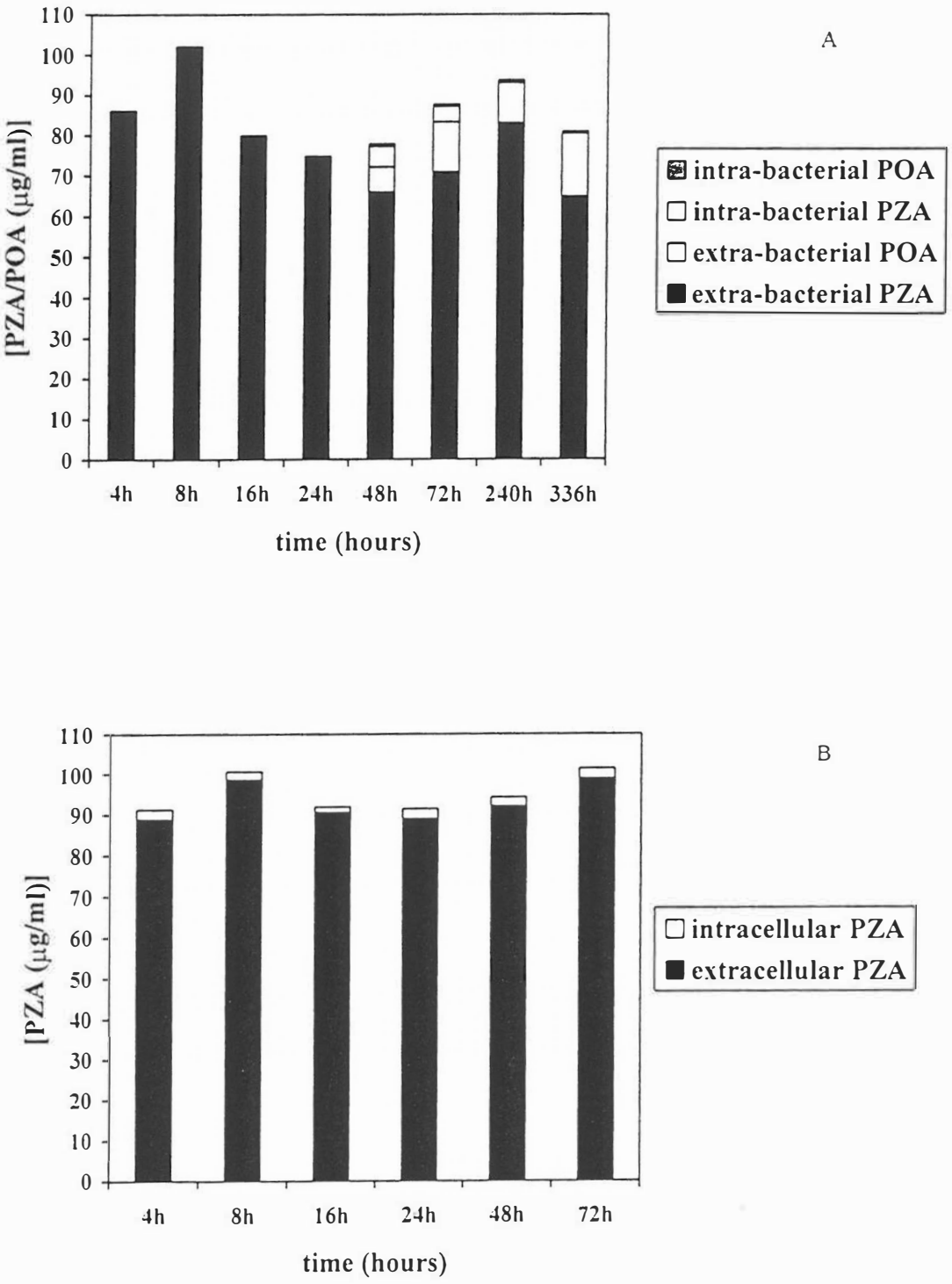


FIG. 4.11. PZA and POA concentrations in a susceptible strain, 10486 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

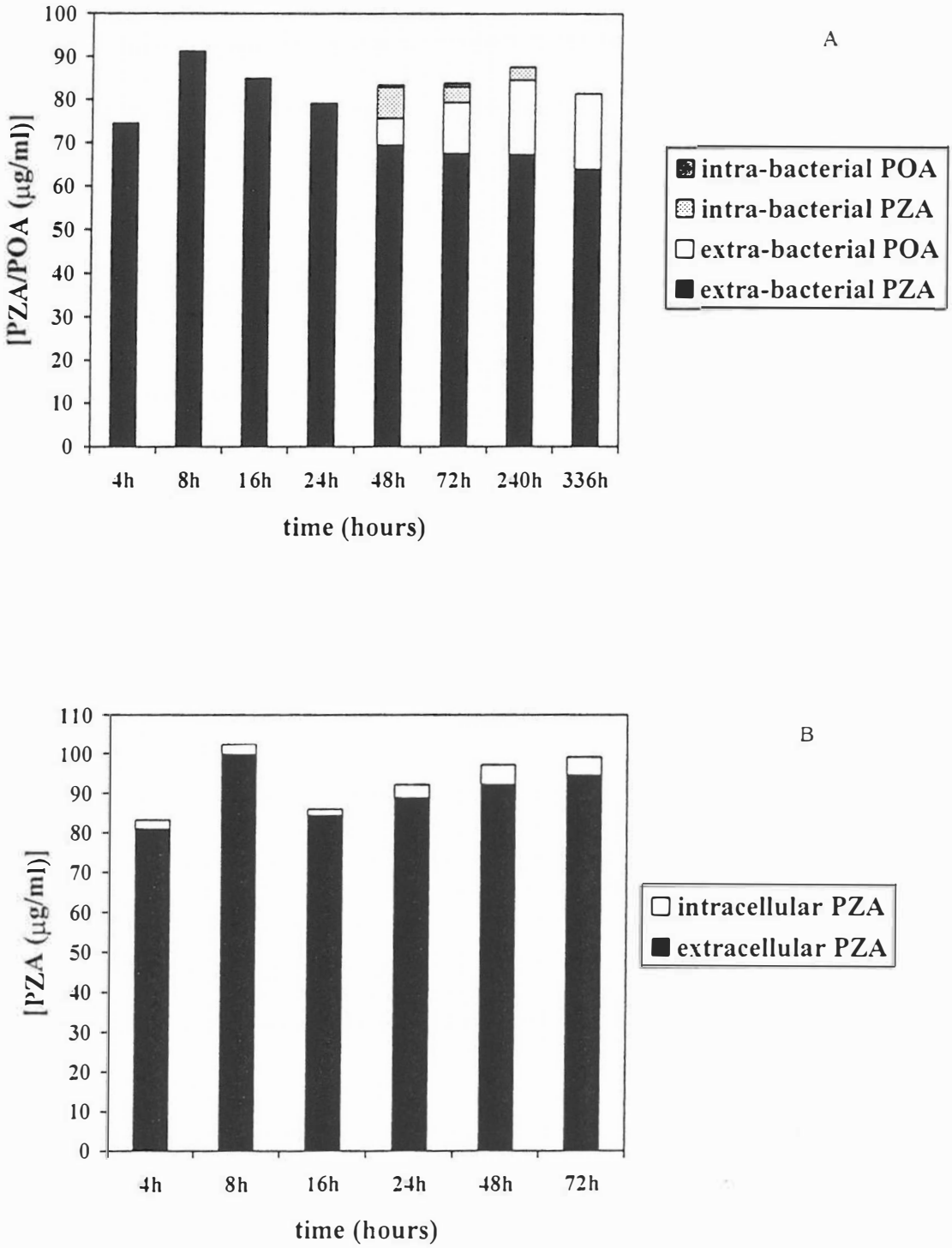


FIG. 4.12. PZA and POA concentrations in a susceptible strain, 1195 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

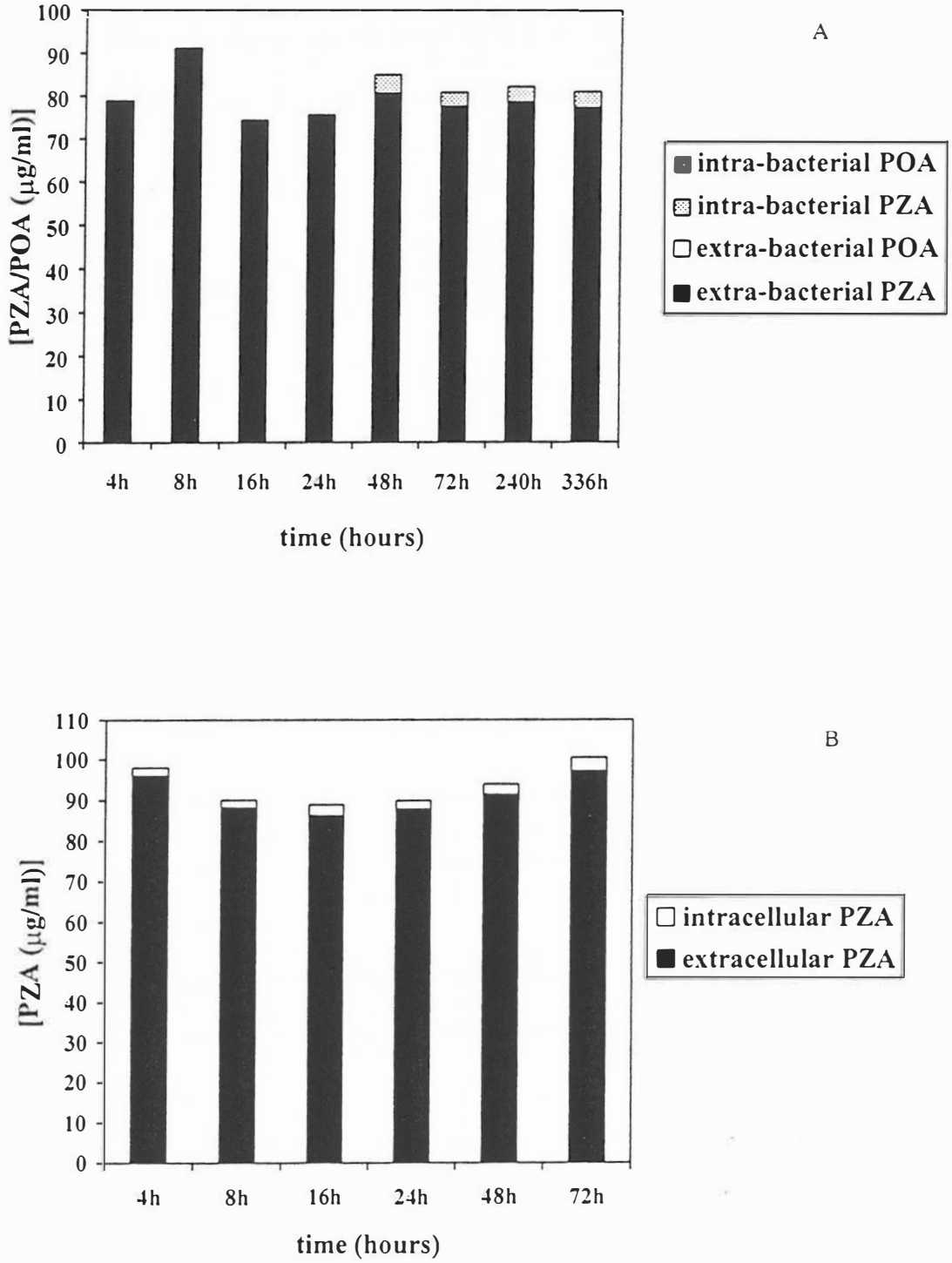


FIG. 4.13. PZA and POA concentrations in *M. bovis*, a mycobacterial strain in which the Pzase enzyme is naturally absent, exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

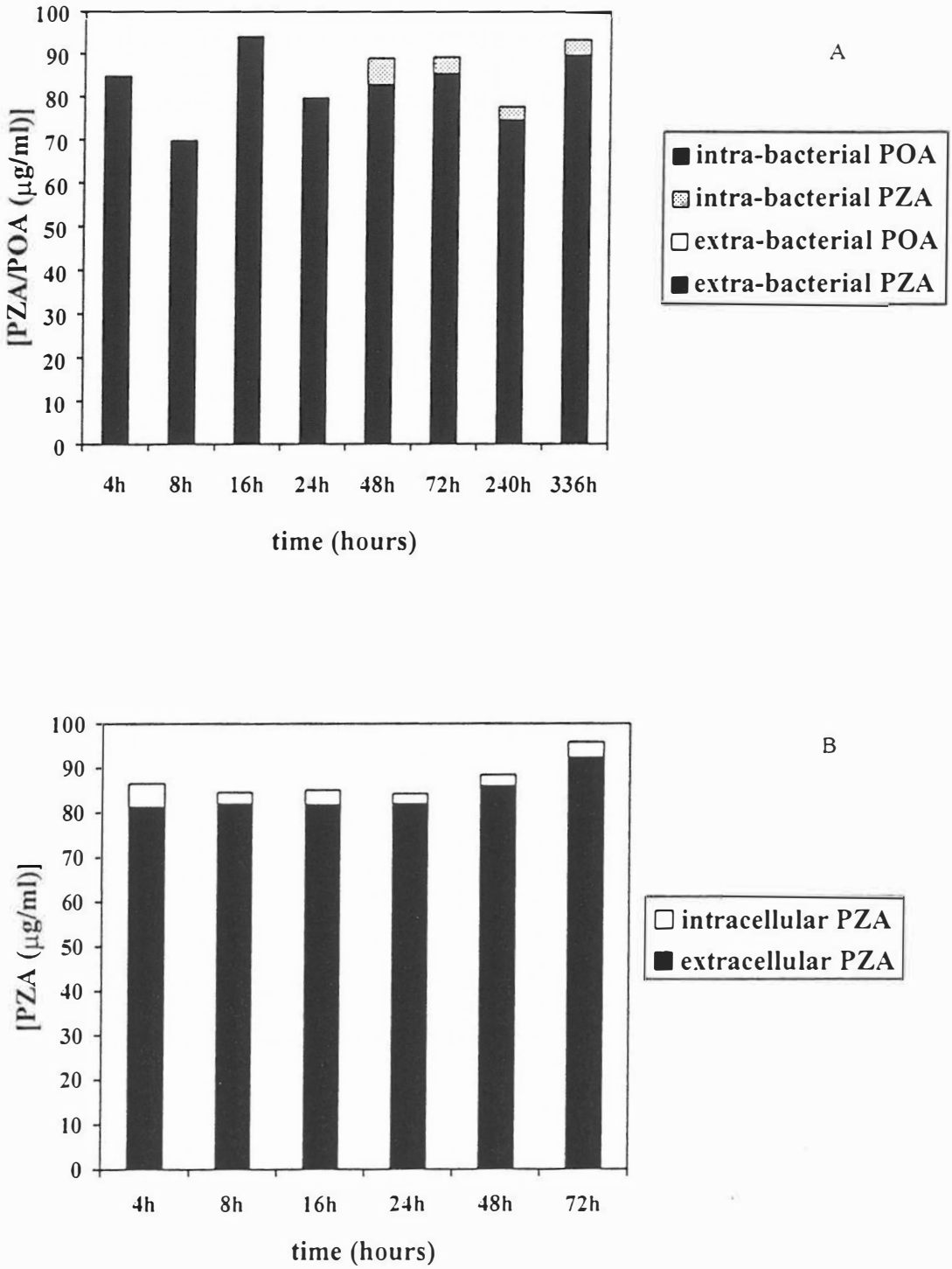


FIG. 4.14. PZA and POA concentrations in a resistant strain, 3886 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

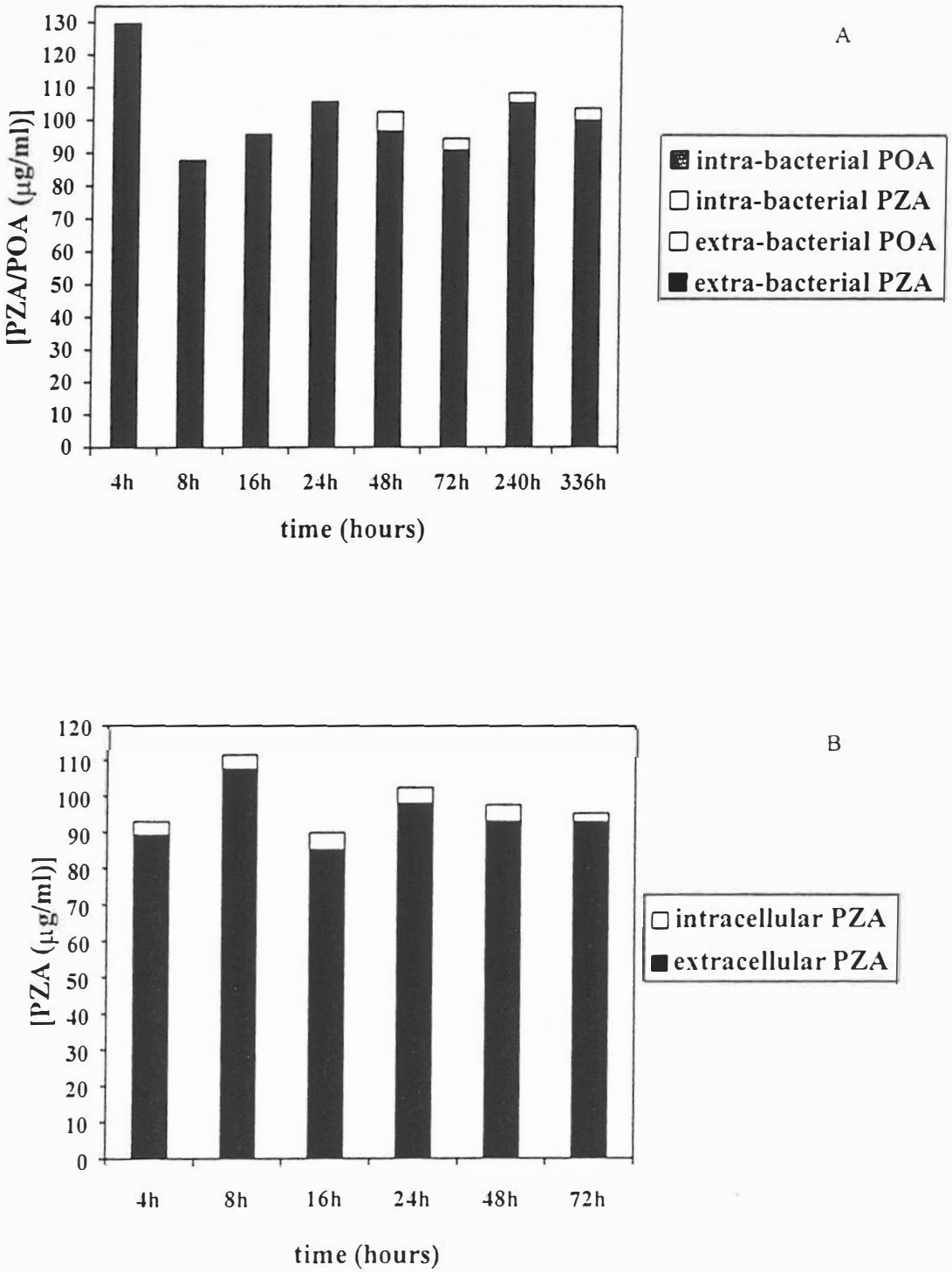


FIG. 4.15. PZA and POA concentrations in a resistant strain, 3732 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

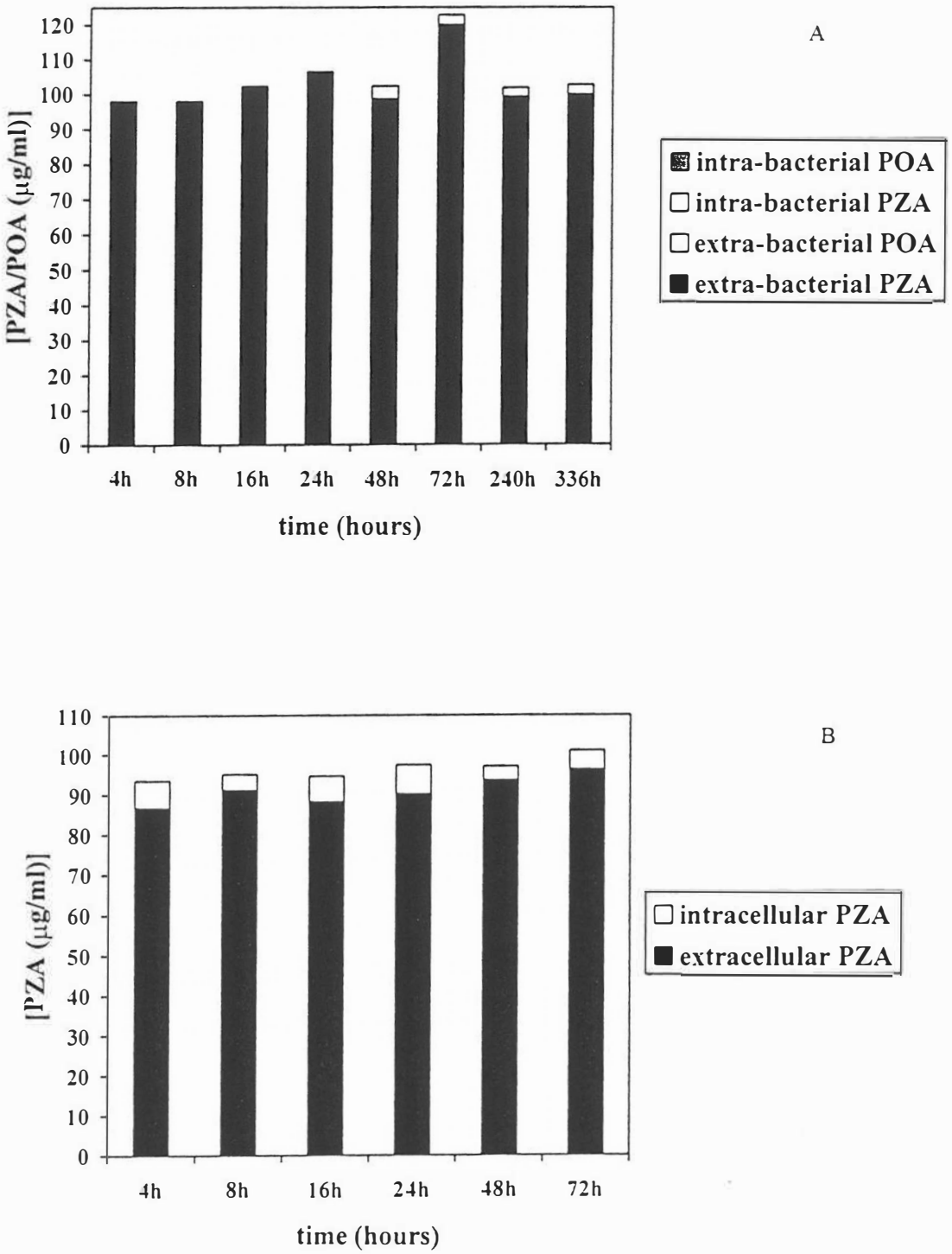


FIG. 4.16. PZA and POA concentrations in a resistant strain, 60650 exposed to 100 µg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

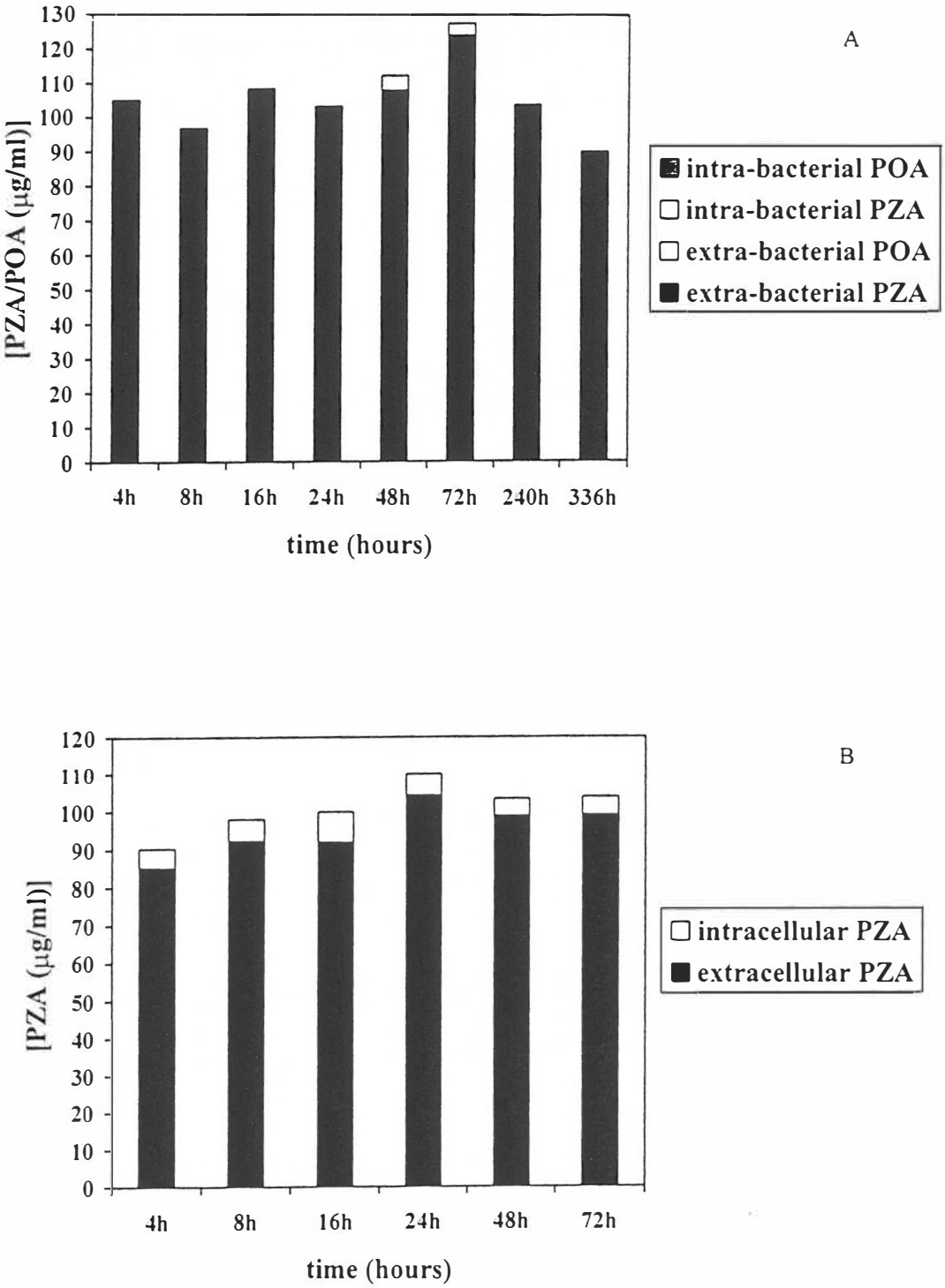


FIG. 4.17. PZA and POA concentrations in a resistant strain, 79386 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

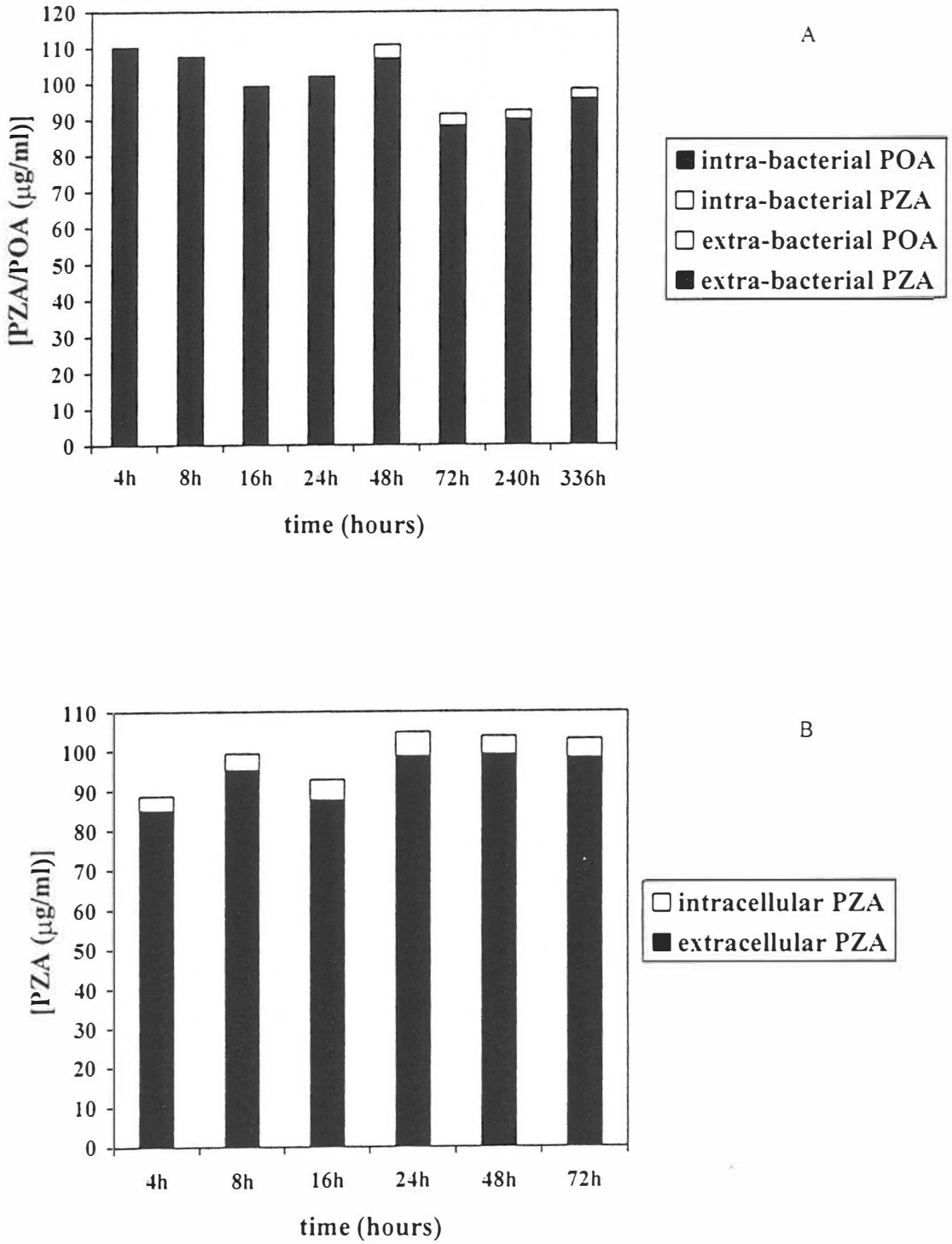


FIG. 4.18. PZA and POA concentrations in a resistant strain, 6114 exposed to 100 μg/ml PZA in (A) RPMI 1640 and (B) infected macrophages.

