Purification and characterisation of the lipid macroamphiphiles of propionibacterium acnes.

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PURIFICATION AND CHARACTERISATION OF THE LIPID MACROAMPHIPHILES OF PROPIONIBACTERIUM ACNES

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ABSTRACT

Purification and Characterisation of the

Lipid Macroamphiphiles of *Propionibacterium acnes*.

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Lipid macroamphiphiles (LMAP's) are cell envelope components of bacteria that have been extensively associated with the pathogenesis of disease. It has been reported that these components can also influence the *in vivo* metabolism of lipids in mammals. For the onset of the skin condition *acne vulgaris*, in addition to the presence of *Propionibacterium acnes* on the skin, changes in the composition of skin lipids has been identified as an important etiological factor in the development of the disease. To date, the LMAP of *P. acnes* has not been purified and for this reason, the significance of these components remains unclear. In order to study their significance, purification of the LMAP was essential for resolving their structures and diverse biological effects.

Following the extraction and consequent purification of lipoteichoic acid (LTA) from *S. aureus*, by hydrophobic interaction chromatography (HIC) a similar method was applied to an extract from *P. acnes*. The resulting amphiphilic fractions were analysed using SDS-PAGE and found to contain two discrete components. To facilitate the further characterisation, these components were separated on a larger scale by preparative SDS-PAGE and purified from the gel via electro-elution. The purified components were designated LMAP1 (running in the 24-29kDa region) and LMAP2 (running in the 14-20kDa region).

Analysis of the LMAP species suggested a lipoglycan structure and revealed evidence for a lipid anchor based on phosphatidylinositol with fatty acids that were comparable to the whole cell fatty acid profile. The polysaccharide moiety of both LMAP1 and LMAP2 contained different relative amounts of mannose, glucose and galactose and it was these differences that affected the size and hydrophobicity of the two lipoglycan components. Additionally, an amino sugar was detected that was suspected of being a diaminohexuronic

acid. This component has been previously identified as a component of the *P. acnes* cell wall polysaccharide. Consequently, it is proposed that a relationship might exist between these distinctive cell envelope polymers.

The effects on lipid metabolism were investigated in an *in vitro* system using the combined lipoglycan from *P. acnes* and compared to both LTA and lipopolysaccharide (LPS). All LMAP components investigated inhibited the uptake of fatty acid by hepatocytes. Furthermore, secretion of lipid from hepatocytes was also inhibited following incubation with each of the LMAP components. However, LTA only had a marginal effect in comparison to LPS and lipoglycan. It was also shown that both LPS and lipoglycan had a direct effect on adipocytes by increasing the uptake of fatty acid. Additionally, lipid secretion within these cells was also stimulated, which propounded an increase in fatty acid secretion. This evidence suggested that the lipoglycan of *P. acnes* can significantly influence the metabolism of lipids by mammalian cells and therefore these lipoglycans might play a crucial role in the pathogenecity of *P. acnes* and thus in the development of disease.

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Finally, I would like to thank my wife, Michelle, for her patience and understanding during many evenings and weekends spent alone. Without her encouragement, through the sometimes difficult and despairing times, this undertaking would remain incomplete and so it is to Michelle that I dedicate this thesis.

DECLARATION

I declare that this thesis has been composed entirely by myself and that it has not been accepted in any previous application for a degree. The work, of which it is a record, has been completed by myself. Sources of information have been specifically acknowledged.

Gary A. Whale

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ABBREVIATIONS

[^{14}C]C50-P-Ara	[^{14}C]Decaprenol-Phospho-Arabinose
2D-TLC	Two-Dimensional Thin Layer Chromatography
ACC	Alternative Complement Cascade
ACP	Acyl Carrier Protein
AG	Arabinogalactan
AraLAM	Arabinofuranosyl Terminated LAM
BCA	Bicinchoninic Acid
BCIP/NBT	5-Bromo-4-Chloro-3-Indolyl Phosphate/Nitroblue Tetrazolium
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
C35-P-Ara	Heptaprenol-Phosphoarabinose
C35-P-Man	Heptaprenol-Phosphomannose
C50-P-Ara	Decaprenol-Phosphoarabinose
C50-P-Man	Decaprenol-Phosphomannose
cAMP	Cyclic Adenosine Monophosphate
CDP-DAG	Cytidine Diphosphate Diacylglycerol
CDP-DP-DAG	Cytidine Diphosphate-Dipalmityl-Diacylglycerol
CDP-glycerol	Cytidine Diphosphate Glycerol
CDP-NBD-DAG	Cytidine Diphosphate-1,2-[Oleyl,(N-(nitrobenzo-2-oxa-1,3-Diazole)aminocaproyl)]diacylglycerol
CMC	Critical Micellar Concentration
Con A	Concanavalin A
DAG	Diacyglycerol
Dcl	D-Alanine-Dcp Ligase
Dcp	D-Alanyl Carrier Protein
DEAE	Diethylaminoethyl Anion Exchange
DHBP	3, 4 Dihydroxybutyl-1-Phosphanate
DI	Deionised
DMAB	Dimethylaminobenzaldehyde
DMEM	Dubelco's Modified Eagle's Medium
DPA	Diphenylamine
ECACC	European Cell Culture Collection
EF-water	Endotoxin-Free Water

