GOODRUM, M A

MYCOLIC ACID ANTIGENS IN TUBERCULOSIS

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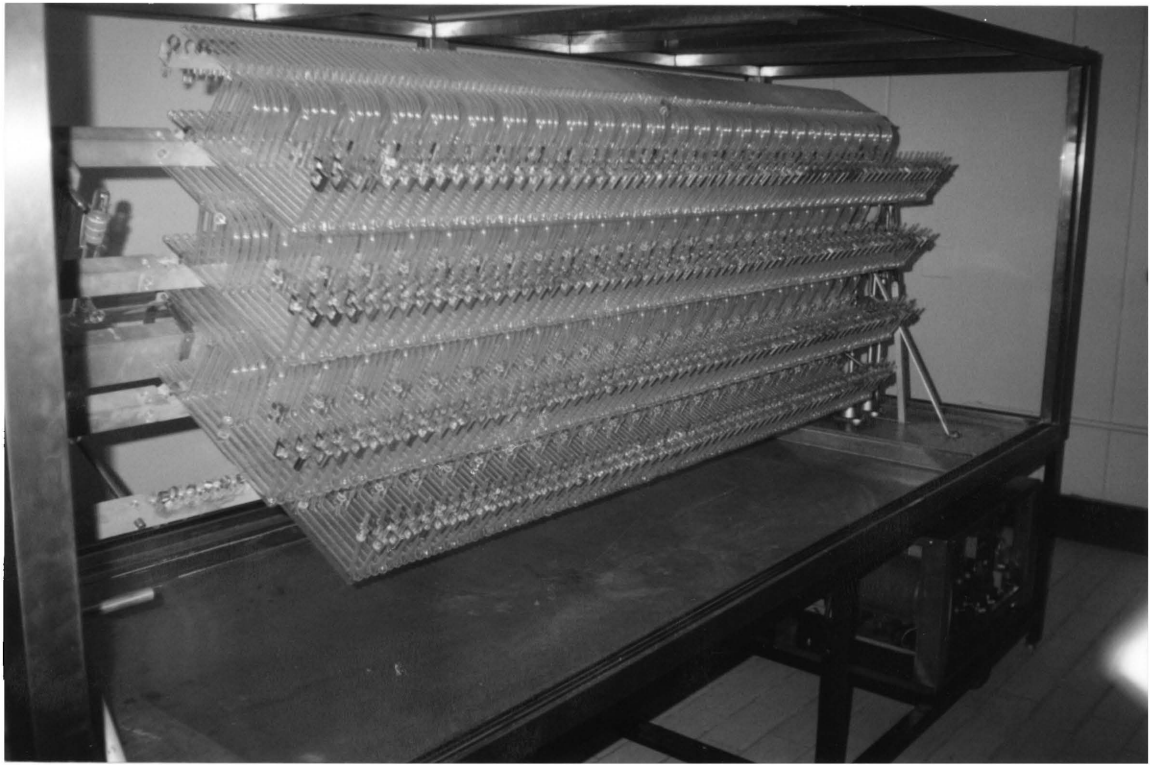
Mycolic acid antigens in tuberculosis

by

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Abbreviations

A_{486} -	Absorbance at 486 nm
AIDS -	acquired immune deficiency syndrome
Adj -	Adjuprime
ATCC -	American Type Culture Collection
APC -	antigen presenting cells
B-cells -	B-lymphocytes
BC -	before Christ
BCG -	Bacille Calmette Guerin
BSA -	Bovine serum albumin
BSA-MA -	Bovine serum albumin - mycolic acids conjugate
cas/PBS -	0.5 % casein in PBS (pH 7,4)
CC -	countercurrent
CCD -	countercurrent distribution
CD 1 -	cluster of differentiation number 1
CD 4 -	cluster of differentiation number 4
CD 8 -	cluster of differentiation number 8
CDC -	Centre for Disease Control
CMI -	cell mediated immunity
CO_2 -	carbon dioxide
cpm -	counts per million
CPR -	cycling probe reaction
DNA -	deoxyribonucleic acid
DTH -	delayed type hypersensitivity
DN -	double negative T-cells
EIA -	enzyme immunoassay
ELISA -	enzyme linked immunosorbent assay
GM-CSF -	granulocyte-macrophage colony stimulating factor
HIV -	human immunodeficiency virus

HPLC -	high pressure liquid chromatography
HRP -	horse radish peroxidase
FN γ -	interferon γ
Ig -	immunoglobulin (IgG, IgM etc)
IL1 -	interleukin 1
IL2 -	interleukin 2
IL4 -	interleukin 4
IL12 -	interleukin 12
IR -	infrared
K -	partition coefficient
Kd -	distribution constant
LAM -	lipoarabinomannan
LCR -	ligase chain reaction
LJ -	Lowenstein Jensen
M. avium -	*Mycobacterium avium*
M. bovis -	*Mycobacterium bovis*
M. chelonei -	*Mycobacterium chelonei*
M. fortuitum -	*Mycobacterium fortuitum*
M. kansasii -	*Mycobacterium kansasii*
M. gordonae -	*Mycobacterium gordonae*
M. leprae -	*Mycobacterium leprae*
M. tuberculosis -	*Mycobacterium tuberculosis*
M. ulcerans -	*Mycobacterium ulcerans*
MAC -	*M. avium* complex
McF -	McFarland
MDR -	multidrug resistance
MHC -	major histocompatibility complex
MTB -	*M. tuberculosis*
MTD -	*M. tuberculosis* direct test
μg -	microgram

Abbreviations

μl -   microliter

μm -   micrometer

OPD -   p-phenylene diamine

PCR -   polymerase chain reaction

PHA -   phytohemagglutinin

PMP -   phenolphthalein monophosphate

PPD -   purified protein derivative

% -   percent

RBC -   red blood cell

RFLP -   restriction fragment length polymorphism

RIA -   radioimmunoassay

RNA -   ribonucleic acid

rRNA -   ribosomal RNA

RNase H -   ribonuclease H

T-cells -   T-lymphocytes

TB -   tuberculosis

TDM -   trehalose dimycolate

TLC -   thin layer chromatography

TNF -   tumour necrosis factor

UK -   United Kingdom

UP -   urea peroxide

USA -   United States of America

US $ -   United States dollar

UV -   ultra violet

v/v -   volume / volume

WHO -   World Health Organisation

X - rays -   radiographs

Abbreviations

Chapter 1:
Introduction

1.1. Tuberculosis, the disease

Tuberculosis is a disease which occurs commonly throughout the world, with an exceptionally high incidence in South Africa and of which the earliest symptoms include weight-loss, coughing, night sweats and sputum that may be green, yellow or blood streaked (Fenton and Vermeulen, 1996). It is caused by *Mycobacterium tuberculosis (M. tuberculosis)*, one of the strictly pathogenic species of the genus *Mycobacterium*. Most *Mycobacteria* are soil or water dwelling saprophytes, although opportunistic pathogens of the genus include *M. leprae, M. avium, M. intracellulare, M. africanum, M. kansasii* and rare opportunistic pathogens like *M. fortuitum* and *M. chelonei*.

It is generally accepted that the primary infection with *M. tuberculosis* occurs by inhalation of very small numbers of virulent bacilli into the respiratory tract, that reach the surface of the alveoli in the lung (Nardell, 1995). Large droplets containing *M. tuberculosis* are generally deposited in the trachea and bronchi, expelled by the cilia on the respiratory epithelium, swallowed and thus rendered harmless (Rich and Ellner, 1994). Small droplets (1-2 μm in size) containing no more than 2-3 bacilli are sufficient for infection. The alveolar macrophages which line the alveoli, mount the first defense against infection (Nardell, 1995). The initial response is, in many cases, crucial to the further development of disease. The bacteria are either killed by alveolar macrophages that ingest and destroy them within the phago-lysosomes before infection is established, or they survive in the macrophagal vacuoles and destroy their host macrophages within a period of 2 to 3 weeks, releasing more bacteria (Fenton and Vermeulen, 1996). Virulent bacteria may impair the innate ability of the macrophages to destroy the bacteria by inhibiting lysosome-phagosome fusion or destroying the phagosome membrane. Two components of the mycobacterial cell wall have been implicated in these processes namely trehalose di-mycolate (TDM) also known as cord factor and sulpholipids (Rich and Ellner, 1994).

If infection occurs, cytotoxic T-cells causing delayed type hypersensitivity (DTH) and macrophages are stimulated, i.e. cell-mediated immunity (CMI) is elicited. The alveolar macrophages which are destroyed by *Mycobacteria*, release surviving bacteria and chemokines, which attract other leucocytes like circulating monocytes, lymphocytes and neutrophils, none of which destroy the released bacteria very effectively (Fenton and Vermeulen, 1996).

The accumulation of lymphocytes and mononuclear cells, which fuse to form multi-nuclear giant cells derived from macrophages, leads to the formation of granuloma called tubercles which encapsulate the pathogens and prevent them from spreading. This constitutes the beginning of the development of cell-mediated immunity. If bacteria escape and spread to the blood, they may move to other organs where they are either destroyed by macrophages, form granulomas or may remain dormant in tissue macrophages for up to ten years (Friedman, 1994).

In the lung a zone of lymphocytes accumulates on the outside of the tubercle where it forms fibrous tissue, while an area of necrosis appears in the centre of the lesion, called caseous necrosis, and is characterised by a high lipid content. After about three months, calcium may begin to be deposited in the necrotic area. In most instances these lesions undergo spontaneous healing, shrinking and calcifying. If the lesion does not undergo spontaneous healing and the infection continues to the extent that symptoms arise, the person moves from the infected to the diseased state. This happens in about 5 % of infected people, when the dormant bacteria start to replicate. The caseous lesions undergo softening and liquefication, causing the bronchial wall to disintegrate and drain the liquid from the necrotic lung. A cavity is formed in this destroyed area of the lung, which contains large numbers of bacteria (more than 10^8 bacteria). The fibrous tissue which forms on the periphery of the cavity is unable to prevent liquefication which may continue for many years.

If the disease is untreated, leading to the infected area of the lung being converted to an empty shell of dense fibrous tissue, haemorrhaging occurs where a blood vessel becomes exposed. Further spread of the disease can involve almost all organs of the body (Fenton and Vermeulen, 1996).

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1.2. The world wide problem of tuberculosis

Tuberculosis is probably the most important plague of mankind. The World Health Organization (WHO) has estimated that a third of the world's population is infected with tuberculosis (WHO, 1997).

No other disease has been as prevalent and widespread over such a long period of time as tuberculosis. The slow progression of the disease resulted in it not being generally considered as an epidemic disease in many societies. For example, in Creighton's massive two volume publication, *A History of Epidemics in Britain*, published in 1890, tuberculosis was not mentioned as an epidemic.

The earliest evidence of tuberculosis in humans came from studies of the lesions caused by the uncommon manifestation of tuberculosis in the remains of skeletal bones of infected individuals. A skeleton of a Neolithic man with such lesions was found near Heidelberg in Germany, dating from approximately 5 000 before Christ (BC), and was suggestive of healed spinal tuberculosis although the possibility cannot be excluded that the lesions could have been caused by other factors. Skeletal remains from Egypt dating from 3 700 - 1 000 BC also show possible evidence of tuberculosis. The earliest definitive evidence of tuberculosis was found in Egypt in a mummy of a five year old child dating from 3 400 BC. Evidence of pulmonary and skeletal tuberculosis was supported by the presence of acid fast bacilli found in the vertebral bone.

Pulmonary tuberculosis is thought to have killed more people in the 19th century than typhus, scarlet fever, measles, smallpox and whooping cough combined. In France, one estimate claims that tuberculosis caused 9 million deaths in the 19th century, many more than that caused by war (2 million) and a cholera epidemic (400 000). In 1889, a quarter of all deaths in Paris were ascribed to tuberculosis. Although the incidence of and mortality caused by the disease in Europe has since declined, it is still a very big problem in other areas of the world including South Africa (Metcalf, 1991).

Tuberculosis is still the leading cause of death due to infectious diseases globally and the incidence is expected to rise over the next ten years due to the interaction between the tuberculosis and the human immunodeficiency virus (HIV) epidemics and the emergence of multi-drug resistance (WHO,

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1997). It is estimated that there will be 88 million new cases of tuberculosis, 8 million attributable to HIV between 1990 and 1999. Thirty million deaths are predicted for the same period of which 2,9 million are expected to be HIV infected. The estimates given above are conservative since the cases of tuberculosis are generally under-reported (Dolin *et al.*, 1994).

It is estimated that 8 million people became sick with tuberculosis in 1996, that someone is infected with tuberculosis every second and that up to 50 million people may be infected with multi-drug resistant tuberculosis (WHO,1997).

Pakistan has 210 700 new cases of tuberculosis annually of which only 25 % are ever diagnosed. Poor health facilities contribute to the increasing problem while more efficient diagnosis would probably not be useful as the disease would not be effectively treated after identification, since only one in seven physicians was able to prescribe effective treatment for tuberculosis.

India has approximately 900 million people in the country, of which 50 % are predicted to be infected with tuberculosis, leading to more than 2 million active tuberculosis cases and 500 000 deaths annually.

The high incidence of AIDS in Thailand contributed to the return of tuberculosis in a country where anti-tuberculosis drugs can be bought without prescription, allowing people to treat themselves without instruction or supervision, leading to an increase in multi-drug resistant tuberculosis cases. It is predicted that there are 436 500 new cases of tuberculosis annually in this country.

In the Philippines, approximately 22 million people are currently infected with tuberculosis of which about 270 000 have the disease. At present approximately 270 300 new cases of tuberculosis occur annually, which is one of the highest rates of spread of tuberculosis in the world.

Although China no longer has as large a problem as other countries, 1 038 200 new cases of tuberculosis currently occur annually. This figure is not expected to increase due to a cure rate of 98 %.

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Tuberculosis is prevalent in Brazil, Mexico, Russia, Ethiopia and Zaire where tuberculosis programmes and health care are non-existent or badly implemented. Tuberculosis rates in Russia are returning to levels of twenty years ago, leading to one of the highest mortality rates in Europe (WHO, 1997).

1.3. The tuberculosis problem in South Africa

The tuberculosis problem is largest in developing countries. It is predicted that the number of new tuberculosis cases in sub-Saharan Africa will double by the year 2 000.

Poor living conditions and overcrowding contributed to the spread of tuberculosis in southern Africa in the late 19th and early 20th century. The introduction of anti-tuberculosis drugs in the 1950's resulted in a sharp decline in tuberculosis death rates although the incidence rates continued to rise (Metcalf, 1991).

The tuberculosis epidemic in South Africa did not affect the working class evenly but largely affected black workers. It is argued that inefficient treatment programmes have produced a growing pool of half-cured, potentially infective cases which contribute to the rise of tuberculosis and the development of drug resistance (Packard, 1991). In portions of the Western Cape, where the health statistics are considered to be the most complete, the annual case rates are 682 per 100 000 people, more than twice the national average (Figure 1.1). South Africa has one of the highest recorded tuberculosis incidence rates in the world. It is thought that at least half of the 42 million people in the country are infected with tuberculosis, of which 10 % would normally develop tuberculosis. The high incidence of acquired immune deficiency syndrome (AIDS), especially amongst black communities, leads to much higher tuberculosis figures. This does not bode well for the future of tuberculosis control in South Africa where the health authorities predict a 10-20 percent yearly increase of tuberculosis due to the presence of HIV in the population (WHO, 1997).
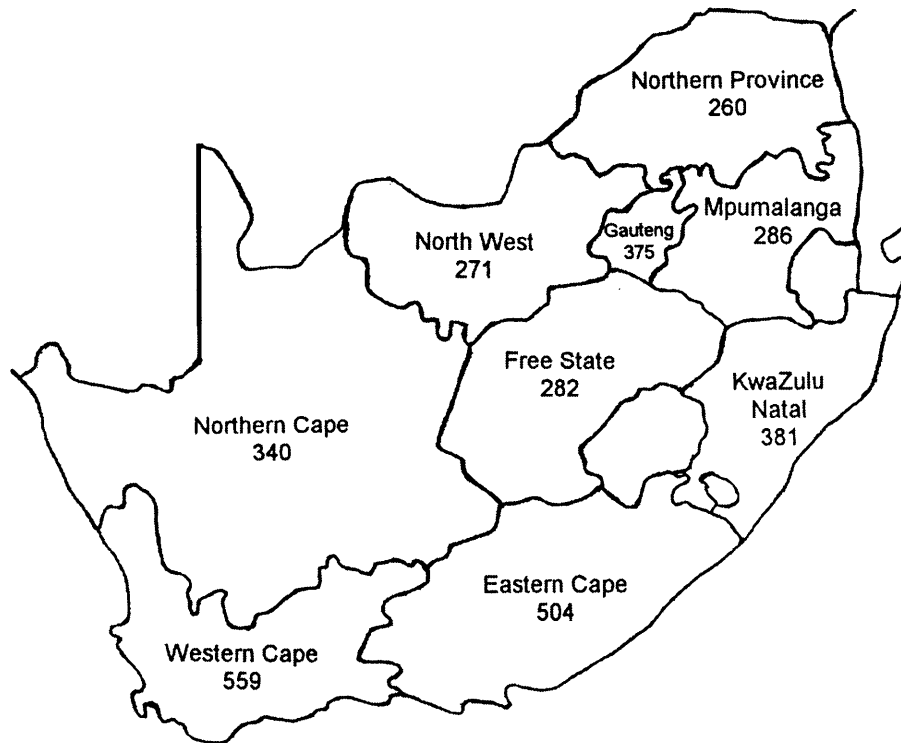
<u>Figure 1.1</u>   Estimated tuberculosis cases per 100 000 in South Africa (Singer, 1997).

1.4. Complicating factors of tuberculosis: MDR and HIV

The emergence of multi-drug resistant (MDR) strains of *Mycobacteria* has had a large effect on the increase of incurable tuberculosis cases. Drug resistance is magnified by patients who fail to take the chemotherapeutic drugs for the prescribed period. This allows the infecting *Mycobacteria* to survive, mutate and become drug resistant. Mycobacterial drug resistance was first recognised in 1946 after the introduction of streptomycin for the treatment of tuberculosis. When given alone, the drug would initially reduce the numbers of bacilli in the patient, resulting in an improvement of clinical symptoms. However, the bacilli would then start to increase and grow, unaffected by the drug. As a result of this phenomenon, streptomycin is now only given as a component of a combination of anti-tuberculosis drugs (Gangadharam, 1993).

In some African countries, it is estimated that 60% of tuberculosis sufferers are HIV positive (Efrat, 1995). Epidemiological studies have shown that HIV infection significantly increases the chances of reactivation of tuberculosis in people previously infected with *M. tuberculosis* and may also increase the chances of primary infection with *M. tuberculosis*. It has been observed that HIV infected people who become secondarily infected with *M. tuberculosis* often progress rapidly to clinical disease,

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usually involving multiple organs (Hopewell, 1993).

HIV affects blood monocytes and macrophages and may therefore, limit the initial non-specific response to *M. tuberculosis* particularly at later stages of HIV infection. The antigen presenting ability of the cells may also be impaired, reducing the ability to initiate an effective immunological response. The main cause of an impairment of resistance to *M. tuberculosis*, however, is most likely due to the influence of HIV on lymphocytes. A reduction in the numbers and functional impairment of the main HIV target, cluster of designation 4 positive ($CD4^+$) T-helper cells, will lead to a reduction in the response of these cells to mycobacterial antigens resulting in reduced clonal proliferation and limited interleukin 12 (by IL12) and interferon γ (by IFN γ) secretion. A reduction in these factors leads to a reduction in the specific activation of macrophages (IFN γ) and T-cell proliferation (IL12). HIV impairs the mechanisms by which new tuberculosis infection is contained, thereby increasing the risk of direct progression to the diseased state (Hopewell, 1993).

It is clear that tuberculosis can no longer be ignored by developed countries in the light of the emergence of AIDS and drug resistance (Gangadharam, 1993).

The rapid diagnosis of tuberculosis is essential, since it is one of the most important factors allowing prompt treatment of the disease and as such could lead to a reduction in the spread of the disease (Godfrey-Faussett, 1994). Testing of people in close contact with known tuberculosis-infected people is also important as it allows the early identification of infection, tracing and prevention of the further spread of tuberculosis. An important factor of a diagnostic test for tuberculosis is the cost, since in the poor developing countries of the world, where tuberculosis is a big problem, the finances available per person for medical treatment are extremely low. For example, the government of Zaire spends less than US$ 2 per person, per year, on health (WHO, 1997).

1.5. Diagnosis of tuberculosis

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The diagnosis of tuberculosis is hampered by various factors, which includes the slow replication of *M. tuberculosis*, which only divides once every 18-21 hours (Bates, 1994). The slow growth of the bacteria makes identification by culturing a long process which requires several weeks. The high degree of cross-reactivity of antibodies directed against Mycobacterial antigens, between various species of *Mycobacteria*, complicates both the detection of tuberculosis patient antibodies, as well as the use of antibodies to detect specific mycobacterial antigens in clinical samples. Reduced levels of antibodies due to tuberculosis, as observed in anergy, further complicates the use of serological methods for the diagnosis of tuberculosis.

Current diagnosis of tuberculosis is based on combined clinical assessment, chest radiographs (X-rays) and laboratory tests, since no single definitive, simple test is available for diagnosis (Bloomfield, 1995).

Chest X-rays are widely used by physicians in suspected cases but in children and HIV patients with other side effects, diagnosis is difficult. Normal chest radiographs have, for example been obtained from HIV-seropositive patients with confirmed pulmonary tuberculosis. Other infections and lung fibrosis may also give similar radiographs to those obtained in tuberculosis patients. Current or previous infection are also indistinguishable (Godfrey-Faussett, 1994).

Three general approaches exist for the detection of tuberculosis:

- Direct identification of *M. tuberculosis* by culture and microscopy (Bloomfield, 1995).
- Detection of the host's response to the infection.
- Detection of constituents of the bacillus itself in clinical samples (Godfrey-Faussett, 1994).

1.5.1. Direct identification of *M. tuberculosis*

1.5.1.1. Culturing

Conventional media for culturing sputum samples include solid (Lowenstein-Jensen (LJ)) or liquid (Middlebrook 7-H-10) media. Culturing is highly specific but it may take from 3-6 weeks to grow *M. tuberculosis* and a further 3-6 weeks are required for drug susceptibility testing.

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Treatment is usually begun long before these test results are received for patients with suspected tuberculosis.

Two commercial culturing methods are available which provide faster results than conventional culturing with a higher detection rate.

The Bactec system which is used world-wide, automatically measures the production of radiolabelled CO_2 by *Mycobacteria* when grown in liquid medium containing ^{14}C - labelled palmitic acid and can identify *M. tuberculosis* growth in approximately 12 days.

The MB-Check system makes use of a biphasic culture system in liquid medium and is a less expensive alternative but requires longer culturing time than Bactec (Bloomfield, 1995).

The fastest and most reliable detection system for *M. tuberculosis* combines these "fast" culturing methods with the use of nucleic acid probe-based identification from culture (Doern, 1996).

1.5.1.2. Microscopy

The most rapid conventional method of detecting *M. tuberculosis* requires special staining of sputum smears and analysis of the smears under a light microscope. The fuchsin-based Ziehl-Nielsen staining technique forces the stain into the cells by heat treatment followed by treatment with acid. *Mycobacteria* retain the dye due to their thick cell walls and pink bacilli can be seen, they are therefore, known as acid-fast. An alternative method of staining exists using auramine, a fluorescent dye requiring fluorescent microscopy under UV light for analysis.

The advantage of microscopy is that it is inexpensive and fast, allowing tentative results to be available on the same day as the sample is submitted. The technique is, however, not very sensitive, with positive smear tests being useful for tentative diagnosis but negative tests not indicating the absence of disease (Bloomfield, 1995). Although species may vary in

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morphology, microscopy is not sufficient for species differentiation. Diagnosis is often made more difficult in patients with HIV infection, due to the presence of both *M. tuberculosis* and *Mycobacterium avium (M. avium)* (Godfrey-Faussett, 1994).

1.5.2. Detection of host response

Most attempts to detect the host's response to tuberculosis infection have centred on the reactivity of patient antibodies against various *M. tuberculosis* culture medium extracts or purified antigens. Others have concentrated on cytokines generated by the cellular immune response of the host to *M. tuberculosis*.

1.5.2.1. The immunology of tuberculosis

Macrophages which play an important role in generating an immune response, are activated in response to contact with *Mycobacteria* in a non-antigen-specific manner. Tumour necrosis factor (TNF) appears to mediate the movement of circulating macrophages, i.e. monocytes, into the tissues at the site of infection. These aggregation to form granulomas in which some of the cells fuse to form giant cells (Daniel and Ellner, 1993). Macrophages phagocytose, process and present antigens to T-lymphocytes (T-cells) in conjunction with major histocompatibility molecules (MHC) and other antigen presenting molecules (CD1). Helper type T-lymphocytes are activated in an antigen-specific manner in response to IL1, secreted by the macrophages. IL1 appears to stimulate IL2 production by T-cells which in turn stimulates T-cell proliferation. Activated T-cells release lymphokines (including IFN γ) resulting in positive feedback that increases the reactivity and differentiation of macrophages, leading to antigen-specific granuloma formation. T-helper cells furthermore activate the B-cell dependent humoral, DTH and cytotoxic T-cell immune responses in an antigen-specific manner. This leads to differentiation of the B-cells into plasma or antibody producing cells (Edwards and Kirkpatrick, 1986).

B-lymphocyte mediated humoral responses to mycobacterial antigens, occur in tuberculosis patients, although they have no clear role in disease pathogenesis. Pathogenesis is almost

exclusively determined by the host T-lymphocyte-mediated cellular immune response. Antibodies to mycobacterial antigens have been repeatedly demonstrated in tuberculosis patient sera, but these studies have not contributed significantly to the understanding of the disease or to the development of a serodiagnostic test (Daniel and Ellner, 1993).

Cell-mediated immunity, especially DTH, has been shown to be important in protective immunity to tuberculosis, with the critical cell type being T-lymphocytes. The protective immune response is dependent on both CD4 and CD8 T-lymphocytes of which CD4 cells provide a more sustained effect. The ability of these cells to provide protection against various species of *Mycobacteria* suggests that the protective immune response is directed against cross-reactive antigens and is thus important in natural acquired immunity and vaccination. $\gamma\delta$ T-cells have recently been shown to proliferate in response to mycobacterial antigens and may play an important role in the initial immune response, particularly to antigens shared between species and may represent an intermediate response between non-specific and specific immune responses. Healthy *M. tuberculosis*-infected people with a positive PPD skin test, are relatively immune to reinfection and their T-cells have been used in studies to determine the nature of "protective T-cells". Exposure of the peripheral blood mononuclear cells of the individuals to live *M. tuberculosis* (H37Ra) leads to selective expansion of $\gamma\delta$ T-cells while heat killed or soluble protein antigens stimulate $\alpha\beta$ T-cells expansion (indicating the presence of memory). Stimulation of $\gamma\delta$ T-cells by live bacteria and the ability of live *M. tuberculosis* organisms to induce protective immunity while killed vaccines were only able to provide non-specific resistance and DTH, indicate the importance of $\gamma\delta$ T-cells in protection against tuberculosis (Daniel and Ellner, 1993).

1.5.2.2. Tuberculosis and anergy

Anergy is the inability of the infected person to mount an immune response, despite the presence of antigen. It is commonly observed in untreated tuberculosis patients in the initial stages of infection. However, during treatment of the disease, reactivity to mycobacterial antigens returns. Reduced responsiveness to mycobacterial antigens has been noted from

various geographic areas and seems independent of prior immunisation with the generally used anti-tuberculosis vaccine, Bacille Calmette Guerin (BCG). Several studies have shown mycobacterial-antigen-specific anergy, as the immune responses to non-mycobacterial antigens are intact. Antigen-specific and/or non-specific anergy during active pulmonary tuberculosis may be caused by the lack of T-cell response to mycobacterial antigens which may be due to the way the molecules are presented to the lymphocytes (Schwartz, 1993). T-cell dysfunction, suppression of T-cells by other cells or by-products of *M. tuberculosis* (Rich and Ellner, 1994) or the absence of co-stimulatory signals could contribute to anergy (Janeway and Travers, 1994).

The significance of T-cell suppression (anergy) may play a role in the pathogenesis of reactivated tuberculosis or may minimise possible negative effects due to a local infection (such as septic shock) (Rich and Ellner, 1994). Blood lymphocytes of patients with active tuberculosis have reduced expression of IL2 upon stimulation with purified protein derivative (PPD - a mixture of *M. tuberculosis* antigens used in tuberculin skin tests), while IFN γ production and blastogenesis by blood mononuclear cells are also reduced. These cytokine levels have been shown to return to normal during treatment (Edwards and Kirkpatrick, 1986).

The detection of the host response against tuberculosis encountered several difficulties which have prevented the development of a definitive diagnostic test based on this approach. The majority of mycobacterial species are saprophytes found in the environment. Prior contact with environmental *Mycobacteria* could generate memory cells that may direct the immune response to common mycobacterial antigens, upon subsequent *M. tuberculosis* infection, rather than to species-specific antigens. Prior contact would cause an accelerated response to common antigens and may suppress the formation of species-specific antibodies. One study showed that the antibody response of patients varied considerably for three different mycobacterial glycolipid antigens and may indicate a genetic predilection to produce antibodies to species-specific or common epitopes (Grange, 1984).

Detecting the hosts immune response to crude antigen preparations may be complicated by the

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presence of shared epitopes with other environmental species of *Mycobacteria*. The usefulness of a particular antigen may be hindered by the fact that the antigen may not be expressed to the same degree by the organism, in all patients. Therefore, patients may not have the same level of immune response to the antigen, or any response at all.

Antibody responses may persist for long periods, making it difficult to distinguish between current and old infection. The phenomenon of anergy in untreated tuberculosis patients would result in false negative results in 17-25% of patients with active tuberculosis, who are unresponsive to PPD skin tests (Rich and Ellner, 1994). A similar problem is encountered for HIV-infected individuals that may lack specific B-cell responses or polyclonal B-cell activation, rendering this kind of test of little use (Godfrey-Faussett, 1994).

Despite the various problems encountered in the detection of the host response to tuberculosis, it is the most useful approach for the detection of latent tuberculosis infection, which excludes culturing due to the low numbers of bacteria present in circulation. The mechanism by which latent tuberculosis infection is contained is not well understood, but clearly involves cell-mediated immunity (CMI), which can be detected with skin tests (Hopewell, 1993). Cytokine profiles and anti-tuberculosis antibody levels of individuals infected with tuberculosis (either latent or active) differ from those of uninfected people and could be used to detect infection.

Improvements in the specificity of assays detecting the host response to tuberculosis are taking place, increasing the possibility of a fast, inexpensive tuberculosis detection test.

1.5.2.3. Tuberculin skin test

This is the oldest of the tuberculosis tests, developed about 100 years ago. It is still the most frequently used method of detecting latent infection, when the amount of bacteria in the body are much lower than in active tuberculosis. The tuberculin test is of little value in diagnosing active tuberculosis due to the high rate of false-positive results, mostly as a result of BCG vaccination.

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The test is based on injecting *M. tuberculosis* antigens intradermally. Patients who have been exposed to *M. tuberculosis* develop a skin reaction caused by the cell-mediated immune response which is elicited and forms an induration and inflammation in a delayed-type hypersensitivity reaction.

Two types of antigens are used, namely old tuberculin which consists of a heat treated preparation of medium from human or bovine *M. tuberculosis* cultures and purified protein derivative (PPD) which is prepared by either ammonium sulphate or trichloracetic acid precipitation of culture filtrates.

Two types of skin tests are generally performed, the Montoux Test where antigens are injected intradermally, usually in the forearm and the multiple puncture test where four or more needles covered in antigen are pressed firmly into the skin. The amount of tuberculin administered with the latter method cannot be precisely controlled which makes interpretation of the reaction size difficult (Friedman, 1994).

Interpretation of the results of skin tests is complex, due to factors such as medical history and risk of contact with active tuberculosis, which must be considered. BCG immunised individuals for example, may be indistinguishable from individuals with active tuberculosis. A further limitation of the test is in HIV infected individuals, who develop anergy (depressed cell-mediated immune responses) and as such a negative test result does not exclude the possibility of infection (Bloomfield, 1995). During early HIV infection, the tuberculin skin test may show some reactivity which decreases to very low, or no reactivity as HIV infection progresses and can be an indication of the stage of HIV infection. Control antigens such as from *Candida*, mumps virus or tetanus toxoid should therefore, be included in the skin test to determine if a negative test result is due to immunosuppression or a truly negative result (Hopewell, 1993).

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1.5.2.4. Serological tests detecting tuberculosis patient antibodies

A serological tuberculosis test, as used in most diagnostic tests for infectious diseases, would have considerable potential for the diagnosis of tuberculosis, especially where conventional means of diagnosis fail, as for smear-negative patients and especially for extra-pulmonary disease. The prerequisites for an ideal diagnostic test could be fulfilled with a serological test such as enzyme-linked immunosorbant assay (ELISA), as it is inexpensive, reliable, easy to read, simple and can be performed rapidly without requiring expensive equipment (Bothamley, 1995).

Some of the problems which are encountered with the use of a serodiagnostic test are that most people infected with tuberculosis do not suffer from active disease and as such the presence of antibodies does not necessarily indicate the presence of active disease. BCG-vaccinated individuals may have cross-reactive antibodies but no tuberculosis infection, and since BCG vaccination was widely applied in developing countries such as South Africa, this may limit the efficacy of such a test in many countries. In addition, *M. tuberculosis* antigens which may be present in the blood of infected individuals, may interfere with assays based on antibodies (Bloomfield, 1995).

Various serological methods have been applied to the diagnosis of tuberculosis without much success, including complement fixation, agglutination of *M. tuberculosis*, haemagglutination, haemaggregation, precipitation and gel diffusion, fluorescent antibody tests and radio-immunoassay. Although the majority of the methods showed a high degree of positive results for confirmed tuberculosis patients, the numbers of false positive and negative results were unacceptably high. High cost and radiation hazard of methods such as radio-immunoassay also reduced the suitability of this approach to diagnosis (Grange, 1984).

Several serologically based tests which are inexpensive and produce results within one day, are on, or approaching the market. InflaZyme is an ELISA based test designed to detect antibodies which recognise a synthetic structural analogue of the natural glycolipid, cord

factor, a major mycobacterial cell wall component (6,6' di-o-mycoloyltrehalose). Leuko Scan is an imaging agent that detects infected areas by disclosing accumulation of white blood cells. MycoAKT can differentiate between *M. tuberculosis*, *M. avium* and *M. kansasii* cultures obtained from clinical samples. It uses monoclonal antibodies to detect an intracellular protein of *M. tuberculosis* and specific phosphoglycolipids of *M. avium* and *M. kansasii* in cultures and takes approximately 1,5 hrs to complete. MycoDOT is a simple, rapid test which detects antibodies to the lipoarabinomannan (LAM) antigen of *Mycobacteria*, in serum or whole blood within 20 minutes. The LAM used in the kit is 97-98% pure and highly immunogenic. Both extra-pulmonary and pulmonary tuberculosis can be detected by the test (Bloomfield, 1995). The MycoDOT test is the fastest of these tests and only requires that the LAM coated comb is incubated in diluted serum, followed by washing and incubation in signal generating reagent and is therefore, the most suitable method for use in developing countries, as no additional equipment and no specimen culturing from patient samples is required.

1.5.2.5. Detection of latent *M. tuberculosis* infection

CSL is an interferon gamma test developed in Australia that measures the amount of IFN γ released by T-cells in response to *M. tuberculosis* proteins. Higher amounts of the cytokine are produced by people previously exposed or currently infected with the organism than in unexposed individuals. Only one visit and one blood sample is required for testing, producing results within one day. The test is designed to replace the Montoux test and has shown 97% agreement with the conventional test. Antigens specific for *M. avium* and *M. bovis* are included in the kit (Bloomfield, 1995).

1.5.3. Detection of *M. tuberculosis* components in clinical samples

Tests based on the detection of the mycobacterial cell components have concentrated either on nucleic acid probes, often using the polymerase chain reaction (PCR) or on the detection of either cytoplasmic, secreted or structural antigens (Godfrey-Faussett, 1994).

1.5.3.1. Nucleic acid based tests

Nucleic acid based tests detect specific sequences in genetic material, (ribonucleic acid (RNA) or deoxyribonucleic acid (DNA)) which are specific to an infectious organism. The advantages of these tests are that they are highly specific, do not require the presence of viable or whole *Mycobacteria* and provide more rapid results than conventional culturing, directly from clinical samples or from cultures of clinical samples. These diagnostic tests are, however, more expensive than conventional or serological methods and require considerable technical training.

Genetic probes are short segments of DNA or RNA complementary to specific target sequences of the specific *Mycobacterium* species, labelled with chemiluminescence, fluorescence or colourimetric markers, which are used to detect the binding of the probes to the pathogen nucleic acids. Genetic probe tests are not extremely sensitive and require 10^6 organisms for detection, and as such are mainly used in conjunction with conventional culturing (Bloomfield, 1995). The presence of inhibitors as well as very low levels of *M. tuberculosis* can contribute to the lack of sensitivity (Nightingale, 1996). In an attempt to make the procedure more sensitive, targets in RNA, particularly ribosomal RNA (which cells have many copies of) or repeated sequences within the DNA are commonly used. The Gen-Probe and the SNAP probe systems which make use of ribosomal RNA targets are highly specific (Bates, 1994), but should be performed in conjunction with conventional culturing methods to confirm the absence of tuberculosis in the case of a negative result (Nightingale, 1996).

DNA fingerprinting makes use of DNA isolated from cultures of clinical samples, analysed by restriction fragment-length polymorphism (RFLP). The DNA is digested with restriction enzymes, separated by agarose electrophoresis, according to size and Southern-blotted onto a membrane. The products are detected by hybridisation with specific, labelled DNA probes. The pattern obtained is characteristic of the organism and even the specific isolate of the organism. The most commonly used method, targets a putative sequence designated IS6110,

which is found in multiple copies in the DNA of *M. tuberculosis* and is able to move about within the chromosome, resulting in each unrelated wild isolate having a different number of copies and/or positions for the sequence (Bates, 1994). This technology is particularly important in epidemiology studies as described by Van Soolingen, Herman and co-workers (Van Soolingen *et al.*, 1991) and to follow infection by specific isolates to investigate strain diversity of the *M. tuberculosis* epidemic in Cape Town (Warren *et al.*, 1996). A potential application of this technique could be the ability to treat a newly infected person with the most effective drugs for the particular bacterial clonal pathogen that is identified as a known drug-sensitive isolate, without the need for further drug susceptibility tests.

Nucleic acid amplification produces multiple copies of sequences from clinical samples and includes three main steps, namely extraction of nucleic material, amplification of the material by the polymerase chain reaction (PCR) and detection of the amplification products. Commercial polymerase chain reaction methods, using non-radioactive probes are available for detection of tuberculosis. The polymerase chain reaction amplifies copies of DNA, allowing detection of either DNA or RNA (after reverse transcription) by hybridisation with labelled probes in samples of pleural fluid, bronchial washes, biopsies and blood samples. The technique involves denaturation of double-stranded DNA or reverse transcription of RNA, annealing of specific complementary primers and amplification of the regions between primers, using heat-stable polymerases. The PCR methods have the potential for automation and the added advantages of speed, sensitivity and specificity, but require a higher level of technical expertise than most diagnostic tests and are expensive. Studies have shown a large amount of variation between laboratories using PCR on identical samples. Further problems encountered are contamination and carry-over from other samples, producing false-positive results. These aspects seem to indicate that the PCR methods are likely to be limited to specific situations and not to routine laboratory diagnosis (Bloomfield, 1995).

The Amplicor *Mycobacterium* system is based on the PCR amplification of a 584 bp segment of 16S rRNA (Doern, 1996) using biotinylated primers. Amplified DNA is detected in a standard microtitre plate by hybridization with species-specific probes for *M. tuberculosis*,

M. avium and *M. intracellulare* in a final colourimetric reaction (Ichiyama *et al.*, 1996). The method takes 6 hours to complete and requires a thermocycler. The Gen-Probe Amplified *Mycobacterium tuberculosis* Direct testing system (AMTD) takes 4 hours and is based on the transcription-mediated amplification of a 16S rRNA target sequence via DNA intermediates and does not require the use of a thermocycler (Doern, 1996). The amplified rRNA sequence is detected by hybridization with a chemiluminescently labelled probe and read in a luminometer (Ichiyama *et al.*, 1996). The sensitivity of these two methods is high and it has even been considered that they may be more sensitive than conventional culturing which has thus far been used as the definitive test for *M. tuberculosis*. The cost is a disadvantage, at approximately US $ 27 per sample, which is too high for use in developing countries or for routine screening (Doern, 1996).

Another nucleic acid amplification method that is being developed, makes use of ligase chain reaction amplification of target nucleic acid segments. It amplifies smaller DNA regions by employing a DNA ligase which binds two complementary, labelled probes after they bind to adjacent areas on the organism's DNA and utilises the ligated product as the new template for amplification. The method is faster than conventional PCR and in some cases has a higher specificity. It is especially useful in cases where the target DNA is damaged (Bloomfield, 1995). A further advance towards improving the sensitivity of PCR called "sequence capture-PCR", on clinical samples was developed by Mangiapan and co-workers, involving the specific capture of mycobacterial DNA from clinical samples prior to amplification, thereby concentrating the target DNA and removing contaminating DNA (Mangiapan *et al.*, 1996).

A number of other nucleic acid amplification techniques are being investigated, all with the advantage of providing results within one day with a high specificity and sensitivity but at a high cost and requiring sophisticated infrastructure and technical training of operators.

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1.5.3.2. Structural components

Gas chromatography and mass spectrometry have been used to detect mycobacterial cell-wall constituents such as tuberculostearic acid but are cumbersome and require expensive equipment. High performance liquid chromatography (HPLC) has been used to detect mycolic acids in cultured clinical samples from, for example, sputum and is helpful with species identification. These methods require technologically advanced equipment and therefore, a test based on these techniques is of little use in developing countries (Godfrey-Faussett, 1994).

1.6. Immunogenicity of mycolic acids

Mycolic acids are long chain fatty acids which constitute up to 40 % of the cell-wall of *Mycobacteria* (Petit and Lederer, 1984) and vary structurally between different mycobacterial species. Mycolic acids form part of trehalose-6-6'-dimycolate or cord factor which has been shown to be immunogenic, as a number of researchers found antibodies directed against cord factor in human tuberculosis patients (Kato, 1972) and have discussed the possibility of utilising them in an ELISA diagnostic test (He *et al.*, 1991 and Maekura *et al.*,1993). Mycolic acids were mainly considered useful for the identification of different *Mycobacteria* species (Butler, 1985) but not particularly useful for diagnosis, prevention or treatment of tuberculosis. This opinion was partly due to the assumption that mycolic acids would, like other lipids, have very weak and/or limited immunogenic properties due to the aliphatic structure of the molecule and the absence of aromatic groups.

The recent discoveries that mycolic acids, presented on CD1 molecules, are able to stimulate double negative (DN) T-cells proliferation (Beckman *et al.*,1994) have shown that mycolic acids may prove to be more important in the fight against tuberculosis than anticipated. Furthermore, it was shown that not all mycolic acids were recognised by the DN T-cells, suggesting that the CD1 system is able to distinguish between differences in lipid structure (Jullien *et al.*, 1996). An immune response to mycolic acids presented on CD1 may therefore, be able to distinguish between species of *Mycobacteria* on the basis of their differing mycolic acids.

The International Patent Application No PCT/JP89/01341 relates to a method for the identification of antibodies directed against mycolic acids, their salts or esters for the detection of infection by acid fast bacteria and was the first approach described where these molecules were to be used for the detection of tuberculosis.

The proposal presented by Dr Sandra Bye to Adcock Ingram Critical Care, for a diagnostic kit utilising anti-mycolic acid antibodies, was forwarded to Prof J.A. Verschoor for evaluation. The method proposed the immunisation of rabbits with partially purified mycolic acids conjugated to a carrier, with the aim of producing antibodies directed against mycolic acids. These antibodies would then be used to detect the presence of *M. tuberculosis* in human tuberculosis clinical samples.

Since the mycolic acids used were only semi-purified, and the immunogenicity of fatty acids such as mycolic acids is generally considered to be low, the potential for isolating mycolic acids-specific antibodies using the proposed method was questioned.

In an attempt to validate and improve the methods used by Dr Bye, the chemical and biological properties of mycolic acids were investigated, with the aim of producing a tuberculosis diagnostic kit based on the detection of mycolic acids. Firstly a method for the large scale purification of mycolic acids had to be developed. Purified mycolic acids were then to be used for the preparation of conjugates to immunise mice with, in an attempt to produce anti-mycolic acids antibodies.

Chapter 1

Chapter 2:
Purification of mycolic acids from
Mycobacterium tuberculosis

2.1. Introduction:

2.1.1. The mycobacterial cell wall

The mycobacterial cell wall, like that of most other bacteria, contains the compound peptidoglycan which is the polymer conferring rigidity to the cell and determining its shape. Peptidoglycan is made up of alternating residues of N-acetyl-D-glucosamine and N-acetyl-D-muramic acids. The separate chains are cross-linked to varying degrees by short peptide bridges. The peptide bridges consist of four amino acids attached to the lactyl moiety of the muramic acid residues. The amino acids are arranged in a characteristic sequence of L-alanyl, D-glutamyl, diaminopimelic acid (in *Mycobacteria*) and D-alanine (Dawes and Sutherland, 1976).

The muramic acid residues in *Mycobacterium* and *Nocardia* are N-glycosylated while in all other bacteria they are N-acetylated. Polysaccharide side chains that are esterified at their distal ends with mycolic acids, are linked to the peptidoglycan layers (Brennan and Nikaido, 1995).

The average composition of the cell wall throughout the genus of *Mycobacterium* is between 20 - 40 % mycolic acids and 30-40 % neutral sugars which include D-arabinose and D-galactose in a ratio of 5:2 (Petit and Lederer, 1984).

Mycobacteria contain normal saturated fatty acids, mostly palmitic acid, which are rapidly incorporated into triglycerides. Normal C_{16}, C_{18}, C_{24} and C_{26} fatty acids exist as esters and provide the hydrophobic moieties of phospholipids, glycolipids and lipoproteins. These moieties are essential for the structure and function of the cytoplasmic membrane and cell envelope and are thought to be the precursors of mycolic acids (Takayama and Qureshi, 1984).

2.1.2. Mycolic acids

Mycolic acids are high molecular weight, α-branched, β-hydroxy fatty acids that are present in all *Mycobacteria* and can be divided into three catagories, varying between families and species (Merck Index, 1989).

1 - corynomycolic acids - found in *Corynebacteria* (C_{28} - C_{40})

2 - nocardic / nocardomycolic acids - produced by *Nocardia* (C_{40} - C_{60})

3 - mycobacterial mycolic acids (C_{60} - C_{90}).

Mycobacterial mycolic acids are covalently bound to arabinogalactan by means of ester bonds. They appear mostly as tetra-mycolyl penta-arabinosyl clusters (see Figure 2.1) and as extractable lipids such as trehalose 6, 6' - dimycolate (TDM). There is evidence that two-thirds of the available 5-OH groups of the cell wall penta-arabinosyl residues, act as attachment points for the mycolate residues (McNeil *et al.*, 1991).

Most of the hydrocarbon chains of mycolic acids assemble to form an exceptionally thick asymmetric bilayer. It is suggested that the inner bilayer has an exceptionally low fluidity that increases to the outer surface. Differences in mycolic acid structures may affect the fluidity and permeability of the cell wall and may explain different levels of sensitivity of various mycobacterial species to antibacterial agents, disinfectants, alkali and to drying (Brennan and Nikaido, 1995). Mycolic acids also occur as components of soluble trehalose dimycolate or cord factor and trehalose monomycolate in the cellular matrix (Besra *et al.*, 1994).
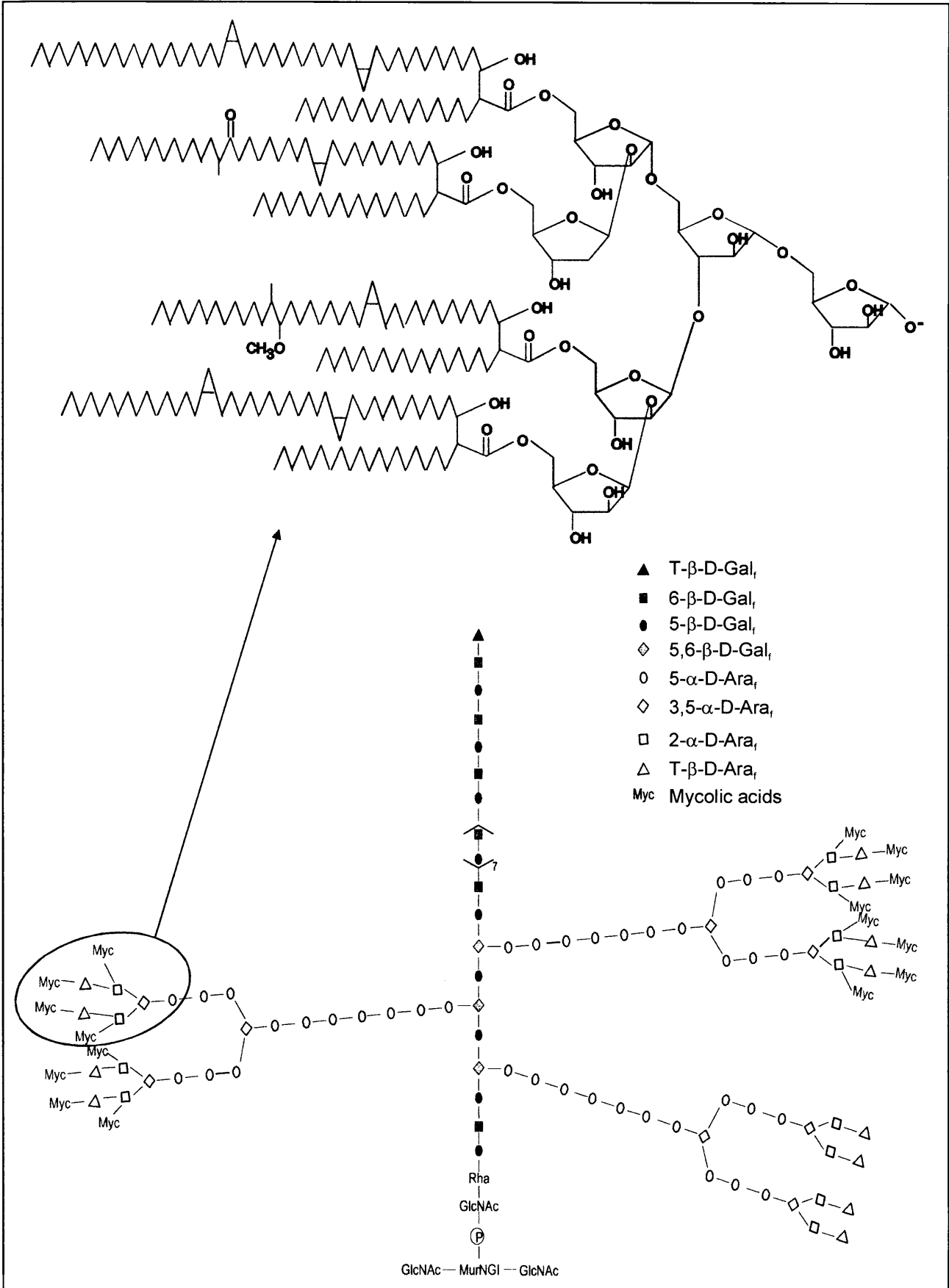
Figure 2.1 Mycolic acids bound via arabinogalactan to the muramic acids of peptidoglycan,

T = terminal (Brennan and Nikaido, 1995).