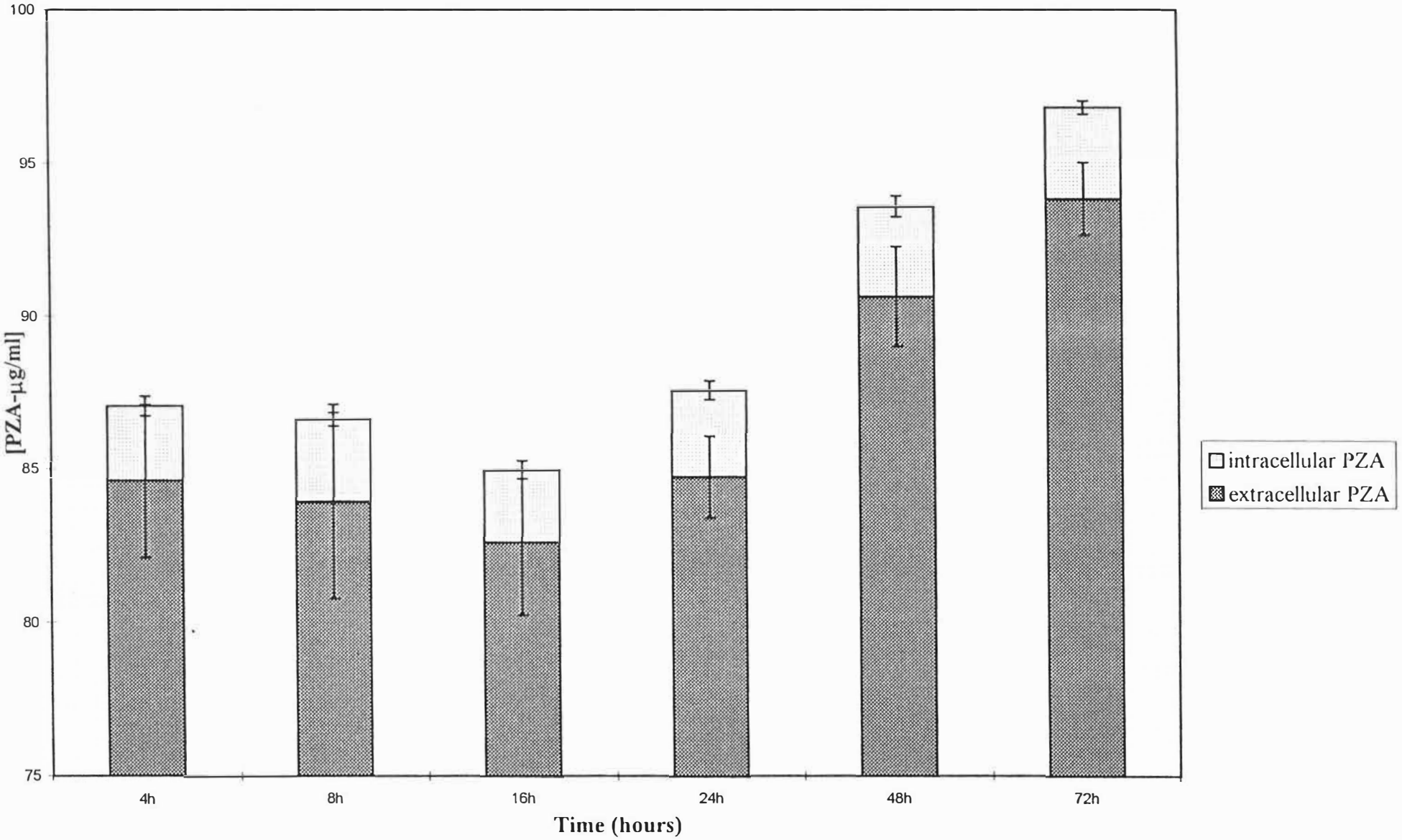


FIG. 4.19 Mean values of intracellular and extracellular concentrations of PZA in macrophages infected individually with 10 susceptible *M. tuberculosis* strains. Intracellular PZA concentrations were lower than extracellular PZA concentrations, indicating minimal entry of PZA into macrophages.

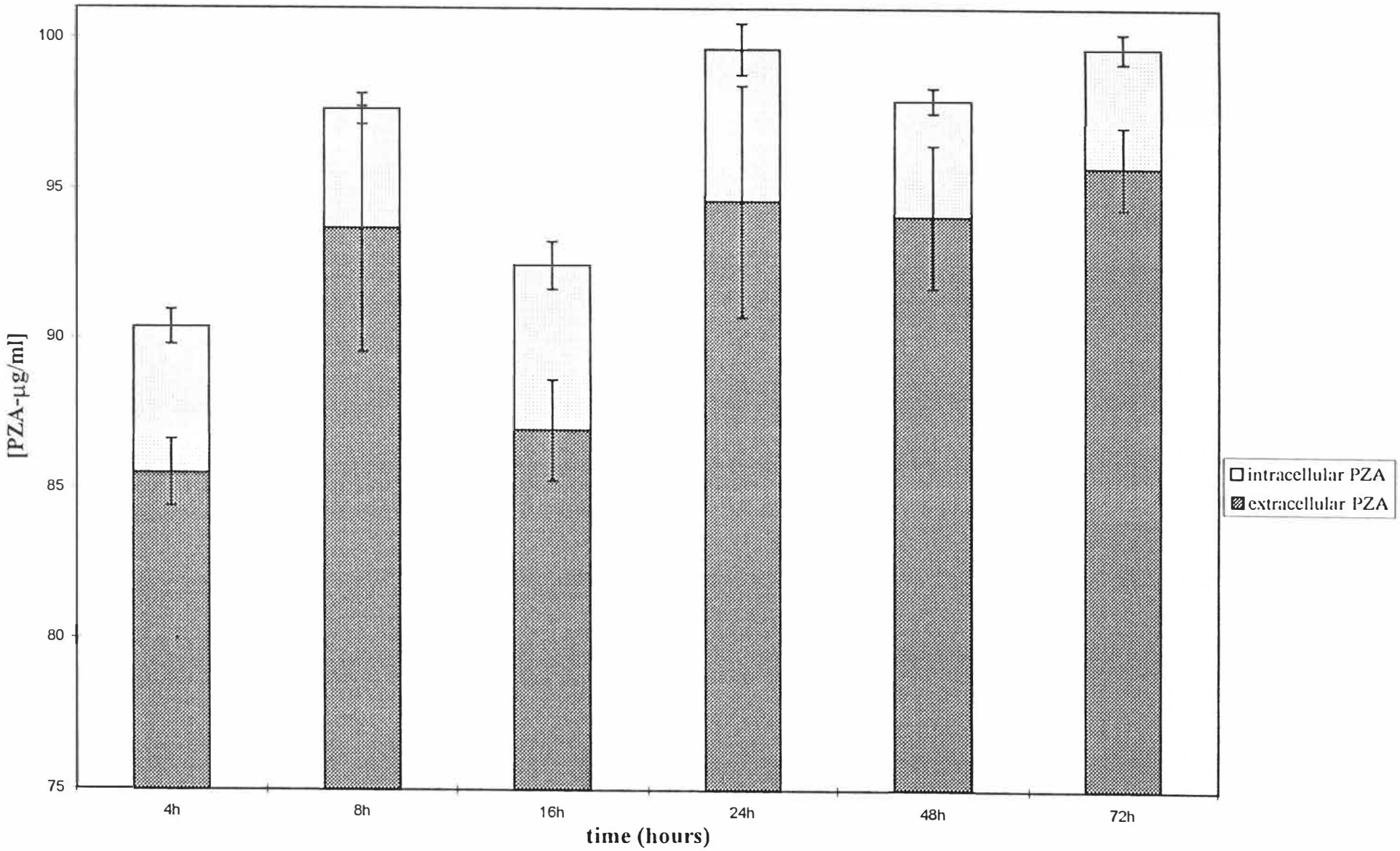


FIG. 4.20 Mean values of intracellular and extracellular concentrations of PZA in macrophages infected individually with 5 resistant *M. tuberculosis* strains. There is also minimal entry of PZA into the infected macrophages, indicating that the Pzase status of the intracellular mycobacteria does not promote or restrain its uptake significantly.

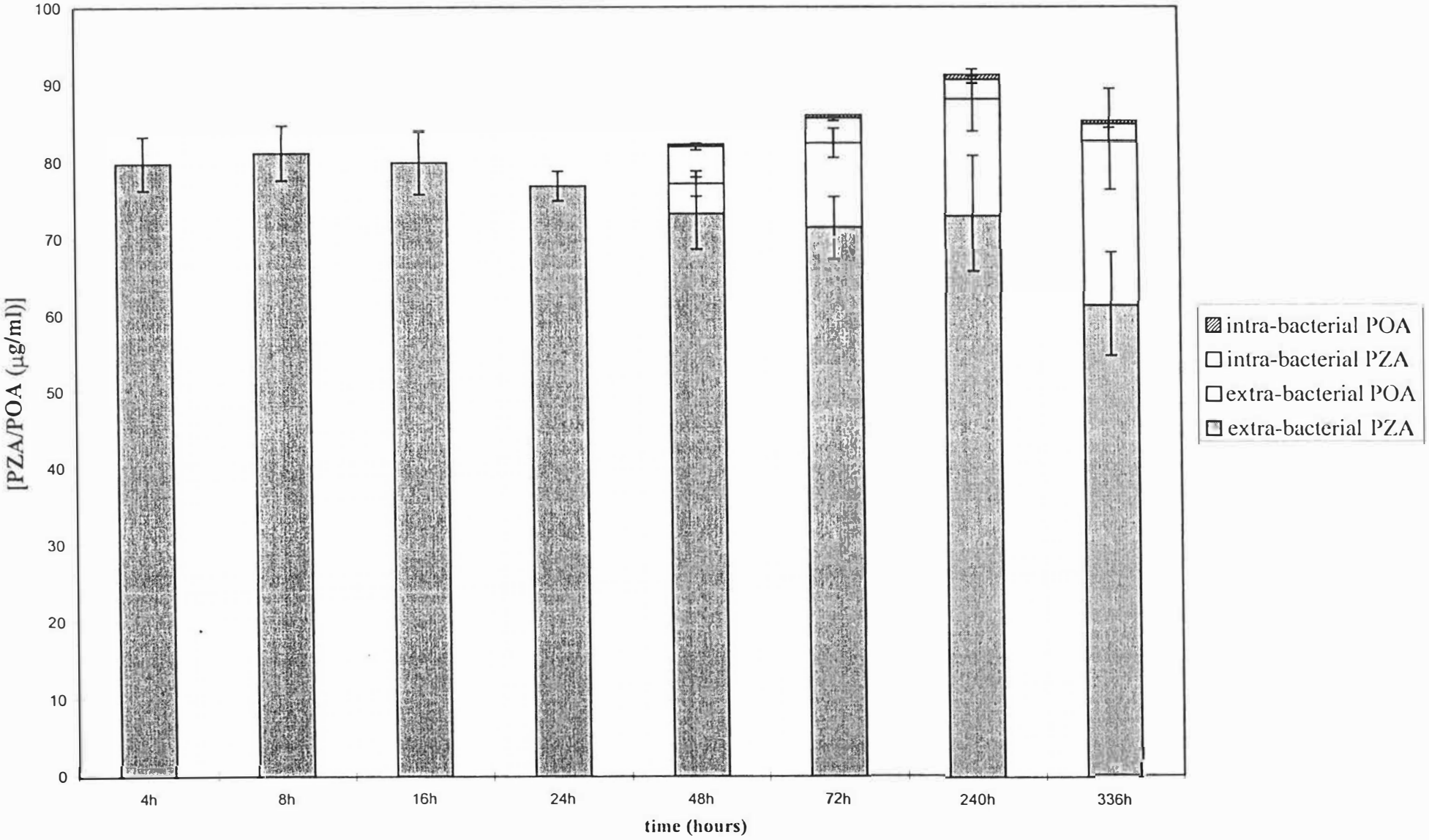


FIG. 4.21 Mean values of PZA and POA concentrations in Pzase positive *M. tuberculosis* cultures incubated in RPMI 1640 supplemented with PZA. PZA converted to POA was detected in higher concentrations in the extrabacterial fraction compared to the intrabacterial fraction. The highest concentration of PZA was detected in the extrabacterial fraction.

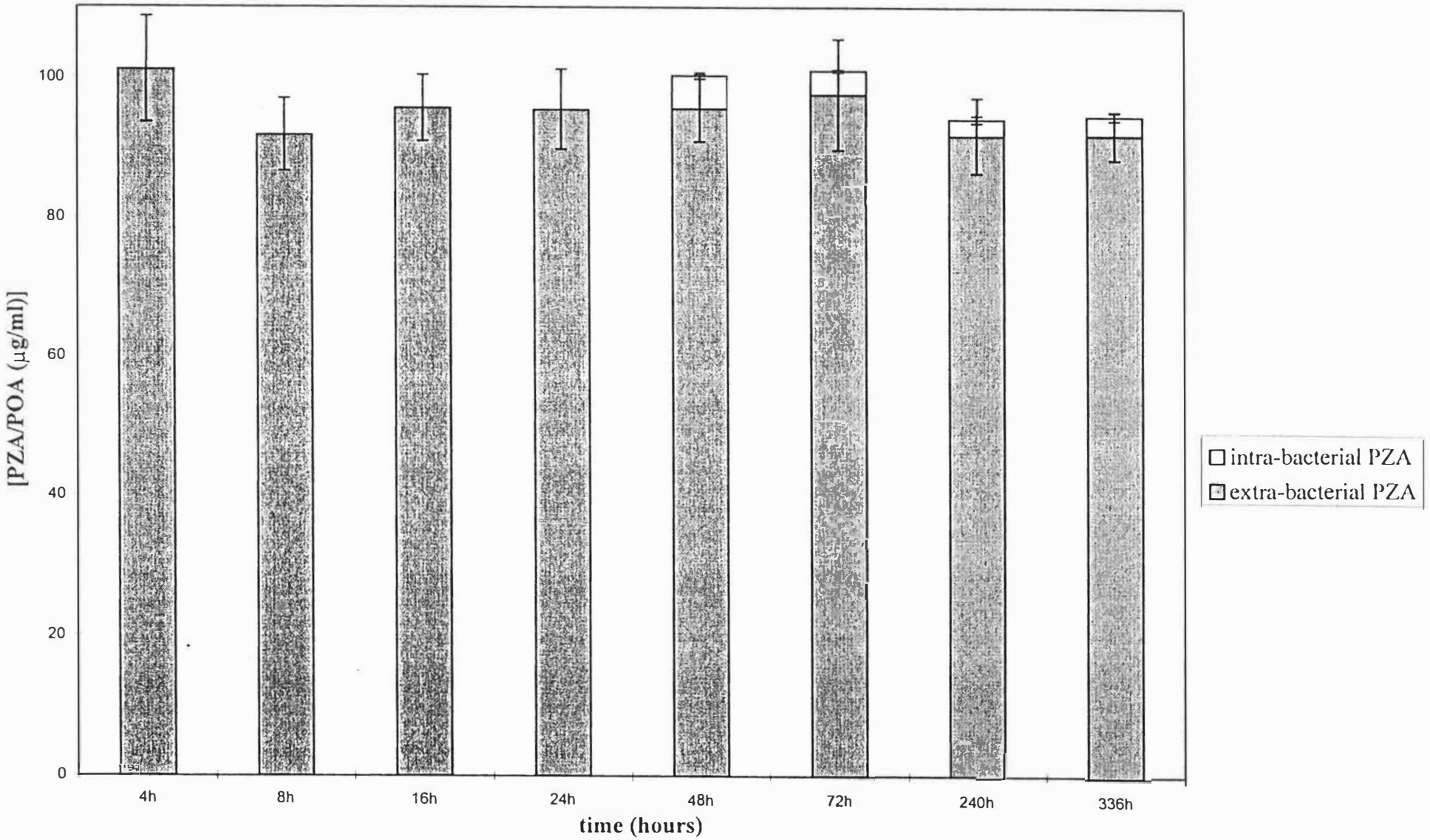


FIG. 4.22 Mean values of PZA concentrations for 5 Pzase negative *M. tuberculosis* strains incubated in RPMI 1640 supplemented with PZA. No POA was detected as these are PZA resistant strains. There was minimal entry of PZA into the intrabacterial fractions.

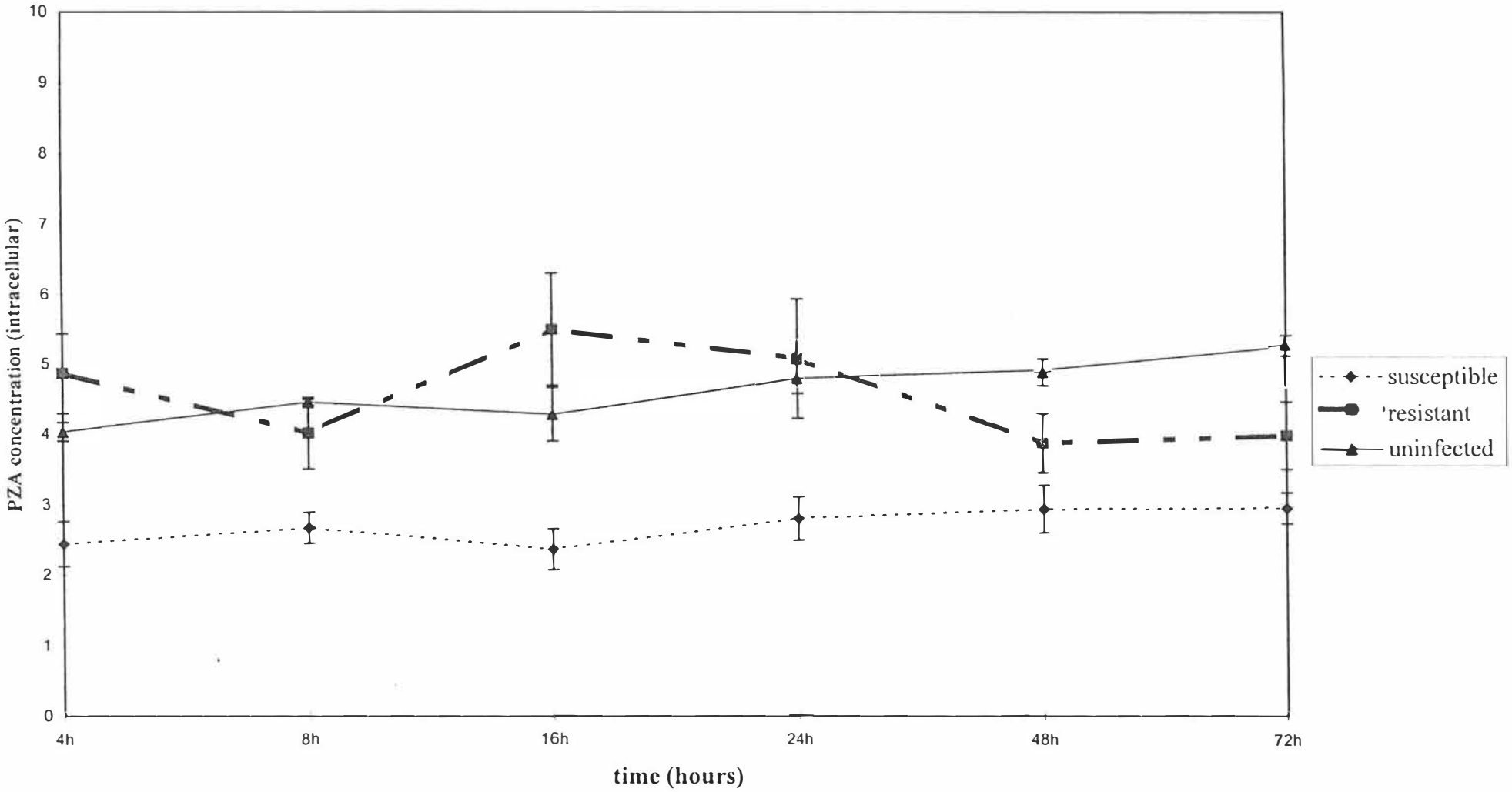


FIG. 4.23 Mean intracellular PZA concentrations in uninfected macrophages as well as in macrophages infected with susceptible and resistant strains. The macrophages infected with the resistant strains showed similar results to uninfected macrophages, while the macrophages infected with susceptible strains showed lower intracellular PZA concentrations.

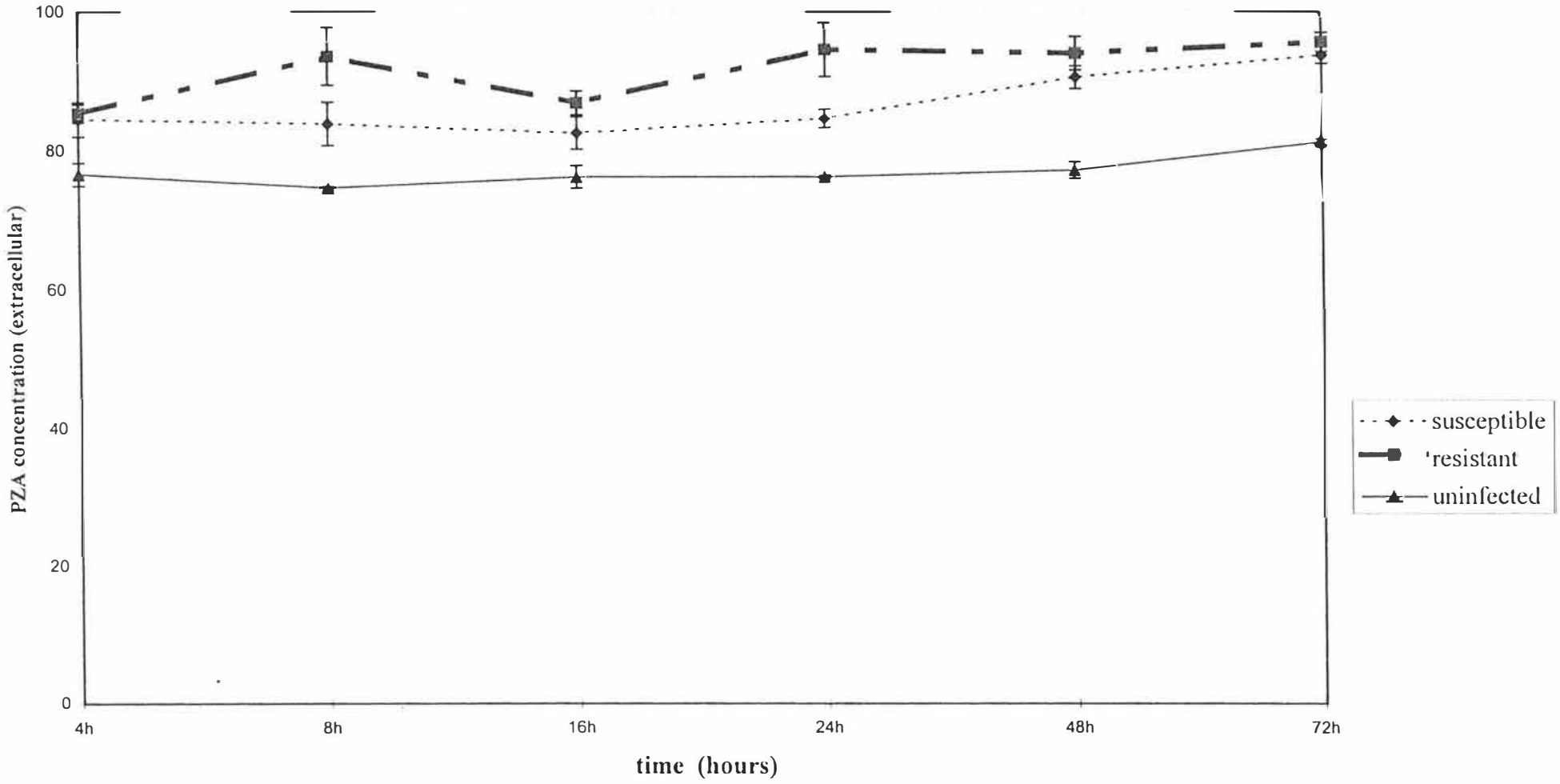


FIG. 4.24 Mean extracellular PZA concentrations in uninfected macrophages as well as in macrophages infected with susceptible and resistant strains. The macrophages infected with the resistant strains and susceptible strains showed similar results, while the uninfected macrophages showed lower extracellular PZA concentrations.

4.4 DISCUSSION

Once the HPLC methodology was established for the simultaneous detection of PZA and POA (chapter 3), 10 Pzase positive and 5 Pzase negative clinical isolates were investigated. This involved exposing the mycobacteria as non cell associated cultures in RPMI 1640 to 100 μg/ml PZA. Also, peripheral blood derived macrophages were infected with the mycobacterial strains and exposed to PZA. After incubating over a designated period, the various fractions were separated *i.e.*, extracellular, intracellular and intrabacterial fractions. Each fraction was quantified for PZA and POA in an effort to determine which fraction had the highest concentration of antibiotic.