ELISA	Enzyme-Linked Immunosorbent Assay
EU	Endotoxin Units
FAB-MS	Fast Atom Bombardment-Mass Spectroscopy
FAME	Fatty Acid Methyl Ester
FCS	Foetal Calf Serum
FID	Flame Ionisation Detection
FPLC	Fast Protein Liquid Chromatography
GC	Gas Chromatography
G-C ratio	Guanine-Cytosine % Molar Ratio
GLA	GPI-Linked Anchor
GPI-protein	Glycosylphosphatidylinositol-Anchored Protein
HIC	Hydrophobic Interaction Chromatography
IEC	Ion-Exchange Chromatography
IFN	Interferon
IL	Interleukin
IMP	Inositol Monophosphatase
iNO synthase	Inducible NO Synthase
LAL	Limulus Amebocyte Lysate
LAM	Lipoarabinomannan
LBP	Lipopolysaccharide-Binding Protein
LDH	Lactate Dehydrogenase
LM	Lipomannan
LMAP	Lipid Macroamphiphile
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
mAGP	Mycolyl Arabinogalactan-Peptidoglycan
ManLAM	Mannose-Capped LAM
MCP-1	Monocyte Chemotactic Protein-1
MEM	Minimum Essential Medium
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein-1α
Mlta	Membrane-Bound LTA
MR	Mannose Receptor
MS	Mass Spectrometer
MWCO	Molecular Weight Cut-Off

NEAA	Non-Essential Amino Acids
NF-κβ	Nuclear Factor κβ
NO	Nitric Oxide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PG	Phosphatidylglycerol
PG	Peptidoglycan
PGP	Poly(Glycerophosphate)
PI	Phosphatidylinositol
PIM	Phosphatidylinositol Mannoside
PIM_2	Phosphatidylinositol Dimannoside
PKC	Protein Kinase C
PTGDAG-lipids	Phosphatidylglycosyldiacylglycerolipids
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis
SEM	Standard Error of the Mean
TAG	Triacylglycerol
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor-α
UF	Ultrafiltration
VAIN	Variable Atmosphere Incubator
VLDL	Very Low-Density Lipoprotein
WCW	Wet Cell Weight

CHAPTER 1

Introduction

1.1 General Introduction

The majority of bacteria can be classified into one of two groups, based on the Gram's Iodine test, devised by Christian Gram in 1884. The test is dependent on major differences between the bacterial cell wall of each type, the bacterial groups are designated as being either Gram-positive or Gram-negative. The test relies on the stain, Crystal Violet, which binds peptidoglycan within the bacterial cell wall. The structure of Gram-positive bacteria is relatively simple and is mainly composed of a thick layer of peptidoglycan. This layer can play host to a wide range of molecules such as teichoic acid, lipoteichoic acid (LTA), lipoglycan, teichuronic acid and numerous proteins, all of which are found in differing amounts, depending on the bacterial species [Alcamo, 1994].

In contrast, Gram-negative bacterial cell walls are generally more complex with a layer of lipopolysaccharide (LPS) coated phospholipid that is anchored to only a thin layer of peptidoglycan, via lipoproteins. Additional constituents of the cell wall are also present such as porins and proteins, both of which are anchored among the phospholipids. Recent evidence suggests that the proteins present are unique for each individual species of bacteria [Madigan, Martinko & Parker, 1997].

1.1.1 Lipopolysaccharide of Gram-negative Bacteria

There is increasing interest in the literature in how the cell wall components of pathogenic bacteria can lead to exacerbations in mammalian metabolism, the most commonly researched component to date being LPS, the endotoxin of Gram-negative bacteria.

The effects of LPS are far reaching and many of these effects tend to be associated with the toxicity of the lipid A portion of LPS, a degradation product of LPS initiated by the immune system (See Figure 1.1).

