The University of Hull

Studies in the iron uptake mechanism of *Mycobacterium smegmatis* :

Identification of components of the iron uptake system

being a thesis submitted for the Degree of

Doctor of Philosophy

In the University of Hull

By

Lynn G. Dover BSc (Sunderland)

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To my Mam, Dad and Sarah.

Armenian clay, borax and verdigris,
Earthen and glass-ware vessels piece by piece,
Our urinals, our pots for oil extraction,
Crucibles, pots for sublimative action,
Phial, alembic, beaker, gourd-retort,
And other useless nonsense of the sort,
Not worth a leek, needless to name them all;
........Our lamps - we had them burning day and night
To help us succeed, if we but might !
Our furnace too for calcifying action,
Our waters in a state of albefaction,
Chalk, quicklime, ashes and the white of eggs,
Various powders, clay, piss, dung and dregs,
Waxed bags, saltpetre, vitriol and a whole
Variety of fires of wood and coal;
Alkali, tartar, salt in preparation,
Matters combust or in coagulation,
Clay mixed with horse-hair, sometimes with my own,
Crystallised alum, oil of tartar thrown
With tartar crude and unfermented beer,
Yeast and a dozen more than you shall hear,
Realgar, various absorbent batters
And, I may add, incorporative matters;
Our silver in a state of citrination,
Things sealed in wax and things in fermentation,
Our moulds, our vessels for assaying metal
And many other things I learnt to settle
I'll tell you as I was taught, if you want more.
.........And still I have forgotten my intention
To speak of iron filings and to mention
Corrosive liquids, ways of mollifying
Or hardening substances, or of supplying
Oils and ablutions, stores of fusible metal,
And so on. Its beyond a book to settle
These matters, any book; it would be best
To give this catalogue and myself a rest
For I imagine I have said enough
To raise the devil, be he never so rough.

The Canon's Yeoman's Tale,

Geoffrey Chaucer

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Summary

The research in this thesis is concerned with the identification of the envelope proteins involved in the uptake of ferri-exochelin MS by *M. smegmatis*. Analysis of envelope protein profiles under iron sufficiency and iron deficiency identified a family of seven proteins with increased expression under iron-limiting conditions. The results of this study were in broad agreement with earlier observations by other workers. The expression of these seven iron-regulated envelope proteins (IREPs) was not increased on heat shock treatment or on zinc limitation demonstrating that their elaboration was a specific response to iron deficiency. The 29 kDa envelope protein suggested as a putative receptor protein by earlier studies, however, was found to be constitutively expressed at a high level irrespective of the iron status of the culture in our hands.

The majority of the work in this thesis is concerned with the isolation of a ferri-exochelin MS receptor protein. A suite of techniques was developed to identify a receptor via the formation of ferri-exochelin MS-IREP complexes.

The 29 kDa IREP was implicated as the major ferri-exochelin MS-binding protein (FEBP) of *M. smegmatis* by every protocol attempted:

i   a radio-labelled ferri-exochelin-29 kDa complex was formed *in situ* in a crude envelope preparation, extracted and separated by electrophoresis.

ii   a 29 kDa protein was purified from crude envelope extracts of the bacterium to homogeneity by affinity chromatography on ferri-exochelin MS-Sepharose.

iii   a ^{55}Fe-labelled-29 kDa ferri-exochelin was isolated from a crude envelope extract by anion-exchange chromatography and gel filtration chromatography.

Anion exchange and affinity chromatography fractionations also provided evidence that a 25 kDa IREP may be involved in the ferri-exochelin uptake mechanism as a component of a receptor complex along with the 29 kDa FEBP.

These techniques were hindered, however, by a lack of sensitivity. In order to address this problem extracted envelope proteins were incorporated into a simple liposome preparation. The ferri-exochelin MS-binding activity of the resulting proteo-liposome suspension was 133-fold greater than that exhibited by extracted envelope proteins in a buffered detergent solution. This activity was protein-dependent and irreversible on dialysis suggesting a stable binding event, rather than a non-specific association, with the proteo-liposome had occurred. A stable [55]Fe-labelled ferri-exochelin-protein complex was extracted from the proteo-liposomes and isolated by gel filtration chromatography. A molecular size of 57 ± 4 kDa was estimated for the complex but the size of its components could not be reliably determined due to their abnormal electrophoresis on SDS-PAGE. The estimated size of this complex was consistent with the proposed hypothesis that a 29 : 25 kDa heterodimer acts as the ferri-exochelin receptor of *M. smegmatis*.

Abbreviations

ABTS	2,2'-Azinobis(3-ethylbenz-thiazolinesulfonic acid)
CHAPS	(3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate
DCPIP	2,6-Dichlorophenol-indo-phenol
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
FEBP	Ferri-exochelin-binding protein
IREP	Iron-regulated envelope protein
NCIMB	National collection of industrial and marine bacteria
OPA	*o*-phthaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
PEI	Polyethyleneimine
PMS	Phenazine methosulphate
PMSF	Phenymethylsulphonyl fluoride
SDS	Sodium dodecyl sulphate
%T	Total monomer concentration of PAGE gel
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
U	Units

Contents

List of figures

List of Tables

I.   Introduction

A.   The biological relevance of iron.

The participation of iron in a wide range of fundamental biochemical processes makes it an absolute requirement for almost all forms of life. Iron plays a central role in the metabolism of prokaryotes and eukaryotes catalysing reactions involved in O_2 metabolism, peroxide and superoxide metabolism, electron transport, photosynthesis, N_2 fixation and nucleic acid synthesis (Griffiths, 1987; Weinberg, 1989). Certain lactobacilli, however, have no requirement for the element and have evolved using other metals to fulfil some of the catalytic functions mediated by iron in other organisms (Archibald, 1983), examples being a Co(II)-cofactored ribonucleotide reductase (Blakley *et al.*, 1965), a mangano-catalase (Johnston and Delwiche, 1965) and a Mn-cofactored, DNA-dependent RNA polymerase (Stetter and Zillig, 1974).

The diversity of metabolic functions requiring iron emphasises the versatility of the metal as a biological catalyst. This versatility is, in part, due to the ability of iron to adopt several electronic configurations. Iron possesses unfilled *d* orbitals and is able to undergo a reversible change in oxidation state, $Fe^{2+} \leftrightarrow Fe^{3+}$. Thus, iron is able to co-ordinate electron donors and participate in redox reactions (Griffiths, 1987). The redox properties of iron depend entirely on the co-ordinating ligand (Table 1). In a macromolecular environment, due to the rigidity of the structures such as porphyrin groups which bear the co-ordinating groups, the interaction between iron and its ligands tends to be restricted with respect to bond length and bond angle. This rigidity leads to the stabilisation of one of the oxidation states of the metal. The extent of this stability is dependent upon the electronegativity of the ligand groups involved and the manner in which they affect the distribution of electrons within the 5*d* orbital shell. Thus, the geometry of the iron-binding centre determines the redox potential of the complex (Griffiths, 1987). This definition of the redox potential of iron complexes by the ligand-metal interaction thus specifies the catalytic properties of the complex and

1

Table 1. **Potentials for iron reduction with various ligands.**
Water, cyanide, *o*-phenanthroline as $\mathcal{E}^0$ (Spiro and Saltman, 1974),
ferrichrome A, ferrioxamine B and enterochelin as $\mathcal{E}_h$
(Cooper *et al.*, 1978).

Half reaction	$\mathcal{E}$
$Fe(H_2O)_6^{3+} + e^- \rightarrow Fe(H_2O)_6^{2+}$	+0.77 V
$Fe(CN)_6^{3-} + e^- \rightarrow Fe(CN)_6^{4-}$	+0.36 V
$Fe(Phen)_3^{3+} + e^- \rightarrow Fe(Phen)_3^{2+}$	+1.12 V
Fe^{3+}Ferrichrome A $+ e^- \rightarrow Fe^{2+}$Ferrichrome A	-0.446 V
Fe^{3+}Ferrioxamine B $+ e^- \rightarrow Fe^{3+}$Ferrioxamine B	-0.454 V
Fe^{3+}Enterochelin $+ e^- \rightarrow Fe^{3+}$Enterochelin	-0.986 V

explains the wide variety of reactions catalysed by iron.

The catalytic properties of iron, however, can have unfavourable consequences in biological systems. Unbound iron can catalyse the formation of highly reactive hydroxyl radicals from H_2O_2 (Halliwell, 1982). These free radicals react at very high rates with most of the organic molecules found in cells. Important consequences of hydroxyl radical formation are lipid peroxidation, which has very serious effects on membrane integrity (Abok *et al.*, 1983; Blake *et al.*, 1981), and DNA scission (Weinberg, 1989). The vast majority of iron in biological systems is tightly bound to proteins and thus the availability of free iron for such radical formation is very limited.

B.   The bioavailability of iron.

Iron is one of the most abundant metals in the earth's crust contributing 4 to 5% of the total mineral component of soils (Crowley *et al.*, 1987). The tendency of iron to oxidise, hydrolyse and polymerise forming insoluble and colloidal ferric hydroxide and oxyhydroxide polymers under physiological conditions makes the metal generally unavailable to biological systems despite its abundance. Soluble ferrous iron is quickly oxidised in the extracellular environment of most aerobic cells (Neilands *et al.*, 1987). The solubility product for Fe(III) hydroxide is estimated to be around 10^{-38} at pH 7.0. Therefore, the free iron concentration from the dissociation of this compound can be calculated thus,

$$K_{sol} = [Fe^{3+}] \cdot [OH^-]^3$$

$$[Fe^{3+}] = 10^{-38}/[OH^-]^3$$

$$[Fe^{3+}] = 10^{-38}/(10^{-7})^3$$

$$[Fe^{3+}] = 10^{-17}M.$$

As a consequence of this extreme insolubility aerobic organisms require very efficient uptake mechanisms to accumulate the metal. The insoluble nature of iron and its potential toxicity necessitates that in biological systems the metal is bound to ligands, as in the haemo- and haemi-proteins, the transport protein transferrin and the related lactoferrin and ovotransferrin, or is sequestered using the iron storage proteins, ferritin and the bacterioferritins (Griffiths, 1987). Indeed the concentration of free iron in human body fluids is very low at around $10^{-18}M$, a concentration which is regarded as being too low to support bacterial growth (Neilands *et al.*, 1987). Whether bacteria are in the body of a host organism or in the environment generally, they therefore require highly efficient methods for the acquisition of this important metal either from the solubilisation of ferric hydroxide or by removal of iron from host complexes.

Bacteria are generally considered as possessing two forms of iron uptake system utilising both low and high affinity uptake mechanisms. The low affinity mechanism can cope with the iron demand of the bacterium, despite the insolubility of ferric iron, if it is plentiful. Little is known about the mechanism of low affinity systems. Pollack *et al.* (1970) demonstrated that mutants of *Salmonella typhimurium*, lacking components of their high affinity iron uptake system, could grow well on a glucose-mineral salts medium. The addition of citrate, which can chelate iron, inhibited the growth of these mutants suggesting that the low affinity uptake system of *S. typhimurium* cannot function adequately in the presence of chelating agents. Under iron-limiting conditions bacteria, though, must rely upon high-affinity uptake systems. The mechanisms employed in high affinity iron uptake systems are discussed in section D.

C. The relationship between iron and bacterial pathogenicity.

The genus *Mycobacterium* incorporates both saprophytic and pathogenic organisms. Amongst the pathogens are *Mycobacterium tuberculosis* and *M. leprae*, the aetiological agents of tuberculosis and leprosy, respectively. Each year around 10 million new cases of tuberculosis are diagnosed and 3 million of these infections prove fatal (Kaufmann and van Embden, 1993). Grange (1989) has estimated that there are

also about 11 million people suffering with leprosy, most of whom are eventually killed by secondary complications. The emergence of multi-drug resistance in mycobacteria and the increasing incidence of AIDS are both contributing towards an increase in the occurrence of mycobacterial infection throughout the world (Kaufmann and van Embden, 1993). If efforts to decrease the incidence of mycobacterial disease are to be successful it is important that the host-microbe interaction is fully understood.

Microbial pathogenicity is a multifactorial phenomenon. In order to initiate an infection, a pathogenic microorganism must first attach itself to the mucosal surfaces of the respiratory, alimentary or urinary tract or penetrate the skin via wounds or vector bites. The microbe must then penetrate the mucosal surface and multiply in the host tissue, resisting the specific and non-specific defence mechanisms of the host organism (Smith, 1984).

The availability of nutrients has a profound effect upon the virulence of bacteria. The growth of *Corynebacterium renale* and *Proteus mirabilis* is greatly stimulated by urea; hence, they tend to be implicated amongst the aetiological agents of pyelonephritis (Smith, 1984). Similarly, brucellosis is a much more severe infection in gestating cattle, sheep, pigs and goats than in other mammals due to the presence of erythritol, a growth stimulant for *Brucella* spp., in the foetal tissues of these animals (Smith, 1984). The corollary is that the restricted availability of a nutrient should limit the extent of an infection. Hatch (1975) demonstrated that competition for isoleucine with the host was the limiting factor in the growth of *Chlamydia psittaci* within mouse fibroblast cells.

Many body fluids contain the iron-binding glycoproteins, transferrin and lactoferrin. Transferrin is present in the blood and lymph while lactoferrin is present in external secretions such as milk and mucus. These proteins bind two ferric ions with the incorporation of two bicarbonate ions per molecule (van Snick *et al.*, 1974). Both proteins have a high association constant for iron (around 10^{36}) and are normally, and importantly, only partly saturated with iron (Bullen *et al.*, 1974).

As a response to inflammation, the low free iron content of body fluids is maintained by the release of desferri- or apo-lactoferrin from polymorphonuclear leucocytes. The resulting holo- or ferric-lactoferrin is taken up by macrophages and the

iron is ultimately deposited in ferritin complexes in the reticulo-endothelial system and the liver (van Snick *et al.*, 1974; Brock *et al.*, 1984; LaForce and Boose, 1987).

The evidence that pathogenic bacteria encounter iron-limiting conditions within a host organism is two-fold. Firstly, increased virulence has been demonstrated on the administration of iron compounds to experimental infection systems and secondly, components of iron-starvation responses expressed by the bacteria *in vitro* have been recognised *in vivo* .

Miles *et al.* (1979) measured the effect of iron on the infectivity of 120 bacterial species (representing 17 genera) in the skin of guinea pigs. Iron was administered either systemically, by intravenous injection, or locally by injection of small amounts of iron into the skin. Iron, administered systemically, enhanced the infectivity of 23% of strains while locally administered iron enhanced 49% of the strains. The degree of this enhancement was measurable but small (2- to 8-fold) for some of the strains including near saprophytes such as *Mycobacterium smegmatis, M. phlei, Bacillus cereus* and *Clostridium bifermentans.* Substantial (14- to 50-fold) enhancement was observed with the recognised animal pathogens including *M. tuberculosis, M. bovis* BCG, the gas-gangrene clostridia, *Corynebacterium ovis* and *C. murium*, the group C streptococci and several Gram-negative pathogens. The only natural guinea pig pathogens used in the study were the group C streptococci.

The smaller enhancement of infectivity observed with the "non-pathogenic" strains is indicative of the involvement of other host factors in the inhibition of their growth. The recognised pathogens resumed vigorous growth on the satiation of their iron demand due to their ability to evade the other defences of the host organism.

Further evidence that pathogenic bacteria encounter iron-limitation as a consequence of their environment was gained by the investigation of their *in vivo* physiology. The work of Griffiths and his co-workers has demonstrated that the components of high affinity iron uptake systems of *Escherichia coli* that are expressed as a specific response to iron-starvation *in vitro* are also expressed *in vivo*.

Griffiths and Humphreys (1980) demonstrated the presence of a low molecular weight ferric iron-solubilising compound, or siderophore (discussed in Section E),

enterochelin, in the peritoneal washings of lethally infected guinea pigs. Analysis of the outer membrane protein population of similarly cultured *E. coli* by SDS-polyacrylamide gel electrophoresis showed that proteins expressed specifically as a result of iron-limitation *in vitro*, including siderophore receptors, were also expressed *in vivo* (Griffiths *et al.*, 1983). The immunogenicity of these iron-regulated, outer membrane proteins (IROMPs) was demonstrated by Griffiths *et al.* (1985) who observed that serum taken from some healthy adult humans contained IgG antibodies which reacted strongly with these IROMPs.

Work by Brown and colleagues at Aston University, UK, established that *Pseudomonas aeruginosa* also encounters iron-restriction in the lungs of cystic fibrosis patients. Brown *et al.* (1984) observed the *in vivo* expression of IROMPs in the bacterium when isolated from the sputum samples of cystic fibrosis sufferers. This constituted the first biochemical evidence for the iron-limited status of bacteria in a natural infection system. Anwar *et al.* (1984) found these proteins to be immunogenic. Ward *et al.* (1988) also found IROMP-reactive immunoglobulins in the serum of patients who had suffered *Pseudomonas aeruginosa* infection of burn wounds. Haas *et al.* (1991) demonstrated the presence of the pseudomonad siderophore pyoverdine in the sputa of cystic fibrosis sufferers. The siderophore was 50 % saturated with ferric iron indicating its ability to mobilise iron in the cystic fibrosis lung.

Shand *et al.* (1985) demonstrated the iron-limiting nature of the urinary tract by analysis of outer membrane protein profiles of isolates from urinary tract infection and their comparison with profiles from the isolates after sub-culture and cultivation *in vitro* under iron-limited and iron-replete conditions.

These studies have shown that iron-limitation is a recurrent problem encountered by potential bacterial pathogens. To successfully initiate an infection bacteria require high affinity iron uptake systems through which they can remove the metal from the iron-protein complexes of the host organism. Several mechanisms have evolved which allow bacteria to acquire iron in these environments.

D. Concepts and mechanisms for bacterial high affinity iron transport systems.

Several strategies could be employed by bacteria to solve the problem of the poor bioavailability of iron. In all possible scenarios, the solubility of iron must be increased. This may be achieved either directly, by the chemical modification of the extracellular environment, or indirectly, by the formation of metal chelates. The synthesis of an iron-chelating agent may be circumvented by the acquisition and the possible disassembly of the iron complexes formed by a host organism. Iron solubilised by any of the strategies outlined below could then be presented at the cell surface for transport into the cell.

1. The chemical modification of the extracellular environment.

The solubility of iron can be increased by its reduction to the ferrous ion and/or by a decrease in pH. The release of acids and reductants by bacteria, particularly in a microaerophilic or anaerobic environment, would therefore cause an increase in the concentration of solubilised iron (viz. at pH 2, now $[Fe^{3+}]$ = 10 mM. See page 3). The prevalence of reducing conditions would also have an effect upon the ferric iron complexes of a host organism. Ferrous iron, formed by the reduction of complexed ferric iron, would exhibit a lower affinity for the complexing ligands. This decrease in affinity may be enough to cause the release of soluble ferrous ions which could then be transported into the cell.

Listeria monocytogenes secretes a soluble reductant *in vitro* which can facilitate the release of iron from transferrin. This reductant causes the conversion of ferric to ferrous ions for which the protein has a decreased affinity (Cowart and Foster, 1985). The growth of *Clostridium perfringens in vivo* leads to a progressive decrease in the E_h of the surrounding tissue with the E_h value finally reaching -400 mV at pH 6.5. These highly reducing conditions and the acidic pH facilitate the release of iron from transferrin and promote the growth of the pathogen (Rogers *et al.*, 1970).

8

In both of these situations, ferrous ions would be stabilised in solution and would then presumably be available for uptake by the bacterium. An uptake system for ferrous iron has been demonstrated in *E. coli* K12 and mutants devoid of this system were also isolated (Hantke, 1987). Although this bacterium does not promote the formation of reducing conditions in its environment, Fe^{2+} may be an important iron source *in situ* in the gut, the major habitat of *E. coli*.

2.   The formation of iron chelates.

Iron can be solubilised from its polymeric or colloidal forms via the action of chelating agents. The production and secretion of iron-specific chelating agents and the subsequent uptake of their ferric complexes could also be the basis of a bacterial iron acquisition mechanism. It is also possible that such a molecule with a very high affinity for iron could remove it from host complexes and mediate its transfer to pathogenic microorganisms.

The most studied bacterial iron acquisition systems are those which transport the metal into the cell via the agency of siderophores. These are low molecular weight, iron chelating agents which exhibit a high affinity for ferric ions. These molecules and their uptake mechanisms will be discussed in detail later (Section E.).

Hydroxy acids, such as citrate and malate, also form complexes with polymeric iron. Citrate, when present in a large excess over iron (molar concentration ratio 20:1), forms the ferric dicitrate anion $[Fe(cit)_2]^{5-}$ (Spiro *et al.*, 1967). Frost and Rosenberg (1973) characterised an inducible, citrate-dependent iron transport system in *Escherichia coli*. This system could operate adequately in mutant strains of the bacterium that lacked functional siderophore-dependent systems. Both systems could operate simultaneously in the wild type organism.

Wagegg and Braun (1981) demonstrated that an outer membrane protein, designated FecA, enhanced the binding of radioactive iron to the outer membranes of *E. coli* in the presence of citrate. The extent of this binding was dependent upon the concentration of FecA in the membranes. Zimmermann *et al.* (1984) demonstrated that

mutant strains unable to transport iron via citrate due to a mutation in *fec*B retained the ability to induce the synthesis of FecA suggesting that induction is dependent upon the presence but not the uptake of ferric-citrate. No evidence has been found to suggest that co-transport of the acid is necessary for the uptake of iron by this route. The mechanism of ferric-citrate transport is similar to those for ferri-siderophores in *E. coli* and is further discussed in section E.

3.   The acquisition of host iron complexes.

Rather than form their own chelating agents bacteria could target iron that had already been complexed by a host organism. Iron complexes such as haem or transferrin could be procured by the bacterium and then processed to release their iron for bacterial metabolism.

a.   Transferrin and lactoferrin as iron sources.

Another method of iron acquisition utilised by bacteria involves the direct binding of transferrin and/or lactoferrin to distinct, specific receptor proteins at the cell surface which are expressed *in vitro* as a response to iron-limitation. This method of iron acquisition has been observed in *Neisseria meningitidis* (Tsai *et al.*, 1988), *N. gonorrhoeae* (McKenna *et al.*, 1988), *Bordetella pertussis* (Redhead and Hill, 1991), *Haemophilus influenzae* (Morton and Williams, 1989, 1990), *Pasteurella haemolytica* (Ogunnariwo and Schryvers, 1990), *Actinobacillus pleuropneumoniae* (Gerlach *et al.*, 1992) and various staphylococci (Modun *et al.*, 1994).

Alcantara *et al.* (1993) found that the human transferrin receptors from *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae* bind to the glycosylated C-lobe of the protein but the method by which the iron is taken up by the bacterium is still poorly understood. A ferric iron-binding protein, Fbp, expressed in pathogenic *Neisseria* spp. binds one ferric ion per molecule, is localised to the periplasm and possesses properties that suggest a role as a periplasmic transport protein, presumably mediating the

transport of iron from the transferrin/receptor complex to a cytoplasmic permease (Chen *et al.*, 1993). A periplasmic ferric-iron-binding protein has also been reported in *Serratia marcescens* (Angerer *et al.*, 1992). *Haemophilus influenzae* possesses two iron-repressed periplasmic proteins, one of which reacted with mono-specific antisera raised against the Fbp from *Neisseria* (Harkness *et al.*, 1992). This may suggest that a common mechanism is possessed by these distantly related bacteria for the acquisition of iron from transferrin.

These methods of iron uptake are siderophore-independent. Many of the bacteria mentioned above do not synthesise these compounds. *B. pertussis*, however, does produce a siderophore but iron uptake via this route is less efficient than that via the direct binding of transferrin (Redhead and Hill, 1991).

A theme seen throughout these investigations is that the pathogen is often limited to the use of the transferrin/lactoferrin of its host organism by the specificity of its receptor. It seems that these bacteria have limited their host range in order to achieve successful growth in a single host species by the efficient acquisition of transferrin or lactoferrin bound iron.

b.   Haemin as an iron source.

Haemin and haemin-protein complexes can act as a source of iron for pathogenic organisms such as *Yersinia enterocolitica* (Stojiljkovic and Hantke, 1992), *Neisseria gonorrhoeae* (Dyer *et al.*, 1987), *Plesiomonas shigelloides* (Daskaleros *et al.*, 1991), *Vibrio cholerae* (Stoebner and Payne, 1988), *Bacteroides fragilis* (Otto *et al.*, 1990) and *Streptococcus pneumoniae* (Tai *et al.*, 1993).

Haemophilus influenzae lacks a complete porphyrin synthesis pathway (White and Granick, 1963) but can grow using haemin as a joint iron and porphyrin source (Pidcock *et al.*, 1988) which suggests that haemin is transported as an intact molecule. Further evidence for the co-transport of the porphyrin moiety was gained from the transfer of the DNA sequences from *Plesiomonas shigelloides* necessary for growth with haemin as a sole iron source into mutants of *E. coli* defective in porphyrin

11

biosynthesis. The resulting recombinant strains gained the ability to grow using haemin and haemoglobin as sole iron sources (Daskaleros *et al.*, 1991).

Using affinity chromatography techniques, Lee (1992) demonstrated the presence of a haemin-binding protein in the outer membrane of *H. influenzae* and a haemoglobin-binding protein in the outer membrane of *N. meningitidis* (Lee and Hill, 1992). Protoporphyrin IX , ferric nitrate and holo-transferrin failed to inhibit the binding of haemin and haemoglobin by their respective receptors.

Free haemin in the blood stream is quickly bound by the carrier proteins haemopexin and albumin. Dyer *et al.* (1987) found that *N. meningitidis* and *N. gonorrhoeae* could not utilise bound haemin. As already mentioned, *H. influenzae* requires haemin not only as an iron source but also as a porphyrin source and has overcome the problems presented by this sequestration of haemin by producing receptors which bind the haemin-haemopexin complex. Two receptors have been characterised: Wong *et al.* (1994) discovered a receptor consisting of three proteins of 29, 38 and 57 kDa (or possibly 3 receptors) which were expressed under iron-limiting conditions only and Hanson *et al.* (1992) reported a 100 kDa receptor which was not necessary for virulence, was not regulated by iron availability but was induced by anaerobiosis. Some strains of this bacterium appear to secrete this 100 kDa protein in a soluble form to the medium (Cope *et al.*, 1994). The significance of this phenomenon, however, is not yet known.

The use of haemoglobin as an iron source can be inhibited by the binding of haptoglobin to the protein. *Vibrio vulnificus*, however, is able to acquire iron from the haemoglobin-haptoglobin complex (Zakaria *et al.*, 1988) while *Campylobacter jejuni* is able to use this iron source as well as haem-haemopexin (Pickett *et al.*, 1992).

The outer membrane receptors of many haemin / haemoglobin utilising Gram-negative bacteria have now been characterised but less information concerning the later stages of their transport mechanisms is available. Stojiljkovic and Hantke (1992), however, not only identified an iron-regulated haemin receptor, HemR, in the outer membrane of *Y. enterocolitica* but have also cloned and characterised a haemin-specific periplasmic binding-protein-dependent transport system located in the cytoplasmic

membrane of this organism (Stojiljkovic and Hantke, 1994). This system consists of a hydrophobic component, HemU, which probably serves as a cytoplasmic permease, a hydrophilic component with ATP-binding motifs, HemV, and a periplasmic binding protein, HemT. The cloning of the system into *E. coli* revealed that a further gene, *hemS*, was necessary to protect the bacterium against haemin-induced toxicity. Mutants in *Y. enterocolitica hemS* could not be obtained suggesting an essential role for the gene product in the iron metabolism of *Yersinia*. It is likely that HemS carries an activity involved in the release of iron from the porphyrin moiety, probably an haemin oxygenase.

E.   Siderophore-dependent iron uptake systems.

Siderophore-dependent iron uptake systems are widespread among the bacteria and fungi and are the most extensively studied of the microbial high affinity uptake mechanisms. Siderophores are defined as low molecular weight (< 1000 Da), ferric specific ligands produced for the solubilisation and transport of iron in microbial species (Neilands, 1984). The synthesis of these compounds is derepressed when the organisms encounter iron-limiting conditions and they are secreted into the extracellular milieu where they eventually form ferri-siderophore complexes by binding iron from a variety of sources. Siderophores possess high association constants for ferric iron and are capable of solubilising polymeric ferric hydroxide (Neilands, 1984).

Tidmarsh and Rosenberg (1981) demonstrated the removal of ferric iron from transferrin by enterochelin, the catecholate siderophore of *Salmonella paratyphi* B. Even though the transferrin was sequestered in a dialysis bag it was still accessible as an iron source to cultures of the bacterium able to synthesise enterochelin but not to those unable to produce the siderophore.

Ferri-siderophores are transported into the cell in an energy dependent manner via cognate receptor proteins at the bacterial outer surface (see Section E.3) and cytoplasmic membrane transport proteins. The iron is finally removed from the

13

OUTSIDE   INSIDE

microbial
cell

Fe (III)
siderophore

receptor
protein

Source of iron

(insoluble ferric
hydroxide or
transferrin,
ferritin etc.)

solubilization

uptake

release
mechanism
(reduction)

secretion

desferri
siderophore

Fe II → to
porphyrins,
apoproteins
etc.

Synthesis
(triggered by
deficiency of iron)
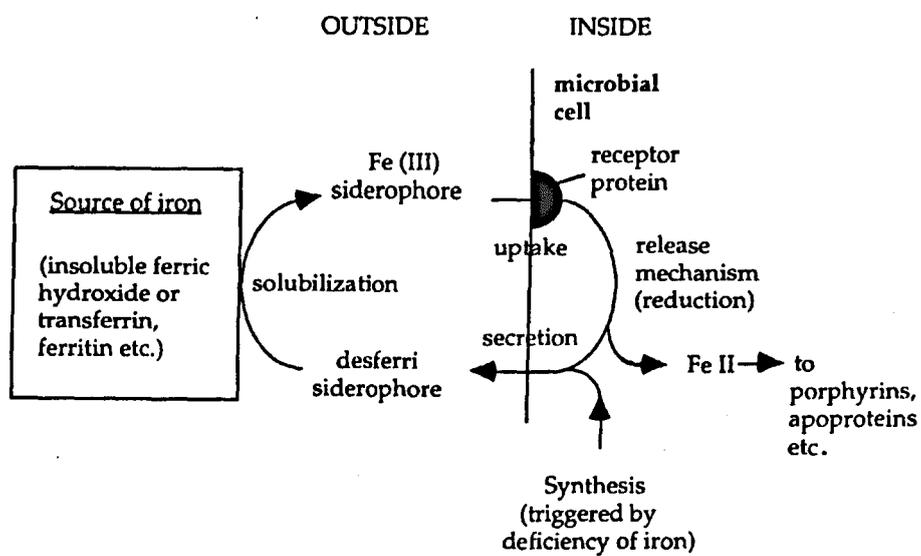
Figure 1. **A generalised scheme for siderophore-mediated iron acquisition in bacteria.**

siderophore in the cytoplasm by reduction to the ferrous form for which the ligand groups have a much decreased affinity (see Section E.4). The iron is then either stored or inserted into co-ordinating groups to fulfil a catalytic function. This general scheme for bacterial siderophore-mediated iron uptake is summarised in Fig. 1.

1.   The structure and classification of siderophores.

Siderophores are generally classified according to their complement of ligand groups. These ligand groups are commonly hydroxamic acid or catecholate moieties, however, some siderophores contain ligand groups with relatively inferior chelating properties which are supported in the iron binding centre by hydroxamates or catecholates. These are classed as mixed-ligand siderophores. The iron-binding centres of most siderophores are composed of three sets of bidentate ligands suitably arranged around a central ferric ion to yield a six co-ordinate system with octahedral geometry. Some siderophores do not contain three bidentate ligand groups and act as dimers or trimers, examples being rhodotorulic acid and 2,3-dihydroxybenzoic acid. These siderophores are usually relatively inferior chelating agents (Persmark *et al.*, 1992). Table 2 details the ligand groups found in siderophores, their structure and examples of siderophores which contain these groups. Fig. 2 shows the structures of a selection of ·
siderophores from each class.

2.   The use of exogenous siderophores.

The ability to utilise the ferric complexes of siderophores produced by other microorganisms is not uncommon among bacteria. Specific surface receptor proteins are synthesised by the non-producing organism in order to transport these siderophores. *E. coli*, for example, is able to acquire a range of ferric-hydroxamate siderophores produced by fungi. Ferrichrome, ferrichrysin, ferricrocin and albomycin all bind to the outer membrane receptor, FhuA, while FhuE acts as receptor for coprogen and rhodotorulic acid (Hantke, 1983; Braun *et al.*, 1987). The use of exogenous

Table 2.   Common ligand groups found in siderophore iron binding centres.

Ligand group	Structure	Occurrence in siderophores	Producing organisms	Reference
Catecholate		Enterochelin Parabactin Agrobactin Yersiniabactin	*E. coli* *Paracoccus* *Agrobacterium* *Yersinia*	O'Brien & Gibson, 1970 Bergeron, 1987 Bergeron, 1987 Haag *et al.*, 1993
Hydroxamate		Ferrichrome Coprogen Rhodotorulate Exochelin MS Aerobactin	*Ustilago* *Neurospora* *Rhodotorula* *Mycobacterium* *Aerobacter*	van der Helm, *et al.*, 1980 Wong *et al.*, 1983 Wong *et al.*, 1983 Sharman *et al.*, 1995. Gibson & Magrath, 1969
α-hydroxy acid		Aerobactin Pseudobactin Schizokinin Arthrobactin	*Aerobacter* *Pseudomonas* *Bacillus* *Arthrobacter*	Gibson & Magrath, 1969 Teintze *et al.*, 1981 Neilands, 1984 Neilands, 1984
2-(2-hydroxyphenyl)-oxazoline		Agrobactin Parabactin Mycobactin Vulnibactin	*Agrobacterium* *Paracoccus* *Mycobacterium* *Vibrio*	Bergeron, 1987 Bergeron, 1987 Snow, 1970 Okujo *et al.*, 1994
Fluorescent quinolinyl chromophore		Pseudobactin	*Pseudomonas*	Teintze *et al.*, 1981
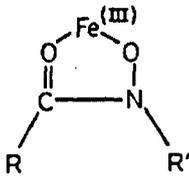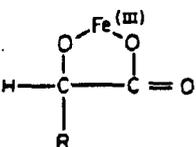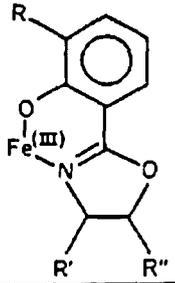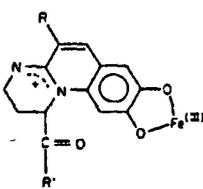
Catecholate siderophores
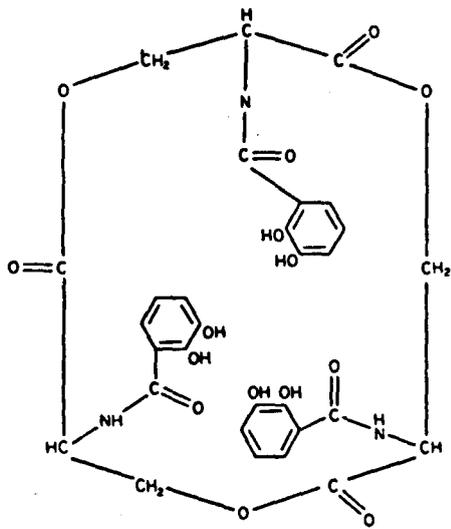
Enterochelin
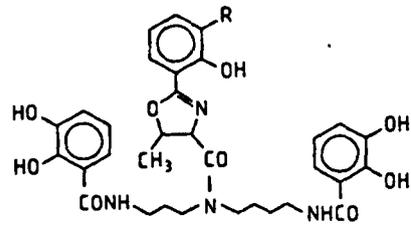
R = OH, Agrobactin
R = H, Parabactin

Hydroxamate siderophores
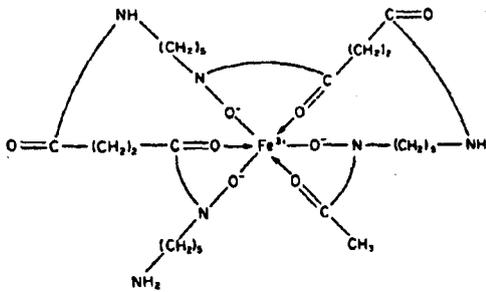
Ferrioxamine B
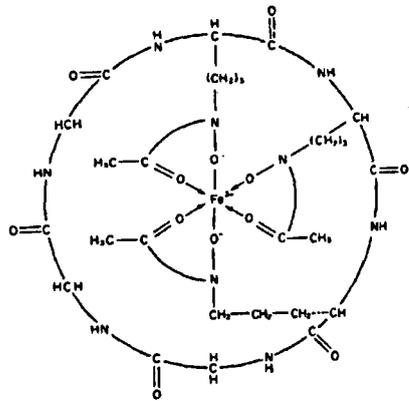
Ferrichrome

Figure 2. Structures of representative siderophores from the catecholate, hydroxamate, mixed ligand and non-hexadentate classes.

Mixed ligand siderophores
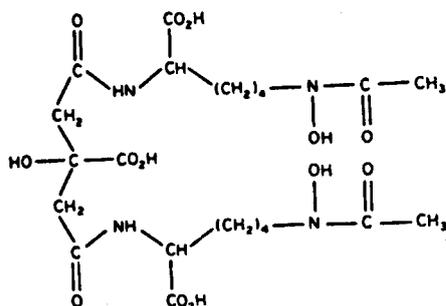
Aerobactin

Non-hexadentate siderophores
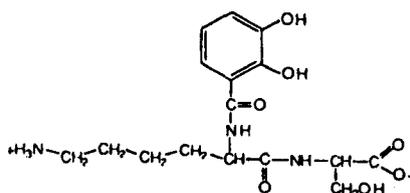
Chrysobactin
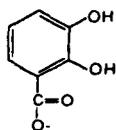
2,3-dihydroxybenzoic acid
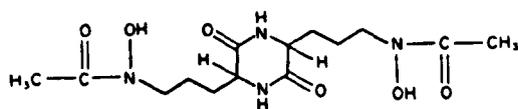
Rhodotorulic acid

siderophores has also been demonstrated in *Haemophilus* spp. (Williams *et al.*, 1990), *Campylobacter* spp. (Baig *et al.*, 1986), *Yersinia enterocolitica* (Brock and Ng, 1983; Robins-Browne and Prpic, 1985), *Staphylococcus aureus* (Brock and Ng, 1983) and *Pseudomonas* spp. (Meyer, 1992; Jurkevitch *et al.*, 1994). The porin, OprF, has been implicated in the outer membrane transport of some of the exogenous siderophores used by *Pseudomonas aeruginosa*, ferrioxamine B, ferrioxamine E, ferrichrysin, ferricrocin (Meyer, 1992). This porin has a large pore size capable of allowing the passage of molecules with molecular masses in the region of 3000 Da (Nikaido *et al.*, 1991). Other porins from Gram-negative bacteria have smaller pore diameters and are thus incapable of transporting siderophores. The efficiency of iron uptake via this porin route was significantly lower than in those involving specific outer membrane receptor proteins (Meyer, 1992).

The genetic investment made by these bacteria in order to benefit from this opportunistic siderophore "piracy" further highlights the importance of iron in bacterial metabolism.

3. Ferri-siderophore transport proteins.

Along with the increased synthesis of siderophores under iron-limiting conditions, there is also a simultaneous expression of several iron-regulated proteins in the envelope layers of bacteria. These proteins interact to facilitate the energy-dependent uptake of ferri-siderophores. For convenience and brevity, the siderophore uptake mechanisms of *E. coli* will be used to illustrate the main features of these systems.

a. The iron-regulated outer membrane receptor proteins.

Several outer membrane proteins are synthesised as a response to iron-limitation in *E. coli* (Table 3). Most of these proteins are also used as receptors by colicins and

bacteriophage and, because of this, resistance to particular colicins or phage has proven to be a convenient phenotype when selecting mutants lacking the receptor of interest.

FhuA and FhuE exhibit a broad specificity and are each able to facilitate the transport of a range of structurally related hydroxamate siderophores. Zhou *et al.* (1993) demonstrated that the ferric dicitrate receptor, FecA, binds the catecholate siderophore enterochelin. The receptor did not bind the hydroxamate siderophore, ferrichrome A, suggesting that the enterochelin binding is a specific event. The binding of enterochelin to FecA is enhanced in the presence of citrate and *in vivo* uptake studies with various *E. coli* phenotypes suggest that FecA has a role in ferri-enterochelin transport (Zhou and van der Helm, 1993).

It seems likely that the Cir and Fiu proteins are involved in the uptake of ferric chelates of simple catechols such as 2,3-dihydroxybenzoic acid, an intermediate in enterochelin synthesis, and 2,3-dihydroxybenzoylserine, a degradation product of the siderophore (Nikaido and Rosenberg, 1990). Hancock *et al.* (1977) demonstrated the uptake of ferric-2,3-dihydroxybenzoate complexes in *E. coli*. The use of similar ferric chelates as siderophores has been observed in several bacteria (Barghourthi *et al.*, 1989; Lopez-Goni *et al.*, 1992; Persmark *et al.*, 1992).

b.   TonB and the transport of ferri-siderophores into the periplasm.

The transport of solutes which can occur at very low extracellular concentrations requires a high affinity receptor system at the cell surface and an energy input in order to transport the substrate through the outer membrane, thereby concentrating it in the periplasm (Nikaido, 1993). Due to its inclusion of the relatively non-specific channels formed by porins, the outer membrane cannot be energised and, therefore, a means of energy transduction from the cytoplasmic membrane is required for the energy-dependent, ferri-siderophore uptake processes (Postle, 1990).

Using the competitive binding of phage T5 and ferrichrome to FhuA, Hantke and Braun (1978) demonstrated that mutants of *E. coli* lacking TonB function were

Table 3.   The iron-regulated outer membrane proteins of *Escherichia coli*.

Protein	Molecular size	Ligands	Reference
FepA	81 kDa	Enterochelin	Hollifield & Neilands, 1978
FhuA	78 kDa	Ferrichrome Ferrichrysin Ferricrocin Albomycin	Hantke & Braun, 1978 Braun *et al.*, 1987
FhuE	76 kDa	Coprogen Rhodotorulate	Hantke, 1983
IutA	74 kDa	Aerobactin	Grewal *et al.*, 1982
FoxB	66 kDa B_1 26 kDa B_2	Ferrioxamine B	Nelson *et al.*, 1992
Cir	74 kDa	Catechols	Nikaido & Rosenberg, 1990
Fiu	83 kDa	Catechols	Nikaido & Rosenberg, 1990
FecA	80 kDa	Ferric dicitrate Enterochelin	Waggeg & Braun, 1981 Zhou *et al.*, 1993

able to bind ferrichrome via the FhuA receptor but were unable to transport the siderophore into the periplasmic space. The same was true for unenergised $tonB^+$ cells (Hantke and Braun, 1978). Uptake systems for ferri-siderophores, ferric citrate and vitamin B_{12} (cobalamin) are all TonB-dependent in *E. coli* (Postle, 1990). The TonB protein is anchored in the cytoplasmic membrane via its N-terminal, signal-like domain (Karlsson *et al.*, 1993a) and appears to span the periplasm linking the outer and cytoplasmic membranes, interacting directly with TonB-dependent receptors (Schöffler and Braun, 1989). Evidence for a direct physical interaction between TonB and FepA was gained using formaldehyde as a cross-linking agent to form a stable, covalently linked TonB/FepA complex (Skare *et al.*, 1993). TonB contains a 33-residue peptide fragment which adopts a rigid structure which may be important in inter-membrane energy coupling (Brewer *et al.*, 1990; Evans *et al.*, 1992). It is suggested that TonB induces a conformational change in the receptor through a mechanical connection, which facilitates the transport of the siderophore (Schöffler and Braun, 1989; Skare *et al.*, 1993). Analysis of the predicted amino acid sequences of a number of TonB-dependent, outer membrane proteins and colicins revealed the presence of a conserved pentapeptide sequence, the TonB box, located close to their N-termini (Schramm *et al.*, 1987). Tuckman and Osburne (1992) demonstrated that a synthetic TonB box pentapeptide (sequence derived from FhuE) caused the *in vivo* inhibition of several TonB-dependent processes in *E. coli* : growth in low-iron media, bacteriophage φ80 infection and killing by colicins B and Ia. This conserved pentapeptide sequence may be the site of the TonB/receptor interaction, the inhibition being caused by competition between the receptors and the pentapeptide for TonB-binding (Tuckman and Osburne, 1992).