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Mycobacterial mycolic acids are distinguishable from other mycolic acids as:

a)   they are the largest of the mycolic acids;

b)   they have the longest α branch (shorter branch) [C_{20} - C_{25}];

c)   the main chain or meromycolic acid contains one or two chemical groups that are capable of

producing bends in the molecule, namely double bonds or cyclopropane rings;

d)   they may contain additional oxygen groups other than the β OH groups;

e)   they may have methyl branches in the main carbon backbone (Brennan and Nikaido, 1995).

M. tuberculosis mycolic acids can be divided into three groups namely α - mycolates, ketomycolates

and methoxymycolates (see Figure 2.2).
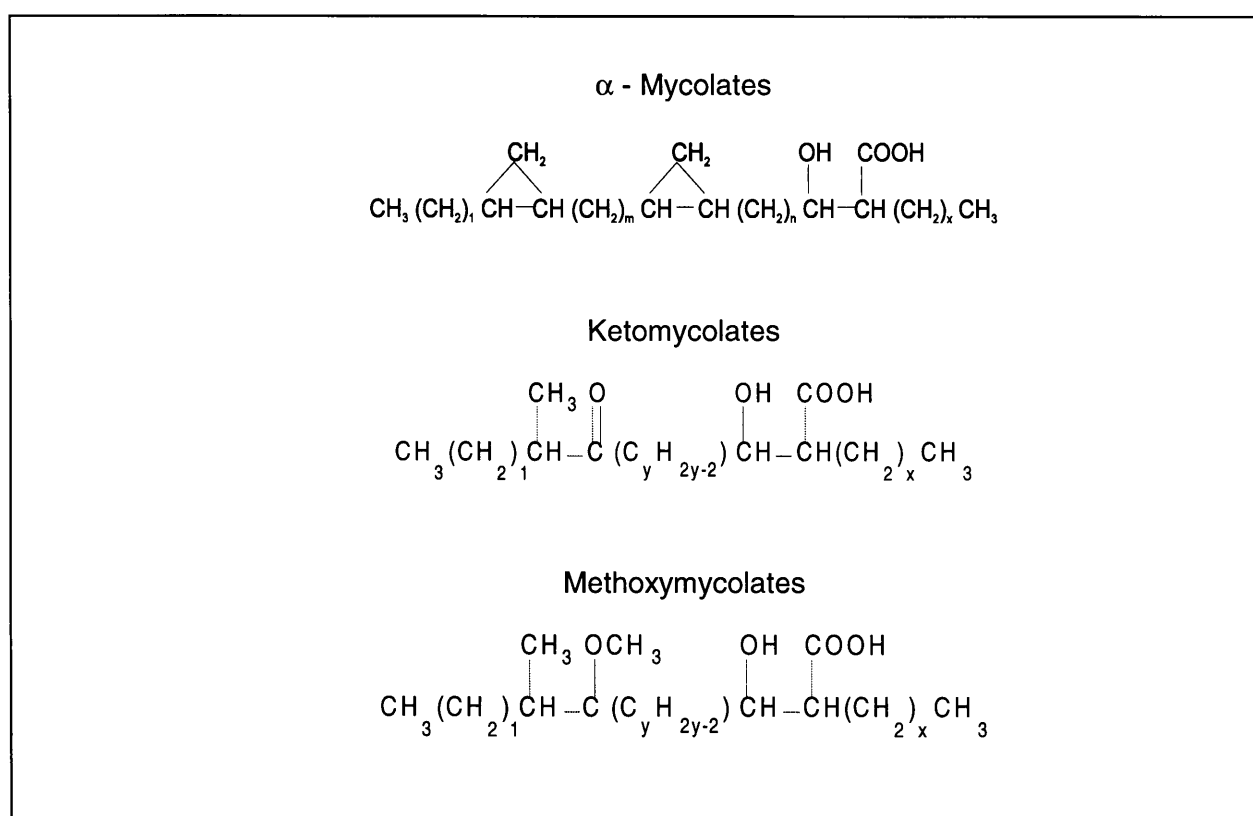
Figure 2.2   The general structure of the three *M. tuberculosis* mycolic acid groups

(Minnikin *et al.*, 1984).

2.1.3. Chromatographic analysis of mycolic acids

2.1.3.1. Thin layer chromatography

Thin layer chromatography (TLC) of mycolic acids allows the separation of the three subclasses

of mycolic acids i.e. α-mycolates, ketomycolates and methoxymycolates, using a silica gel thin-

layer plate. The mycolic acids spotted on the plate are developed in *n*-hexane-diethyl ether (80:20 v/v) solution and the mycolic acid methyl esters located with iodine vapour. Each subclass can be recovered from the thin layer plate with chloroform. TLC analysis of a variety of *Mycobacterium* species separated in this manner is shown in Figure 2.3 (Kaneda *et al.*, 1986).
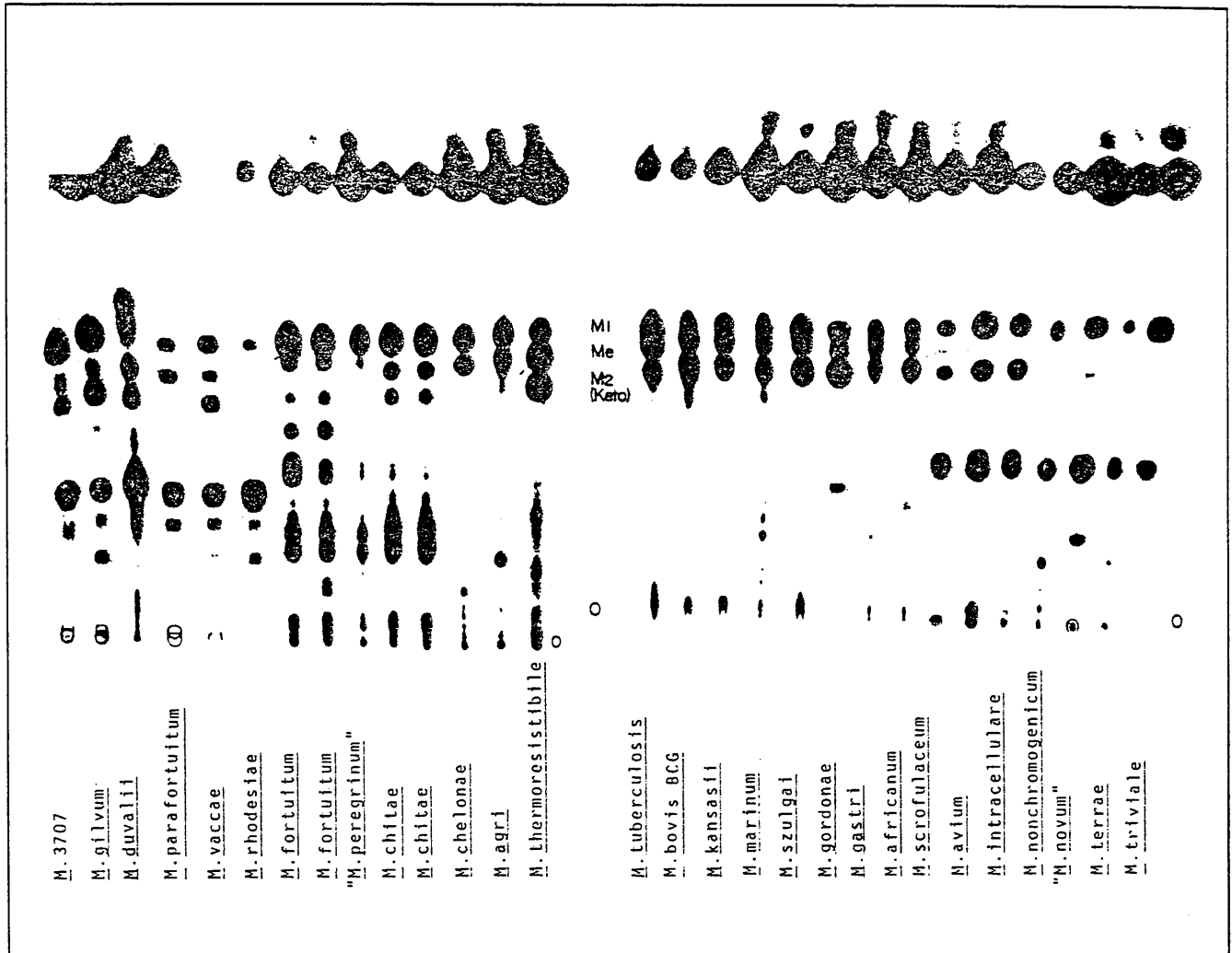
Figure 2.3   Thin layer chromatographic separation of the various groups of mycolic acids of a number of species of *Mycobacteria* by TLC (Kaneda *et al.*, 1986).

M1 marks the alphamycolates while Me represents the methoxymycolates and M2 the ketomycolates in *M. tuberculosis*.

2.1.3.2. High performance liquid chromatography

A complex mixture which includes mycolic acids can be extracted from the bacterial cells by saponification at a minimum temperature of 85°C for 60 minutes but autoclaving is suggested for safety reasons. Saponification involves the breaking of the ester bonds between the mycolic acids and the arabinose moiety by the strong alkali, potassium hydroxide, upon heating as shown in Figure 2.4.
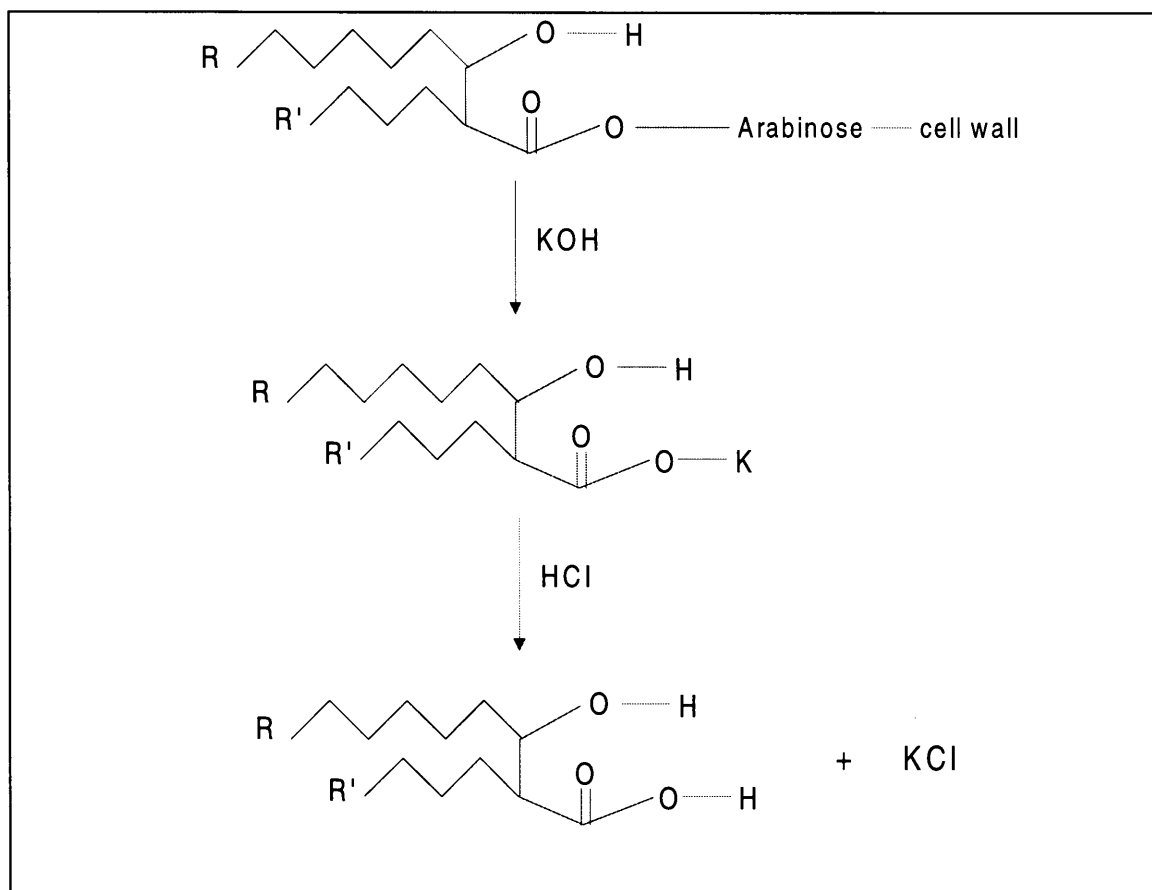
__Figure 2.4__   Saponification of the ester bond binding mycolic acids to the arabinose of the cell wall.

An adjustment of the pH is necessary to convert the carboxyl anion to a less polar COOH moiety which then becomes extractable in chloroform. After saponification and extraction of mycolic acids from *Mycobacteria*, they are derivatised to convert them (and any other fatty acids present in the mixture) to UV-absorbing bromophenacyl esters which are detected at 260 nm. The derivatisation process is rapid, producing pure derivatives without interfering or unwanted by-products. The first step of derivatisation, as described by Butler, involves the addition of potassium bicarbonate, which provides potassium ions, which along with a catalyst, dicyclohexyl-18-crown-6 ether are necessary for the reaction to take place. Crown ether

enhances the solubility of the potassium ion in the organic chloroform solvent, thereby

increasing the reactivity of the carboxylated anion with the p-bromophenacyl bromide,

producing p-bromophenacyl esters (Butler, 1985). This derivatisation method is highly selective

for the analysis of mycolic acids and other chloroform soluble fatty acids with free carboxyl

groups as shown in the diagram adapted from Butler in Figure 2.5.
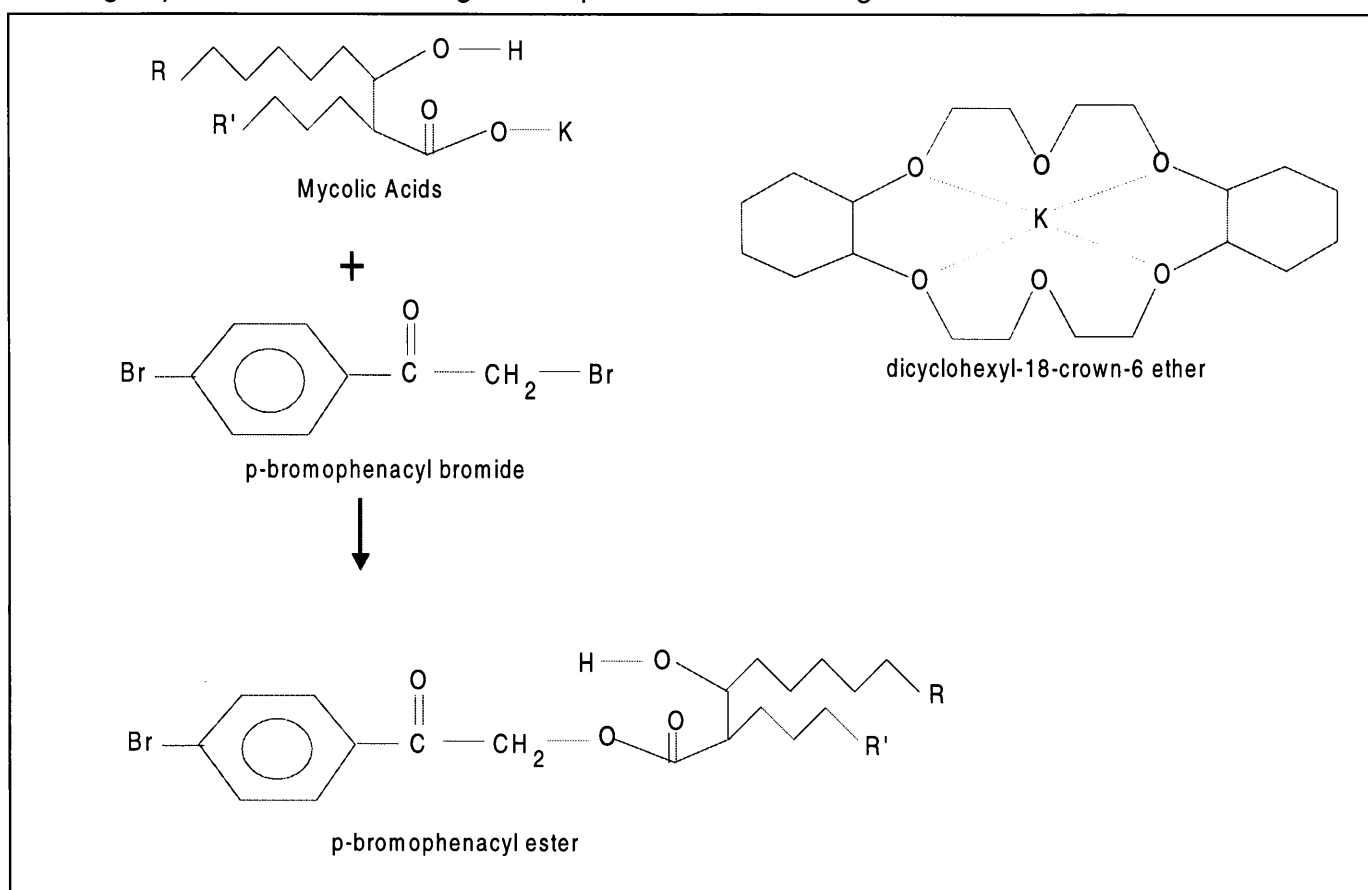
Figure 2.5   The derivatisation reaction of fatty acids including mycolic acids for HPLC analysis

(Butler, 1985).

Separation of mycolic acids with different functional groups by adsorption chromatography as

p-bromophenacyl esters, using high performance liquid chromatography (HPLC), was initially

demonstrated by Steck *et al.*(1978), using a silica column. The later use of a C-18 column

made it possible to separate the functional groups into what was then thought to be their

individual chemical entities by reverse phase chromatography. The HPLC profiles of the

mycolic acids of various *Mycobacteria* were determined and it was noted that the peaks

represented unresolved mixtures of various mycolic acids (Butler *et al.*, 1991). The species-

specific variations in the HPLC profiles (produced by combinations of the different functional

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groups and lengths of the carbon chains) proved to be chemotaxonomically significant. The

HPLC profiles of the mycolic acids of a few mycobacterial species are shown in Figure 2.6.
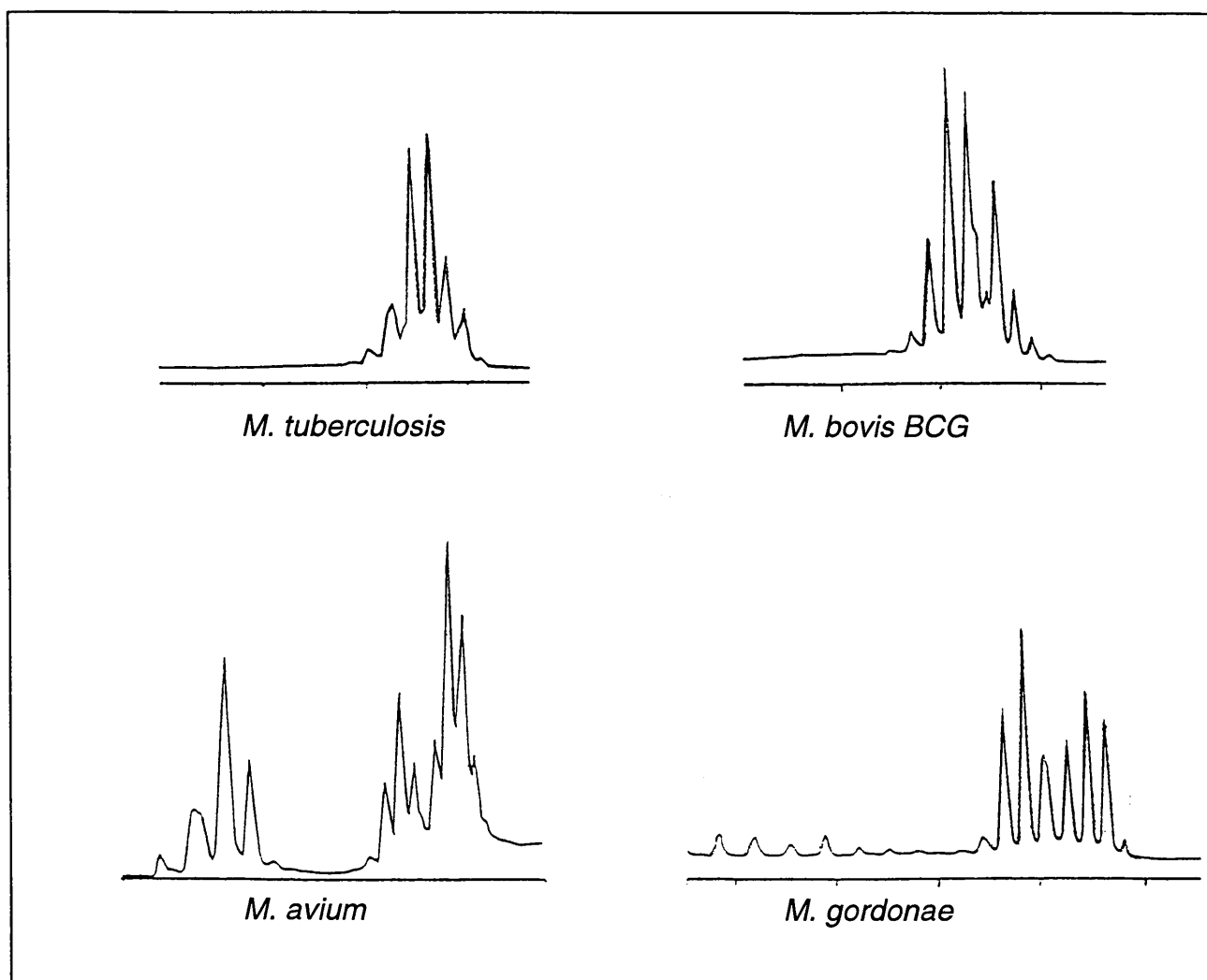
Figure 2.6 HPLC profiles of the mycolic acids of a few *Mycobacterium* species

(Butler *et al.*, 1991).

Culturing of clinical samples is needed to provide enough bacteria to enable the extraction of

sufficient quantity of material for HPLC detection of mycolic acids. The advantages of using

HPLC analysis for the identification of mycobacterial species by comparing the mycolic acids

profiles, are that the method is reliable and rapid, but the disadvantage is that it is often

insufficient to identify mycobacterial species in patients with multiple infections.

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An internal standard which contains molecules with 110 carbons atoms is often included in mycolic acid HPLC samples. The internal standard elutes as the last component in the analysis, after the mycolic acids and is used for the quantification of mycolic acids.

The peaks obtained by HPLC of the p-bromophenacyl ester of the mycolic acids from *M. tuberculosis* do not represent pure chemical entities, rather they are mixtures of the mycolic acids types characteristic of a particular species. The typical profile obtained for *M. tuberculosis* contains 9 major peaks of which only peaks 1, 2 (keto mycolic acids) and 9 (α-mycolic acids) are pure. The other peaks contain a mixture of all three types of mycolic acids. This becomes clear when the mycolic acids are separated into three major structural types by normal-phase HPLC and each type analysed on reverse phase HPLC as shown in Figure 2.7 (Butler, 1985).

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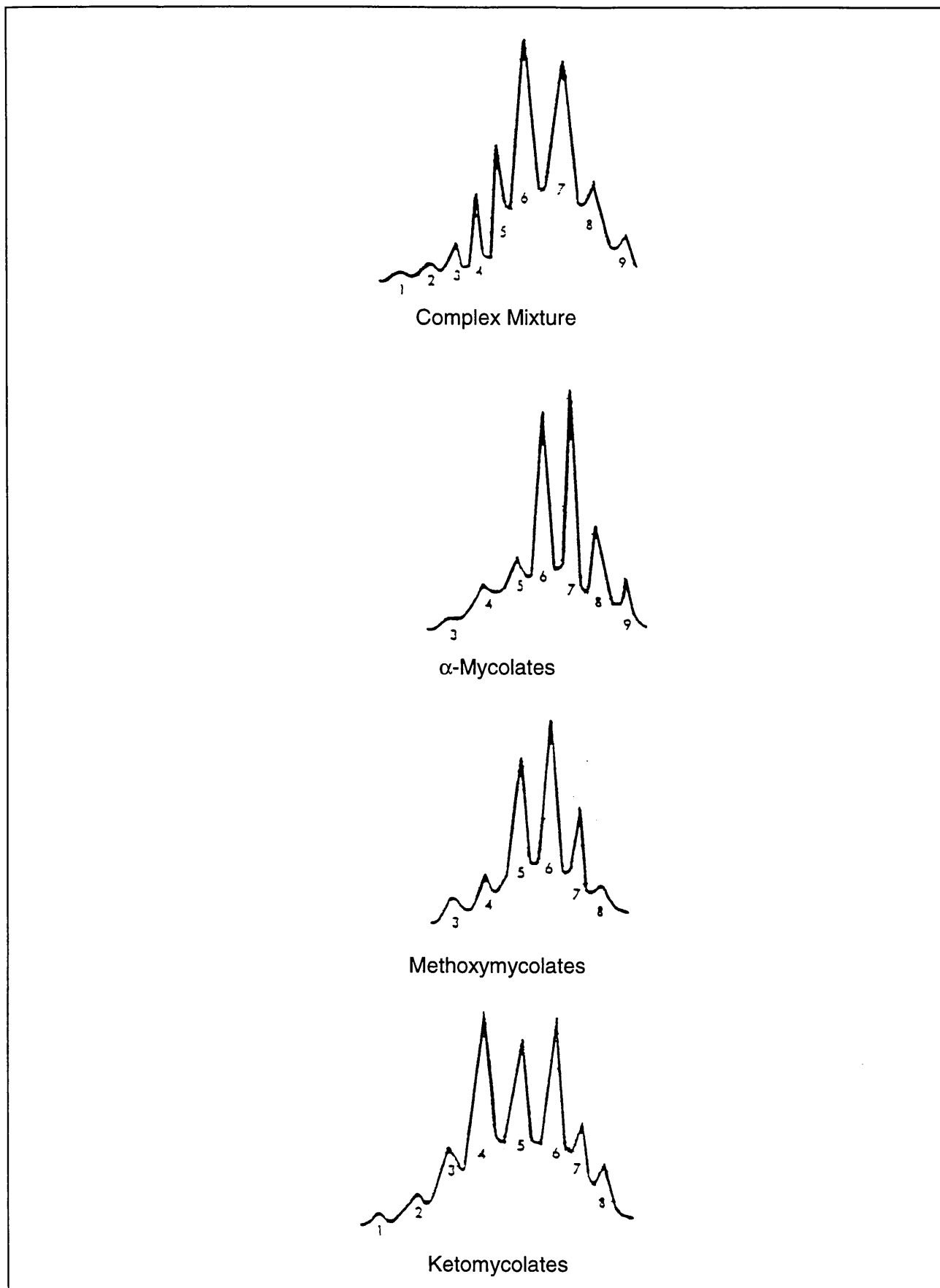
Figure 2.7   The HPLC profiles of each of the three groups of *M. tuberculosis* mycolic acids

(Butler, 1985).

2.1.4. Structural analysis of mycolic acids with infrared spectroscopy

The involvement of the relatively simple functional groups on mycolic acids in inter- and intramolecular bonding can be detected using infrared spectroscopy. The infrared (IR), part of the electromagnetic spectrum includes the region with a wavelength of 0,75 - 25 μm and a frequency of 13 333 - 400 cm^{-1}, which encompasses the frequencies at which molecular vibration by stretching and bending occurs. The frequency range of the infrared spectrum that is useful for structural analysis is between 4 000 and 660 cm^{-1}. Absorption bands are detected in the infrared spectrum due to energy changes caused by bond stretching and bending within compounds. A blank sample containing only the solvent is analysed first. This spectrum is subtracted from the subsequent sample spectra. The liquid or dissolved samples are analysed in the form of a thin film between two infrared-transparent discs (such as polished sodium chloride discs) which are placed in the path of the infrared beam. Powdered samples can be examined as pressed alkali halide discs of potassium bromide (KBr). The sample is intimately ground with pure, dry KBr, the mixture inserted into a special die and subjected to pressure under vacuum. A spectrum is also obtained from a blank disc and subtracted from the spectra of all samples tested (Vogel, 1989). A simplified correlation chart of the absorption positions of important bonding types is shown in Figure 2.8.
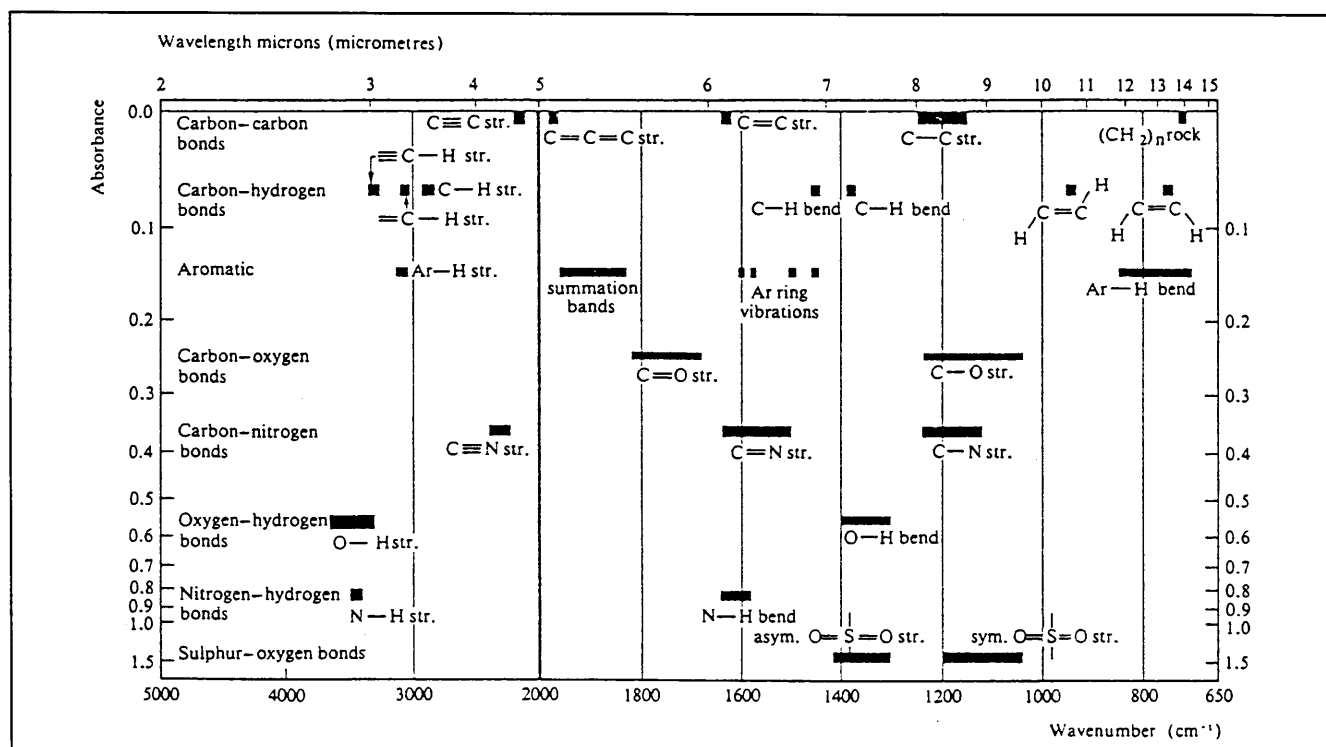
Figure 2.8   A correlation chart of the positions of the IR absorption regions of important bonds (Vogel, 1989).

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An infrared profile of partially purified methyl mycolates isolated from *M. tuberculosis*, harvested

from lungs of infected mice is shown in Figure 2.9. The split absorption band at 1720 cm^{-1} is that of

the carbonyl ester which is usually situated at 1740 cm^{-1} but moves due to intramolecular hydrogen

bonding between the carbonyl group and the β-hydroxyl group which is responsible for the rather

broad absorption band at 3626 cm^{-1} and 3534 cm^{-1}. The presence of cyclopropane substituents is

indicated by the presence of an absorption band at 1025 cm^{-1}, as well as the band at 3060 cm^{-1}, the

intensity of which corresponds to the number of cyclopropane rings (Goren, 1979). The sharp peak at

approximately 3570 cm^{-1} within the broad band is due to free OH groups (Lin-Vien *et al.*, 1991).
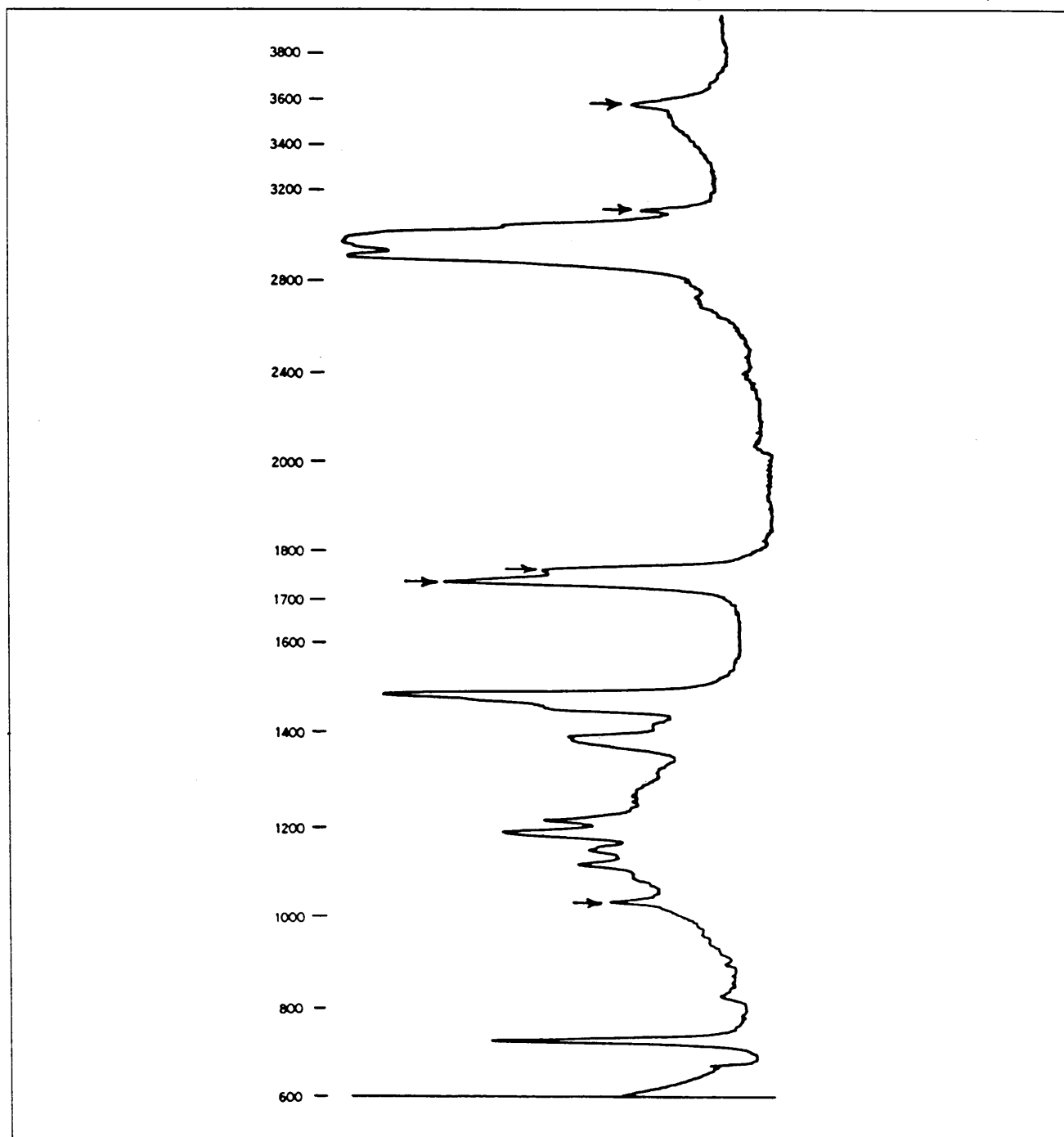
Figure 2.9 Infrared spectrum of methyl mycolates from *Mycobacterium tuberculosis* (Goren, 1972).

2.1.5. Countercurrent distribution as a purification tool

Separation of chemical substances by extraction is a commonly used method, usually utilising a separation funnel to separate substances with very different partitioning properties. Countercurrent distribution (CCD) utilises a similar principle to separate substances by carrying out repetitive extractions involving many 'funnels' or tubes to exploit small differences in the partitioning properties of the substances in a discontinuous equilibrium process. A distinctive feature of the process is that one can predict the distribution of substances in the tube train and thus determine the number of cycles required to purify the isolated product. The segregation of components in aqueous two phase systems has been used to separate biological materials such as proteins, nucleic acids as well as cells, organelles, bacteriophages, viruses and membranes (Walter and Johansson, 1986).

The principle used in CCD is the selective transport of solutes, effected by the flow of liquid upper phase over a stationary liquid lower phase. The rate at which the solutes are transported along the train depends on the differential solubility of the solutes in the two phases. The partition coefficient can be described as the ratio of the amount of solute dissolved in the upper phase to that dissolved in the lower phase of a biphasic system. If the fractional amounts of solute dissolved in the upper and lower phases are designated p and q respectively, where $p + q = 1$, then $K = p / q$.

The optimal phase system provides a partition coefficient (K) of approximately one and allows molecules to be easily separated. Such solutes would then be situated in the middle of the tube train in which the separation occurred. For example, samples with partition constants of 20 and 21 are more difficult to separate than molecules with distribution constants of 1 and 2. A substance with a partition coefficient lower than 1,0 would imply that it would be situated towards the beginning of the tube train, while substances with partition coefficients above 1,0 would be situated towards the end of the tube train over which the distribution was done. Molecules with similar distribution constants in a specific phase system are difficult to separate (Te Piao and Lyman, 1962).

2.1.6. Methods for obtaining purified mycolic acids

Beckman and co-workers previously purified mycolic acids from sonicates of *M. tuberculosis* (H37Ra) by extraction (4:1 v/v organic to aqueous) with chloroform and methanol (2:1 v/v), followed by drying of the organic phase, re-suspension in hexane and chromatography over a cyano (CN)-bonded silica solid phase extraction column. Mycolic acids eluted from the column at 85 % chloroform in hexane, were saponified with methanolic KOH (25 % KOH) at 85 °C for 3-5 hours, acidified with HCl, extracted with chloroform, neutralised and derivatised with p-bromophenacyl bromide before being analysed by HPLC on a C18 reverse phase column. The mycolic acid fraction was resaponified to remove the derivatising agent and restore antigenic activity. Mycolic acids were also isolated from trehalose 6-6' dimycolate (cord factor) obtained from *M. tuberculosis* (H37Ra) and from commercially available (Sigma) trehalose 6-6' dimycolate by saponification, derivatisation with p-bromophenacyl bromide, reverse phase HPLC and resaponification (Beckman, 1994). Mycolic acids can also be chemically synthesised as described in the International Patent Application Number PCT/JP89/01341 by Sawai Pharmaceutical Co. Ltd. and Medisa Shinyaku Inc. allowing the production of specific mycolic acid molecules (Sawai, 1989).

2.1.7. The presentation of mycolic acids on CD1 and stimulation of CD4⁻CD8⁻ T-cells

CD1 is a surface glycoprotein that is non-covalently associated with β microglobulin and has structural homology to the MHC molecules. This led to the suggestion that they may have an antigen presenting function. CD1 molecules, however show little amino acid homology with MHC molecules, have a different predicted secondary structure and are more hydrophobic than MHC molecules. Expression of the CD1 molecule is induced in antigen presenting cells upon treatment with GM-CSF and IL4. CD1 is able to present antigens to double negative T-cells, leading to their activation and proliferation (Tanaka *et al.*, 1996).

Porcelli showed that human double negative T-cells (lacking the CD4 and CD8 surface markers) proliferated in response to antigens presented on CD1a and CD1c and secreted IL2, in a similar manner to T-cells activated by antigen presented on MHC (Porcelli *et al.*, 1989). Although DN T-cells are present in low numbers in peripheral blood (< 2 %), they have been found in the human skin and

are present in murine lymphoid organs as well as bone marrow where they can represent a majority of the total T-cell population. DN T-cells in circulation appear to be mature and functional T-cells with cytolytic activity (Niehues *et al.*, 1994). Further studies by Porcelli and co-workers lead to the discovery that αβ CD4⁻CD8⁻ T-cells were stimulated by a mycobacterial antigen in a CD1b-restricted manner and showed cytolytic activity (Porcelli *et al.*, 1992). The antigen presented on CD1b was identified as mycolic acids and provided the first indication of antigen processing and presentation of a fatty acid to T-cells. This discovery showed for the first time, that the αβ TCR was able to recognise molecules other than proteins (Beckman *et al.*, 1994). Lipoarabinomannan (LAM) presented by CD1 was also found to stimulate αβ DN T-cell proliferation and lead to the idea that CD1 may present non-peptide ligands to T-cells (Sieling *et al.*, 1995). The work by Tangri showed that CD1 molecules were also able to present hydrophobic peptides to CD8⁺ T-cells in mice, which indicated that they were able to present hydrophobic peptides, LAM and lipids to T-cells (Tangri *et al.*, 1995).

The isolation of γδ CD4⁻CD8⁻ T-cells from synovial tissue of patients with rheumatoid arthritis, which could be activated by unmodified self-CD1c and the isolation of such T-cells (reactive to CD1a) from the blood of a systemic lupus erythematosus patient, may suggest a link between CD1 reactive DN T-cells and autoimmune disease (Porcelli, 1989). DN T-cells have also been implicated in immune suppression. This observation was supported by the work of Niehues who showed that the supernatant of stimulated DN cells contained IL10 (secreted by DN cells), which inhibits the antigen presenting capacity of monocytes, as well as reduces the proliferative response of T-cells (Niehues *et al.*, 1995). This subset of T-cells has therefore, been implicated in both autoimmunity (rheumatoid arthritis) and anergy, phenomena which are both observed in tuberculosis infection. DN T-cells may therefore, play an important role in the immunology and pathology of tuberculosis.

2.1.8. Aims and rationale for the purification of mycolic acids

Pure and biologically active mycolic acids were required in order to repeat and evaluate the experiments done by Dr S. Bye with the aim of producing antibodies specific for mycolic acids in experimental animals for use in the development of a diagnostic kit. Pure mycolic acids were not commercially available when this research started in 1994 and only became commercially available from Sigma (98% pure) in 5 and 25 mg quantities in 1995. This source of mycolic acids is however rather costly and therefore, a cheaper method with the potential for scaling-up for industry would be beneficial. The work reported in this thesis is part of the Tuberculosis Project at the Department of Biochemistry, which aims at a pharmaceutical application of mycolic acids for the diagnosis, treatment or prevention of tuberculosis. If such an application were found, it would be important to have an inexpensive source of pure, biologically active, mycolic acids.

2.2. Materials:

2.2.1. Culture and media

Mycobacterium tuberculosis H37Rv - a virulent strain, originally isolated from an infected human lung was used in the experiments.

The culture was purchased in lyophilised form from the American Type Culture Collection
(ATCC 27294), Maryland, USA.

The following media were used for the cultivation of *Mycobacterium* cultures:

    Liquid medium: Dubos broth

    Solid media: Löwenstein-Jensen (LJ) medium

                    Middlebrook 7H-10 medium

A detailed composition of the ingredients necessary for the preparation of these media, as well as the conditions recommended for their sterilisation, are given in the Laboratory Manual of Tuberculosis Methods, Tuberculosis Research Institute of the SA Medical Research Council (1980, Chapter 6, pp 83-105; Second Edition, revised by E.E. Nel, H.H. Kleeberg and E.M.S. Gatner). The media were prepared by the Department of Medical Microbiology at the Institute of Pathology of the University of Pretoria.

2.2.2. Reagents

HPLC grade methanol and double-distilled de-ionised water were used for the preparation of the reagents used for the extraction, derivatisation and high-performance liquid chromatography (HPLC) analysis of mycolic acids. Reagents A, B, C and E were prepared fresh prior to experiments, taking all the necessary safety precautions.

Reagent A: Potassium hydroxide (25 %) dissolved in methanol-water (1:1).

Potassium hydroxide (62,5 g - Univar A.R. grade - SAARCHEM[1]) was dissolved in 125 ml double distilled water and 125 ml methanol (HPLC grade - BDH[2]) was added.

Reagent B: Concentrated hydrochloric acid (Analytical grade - BDH[2]) diluted 1:1 with water.

Reagent C: Potassium bicarbonate (2 %) dissolved in methanol-water (1:1).

Potassium bicarbonate (10 g - Analytical grade - BDH[2]) was dissolved in 250 ml water and 250 ml methanol (HPLC grade - BDH[2]) was added.

Reagent D: A solution of para-bromophenacylbromide and Crown Ether in a 20:1 molar ratio (Pierce Chemical Co[3], Cat. No 48891) in acetonitrile. This was dispensed in 1 ml quantities into small amber-coloured screw cap vials with teflon-coated septa. The caps were tightened and the vials were wrapped with Parafilm. Reagent D was stored at 4°C.

Reagent E: Reagent E was prepared by mixing reagent B 1:1 with methanol (HPLC grade-BDH[2])

HPLC Standard: High molecular weight internal standard (C-100) from Ribi ImmunoChem Research Company, Cat No R-50. The standard, 1 mg, was suspended in 20 ml chloroform (Univar A.R. grade - SAARCHEM[1]) and aliquots of 100 µl (5 µg) were dispensed into 200 small screw-cap vials. The chloroform was allowed to evaporate, the vials were sealed with Teflon[TM] septa-containing caps and stored at 4°C.

Acetone (Unilab C.P. grade - SAARCHEM[1])

Chloroform (Chemically pure grade, SAARCHEM[1])

Chloroform (Univar A.R. grade - SAARCHEM[1])

Methanol (Unilab C.P. grade - SAARCHEM[1]) for countercurrent purification

Methanol (HPLC grade - BDH[2])

Methylene chloride (HPLC grade - BDH[2])

Tween 80 (Chemically pure grade – Merck[4])

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[1] SAARCHEM; P.O. Box 1124; Krugersdorp; 1740

[2] BDH, supplied by Merck; P.O. Box 1998; Midrand; 1685

[3] Pierce Chemical Company, supplied by Separations; P.O. Box 4181; Randburg; 2125

[4] Merck; P.O. Box 1998; Midrand; 1685

Chapter 2

2.3. Methods:

2.3.1. Cultivation of bacterial strains

M. tuberculosis bacilli were inoculated into Dubos liquid medium and incubated until growth was observed. Aliquots of this culture were inoculated onto LJ slants and allowed to grow at 37°C in an aerobic environment. After four weeks of growth the slants were usually sufficiently covered to allow either scraping for extraction or inoculation of further LJ slants. Inoculation of slants was done by preparing a suspension of *Mycobacteria* in sterilised 0,9 % m/v saline containing 0,01 % v/v Tween 80 and inoculating further LJ slants with aliquots of the suspension. A standard curve of the McFarland (McF) standards' absorbance at 486 nm was set up (Barlows, 1991).

2.3.2. Preparation of crude mycolic acids extracts of bacterial samples

The preparation of bacterial samples comprised three steps:

harvesting of the *Mycobacteria* cells;

saponification and

extraction of crude mycolic acids

The saponification, extraction and derivatisation of mycolic acids were carried out as described by Butler, Jost and Kilburn (1991), with minor modifications as are described under the relevant headings.

Glassware used for the extraction, derivatisation and HPLC analyses of mycolic acids was washed in 2% (v/v) Contrad (Merck[1]), rinsed in water, followed by rinsing in chloroform, water, methanol, water and finally in double distilled water. The washed glassware was dried in an oven.

Harvesting was done by scraping the bacterial growth from the surface of media slants (using sterile plastic loops). Homogenous bacterial suspensions were prepared in Reagent A by shaking or vortexing the harvested cells with sterile glass beads.

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[1] Merck; P.O. Box 1998; Midrand; 1685

Saponification was done by autoclaving the suspension of *Mycobacteria* in reagent A adjusted to a McFarland standard No.4 density ($A_{486} = 1$) at 121°C, for 1 hour in sealed vials.

Extraction of mycolic acids comprised the following:

The saponification mixture was allowed to cool and 1,5 ml reagent B was introduced per 2 ml reagent A in the mixture. After vortexing, the pH was checked and if necessary, adjusted to pH 1 with reagent B. Chloroform (2,0 ml) was added per 2 ml reagent A and the mixture vigorously shaken in a Schott bottle for two minutes. The mixture was then transferred to a separation funnel and the layers were allowed to separate. The bottom layer was removed and transferred to a round-bottomed flask and the chloroform evaporated off on a Buchi rotary evaporator under vacuum at 80°C. Reagent C (100 µl) was added per 2 ml reagent A initially used and the mixture dried at 80°C on a Buchi rotary evaporator under vacuum. Samples of crude extract of mycolic acids were stored under acetone in amber WISP vials at 4°C until use.

2.3.3. Derivatisation of crude extracts, HPLC analysis and quantification of mycolic acids

Crude mycolic acid extracts were derivatised for HPLC analysis as follows:

Samples of the crude extract were dissolved in chloroform (approximately 2 ml chloroform per 50 mg crude extract), a portion aliquoted into a clean WISP vial and the chloroform evaporated off on a heat block evaporator at 80°C under a stream of nitrogen. To each cooled sample, 1,0 ml chloroform was introduced, followed by the addition of 100 µl of reagent D. The capped samples were vortexed for 30 seconds, heated for 20 minutes at 80°C in a heat block-evaporator, 1,0 ml of reagent E added, vortexed for 30 seconds and the layers allowed to separate. The bottom layers were removed with Pasteur pipettes and transferred to WISP-vials containing 5 µg Ribi internal standard. The vials were placed in a heat block evaporator and their contents evaporated to dryness at 80°C, using a stream of nitrogen.

The residues were resuspended in 160 µl methylene chloride, capped and vortexed. Each reconstituted sample was filtered through a 0.22 µm Millipore GV$_4$ Durapore (PVDF membrane and polyethylene

housing) membrane filter into a 300 µl conical insert, in an amber-coloured WISP-vial. The re-capped vials were wrapped in parafilm[TM] and stored at 4°C until ready for HPLC analysis, which was usually done within 1 or 2 days of sample preparation.

For the HPLC analysis 5 or 10 µl from each sample was injected. Control samples, *i.e.* 5 or 10 µl of filtered methylene chloride, were injected and run prior to each set of samples analysed. In order to validate the reliability of the HPLC apparatus when a large number of samples were analysed, control samples were run after every eight to ten test samples.

The reverse-phase HPLC analyses were initially carried out using a System Gold High Performance Liquid Chromatography unit (Beckman, South Africa) consisting of:

Pumps (Beckman 110 B Solvent Delivery Module);

Detector (Programmable detector module 166 or 168);

Column (Nova-Pak C18 4 µm 3,9 x 150 mm);

Column temperature regulator (RKC Rex - C4);

and later a Waters High Performance Liquid Chromatography (Microsep, South Africa) apparatus consisting of:

Pumps (WATERS 600E system controller);

Detector (WATERS 486 tunable absorbance detector);

Integrator (Microsep Data Module M741);

Autosampler (WATERS 712 WISP autosampler);

Autosampler hydraulic gas (Afrox, Nitrogen gas);

Solvent sparging gas (Afrox, Helium gas, Instrument grade).