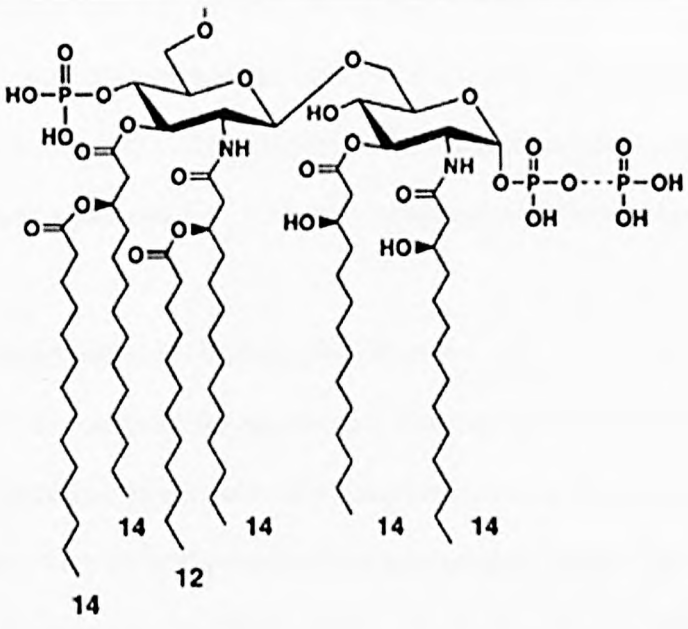


Figure 1.1 A Schematic Representation of the Lipid A Portion of LPS

The fatty acids vary in chain length and this is indicated at the end of each of the fatty acids. The unattached oxygen atom on the first diglucosamine molecule attaches the lipid A moiety to the 2-keto-3-deoxy-*manno*-octonate of the core polysaccharide.

The toxicity of lipid A resides with six fatty acids bound to a specific diglucosamine backbone, which is diphosphorylated at one end and monophosphorylated at the other [Brandenburg *et al.*, 1996]. It is this structure that binds LPS-reactive mononucleated cells of the mammalian immune system [Brandenburg *et al.*, 1996]. In binding these host defense cells, immune-mediating proteins are released in the form of cytokines that include interleukins (IL) and tumour necrosis factor-α (TNF-α). These cytokines are potent stimulators of the alternative complement cascade (ACC) and a massive immune response is mounted. Following the stimulation of ACC, disseminated vascular intracoagulation then occurs, while the TNF-α acts on the hypothalamus to produce a large elevation in body temperature. Infections of this kind can easily cause death if treated incorrectly i.e. treatment of the infection with antibiotics that target the bacterial cell wall, as this can result in the

release of large amounts of the lipid A toxiphore and thus accelerating the effects of the condition termed 'Toxic Shock Syndrome' [Mims *et al.*, 1995].

The lipid A portion is the primary toxiphore of LPS, but it is thought that the polysaccharide portion plays an important role in the solubility of the whole molecule, which reduces the formation of LPS aggregates and thus, increases the biological activity [Mims *et al.*, 1995].

1.1.2 Lipid Macroamphiphiles of Gram-positive Bacteria

Gram positive bacteria do not possess LPS, but they do have structures that are thought to play a similar role in the bacterial pathogenesis of such organisms [Renzi & Lee, 1995]. These structures are generally composed of a hydrophilic portion joined to a lipid anchor, which holds various types of fatty acid. The whole structure can display considerable variation between taxonomic order, but there are characteristics emerging through the structural analysis of these components that demonstrates certain traits across the taxonomic genera of bacteria [Fischer, 1990]. A general trait observed is that classes of bacteria possessing a low Guanine-Cytosine % molar ratio (G-C ratio), in terms of DNA base comparisons, tend to have lipoteichoic acids (LTAs) as a cytoplasmic membrane component. Those with that have a higher G-C ratio, usually >55%, have been found to contain a lipoglycan structure [Fischer, 1994a; Fox *et al.*, 1980; Sutcliffe, 1994a]. These structures are thought to replace each other functionally between the types of bacteria, but are never found together in the same bacterial cell [Fischer, 1994a; Fox *et al.*, 1980]. Collectively, both LTAs and lipoglycans can be called lipid macroamphiphiles (LMAPs)

1.2 Structure and Diversity among Lipoteichoic Acids (LTA)

LTAs have been found to occur in many taxa of bacteria including *Listeria* spp. *Bacillus* spp. *Staphylococcus* spp. and some *Streptococcus*, all of which possess a G-C ratio of well below 50% [Fischer, 1994a; Fox *et al.*, 1980].