Bradbeer (1993) treated *btu*C and *atp* mutants of *E. coli*, which lacked inner membrane components for cobalamin transport and membrane-bound ATP synthase respectively, with cyanide and 2,4-dinitrophenol and demonstrated that the TonB-dependent transport of the vitamin across the outer membrane was driven by the proton motive force.

Two accessory proteins, ExbB and ExbD, are required for TonB function. ExbB acts as a chaperone-like protein and stabilises TonB during its synthesis in the cytoplasm and, along with ExbD, forms a stable, cytoplasmic membrane-bound complex with the N-terminal domain of TonB (Karlsson et al., 1993b)

c.   TonB-dependent outer membrane receptors as gated channels.

The porins and TonB-dependent receptor proteins of the outer membranes of Gram-negative bacteria share several structural features. Both classes possess similar charge distributions, have the same mean hydropathies and both are predicted to have a high β-sheet content (Nau and Konisky, 1989; Rutz et al., 1992). Porins are commonly constructed from three identical subunits each consisting of 16 β-strands forming an anti-parallel barrel. Inter-β-strand hydrophilic loops extend into the centre of the barrel thereby restricting the effective internal diameter of the pore to around 10 Å (Weiss et al., 1991; Nikaido and Saier, 1992).

The construction of a topology model of the ferrioxamine receptor, FoxA, of *Yersinia enterocolitica* predicted a pore-like structure in which three large extracellular loops were suggested as potential binding domains for the siderophore (Bäumler and Hantke, 1992). Liposome swelling and black lipid membrane studies suggested that the receptor did not form an open pore. These loops may cover the channel from the outside forming a closed pore structure (Bäumler and Hantke, 1992) (Fig. 3). The deletion of a 140 amino acid loop from FepA of *E. coli*, that had been implicated in ligand-binding (Murphy et al., 1990), transformed this high-affinity receptor into a non-specific, TonB-independent channel, effectively a porin (Rutz et al., 1992). The observed properties of this modified FepA protein appear to confirm the predictions made in the *Y. enterocolitica* FoxA model.

The mechanism envisaged for TonB-dependent outer membrane translocation of enterochelin is one in which an interaction with TonB is facilitated as a result of the binding of the siderophore by the receptor. The TonB/receptor interaction would then initiate a conformational change in the receptor causing the release of the ligand into the
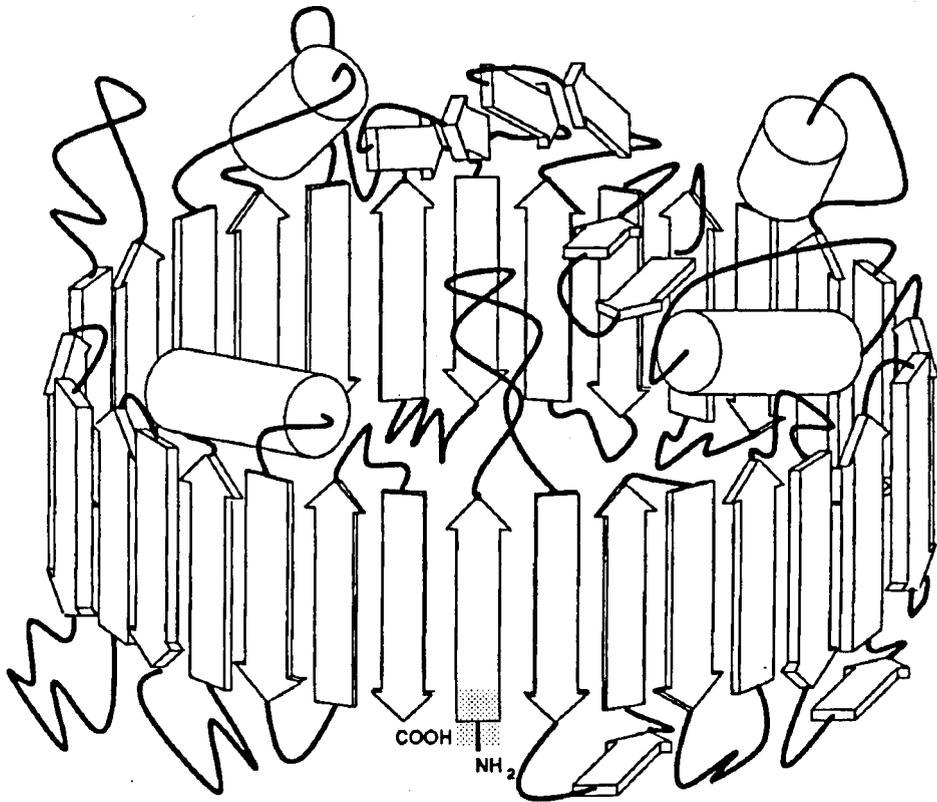
Figure 3. The proposed topology model of the outer membrane ferrioxamine receptor, FoxA, of *Y. enterocolitica*. Putative β-sheets are presented as arrows and α-helices as barrels. The proposed structure is a three dimensional barrel, forming an outer membrane pore, blocked by extracellular loops which are also the probable site of siderophore-binding. The hatched area is the region which corresponds to the TonB box consensus pentapeptide. Adapted from Bäumler and Hantke (1992).

trans-membrane channel and ultimately into the periplasm (Rutz *et al.*, 1992). However, no *in vivo* conformational changes have yet been demonstrated in FepA or in any other outer membrane proteins (Klebba, 1993).

Significant homologies have been demonstrated between the TonB-dependent receptors of *E. coli* and ferri-siderophore receptors from *Yersinia* spp. (Bäumler and Hantke, 1992; Koebnik *et al.*, 1993) and *Pseudomonas putida* (Bitter *et al.*, 1991) suggesting that ferri-siderophore receptor topology and their outer membrane translocation mechanisms may be conserved throughout the Gram-negative bacteria.

d.   Cytoplasmic membrane transport of ferri-siderophores.

The transport of several nutrients, such as sugars and amino acids, across the cytoplasmic membrane of the Gram-negative bacteria is facilitated by periplasmic binding protein-dependent transport systems which are composed of a binding protein, a trans-membrane diffusion complex consisting of two hydrophobic domains, and two hydrophilic membrane associated domains which contain nucleotide-binding-folds (Nikaido and Saier, 1992).

Three *E. coli* periplasmic proteins have been implicated in iron transport: the FecB protein of the iron(III)-dicitrate transport system (Staudenmaier *et al.*, 1989), the FepB protein of the ferri-enterochelin transport system (Elkins and Earhart, 1989) and the FhuD protein of the ferric hydroxamate uptake system (Köster and Braun, 1990a).

The hydroxamate siderophores, ferrichrome, aerobactin and coprogen, differ structurally and, as a consequence of their relatively low extracellular concentration, require high affinity and thus specific receptors to effect their transport into the periplasm. The molecules are concentrated in the periplasmic space, however, where a binding-protein with a broader specificity and lower affinity for the ferri-hydroxamates is adequate and probably energetically favoured. The binding of the ferric complexes of ferrichrome, aerobactin and coprogen by FhuD has been demonstrated by Köster and Braun (1990a).

Each system also includes two hydrophobic domains located in the cytoplasmic membrane which form a diffusion channel (Köster and Braun, 1990b; Staudenmaier *et al.*, 1989; Shea and McIntosh, 1991; Chenault and Earhart, 1991) and a protein containing ATP-binding motifs which associates with this permease (Becker *et al.*, 1990; Staudenmaier *et al.*, 1989; Chenault and Earhart, 1991). The envisaged mechanism for the transport of nutrients via binding protein-dependent systems involves an initial binding to the periplasmic binding-protein which then presents the ligand to a specific cytoplasmic permease. Conformational changes caused by the interaction of the permease and the binding-protein allow the binding and hydrolysis of ATP by the associated ATPase. The transduction of the energy released from this hydrolysis facilitates the release of the ligand by the binding protein and its entry into the trans-membrane channel of the permease (Nikaido and Saier, 1992).

e.   Ferri-siderophore uptake in Gram-positive bacteria.

Binding protein-dependent uptake systems also occur in Gram-positive bacteria. These systems are similar in structure and organisation to the periplasmic binding protein-dependent systems of Gram-negative organisms. The membrane-bound, hydrophobic domains, which form the permeases, and their associated ATP-binding domains are similar in both classes of bacterium and indeed exhibit significant homology to the components of ATP-driven transport systems in higher eukaryotes (Nikaido and Saier, 1992).

The binding proteins also exhibit homology with their Gram-negative counterparts but, as the Gram-positive bacteria do not possess an outer membrane, the binding protein must be anchored to the cell to effectively concentrate nutrients at the cell surface (Nikaido and Saier, 1992).

The hydroxamate uptake systems of *Bacillus subtilis* have recently been investigated by Schneider and Hantke (1993). Binding proteins exhibiting higher specificity than those found in the periplasm of *E. coli* appear to be necessary,

presumably as a higher affinity for the ligand is required to achieve adequate uptake kinetics.

Two classes of mutant, *fhu*D and *fox*D, were isolated which were unable to transport ferrichrome and ferrioxamine respectively, and others, *fhu*BC, which were unable to transport either siderophore. The *fhu*D gene was cloned and sequenced, and analysis of the predicted amino acid sequence of the product revealed significant homology to the FepB, FecB and FhuD periplasmic binding-proteins of *Escherichia coli*. The N-terminal 23 amino acids of the protein possessed the characteristics of an N-terminal lipoprotein signal sequence. Evidence for post-translational modification within this sequence to form a glyceryl-cysteine-lipid residue, which could serve to anchor the protein in the membrane, was found (Schneider and Hantke, 1993). The other components of the ferri-hydroxamate uptake system of *B. subtilis* have yet to be defined.

The major similarities and differences between the hydroxamate uptake system of *Escherichia coli* and *Bacillus subtilis* are presented in Fig. 4.

4.   The release of iron from ferri-siderophores in the cytoplasm.

a.   Ferri-siderophore reductases.

Compared to the study of the uptake of ferri-siderophores to the cytoplasm, very little work has been carried out to investigate the intracellular release of iron from these molecules. Siderophores have a much lower affinity for ferrous iron and the reductive release of iron from the ferri-siderophores has been demonstrated with extracts from several organisms in the presence of flavins and suitable acceptor molecules for ferrous iron (Ratledge, 1971; Ernst and Winkelmann, 1977; Arceneaux and Byers, 1980; Gaines *et al.*, 1981; Lodge *et al.*, 1982).

Recently, Fontecave *et al.* (1994) have proposed that ferri-siderophore reductases are, in fact, flavin reductases. They suggest that free flavin moieties reduced by these enzymes are responsible for the reduction of ferri-siderophore-bound iron.
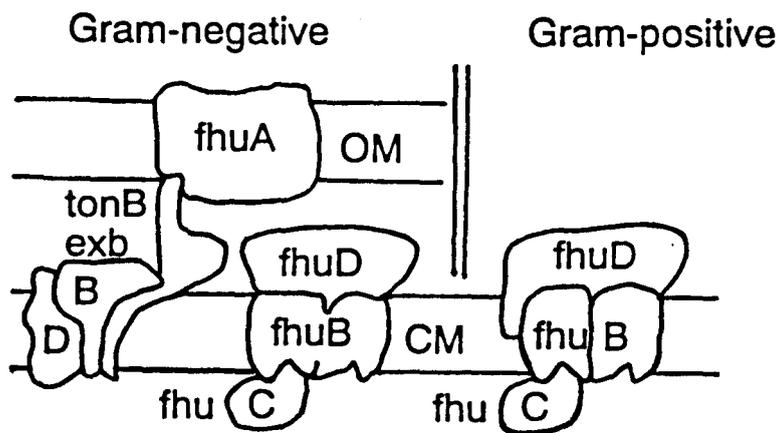
Figure 4. **Comparison of the hydroxamate uptake systems of Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria.** The proteins necessary for the uptake of ferrichrome are shown in both cases. As the Gram-positive organism has no outer membrane then only one transport system is necessary and, conseqently, homologues of the TonB and ExbBD proteins are not present. The binding protein, FhuD, of *B. subtilis* exhibits significant homology with its Gram-negative counterpart but is anchored in the cytoplasmic membrane. The FhuBC complex of *B. subtilis* has not yet been fully determined but is thought to be very similar to that of *E. coli*. **CM** cytoplasmic membrane, **OM** outer membrane, **Fhu** Ferric hydroxamate uptake. After Schneider and Hantke (1993)

This mechanism implies a lack of specificity in ferri-siderophore reduction. The reductive release of iron from ferri-enterobactin in the presence of NADH and flavin mononucleotide by extracts of *Bacillus subtilis* (Lodge *et al.*, 1980), a bacterium which cannot utilise this siderophore *in vivo*, and the reduction of ferri-mycobactin by extracts of *Candida utilis* and *E. coli* (McCready and Ratledge, 1979) are certainly consistent with this hypothesis.

The ferrous iron released from siderophores enters the low molecular mass iron pool of the cytoplasm from which it can be sequestered for storage, inserted into a functional group to serve a catalytic function and also serve to regulate gene expression. This "transit" pool of ferrous iron presumably exists as small soluble complexes in the cytoplasm (Fontecave and Pierre, 1991). This area of iron metabolism, however, remains poorly characterised.

b.   Post-reduction modification of siderophores.

The removal of iron from ferri-siderophores results in the presence of a potentially deleterious ligand in the cytoplasm. Hartmann and Braun (1980) found evidence for the acetylation of one of the *N*-hydroxyl groups of ferrichrome in the cytoplasm of *E. coli*. The acetylated ferrichrome was then secreted to the medium. This modified compound had a much diminished affinity for ferric iron and as such would be unlikely to interfere in the intracellular iron transport of the bacterium.

Escherichia coli possesses a ferric enterochelin esterase which is necessary for the growth of the bacterium using ferri-enterochelin as the sole iron source (Langman *et al.*, 1972). The iron of ferric enterochelin has a low E_h of -986 mV at neutral pH (Cooper *et al.*, 1978) but this is of little metabolic consequence and is erroneously interpreted as evidence for the need to hydrolyse the siderophore to its more readily reduced dihydroxybenzoylserine monomers in order to remove the iron. The reductive release of iron by bacterial extracts from ferri-MECAM, a synthetic analogue of enterochelin which is insensitive to the esterase, has been observed (Lodge *et al.*, 1980), however, suggesting that the hydrolysis of enterochelin is not necessary for the removal

of the iron. Mutants of *E. coli* lacking enterochelin esterase activity are unable to grow using this siderophore and this may reflect a need for the modification of enterochelin in the cytoplasm to maintain efficient growth.

5. The regulation of expression of siderophore-dependent iron uptake systems.

The biosynthesis of siderophores and their associated uptake proteins is coordinately regulated by the intracellular iron concentration (McIntosh and Earhart, 1977; Bagg and Neilands, 1987). Mutants of *E. coli*, designated *fur*, were isolated which constitutively expressed β-galactosidase fusions of the otherwise iron-regulated outer membrane proteins FhuA, FepA and Cir (Hantke, 1981). The *fur*, ferric uptake regulation, gene was cloned by complementation in *fur* mutants and an 18 kDa protein was identified as the gene product (Hantke, 1984). It is proposed that Fur acts as a classical transcriptional repressor protein, binding to iron-regulated promoters as a dimer in the presence of ferrous iron (Neilands *et al.*, 1987).

de Lorenzo *et al.* (1988) conducted DNAase I protection experiments to identify the Fur operator sequences in the aerobactin operon of the ColV-K30 plasmid. The operator sequences appear to be well conserved across several bacterial genera (Dale and Patki, 1990) and homologous Fur systems have now been identified in *Y. pestis, Y. enterocolitica* (Staggs and Perry, 1992) and *Neisseria gonorrhoeae* (Berish *et al.*, 1993).

F. The mycobacterial cell envelope.

As well as conferring shape and rigidity upon the cell, the envelope layers of a bacterium also control the transport of various molecular species both into and out of the cell. The chemical nature of its components and their physical organisation together define the effectiveness of the bacterial envelope as a permeability barrier.

The mycobacterial envelope is extremely important in terms of the pathogenicity of the mycobacteria, protecting against and circumventing the initiation of the oxidative bursts of macrophages (Pabst *et al.*, 1988). It is also generally believed to be responsible for the intrinsic resistance of the pathogenic mycobacteria to a wide range of antibiotics. The physiology of the mycobacteria is basically similar to that of most other aerobic bacteria and thus, although it is an over-simplification, they can be thought of as "*E. coli* wrapped in a fur coat" (Ratledge and Stanford, 1982). The structure of the mycobacterial envelope obviously affects the uptake of nutrients into the cell and must be seen as the context within which the interactions of the components of the iron stress response of the bacterium constitute the ferri-exochelin uptake process.

1.   Envelope ultrastructure.

The cytoplasmic membrane of the mycobacteria is a classic lipid bilayer in which phosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol are likely to be the main structural amphipathic polar lipids. These lipids are commonly found in the cytoplasmic membranes of many organisms (Minnikin, 1982). The membrane, which also incorporates the lipopolysaccharides lipomannam, lipoarabinomannam and the phosphatidylinositolmannosides, is asymmetric and there is evidence to suggest that these lipopolysaccharides may be present only in the outer leaflet of the bilayer (Brennan and Draper, 1994).

The cytoplasmic membrane is surrounded by a cell wall constructed chiefly of peptidoglycan arabinogalactan mycolate. The peptidoglycan, which composes the inner layer of the cell wall, closely resembles that of other bacteria (Brennan and Draper, 1994) and is linked at intervals to the arabinogalactan matrix via diglycerophosphoryl bridges (McNeil *et al.*, 1990). The outermost layer of the cell wall "skeleton" is comprised of mycolic acids which are 1-alkyl branched, 2-hydroxy fatty acids usually containing 50 to 70 carbon atoms, the branch commonly being about 24 carbon atoms in length (Brennan and Draper, 1994). These are covalently bonded to the underlying

arabinogalactan matrix via ester linkages to 5-hydroxyl functions of terminal- and 2-linked arabinofuranosyl residues (McNeil *et al.*, 1991). The flexibility of these bonds allows the mycolic acids to be arranged in a highly ordered structure. Nikaido *et al.* (1993) used X-ray diffraction to demonstrate that the hydrocarbon chains of the cell wall of *M. chelonei* exist in a tightly packed, parallel, quasi-crystalline array perpendicular to the plane of the cell wall (Fig. 5).

An outer layer of polar lipids was anticipated and the results of quantitative analyses of solvent extractable wall-associated lipids was consistent with the presence of a highly asymmetric lipid bilayer as the outer layer of the mycobacterial cell wall (Nikaido *et al.*, 1993). The wall-associated lipids included molecules with medium length (C_{24} to C_{36}) and short length (C_{14} to C_{18}) fatty acyl groups and it is proposed that those with medium length fatty acyl groups intercalate between the branches of the mycolic acids while those with shorter fatty acyl chains form the remainder of an irregular outer layer (Brennan and Draper, 1994)(Fig. 5). This asymmetric lipid bilayer model is further supported by observations of a second freeze fracture plane, other than that of the cytoplasmic membrane, in mycobacterial envelopes (Minnikin, 1982).

2. The permeability of the mycobacterial wall.

The ultrastructure of the cell envelope of the mycobacteria bears some similarities to that of the Gram-negative bacteria in that the outer layers of both comprise a highly asymmetric lipid bilayer. The outer leaflet of the Gram-negative outer membrane is composed exclusively of lipopolysaccharide (LPS) in which 6 or 7 fatty acyl chains are linked to a common head group. The head groups of neighbouring LPS molecules interact strongly with each other in the presence of inorganic (Na^+, K^+, Mg^{2+} and Ca^{2+}) and organic (polyamines) counterions (Nikaido and Vaara, 1985). The fatty acyl chains of LPS adopt a highly ordered conformation, being tightly packed in a dense hexagonal lattice (Labischinski *et al.*, 1985), and are unsaturated which further decreases the mobility of the chains and, consequently, the fluidity of the lipid interior.
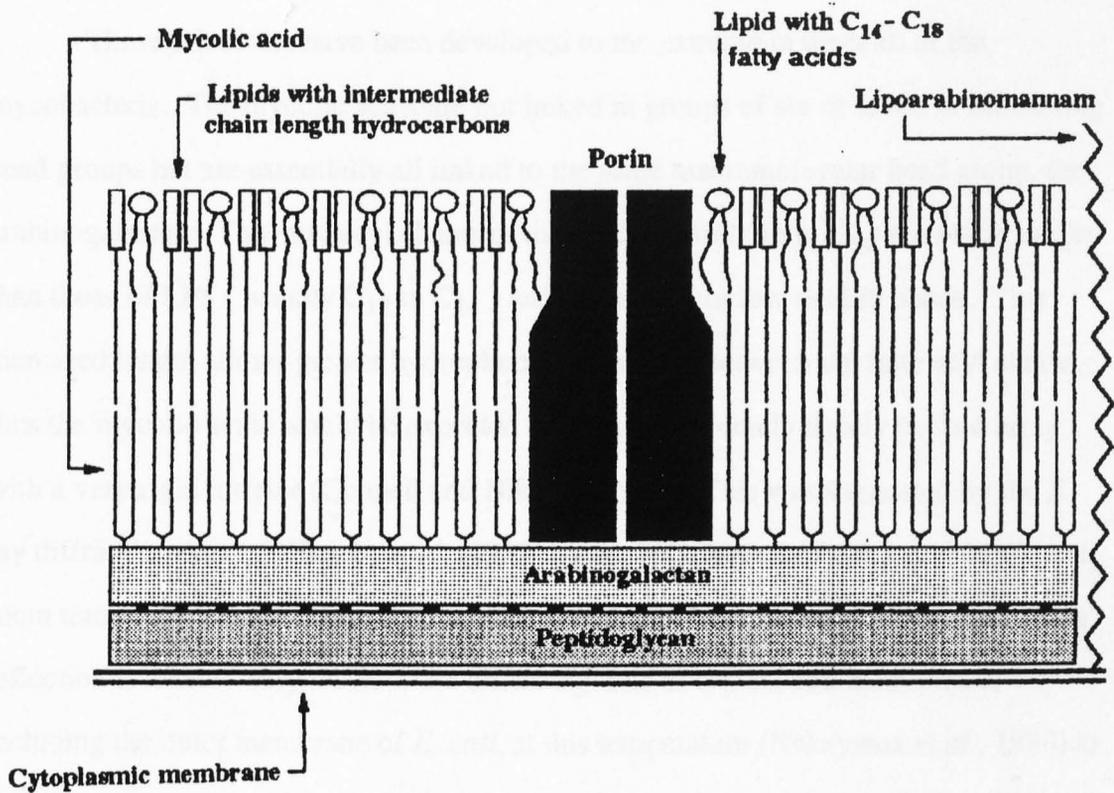
Figure 5. **A representation of the ultrastructure of the mycobacterial cell wall.**
The mycolic acids anchored to the underlying arabinogalactan-peptidoglycan matrix form a densely packed, quasi-crystalline array which appears to be the basis for a highly asymmetric lipid bilayer constituting a very effective permeability. Putative hydrophilic diffusion pathways for the entry of nutrients are formed by the mycobacterial porin and lipoarabinomannam. After Brennan and Draper (1994).

This decreased fluidity contributes to the low permeability of the outer membrane to hydrophobic molecules (Nikaido and Vaara, 1985). The outer membrane is estimated to be 50- to 100-fold less permeable to hydrophobic molecules than a typical glycerophospholipid bilayer (Connell and Nikaido, 1994).

These principles have been developed to the extreme in the wall of the mycobacteria. The mycolic acids are not linked in groups of six or seven to interacting head groups but are essentially all linked to the same macromolecular head group, the arabinogalactan. The fatty acyl chains of the mycolic acid (up to C_{50}) are much longer than those of LPS (usually C_{12} to C_{14}) and contain no or few double bonds. This increased length allows greater hydrophobic interaction between the fatty acyl chains, thus the mycolic acids would be expected to form an extremely tightly packed array with a very rigid interior (Connell and Nikaido, 1994). This was suggested by the X-ray diffraction data of Nikaido *et al.* (1993) by the prominence of a 4.2 Å reflection at room temperature indicating a high degree of order in the lipid array of the wall. This reflection is usually very weak in the diffractograms of typical cell membranes, including the outer membrane of *E. coli*, at this temperature (Nakayama *et al.*, 1980) as their lipids are mostly in a relatively disordered state.

Jarlier and Nikaido (1990) measured the permeability coefficients for a range of cephalosporins and small nutrient molecules in the wall of *M. chelonei*. Comparisons with those for *Pseudomonas aeruginosa* and *E. coli* confirmed the effectiveness of the mycobacterial envelope as a permeability barrier. The coefficients were at least 1 and 3 orders of magnitude lower than those for *P. aeruginosa* and *E. coli* respectively. The limited effect of temperature upon the diffusion rates of these compounds was considered to suggest a mainly hydrophilic route through the wall.

Trias *et al.* (1992) have demonstrated the presence of a porin in the cell wall of *M. chelonei*. A 59 kDa minor cell wall protein formed a water-filled, ion-permeable channel of estimated diameter 22 Å which may constitute the main pathway for the diffusion of hydrophilic molecules through the mycobacterial cell wall. The porin was cation-selective, voltage-gated (closing at applied potentials ≥ 40 mV) and exhibited a low specific activity despite its large pore diameter. The porin could potentially allow

the permeation of hydrophilic molecules with molecular masses of up to 2000-3000 Da (Trias and Benz, 1993). A similar porin has recently been reported in *M. smegmatis* (Trias and Benz, 1994) with an estimated channel diameter of 3 nm, cation selectivity and a closing potential in the range 20 to 30 mV.

The lipoarabinomannam of the cytoplasmic membrane spans the whole envelope structure (Wheeler, 1990). The polysaccharide chain of this lipopolysaccharide may interact with non-mycolated terminal arabinofuranosyl residues of the arabinogalactan to form another putative hydrophilic diffusion pathway (McNeil *et al.*, 1991).

G.   The acquisition of iron by the mycobacteria.

The metabolism of the mycobacteria, like that of all aerobic bacteria, is heavily dependent upon the catalytic properties of iron. When faced with iron-limiting conditions, mycobacteria elicit a coordinated response involving the synthesis of several iron-binding compounds and a series of envelope proteins. Although the iron uptake mechanisms of these bacteria have several features in common with the siderophore-dependent uptake systems of other bacteria, the genus exhibits interesting variations upon this theme.

Most mycobacteria produce at least three separate iron-binding compounds under iron limiting conditions, the mycobactins, exochelins and either salicylic acid or 6-methyl-salicylate. The functions of the mycobactins and exochelins have been resolved but the involvement of salicylic acid has yet to be fully defined.

1.   Mycobactin.

The mycobactins, which were discovered as a growth factor for *Mycobacterium paratuberculosis*, form an unusual family of bacterial siderophores. Unlike most other siderophores characterised to date, mycobactin does not chelate iron as a cell free entity in the extracellular milieu but is lipid-soluble and occupies an intracellular location.

Snow (1970) and colleagues isolated mycobactins from several mycobacteria and deduced their structures. The basic structure for the mycobactins is given in Fig. 6. The siderophore produced by each species of *Mycobacterium* varies slightly from this structure with respect to their side groups such that HPLC analysis of extracted mycobactins can be a useful taxonomic tool (Ratledge and Hall, 1984). The three dimensional structure of ferri-mycobactin P was determined using X-ray crystallography (Hough and Rogers, 1974).

The mycobactin of *M. smegmatis* has been localised by electron microscopy after labelling with vanadate (Ratledge *et al.*, 1982). The molecule was seen to occur close to, or even partially in, the cytoplasmic membrane of the cell. Mycobactin may contribute up to 10% of the dry weight of the cell in some species (Ratledge, 1987). Studies with liposomes, however, showed that membrane integrity was lost with the inclusion of only about 2% mycobactin (C. Ratledge and M.J. Moore, unpublished work). Thus, it is likely that mycobactin is located outside the cell membrane but is contained within the extremely lipophilic cell wall (Ratledge, 1987). The extent of mycobactin production by mycobacterial pathogens *in vivo* has not been quantified.

Some species of *Mycobacterium*, such as *M. paratuberculosis* and some strains of *M. vaccae*, appear to be devoid of mycobactin. *M. vaccae*, though, produces an exochelin (see below) and can grow adequately in iron-deficient media without a mycobactin supplement. *M. paratuberculosis*, however, requires an input of iron from either ferri-mycobactin, ferri-exochelin or ferric ammonium citrate for growth. On receiving this initial iron dose, the bacterium can grow and also produce its own exochelin. The ability to produce mycobactin, however, is not recovered. The repeated subculture of mycobactin-dependent strains of *M. avium* has resulted in these strains gaining the ability to produce their own mycobactin suggesting that their initial lack of the siderophore was due to the super-repression of the operon encoding its biosynthesis (Barclay and Ratledge, 1983). A similar, potent repression may also explain the lack of mycobactin biosynthesis in *M. paratuberculosis*. As some strains of *M. vaccae* can grow adequately in the absence of mycobactin it seems that the siderophore is not essential for iron uptake but plays a more subtle role in mycobacterial iron metabolism

Figure 6. The structure of mycobactin, the intracellular siderophore of the mycobacteria. Substituents: R_1, alkyl chain up to C_{19}; R_2, -H or -CH_3; R_3, -H or -CH_3; R_4, usually -CH_3 or -C_2H_5; R_5, -H or -CH_3. For *M. smegmatis*, $R_1 = C_{17}$, $R_2 = R_3 = R_5 =$ -H and $R_4 =$ -CH_3. After Ratledge (1987).

though evidently the role of exochelin in these strains may be adequate for iron acquisition.

A comparison of the closely related species, *M. vaccae* and *M. neoaurum*, carried out by Hall and Ratledge (1986) shed light upon the possible function of mycobactin. In an iron-limited, mixed culture, *M. vaccae* outgrew *M. neoaurum*, and this was suggested as being due to the increased energy expenditure required for mycobactin synthesis in the latter species. However, when iron was presented suddenly in mid-growth phase then *M. neoaurum* outgrew *M. vaccae*.

This observation could be explained by looking at how the iron is stored by the cell before it can be utilised. Porphyrin synthesis is repressed in the mycobacteria under conditions of iron starvation (McCready, 1978). This is not the case in other genera which tend to accumulate porphyrins and thus, on the sudden presentation of iron, the metal can rapidly be incorporated into the available porphyrins (Light and Clegg, 1974). The likely function of mycobactin then seems to be to act as a temporary store of iron. The sudden presentation of iron to iron-starved mycobacteria causes the rapid formation of ferri-mycobactin. This can be seen as the cells develop a light red colouration within two minutes of the presentation of the iron (McCready and Ratledge, 1979). Thus in such a situation, *M. neoaurum* by virtue of its iron storage potential in mycobactin, would have an advantage over *M. vaccae*.

The removal of iron from mycobactin by a reductive step catalysed by a ferri-mycobactin reductase activity utilising NADH or NADPH as a cofactor has been demonstrated (Ratledge, 1971; Brown and Ratledge, 1974). Salicylate acted as an acceptor of the ferrous ion from ferro-mycobactin *in vitro* and could possibly mediate its transfer to porphyrins *in vivo*.

Thus the following sequence can be envisaged for the transfer of iron from its primary storage location, mycobactin, into the haem moiety.

$$Fe(III)mycobactin + NADH \rightarrow Fe(II)mycobactin + NAD^+$$
$$Fe(II)mycobactin + salicylate \rightarrow mycobactin + Fe(II)salicylate$$
$$Fe(II)salicylate + porphyrin \rightarrow Fe(II)haem + salicylate$$

(McCready and Ratledge, 1979)

38
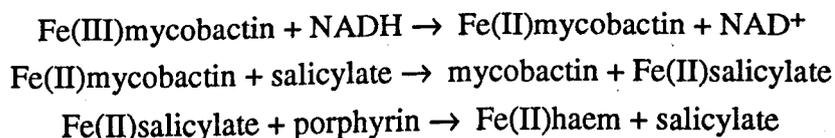
This scheme was never proven due to problems encountered in the assay of ferrochelatase activity which would presumably be responsible for the insertion of the metal into the porphyrin ring.

2.   Salicylate.

Analysis of culture filtrates from iron-starved mycobacteria show an accumulation of either salicylic acid or 6-methylsalicylic acid (6-MSA)(Ratledge and Winder, 1962). These compounds are aromatic precursors of their respective mycobactins in *M. smegmatis* and *M. phlei*. If group R_2 (Fig. 6) is an H atom then salicylate is present in the culture filtrate or if R_2 is a CH_3 group then 6-MSA accumulates (Ratledge, 1987). Analysis of a salicylate-requiring auxotrophic mutant of *M. smegmatis* revealed that it could not be satisfied by the addition of mycobactin (Ratledge and Hall, 1972). Assuming that the mycobactin could gain access to an active location in the cell, which is likely as it serves as a growth stimulant for *M. paratuberculosis*, then salicylate, as well as being a precursor of mycobactin, would appear to have a second role in mycobacterial physiology. The mycobactin-lacking species *M. vaccae*, however, does not accumulate salicylate or 6-MSA in culture filtrates (Messenger and Ratledge, 1986) suggesting that the second role of this substance is linked in some way with mycobactin.

It was originally thought that salicylate could act as a siderophore and, indeed, rapid uptake of ^{55}Fe from ferri-salicylate could be demonstrated (Ratledge and Marshall, 1972). However, it was subsequently discovered that in the presence of phosphate ions, salicylate was incapable of holding iron in solution and could then no longer act as a siderophore. As phosphate ions are constituents of laboratory media and host tissues, the hypothesis that salicylate was a siderophore had to be abandoned (Ratledge *et al.*, 1974). The work of McCready and Ratledge (1979)(Section G.1) suggests a role for salicylate in the removal of ferrous iron from ferro-mycobactin.

The anti-tubercular drug, *para*-aminosalicylic acid (PAS), was previously believed to act as an inhibitor of folic acid biosynthesis but Brown and Ratledge (1975)

39

showed that it interfered with the iron metabolism of the mycobacteria: yields of mycobactin were diminished in PAS-treated cells. As a structural analogue of salicylate, the drug may behave as an anti-metabolite interfering with either of the two putative functions of salicylate: as an inhibitor of mycobactin biosynthesis or interfering with the intracellular transport of iron. Such a proposal has not been followed up in these laboratories or elsewhere.

3.   The exochelins.

After the finding that salicylate could not function as a siderophore in the presence of phosphate, other possible siderophore molecules were sought. A water-soluble group of iron chelating agents capable of transporting iron into the mycobacteria was discovered and termed exochelins (Macham and Ratledge, 1975). Exochelins have now been discovered in all species of mycobacteria and fall into two major groups, both of which appear to be synthesized to different extents in many species. The group A exochelins are prevalent in culture filtrates of the fast growing saprophytic mycobacteria typified by *M. smegmatis, M. phlei, M. vaccae* and *M. neoaurum*. These siderophores are water-soluble in both the ferri- and desferri-forms and cannot be extracted into chloroform. The group B exochelins which are the prevalent exochelins of the slow-growing pathogenic mycobacteria such as *M. avium, M. tuberculosis, M. intracellulare, M. paratuberculosis, M. scrofulaceum* and *M. bovis* BCG, however, are water-soluble but can be extracted into chloroform after acidification of the aqueous phase, presumably as a result of the protonation of a carboxylic acid group. The ferri-form of these exochelins partition into the organic phase more readily than do the desferri-forms (C. Ratledge, pers.comm.).

Exochelins of both types are produced at elevated concentrations under iron deficient conditions. They solubilise iron in the presence of phosphate and reverse the bacteriostatic effect of serum, probably by removing iron from transferrin (Macham *et al.*, 1975).

a.   The structure of the exochelins.

The structure of exochelin MS, the group A exochelin of *M. smegmatis*, has recently been determined (Sharman *et al.*, 1995)(Fig. 7) by the analysis of desferri-, ferri- and the gallium complexes of exochelin using ^{1}H-n.m.r. Exochelin MS is a linear, formylated pentapeptide: N-(δN-formyl, δN hydroxy-R-ornithyl)–β-alaninyl-δN-hydroxy-R-ornithyl-R-allo-threoninyl-δN-hydroxy-S-ornithine. Although there are two peptide bonds in the structure, these involve the three R (=D) amino acids and thus the molecule contains no conventional peptide bonds and as a consequence is probably resistant to hydrolysis by peptidases (Sharman *et al.*, 1995). The coordination centre forms an octahedral structure with ferric iron and is hexadentate, the ligand groups being three hydroxamic acid groups. A gene which could possibly encode the enzyme responsible for the N-formylation of the terminal hydroxyornithine of exochelin MS has recently been isolated (Fiss *et al.*, 1994) by the complementation of an exochelin-lacking mutant of *M. smegmatis* with a cosmid library containing genomic DNA from the bacterium. The predicted product of this gene, *fxb*A, shares amino acid homology with *E. coli* and rat formyltransferases and contains a consensus sequence for folate binding. It is possible, therefore, that PAS may inhibit the iron metabolism of the mycobacteria at yet another point (see Section G.2), as a putative anti-metabolite in folic acid biosynthesis (the original hypothesis), if folate is necessary for exochelin biosynthesis. This possibility will soon be examined in this laboratory. The *M. smegmatis* exochelin-lacking mutant was also complemented by a cosmid containing a DNA fragment from *M. bovis* BCG Pasteur which suggests the involvement of *N*-formylation in the synthesis of exochelins of the slow-growing pathogenic mycobacteria.

The group A exochelin of *M. neoaurum*, exochelin MN, differs structurally from that of *M. smegmatis* and contains an unusual β-hydroxyhistidine residue. The structure of this molecule is now known but has not yet been presented for publication (C. Ratledge, pers. comm.).

NH₂

$$NH_2$$

CH-C=O

CH₂   NH

CH₂   CH₂

H   CH₂   CH₂

C=O   HO-N   O=C

N-OH   O=C   HO-N

CH₂   HC—CH—OH   CH₂

CH₂   NH   CH₂

CH₂   CH₃   CH₂

CH₂   C=O   CH-NH₂

CH   COOH

NH₂
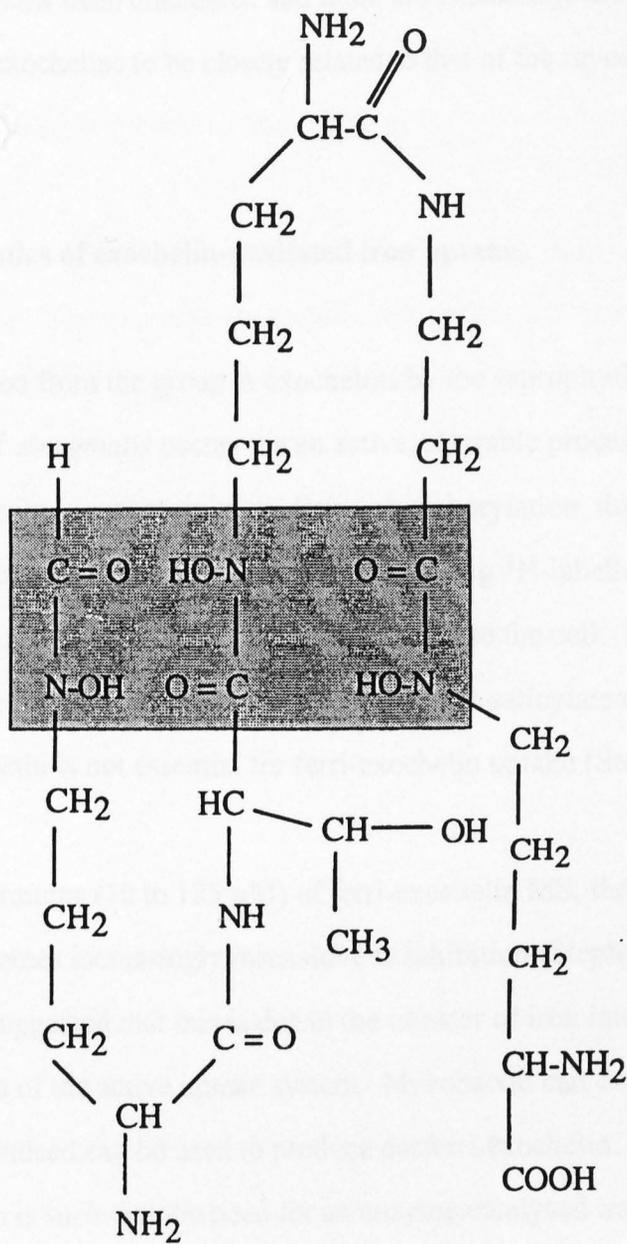
Figure 7. The structure of exochelin MS, the group A extracellular siderophore of *Mycobacterium smegmatis*. The three hydroxamate groups which comprise the iron-binding centre are those within the shaded area.

The structure of the chloroform-extractable exochelins, prevalent in the pathogenic species, has now been elucidated and these are chemically distinct to the water-soluble group A exochelins to be closely related to that of the mycobactins (C. Ratledge, pers. comm.).

b.   The characteristics of exochelin-mediated iron uptake.

The uptake of iron from the group A exochelins by the saprophytic mycobacteria such as *M. smegmatis* occurs via an active, saturable process which is inhibited by energy poisons, uncouplers of oxidative phosphorylation, thiol group inhibitors and anaerobiosis. The results of uptake assays using ^{3}H-labelled ferri-exochelin suggested that the whole complex is transported into the cell. The system was not inhibited by the pre-saturation of mycobactin by ferri-salicylate suggesting that the presence of mycobactin is not essential for ferri-exochelin uptake (Stephenson and Ratledge, 1979)

At high concentrations (10 to 125 μM) of ferri-exochelin MS, the uptake of iron into *M. smegmatis* becomes increasingly insensitive to inhibition (Stephenson and Ratledge, 1979). It is suggested that this is due to the transfer of iron into mycobactin caused by the saturation of the active uptake system. Mycobactin can extract iron from exochelin directly and indeed can be used to produce desferri-exochelin. The ease of transfer of the ferric ion is such that the need for an enzyme-catalysed transfer is not envisaged (Ratledge, 1987).

In summary, two routes for the uptake of iron from ferri-exochelin appear to operate. The first is a high affinity, low capacity, active uptake system mediated via iron-regulated envelope proteins which transports the ferri-siderophore complex and the second is a low affinity, high capacity, passive uptake system based upon the transfer of iron from ferri-exochelin to cell-bound desferri-mycobactin (Fig. 8).

The uptake of iron by the pathogenic species from their group B exochelins, however, is insensitive to inhibition and appears to be a system based upon facilitated

diffusion. (Macham *et al.*, 1977). Further work is necessary, however, to characterise this process.

c.   Specificity of exochelin-mediated iron uptake.

Both Ferri-exochelin MS and group B exochelins from various other mycobacterial species are able to reverse the bacteriostatic effect of serum on the growth of *M. smegmatis* (Macham *et al.*, 1975). The uptake of iron from group B ferri-exochelins was not affected by the addition of various inhibitors (Stephenson and Ratledge, 1980) in all of the mycobacteria tested. The pathogenic species *M. bovis* BCG and *M. intracellulare*, however, were unable to acquire iron via ferri-exochelin MS the group A exochelin of *M. smegmatis*. It appears that the mycobacteria possess a universal facilitated diffusion uptake system for the group B exochelins and that the saprophytic species especially possess an active, and more specific, ferri-exochelin transport system.