The same type of column, column temperature regulator and running conditions were used throughout.

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Running conditions were:

Mobile phase:

Solvent A: HPLC grade methanol

Solvent B: HPLC grade methylene chloride

Flow Rate: 2,5 ml/min

Column temperature: $30^{o}C$

Detector wavelength: 260 nm.

The HPLC gradient initially comprised 98 % (v/v) methanol (Solvent A) and 2 % (v/v) methylene chloride (Solvent B). The gradient was increased linearly to 80 % solvent A and 20 % solvent B over a period of one minute; 35 % solvent A and 65 % solvent B over a period of ten minutes, held at these conditions for 50 seconds and then decreased over 10 sec back to 98% solvent A and 2 % solvent B. This ratio was maintained for 6 minutes, to allow for regeneration of the system, prior to injection of the next sample.

Mycolic acids were quantified by comparing the area of the mycolic acids cluster of peaks, to the peak area of the high molecular weight internal standard, as follows:

Mass of mycolic acids = $\dfrac{\text{Area of mycolic acids}}{\text{Area of internal standard}}$ x   Mass of internal standard

2.3.4. Countercurrent purification of mycolic acids from crude mycolic acid extracts

2.3.4.1. Countercurrent apparatus

The countercurrent (CCD) apparatus (H O POST, Instrument Company Inc., Middle Village, New York) used is shown in Fig. 2.10, and consists of 2 X 250 inter-connected tubes.
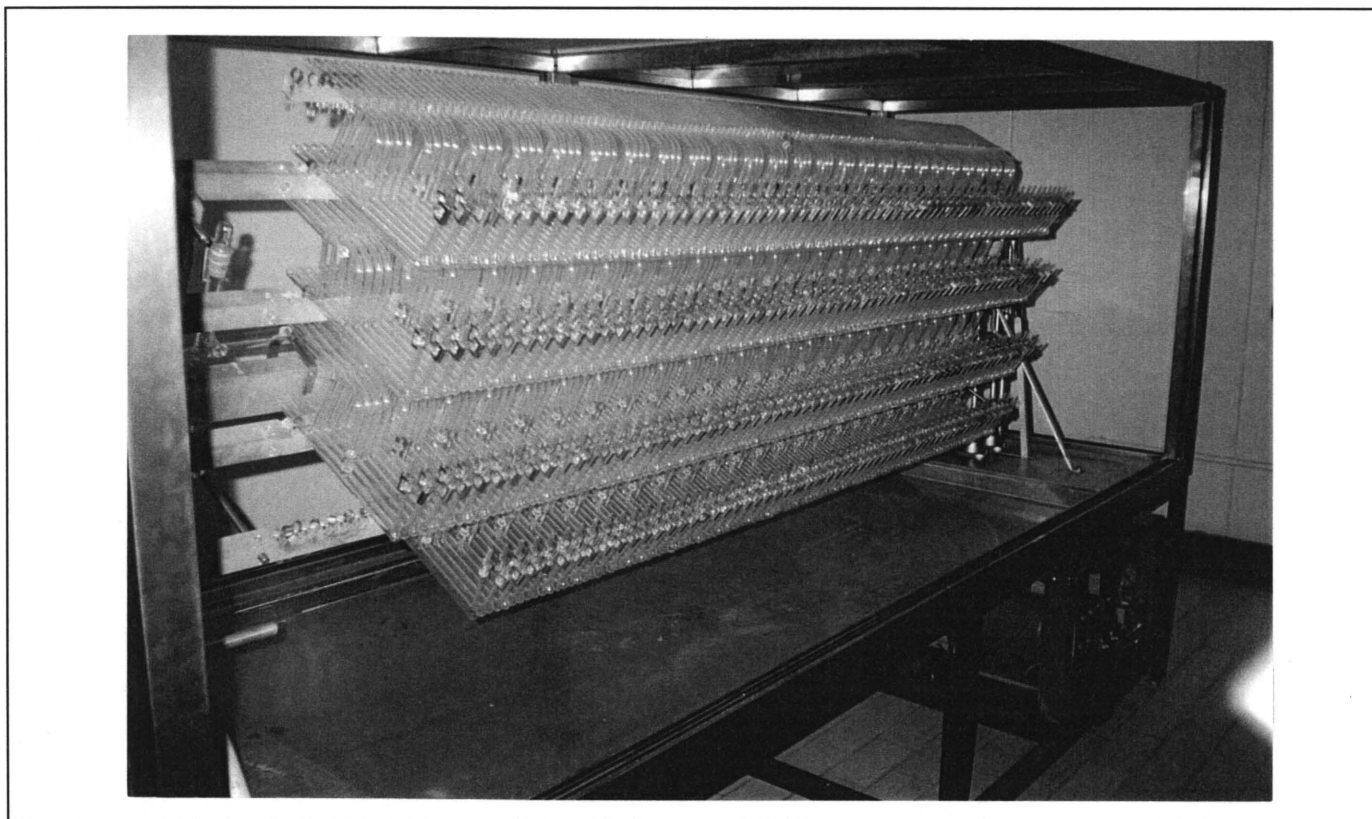
Figure 2.10   The countercurrent distribution apparatus.

2.3.4.2. Development and optimisation of a solvent system for the purification of mycolic acids on the countercurrent apparatus

Previous studies describing HPLC analysis of mycolic acids showed that mycolic acids dissolved in chloroform and dichloromethane as well as in 65 % mixtures of either of these solvents with methanol.

In order to develop a bi-phasic separation system, based on the use of these solvents, in which the highest degree of solubility could be obtained, water was added as a third component (chloroform and water would thus constitute the non-miscible components and methanol, the fully miscible integrator component of the system).   Differing ratios of chloroform and water were titrated with methanol to determine the phase diagram.

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This experiment was carried out in the following manner:

Nineteen concentrations of chloroform were prepared in double-distilled de-ionised water, starting from 5 % chloroform and 95 % water, and increasing the concentration of chloroform in 5 % increments (thus 5, 10, 15 and 20 up to 95 % chloroform). Into identical volumes (100 ml) of each of these concentrations, methanol was introduced until disappearance of the turbidity. The exact volume of methanol necessary to obtain complete clarity (one phase) of the chloroform-water mixture was noted for each titration. Titrations were carried out at the regulated temperature of 24,5 °C, prevalent in the laboratory housing the countercurrent apparatus.

2.3.4.3. Determination of the ideal phase composition for countercurrent distribution

Countercurrent distribution provides optimum separation when a phase system is used which separates the solutes of the sample equally between the upper and the lower phases.
($K = 1$, or $p = q$).

This experiment had a dual aim, namely to determine the mass distribution of the dissolved, extracted material in both phases of the solvent system and to measure the levels of mycolic acids present in these phases.

A biphasic solvent system, chosen for this experiment comprised:

45% methanol: 30% chloroform: 25% (v/v) double distilled de-ionised water.

This composition was derived after studying the solubility behaviour of the crude extract in similar solvent systems.

Two portions of the crude extract (6.9 mg and 5.7 mg), extracted from *M. tuberculosis* H37 Rv were dried on a heat block evaporator at 80°C, under a stream of nitrogen. The samples were suspended separately in each phase of the equilibrated solvent system to a concentration of 5 mg sample / ml, using 1,38 ml of the upper phase and 1,14 ml of the lower phase, respectively. The top and bottom phase suspensions were then mixed.

After equilibration, the two phases appeared clear, with only a small amount of interphase visible between the two phases. The top, bottom and interphase layers were individually transferred to WISP-vials, the mass of which was previously gravimetrically determined. The three samples were dried on a heat block evaporator at 80°C, under a stream of nitrogen. The dry mass of each of the samples was obtained by calculating the weight differences of the WISP-vials, before and after the introduction of the samples.

The contents of the WISP-vials were derivatised and analysed by the HPLC, as described under 2.3.3.

As the solvent system described above resulted in a mass distribution constant approaching $K = 1$, the phase-diagram was interpreted to derive a solvent composition of chloroform 42%, methanol 39% and water 19%, which gave equal volumes of the two phases. After equilibration, the composition of the upper and lower phases was determined according to the approach described by Alders (1959). This solvent system was subsequently used in the countercurrent separation.

2.3.4.4. Countercurrent (CCD) purification of mycolic acids from crude mycolic acid extracts and calculation of the distribution constants of mycolic acids

A portion of the countercurrent distribution train comprising 25 tubes, numbered 0-24, was used in the experiment. Into the buffer reservoir approximately 900 ml of the upper phase was introduced.

Into tube number 0, a portion of the crude cellular extract of *M. tuberculosis* obtained from a large-scale extraction experiment (30 - 150 mg), dissolved in 10 ml of the lower phase and 10 ml of the upper phase was introduced. Into the remaining 24 tubes, aliquots of 10 ml of the lower phase were introduced. Upper phase was automatically dispensed into tube number 0, in volumes of 10 ml per cycle, repeatedly over 25 cycles as the upper phase moved along the tube train resulting in an approximately 16 hour operation. Thus, twenty- five countercurrent cycles

were performed, with each cycle consisting of 20 mixing pendula and 40 minutes phase separation time. With each transfer, any solute originating from the sample and present in the upper phase was carried into the succeeding tube. After the completion of twenty-five transfers, the separated solute fractions were distributed along the train of 25 tubes.

The process is graphically illustrated in Fig. 2.11.
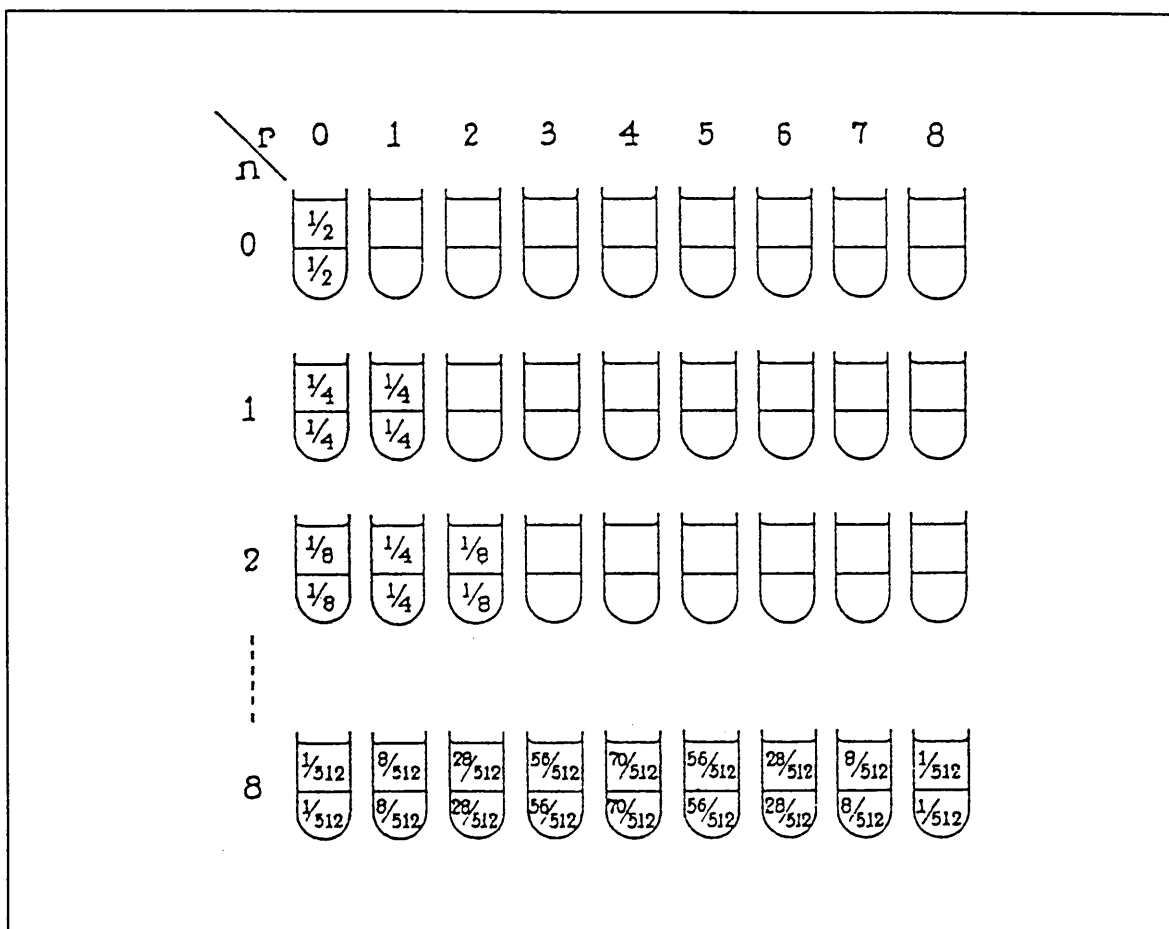
Figure 2.11   A schematic representation of countercurrent distribution of a solute with

Kd =1,0 over nine tubes during 8 cycles (Te Piao and Lyman, 1962).

The countercurrent-separated material was withdrawn from the tubes using a 50 ml glass syringe, with Teflon^{TM} tubing attached. Fractions of the material were dried individually under vacuum in a Buchi evaporator at 80°C, the dried material re-dissolved in chloroform or methanol / water (in approximately 5 ml) for the more polar fractions, transferred into amber WISP-vials, dried on the heat block and stored at 4°C until required.

Each fraction obtained from CCD was dissolved in 4 ml chloroform and vortexed, a volume corresponding to 0,5 mg mycolic acids of the first fraction was withdrawn from each vial and transferred into new amber WISP-vials and dried on a heat block. The samples were derivatised and analysed by HPLC, as described under 2.3.3.

The distribution constants for the fractions were calculated using the following formula:

$$Kd = \frac{r_{max} + 1}{n - r_{mx}}$$

where n = total number of tubes over which the separation was done

r_{max} = the number of the tube containing the maximum amount of material.

2.3.5. De-esterification (saponification) of mycolic acids methyl esters

One aliquot (1 mg) of CCD-purified mycolic acids was dissolved in 3 ml chloroform and 66,6 µl

(200 µg) transferred to each of two vials. One sample was subjected to a resaponification step in order to de-esterify the methanol group from the carboxylic acid group of the fatty acid, while the other was merely derivatised without resaponification.

Resaponification: 2 ml reagent A was added to the mycolic acids sample, which was vortexed for

30 seconds, and autoclaved for 1 hr at 121 °C. The sample was then allowed to cool,

1,5 ml reagent B added, vortexed and the pH tested and adjusted to pH ≈1.

Chloroform (2 ml) was added for extraction, the lower chloroform phase transferred to

another vial and dried on a heat block at 85 °C under a stream of nitrogen.

Reagent C (100 µl) was added and the sample vortexed and dried on the heat

block under a stream of nitrogen.

Derivatisation: For HPLC analysis 1 ml chloroform and 100 µl of reagent D were added to the

mycolic acids, vortexed for 30 seconds, sealed and heated on a heat block at 85 °C

for 20 minutes. After cooling, 1 ml reagent E was added and the sample vortexed for

30 seconds. The lower phase was transferred to a clean vial, dried, re-dissolved in

160 µl dichloromethane, 5 µg internal standard added, filtered and loaded on the

HPLC as described in 2.3.3.

A modification to the de-esterification process was tested when 1 mg aliquot of mycolic acid was divided into two identical samples containing 0,5 mg mycolic acids each. One sample was resaponified using the protocol described above and the second sample was treated at room temperature instead of at 121 °C with reagent A. The two samples were vortexed, extracted and derivatised as described above.

2.3.6. Determination of the chemical purity of mycolic acids

The acetone, under which crude extracts of mycolic acids were stored in WISP vials, was removed by evaporation, on a heat block at 85 °C under a stream of nitrogen. Crude extract (478,8 mg) was dissolved in 44 ml of lower and 44 ml upper phase for the countercurrent and loaded in tubes 0-3 of the countercurrent apparatus (4 tubes, each with 10 ml upper and 10 ml lower phase). One tenth of the volume of dissolved crude extract was retained (4 ml upper and 4 ml lower phase), dried and stored under acetone for HPLC analysis of crude extract versus purified mycolic acids. The countercurrent was run for twenty-eight cycles overnight in the dark.

A quantity of crude extract (6,86 mg) that was expected to yield 0,5 mg of mycolic acids (as calculated from the percentage mycolic acid yield obtained gravimetrically after countercurrent purification) was derivatised and analysed by HPLC to determine the yield of mycolic acids. Internal standard (5,01 µg) was added to the sample to allow quantification of the mycolic acids obtained. The percentage yield of mycolic acids gravimetrically determined after CCD purification from crude extract and the percentage yield of mycolic acids present in crude extract as determined by HPLC, were compared to determine the percentage purity of the mycolic acids obtained by countercurrent purification.

2.3.7. Removal of remaining contaminants by rinsing mycolic acids with acetone

Although the percentage purity, determined in 2.3.6., was found to be high, the HPLC profiles showed the presence of small contaminating peaks. It was observed that the acetone under which the countercurrent-purified mycolic acids were stored, became green which indicated that contaminants were acetone soluble. This observation provided acetone rinsing as a possible method by which the HPLC contaminant peaks could be removed.

An experiment was designed to determine the purity of mycolic acids by HPLC at different steps of the extraction and purification process. The contribution of the culture medium (LJ slant) and reagents to contaminant peaks was also tested by treating samples of these in the same manner as the bacteria scraped off LJ slants.

The procedure followed is depicted in the flow diagram in Fig 2.12.
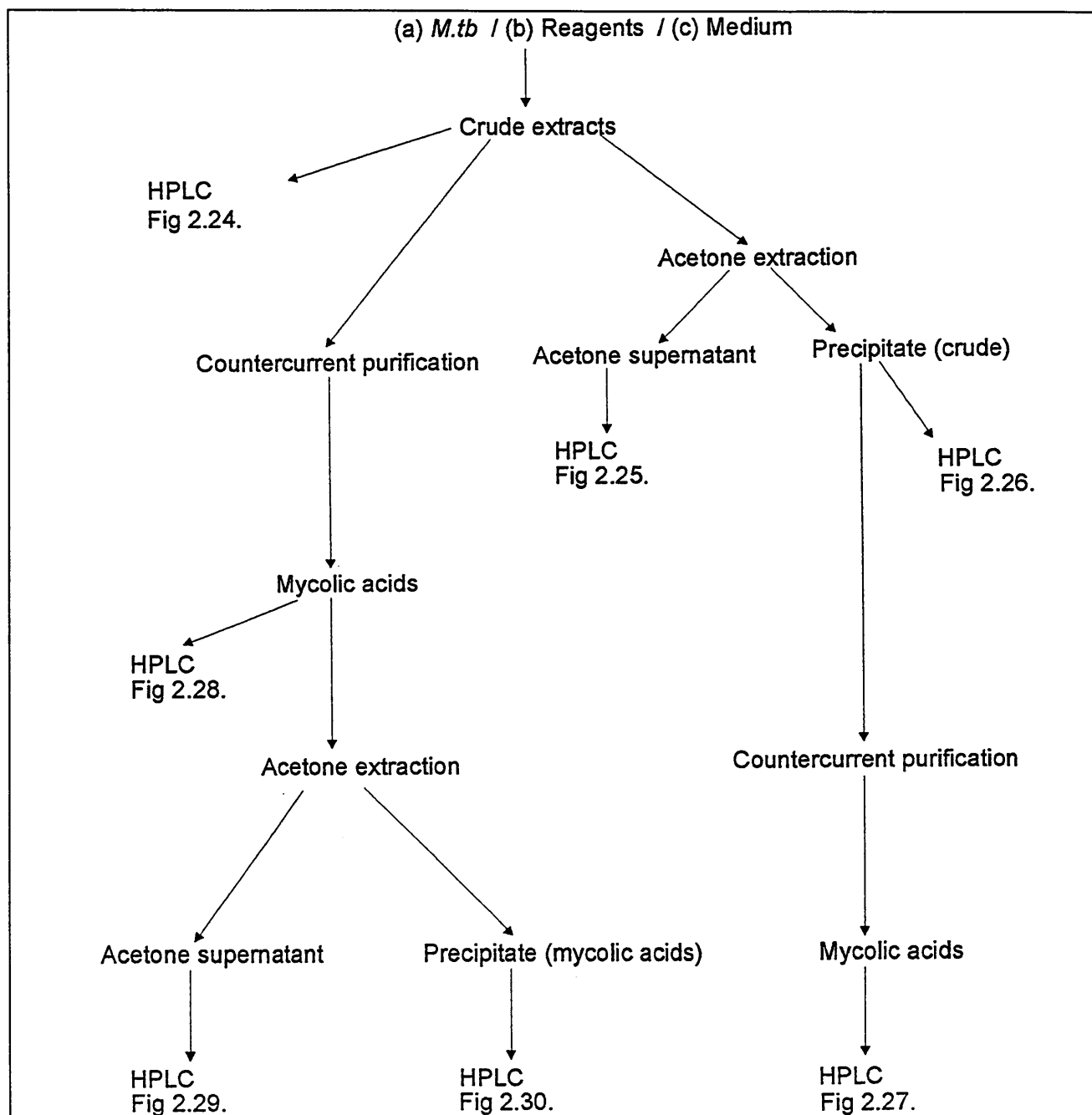
Figure 2.12   Flow diagram of the experimental method used to determine the purity of mycolic acids at different steps of the purification process as well as the contribution of the *M. tuberculosis* culture medium and extraction reagents to the HPLC profile.

The growth from one optimally and three sub-optimally-covered *M. tuberculosis* inoculated LJ slants were scraped off, and suspended in reagent A to a turbidity of just less than a McFarland Standard number 5 and a final volume of 120 ml. Medium obtained from a LJ slant was suspended in reagent A to a similar turbidity and volume. The same volume of pure reagents was prepared.

The extraction procedure was followed until after addition of reagent C and drying.

2.3.8. Removal of contaminants by selective precipitation of mycolic acids with acetone

Countercurrent purified mycolic acids (92 mg) were dissolved in 1 ml chloroform and transferred to a round-bottom flask. The vial was rinsed twice with 1 ml chloroform and added to that in the round-bottom flask. The procedure was carried out at room temperature. Acetone was added to the dissolved mycolic acids in 500 μl aliquots and the precipitation of mycolic acids observed. Samples taken before and after selective precipitation were saponified, derivatised and analysed by HPLC to determine if contaminants were successfully removed by the procedure.

2.3.9. HPLC analysis of purified mycolic acids to detect contaminants

In another approach to determine the presence of contaminants in purified mycolic acids, a mycolic acids sample prepared for HPLC was monitored at two additional wavelengths.

Countercurrent-purified, acetone-precipitated mycolic acids (0,2 mg) were resaponified, derivatised and analysed by HPLC at detector wavelengths of 260 nm, 230 nm and 280 nm to determine the presence of possible contaminants such as proteins (peptide bonds) and aromatic amino acids. The baseline was also run at 230 and 280 nm to determine the influence of the solvents used in the gradient, at these wavelengths.

2.3.10. Infrared analysis of CCD purified mycolic acids, before and after saponification and after storage

Countercurrent-purified mycolic acids were analysed by infrared either without further treatment, freshly saponified (2 ml reagent A per 5 mg mycolic acids) as described in 2.3.5, or saponified and stored for three weeks (in the presence of 100 µl chloroform at 4°C) and used in cell proliferation studies. Approximately 4 mg mycolic acids were used for IR analysis while approximately 10 mg mycolic acids were used for cell proliferation studies.

In addition, the KBr pellets prepared for IR analysis of the 'freshly' saponified and unsaponified mycolic acids were re-analysed in the same manner, three weeks later.

Mycolic acids were prepared for IR analysis, by dissolving 1 mg mycolic acids in 1 ml chloroform. This was added to 200 mg KBr and mixed well, the chloroform was allowed to evaporate before preparing a pellet of mycolic acids in KBr using a Shimadzu tablet die and placing a 10 kN pressure on the sample for 10 minutes. A control pellet was prepared with chloroform (no mycolic acids added) and was used to determine the background IR spectrum. The spectrum was analysed on a Perkin Elmer 1600 series FT-IR system and plotted on a Roland Digital Group X-Y Plotter DXY-1200.

2.3.11. Biological activity of mycolic acids measured by double negative-T-cell proliferation studies

To test the biological activity of CCD purified mycolic acids, unsaponified (10,2 mg), freshly saponified (10,4 mg) and saponified and stored (10,4 mg) mycolic acids were sent to Dr Niehues (Heinrich Heine Children's Hospital, Düsseldorf, Germany) to perform double negative T-cell (DN T-cell) proliferation studies. His method (Niehues et al., 1994) briefly entailed the following:

Peripheral human blood leukocytes (PBL) were obtained from freshly donated blood from the blood bank. From this, $\alpha\beta$ CD4⁻ single positive, CD8⁻ single positive and CD4⁻, CD8⁻ double negative (DN) T-cells were purified using magneto-labelled monoclonal antibodies and used in a T-cell proliferation assay. Antigen presenting cells (APC) were induced to express CD1 on their cell surface by treatment

with GM-CSF and IL4 (Porcelli *et al.*, 1992). Fluorescence activated cell sorting (FACS) was used to test for expression of CD1. Single positive and DN T-cells were plated in triplicate in 96 well microtitre plates and stimulated with human AB serum containing mycolic acids (5, 25 and 50 µg per ml of culture) and phytohemagglutinin (PHA − 3,3 µg/ml), a mitogen, for 72 hours. Proliferation was tested by pulsing cells with ^{3}H-thymidine (0.5 mCi/well) and testing for incorporation of radio-labelled thymidine, measured by scintillation counting. Dose response curves were generated from the data obtained.

Mycolic acids were added to the serum in the following manner:

Mycolic acids were dissolved in chloroform (2 % of the serum volume) and added to filter sterilised human type AB serum in a sterile environment. The samples were then sonicated using a Branson sonifier with an autoclaved tip for 50 pulses at 20% duty cycle and an output of 2, at room temperature. Nitrogen gas was bubbled through the solution until no odour of chloroform could be detected in the solution. The highest concentration of mycolic acids was prepared and diluted with human type AB serum to obtain the concentration range used in the cultures.

2.4. Results:

2.4.1 Cultivation of bacteria

Growth was scraped off four-week old cultures on LJ slants for extraction purposes. Suitably cultured slants contained growth with a coarse texture and a creamy colour. Cultures dried out when too old and were difficult to remove from the slant without scraping off some of the medium. An example of an LJ slant is shown in Fig 2.13.

Figure 2.13   LJ slant containing four week old *M. tuberculosis* growth.

2.4.2. Preparation of crude mycolic acids extracts from bacterial samples

Bacterial suspensions in reagent A were prepared at turbidities equivalent to McFarland standards. Initially, this was done visually by comparing the McFarland standard to the bacterial suspension. To improve this method, the absorbances of the McFarland standards were determined spectrophotometrically at 486 nm.

The following results were obtained for McFarland standards number 1-5 suspensions:
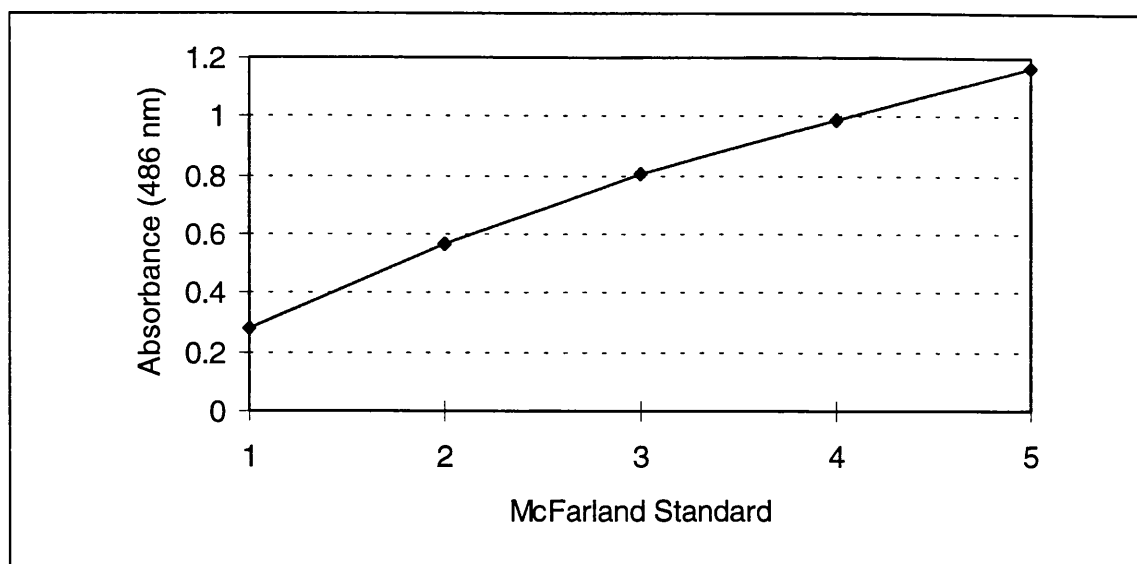
Figure 2.14   Standard curve obtained for McFarland standards at 486 nm.

The graph obtained when the absorption values at 486 nm were plotted against the McFarland standard approached a straight line. For all subsequent extractions the bacterial suspensions were prepared to an absorbance of 1 at 486 nm, which corresponds to a McFarland standard number 4 on the standard curve.

2.4.3. HPLC analysis and quantification of mycolic acids

The reagent baseline at 260 nm, under the conditions at which the HPLC analyses were done, was obtained before any sample was analysed by injecting 10 µl of pure filtered methylene chloride.
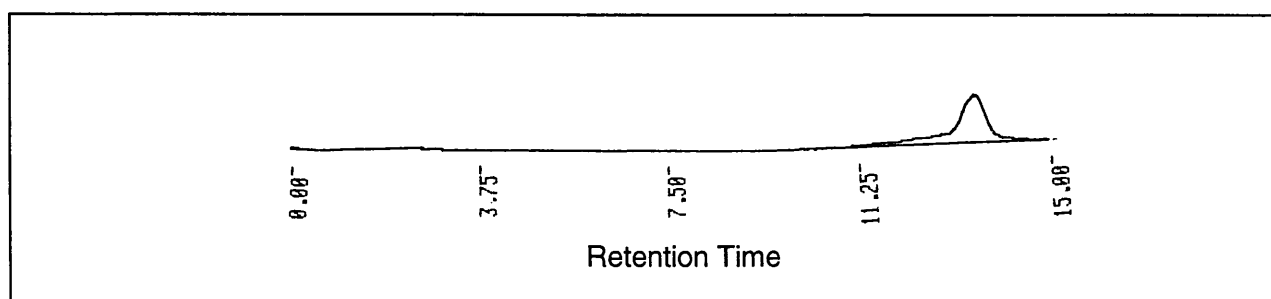
Figure 2.15   The baseline HPLC profile, obtained when injecting filtered methylene chloride.

The profile in Figure 2.15 indicates a reagent mixing peak at a retention time of 13,5 min which is due to the rather sudden change from 35% methanol / 65% dichloromethane to 98% methanol / 2% dichloromethane producing mixing vortices detected at 260 nm. As all components of the samples eluted before this reagent peak, the end time of all subsequent profiles was set at 12,5 min.

The HPLC profile of only the derivatising agent and internal standard, follows in Figure 2.16.
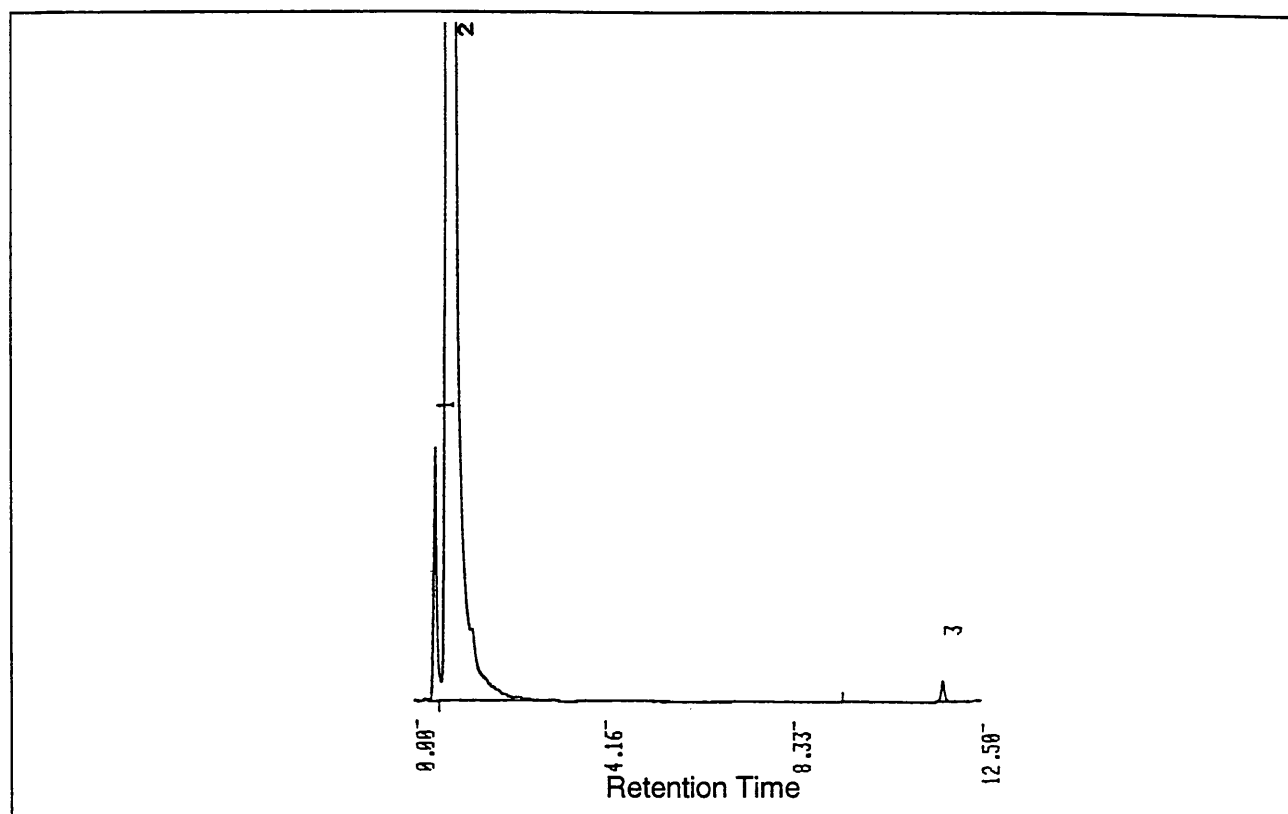
Figure 2.16   HPLC profile of the internal standard and derivatising agent.

Peaks 1 and 2 are accounted for by the injection peak, and derivatising agent respectively and peak 3 represents the internal standard.

Crude extract of *M. tuberculosis* was prepared by suspending the bacteria in reagent A to a turbidity corresponding to a McFarland Standard number 4, followed by extraction and derivatised as described in 2.3.2. and 2.3.3. The sample was injected into the HPLC system for analysis, to determine the HPLC profile before purification of mycolic acids.

The HPLC profile obtained of the derivatised crude extract is shown in Fig 2.17.
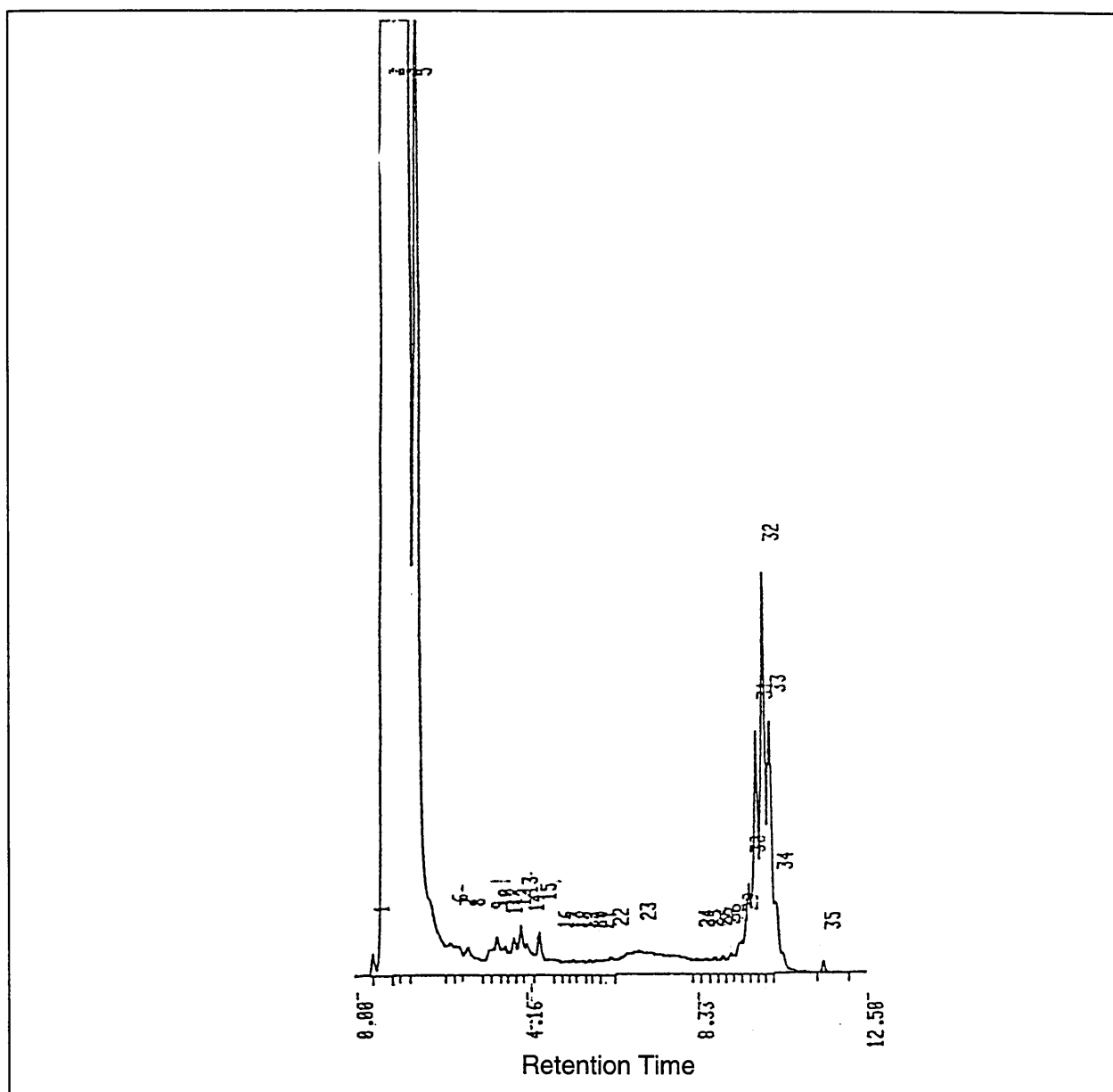
Figure 2.17   Typical HPLC profile for crude extract from *M. tuberculosis*.

Peaks 6 - 23 represent derivatisable short chain fatty acids present in the crude extract while peaks 24 - 34 are the mycolic acids peaks. The mycolic acids (0,524 mg) were quantified by comparing the integrated areas of these peaks to that of the internal standard according to the formula presented in 2.3.3. resulting in a yield of 7,64 % from 6,86 mg crude extract.

2.4.4. Countercurrent purification of mycolic acids from crude mycolic acids extracts

2.4.4.1. The phase diagram for chloroform, water and methanol

A phase diagram was prepared by plotting the results of the titrations done for methanol, chloroform and water (Figure 2.18). The dashed curve represents the solvent compositions at which the titration mixtures became one phase. All areas below the line, represent mixtures of the solvents at which two phases are formed, while above the line, one phase is formed.

Point [1] represents the phase composition at which preliminary experiments were done.

The point numbered [2] represents a phase composition of

45 % methanol : 25 % water : 30% chloroform

and is the point in the phase diagram at which the experiment described in 2.3.4.3. was done.

The phase composition at point [2] was chosen for further procedures to increase the distribution constants of other fatty acids with distribution constants similar to that of mycolic acids at point [1], thereby increasing the separation of contaminants from the mycolic acids which have distribution constants approaching zero at both [1] and [2].

The phase composition of the biphasic system described above [2] was determined to be 15 % chloroform, 52 % methanol and 33 % water in the upper phase and 68 % chloroform, 27 % methanol and 5% water in the lower phase. Line [PQ] was plotted on the phase diagram in Figure 2.18, where [P] represents the composition of the upper phase and [Q] represents the composition of the lower phase. The point labelled [M] represents the point at which the upper and lower phases have equal volume since it is the point half-way between [Q] and [P] on the [PQ] line. The phase composition at this point (42 % chloroform, 39 % methanol and 19 % water) was selected for the purification of mycolic acids by countercurrent distribution as described in 2.3.4.4.

Methanol
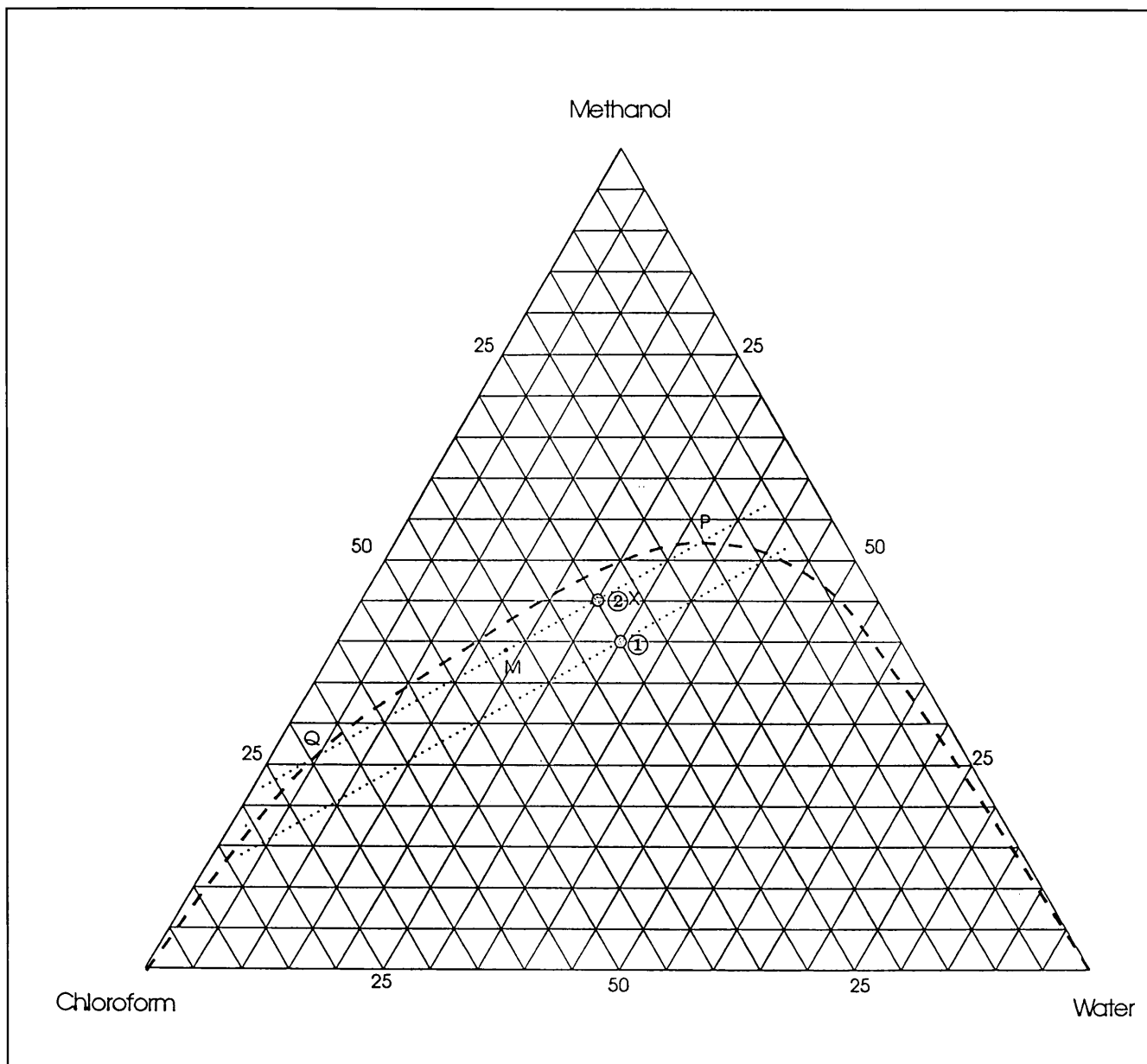
Chloroform

Water

Figure 2.18   The phase diagram obtained for methanol, chloroform and water.

2.4.4.2. Determination of the ideal phase composition for countercurrent distribution

Crude mycobacterial extract was dissolved in equal volumes of upper and lower phases of a biphasic system composed of 45 % methanol: 30 % chloroform: 25 % water to determine if the phase system was suitable for the purification of mycolic acids on the countercurrent apparatus. The mass of crude extract dissolved in the upper, lower and inter-phase of the biphasic system was determined to be 6,8 mg in the upper phase (54 %), 0,9 mg in the inter-phase (7 %) and 4,9 mg in the lower phase (39 %). The mycolic acid's distribution in these phases was determined by HPLC analysis, shown in the Figure 2.19.
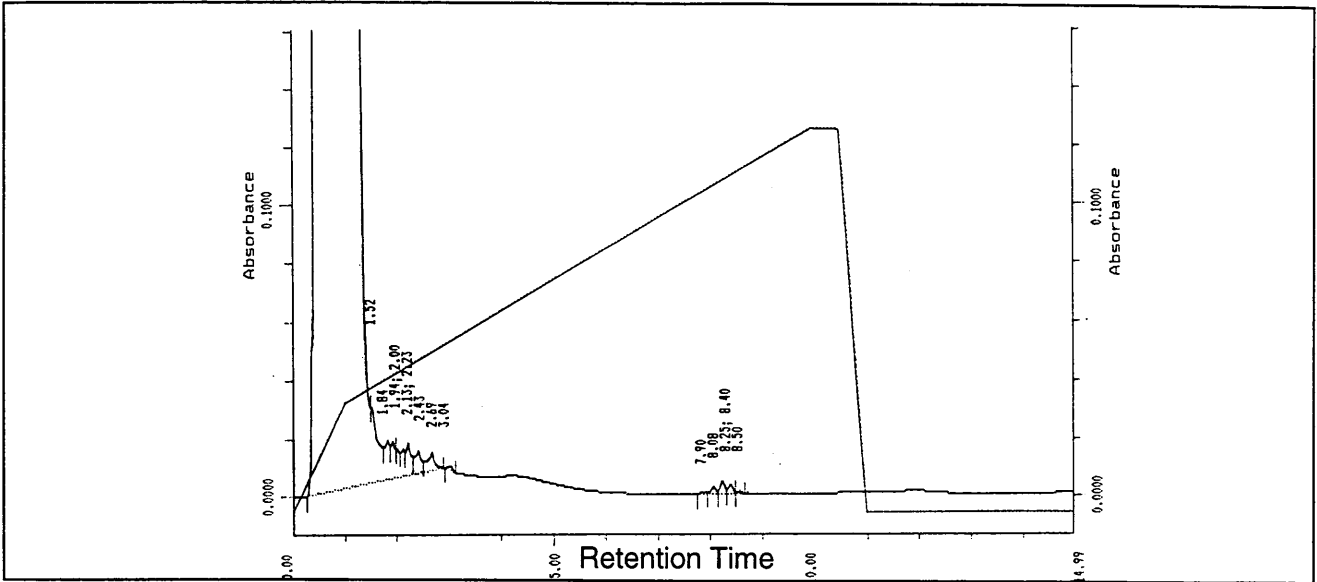
Figure 2.19 (a) HPLC profile of crude *M. tuberculosis* extract dissolved in the upper phase.
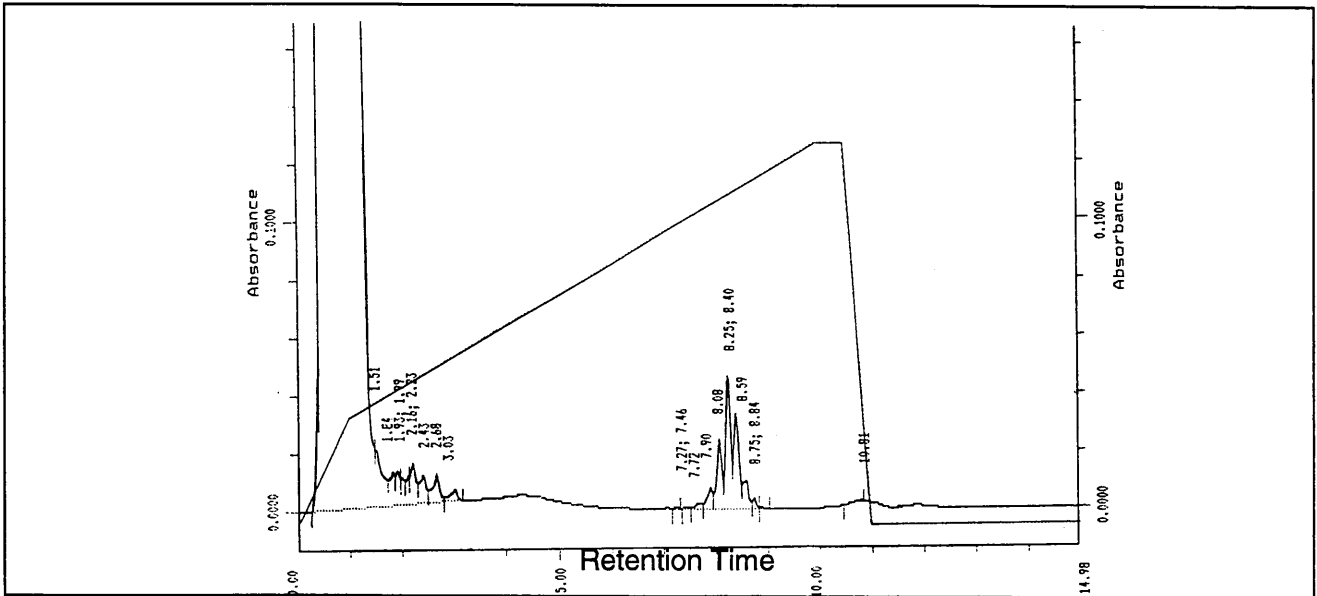
Figure 2.19 (b) HPLC profile of crude *M. tuberculosis* extract dissolved in the inter-phase.
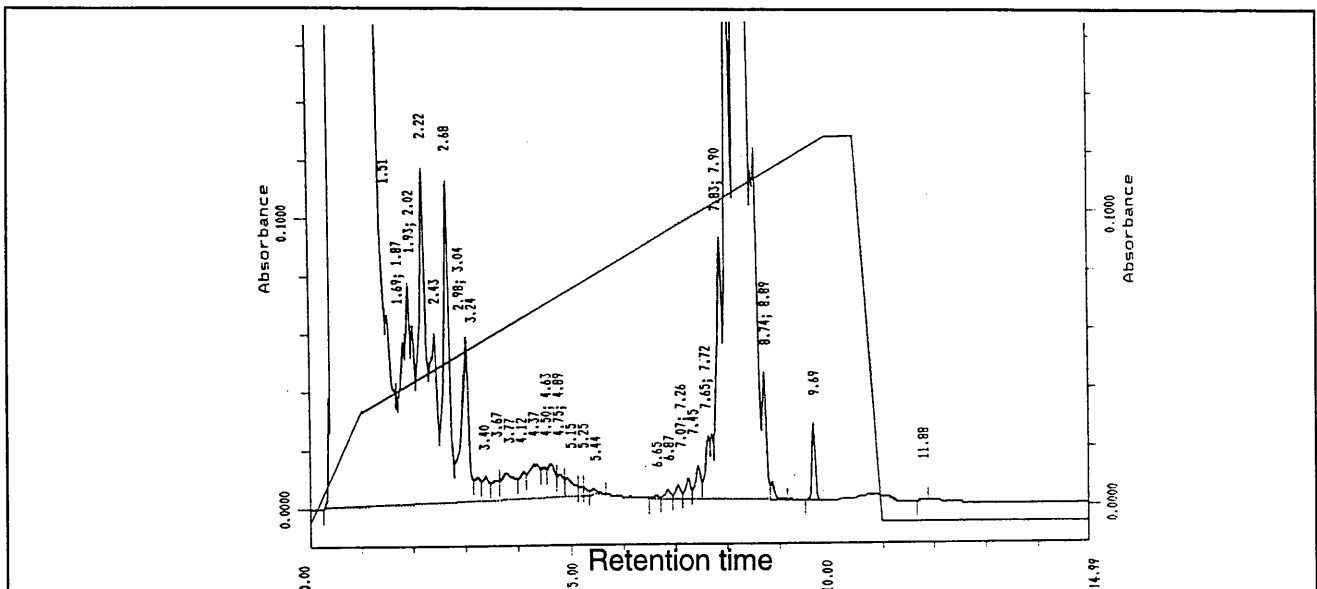
Figure 2.19 (c) HPLC profile of crude *M. tuberculosis* extract dissolved in the lower phase.