The LTA structures that each class contains can vary significantly, but in general terms the LTA consists of a hydrophobic moiety linked via a phosphodiester bond to an alditolphosphate-containing hydrophilic chain [Fischer, 1994a; Fox *et al.*, 1980].

1.2.1 Variation in the Hydrophilic Chain

1.2.1.1 Poly(glycerophosphate) Hydrophilic Structures

The most predominant hydrophilic structure found within LTA, consists of a 1,3-linked poly(glycerophosphate) (PGP) polymer as the hydrophilic chain [Fischer, Mannsfeld & Hagen, 1990]. PGP LTAs generally comprise a single, unbranched glycerol phosphate chain linked to the hydrophobic glycolipid via a phosphodiester bond. [Fischer *et al.*, 1990]. The length of the chain can vary between genus, but remains reasonably constant among strains of the same species [Fischer *et al.*, 1990]. The chains can also be substituted at the C2 position of the glycerophosphate polymer substituting the hydroxyl residue [Fischer *et al.*, 1990]. Substitution has only been seen at this position along the chain, but the type and frequency of substitution can vary considerably [Fischer *et al.*, 1990]. Generally, there are four categories of substitution that have been observed to date, the first category consists of LTA with no substitution along the hydrophilic chain. The second and third categories consists of D-alanyl substitution and glycosyl substitution respectively and the fourth category contains both D-alanyl and glycosyl substitution. At present, only D-alanine amino acids have been observed as substituents. The glycosyl substituents can take the form of either a common monosaccharide, such as glucose, mannose and galactose or *N*-acetylglucosamine [Fischer, 1988; Fischer & Rosel, 1980]. Using step-wise enzymatic degradation with phosphomonoesterase allowed the degradation of each polymeric unit one at a time and then each monomer was analysed for substitution. The nature of the substitution along the chain was found to be regular in some places and of random arrangement in others [Fischer *et al.*, 1980b]. Further information was provided by ^{31}P nuclear magnetic resonance (NMR) spectroscopy studies and has indicated that substitution

occurs in more of a random fashion as opposed to a regular and predetermined order [Batley, Redmond & Wicken, 1987].

LTAs displaying a high degree of glycosylation such as the LTA from *Enterococcus faecalis* [Wicken & Baddiley, 1963] suggest that alanine substitution can be in part attached to glycosyl residues. This data is further supported by the unpublished observations of Fischer in 1988, where it was claimed that after removal of the D-alanine residues from the LTA of *Lactococcus lactis*, the LTA was applied to anion exchange chromatography where it was found that unsubstituted LTA did not appear, but eluted in the glycosyl-substituted fractions [Fischer, 1988].

1.2.1.2 Poly(diglycosylglycerophosphate) Hydrophilic Structures

A very different hydrophilic chain structure was identified after analysis of the LTA from *Lactococcus garvieae*. On analysis, it was found that not only was the glycerophosphate consistently substituted at the C2 position with monogalactosyl residues, but also intercalating between the glycerophosphate residues along the LTA backbone were digalactosyl moieties. The structure of this chain bears some resemblance to a cell wall teichoic acid, but with the addition of glycero-1-phosphate units [Koch & Fischer, 1978].

1.2.1.3 Pneumococcal (Forssman) LTA

The first LTA to be characterised was not a polyglycerophosphate LTA, such as those already discussed, but a carbohydrate containing LTA. This unusual LTA belonged to *Streptococcus pneumoniae* and was first identified in 1935 where it was described as a Forssman antigen, due to its lipocarbohydrate structure and that it evoked cell hemolysins when injected into rabbits [Buchbinder, 1935]. Subsequently, the Forssman antigen was renamed as the Pneumococcal F antigen [Goebel *et al.*, 1943]. The complex carbohydrate hydrophilic chain of the pneumococcal LTA consists of, on average, six repeating chains. Each repeat contains a tetrasaccharide, which is glycosidically linked to O-1 of D-ribitol-5-

phosphate. The tetrasaccharide itself is substituted in two places at the O-6 position of the *N*-acetylgalactosaminyl residues with phosphocholine residues [Behr *et al.*, 1992]. The repeats are joined together by phosphodiester bonds [Behr *et al.*, 1992]. Interestingly, the teichoic acids of *S. pneumoniae* shares the same chain structure as that of the LTA, but are bound to peptidoglycan via a sugar-phosphate linkage unit, instead of the lipid anchor as in the LTA [Fischer, 2000]. This is also different to other bacteria as the LTA and teichoic acids tend to be structurally and biosynthetically distinct from one another. There is evidence to suggest that the two molecules are metabolically independent. This was demonstrated by following the incorporation of choline in pulse-chase experiments. However, the possibility that these components share common precursors cannot be ruled out [Fischer *et al.*, 1993]. The structures of the repeating units are shown in Figure 1.2.