The endogenous iron uptake systems of *M. leprae* remain uncharacterised due to the current inability of microbiologists to culture this bacterium on artificial media. The uptake of iron from the ferri-exochelins of *M. neoaurum* and a culturable armadillo-derived mycobacterium (ADM) by *M. leprae*, however, has been demonstrated (Hall and Ratledge, 1987). ADM can occur along with *M. leprae in vivo* and may act as an "exochelin donor" in a mixed infection, however, it is equally possible that the leprosy bacillus produces its own exochelin which may possess structural similarities to those of the ADM and *M. neoaurum* which permits this cross-feeding (Hall and Ratledge, 1987).
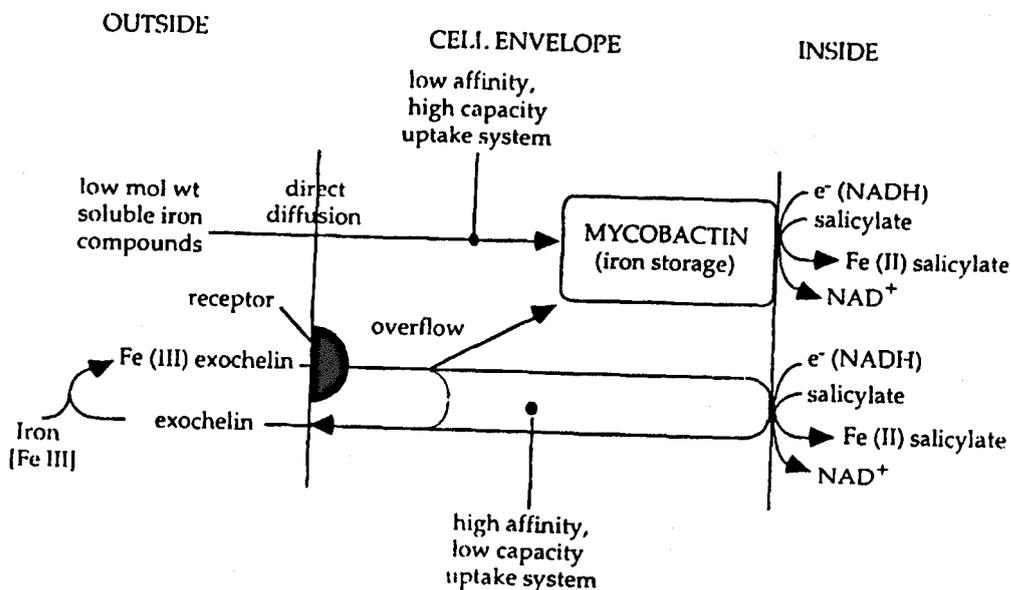
Figure 8. **Iron uptake mechanisms in *Mycobacterium smegmatis*.** The iron of ferri-exochelin MS is transported into the cell primarily as the intact ferri-siderophore complex via a high affinity active uptake system. This is a saturable system and iron from any excess ferri-exochelin is removed from the siderophore and is stored temporarily in mycobactin. Mycobactin may also chelate soluble iron compounds and store this iron in the same manner. The iron is removed from both siderophores by a reductive mechanism. (After Ratledge and Wheeler, 1994)

4. Other iron uptake systems in mycobacteria.

Messenger and Ratledge (1982) demonstrated the sequestration of iron from a ferric-dicitrate complex in *M. smegmatis*. Studies with ^{14}C-labelled citrate revealed that there was no co-transport of the citrate moiety. The system was insensitive to the effects of various inhibitors and is independent of the exochelin system. As a similar system was also observed in *M. vaccae*, it is unlikely that mycobactin is involved in this uptake mechanism (Messenger *et al.*, 1986).

The uptake of iron from rhodotorulic acid, a siderophore produced by the yeast *Rhodotorula pilimanae*, has been observed in *M. smegmatis* (A.J.M. Messenger, unpublished work). Although acquisition of iron via this siderophore was not inhibited by energy poisons and thiol group inhibitors; it was, however, dependent upon the iron status of the cells. Uptake of iron could be followed into iron deficient cells but not cells grown iron sufficiently. Studies with *M. vaccae* revealed that this species has no capability for the uptake of iron from ferri-rhodotorulate but whether this observation is linked to the absence of mycobactin in the species is not known (Ratledge, 1987).

5. Iron-regulated envelope proteins

Several iron-regulated envelope proteins (IREPs) have now been observed in mycobacterial preparations (Hall *et al.*, 1987; Sritharan and Ratledge, 1990). The coordinate expression of these proteins, along with exochelin and mycobactin synthesis was demonstrated in *M. neoaurum* (Sritharan and Ratledge, 1989). The synthesis of envelope proteins of molecular mass 14, 25, 29, 84 and 180 kDa was observed in *M. smegmatis* when grown under iron-limiting conditions but not under iron-sufficient conditions (Sritharan, 1988). Similarly sized proteins were expressed by *M. avium* and *M. leprae* grown *in vivo* (Sritharan and Ratledge, 1990).

a.   Potential ferri-exochelin receptors.

Polyclonal antisera were raised against the 25, 29, 84 and 180 kDa IREPs and incorporated into ^{55}Fe-exochelin uptake experiments. Only the antisera raised against the 29 kDa IREP caused a marked inhibition of the uptake of iron from ferri-exochelin into iron deficient cells. This inhibition, uptake occurred at 30% of the control rate, was not observed when antisera raised against the other IREPs were used or in uptake studies using iron sufficient cells. This evidence suggests that the 29 kDa protein is exposed at the cell surface and as such is a candidate as a primary receptor molecule for the ferri-exochelin complex (Sritharan, 1988). Attempts to label the IREPs with ^{55}Fe-exochelin and recover the ligand-protein complex, however, proved unsuccessful. This lack of progress was attributed to the exposure of the envelope proteins to sodium dodecyl sulphate during extraction and subsequent electrophoretic separations (Sritharan, 1988).

M. leprae is known to produce a strongly antigenic 28 kDa protein but the possible equivalence of this protein to the 29 kDa IREP recognised by Sritharan and Ratledge (1990) is, as yet, uncertain. The gene encoding the 28 kDa antigen of *M. leprae* has been cloned and sequenced (Cherayil and Young, 1988), the predicted amino acid sequence has revealed two hydrophobic domains at the termini of the mature protein after the cleavage of a putative signal sequence, a structure consistent with the location of this protein in the lipid rich wall of the bacterium. Analysis of the upstream sequence of this gene revealed a sequence demonstrating significant homology to the consensus Fur binding site (Dale and Patki, 1990) which overlapped with the -35 sequence for RNA polymerase binding. The antigenicity, predicted structure and location, and the possibility of transcriptional regulation controlled by intracellular iron concentration suggest that the 28 kDa antigen of *M. leprae* may participate in the early stages of an iron acquisition system in this bacterium. If one considers the similarity in size to the 29 kDa IREP described by Sritharan (1988), it is reasonable to suggest that this protein may have a role in the uptake of a ferri-exochelin in *M. leprae*. This hypothesis is yet to be rigorously examined.

b.   A periplasmic binding protein-dependent uptake system.

Fiss *et al.* (1994) recently cloned a DNA fragment from *M. smegmatis* which complemented siderophore deficient mutants of the bacterium. Along with the sequence complementing the mutation, three other genes were cloned, *fxu*ABC which possess a promoter containing a sequence exhibiting homology with the operator site of the iron-dependent repressor protein of *Corynebacterium diphtheriae*, DtxR. The predicted amino acid sequences of these three genes exhibit homology with those for components of the periplasmic binding protein-dependent uptake system for enterochelin in *E. coli*. FxuA and FxuC share amino acid homology with FepG (48% identity) and FepD (43% identity), which suggests that together they form a membrane bound diffusion channel, while FxuB shares amino acid homology with FepC (56% identity) and contains ATP-binding motives suggesting that this protein forms the membrane-associated ATPase. No genes that could potentially encode a binding protein, however, were found within this operon.

H.   Aims of the study.

The aim of this study was to investigate the function of the iron-regulated envelope proteins of *Mycobacterium smegmatis* in the uptake of ferri-exochelin, in particular, the identification and isolation of a ferri-exochelin receptor from the wall of the bacterium was to be attempted.

The first aim of the study was the definition of the iron-regulated envelope proteins of *M. smegmatis*. Although a series of IREPs had previously been reported in the bacterium (Hall *et al.*, 1987) the effect of other physiological stresses on their expression had not been fully assessed. The effect of heat shock and zinc-limitation, as well as iron starvation, on the envelope protein profile of the bacterium were to be investigated in order to define the proteins which were expressed specifically as a response to iron limitation.

The identification of the ferri-exochelin MS receptor protein was to be attempted via the direct binding of the radiolabelled ferri-siderophore. The binding of ferri-siderophores to proteins has been reported with other systems. Fiss *et al.* (1982) demonstrated the binding of ferric enterochelin to purified FepA of *E. coli* and the binding of ferrichrome, coprogen and aerobactin have been demonstrated by the cloned periplasmic hydroxamate-binding protein, FhuD, also of *E. coli* (Köster and Braun, 1990a). Siderophores, derivatised using bifunctional agents, have also been used to identify siderophore receptors in the envelopes of *Pseudomonas aeruginosa* (Sokol and Woods, 1983) and *E. coli* (Nelson *et al.*, 1992).

For these studies a preparation of pure, biologically active exochelin MS was required. Biological activity was to be assessed by performing ^{55}Fe(III)exochelin MS uptake assays. The interaction of envelope proteins from *M. smegmatis* and ferri-exochelin MS was to be studied *in situ* in crude envelope fractions, after extraction from the envelope with non-denaturing detergents and in a proteo-liposome suspension in order to identify and characterise any stable complexes formed.

II. Materials and Methods

A.   Maintenance and cultivation of *Mycobacterium smegmatis* NCIMB 8548

The entire work of this thesis has been conducted with *Mycobacterium smegmatis* NCIMB 8548.

1.   Maintenance

Cultures were maintained on slopes prepared from Sauton's medium and stored at 4°C.

2.   The preparation of glassware.

All glassware used in medium preparation and for the growth of cultures was treated to remove any traces of iron prior to use. The glassware was filled with alcoholic KOH (2% KOH in methylated spirit) and was allowed to stand overnight. The glassware was then rinsed thoroughly with distilled water before being filled with 8M HNO_3. Again the glassware was left to stand overnight before washing three times . with deionised water.

3.   The preparation of bacteriological growth media.

M. smegmatis was grown in a chemically defined medium consisting of:

KH_2PO_4	@	5 g/l	Zn^{2+}	@	452.0 µg/l
L-asparagine	@	5 g/l	Mn^{2+}	@	98.30 µg/l
D-glucose	@	10 g/l	Mg^{2+}	@	39.62 mg/l

The KH_2PO_4 and L-asparagine were dissolved in deionised water at a concentration of 5.5 g/l and the pH was adjusted to pH 7.0 with 5M NaOH.

Aluminium oxide was added to a concentration of 5 g/l. The medium was then sterilized by autoclaving for 15 minutes at 121°C. The glucose was prepared separately by dissolving in deionised water to a concentration of 10% (w/v) and then adjusting to pH 7.0 with 5 M NaOH. Aluminium oxide was added to 5 g/l and the glucose was sterilised as above. While cooling, the glucose and the medium were shaken frequently in order to circulate the insoluble aluminium oxide allowing it to bind any traces of iron present. Both solutions were filtered through Whatman No. 541 hardened ashless filter paper to remove the aluminium oxide. The medium was dispensed into 90 ml aliquots in 250 ml iron-free Erlenmeyer flasks and the glucose was dispensed as 10 ml aliquots into iron-free universal bottles. The medium and glucose were once again sterilized separately by autoclaving and then combined immediately before inoculation.

a.   Metal supplements.

i.   General metal supplements.

A stock solution of the sulphate salts of zinc, magnesium and manganese for addition to the medium was formulated:

$ZnSO_4.7H_2O$ (Analar)   20.3 mg

$MnSO_4.5H_2O$ (Analar)   4.4 mg

$MgSO_4.7H_2O$ (Analar)   4.1 g.

These salts were dissolved in 100 ml of deionised water, dispensed into iron-free bottles and autoclaved. For zinc-limitation studies, Zn^{2+} was omitted from the stock solution. 1 ml of the supplement was added to each 100 ml of medium at inoculation. Further metal supplements were prepared and dispensed as above for studies investigating the regulation of expression of the ferri-exochelin-binding protein. These will be detailed in the results sections.

ii. Iron supplements.

The medium was supplemented with 2.0 µg Fe/ml for iron sufficient growth and ≥0.1 µg Fe / ml for iron-limited growth. Two iron stock solutions were formulated:

High iron stock solution:

99 mg $FeSO_4.7H_2O$ (Analar) were dissolved in 1 ml conc. H_2SO_4, the volume then being made up to 100 ml with deionised water.

Low iron stock solution:

5 ml of the high iron stock solution was made up to a volume of 100 ml with deionised water.

Both stock solutions were stored in iron-free glass bottles and were sterilized by autoclaving.

4. Inocula.

For each experimental flask, a 1% inoculum, i.e., 1 ml from a two day old starter culture was used. Two successive starter cultures were used; the first culture was inoculated from an agar slope culture, grown for two days and then used to provide a 1 % inoculum for a second starter culture which, after two days of growth, was inoculated into the flasks.

5. Culture conditions.

All cultures were grown at a temperature of 37°C for 4 to 6 days with shaking (180 rpm) unless otherwise stated.

B.   The extraction of proteins from the envelope of *Mycobacterium smegmatis*.

1.   The preparation of cell envelope fractions.

Cultures were usually harvested after 4 to 6 days of growth by centrifugation at 16,300 g for 10 minutes at 4°C and the spent medium was used for the preparation of exochelin. The cells were resuspended and washed twice with 50 mM KH_2PO_4 / NaOH pH 7.1 and were finally resuspended in 5% of their original volume in the above buffered solution containing 1mM benzamidine and 1mM PMSF. Cells were disrupted, in 15 ml lots, by sonication using a Dawe Soniprobe Type 7533A. Sonication was performed on ice for 10 minutes on a 50% duty, pulsed operation cycle. The sonicated suspension was then centrifuged at 6000g for 10 minutes at 4°C to remove cell debris which was re-sonicated once, as before, to increase yields.

Attempts were made to separate wall and membrane fractions by the differential centrifugation method of Sritharan (1988) : the supernatant fluid derived from the post-sonication centrifugation step was centrifuged at 20,000 g for 30 minutes at 4°C to sediment the wall fraction. The supernatant fluid from this step was then centrifuged at 105,000g for 90 minutes at 4°C to deposit the membrane fraction.

The pellets representing both fractions were washed, by resuspending on ice with a small Potter homogeniser, and re-centrifuged under the stated conditions a further twice.

Cell envelope fractions were prepared as above but omitting the 20,000g centrifugation step thus depositing all of the envelope material in the 105,000g centrifugation step. The pellets were washed in the same way as stated for the wall and membrane fractions

The pellets were then either resuspended in 62.5 mM Tris/HCl pH 6.8, for analysis by SDS-PAGE, or were solubilised using the non-denaturing detergent, CHAPS, for functional studies.

2. Assay of succinate dehydrogenase as a cytoplasmic membrane marker enzyme.

In order to assess the extent of the separation of wall and membrane fractions after differential centrifugation, the method of Schwitzgeubel (1981) was used to assay succinate dehydrogenase, a cytoplasmic membrane marker enzyme, in the wall and membrane fractions. The following reagents were mixed in a cuvette at 25°C:

Buffer 200mM K phosphate, pH 7.2	500 μl
Bovine serum albumin, 3 mg/ml	1.0 ml
NaN_3, 1M	15 μl
Triton X-100, 1% (v/v)	30 μl
EDTA, 10 mM	30 μl
DCPIP, 1.32 mM	15 μl
PMS, 14 mM	30 μl

Water and extract to a volume of 2.94 ml.

Endogenous reaction rates were recorded at 600nm for approximately 2 minutes and then the assay was initiated by the addition of 60 μl 1M sodium succinate.

3. Solubilisation of envelope material in the non-denaturing detergent, CHAPS.

Envelope fractions were resuspended on ice in a small volume (approx 2 ml) of 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH pH 7.1 by gentle agitation with a glass rod. The material was transferred to a vial and the suspension was mixed on a magnetic stirrer overnight at 4°C. The solubilised protein was separated by centrifugation at 105,000g for 30 min at 4°C. The supernatant fluid containing the CHAPS-solubilised envelope proteins was taken and the sedimented envelope material was discarded.

4.   Protein determination.

The bicinchoninic acid (BCA) assay (Pierce, Rockford USA) was used for protein determinations. The manufacturer's instructions were followed exactly. γ-globulins were used as a protein standard for these determinations.

C.   Polyacrylamide gel electrophoresis.

1.   SDS-PAGE.

The method of Laemmli (1970) was followed: 5 to 20% linear gradient gels and 10% homogeneous gels were used in the study. The gels (1.5 x 16 x 14cm) were cast using the stock solutions described by Hames (1981) and were electrophoresed for approximately 3 h at a constant current of 35 mA at 4°C.

Samples were prepared for electrophoresis by mixing with an equal volume of 62.5 mM Tris/HCl pH 6.8; 2%(w/v) SDS; 10%(w/v) glycerol; 0.002%(w/v) Bromophenol blue. The samples were boiled for 3 minutes and were then rapidly cooled.

2.   CHAPS-PAGE.

This non-denaturing gel system was assessed as a method to separate and identify a radiolabelled ferri-exochelin-receptor complex. The method of Cavinato *et al.* (1988) was used. 10% gels were cast using the same stock buffer solutions as were used for SDS-PAGE. Samples were prepared for electrophoresis by diluting with an equal volume of 62.5 mM Tris/HCl pH 6.8; 3.5 mM CHAPS; 10%(w/v) glycerol; 0.002%(w/v) Bromophenol blue. The gels were run at a constant current of 35 mA at 4°C for 3 h. Protein bands were visualised by silver staining.

3. Triton X-100-PAGE.

The electrophoretic conditions were exactly the same as used for SDS-PAGE other than 0.1% Triton X-100 was substituted for 0.1% SDS. Samples were prepared for electrophoresis by diluting with an equal volume of 62.5 mM Tris/HCl pH 6.8; 2% Triton X-100; 10%(w/v) glycerol; 0.002%(w/v) Bromophenol blue. Proteins bands were visualised by silver staining and autoradiography.

4. Visualisation of protein bands in polyacrylamide gels.

Gels used in the iron-regulated envelope protein (IREP) expression studies were stained using Serva Blue G; however, gels used to identify proteins in purification fractions were routinely silver stained due to the greater sensitivity of this method.

a. Staining of gels with Serva Blue G.

Gels were stained for 3 h in a solution comprising 0.05%(w/v) Serva Blue G in glacial acetic acid/methanol/water (1:5:5 by vol) and were then destained overnight using 10% methanol, 7.5% (v/v) acetic acid in distilled water.

b. Silver staining.

Gels were fixed by soaking for 2 x 10 min in 10% (v/v) ethanol / 5% (v/v) acetic acid and were then transferred to oxidising solution, 3.4 mM $K_2Cr_2O_7$; 3.2 mM HNO_3, for 10 min. The gel was washed in several changes of distilled water until it was no longer yellow (approx. 1 h) and was then incubated for 30 min in the dark with freshly prepared 12 mM $AgNO_3$. The gel was then washed for 2 min in distilled water before the stain was developed. Gel development was standardised by using three strictly timed washes in 0.28 M Na_2CO_3; 0.005%(v/v) 40% formaldehyde for 1, 5 and 3 minutes, each with fresh developing solution. The developing reaction was stopped by the addition of 5% (v/v) acetic acid.

c. Autoradiography.

Polyacrylamide gels used to separate protein-bound and free ^{55}Fe(III)exochelin were visualised by autoradiography. The gels were placed between a lower sheet of Whatman No. 1 filter paper and an upper sheet of cling film on a flat-bed gel drier and were dried under vacuum at 80°C for approximately 2 h. The dried gels were removed from the gel drier and overlaid with a sheet of ^{3}H-Hyperfilm (Amersham) in an X-ray cassette for periods of up to 1 week. Films were developed and fixed using standard protocols.

D.   The preparation and assay of exochelin and mycobactin.

1.   The purification of exochelin MS from *M. smegmatis* culture supernatants.

The method of Sharman *et al.* (1995) was used to purify exochelin MS. Culture supernatants from at least 30 x 100ml iron-limited cultures of *M. smegmatis* were pooled and adjusted to pH 7.0 with HCl and a small volume of a saturated solution of ferric chloride was added until the precipitation of proteins in the spent media was evident. The medium was stirred for approximately 1 h to allow the complete formation of ferri-exochelin and was then filtered through Whatman No.1 filter paper at 4°C overnight. The filtrate was run through a Biorad AG50W-X8 cation exchange column (8 cm x 3 cm; 100-200 mesh in the NH_4^+ form; flow rate = 2 ml/min) in 600 ml aliquots overnight at 4°C. Ferri-exochelin was retained by the resin and the column was washed with 200ml distilled water. The ferri-exochelin was eluted with 1.0 M NH_4Cl / NH_4OH pH 9.5. The eluted ferri-exochelin solution was evaporated to a volume of approximately 10 ml using a rotary vacuum evaporator. Any precipitated salts were removed by filtration through Whatman No. 1 filter paper. The solution was further desalted by gel filtration through Sephadex G-10 column (48 cm x 3 cm; flow rate = 1.2 ml/min) eluting with distilled water and collecting 10 ml fractions. The

conductivity of eluted fractions of the crude ferri-exochelin was measured using a PTI F8 digital conductivity meter. Those fraction with conductivity $< 5\mu S/cm$ were pooled for further purification and those with higher conductivity readings were desalted once more. The crude, salt-free, ferri-exochelin was further fractionated by ion exchange chromatography using a BioRad AG50W-X4 cation exchange column (40 x 2 cm; 200-400 mesh in the NH_4^+ form; flow rate = 2 ml/min) pre-equilibrated with 0.1M NH_4OH / acetic acid pH 6.0. The exochelin was eluted by running a gradient from 0.1 M NH_4OH / acetic acid pH 6.0 to 1.0 M NH_4OH / acetic acid pH 9.0. 10 ml fractions were collected and elution was monitored by A_{420nm}, the λ_{max} of ferri-exochelin MS. The active ferri-exochelin MS fractions were collected (Fig. 9), evaporated to a volume of approximately 3 ml and were then desalted by gel filtration until the conductivity of all eluted fractions $< 5\mu S/cm$.

The final step in the ferri-exochelin purification process was by h.p.l.c. fractionation passing 200-300 μl samples of the salt-free ferri-exochelin solution through a Lichrosorb RP8, 10μm, (25 cm x 1 cm) column with the following elution profile: solvent system (%,v/v) at 0 min, 100% water; 15 min, 100% water; 20 min, 100% 0.1% trifluoroacetic acid; 45 min, 90% 0.1% trifluoroacetic acid in water/10% 0.1% trifluoroacetic acid in methanol; 60 min, as for 45 min. The flow rate was 2 ml/min and the eluate was continuously monitored at 220nm. The ferri-exochelin MS which eluted at about 28 min was collected, freeze-dried and dissolved in 0.5 ml water. A 20 μl sample was assessed for purity on the same h.p.l.c. column using the following solvent system at a flow rate of 2 ml/min: (%,v/v) at 0 min, 100% 0.1% trifluoroacetic acid in water; 35 min, 90% 0.1% trifluoroacetic acid in water/10% 0.1% trifluoroacetic acid in methanol; 60 min, as for 35 min.
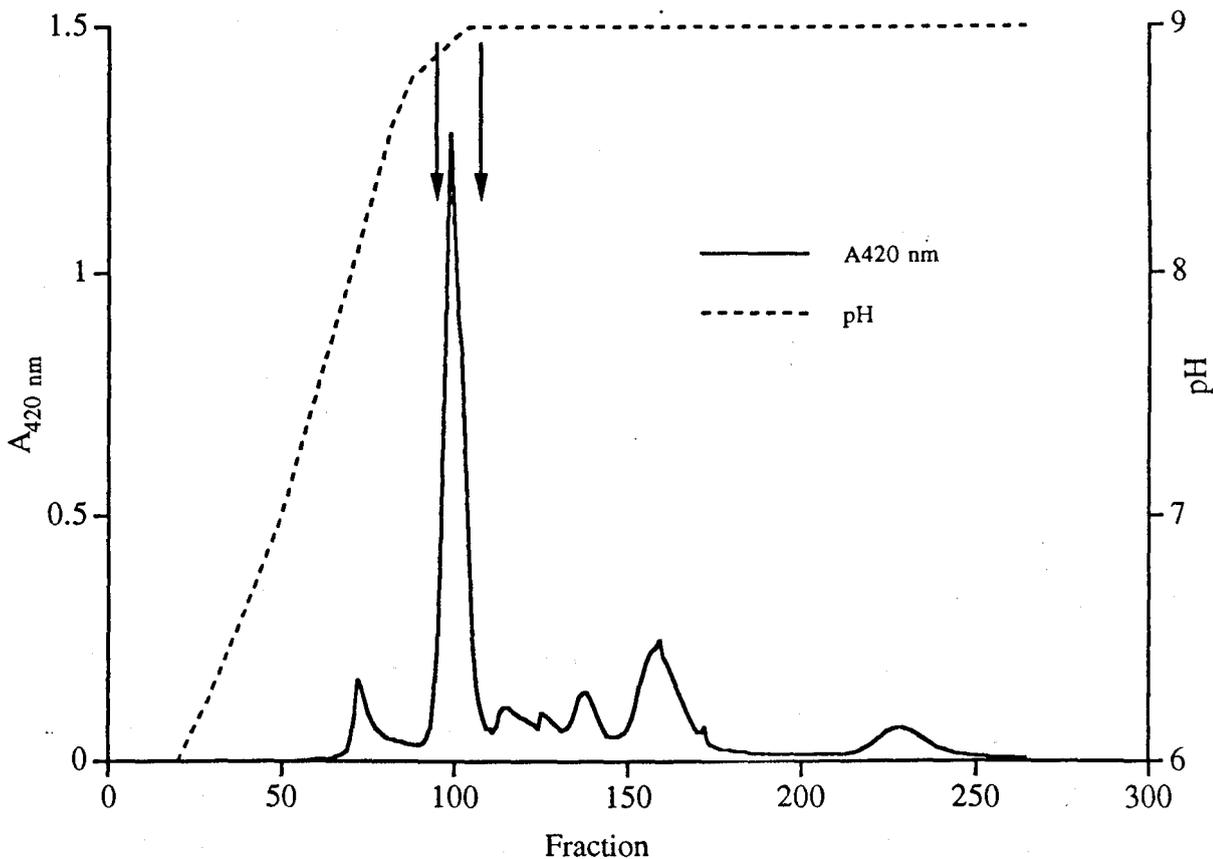
Figure 9. Fractionation of ferri-exochelin MS solution by cation exchange chromatography. Crude ferri-exochelin MS was fractionated on a BioRad AG50W-X4 cation exchange column (40 x 2 cm; 200-400 mesh in the NH_4^+ form; flow rate 2 ml / min). A gradient from 0.1 M NH_4OH / acetic acid pH 6.0 to 1.0 M NH_4OH / acetic acid pH 9.0. 4 ml fractions were collected and elution was monitored at 420nm. The arrows indicate the first and last fractions retained for further purification by h.p.l.c.

2. The preparation of desferri-exochelin.

The purified ferri-exochelin sample was decreased to approximately 3 ml in a rotary evaporator and was then added to a solution of desferri-mycobactin in chloroform. Ethanol was added until the phases became miscible and then the solution was mixed by shaking in a separating funnel. The mixture was held at ambient temperature overnight and then the aqueous and organic phases were separated by the addition of iron-free water. The lower, organic phase was removed and the aqueous phase was washed with redistilled chloroform to remove any traces of mycobactin.

This process was repeated until the mycobactin-containing organic phase failed to become coloured by extracting iron from exochelin. The desferri-exochelin was concentrated in a rotary evaporator and stored at -20°C in an iron-free glass vial.

3. The labelling of exochelin with ^{55}Fe and ^{59}Fe.

The amount of exochelin present in the sample was estimated by titrating siderophore with a known amount of $FeCl_3$. Ferri-exochelin formation was monitored by A_{430} against an iron-equivalent $FeCl_3$ reference solution. An appropriate amount of radioisotope, as $FeCl_3$ in 0.1 M HCl, was added to a suitably buffered, desferri-exochelin of known concentration and was held at ambient temperature and neutral pH for 2 h. The amounts of phosphate buffer required to maintain a neutral pH was determined by titration with 0.1M HCl for each labelling experiment. Enough non-radioactive $FeCl_3$ was added to saturate the exochelin to 95%. The solution was held at ambient temperature for a further two hours and was then stored at 4°C overnight. The solution was centrifuged at 16,000g for 10 min at ambient temperature in order to deposit any polymeric or colloidal ferric salts.

4. Liquid scintillation counting and the estimation of counting efficiency.

Radioactivity in liquid samples was determined by liquid scintillation counting. Each sample was mixed with 10 ml of Ecoscint A liquid scintillant and was then counted in a Beckmann LS9000 scintillation counter. The degree of quenching in each sample was determined automatically, using an external ^{137}Cs standard of known activity, and was expressed as an H-number.

To estimate the counting efficiency for $^{55}Fe(III)$exochelin, duplicate samples of known activity were deliberately quenched by the sequential addition of water and bromophenol blue to the scintillation vials. The H-numbers of these quenched samples were plotted against the counting efficiency for each sample to produce a calibration graph.

5. Ferri-exochelin uptake assays.

Cultures were harvested by centrifugation at 6000g for 10 minutes at 4°C. and the iron status of the cultures was verified by the reaction of $FeCl_3$ with the spent medium; the spent medium from iron-limited cultures turned a characteristic orange colour due to the formation of ferri-exochelin MS.

The cells were washed in 50 mM KH_2PO_4 / NaOH pH 7.1, centrifuged once more and then thoroughly resuspended in the same buffer, using a Potter homogeniser on ice, to a concentration of approximately 30 g dry weight/ml. This suspension was then stored on ice for 15 minutes to allow any remaining large clumps of mycobacteria to settle out. The suspended cells were decanted into another bottle and stored on ice. Triplicate samples of the suspension were taken, centrifuged, washed twice in distilled water and then dried to constant weight under vacuum in a pre-weighed vial to determine the dry weight of cells taken in each assay sample.

The assays were carried out in a jacketed flask maintained at 37°C by a pumping water bath. The assay suspension was mixed gently using a magnetic stirrer. 1.25 ml of the cell suspension were introduced into the flask and diluted with 50 mM

KH$_2$PO$_4$ / NaOH pH 7.1 to the final assay volume of 10 ml. The cells were then allowed to equilibrate at the assay temperature for 10 minutes.

The experiment was initiated by the addition of enough labelled ferri-exochelin to give an initial free ferri-exochelin concentration of 1 μM. 0.5 ml samples were withdrawn from the flask over a 20 to 25 minute period and were immediately transferred to test tubes containing 2 ml of 50 mM Na$_2$EDTA pH 6.0. The contents of these tubes were then filtered by suction through Whatman GF/C filters which were then washed twice with 2 ml aliquots of the EDTA solution.

The filters were then placed in plastic scintillation vials and were each treated with 0.5 ml Soluene tissue solubiliser. After 2 hours' incubation at 50°C 50 μl of glacial acetic acid was added to neutralise each sample. 10 ml of Ecoscint A was added to each vial and the iron retained in each sample was then quantified by scintillation counting.

6.   Extraction and purification of mycobactin.

Moist cells from approximately 25 x 100 ml, 4 to 6 day old iron-deficient cultures were held at ambient temperature in approximately 200ml ethanol for 24 h to extract desferri-mycobactin from the lipid matrix of the cell wall. The mixture was then filtered through Whatman no.1 filter paper and the residues discarded. The mycobactin was converted to the ferri-form by the drop-wise addition of a saturated solution FeCl$_3$ resulting in the development of a deep red colouration. An equal volume of chloroform was added followed by enough distilled water to cause the organic and aqueous phases to separate. The aqueous layer was discarded and the chloroform extract was washed with distilled water to remove excess FeCl$_3$. After washing the extract was dried over MgSO$_4$ overnight, filtered through Whatman no.1 filter paper and then evaporated to dryness in a rotary vacuum evaporator. This dried material was extracted with 100 ml methanol to remove methanol-insoluble species such as phopholipids and waxes. The methanol extract was evaporated to dryness, dissolved in 100 ml cyclohexane and absorbed onto neutral grade alumina in a beaker.

This was washed twice with 50 ml lots of cyclohexane, twice with 75 ml lots of petroleum spirit, once with 75 ml toluene and three times with 75 ml lots of diethylether; in each case the washings were discarded. The ferri-mycobactin was eluted from the alumina using chloroform:acetone (1:1), evaporated to dryness and redissolved in ethanol.

7. The production of desferri-mycobactin.

Concentrated HCl was added dropwise to a solution of ferri-mycobactin in ethanol until it was completely decolourised. Redistilled chlorform was added until the aqueous and organic phases separated. After a short time, to allow the phases to fully separate, the aqueous layer was removed. The chloroform solution was repeatedly washed with iron-free water until the pH of the aqueous phase remained neutral after washing. The "iron-free" water often contained traces of iron which would be extracted by the desferri-mycobactin during washing. In these instances the water from later washes, with near neutral pH, were stored. The mycobactin was evaporated to dryness using a vacuum rotary evaporator and was redissolved in ethanol, acidified and the procedure repeated using the iron-free water from the previous washes to extract the acid from the organic phase.

8. Derivatisation of ferri- and desferri-exochelin using *o*-phthaldialdehyde.

o-Phthaldialdehyde reacts with amino acid in alkaline conditions in the presence of 2-mercaptoethanol giving rise to strongly fluorescing compounds (λ_{ex} = 340 nm; λ_{em} = 455 nm). The high sensitivity of the system allows analysis of amino acids in the nanomole range. The procedures of Roth (1971) were used to analyse the reaction of this reagent with desferri- and ferri-exochelin.

A buffered solution of *o*-phthaldialdehyde was prepared each day:
1.5 ml of *o*-phthaldialdehyde solution (10 mg/ml in ethanol) and 1.5 ml of
2-mercaptoethanol solution (5 μl/ml in ethanol) were added to 90 ml 50mM $Na_2B_4O_7$ /

NaOH pH 9.5.

For each assay 100 μl of sample, either ornithine, desferri- or ferri-exochelin. were mixed with 3 ml of the buffered reagent solution and fluorescence was monitored using a Perkin-Elmer LS-5B luminescence spectrometer between 5 and 25 minutes of initiating the reaction.

E.   Affinity chromatography techniques.

1.   Immobilisation of ferri-exochelin on Sepharose 4B.

An affinity matrix of 6-aminohexanoic acid *N*-hydroxysuccinimide ester-Sepharose 4B was used to immobilise ferri-exochelin. This matrix incorporates a C_6 spacer arm to improve the accessibility of small ligands and will react with free NH_2 groups of peptides or amines. The exochelin from *M. smegmatis* was considered to have free NH_2 groups and this was confirmed by the ligation of the ferri-exochelin to the support causing it to change colour, from white to orange. The occurence of three NH_2 groups in exochelin MS has now been confirmed by structural analyses (Sharman *et al.*, 1995). The manufacturer's instructions for the binding of ligands to the Sepharose support were followed exactly. After five cycles of consecutive high and low pH washes the matrix remained orange indicating the ferri-siderophore was covalently bound to the support. This procedure was repeated exactly, except that Tris was used in place of ferri-exochelin in order to produce a control column in order to assess the specificity of envelope protein-binding by the ferri-exochelin-Sepharose.

2. Analysis of the binding properties and interactions of envelope proteins by affinity chromatography on ferri-exochelin-Sepharose 4B.

Samples of CHAPS-solubilised envelope proteins (1 mg protein in 300 μl) derived from both high and low iron cultured cells were loaded on to small columns (bed volume = 0.4 ml) of ferri-exochelin-Sepharose (see E.1). The flow of the solution was stopped for 90 minutes once all of the solution had entered the column to increase the extent of the binding of proteins to the affinity matrix. Non-binding proteins were then eluted by washing the columns with 10 bed volumes of 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH pH 7.1.

The affinity elution of IREPS was attempted using combinations of 1mM ferri-exochelin, 1mM ferri-exochelin in various concentrations of NaCl and 10 mM ATP, all in buffered CHAPS, as eluents. To assess the specificity of the elution profiles gained from these affinity elutions a non-specific control was run using a solution of 0.5 mM ornithine; 0.5 mM glutamine was used as the eluent. The eluents were applied to the column in 0.5 ml and allowed to elute for 30 min. The column was then centrifuged at 3000g at room temperature for 2 min to remove the final traces of the eluent. Each eluate was stored at -20°C. After the completion of each elution scheme, any remaining proteins still bound to the column were eluted with either 2% SDS in 50 mM Tris / HCl pH 6.8 or increasing concentrations of guanidine.HCl.

Eluates were analysed either directly by gel filtration chromatography on Sephadex G-100 or by SDS-PAGE following lyophilisation to concentrate the sample. After the final elution the columns were washed with 50 mM KH_2PO_4 / NaOH pH 7.1 to remove any residual SDS or guanidine to regenerate the column.

F. Ion exchange chromatography of ferri-exochelin-protein complexes on polyethyleneimine-cellulose.

Samples of CHAPS-solubilised envelope proteins (1 mg protein) were mixed with 50 nmol ^{55}Fe(III)exochelin in a 1 ml final volume and held at 37°C for 30

minutes. (Final detergent and buffer concentrations were 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH pH 7.5.) This mixture was then loaded on to a small polyethyleneimine (PEI)-cellulose anion exchange column (5 x 1 cm), pre-equilibrated with 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH pH 7.5, which was then washed with 30 ml of the same solution to elute any non-protein-bound ferri-exochelin. The adhered envelope proteins were then displaced with NaCl: (i) 20 ml 0.1 M NaCl in 8 mM CHAPS; 50 mM KH_2PO_4 / NaOH buffer pH 7.5, (ii) 10 ml of 1.0 M NaCl in 8 mM CHAPS; 50 mM KH_2PO_4 / NaOH buffer pH 7.5. One ml fractions were collected and 100 µl samples of each were quantified by scintillation counting. The remainder of each fraction was lyophilised to concentrate the protein for subsequent analysis by SDS-PAGE.

G.   Study of the ferri-exochelin-binding activity of CHAPS-extracted envelope proteins in liposomes.

1.   Incorporation of CHAPS-solubilised envelope protein into liposomes.

A sample of CHAPS-solubilised envelope proteins (20 mg)derived from low-iron cultured cells was dialysed against 400 volumes of 50 mM KH_2PO_4 / NaOH pH 7.1 overnight at 4°C to remove the detergent. This protein was then used for binding analyses in liposomes.

The liposomes were constructed using three constituents, cholesterol, phosphatidylcholine and dipalmitoylphosphatidic acid (all approx. 99% pure) in molar ratios 10:10:1. These were mixed in chloroform,then 300 µg was dried on to the walls of a test tube under N_2 unless otherwise stated. Dialysed envelope proteins in buffer were added to the lipid-coated test tubes and the volume was made up to 1 ml with 50 mM KH_2PO_4 / NaOH pH 7.1. Crude proteo-liposomes were formed by shaking the mixture vigorously for 30 seconds. A cloudy suspension of proteo-liposomes was formed. This suspension was incubated with 50 µM [55]Fe(III)-exochelin (1.09×10^5 Bq/nmol) for 30 min at 37°C. Bound and unbound [55]Fe was separated by filtering

66

through Millipore GVWP filters (0.22 μm pore size), on which the proteo-liposomes were retained, and subsequent washing with 2 ml of the same buffer. Radioactivity on the filters was then determined by scintillation counting. Bound and unbound ^{55}Fe was also separated by gel filtration through Sephadex G 200-120 column (see H.2). Samples were assayed for turbidity (A $_{628nm}$), protein content, by the bicinchoninic acid assay, and radiolabel retention by scintillation counting in Ecoscint A.

2. Complex release from liposomes by digestion with various detergents.

Larger preparations of proteo-liposomes were made containing 4 mg lipids: 1 mg envelope protein and labelled as before. Non-specific label retention was removed by overnight dialysis against 400 volumes of 50 mM KH_2PO_4 / NaOH, pH 7.1, at 4°C. Specific ^{55}Fe retention was assessed by scintillation counting. Various detergents were used to assess the degree of solubilisation of protein-ferri-exochelin complex release: (i) 8 mM CHAPS, (ii) 8 mM CHAPS + 1mM taurodeoxycholic acid, (iii) 8mM CHAPS + 0.5 M NaCl and (iv) 0.5% (w/v) Triton X-100. Proteo-liposomes were incubated with the detergents overnight at 4°C. Solubilisation was quantified by scintillation counting of a sample of the supernatant fluid after centrifugation at 105,000g for 30 minutes at 4°C. The diffusibility of the released label was assessed by recounting after overnight dialysis against 100 volumes of deionised water.

H. The analytical use of gel filtration chromatography.

1. Separation of bound and unbound radiolabel.

Sephadex G-200-120 was used to separate bound and unbound ^{55}Fe in the studies of envelope protein/ferri-exochelin complex formation in liposomes. The column had dimensions 160 cm x 1.5 cm, was loaded with 1 ml samples and eluted with 50 mM KH_2PO_4 / NaOH pH 7.1. 10 drop samples (= 650 μl) were collected for

analysis.

2. Separation of ferri-exochelin-envelope protein complexes.

Two columns were used in attempts to isolate solubilised ferri-exochelin-envelope protein complexes and estimate their molecular weight. Initially a Sephadex G-100-superfine (37 cm x 1.5 cm; flow rate = 0.3 ml/min) was used. Samples, usually 1 ml was loaded, were eluted with 8 mM CHAPS; 50 mM KH_2PO_4 / NaOH pH 7.1 collecting 20 drop samples (= 0.6 ml). This column was run at ambient temperature; ideally this work should have been carried out at 4°C but, as ^{59}Fe was to be separated on the column, communal areas, such as the cold room, could not be used.

For later work a Sephacryl S-100-HR column was used (60 x 1.5cm) to separate [55]Fe-containing ferri-exochelin/envelope protein complexes at 4°C.

3. The calibration of gel filtration columns.

The Sephadex S-100-HR column was calibrated using a mixture of proteins, each at 1 mg/ml: Albumin, bovine serum (66,000 Da); Carbonic anhydrase, bovine erythrocytes (29,000 Da); Cytochrome c, Horse heart (12,400 Da) and Aprotinin, bovine lung (6,500 Da). The elution of these proteins was monitored by A_{280nm}.

The Sephadex G-100-superfine column was calibrated using a mixture of marker enzymes:

Pyruvate kinase	50U	235 kDa
Aldolase	10U	158 kDa
Alcohol dehydrogenase	800U	141 kDa
Lactate dehydrogenase	50U	130 kDa
Citrate synthase	5U	87 kDa
Peroxidase	190U	40 kDa
Myokinase	50U	22 kDa.

^{55}Fe(III)exochelin was also used as a marker (0.64 kDa), elution being monitored by scintillation counting.

LDH, ADH, aldolase, pyruvate kinase and myokinase were assayed using methods from Bergmeyer (1978).

a.   Assay for peroxidase.

        50 μmol KH_2PO_4/K_2HPO_4 pH 7.0

        2.5 μmol ABTS, Na salt

        30 μl peroxidase sample

        Water to 2.9 ml

The reaction was initiated by the addition of 100 μl 150 mM H_2O_2 and was monitored spectrophotometrically at 412nm.

b.   Assay for citrate synthase.

        1ml 1M Tris.HCl pH 7.0

        10 μl DTNB in buffer

        100 μl enzyme sample

        10 μl 5mM acetyl CoA

        Water to 3.0 ml

The reaction was initiated by the addition of 5 μl 50 mM potassium oxaloacetate and was monitored spectrophotometrically at 412nm.

The void volume of each column was determined by monitoring the elution of blue dextran.

I. Sources of materials.

All general biochemicals, enzymes, electrophoresis products and chromatography stationary phases were supplied by the Sigma Chemical Company (U.K.), BioRad (U.K.) BDH (U.K.) and Pharmacia. The Lichrosorb RP8, h.p.l.c. column was supplied by Jones Chromatography. Ecoscint A was supplied by National Diagnostics (U.K.) and all radioisotopes and autoradiography film were supplied by Amersham (U.K.).

III   The identification of iron regulated envelope proteins in *Mycobacterium smegmatis*

A.   Introduction

In attempts to identify the components of the ferri-siderophore uptake systems of the mycobacteria, previous studies have compared the envelope protein profiles of several mycobacterial species grown under iron-replete and iron-limiting conditions (Hall *et al.*, 1987; Sritharan, 1988; Sritharan and Ratledge, 1989 and 1990). It remained a possibility, however, that some of these proteins may have been expressed as a response to general physiological stress rather than as a specific response to iron availability. It was considered important, therefore, that the specificity of the iron stress response observed in earlier studies was examined by analysing the changes in the envelope protein profile of *M. smegmatis* elicited as response to other stresses.

All cells when subjected to a sudden rise in temperature, irrespective of the initial temperature (Jerez, 1988), respond by increasing the expression of a family of proteins known as the heat shock proteins (hsps). This response appears to be universal and, moreover, components of the response are highly conserved among divergent organisms (Lindquist, 1986; Garsia *et al.*, 1989). Several of these proteins are polypeptide chain binding proteins and are thought to be involved in the process leading to the correct folding of proteins in the cell (Rothman, 1989). It is thought that during heat shock an increase in the protein folding capacity of the cell is required in order to re-fold those proteins denatured during the temperature shift and that, as catalysts of protein folding, the expression of the heat shock proteins is increased accordingly. Heat shock proteins, however, are also elicited after exposure to stresses other than heat shock including exposure to ethanol, hydrogen peroxide, cadmium, zinc, copper and mercury, sodium arsenite and anaerobiosis (Lindquist, 1986) and therefore a more apt description of this group may be the stress proteins.