Only a small fraction of the 100 μg/ml PZA to which the organisms were exposed entered into Pzase positive and negative mycobacteria grown in the absence of macrophages *i.e.*, non cell associated cultures (FIG. 4.2A-FIG. 4.18A). This would reflect the need for a high diffusion gradient or limited capacity of a transmembrane transport mechanism. POA was detected in minute quantities (0.67 μg/ml) in the intra bacterial fraction of Pzase positive strains. Most of the PZA and POA was found in the extrabacterial fraction of the non cell associated cultures. There are two explanations as to where the conversion of PZA to POA takes place, firstly PZA could be converted to POA by the mycobacterial Pzase in the extrabacterial fraction and secondly the conversion could take place in the intrabacterial fraction of the cultures after which POA diffuses back into the extracellular fraction. Raynaud *et al.* (1999) studied the localisation and distribution of Pzase in *M. tuberculosis* and other mycobacteria. These authors used whole cells as well as material extracted by gentle mechanical treatment to investigate Pzase localisation. Pzase activity, as tested by the Wayne's test, was not detectable in any of the surface exposed materials examined, whilst easily found in whole cells of *M. tuberculosis*. Thus, Raynaud *et al.* (1999) found that Pzase is not located on the surface of the mycobacteria examined but may be present in inner compartments of both PZA susceptible and resistant strains. Thus, for the experiments in this study, the conclusion is that PZA must diffuse into the mycobacterial cells to be converted to POA and thus the second explanation must be accepted.

Uninfected macrophages have the ability to take up PZA. A consistent amount of PZA is taken up by the macrophages over the 72h period studied (FIG. 4.1). Also, the extracellular PZA remains constant over this 72h period. No POA was detected in the extracellular or intracellular fraction of the uninfected macrophage. However, 20 μg/ml cannot be accounted for in the uninfected macrophages and could be complexed in internal compartments of the macrophages and thus undetectable. This could be as PZA or POA. Therefore, production of amidases that have the ability to convert intracellular PZA to POA, cannot be excluded.

There is also limited entry of PZA into macrophages infected with Pzase positive and negative strains (FIG. 4.2B-FIG. 4.18B). However, there is no POA detected in any of the fractions *i.e.*, extracellular, intracellular or intrabacterial in macrophages infected with Pzase positive or negative strains (FIG. 4.2B-FIG.4.12B). Thus, if PZA is converted to POA by Pzase positive strains, this POA is bound in some compartment of the macrophage or internalised mycobacteria, or the amount is too small to be detected by HPLC. With the reference strain, H37Rv, a total of approximately 80 μg/ml PZA is detected in the extracellular and intracellular fractions over the time period 4-72h studied. Thus, 20 μg/ml cannot be detected compared to clinical susceptible isolates where only a total of 5 μg/ml was undetected at 72h. Because the macrophages used were from the same source, this phenomenon seems to be strain dependent. H37Rv might have a more effective transport mechanism but it seems more likely that it has a increased capacity to bind PZA or POA. The amidase seems to play a role because no loss is seen with Pzase negative strains. Raynaud *et al.* (1999), showed that the uptake of PZA was by a ATP-dependent transport system. It was shown that one of the mechanisms of resistance to PZA resides in the failure of strains to take up the drug, which indicated that susceptibility to PZA in mycobacteria requires both the presence of functional Pzase and a PZA transport system. Thus, in this PhD study one could imply that although the clinical isolates have Pzase activity, the ATP-dependent transport system present in the naturally Pzase positive reference strain, H37Rv, is much superior than those found in the clinical isolates depending on the MICs of the isolates. Thus, macrophages infected with H37Rv are able to take up

much more PZA and this PZA stays bound in the intracellular compartments of the macrophages and mycobacteria. The clinical isolates may not have a PZA transport system that is as efficient as H37Rv.

Chan *et al.* (1986) used HPLC to determine PZA concentration in cerebrospinal fluid (CSF) and plasma in rabbits. The HPLC methodology differences have being discussed in chapter 3. PZA was extracted from CSF and plasma using a mixture of dichloromethane-diethyl ether (2:3). This extraction protocol was first used on samples used, however, the extraction procedure was not suitable for macrophages grown in RPMI 1640. No PZA or POA could be detected in RPMI 1640 spiked with PZA or POA. Thus, the extraction procedure was not used for any of the samples. At 0.25 h 20.9 μg/ml was recovered from plasma of rabbits after oral administration of 30 mg/kg. Over a 24 h period studied the concentration of PZA progressively decreased with 0.4 μg/ml been detected at 24 h. The absorption of PZA was very rapid, also the drug was widely distributed in body tissues as it was still detectable at 24 h (Chan *et al*, 1986). There was minimal entry of PZA into the macrophages even with a higher starting concentration of PZA. Therefore, this rapid distribution seems not to include the intra-macrophage compartment. The question if the limited intracellular amount is sufficient to provide antimicrobial effect remains to be answered.

In another study, Woo (1987) used HPLC to simultaneously determine PZA and rifampicin concentration in serum samples from patients with tuberculous meningitis. With a dose of PZA of 25-35 mg/kg body weight, 42.96 μg/ml PZA was detected in the serum of patients at 2 h and decreased over time to 27.4 μg/ml at 8 h. In this PhD study with infected macrophages, only a fraction of intracellular PZA could be detected even with an initial concentration of 100 μg/ml of PZA added to the macrophage-mycobacterium co-culture system. This does not exclude that this fraction is enough to exert antibacterial activity. There was no activity in the 72h period studied.

Yamamoto *et al.* (1987b) also used HPLC to determine PZA and its metabolites concentrations in human plasma. Initially plasma was spiked with various concentrations of PZA, POA, 5 hydroxypyrazinamide and 5 hydroxypyrazinoic acid, extracted and run on HPLC to determine percentage recovery. Good recovery was obtained after which a healthy volunteer was given 3 g of PZA and the volunteer's plasma tested for PZA and its metabolites. The highest concentration obtained was for PZA (67.60 μg/ml) followed by pyrazinoic acid (4.37 μg/ml) and 5-hydroxypyrazinamide (2.30 μg/ml). The PZA concentration of 67.60 μg/ml obtained is comparable to the extrabacterial PZA concentration of 61.48 μg/ml obtained at 336h for non cell associated mycobacteria. The pyrazinoic acid concentration of 4.37 μg/ml is comparable to the extrabacterial POA concentration obtained at 48h. Although 0.02 M KH_2PO_4 (pH 2.56) has been reported to resolve POA by HPLC (Yamamoto *et al.*, 1987a), the chromatograph obtained using this mobile phase was unsatisfactory. Thus, this mobile phase was not used. Although the reported extraction procedures (Chan *et al*, 1986, Woo, 1987, Yamamoto *et al*, 1987) gave good recovery of antibiotics in plasma, they were not suitable to efficiently recover PZA or POA from the *in vitro* system set up in my experiments. However, the silicone oil density gradient centrifugation technique used was very efficient to separate extracellular and intracellular antibiotic.

Walubo *et al.* (1994) also used HPLC to detect PZA, rifampicin and isoniazid in human plasma by HPLC. They also found good recovery of the antibiotics from the plasma. They did not evaluate the method for *in vitro* assays.

There is no reports on the *in vitro* assessment of PZA concentrations in non cell associated mycobacteria or on macrophages infected with mycobacteria. Thus, this study is unique in that intracellular, extracellular and intrabacterial PZA has been quantified in *in vitro* assays in an attempt to understand the mode of action of PZA. Most of the *in vitro* studies involving PZA have only assessed the effect on intracellular growth using colony forming units as discussed in chapter 2. All the *in*

vivo studies using HPLC to quantify PZA showed that the concentration of PZA *in vivo* is below 100 μg/ml. If the *in vivo* situation mimicks the *in vitro* situation where minute quantities of PZA was detected in infected macrophages exposed to 100 μg/ml PZA, this could suggest that the activity is mainly extracellular.

Composite graphs of the 10 PZA susceptible clinical isolates (FIG. 4.19) shows that there is an influx of PZA into the macrophages (mean values) from 4-16 h after which an efflux into the extracellular environment is observed. This observation is further enhanced by statistical analysis using the general linear models procedures where over the time period studied (72 h) the macrophages infected with susceptible strains show significant differences to uninfected macrophages at every time point ($p=0.0001$). The macrophages infected with susceptible strains are significantly different from those with resistant strains at each time point until 16h ($p=0.0005$), then the PZA concentration for the resistant strains decreases and the differences between macrophages infected with susceptible and resistant strains become insignificant at 48h ($p=0.12$). The mean intracellular PZA concentration remains almost constant throughout the time period studied. The PZA detected in macrophages infected with resistant strains compared to the uninfected macrophages was statistically not significant ($p=0.79$), while a significant difference was found when comparing the PZA concentration detected in macrophages infected with resistant strains compared to macrophages infected with susceptible strains ($p=0.001$) (FIG. 4.23). Also, a significant difference was found when macrophages infected with susceptible strains were compared to uninfected macrophages ($p=0.001$). This suggests that the macrophage has the ability to absorb only a certain percentage of antibiotic, thus once the macrophage is saturated the excess antibiotic is found in the extracellular environment.

The significant difference between intracellular PZA in macrophages infected with resistant and susceptible strains ($p=0.001$) implies that susceptible strains do enhance the uptake of PZA intracellularly, however, extracellular PZA concentrations in this

systems with both resistant and susceptible strains do not differ significantly (p=0.07). This suggests that the macrophage infected with susceptible bacteria has a better PZA transport system as shown by Raynaud *et al.* (1999) and has the ability to enhance uptake of PZA. Over the 72 h period studied the constant ratio of intracellular PZA concentration/extracellular PZA concentration is 0.03 suggests that uptake is by diffusion gradient across the macrophage membrane. The lower intracellular PZA concentration found in macrophages infected with susceptible strains compared to resistant strains and their statistically significant difference (p=0.001) suggest that a fraction of intracellular PZA is converted to POA. However, this POA is bound in cellular compartments of the bacteria or macrophage and cannot be detected. The significant difference between intracellular PZA in susceptible and uninfected macrophages (p=0.001) further suggest that intracellular PZA is being converted to POA which is bound in some cellular component. The intracellular PZA in uninfected macrophages does not differ significantly from that in macrophages infected with resistant strains (p=0.79), thus it can be suggested that infection of macrophages in itself does not enhance uptake of PZA but that a PZA transport system must be present.

Composite graphs of 5 PZA resistant strains (FIG. 4.20) show an influx of PZA (mean values) into the macrophages. However, a distinct pattern is not observed. Statistical analysis showed that the intracellular PZA concentration detected in macrophages infected with resistant strains does not differ significantly at each time period compared to uninfected macrophages (p=0.79). There is no time effect on the amount of PZA detected in the intracellular compartment of the uninfected macrophage or the macrophages infected with susceptible or resistant strains. The concentration of intracellular PZA remains fairly constant over the 72 h period studied. Since these are Pzase negative strains, the absence of POA could be expected. The total recovery of intracellular and extracellular PZA equals approximately 100 μg/ml at 72 h. Although there is an influx of PZA into the macrophage, there seems to be unrestrained binding of the antibiotic to cellular components (16 h), where the extracellular PZA concentration is lower. Thereafter,

(24-72 h) there is an efflux into the extracellular environment, with the extracellular PZA concentration increasing. This implies that two pump mechanisms operate. The first pump mechanism operates between the internalised mycobacteria and the cytoplasm of the macrophages. Once the PZA reaches saturation concentration within the internalised mycobacteria, the excess PZA is pumped across the mycobacterial membrane into the macrophage cytoplasm. The second pump mechanism exists between the macrophage cytoplasm and the extracellular environment. The PZA pumped out of the internalised mycobacteria into the cytoplasm is finally pumped out into the extracellular environment. The composite graph (FIG.4.19) showing the increase in the extracellular PZA concentration at 72 h supports the theory that pump mechanisms operates pumping excess PZA into the extracellular environment. The amount of PZA that can be retained within the macrophage cytoplasm is limiting.

The concentration of PZA in macrophages was lower for those infected with Pzase positive strains as compared to Pzase negative strains. Although POA was not detected, this finding suggest that this difference was the result of partial conversion by the enzyme producing organisms. The fact that POA was not detected could be because the concentration was below the detection limit of the HPLC methodology, (0.5 μg/ml), or because this forms complexes with other molecules in the bacteria or the macrophage. With the reference strain, H37Rv, the amount of undetectable drug (PZA or POA) was higher as compared to the susceptible clinical isolates. This might reflect the very high Pzase activity of this strain as well as the efficient PZA transport system. The fact the the conversion into POA did not result in a compensating influx of PZA into the macrophage, indicates that the POA still influences the concentration gradient. This goes against the hypothesis of complex formation. Another possibility is the accumulation in a biological structure.

Comparing the extracellular PZA concentrations statistically (FIG. 4.24) it was found that the uninfected macrophages behave significantly different from the macrophages infected with susceptible strains ($p=0.008$) and resistant strains ($p<0.0001$), however, there was no statistically significant difference between the extracellular PZA found in the experiments with macrophages infected with susceptible and resistant strains

(p=0.07). Over each time interval studied the difference between macrophages infected with susceptible strains and uninfected macrophages increase over time (p=0.0008). There is also significant difference between macrophages infected with resistant strains and uninfected macrophages at each time point (p=0.0001). There is no significant difference between extracellular PZA in susceptible and resistant strains over each time interval (p=0.08). It can be inferred that susceptible strains have a better PZA transport system, which allows for more PZA to be taken up by the infected macrophages. However, the efflux system into the extracellular environment is independent of the influx transport system. The efflux system pumps outs excess PZA once the macrophage is saturated irrespective of the Pzase status of the intracellular mycobacteria.

There is significant difference between the extracellular PZA in macrophages infected with resistant strains and uninfected macrophages ($p<0.0001$) and macrophages infected with susceptible strains and uninfected macrophages (p=0.008). The extracellular PZA concentration of uninfected macrophages is lowest followed by macrophages infected with susceptible and resistant strains. The inability to detect PZA or POA in intracellular mycobacteria may be explained by the following mathematically calculations. Consider the mycobacteria to be a cylinder and use the equation πr^2 x height to calculate its volume. Mycobacteria have a diameter of 0.2-0.6 μm and a length of 1.0-10 μm, mid range values were used to calculate the volume of mycobacteria. Thus the volume occupied by one mycobacterium is $\pi (0.2)^2 \times 5 = 0.628\ \mu m^3$. This volume is Y. Considering the macrophage to be a sphere the formula $4/3\ \pi r^3$ was used to calculate the total volume of macrophages with the diameter of macrophages being between 25-50 μm. Thus volume occupied by one macrophage equals $4/3\ \pi (17.5)^3 = 22449\ \mu m^3$. This volume is Z. Total volume of intracellular mycobacteria is equal to the average intracellular colony forming units x volume of one mycobacterium = $Y \times 5 \times 10^6$ CFU/ml = $3.14 \times 10^6\ \mu m^3$. Therefore the total volume of 10^5 macrophages planted equals $Z \times 10^5 = 2.24 \times 10^9\ \mu m^3$. Thus the ratio of volume of macrophage/volume of mycobacteria $R = Z \times 10^5 / Y \times 5 \times 10^6 = 2.24 \times 10^9 / 3.14 \times 10^6 = 713$. Thus, the intrabacterial PZA/POA is 713x dilute in the

macrophage volume and thus is too dilute to detect within the detection limits set by HPLC. If one assumes that the extrabacterial POA detected at 72h for non cell associated mycobacteria is the same for macrophage bound mycobacteria at the same time then the final concentration of POA in the macrophage lysate would be 10.81/713= 0.01 μg/ml which is below the detection limit set by HPLC.

Conclusions

* The new HPLC methodology developed was successfully used to quantify PZA and POA in non cell associated tuberculosis cultures as well as in macrophage bound cultures.

* A small fraction of PZA entered Pzase positive and negative mycobacteria grown in RPMI 1640, with most of the drug remaining in the extrabacterial fraction.

* Since Raynaud *et al.* (1999) showed that Pzase is located on the inner compartments of both PZA susceptible and resistant strains, it was concluded in this study that PZA must diffuse into the mycobacterial cells to be converted to POA, then diffuses into the extrabacterial environment. Thus, PZA and POA concentrations are higher in the extrabacterial fraction.

* Macrophages infected with Pzase positive and negative strains showed limited entry of PZA, with no POA being detected in any fraction.

* The reference strain, H37Rv, seems to have a more effective transport mechanism as 20 μg/ml PZA remained undetected at 72h compared to 5 μg/ml PZA undetected at the same time in clinical isolates.

* It was also shown by a mathematically model that the intrabacterial PZA/POA was diluted and thus could not be detected by HPLC.

* The significant difference between intracellular PZA in macrophages infected with resistant and susceptible strains ($p=0.001$) implies that susceptible strains do enhance the uptake of PZA intracellularly.

* There was no significant difference between the extracellular PZA found in the macrophages infected with susceptible and resistant strains ($p=0.07$). This suggests that the efflux system pumps out excess PZA once the macrophage is saturated irrespective of the Pzase status of the intracellular mycobacteria.

CHAPTER 5

RAISING POLYCLONAL ANTIBODIES TO PZA IN RABBITS

5.1 INTRODUCTION

The objective of this chapter is to give a detailed account of the production of antibodies to PZA which were used for immunogold labelling studies (chapter 5) to locate the site of action of PZA. Since there are no commercially available PZA antibodies that could have been used, we produced polyclonal antibodies to PZA in rabbits.

Antibodies can be generated by inoculation of an animal with any foreign protein. Molecules other than proteins are less immunogenic. The sera of immunised animals contain a mixture of antibodies produced by different lymphocytes, that react against multiple sites on the immunising antigen. Antibodies can be used in a variety of ways to detect proteins in cell extracts and can also be used to visualise proteins within cells, as well as in lysates (Cooper, 1997).

In the synthesis of drug-protein conjugate the choice of carrier molecule is important. Almost any immunogenic protein may be used as a carrier for an haptenically coupled drug. The functional groups in the carrier molecules to which the drugs may be conjugated are most frequently the amino, carboxyl or phenolic groups. The method of conjugation is also important. In the method selected for the conjugation of a drug to a carrier, the chemical conditions must entail minimal structural alterations of the hapten and must not cause sufficient denaturation of the carrier molecule to render it insoluble (Butler, Jr, 1978).

Large chemically complex molecules are good immunogens. The general rule for the production of antisera to a antigen is that the antigen has to be in pure form or antibodies will be produced to the contaminants as well, which may interfere in antibody assays. There are many examples of antisera being produced against single antigens linked to other molecules. Molecules of a molecular weight less than 1000 daltons (D) such as many drugs or hormones need to be linked to immunogenic carriers and immunisation may lead to production of high affinity antibodies to the drug (English, 1994).

There are many immunogenic carriers, including keyhole limpet haemocyanin (KLH), bovine serum albumin (BSA), egg albumin (ovalbumin) and fowl gamaglobulin (English, 1994). KLH is a carrier molecule that has good epitopes to activate T-helper cells which produce cytokines to activate antigen specific B-cells. This results in clonal proliferation and class switching resulting in good quality antibodies.

The methodology for polyclonal antibody production is relatively straight-forward. The animals are given a priming injection followed usually by a number of booster injections. Serum is collected throughout this process (test bleeds) to check the quantity of the antibodies produced and a final collection of a large volume of antiserum is made when the antibody level is satisfactory (English, 1994).

The preferred animals for production of polyclonal antibodies are large such as rabbits and sheep because of the quantity of serum that can be obtained. Mice and rats are good species for the production of antisera, however, the volumes of antisera collected from such animals are small. The animal of choice for most polyclonal antibody production is the rabbit. Most rabbits will provide a minimum of 100 ml of antiserum. However, because they are outbred animals, individual rabbits respond differently and it is always wise to include about 3 rabbits in the immunisation protocol (English, 1994).

In general, particulate antigens are good immunogens and can often be injected alone into the animals. For soluble materials and small molecules such as drugs, a much

improved response can be obtained by the use of adjuvants. These are non-specific stimulators of the immune response and have a two-fold effect. Firstly, they form a local deposit of the conjugated antigen which is slowly released to the immune system and secondly, they stimulate the action of antigen presenting cells and cause an inflammatory reaction at the site of injection. The most commonly used adjuvant, Freund's adjuvant, is an example of the former being a water-in-oil emulsion containing the immunogen. Freud's adjuvant is available in two forms: complete Freud's adjuvant (CFA) and incomplete Freud's adjuvant (IFA). CFA does include heat killed bacteria such as *Bordetella pertussis* or *M. tuberculosis*, which stimulate the production of different cytokines which promote the inflammatory reaction and the stimulation of antigen presenting cells. The antigen is incorporated in the aqueous phase of a stabilised water in paraffin oil emulsion for IFA (English, 1994).

The dose and route that the immunogen is administered to the animal varies considerably depending on the immunogenicity of the antigen. Usually between 50-1000 ug/ml of immunogen is administered to rabbits. In rabbits and larger animals large volumes of immunogen are best given subcutaneously. Animals are bled at 6 weeks and final bleeds from rabbits are done from the heart under terminal anaesthesia, a process known as exsanguination. The collected blood is allowed to stand at room temperature to clot. The clot is detached from the sides of the vessel and kept in the refrigerator overnight. During this period the clot retracts exuding the serum, which is separated from the clot by centrifugation. The serum can then be tested for antibodies produced (English, 1994).

5.2 MATERIAL AND METHODS

5.2.1 CONJUGATION OF PZA TO N-SUCCINIMIDYL 4-(N-MALEIMIDOMETHYL) CYCLOHEXANE-1-CARBOXYLATE

2 mg of PZA was dissolved in 300 µl of 0.1 M sodium phosphate buffer, pH 7.0. 5 mg of N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) was dissolved in 90 µl of N-N-dimethyl formamide (DMF). The SMCC solution was warmed at 30°C for 1-2 mins before adding to the PZA solution as this prevents precipitation of the reagent. The reagent solution was added to the PZA solution and the mixture incubated at 30°C for 30 mins with continuous stirring. The reaction mixture was centrifuged to remove excess precipitated reagent and the clear supernatant applied to a Sephadex G-25 column that was pre-equilibrated with 0.1 M sodium phosphate buffer, pH 6.0. 1 ml aliquots were collected and absorbance read at 280 nm. The aliquot that showed a high absorbance reading was the conjugate which was transferred to a collodion bag and concentrated in the cold.

5.2.2 CONJUGATION OF KEYHOLE LIMPET HAEMOCYANIN OR BOVINE SERUM ALBUMIN TO S-ACETYL MERCAPTOSUCCINIC ANHYDRIDE

2 mg bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH) was added to 900 µl of 0.1 M sodium phosphate buffer, pH 6.5. The BSA and KLH solutions were mixed with 25 X excess S-acetyl mercaptosuccinic anhydride (SAMSA) dissolved in 10 µl of DMF. The BSA-SAMSA and KLH-SAMSA solutions were incubated at 25°C for 30 mins. The following was then added to each of the solutions: 200 µl of Tris-HCl buffer, pH 7.0, 20 µl of 0.1 M EDTA, pH 7.0 and 200 µl of 1 M hydroxylamine, pH 7.0 and incubated at 30°C for 30 mins. Each of the mixtures were applied to a Sephadex G25 column that was pre-equilibrated with 0.1 M sodium phosphate buffer, pH 6.0. 1 ml aliquots were collected and absorbance measured at

280 nm. The aliquot that showed a high absorbance reading was the conjugate. This fraction was the transferred to a collodion bag and concentrated in the cold.

5.2.3 CONJUGATION OF PZA-SMCC TO KLH-SAMSA OR BSA-SAMSA

The PZA-SMCC mixture was added to the KLH-SAMSA or the BSA-SAMSA mixture in a 1:1 ratio. The PZA-SMCC-SAMSA-KLH conjugate was inoculated into 3 rabbits for production of PZA antibodies. The PZA-SMCC-SAMSA-BSA conjugate was used to coat enzyme linked immunosorbent assay (ELISA) plates to determine the specificity of the PZA antibodies formed. A schematic representation of the conjugation procedure is illustrated in FIG. 5.1.

5.2.4 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO DETERMINE SPECIFICITY OF PZA ANTIBODIES RAISED

After 6 weeks the rabbits were bled. The ELISA assay was carried out on the pre-bleed and test bleed serum to determine if antibodies to PZA were produced in the rabbits. The ELISA plates were coated with various dilutions of the PZA-SMCC-SAMSA-BSA conjugate in coating buffer (0.5 M Tris-Cl, 0.1 M NaCl, pH 9.6) and incubated at room temperature overnight. ELISA plates were washed with 500 ml Tris-saline buffer supplemented with tween 20 (TST). The plates were centrifuged in an inverted position to remove all wash buffer. 50 µl of the pre-bleed and test bleed serum was added to each well and the plate incubate at 42°C for 1h. The serum was diluted in 5 % lactogen milk powder in Tris-saline buffer. After 1 h the plate was washed with 500 ml TST and dried as described above. 20 µl sheep anti-rabbit IgG diluted in lactogen (1/100) were added to the wells and incubated for 0.5 h at 42°C. 50 µl of alkaline phosphatase was added to each well and the plate incubated in a dark drawer for 0.5 h after which the reaction was stopped with 100 µl of sulphuric acid. ELISA reactions were read with an ELISA reader with the measuring filter at 492 nm and the reference filter at 620 nm.

5.2.5 PURIFICATION OF ANTISERA

Solid Na_2S04 was added slowly to the antiserum at a final concentration of 14 % and mixed on a magnetic stirrer for 15 mins. The solution was centrifuged and transferred to dialysis tubing and dialysed at 4°C against distilled water. The dialysate was passed through a protein A column. Fractions were collected and absorbance measurements were read at 280 nm. The antibodies were in the fractions that gave high absorbance readings at 280 nm. These tubes were pooled and dialysed in the cold against Tris-saline buffer, pH 8.0. ELISA was repeated to confirm that PZA antibodies were present in the pooled fractions. Once this was confirmed the antibodies were stored at -20°C for use in immunogold labelling experiments. The highest yield of PZA antibodies was obtained from rabbit 2814, this purified serum was used in all immunogold labelling experiments.

5.3 RESULTS

PZA was conjugated to a carrier molecule KLH and used as inoculum to inoculate rabbits. FIG. 5.1. illustrates the conjugation procedure schematically. ELISA was performed on pre-bleed and test bleed samples to determine if PZA antibodies were being produced (TABLE 5.1.). Sera was purified before use using a protein A column (TABLE 5.2.). A second ELISA was performed to confirm that PZA activity was maintained (TABLE 5.3.). The purified antibodies were used in all immunogold labeling experiments as detailed in chapter 6.