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The HPLC profile of the upper phase (Figure 2.19 a) shows the presence of a small quantity of mycolic acids (retention time 7,9 - 8,5) as well as other short chain, derivatised fatty acids (retention time 1,52 - 3,04).

The HPLC profiles indicated that most of the mycolic acids as well as other derivatisable short chain fatty acids dissolve in the lower phase while a small quantity was present in the interphase. A portion of the mycolic acids present in both the interphase and upper phase could be due to mycolic acids adhering to the glass of the separation funnel. Therefore, the phase composition chosen for this experiment semi-purifies the mycolic acids (lower phase) from the more water soluble, underivatisable components, (upper and inter-phase) which constitute 61 % of the mass of crude mycobacterial extract.

2.4.4.3. Countercurrent purification of mycolic acids from crude mycobacterial extracts

The mass distribution of approximately 30 mg of crude extract, separated over 25 tubes of the countercurrent apparatus after 24 cycles, was determined, the distribution constants for each fraction calculated and the emulsion pattern in the tubes, immediately after the mixing step, was noted. The performance of the countercurrent distribution technique for purification of mycolic acids from crude extract was quantified.

The results obtained are summarised in Figure 2.20.

% Mass distribution chart — Fraction values:
- 1: 8
- 2: 1.3
- 3: 0
- 4: 2.7
- 5: 35.5
- 6: 41.6
- 7: 4.5

Fraction number	1		2	3	4	5	6	7
Tube Number	0	1	2 - 4	5 - 7	8 - 11	12 - 19	20 - 22	23 24
Yield (mg) [total 35,1 mg]	3,0		0,5	0	1,0	13,3	15,6	1,7
Distribution constant (Kd)	0,008			- -	1,26		5,5	16,3
Emulsification pattern	Lower phase			None	Lower phase	Upper phase	None	None
Physical nature	Mycolic acids			None	Soap (saponified fatty acids)		Crystalline compound	Non-crystaline compound
Soluble in:	Chloroform			- -	Chloroform : methanol (3:7 v/v)		50 % methanol-water	50 % methanol-water

Figure 2.20   Countercurrent separation of components in the crude mycolic acids extract of

M. tuberculosis.

The various fractions were termed Mycolic Acid, Clear, Acidic Soaps, Basic Soaps and Crystalline.

The HPLC profile obtained for fraction1 is shown in Figure 2.21.
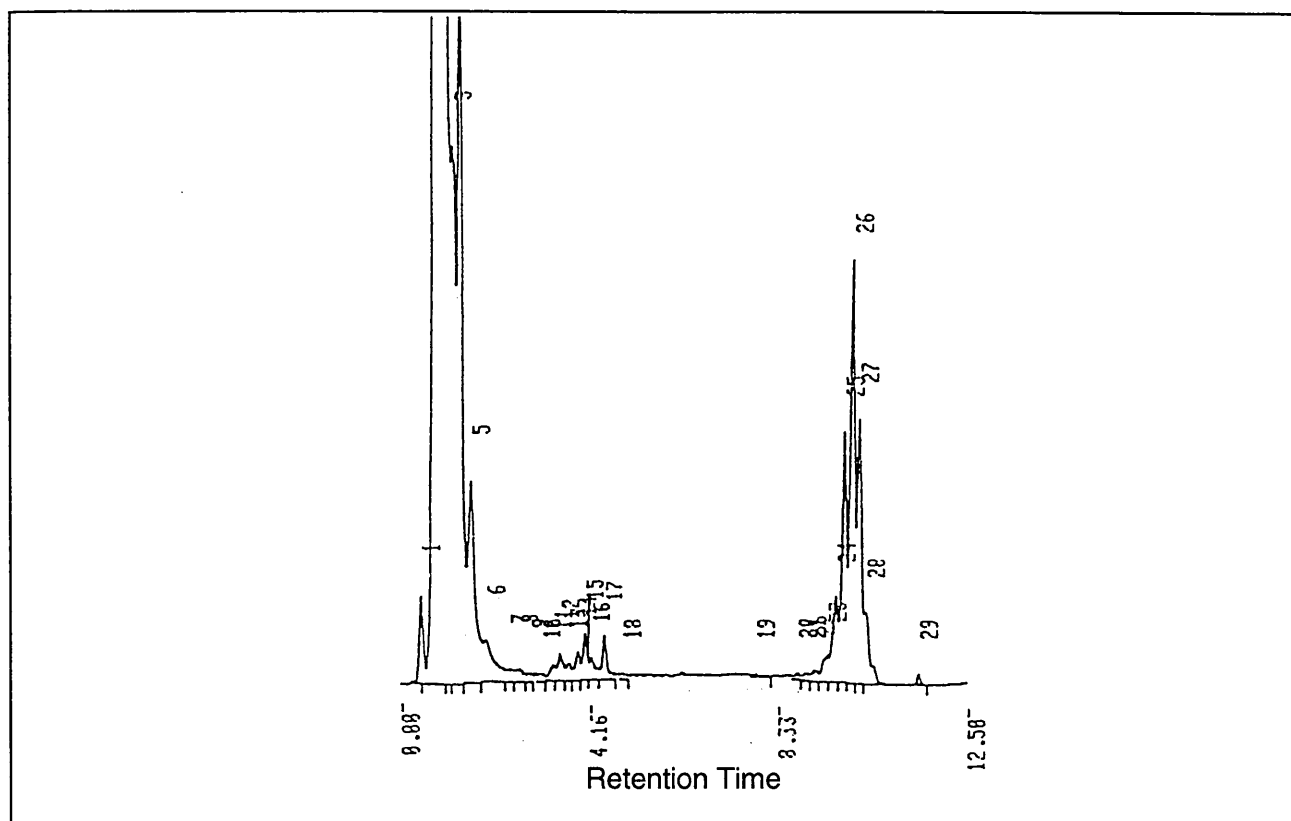
Figure 2.21   HPLC profile of mycolic acids in Fractions 1 and 2.

The HPLC profiles indicated that the mycolic acids occurred only in the first two fractions. The other components of the crude mycobacterial extract have various distribution constants, higher than that of mycolic acids, and thus moved further along the tube train of the countercurrent apparatus.

2.4.5. Infrared analysis of countercurrent purified mycolic acids

Infrared analysis of countercurrent purified mycolic acids was performed to investigate the purity as well as the degree of saponification of the reactive carboxyl group (used for derivatisation) of mycolic acids. The FT-IR profile obtained for mycolic acids purified by CCD is shown in Figure 2.22.
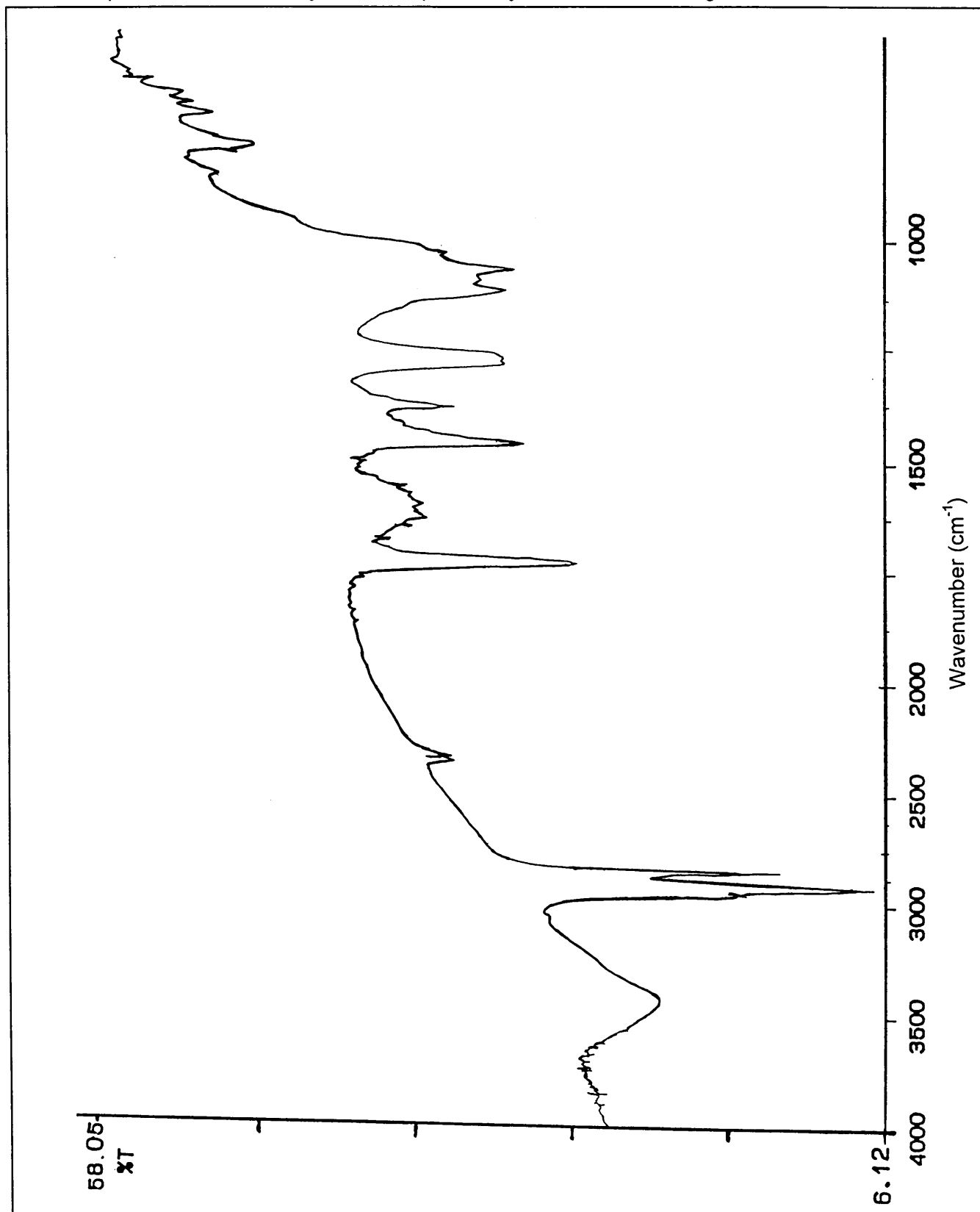
Figure 2.22   Infrared profile for CCD purified mycolic acids obtained by FT-IR.

The infrared spectrum of countercurrent-purified mycolic acids was expected to show the mycolic acids in a carboxylic acid form, since the molecule was saponified by KOH in the initial crude extraction procedure. A carboxylic acid should have shown a very broad absorption band between 3 500 cm^{-1} and 2500 cm^{-1} (Vogel, 1989). This was not present in this profile (Figure 2.22) which looks similar to that of a methyl ester of mycolic acids in Figure 2.9 and suggests that the molecules were in an esterified form.

The broad absorption band between 3 600 cm^{-1} and 3 100 cm^{-1} is indicative of O-H stretch, involved in intramolecular hydrogen bonding (Vogel, 1989). The sharp peak in Figure 2.9 within this broad band (at approximately 3570 cm^{-1}) is however absent. The strong double absorption band present in the region of 3000 cm^{-1} - 2800 cm^{-1} shows the presence of CH_3 and CH_2 stretch (Lin-Vien *et al.*, 1991). The weak absorption bands in the region of 2 400 cm^{-1} were present in the blank sample analysed and are thus not due to the mycolic acids. The sharp absorption band at 1750 cm^{-1} indicates C=O stretch due to the presence of either carboxylic acid or ester bonds.

The absence of strong absorption bands at 850 cm^{-1} - 730 cm^{-1} and two to three bands of medium intensity at 1600 cm^{-1} - 1500 cm^{-1} eliminates the presence of aromatics in the sample. Furthermore, the lack of sharp and relatively weak bands in the 3 500 cm^{-1} - 3 000 cm^{-1} region indicates the absence of amines. Amides are not present in the sample since there are not two sharp bands in the 3 450 cm^{-1} – 3 300 cm^{-1} region. These observations support the data in 2.4.7 and show the absence of contaminants which contain these chemical groups in the purified mycolic acid sample (Vogel, 1989).

Countercurrent-purified mycolic acids could not be derivatised with reagent D, which forms an ester with the carboxylic acid of the fatty acid during derivatisation and could account for the apparent low yield of purified mycolic acids obtained by HPLC analysis, after CCD purification (as shown in 2.4.6.). In order to derivatise CCD-purified mycolic acids, they had to be de-esterified or re-saponified prior to derivatisation. This supports the idea that mycolic acids obtained after countercurrent purification are esterified, more than likely to the methanol in the biphasic solvent system, forming a mycolic acid methyl ester.

Mycolic acids are large molecules containing up to 90 carbon molecules and only one carboxyl group. The contribution of the carboxyl as well as the OH groups, to the infrared spectrum are therefore, relatively small, accounting for the weak bands generated by these groups compared to the bands produced by CH_3 and CH_2.

2.4.6. De-esterification (saponification) of mycolic acid methyl esters

CCD-purified mycolic acids were subjected to saponification in an autoclave as done during the crude extraction process, to determine if methyl esterification was the cause of the low yield on HPLC. A control sample was prepared of pure mycolic acids that were derivatised for HPLC without re-saponification.
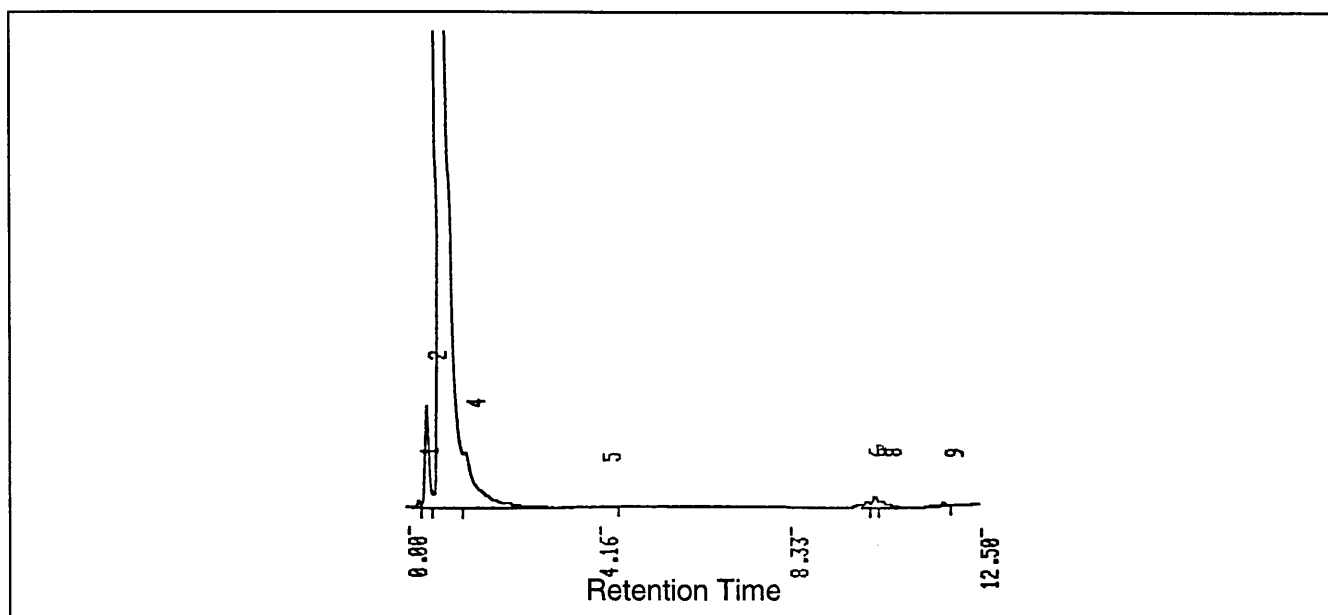
Figure 2.23 (a)   HPLC profile of CCD purified mycolic acids not subjected to resaponification.
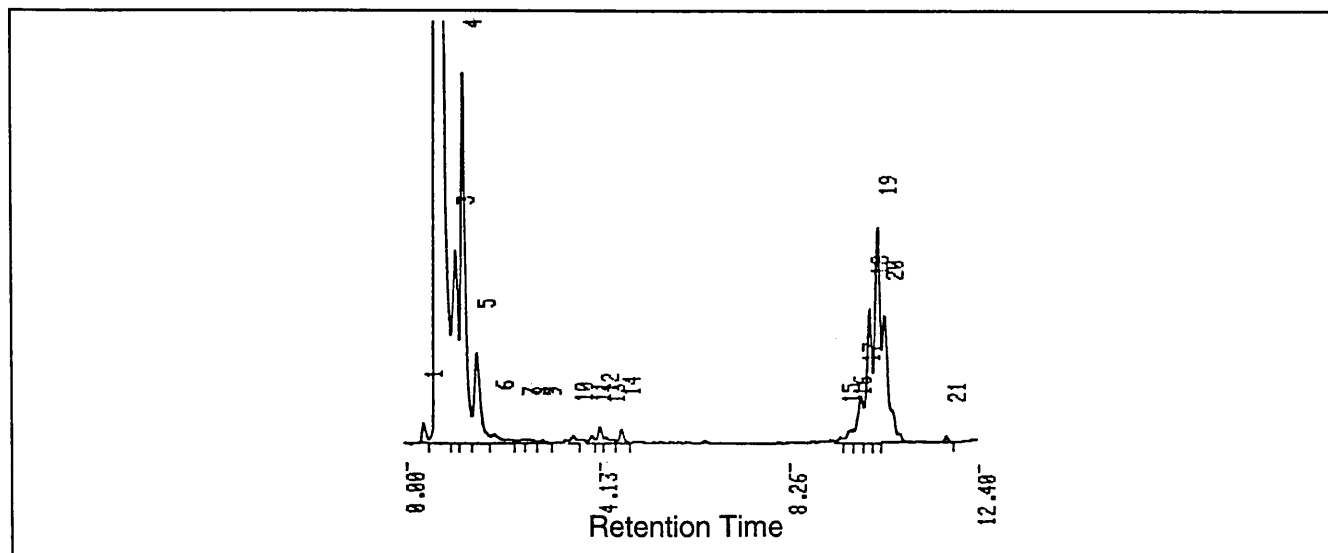
Figure 2.23 (b)   HPLC profile of CCD purified mycolic acids subjected to resaponification.

The HPLC profiles presented in Figure 2.23 confirm the hypothesis that the low yield of mycolic acids detected after CCD purification was due to esterification of the reactive mycolic acids carboxyl group during the purification process. CCD-purified mycolic acids (approximately 0,5 mg) resaponified before derivatisation and HPLC analysed, yielded 371,8 µg (approximately 74,4 %) while the equivalent sample (approximately 0,5 mg), derivatised and HPLC analysed without prior resaponification, yielded only 18,7 µg (3,74 %). These results showed that resaponification was essential for accurate detection of CCD-purified mycolic acids by HPLC.

A modification of the saponfication process was tested to determine whether autoclaving was necessary. One sample was saponified in the autoclave while the other was saponified at room temperature. There was no difference between the degree of saponification of the two samples, indicating that autoclaving is not necessary for saponification of CCD-purified mycolic acids.

2.4.7. Determination of the chemical purity of mycolic acids

The purity of CCD purified mycolic acids was calculated by comparing the gravimetric yield of pure mycolic acids obtained from crude mycobacterial extract with the amount of mycolic acids present in the crude extract, determined by HPLC quantification.

A yield of 7,28 % of purified mycolic acids (exactly 34,9 mg) was obtained from crude mycobacterial extract (exactly 478,8 mg) after countercurrent purification. The total quantity of mycolic acids (524 µg) present in a sample of this crude mycobacterial extract (exactly 6,85 mg) was determined by HPLC quantification relative to the internal standard. Thus, the yield of mycolic acids in the crude mycobacterial extract, determined by HPLC amounted to 7,64 %. The purity of the CCD-purified mycolic acids relative to the total amount of mycolic acids in crude extract was therefore 95 %. Mycolic acid purity varied from 90 % to 98% in subsequent experiments.

$$\% \, purity \quad = \quad \frac{7,28}{7,64} \times 100$$

$$= \quad 95\,\%$$

2.4.8. Removal of remaining contaminants by acetone rinsing of mycolic acids

Figure 2.23 (b) shows that some contamination (peaks 7-14) remained after CCD purification. Acetone rinsing was considered as a further purification step to remove contaminants from mycolic acids.

Triplicate samples of crude mycobacterial extract were retained for HPLC analysis (10,9 mg-Figure 2.24 (a)) and the remaining crude extract (109,6 mg) was divided in half (54,8 mg each). One half was rinsed with acetone and samples of the acetone supernatant and the rinsed crude extract were retained for HPLC analysis (Figure 2.25 (a) and Figure 2.26 (a)). Mycolic acids (in tubes 0-4) were purified from both the acetone-rinsed and unrinsed extracts by CCD purification after 26 cycles. Samples of the mycolic acids fraction purified from unrinsed crude extract were removed for HPLC analysis (Figure 2.28 (a)). The mycolic acids were then rinsed with acetone and both the acetone (Figure 2.29 (a)) and the rinsed mycolic acids (Figure 2.30 (a)) retained for HPLC analysis. The mycolic acids purified from acetone rinsed crude extract were also retained for HPLC analysis (Figure 2.27 (a)).

The same procedure was simultaneously followed for reagents (Figures 2.24 (b) - 2.30 (b)), and crude LJ medium extract (Figures 2.24 (c) - 2.30 (c)), prepared in the same manner as crude mycobacterial extract. All HPLC samples were prepared in triplicate with the average mass, quantified by HPLC, used to determine the mycolic acid yield at each step of the procedure, (the flow diagram in Figure 2.12 was followed).

The HPLC profiles of crude mycobacterial extract, reagents and medium before and after acetone rinsing are shown in Figures 2.24 (a-c) and 2.26 (a-c). The HPLC profiles of the acetone used to rinse the crude extract, reagents and medium are shown in Figure 2.25 (a-c).

Mass weighed: 10,9 mg

Mycolic acids (HPLC quantified): 0,78 mg

% Yield of mycolic acids: 7,1 %
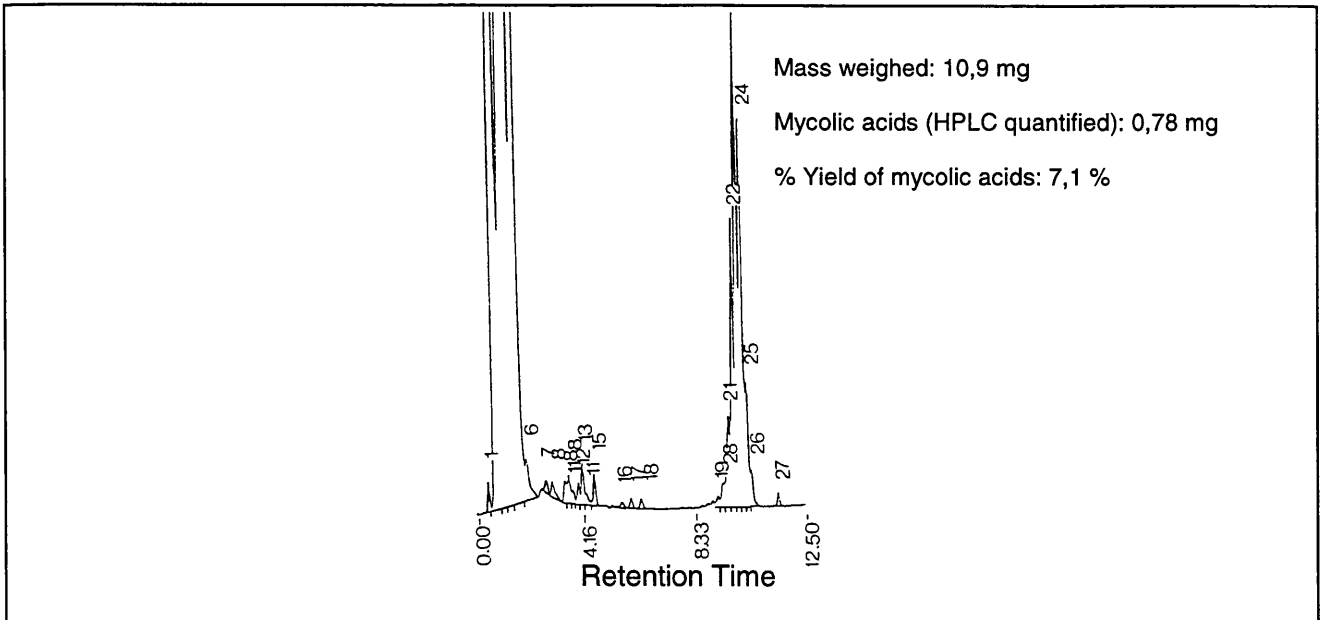
Figure 2.24 (a) HPLC profile of crude extract of *M. tuberculosis.*
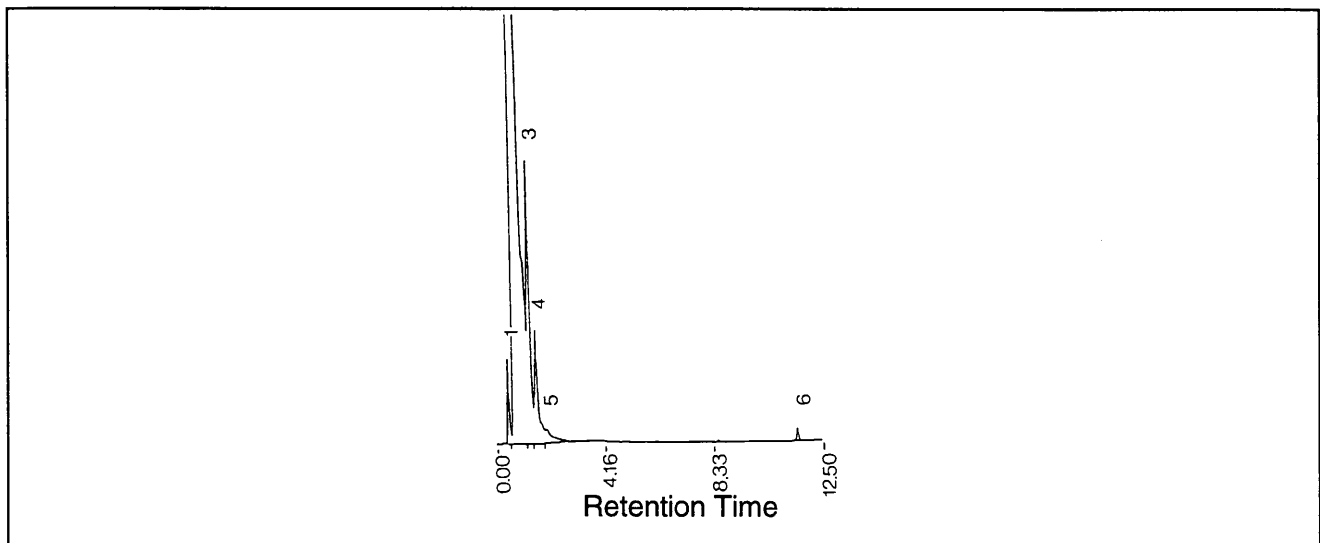
Figure 2.24 (b) HPLC profile of crude extract of reagents.
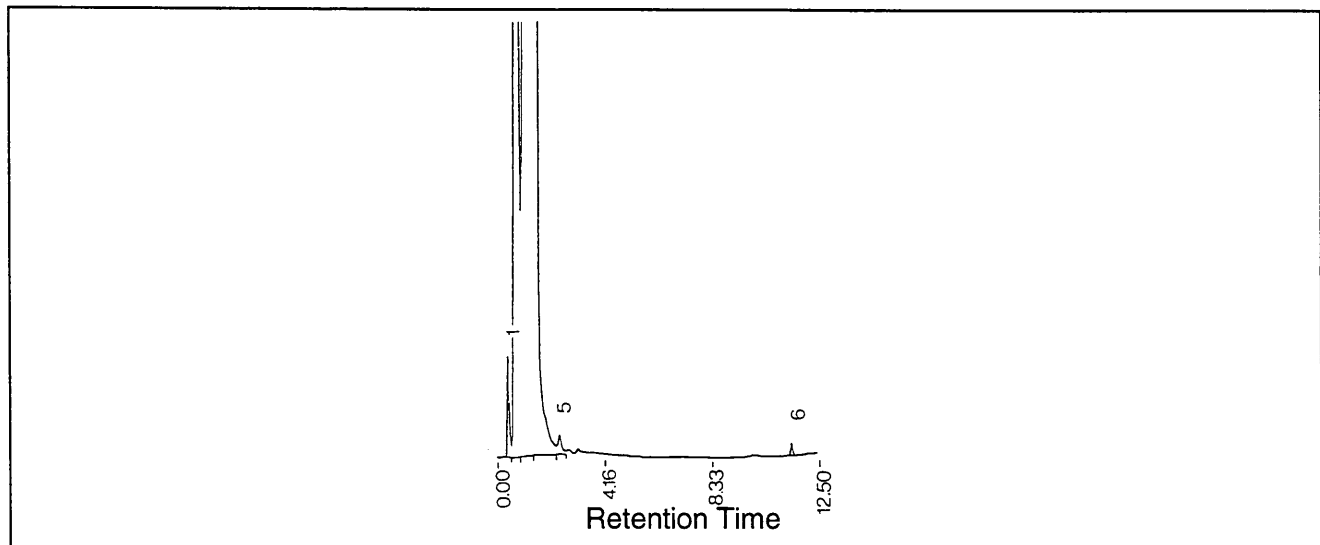
Figure 2.24 (c) HPLC profile of crude extract of LJ medium.

Many contaminants were present in the crude extract (Figure 2.24 (a)) including a large quantity of short chain fatty acids (peaks 3-6) which eluted in the region of the p-bromophenacyl bromide peak. Mycolic acids constitute 7,1 % of the mass of crude mycobacterial extract.

Figures 2.24 (b) and (c) contain a number of peaks (peaks 3-5 in each case) which elute in the same region as some of the contaminating peaks in Figure 2.24 (a). A portion of the contaminants may therefore originate from the medium or reagents used for the extraction of the *Mycobacteria*.

A portion of the mycolic acids present in the crude mycobacterial extract was removed by the acetone used to rinse the extract. The acetone contained some contaminants originating from the crude extract as shown in Figure 2.25 (a) (peaks 3-5 and in the region of peak 6).

Figures 2.25 (b) and (c) also show the removal of contaminants from both extracted reagents and medium. A number of contaminant peaks (peaks 3-5) were present in the acetone used to rinse the medium extract (Figure 2.25 (c)), indicating that acetone rinsing before countercurrent purification may be useful for the removal of contaminants.
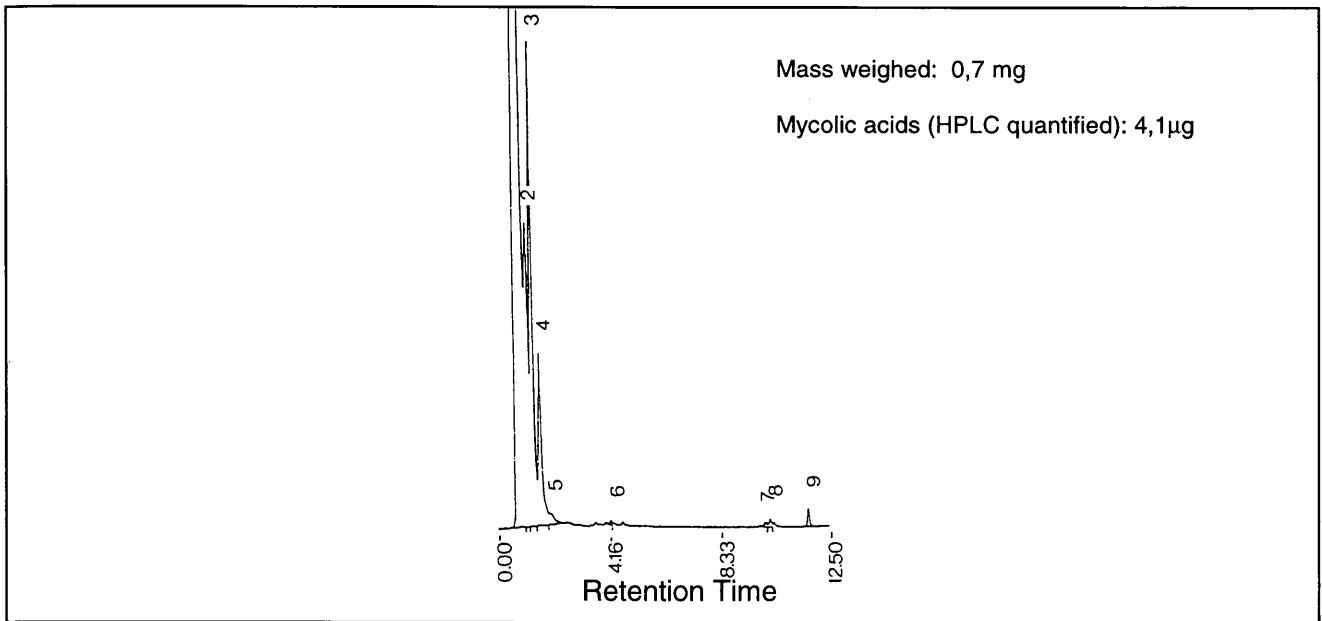
Figure 2.25 (a) HPLC profile of acetone supernatant of rinsed crude extract of *M. tuberculosis.*
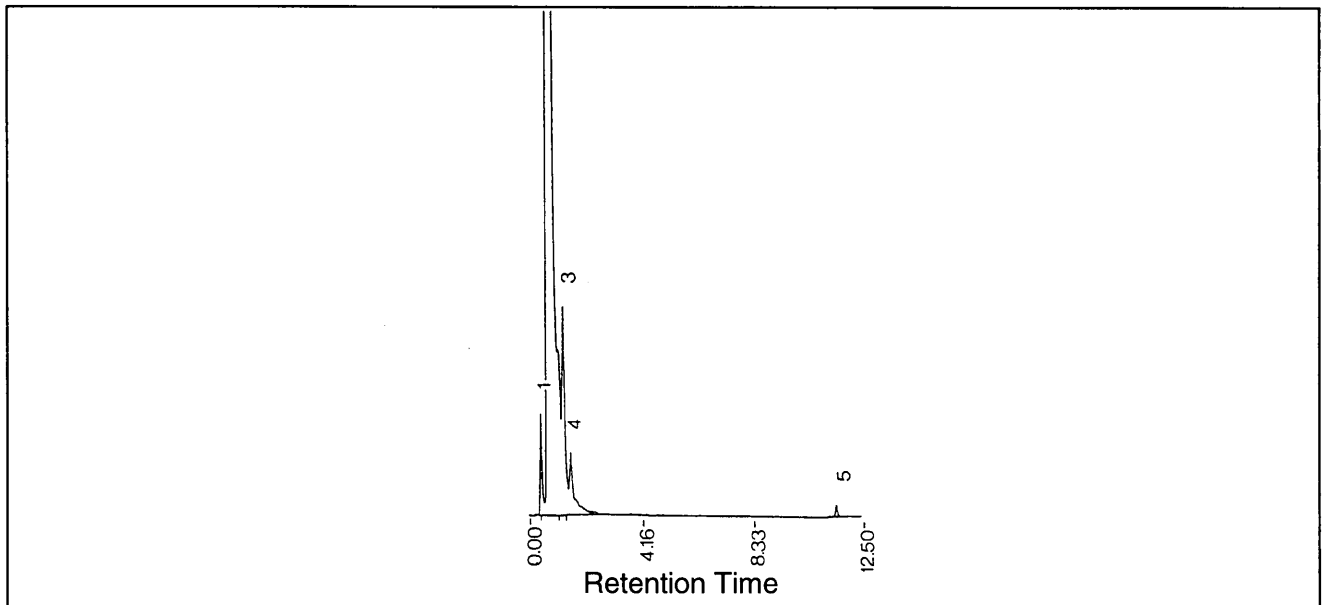
Figure 2.25 (b) HPLC profile of acetone supernatant of rinsed crude extract of reagents.
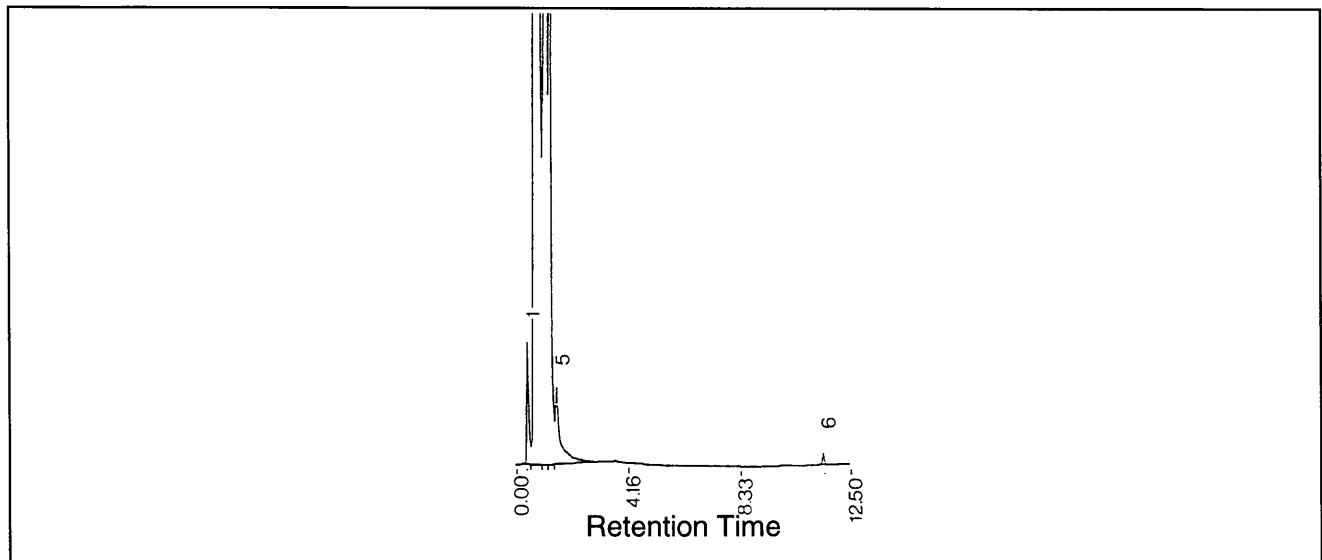
Figure 2.25 (c) HPLC profile of acetone supernatant of rinsed crude extract of LJ medium.

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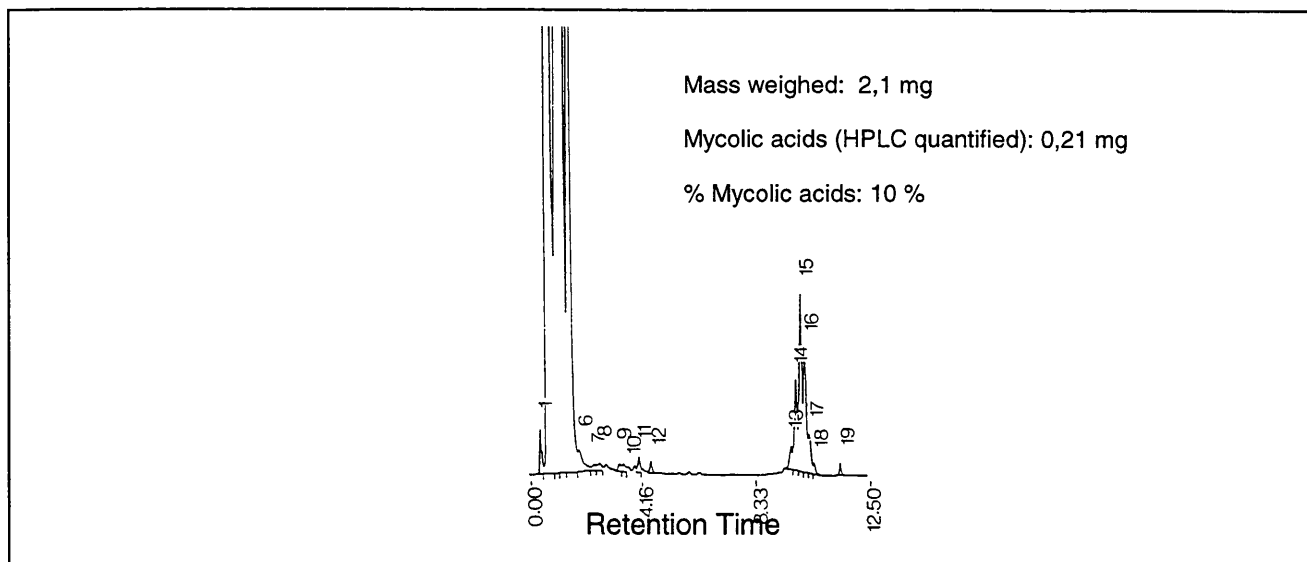
Figure 2.26 (a) HPLC profile of acetone-rinsed crude extract of *M. tuberculosis*.
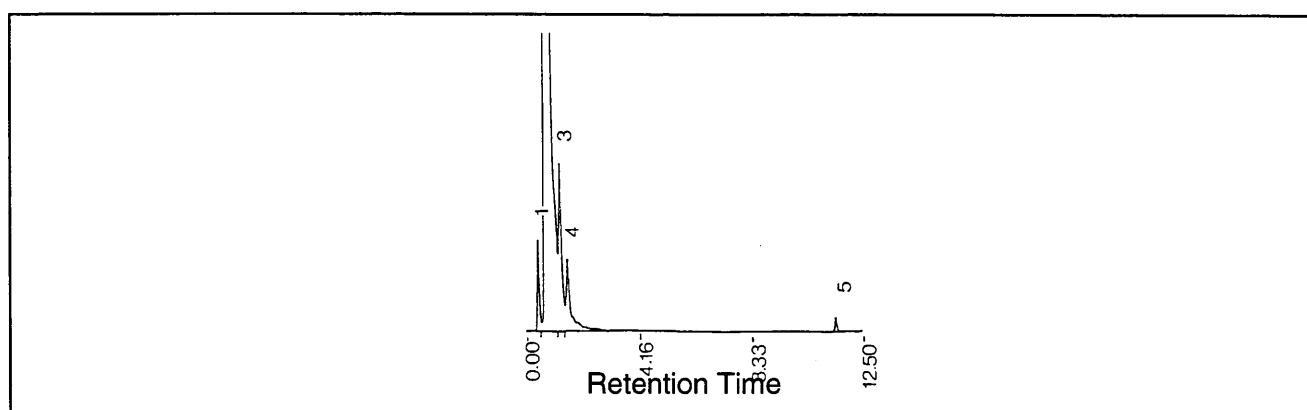
Figure 2.26 (b) HPLC profile of acetone-rinsed crude extract of reagents.
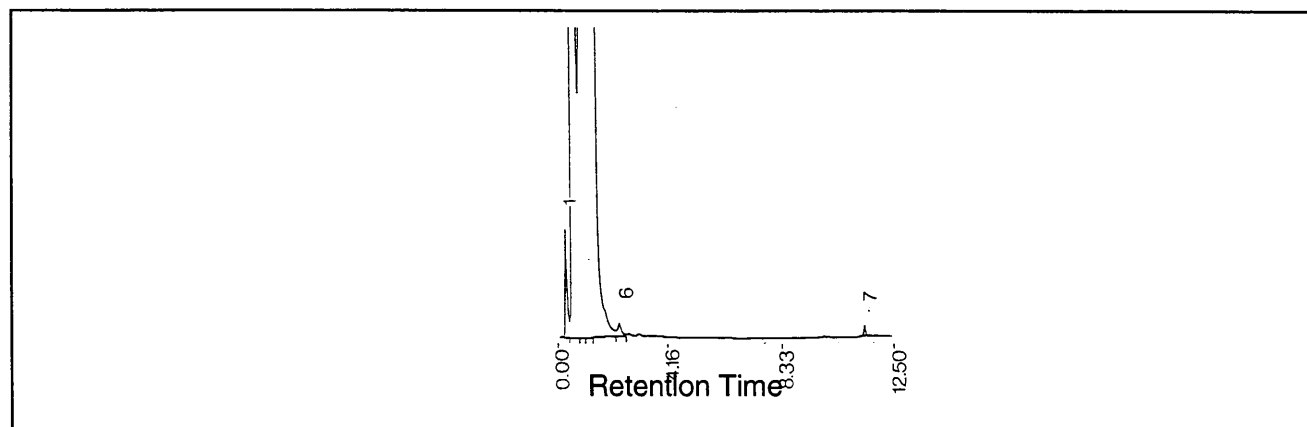
Figure 2.26 (c) HPLC profile of acetone-rinsed crude extract of LJ medium.

The HPLC profile of crude mycobacterial extract, rinsed with acetone (Figure 2.26 (a)), did not differ much from crude extract, prior to acetone rinsing (Figure 2.24 (a)). Similarly, the HPLC profiles of acetone rinsed reagents and medium (Figures 2.26 (b) and (c)), did not differ significantly from profiles before acetone rinsing (Figures 2.24 (b) and (c)).

Predicted mass: 0,3 mg

Mycolic acids (HPLC quantified): 0,23 mg
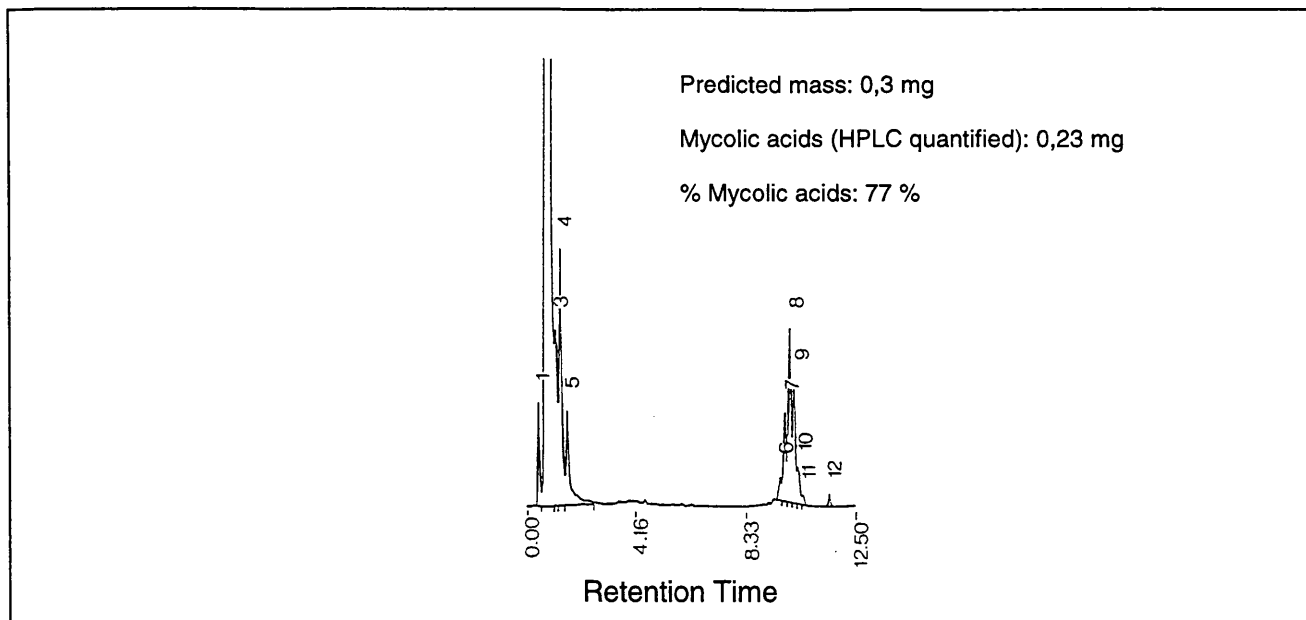
% Mycolic acids: 77 %

Figure 2.27 (a) HPLC profile of CCD-purified mycolic acids from acetone-rinsed crude extract of *M. tuberculosis.*
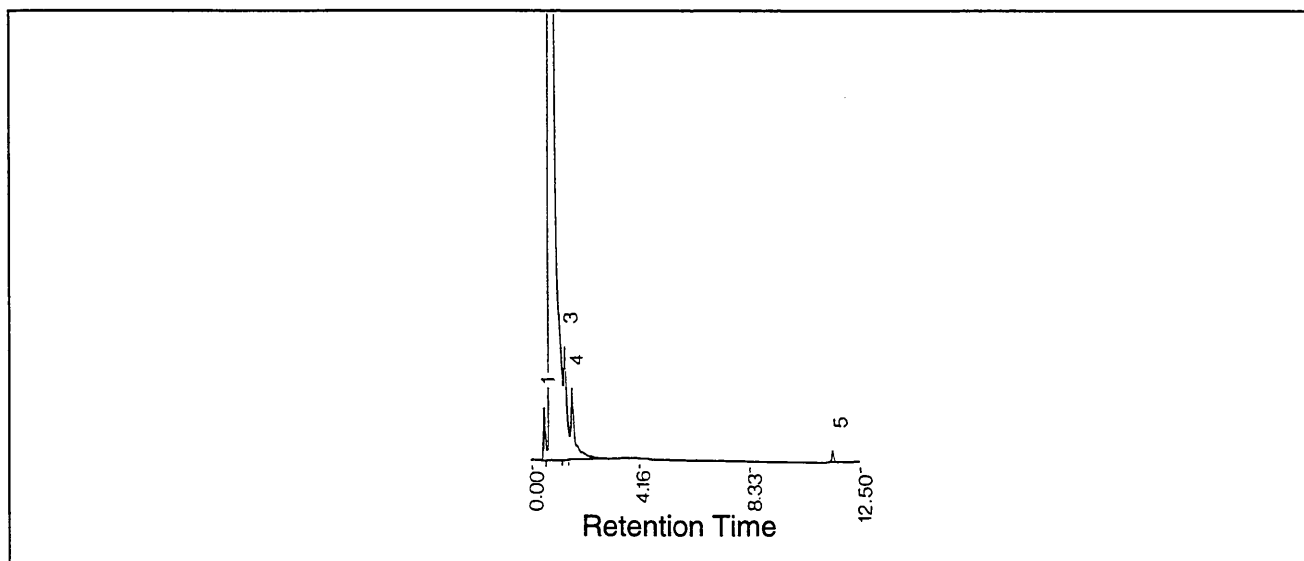
Figure 2.27 (b) HPLC profile of CCD purified first fraction of acetone rinsed crude extract of reagents.
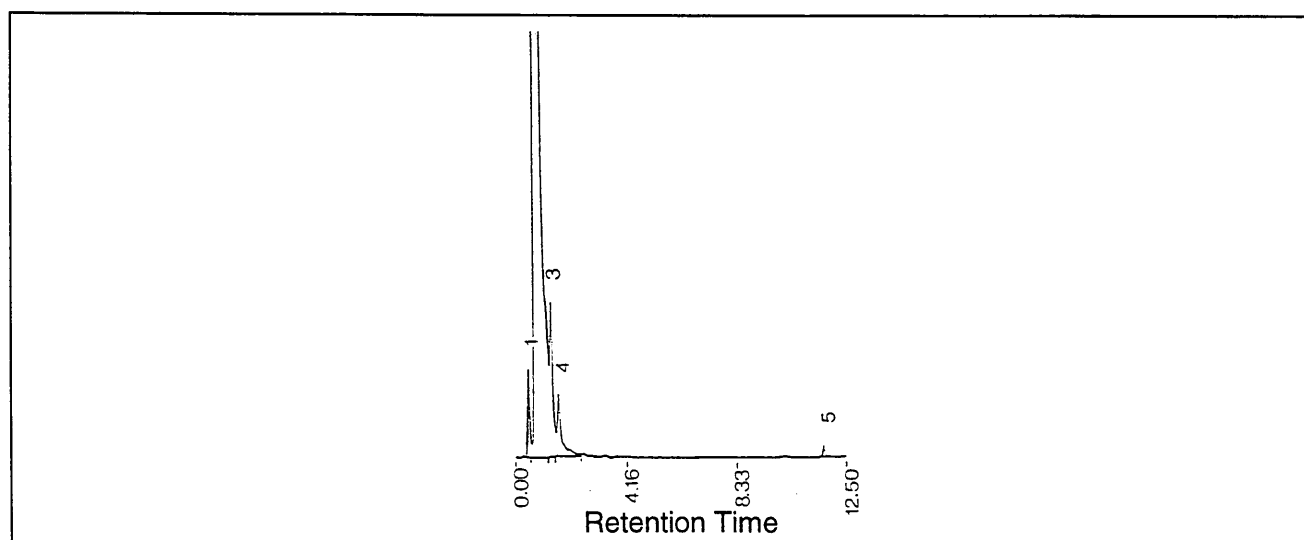
Figure 2.27 (c) HPLC profile of CCD-purified, first fraction of acetone-rinsed crude extract of LJ medium.