Like iron-regulated outer membrane proteins generally (see Section I.C and references therein) and also the IREPs of the pathogenic mycobacteria (Sritharan and Ratledge, 1990) stress proteins have been shown to be expressed by pathogenic organisms *in vivo* (Buchmeier and Heffron, 1990; Rey-Ladino and Reiner, 1993) and are often immuno-dominant antigens (Buchmeier and Heffron, 1990; Young *et al.*, 1988, Esaguy *et al.*, 1991; Águas *et al.*, 1990).

A component of the heat shock response of the mycobacteria, the immuno-dominant P64 antigen, has been shown to be over expressed during zinc starvation (De Bruyn *et al.*, 1989) which suggests that nutrient limitation may also be involved in the induction of the stress response. It was important, therefore, to determine if any of the previously reported IREPS of *M. smegmatis* were expressed as a response to general physiological stresses rather than as a specific response to iron limitation.

B.   The effect of nutrient limitation upon the envelope protein profile of *M. smegmatis*.

Mycobacterium smegmatis was grown under three nutrient regimes: iron/zinc sufficiency (2 μg Fe/ml; 0.45 μg Zn/ml), iron limitation (0.1 μg Fe/ml; 0.45 μg Zn/ml) and zinc limitation (2 μg Fe/ml; no added zinc). Cultures were grown for 6 days at 37°C, were harvested and envelope fractions were prepared as described in Section II.B.1. The protein profiles of the envelope fractions were then analysed by SDS-PAGE (Fig. 10).

Comparison of lanes A&B, samples derived from different iron-limited cultures, with lane C (Fig. 10) revealed the increased expression of several proteins under iron limitation (viz. 180, 84, 70, 54, 25, 21 and 14 kDa). Although some of the differences were minor they were consistently observed on many gels. These observations broadly agreed with the findings of earlier studies (Hall *et al.*, 1987; Sritharan, 1988). Three of the proteins, however, had not been previously described in *M. smegmatis*. The 29 kDa protein IREP described by Hall *et al.* (1987), however, was now expressed equally under iron-sufficient and iron-deficient conditions. Moreover, this protein now represented the

72

major envelope protein of the cell. This 29 kDa protein had been suggested as a putative ferri-exochelin receptor molecule on the basis of the inhibition of ^{55}Fe(III)exochelin uptake by antisera raised against the purified protein (Hall *et al.*, 1987). The apparent lack of regulation of the expression of this protein by the iron status of the culture appeared inconsistent with this role.

The effect of zinc limitation upon the envelope protein profile of *M. smegmatis* (Fig. 10, Lane D) was observed as an increase in the expression of 57.5 and 29 kDa proteins. None of the proteins elicited as a response to iron limitation in this study were over expressed in zinc-limited cultures of *M. smegmatis* suggesting that the IREPs observed in this study were indeed expressed as a specific response to iron limitation. The expression of a 29 kDa protein appeared to be increased slightly during zinc limitation; whether this is a result of the over expression of the 29 kDa protein described by Sritharan (1988) or the co-migration of a similarly sized protein is not known.
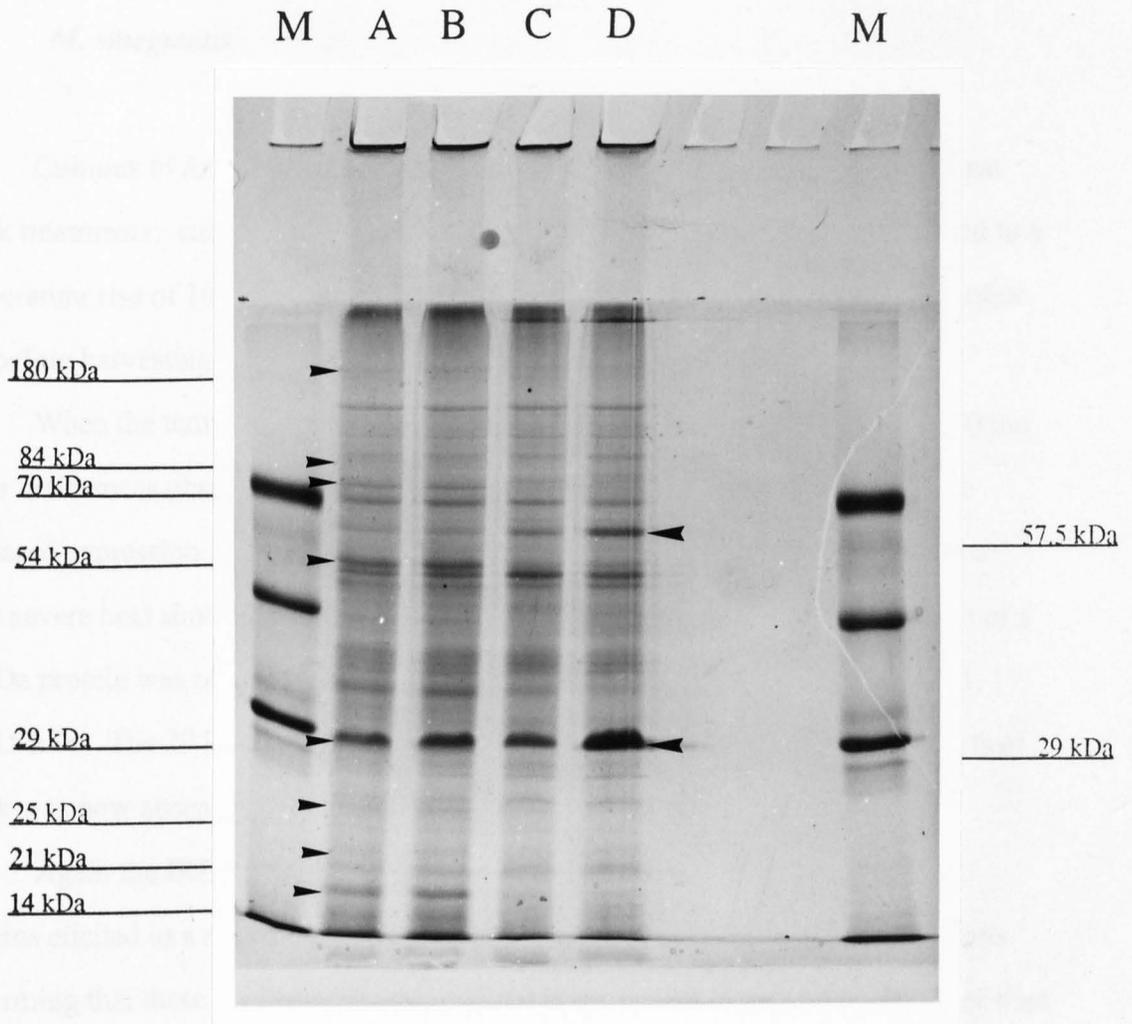
Figure 10. The effect of nutrient limitation upon envelope protein expression in _M. smegmatis_. SDS-PAGE analysis using 10% resolving gel of envelope extracts from _M. smegmatis_ NCIMB 8548 grown at 37°C and cultivated in:
  Lanes A&B iron limited medium (0.1µg Fe/ml added)
  Lane C   nutrient sufficient medium (2µg Fe/ml; 0.45µg Zn/ml added)
  Lane D   zinc-limited medium (2µg Fe/ml; no zinc added)
  Lanes M molecular size standards: 66, 37.5, 29 and 12.4 kDa
  100 µg protein was loaded in each lane.
The gel was visualised by Coomassie staining.

C.   The effect of heat shock upon the envelope protein profiles of
M. smegmatis

Cultures of *M. smegmatis* grown iron sufficiently were subjected to two heat shock treatments: cultures grown at 30°C and 37°C for 3 days were each subjected to a temperature rise of 10°C, to 40°C and 47°C respectively, and were grown for a further 24h before harvesting and envelope fractions were prepared.

When the temperature was raised from 30°C to 40°C (Fig. 11, Lanes A&B) the major differences observed in the envelope protein profile on SDS-PAGE were the increased expression of 16 and 29 kDa proteins. When cultures were subjected to a more severe heat shock (37°C to 47°C)(Fig. 11, Lanes C&D) increased expression of a 16 kDa protein was observed along with proteins of molecular weight 63, 48.5, 33, 17 and 15 kDa. The 29 kDa protein which had been highly expressed at 37°C before heat shock was now absent.

Again the IREPs recognised in this study did not correspond to any of the proteins elicited as a response to heat shock in the envelope fraction of *M. smegmatis* confirming that these proteins were expressed as a response to iron limitation rather than to general physiological stress.

D.   The expression of the 29 kDa protein.

The studies of Hall *et al.* (1987) and Sritharan (1988) identified a 29 kDa protein as an IREP. Incorporation of antisera raised against purified IREPs into ^{55}Fe(III) exochelin uptake assays demonstrated that only antisera raised against the 29 kDa protein caused the inhibition of ^{55}Fe accumulation.

In this study, however, the protein was seen to be expressed as the major envelope protein of the cell irrespective of the iron status of the culture (Fig. 10). The expression of the protein may also have been further increased during zinc limitation (Fig. 10) and after the less severe of the heat shock treatments (Fig 11).
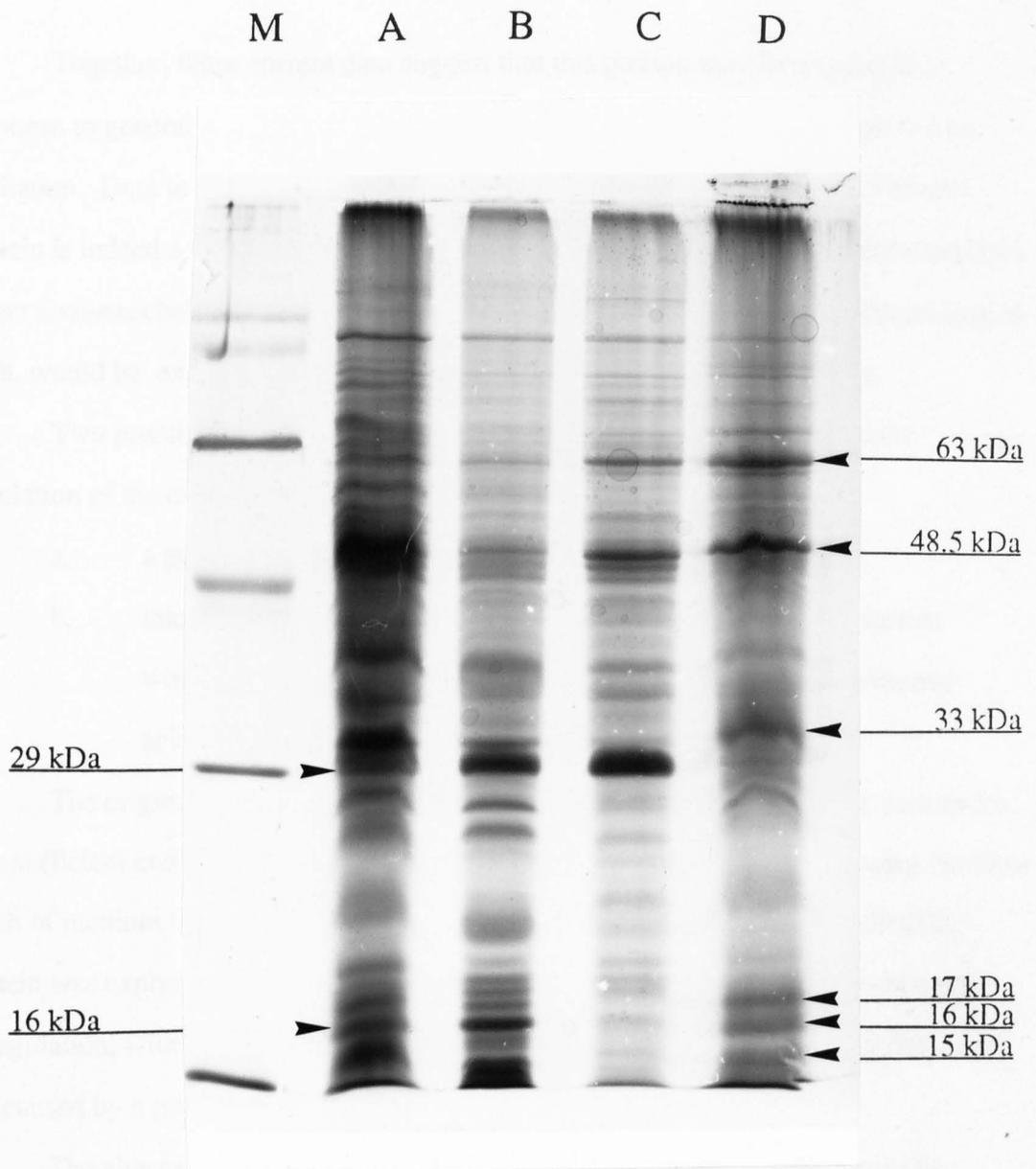
Figure 11. The effect of heat shock upon envelope protein expression in
M. smegmatis. SDS-PAGE analysis using 10% resolving gel of envelope
extracts from *M. smegmatis* NCIB 8548 cultivated in nutrient sufficient
medium for three days at initial temperature T_i and then moved to the shock
temperature, T_s, for a further 24h:
Heat shock 1: $T_i = 30°C$ (Lane A); $T_s = 37°C$ (Lane B)
Heat shock 2: $T_i = 40°C$ (Lane C); $T_s = 47°C$ (Lane D)
Lane M   molecular size standards: 205, 116, 97.6, 66, 45, 29 and 14 kDa
100 µg protein was loaded in each lane.
The gel was visualised by Coomassie staining.

Together, these current data suggest that this protein may be expressed as a response to general physiological stress rather than specifically as a response to iron limitation. Data to be presented later in this thesis, however, demonstrates that this protein is indeed a ferri-exochelin binding protein which appears to be a component of a larger ferri-exochelin receptor complex situated in the envelope of the bacterium and, as such, would be expected to be essential for exochelin-mediated iron uptake.

Two possibilities were considered to explain the apparent change in the regulation of the expression of this protein:

a.   a genetic mutation in an upstream regulatory sequence, or

b.   that the protein was involved in the acquisition of another nutrient which was now limiting in culture, possibly as a result of continued acid washing of laboratory glassware.

The original strain received from the NCIB culture collection was grown under iron sufficient and iron deficient conditions alongside the working isolate, using the same batch of medium. Analysis of the envelope extracts demonstrated that the 29 kDa protein was expressed as the major envelope protein in all cases. Thus, the apparent deregulation, with respect to iron status, of the 29 kDa IREP of Sritharan (1988) was not caused by a genetic mutation.

The alternative hypothesis was that the expression of this protein could be regulated not only by iron but also by another nutrient, possibly another metal. Nine other metals were added to iron sufficient medium separately and as a "cocktail" (Ba, Cd, Ca, Co, Cu, Cr, Ni, Mo, all at $0.2\ \mu M$ and V at $0.02\ \mu M$). After 6 days' growth, as before, the envelope protein profiles of the cultures were analysed by SDS-PAGE which revealed that the 29 kDa protein remained over expressed in all cases. The cause of this apparent deregulation of the 29 kDa protein was not pursued further due to the need to proceed with functional studies on the IREPs.

E.   Validity of the fractionation of cytoplasmic membrane and cell wall

fractions of *M. smegmatis* by differential centrifugation.

Sritharan used a differential centrifugation protocol in order to separate the envelope layers of mycobacteria in to cell wall and cytoplasmic membrane fractions (See section II.B.i). To assess the validity of this fractionation procedure, succinate dehydrogenase was assayed as a cytoplasmic membrane marker enzyme. Similar specific activities were present in both fractions (cyto. membrane, 8.7 nmol/mg/min; cell wall, 7.8 nmol/mg/min) suggesting that the differential centrifugation process was not separating fractions on the basis of their density but was merely depositing large fragments of envelope material in the first centrifugation step and the remainder in the 105,000g centrifugation step. The cellular locations of proteins predicted on the basis of this technique (Sritharan, 1988) were considered to be unproved. It was considered prudent, therefore, to continue this investigation using crude envelope fractions which would be more likely to contain all the necessary components for the formation of a stable ferri-exochelin/envelope protein complex.

F.   Summary

The changes in envelope protein expression observed as a result of the nutrient limitation and heat shock treatments presented in this chapter are summarised in Table 4. The IREPs recognised by previous studies are also included for comparison.

None of the IREPs recognised in this study corresponded to proteins that were elicited as a response to zinc limitation or the two heat shock regimes, suggesting that these are genuine IREPs and do not form part of a general stress response.

Previous studies, however, had not identified 70, 54 and 21 kDa IREPs in *M. smegmatis* (Hall *et al.*, 1987; Sritharan, 1988). A 21 kDa IREP was reported in *M. neoaurum, M. leprae* and *M. avium* (Sritharan and Ratledge, 1988, 1989, 1990), however. Although the two larger proteins were not identified as IREPs by Sritharan (1988) her study did provide evidence to suggest an association of these proteins with

the IREPs in *M. smegmatis*. Sritharan raised polyclonal antibodies against purified IREPs from *M. neoaurum* and these were used to probe Western blots of the envelope preparations of several iron-starved mycobacteria. When samples from *M. smegmatis*, *M. neoaurum*, *M. avium* and an Armadillo-derived mycobacterium were probed with antisera raised against 180, 120, 21 and 14 kDa, a 54 kDa protein was recognised in every case. When antisera raised against the 180 kDa IREP of *M. neoaurum* was used to probe Western blots of iron starved *M. smegmatis* envelope protein preparations then a 70 kDa protein was recognised (Sritharan, 1988). In this series of immuno-blotting experiments the only cross-reactions seen with IREP antisera were with other IREPs and these 70 and 54 kDa envelope proteins. Taken together with the identification of 70 and 54 kDa IREPs in this study it is possible to speculate that these cross reactions were the result of specific interactions. Common epitopes may be present in all of these proteins which may represent important structural or functional determinants.

It is also possible that these sequences have a common origin and that some of the proteins are the degradation products of a larger IREP. The 70 kDa protein was only recognised with antisera from the 180 kDa IREP (Sritharan, 1988) which may suggest that the 70 kDa protein is the product of the proteolysis of the larger molecule. The 54 kDa protein cross-reacted with antisera raised against all of the IREPs tested (180, 120, 21 and 14 kDa). It seems unlikely that all of these proteins are proteolytic cleavage products of the 180 kDa protein and therefore it is considered that the 54 kDa protein is a genuine IREP.

If the IREPs do contain similar sequences then this degree of conservation may reflect an essential functional or structural role. It is likely that, as putative iron transport proteins, some of the IREPs would be required to interact with each other in order to facilitate the binding and translocation of iron across the envelope layers of the cell. A conserved sequence may be expected to facilitate some of the protein interactions necessary for the uptake of iron. A precedent for this hypothesis does exist: a conserved sequence appears to be important as a site of the physical interactions that occur between TonB and its dependent receptor proteins in ferri-siderophore transport in the

Table 4. Summary of the proteins elicited as a response to iron limitation, zinc limitation and heat shock in *M. smegmatis*.

Protein size kDa				
Iron limitation: This study	Iron limitation: Sritharan (1988)	Zinc limitation:	Heat shock: 30°C→40°C	Heat shock: 37°C→47°C
180	180			63
84	84			48.5
70		57.5		33
54				
	29	29	29	
25	25			
21				
				17
			16	16
				15
14	14			

Enterobacteriaceae (Skare et al., 1993). However, in the absence of amino acid sequence data for the IREPs then this area is entirely speculative.

The major discrepancy between the IREP repertoires observed in this and previous studies is the expression of the 29 kDa envelope protein. In this study the 29 kDa envelope protein was not only expressed constitutively but was also the major envelope protein of the bacterium. In the study of Sritharan (1988) the expression of the protein was strictly regulated by iron availability. Although a genetic mutation does not appear to be the basis of this apparent deregulation the cause of this discrepancy remains unresolved. Evidence presented later in this thesis, however, demonstrates a role for this 29 kDa protein in the ferri-exochelin uptake process as a ferri-exochelin binding protein. It appears that the regulation of expression of this presumably vital component of the ferri-exochelin uptake system is more complex than that for the other IREPs.

IV The formation and analysis of ferri-exochelin-envelope protein complexes using CHAPS-solubilised proteins

A. Confirmation of the biological activity of purified ferri-exochelin MS.

The identification and isolation of a ferri-exochelin receptor protein would rely upon the formation of a stable ferri-exochelin-envelope protein complex and the development of a suitable suite of techniques to detect and analyse the complex(es).

A pure, biologically active, radio-labelled ferri-exochelin preparation was required to form the complexes. A new purification protocol had been developed in this laboratory which produced ferri-exochelin MS at > 98% purity (see Section II.D.1). The iron of this ferri-exochelin MS was removed by washing with desferri-mycobactin and was replaced with ^{55}Fe. The biological activity of this labelled siderophore could now be assessed by the analysis of its uptake into a washed suspension of iron-starved *M. smegmatis*. The kinetics of uptake (Fig. 12) were in accordance with those reported by Stephenson and Ratledge (1979): iron accumulation occuring at a rapid initial rate which decreased after approximately 10 min when 150 nmol Fe / g dry weight had been accumulated. Similarly, the uptake of ^{55}Fe from ferri-exochelin was inhibited by the pre-incubation of the cell suspension with 30 mM NaN_3 as was expected of this energy-dependent uptake process (Fig. 12).

B. Resolution of envelope proteins by CHAPS-PAGE.

In order to preserve the activity of the envelope proteins during electrophoretic separations the non-denaturing polyacrylamide gel electrophoresis system of Cavinato *et al.* (1988) was used in which the non-denaturing detergent CHAPS, a zwitterionic bile salt derivative, stabilises the hydrophobic proteins in solution. Proteins were extracted from the envelope of *M. smegmatis* using 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH, pH7.1 and were then separated on a 10 % CHAPS-PAGE resolving gel. The resolution acheived using this system was not ideal, the mobility of samples in the gels

□ ^{55}Fe(III) exochelin uptake in iron starved cells

O ^{55}Fe(III) exochelin uptake in azide-treated iron starved c
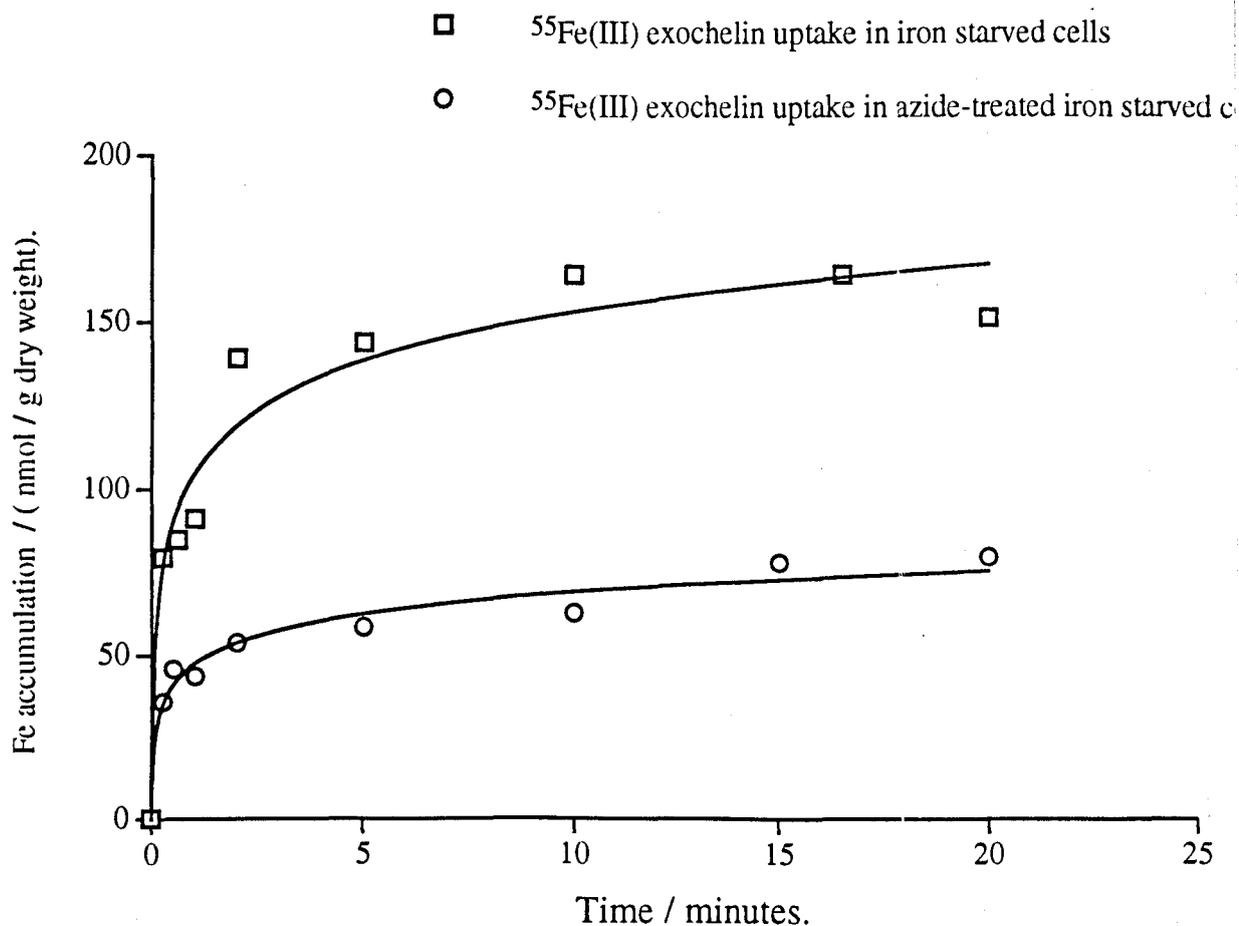
Figure 12.   The uptake of ^{55}Fe from ferri-exochelin by iron-starved
M. smegmatis. The uptake of ^{55}Fe from ferri-exochelin by a washed
suspension of iron-starved *M. smegmatis* was monitored. In each assay,
at time t=0, ^{55}Fe(III)exochelin (Specific activity 2947 Bq / nmol) was
added to a 10 ml suspension (2.35 mg dry weight / ml) at a final
concentration of 1μM. Duplicate 0.5 ml samples were taken at intervals
from the assay mixture, filtered, washed on the filter and lysed. The
lysed cells were suspended in Ecoscint A scintillation fluid and were
then analysed for ^{55}Fe by scintillation counting. The effect of pre-
incubation of the suspension with NaN_3 at 30 mM for 10 min on the
uptake of ^{55}Fe(III)exochelin by a similar suspension from the same
preparation of *M. smegmatis* was also determined. See Section II.D.5.
for protocol.

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was poor (Fig. 13). After silver staining, distinct bands were visible when $R_f > 0.47$ but were indistinguishable when $R_f < 0.47$. The majority of the loaded protein remained unresolved near the boundary between the stacking and resolving gels, presumably as a result of the aggregation and precipitation of the proteins in the gel system. The concentration of CHAPS in the reservoir buffer was increased 10- and 100-fold but this did not result in improved resolution of proteins.

It was considered that the extent of precipitation of protein observed within this gel system rendered it unsuitable for the analysis of the expression of iron-regulated proteins. As some resolution of proteins was achieved at $R_f > 0.47$, it was felt that the CHAPS-PAGE system probably represented the best available system to separate bound and unbound [55]Fe(III)exochelin in envelope protein labelling experiments.

C.   Analysis of ferri-exochelin-binding activity in native envelope.

It was judged that the ferri-exochelin-binding activity of the envelope proteins would be greatest in the native envelope and that extraction with detergents would inevitably cause conformational changes in the proteins which would be likely to diminish this activity. The first attempts made to identify a ferri-exochelin receptor complex, therefore, were carried out using a native envelope preparation. It was likely, however, that a native envelope fraction from iron-starved cells would contain desferri-mycobactin, which could remove the [55]Fe from radio-labelled ferri-exochelin thereby compromising the sensitivity of the system.

To counteract the possible interference by mycobactin, an envelope sample containing 100 μg protein which was to be labelled was pre-incubated with 5 μl 0.1 mM ferri-salicylate for 10 minutes at ambient temperature in order to saturate any desferri-mycobactin with iron. The labelling reaction was then carried out at ambient temperature for 20 min with 50 nmol [55]Fe(III)exochelin (8332 Bq/nmol). The envelope proteins were extracted from the envelope preparation with 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH, pH 7.1. The non-solubilised material was sedimented by centrifugation (105,000 x g, 30 min, 4°C) and the solubilised proteins were then

A   B   M

$R_f = 0.47$ ————➤
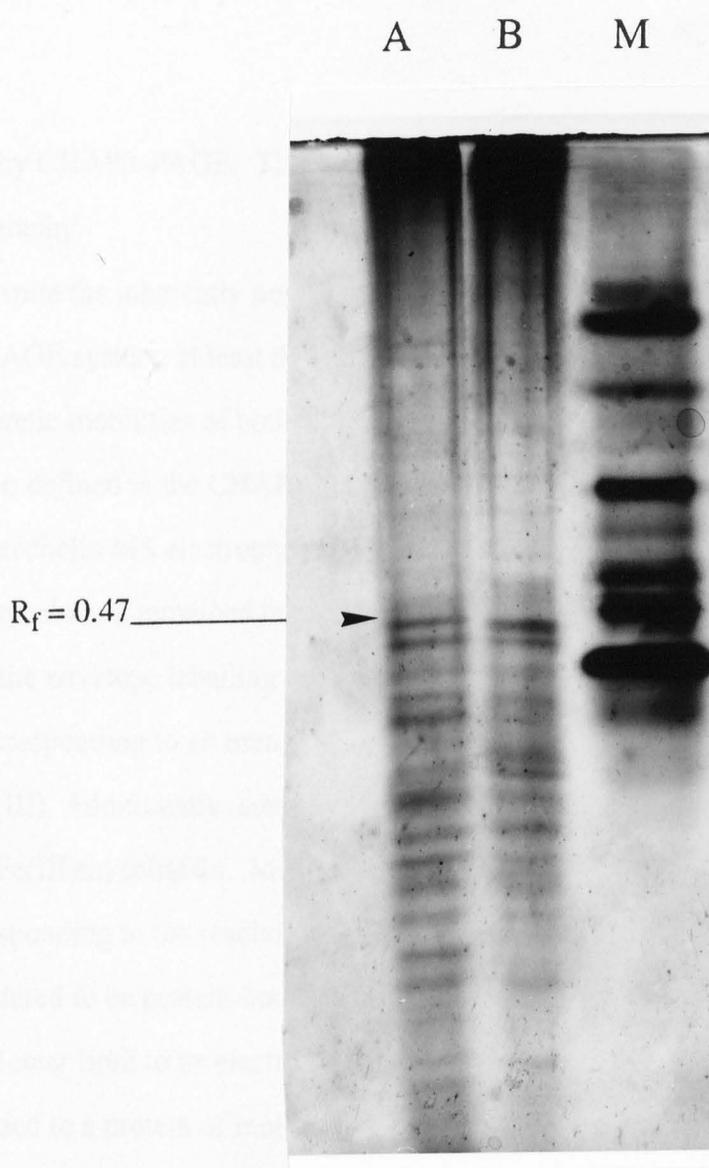
Figure 13. The separation of envelope proteins from *M. smegmatis* by CHAPS-PAGE. A cell envelope fraction of *M. smegmatis* was prepared by sonication and was separated from intact cells and the cytoplasmic fraction by differential centrifugation. The envelope fraction was incubated for 16 hours at 4°C with 8 mM CHAPS in buffer to extract protein. Soluble and insoluble material were then separated by centrifugation (105,000 x g, 30 min, 4°C). The soluble extract was then analysed by CHAPS-PAGE chromatography on a gel (14 x 16 cm) with composition 10 %T at 35 mA constant current for 2-3 hours. The gel was then fixed and visualised by silver staining.
Lane A, 100 µg extracted envelope protein from iron-starved cells;
Lane B, 100 µg extracted envelope protein from iron-replete cells.
Lane M, Molecular size standards.

separated by CHAPS-PAGE. The gel was dried under vacuum and then visualised by autoradiography.

Despite the inherently poor resolution of *M. smegmatis* envelope proteins in the CHAPS-PAGE system, at least three ^{55}Fe species were separated (Fig. 14). The electrophoretic mobilities of both ^{55}Fe(III)exochelin and ^{55}Fe(III)mycobactin had earlier been defined in the CHAPS-PAGE system: as anticipated, the soluble ^{55}Fe(III)exochelin MS electrophoresed freely at $R_f = 1$ and the water insoluble ^{55}Fe(III)mycobactin remained in the sample loading well.

In the envelope labelling experiment, a small amount of ^{55}Fe was observed at $R_f = 1$, corresponding to an orange band on the original gel, indicative of free ferri-exochelin(III). Additionally, some ^{55}Fe remained in the sample loading well suggesting it to be ^{55}Fe(III)mycobactin. Most of the ^{55}Fe, however, appeared as a streak in the area corresponding to the resolving gel and, as this had not electrophoresed freely, it was considered to be protein-bound ^{55}Fe-exochelin. This protein-bound ^{55}Fe exhibited a definite lower limit to its electrophoretic mobility at $R_f = 0.56$ (Fig. 14) which corresponded to a protein of molecular mass 29 kDa. It is considered that this result demonstrated a ^{55}Fe(III)exochelin-binding activity resident on a 29 kDa envelope protein of limited solubility in the CHAPS-PAGE system and that the aggregation and precipitation of this protein, with bound ^{55}Fe, caused the streaking observed on the autoradiograph.

The outer membrane ferri-siderophore receptors of the Enterobacteriaceae mostly fall within the size range 70-80 kDa (See Table 3). It was thought, therefore, that a 29 kDa protein acting as a monomer may have been too small to mediate the transport of ferri-exochelin across the outer membrane of the cell and that this protein may form part of a complex which was necessary to conduct ferri-exochelin transport. It is possible that complexes, larger and more elaborate in composition than this putative ferri-exochelin-29 kDa receptor complex, may have been formed during the labelling reaction and that detection of these was hindered by the obscufation of detail in the sample lane at $R_f < 0.56$ caused by this protein precipitation.
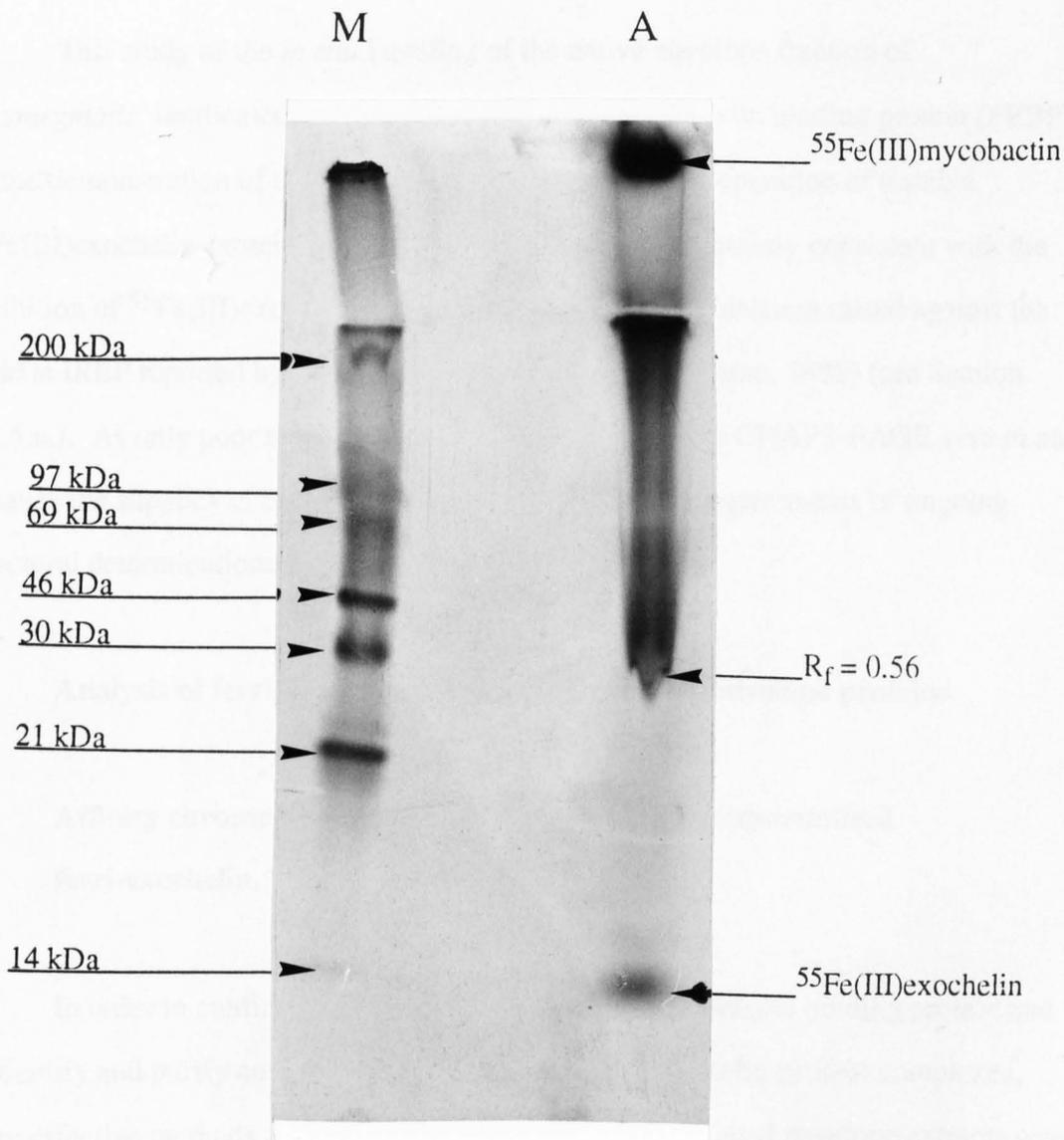
Figure 14. Autoradiograph of ^{55}Fe(III)exochelin-labelled envelope proteins separated by CHAPS-PAGE. A ferri-exochelin-receptor protein complex was formed by incubation of a sample of native envelope, containing 100 μg protein, with ^{55}Fe(III)exochelin (8332 Bq/nmol). The complex was then extracted using 8 mM CHAPS for 16 hours at 4°C. The complex was separated by CHAPS-PAGE on a 10 % resolving gel at 35 mA for 2-3 hours. The gel was then dried and visualised by autoradiography. Lane A, ^{55}Fe(III)exochelin envelope complex; Lane M, ^{14}C Molecular weight marker proteins.

This study of the *in situ* labelling of the native envelope fraction of
M. smegmatis implicated a 29 kDa protein as a ferri-exochelin binding protein (FEBP)
by the demonstration of the formation and electrophoretic separation of a stable
^{55}Fe(III)exochelin-protein complex. This observation is certainly consistent with the
inhibition of ^{55}Fe(III)exochelin uptake in *M. smegmatis* by antisera raised against the
29 kDa IREP reported by Sritharan (Hall *et al.*, 1987; Sritharan, 1988) (see Section
I.G.5.a.). As only poor resolution could be gained using the CHAPS-PAGE system and
because the supplies of exochelin were limited due to the requirements of ongoing
structural determinations this experiment was not repeated.

D.   Analysis of ferri-exochelin-binding by extracted envelope proteins.

**1.   Affinity chromatography of envelope proteins on immobilised
         ferri-exochelin.**

In order to confirm the 29 kDa protein as a ferri-exochelin binding protein and
to identify and purify any larger, more elaborate ferri-exochelin-protein complexes,
more effective methods than CHAPS-PAGE analysis of labelled envelope extracts were
necessary. Affinity chromatography on ferri-exochelin-Sepharose 4B and anion
exchange chromatography on polyethyleneimine-cellulose, both utilising CHAPS-
solubilised envelope proteins from iron-starved *M. smegmatis*, were then used.

**a.   Affinity elution of proteins from ferri-exochelin-Sepharose with
         ferri-exochelin.**

Affinity chromatography appeared to offer a powerful method for the formation
and analysis of ferri-exochelin receptor complexes. Ferri-exochelin, although the
structure was at the time only partially resolved (see Chapter VI), was thought to
contain a terminal amino group. This group, it was thought, could be used to bind the

molecule to the activated Sepharose support (see Section II.E). To decrease the effect of steric hindrance upon the binding of the immobilised ferri-exochelin by envelope proteins a support with a C_6 spacer arm (6-aminohexanoic acid N-hydroxysuccinimide ester-Sepharose 4B) was used. The ferri-exochelin MS was covalently bound to the support material. This binding, its implications and application to the study of the structure of the ferri-exochelin molecule are discussed further in Chapter VI. In the event, exochelin MS proved to have three free NH_2 groups (Sharman *et al.*, 1995).

CHAPS-solubilised envelope proteins could be loaded onto the column and, as long as the amino group used to form the covalent linkage to the support was not essential for interaction with a receptor, an immobilised ferri-exochelin receptor protein complex could be formed. Extensive washing of the support could then be used to remove non-binding proteins and the conditions for the elution of the remaining protein(s) could be studied.

Samples, 1 mg of CHAPS-solubilised envelope proteins from both iron-starved and iron-replete cells, were passed through separate ferri-exochelin-Sepharose 4B affinity columns (0.4 ml bed volume). The flow of each sample was halted for 90 min when the sample had completely entered the matrix to maximise binding. Non-binding proteins were then eluted by washing the columns with 10 bed volumes of 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH, pH 7.1. Analyses of the washes by SDS-PAGE demonstrated that all non-binding proteins had been eluted from the column by this washing protocol.

The columns were then washed with 0.5 ml 1mM ferri-exochelin in 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH, pH 7.1. The flow of eluent was stopped for 30 minutes before a further 0.5 ml of the same eluent was applied to the columns. All eluates were lyophilised in order to concentrate the protein and were analysed by SDS-PAGE.

Of the proteins eluted with ferri-exochelin (Fig. 15), only the 25 kDa protein corresponded to the size of a previously reported IREP in *M. smegmatis*. The other proteins present in these eluates did not coincide with the molecular sizes of previously
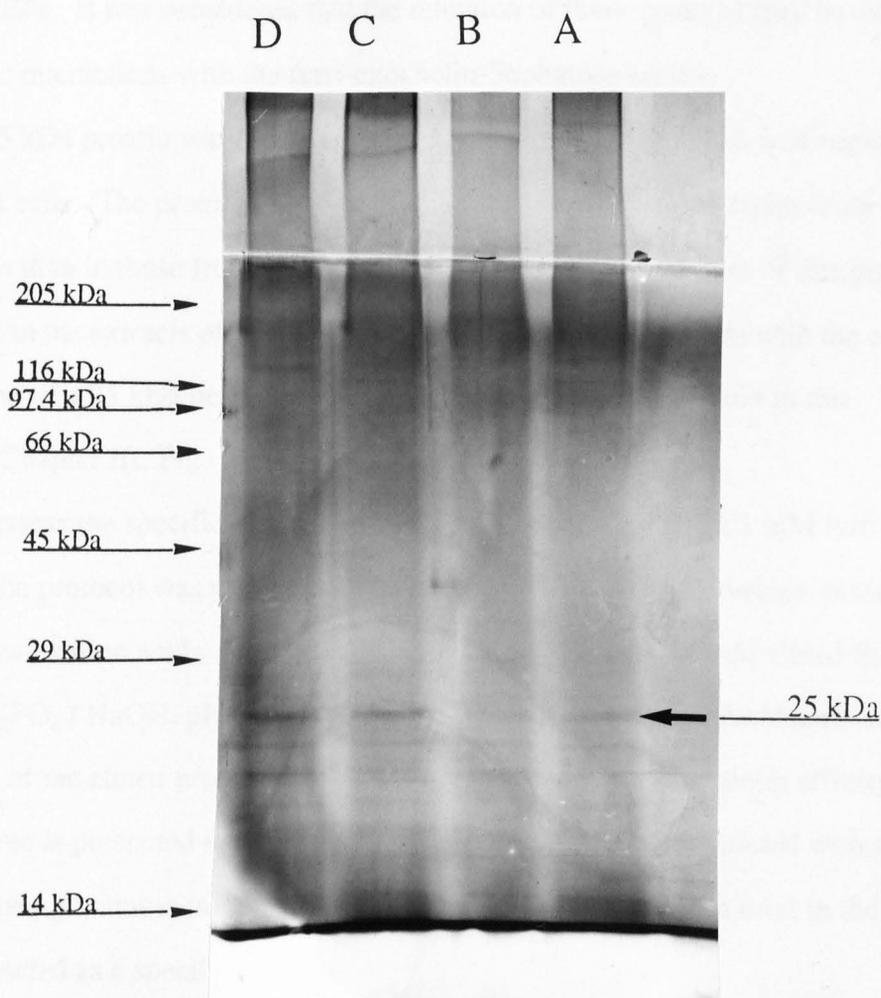
Figure 15. **Affinity elution of bound *M. smegmatis* envelope proteins from ferri-exochelin-Sepharose using 1 mM ferri-exochelin.**

Protein samples (1 mg) extracted with CHAPS from the cell envelopes of iron-starved and iron-replete *M.smegmatis* were loaded onto ferri-exochelin-Sepharose columns. After the removal of non-binding proteins by washing with buffered CHAPS, 1mM ferri-exochelin was used as an affinity eluent.