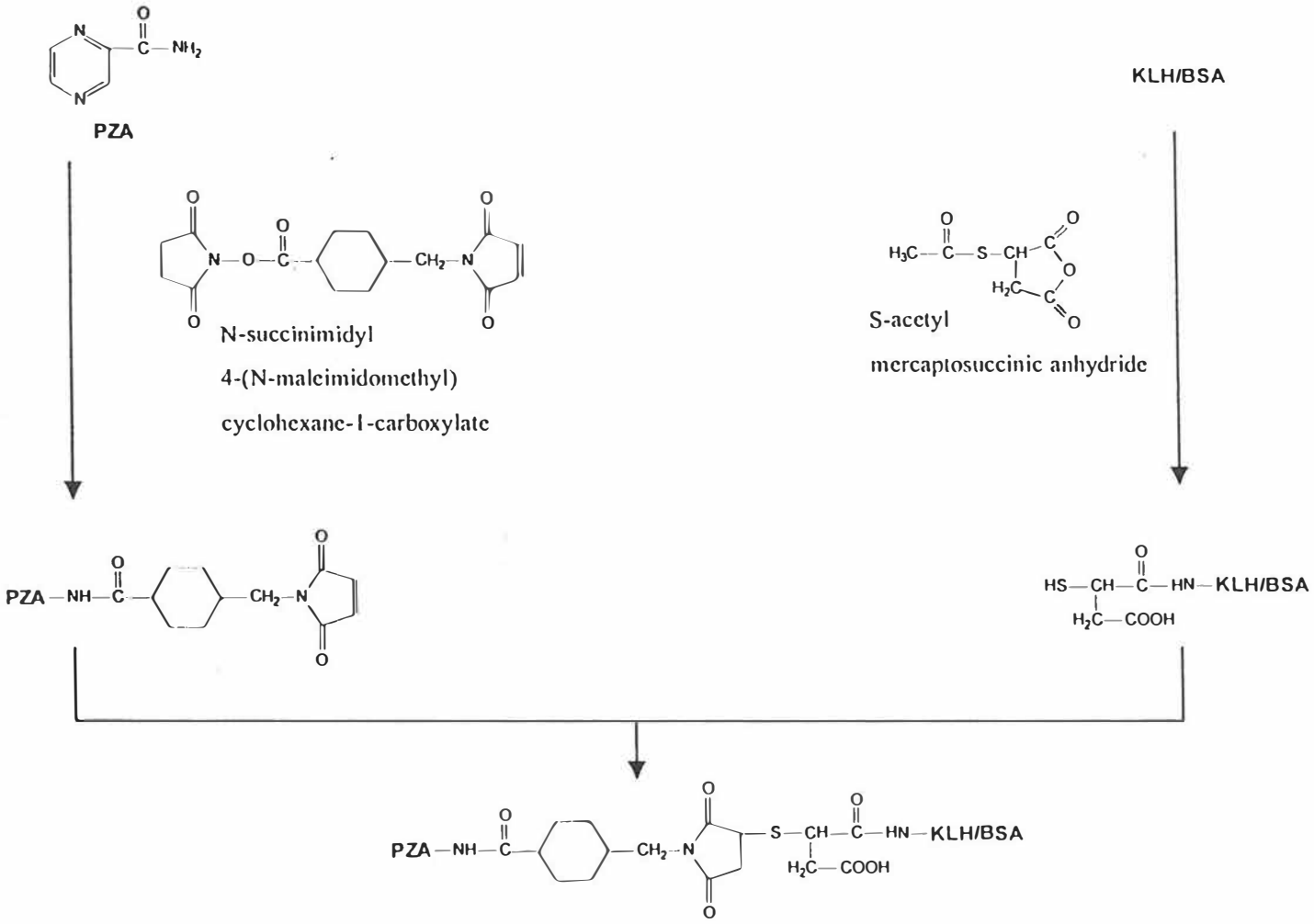


FIG. 5.1 Schematic representation of the conjugation of PZA to KLH or BSA

TABLE 5.1. Pre-bleed and test-bleed serum from 3 rabbits tested for PZA antibodies by ELISA.

Prebleed							Test bleed						
coating buffer dilutions	neat serum	neat serum	1/20	1/20	1/100	1/100	neat serum	neat serum	1/20	1/20	1/100	1/100	Rabbit number
1/20	0.241	0.289	0.052	0.053	0.074	0.094	**2.737**	**2.443**	**2.489**	**2.498**	**2.066**	**2.153**	**2814**
1/100	0.333	0.295	0.061	0.098	0.089	0.111	**2.083**	**2.350**	**2.041**	**1.773**	**1.104**	**0.954**	**2814**
1/20	0.275	0.229	0.072	0.099	0.103	0.104	2.181	2.214	1.313	1.395	0.498	0.531	2815
1/100	0.273	0.242	0.117	0.078	0.091	0.081	1.669	1.724	0.508	0.526	0.201	0.220	2815
1/20	0.209	0.288	0.071	0.085	0.066	0.132	2.318	2.423	1.995	2.153	1.195	1.226	2816
1/100	0.289	0.143	0.076	0.093	0.055	0.077	1.881	1.878	1.086	1.148	0.606	0.467	2816

TABLE 5.2. Purification of PZA antibodies by passage through a Prosep protein A column.

Key: Underlined: unbound antibodies Bold: Bound PZA antibodies

Test Tube Number	Absorbance	Test tube Number	Absorbance
1	-.007	21	0.018
2	-	22	-
3	0.093	23	0.014
4	-	24	-
5	1.876 (unbound)	25	0.016
6	1.841 (unbound)	26	-
7	1.112 (unbound)	27	0.015
8	0.370	28	-
9	0.123	29	0.039
10	-	**30**	**1.445 (IgG-PZA)**
11	0.038	**31**	**2.950 (IgG-PZA)**
12	-	**32**	**2.661 (IgG-PZA)**
13	0.032	**33**	**1.337 (IgG-PZA)**
14	-	34	0.568
15	0.027	35	0.291
16	-	36	0.177
17	0.023	37	0.121
18	-	38	0.091
19	0.019	39	0.072
20	-	40	0.062

TABLE 5.3. ELISA absorbance readings indicating the fractions that contain the IgG-PZA antibodies.

COATING BUFFER DILUTIONS	UNBOUND PZA ANTIBODIES				IgG-PZA ANTIBODIES			
	neat serum	1/20	1/40	1/100	neat serum	1/20	1/40	1/100
1/100	0.380	0.008	0.015	0.006	2.750	1.037	0.755	0.629
1/100	0.359	0.009	0.009	0.009	2.835	1.158	0.712	0.585
1/100	0.329	0.008	0.008	0.011	2.784	1.136	0.942	0.649

5.4 DISCUSSION

After HPLC analysis of intracellular, extracellular and intra-bacterial concentrations of PZA and POA was assessed an attempt was made to further quantify PZA activity by immunocytochemistry. Since there are no commercially available gold labelled PZA antibodies, theses antibodies had to be raised.

The structure of PZA and POA are very similar, thus we could not raise antibodies that would distinguish between them. PZA was conjugated to KLH and used as inoculum to inject rabbits that were used to raise PZA antibodies. Because PZA is a very small molecule, it would not serve as a good immunogen on its own. Therefore, a carrier molecule was used. PZA conjugated to BSA was used to coat the ELISA plates. Thus, if antibodies were produced against KLH instead of PZA, the ELISA would be negative as the ELISA plates have BSA instead of KLH attached to PZA. A positive ELISA would indicate that antibodies produced are indeed against PZA only.

ELISA was carried out on pre-bleed and test bleed samples of 3 inoculated rabbits Coating buffer as well as serum was diluted to obtain good absorbance readings and served as a technically necessity. The ELISA readings for the pre-bleed samples were low over the entire dilution range, thus indicating that there was no interfering substances present in the serum prior to inoculating the PZA-KLH conjugate. ELISA readings obtained for the test bleeds indicated that PZA antibodies were produced in the rabbits. Sera obtained from rabbit 2814 gave the highest absorbance readings and was used for all further experiments (TABLE 5.1).

The antibodies were a crude extract, thus they had to be purified. Purification involved passing the antibody solution through a Prosep protein A column (TABLE 5.2) and collection of 1 ml aliquots. The absorbance was read on every alternate aliquot until a high absorbance reading was obtained, then every aliquot was read. There were 2 sets of aliquots that showed high absorbance readings. These aliquots were pooled and ELISA repeated to determine which pooled aliquot had the PZA antibodies.

The second ELISA was carried out on the 2 sets of pooled aliquots (TABLE 5.3). the pooled aliquot that was eluted first contained the non-specific antibodies. The aliquot (test tubes 30-33) eluted second contained the purified PZA antibodies.

The purified PZA antibodies were stored in small aliquots at -20^{0}C. This was used for immunogold labelling experiments as discussed in detail in chapter 6.

CHAPTER 6

AN IMMUNOCYTOCHEMISTRY PERSPECTIVE ON THE MODE OF ACTION OF PZA

6.1 INTRODUCTION

The objective of this chapter is to show by means of immunogold labelled PZA antibodies and transmission electron microscopy (TEM) the exact location of PZA activity in infected macrophages. Since PZA antibodies are commercially unavailable, these antibodies were raised in rabbits as described in detail in chapter 5. The structure of PZA and POA are very identical, thus the antibodies raised do not give distinction between PZA and POA.

TEM differs markedly and in many respects from the optical microscopic techniques. TEM provides very high levels of magnification because of the much higher resolution obtainable with the extremely short wavelength of the electron beam used to magnify the specimen. With TEM employing 60-80 kV electrons the wavelength is only 0.05 angstrom. The resolving power of the TEM is more than 100 times that of the light microscope. For TEM, specimens to be examined must be prepared in extremely thin dry film on grids. The grids are introduced into the instrument at a point between the magnetic condenser and the magnetic objective. To make observations of intracellular structures, the specimen must be extremely thin. An intact microbial cell is too thick to allow distinct visualisation of its internal fine structure. Techniques are available for bacterial cells to be embedded in resin and ultrathin sections to be cut. The sections reveal cells at different levels and at different angles (Pelczar *et al*, 1986).

In 1971, Faulk and Taylor introduced their "immunocolloid" method for the study of cell surface antigens. Specific antibodies adsorbed to colloidal gold particles were used to visualise *Salmonella* antigens by TEM. Since then a large number of studies have documented the potential of colloidal gold as a marker for immunocyto-chemistry. Colloidal gold particles are particularly interesting as markers for immunocytochemistry because they can be used both at high and low resolution levels. Due to their electron dense properties, they are easily detected by TEM. The specificity and sensitivity of an immunocytochemical technique is limited by the specific activity of the antibodies used in the different steps of the procedures (Waele *et al*, 1983).

6.2 MATERIALS AND METHODS

6.2.1 LOCATION OF PZA/POA IN NON CELL ASSOCIATED MYCOBACTERIA

A McFarland No 1 standard of the reference strain, H37Rv, as well as all PZA susceptible and resistant clinical mycobacterial isolates was exposed to 100 μg/ml PZA in RPMI 1640 tissue culture medium. After exposure for 24 h, the mycobacterial isolates were fixed with 2.5 % glutaraldehyde (made in RPMI 1640 medium) at 4°C overnight. The bacteria were then processed for transmission electron microscopy.

6.2.2 LOCATION OF PZA/POA IN MACROPHAGES INFECTED WITH MYCOBACTERIA

Macrophages were exposed to the mycobacteria in the ratio 1:10 for 1 h, after which all unbound mycobacteria were washed off. Fresh RPMI 1640 supplemented with 100 μg/ml PZA was added to the petri dishes. After exposure to PZA for 24 h, the infected macrophages were fixed with 2.5 % glutaraldehyde (made in RPMI 1640

medium) at 4°C overnight. The infected cells were gently scraped off 3 petri dishes using Sterilin quadloops, pooled and processed for TEM.

6.2.3 PREPARATION OF SAMPLES FOR TEM

The macrophage infected cells as well as the free mycobacterial isolates were centrifuged, resuspended in 1 ml of glutaraldehyde and transferred to an eppendorf tube. The cells were washed twice with cacodylate buffer, pH 7.2. The pellets were dehydrated for 10 mins each through alcohol in an ascending concentration (70 %, 90 %, 3X100 %). The cells were not subject to osmium tetroxide treatment as this would mask the results of immunogold labelling experiments. A final dehydration was carried out with propylene oxide for 20 mins. At each of the dehydration step the pellets were resuspended in the alcohol. After dehydration cells were infiltrated stepwise with Spurr resin in propylene oxide. This was achieved by suspending the cells first in 25 % resin for 1 h, 50% resin overnight, 75 % resin for 6 h and finally 100 % for 1 h. All steps took place at room temperature apart from the last one during which the cells were incubated at 60 ^{0}C. At each infiltration step the pellet was carefully resuspended to ensure maximum penetration of the resin into the cells. The pellets were then embedded in 100 % resin, centrifuged to allow all the cells to form a pellet and cured at 60°C for 24 h.

6.2.4 IMMUNOGOLD LABELLING OF SECTIONS

Sections of 50-60 nm in thickness were cut using a Sorvall Ultramicrotome MT 5000 with glass knives. Nickel grids with sections were placed onto 40 µl droplets of 5 % H_2O_2 for 3 mins. The grids were washed with distilled water, dried and placed on 40 µl droplets of commercially available blocking agent for 30 mins in a humidity chamber. The blocking agent was drained, the grids placed onto PZA antibody (raised in rabbits-chapter 4) solution at a dilution of 1/75 and incubated at room temperature in a humidity chamber for 3 h. Thereafter, the grids were washed thoroughly with 3

% bovine serum albumin (BSA) in phosphate buffered saline (PBS), dried and placed onto 40 µl droplets of 1/75 dilution of 10 nm gold labelled anti rabbit IgG, which was incubated for 1 h at room temperature in a humidity chamber (Beatty *et al.*, 1994). The grids were washed with 3 % BSA in PBS, followed by washes in PBS, then distilled water. This was following by uranyl acetate staining (Appendix II). For this, the grids were placed onto 40 µl droplets of 2 % uranyl acetate for 4 mins, after washing with distilled water, they were dried on filter paper and counterstained using Reynolds lead citrate (Appendix II) for 4 mins. The grids were again washed with distilled water dried, on filter paper and stored in eppendorf tubes until viewed using a Jeol 1010 TEM.

6.3 RESULTS

Uninfected macrophages as they appear prior to infection are showed in FIG. 6.1 (A-D). Micrographs of the infected macrophages are shown in FIG. 6.2, A shows infection at low power, B a single internalised mycobacterium at high power and C a phagosome at high power. Controls treated with PBS instead of the PZA antibodies are indicated in FIG 6.3 (A-C). This shows that no non-specific binding occurred as no gold label is observed in any of the 3 regions of randomly selected areas of the macrophage. FIG 6.4-6.20 show the TEM micrographs of all the *M. tuberculosis* strains used. The arrows indicate gold label. FIG 6.4-6.14 (A-D) are micrographs showing PZA gold label distribution in macrophages infected with susceptible *M. tuberculosis* strains, while FIG. 6.4-6.14 (E, F) show the distribution of PZA gold label in non-cell associated free susceptible mycobacteria. PZA gold label distribution in macrophages infected with resistant strains are showed in FIG. 6.15-6.20 (A-D), while FIG. 6.15-6.20 (E, F) show gold label distribution in non cell associated free resistant mycobacteria.

Analysis of gold label in the different compartments of the infected macrophages as well as in free mycobacteria were carried out using the Zeiss Kontron 300 Image Analyser. The calculation methodology is indicated in Appendix 4. The mean values

of gold label as calculated for susceptible and resistant strains are represented graphically FIG. 6.21-6.25. FIG. 6.21 gives a graphical representation of PZA gold label distribution in free susceptible and resistant *M. tuberculosis* strains. The most gold label is located in free susceptible bacteria followed by free resistant bacteria. FIG. 6.22, 6.23 and 6.24 shows the distribution of PZA gold label in the cytoplasm, membrane bound mycobacteria and nuclei, respectively, of macrophages infected with susceptible and resistant *M. tuberculosis* strains. Distribution of gold label is higher in the cytoplasm (FIG. 6.22) and membrane bound mycobacteria (FIG. 6.23) for macrophages infected with susceptible strains of *M. tuberculosis* compared to resistant strains. However, more gold label is located in the nucleus of macrophages infected with resistant strains compared to macrophages infected with susceptible strains (FIG. 6.24). No gold label was located in lysosomes (bacteria free vesicles) in macrophages infected with susceptible or resistant strains. FIG. 6.25 is a composite representation of the PZA gold label in the different components of the infected macrophages.

The immunogold labelling results were analysed statistically to assess whether the differences found were significant. A non-parametric test was used because the number of particles/ area was not evenly distributed. The test used was the Wilcoxon rank sum test. Non cell associated free susceptible mycobacteria showed the most accumulation of gold, followed by free resistant strains (FIG. 6.21). Comparisons were made between resistant and susceptible strains for each compartment of the macrophage. There is a slight graphical difference between the gold label found in macrophage bound susceptible and resistant strains (FIG. 6.23), however, statistical analysis showed that there was no significant difference ($p=0.23$). Infection of the macrophage with Pzase positive mycobacteria does not seem to drastically increase the accumulation of PZA by internalised mycobacteria.

The amount of label detected in the cytoplasm of macrophages infected with susceptible strains is higher than when infected with resistant strains (FIG. 6.22). This difference is statistically significant ($p=0.001$). The amount of label in the nucleus of macrophages infected with susceptible strains is lower than the nucleus of

macrophages infected with resistant strains by graphically representation, (FIG. 6.24), however, statistically this difference is not significant ($p=0.07$).

To compare significant differences among the different compartments of macrophages infected with susceptible and resistant strains as well as with free mycobacteria, the Kruskal-Wallis test (a non-parametric test) with adjustment for multiple comparisons were used. The overall comparison showed statistically significant differences among the compartments for both susceptible and resistant strains. Pairwise comparisons were made to identify more specifically where the differences could be found. A significant value of $p<0.007$ was used to adjust for multiple comparisons. The composite graph (FIG 6. 25) gives a clear indication of the distribution of PZA in all the different components of the macrophages. It is clear that the most label is present in the mycobacteria with the free mycobacteria (FIG. 6.21) having more label than the macrophage bound mycobacteria (FIG. 6.23), followed by the cytoplasm and nucleus.

The gold label located over the free susceptible mycobacteria was significantly different from the cytoplasm, nucleus and membrane bound mycobacteria of macrophages ($p<0.0001$). Within the compartments of the macrophages infected with susceptible mycobacteria, the gold label located over the membrane bound mycobacteria is significantly different from the gold label located in the cytoplasm and nucleus ($p<0.0001$). However, the gold label located in the nucleus and cytoplasm of these macrophages is not significantly different. The gold label located over free resistant mycobacteria is significantly different from the gold label located in the cytoplasm, nucleus and membrane bound mycobacteria in macrophages ($p<0.0001$). The gold label located over membrane bound mycobacteria in macrophages infected with resistant mycobacteria is significantly different from the cytoplasm ($p<0.0009$) but it is not significantly different from the nucleus.

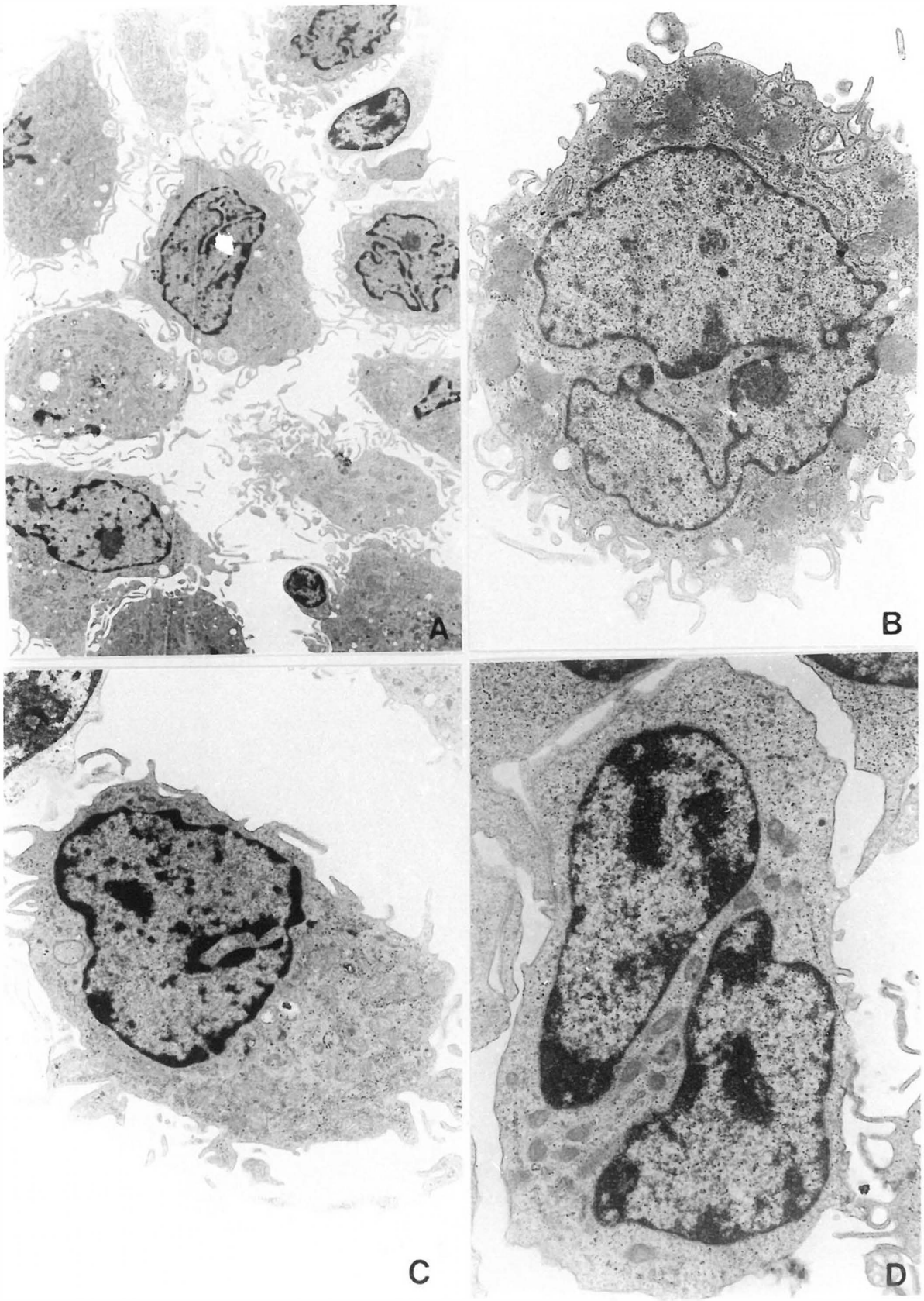

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FIG. 6.1 Micrographs of uninfected macrophages prior to infection with the different *M. tuberculosis* strains, (A) 2.3K, (B) 7K, (C) 6.5K, (D) 11K.

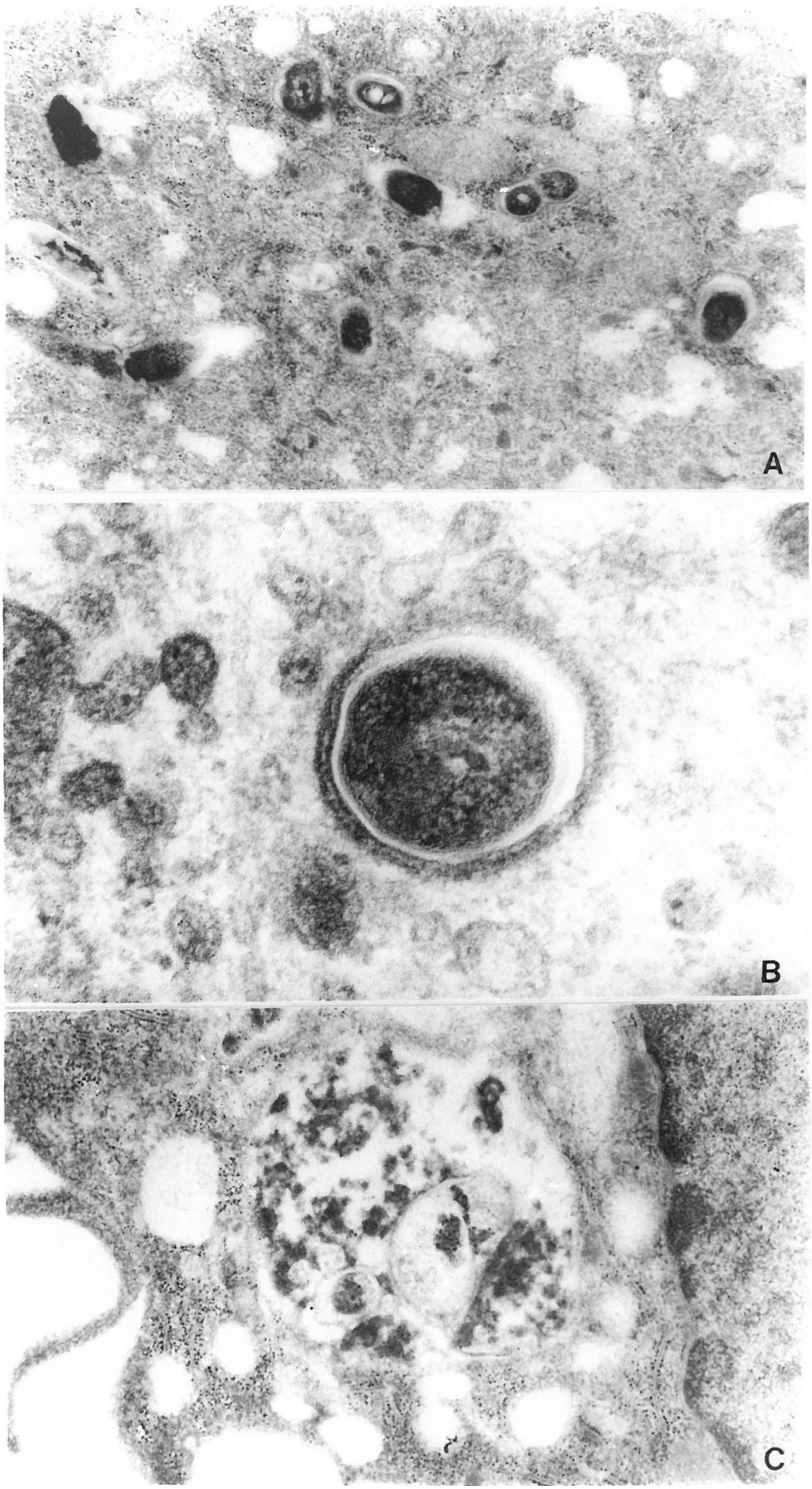
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FIG. 6.2 (A) Infection of macrophage with *M. tuberculosis* as observed at low power 2.8K, (B) internalised *M. tuberculosis* at high power 140K, (C) vesicle bound mycobacteria 30K.

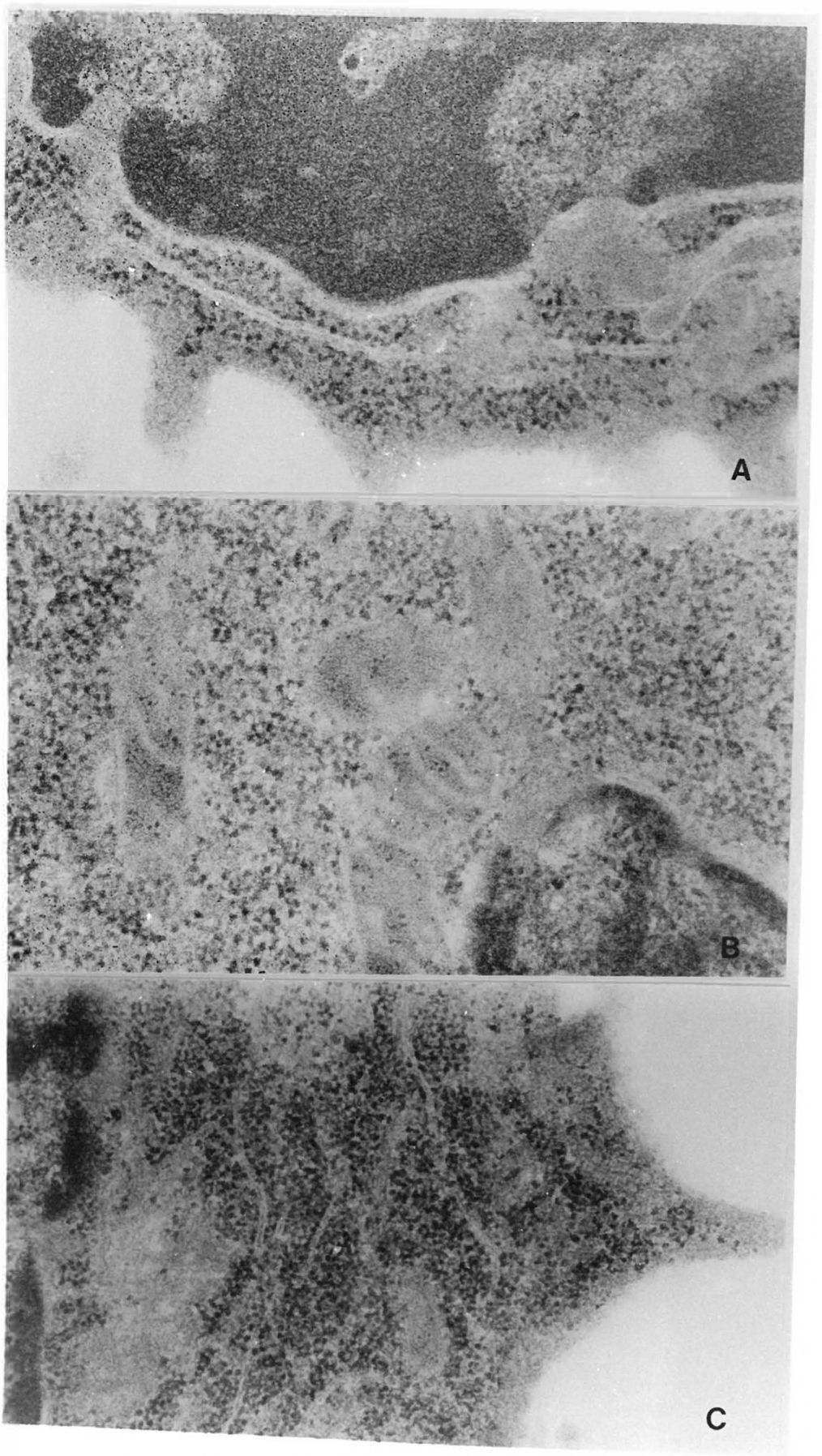
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FIG. 6.3. Random areas of macrophages to indicate method controls incubated with PBS instead of PZA antibodies. No non-specific label is observed (A) 51K, (B) 46K, (C) 55K.