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The HPLC profile of mycolic acids purified by countercurrent distribution from crude mycobacterial extract rinsed with acetone is shown in Figure 2.27 (a). Contaminant peaks (peaks 3-5) were still present in the CCD-purified sample as well as some small peaks in the region of retention time of 4,16 minutes. Peaks 3 and 4 of Figures 2.27 (b) and (c) appear to originate from the reagents used for the extraction reactions and may account for peaks 4 and 5 of Figure 2.27 (a). Mycolic acids purified from acetone-rinsed crude extract by countercurrent distribution are 77 % pure if it is assumed that no loss occurred during CCD purification and the predicted mass of 0,3 mg is considered as the total mass of the sample. Previous studies showed 90 % purity, therefore it is thought that some mycolic acids were lost during acetone rinsing of the crude extract and on the surfaces of the countercurrent tubes, syringe used for loading the CCD and other vials used in the procedure.

The HPLC profile shown in Figure 2.28 (a) is that of mycolic acids purified from crude mycobacterial extract, not rinsed with acetone. The few contaminant peaks present were not significantly more numerous than those obtained from CCD purified mycolic acids from acetone rinsed crude-extract (Figure 2.27 (a)). Although a small quantity of contaminants were removed from the crude extract (Figure 2.25 (a)), by acetone rinsing, it is not an effective method of removing contaminants when applied before countercurrent purification of mycolic acids. Mycolic acids purified in this manner are 90 % pure if it was assumed that no loss occurred during CCD purification and the predicted mass of 0,3 mg was considered as the total mass of the sample. Small contaminant peaks were still present in the region of retention time 4,16 minutes.
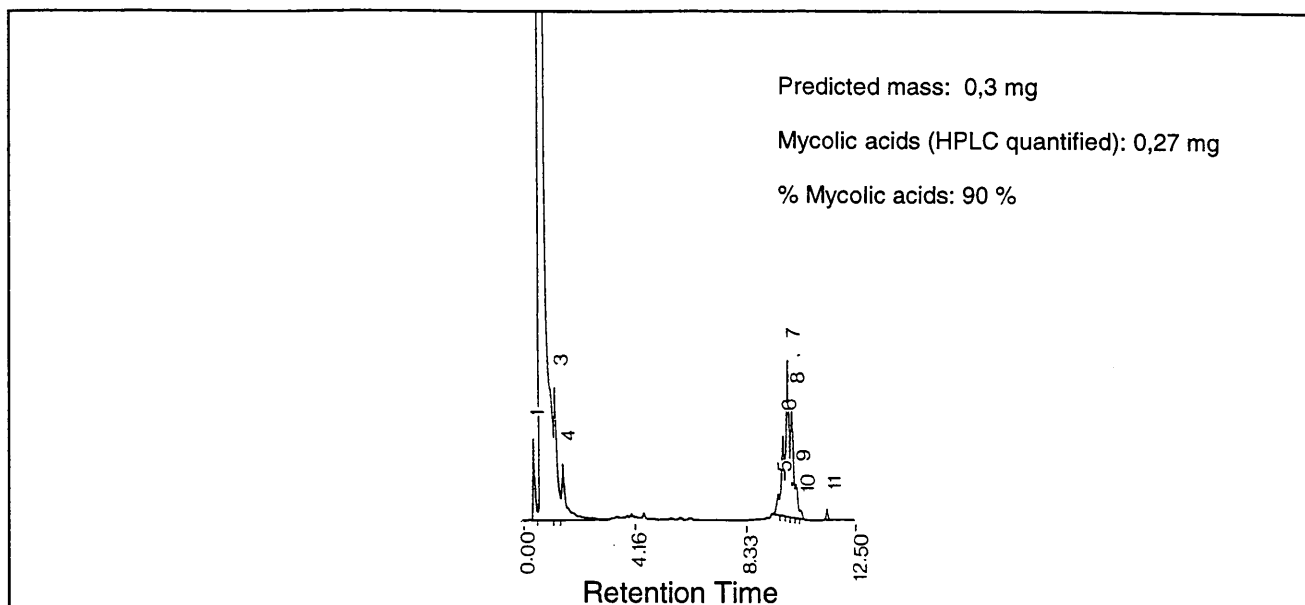
Figure 2.28 (a) HPLC profile of countercurrent purified mycolic acids from *M. tuberculosis* with no prior acetone rinsing of the crude extract.
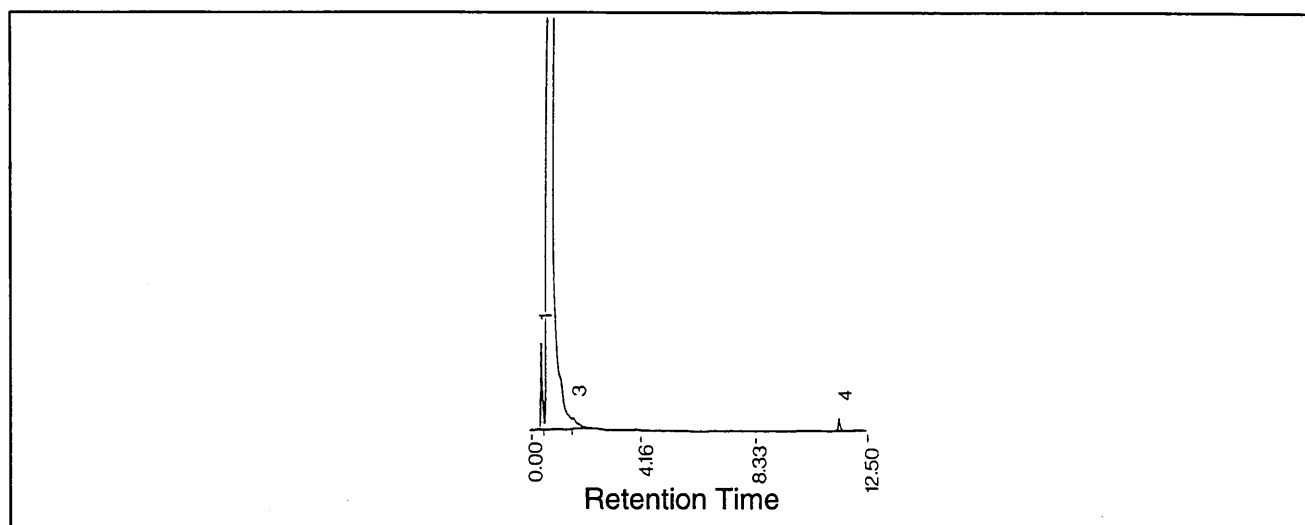
Figure 2.28 (b) HPLC profile of countercurrent purified reagents (first fraction) with no prior acetone rinsing of the crude extract.
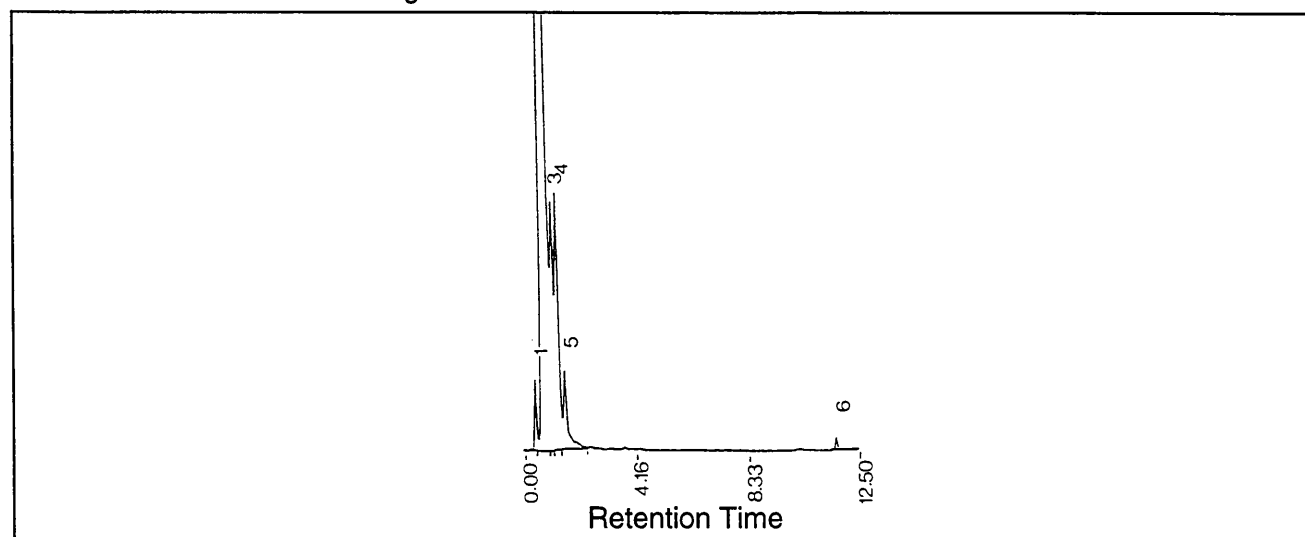
Figure 2.28 (c) HPLC profile of countercurrent purified LJ medium (first fraction) with no prior acetone rinsing of the crude extract.

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Mass weighed: 0,9 mg

Mycolic acids (HPLC quantified): 32,9 µg
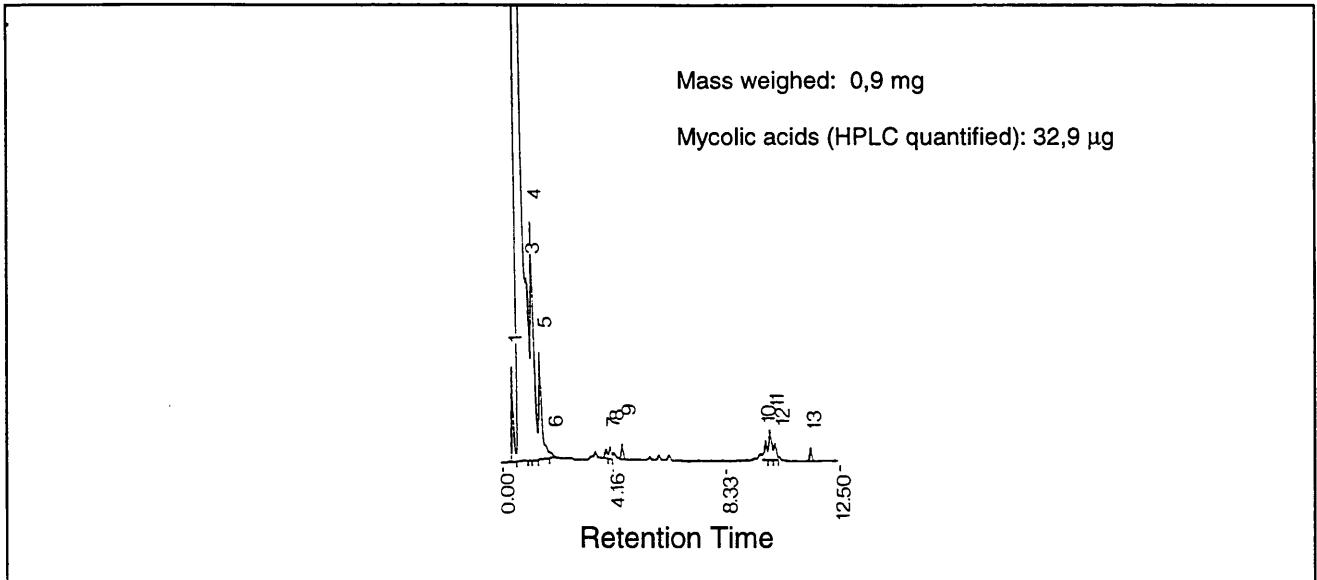
Figure 2.29 (a) HPLC profile of acetone supernatant of rinsed,CCD-purified mycolic acids from *M. tuberculosis.*
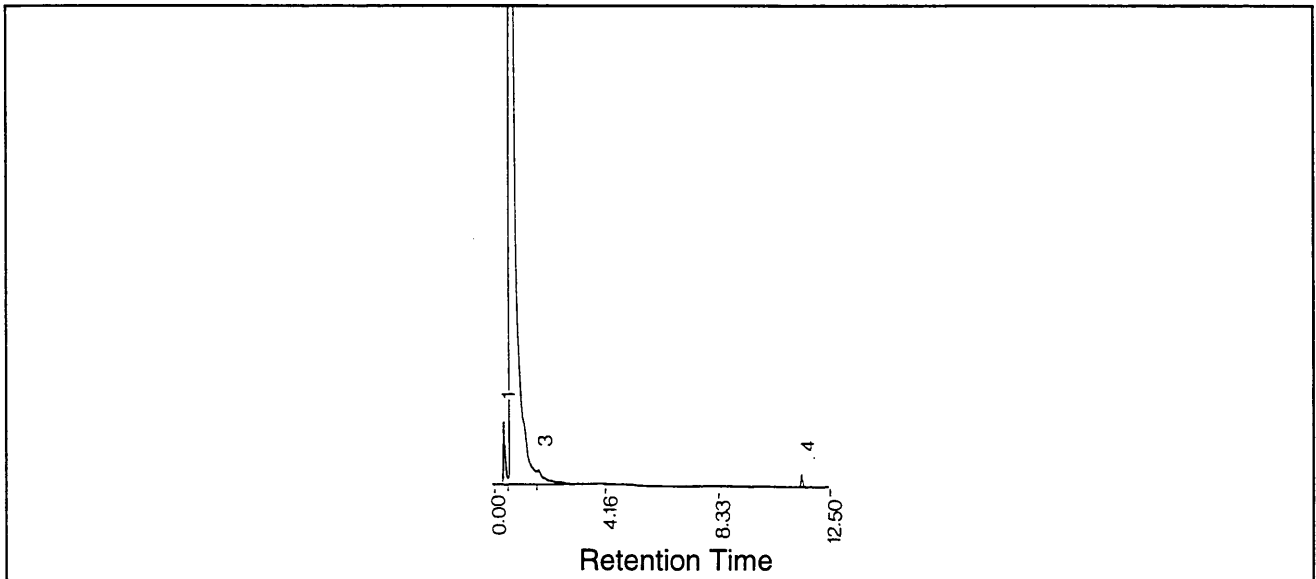
Figure 2.29 (b) HPLC profile of acetone supernatant of rinsed, CCD-purified reagents only.
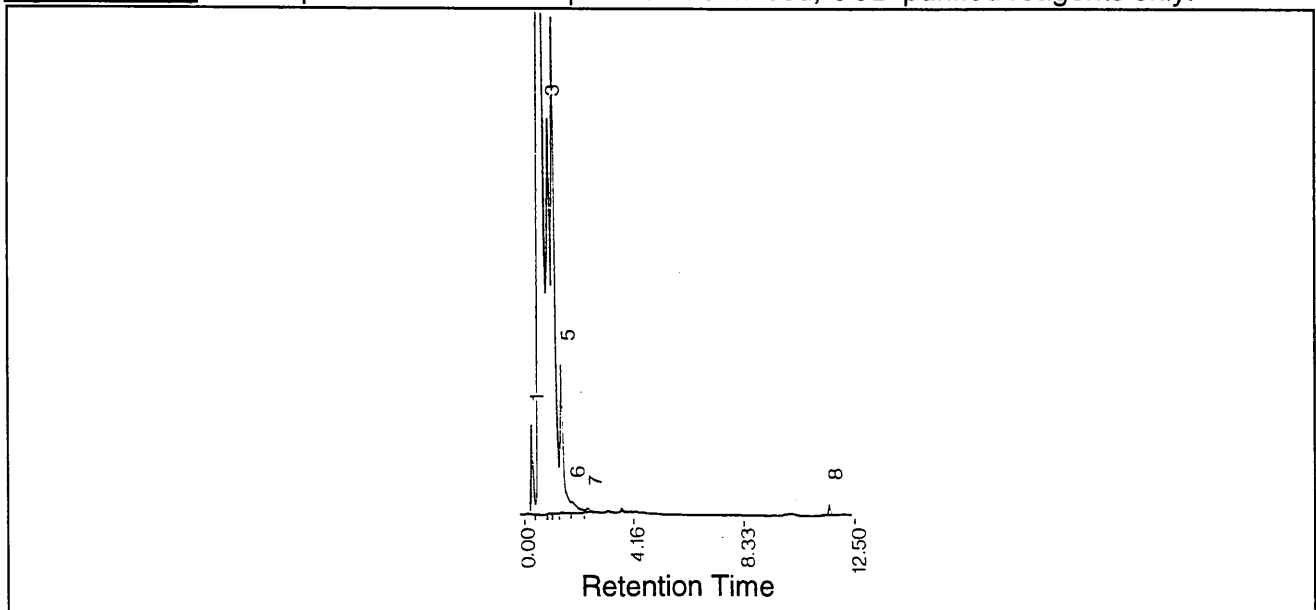
Figure 2.29 (c) HPLC profile of acetone supernatant of rinsed, CCD-purified LJ medium.

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Mycolic acids were purified from crude mycobacterial extract, which was not rinsed with acetone prior to CCD-purification. The purified mycolic acids were rinsed with acetone and analysed by HPLC. A number of contaminants were removed by acetone rinsing of CCD-purified mycolic acids. The peaks in the region of 4,16 minutes retention time (Figure 2.29 (a)) were relatively large, indicating that the contaminants which eluted in this region were efficiently removed by the acetone. Some mycolic acids were removed by the acetone in the process of rinsing, however this would be acceptable if the amount was not too large and contaminants were successfully removed. The acetone used to rinse extracted and countercurrent-purified LJ medium also contained a number of contaminants (Figure 2.29 (c) Peaks 3-7), removed from the medium.

Figure 2.30 (a) shows that the mycolic acids were greatly purified in this manner with no contaminants in the region of 4,16 minutes retention time and greatly reduced peaks 3 and 4 which also appear in the reagents profile. Although the mycolic acids were determined to be only 65 % pure, compared to 90 % of the pre-acetone-rinsed CCD purified material (Figure 2.28 (a)), it is predicted that the purity of these mycolic acids is higher than 90 %, as more contaminating peaks were present in the HPLC profile before acetone treatment (Figure 2.28 (a)). Apparently a larger degree of mass loss occurred during this procedure for obtaining mycolic acids of higher purity.

Predicted mass: 0,3 mg

Mycolic acids (HPLC quantified): 0,19 mg
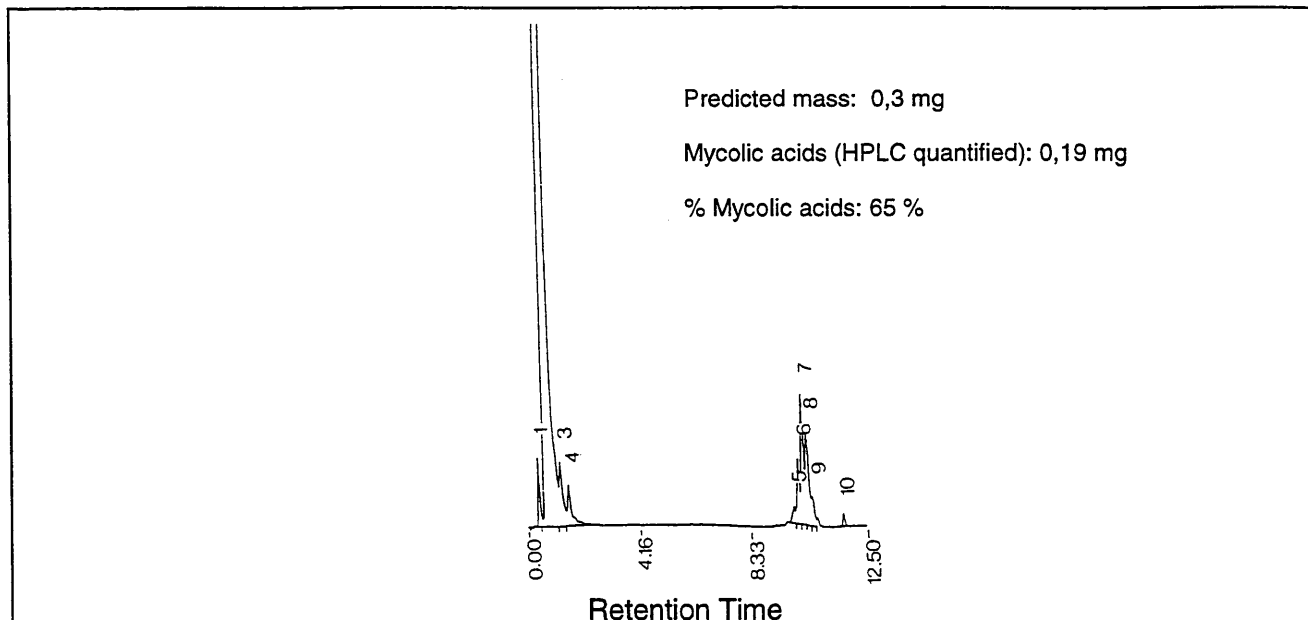
% Mycolic acids: 65 %

Figure 2.30 (a) HPLC profile of acetone rinsed, CCD-purified, mycolic acids from *M. tuberculosis.*
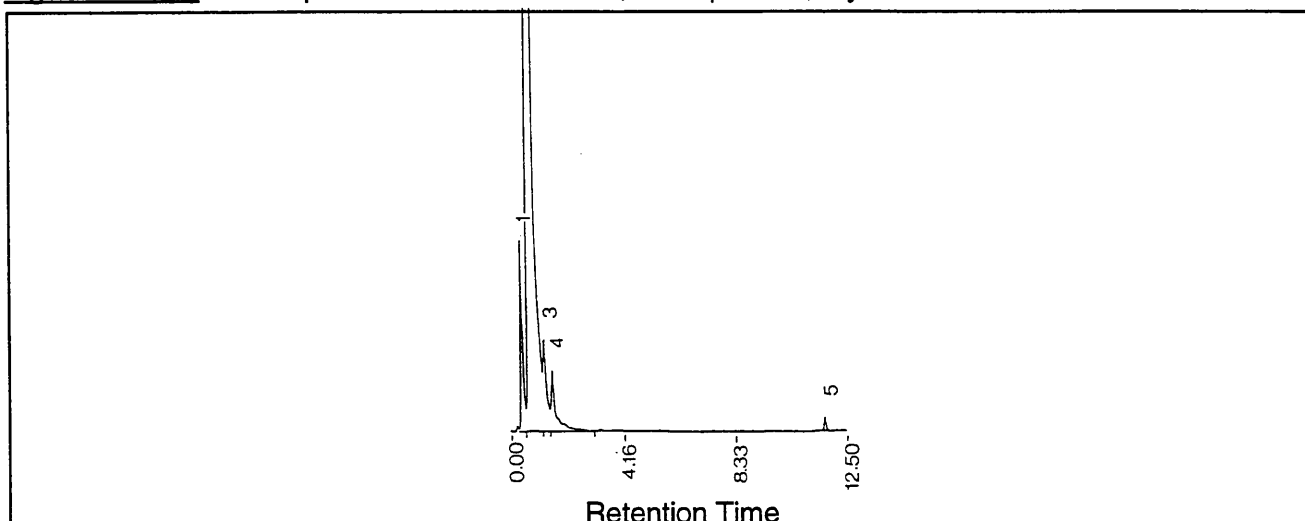
Figure 2.30 (b) HPLC profile of acetone rinsed, CCD-purified, reagents (first fraction).
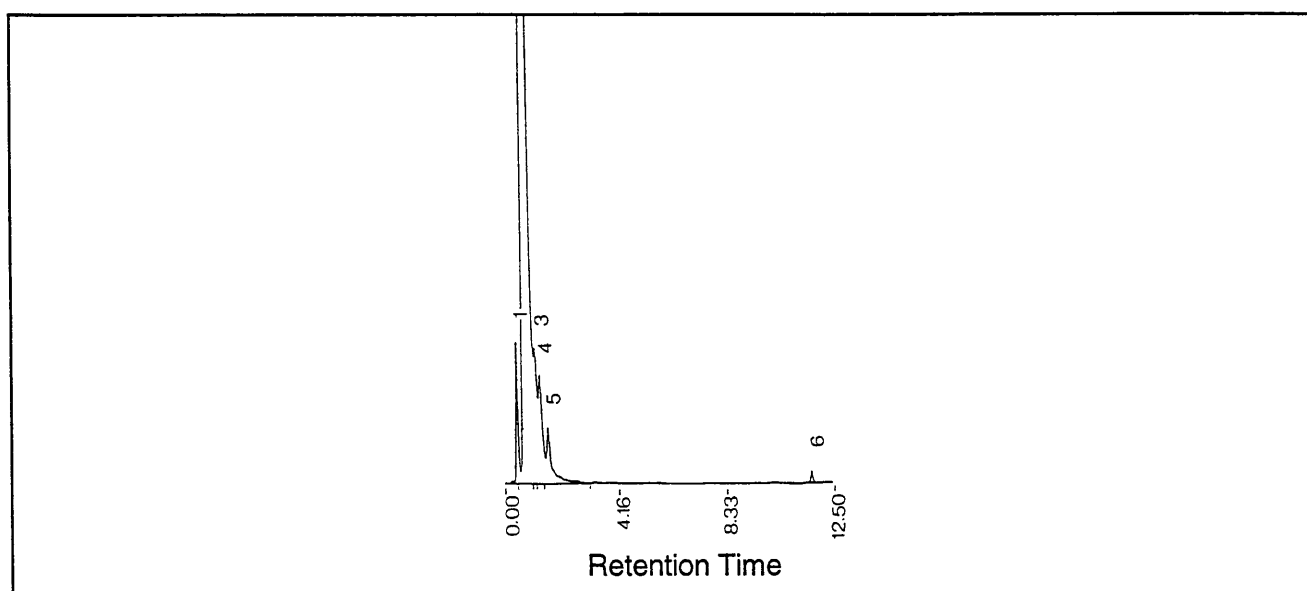
Figure 2.30 (c) HPLC profile of acetone rinsed, CCD-purified, LJ medium (first fraction).

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2.4.9. Removal of contaminants by selective precipitation of mycolic acids out of solution with acetone

It was observed that for small amounts of mycolic acids, rinsing with acetone was acceptable for the removal of contaminants. However, when the procedure was applied to larger amounts of mycolic acids, not all the contaminants were removed due to the thick, waxy layer of mycolic acids, which trapped the contaminants. Selective precipitation of mycolic acids out of solution was considered, as this would allow the contaminants to remain in solution.

From a 3 % solution of mycolic acids in chloroform (3 ml), mycolic acids started to precipitate out in milky wisps after 1 500 µl acetone had been added and precipitated out completely after 2 000 µl had been added. No further precipitation occurred upon subsequent addition of a large excess of acetone (26 ml). The light, white flakes of mycolic acids were easily disturbed and could not be efficiently centrifuged. The acetone supernatant was removed after the flakes had settled to the bottom of the flask. The mycolic acids were rinsed twice with 20 ml acetone, the supernatant removed and the flakes allowed to air-dry to form a fine, white powder. Since the acetone had a green shade, it was anticipated that the contaminants may include malachite green (present in the LJ medium). To ensure that malachite green remained in solution under the mycolic acid-precipitation conditions, the procedure was repeated with 1 mg malachite green dissolved in chloroform. Malachite green remained in solution, after the addition of 150 ml acetone, indicating that any contaminating malachite green, that may be present after CCD purification, would be removed after acetone precipitation of the mycolic acids.

2.4.10. HPLC and spectrophotometric analysis of purified mycolic acid to detect contaminants

Countercurrent-purified, acetone-precipitated mycolic acids derivatised with p-bromophenacyl bromide were analysed by HPLC at 230 and 280 nm wavelengths to detect the presence of any protein or aromatic contaminants. The HPLC profile in Figure 2.31 is the profile of the mycolic acids at 260 nm.
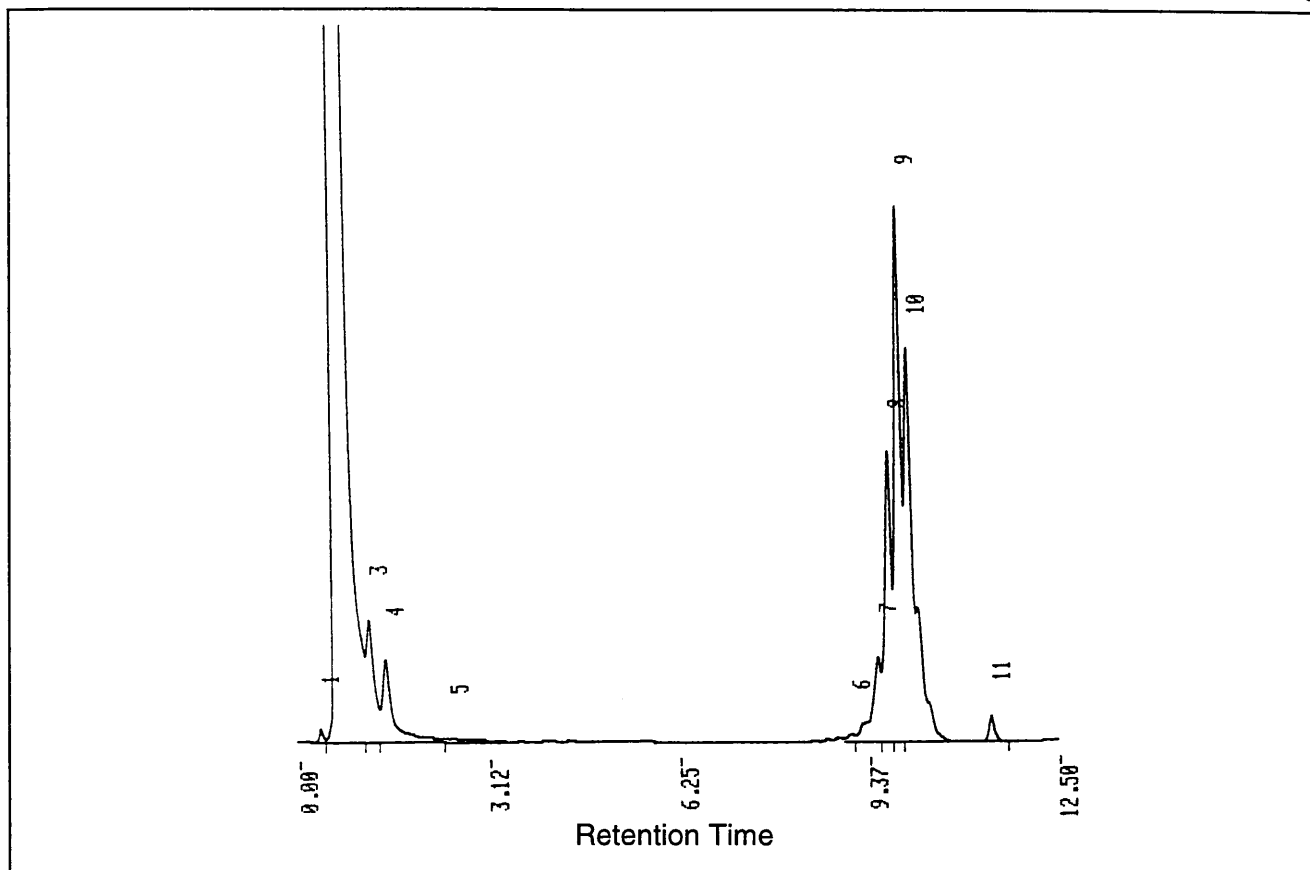
Figure 2.31   HPLC profile of purified mycolic acids at 260 nm.

The HPLC profile of the reagent gradient, detected at 260 nm and 280 nm were both straight baseline graphs and were therefore not included. The HPLC profile in Figure 2.32(a) was that of the reagent gradient, detected at 230 nm. In Figure 2.32(b) the excess derivatising agent was detected at 230 nm in the form of peak 1 and the mycolic acids in peaks 2-5.
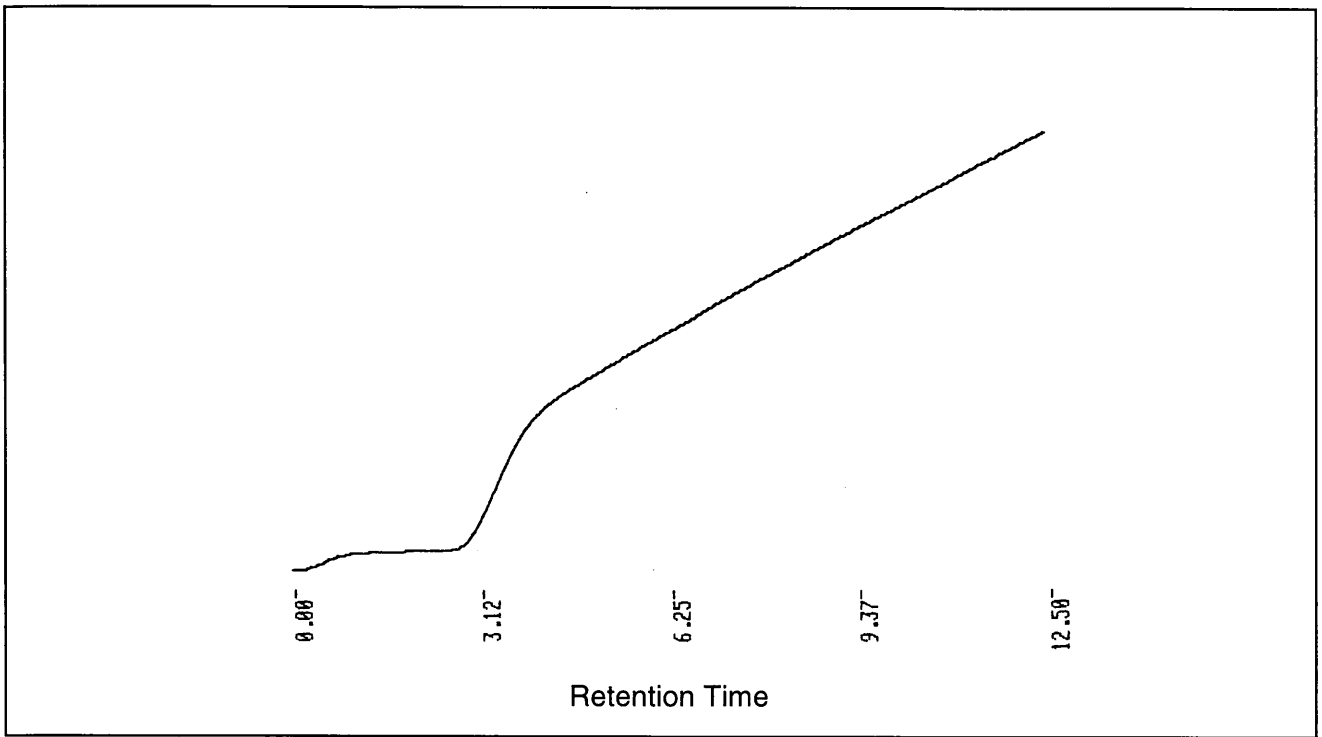
Figure 2.32   Profile of the HPLC gradient detected at 230 nm.
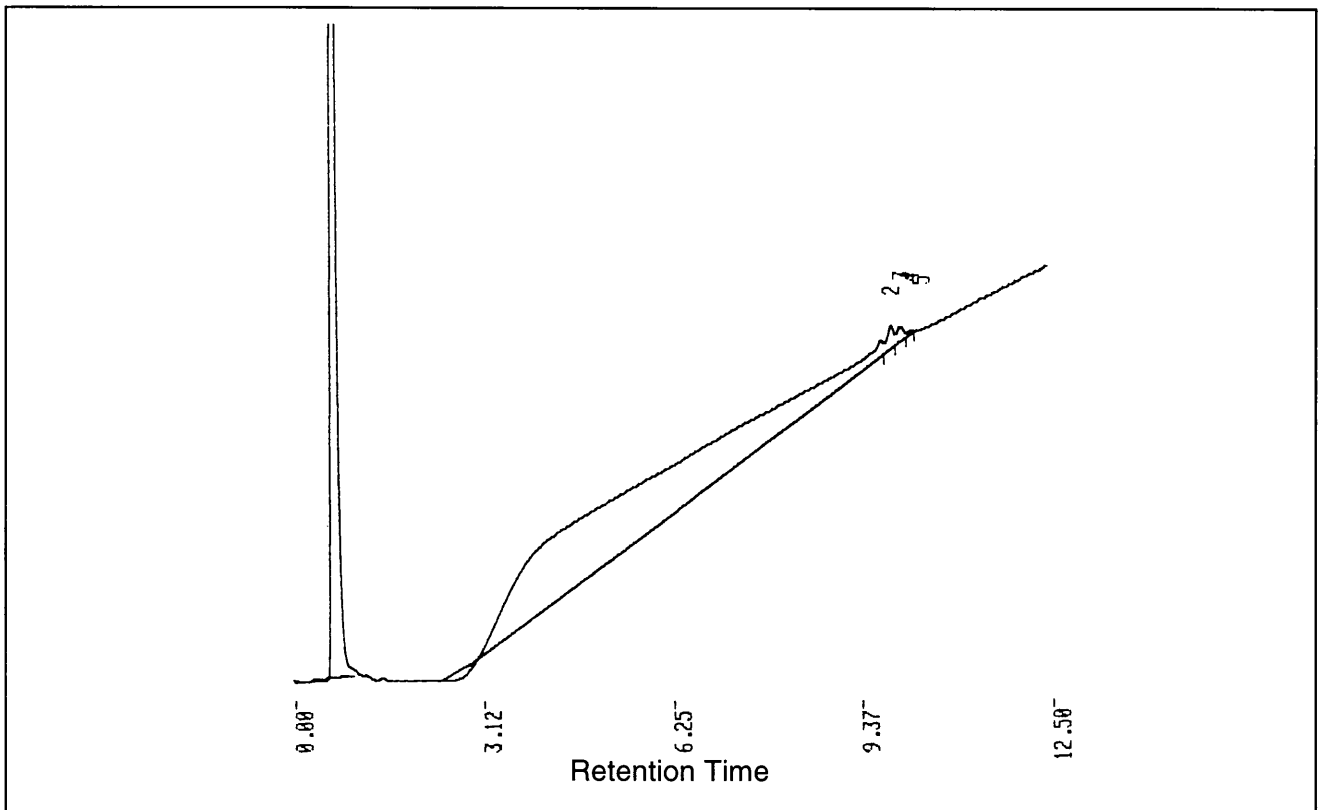
Figure 2.33   HPLC profile of purified mycolic acids at 230 nm.

The excess p-bromophenacyl bromide derivatising agent was also detected at 280 nm, as peak 1 and that bound to mycolic acids as peaks 4-8.
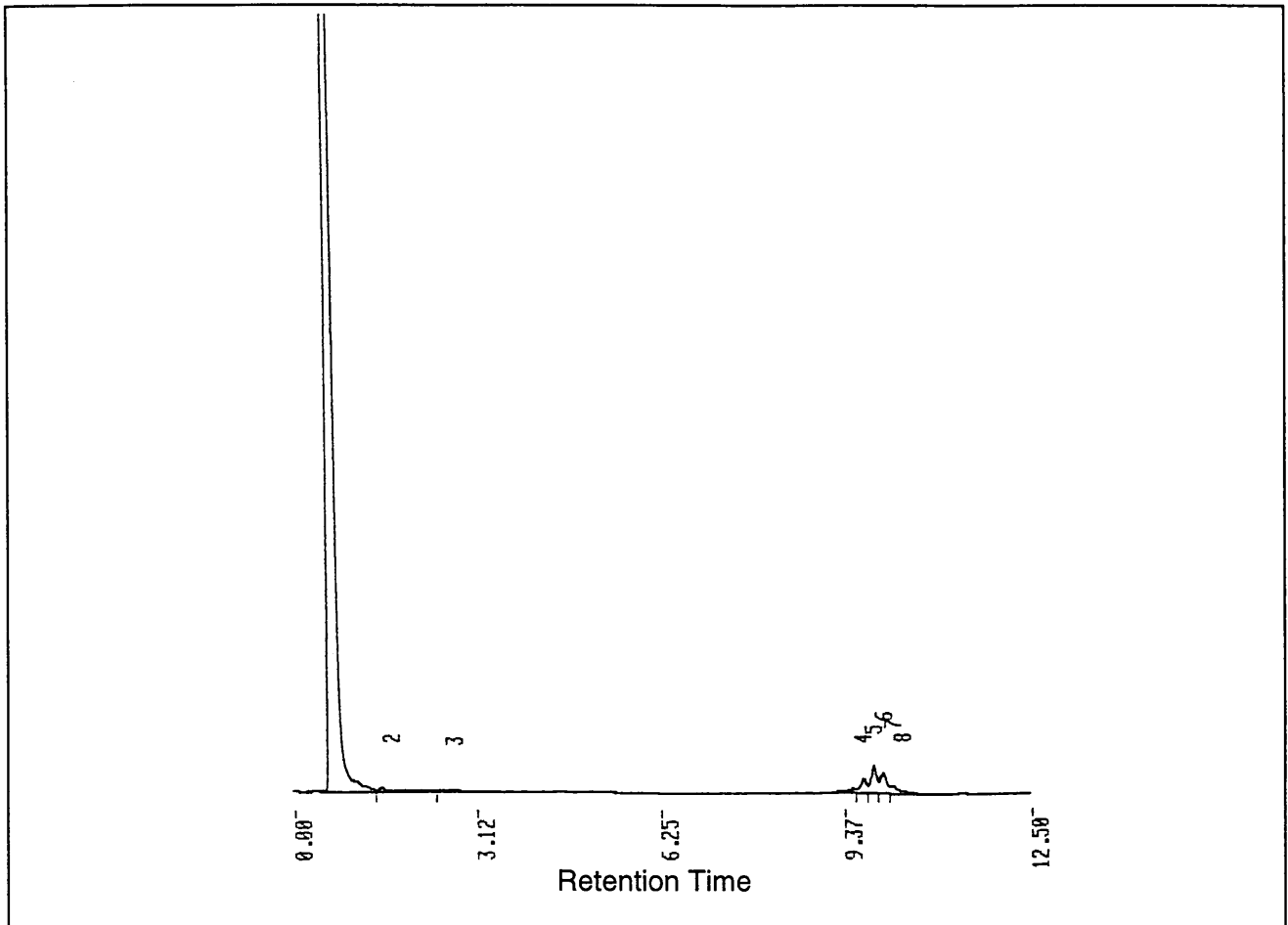
Figure 2.34   HPLC profile of purified mycolic acids at 280 nm.

The peaks in Figures 2.32 - 2.34 were small due to a less sensitive setting of the integrator than for Figure 2.31. The mycolic acids analysed did not appear to contain protein or aromatic contaminants as is evident from the profiles of Figures 2.31 - 2.34, where only derivatising agent and derivatised mycolic acids were detected above background.

2.4.11. Infrared analysis of CCD purified mycolic acids, before and after saponification and after storage

Countercurrent-purified, acetone-precipitated mycolic acids (3,9 mg) were analysed by infrared spectroscopy (IR), in the unsaponified form. A further 10,2 mg mycolic acids were saponified and either IR analysed in the freshly saponified form (3,9 mg) or stored for three weeks before IR analysis (4,2 mg) to determine whether saponification and storage had any effect on the IR spectrum of mycolic acids.
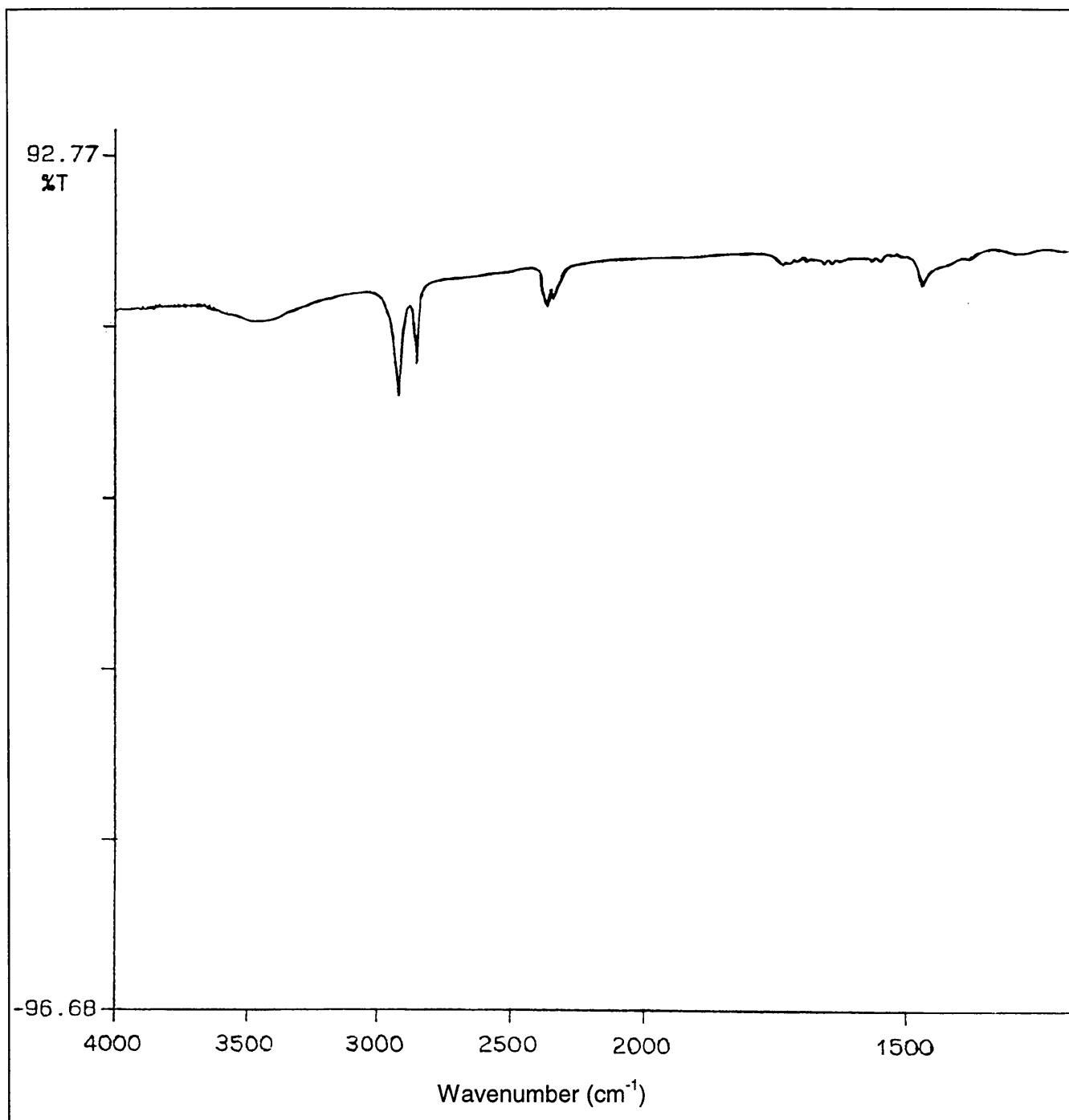
<u>Figure 2.35</u>   An IR spectrum of the 'blank' (chloroform added to KBr).