Lane A First affinity elution of samples from iron-replete cells.

Lane B Second affinity elution samples from iron-replete cells.

Lane C. First affinity elution of samples from iron-starved cells.

Lane D Second affinity elution of samples from iron-starved cells.

The gel was visualised by silver staining.

reported IREPs. It was considered that the retention of these proteins may be caused by non-specific interactions with the ferri-exochelin-Sepharose matrix.

A 25 kDa protein was detected in affinity eluates derived from iron-replete and iron starved cells. The protein band stained more intensely in the samples from iron-starved cells than in those from iron-replete cells suggesting that more of this protein was present in the extracts of the iron-starved cells. This is consistent with the earlier identification of a 25 kDa protein as an iron-regulated envelope protein in this bacterium (Chapter III; Fig. 11).

To assess the specificity of the elution of the 25 kDa IREP in 1 mM ferri-exochelin, the protocol was repeated using an aliquot of the same envelope extract and a mixture of two amino acids, ornithine and glutamine at 1 mM in 8 mM CHAPS; 50 mM KH_2PO_4 / NaOH, pH 7.1, in place of the ferri-exochelin as the eluent. A comparison of the eluted proteins detected after this and a ferri-exochelin affinity elution regime is presented in Table 5. The 25 kDa protein was not eluted with the 1 mM ornithine / glutamine mixture suggesting that the ferri-exochelin used in the earlier experiment acted as a specific eluent (Fig. 16).

As the elution of the 25 kDa IREP with ferri-exochelin appeared to be specific, the nature of the eluate was investigated by using radio-labelled ferri-exochelin and analysing the elution of radioactive species by gel filtration chromatography. With another identical preparation 1 mM $^{59}Fe(III)$-exochelin in phosphate-buffered CHAPS was used as an eluent. Subsequent analysis of the eluate by gel filtration on Sephadex G100-superfine revealed that the elution profile was identical to that previously determined for free ferri-exochelin (Fig 17). Thus, no stable ferri-exochelin-protein complexes were formed during this elution. A small radioactive peak did elute in the void volume in both profiles but this contained no detectable protein and could, therefore, have corresponded to a trace of colloidal iron. These results were confirmed by repeating the protocol a further two times.

The lack of a stable association between the 25 kDa IREP and ferri-exochelin was also compatible with the result demonstrated in Fig 14 in which the limit of mobility of bound ^{55}Fe in the CHAPS-PAGE system, corresponding to the smallest

A B C D M

116 kDa

66 kDa

47 kDa

45 kDa

39 kDa

33.5 kDa

29 kDa

14 kDa
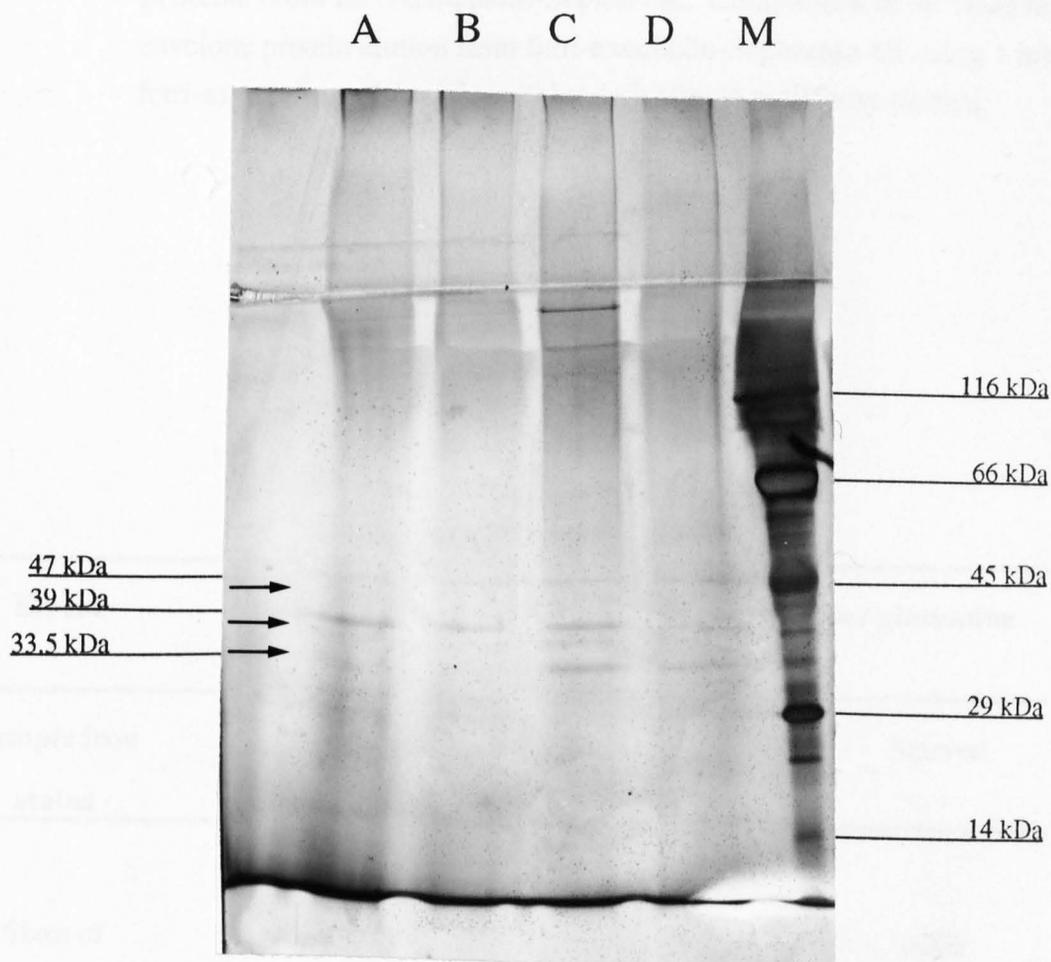
Figure 16. **The elution of bound envelope proteins from ferri-exochelin-Sepharose using an amino acid mixture as an affinity eluent.**

Protein samples (1mg) extracted with CHAPS from the cell envelopes of iron-starved and iron-replete *M.smegmatis* were loaded onto ferri-exochelin-Sepharose columns. After the removal of non-binding proteins by washing with buffered CHAPS 1mM ornithine/glutamate was used as an affinity eluent.

Lane A First elution of samples from iron-replete cells.

Lane B Second elution samples from iron-replete cells.

Lane C. First elution of samples from iron-starved cells.

Lane D Second elution of samples from iron-starved cells.

Lane M Molecular size standards.

The gel was visualised by silver staining.

92

Table 5. Specificity of ferri-exochelin elution of *M. smegmatis* envelope proteins from ferri-exochelin-Sepharose. Comparison of *M. smegmatis* envelope protein elution from ferri-exochelin-Sepharose 4B using 1 mM ferri-exochelin and 1 mM ornithine / glutamine as affinity eluents.

Eluent	1 mM ferri-exochelin		1mM ornithine / glutamine	
Sample iron status	Replete	Starved	Replete	Starved
Sizes of	25	25		>205
eluted	22	22	47	47
proteins	19	19	39	39
(kDa)		18		37
				33.5

Figure 17 Size determination of ^{59}Fe species in an affinity eluate from an
envelope protein-loaded ferri-exochelin-Sepharose column by gel
filtration chromatography. CHAPS extracted envelope proteins derived
from iron-starved cultures of *M. smegmatis* were loaded onto ferri-
exochelin-Sepharose. 1 mM [59]Fe(III)exochelin was used as an eluent and
the resulting eluate was separated on Sephadex G-100-superfine, eluting
with buffered CHAPs and collecting 0.6 ml fractions. 100 µl samples of
each fraction were taken for liquid scintillation counting.

A B C D M

29 kDa

25 kDa
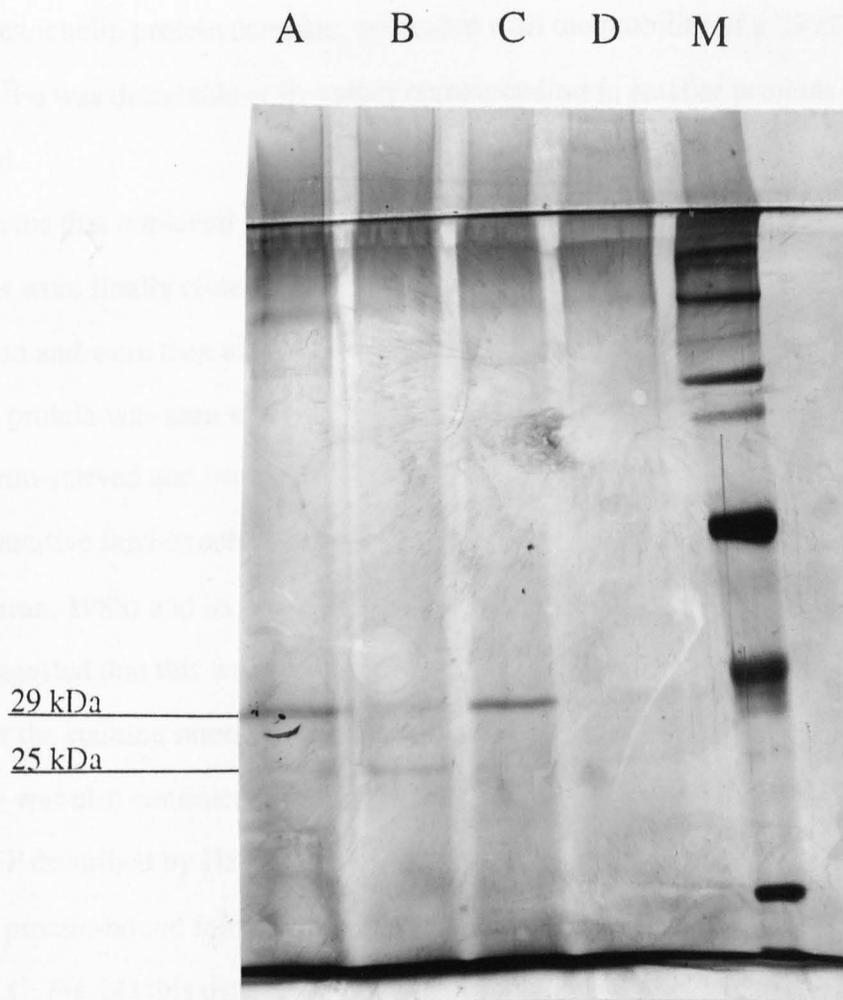
Figure 18.   The elution of the remaining, bound protein from ferri-exochelin-Sepharose after affinity elution.

Protein samples (1 mg) extracted with CHAPS from the cell envelopes of iron-starved and iron-replete *M. smegmatis* were loaded onto ferri-exochelin-Sepharose columns. After the removal of non-binding proteins and further proteins by affinity elution with 1 mM ferri-exochelin, 2% SDS was used to remove the remaining proteins.

Lane A   First elution with 2% SDS of iron-starved samples.

Lane B   Second elution with 2% SDS of iron-starved samples.

Lane C. First elution with 2% SDS of iron-replete samples.

Lane D   Second elution with 2% SDS of iron-replete samples.

Lane M   Molecular weight markers.

The gel was visualised by silver staining.

stable ferri-exochelin protein complex, coincided with the mobility of a 29 kDa protein. No bound ^{55}Fe was detectable at R_f values corresponding to smaller proteins (i.e. a 25 kDa protein).

Proteins that remained bound to the affinity column after the ferri-exochelin elution steps were finally eluted using 2% SDS. The eluates were concentrated by lyophilisation and were then analysed by SDS-PAGE (Fig. 18). A band corresponding to a 29 kDa protein was seen which stained to a similar intensity in samples derived from both iron-starved and iron-replete cells. The size of this protein corresponded to that of the putative ferri-exochelin receptor protein proposed by Sritharan (Hall *et al.*, 1987; Sritharan, 1988) and its retention on the ferri-exochelin-Sepharose column strongly suggested that this was a ferri-exochelin binding protein (FEBP). The similarity of the staining intensity of the band in samples from iron-starved and iron-replete cells was also consistent with the apparent de-regulation (Chapter III) of the 29 kDa IREP described by Hall *et al.* (1987). Together with the limit of electrophoretic mobility of protein-bound ferri-exochelin observed in the CHAPS-PAGE system (Section IV.C, Fig 14) this data represented compelling evidence that the 29 kDa envelope protein of *M. smegmatis* was a ferri-exochelin binding protein.

The 25 kDa IREP was also observed in the SDS eluates of samples from iron-starved cells demonstrating that this protein was only partially eluted from the matrix with 1 mM ferri-exochelin. As the elution of this protein with 1 mM ferri-exochelin appeared to be specific, then the absence of a stable radio-labelled ferri-exochelin-IREP complex on gel filtration of the affinity eluate, may suggest that the 25 kDa protein can only associate with ferri-exochelin in the presence of the 29 kDa FEBP or that the smaller protein formed no direct association with the ferri-siderophore but was retained on the ferri-exochelin MS-Sepharose via an interaction with the 29 kDa protein.

A few other bands were seen on these gels which were not observed consistently on repetition of the protocols (n=3).

b.   Purification of a 29 kDa Ferri-exochelin binding protein by affinity chromatography.

Unsuccessful attempts had been made to elute the 29 kDa FEBP from ferri-exochelin-Sepharose by affinity elution using ferri-exochelin. However, purification of ferri-exochelin MS was time consuming and yields from *M. smegmatis* were relatively low (~ 2 mg / l) and, as structural studies on the molecule were still in progress, supplies of the siderophore were limited at this time. It was not feasible to increase the concentration of ferri-exochelin above the 1 mM already used. Therefore, the release of the 29 kDa FEBP from the immobilised siderophore matrix was attempted using 1 mM ferri-exochelin MS in 0, 0.1, 0.5 and 1 M NaCl as it was considered that the increasing ionic strength of the buffer would remove proteins which had been retained on the column by non-specific interactions with the ferri-exochelin-Sepharose matrix.

A sample containing 1 mg CHAPS-solubilised envelope proteins from iron-starved *M. smegmatis* was loaded onto the column and, after washing away non-binding proteins with phosphate-buffered CHAPS, the eluents were applied to the column in a series of ascending NaCl concentration (2 x 0.5 ml washes of each concentration of NaCl were added and then combined for analysis). The eluates from these washes were lyophilised and analysed by SDS-PAGE (Fig. 19). The 29 kDa FEBP was not eluted with 1 mM ferri-exochelin in 1M NaCl and still required 2% SDS for elution from the immobilised ligand. No proteins were detected by silver staining in the lanes corresponding to the 1 mM ferri-exochelin / NaCl washes; presumably these proteins were eluted gradually throughout the scheme and were present at concentrations below the limits of detection of the silver stain. The 29 kDa protein was now the only protein detected in the final SDS eluate confirming that this had been the last protein released from the ferri-exochelin affinity matrix and presumably, therefore is the major ferri-exochelin binding protein of *M. smegmatis*. The retention of this protein on the affinity column despite stringent washing regimes suggests that the FEBP exhibits a strong affinity for ferri-exochelin MS which is consistent with its perceived role as a ferri-siderophore receptor protein and presumably an impractically high concentration of the
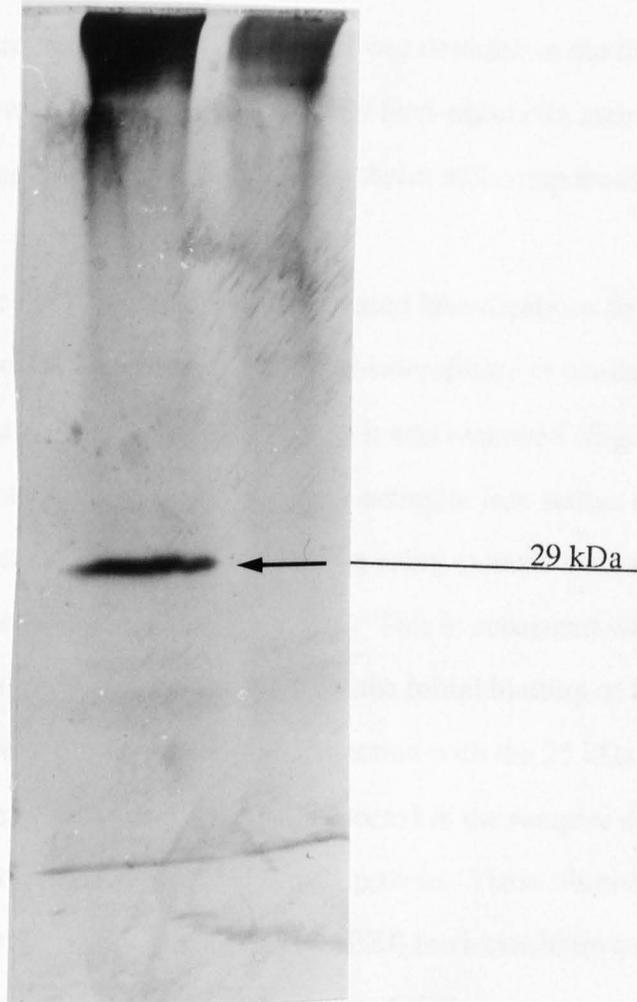
29 kDa

Figure 19. **Purification of the 29 kDa ferri-exochelin binding protein by affinity chromatography on ferri-exochelin-Sepharose.**

1 mg CHAPS-solubilised envelope protein, from iron-starved *M. smegmatis*, were loaded onto ferri-exochelin-Sepharose and washed with 1 mM ferri-exochelin in buffered CHAPS with up to 1M NaCl. The remaining protein was eluted using 2 % SDS. The SDS-eluate was dialysed, concentrated by lyophilisation and then runwere on a 10% SDS-PAGE gel which was visualised by silver staining.

ferri-exochelin MS would be required to effect the affinity elution of the protein from the column.

To confirm that the retention of the 29 kDa FEBP on ferri-exochelin-Sepharose was mediated by a specific interaction between the protein and the immobilised ferri-siderophore an identical extract was loaded onto a Tris-Sepharose matrix and the protocol was repeated exactly. In this case no FEBP was detected in the final SDS-eluate suggesting that its retention on the immobilised ferri-exochelin matrix was facilitated by a specific interaction with the ferri-exochelin MS component of the matrix.

In summary, these affinity chromatography-based investigations demonstrated that the 29 kDa FEBP bound to the immobilised ferri-siderophore in similar amounts irrespective of the iron status of the cells from which it was extracted (Fig. 18). The binding of the 25 kDa protein, however, did vary according to iron status: more protein was retained on the ferri-exochelin-Sepharose column using extracts from iron-starved cells than when using extracts from iron-replete cells. This is consistent with the identification of a 25 kDa IREP in this bacterium. If the initial binding of ferri-exochelin by the FEBP was dependent upon an interaction with the 25 kDa IREP, then a decrease in the retention of the FEBP would be expected in the samples derived from iron-replete cells which contained less of the smaller protein. These observations, therefore, suggest that the formation of the 29 kDa FEBP-ferri-exochelin complex *in vitro* is not dependent upon the presence of the 25 kDa IREP.

These data demonstrated that the 29 kDa FEBP was able to remain firmly attached to the ferri-exochelin-Sepharose affinity matrix after all other proteins had been removed and may also suggest that the 29 kDa-ferri-exochelin complex was formed without the need for further interaction with other IREPs. Analysis of a radio-labelled affinity eluate (^{59}Fe(III)exochelin as eluent) by gel filtration demonstrated that the 25 kDa protein did not bind ferri-exochelin MS in the absence of the FEBP and may suggest that the smaller protein interacts with the FEBP rather than with the ferri-siderophore.

2. Implication of the 25 and 29 kDa proteins as siderophore-receptor complex components using ion-exchange chromatography.

Samples (2 mg) of CHAPS-solubilised envelope proteins, from iron-starved cells, were incubated with 50 nmol ^{55}Fe(III)-exochelin (8332 Bq/nmol, 37°C, 30 min). The entire labelling mixture was then loaded onto a polyethyleneimine (PEI-)-cellulose anion exchange column equilibrated at pH 7.5. The column was initially washed with 8 mM CHAPS in 50 mM KH_2PO_4 / NaOH, pH 7.5, to remove free ferri-exochelin, which is cationic at this pH and therefore eluted in the void volume. Retained material was eluted with a stepped series of 0.1 M to 1 M NaCl collecting 650 µl fractions. ^{55}Fe in each fraction was quantified by the scintillation counting of a 100 µl sample.

Three radioactive peaks were observed in the elution profile (Fig. 21). The quantification of protein in each fraction revealed that Peak II exhibited the highest specific activity per unit protein (Table 6).

The proteins eluted in the fractions corresponding to the peaks of ^{55}Fe elution from the PEI-cellulose were analysed by SDS-PAGE (Fig. 22)(summarised in Table 6). Of the proteins detected in these experiments, only the 25 and 29 kDa proteins of Peak II correspond to sizes of IREPs in *M. smegmatis* (Chapter III). The elution of ^{55}Fe within peaks I & III was not investigated further.

Analysis of this Peak II eluate by gel filtration on Sephadex G100-superfine was carried out. Fractions (2 ml) of the eluate from the gel filtration column were collected
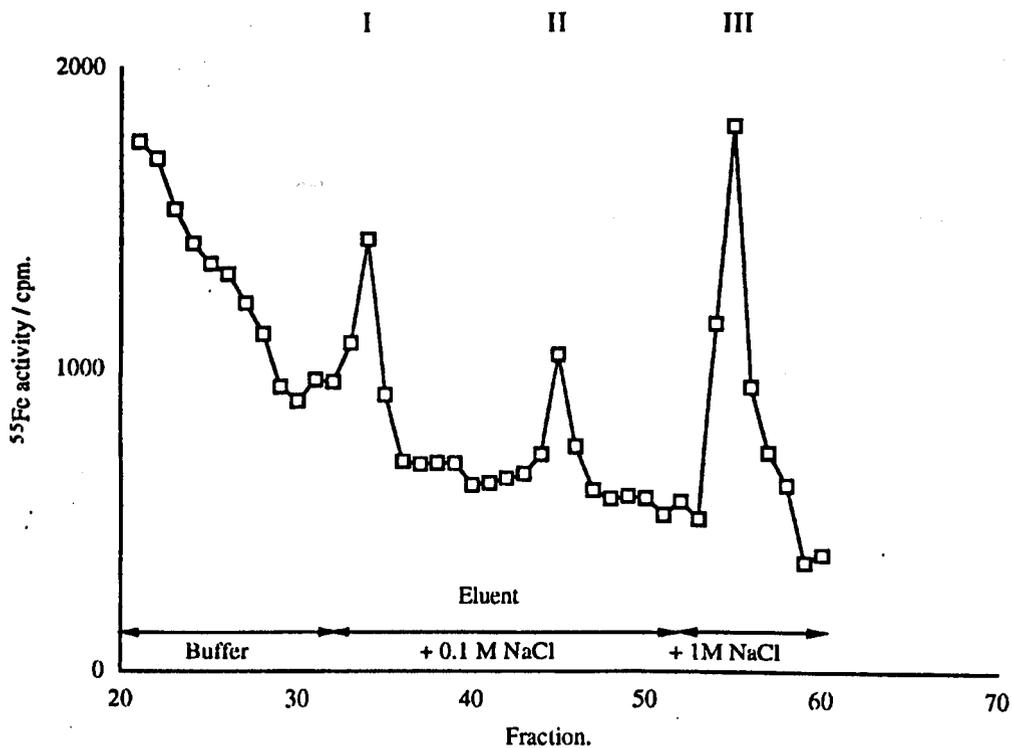
Figure 21. **The separation of ^{55}Fe-labelled envelope proteins by anion exchange chromatography on PEI-cellulose.**

CHAPS-extracted envelope proteins were incubated at 37°C for 30 min with 50 nmol ^{55}Fe(III)exochelin (8332 Bq/nmol) before application to the anion exchange column. Unbound ferri-exochelin was eluted from the column in the void volume. 0.1M and 1M NaCl, in buffer, were used to elute labelled proteins from the PEI-cellulose. 1 ml fractions were collected and ^{55}Fe elution was quantified by scintillation counting of a 100 µl sample from each fraction.
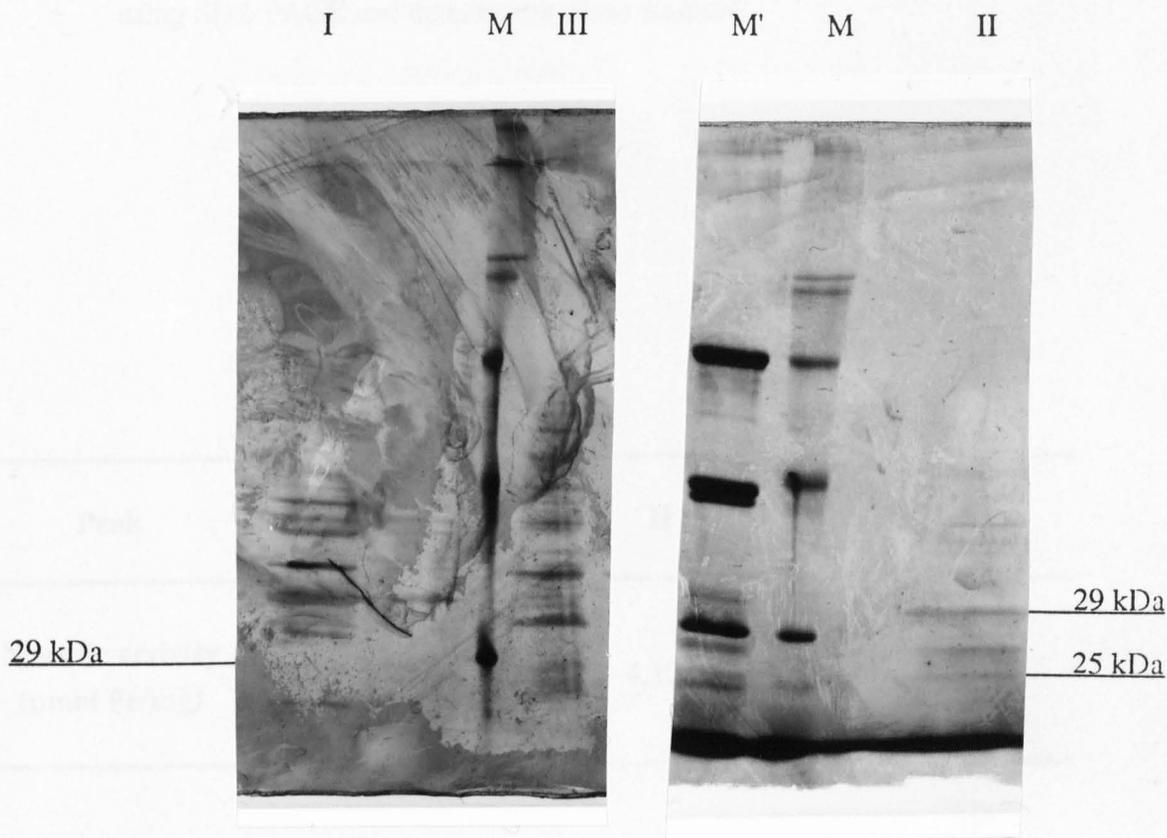
Figure 22. Proteins co-eluted with ^{55}Fe in a step gradient of NaCl from PEI-cellulose.

^{55}Fe(III)exochelin labelled, CHAPS-solubilised, envelope proteins from iron-starved *M. smegmatis* were retained by PEI-cellulose. 0.1M and 1M NaCl, in buffer, were used to elute labelled proteins from the PEI-cellulose. The proteins in the fractions of three radioactive peaks were lyophilised and analysed by SDS-PAGE. The gels were silver stained.

Lane I   eluate peak I

Lane II   eluate peak II

Lane III eluate peak III

Lane M   Molecular size standards: 205, 116, 97.4, 66, 45, 29 and 14 kDa

Lane M´ Molecular size standards: 66, 37.5, 29 and 12.4 kDa

Table 6. Proteins co-eluted with ^{55}Fe in a step gradient of NaCl from PEI-cellulose. The fractions of eluate from the anion exchange column corresponding to Peaks I, II and III (Fig. 21) were lyophilised, separated using SDS-PAGE and detected by silver staining.

Peak	I		II	III	
Specific activity (nmol Fe/mg)	2.692		4.129	0.166	
	43	31	46	76	44
Sizes of	41	30	39	67	41
co-eluting	39	27	29	66	39
proteins	38		26	60	37
(kDa)	36		25	52	35
	34			51	31
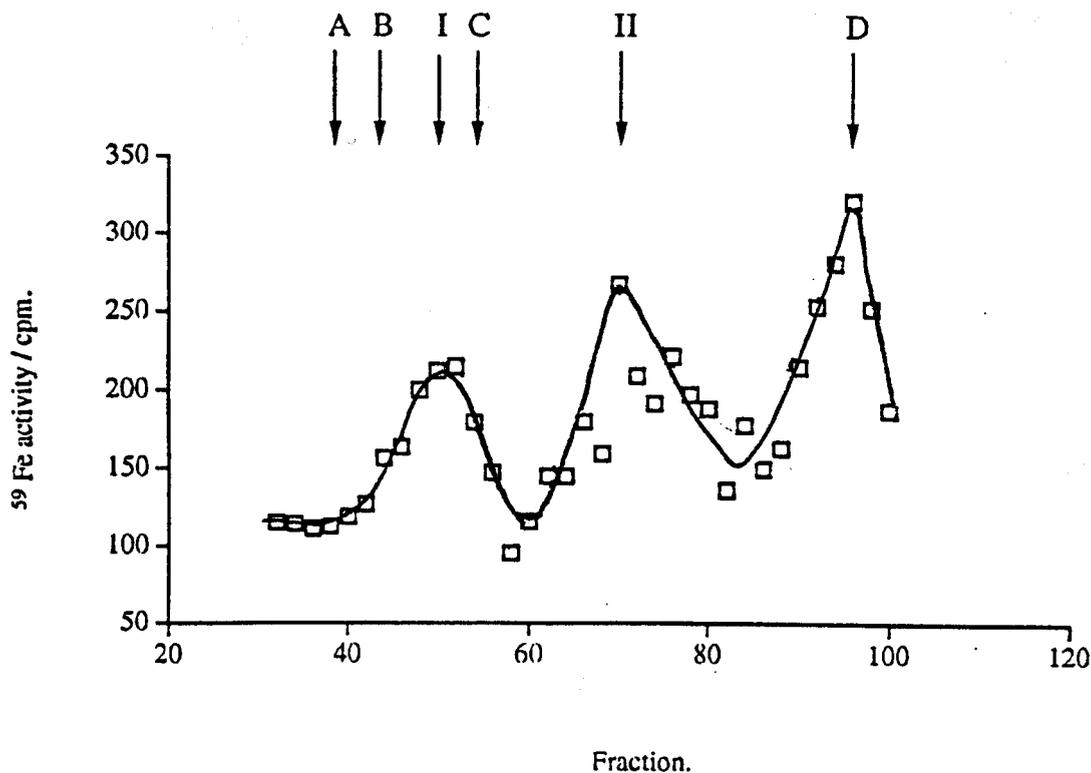
Figure 23. **The fractionation of the components of the PEI cellulose eluate,** **Peak II, by gel filtration chromatography.**

The fractions in Peak II from the PEI-cellulose fractionation experiment were loaded on to a Sephadex G-100-superfine column and were then eluted with 8 mM CHAPS in 50 mM KH_2PO_4 /NaOH, pH 7.1 collecting 2 ml fractions. [55]Fe elution was monitored by liquid scintillation counting of 100 µl samples from each fraction.

A, B and C represent the elution volumes of marker enzymes of 87, 40, 22 kDa respectively;

D represents the elution volume of ferri-exochelin (0.64 kDa).

Peak I corresponds to a protein of size 28.8 ± 2.5 kDa.

Peak II corresponds to a molecule of size 5.7 ± 0.3 kDa.

and 100 µl samples were taken for scintillation counting. Two peaks of low activity were observed (Fig. 23).

Repetition of the entire experiment demonstrated that these elution profiles were reproducible. Calibration of the gel filtration column with marker enzymes revealed that these two peaks eluted as proteins of 28.8 ± 2.5 kDa and 5.7 ± 0.3 kDa. The identity of the smaller molecule has not been resolved but once again a direct association between a protein of approximately 29 kDa and ferri-exochelin was demonstrated. No evidence of a larger labelled molecule was observed.

Although a 25 kDa envelope protein again co-eluted with the 29 kDa FEBP-ferri-exochelin complex, evidence of a larger complex incorporating this protein was not observed on gel filtration of the eluate. This co-elution, however, is consistent with the formation of a more elaborate ferri-exochelin envelope protein complex incorporating the 25 kDa IREP which was unstable in the 0.1 M NaCl eluent and dissociated on elution with only the ferri-exochelin-29 kDa FEBP complex persisting.

V   The labelling of liposome-bound envelope proteins with ^{55}Fe(III)exochelin.

Due to either a lack of sensitivity and / or resolution, the techniques used thus far in the study had allowed only limited analyses of complexes formed by the interaction of ferri-exochelin MS with the putative components of its uptake system. The formation of ^{55}Fe(III)exochelin-receptor complexes *in situ* implicated a 29 kDa protein as a ferri-exochelin binding protein but further interactions, forming more elaborate complexes, may have been obscured because of the inherently poor resolution of the CHAPS-PAGE system (see fig.14). The co-elution of a 25 kDa iron-regulated envelope protein with the 29 kDa FEBP from polyethyleneimine-cellulose, and the association between these proteins inferred by affinity chromatography on ferri-exochelin-Sepharose, suggested an accessory role for the 25kDa protein in the ferri-exochelin uptake process. These techniques, however, were impaired by a lack of sensitivity, as complexes were formed at concentrations approaching the limits of detection of proteins by silver staining. An identification of more elaborate complexes which probably would be less stable than the ferri-exochelin-29 kDa protein complex, was thought to be unlikely.

The insensitivity of these techniques might be caused by a decrease in the ferri-exochelin binding activity of the FEBP as a result of conformational changes within the protein induced by its extraction from the extremely hydrophobic environment of the mycobacterial cell wall into a detergent solution. It was considered that enhanced sensitivity might be gained by incorporating mycobacterial envelope proteins into a matrix which allowed the FEBP to adopt a conformation resembling its native structure. It seemed likely, therefore, that greater ferri-exochelin binding activity might occur if envelope extracts were incorporated into liposomes rather than just solubilised in CHAPS.

Accordingly, CHAPS-extracted envelope proteins from iron-starved cultures of *M. smegmatis* were dialysed overnight at 4°C to remove the detergent and were then

incorporated into a simple liposome preparation in order to study their ferri-exochelin binding properties in an artificial membrane system. The resulting proteo-liposomes were incubated with ^{55}Fe(III)exochelin and then analysed to investigate any ^{55}Fe(III)exochelin binding.

Several controls were required to determine the specificity and source of any ^{55}Fe retention in a liposome system observed in the proteo-liposome system. Although it was unlikely, there remained a possibility that desferri-mycobactin, perhaps extracted by CHAPS from the mycobacterial envelope, could be incorporated into liposomes and could then remove ^{55}Fe from ^{55}Fe(III)exochelin and hence mimic, or obscure, specific ferri-exochelin-binding by the incorporated envelope proteins. Similarly, the non-specific binding of ^{55}Fe, or ^{55}Fe(III)exochelin, by the components of the liposomes was also an important consideration.

A more likely scenario for any spurious association of ferri-exochelin and the proteo-liposomes was the inclusion of a homologue of the porin that had recently discovered in the cell wall of *M. chelonei* (Trias *et al.*, 1992). The large estimated pore size and cation selectivity of the channel (Trias and Benz, 1993) suggested that passive ferri-exochelin transport, which is cationic at neutral pH, via this route could be a possibility. It was considered that such a channel may present a route through which ^{55}Fe(III)exochelin could enter the lumen of the vesicles, thus mimicking, or obscuring, a specific ferri-exochelin binding event. A cation selective porin has since been discovered in the wall of *M. smegmatis* which forms an even larger pore, 3 nm in diameter, than that of *M. chelonei* (Trias and Benz, 1994) suggesting that the unhindered diffusion of ferri-exochelin through a water-filled channel in these artificial membranes would have to be considered.

A.   Protein content dependency and heat-lability of ^{55}Fe retention in ultrafiltration assays.

Proteo-liposomes were formed with up to 75 μg envelope protein in a 300 μg lipid suspension (phophatidyl choline, dipalmitoyl phosphatidic acid and cholesterol) in 1ml 50 mM KH_2PO_4 / NaOH, pH 7.1. The liposome preparation technique used was unrefined giving rise to a suspension of vesicles which varied markedly in size and were presumably multi-lamellar (Fig. 24). The technique was not optimised but the results gained were adequate for the purpose of these investigations.

The proteo-liposomes were incubated with 50 nmol ^{55}Fe(III)exochelin (15 μl, 1.087 x 10^5 Bq/nmol) for 30 min at 37°C. Any non-bound ^{55}Fe was removed by filtering through Millipore GVMP filters (0.22 μm) and washing with 50 mM KH_2PO_4 / NaOH pH 7.1 (Fig. 32.a). The extent of ^{55}Fe retention, quantified by scintillation counting of the washed filters, was observed to increase with the envelope protein content of the proteo-liposomes (Table 7). This, however, was not a directly proportional relationship.

These proteo-liposomes were likely to have been multi-lamellar vesicles and it is, therefore, possible that some of the incorporated protein would have been included into lamellae beneath the surface of the vesicle and, as such, would have been inaccessible for ferri-exochelin binding. It is likely, therefore, that a decreasing crypticity of liposome-bound protein was a factor in the generation of this non-linear relationship.

When 75 μg envelope protein samples, which had previously been held at 100°C for 5 minutes in order to denature the protein, were incorporated into proteo-liposomes, a decrease in the ^{55}Fe binding capacity of the proteo-liposomes, to 25% of that of proteo-liposomes bearing unheated samples, was observed (Table 7). This decrease in activity, as would be expected, is probably associated with the heat denaturation of the envelope proteins before their incorporation into the proteo-liposomes The residual activity of these proteo-liposomes was taken as representing non-specific iron retention
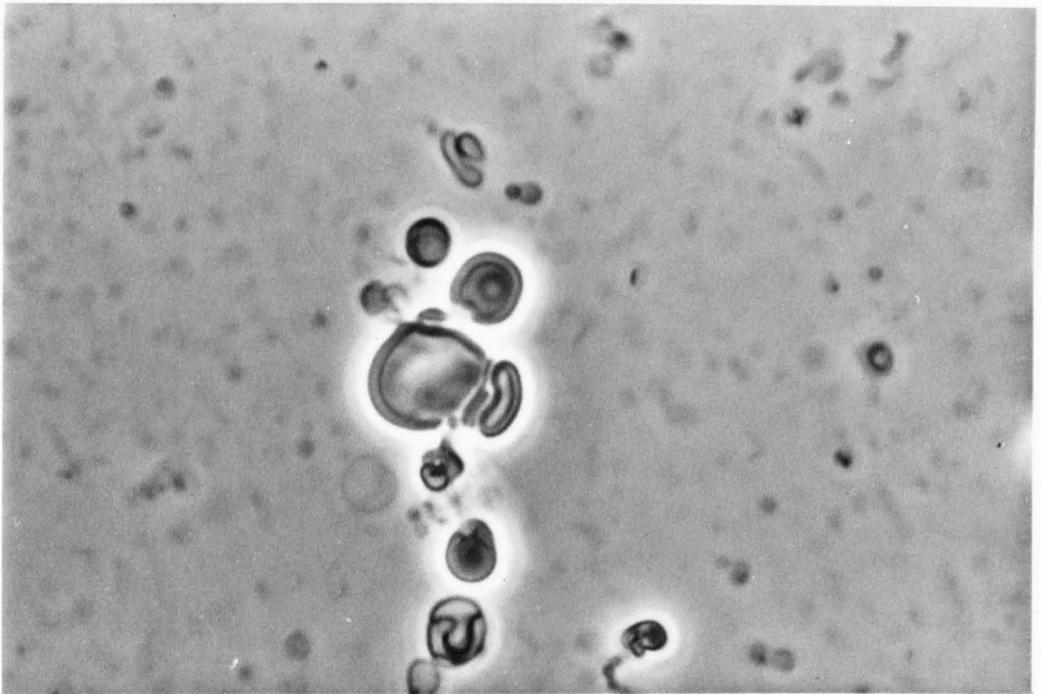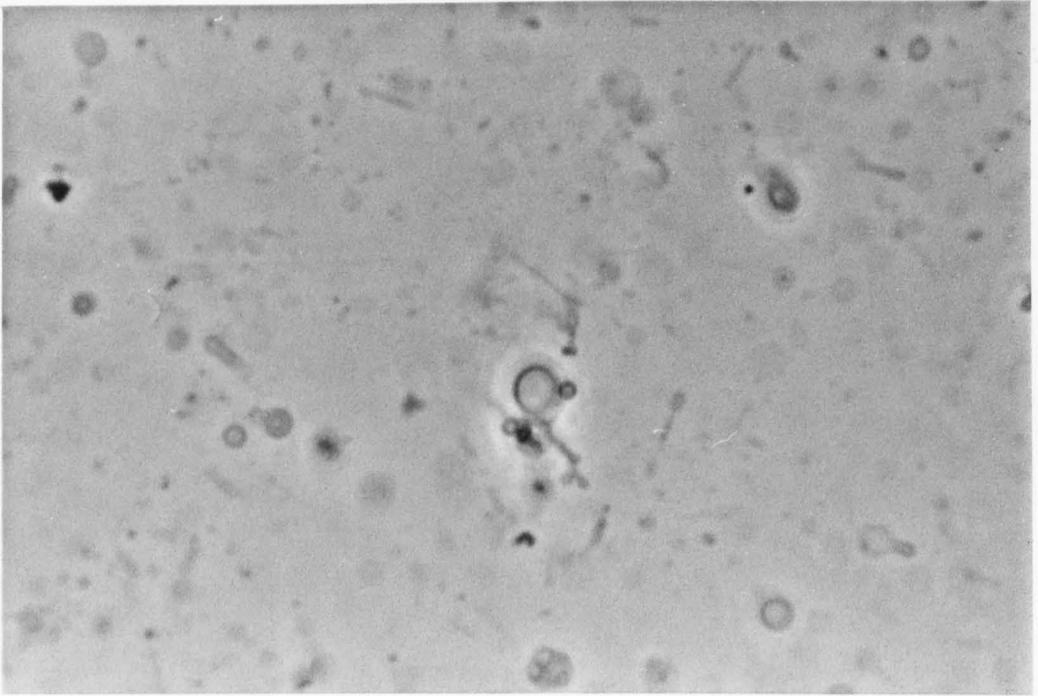
Figure 24. **The variation in size of the vesicles produced by the simple preparation method as visualised by phase contrast microscopy.** (Magnification x 2000). The liposomes were prepared by mixing the constituent phospholipids and cholesterol (see Section II.G.1) in chloroform, drying under N_2 and then resuspending by vortex mixing for 30 s in 50 mM KH_2PO4 / NaOH, pH 7.1. For the production of proteoliposomes the protein to be incorporated into the membranes was added with the buffer. A sample of the liposome suspension was taken for analysis by phase contrast microscopy.

Table 7. **The dependence of iron retention upon the non-denatured envelope protein content of proteo-liposomes.** The retention of [55]Fe, administered as [55]Fe-exochelin (50 nmol in 1 ml labelling reaction; specific activity, 1.087×10^5 Bq/nmol), by a buffered proteo-liposome suspension after labelling at 37°C for 30 minutes and ultrafiltration. Apparent retention was calculated from the [55]Fe activity retained on the filter. Corrected retention was calculated by subtracting the apparent iron retention of assays using non-protein bearing liposomes only. The results presented are the mean values of triplicate samples. The experiment was repeated a further three times, the results in each case were similar.