FIG. 6.4. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain H37Rv: (A) nucleus [44K], (B) cytoplasm with membrane bound mycobacteria [48K], (C) internalised mycobacterium [50K], (D) membrane bound mycobacteria [49K] and (E, F) non cell associated H37Rv, (E) 80K and (F) 55K.

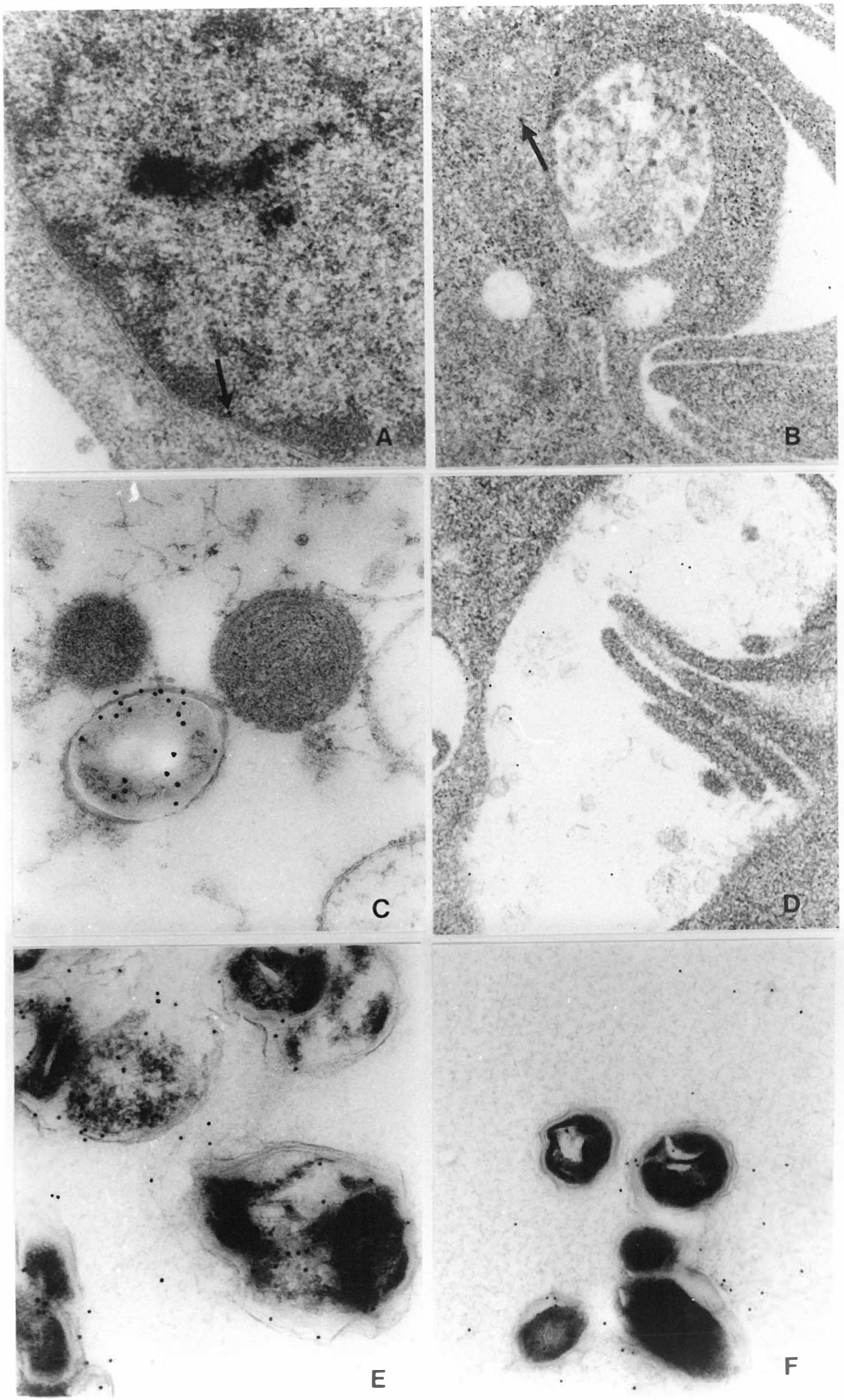
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FIG. 6.5. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 200R: (A) nucleus and cytoplasm [52K], (B) membrane bound mycobacteria [83K], (C) internalised mycobacteria and cytoplasm [48K], (D) internalised mycobacteria and cytoplasm [43K] and (E, F) non cell associated 200R, (E) 51K and (F) 46K.

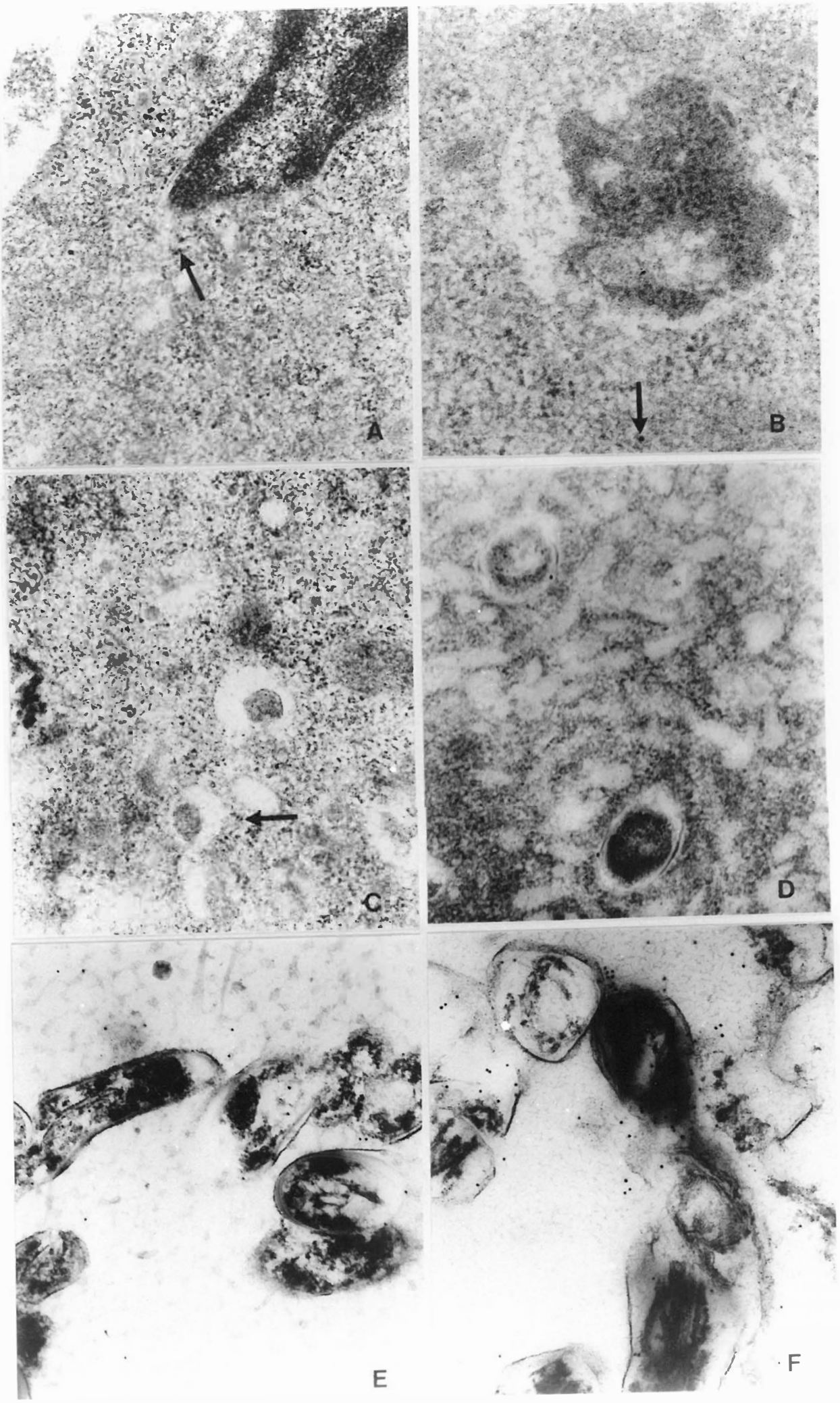
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FIG. 6.6. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 100R: (A) nucleus [45K], (B) membrane bound mycobacteria [47K], (C) cytoplasm [49K], (D) internalised mycobacterium in cytoplasm [68K] and (E, F) non cell associated 100R, (E) 60K and (F) 84K.

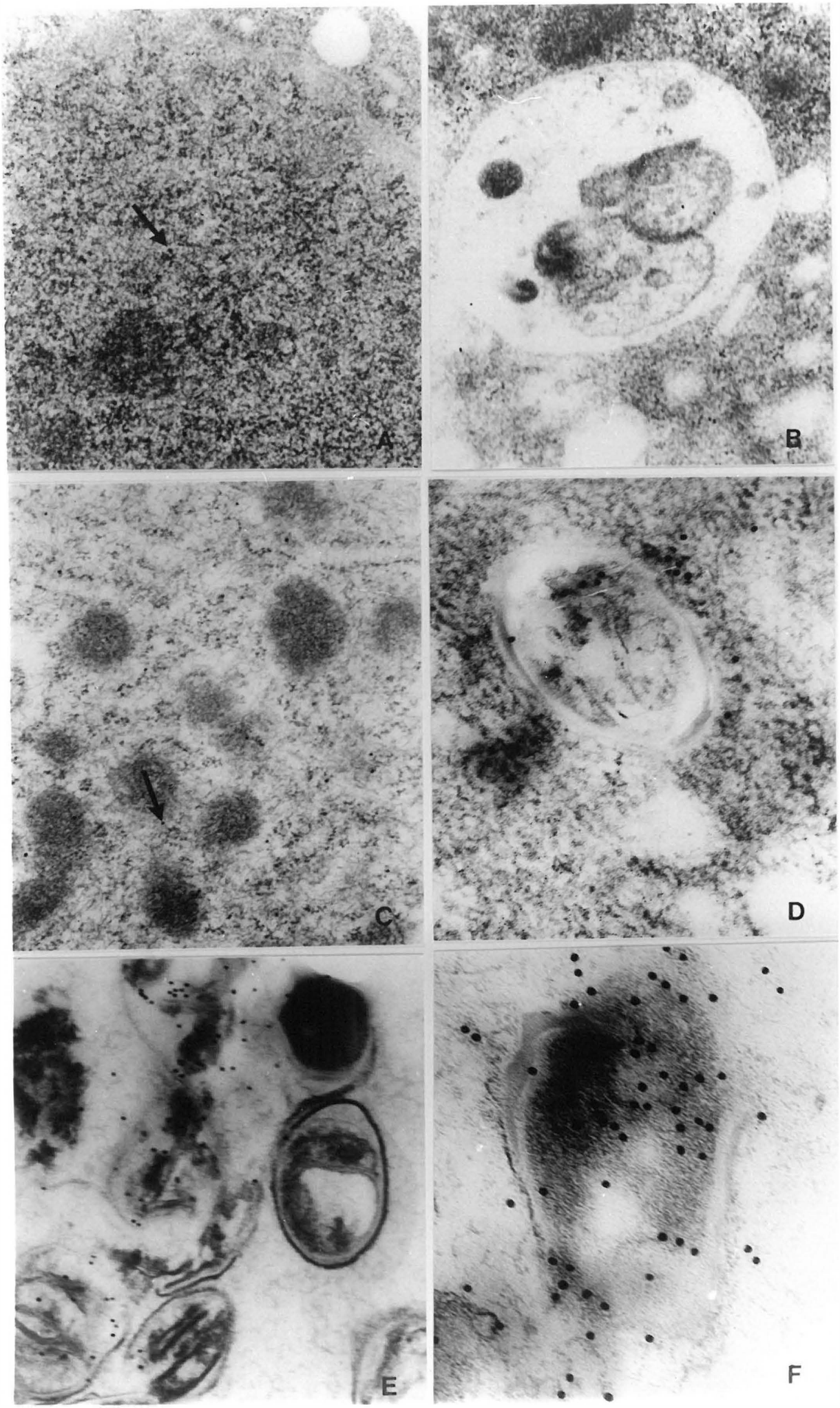
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FIG. 6.7. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 11344: (A) nucleus and cytoplasm [47K], (B) nucleus [90K], (C) internalised mycobacteria in cytoplasm [51K], (D) internalised mycobacterium in cytoplasm [45K] and (E, F) non cell associated 11344, (E) 48K and (F) 54K.

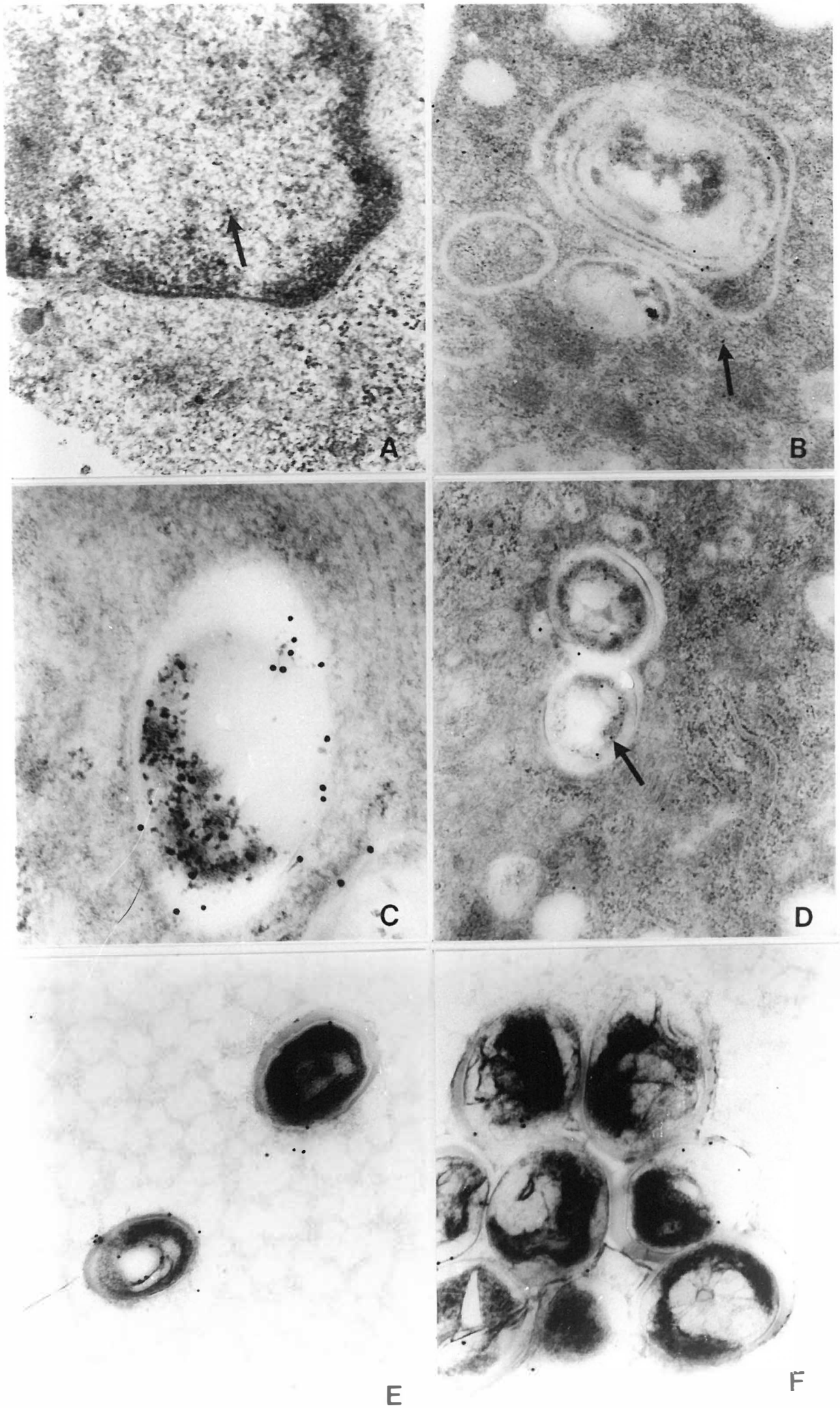
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FIG. 6.8. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 90s: (A) cytoplasm [45K], (B) membrane bound mycobacteria [45K], (C) internalised mycobacteria in cytoplasm [71K], (D) nucleus and internalised mycobacterium in cytoplasm [60K] and (E, F) non cell associated 90s, (E) 56K and (F) 37K.

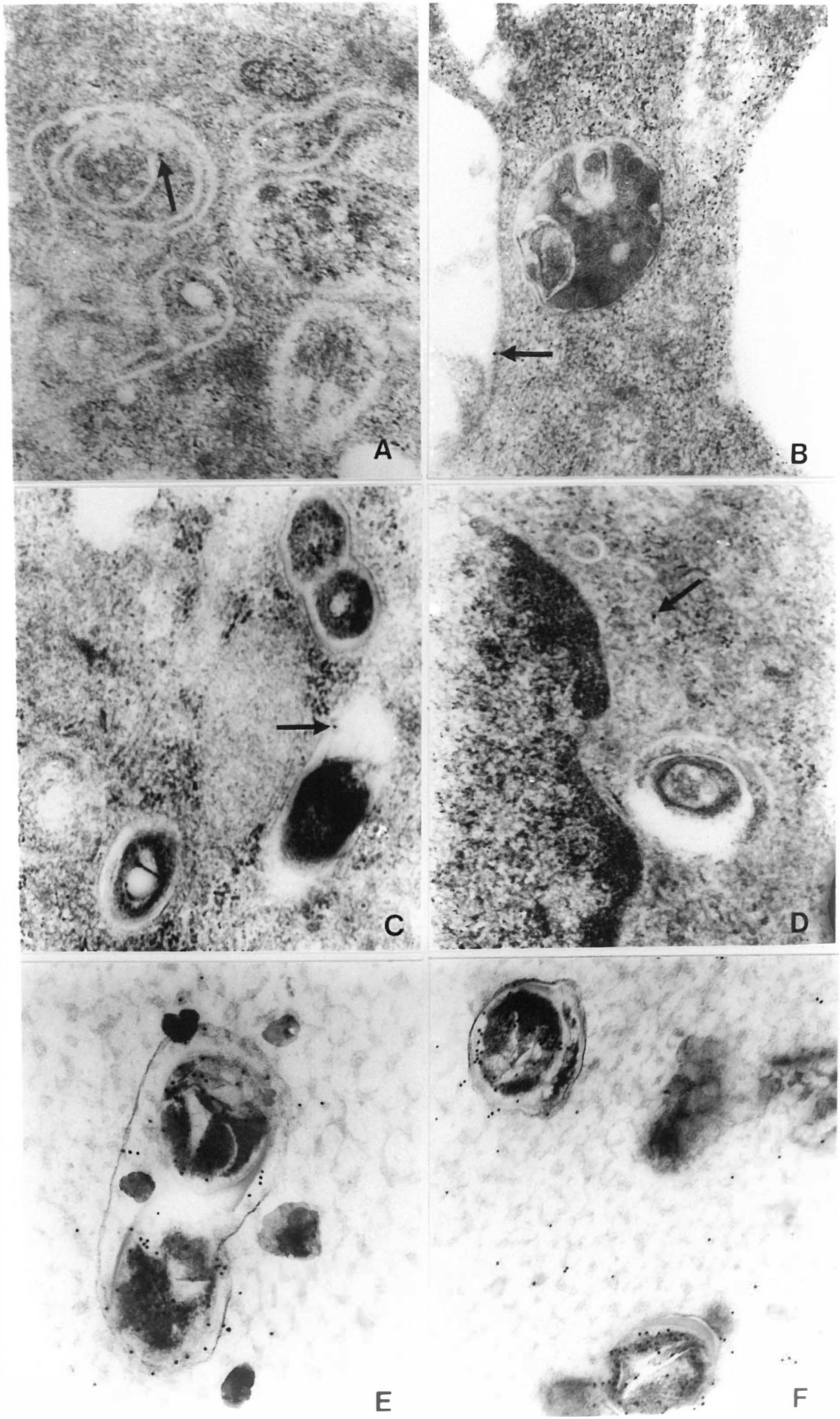
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FIG. 6.9. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 11102: (A) nucleus and cytoplasm [44K], (B) cytoplasm [48K], (C) internalised mycobacterium in cytoplasm [75K], (D) internalised mycobacteria in cytoplasm [47K] and (E, F) non cell associated 11102, (E) 50K and (F) 42K.

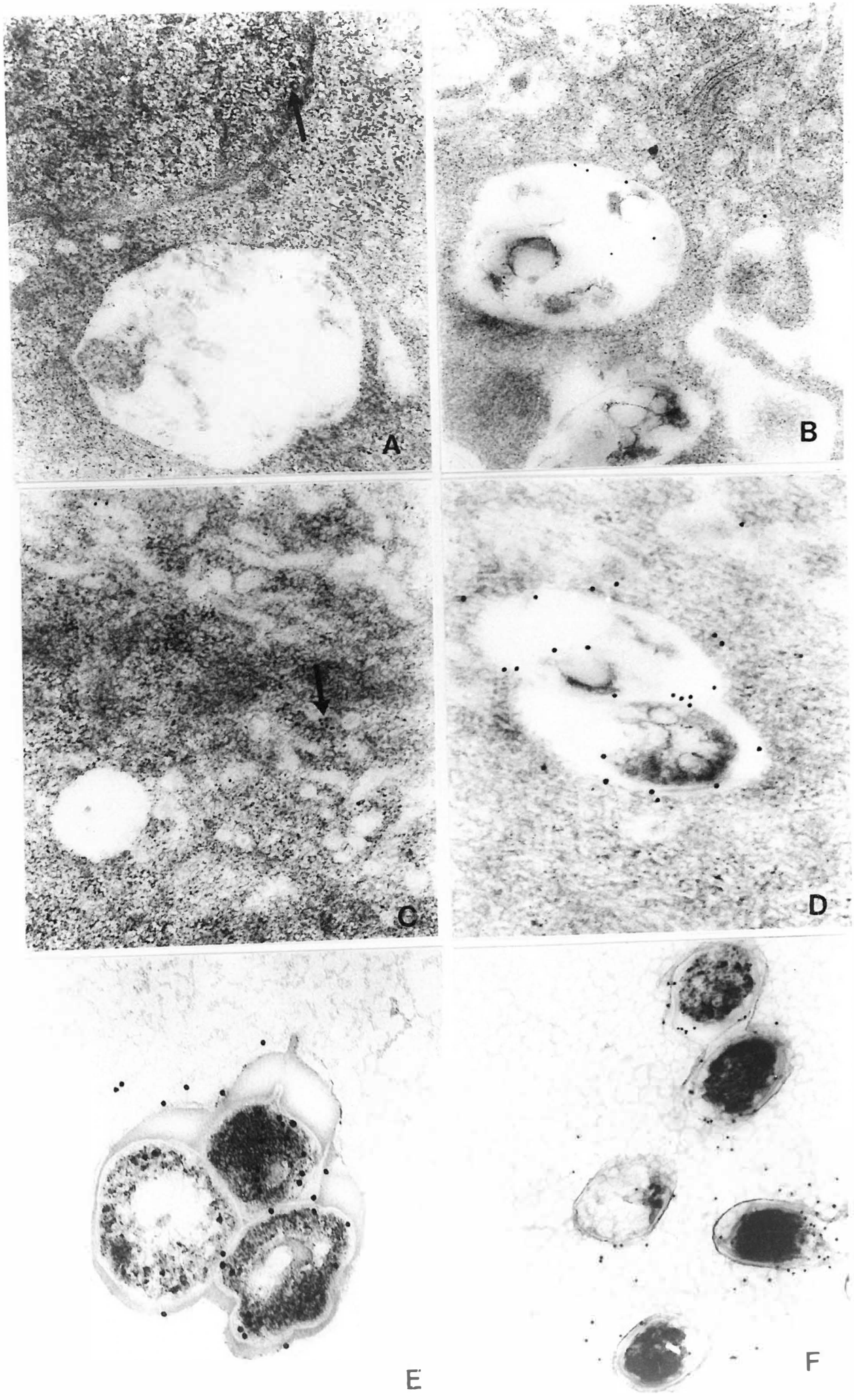
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FIG. 6.10. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 11191: (A) nucleus, membrane bound mycobacteria and cytoplasm [51K], (B) membrane bound mycobacteria and cytoplasm [45K], (C) cytoplasm [57K], (D) internalised mycobacteria in cytoplasm [75K] and (E, F) non cell associated 11191, (E) 75K and (F) 41K.

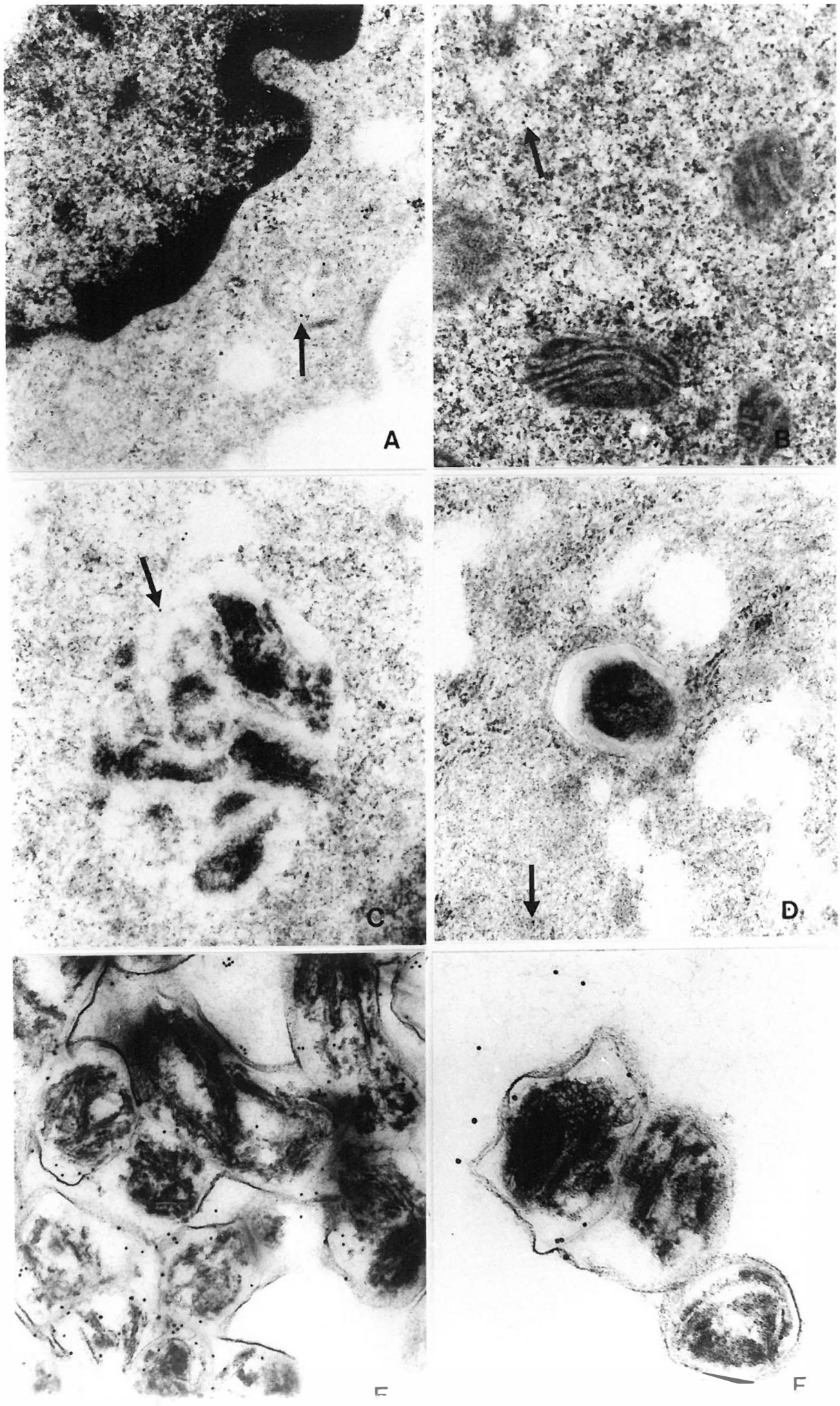
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FIG. 6.11. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 11341: (A) nucleus and cytoplasm [46K], (B) cytoplasm [46K], (C) membrane bound mycobacteria and cytoplasm [47K], (D) internalised mycobacterium in cytoplasm [50K] and (E, F) non cell associated 11341, (E) 56K and (F) 77K.