Figure 2.35 shows the IR profile of chloroform, which served as the blank profile which was subtracted from all subsequent profiles by the FT-IR apparatus.
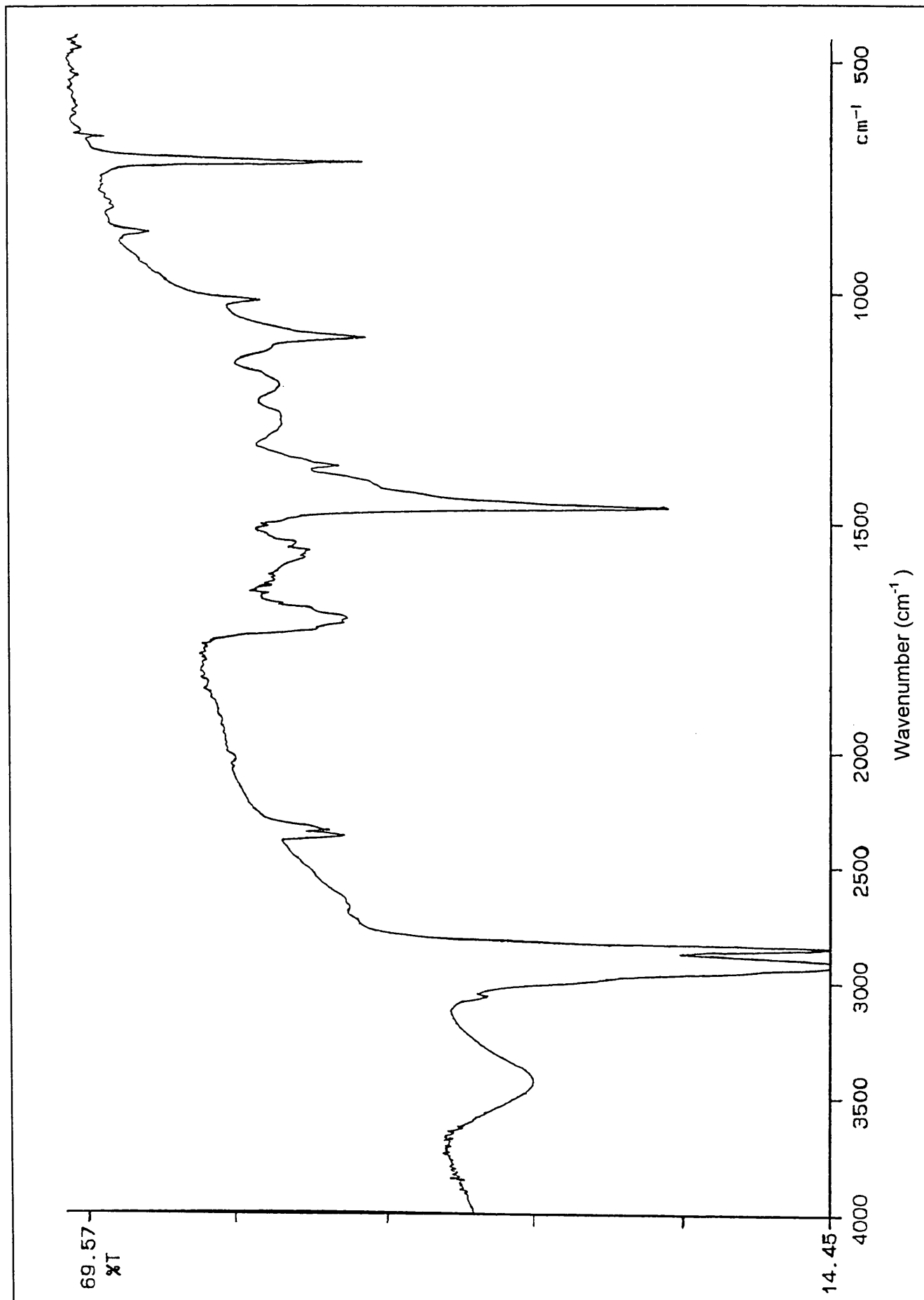
Figure 2.36   IR spectrum of fresh, unsaponified, acetone-precipitated mycolic acids.
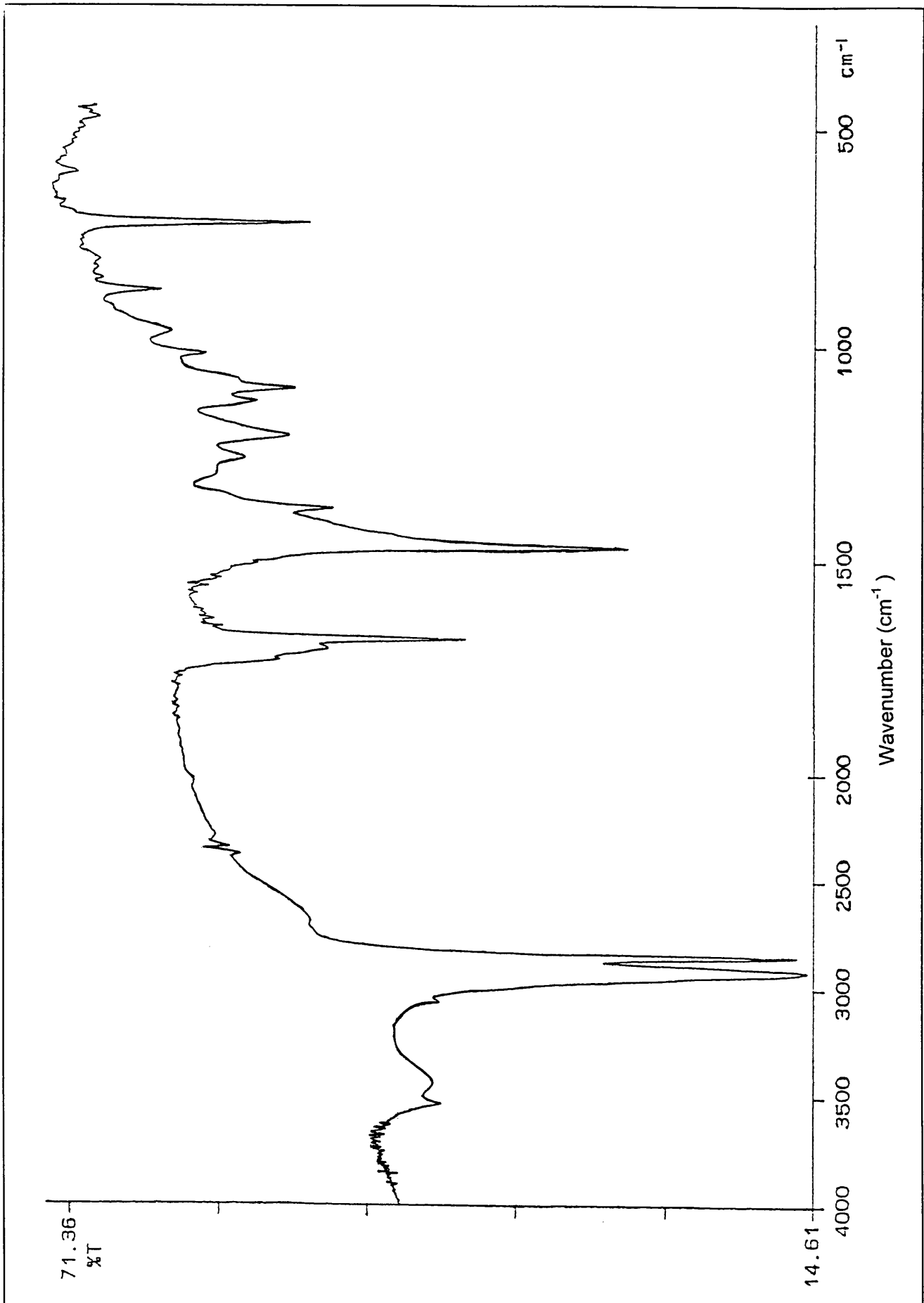
Figure 2.37   IR spectrum of freshly saponified, acetone-precipitated mycolic acids.

The blank sample (Figure 2.35) showed small peaks in the 3 000 cm^{-1} region which are probably due to C-H stretch in the chloroform used while preparing the KBr pellet. A second set of peaks situated in the 2 400 cm^{-1} region, are background peaks and are therefore not important.

The IR scan of the 'fresh', unsaponified CCD-purified mycolic acids (Figure 2.36) looked similar to the profile obtained previously for mycolic acids directly after countercurrent purification in Figure 2.22. The profile showed a broad band of O-H stretch between 3 600 cm^{-1} and 3 100 cm^{-1} (Vogel, 1989), the intense double peak of CH$_2$ and CH$_3$ stretch between 3 200 cm^{-1} and 2 800 cm^{-1} (Lin-Vein *et al.*, 1991) as well as the presence of the carboxyl group (C=O) of mycolic acids (the slightly jagged band at 1 750 cm^{-1}). The absence of a broad peak between 3 500 cm^{-1} and 2 500 cm^{-1} suggests that free carboxylic acids are not present (Vogel, 1989). The strong peak at approximately 1 470 cm^{-1} and the smaller one at its shoulder are caused by a large amount of CH$_3$, CH$_2$ deformation (Lin-Vein *et al.*, 1991) as expected for such long fatty acids. The jagged, broad band between 1 620 cm^{-1} and 1 500 cm^{-1} was not present in the profile of Figure 2.9, but was present in the IR profile of Figure 2.22. The molecular interactions responsible for this band could not be elucidated. This band is however absent from the IR profile of the carboxylic acid form of mycolic acids (Figure 2.37) and may be due to inter- or intra-molecular interactions of the methyl ester, as seen for enols of 1,3-diketone which results in a band between 1 650 cm^{-1} and 1 570 cm^{-1} (Lin-Vein *et al.*, 1991 & Colthup *et al.*, 1990). The similarity of this IR spectrum with that in Figure 2.22 indicates that the acetone precipitation procedure does not significantly affect the chemical state of mycolic acids'.

The 'freshly' saponified sample was analysed one week after saponification and some changes may have occurred in this delay. The sample of mycolic acids that was 'freshly' saponified before the IR analysis showed very slight differences from the unsaponified sample. Figure 2.37 shows a decrease in the over-all size of the broad O-H band between 3 600 cm^{-1} and 3 100 cm^{-1} but an increase of one specific peak within this broad band, at approximately 3 540 cm^{-1}. This peak is caused by free hydroxyl groups (Lin-Vein *et al.*, 1991). The lack of free carboxylic acids (a broad peak between 3 500 cm^{-1} and 2 500 cm^{-1}) was at first surprising since the process of saponification was expected to free the carboxylic acid from the esterified state after CCD-purification. The ratio of carboxylic acids

in the mycolic acids molecules relative to CH_2 and CH_3 groups, is however very low, which could result in the large CH_2 and CH_3 stretch band between 3 200 cm^{-1} and 2 800 cm^{-1}, hiding the broad but shallow carboxylic acid band. The fact that CCD-purified mycolic acids could only be derivatised for HPLC after re-saponification, provides proof that the re-saponified form was in the carboxylic acid form. The infrared profile of re-saponified CCD-purified mycolic acids in Figure 2.37 looks identical to the profile of Goren (1972) in Figure 2.9, described as partially purified methyl mycolates. This indicates that the profile described by Goren may have been of the carboxylic acid form of the molecule, after saponification, and not the methyl ester form.
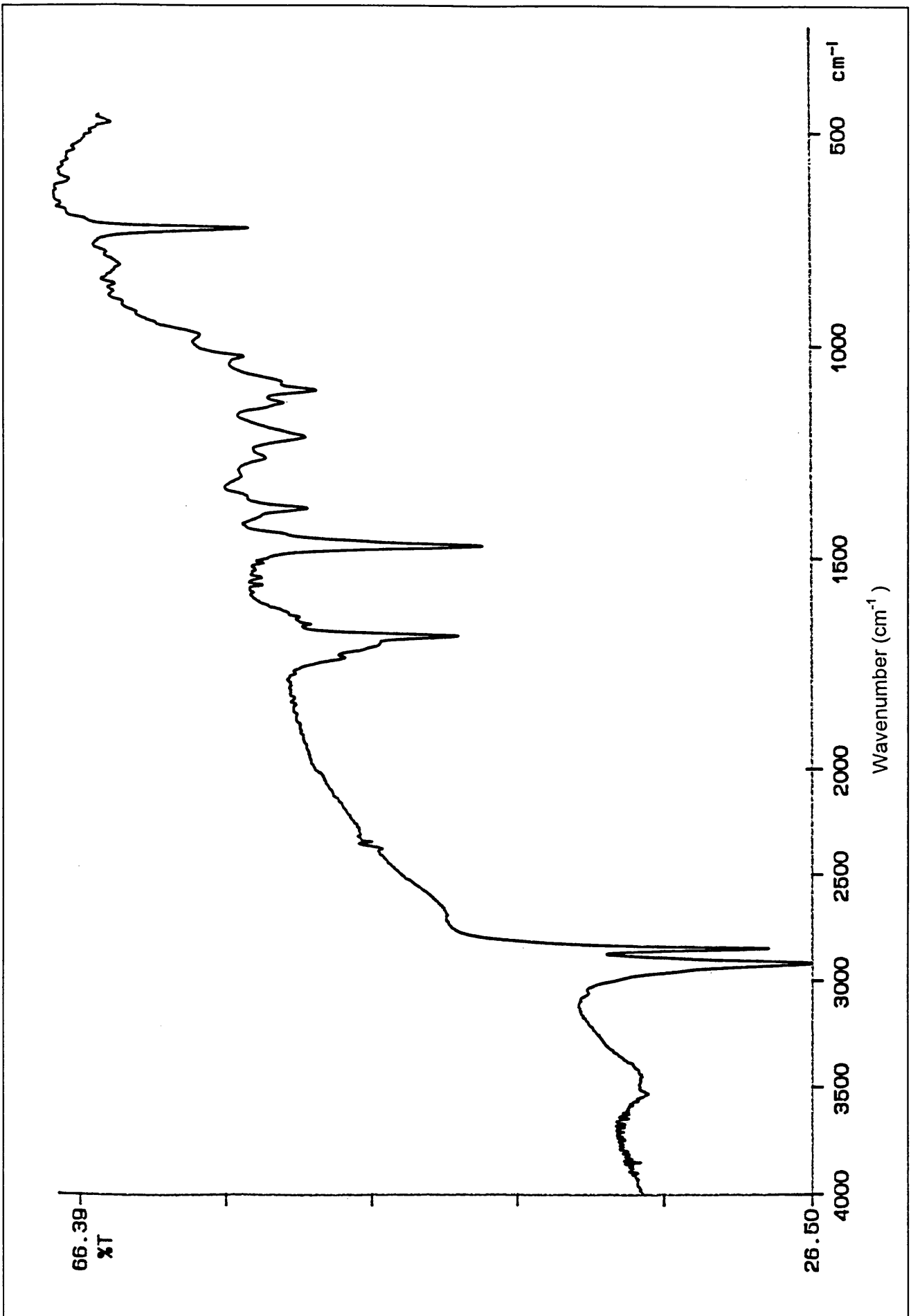
Figure 2.38   IR spectrum of saponified, acetone-precipitated mycolic acids stored for 3 weeks.
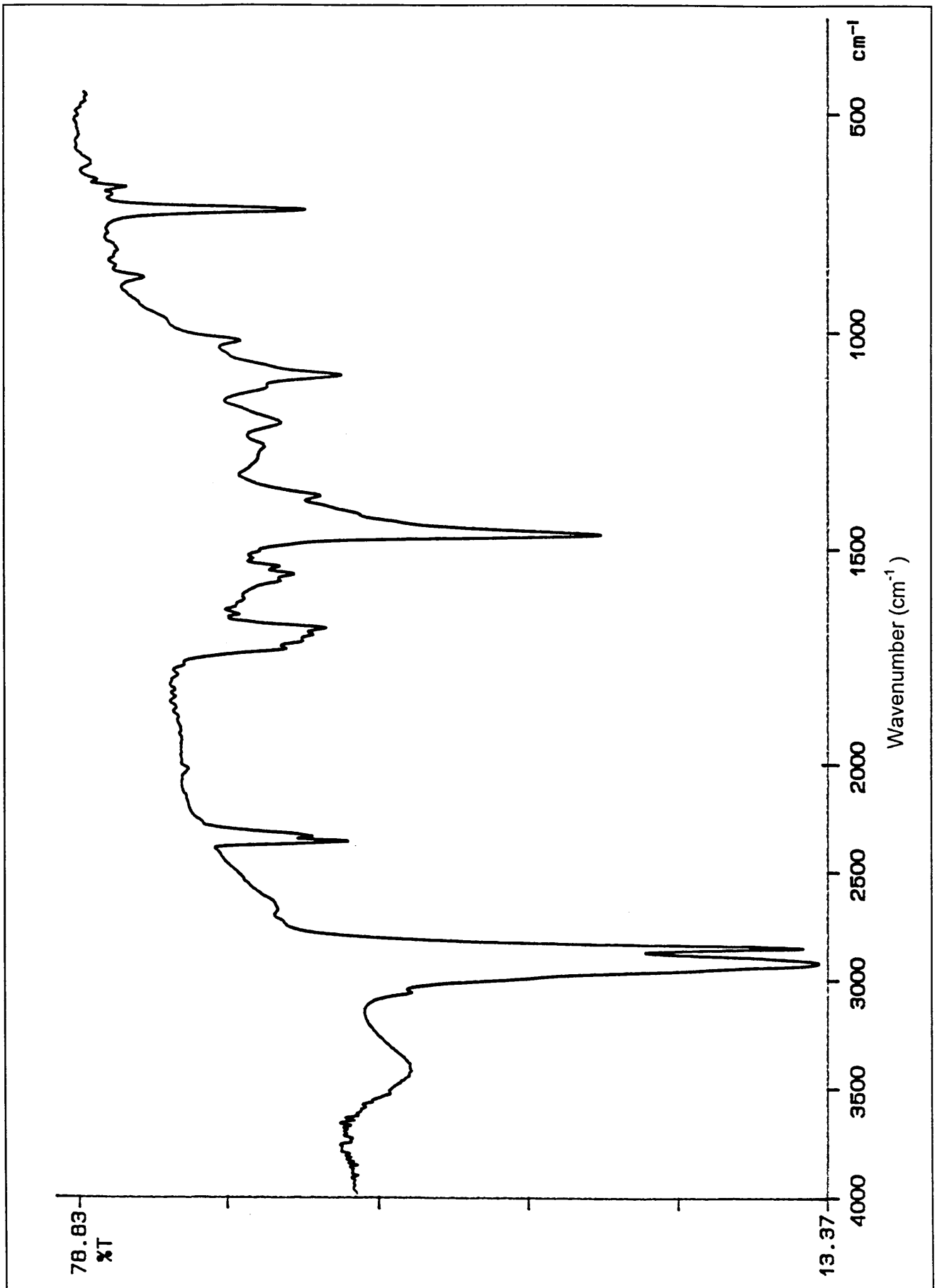
<u>Figure 2.39</u>   IR spectrum of the KBr pellet of unsaponified, acetone-precipitated mycolic

acids, re-analysed three weeks later.

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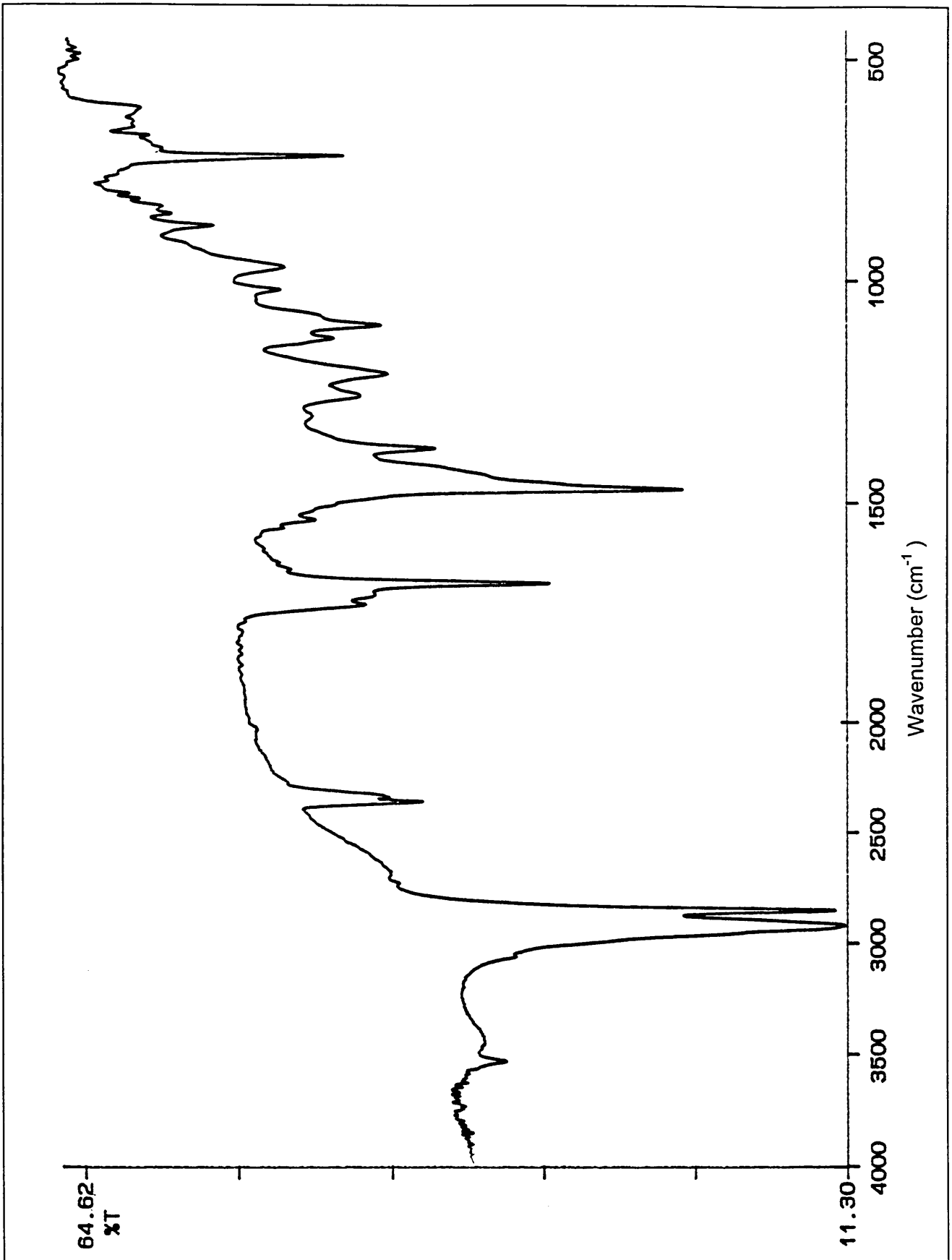
Figure 2.40   IR spectrum of the KBr pellet of freshly saponified, acetone-precipitated mycolic

acids, re-analysed three weeks later.

The IR scan of saponified mycolic acids stored for three weeks (Figure 2.38), slightly wet with chloroform, was very similar to that of 'freshly' saponified mycolic acids (Figure 2.37). Therefore the interactions that occurred in the sample analysed by IR, one week after saponification did not undergo a significant change during three weeks of storage in the presence of chloroform.

The unsaponified mycolic acid sample (Figure 2.36), re-analysed three weeks after the initial IR scan (Figure 2.39), showed a slight change in the profile. A very small peak appeared within the broad O-H stretch band (between 3 600 cm^{-1} - 3 100 cm^{-1}), that was more prominent in the saponified sample in Figure 2.37. The carbonyl peak in Figure 2.39 at 1 750 cm^{-1} became more jagged with a flatter base than in Figure 2.36. These were the only changes, which occurred after three weeks of storage, which indicated that the unsaponified mycolic acids were stable when stored dry.

The 'freshly' saponified mycolic acids used for IR analysis (Figure 2.37) were re-analysed three weeks later and the profile (Figure 2.40) showed only subtle changes in the IR spectrum indicating that very slight changes occur in samples of saponified mycolic acids, over time, when the samples are stored dry.

2.4.12. Cell proliferation studies of mycolic acids prepared in conjunction with samples prepared for infra red in 2.3.10 to test for biological activity

To test the biological activity of countercurrent purified mycolic acids, preliminary cell proliferation studies were done using saponified mycolic acids adsorbed onto human serum. Cell proliferation of CD4 and CD8 cells as well as double negative T-cells in the presence of APC was tested. A second experiment was performed in a similar manner where the APC were induced to express CD1 by adding IL4 and GM-CSF. Figure 2.41 shows that low CD4 cell proliferation was induced when APC were used that did not express CD1. CD4 proliferation was much more pronounced and double negative T-cells were also stimulated to almost the same extent by saponified mycolic acids when APC expressed CD1 (Figure 2.41). The concentration of mycolic acids was found to be critical for the stimulation of CD4 and DN T-cell proliferation as shown in Figure 2.41. Inhibition of proliferation of both CD4 and DN T-cells was observed at higher concentrations of mycolic acids. The results indicate that mycolic acids are biologically active, as they are able to stimulate T-cell proliferation when presented on CD1$^+$-antigen presenting cells. High concentrations of mycolic acids, however, have an inhibitory effect on proliferation, possibly due to toxicity, although the cells appeared viable as determined by trypan blue exclusion. CD8$^+$ T-cells were not stimulated by mycolic acids in any specific way.
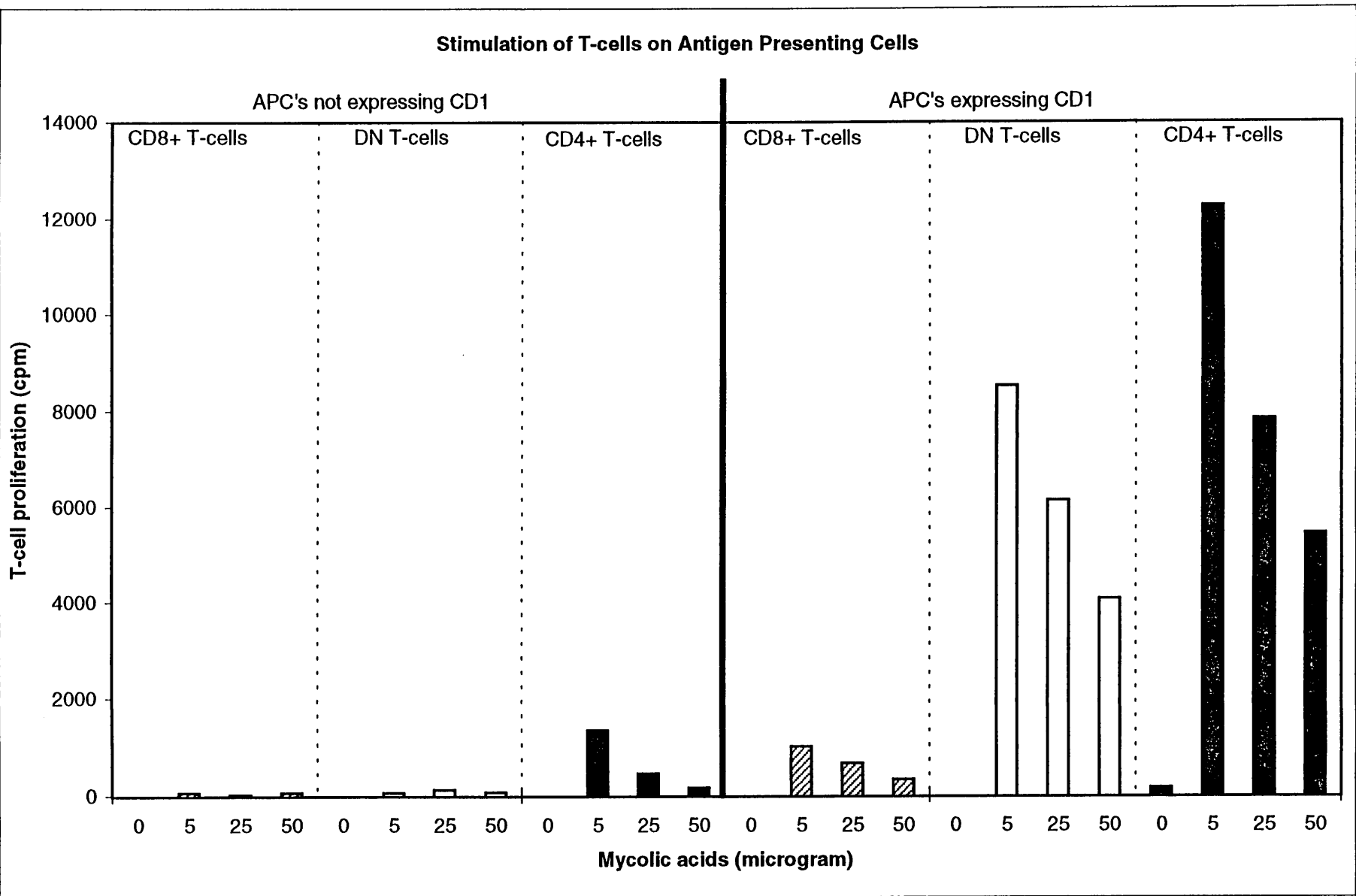
Figure 2.41 Stimulation of T-cell proliferation, by saponified mycolic acids, in the presence of APC, either expressing or not expressing CD1.

Further cell proliferation studies were done using the mycolic acids that were analysed by infrared in 2.3.11. The mycolic acids were CCD-purified, acetone-precipitated and aliquoted. Aliquots were maintained unsaponified, freshly-saponified or saponified-and-stored before cell proliferation studies to determine whether these forms, which had different IR spectra, were all biologically active and had the same effect on T-cell proliferation. Each of the samples mentioned, i.e. unsaponified (Figure 2.42), freshly saponified (Figure 2.43) and saponified and stored (Figure 2.44) stimulated proliferation of both CD4 and DN T-cells in the presence of antigen presenting cells that express CD1.

A similar concentration effect as in Figure 2.41 was also observed in each experiment, where the lowest (5 µg/ml) mycolic acids concentration, resulted in the most T-cell proliferation. Freshly saponified (Figure 2.43) and saponified and stored (Figure 2.44) mycolic acids stimulated proliferation of DN T-cells to a larger extent (1489.93 cpm and 1586.27 cpm respectively) than unsaponified mycolic acids (964.3 cpm), in the presence of CD1 at a concentration of 5 µg mycolic acids/ml. The significance of this difference becomes doubtful as the inverse situation is observed at a higher (25 µg/ml) concentration. The degree of DN T-cell proliferation decreased with increasing mycolic acids concentration. The stimulation of CD4 T-cell proliferation, in the presence of CD1, was higher with unsaponified mycolic acids (1408.4 cpm) and saponified and stored (1307.73 cpm) mycolic acids, than with freshly saponified mycolic acids (918.47 cpm) at 5 µg/ml mycolic acids, but this was also reversed at a higher concentration (25 µg/ml).

The results show that the CCD-purified mycolic acids stimulate the proliferation of DN T-cells and CD4 T-cells, but not CD8 T-cells, when presented on CD1$^+$ APC, irrespective of the saponified or methyl ester state of the mycolic acids.

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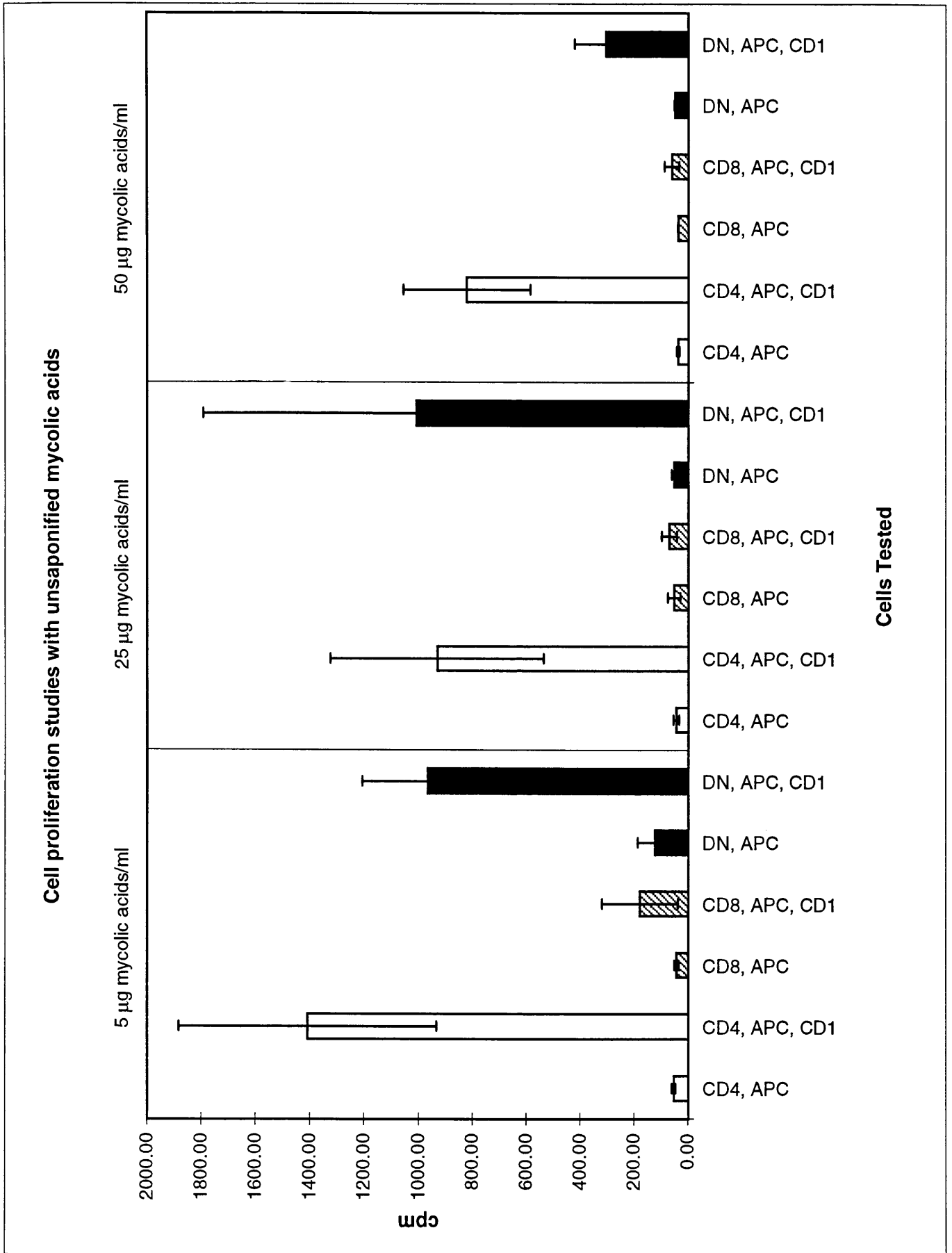
Figure 2.42 Stimulation of T-cell proliferation, by unsaponified mycolic acids (methyl ester).
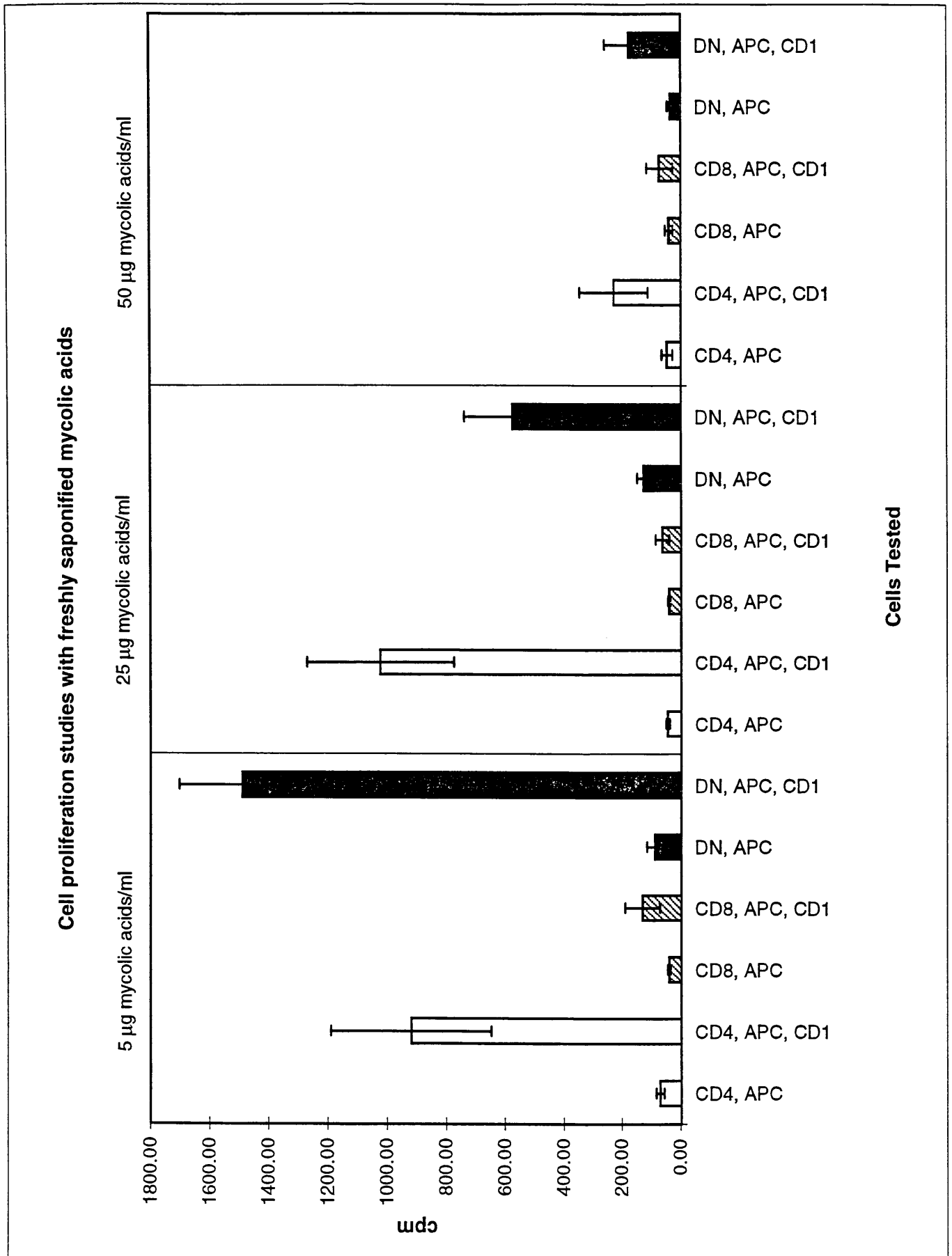
Figure 2.43 Stimulation of T-cell proliferation, by freshly saponified mycolic acids.
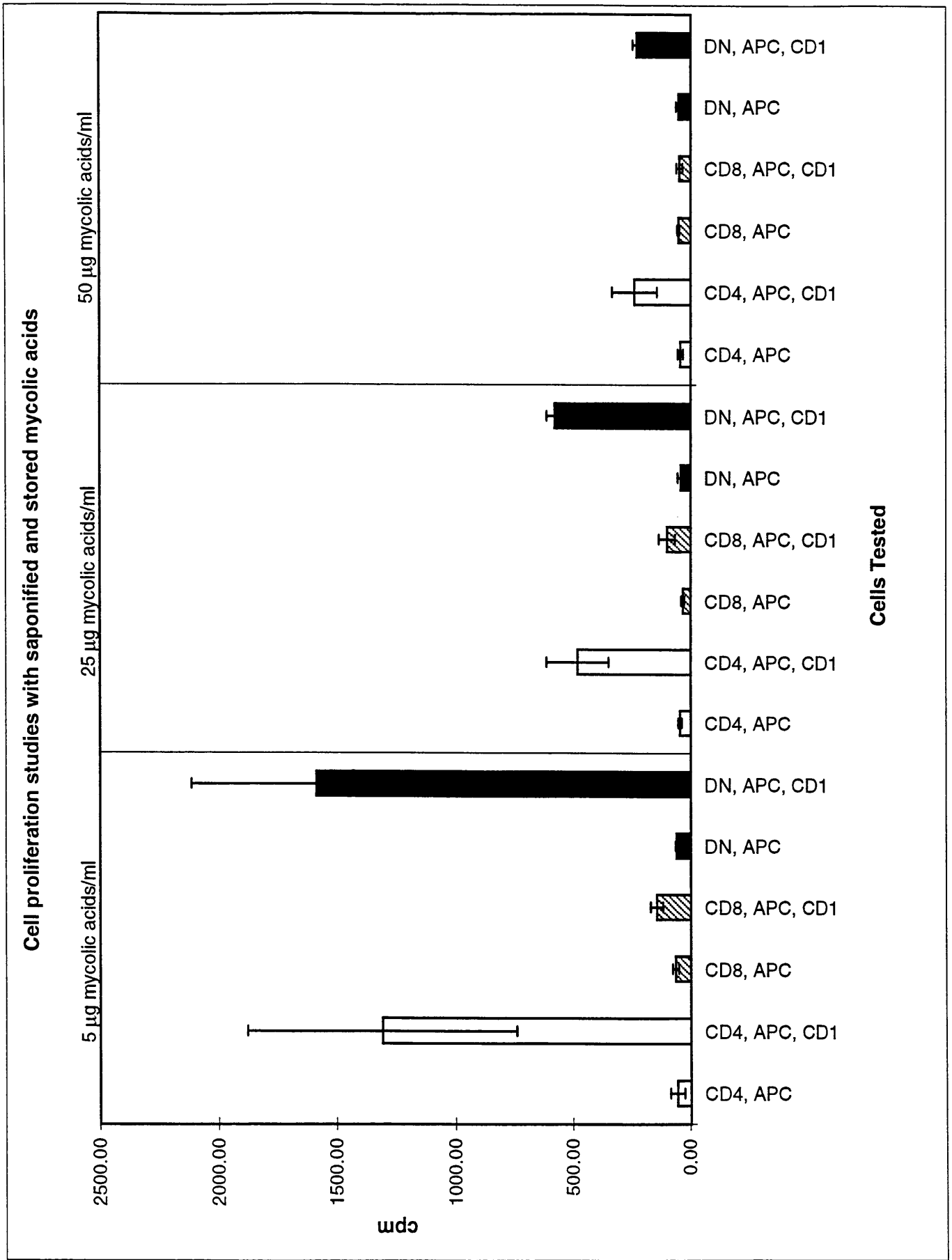
Figure 2.44 Stimulation of T-cell proliferation, by saponified and stored mycolic acids.

2.5. Discussion

The tuberculosis diagnostic kit proposed by Dr S. Bye, formed the basis of the hypothesis that it is possible to generate anti-mycolic acids antibodies. The method used by Dr S. Bye to isolate mycolic acids (by saponification of *M. tuberculosis* with methanolic KOH as described in 2.3.2.) was evaluated and it was determined that the mycolic acids used by Dr S. Bye for the production of anti-mycolic acids antibodies were, at most, 10 % pure.

To validate the provisional patent claims, the purification of mycolic acids from crude mycobacterial extract before immunisation of mice constituted the first aim. Previous methods for the isolation of mycolic acids were costly and yielded limited amounts of purified mycolic acids. They involved partial purification from mycobacterial sonicates or saponification of *M. tuberculosis* trehalose dimycolate (TDM) or commercially available TDM, derivatisation, HPLC purification and saponification (Beckman *et al.*, 1994). The p-bromophenacyl group removed by saponification remained in the mixture after saponification and had to be removed before pure mycolic acids could be obtained. This method of obtaining pure mycolic acids is costly and time consuming. It is unsuitable for large scale application in research or disease control, since the amount of mycolic acids that can be purified in this manner is determined by the capacity of the HPLC column.

Purified mycolic acids became commercially available after this research had been started in 1994. This source was, however, rather costly compared to the cost of producing pure mycolic acids locally. Chemical synthesis of mycolic acids was not considered, as the use of a mixture of the different mycolic acids present in *M. tuberculosis* would allow antibody production against the most immunogenic of the mycolic acids.

Countercurrent purification of mycolic acids, using a biphasic, tricomponent system, resulted in baseline separation of mycolic acids from the other components of the crude mycobacterial extract. Mycolic acids were found in the first of five fractions, clearly distinguished by their emulsification pattern after the mixing phase of each CCD cycle. The emulsification pattern allowed easy identification of the various fractions. Although further improvements of the CCD purification for scaling up were not pursued,

various possibilities exist, which are currently under investigation. A scaled up process, improved by chemical engineers, could improve the cost effectiveness, if a pharmaceutical application was found for purified mycolic acids.

Initially, countercurrent-purified mycolic acids were not efficiently detected by HPLC due to the formation of methyl esters during CCD purification. Resaponification, which could be done at room temperature, solved the problem and allowed efficient derivatisation and quantification of mycolic acids by HPLC. Although it was not tested, it may also be possible to saponify p-bromophenacyl bromide derivatised mycolic acids at room temperature, instead of at 85 °C for 3-5 hours (Beckman *et al.*, 1994). The mass of mycolic acids, determined gravimetrically, corresponded to the amount quantified by HPLC, thus showing the mycolic acids to be pure. Contaminants detected in the HPLC profile of purified mycolic acids, which did not significantly contribute to the mass of the purified mycolic acids, were removed by acetone rinses and later by selective acetone precipitation of larger amounts of mycolic acids dissolved in chloroform.

The infrared spectra obtained for saponified mycolic acids showed that inter- or intra- molecular interactions occurred which were influenced by the free or esterified state of the mycolic acids. The profile obtained by Goren (1972) for the methyl-mycolates corresponded to that observed with the saponified form of the mycolic acids. Saponification increases the amount of free OH groups in mycolic acids. Intra-molecular interactions in saponified mycolic acids could occur from hydrogen bonding between the acid carbonyl, the β-OH-group, or to other –OH groups in the long meromycolate chain.

There is much to be learned about the interactions and reactions that the mycolic acids molecules undergo in their saponified or esterified configurations. A better understanding of the molecule would improve future investigations into the possible utilisation of mycolic acids in the treatment or detection of tuberculosis.

Cell proliferation studies showed that unsaponified, freshly saponified and saponified and stored, CCD-purified, acetone-precipitated mycolic acids were biologically active as they were able to stimulate DN T-

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cell proliferation in the presence of antigen presenting cells expressing CD1, similar to the discovery made by Beckman *et al.* which was published in 1994. The mycolic acids used for the immunisation of experimental mice were thus not only pure, but also biologically active. In addition to the stimulation of DN T-cell proliferation, mycolic acids were able to stimulate $CD4^+$ T-cells which has not been reported previously. The stimulation of CD4 cells by mycolic acids was largely dependent on the expression of CD1 on the antigen presenting cells, corroborating the results of Beckman *et al.* (1994). The methyl ester as well as the saponified forms of mycolic acids, were both biologically active in spite of differences in their IR spectra.

In all cases, there appeared to be an inhibitory effect at high mycolic acids concentrations, possibly due to some toxicity. The results indicate that a very low concentration (< 5 µg) of mycolic acids is required to stimulate the proliferation of CD4 and DN T-cells.

Since mycolic acids could now be purified to better than 90 % purity, and they were shown to be biologically active and immunogenic, they could be used in further studies to determine whether they could be useful in the development of an antibody based diagnostic test for tuberculosis.

Chapter 3:

Developing a serological test for the

diagnosis of tuberculosis

3.1. Introduction:

3.1.1. Mycolic acids as antigen

Mycolic acids form an integral part of the cell wall of *Mycobacteria* and have an important structural function as demonstrated by the antibacterial action of isoniazid, which inhibits mycolic acids synthesis. The formation of a defective cell wall, lacking mycolic acids, results in the loss of acid fastness, the possible loss of other cell-wall components and ultimately the death of the organism (Goren, 1979). There is thus no doubt regarding the essential functional role that mycolic acids fulfil in the survival of the organism. In addition, mycolic acids constitute up to 40 % of the cell wall of *M. tuberculosis* and are therefore, the most abundant components of the outer surface of the organism. Besides the structural function of mycolic acids, they have been thought to interact directly with the host immune response in various ways. Firstly, they induce granuloma formation as part of glycolipids such as cord factor (trehalose dimycolate-TDM) (Goren, 1979). Secondly, lipids such as lipid A from *Escherichia coli* and from *Salmonella minnesota* have been shown to be the active adjuvant entity of lipopolysaccharides often used as adjuvants (Johnson, 1994). Similarly, mycolic acids may also be the functional entity in cord factor, which is thought to have adjuvant activity (Rastogi and David, 1988). Thirdly, mycolic acids have been shown to be presented by CD1b on antigen presenting cells thereby stimulating double negative T-cells (see chapter 2). Last but not least, mycolic acids have been indirectly implicated as a possible antigen to evoke mycolic acids specific antibodies. Cord factor has been used in the development of a serodiagnostic test to detect IgG antibodies specific for TDM in human tuberculosis patient sera (Maekura *et al.*, 1993). Although mycolic acids were not considered to be the antigenic determinant of TDM (Kato, 1972), probably due to the low immunogenicity of lipid and fatty acid molecules, the possibility that antibodies directed against mycolic acids could arise in mice immunised with cord factor, is mentioned in International Patent Application number PCT/JP89/01341 of Sawaii Pharmaceutical Co. Ltd. and Medisa Shinyaku Inc. This patent describes a method for

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detecting antibodies against an acid fast bacterial antigen which comprises mycolic acids, mycolic acid salts, mycolic acid esters or mono- or disaccharide esters of non-mycolic acid fatty acids having a carbon number of 14 or more. In this patent, mycolic acids were not used as immunogen, and *M. tuberculosis* derived mycolic acids were either very weakly, or not at all antigenic in ELISA, in contrast to those derived from *Nocardia spp.*, the source from which the trehalose dimycolate (TDM) immunogen was obtained (Sawai, 1989).

3.1.2. The need for the development of a detection test for tuberculosis

Numerous serological techniques for the detection of patient antibodies to *M. tuberculosis* were tested before the extent of inter-species cross-reactivity of mycobacterial antigens was realised. As a result of the cross-reactivity and other factors, the tests were found to be highly variable. The greater the cross-reactivity of the test, the lower the specificity. No serological test developed thus far has satisfied the criteria of both specificity and sensitivity. Although serological tests can be used for detecting tuberculosis in early HIV infection, they cannot be used when the disease progresses to acquired immune deficiency syndrome (AIDS), as antibody responses to mycobacterial antigens may be absent in such patients.

An alternate approach, which could provide a better indication of the existence of disease, involves the detection of mycobacterial antigens in clinical samples. This would require monoclonal or polyclonal antibodies to the specific antigen, which have minimal cross-reactivity between mycobacterial species. The induction of such antibodies in experimental animals, could be brought about by immunising with purified antigen, or antigen conjugated to a suitable carrier to enhance the antigenicity of haptens. Such a test would not rely on the immune system of the patient to develop antibodies to the antigen, which is a problem in HIV infected individuals. It would depend only on the detection of *M. tuberculosis* in the infected individual.

Intracellular antigens would be of limited use as targets for such a serodiagnostic test since detection of the antigens depends on exposure of the antigen to the antibodies, which would require rupturing of the *M. tuberculosis* cell wall. This would not be necessary for antigens present on the surface of

M. tuberculosis.

Since mycolic acids were found to be immunogenic and able to stimulate T-cell proliferation, it is conceivable that antibodies to mycolic acids could be generated. Mycolic acids have contributed to a new vision in cellular immunity, thereby warranting research into its possible role in the humoral antibody response and its potential as an antigen target in the serodiagnosis of tuberculosis. However, due to the hydrophobicity of mycolic acids, a high degree of cross-reactivity of antibodies against mycolic acids from *M. tuberculosis* with mycolic acids from other mycobacterial species can be anticipated. Antibodies generated against purified mycolic acids may also not recognise the mycolic acids in their native state on the surface of mycobacteria. This would require processing of patient samples to extract mycolic acids, which in turn, would reduce the sensitivity of the test.

Despite all these problems that were foreseen, the aim was to make antibodies specifically targeted to mycolic acids and to use these antibodies to develop a kit to detect tuberculosis organisms in fluid samples of tuberculosis patients.

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3.2. Materials:

3.2.1. Reagents used for the preparation of conjugates

Bovine serum albumin (BSA) - Sigma[1], (Cohen fraction IV)

Mycolic acids extracted from *M. tuberculosis* H37Rv ATCC 27294 and purified by CCD (see Chapter 2)

$NaHCO_3$ - Univar A.R. Grade - SAARCHEM [2]

Phosphate buffered saline (PBS):

      8 g NaCl - Unilab C.P. Grade - SAARCHEM [2]

      0,2 g KCl - Merck [3]

      0,24 g KH_2PO_4 - Merck [3]

      1,44 g $Na_2HPO_4.12H_2O$ - Merck [3]

      Citric Acid - Merck [3]

      Tri-sodium citrate - Merck [3]

Adjuprime - Pierce Chemical Company[4]   Chloroform - Univar A.R. Grade - SAARCHEM[2]

Acetone - Unilab C.P. Grade - SAARCHEM[2]   Methanol - Univar A.R. Grade - SAARCHEM[2]

Gelatin - Serva[7] Reinst-Research Grade

Double distilled water

3.2.2. Reagents used for the ELISA assays

Casein (prepared according to Hammersten *) - Merck [3]

Goat anti-mouse (heavy + light) antibody - peroxidase conjugate - Cappel[5]

Sheep anti-mouse, isotype specific antibodies - peroxidase conjugates- Sigma [1]

Rabbit anti-human (heavy + light chain) antibody - peroxidase conjugate - Sigma [1]

Substrate: 8 mg urea hydrogen peroxide (UP) - BDH[6]

                  10 mg o-Phenylenediamine (OPD) - Sigma [1]

                  10 ml 0,1M Citrate buffer

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* O. Hammersten (1883) Z. Physiol. Chem.
[1] Sigma; P.O. Box 12202; Vorna Valley; 1686
[2] SAARCHEM; P.O. Box 1124; Krugersdorp; 1740
[3] Merck; P.O. Box 1998; Midrand; 1685
[4] Pierce Chemical Company, supplied by Separations; P.O. Box 4181; Randburg; 2125
[5] Cappel, supplied by Separations; P.O. Box 4181; Randburg; 2125
[6] BDH, supplied by Merck; P.O. Box 1998; Midrand; 1685

3.2.3. Experimental animals

Six weeks old, female Balb-C and C57Bl/6 mice were used in immunisation experiments (these mice were not inbred). The mice were bred by the Animal Centre at the South African Institute for Medical Research (SAIMR[1]). Up to five mice were kept per cage and the cages were washed weekly with diluted Dettol[2] antiseptic and new sawdust bedding added. Fresh food and water were provided every second day.