Envelope protein added (μg)	Apparent Fe retention (pmol)	Corrected Fe retention (pmol)
0	639	0
25	656	18
50	747	107
75	878	239
75 Heated	672	66

possibly caused by an increase in the permeability of the liposomes to ferri-exochelin as a result of the inclusion of the denatured protein.

B. **Analyses for desferri-mycobactin in CHAPS envelope protein extracts derived from iron-starved cultures.**

Although the heat-labile nature of the ^{55}Fe binding activity exhibited by the proteo-liposomes suggested the involvement of proteins, it was important to eliminate desferri-mycobactin as the source of this activity. No precautions had been made to remove iron from the solutions used for the envelope extractions or proteo-liposome assays and so mycobactin-dependent ^{55}Fe retention was very unlikely as any traces of iron present would have quickly been bound by any desferri-mycobactin in the CHAPS-solubilised envelope extract. Any mycobactin present should, therefore, have already been in the ferri-form and, as a consequence, would have been unavailable for exochelin-mediated ^{55}Fe retention in the proteo-liposomes. Indeed, the envelope material turned red during preparation which is considered indicative of ferri-mycobactin formation.

Mycobactin is fluorescent in the desferri- form and this property was exploited in the analysis of several CHAPS envelope extracts. Analysis of the emission spectra of CHAPS extracts of envelope preparations at excitation wavelengths of 275 nm and 340 nm revealed no detectable fluorescence. Analyses for mycobactin using spectrophotometry were also carried out. The simultaneous addition of $FeCl_3$, in equimolar concentrations, to a CHAPS envelope extract and a reference sample of the extraction buffer showed no increase in the absorbance of the extract at 450 nm(λ_{max} of ferri-mycobactin) relative to the buffer, only an increased turbidity, caused by the precipitation of proteins, with increasing ionic strength.

Desferri-mycobactin, therefore, was not detected in the CHAPS envelope extracts by fluorescence or spectrophotometry. This, however, did not exclude the possibility that very small amounts of desferri-mycobactin, below the limits of detection

of these techniques, could be responsible for some of the ^{55}Fe binding of the proteo-liposomes.

The ^{55}Fe retention activity of the proteo-liposomes was known to be heat-labile Section VI.A) and in order to eliminate mycobactin as the source of this activity it was important to relate this finding to the properties of desferri-mycobactin. The heat stability of desferri-mycobactin was investigated, therefore, by determining the iron-binding activity of heated and unheated samples of the siderophore by comparing ΔA_{450nm} on the addition of $FeCl_3$. Two identical samples of desferri-mycobactin were dried from solution in chloroform and one was held at $100°C$ for 10 minutes. Both samples were then redissolved in ethanol. The A_{450nm} of the desferri-mycobactin was then read and $FeCl_3$ added to form ferri-mycobactin. The amount of ferri-mycobactin formed, determined by measuring A_{450nm} against an ethanol reference solution to which an equal amount of $FeCl_3$ had been added, was the same in both the heated and unheated samples (Table 8). These data demonstrated that the iron binding activity of desferri-mycobactin was not heat-labile.

These results confirmed that the iron binding activity of the proteo-liposomes was not mycobactin-dependent as: (i) desferri-mycobactin could not be detected in the envelope extracts either spectrophotometrically at 450 nm by probing with $FeCl_3$ or (ii) using fluorimetric techniques and (iii) the activity was heat-labile, and thus inconsistent with the observed properties of mycobactin. The iron binding activity of the proteo-liposomes was, therefore, attributed to the presence of proteins derived from the cell envelope of iron-starved *M. smegmatis*.

Table 8. **The heat stability of desferri-mycobactin.** The investigation of the iron-binding capacity of desferri-mycobactin with and without heating at $100°C$ for 10 minutes. The iron-binding capacity was quantitated by comparison of ΔA_{450nm} of heated and unheated samples of the siderophore with an iron-equivalent ethanol reference solution.

Sample treatment	A_{450nm}	A_{450nm} after adding $FeCl_3$	ΔA_{450nm}
Desferri-mycobactin	0.0385	1.5395	1.501
Heated desferri-mycobactin	0.0565	1.5675	1.511
Ethanol reference	0.0000	1.2660	1.2660

C. Separation of proteo-liposome-bound and free ^{55}Fe(III)-exochelin by gel filtration.

In the proteo-liposome filtration assays (Section VI.A), high background counts of ^{55}Fe were recorded for liposomes which did not contain *M. smegmatis* envelope proteins. This was not a great problem for the simple investigations given thus far but it was considered that a high background count could mask important data. A more practical method of label separation was required. In subsequent investigations, the bound and unbound ^{55}Fe(III)exochelin were separated by gel filtration chromatography through a Sephadex G200-120 column (Fig. 32.b).

The elution volumes of each of the components of the liposome-based ferri-exochelin binding system required definition so liposomes, proteo-liposomes, free protein and ferri-exochelin were all passed through the column separately and their elution monitored. Liposome elution was monitored turbidimetrically at 625 nm while ferri-exochelin elution was monitored by the detection of ^{55}Fe by scintillation counting. These elution profiles are presented in Fig. 25. The elution volumes of the CHAPS-solubilised and liposome-bound envelope proteins of iron-starved *M. smegmatis* were also monitored separately using the BCA protein assay. The elution profiles are compared in Fig. 26. The bulk of both the protein samples eluted from the column in the early fractions, as was expected. The protein concentrations observed in the later fractions of the elution profiles were higher with the free envelope protein than with the proteo-liposome elution. This was taken as suggesting that most of the protein in the proteo-liposome preparation was vesicle-bound.

The labelling reactions were exactly the same as those for the filtration assay (Section VI.A): 50 nmol ^{55}Fe(III)exochelin (specific activity, 1.087×10^5 Bq/nmol), per assay, was incubated with liposomes, proteo-liposomes or free proteins in a 1 ml final volume of KH_2PO_4 / NaOH, pH 7.1 for 30 min at 37°C. The entire mixture was then passed through the Sephadex G200-120 gel filtration column and the elution of ferri-exochelin monitored, as ^{55}Fe, by scintillation counting.

When liposomes, with no incorporated envelope proteins, were labelled as above and passed through the gel filtration column, the radioactive iron eluted as free ferri-exochelin (Fig. 27). No [55]Fe eluted in the fractions associated with liposome elution (Fig. 25) suggesting that there was no interaction between the ferri-exochelin and the liposome vesicles. The ferri-siderophore had not bound to the phospholipids nor had it diffused into the interior of the vesicles.

When proteo-liposomes, containing 75 μg envelope protein from iron-starved *M. smegmatis*, were labelled with 50 nmol [55]Fe(III)exochelin (specific activity, 1.087 x 10^{5} Bq/nmol) and separated as above, a peak of [55]Fe elution was observed in the fractions associated with liposome elution (Fig. 28). This result, along with those from the ultrafiltration assays (Section VI.A), clearly showed that proteo-liposomes, bearing an *M. smegmatis* envelope protein extract, were able to retain the [55]Fe of ferri-exochelin, presumably without its release from the siderophore.

Investigation of the [55]Fe(III)exochelin-binding (50 nmol, 1.087 x 10^{5} Bq/nmol) activity of 75 μg envelope protein in CHAPS (Fig.29), without their incorporation into liposomes, led to a broader elution of the ferri-exochelin but there was no evidence of a detectable, specific binding of ferri-exochelin to protein. This indicated that the ferri-exochelin binding activity of the envelope proteins was enhanced when incorporated into a liposome preparation suggesting that the conformation of the FEBP was altered in the lipid environment allowing the binding of ferri-exochelin.

All of these investigations were repeated once with similar results.

1. The stability of ferri-exochelin-envelope protein complexes formed in proteo-liposomes.

The observed [55]Fe retention of the proteo-liposomes in proteo-liposomes may have been facilitated in several ways besides the obvious proposition of the direct binding of the [55]Fe(III)exochelin. The transfer of [55]Fe to desferri-mycobactin has already been excluded but an increase in the permeability of the vesicles, either specifically via the inclusion of mycobacterial porins, or non-specifically, by localised

disruption of the membrane structure around the interface between proteins and phospholipids was possible. The size of the ferri-exochelin molecule, however, made the porin route appear more plausible, especially as the size of the pore created by the recently discovered mycobacterial porin is potentially large enough to allow the passage of ferri-exochelin (Trias and Benz, 1993 & 1994). The entry of ferri-exochelin to the interior of proteo-liposomes via these non-specific routes, however, should be reversible on changing the concentration gradient. The stability of the putative ^{55}Fe(III)exochelin-proteo-liposome binding protein (PLBP) complex, therefore, was investigated by overnight dialysis of the complex before gel filtration. The elution profile (Fig. 30) showed that free ^{55}Fe(III)exochelin was not present after the dialysis and that all of the remaining ^{55}Fe eluted in fractions coincident with liposome elution. The activity, however, had decreased to about 70% of that observed before dialysis (Fig. 28). This 30 % decrease in ^{55}Fe could have been due to either the diffusion from the system of free ferri-exochelin which had previously been retained within the liposome, presumably entering via a porin, or to a limited dissociation of a protein-ferri-exochelin complex. A significant amount (70%) of proteo-liposome-associated ^{55}Fe was retained, presumably as a ferri-exochelin-protein complex.

These results demonstrated that the majority of the ^{55}Fe retention by the proteo-liposome was stable and was not mediated by the inclusion of a mycobacterial porins. ^{55}Fe retention by these proteo-liposomes, presumably in the form of ferri-exochelin, appears to be the result of a specific binding event rather than a non-specific retention by the vesicles.
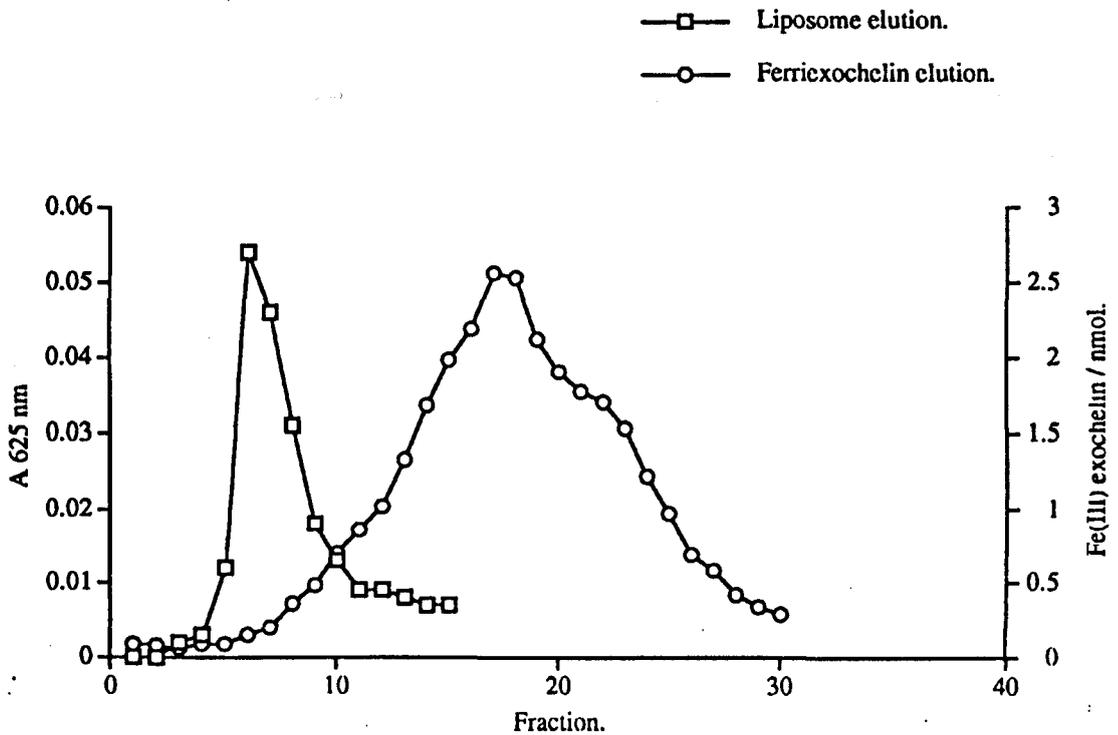
Figure 25. **A comparison of the separate elution profiles of ferri-exochelin and liposomes from Sephadex G200-120.** 50 nmol ^{55}Fe(III)exochelin (specific activity, 1.087×10^5 Bq/nmol) was diluted to 1 ml in 50 mM KH_2PO_4 / NaOH, pH 7.1 to simulate a labelling protocol and was then passed through a Sephadex G200-120 column (16 x 1.5 cm). Elution was monitored by the scintillation counting of ^{55}Fe activity.

A liposome suspension was formed by the resuspension of the constituent phospholipids and cholesterol in 1 ml buffer by vortex mixing for 30 s. The suspension was separately passed through the same gel filtration column and elution was monitored turbidimetrically at 625 nm.
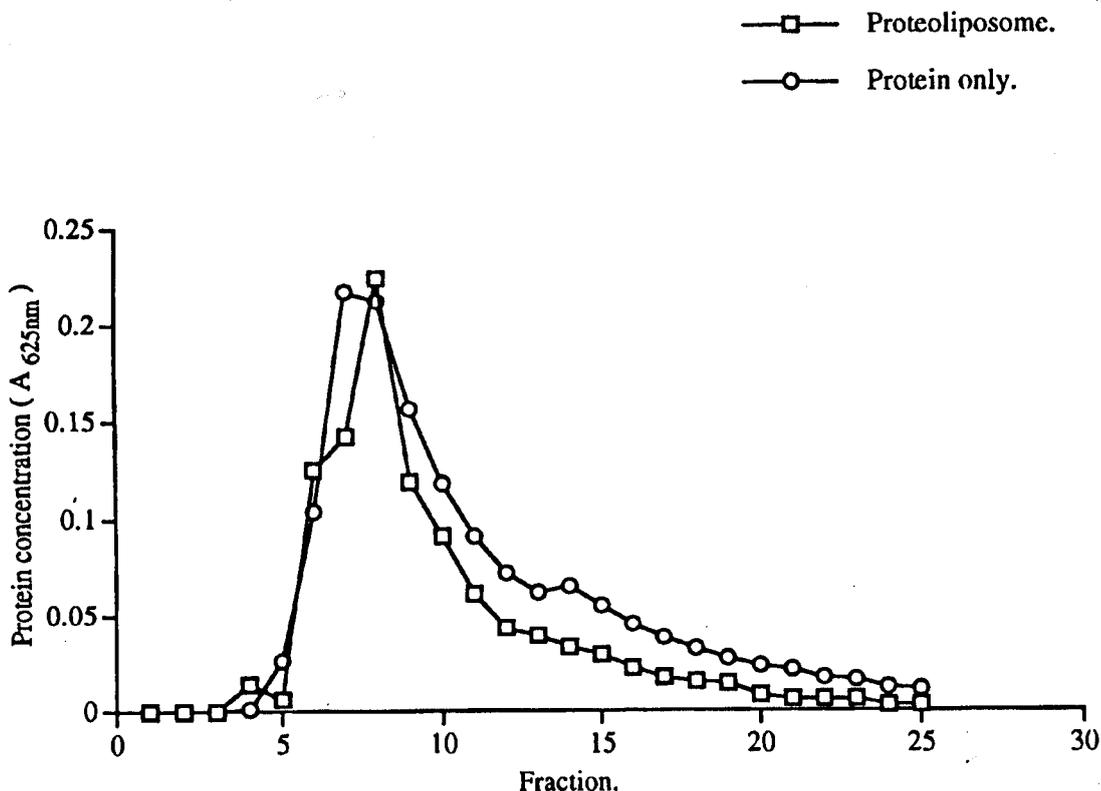
Figure 26. **A comparison of the separate elution profiles of free envelope protein and proteo-liposomes from Sephadex G200-120.** The separate elution of free and liposome-bound envelope protein from the Sephadex G200-120 gel filtration column (16 x 1.5 cm). In all cases dialysed envelope proteins, extracted using CHAPS, from iron-starved cultures of *M. smegmatis* were used. Proteo-liposomes were formed, incorporating 1 mg protein in 4 mg lipid, by resuspension of the dried phospholipid and cholesterol substituents in 1ml 50 mM KH_2PO_4 containing the protein. This suspension was loaded directly on to the column and was eluted with 50 mM KH_2PO_4 / NaOH, pH7.1. The free protein (1mg) was diluted to 1 ml in the same buffer and passed through the column separately using the same buffer as eluent. In both cases the elution of protein was monitored using the BCA protein assay to monitor 100 μl samples of fractions (Pierce).
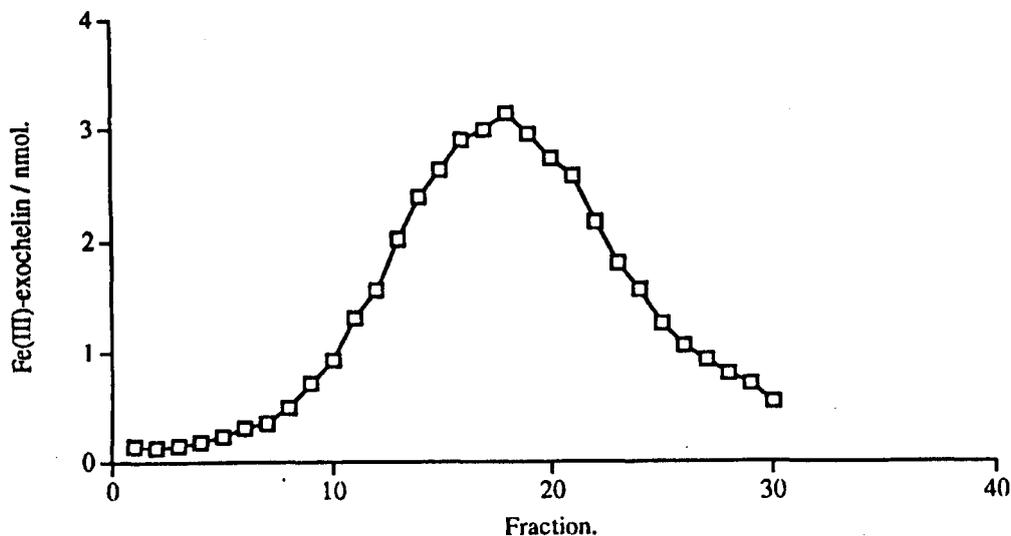
Figure 27. **The elution of a mixture liposomes and ferri-exochelin from Sephadex G200-120.** A 1ml liposome suspension, containing 4 mg lipid constituents with no incorporated protein, in buffer was incubated for 30 min with 50 nmol [55]Fe(III)exochelin (specific activity, 1.087 x 10^5 Bq/nmol) at 37°C. The 1 ml labelling mixture was then passed through the Sephadex G200-120 gel filtration column (16 x 1.5 cm) eluting with 50 mM KH_2PO_4 / NaOH, pH 7.1. 0.65 ml fractions were collected and [55]Fe(III)exochelin elution was monitored by taking 100 µl samples of each fraction for scintillation counting.

Figure 28. **The elution of a mixture of ferri-exochelin and proteo-liposomes containing *M. smegmatis* envelope proteins from Sephadex G200-120.** A 1 ml proteo-liposome suspension, containing 4 mg lipid constituents and 1mg CHAPS-extracted, envelope protein from iron-starved *M. smegmatis*, in buffer was incubated for 30 min with 50 nmol ^{55}Fe(III)exochelin (specific activity, 1.087 x 10^5 Bq/nmol) at 37°C. The 1 ml labelling mixture was then passed through the Sephadex G200-120 gel filtration column (16 x 1.5 cm) eluting with 50 mM KH$_2$PO$_4$ / NaOH, pH7.1. 0.65 ml fractions were collected and ^{55}Fe(III)exochelin elution was monitored by taking 100 µl samples of each fraction for scintillation counting.

Figure 29. The elution of a mixture of ferri-exochelin and CHAPS-solubilised envelope proteins from Sephadex G200-120. 1mg dialysed, CHAPS-extracted, envelope protein form iron-starved *M. smegmatis* in 1 ml 50 mM KH_2PO_4 / NaOH, pH7.1 was incubated for 30 min with 50 nmol $^{55}Fe(III)$exochelin (specific activity, 1.087×10^5 Bq/nmol) at 37°C. The 1 ml labelling mixture was then passed through the Sephadex G200-120 gel filtration column (16 x 1.5 cm) eluting with 50 mM KH_2PO_4 / NaOH, pH7.1. 0.65 ml fractions were collected and $^{55}Fe(III)$exochelin elution was monitored by taking 100 µl samples of each fraction for scintillation counting. The data from Fig 28 is overlaid for comparison.
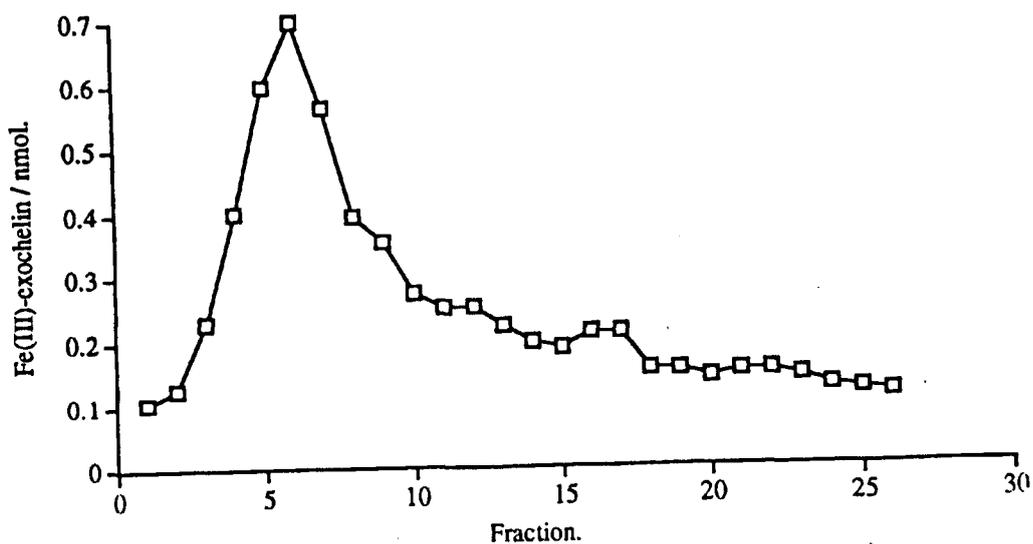
Figure 30. **The elution of dialysed, ferri-exochelin-labelled proteo-liposomes from Sephadex G200-120.** A proteo-liposome suspension, labelled with [55]Fe(III)exochelin (50 nmol, 1.087×10^5 Bq/nmol) by incubation for 30 minutes at 37°C, was dialysed overnight in order to remove any free [55]Fe(III)exochelin. The retentate was passed through the Sephadex G200-120 gel filtration column (16 x 1.5 cm) eluting with 50 mM KH_2PO_4 / NaOH, pH7.1. 0.65 ml fractions were collected. The elution of the remaining [55]Fe(III)exochelin was monitored by taking 100 μl samples of each fraction for scintillation counting.

2. **Calculation of the enhancement of ferri-exochelin-binding by envelope proteins on inclusion into liposomes.**

Proteo-liposomes containing 75 µg envelope protein, which had been labelled according to the standard protocol, retained 3.5 nmol ferri-exochelin after overnight dialysis (Fig. 30; Section V.C.1). This [55]Fe retention was assumed to be the product of a specific binding event and, as such, was comparable to the [55]Fe(III)exochelin co-eluted with the 29 kDa FEBP from PEI cellulose in Peak II (Section IV.D.2, Fig. 21). In the latter case, only 0.7 nmol ferri-exochelin was eluted in Peak II after labelling 2 mg CHAPS-solubilised envelope protein. The enhancement of ferri-exochelin-binding by envelope proteins, observed when incorporated into liposomes, can be quantified by calculating specific ferri-exochelin-binding activities for the proteo-liposomes and the free proteins in CHAPS.

Binding activity of proteo-liposome   =   3.5 / 0.075   = 46.7 nmol Fe/mg protein

Binding activity of PEI Peak II   =   0.7 / 2.000   = 0.35 nmol Fe/mg protein

46.7 / 0.35 = 133

An estimated 133-fold increase in ferri-exochelin-binding activity was associated with the incorporation of *M. smegmatis* envelope protein extracts into liposomes as compared to the binding activity exhibited in CHAPS solution.

D.   Attempts to characterise ferri-siderophore-receptor complexes formed in liposomes.

Having established that a stable, non-dialysable ferri-exochelin-envelope protein complex was formed in liposomes, attempts were made to isolate the complex(es) by detergent extraction from labelled proteo-liposomes. Larger preparations of proteo-liposome were prepared incorporating 1 mg envelope protein from iron-starved cultures into 4 mg lipids. These were resuspended to form the vesicles by vortex-mixing for 30 s in 1 ml 50 mM KH_2PO_4 / NaOH, pH 7.1 as before, and were then labelled with 50 nmol ^{55}Fe(III)-exochelin (15 μl, 1.087 x 10^5 Bq/nmol) at 37°C for 30 minutes. Four different detergent regimes were assessed for efficiency of releasing protein-bound ^{55}Fe from labelled proteo-liposomes (see Table 9).

In each case, the vesicles were extracted with the appropriate detergent overnight at 4°C, with stirring, and then the solubilised and unsolubilised ^{55}Fe were separated by centrifugation. The solubilised ^{55}Fe was quantified by the scintillation counting of a small sample of the supernatant.

Approximately 10% of the total proteo-liposome-bound ^{55}Fe was solubilised by the CHAPS-based regimes. The Triton X-100 treatment, however, resulted in a significantly greater degree of solubilisation, corresponding to 33% of the original ^{55}Fe bound to the proteo-liposome (Table 9). In each case, the remainder of the original ^{55}Fe bound to the proteo-liposome was deposited during the centrifugation step, presumably as the unchanged and still intact ferri-exochelin-protein complex.

It was possible, however, that the ^{55}Fe released from the proteo-liposomes during the detergent extraction was not in the form of a solubilised complex but was merely free ferri-exochelin released by the dissociation of the complex. As free ferri-exochelin would be removed by dialysis each detergent extract was dialysed overnight. A sample of the retentate was counted to quantify the amounts of high molecular weight, i.e. non-dialysable, protein-bound, ^{55}Fe released by the solubilisation regime.

Table 9. **Recovery of high molecular weight, solubilised ^{55}Fe from labelled proteo-liposomes.** Four digestion regimes were assessed to separate high and low molecular weight ^{55}Fe, i.e. ferri-exochelin protein complexes and free ferri-exochelin. The total for ^{55}Fe binding was measured from a sample of a pre-digested, dialysed proteo-liposome suspension by scintillation counting (7.85×10^5 cpm). Each digestion procedure was conducted at 4°C for 16 h with continuous stirring. Non-solubilised ^{55}Fe, probably in the form of an intact ferri-exochelin protein complex, was deposited by centrifugation. Solubilisation of ^{55}Fe was determined by the scintillation counting of a sample of the supernatant the bulk of which was then dialysed to remove any free ferri-exochelin. Samples of the retentates were counted to quantify the high molecular weight, soluble ^{55}Fe. The figures in parentheses are expressed as percentages of the total binding activity of the pre-digested proteo-liposome suspension.

Detergent regime	Soluble ^{55}Fe activity (cpm)	High molecular weight, soluble ^{55}Fe (cpm)
8 mM CHAPS	7.11×10^4 (9.1)	5.18×10^4 (6.6)
8 mM CHAPS + 1 mM taurodeoxycholic acid	8.27×10^4 (10.5)	6.31×10^4 (8.0)
8 mM CHAPS + 0.5 M NaCl	8.79×10^4 (11.2)	7.03×10^4 (9.0)
0.5% (w/v) Triton X-100	2.61×10^5 (33.3)	1.44×10^5 (18.4)

The results of the assessment (Table 9) showed that the greatest recovery of a high molecular weight-solubilised ^{55}Fe, presumably a ferri-exochelin-receptor complex, was achieved using 0.5% Triton X-100 as the solubilising agent. The recovery, though far from ideal, was, at approximately 20% of the original ^{55}Fe in the proteo-liposomes, probably good enough to allow the characterisation of a complex by electrophoresis coupled with autoradiography.

E.   Analysis of high molecular weight solubilised ^{55}Fe by Triton X-100-PAGE.

The high molecular weight ^{55}Fe released from proteo-liposomes by Triton X-100 digestion, the putative ferri-exochelin-binding protein complex, required further separation in order to confirm the molecular size of the ferri-exochelin-binding protein. Potentially such a separation may also have demonstrated complexes which were larger and more elaborate than the simple ferri-exochelin-FEBP complex which might have been formed by interactions between the ferri-exochelin-FEBP and other IREPs. Neither of the electrophoresis methods used thus far, i.e. CHAPS and SDS-PAGE, could be used for this separation as the complex was known to be relatively insoluble in the CHAPS-PAGE system (Section IV.C) and affinity chromatography on ferri-exochelin-Sepharose had demonstrated that the binding of ferri-exochelin by the 29 kDa binding protein was disrupted in the presence of SDS (Section IV.D.1.a&b). As the complex was relatively stable in 0.5% Triton X-100 a final separation was attempted using Triton X-100-PAGE.

The previous procedure was repeated. Dialysed, CHAPS-extracted envelope proteins from iron-starved cultures of *M. smegmatis*, were incorporated into proteo-liposome preparations in a ratio of 0.25 mg protein : 1 mg phospholipid. Initially, the proteins were not labelled with ^{55}Fe(III)exochelin, but were extracted from the proteo-liposomes by an overnight incubation in 0.5% Triton X-100. The extract was clarified by centrifugation (105,000g, 30 min), dialysed and then the soluble material in the supernatant was separated by Triton X-100-PAGE on 10% resolving gels. Visualisation of the gel by silver staining revealed the presence of many protein bands.

Resolution, however, was not ideal (Fig 31.A) but was superior to that achieved in the CHAPS-PAGE system(Fig. 13).

The protocol was then repeated with the inclusion of a labelling step consisting of a 30 minute incubation of the proteo-liposomes with 50 nmol Fe(III)-exochelin in 1 ml 50 mM KH_2PO_4 / NaOH, pH 7.1, at 37°C followed by the overnight detergent digestion at 4°C (Fig. 32.c.i) and dialysis. After centrifugation, the electrophoresis was repeated, the gels were dried and autoradiographed. The autoradiography revealed that the majority of the ^{55}Fe had electrophoresed at the dye front, i.e. with $R_f = 1$, indicative of free ferri-exochelin (Fig 31.B). A small amount of ^{55}Fe had been retained in the well probably as a result of the aggregation of envelope protein. The majority of the high molecular weight ^{55}Fe that had been applied to the gel system appeared to have dissociated allowing the ferri-exochelin to electrophorese freely and thus the ferri-exochelin-receptor complex appeared to be unstable under the conditions of Triton X-100-PAGE.

To avoid this dissociation, it was considered that the above protocol should be repeated exactly but that the final separation should be performed using gel filtration chromatography. The solubilised complex, in this case, was passed through a Sephacryl S-100-HR column (Section II.H.b; Fig. 32.c.ii) eluting with 0.1% Triton-X-100 in 50 mM KH_2PO_4 / NaOH, pH 7.1, collecting 2 ml fractions. Analysis of eluted ^{55}Fe, by scintillation counting of 100 µl samples of the fractions, demonstrated the presence of a single radioactive peak in the elution profile (Fig. 33). The peak was centred at fraction 71, eluting, relative to markers, as a protein with apparent molecular mass of 57.5 ± 4.0 kDa. The radioactive fractions, 69 to 79 inclusive, were pooled and concentrated using a Millipore UF-CL low binding cellulose filter (nominal molecular weight limit = 5000 Da). The residue was then analysed by SDS-PAGE to define the components of the complex. The resolution of the proteins in the gel, however, was poor (Fig. 34) with much of the protein apparently electrophoresing abnormally. Three bands, however, were visible corresponding to proteins of apparent molecular mass 38, 32 and 29 kDa. It is considered, however, that these sizes may be slightly inaccurate due to the abnormal behaviour of the complex under electrophoresis.
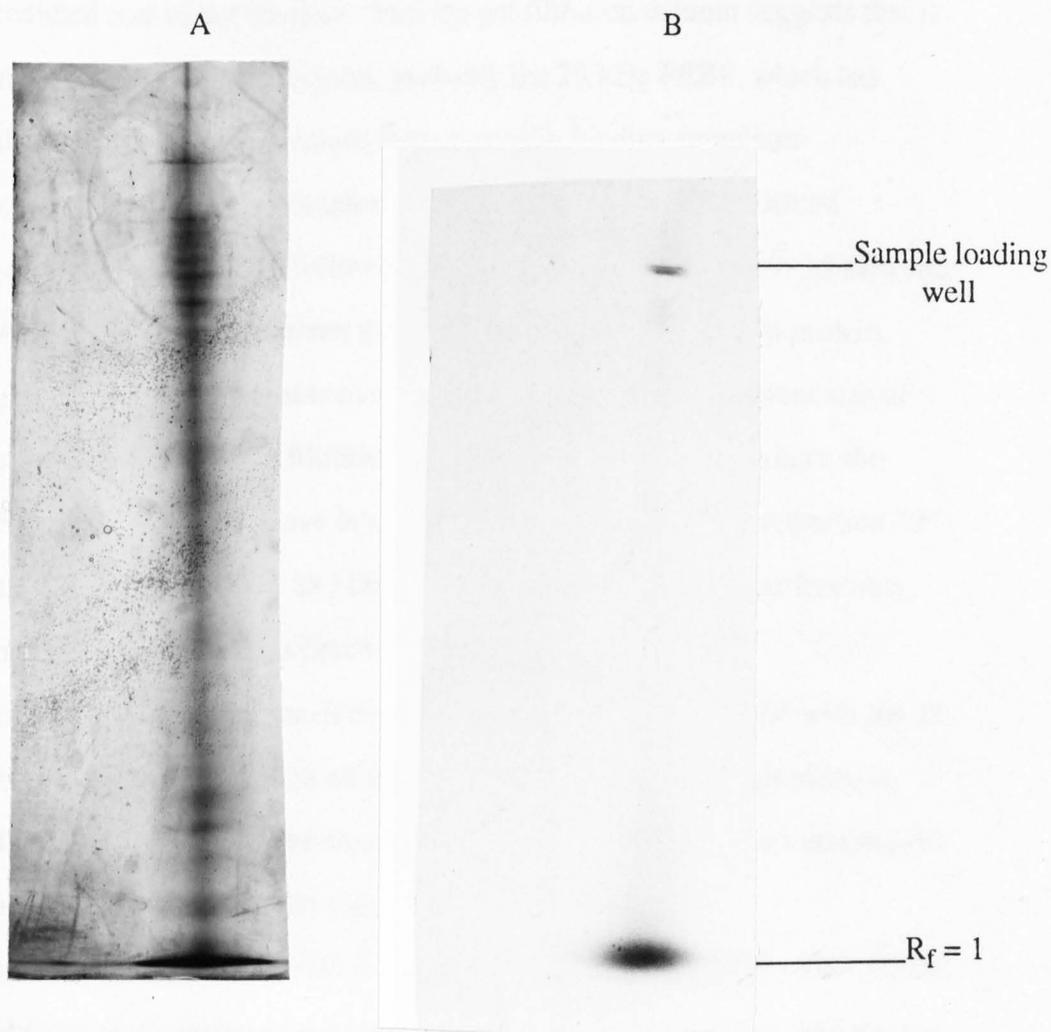
Figure 31. **The separation of *M. smegmatis* envelope proteins and a ferri-exochelin-envelope protein complex using Triton X-100-PAGE.**
The envelope proteins and ferri-exochelin-envelope protein complex, formed as before, were both extracted from the vesicles by digestion in 0.5% Triton X-100, dialysed to remove free ferri-exochelin and were separated on 10 % Triton X-100-PAGE gels. (A) shows the separation of envelope protein extracted from unlabelled proteo-liposomes while (B) shows the autoradiograph of an extract from a ^{55}Fe(III)exochelin-labelled proteo-liposome separated by this method (B).

The predicted size of the complex from the gel filtration column suggests that it is possibly formed by two of these proteins, probably the 29 kDa FEBP, which has already been demonstrated to be the major ferri-exochelin binding protein of *M. smegmatis,* and one other. The complex could, therefore, have been formed between ferri-exochelin MS and the following components: 29+25 kDa, 29+38 kDa and 29+29 kDa. A complex formed between the 29 kDa FEBP and the 38 kDa protein, however, would have a molecular mass over 10 kDa in excess of the apparent size of the complex estimated from the gel filtration analysis. Relative to the markers, the elution of a 38 kDa protein would have been predicted in a peak centred at fraction 79 and, therefore, it is possible that the 38 kDa protein merely co-eluted in the fractions that were combined for analysis as a function of its own molecular mass.

The co-purification of the non-ferri-exochelin binding 25 kDa IREP with the 29 kDa FEBP-ferri-exochelin complex in all of the protocols attempted in this study is consistent with the hypothesis that the smaller protein is a receptor complex component which associates with the FEBP rather than the ferri-siderophore.

The problems encountered in the final separation of the complex by SDS-PAGE are considered to be attributable to the persistence of some of the liposome components (phospholipids and/or cholesterol) after the Triton X-100 digestion. Proteo-liposomes, exactly the same as those used before, which had not been labelled with $^{55}Fe(III)$exochelin were used in attempts to improve the resolution of the complex in the SDS-PAGE system. The pre-digestion of the protein extracts with phospholipase A from rattlesnake venom before electrophoresis did not improve resolution in SDS-PAGE suggesting that it was the cholesterol component of the liposomes rather than the phospholipids which was possibly the cause of this problem.

This protocol appeared to yield an almost homogeneous and easily detected preparation of a ferri-exochelin-receptor protein complex and thus provides the best available means to resolve the identity of the complex components. The problems encountered in electrophoresis of the complex may possibly be overcome by altering the composition of the lipid constituents of the liposome suspension.

CHAPS-extracted envelope proteins
from iron-starved cells.

|
| + phospholipids and
| cholesterol.
↓

Proteo-liposomes

|
| + ^{55}Fe(III)exochelin
| 37°C; 30 minutes.
↓

^{55}Fe-labelled proteo-liposomes

(a) Ultrafiltration assays   (b) Gel filtration   (c) Triton X-100 digestion

Ultrafiltration   Sephadex G-100-120

Centrifugation
105,000g; 30 min

Count sample as total   ←   Supernatant
solubilised ^{55}Fe.

|
| Dialysis
| 4°C; overnight
↓

Count sample as high   ←   Retentate
mol. wt. solubilised ^{55}Fe.

(i) Triton X-100-PAGE   (ii) Gel filtration

Complex dissociation   Complex purification

Figure 32.   Scheme for the purification and analysis of ferri-exochelin-envelope
protein complexes formed in proteo-liposomes.

132
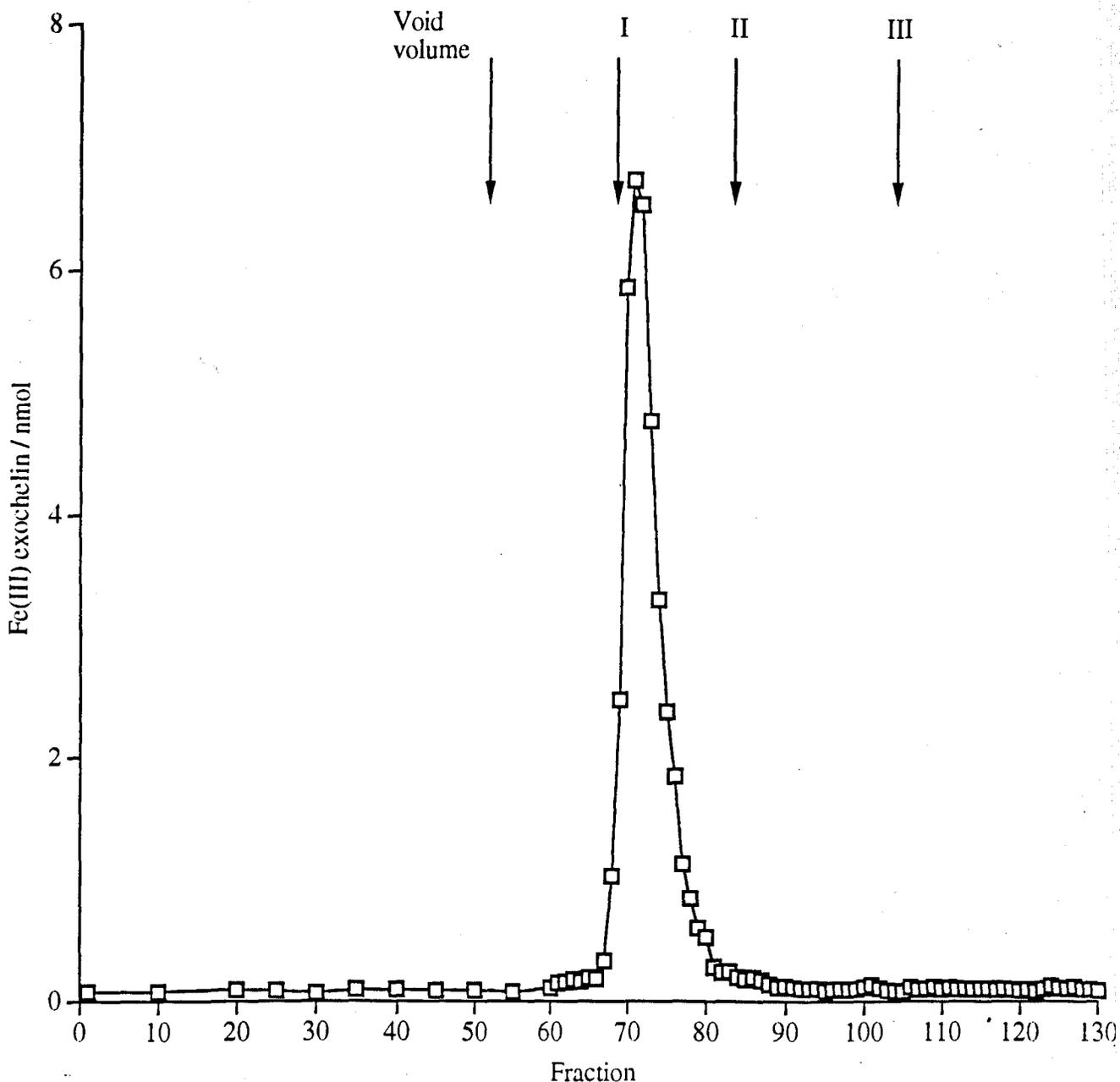
Figure 33. **The elution of a ferri-exochelin protein complex, formed in proteo-liposomes, from Sephacryl S-100-HR.**

Proteo-liposomes containing 1mg envelope proteins from iron starved *M. smegmatis* were labelled using 50 nmol [55]Fe(III)exochelin (15 μl, 1.087 x 10^{5} Bq/nmol) for 30 min at 37°C. The labelled proteo-liposomes were digested overnight in 0.5% (w/v) Triton X-100 at 4°C. Soluble [55]Fe-containing species were recovered from the supernatant fluid after centrifugation (105,000g,30 min, 4°C) and dialysed overnight against buffered CHAPS. The retentate was loaded onto a Sephacry-S100-HR column and eluted with 50 mM KH_2PO_4/NaOH pH 7.1, collecting 2 ml fractions. 100 μl samples of each fraction were taken for liquid scintillation counting in order to quantify [55]Fe.
Markers: **I**, 66 kDa; **II**, 29 kDa; **III**, 12.4 kDa.