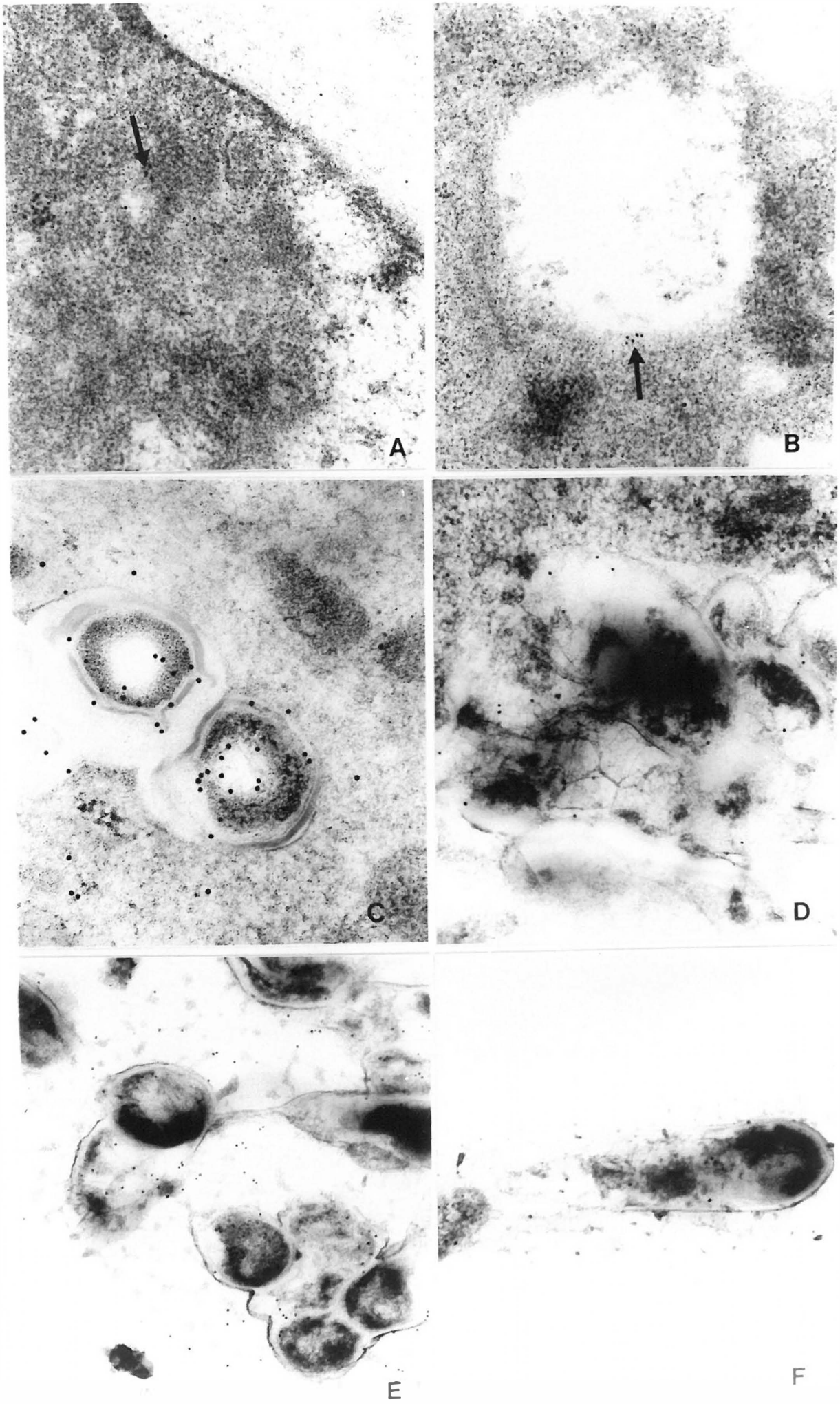
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FIG. 6.12. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 11851: (A) nucleus and cytoplasm [50K], (B) membrane bound mycobacteria and cytoplasm [50K], (C) internalised mycobacteria in cytoplasm [51K], (D) internalised mycobacteria in cytoplasm [49K] and (E, F) non cell associated 11851, (E) 48K and (F) 49K.

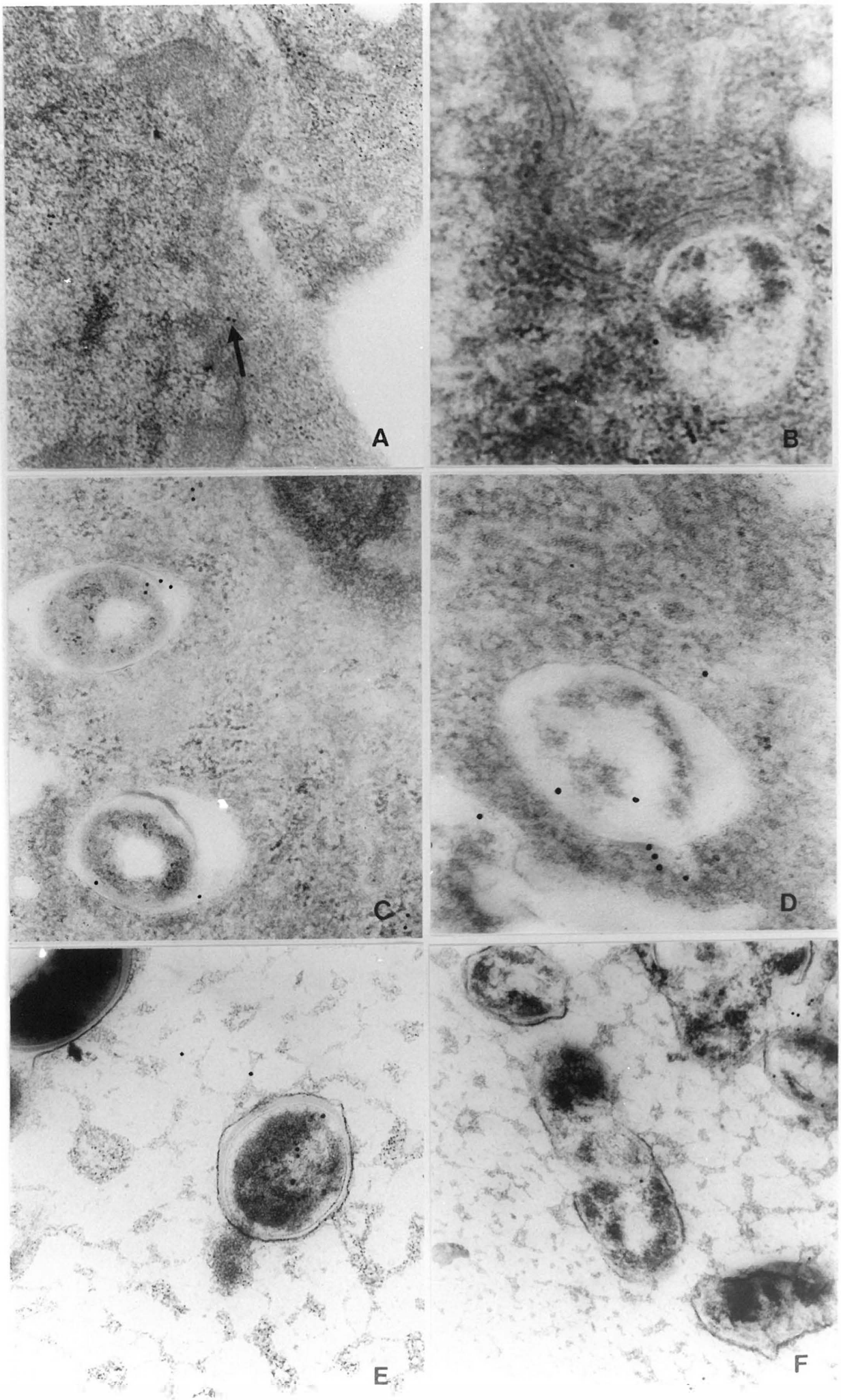
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FIG. 6.13. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 10486: (A) nucleus and cytoplasm [52K], (B) cytoplasm [66K], (C) internalised mycobacteria in cytoplasm [66K], (D) internalised mycobacterium in cytoplasm [54K] and (E, F) non cell associated 10486, (E) 75K and (F) 48K.

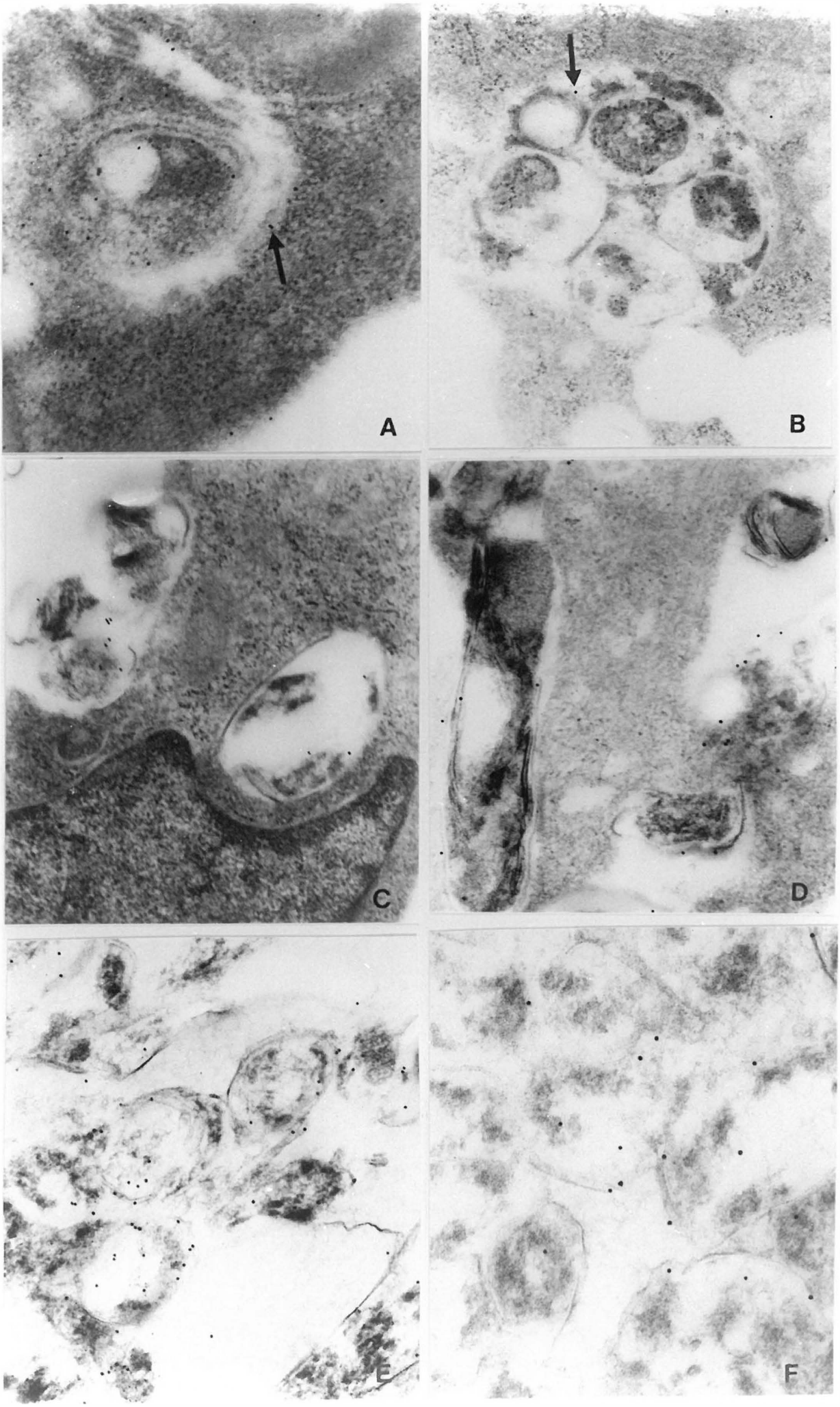
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FIG. 6.14. Distribution of PZA gold label in (A-D) macrophages infected with susceptible strain 1195: (A) cytoplasm [57K], (B) membrane bound mycobacteria and cytoplasm [48K], (C) nucleus and internalised mycobacteria in cytoplasm [49K], (D) internalised mycobacterium in cytoplasm [52K] and (E, F) non cell associated 1195, (E) 56K and (F) 60K.

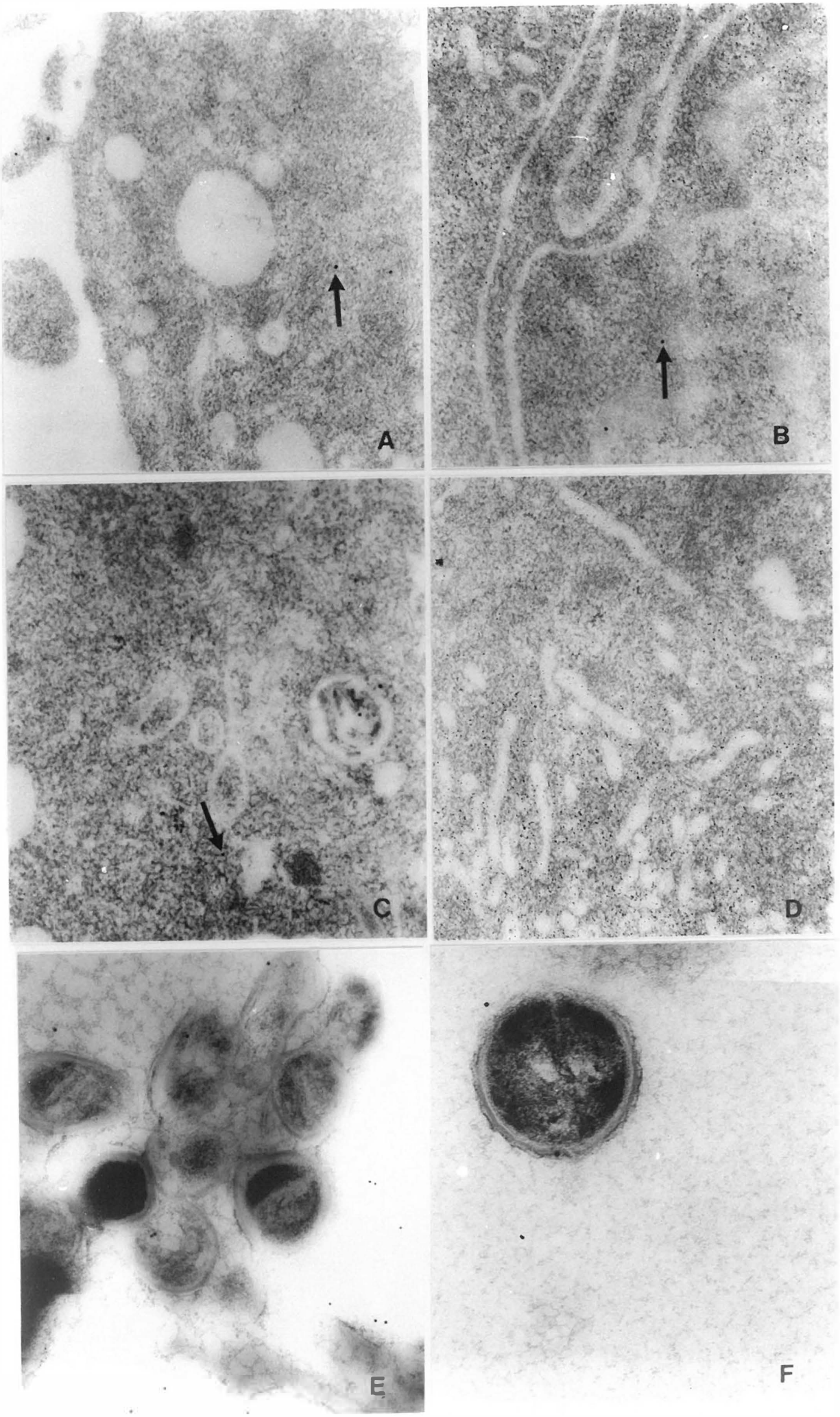
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FIG. 6.15. Distribution of PZA gold label in (A-D) macrophages infected with reference strain (Pzase negative) *M. bovis*: (A) cytoplasm [48K], (B) nucleus and cytoplasm [45K], (C) internalised mycobacterium in cytoplasm [44K], (D) cytoplasm [45K] and (E, F) non cell associated *M. bovis*, (E) 46K and (F) 85K.

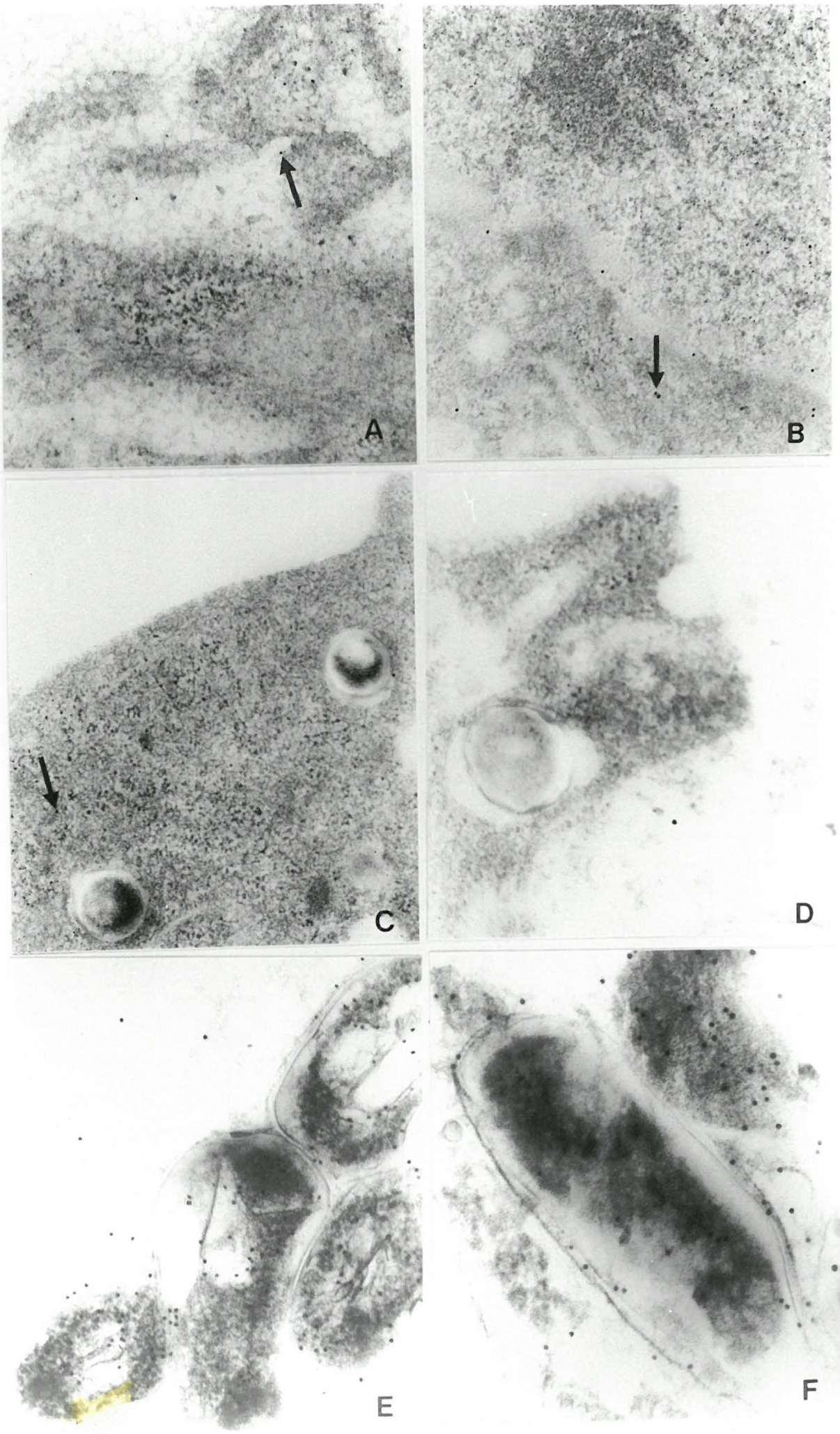
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FIG. 6.16. Distribution of PZA gold label in (A-D) macrophages infected with resistant strain 3886: (A) cytoplasm [50K], (B) nucleus and cytoplasm [50K], (C) internalised mycobacteria in cytoplasm [38K], (D) internalised mycobacterium in cytoplasm [46K] and (E, F) non cell associated 3886, (E) 72K and (F) 90K.

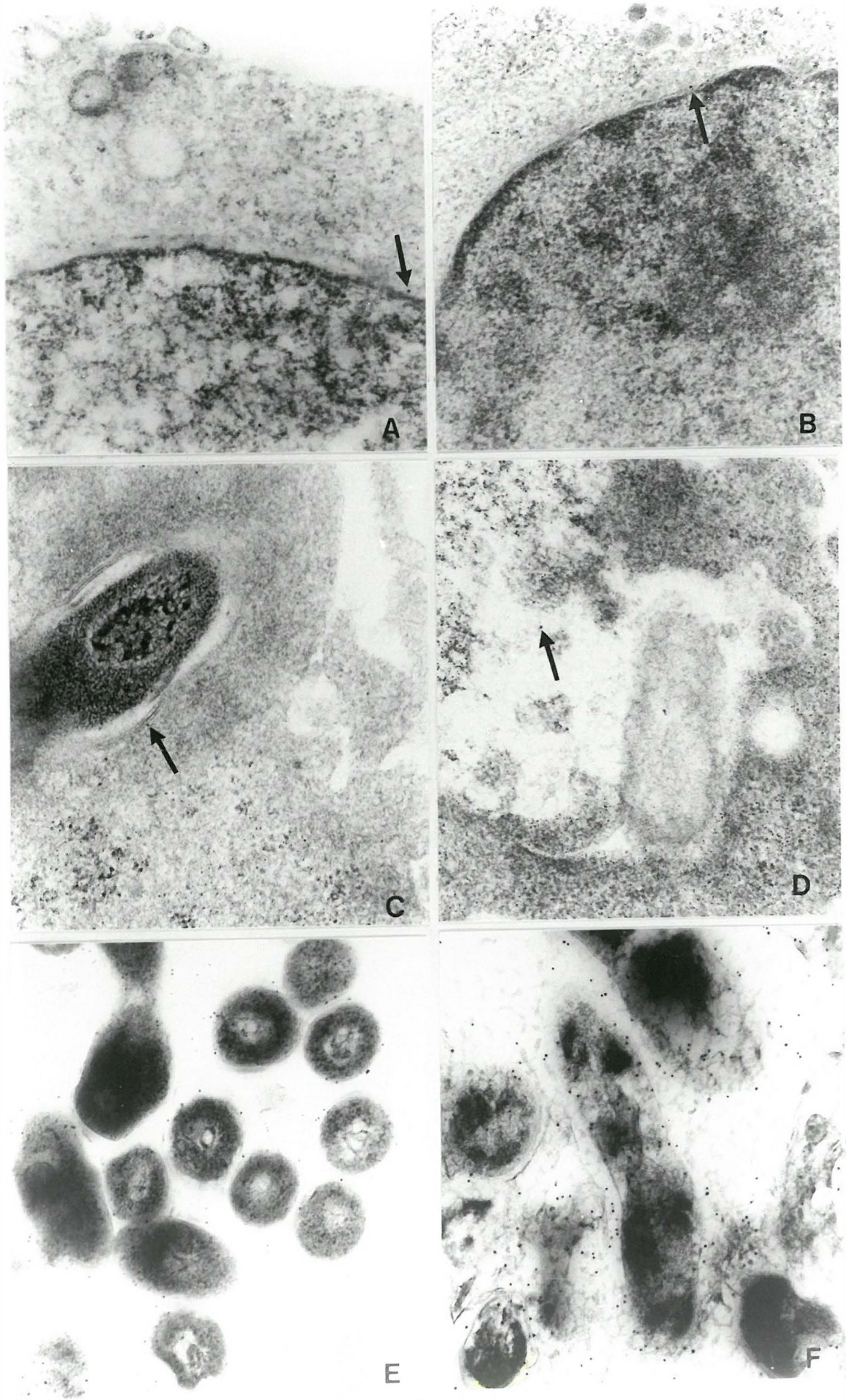
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FIG. 6.17. Distribution of PZA gold label in (A-D) macrophages infected with resistant strain 3732: (A) cytoplasm and nucleus [44K], (B) nucleus and cytoplasm [41K], (C) internalised mycobacterium in cytoplasm [49K], (D) membrane bound mycobacteria and cytoplasm [48K] and (E, F) non cell associated 3732, (E) 38K and (F) 47K.