3.3. Methods:

3.3.1. Preparation of BSA- and gelatin - mycolic acid conjugates

Due to the insolubility of mycolic acids in aqueous solution, initial experiments were undertaken to develop a monophasic solution in which a protein carrier (BSA or gelatin) and mycolic acids could be combined to form a conjugate. The solubility of BSA and gelatin in solutions containing methanol and chloroform was investigated.

3.3.1.1. Solubility of BSA

The maximum volume of methanol, which could be tolerated by BSA before denaturation, was determined by adding 5 ml methanol to 20 ml of BSA solution (25 mg/ml in 0,1 N $NaHCO_3$ (pH 8,63)). Additional methanol was added until precipitation (denaturation) of BSA was observed. To determine the effect of chloroform on the solubility of BSA, an aqueous solution of 20 ml BSA (25 mg/ml) containing 53 % v/v methanol (23 ml) was prepared and chloroform added in increments until partial denaturation of the protein was observed (a milky suspension). Ice cold acetone was added until the BSA precipitated out.

3.3.1.2. Solubility of gelatin

The maximum volume of methanol and chloroform, which could be tolerated by 500 µl gelatin (25 mg/ml in 0,1 N $NaHCO_3$ (pH 8,63)), before denaturation, was tested by adding methanol and chloroform in various increments, until denaturation of gelatin was observed.

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[1] SAIMR; P.O. Box 28999; Sandringham; 2131
[2] Supplied by R & C Pharmaceuticals (Pty) Ltd; South Coast Rd; Mobeni; 4052

3.3.1.3. Preparation of mycolic acids-BSA conjugate for the immunisation of mice

3.3.1.3.1. Preparation of acetone precipitated mycolic acids-BSA conjugate

Methanol (600 µl) was added to 500 µl of BSA solution (10 mg/ml in 0.1 N NaHCO$_3$ (pH 8,63)). Countercurrent-purified mycolic acids (2 mg) dissolved in 200 µl chloroform, were added over a period of 1 min 30 sec, at room temperature, while vortexing. The sample was vortexed for an additional 30 seconds and placed on ice for 15 minutes. Ice-cold acetone (2 ml) was added over a period of two minutes, while the sample was maintained on ice. The mixture was left to stand for an additional half an hour on ice to allow complete precipitation of the conjugate.

To determine if the method of BSA-MA conjugate preparation was successful, a portion of the suspension (500 µl) was transferred to a new vial and dried on a heat block at a temperature of 80-85°C, under a stream of nitrogen for HPLC analysis. The remainder of the sample was centrifuged with three 10 second centrifugation pulses, the pellet rinsed twice with acetone and allowed to air-dry. Samples were stored at 4°C in the dark.

Mycolic acids-BSA conjugate (1 mg) was added to AdjuPrime Immune Modulator (10 mg) (adjuvant) and the powders ground together to obtain a homogenous mixture. Of this, 1 mg was weighed off and dissolved in 1 ml sterile PBS for immunisation of mice. The remaining AdjuPrime / BSA-mycolic acids mixture was sealed under vacuum and stored at 4 °C in the dark.

The antigen / adjuvant solution was sonified on ice, using a Branson Sonifier - Cell Disrupter B-30 for ten 10 second pulse cycles with 10 second intervals, followed by one 30 second pulse, to ensure a homogenous suspension. The solution was left at 4° for four hours prior to immunisation into mice.

3.3.1.3.2. Freshly prepared and freeze-dried mycolic acids - BSA conjugates

In an attempt to test whether a stock of mycolic acid-BSA conjugates could be prepared at one time and stored, mycolic acid-BSA conjugate was prepared, freeze-dried and immunised in parallel with

freshly prepared conjugate. It was predicted that the mycolic acids content of the freeze-dried conjugates would be the same for each immunisation, leading to a more consistent dose of antigen while the mycolic acid content of the freshly prepared conjugate may vary between preparations.

3.3.1.3.2.1. Preparation of fresh BSA and BSA-MA conjugates

Fresh BSA-MA and BSA conjugates were prepared prior to each scheduled immunisation. Countercurrent purified mycolic acids (0,5 mg), re-saponified 5-7 days prior to preparation of conjugates, were dissolved in 4 µl chloroform and added to 200 µl BSA (5 mg /ml). The mixture was sonicated on a Branson Cell Disrupter at 20% duty cycle and an output control of 2 for 50 pulses at room temperature. The sonicator tip was washed with soap, water and chloroform between samples. After sonication, the samples were left to stand for 30 minutes at room temperature. The remaining chloroform was then evaporated off, by bubbling nitrogen gas through the samples, until no odour of chloroform could be detected. Samples were reconstituted to 200 µl with ddH$_2$O.

Control BSA samples were prepared similarly, but without mycolic acids being dissolved in the 4 µl chloroform added to the BSA solution. A portion (80 µl) of each conjugate prepared for immunisation was analysed by HPLC to confirm the presence of mycolic acids in the conjugate.

3.3.1.3.2.2. Preparation of freeze-dried BSA and BSA-MA conjugates

Ten samples of freeze-dried conjugates of BSA-MA and BSA were prepared using the same method described in 3.3.1.3.2.1., but frozen at - 70 °C, freeze-dried overnight and stored at 4°C until required for immunisation. Each sample was dissolved in 200 µl ddH$_2$O and used for the preparation of antigen for immunisation. A portion of each sample (80µl) used for immunisation, was retained for HPLC analysis to confirm the presence of mycolic acids in the conjugate used for immunisation.

3.3.1.3.2.3. Preparation of antigens for immunisation

Adjuprime immune modulator (1,82 mg) was added to 73 µl conjugate (containing 182 µg conjugate) and reconstituted to a total volume of 2 ml with sterile PBS, prior to immunisation. The suspension was mixed using a 1 ml syringe and a 25 gauge needle until a homogenous suspension was obtained. The suspension was stored at 4 °C for four hours prior to immunisation and mixed well before and between immunisations.

Per mouse, 100 µl of the suspension was immunised intra-dermally in the foot pads for the first immunisation and thereafter subcutaneously under the skin of the neck.

3.3.1.4. Preparation of mycolic acids-gelatin conjugates for coating ELISA plates

3.3.1.4.1. Preparation of acetone precipitated mycolic acids-gelatin conjugate

Methanol (600 µl) was added to 500 µl gelatin solution (10 mg/ml in 0,1 N $NaHCO_3$ (pH 8,63)). Mycolic acids (2 mg) were dissolved in 200 µl chloroform and added to the gelatin solution over a period of 1 min 30 sec, while vortexing. The sample was vortexed for an additional 30 sec, placed on ice for 15 minutes, transferred to a 100 ml Schott bottle, 80 ml of ice-cold acetone added and left on ice for 30 minutes. The precipitate comprising mycolic acids adsorbed to gelatin was rinsed twice with acetone and 25 % of the suspension retained for HPLC analysis. The remainder of the precipitate was allowed to settle, the acetone decanted off and the precipitate allowed to air dry before storage at 4°C in the dark.

3.3.2. HPLC analysis of the mycolic acids conjugates (BSA and gelatin)

3.3.2.1. HPLC analysis of acetone precipitated mycolic acids-BSA and mycolic acids-gelatin conjugates

A portion (25 % v/v) of the acetone precipitated conjugate suspension prepared as described in 3.3.1.3.1. (which should contain 0,5 mg mycolic acids) was retained for HPLC analysis. Mycolic acids were removed from their protein carriers by saponification with 2 ml reagent A, extracted using 1,5 ml reagent B and 2 ml chloroform, 100 µl reagent C added, and derivatised with 50 µl reagent D, followed by the addition of 1 ml reagent E. Analysis was performed by HPLC as described in 2.3.3.

3.3.2.2. HPLC analysis of fresh and freeze-dried BSA-MA conjugates

A portion of the reconstituted conjugate solutions (80 µl) prepared for immunisation of mice (theoretically containing 0,2 mg MA) was transferred to a vial containing 5 µg HPLC internal standard. Mycolic acids were removed from their protein carriers by saponification with 2 ml reagent A, extracted using 1,5 ml reagent B and 2 ml chloroform, 100 µl reagent C added, and derivatised with 50 µl reagent D, followed by the addition of 1 ml reagent E. Analysis was by HPLC as described in 2.3.3.

3.3.3. Immunisation of mice

Initially, only female Balb/C mice were immunised with BSA and acetone precipitated BSA-mycolic acids conjugate. For the subsequent immunisation protocol with freshly prepared and freeze-dried BSA and BSA-mycolic acids both Balb/C and C57Bl/6 female mice were used, to increase the possibility that anti-mycolic acid antibodies would be obtained. Normally, Balb/C mice are used for the production of antibodies against *Mycobacteria* but the use of different immunisation protocols (Wright *et al.*, 1989) and different strains of mice could be advantageous (Galfrè, Milstein, 1981). Subsequent immunisation was done subcutaneously under the skin on the back of the mice.

The second immunisation (first booster) was given 14 days after the first immunisation, in order to boost the initial immune response at the time when the serum IgM levels have dropped. Subsequent immunisations were done in approximately 28 day intervals to allow the serum levels of the expected IgG

antibodies to return to normal, thereby reducing the possibility of circulating antibodies binding to and removing the immunised antigen (Harlow and Lane, 1988).

3.3.3.1. Immunisation of mice with BSA and acetone precipitated BSA and BSA-MA conjugates

The first immunisation experiment involved the immunisation of five female Balb/C mice with 100 µl of the acetone-precipitated mycolic acids-BSA / AdjuPrime mixture. As a control experiment, a group of two mice was injected with BSA and the adjuvant (AdjuPrime).

Day	Treatment/ Dose	Site of immunisation / treatment
1	50 µl per foot of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	hind foot pad
14	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
21	first bleeding	tail vein
28	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
35	second bleeding	tail vein
49	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
59	third bleeding	tail vein

Subsequent immunisations were done at 28 day intervals and mice bled from the tail vein ten days after immunisation for ELISA analysis.

3.3.3.2. Immunisation of mice with freeze-dried or freshly prepared BSA and BSA-MA conjugates

The second immunisation experiment involved the immunisation of freshly prepared BSA-MA or BSA conjugates and freeze-dried BSA-MA or BSA conjugates with Adjuprime adjuvant into five female Balb/C and five female C57Bl/6 mice per conjugate (i.e. 40 mice in total).

Day	Treatment/ Dose	Site of immunisation / treatment
1	50 µl per foot of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	hind foot pad
14	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
42	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
69	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
77	first bleeding	tail vein
101	100 µl of 1 mg/ml BSA / BSA-MA: Adjuprime (1:10)	sub-cutaneous
108	second bleeding	tail vein

3.3.4. Monitoring of the immune response of immunised mice

3.3.4.1. Preparation of anti-sera

Each immunised mouse was bled from the tail vein, the blood left at 4 °C for 4 hours to allow coagulation and centrifuged for 15 minutes in a bench top Eppendorf centrifuge. The serum drawn off was used within 2 days or stored in 10 % v/v glycerol at -20°C. Serum was diluted 1:50 for ELISA to determine the antibody response of mice immunised with acetone-precipitated BSA-MA or BSA and 1:10 for mice immunised with fresh or freeze-dried BSA-MA or BSA.

3.3.4.2. Enzyme linked immunosorbant assay (ELISA) of mouse sera

ELISA plates (96 well microtitre plates), used to assay the immune response of mice immunised with acetone precipitated BSA-MA, were coated with 50 µl of a 10 µg/ml BSA, BSA-mycolic acids, gelatin or gelatin-mycolic acids conjugate solutions in PBS buffer (pH 7,4) and incubated at room temperature for one hour. Microtitre plates prepared to assay the immune response of Balb/C and C57Bl/6 mice immunised with freeze-dried or freshly prepared BSA-MA or BSA were coated with 3 µg mycolic acids per well in the same way as ELISA plates used for the detection of human anti-mycolic acid antibodies, described in 3.3.5.1. The coating antigen solutions were flicked out and 200 µl, 0,5 % m/v casein in PBS buffer (pH 7,4) (cas/PBS) as blocking agent added to each well. The microtitre plates were incubated (blocked) for one hour at room temperature to block the plates. The blocking solution was flicked out and the wells aspirated to remove excess cas/PBS.

The antisera obtained from mice immunised with BSA and acetone-precipitated BSA-MA were diluted in 0,5% m/v casein in PBS buffer, loaded into the ELISA plate (50 µl per well) in triplicate at a dilution of 1:50, incubated at room temperature for 1 hour, the well contents flicked out and the plates washed three times with cas/PBS using an Anthos Autowash. Antiserum from mice immunised with freshly prepared or freeze-dried BSA or BSA-MA was diluted 1:10 (due to their low ELISA signals) and treated in the same manner.

Goat anti-mouse (heavy + light chain) peroxidase conjugated monoclonal antibodies, diluted 1:4 000 or isotype specific goat anti-mouse immunoglobulin-peroxidase conjugates, diluted 1:1 000, were introduced into the microtitre plates (50 µl per well), the plates incubated for 1 hour at room temperature, the conjugate solution flicked out and the plates washed three times with cas/PBS. The substrate (OPD and UP) was freshly prepared as described in 3.2.2. and added to the microtitre plates (50 µl per well). Plates were incubated in the dark and the colourimetric reaction in each well read at 450 nm with a 340 ATC ELISA reader (Labinstruments Austria, SLT).

3.3.4.3. Inhibition ELISA of mouse serum

BSA-MA prepared as described in 3.3.1.3.1. was dissolved in cas/PBS to a concentration of 0.33 mg / ml and 25 µl added to 25 µl antiserum (diluted 1:25) from mice immunised with acetone precipitated BSA-MA, resulting in a final serum dilution of 1:50. The diluted serum was mixed, added to microtitre wells coated with either gelatin-mycolic acids, or gelatin. The ELISA was completed as described in 3.3.4.2. Uninhibited serum was diluted 1:50 in cas/PBS and treated in the same manner.

3.3.5. Detection of mycolic acids-specific antibodies in human tuberculosis patient sera

Antibodies against mycolic acids were observed in human tuberculosis patients (*Pretorius, 1999* – see appendix A). To confirm that the antibodies recognised mycolic acids specifically, an inhibition ELISA was performed using mycolic acids adsorbed to naive mouse serum as competing antigen.

3.3.5.1. Preparation of mycolic acids coated ELISA plates

PBS (27 ml) was pre-warmed to 85 °C in a 50 ml tube and 2 ml added to 1 mg unsaponified, countercurrent purified mycolic acids. After rigorous mixing the solution was transferred back to the hot PBS in the 50 ml tube. The procedure was repeated four times while maintaining the tubes in an 85 °C water bath and mixing continuously. The solution of melted mycolic acids in hot PBS was loaded onto microtitre plates (100 µl per well, i.e. approximately 3 µg mycolic acids per well) and allowed to cool to room temperature. After the microtitre plates were maintained at 4°C overnight, the contents were flicked out.

3.3.5.2. Preparation of competing antigen

Unsaponified countercurrent-purified mycolic acids were adsorbed onto naive Balb/C mouse serum at a concentration of 2,5 mg/ml by adding mycolic acids dissolved in chloroform to the serum (2 % v/v of the serum) and sonicating the suspension using a Branson Cell Disrupter at 20% duty cycle and an output control of 2 for 50 pulses while on ice. Samples were kept at 4°C overnight. The chloroform was subsequently evaporated off under a stream of nitrogen until no chloroform odour remained. The samples were re-constituted to their original volumes with sterile ddH$_2$O. Competing antigen was

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mixed in a ratio of 1:1 with the human tuberculosis patient's and control serum during the inhibition ELISA. Control antigen lacking mycolic acids was prepared similarly.

3.3.5.3. Inhibition ELISA of human tuberculosis patient serum

Mycolic acids-mouse serum (75 µl) or control mouse serum (75 µl) was added to both tuberculosis patient serum and control human serum (75 µl) samples, mixed well and incubated at room temperature for 1 hour. Samples were made up to 750 µl with Cas/PBS, resulting in a 10 fold final dilution of human sera. Mycolic acids-coated and uncoated wells were loaded with 50 µl of the sera in quadruplicate and incubated on an ELISA plate shaker for 1 hour at room temperature. The contents of the plates were flicked out, washed three times with cas/PBS using an Anthos Autowasher and the wells aspirated. Anti-human gamma chain specific IgG peroxidase conjugate was diluted 1:1000 in cas/PBS, added to the plates (50 µl per well) and incubated at room temperature for 30 minutes. The conjugate was flicked out, the plates washed three times with cas/PBS and the wells aspirated. Substrate (OPD and UP as described in 3.2.2.) was freshly prepared, loaded onto the plate (50 µl per well) and incubated in the dark prior to reading the signal at 450 nm on a 340 ATC ELISA reader (Labinstruments, Austria, SLT).

3.4. Results:

3.4.1. Preparation of BSA - and gelatin - mycolic acid conjugates

Since mycolic acids are insoluble in aqueous solutions and are most soluble in chloroform or dichloromethane, an aqueous protein solution had to be prepared which could accommodate some chloroform (containing mycolic acids) without significantly denaturing the protein. Methanol, which can facilitate a monophasic solution containing both chloroform and water was added to a solution of protein in 0,1 N $NaHCO_3$ to determine the amount of methanol that could be tolerated by BSA before complete denaturation.

3.4.1.1. Solubility of BSA in water/methanol/chloroform

On the introduction of methanol into the solution of BSA in 0,1 N $NaHCO_3$, the following results were observed:

Table 3.1: Solubility of BSA in the presence of methanol

Total volume of methanol added (ml) to 20 ml of BSA solution (25 mg/ml)	Reaction observed
5,0	Clear solution
6,0	Slightly milky solution
9,0	Milky strings formed at the upper half of the tube which subsequently dissolved
9,2	Milky jelly in upper half of tube, which subsequently dissolved
11,0	Milky solution throughout the tube
13,0	A suspension of fine flakes formed
16,0	Larger flakes formed and the mixture separated into two phases after standing for a few minutes. The lower phase was jelly-like.
> 63,0	Precipitation of protein

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Proteins usually precipitate out of solution, upon addition of the methanol, in the form of a denatured, unmanageable "protein gumball". Instead, BSA formed flakes that remained in suspension for a few minutes. As BSA did not precipitate out completely until the final solution contained more than 76 % v/v methanol, it was concluded that methanol was a compatible solvent to be used in the process of adsorption of mycolic acids from chloroform onto BSA.

The introduction of chloroform to a BSA solution (43 ml) containing 53% methanol (23 ml), could be expected to cause the immediate denaturation of BSA resulting in its precipitation from the solution. Instead, the introduction of a few drops of chloroform resulted in breaking up of the jelly-like texture of the BSA. Precipitation of the protein occurred when the concentration of chloroform added was 14 % v/v (7 ml chloroform introduced) when the solution remained milky with flakes in suspension and the jelly-like phase disappeared. This implied that a solution of mycolic acids in chloroform could be added to a BSA solution containing methanol (53 %) to a final concentration of up to 14 % v/v chloroform.

The final reaction mixture, in which BSA could still be maintained in homologous suspension was found to comprise: 14 % v/v chloroform, 46 % v/v methanol and 40 % v/v 0,1 N $NaHCO_3$.

Upon the introduction of ice-cold acetone (final volume of 50 ml), BSA precipitated out as re-dissolvable, fine, light, white flakes. The mixture was allowed to stand for about 10 minutes, which resulted in the formation of a soft, white flocculent precipitate.

3.4.1.2. Solubility of gelatin in water/methanol/chloroform

On the introduction of methanol and chloroform in various combinations to a solution of gelatin in 0,1 N $NaHCO_3$, the following results were observed:

Methanol was added to 500 µl of gelatin solution (25 mg/ml) in increments. After the addition of 1300 µl methanol, the solution started to turn milky, after a total of 2000 µl methanol was added, white flakes started to form. Upon the addition of a further 100 µl methanol, a denatured protein "gumball" formed which adhered to the glass of the test tube. As this occurred only at a final methanol concentration of

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76 % v/v, gelatin was considered to be quite tolerant of methanol.

Chloroform (50 μl) was added to a second 500 μl aqueous gelatin solution (25 mg/ml) resulting in a chloroform droplet floating on the aqueous solution. Methanol (100 μl) was added, causing the chloroform droplet to turn milky and drop to the bottom of the vial. The chloroform droplet decreased in size upon further additions of methanol and finally dissolved after 500 μl methanol had been added. Further additions of methanol (total volume of methanol added was 1800 μl) caused the solution to become milky, and white flakes formed after the addition of 2500 μl methanol. The white flakes started to stick together and form a denatured protein "gumball" after a total of 2600 μl methanol had been added.

In an attempt to prepare a solution containing gelatin in the presence of methanol, in which the protein remained in solution, methanol (500 μl) and chloroform (50 μl) were added to 500 μl aqueous gelatin solution (25 mg/ml). Two phases were observed indicating that more methanol was required to draw the chloroform into solution. Upon addition of a further 100 μl methanol, the chloroform dissolved and a clear solution was obtained. Acetone precipitation with 500 μl ice cold acetone, resulted in the formation of a denatured, protein "gumball" which could not be used further. The experiment was repeated with 500 μl gelatin solution (25 mg/ml), 600 μl methanol and 50 μl chloroform, followed by acetone precipitation with the quick addition of 20 ml ice cold acetone while vortexing, which resulted in the formation of light, white flakes of precipitated protein.

On the basis of this experiment it was concluded that:

i)   gelatin is tolerant of methanol (up to 75% v/v) and precipitates out as flakes if cold acetone is added to a gelatin solution containing methanol and chloroform (final acetone concentration, 17 % v/v).

ii)   an optimum composition at which a monophasic solution is observed required a ratio of 43 % aqueous gelatin solution, 52 % methanol and 5 % chloroform. Further experiments showed that additional chloroform was needed to dissolve sufficient mycolic acids for conjugate preparation, thus a final ratio of 46 % gelatin, 50 % methanol and 14 % chloroform (as described for BSA-MA conjugate preparation) was used for conjugate preparation.

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iii)   fast addition of a large excess of ice cold acetone (while vortexing the solution) results in gelatin precipitating out of the solution in light white flakes instead of a denatured "gumball".

3.4.2. HPLC analysis of mycolic acids conjugates

3.4.2.1. HPLC analysis of acetone precipitated mycolic acids-BSA and mycolic acids-gelatin conjugates

Conjugates for immunisation of mice were prepared by adsorbing 2 mg mycolic acids to 5 mg BSA (3.3.1.3.1.). A quarter of the resulting conjugate suspension (which should contain 0,5 mg mycolic acids) was retained for HPLC analysis.

The mass of mycolic acids present on the analysed conjugate was determined by HPLC quantification to be 0,125 mg, therefore, the total amount of mycolic acids present on 5 mg BSA was calculated to be 0,5 mg (0,125 x 4). The epitope density in the mycolic acids conjugates used for the immunisation of the experimental animals, *i.e.* the ratio of mycolic acids molecules attached to each molecule of BSA or gelatin, was calculated according to the formula and example given below.

$$n = \frac{m}{mol.\ wt.}$$

where: n - number of moles
m - mass (g)
mol. wt. - molecular weight (g/mol)

$$n\ (mycolic\ acids) = \frac{0,5 \times 10^{-3}}{1200} = 4,833 \times 10^{-7} \qquad 0,5\ mg\ mycolic\ acids$$

$$n\ (BSA) \quad = \frac{5 \times 10^{-3}}{66000} = 7,5757 \times 10^{-8} \qquad 5\ mg\ BSA$$

Epitope density represents the number of molecules of mycolic acids (MA) per molecule of BSA:

$$Epitope\ density \qquad \frac{n\ (MA)}{n\ (BSA)} = \frac{4,833 \times 10^{-7}}{7,5757 \times 10^{-8}} = 6,38$$

Therefore, there were 6,38 mycolic acids molecules adsorbed to each BSA molecule.

The same approach was followed for determining the epitope densities of gelatin-MA conjugates.

HPLC analysis of the mycolic acids-protein conjugate required re-saponification and extraction, which may have lead to the loss of some mycolic acids in the procedure resulting in an under-estimation of the epitope density. The mass of mycolic acids adsorbed to protein was indirectly assessed by determining the amount of mycolic acids remaining in the vial used for the preparation of the conjugate. Epitope densities were then calculated to be 16 and 12 mycolic acids molecules adsorbed to BSA and gelatin, respectively.

3.4.2.2. HPLC analysis of fresh and freeze-dried mycolic acids-BSA conjugates

The amount of mycolic acids present in the freshly prepared and freeze-dried conjugates used for immunisation of both Balb/C and C57Bl/6 mice was determined in a similar manner to that of acetone precipitated conjugates. A portion (80 µl) of each of the conjugates used for immunisation (80 µl should contain 0,2 mg mycolic acids - 3.3.1.3.2.) was retained for HPLC analysis, saponified and the amount of mycolic acids present determined by HPLC. The mycolic acids contents of the analysed portion of the samples for each immunisation ranged from 50 µg mycolic acids and higher. The total amount of mycolic acids present on 1 mg BSA in the conjugate was calculated to be 125 µg and the epitope density was calculated as shown below.

$$n = \frac{m}{mol.\ wt.}$$

where: n - number of moles
m - mass (g)
mol. wt. - molecular weight (g/mol)

$$n\ (mycolic\ acids) = \frac{0{,}125 \times 10^{-3}}{1200} = 1{,}04 \times 10^{-7} \qquad 0{,}125\ mg\ mycolic\ acids$$

$$n\ (BSA) = \frac{1 \times 10^{-3}}{66000} = 1{,}5151 \times 10^{-8} \qquad 1\ mg\ BSA$$

Epitope density - number of molecules of mycolic acids (MA) per molecule of BSA:

$$Epitope\ density \qquad \frac{n\ (MA)}{n\ (BSA)} = \frac{1.04 \times 10^{-7}}{1.5151 \times 10^{-8}} = 6{,}86$$

Therefore, there were at least 6,86 mycolic acids molecules adsorbed to each BSA molecule.

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3.4.3. Monitoring the immune response of immunised mice

3.4.3.1. The immune response of mice immunised with acetone precipitated BSA-mycolic acids and BSA

The immunisation protocols involved an initial immunisation in the foot pads of the mice i.e. intra-dermally, to introduce antigen in the vicinity of the Langerhans cells. Langerhans cells are mature dendritic cells in the skin which are capable of priming naive T-cells and are therefore, important in the priming of naive animals by processing antigen, moving to the draining lymph nodes where they become interdigitating cells and present antigen to naive T-cells (Janeway and Travers, 1994).

The results from ELISA experiments carried out on gelatin / gelatin-mycolic acid coated microtitre plates after the first and second bleedings, showed that no detectable immune response was elicited against mycolic acids, 21 days into the immunisation programme (results not shown). ELISA plates were coated with conjugates using gelatin rather than BSA as the carrier protein to exclude the possibility of the mouse antisera recognising the carrier protein as may be the case with other albumins since the carrier of the immunised conjugate was BSA. Other proteins such as casein were not considered as they may form part of the diet of the mice and antibodies directed against them may form part of the polyclonal repertoire of the mice. The control mice, immunised with pure BSA added to Adjuprime, (not treated with chloroform, methanol and acetone) were included to show whether the immunisation was successful i.e. elicited an immune response in the form of anti-BSA antibodies. After 37 days a slight anti-mycolic acid antibody signal was obtained by ELISA on the plates coated with gelatin-MA and gelatin where the signal for mice immunised with acetone precipitated BSA-MA and Adjuprime was slightly higher on gelatin-MA than on Gelatin (9 - 23 % higher than the signal on gelatin) in all except mouse 5 (Figure 3.1).
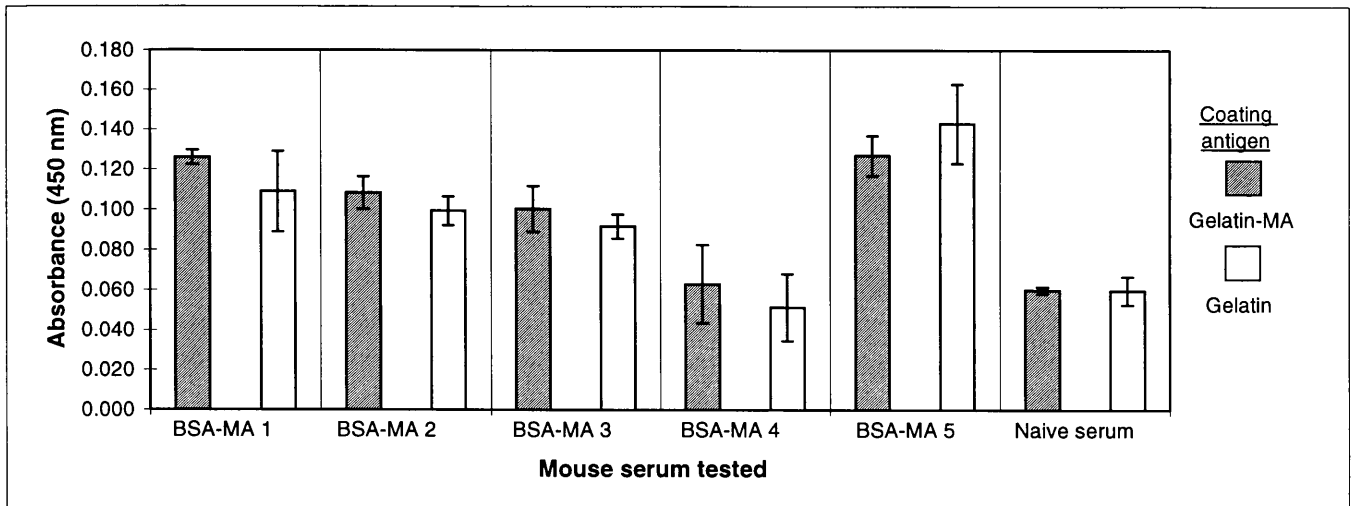
Figure 3.1   Antibody response of individual Balb/C mice immunised with acetone precipitated BSA-MA / Adjuprime, on day 37, measured by ELISA. ELISA plates were coated with gelatin and gelatin-MA. The bars represent the average signal of duplicate samples of 1:50 diluted serum.

The ELISA signals of sera obtained from mice immunised with acetone precipitated BSA-MA and BSA on microtitre plates coated with BSA (Figure 3.2.) were moderate and similar, indicating a good degree of immunisation detectable in all mice shown by the presence of anti-BSA antibodies.
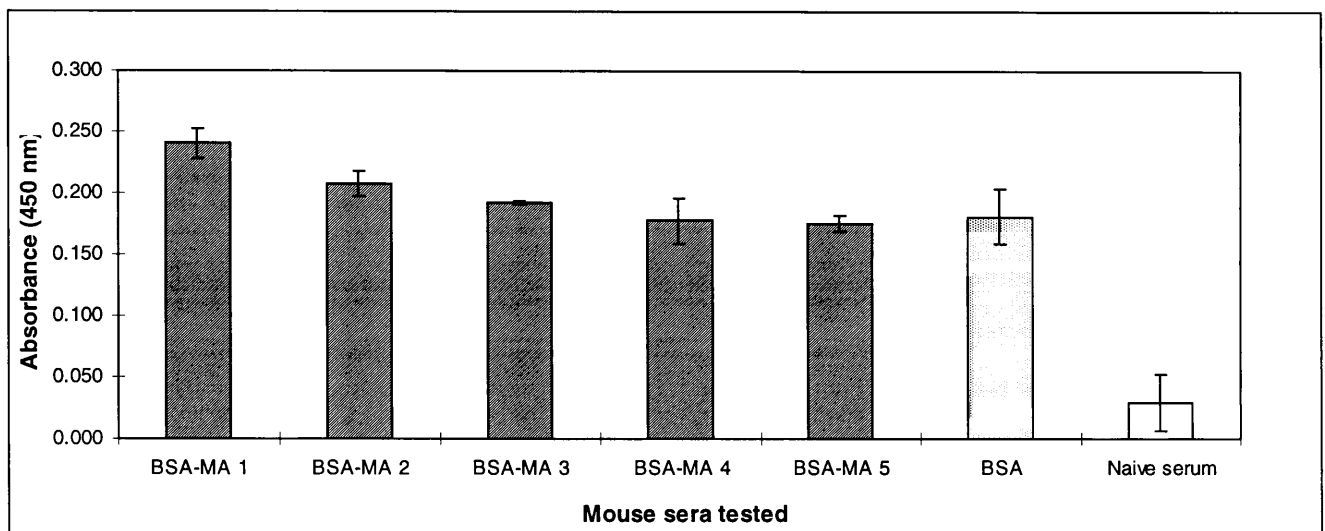
Figure 3.2   Immune response of individually bled Balb/C mice, immunised with BSA or acetone precipitated BSA-MA / Adjuprime after day 37, tested on ELISA plates coated with BSA. The bars represent the average signal of duplicate samples of 1:50 diluted serum.

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Antibody specificity for mycolic acids was tested by inhibition ELISA on antiserum from day 80 of the immunisation schedule (Figure 3.3.). Sera from mice immunised with BSA and BSA-MA in the presence of Adjuprime were analysed by inhibition ELISA on microtitre plates coated with gelatin and gelatin-mycolic acids, as described in 3.3.4.3. If antibodies present in the serum from BSA-MA immunised mice, specifically recognised mycolic acids on gelatin-MA, then inhibition of the ELISA signal on gelatin-MA would occur upon the addition of a competing antigen containing mycolic acids (BSA-MA). No inhibition should be observed on the gelatin coated wells as mycolic acids-specific antibodies would not recognise gelatin and the ELISA signal on these wells should be low.
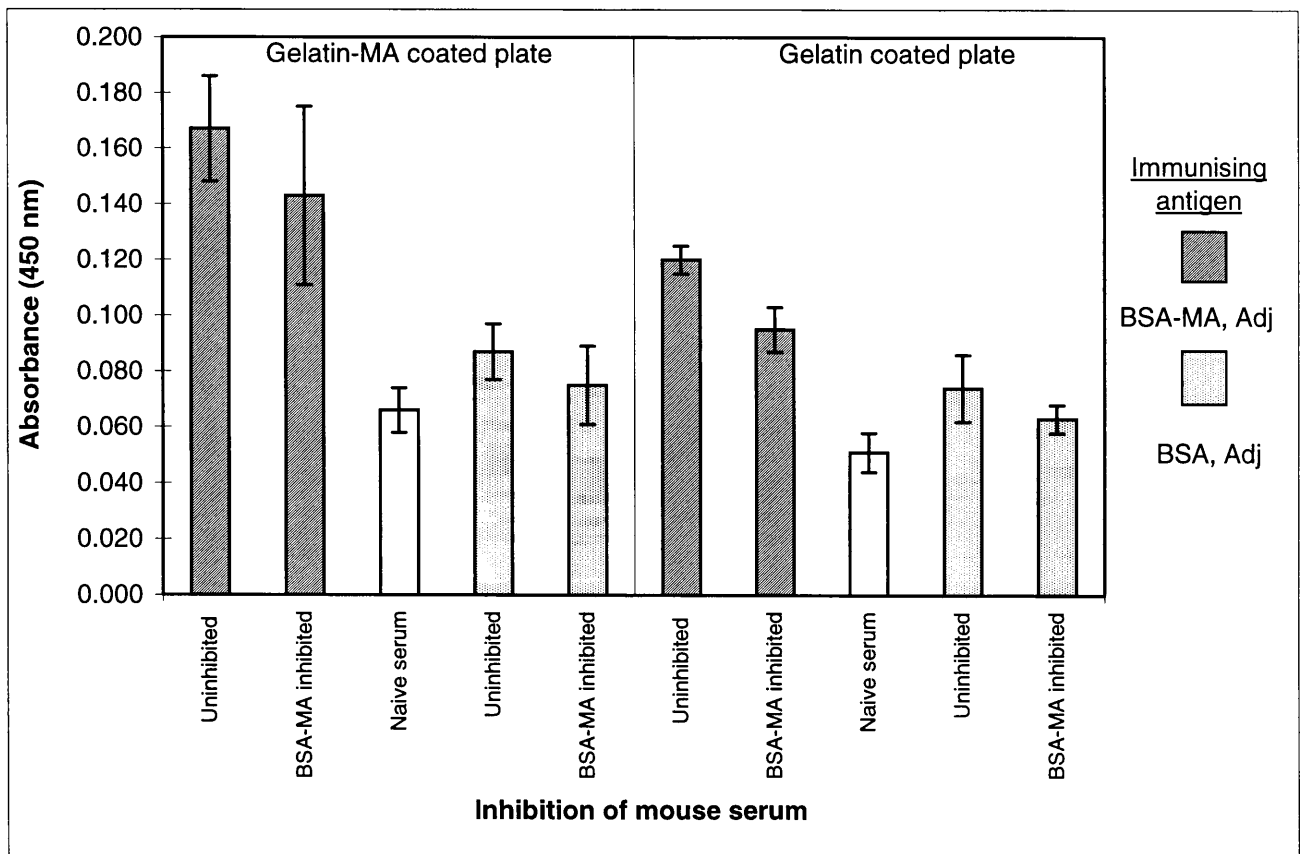
Figure 3.3   Competition ELISA of pooled serum of 3 Balb/C mice, immunised with BSA /Adjuprime or acetone precipitated BSA-MA / Adjuprime after day 80, tested on ELISA plates coated with gelatin and gelatin-MA. The bars represent the average signal of triplicate samples of 1:50 diluted serum, inhibited with BSA-MA in some cases.

The average ELISA signal (Figure 3.3.) of uninhibited sera from mice immunised with acetone precipitated BSA-MA, was 39 % higher on gelatin-MA than on gelatin coated wells, indicating the presence of antibodies recognising mycolic acids. The ELISA signals of uninhibited serum from naive and control mice

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immunised with BSA were 29 % and 17 % higher on gelatin-MA than on gelatin respectively, indicating some non-specific interaction of mouse antibodies with gelatin-MA.

The addition of the competing antigen (BSA-MA) resulted in a 14 % inhibition of serum from BSA-MA / Adjuprime-immunised mice on gelatin-MA coated ELISA plates and a 20 % inhibition on gelatin coated plates. These results indicate that the antibodies generated against BSA-MA are not very specific for mycolic acids and that they may recognise denatured protein (gelatin or BSA) and / or a combination of the denatured protein and mycolic acids (gelatin-MA or BSA-MA). The antibodies may show a greater affinity for denatured protein conjugated to mycolic acids than without mycolic acids, thus having a greater affinity for the competing BSA-MA than for the gelatin coating the ELISA plate, resulting in the slightly greater inhibition of the ELISA signal on gelatin than gelatin-MA, where both the coating and competing antigen contain mycolic acids.

The ELISA signal of the BSA-MA / Adjuprime immunised mouse sera, inhibited with BSA-MA was 50 % higher on gelatin-MA than on gelatin (compared to 39 % for uninhibited serum). Since the serum was mixed with the competing antigen prior to loading onto the ELISA plate, more non-specific antibodies may bind to the competing antigen (BSA-MA) leaving more specific antibodies to bind to the gelatin-MA and give a higher ELISA signal than on gelatin coated plates. The presence of mycolic acids specific antibodies in the BSA-MA immunised animals therefore remain possible, despite the outcome of the inhibition experiment. The inhibition of IgM with ligand is not easily measured by ELISA due to its multivalency and could also be the reason for the results obtained in Figure 3.3.

Three of the five mice immunised in these experiments had been killed by this stage and used for fusion experiments in an attempt to produce monoclonal IgG antibodies specific for mycolic acids. The hundreds of clones obtained from the fusions did not produce monoclonal antibodies of the desired specificity.

The sera from the two remaining mice, immunised with BSA-MA and Adjuprime were analysed by ELISA on gelatin and gelatin-MA coated microtitre plates, 202 days into the immunisation schedule. The average signal obtained for the pooled mouse sera was compared with the ELISA signal obtained after 80 days in Figure 3.4. The ELISA signal for mice immunised with BSA-MA was 39 % higher on gelatin-MA than on

gelatin after 80 days and 62 % higher after 202 days. This may indicate an increase in the quantity or affinity of antibodies, which recognise mycolic acids.
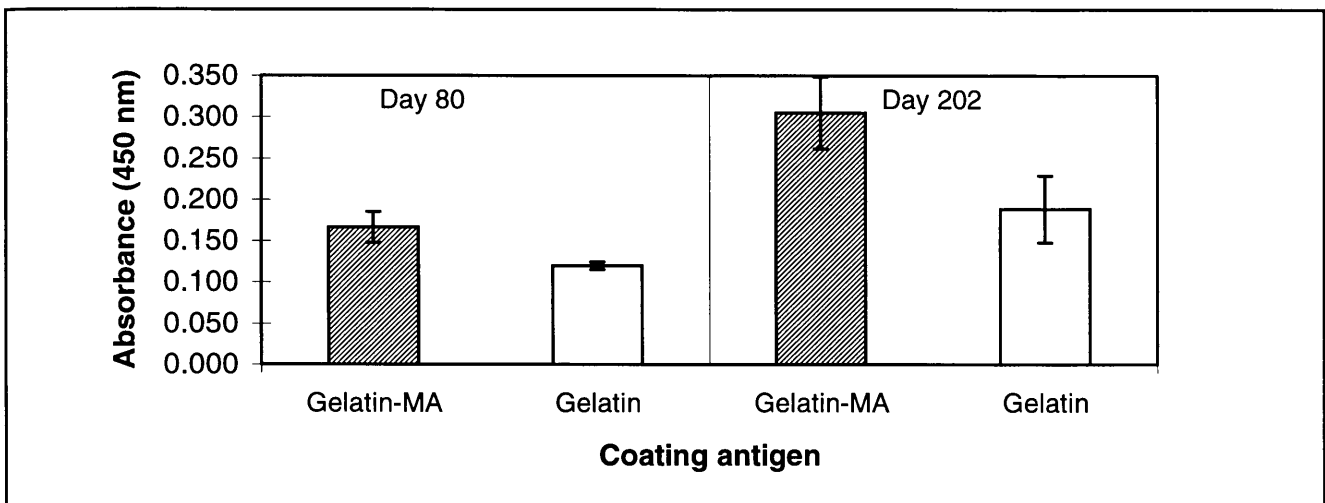
<u>Figure 3.4</u>   The ELISA signal of BSA-MA / Adjuprime immunised mice tested on day 80 (3 mice) and day 202 (2 mice) on microtitre plates coated with gelatin and gelatin-MA. The bar represents the average signal of triplicate samples of 1:50 diluted serum.

The antibodies present in the mouse serum 202 days into the immunisation programme were isotyped to determine whether isotype conversion from IgM to IgG had occurred (Figure 3.5.). Isotype specific peroxidase conjugates were used at a dilution of 1:1000 to test sera from naive mice and mice immunised with BSA-MA /Adjuprime. There was a general increase in signal above that of naive serum, for each of the immunoglobulin classes tested, with a 123 % increase in the amount of IgG1, a 134 % increase in the amount of IgG2 a and only an increase of 26,6 % in the amount of IgM. This would indicate a certain degree of maturation of the immune response towards the production of IgG antibodies.

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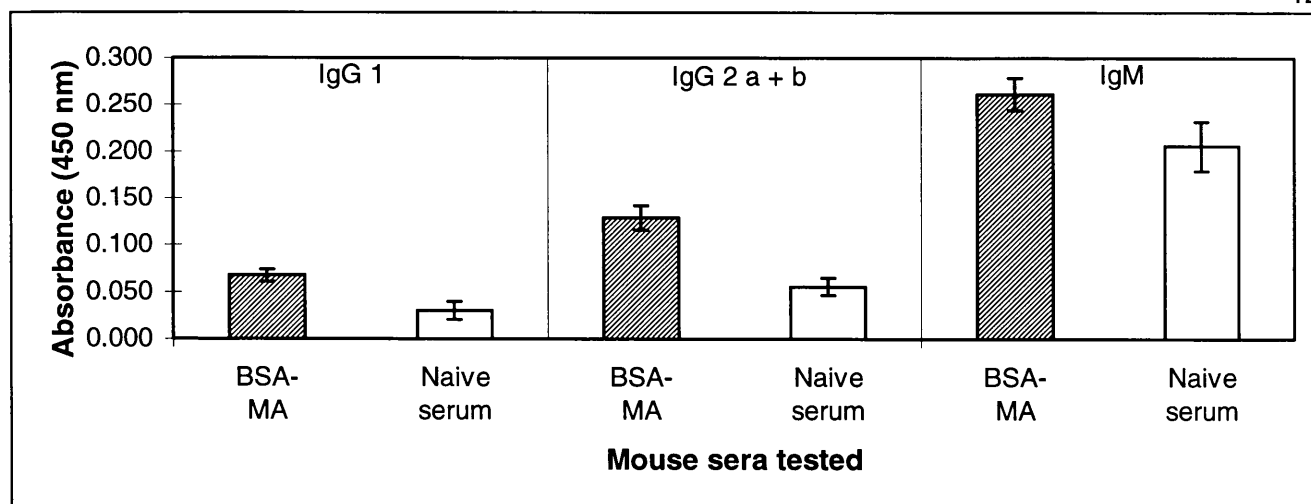
<u>Figure 3.5</u>   ELISA for immunoglobulin isotyping of pooled serum of Balb/C mice, immunised with acetone precipitated BSA-MA / Adjuprime, tested on day 202. ELISA plates were coated with gelatin-MA. The bars represent the average signal of six wells of 1:50 diluted serum.

3.4.3.2. Inhibition ELISA of human tuberculosis patient serum

In an attempt to develop an ELISA method to detect antibodies specific for mycolic acids, pure mycolic acids were melted into hot PBS and used to coat microtitre wells in the absence of a protein carrier, as described in 3.3.5.1. This experiment was done by other members of the research group working with human tuberculosis patient sera to determine whether anti-mycolic acids antibodies were present in human tuberculosis patient serum (Pretorius, 1999).

The immune system of a human tuberculosis patient is exposed to a large number of *M. tuberculosis* bacteria which it attempts to destroy. Since a large proportion of the *M. tuberculosis* cell wall consists of mycolic acids, it was hoped that antibodies against mycolic acids may be present in human tuberculosis patient sera. Human tuberculosis patient sera were obtained from Dr Bernard Fourie, Director of the Tuberculosis Research Institute of the Medical Research Council and analysed by ELISA on microtitre plates coated with mycolic acids. Of the 100 sera tested, only one (1%) showed antibodies which recognised mycolic acids on the ELISA plate (see appendix A) (Pretorius, 1999).

The specificity of the antibodies present in the serum of the tuberculosis patient was determined by inhibition or competitive ELISA as described in 3.3.5.3. using mycolic acids adsorbed onto mouse serum as the competing antigen. The control competing antigen was prepared by treating mouse serum similarly, but without including mycolic acids. Figure 3.6. shows that the human tuberculosis patient serum

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gave a signal of 0,84 on mycolic acids coated wells and only 0.07 on uncoated wells indicating a strong anti-mycolic acids immune response. Human control serum gave a signal of 0,24 on mycolic acids coated wells and 0,11 on uncoated wells, indicating the absence of anti-mycolic acids antibodies. The addition of control mouse serum to the patient's serum caused no decrease in the signal on mycolic acids coated plates, although an increase in signal on both mycolic acids-coated and uncoated wells was observed. The addition of the competing serum-MA to the human tuberculosis patient's serum resulted in a decrease of the signal from 0,84 to 0,29 (70 % decrease) on mycolic acids coated plates and a slight decrease of signal on uncoated plates. These results show that the tuberculosis patient produced mycolic acids-specific antibodies, most probably of the IgG isotype and therefore imply that anti-mycolic acids antibodies can be elicited in human tuberculosis patients during the process of infection.

Although most tuberculosis patients do not generate anti-mycolic acids antibodies (Pretorius, 1999), some do. The reason for this is unknown. The antibodies present in the human tuberculosis patient's serum were able to recognise and bind specifically to pure mycolic acids in the absence of protein, unlike those elicited in mice immunised with acetone-precipitated BSA-MA. In mice, the antibodies appeared to recognise mycolic acids but the specificity could not be confirmed by inhibition ELISA. Such a result could imply an IgM response in the mouse, in contrast to an IgG response in the human tuberculosis patient.
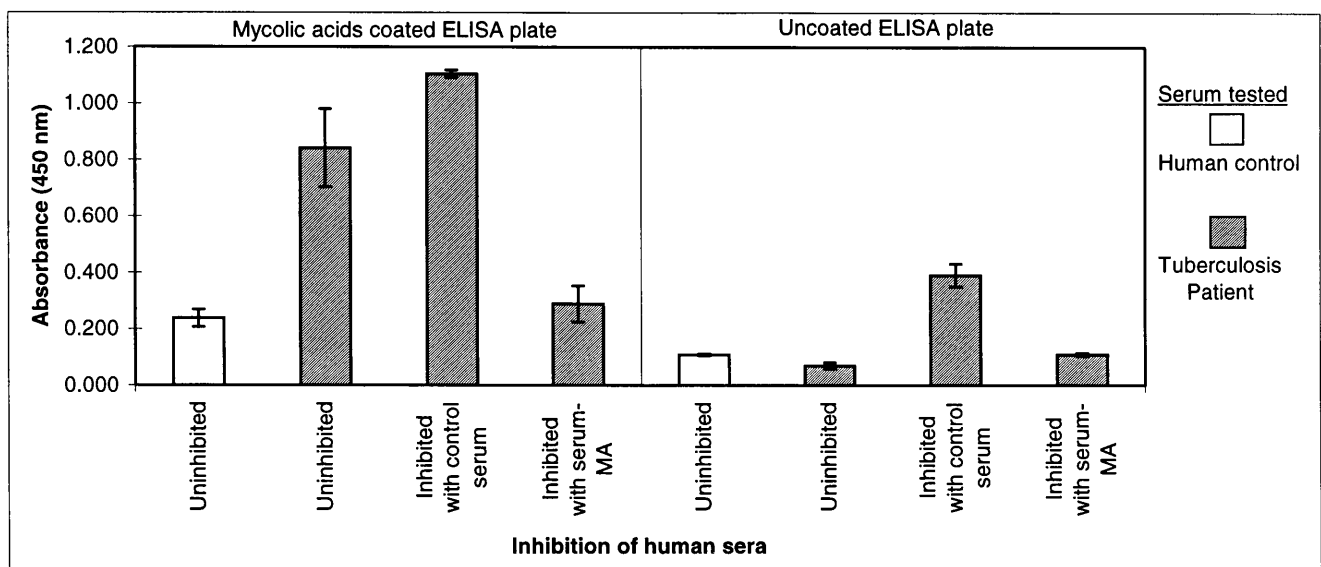
Figure 3.6   Inhibition ELISA of serum from a human tuberculosis patient and human control serum on mycolic acids coated and uncoated plates. The bars represent the average signal of four wells of 1:10 diluted serum.