133
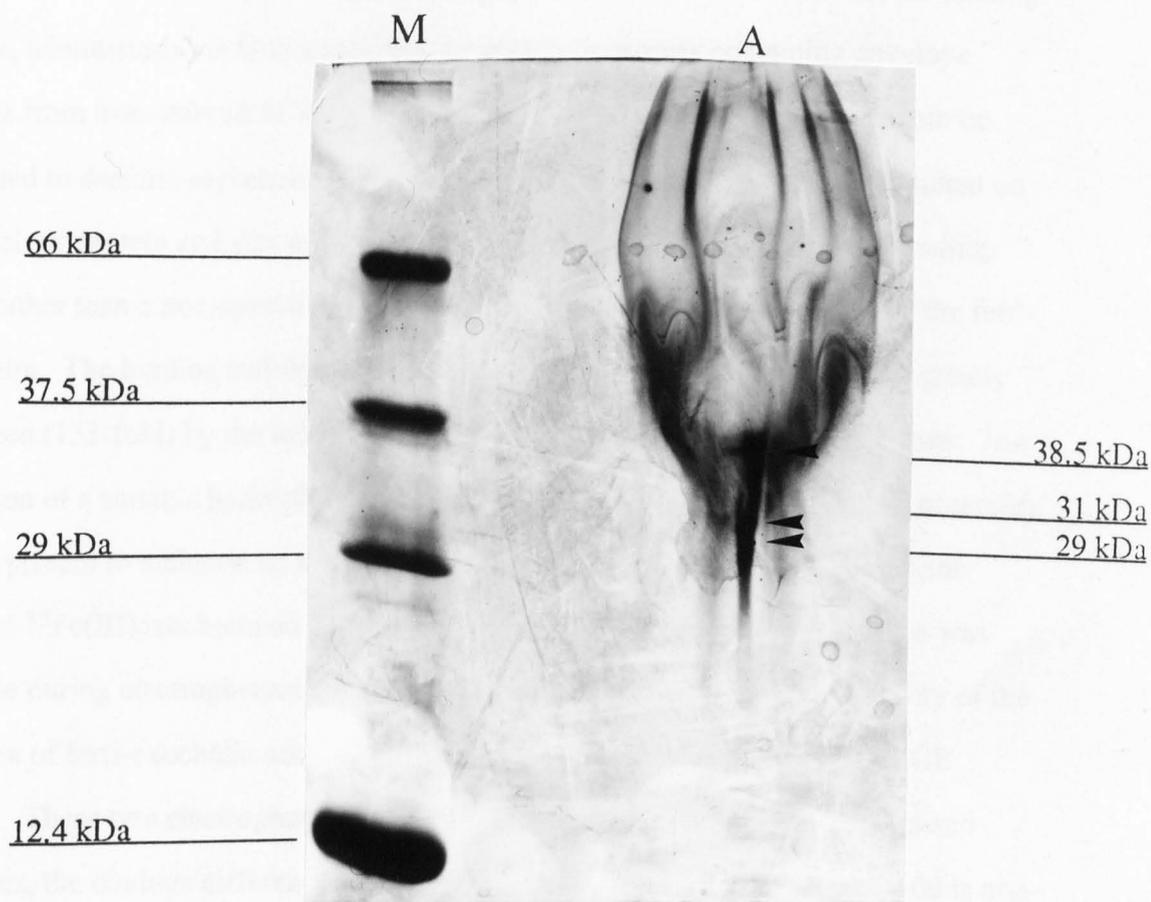
Figure 34. **The analysis of the components of the ferri-exochelin-envelope protein complex formed in proteo-liposomes by SDS-PAGE.** The fractions corresponding to the elution of the 57.5 kDa complex from the Sephacryl S-100-HR column were pooled and concentrated using a Millipore UF-CL low binding cellulose filter. The residue was analysed using SDS-PAGE on a 10 % resolving gel. The gel was visualised by silver staining.

Lane A   complex components

Lane M   Molecular size standards

F.   Conclusions.

The conclusions drawn from these liposome-based studies were that the binding of ^{55}Fe, administered as ferri-exochelin, by proteo-liposomes containing envelope extracts from iron-starved *M. smegmatis* was heat-labile and therefore could not be attributed to desferri-mycobactin. The binding activity would appear to be resident on an envelope protein and was not reversible by dialysis suggesting a specific binding event rather than a non-specific association between the proteo-liposomes and the ferri-exochelin. The binding activity of CHAPS-solubilised envelope proteins was greatly enhanced (133-fold) by the incorporation of these proteins into proteo-liposomes. The provision of a suitable hydrophobic environment for the protein appears to be necessary for the protein to maintain an active binding conformation. Although the proteins retained ^{55}Fe(III)exochelin on solubilisation with Triton X-100 this association was unstable during electrophoresis. This observation is in contrast with the stability of the complex of ferri-exochelin and the 29 kDa binding protein in the CHAPS-PAGE system. These two electrophoresis systems are both based on the same buffers and solutions, the obvious difference being the nature of the detergent; Triton X-100 is non-ionic whereas CHAPS is a zwitterionic detergent. It is possible that the zwitterionic detergent stabilises the complex in an electric field by a mechanism, one would assume, that cannot be mimicked by its non-ionic counterpart.

An effective purification was observed by passing the Triton X-100 proteo-liposome extract through a Sephacryl S-100-HR gel filtration column. A single radio-labelled moiety eluted from the column with an apparent molecular mass of 57.5 ± 4 kDa. Analysis of the pooled fractions contributing this peak by SDS-PAGE revealed the presence of three detectable proteins on silver staining. These proteins were assigned molecular weights of 29, 32 and 38 kDa, although these sizes are possibly unreliable due to the abnormal electrophoretic separation observed in this instance. It is possible, however, that the 38 kDa protein may have merely co-eluted with a complex composed of the two smaller proteins. The other two proteins may be the 25 and 29 kDa proteins respectively with slight distortions in molecular mass due to the

retention of small amounts of lipid material from the liposomes. If this is so then once again we have evidence for a ferri-exochelin-protein complex being formed with the 25 and 29 kDa proteins. This then would account for the size of the complex recovered from the Sephacryl S-100-HR step.

VI   Studies in the derivatisation and structure of ferri-exochelin MS

The molecular structure of exochelin MS has only recently been determined (Sharman *et al.*, 1995). At the beginning of this study little was known of the structure. On the basis of the amino acid composition of the molecule a model had been suggested in which the molecule consisted of a conventional linear pentapeptide with terminal and central hydroxyornithine residues interspersed with β alanine and threonine residues. with the hydroxyl groups of the hydroxyornithine residues forming the ligand groups. The ferric complex was envisaged as a dimer with a central ferric ion (Fig. 34). This model, however, did not accommodate all of the observed properties of ferri-exochelin MS: the model predicted a zwitterionic molecule yet ferri-exochelin MS was known to be basic and the proposed iron-chelating ligand groups were novel and unsupported by effective ligands such as hydroxamates or catecholates.

The production of immobilised ferri-exochelin was considered an important aim of the study as it would provide a means to test this dimer hypothesis and also act as an affinity chromatography medium for the identification of a ferri-exochelin MS receptor protein.

A.   The derivitisation of ferri-exochelin MS using *o*-phthaldialdehyde

Ferri-exochelin MS was thought to possess two free NH_2 groups via which the molecule could possibly be immobilised. In order to examine the structural dimer hypothesis a sensitive analytical method for the detection of peptides in the presence of reducing agents was required. The use of *o*-phthaldialdehyde (OPA) to derivatise ferri-exochelin MS fulfilled all of these requirements. This agent reacts strongly with primary amino groups of amino acids and peptides in the presence of reducing agents to produce derivatives that are detectable in the nanomole range by fluorescence (Roth, 1971; Joseph and Marsden, 1986). The derivatisation reaction was carried out in accordance with the method of Roth (1971) but determination of excitation and emission spectra for
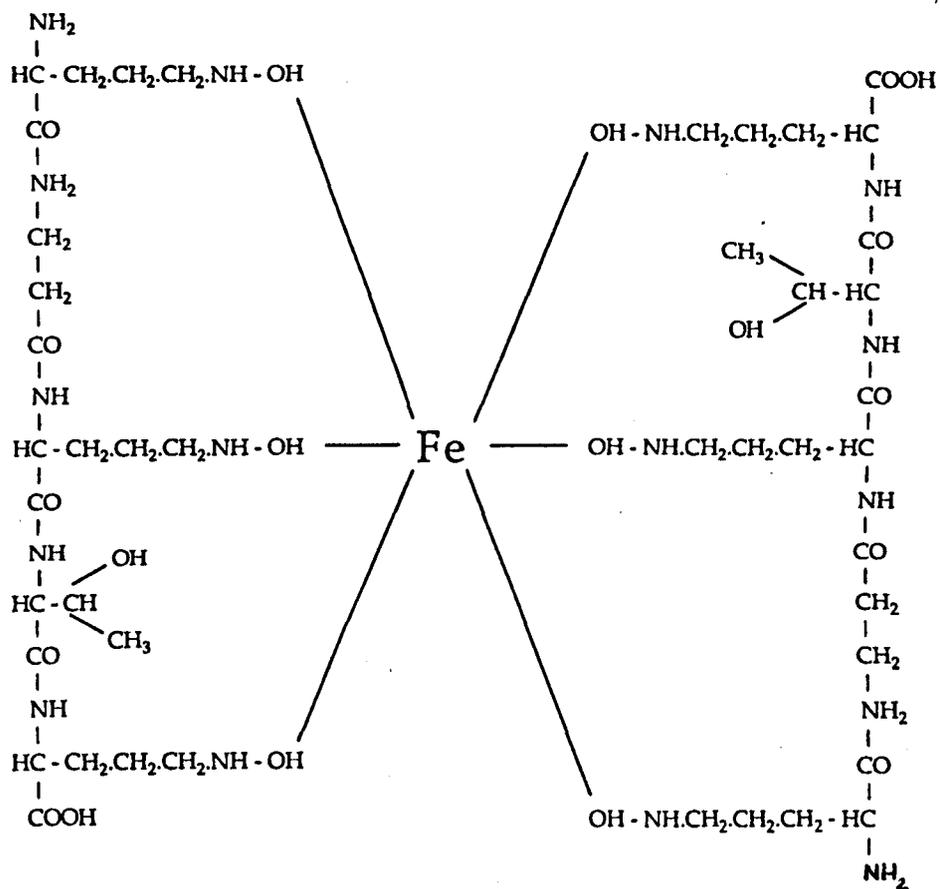
Figure 34. The proposed structure of ferri-exochelin MS current at the beginning of the study.

the derivatised exochelin demonstrated a shift in the maxima to 410 nm and 490 nm respectively.

Using these parameters, a concentration dependent enhancement of fluorescence was demonstrated upon the addition of h.p.l.c.-purified ferri-exochelin MS (>99% pure) to the buffered OPA (Table 10.). This was taken as demonstrating the generation of a derivatised exochelin MS molecule and thus indicative of the existence of a derivatisable amino group. The detection of exochelin MS had also been demonstrated in the low nanomole range.

B.   The immobilisation of ferri-exochelin MS on activated Sepharose 4B.

Following the demonstration of a derivatisable amino group in ferri-exochelin MS it was considered likely that the siderophore could be immobilised on an amino-specific activated chromatography support. To test the hypothesis that ferri-exochelin existed as a dimeric structure it was important that the matrix was fully saturated with the siderophore such that only one of the amino groups from each complex was covalently linked to the matrix and that the other exochelin MS monomer was retained on the matrix by its association with the central ferric ion.

6-amino-hexanoic acid N-hydroxysuccinimide ester-Sepharose 4B was chosen as the affinity support material as it was amino-specific and also possessed a six carbon spacer arm between the activated group and the agarose support which would allow greater mobility of the immobilised ferri-exochelin MS thus increasing the probability of successful receptor-ligand interaction when used as an affinity chromatography support.

0.4 g of activated Sepharose with a maximum binding capacity of 16 μmol ferri-exochelin was incubated with 16 μmol h.p.l.c. purified ferri-exochelin MS (>99% pure) according to the manufacturers' instructions for 1 hour at room temperature. The exochelin had been quantified with respect to iron content by titration of desferri-exochelin MS so the exochelin monomer concentration would have been double the binding capacity of the column if the dimer hypothesis were correct. When the reaction

Table 10. **The concentration-dependent enhancement of OPA fluorescence with ferri-exochelin MS.** 100 µl of ferri-exochelin MS (h.p.l.c.-purified, > 99% pure) was added to 3ml borate-buffered OPA pH 9.5. Fluorescence was measured using $\lambda_{ex} = 410$nm $\lambda_{em} = 490$nm on a Perkin Elmer spectrofluorimeter with slit widths set at 2.5 nm against an OPA reference. Readings presented are means of duplicate samples.

Ferri-exochelin MS added (nmol)	Fluorescence at 490 nm
0	1
17.5	193
70	752

mixture was then filtered through a glass sinter the Sepharose support was observed to be red and the reading of $A_{430\ nm}$ of the filtrate showed that 98% of the ferri-exochelin had been retained by the support. The degree of saturation of the matrix was not tested by the addition of further ferri-exochelin MS due to the limited supply of the siderophore at that time. The Sepharose remained red after five consecutive low and high pH washes demonstrating the covalent linkage of the ferri-exochelin MS to the activated resin via its amino groups.

The immobilised ferri-exochelin MS could now be used to test the dimer hypothesis and also as an affinity chromatography support matrix. The success of this latter application would, however, be dependent upon the non-participation of the immobilised amino group in the receptor-ligand interaction. In the event, ferri-exochelin MS was found to contain three free amino groups, all of which were presumably available for the immobilisation reaction producing a population of different ferri-exochelin MS-Sepharose moieties thus increasing the probability of successful receptor-siderophore interactions.

C.   Testing of the validity of the dimer hypothesis of ferri-exochelin MS structure

A ferri-exochelin MS saturated agarose matrix had been constructed and the detection of exochelin MS at nanomole levels in the presence of reducing agents had been demonstrated. Together these factors allowed the testing of the dimer hypothesis of ferri-exochelin structure. In theory, 8 μmol exochelin monomers were retained on the agarose matrix solely by their interaction with coordinated ferric iron. The *in vivo* dissociation of ferri-siderophore complexes is achieved by a reductive mechanism; thus the addition of a reducing agent to this system would cause the concomitant release of exochelin MS monomer and ferrous iron from the matrix.

1 ml of 1 mM thioglycollic acid was applied to a ferri-exochelin MS-Sepharose column (0.4 g Sepharose containing 16 μmol ferri-exochelin MS). As the reducing agent entered the column bed the mobile phase immediately became purple which was taken as

indicative of the co-ordination of ferrous iron, derived from ferri-exochelin MS, by the thioglycollic acid.

100 ml samples of the eluate were then analysed by reaction with OPA. No enhanced fluorescence was observed with this eluate but a control assay containing 5 nmol ferri-exochelin MS in 100 µl 1 mM thioglycollic acid did cause enhanced fluorescence with OPA (Table 11). The release of exochelin monomers would have been expected to exceed this level if the hypothesis and the assumption regarding matrix saturation were correct. These observations were, therefore, taken as suggesting that no release of peptide material had occurred with the observed release of iron on the reduction of the ferri-siderophore complex by thioglycollic acid and, by implication, the dimer hypothesis of ferri-exochelin MS structure was incorrect.

Table 11. **The effect of thioglycollic acid on immobilised ferri-exochelin MS.**

1 ml 1mM thioglycollic acid was applied to a ferri-exochelin MS-Sepharose column. 100 µl of the eluate was added to 3ml borate-buffered OPA pH 9.5. Fluorescence was measured using λ_{ex} = 410nm λ_{em} = 490nm on a Perkin Elmer spectrofluorimeter with slit widths set at 2.5 nm. Readings presented are means of duplicate samples.

Sample assayed	Fluorescence at 490 nm
100 µl water	0
100 µl eluate	-19.1
5 nmol ferri-exochelin in thioglycollic acid	93.15

VII   Further Discussion

A.   The iron-regulated envelope proteins of *Mycobacterium smegmatis*.

The effect of physiological stresses upon the expression of envelope proteins in *Mycobacterium smegmatis* was studied in order to identify those proteins that were expressed as a specific response to iron starvation. A family of seven proteins was identified which exhibited an increased expression on iron limitation (viz. 180, 84, 70, 54, 25, 21 and 14 kDa; see Fig. 10). The expression of these proteins was not increased, however, on zinc limitation (Fig. 10) or during two heat shock regimes (Fig 11) and, therefore, it was concluded that they were not expressed as part of a general stress response. These proteins, therefore, could legitimately be described as iron-regulated envelope proteins (IREPs).

Although 70, 54 and 21 kDa had not been identified as IREPs of *M. smegmatis* in previous studies (Hall *et al.*, 1987; Sritharan, 1988) 21 kDa IREPs had been reported in *Mycobacterium neoaurum*, *M. leprae* and *M. avium* (Sritharan, 1988; Sritharan and Ratledge, 1989 & 1990) and there was immunological data to suggest that the 70 and 54 kDa proteins may be genuine IREPS (Sritharan, 1988). Polyclonal antisera raised against purified IREPs of *M. neoaurum* were used to probe Western blots of envelope protein preparations from several iron-starved mycobacteria. When samples from *M. smegmatis*, *M. neoaurum*, *M. avium* and an Armadillo-derived mycobacterium were probed with antisera raised against the 180, 120, 21 and 14 kDa proteins, a 54 kDa protein was recognised in every case. When antisera raised against the 180 kDa IREP of *M. neoaurum* was used to probe Western blots of envelope protein preparations from iron starved cultures of *M. smegmatis* then a 70 kDa protein was recognised (Sritharan, 1988). In this series of immunoblotting experiments the only cross-reactions seen with IREP antisera were with other IREPs and these 70 and 54 kDa envelope proteins. The antisera used did not cross react with any other proteins which suggested that these were specific interactions and may suggest that the 70 and 54 kDa proteins, although not previously reported, may be genuine IREPs. This degree of cross

144

reaction may suggest that the IREPs contain common epitopes. The amino acid sequences forming such epitopes could, therefore, represent important structural or functional determinants of the IREPs. It is likely that, as putative iron transport proteins, some of the IREPs would be required to interact with each other in order to facilitate the binding and translocation of iron across the envelope layers of the cell and, therefore a conserved sequence may be expected to facilitate some of the protein interactions necessary for the uptake of iron. A precedent for this hypothesis does exist: a conserved sequence appears to be important as a site of the physical interactions that occur between TonB and its dependent receptor proteins in ferri-siderophore transport in the Enterobacteriaceae (Skare *et al.*, 1993).

The possibility remains, however, that these sequences simply had a common origin and that some of the proteins were degradation products of a larger IREP. The 70 kDa protein was only recognised with antisera from the 180 kDa IREP (Sritharan, 1988) which may suggest that the 70 kDa protein was the product of the proteolysis of the larger molecule. The 54 kDa protein, however, cross-reacted with antisera raised against all of the IREPs tested (180, 120, 21 and 14 kDa). It seems unlikely that all of these proteins are proteolytic cleavage products of the 180 kDa protein and, therefore, it was considered that the 54 kDa protein was a genuine IREP.

A major discrepancy occurred between the IREPs observed in this study and those reported previously in *M. smegmatis* (Hall *et al.*, 1987; Sritharan, 1988). Previously a 29 kDa protein had been described as an IREP. Expression of this protein appeared to be strictly regulated by iron and indeed the results from a series of experiments using antisera raised against the IREPs of *M. smegmatis* as components of ^{55}Fe(III)exochelin uptake assays had been taken as suggesting that the 29 kDa IREP was a putative ferri-exochelin receptor protein (Hall *et al.*, 1987; Sritharan, 1988). In this study, however, the 29 kDa envelope protein was not only expressed constitutively in *M. smegmatis* grown at 37°C but was also the major envelope protein of the bacterium. The changes in the envelope protein profiles seen during the heat shock experiments suggested the involvement of a temperature-dependent factor in the regulation of expression of this protein. Despite the fact that the constitutive expression

of this protein appeared inconsistent with its putative role as a ferri-siderophore receptor, subsequent investigations confirmed that the 29 kDa protein of *M. smegmatis* was a ferri-exochelin MS-binding protein.

Conceivably, a mutation in the promoter region of the gene encoding the 29 kDa protein may have affected the regulation of its expression so this possibility was addressed by comparing the envelope protein profiles of the working strain and a new culture of the original strain, NCIMB 8548. SDS-PAGE analysis of envelope protein extracts from iron starved and iron replete cultures of each bacterium revealed that there were no significant differences between the protein profiles of the cell envelope of these bacteria. The 29 kDa protein was constitutively expressed at a high level in iron replete cells in both cases and thus there was no genetic basis for this de-regulation of the expression of the protein. It was thought that this change in regulation could have had a nutritional basis so the bacterium was grown in iron replete medium supplemented with 9 other metals, singly and as a mixture, but the regulation of the expression of the 29 kDa protein by iron was not regained. This discrepancy remains to be resolved.

B. The identity of the major ferri-exochelin binding protein in the cell `envelope of *Mycobacterium smegmatis*.

In an effort to identify the protein, or proteins, involved in the binding of ferri-exochelin at the cell surface, the putative first event in the siderophore-mediated transport of iron into the bacterium, a suite of techniques was used to form and separate complexes incorporating ferri-exochelin and envelope proteins from iron-starved cultures of *Mycobacterium smegmatis*. The formation and analysis of ferri-exochelin-envelope protein complexes was attempted as follows:

1. *In situ* in native cell envelope labelling with ^{55}Fe(III)exochelin. Separation of the proteins was attempted using CHAPS-PAGE.

2. using CHAPS-solubilised envelope proteins:
   a. affinity chromatography on ferri-exochelin MS-Sepharose and
   b. using ^{55}Fe(III)exochelin separating labelled molecules by anion exchange chromatography.

1. **Analysis of the ferri-exochelin MS-binding activity of *M. smegmatis* envelope proteins *in situ***

Despite the poor resolution gained when separating *M. smegmatis* envelope proteins on CHAPS-PAGE (Fig. 13), the first direct evidence for the involvement of the 29 kDa envelope protein in the uptake of ferri-exochelin MS was gained using this separation technique. A native envelope preparation from iron starved *M. smegmatis* cultures was incubated with ^{55}Fe(III)exochelin and the proteins extracted using the non-denaturing detergent CHAPS. The solubilised proteins were then separated on CHAPS-PAGE and ^{55}Fe-labelled complexes were visualised by autoradiography of the dried gel (Fig. 14). Instead of forming a distinct band on the autoradiograph that corresponded to a well-resolved protein band in the gel, ^{55}Fe had been deposited in an almost uniform

147

manner in the lane down to a definite limit at R_f 0.56. It was thought that the ^{55}Fe in the upper part of the gel ($R_f < 0.56$) was probably protein-bound and that this had not resolved to distinct bands because of the aggregation and precipitation of the *M. smegmatis* envelope proteins in the resolving gel that was common on CHAPS-PAGE. This protein-bound ^{55}Fe did, however, exhibit a definite limit of mobility at R_f 0.56 which corresponded to a 29 kDa protein beyond which no ^{55}Fe, other than a small amount of unbound ^{55}Fe(III)exochelin, had migrated. This result, therefore, was considered as demonstrating the association of ^{55}Fe(III)exochelin with a 29 kDa envelope protein of limited solubility in the CHAPS-PAGE gel system.

This observation was consistent with the work of Sritharan (1988) in which the pre-incubation of a suspension of iron-starved *M. smegmatis* with polyclonal antisera raised against the 29 kDa IREP caused ^{55}Fe(III)exochelin uptake to be inhibited to 30 % of the control rate.

Recent advances in the study of the mycobacterial cell wall structure which suggest the existence of a highly ordered, asymmetric, outer membrane (see Section I.F.1) may indicate that the ferri-exochelin receptor of *M. smegmatis* may be required not only to bind the ferri-siderophore, but as with Gram-negative bacteria, also form a pore to facilitate the translocation of the molecule across this outer membrane layer. As the outer membrane ferri-siderophore receptor proteins of *Escherichia coli* all have molecular sizes in the range 70-80 kDa (see Table 3) then, assuming that the size of the *E. coli* receptors is near optimal for their function, this 29 kDa ferri-exochelin binding protein (FEBP) appeared to be too small to fulfil both of these functions. It was considered likely, therefore, that this protein would be required to either function as a dimer (at least) or associate with at least one other protein in order to form a complete ferri-exochelin receptor. The precipitation of the ferri-exochelin-FEBP complex in the CHAPS-PAGE gel system obscured detail in the upper part of the lane and thus made the identification of larger, more elaborate complexes impossible. Due to a shortage of exochelin MS at this time, because of the needs of ongoing structural determinations, and also the poor resolution gained on CHAPS-PAGE this experiment was not repeated.

2. The analysis of the ferri-exochelin MS-binding activity of CHAPS-extracted envelope proteins

The next approach used to confirm the identity of the FEBP was the use of envelope proteins that had been extracted from the mycobacterial envelope using the non-denaturing detergent CHAPS to form ferri-exochelin-protein complexes.

a. Affinity chromatography on ferri-exochelin MS-Sepharose.

The first technique used was affinity chromatography. A ferri-exochelin MS-Sepharose 4B chromatography medium was constructed and used to fractionate CHAPS-extracted envelope proteins from iron-starved and iron-replete cultures of *M. smegmatis*. Total envelope extracts were loaded onto the column which was then washed with various eluents. The eluates were analysed by SDS-PAGE. A 29 kDa protein was repeatedly retained on the immobilised ferri-siderophore matrix and could only be eluted using 2 % SDS. A similar amount of 29 kDa protein was bound to the column irrespective of the iron status of the culture from which they were derived, an observation which was consistent with the apparent deregulated expression of the 29 kDa IREP in our hands. When the column was washed with 1M NaCl, after the loading of the extracted envelope proteins, only the 29 kDa protein was retained on the matrix demonstrating that this protein was the major ferri-exochelin binding protein of the *M. smegmatis* cell envelope. When the column was washed with 1 mM ferri-exochelin in 1M NaCl, after the loading of the extracted envelope proteins, only the 29 kDa protein was retained on the affinity matrix (Fig. 19). Exact repetition of this washing protocol using a Tris-Sepharose column, however, did not result in the retention of the 29 kDa protein. The retention of the FEBP on ferri-exochelin-Sepharose was demonstrated, therefore, to be mediated by an interaction with the immobilised siderophore rather than the supporting agarose or the spacer arm. The 29 kDa protein had thus been demonstrated to be the major ferri-exochelin-binding protein of the *M. smegmatis* envelope.

Although some evidence was gained from these experiments which implicated the 25 kDa IREP in the ferri-exochelin uptake process no direct physical evidence for a larger ferri-exochelin-protein complex was seen.

b.   Anion-exchange chromatography on PEI-cellulose.

The isolation of a stable ferri-exochelin MS-protein complex was attempted by incubating an envelope protein solution, in CHAPS, with ^{55}Fe(III)exochelin and then separating the complexes formed by anion exchange chromatography on polyethyleneimine cellulose. After washing away the unbound ^{55}Fe(III)exochelin, which is cationic, three peaks of radioactivity were eluted from the PEI-cellulose column with a step gradient of increasing NaCl concentration. The fractions corresponding to the second peak, which exhibited the greatest specific activity, contained five proteins that could be detected by silver staining after SDS-PAGE. Of these proteins two, 25 and 29 kDa, corresponded to previously reported IREPs. When the fractions corresponding to the elution of this peak were passed through a gel filtration column three peaks of ^{55}Fe elution were observed. The first eluting peak corresponded to a molecule of 28.8 ± 2.5 kDa in size. The identity of the second eluting species (5.7 ± 0.3 kDa) is unknown while the third species eluted at the elution volume of free ferri-exochelin. Again evidence of a direct association of ^{55}Fe, administered as ferri-exochelin, and a 29 kDa protein had been demonstrated, confirming the identity of the FEBP, but although the 25 kDa IREP had co-purified with this complex no evidence of a larger species was found using this approach.

C. Evidence suggesting that the ferri-exochelin receptor may be a heterodimer: the likely role of the 25 kDa IREP in the iron metabolism of *M. smegmatis*.

Although the identity of the FEBP had been confirmed by the demonstration of a ferri-exochelin-FEBP complex using a variety of techniques, no direct evidence had been found for the formation of a larger, more elaborate complex which was though likely to be a requirement for the translocation of the ferri-siderophore across the wall of the bacterium *in vivo*. Some evidence for the involvement of the 25 kDa IREP in the uptake of ferri-exochelin, however, had been gained from the fractionation of free [55]Fe-protein complexes by anion exchange chromatography on PEI-cellulose and the elution profiles observed during the affinity chromatography fractionations of envelope proteins on ferri-exochelin-Sepharose 4B.

As mentioned in the previous section, the fractionation of [55]Fe(III)exochelin-envelope protein complexes on PEI-cellulose using a step gradient of increasing NaCl concentration resulted in the elution of three radioactive peaks from the column after the removal of excess free [55]Fe(III)exochelin. Two of these peaks were eluted using 100 mM NaCl while the third peak was eluted with 1 M NaCl. The second peak exhibited the greatest specific activity. The fractions corresponding to this second radioactive peak were pooled and then analysed by SDS-PAGE revealing 5 proteins after silver staining (viz. 25, 26, 29, 39 and 46 kDa; see Fig. 22), of which, only the 25 and 29 kDa proteins coincided with the sizes of previously recognised IREPs. At this stage, the co-elution of a 25 kDa protein with the ferri-exochelin-FEBP complex could have been coincidental as is presumably the case for the other three co-eluting proteins. It was also equally tenable that this smaller protein could have eluted as part of a more elaborate ferri-exochelin-protein complex that was unstable in 0.1 M NaCl / CHAPS solution used as the eluent and thus dissociated on elution from the PEI-cellulose with only the smaller ferri-exochelin-FEBP complex persisting.

Further evidence for the involvement of the 25 kDa IREP in ferri-exochelin uptake was gained from the affinity chromatography experiments. When 1 mM ferri-exochelin in buffered CHAPS was used as an eluent, after non-binding proteins had been removed from the loaded columns by washing with buffered CHAPS, analysis of the eluate by SDS-PAGE revealed the elution of several proteins. When envelope protein extracts from iron-replete cells were used three proteins, 25, 22 and 19 kDa, were eluted, when using extracts from iron-starved cells an 18 kDa protein was also eluted (Fig. 15). Of these, only the 25 kDa protein coincided with the size of a previously recognised IREP and the band on the gel corresponding to this protein appeared to stain more strongly in the samples derived from iron-starved cells which is consistent with the observed iron-regulated synthesis of this protein and its identification as an IREP (Fig. 10).

When 1 mM [55]Fe(III)exochelin was used as the eluent while analysing extracts from iron-starved cultures, gel filtration of the eluate provided no evidence for the formation of a ferri-exochelin-protein complex (Fig. 17). This observation was consistent with earlier results in that the size of the smallest stable complex formed *in situ* in native envelope was determined as 29 kDa, not 25 kDa, on CHAPS-PAGE analysis (Fig. 14). If the 25 kDa IREP forms a specific association with ferri-exochelin MS then the resulting complex appears unstable and relatively ephemeral.

The removal of the remaining proteins from the affinity chromatography columns, after the ferri-exochelin elution steps, by washing with 2 % SDS and analysis of the eluate by SDS-PAGE demonstrated only a partial elution of the 25 kDa protein had occurred with ferri-exochelin and that some of the protein had remained bound to the affinity matrix along with the FEBP (Fig. 18). As any association between the 25 kDa IREP and ferri-exochelin MS appeared to be weak then this was considered as suggesting that the 25 kDa IREP may have interacted with the FEBP rather than the ferri-siderophore or that the FEBP may have stabilised the association of the 25 kDa protein with the ferri-exochelin-FEBP complex. If the two proteins were to cooperate in the binding of the ferri-exochelin then it is possible that both proteins would be required to effect the binding of the molecule. The purification of the FEBP on ferri-

exochelin-Sepharose demonstrated only that this protein was able to retain the siderophore, in isolation, once the complex had been formed on the matrix, and yielded no information about the involvement of other proteins in the initial binding of the ligand.

When samples from iron-starved and iron-replete cultures were analysed by affinity chromatography, however, similar amounts of FEBP were retained even though the amounts of the 25 kDa IREP varied due to its iron-regulated synthesis (Fig. 15). Although investigation of the binding properties of an active, purified FEBP would be required to resolve this question these data do suggest that the ferri-exochelin-FEBP complex may be formed without the involvement of the 25 kDa IREP.

After non-binding proteins from an envelope extract of iron-starved *M. smegmatis* had been washed from a ferri-exochelin-Sepharose column, washing with 10 mM ATP in buffered CHAPS caused the elution of the 29 kDa FEBP and a 25 kDa protein (data not shown). Again the co-elution of these two proteins suggested that a physical association may have existed between them. Interestingly, this result also represented the only observed elution of bound FEBP from ferri-exochelin-Sepharose in the absence of SDS. Although this experiment requires validation by several repetitions, all using fresh extracts, this observation may suggest the involvement of ATP in the release of ferri-exochelin MS from the FEBP *in vivo* and may thus prove valuable in the study of the later stages of the uptake mechanism.

In summary, the evidence from the PEI-cellulose fractionations and affinity chromatography experiments suggested that the 25 kDa IREP may be involved in the transport of ferri-exochelin, possibly as a receptor component along with the FEBP, but that it does not appear to be a ferri-exochelin binding protein. The association of two transport proteins to form a functional unit is consistent with our current understanding of membrane transport systems in general. Transport systems are modular in organisation; their components can be expressed as distinct domains on one protein or as a series of interacting proteins (Nikaido and Saier, 1992). Indeed, Köster and Braun (1990b) demonstrated that the N- and C- terminal halves of the cytoplasmic membrane-associated ferri-hydroxamate transport protein, FhuB, could combine to restore iron

supply to *fhu*B⁻ mutants of *E. coli* even when expressed on separate plasmids when neither half was sufficient to restore iron supply in isolation

D.   The ferri-exochelin MS- binding activity of extracted envelope proteins in proteo-liposomes

The use of extracted envelope proteins in the formation of ferri-exochelin-receptor complexes was not ideal as it was thought unlikely that the proteins would be present in their native conformations. This was probably the major factor that caused the inherent lack of sensitivity of the PEI-cellulose and affinity chromatography based protocols. The extracted envelope proteins were incorporated into liposomes in order to simulate a more natural situation in which the proteins may have adopted a conformation approximating their native structure. The proteo-liposomes possessed a ^{55}Fe(III)exochelin-binding activity which was protein concentration dependent and heat-labile, thus precluding desferri-mycobactin as the source of the activity. Comparison of the elution of ^{55}Fe(III)exochelin from a gel filtration column alone and after mixing with liposomes and proteo-liposomes demonstrated that the ferri-exochelin associated only with proteo-liposomes. A comparison of the extent of ferri-exochelin binding of proteo-liposomes and CHAPS-solubilised envelope proteins (Peak II of the PEI-cellulose fractionation) revealed a 133-fold enhancement of the ferri-exochelin binding activity of CHAPS-extracted envelope proteins once they had been incorporated into liposomes.

70 % of bound ^{55}Fe(III)exochelin was retained by labelled proteo-liposomes after overnight dialysis which was taken as suggesting that the retention of ^{55}Fe was a specific event and that it was not a porin-mediated phenomenon. Attempts were then made to solubilise this bound ferri-exochelin which was presumably in the form of a ferri-exochelin-protein complex. A range of detergent treatments were used and, after a dialysis step and liquid scintillation counting of the retentate, Triton X-100 was found to effect the greatest release of a high molecular weight soluble ^{55}Fe species, i.e. a complex rather than free ferri-exochelin, from the liposomes. Although this complex

was stable in a buffered Triton X-100 solution, electrophoresis of this material on Triton X-100-PAGE resulted in the dissociation of the complex (Fig. 31). The ferri-exochelin-FEBP complex, however, was known to be stable under the conditions prevalent in the CHAPS-PAGE electrophoresis system (Fig. 14), the only difference between the two electrophoresis systems being the nature of the detergent: CHAPS is zwitterionic whereas Triton X-100 is a non-ionic detergent. It appears that CHAPS may able to stabilise the ferri-exochelin-FEBP complex in an electric field in a manner in which its non-ionic counterpart can not mimic.

The estimation of the molecular weight of the Triton X-100 solubilised complex was then attempted using gel filtration chromatography instead of electrophoresis (Fig. 33). Analysis of the eluate from a Sephacryl S-100-HR column revealed the elution of a single radioactive peak corresponding to a protein of 57.5 ± 4 kDa. The fractions corresponding to this peak were pooled, concentrated by lyophilisation and then analysed by SDS-PAGE. This electrophoretic separation ran abnormally, presumably due to the persistence of liposome components in the protein preparation. Three proteins were detected by silver staining with apparent molecular weights of 29, 32 and 38 kDa. As this electrophoresis was abnormal it was considered that these sizes were unreliable. Attempts to improve this last electrophoretic step have, thus far, failed. It is thought that the 38 kDa protein does not form part of the complex and simply co-eluted in the later fractions pooled for electrophoresis. This protein also appears too large to form a dimeric complex of 57.5 kDa with either of the smaller proteins, therefore, the complex was presumably formed from the 29 and 32 kDa proteins. To this point, all ferri-exochelin-protein complexes that had been recognised had contained the 29 kDa FEBP and often the 25 kDa IREP had co-purified with the complex. It is possible that the proteins that formed this 57.5 kDa complex are the 29 FEBP and 25 kDa IREP which electrophoresed abnormally with slight distortions to their apparent molecular sizes due to the retention of small amounts of lipid material from the liposomes.

As attempts to improve the final electrophoretic step failed, it is considered that a conclusive result may be gained if the composition of the liposomes were altered and

the protocol repeated. Failing this, monoclonal antibodies raised against the complex could possibly be used to probe Western blots of envelope protein profiles in order to identify its components .

E.   Further studies in mycobacterial iron assimilation.

1.   Are the 29 kDa IREPs of the pathogenic mycobacteria ferri-exochelin receptors ?

Although the work of this thesis was carried out solely using the saprophyte, *Mycobacterium smegmatis*, the progress made in the understanding of ferri-exochelin MS binding at the cell surface may have important implications to our understanding of the in vivo growth of the pathogenic mycobacteria. The analysis of the gene encoding the 28 kDa antigen of *M. leprae* suggested that the gene product is an envelope-associated, iron-regulated protein (Dale and Patki, 1990) and, considering its similarity in size to the FEBP of *M. smegmatis*, then it is reasonable to speculate that this protein may also be a ferri-exochelin-binding protein. The molecular cloning of the gene encoding the FEBP of *M. smegmatis* would not only facilitate structural and functional analyses of the protein but would also allow its comparison with the gene encoding the 28 kDa antigen of *M. leprae*.

A DNA fragment has recently been isolated from a *M. tuberculosis* gene library using the promoter region of the *M. leprae* 28 kDa antigen gene as a probe (O. Dellagostin, unpublished work). Sequence analysis of a sub clone from this DNA fragment demonstrated that the *M. tuberculosis* DNA contained areas of significant sequence homology with the *M. leprae* gene. The putative regulatory protein operator site overlapping the RNA polymerase binding motif was highly conserved (85% sequence identity) and the predicted N-terminal amino acid sequence of the gene products also exhibited a high degree of homology (L. Dover, unpublished work). This *M. tuberculosis* gene was truncated, however, and the rest of the gene is currently being cloned in this laboratory.

A 29 kDa IREP has also been reported in *M. avium* which was also expressed in bacteria recovered from infected mice (Sritharan and Ratledge, 1990; Sritharan, 1988). It appears that 28/29 kDa iron-regulated envelope proteins may be present in all pathogenic mycobacteria and, if these were also ferri-exochelin-binding proteins, then their *in vivo* expression could suggest that group A exochelins are important for the growth of the pathogenic mycobacteria *in vivo*.

Cross-feeding studies have shown that suspensions of *M. leprae*, isolated from infected tissue, were able to assimilate iron from ferri-exochelin MN, the group A exochelin from the saprophyte *M. neoaurum* (Hall *et al.*, 1983; Sritharan, 1988). This may suggest that the leprosy bacillus synthesises a group A exochelin related structurally to exochelin MN and a cognate receptor protein *in vivo*. Analysis of envelope extracts from *M. leprae* by affinity chromatography on ferri-exochelin MN-Sepharose has been attempted (K.B. Kannan, unpublished work) but these studies proved inconclusive due to the difficulties inherent to work with this bacterium: the technique lacks sensitivity when working with relatively abundant envelope material from the fast-growing *M. smegmatis*.

The expression of the *M. leprae* 28 kDa antigen in *M. smegmatis* could provide a means by which significant amounts of this protein could be analysed *in vitro*. Antibodies raised against the recombinant protein could possibly be used in the immuno-affinity purification of the native protein from extracts of *M. leprae*-infected tissue for use in binding studies with ferri-exochelin MN.

2.   Further studies in the ferri-exochelin MS uptake mechanism of *M. smegmatis*.

Although the data presented in this thesis suggests that the 25 kDa IREP may interact with the FEBP-ferri-exochelin complex, a complex containing all three components has not been demonstrated. A 57.5 kDa ferri-exochelin-protein complex was isolated from ^{55}Fe(III)exochelin-labelled proteo-liposomes but its components have not been reliably identified. Two of the protocols developed in this study may,

with further development, potentially lead to the resolution of the structure of this complex. The alteration of the liposome constitution by omission of the cholesterol may be lead to improved resolution of the complex components on SDS-PAGE. Alternatively, if the elution of the FEBP and 25 kDa IREP from ferri-exochelin MS-Sepharose can be achieved reproducibly with ATP then the interaction of ^{55}Fe(III)exochelin MS with the dialysed eluate could be investigated using gel filtration chromatography.

The molecular cloning and sequence analysis of the gene encoding the FEBP of *M. smegmatis* would yield valuable data which would allow the protein-ligand interaction to be studied through a directed mutagenesis program. The production of stable derivatives of exochelin MS which could be useful in the study of ferri-exochelin recognition and binding may also now be possible now that the structure of the siderophore has been resolved.

The role of the putative ferri-exochelin transport genes isolated from *M. smegmatis* by Fiss *et al.* (1994) requires investigation. These genes were found to exhibit homology with the cytoplasmic membrane-associated components of the ferri-enterobactin periplasmic binding protein-dependent transport system from *E. coli*. A gene encoding a ferri-exochelin MS-binding protein was not discovered. This lack of a · binding protein may be of little significance until more of the iron-regulated genes of the bacterium are isolated and characterised. The genetic linkage of the *fxb*A gene, which appears to encode the enzyme responsible for the addition of the terminal N-formyl group to exochelin MS, to these putative transport genes is interesting. It is thought likely that this formylation reaction represents the last stage in the synthesis of the siderophore by forming the last of the hydroxamate groups responsible for iron binding. If the completed molecule were then released into the cytoplasm before export to the medium then one could envisage either the disruption of intracellular iron metabolism by this potent chelator or a considerable burden being exerted upon the NAD(P)H pool of the cell to remove iron from exochelin by reduction. These situations would obviously be undesirable especially in an already stressed cell. It is therefore interesting to speculate that the putative transport genes could be involved in

the export of exochelin MS from the cell. The *fxb*A gene product could possibly retain the completed siderophore and then present the molecule to the ATP-driven permease. The hydrolysis of ATP could then facilitate the release of the siderophore from the enzyme and allow its export through the membrane. Such a sequestration mechanism could provide a means by which the iron-starved cell prevented disruption of its metabolism by exochelin. Analysis of the phenotypes of mutants in *fxu* is now necessary to resolve their role in mycobacterial iron metabolism..

VIII   References.

Abok, K., Blomquist, E., Ericsson, J. and Brunk, U. 1983. Macrophage radiosensitivity in culture as a function of exposure to ionic iron. Virchows Arch. [Cell pathol.] **42** : 119-129.

Águas. A.P., Esaguy, N., Sunkel, C.E. and Silva, M.T. 1990. Cross-reactivity and sequence homology between the 65-kilodalton mycobacterial heat shock protein and human lactoferrin, transferrin and DR$_\beta$ subsets of major histocompatabilty complex class II molecules. Infect. Immun. **58** : 1461-1470.

Alcantara, J., Yu, R.H. and Schryvers, A.B. 1993. The region of human transferrin involved in binding to bacterial transferrin receptors is localized in the C-lobe. Mol. Microbiol. **8** : 1135-1143.

Angerer, A., Klupp, B. and Braun, V. 1992. Iron transport systems of *Serratia marcescens*. J. Bacteriol. **174** : 1378-1387.

Anwar, H., Brown, M.R.W., Day, A. and Weller, P.H. 1984. Outer membrane antigens of mucoid *Pseudomonas aeruginosa* isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiol. Lett. **24** : 235-239.

Arceneaux, J.E.L. and Byers, B. 1980. Ferrisiderophore reductase activity in *Bacillus megaterium*. J. Bacteriol. **141** : 715-721.

Archibald, F. 1983. *Lactobacillus plantarum*, an organism not requiring iron. FEMS. Microbiol. Lett. **19** : 29-32.

Bagg, A. and Neilands, J.B. 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol. Rev. **51** : 509-518.

Baig, B.H., Wachsmuth, K. and Morris, G.K. 1986. Utilisation of exogenous siderophores by *Campylobacter* spp. J. Clin. Microbiol. **23** : 431-433.

Barclay, R. and Ratledge, C. 1983. Iron-binding compounds of *Mycobacterium avium, M. intracellulare, M. scrofulaceum* and mycobactin-dependent *M. paratuberculosis* and *M. avium*. J. Bacteriol. **153** : 1138-1146.

Barghouthi, S., Young, R., Olson, M.O.J., Arceneaux, J.E.L., Clem, L.W. and Byers, B.R. 1989. Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. J. Bacteriol. **171** : 1811-1816.