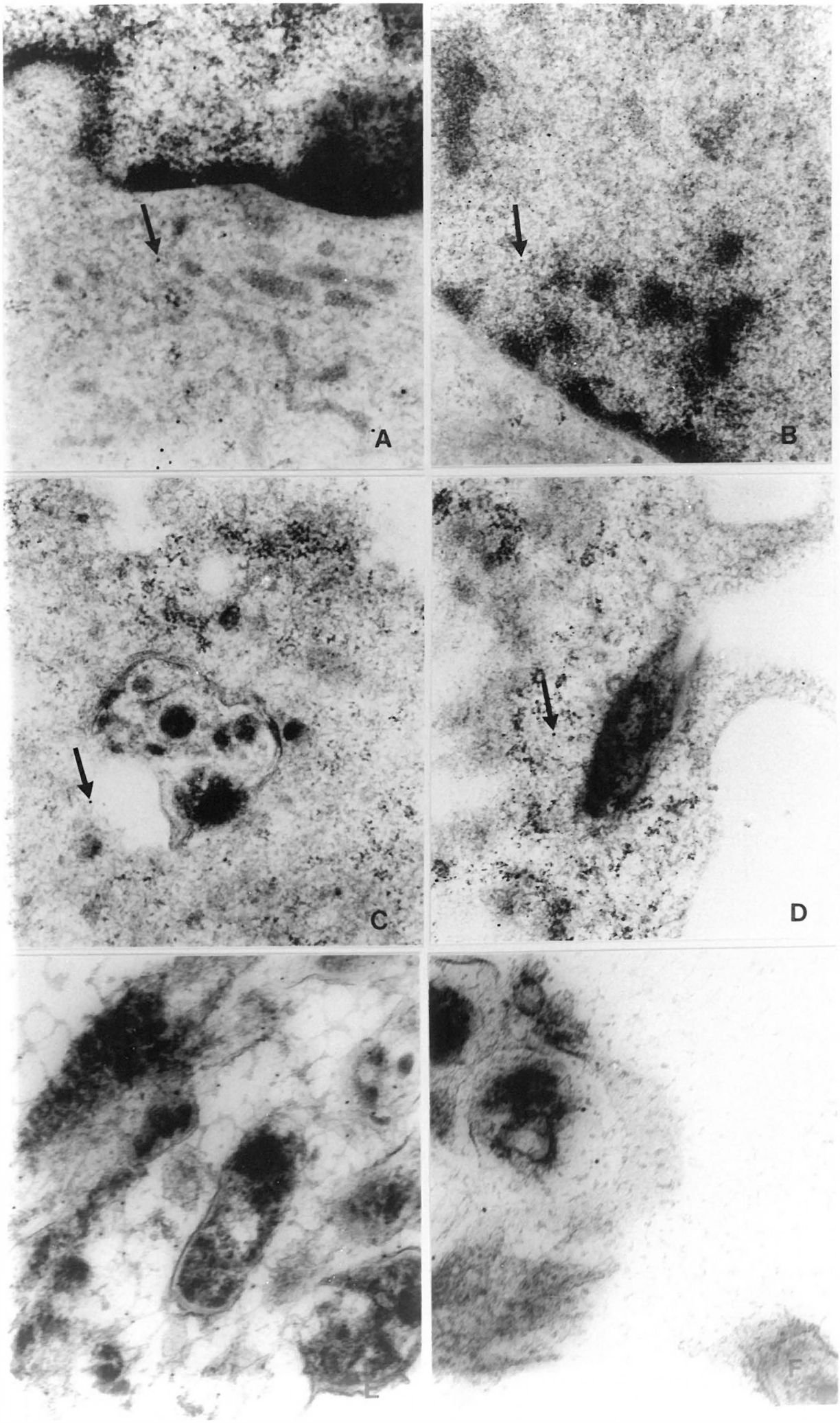
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FIG. 6.18. Distribution of PZA gold label in (A-D) macrophages infected with resistant strain 60650: (A) cytoplasm and nucleus [51K], (B) nucleus and cytoplasm [42K], (C) membrane bound mycobacteria and cytoplasm [44K], (D) internalised mycobacterium in cytoplasm [41K] and (E, F) non cell associated 60650, (E) 43K and (F) 75K.

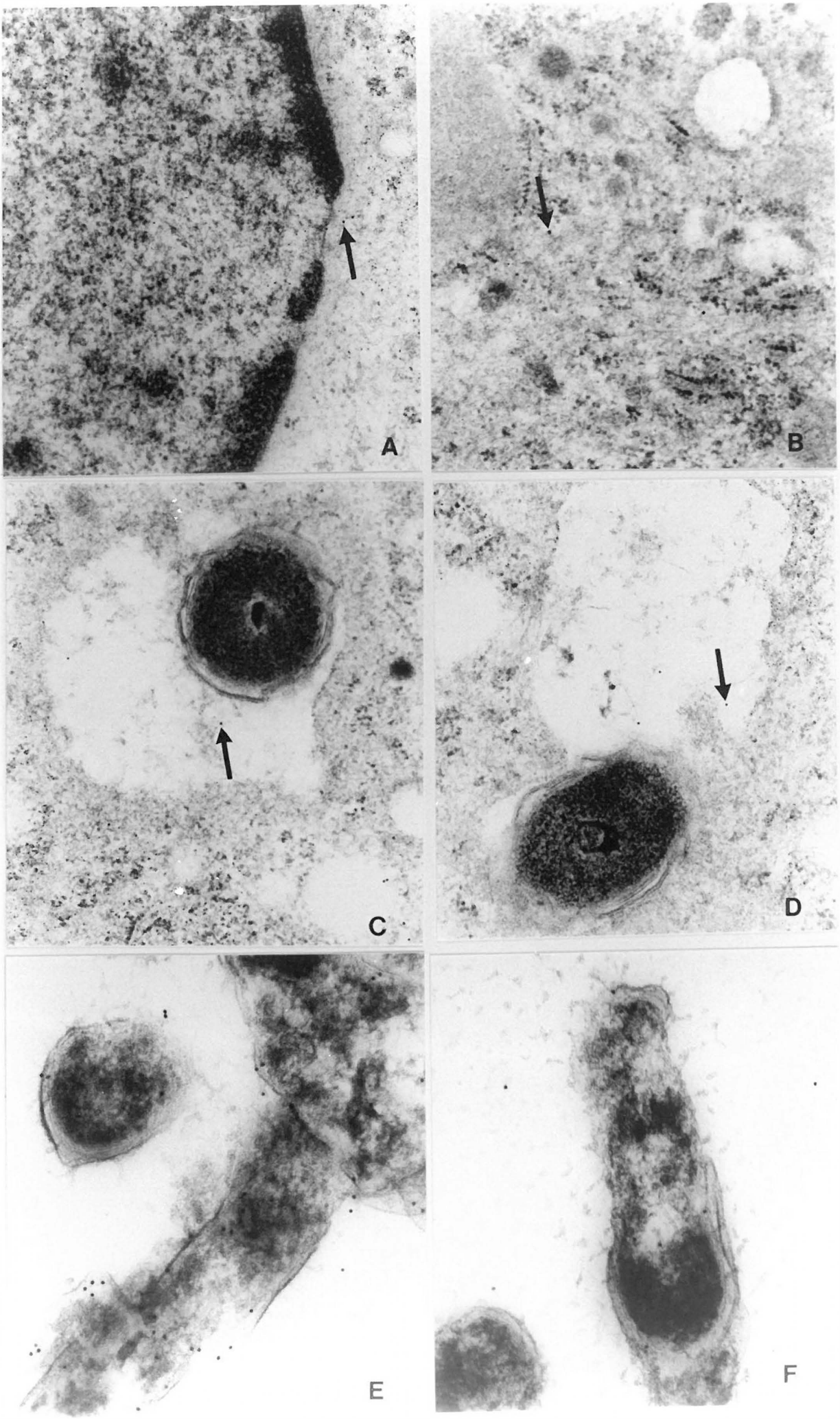
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FIG. 6.19. Distribution of PZA gold label in (A-D) macrophages infected with resistant strain 79386: (A) cytoplasm and nucleus [41K], (B) cytoplasm [50K], (C) membrane bound mycobacteria and cytoplasm [44K], (D) mycobacterium entering vesicle and cytoplasm [50K] and (E, F) non cell associated 79386, (E) 74K and (F) 66K.

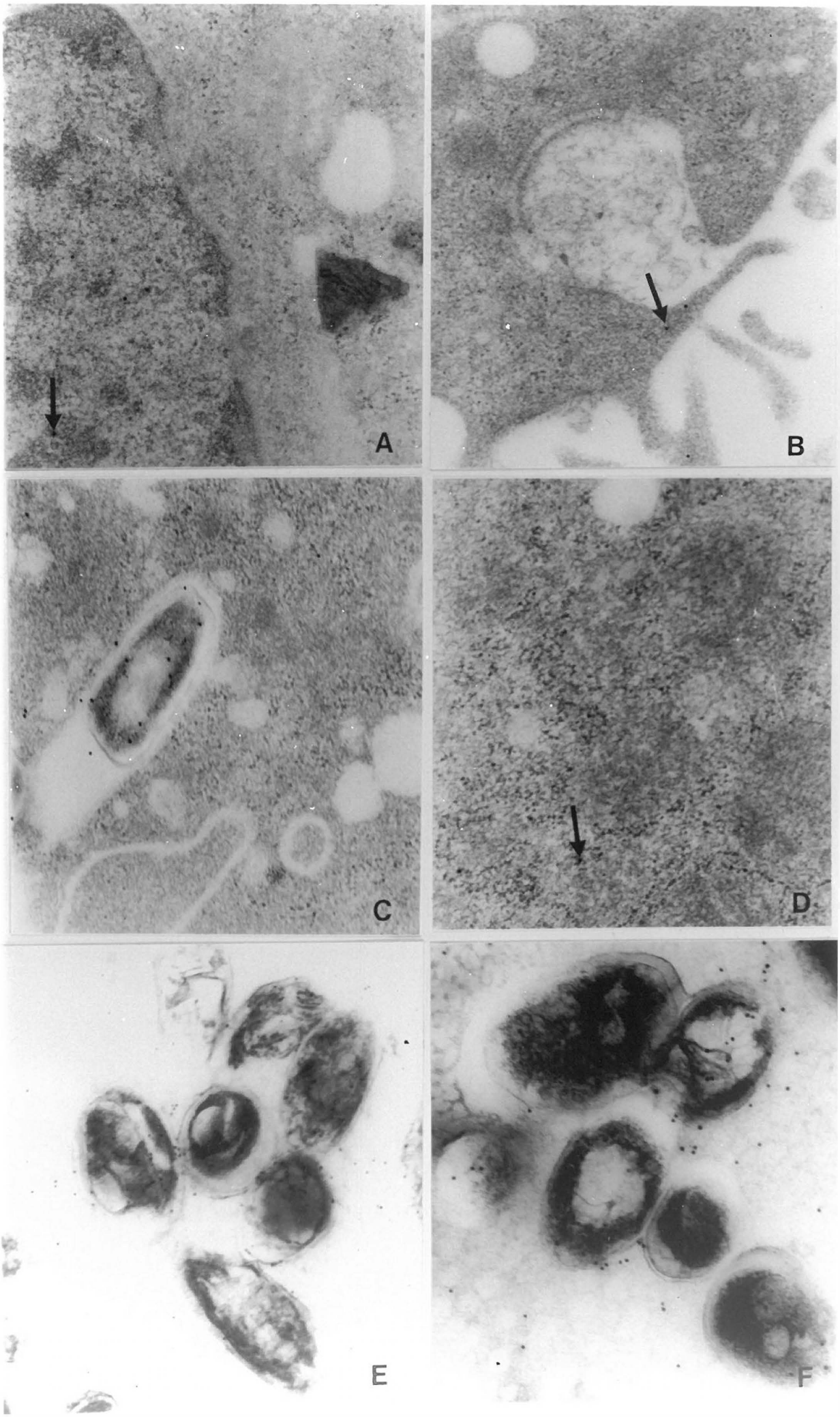
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FIG. 6.20. Distribution of PZA gold label in (A-D) macrophages infected with resistant strain 6114: (A) cytoplasm and nucleus [41K], (B) membrane bound mycobacteria and cytoplasm [53K], (C) mycobacterium internalised in cytoplasm [48K], (D) cytoplasm [47K] and (E, F) non cell associated 6114, (E) 42K and (F) 58K.

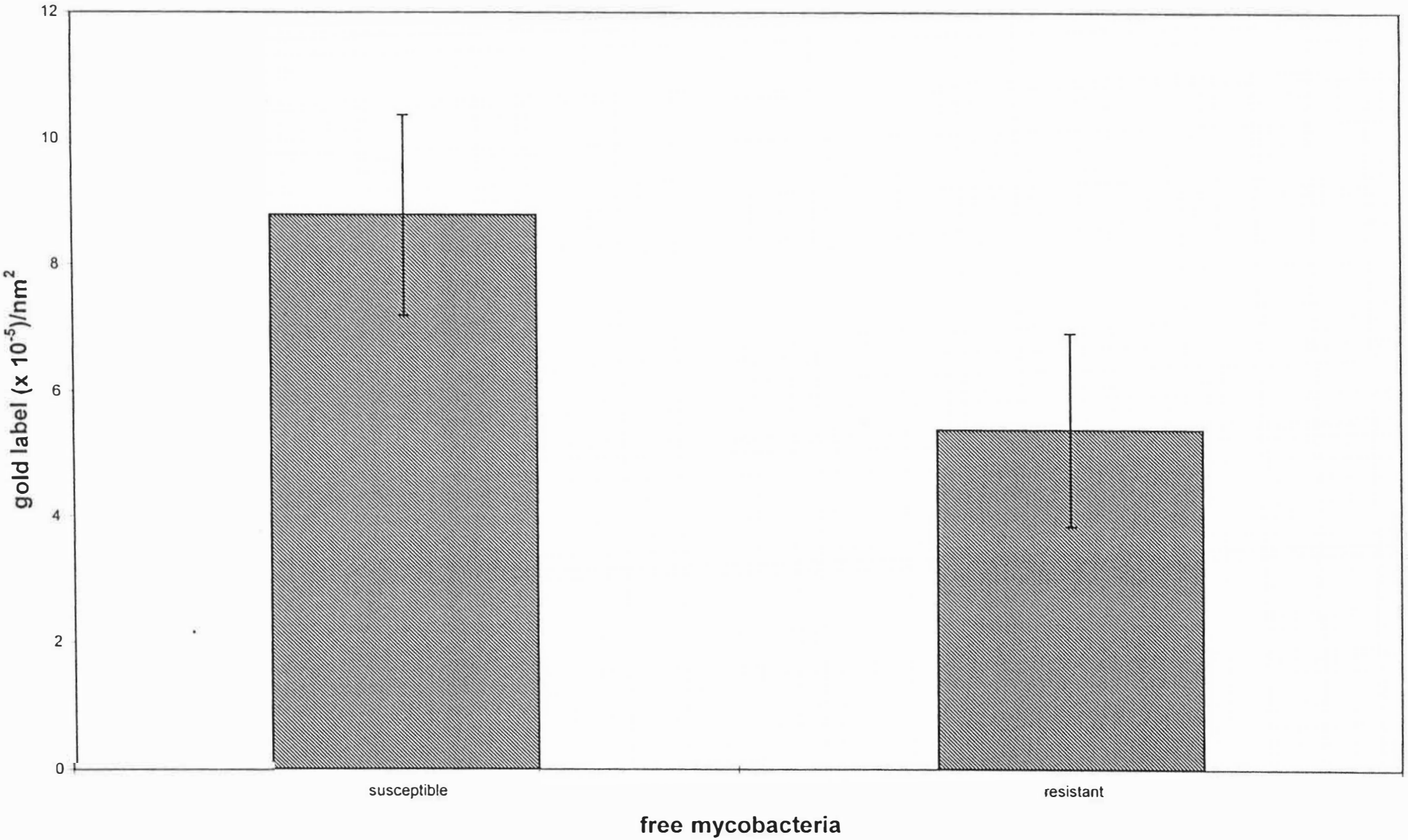

FIG. 6.21 Mean values for gold label distribution for free susceptible and resistant *M. tuberculosis* strains.

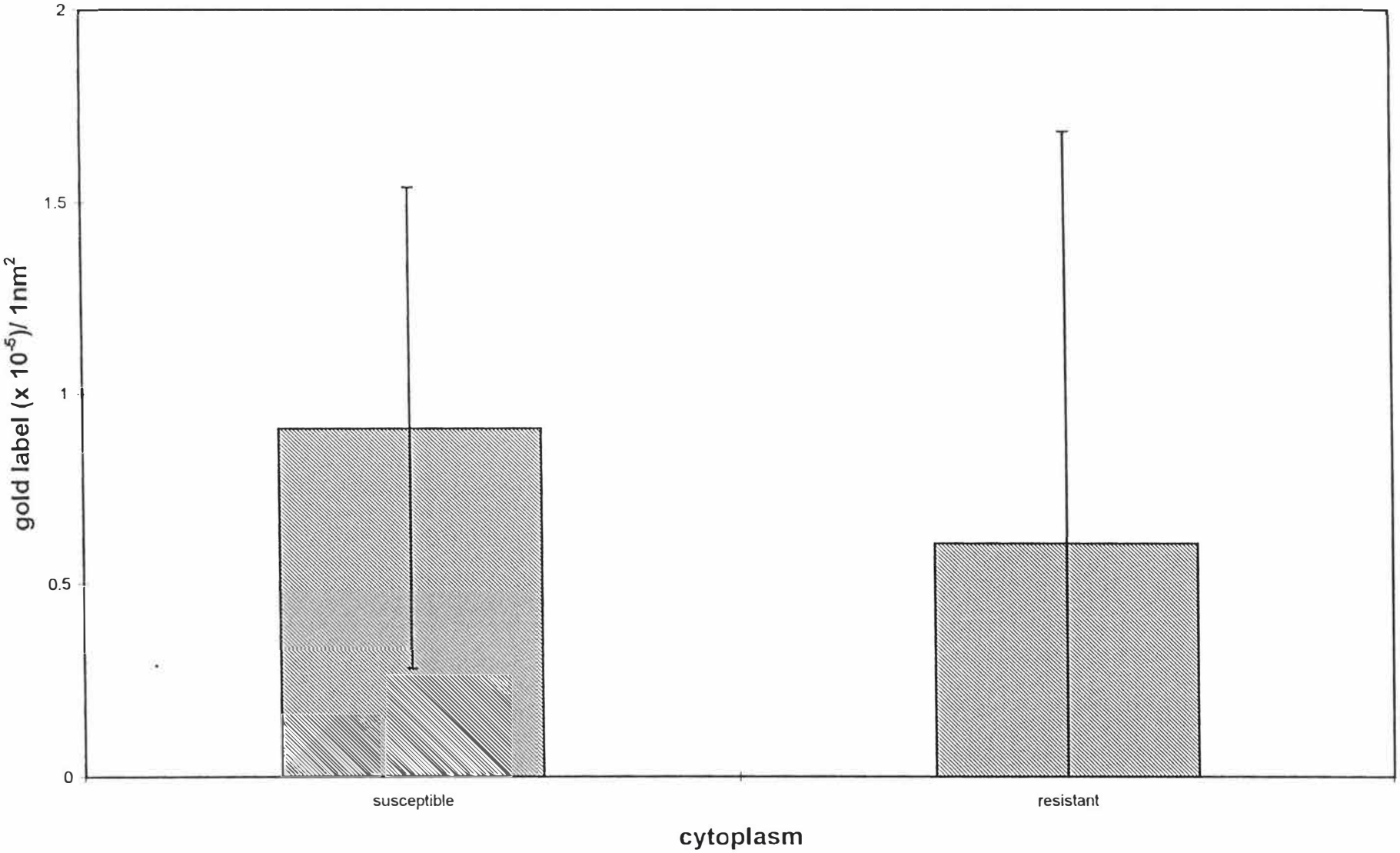

FIG. 6. 22 Mean values for gold label distribution in cytoplasm of macrophages infected with susceptible and resistant strains of *M. tuberculosis*.

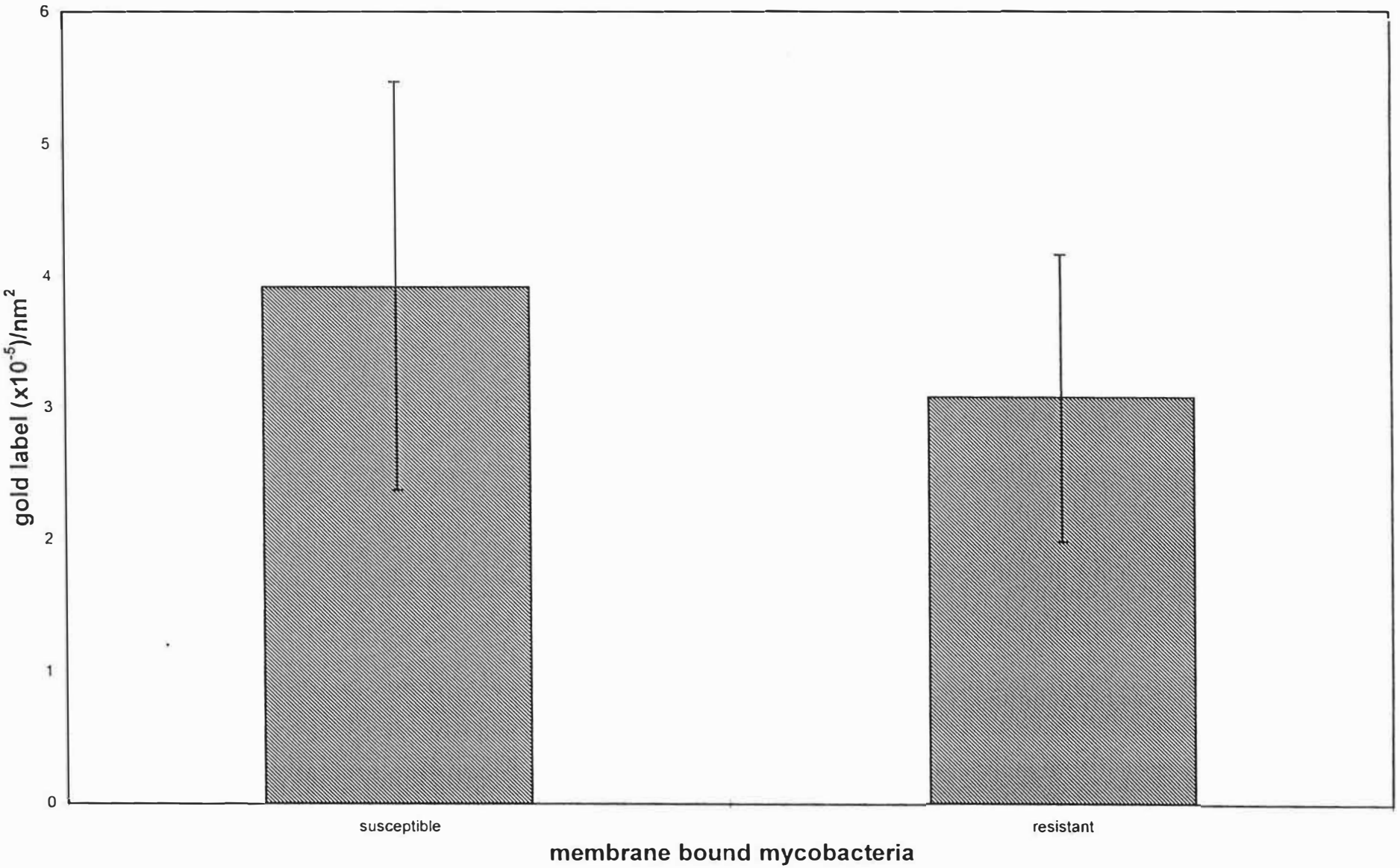

FIG.6.23 Mean values for gold label distribution in membrane bound mycobacteria in macrophages

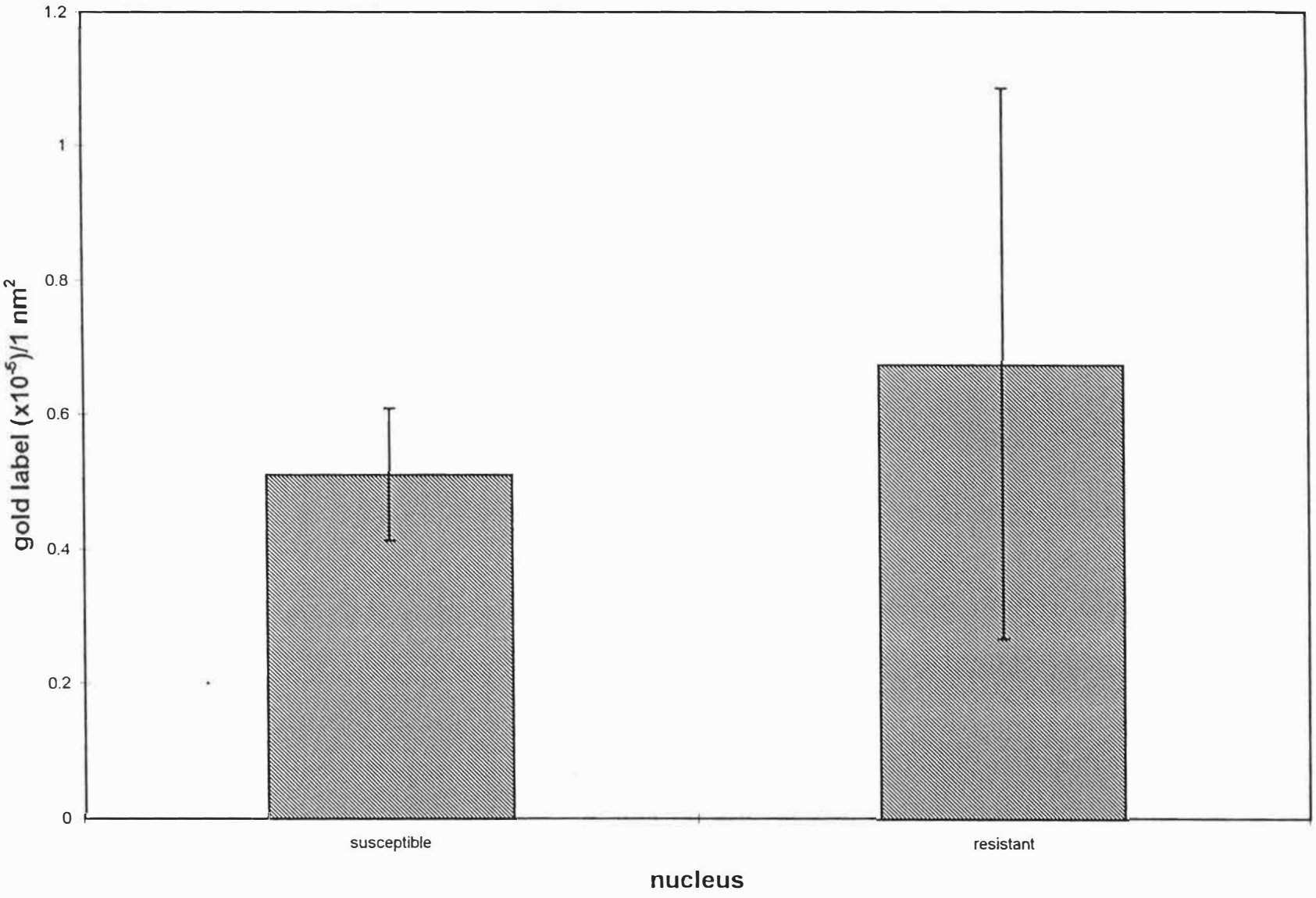

FIG. 6. 24 Mean values for gold label distribution in nucleus of macrophages infected with susceptible and resistant strains of *M. tuberculosis*.

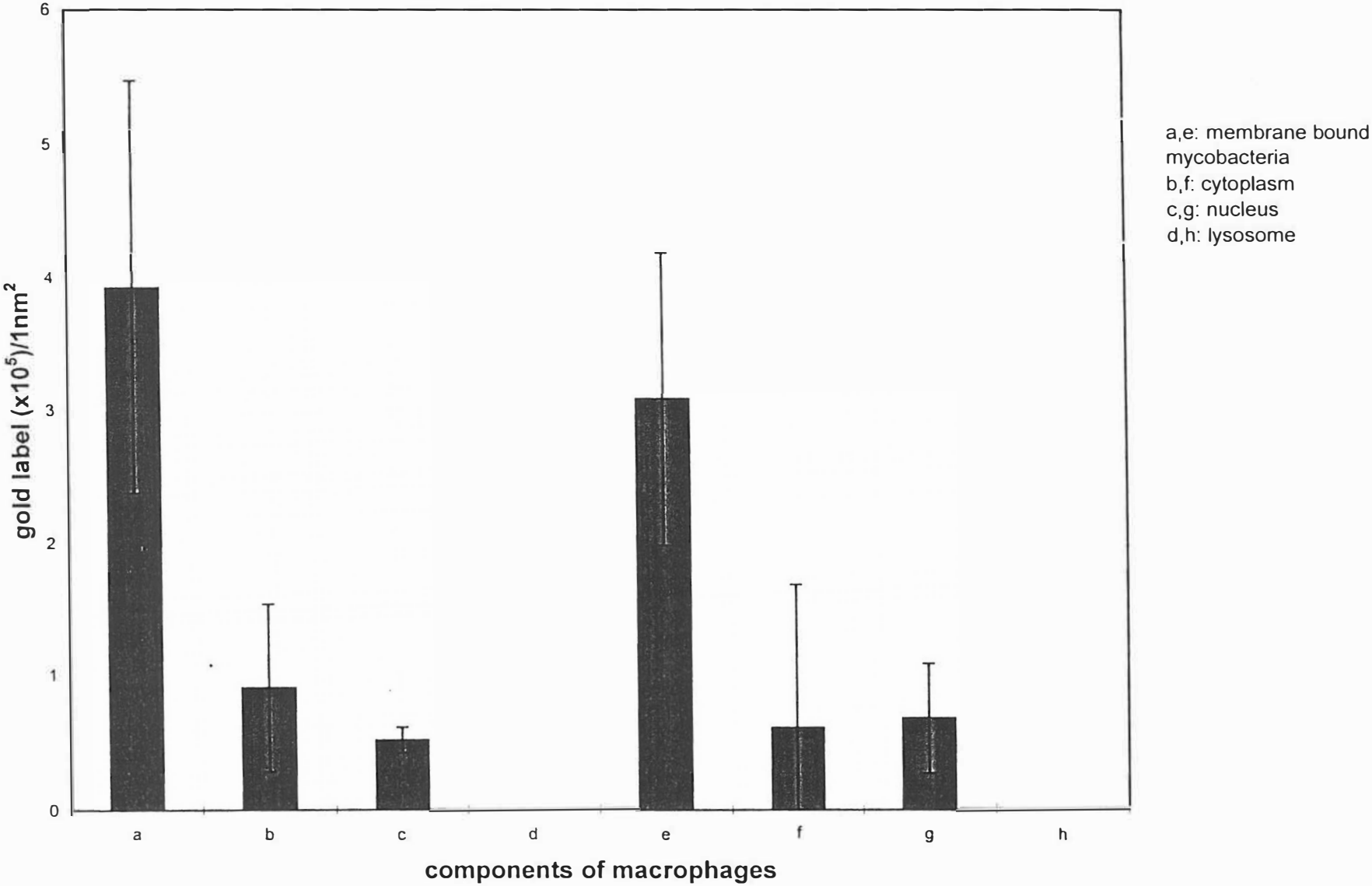

FIG. 6.25 Composite representation of the mean values for gold label analysis in the different compartments of the macrophages infected with susceptible (a-d) and resistant (e-h) *M. tuberculosis* strains.