3.4.3.3. Immune response of mice immunised with fresh and freeze-dried mycolic acids-BSA

The presence of anti-mycolic acids antibodies in the serum of a human tuberculosis patient indicated that it is possible to elicit an antibody response to this fatty acid and led to further attempts to elicit such antibodies in mice. An alternate method for the production of mycolic acids-protein conjugates, similar to that used by Beckman and co-workers for the studies into CD1 presentation of mycolic acids, was used. Beckman's method involved the use of dry mycolic acids which were sonicated into serum in a sonicating bath (Beckman *et al.*, 1994). This approach was modified by adding saponified mycolic acids (in a small volume of chloroform) to an aqueous protein solution before sonication. The chloroform was removed by bubbling nitrogen though the solution. Although the addition of chloroform made an additional step necessary, the fact that the mycolic acids were in solution when added, instead of dry, may enhance the even distribution of the fatty acids onto the proteins. Saponified mycolic acids were used in an attempt to utilise the increased polarity of the fatty acids in the hope that they would be more immunogenic than the methyl ester form of the molecule, in analogy to the increased immunogenicity of more polar proteins (Farmer *et al.*, 1993). The variability of the amount of mycolic acids coated on the conjugates made it necessary that each sample prepared for immunisation was analysed by HPLC to confirm the presence of mycolic acids.

BSA-MA samples were either prepared in one batch and freeze-dried or prepared fresh prior to immunisation of both Balb/C and C57Bl/6 mice, the latter which are reported to be more resistant to infection with *M. tuberculosis* (Flynn *et al.*, 1995) than Balb/C mice. Both freshly prepared BSA-MA and freeze-dried BSA-MA were immunised into mice to test the possibility of freeze-drying protein-mycolic acids conjugates without affecting the antigenicity of the mycolic acids and to facilitate the use of a standard conjugate preparation for immunisation which could be stored in a stable form. It was hoped that the more resistant C57Bl/6 may produce anti-mycolic acids antibodies more readily than the Balb/C mice used previously. Microtitre plates were coated with mycolic acids in hot PBS, in a similar manner to that used for the ELISA of human tuberculosis patient inhibition ELISA, in an attempt to reduce cross-reactivity of serum with protein carriers (e.g. gelatin), thereby detecting only anti-mycolic acids antibodies. The hypothesis was that both freshly prepared and freeze-dried BSA-MA conjugates,

would elicit a similar immune response in mice, possibly inducing a stronger anti-mycolic acids immune response in C57Bl/6 mice which are more resistant to *M. tuberculosis* infection than in Balb/C mice, particularly if such antibodies are beneficial in protection against tuberculosis.
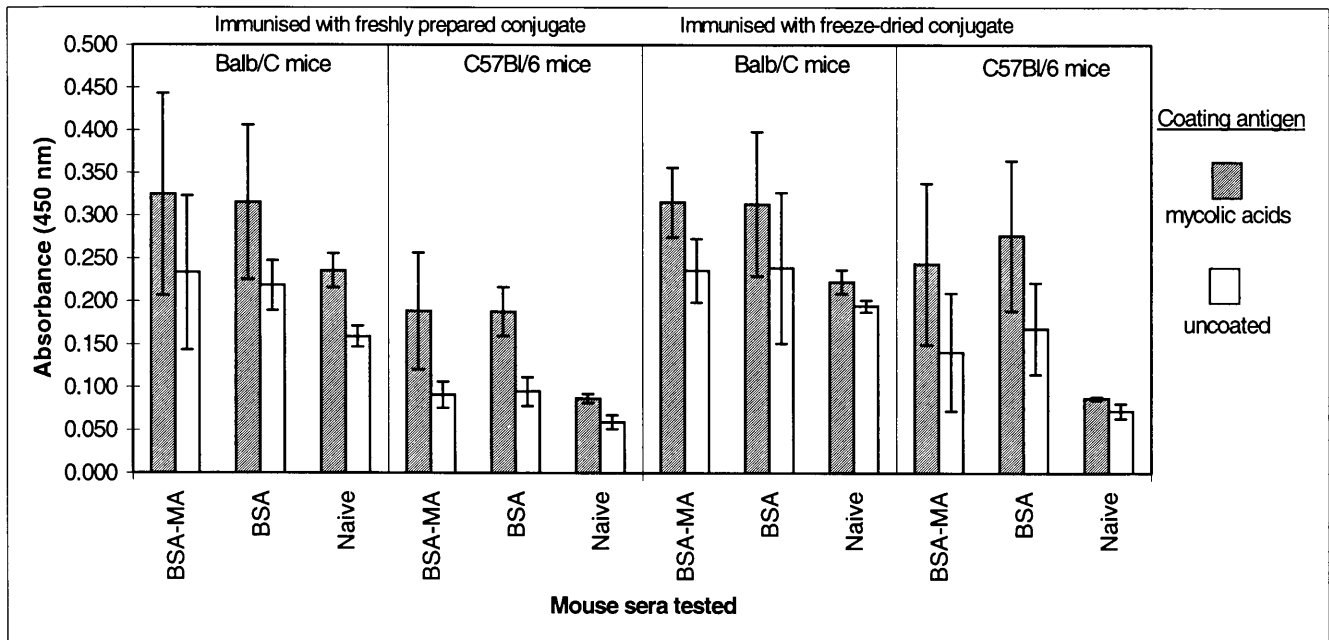
<u>Figure 3.7</u> Average ELISA signal of sera from Balb/C and C57Bl/6 mice, immunised with freshly prepared or freeze-dried BSA or BSA-MA on day 77, tested on ELISA plates coated with mycolic acids or uncoated.

On day 77 of the immunisation schedule (Figure 3.7), the average immune response of the five experimental Balb/C mice immunised with freshly prepared BSA-MA was 39 % higher on mycolic acids-coated wells (0.325 ± 0,118) than on uncoated wells (0.233 ± 0.09). The control mice immunised with freshly prepared BSA had a signal on mycolic acids-coated wells (0.315 ± 0.09) that was 44 % higher than the signal on uncoated wells (0.218 ± 0.029). Similarly, naive serum had a relatively high signal on mycolic acids-coated wells (0.235 ± 0.02) which was 47 % higher than the signal on uncoated wells (0.159 ± 0.012). The ELISA signal of sera from Balb/C experimental mice and control mice were similar on both mycolic acids-coated and uncoated wells. Furthermore, both these groups and the naive mice had ELISA signals that were 39 - 47 % higher on mycolic acids coated wells than on uncoated wells. These results indicate the absence of mycolic acid specific antibodies in Balb/C mice immunised with freshly prepared BSA-MA, at least when tested on plates coated with mycolic acids not conjugated to a carrier protein.

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The average immune response of 5 experimental C57Bl/6 mice immunised with freshly prepared BSA-MA on day 77 of the immunisation schedule (Figure 3.7), was 106 % higher on mycolic acids-coated wells (0.188 ± 0,068) than on uncoated wells (0.091 ± 0.015). The control mice immunised with freshly prepared BSA had a signal on mycolic acids-coated wells (0.188 ± 0.028) that was 97 % higher than the signal on uncoated wells (0.095 ± 0.017). Since the experimental and control mice had the same ELISA signal on mycolic acids-coated wells and the signals were 106 and 97 % higher than on uncoated wells, there appeared to be no difference in the immune response of these two groups and subsequently no anti-mycolic acids response in the C57Bl/6 mice immunised with freshly prepared BSA-MA. Naive serum had a low average signal on both mycolic acids coated (0.087 ± 0.005) and uncoated wells (0.059 ± 0.008).

Balb/C mice immunised with freeze-dried BSA-MA on day 77 of the immunisation schedule (Figure 3.7), had an average ELISA signal on mycolic acids-coated wells (0.315 ± 0,04) that was 33 % higher than on uncoated wells (0.236 ± 0.037). The control mice immunised with freeze-dried BSA had a signal on mycolic acids-coated wells (0.314 ± 0.084) that was 31 % higher than the signal on uncoated wells (0.239 ± 0.088). Since the ELISA signal of the experimental group was similar to the control group on mycolic acids-coated wells and both groups had signals on mycolic acids which were approximately 30 % higher than on uncoated wells, it can be concluded that no mycolic acid-specific immune response was elicited by immunising Balb/C mice with freeze-dried BSA-MA, at least when tested on plates coated with mycolic acids not conjugated to a carrier protein.

Serum from C57Bl/6 mice immunised with freeze-dried BSA-MA, analysed by ELISA on day 77 of the immunisation schedule (Figure 3.7) had an ELISA signal that was 72 % higher on mycolic acids-coated wells (0.243 ± 0,094) than on uncoated wells (0.141 ± 0.069). The control mice immunised with freeze-dried BSA had a signal on mycolic acids-coated wells (0.276 ± 0.088) that was 64 % higher than the signal on uncoated wells (0.168 ± 0.053). Naive serum had a low average signal on both mycolic acids coated (0.087 ± 0.002) and uncoated wells (0.072 ± 0.009). The ELISA signal for the control mice immunised with freeze-dried BSA was higher on mycolic acids than the signal for serum from mice immunised with freeze-dried BSA-MA. The difference between the ELISA signal for both

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groups of mice on mycolic acids-coated wells and on uncoated wells is similar (72 % and 64 %), indicating the absence of antibodies recognising mycolic acids under the conditions selected for these experiments.

Antibodies recognising mycolic acids, similar to that found in one human tuberculosis patient, were not elicited in either Balb/C or C57Bl/6 mice immunised with either freshly prepared or freeze-dried BSA-MA conjugates, 77 days into the immunisation schedule. The ELISA signals of all the sera tested were higher on mycolic acids-coated than on uncoated microtitre wells, possibly due to non-specific, non-polar interaction with mycolic acids. The standard deviation observed for each group of 5 mice was relatively high, indicating that the conjugates and/or protocol did not elicit a consistent response between different mice. All sera were diluted 1:10 for the ELISA procedure which may partially account for the high signals of naive Balb/C serum on both mycolic acids-coated and uncoated microtitre wells. Naive serum from C57Bl/6 mice, however had consistently lower signals. Results from similar experiments done at higher dilutions of antiserum (1:50) showed lower signals but similar results. These results show that the anti-mycolic acids antibody response, if present, is of low affinity.

The Balb/C and C57Bl/6 mice, immunised with freshly prepared or freeze-dried BSA-MA or BSA, were bled on day 108 of the immunisation schedule and ELISAs performed in the same manner as on day 77.
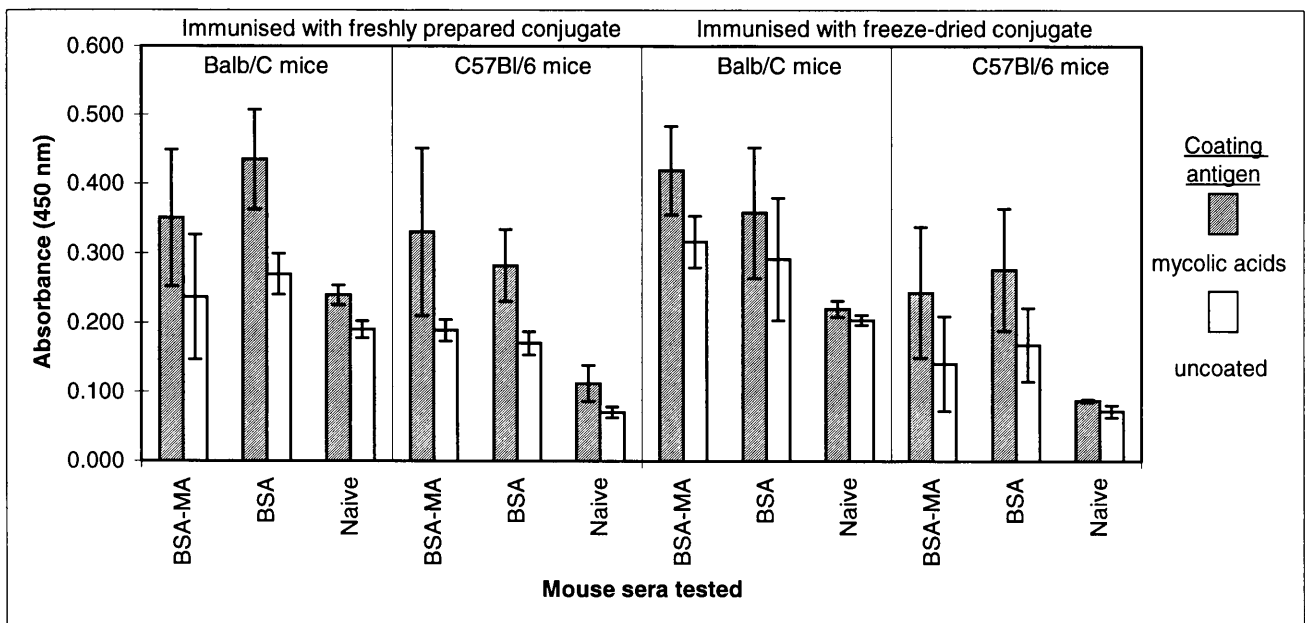
<u>Figure 3.8</u> Average ELISA signal of Balb/C and C57Bl/6 mice, immunised with freshly prepared or freeze-dried BSA or BSA-MA on day 108, tested on ELISA plates coated with mycolic acids or uncoated.

Results obtained in ELISAs performed 108 days into the immunisation schedule were similar to those obtained on day 77. The average ELISA signal for Balb/C mice immunised with freshly prepared BSA-MA and BSA and naive serum were all higher on mycolic acids coated wells (48 , 61 and 26 % respectively) than on uncoated wells. The ELISA signal for control mice immunised with freshly prepared BSA was higher on mycolic acids-coated wells (0.435 ± 0.072) than the signal of experimental mice immunised with freshly prepared BSA-MA (0.351± 0.098) on mycolic acids-coated wells which indicates that no anti-mycolic acids specific antibodies were elicited in these mice.

C57Bl/6 mice immunised with freshly prepared BSA-MA and BSA had ELISA signals 75 and 66 % higher on mycolic acids-coated wells than on uncoated wells. Mice immunised with BSA-MA had signals slightly higher on mycolic acids (0.330 ± 0.121) than BSA immunised mice (0.282 ±. 0.052). The ELISA signal of naive C57Bl/6 serum was also higher (60 %) on mycolic acids-coated wells than on uncoated wells.

Balb/C mice immunised with freeze-dried BSA-MA had an average ELISA signal that was 32 % higher on mycolic acids-coated wells (0.419 ± 0.064) than on uncoated wells and was higher than the average signal for control mice immunised with freeze-dried BSA, on mycolic acids coated wells (0.358 ± 0.094). The ELISA signal for the control mice immunised with freeze-dried BSA, on mycolic acids-coated wells was 23 % higher than on uncoated wells.

C57Bl/6 mice immunised with freeze-dried BSA-MA showed lower ELISA signals on mycolic acids-coated wells (0.243 ± 0.094) than control mice immunised with freeze-dried BSA (0.276 ± 0.088). Both groups of mice immunised with freeze-dried BSA-MA and BSA had ELISA signals which were higher on mycolic acids-coated than on uncoated wells (72 and 64 %).

Balb/C and C57Bl/6 mice immunised with freshly prepared or freeze-dried BSA-MA show very little or no anti-mycolic acids immune response, as they had similar ELISA signals to control BSA immunised mice on mycolic acids-coated wells and on uncoated wells. All the serums tested, including naive sera, showed higher signals on mycolic acids-coated wells than on uncoated wells and most groups had large standard deviations.

These results indicate that no significant anti-mycolic acids immune response was detected in either Balb/C or C57Bl/6 mice, immunised with either freshly prepared or freeze-dried BSA-MA, 108 days into the immunisation schedule. The ELISA signals of naive Balb/C mice or Balb/C mice immunised with either freshly prepared or freeze-dried conjugates were higher on mycolic acids-coated as well as uncoated wells than the ELISA signals of serum from C57Bl/6 mouse sera but seems unrelated to the immunising antigen. No increase in anti-mycolic acids immune response was observed in C57Bl/6 mice, compared to Balb/C mice. The BSA-MA conjugates used as antigen for the immunisation of mice did not elicit anti-mycolic acids antibodies and may not be suitable for this purpose. Other methods by which such an immune response may be elicited may include immunising other laboratory animals such as rabbits or rats with similar conjugates or infecting laboratory animals with *M.tuberculosis* and testing for the presence of anti-mycolic acids antibodies in an attempt to emulate the human tuberculosis patient.

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3.5. Discussion

Mycolic acids are the most abundant and exposed component of the cell wall of *M. tuberculosis* and an antibody directed against these fatty acids could make it possible to detect low quantities of these bacteria in clinical samples. The development of antibodies which recognise different types of mycolic acids could be beneficial in species identification, as the mycolic acids repertoire of mycobacterial species differ (Minnikin *et al.*, 1984). The hydrophobic nature of mycolic acids may however result in cross-reactivity of such antibodies with mycolic acids from other mycobacterial species or even with other long chain fatty acids in clinical samples (Janeway and Travers, 1994; Miller and Levinson, 1996).

The presence of anti-mycolic acids antibodies in mice or human patient serum may prove to be beneficial in protection against tuberculosis and the antigen used to elicit such an immune response could therefore be useful as a vaccine. In addition, anti-mycolic acids antibodies could be used to detect mycobacterial mycolic acids in patient clinical samples for the detection of tuberculosis. Beckman and co-workers recently showed that mycolic acids were able to stimulate DN T-cell proliferation when presented on CD1 molecules, thereby indicating that these fatty acids have some degree of immunogenicity (Beckman *et al.*, 1994). Despite the reported immunogenicity of mycolic acids, to T-cells, antibodies to mycolic acids or other fatty acids have not been reported and as such the probability of inducing antibodies to mycolic acids was not considered to be high. The immunogenicity of cord factor (trehalose dimycolate - TDM) has lead to the development of a serodiagnostic test for the detection of antibodies to TDM in patient serum (Maekura *et al.,* 1993). Mycolic acids may contribute to the immunogenicity of TDM as they comprise a large component of these molecules. In the work done by Kato, the α-D-trehalose was found to be the antigenic determinant of the cord factor molecule and not the mycolic acids component (Kato, 1972).

The patent application by Sawaii Parmaceutical Co. describes antibodies generated by injecting mice with trehalose-dimycolate from *Norcardia rubra*, which recognised mycolic acids methyl esters from the same organism (Sawai, 1989). This supports the idea that antibodies could be generated against mycolic acids of M*ycobacterium tuberculosis.*

A suitable antigen, incorporating the mycolic acids as ligands had to be developed for immunisation into mice. Initially only Balb/C mice which are susceptible to tuberculosis were used, but the more resistant C57Bl/6 mice were later included in anticipation that the immune system of one of the two strains would be more suited to the production of antibodies to mycolic acids. Mycolic acids purified from *M. tuberculosis* by countercurrent distribution were used in an attempt to produce antibodies directed at mycolic acids. It was found that these mycolic acid molecules were in an esterified state, most probably the methyl ester. Mycolic acids, which have limited solubility, were introduced into an aqueous proteinaceous solution (BSA) in the presence of methanol to produce a conjugate of mycolic acids to a suitable protein carrier (BSA-MA). The ELISAs of the initial experiment used gelatin-mycolic acid conjugates as ELISA coating antigens. These were prepared in a similar manner as the BSA-MA immunising antigen. A certain degree of cross-reactivity between gelatin and BSA was observed with anti-BSA-MA and anti-BSA sera. Some indication was obtained of antibodies that reacted with mycolic acids in the immunised mice, but the specificity could not be confirmed with an inhibition assay. In addition, the affinity of the antibodies to mycolic acids was very low. This reminds of IgM antibodies which are usually of relatively low affinity (Goding, 1986).

A further immunisation protocol was developed in which both C57Bl/6 and Balb/C mice were immunised with conjugates prepared with saponified mycolic acids (which are more polar than unsaponified mycolic acids) sonicated into a proteinaceous solution (BSA). In an attempt to reduce the chances of cross-reactivity, an ELISA was developed where pure mycolic acids were directly coated onto the microtitre plate. This method is unique as it takes advantage of the ability of mycolic acids to melt into an aqueous solution at high temperature. The exact conditions at with the microtitre plates was coated, was difficult to control, as well as the exact amount of mycolic acids coated per well. Therefore, although this method reduces the chances of cross-reactivity and increases the chances of detecting specific anti-mycolic acids responses, it should be further optimised. Coating mycolic acids onto microtitre plates in this manner was adequate enough to detect antibodies directed specifically at mycolic acids in a human tuberculosis patient.

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Since no specific immune response was elicited in any of the groups of mice immunised with either freshly prepared or freeze-dried conjugate, it can be concluded that the protocols followed are not suitable for eliciting such a response in either Balb/C or C57Bl/6 mice, or that measuring the response on plates coated with mycolic acids without a carrier protein is not the correct way of binding such antibodies.

The discovery of one tuberculosis patient with antibodies which recognise mycolic acids specifically is a significant breakthrough which has not been reported previously. Since the serum samples provided by the MRC are from anonymous patients and could not be replenished, further tests on the same patient serum were not possible. Numerous tuberculosis patient sera need to be screened and if anti-mycolic acids antibodies are present, the medical history and genetic disposition of the individual should be carefully studied, in an attempt to determine the conditions necessary to elicit such antibodies and their effect on disease development. The presence of anti-mycolic acids antibodies in the human patient may also indicate that the production of these antibodies could be restricted to certain species of animals and that a wider variety of animals should be used to determine the propensity of mycolic acids as an antibody inducing antigen.

The conclusion which can be made from these experiments is that although individual mice showed a slight anti-mycolic acids response, it was not universal and mice may not be the ideal model for eliciting such antibodies. Mycolic acid specific antibodies can however be produced in humans and the approach therefore has merit, although the optimal conditions for the production of such antibodies may be difficult to determine. The immune response against mycolic acids is weak, even in humans, since the human serum was barely diluted to detect a significant signal. The use of polyclonal anti-mycolic acid serum for the detection of these molecules in clinical samples would not be practical due to the limitation of the serum dilution. Monoclonal antibodies would have to be generated to allow the practical use of such a test, but several attempts by my team-members to generate such antibodies have indicated that the affinity of such antibodies, had they existed, was too low to be detected at the dilutions typical of hybridoma culture supernatants.

Chapter 4:
Concluding Discussion

Tuberculosis is a growing world-wide problem and if neglected, could lead to the global spread of the disease and the development of more multi-drug resistant strains which would render the disease incurable. Very few new drugs for the treatment of tuberculosis have been developed in the last 25 years, a matter of concern when considering the development of multi-drug resistance to the commonly used drugs, which is currently emerging in all parts of the world. The interaction of tuberculosis and HIV amplifies the effect of both diseases. It is thought, that by the end of the century, tuberculosis will be the leading cause of death in HIV patients who have a 30 times higher chance of developing tuberculosis than people only infected with tuberculosis bacteria (WHO, 1997).

The diagnostic assays for tuberculosis used in most laboratories were developed early in the last century and have to date only been modified slightly. Most diagnostic assays require the consecutive collection of several clinical sputum samples over a period of one week, since tuberculosis lesions leak into the bronchial tree intermittently, causing one sample to be negative and the next to be positive. These difficulties contribute to the inefficient diagnosis of tuberculosis by current methods, which generally include microscopic analysis and culturing. Microscopy is not very sensitive and cannot differentiate between species of *Mycobacteria.* Although culturing is very sensitive, it takes a few days to obtain results (Smithwick, 1994). Faster methods that are both sensitive and specific would be in popular demand.

Mycobacterial cell walls contain mycolic acids in large abundance, contributing to the resistance of *Mycobacterium spp.* to chemotherapeutic agents and antibiotics (Liu *et al.*, 1996). The HPLC (Butler *et al.*, 1991) and TLC (Minnikin *et al.,* 1984) profiles of the mycolic acids of various species of *Mycobacterium* differ considerably, making these molecules of taxonomical importance (Butler *et al.*, 1991). Due both to their abundance and their uniqueness to mainly *Mycobacteria*, this study aimed at determining the potential use of mycolic acids for the diagnosis or identification of *M. tuberculosis.*

Mycolic acids first had to be isolated in pure form. Crude alkaline methanolytic extracts of *M. tuberculosis* cultures were used as a starting point for the purification of mycolic acids (Butler, 1985).

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Countercurrent distribution, an old technique that has largely been replaced by methods such as HPLC and affinity chromatography, proved to be a useful tool for the purification of mycolic acids from *M. tuberculosis* without necessitating derivatisation, as required for HPLC. A biphasic system comprising methanol, chloroform and water was developed in which the distribution constant of mycolic acids was close to zero since they were negligibly dissolved in the upper phase of the system. The low distribution constant of mycolic acids resulted in the majority of contaminants moving further down the countercurrent train, away from the mycolic acids fraction. The method allowed the separation of mycolic acids from approximately 90% of the contaminants of the crude extract. Further optimization of the solvent system used could result in faster purification of mycolic acids, possibly by the inclusion of an ionic substance in the aqueous phase to reduce the time required for the breakup of the emulsion formed after the mixing step of each countercurrent cycle (Siko, 1999).

The countercurrent distribution system could easily be scaled up to isolate large quantities of pure mycolic acids, providing an inexpensive source of large quantities of these molecules. This will enhance further studies into the potential use of mycolic acids in the treatment and diagnosis of tuberculosis and makes it feasible to use mycolic acids for therapeutic or diagnostic purposes.

A different biphasic system could possibly be developed which may separate the peaks of the mycolic acids cluster during purification or in a subsequent countercurrent distribution step. However, since the mycolic acids have similar hydrophobicity and are only soluble in organic chlorinated solvents, the development of such a biphasic system may be difficult. Moreover, it is known that the individual peaks of the HPLC chromatogram of the mycolic acids cluster, represent mixtures of the different types of mycolic acids that have similar hydrophobicity, due to varying meromycolic acid chain lengths, thereby preventing the possibility to separate the different types of mycolic acids (alpha, keto or methoxy mycolates) (Butler, 1985).

Mycolic acids were previously isolated from crude mycobacterial extracts or saponified trehalose dimycolate by HPLC. The life of HPLC columns, however, is limited by the purity of the mycolic acids loaded (Duffey *et al.*, 1996). Large quantities of mycolic acids can therefore not be purified from crude mycobacterial extracts. Purified mycolic acids became commercially available in 1994, allowing studies of the immunogenic function of mycolic acids to be more readily pursued. However, this source of

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mycolic acids is costly. No commercially available source exists for purified mycolic acids from other bacterial species such as *M. vaccae*, which could prove useful for the development of a vaccine for tuberculosis. Countercurrent distribution using the technique described above could be used for the purification of mycolic acids from a wide variety of mycobacterial species, possibly with small modifications in the solvent system. This would allow researchers to further investigate the role of mycolic acids from different species of *Mycobacterium* in detection, prevention or therapy of disease.

Although mycolic acids are fatty acids which are considered to be of low immunogenicity due to their lack of polarity, they have recently been shown to be presented on human CD1b molecules of antigen presenting cells to stimulate double negative T-cells. The Harvard group of Michael Brenner obtained pure mycolic acids by HPLC from sonified *M. tuberculosis* or from saponified commercially available trehalose dimycolate for the purposes of these experiments (Beckman *et al.*, 1994).

The work done by Brenner and co-workers has lead to the founding of a commercial concern to investigate the CD1 system of lipid antigen presentation with the potential aim of a vaccine against tuberculosis. They initially put their aims on tuberculosis but foresee later applications among other diseases such as malaria and *Salmonella* infections (Business Wire, 1996). The availability of pure fatty acids such as mycolic acids will be an important aspect in these investigations. The development of mutants, which are able to escape the immune system of an individual immunised with a proteinaceous vaccine, is possible by the alteration of individual amino acids of the target molecule. This is not possible if a lipid based vaccine is used, lipids not being directly derived from genes. The pathway by which lipid molecules are synthesised are complicated and the enzymes used in the synthesis would have to be changed by the organism to result in resistant mutants. MHC molecules, which are polymorphic between individuals of a population, can be the cause of the varying efficacy of some protein based vaccines. The presentation of mycolic acids-type vaccines on CD1, with its lack of polymorphism, could therefore provide hope for a more efficient vaccination of a diverse population. This route of presentation, discovered for the mycobacterial mycolic acids antigens, will probably play an important role in the immune response to other pathogens, paving the way to significant progress in the field of vaccination (McCarthy, 1995).

Recent studies have shown that the stimulation of CD1-restricted cytotoxic CD4⁻CD8⁻ (double negative) and CD8⁺ T-cells, both resulted in the lysis of macrophages infected with *M. tuberculosis*. Lysis of the macrophage by double negative T-cells did not affect the viability of the *Mycobacteria*, while that caused by stimulation of CD8⁺ T-cells killed the bacteria (Stenger *et al.,* 1997).   The stimulation of CD8⁺ T-cells by antigen in the context of CD1 may therefore be a more important factor in the combat of tuberculosis than the stimulation of double negative (DN) T-cells.

In this chapter, it was shown that purified mycolic acids could stimulate both DN T-cells as well as CD4 T-cells by CD1 expressing antigen presenting cells, which was not observed in Beckman's studies. These results indicate that mycolic acids may be able to stimulate T-helper cells (CD4) as well as the cytotoxic DN T-cells. Stimulation of CD8⁺ T-cells by mycolic acids under the same circumstances was not observed.

The CD4 T-helper response is composed mainly of the Th1 and Th2 sub-populations of T-cells. T-helper type one (Th1) is stimulated by antigen presented by macrophages and results in the secretion of IFNγ which is especially effective against intracellular viruses, bacteria and parasites, while T-helper type two (Th2) is characterised by being stimulated by antigen presented on B-cells in order to assist the latter in producing antibodies in defense against free living bacteria (Street and Mosmann, 1991).   The stimulation of CD4 T-cells by mycolic acids presented on CD1-expressing antigen presenting cells, particularly macrophages (Porcelli, 1995), may drive the immune response towards a Th1 response.   Th1 is the more effective response against the *Mycobacteria* present in the macrophage host cells.

Peptide antigens which stimulate T-helper cells are presented in the context of MHC class II molecules while cytotoxic T-cells are stimulated by antigen presented on MHC class I molecules (Janeway and Travers, 1994).   The results of this study show that mycolic acids presented on CD1 are able to stimulate both cytotoxic (DN T-cells) and T-helper (CD4) cells.

In some cases the differentiation of T-cytotoxic precursor (Tcp) cells into T-cytotoxic cells requires the assistance of T-helper (Th) cells. This is thought to occur by the binding of both types of cells (Tcp and Th) to the same antigen-presenting cell, which requires both MHC I (for Tcp) and II (for Th) for peptide

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antigens. The activated T-helper cells secrete IL2 and IL6 cytokines, which stimulate the proliferation and differentiation of T-cytotoxic precursor cells to cytotoxic cells (Janeway and Travers, 1994; Keene and Forman, 1982).

The presence of both a cytotoxic response and a Th1 response would make the attack on *Mycobacteria* in macrophage cells more effective and could indicate that mycolic acids play an important role in the immune response to tuberculosis.

This proposed mechanism would attribute immunoregulatory properties to mycolic acids, which may be of significance in the immune response to *Mycobacteria*. It is not clear, however, how the production of antibodies to mycolic acids occurs. Although the presence of such antibodies could be demonstrated in this study in both immunised mice and human tuberculosis patients, the arguments suggesting a mechanism for the immunoregulatory properties of mycolic acids mentioned above do not support antibody stimulation. The immune system of the tuberculosis patient expressing antibodies to mycolic acids may have processed the *Mycobacteria* in a unique way or may have been exposed to degraded bacteria possessing a more immunogenic form of mycolic acids.

Investigations into the frequency of occurrence of anti-mycolic acids antibodies in human tuberculosis patients revealed a very low prevalence (Pretorius, 1999). The general lack of such antibodies in human tuberculosis patients indicates that mycolic acids will not provide a suitable antigen for the diagnosis of tuberculosis infection in an ELISA type serodiagnostic assay. The presence of anti-mycolic acids antibodies in some tuberculosis patients does, however, indicate that such antibodies can be produced under particular conditions and that a diagnostic method utilising such antibodies to detect mycolic acids from *Mycobacteria* in clinical samples may be possible.

In an attempt to produce anti-mycolic acids antibodies, mice were immunised with countercurrent purified mycolic acids adsorbed on a proteinaceous carrier. Although a slight immune response was detected against mycolic acids in individual mice, it was not strong or specific and was not consistent within the experimental group. The possibility exists that an alternative approach may yield specific antibodies of a quality that can be utilized in diagnostic assays, but the low affinity of anti-mycolic acids antibodies in human patients do not provide much hope for a simple solution to this problem.

Chapter 4

Other problems that may be foreseen with the use of antibodies against a fatty acid antigen include the hydrophobicity of the antigen. It may be similar to other hydrophobic molecules and result in a high degree of cross-reactivity of the antibodies with mycolic acids or fatty acids of other bacterial species. This would cause such a test to be unsuitable for general diagnostic purposes (Miller and Levinson, 1996).

The difficulty encountered with the production of anti-mycolic acids antibodies in mice may be due to a number of factors. Since the mycolic acids were adsorbed onto the protein carrier and not covalently linked, the number of molecules presented in an immunogenic form may have been limited and may have varied between each carrier molecule and batch of preparation. Since the antigenicity of mycolic acids is expected to be low, the concentration of mycolic acids should possibly be increased, although the capacity of the carrier molecule for adsorbing these large fatty acids is limited. Particular forms of mycolic acids (alpha, keto or methoxy) may be more antigenic. This should be tested once an effective method for the large-scale separation of each type has been developed. Increasing the concentration of only the most immunogenic mycolic acids could increase the chances of obtaining anti-mycolic acids antibodies.

Animal models other than the mouse should be tested for their ability to produce anti-mycolic acid antibodies, since this ability may vary both among individuals within one species (such as the single human tuberculosis patients among many who produced such antibodies) and between species (Mayer and Walker, 1987).

The availability of pure mycolic acids from *M. tuberculosis* and possibly mycolic acids from other *Mycobacterium spp.* could facilitate further studies into the antigenicity of these fatty acids and their role in the immunology of tuberculosis as well as their potential use in the development of a vaccine against tuberculosis. The specific function of the presentation of these and other non-proteinaceous molecules on CD1 molecules is not yet understood but has lead to the discovery of an entirely new mechanism of the immune system which may prove to be a breakthrough in a number of diseases, particularly tuberculosis.

Chapter 4

The production of anti-mycolic acids antibodies appears to be possible and could lead to the use of such antibodies in a serodiagnostic assay to detect *Mycobacterium spp.* in clinical tuberculosis patient samples. Such antibodies could also serve as important tools in studies of the presentation of mycolic acids on CD1 molecules as well as the processing of these fatty acids in the cells of the immune system. The ability of the immune system to produce antibodies against fatty acids has not been described previously. The significance of this in immunity in general may provide unique clues in the combat against various diseases or further understanding of anergy and autoimmunity as typical harassing side-effects of parasitism.

Chapter 4

Summary

Tuberculosis is recognised as the leading cause of death among infectious diseases world-wide. The incidence of tuberculosis is expected to rise due to the emergence of drug-resistant *Mycobacterium tuberculosis* strains and the interaction between *M. tuberculosis* and the human immunodeficiency virus (HIV). The diagnosis of tuberculosis is complicated by various factors such as the slow growth of the organisms in culture, anergy, and the cross-reactivity of antibodies to *Mycobacterial* antigens. Currently, diagnosis is made by a combination of clinical assessment, chest radiographs and laboratory tests, since no single definitive, simple test is reliable.

Mycolic acids are species-specific, high molecular weight fatty acids which contribute to the resistance of these organisms to antibacterial agents, disinfectants, alkali and drying. Recent discoveries that mycolic acids are presented by CD1 molecules and stimulate double negative (DN) T-cell proliferation, have indicated that an immune response against mycolic acids is possible.

In an attempt to validate a proposal for a diagnostic kit utilising anti-mycolic acids antibodies, the chemical and biological properties of purified mycolic acids were investigated. A countercurrent distribution (CCD) purification method was developed for the large-scale purification of mycolic acids from crude mycobacterial extracts, using a mixture of chloroform, methanol and water. This was followed by acetone precipitation of mycolic acids out of chloroform solution. HPLC studies showed that CCD-purified mycolic acids were 90-98 % pure, were esterified and had to be re-saponified prior to derivatisation to allow quantification by HPLC analysis.

Cell proliferation studies showed that CCD-purified mycolic acids were biologically active and stimulated the proliferation of DN and CD4 but not CD8 T-cells, in the presence of CD1$^+$ antigen presenting cells. This occurred at low concentrations of mycolic acids whereas high concentrations appeared to have an inhibitory effect, possibly due to toxicity.

CCD-purified mycolic acids were used to prepare BSA-mycolic acids conjugates (for immunisation of Balb/C mice) by adsorption of mycolic acids onto BSA in aqueous solution containing methanol. ELISA coating antigen (gelatin-mycolic acids) was prepared similarly. Antibodies of low affinity and specificity to mycolic acids, were detected in the sera of the immunised mice using ELISA.

Additional BSA-mycolic acids conjugates were prepared by sonication into proteinaceous solution and administered to Balb/C and C57Bl/6 mice as freshly prepared and freeze-dried preparations. ELISAs performed on microtitre plates coated with pure mycolic acids (by dissolving them in aqueous solution at high temperature) showed no specific immune response in any of the immunised groups. It may be concluded that the protocols followed in this study were not suitable for eliciting such an immune response.

Antibodies specific for mycolic acids were detected in the serum of one human tuberculosis patient by inhibition ELISA on microtitre plates coated with pure mycolic acids. This significant discovery indicates that antibodies specific for mycolic acids can be elicited, although at low frequency and under unknown conditions. The possibility that such antibodies may be restricted to certain individuals, species or stage of disease of animals must be considered in the design of future studies of this nature.

Opsomming

Tuberkulose word beskou as 'n infektiewe siekte wat verantwoordelik is vir die meeste mortaliteite wêreldwyd. Daar word verwag dat die voorkoms van tuberkulose sal styg, weens die toename van middel weerstandige stamme van *Mycobacterium tuberculosis* en die interaksie tussen *M. tuberculosis* en die HIV virus. Tans word tuberkulose gediagnoseer deur 'n kombinasie van kliniese ondersoeke, bors radiogramme en laboratorium toetse aangesien daar geen enkel, beslissende, betroubare toets bestaan nie. Die diagnose van tuberkulose word deur verskeie faktore gekompliseer soos die stadige groei van die organisme in kultuur, anergie en die kruisreaktiwiteit van teenliggame teen die mikobakteriese antigene.

Mikolsure is spesie-spesifieke, hoë molekulêre massa vetsure wat bydra tot die weerstand van *Mycobacteria* teen antibakteriese agente, ontsmettingsmiddels, alkali en droging. Die onlangse ondekking dat mikolsure deur CD1 molekule gepresenteer kan word en dat dit dan dubbel negatiewe T-sel proliferasie stimuleer, dui daarop dat 'n immuunreaksie teen mikolsure moontlik is.

Die chemiese en biologiese eienskappe van gesuiwerde mikolsure is ondersoek in 'n poging om 'n voorstel vir 'n diagnostiese toets wat anti-mikolsuur teenliggame gebruik, te staaf. 'n Teenstroom suiwerings metode is ontwikkel vir die grootskaalse suiwering van mikolsure vanaf 'n kru mikobakteriese ekstrak. Die mikolsure is daarna uit 'n oplossing van chloroform gepresipiteer met asetoon. Die gesuiwerde mikolsure was verester en moes gesaponifiseer word voor derivatisering om sodoende kwantifisering deur HPLC analise moontlik te maak. HPLC analise het getoon dat die teenstroom gesuiwerde mikolsure 90-98 % suiwer was.

Sel proliferasie studies het getoon dat teenstroom gesuiwerde mikolsure biologies aktief is. Dubbel negatiewe en CD4 T-selle prolifereer in die teenwoordigheid van CD1$^+$ antigeen presenterende selle. Sel proliferasie het voorgekom teen lae mikolsuurkonsentrasies, maar hoë mikolsuurkonsentrasies het 'n inhiberende effek gehad, waarskynlik weens toksisiteit.

Teenstroom gesuiwerde mikolsure is gebruik om BSA-mikolsuur konjugate voor te berei vir die immunisasie van Balb/C muise. Mikolsure is op BSA in 'n waterige oplossing geadsorbeer in die teenwoordigheid van metanol. Antigeen vir die bereiding van ELISA plate (gelatin-mikolsure) is op 'n

soortgelyke wyse voorberei. Teenliggame met 'n lae affiniteit en spesifisiteit is gevind in die sera van die geïmmuniseerde muise d.m.v. ELISA.

Addisionele BSA-mikolsuur konjugate is berei deur sonifisering in 'n waterige BSA oplossing. Dit is toegedien aan Balb/C en C57Bl/6 muise as oplossings van vars voorbereide sowel as gevriesdroogde bereidings. Mikrotiter plate (vir ELISA) is gelaag met gesuiwerde mikolsure in 'n waterige oplossing by hoë temperature. Geen spesifieke immuunreaksies is getoon met ELISAs in enige van die eksperimentele groepe nie. Die protokolle gevolg in hierdie studies was dus nie geskik vir die indusering van anti-mikolsuur teenliggame nie.

Mikolsuur-spesifieke teenliggame is in die serum van een menslike tuberkulose pasiënt d.m.v. inhibisie ELISA op mikolsuur-gelaagde mikrotiter plate gevind. Hierdie betekenisvolle bevinding dui daarop dat teenliggame spesifiek vir mikolsure bestaan, al is dit teen lae frekwensie en onder onbekende omstandighede. Die waarskynlikheid bestaan dat sulke teenliggame beperk is tot sekere individue, spesies of siekte stadiums. Die faktore moet in ag geneem word in die beplanning van toekomstige studies van hierdie aard.

Appendix A
(Pretorius, 1999)

a.

b.

Appendix A
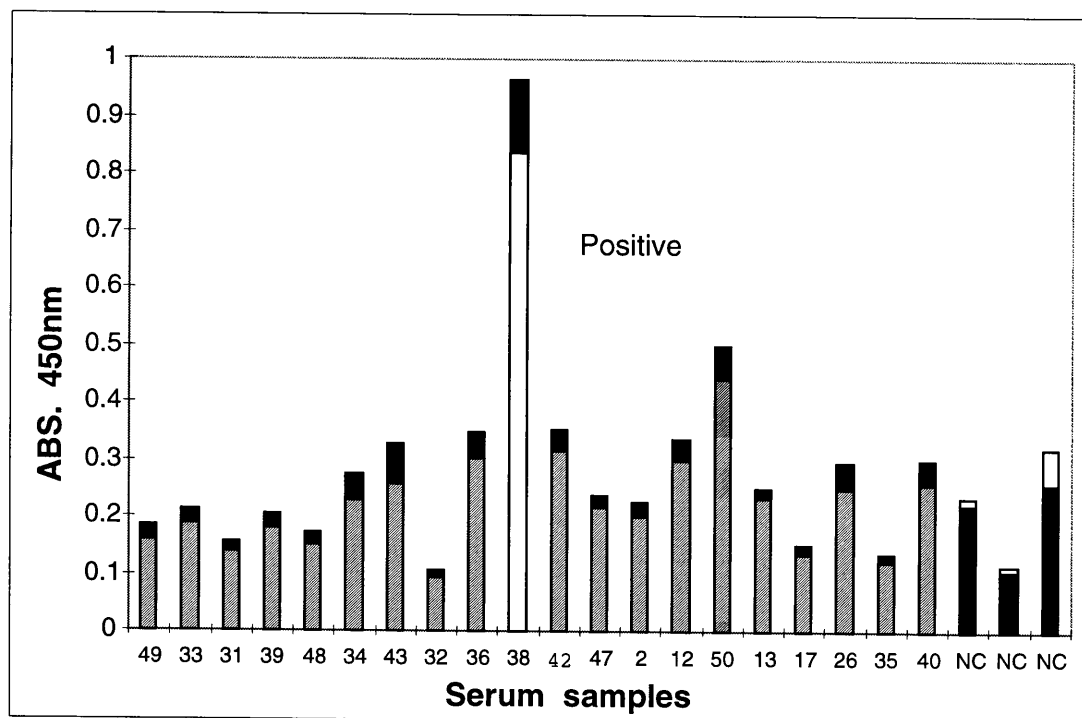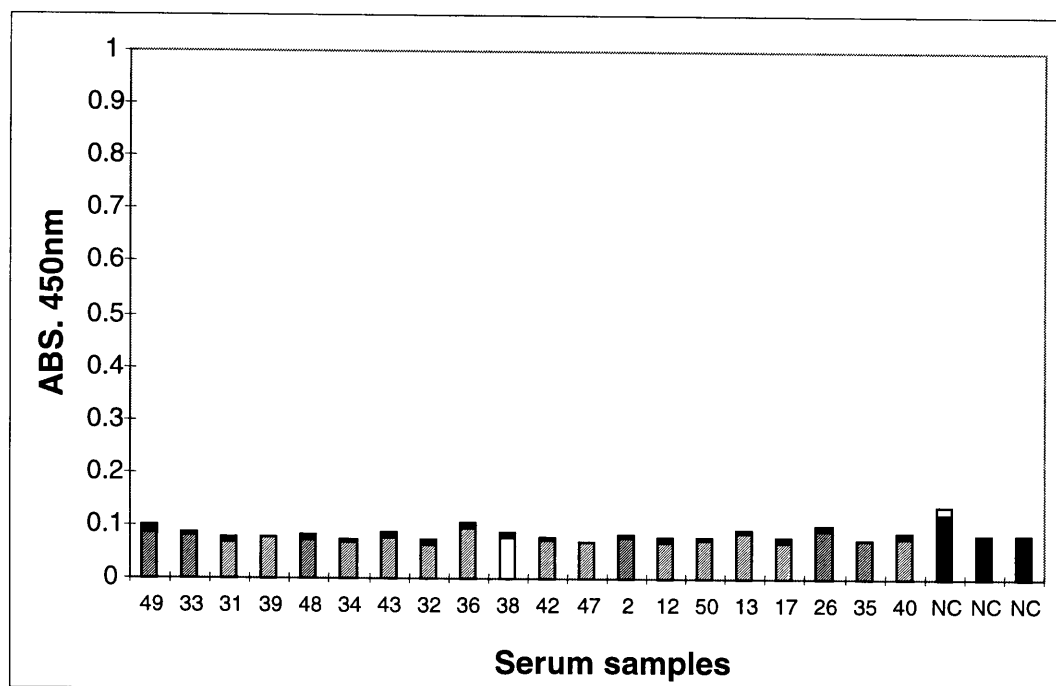
c.
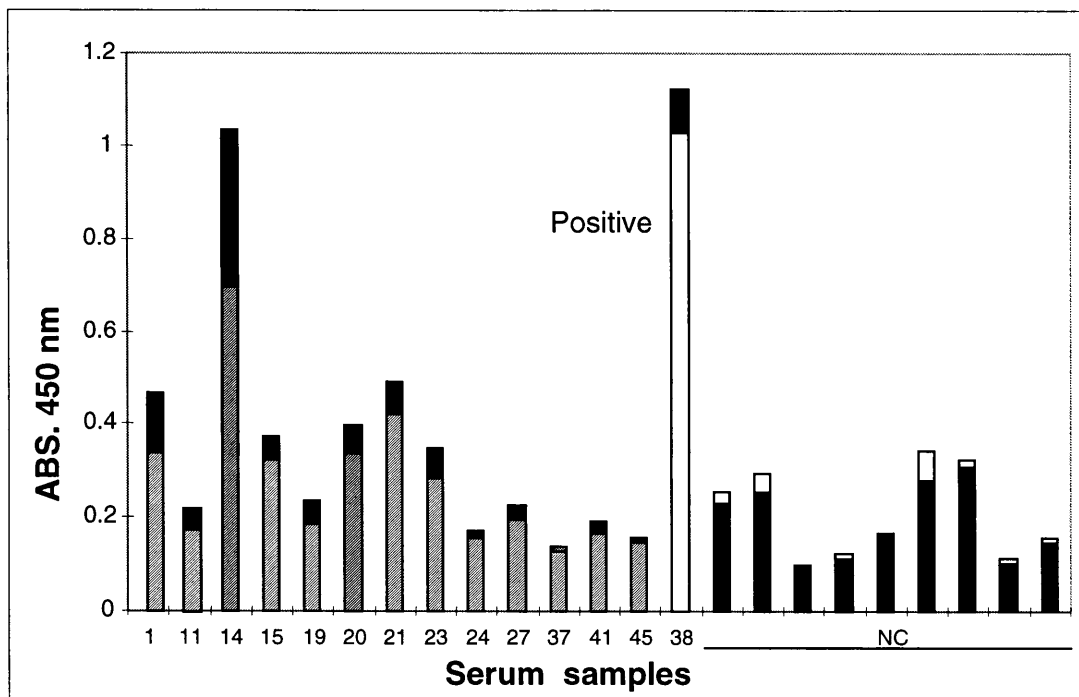
d.
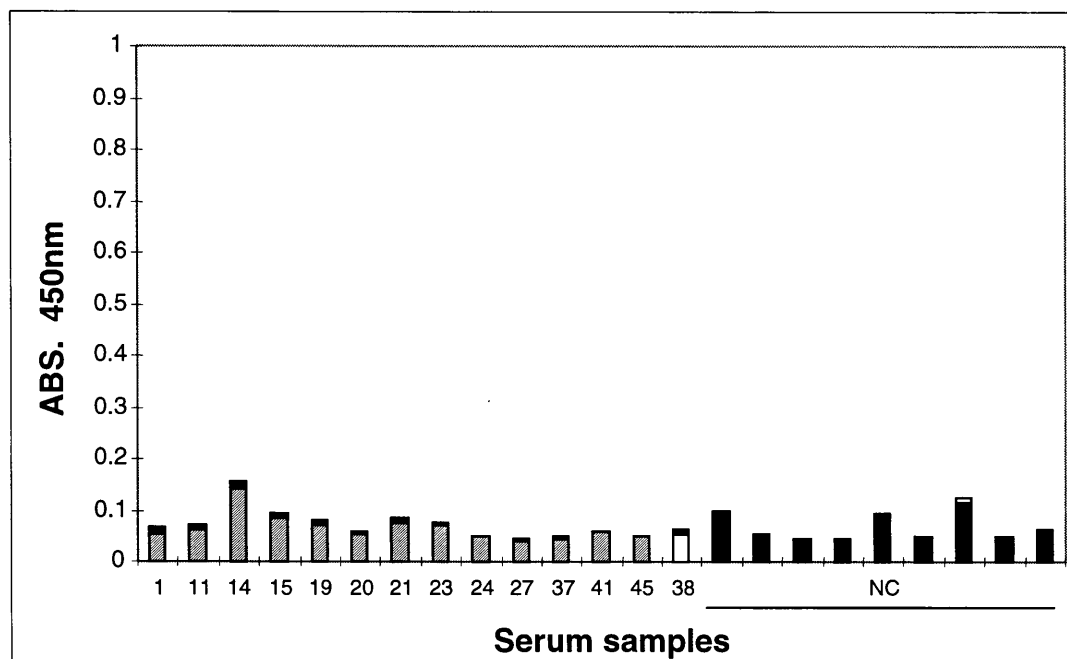
FIG A.1(a,b,c,d). Detection of MA-specific antibodies in various individual human patient sera on mycolic acid coated ELISA plates (A.1 a and c). All sera were diluted 1:10 times. Each patient was allocated a specific number and the negative controls were indicated with NC. The same sera were also screened on uncoated ELISA plates (A.1 b and d).

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