Bäumler, A.J. and Hantke, J. 1992. Ferrioxamine uptake in *Yersinia enterocolitica* : characterisation of the receptor protein FoxA. Mol. Microbiol. **6** : 1309-1321.

Becker, K., Köster, W. and Braun, V. 1990. Iron(III)hydroxamate transport of *Escherichia coli* K12 : single amino acid replacements at potential ATP-binding sites inactivate the FhuC protein. Mol. Gen. Genet. **223** : 159-162.

Bergeron, R.J. 1987. Synthesis and properties of polyamine catecholamide chelators. In G.Winkelmann, D. van der Helm and J.B. Neilands (ed.) Iron transport in microbes, plants and animals. pp.285-315. VCH Publishers (U.K.) Ltd., Cambridge.

Bergmeyer, H.U. 1973. Methods of enzymatic analysis. Verlag Chemie, Weinheim

Berish, S.A., Subbarao, S., Chen, C.-Y., Trees, D.L. and Morse, S.A. 1993. Identification of a *fur* homolog from *Neisseria gonorrhoeae*. Infect. Immun. **61** : 4599-4606.

Bitter, W., Marugg, J.D., de Weger, L.A., Tommassen, J. and Wisbeek, P.J. 1991. The ferric pseudobactin receptor PupA of *Pseudomonas putida* WCS358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. Mol. Microbiol. **5** : 647-655.

Blake, D.R., Hall, N.D., Bacon, P.A., Dieppe, P.A., Halliwell, B. and Gutteridge, J.M.C. 1981. The importance of iron in rheumatoid disease. Lancet. November : 1142-1143.

Blakley, R.L., Ghambeer, R.K., Nixon, P.F. and Vitols, E. 1965. The cobamide-dependent ribonucleoside triphosphate reductase of lactobacilli. Biochem. Biophys. Res. Comm. **20** : 439-445.

Bradbeer, C. 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J. Bacteriol. **175** : 3146-3150.

Braun V., Hantke, K., Eick-Helmerich, K., Köster, W., Preßler, U., Sauer, M., Schäffer, S., Schöffler, H., Staudenmaier, H. and Zimmermann, L. 1987. Iron transport systems in *Escherichia coli*. In G. Winkelmann, D. van der Helm and J.B. Neilands (ed.) Iron transport in microbes, plants and animals. pp.35-51. VCH Publishers (U.K.) Ltd., Cambridge.

Brennan, P.J. and Draper, P. 1994. Ultrastructure of *Mycobacterium tuberculosis*. In B.R. Bloom (ed.) Tuberculosis, pathogenesis, protection and control. pp. 271-284. ASM Press. Washington D.C.

Brewer, S., Tolley, M., Trayer, I.P., Barr, G.C., Dorman, C.J., Hannavy, K., Higgins, C.F., Evans, J.S., Levine, B.A. and Wormald, M.R. 1990. Structure and function of X-pro dipeptide repeats in the TonB proteins of *Salmonella typhimurium* and *Escherichia coli*. J. Mol. Biol. **216** : 883-895.

Brock, J.H., Esparza, I. and Logie, A.C. 1984. The nature of iron released by resident and stimulated mouse peritoneal macrophages. Biochim. Biophys. Acta. **797** : 105-111.

Brock, J.H. and Ng, J. 1983. The effect of desferrioxamine on the growth of *Staphylococcus aureus, Yersinia enterocolitica* and *Streptococcus faecalis* in human serum: uptake of desferrioxamine-bound iron. FEMS Microbiol. Lett. **20** : 439-442.

Brown, K.A. and Ratledge, C. 1975. The effect of *p*-aminosalicylic acid on iron transport and assimilation in mycobacteria. Biochim. Biophys. Acta **385** : 207-220.

Brown, K.A. and Ratledge, C. 1974. Iron transport in *Mycobacterium smegmatis* : ferrimycobactin reductase [NAD(P)H:ferrimycobactin oxido-reductase], the enzyme releasing iron from its carrier. FEBS Letters **53** : 262-266.

Brown, M.R.W., Anwar, H. and Lambert, P.A. 1984. Evidence that mucoid *Pseudomonas aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. FEMS Microbiol. Lett. **21** : 113-117.

de Bruyn, J., Bosmans, R., Nyabenda, J. and van Vooren, J.P. 1989. Effect of zinc-deficiency on the appearance of 2 immunodominant antigens (32 kDa and 65 kDa) in culture filtrates of mycobacteria. J. Gen. Microbiol. **135**: 79-89.

Buchmeier, N.A. and Heffron, F. 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science. **248** : 730-732.

Cavinato, A.G., Macloed, R.M. and Ahmed, M.S. 1988. A non-denaturing gel electrophoresis system for the purification of membrane proteins. Prep. Biochem **18** : 205-215.

Chen, C.Y., Berish, S.A., Morse, S.A. and Meitzner, T.A. 1993. The ferric iron-binding protein of pathogenic Neisseria spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. Mol. Microbiol. **10** : 311-318.

Chenault, S.S. and Earhart, C.F. 1991.Organisation of genes encoding membrane proteins of the *Escherichia coli* ferrienterobactin permease. Mol. Microbiol. **5** : 1405-1413.

Connell, N.D. and Nikaido, H. 1994. Membrane permeability and transport in *Mycobacterium tuberculosis*. In B.R. Bloom (ed.) Tuberculosis, pathogenesis, protection and control. pp. 333-352. ASM Press. Washington D.C.

Cooper, S.R., McArdle, J.V. and Raymond, K.N. 1978. Siderophore electrochemistry: relation to intracellular iron release mechanism. Proc. Nat. Acad. Sci. USA. **75** : 3351-3354.

Cope, L.D., Thomas, S.E., Latimer, J.L., Slaughter, C.A., Müller-Eberhard, U. and Hansen, E.J. 1994. The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae* : structure and localization. Mol. Microbiol. **13** : 863-873.

Cowart, R.E. and Foster, B.G. 1985. Differential effects of iron on the growth of *Listeria monocytogenes* : Minimum requirements and mechanism of acquisition. J. Infect. Dis. **151** : 721-730.

Crowley, D.E., Reid, C.P.P. and Szaniszlo, P.J. 1987. Microbial siderophores as iron sources for plants. In G.Winkelmann, D. van der Helm and J.B. Neilands (ed.) Iron transport in microbes, plants and animals. pp.375-386. VCH Publishers (U.K.) Ltd., Cambridge.

Dale, J.W. and Patki, A. 1990. Mycobacterial gene expression and regulation. In J. McFadden (ed.) Molecular biology of the mycobacteria. pp.173-198. Surrey University Press / Academic Press Ltd., London.

Daskaleros, P.A., Stoebner, J.A. and Payne, S.M. 1991. Iron uptake in *Plesiomonas shigelloides* : cloning of the genes for the heme-iron uptake system. Infect. Immun. **59** : 2706-2711.

Dyer, D.W., West, E.P. and Sparling, P.F. 1987. Effects of serum carrier proteins on the growth of pathogenic Neisseriae with heme-bound iron. Infect. Immun. **55** : 2171-2175.

Elkins, M.F. and Earhart, C.F. 1989. Nucleotide sequence and regulation of the *Escherichia coli* gene for ferrienterobactin transport protein FepB. J. Bacteriol. **171** : 5443-5451.

Ernst, J.F. and Winkelmann, G. 1977. Enzymatic release of iron from sideramines in fungi. NADH:sideramine oxidoreductase in *Neurospora crassa*. Biochim. Biophys. Acta. **500** : 27-41.

Esaguy, N., Águas, A.P., van Embden, J.D.A. and Silva, M.T. 1991. Mycobacteria and human autoimmune disease: direcr evidence of cross-reactivity between human lactoferrin and the 65-kilodalton protein of tubercle and leprosy bacilli. Infect. Immun. **59** : 1117-1125.

Evans, J.S., Levine, B.A., Trayer, I.P., Dorman, C.J. and Higgins, C.F. 1986. Sequence-imposed structural constraints in the TonB protein of *E. coli*. FEBS Lett. **208** : 211-216.

Fiss, E.H., Stanley-Samuelson, P. and Neilands, J.B. 1982. Properties and proteolysis of the ferric enterobactin outer membrane receptor in *Escherichia coli* K-12. Biochemistry **21** : 4517-4522.

Fiss, E.H., Yu, S. and Jacobs, W.R., Jr. 1994. Identifcation of genes involved in the sequestration of iron in mycobacteria: the ferric exochelin biosynthetic and uptake pathways. Mol. Microbiol. **14** : 557-569.

Fontecave, M., Coves, J. and Pierre, J.-L. 1994. Ferric reductases or flavin reductases ? Biometals **7** : 3-8.

Fontecave, M. and Pierre, J.-L. 1993. Iron metabolism: the low-molecular-mass iron pool. Biol. Metals. **4** : 133-135.

Frost, G.E. and Rosenberg, H. 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K12. Biochim. Biophys. Acta. **330** : 90-101.

Gaines, C.G., Lodge, J.S., Arceneaux, J.E.L. and Byers, B.R. 1981. Ferrisiderophore reductase activity associated with an aromatic biosynthetic enzyme complex in *Bacillus subtilis*. J. Bacteriol. **148** : 529-533.

Garsia, R.J., Hellqvist, L., Booth, R.J., Radford, A.J., Britton, W.J., Astbury, L., Trent, R.J. and Basten, A. 1989. Homolgy of the 70-kDa antigens from *Mycobacterium leprae* and *Mycobacterium bovis* with the *Mycobacterium tuberculosis* 71-kilodalton antigen and the conserved heat shock protein 70 of eucaryotes. Infect. Immun. **57** : 204-212.

Gerlach, G.-F., Klashinsky, S., Anderson, C., Potter, A.A. and Willson, P.J. 1992. Characterization of two genes encoding distinct transferrin-binding proteins in different *Actinobacillus pleuropneumoniae* isolates.
Infect. Immun. **60** : 3253-3261.

Gibson, F and Magrath, D.I. 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes*62-I.
Biochim. Biophys Acta **192** : 175-184.

Grange, J.M. 1989. Mycobacterial disease in the world: Yesterday, today and tomorrow. In C. Ratledge, J. Stanford and J.M.Grange (ed.) The biology of the mycobacteria. Vol. 3. Clinical aspects of mycobacterial disease. pp. 3-36. Academic Press Inc., London.

Grewal, K.K., Warner, P.J. and Williams, P.H. 1982. An inducible outer membrane protein involved in aerobactin-mediated iron transport by ColV strains of *Escherichia coli*. FEBS Lett. **140** : 27-30.

Griffiths, E. 1987. Iron in biological systems. In J.J. Bullen and E. Griffiths (ed.) Iron and infection. pp.1-25. John Wiley & sons Ltd., London.

Griffiths, E. and Humphreys, J. 1980. Isolation of enterochelin from the peritoneal washings of guinea pigs lethally infected with *Escherichia coli*. Infect. Immun. **28** : 286-289.

Griffiths, E., Stevenson, P. and Joyce, P. 1983. Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*.
FEMS. Micro. Lett. **16** : 95-99.

Griffiths, E., Stevenson, P., Thorpe, R. and Chart, H. 1985. Naturally occuring antibodies in human sera that react with the iron-regulated outer membrane proteins of *Escherichia coli*. Infect. Immun. **47** : 808-813.

Haag, H., Hantke, K., Dreschel, H., Stojiljkovic, I., Jung, G. and Zähner, H. 1993. Purification of yersiniabactin: a siderophore and possible virulence factor of *Yersinia enterocolitica*. J. Gen. Micro. **139** : 2159-2165.

Haas, B., Kraut, J., Marks, J., Zanker, S.C. and Castignetti, D. 1991. Siderophore presence in sputa of cystic fibrosis patients.   Infect. Immun. **59** : 3997-4000.

Hall, R.M. and Ratledge, C. 1987. Exochelin-mediated iron acquisition by the leprosy bacillus, *Mycobacterium leprae*. J. Gen. Micro. **133** : 193-199.

Hall, R.M. and Ratledge, C. 1986. Mycobactin and the competition for iron between *Mycobacterium neoaurum* and *M. vaccae*. J. Gen. Micro. **132** : 839-843.

Hall, R.M. and Ratledge, C. 1984. Mycobactins as chemotaxonomic markers for some rapidly growing mycobacteria. J. Gen. Micro. **130** : 1883-1892.

Hall, R.M., Sritharan, M., Messenger, A.J.M. and Ratledge, C. 1987. Iron transport in *Mycobacterium smegmatis* : occurrence of iron regulated envelope proteins as potential receptors for iron uptake. J. Gen. Micro. **133** : 2107-2114.

Halliwell, B. 1982. Superoxide and superoxide-dependent formation of hydroxyl radicals are important in oxygen toxicity. Trends. Biochem. Sci. August : 270-272.

Hames, B.D. 1981. An introduction to polyacrylamide gel electrophoresis. In B.D. Hames and D. Rickwood (ed.). Gel electrophoresis of proteins. pp.1-91. IRL Press Ltd., Oxford.

Hancock, R.E.W., Hantke, K. and Braun, V. 1977. Iron transport in *Escherichia coli* K12. 2,3-dihydroxybenzoate-promoted iron uptake. Arch. Microbiol. **114** : 231-239.

Hanson, M.S., Slaughter, C. and Hansen, E.J. 1992. The *hbp*A gene of *Haemophilus influenzae* type b encodes a heme-binding lipoprotein conseved among heme-dependent *Haemophilus* species. Infect. Immun. **60** : 2257-2266.

Hantke, K. 1987. Ferrous iron transport mutants in *Escherichia coli* K12. FEMS Microbiol. Lett. **44** : 53-57.

Hantke, K. 1984. Cloning of the repressor gene of iron-regulated systems in *Escherichia coli* K12. Mol. Gen. Genet. **197** : 337-341.

Hantke, K. 1983. Identification of an iron uptake system specific for coprogen and rhodotorulic acid in *Escherichia coli* K12. Mol. Gen. Genet. **191** : 301-306.

Hantke, K. 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol. Gen. Genet. **182** : 288-292.

Hantke, K. and Braun, V. 1978. Functional interaction of the TonA/TonB receptor system in *Escherichia coli*. J. Bacteriol. **135** : 190-197.

Harkness, R.E., Chong, P. and Klein, M.H. 1992. Identification of two iron-repressed periplasmic proteins in *Haemophilus influenzae*. J.Bacteriol. **174** : 2425-2430

Hartmann, A. and Braun, V. 1980. Iron transport in *Escherichia coli*. Uptake and modification of ferrichrome. J. Bacteriol. **143** : 246-255.

Hatch, T.P. 1975. Competiton between *Chlamydia psittaci* and L cells for host isoleucine pools: a limiting factor in chlamydial multiplication. Infect. Immun. **12** : 211-220.

van der Helm, D., Baker, J.R., Eng-Wilmot, D.L., Hossain, M.B. and Loghry, R.A. 1980. Crystal structure of ferrichrome and a comparison with the structure of ferrichrome A. J. Am. Chem. Soc. **102** : 4224-4231.

Hollifield, W.C. and Neilands, J.B. 1978. Ferric enterobactin transport system in *Escherichia coli* K12. Extraction, assay and specificity of the outer membrane receptor. Biochemistry **17** : 1922-1928.

Hough, E. and Rogers, D. 1974. The crystal structure of ferrimycobactin P, a growth factor for the mycobacteria. Biochem. Biophys. Res. Comm. **57** : 73-78.

Jarlier, V. and Nikaido, H. 1990. Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J. Bacteriol. **172** : 1418-1423.

Jerez, C.A. 1988. The heat shock reponse in meso- and thermoacidophilic chemolithotrophic bacteria. FEMS Microbiol. Lett. **56** : 289-294.

Johnston, M.A. and Delwiche, E.A. 1965. Isolation and characterisation of the cyanide-resistant and azide-resistant catalase of *Lactobacillus plantarum*. J. Bacteriol. **90** : 352-356.

Joseph, M.H. and Marsden, C.A. 1986. Amino acids and small peptides. In C.K. Lim (ed.) HPLC of small molecules: a practical approach. IRL Press Ltd. Oxford. pp 13-28.

Jurkevitch, E., Hadar, Y., Chen, Y., Yakirevitch, P., Libman, J. and Shanzer, A. 1994. Iron uptake and molecular recognition in *Pseudomonas putida*: receptor mapping with ferrioxamine B, coprogen B and their biomimetic analogues. Microbiology **140** : 1697-1703.

Karlsson, M., Hannavy, K. and Higgins, C.F. 1993a. A sequence-specific function for the N-terminal signal-like sequence of the TonB protein. Mol. Microbiol. **8** : 379-388.

Karlsson, M., Hannavy, K. and Higgins, C.F. 1993b. ExbB acts as a chaperone-like protein to stabilise TonB in the cytoplasm. Mol. Microbiol. **8** : 389-396.

Kaufmann, S.H.E. and van Embden, J.D.A. 1993. Tuberculosis: a neglected disease strikes back. Trends Microbiol. **1** : 2-3.

Klebba, P.E. 1993. Response from Rutz *et al*. Trends Microbiol. **1** : 7-8.

Koebnik, R., Hantke, K. and Braun, V. 1993. The TonB-dependent ferrichrome receptor FcuA of *Yersinia enterocolitica* : evidence against a strict co-evolution of receptor structure and substrate specificity. Mol. Microbiol. **7** : 383-393.

Köster, W. and Braun, V. 1990a. Iron(III)hydroxamate transport into *Escherichia coli* : substrate binding to the periplasmic FhuD protein. J. Biol. Chem. **265** : 21407-21410.

Köster, W. and Braun, V. 1990b. Iron(III)hydroxamate transport into *Escherichia coli* : restoration of iron supply by the co-expression of the N- and C- terminal halves of the cytoplasmic membrane protein FhuB cloned on separate plasmids. Mol. Gen. Genet. **223** : 379-384.

Labischinski, H., Barnickel, G., Bradaczek, H., Naumann, D., Rietschel, E.T. and Giesbrecht, P. 1985. High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. J. Bacteriol. **162** : 9-20.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227** : 680-685.

LaForce, F.M. and Boose, D.S. 1987. Release of lactoferrin by polymorphonuclear leukocytes after aerosol challenge with *Escherichia coli*. Infect. Immun. **55** : 2293-2295.

Langman, L., Young, I.G., Frost, G.E., Rosenberg, H. and Gibson, F. 1972. Enterochelin system of iron transport in *Escherichia coli* : mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112** : 1142-1149.

Lee, B.C. 1992. Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type B. Infect. Immun. **60** : 810-816.

Lee, B.C. and Hill, P. 1992. Identification of an outer-membrane haemoglobin-binding protein in *Neisseria meningitidis*. J. Gen. Micro. **138** : 2647-2656.

Lindquist, S. 1986. The heat-shock response. Ann. Rev. Biochem. **55** : 151-1191.

Lodge, J.S., Gaines, C.G., Arceneaux, J.E.L. and Byers, B. 1982. Ferrisiderophore reductase activity in *Agrobacterium tumefaciens*. J. Bacteriol. **149** : 771-774.

Lodge, J.S., Gaines, C.G., Arceneaux, J.E.L. and Byers, B. 1980. Non-hydrolytic release of iron from ferrienterobactin analogs by extracts of *Bacillus subtilis*. Biochem. Biophys. Res. Comm. **97** : 1291-1295.

Lopez-Goni, I., Moriyon, I. and Neilands, J.B. 1992. Identification of 2,3-dihydroxybenzoic acid as a *Brucella abortus* siderophore. Infect. Immun. **60** : 4496-4503.

de Lorenzo, V., Wee, S., Herrero, M. and Neilands, J.B. 1988. Operator sequences of the ferric aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J. Bacteriol. **169** : 2624-2630.

McCready, K.A. and Ratledge, C. 1979. Ferrimycobactin reductase activity from *Mycobacterium smegmatis*. J. Gen. Micro. **113** : 67-72.

McCready, K.A. and Ratledge, C. 1978. Amounts of iron, haem and related compounds in *Mycobacterium smegmatis* grown in various concentrations of iron. Biochem. Soc. Trans. **6** : 421-423.

Macham, L.P. and Ratledge, C. 1975. A new group of water-soluble iron-binding compounds from mycobacteria: the exochelins. J. Gen. Micro. **89** : 379-382.

Macham, L.P., Ratledge, C. and Nocton, J.C. 1975. Extracellular iron acquisition by mycobacteria: Role of the exochelins and evidence against the participation of mycobactin. Infect. Immun. **12** : 1242-1251.

McIntosh, M.A. and Earhart, C.F. 1977. Coordinate regulation of the synthesis of phenolate compounds and three outer membrane proteins in *Escherichia coli.* J. Bacteriol. **131** : 331-339.

McKenna, W.R., Mickelsen, P.A., Sparling, P.F. and Dyer, D.W. 1988. Iron uptake from lactoferrin and transferrin by *Neisseria gonorrhoeae.* Infect. Immun. **56** : 785-791.

McNeil, M., Daffe, M. and Brennan, P.J. 1990. Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. J. Biol. Chem. **265** : 18200-18206.

McNeil, M., Daffe, M. and Brennan, P.J. 1991. Location of the mycolyl ester substituents in the cell walls of the mycobacteria. J. Biol. Chem. **266** : 13217-13223.

Messenger, A.J.M. and Ratledge, C. 1982. Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferric citrate. J. Bacteriol. **149** : 131-135.

Messenger, A.J.M., Hall, R.M. and Ratledge, C. 1986. Iron uptake processes in *Mycobacterium vaccae* R877R, a mycobactin-lacking mycobacterium. J. Gen. Micro. **132** : 845-852.

Meyer, J.M. 1992. Exogenous siderophore-mediated iron uptake in *Pseudomonas aeruginosa*: possible involvement of porin OprF in iron translocation. J. Gen. Micro. **138** : 951-958.

Miles, A.A., Khimji, P.L. and Maskell, J. 1979. The variable response of bacteria to excess ferric iron in host tissues. J. Med. Microbiol. **12** : 17-28.

Minnikin, D.E. 1982. Lipids: complex lipids, their chemistry, biosynthesis and roles. In C. Ratledge and J. Stanford (ed.) The biology of the mycobacteria. Vol. 1. Physiology, identification and classification. pp. 95-184. Academic Press Inc., London.

Modun, B., Kendall, D. and Williams, P. 1994. Staphylococci express a receptor for human transferrin: identification of a 42-kilodalton cell wall transferrin-binding protein. Infect. Immun. **62** : 3850-3858.

Morton, D.J. and Williams, P. 1990. Siderophore-independent acquisition of transferrin-bound iron by *Haemophilus influenzae* type b. J. Gen. Micro. **136** : 927-933.

Morton, D.J. and Williams, P. 1989. Utilization of transferrin-bound iron by *Haemophilus* species of human and porcine origins. FEMS Microbiol.Lett. **65** : 123-128.

Murphy, C.K., Kalve, V.I. and Klebba, P.E. 1990. Surface topology of the *Escherichia coli* K12 ferric enterobactin receptor. J. Bacteriol. **172** : 2736-2746.

Nakayama, H., Mitsui, T., Nishihara, M. and Kito, M. 1980. Relation between growth temperature of *E. coli* and phase transition temperatures of its cytoplasmic and outer membranes. Biochim. Biophys. Acta. **601** : 1-10.

Nau, C.D. and Konisky, J. 1989. Evolutionary relationship between TonB-dependent outer membrane transport proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J. Bacteriol. **171** : 1041-1047.

Neilands, J.B. 1984. Siderophores of bacteria and fungi. Microbiol. Sci. **1** : 9-14.

Neilands, J.B. 1982. Microbial envelope proteins related to iron. Ann. Rev. Microbiol. **36** : 285-309.

Neilands, J.B., Kanopka, K., Schwyn, B., Coy, M., Francis, R.T., Paw, H. and Bagg, A. 1987. Comparative biochemistry of microbial iron assimilation. In G. Winkelmann, D. van der Helm and J.B. Neilands (ed.) Iron transport in microbes, plants and animals. pp 3-33. VCH Publishers (U.K.) Ltd., Cambridge.

Nelson, M., Carrano, C.J. and Szaniszlo, P. 1992. Identification of the ferrioxamine B receptor, FoxB, in *Escherichia coli* K12. Biometals **5** :37-46.

Nikaido, H. 1993. Uptake of iron-siderophore complexes across the bacterial outer membrane. Trends Microbiol. **1** : 5-8.

Nikaido, H., Kim, S.H. and Rosenberg, E.Y. 1993. Physical organisation of the lipids in the cell wall of *Mycobacterium chelonae*. Mol. Microbiol. **8** : 1025-1030.

Nikaido, H., Nikaido, K. and Harayama, S. 1991. Identification and characterisation of porins in *Pseudomonas aeruginosa*. J. Biol. Chem. **266** : 770-779.

Nikaido, H. and Rosenberg, E.Y. 1990. Cir and Fiu proteins in the outer membrane of *Escherichia coli* catalyse the transport of monomeric catechols: study with β-lactam antibiotics containing catechol and analogous groups. J. Bacteriol. **172** : 1361-1367.

Nikaido, H. and Saier, M.H., Jr. 1992. Transport proteins in bacteria: common themes in their design. Science **258** : 936-942.

Nikaido, H. and Vaara, M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49** : 1-32.

O'Brien, I.G. and Gibson, F. 1970. The structure of enterochelin and related 2,3-dihydroxy-*N*-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215** : 393-402.

Ogunnariwo, J.A. and Schryvers, A.B. 1990. Iron acquisition in *Pasteurella haemolytica*: expression and identification of a bovine-specific transferrin receptor. Infect. Immun. **58** : 2091-2097.

Okujo, N., Saito, M., Yamamoto, S., Yoshida, T., Shinichi, M. and Shinoda, S. 1994. Structure of vulnibactin, a new polyamine-containing siderophore from *Vibrio vulnificus*. Biometals 7 : 109-116.

Otto, B.R., Sparrius, M., Verweij-van Vught, A.M.J.J. and Maclaren, D.M. 1990. Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. Infect. Immun 58 : 3954-3958.

Pabst, M.J., Gross, J.M., Brozna, J.P. and Goren, M.B. 1988. Inhibition of macrophage priming by sulfatide from *Mycobacterium tuberculosis*. J. Immunol. 140 :634-640.

Persmark, M., Expert, D. and Neilands, J.B. 1992. Ferric iron uptake in *Erwinia chrysanthemi* mediated by chrysobactin and related catechol-type compounds. J. Bacteriol. 174 : 4783-4789.

Pickett, C.L., Auffenberg, T., Pesci, E.C., Sheen, V.L. and Jusuf, S.S.D. 1992. Iron acquisition and hemolysin production by *Campylobacter jejuni*. Infect. Immun. 60 : 3872-3877.

Pidcock, K.A., Wooten, J.A., Daley, B.A. and Stull, T.L. 1988. Iron acquisition by *Haemophilus influenzae*. Infect. Immun. 56 : 721-725.

Pollack, J.R., Ames, B.N. and Neilands, J.B. 1970. Iron transport in *Salmonella typhimurium* : mutants blocked in the biosynthesis of enterochelin. J. Bacteriol. 104 :635-639.

Postle, K. 1990. TonB and the Gram-negative dilemma. Mol. Microbiol. 4 : 2019-2025.

Ratledge, C. 1971. The uptake of iron by mycobactin in *Mycobacterium smegmatis*. Biochem. Biophys. Res. Comm. 45 : 856-861.

Ratledge, C. 1987. Iron metabolism in Mycobacteria. In G. Winkelmann, D. van der Helm and J.B. Neilands (ed.) Iron transport in microbes, plants and animals. pp.207-222. VCH Publishers (U.K.) Ltd., Cambridge.

Ratledge, C. and Hall, M.J. 1972. Isolation and properties of auxotrophic mutants of *Mycobacterium smegmatis* requiring either salicylate or mycobactin. J. Gen. Micro. 72 : 143-150.

Ratledge, C., Macham, L.P., Brown, K.A. and Marshall, B.J. 1974. Iron transport in *Mycbacterium smegmatis* : a restricted role for salicylic acid in the extracellular environment. Biochim. Biophys. Acta. 372 : 39-51.

Ratledge, C. and Marshall, B.J. 1972. Iron transport in *Mycobacterium smegmatis* : The role of mycobactin. Biochim. Biophys. Acta. 279 : 58-74.

Ratledge, C., Patel, P.V. and Mundy, J. 1982. Iron transport in *Mycobacterium smegmatis* : the location of mycobactin by electron microscopy. J. Gen. Micro. 128 : 1559-1565.

170

Ratledge, C. and Stanford, J.L. 1982. Introduction. In C. Ratledge and J. Stanford (ed.) The biology of the mycobacteria. Vol. 1. Physiology, identification and classification. pp. 1-8. Academic Press Inc., London.

Ratledge, C. and Winder, F.G. 1962. The accumulation of salicylic acid by mycobacteria during growth on an iron-deficient medium.
Biochem. J. **84** : 501-506.

Redhead, K. and Hill, T. 1991. Acquisition of iron from transferrin by *Bordetella pertussis*. FEMS Microbiol. Lett. **77** : 303-308.

Rey-Ladino, J.A. and Reiner, N.E. 1993. Expression of 65- and 67-kilodalton heat-regulated proteins and a 70-kilodalton heat shock cognate protein of *Leishmania donovani* in macrophages. Infect. Immun. **61** : 3265-3272.

Robins-Browne, R.M. and Prpic, J.K. 1985. Effects of iron and desferrioxamine on infections with *Yersinia enterocolitica*. Infect. Immun. **47** : 774-779.

Rogers, H.J., Bullen, J.J. and Cushnie, G.H. 1970. Further experiments with *Clostridium welchii* Type A *in vivo* and *in vitro*. Immunology **19** : 521-538.

Roth, M. 1971. Fluorescence reaction for amino acids. Anal. Chem. **43(7)** : 880-882.

Rothman, J.E. 1989. Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell. **59** : 591-601.

Rutz, J.M., Lin, J., Lyons, J.A., Goranson, J., Armstrong, S.K., McIntosh, M.A., Feix, J.B. and Klebba, P.E. 1992. Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane. Science **258** : 471-475.

Schneider, R. and Hantke, K. 1993. Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as part of a binding-protein-dependent transport system. Mol. Microbiol. **8** : 111-121.

Schramm, E., Mende, J., Braun, V. and Kamp, R. 1987. Nucleotide sequence of the colicin B activity gene *cba* : consensus pentapeptide among TonB-dependent colicins and receptors. J. Bacteriol. **169** : 3350-3357.

Schöffler, H and Braun, V. 1989. Transport across the outer membrane of *Escherichia coli* K12 via the FhuA receptor is regulated by the TonB protein of the cytoplasmic membrane. Mol. Gen. Genet. **217** : 378-383.

Shand, G.H., Anwar, H., Kadurugamuwa, J., Brown, M.R.W., Silverman, S.H. and Melling, J. 1985. *In vivo* evidence that bacteria in urinary tract infection grow under iron-restricted conditions. Infect. Immun. **48** : 35-39.

Sharman, G., Williams, D.H., Ewing, D.F. and Ratledge, C. 1995. Isolation, purification and structure of exochelin MS, the extracellular siderophore from *Mycobacterium smegmatis*. Biochemical Journal **305** :187-196.

Shea, C.M. and McIntosh, M.A. 1991.Nucleotide sequence and genetic organisation of the ferric enterobactin transport system: homology to other periplasmic binding protein-dependent systems in *Escherichia coli*. Mol. Microbiol. **5** : 1415-1428.

Skare, J.T., Ahmer, B.M.M., Seachord, C.L., Darveau, R.P. and Postle, K. 1993. Energy transduction between membranes. TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vivo* to the outer membrane receptor FepA. J. Biol. Chem. **268** :16302-16308.

Smith, H. 1984. The biochemical challenge of microbial pathogenicity. J. App. Bacteriol. **47** : 395-404.

van Snick, J.L., Masson, P.L. and Heremans, J.F. 1974. The involvement of lactoferrin in the hyposideremia of acute inflammation. J. Expt. Med. **140** : 1068-1084.

Snow, G.A. 1970. Mycobactins : iron-chelating growth factors from mycobacteria. Bacteriol. Rev. **34** : 99-125.

Sokol, P.A. and Woods, D.E. 1983. Demonstration of an iron siderophore-binding protein in the outer membrane of *Pseudomonas aeruginosa*. Infect. Immun. **40** : 665-669.

Spiro, T.G., Bates, G. and Saltman, P. 1967. The hydrolytic polymerisation of ferric citrate. II. The influence of excess citrate. J. Am. Chem. Soc. **89** : 5559-5562.

Sritharan, M. 1988. PhD Thesis. University of Hull.

Sritharan, M. and Ratledge, C. 1990. Iron regulated envelope proteins of mycobacteria grown *in vitro* and their occurence in *Mycobacterium avium* and *Mycobacterium leprae* grown *in vivo*. Biol. Metals. **2** : 203-208.

Sritharan, M. and Ratledge, C. 1989. Co-ordinated expression of the components of iron transport, mycobactin, exochelin and envelope proteins in *Mycobacterium neoaurum*. FEMS. Micro. Lett. **60** : 183-186.

Staggs, T.M. and Perry, R.D. 1992. Fur regulation in *Yersinia* species. Mol. Microbiol. **6** : 2507-2516.

Staudenmaier, H., van Hove, B., Yaraghi, Z. and Braun, V. 1989. Nucleotide sequence of the *fec*BCDE genes and locations of the proteins suggest a periplasmic-binding-protein-dependent transport mechanism for Iron(III)dicitrate in *Escherichia coli*. J. Bacteriol. **171** : 2626-2633.

Stephenson, M.C. and Ratledge, C. 1980. Specificity of exochelins for iron transport in three species of mycobacteria. J. Gen. Micro. **116** : 521-523.

Stephenson, M.C. and Ratledge, C. 1979. Iron transport in *Mycobacterium smegmatis*: Uptake of iron from ferriexochelin. J. Gen. Micro. **110** : 193-202.

Stetter, K.O. and Zillig, W. 1974. Transcription in Lactobacillaceae. DNA-dependent RNA polymerase from *Lactobacillus curvatus*. Eur. J. Biochem. **48** : 527-540.

Stoebner, J.A. and Payne, S.M. 1988. Iron-regulated hemolysin production and utilisation of heme and hemoglobin by *Vibrio cholerae*. Infect. Immun. **56** : 2891-2895.

Stojiljkovic, I. and Hantke, K. 1994. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol. Microbiol. **13** : 719-732.

Stojiljkovic, I. and Hantke, K. 1992. Hemin uptake system of *Yersinia enterocolitica* : similarities with other TonB-dependent systems in Gram-negative bacteria. EMBO. J. **11** : 4359-4367.

Tai, S.S., Lee, C.-J. and Winter, R.E. 1993. Hemin utilisation is related to virulence of *Streptococcus pneumoniae*. Infect. Immun. **61** : 5401-5405.

Teintze, M., Hossain, M.B., Barnes, C.L., Leong, J. and van der Helm, D. 1981. Structure of ferric pseudobactin, a siderophore from a plant growth promoting *Pseudomonas*. Biochemistry **20** : 6446-6457.

Tidmarsh, G.F. and Rosenberg, L.T. 1981. Acquisition of iron from transferrin by *Salmonella paratyphi* B. Curr. Microbiol. **6** :217-220.

Trias, J. and Benz, R. 1994. Permeability of the cell wall of *Mycobacterium smegmatis*. Mol.Microbiol. **14** : 283-290.

Trias, J. and Benz, R. 1993. Characterisation of the channel formed by the mycobacterial porin in lipid bilayer membranes. Demonstration of voltage gating and of negative point charges at the channel mouth. J. Biol. Chem. **268** : 6234-6240.

Trias, J., Jarlier, V. and Benz, R. 1992. Porins in the cell wall of mycobacteria. Science **258** : 1479-1481.

Tsai, J., Dyer, D.W. and Sparling, P.F. 1988. Loss of transferrin receptor activity in *Neisseria meningitidis* correlates with inability to use transferrin as an iron source. Infect. Immun. **56** : 3132-3138.

Tuckman, M. and Osburne, M.S. 1992. *In vivo* inhibition of TonB-dependent processes by a TonB box consensus pentapeptide. J. Bacteriol. **174** : 320-323.

Wagegg, W. and Braun, V. 1981. Ferric citrate transport in *Escherichia coli* requires outer membrane receptor protein FecA. J. Bacteriol. **145** : 156-163.

Ward, K.H., Anwar, H., Brown, M.R.W., Wale, J. and Gowar, J. 1988. Antibody response to outer-membrane antigens of *Pseudomonas aeruginosa* in human burn wound infection. J. Med. Microbiol. **27** : 179-190.

Weinberg, E.D. 1989. Cellular regulation of iron assimilation. Quart. Rev. Biol. **64** :2612-90.

Weiss, M.S., Abele, U., Weckesser, J., Welte, W., Schlitz, E. and Schulz, G.E. 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science **254** : 1627-1630.

White, D.C. and Granick, S. 1963. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85** : 842-850.

Wheeler, P.R. 1990. Recent research into the physiology of *Mycobacterium leprae*. Adv. Microb. Physiol. **31** : 71-124.

Williams, P., Morton, D.J., Towner, K.J., Stevenson, P. and Griffiths, E. 1990. Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae, H. parainfluenzae* and *H. paraphrophilus*. J. Gen. Micro. **136** : 2343-2350.

Wong, G.B., Kappel, M.J., Raymond, K.N., Matzanke, B. and Winkelmann, G. 1983. Co-ordination chemistry of microbial iron transport compounds. 24. Characterisation of coprogen and ferricrocin, two ferric hydroxamate siderophores. J. Am. Chem. Soc. **105** : 810-815.

Wong, J.C.Y., Holland, J., Parsons, T., Smith, A. and Williams, P. 1994. Identification and characterisation of an iron-regulated hemopexin receptor in *Haemophilus influenzae* type B. Infect. Immun. **62** : 48-59.

Young, D., Lathigra, R., Hendrix, R., Sweetser, D. and Young, D. 1988. Stress proteins are immune targets in leprosy and tuberculosis. Proc. Nat. Acad. Sci. USA. **85** : 4267-70.

Zacharia-Meehan, Z., Massad, G., Simpson, L.M., Travis, J.C. and Oliver, J.D. 1988. Ability of *Vibrio vulnificus* to obtain iron from hemoglobin-haptoglobin complexes. Infect. Immun. **56** : 275-277.

Zhou, X.H. and van der Helm, D. 1993. A novel purification of ferric citrate receptor (FecA) from *Escherichia coli* UT5600 and a further characterisation of its binding activity. Biometals **6** : 37-44.

Zhou, X.H., van der Helm, D. and Adjimani, J. 1993. Purification of outer membrane iron transport receptors from *Escherichia coli* by fast protein liquid chromatography: FepA and FecA. Biometals **6** : 25-35.

Zimmermann, L., Hantke, K. and Braun, V. 1984. Exogenous induction of the iron dicitrate transport system of *Escherichia coli* K12. J. Bacteriol. **159** : 271-277.

Appendix A

The validity of CHAPS extraction and CHAPS-PAGE techniques for envelope proteins of *M.smegmatis*

CHAPS-extraction of envelope proteins from *M.smegmatis*.

Envelope fractions were prepared from iron starved *M. smegmatis* and the proteins extracted with 8 mM CHAPS at 4°C overnight. The extract was clarified by centrifugation (105,000g; 4°C; 30 min) and the solubilised proteins withdrawn in the supernatant. This preparation was then dialysed against 1000 volumes of 62.5 mM Tris.HCl, pH 6.8 overnight at 4°C to remove the CHAPS and was then analysed by SDS-PAGE. Comparison with SDS-extracted envelope proteins demonstrated that a similar profile was gained from both detergent treatments but that the yield of solubilised protein was lower when using CHAPS (Fig. 36). It appeared that the population of solubilised proteins gained from this protocol was representative of the original membrane population. The extraction of envelope proteins from *M. smegmatis* using CHAPS, therefore, appeared to be a valid preparative method to provide the samples to be used in the later binding studies.

The behaviour of marker proteins on CHAPS-PAGE.

Cavinato *et al.* (1988) demonstrated that globular marker proteins migrated as a function of their molecular weight in the CHAPS-PAGE system. The migration of the ^{14}C molecular weight markers used in the experiment illustrated by Fig. 14 is presented here to demonstrate the relationship between R_f and $\log_{10}$ (molecular weight) observed during this study (Fig. 37). The mobility of the markers representing 21 - 200 kDa correlated well to the predicted linear relationship but the linear relationship degenerated at high R_f values. These molecular weight markers were all globular proteins, however, which may have not interacted with the CHAPS present in the gel system. It is possible that the mycobacterial envelope proteins did interact with the detergent causing modifications to their shape and electrophoretic mobility.

Figure 36. **Comparison of SDS- and CHAPS-extracted proteins from the cell envelope of iron-starved *M. smegmatis* by SDS-PAGE.** Equivalent volumes of SDS- and CHAPS-extracted proteins were run on a 10% SDS-PAGE gel. The gel was stained using Coomassie blue.

A SDS extracted sample

B CHAPS-extracted sample
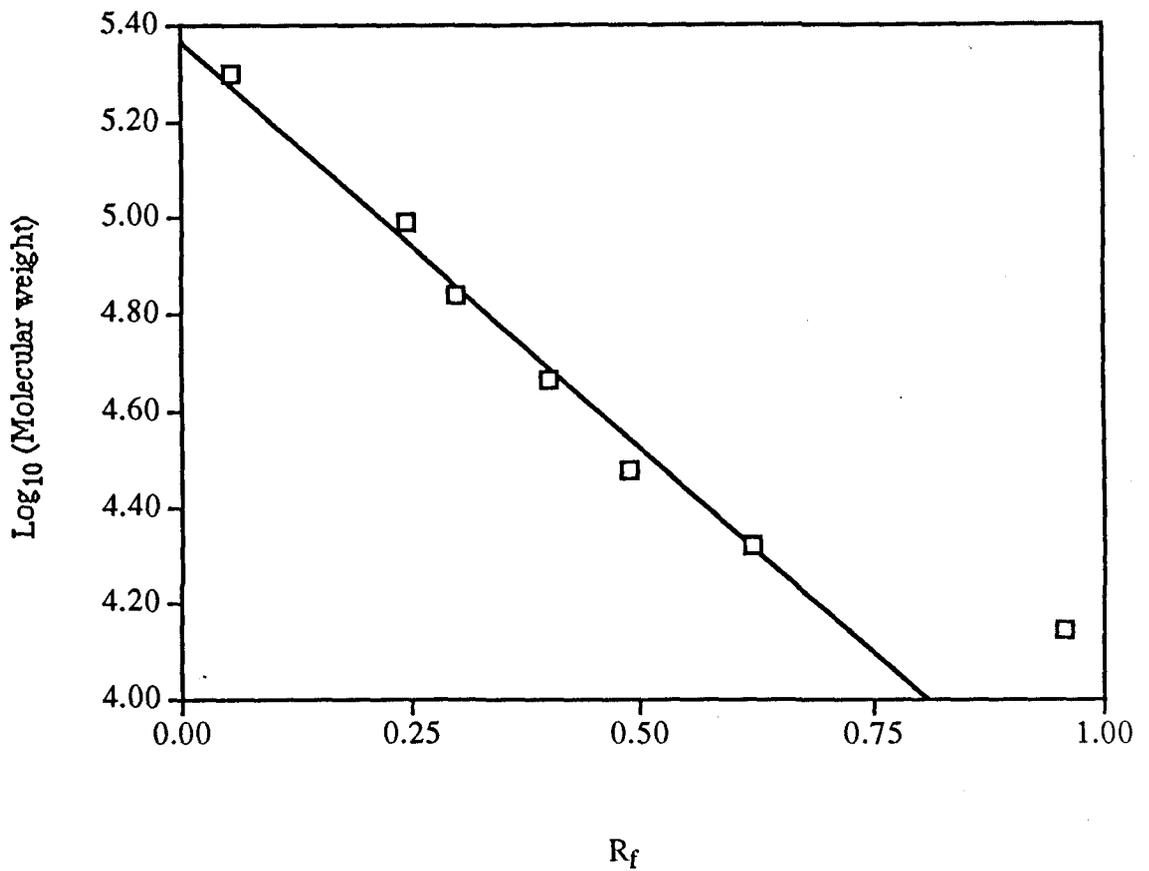
Figure 37. The electrophoretic mobility of globular proteins on CHAPS-PAGE. Globular, ^{14}C-labelled molecular weight markers were separated on a 10% CHAPS-PAGE geland viualised by autoradiography. R_f values were calculated and plotted against $\log_{10}$ (molecular weight).