6.4 DISCUSSION

There have been no reports on the use of gold labelled PZA antibodies to study the site of action of PZA. We successfully produced PZA antibodies in rabbits (as outlined in chapter 5) and performed immunocytochemistry experiments to try to elucidate the site of action of PZA in macrophages. No distinction can be made between PZA and POA by these experiments as the structural features of both are too similar to be able to differentiate between them by means of antibodies.

Each of the susceptible and resistant strains were used to infect macrophages and subject to exposure to PZA. The susceptible and resistant strains are represented in FIGS. 6.4-6.14 and FIGS. 6.15-6.20, respectively. Each strain was studied as internalised mycobacteria and as non cell associated free mycobacteria. Each figure gives an account of the gold label distribution in the nucleus, cytoplasm and membrane bound mycobacteria. However, visual comparison among strains would not be an accurate account of the distribution of gold label. Therefore, the gold label located in the different compartments of the macrophages were statistically analysed.

There is no literature which describes similar studies with PZA antibodies, thus to make comparisons and contrasts is difficult. The gold label located over the free susceptible and resistant mycobacteria was significantly different from the cytoplasm, nucleus and membrane bound mycobacteria ($p<0.0001$). This suggests that the action of PZA is not solely restricted to the macrophage. The significant difference ($p=0.001$) between the accumulation of gold label in the cytoplasm of macrophages infected with susceptible and resistant bacteria suggests that the mycobacteria may influence the uptake of PZA. Susceptible strains may have a better PZA transport system in addition to Pzase positivity, thus they are able to take up more PZA, however, the accumulation is not over the intracellular bacteria but rather in the cytoplasm. The cytoplasm serves as a harbour for the antibiotic until it diffuses into the intracellular bacteria or back into the extracellular environment depending on the saturation capacity of the intracellular mycobacteria and the time period of incubation.

The significant difference between the gold label located over the membrane bound susceptible mycobacteria in macrophages and the nucleus suggests that the PZA could interfere with the DNA nucleotides as PZA and POA is similar in structure. The gold label accumulation between the internalised mycobacteria and nucleus of macrophages infected with resistant strains is not statistically significant, which suggests that only when Pzase activity is present, does the interference at the nuclear level take place, which infers that Pzase activity is important in PZA activity since it seems that the PZA metabolite POA is actually causing the interference at the nuclear level.

Addition of the mean gold label counts/nm^2 within the components of the macrophage equals to 5.34 x 10^{-5} gold label particles/nm^2 for susceptible strains and 4.39 x 10^{-5} gold label/nm^2 for resistant strains. This is lower than the mean gold label count for the free susceptible mycobacteria which is 8.78 x 10^{-5} gold label particles/nm^2 but comparable to free resistant mycobacteria which has a mean count of 5.38 x 10^{-5} gold label particles/nm^2. This suggests that accumulation of PZA takes place best in free Pzase positive mycobacterial strains and that the macrophage may not be the only site of action of PZA.

Although, the PZA and POA could not be found within mycobacteria located in the macrophages by HPLC due to the antibiotic being diluted within the macrophage as shown in calculations in chapter 4, the gold label experiments with PZA antibodies enabled quantification of the intracellular PZA. Thus, there is an accumulation of PZA in intracellular mycobacteria. Also, the high accumulation of PZA antibodies in free mycobacteria questions the theory that PZA acts in macrophages only.

This study does not address the question whether accumulation takes place in phagosome or phagolysosome because clear distinction between the phagosome bound and cytoplasm bound mycobacteria cannot be made on physical characteristics only. Double gold labelling using markers that distinguish phagosome would be needed. It would enable one to ascertain whether the accumulation of PZA is higher in the phagosomes or in cytoplasm enclosed mycobacteria. However, vesicles with no

bacteria (lysosomes) did not have any gold label. This suggests that PZA does not have a specific affinity for such organelles. Also, PZA accumulated over the intracellular bacteria suggesting that the antibody has more affinity for the bacteria. If the membrane bound bacteria fused with the lysosomes, the gold label located here is due to the bacteria and not the organelle.

Conclusions

* The PZA antibodies raised in rabbits was successfully used to locate the sites of action of PZA in macrophages infected with *M. tuberculosis*.

* Due to the similar structural features of PZA and POA, no distinction can be made between them by antibodies.

* The significant difference between gold label located over the free susceptible and resistant mycobacteria compared to cytoplasm, nucleus and membrane bound mycobacteria ($p<0.0001$) suggests that the action of PZA is not solely restricted to macrophages

* The high accumulation of PZA antibodies in the free mycobacteria suggests that the macrophage is not solely the site of action of PZA

* The cytoplasm serves as a harbour for the antibiotic since there is a significant difference between the gold label located in the macrophages infected with susceptible and resistant bacteria.

* Immunocytochemistry was more sensitive than HPLC as PZA could be quantified in intracellular mycobacteria

CHAPTER 7

GENERAL DISCUSSION

PZA has a special place in tuberculosis chemotherapy in that it allows shortening the duration of treatment from the earlier norm of 12-18 months to the current standard of 6 months. It is thought that this results from its ability to kill a population of semi-dormant tubercle bacilli that are not affected by other antituberculous drugs. The drug has long been known to be active in humans with pulmonary tuberculosis and is now for more than a decade used in routine chemotherapy for tuberculosis, however, its mechanism of action is still obscure (Raynaud *et al,* 1999). Heifets (1999) summarises the results of studies that address the question; how PZA affects mycobacteria? PZA is active only against *M. tuberculosis* and *M. africanum*, but does not exhibit any antimicrobial activity against *M. bovis* or any of the non tuberculous mycobacteria. The mode of action of PZA is associated with POA, an enzyme generated product considered the active moiety. Less is known about the mode of action of PZA than about that of any other antimycobacterial agent. At peak concentrations attainable *in vivo* (30-40 μg/ml) PZA activity *in vitro* can only be shown in acidic environments, at pH 5.5 to 5.6. It has therefore been suggested that the effect of PZA *in vivo* takes place in the acidic intracellular environment of the phagolysosome inside macrophages. This study addressed the question: is the site of action of PZA indeed the phagolysosome of macrophages? Experiments were conducted in such cells infected with susceptible and resistant strains of *M. tuberculosis*. An attempt was made to identify in which compartment the drug is active, and this was compared with extracellular bacteria *i.e.*, non macrophage associated mycobacteria. Survival of mycobacteria over time was assessed by colony counts. Intracellular, extracellular and intrabacterial PZA and POA concentrations were measured by HPLC and finally gold labelled antibodies gave an insight into the site of accumulation of the drug.

The colony counts have shown that there is no bactericidal effect over the 72 h period studied with infected macrophages and the 336 h period studied with non cell

associated cultures. There is limited entry of PZA into macrophages although the extracellular PZA concentration (100 μg/ml) is 2-3 times the concentration found *in vivo*. POA was only detected in the experiments with free bacteria. The gold labelling experiments showed an accumulation of PZA and/or POA in mycobacteria, even in the intracellular environment.

When macrophages were infected with reference strain H37Rv, 20 μg/ml of the initial concentration of 100 μg/ml PZA could not be detected. This suggests that the PZA or its metabolite POA is bound in cellular components of the macrophage or in the bacteria. Also, as discussed in chapter 4 the dilution factor of 700 would make any POA undetectable as it would be below the detection limit of 0.5 μg/ml set by HPLC. In macrophages infected with Pzase positive clinical isolates 5 μg/ml of PZA could not be accounted for. This suggests that PZA is converted to POA intracellularly and that the proposed mode of action of PZA could be true. However, this study showed that there is far greater conversion of PZA to POA in cell free susceptible mycobacteria. The PZA did enter non cell associated free mycobacteria and POA was present at a steady amount during the time period studied. However, most of the PZA and POA was found extracellularly. During the 336 h period studied the PZA and POA had no bactericidal effect on the free mycobacteria in RPMI 1640. It was not possible to measure bacteriostasis in this milieu because bacterial replication could not be observed in drug free RPMI 1640.

The HPLC observations in this study suggest that a reverse transport system exists that transports PZA out of the macrophages back into the extracellular environment. This was evident by the disappearance of extracellular PZA followed by an increase in extracellular PZA in macrophage experiments. How this mechanism works is not known but it suggests that the transport mechanism over time changes into a reverse state. Another observation is that a percentage (5-20%) of the drug cannot be accounted for and the exact location of this drug is unknown. However, gold labelling has shown accumulation in intracellular bacteria. The mathematical model showed if the drug is indeed present in the intracellular bacteria it would not be detected by HPLC due to the dilution factor in the cytoplasmic fraction.

So far, HPLC has only been used in pharmacokinetic studies. PZA concentrations were determined in CSF and plasma in rabbits (Chan, 1986). Woo (1987) used HPLC to determine PZA and rifampicin concentration is serum samples from patients with tuberculous meningitis while Yamamoto *et al.* (1987) used HPLC to determine PZA and its metabolites concentrations in human plasma. Walubo *et al.* (1994) used HPLC to detect PZA, rifampicin and INH in human plasma by HPLC. These authors showed that *in vivo* PZA concentration is below 100 μg/ml (20-70 μg/ml). The *in vitro* experiments carried out in this study also showed that the PZA concentration attainable intracellularly in macrophages is low (<5 μg/ml) although the macrophages were exposed to 100 μg/ml PZA. The present study is unique in that a correlation was made between PZA concentrations in the various fractions of the infected macrophage model by HPLC to the effect it has on growth of intracellular mycobacteria as well as with its location in macrophages. All of the studies that have studied PZA activity have investigated these two aspects separately.

There have been studies assessing the effect of PZA on macrophage bound bacteria, however, these studies have only made assessment by using colony counts. Also, more than one drug was included in the experiments, thus combination effects were studied rather than the sole effect of PZA. Carlone *et al.* (1985) investigated the killing capacity of rifampicin, PZA and POA on macrophages that had ingested live *M. tuberculosis*. No rapid increase in the killing effect was observed with the combinations compared to the individual drugs. They found over a 3 to 24h period more rapid, although not statistically significant killing of intracellular bacteria. The study of Carlone *et al.* (1985) differed from this study in that in the latter no combinations of drugs were tested. The infected macrophages were exposed to PZA only. Thus, there was no competition among the drugs for entry into the macrophages. PZA used as a single drug had no bactericidal effect over the 72 h period studied.

Acocella *et al.* (1985) investigated the degree of penetration of rifampicin, PZA and POA in mouse macrophages in different metabolic stages. These authors used radiolabelled drugs and scintillation spectrophotometry to assess penetration into the

macrophage. It was found that stimulation of macrophages had a marked effect on the degree of penetration of rifampicin at higher concentration. It affected PZA penetration only at the lower concentration and seemed not to affect penetration of POA. This study differed from the studies of Acocella *et al.* (1985) in that infected macrophages not stimulated by cytokines were used and that only PZA effect was studied. Also, HPLC was used instead of scintillation spectrometry because this allows differentiation between PZA and POA since the conversion of PZA to POA was of importance.

Crowle *et al.* (1986) studied the effect of PZA on intracellular bacilli by counts of acid fast bacilli and CFUs. The authors comment that PZA could be bacteriostatic or bactericidal against intramacrophage tubercle bacilli depending on its concentration, the donor of macrophages and length of exposure of the infected macrophage to the drug. The donor of the macrophages did not influence the results obtained in this PhD study because a pool of peripheral blood was used to isolate monocytes and cultured to the macrophage stage. These authors found that PZA was active in the macrophage model as it was inhibiting virulent bacilli at a concentration of 20 μg/ml or higher over a 7 day period. The macrophages were exposed to a higher concentration of 100 μg/ml PZA. Inhibition of growth was observed in infected macrophages but no killing of intracellular bacteria was observed in the 72 h period studied. Longer incubation periods could result in a killing effect being observed. The macrophage experiments were only studied for 72 h, due to the HPLC measurements done concurrently to be able to relate this to killing. If RPMI 1640 was renewed and the incubation period prolonged, the true intracellular PZA concentration could not be measured as addition of medium would have an affect on the initial concentration of drug added.

Also, Sbarbaro *et al.* (1986) demonstrated an inhibitory effect of PZA and a quinolone (ofloxacin) on intracellular mycobacteria by colony counts, while Mor and Esfandiari (1997) investigated the synergistic effect of clarithromycin (CLA) and PZA on *M. tuberculosis*. It was shown that CLA tends to enhance the effectiveness of PZA against tubercle bacilli within macrophages. These authors showed that the

combination of drugs does have an effect on the behaviour of PZA intracellularly in macrophages. This study did not assess combination of drugs as it was important to establish the site of accumulation of PZA and POA solely. Although, this study wanted to establish the site of accumulation of PZA and POA, it is evident from other studies like Sbarbaro *et al.* (1986) and Mor and Esfandiari (1997) that PZA may best have its place in combination therapy of tuberculosis.

The immunogold label experiments were able to locate PZA/POA in infected macrophages. It also allowed quantification of PZA within different compartments of the macrophage. However, the drawback of this methodology is that the antibodies can not distinguish between PZA and POA due to the structural similarity. Nevertheless, the gold label studies complement the HPLC studies in an attempt to identify the significant compartments within the macrophages that play a role in the mode of action of PZA. Also, we were able to compare macrophage bound mycobacteria with free mycobacteria. The extracellular antibiotic has to pass through the cytoplasmic membrane to enter the macrophages. The cytoplasmic membrane can either enhance the uptake of PZA or restrict its uptake. The uptake is not by mere diffusion across the cytoplasmic membrane. If uptake was by diffusion, there would be an influx into the macrophages infected with Pzase positive mycobacteria because it was found that these bacteria remove PZA from the cells. However, such influx is not observed. A constant amount remains intracellularly throughout the 72 h period studied. This infers that membrane transport molecules are involved but the actual dynamics of the mechanism is not known. In this study it is not possible to comment whether activity takes place in the phagolysosome as double gold labelling to identify phagolysosome was not used. However, the gold labelling experiments have shown that it is unlikely that activity takes place in the extrabacterial component of phagolysosomes because gold label accumulated inside bacteria and not in bacteria free vesicles. This is further emphasised by the accumulation of gold label over free bacteria where the HPLC methodology was able to quantify POA as it was present in detectable amounts.

The gold label experiments also showed an accumulation of PZA antibodies in the nucleus, when Pzase was present. This suggests that POA acccumulates in the nucleus, which could not be assessed by HPLC. HPLC showed PZA in the total macrophage and also within internalised bacteria. However, large amounts were present extracellularly. This did influence the observation with gold label experiments because the extracellular fluid was removed by washing.

The gold label experiments may provide precise insight into the accumulation of gold label within macrophages by using two antibodies, one identifying the structural feature and the second identifying the drug. Thus, a definite comment can be made as to the accumulation of the drug. Confocal microscopy using double fluorescent antibodies may also serve as an alternative to TEM. Also, pH determinations of the phagosomes in uninfected and infected macrophages will serve to give a complete explanation as to whether pH plays a pivotal role in the site of action of PZA within macrophages. It is unlikely that the drop in pH has an effect on intracellular bacteria in macrophages as the drug accumulates in the bacteria and it is unlikely that enough PZA could be converted to POA to allow for the drop in pH. It is also unlikely to be a natural drop in pH because a natural drop in pH takes place after fusion and activation of the phagosome and lysosome which leads to bacterial kill and would thus make PZA redundant.

The higher extracellular PZA concentration detected by HPLC in the macrophage experiments as well as the higher intrabacterial PZA/POA antibody concentration found in extracellular bacteria by gold labelling experiments serves to question the belief that PZA acts solely in macrophages. The effect of PZA *in vivo* is not restricted to macrophages. It is unclear whether the predominant interaction between PZA and bacteria is taking place intracellularly in macrophages or extracellularly in the acidic environments of the inflammation sites (Heifets, 1999). From this PhD study, the high extracellular PZA concentration found outside macrophages as well as the high extrabacterial PZA and POA concentrations found with non cell associated free bacteria tend to favour the latter part of Heifets (1999) statement.

PZA is important in the therapy of tuberculosis patients in that the length of therapy is reduced when PZA is included in the first 2 months of therapy. This suggests that PZA itself is able to decrease the bacterial load drastically within the 2 month time frame or allows other drugs to do that more effectively. The latter is most likely because early bactericidal assays have shown that *in vivo* kill by PZA alone is rather poor. The mycobacteria maybe in a "so called injured state" and the other drugs like INH and rifampicin are thus able to kill the bacteria more effectively reducing the bacterial load more rapidly and so allowing for short course treatment.

The HPLC methodology developed was able to quantify extracellular and intracellular PZA within macrophages. However, antibiotic found within intracellular bacteria could not be quantified due to a dilution factor (a mathematical model gives the explanation in chapter 4). The immunogold labelling studies were more sensitive as intrabacterial drug could be quantified. Although the intrabacterial PZA accumulation was low, immunogold labelled PZA antibodies have the ability to locate the site of action of PZA. The drawback of these immunogold labelling experiments is that no distinction can be made between PZA and POA because of the similar structural features.

New findings:

* Development of a HPLC methodology *i.e.*, use of methanol and phosphate buffer as a mobile phase to separate PZA and POA.
* Production of polyclonal antibodies to PZA.
* Development of an immunogold labelling technique to locate PZA activity within macrophages and on free mycobacteria using the antibodies produced.
* The HPLC methodology and the immunogold labelling experiments showed that the activity of PZA is not solely restricted to macrophages but is present more significantly in the extracellular environment where free mycobacteria are found.

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APPENDICES

Appendix I: *Mycobacterium tuberculosis* strains

Mycobacterium tuberculosis (strains)	Pyrazinamidase status
H37Rv	positive
200R	positive
100R	positive
11344	positive
90S	positive
11102	positive
11191	positive
11341	positive
11851	positive
10486	positive
1195	positive
M. bovis	negative
3886	negative
3732	negative
60650	negative
79386	negative
6114	negative

Appendix II: Media and Reagents

2.1	Antibiotic stock solutions:	PZA, POA and PARA dissolved in warm distilled water
	HPLC standard solutions:	PZA: 100, 50, 25, 10, 5 μg/ml POA: 50, 25, 10, 5 μg/ml PARA: 100 μg/ml (internal standard)
2.2	Middlebrook 7H10 agar:	suspend 19g in 900 ml distilled water add 5 ml glycerol sterilize 121 °C for 15 mins cool and add 100 ml OADC (oleic acid, albumin fraction V bovine, dextrose, catalase, sodium chloride-Biolab) final pH 6.6
2.3	Middlebrook 7H9 broth:	suspend 4.7 g in 900 ml distilled water add 5 g glycerol sterilize 121 °C for 15 mins cool and add 100 ml OADC final pH 6.6
2.4	Mobile phase:	20% methanol: 80% KH_2PO_4
	Solution A:	10 mM KH_2PO_4, pH 3.5 dissolve 136.09g KH_2PO_4 in 100 ml distilled water and filter sterilise
	Solution B:	methanol (analytical grade) acetonitrile (analytical grade)

2.5 Reynolds lead citrate

(electron microscopy stain): dissolve 1.33g lead citrate
1.76g sodium citrate in 30 ml distilled water

To complete conversion of lead nitrate to lead citrate, shake the mixture vigorously until a uniform milky suspension is observed. Add 8 ml of 1N NaOH until suspension clears

2.6 Uranyl acetate

(electron microscopy stain): Dissolve sufficient uranyl acetate to form a saturated solution in 50 % ethanol

2.7 Washing solution for Immunogold labelling: 3 % BSA diluted in PBS

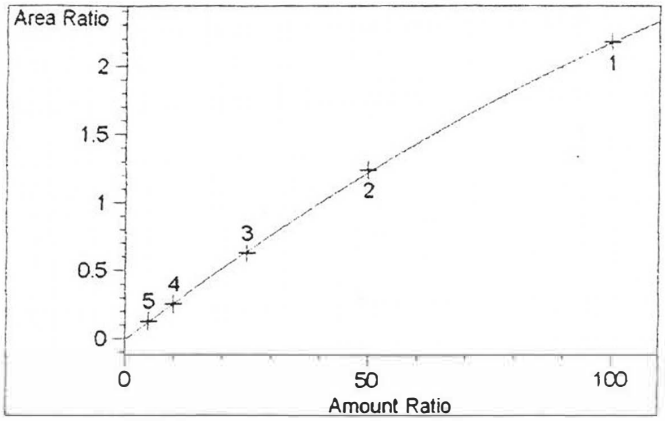
2.8 Wayne's test agar: suspend 1.84 g Middlebrook 7H11 agar
in 400 ml distilled water
Add: 0.08 g PZA
0.8 g pyruvic acid
Autoclave 121 °C for 15 mins

Indicator: 2 % $Fe(NH4)_2(SO4)_2$
reddish brown colour is positive for Pzase

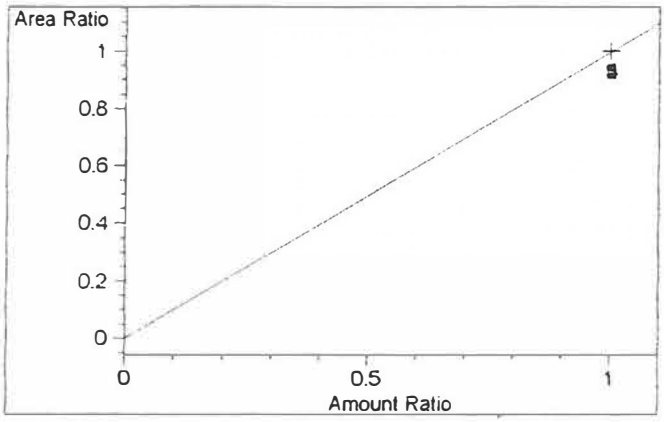
Appendix III: Standard curves for HPLC

The standard curves for PZA (FIG. 3A) and POA (FIG. 3B) were generated automatically using the HP software used to the run the HPLC instrument. The curve for the internal standard is also shown. It is evident from this curve that a constant amount of internal standard was used.

Calibration Curves



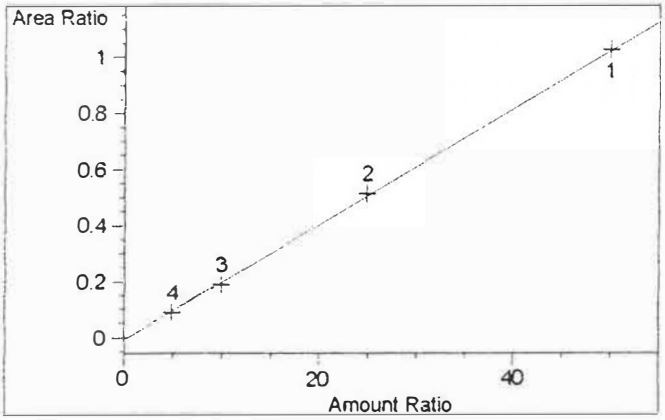
PZA at exp. RT: 4.167
DAD1 A, Sig=265,4 Ref=450,16
Correlation: 0.99993
Residual Std. Dev.: 0.01155
Formula: $y = ax^2 + bx + c$
a: -5.64621e-5
b: 2.75095e-2
c: -1.14030e-2
x: Amount Ratio
y: Area Ratio



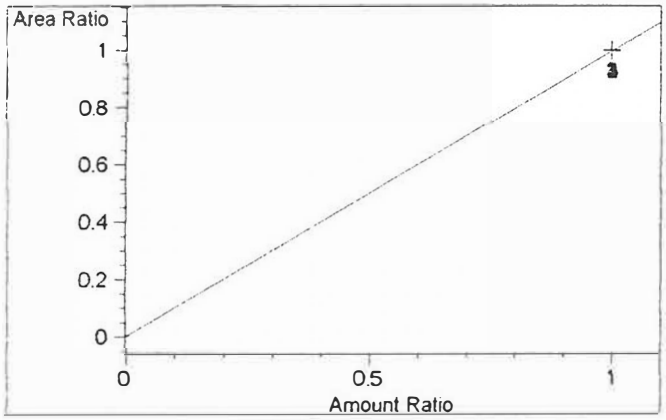
PARA at exp. RT: 5.547
DAD1 A, Sig=265,4 Ref=450,16
Correlation: 1.00000
Residual Std. Dev.: 0.00000
Formula: $y = mx + b$
m: 1.00000
b: 0.00000
x: Amount Ratio
y: Area Ratio

FIG. 3A Standard curve for PZA: 100, 50, 25,10, 5 μg/ml.

Calibration Curves



POA at exp. RT: 2.817
DAD1 A, Sig=265,4 Ref=450,16
Correlation: 0.99992
Residual Std. Dev.: 0.00714
Formula: y = mx + b
m: 2.07060e-2
b: -9.40077e-3
x: Amount Ratio
y: Area Ratio



PARA at exp. RT: 5.542
DAD1 A, Sig=265,4 Ref=450,16
Correlation: 1.00000
Residual Std. Dev.: 0.00000
Formula: y = mx + b
m: 1.00000
b: 0.00000
x: Amount Ratio
y: Area Ratio

FIG. 3B Standard curve for POA: 50, 25, 10, 5 μg/ml.

Appendix IV: Calculation of PZA gold label distribution

4.1 Distribution of PZA gold label in non cell associated and macrophage bound mycobacteria

All measurements were carried out from negatives with a constant magnification of 30K. Using the Zeiss Kontron 300 Image Analyser, surface areas of the bacteria were measured by drawing along the perimeter of the mycobacteria (FIG. 4A). The area of 3-5 mycobacteria per strain were measured. The gold label (10nm black dots) were counted within this area as indicated in FIG. 4A. The average area among the susceptible and resistant strains at 30K were 255001 and 282955 nm^2, respectively. Gold label distribution was then calculated per 1 nm^2 of mycobacterium.

4.2 Distribution of PZA gold label in nucleus and cytoplasm of the infected macrophages

All calculations were carried out from negatives with a 30K magnification. A square with a defined area of 572867nm^2 was drawn as indicated in FIG. 4B. This square was randomly moved in each of the 2 areas of interest *viz*., nucleus and cytoplasm. Gold label (10 nm black dots) were counted within this square in each area. 3-5 blocks were used for each area and strain. The average gold label per 1 nm^2 was calculated for each area within the macrophages infected with susceptible and resistant strains of *M. tuberculosis*.

FIG. 4A Use of the Zeiss Kontron 300 Image Analyser for measurements of the area of the mycobacteria and counting of the PZA gold label.

FIG. 4B Use of the Zeiss Kontron 300 Image Analyser for measurements of areas within the cytoplasm and counting of the PZA gold label.