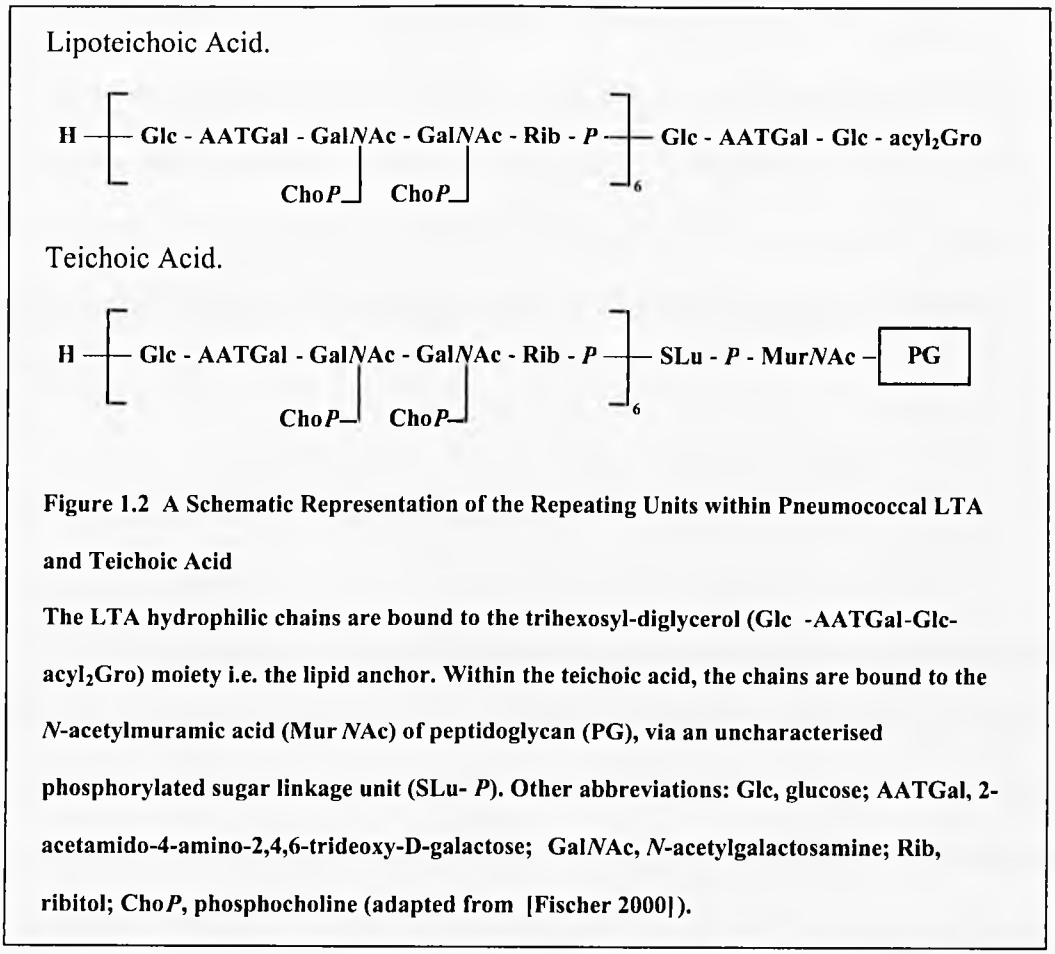


Figure 1.2 A Schematic Representation of the Repeating Units within Pneumococcal LTA and Teichoic Acid

The LTA hydrophilic chains are bound to the trihexosyl-diglycerol (Glc -AATGal-Glc-acyl$_2$Gro) moiety i.e. the lipid anchor. Within the teichoic acid, the chains are bound to the *N*-acetylmuramic acid (Mur *N*Ac) of peptidoglycan (PG), via an uncharacterised phosphorylated sugar linkage unit (SLu- *P*). Other abbreviations: Glc, glucose; AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Gal*N*Ac, *N*-acetylgalactosamine; Rib, ribitol; Cho*P*, phosphocholine (adapted from [Fischer 2000]).

1.2.1.4 Other Streptococcal and Polyribitol LTA

In addition to *S. pneumoniae* other Streptococci within Group B, such as *S. agalactiae* (serotype II), also contains a 'lipocarbohydrate', but the phosphocholine appears to be absent. This amphiphile contains galactose, glucose, glucosamine, phosphorus and fatty acids and is therefore more appropriately described as a lipoglycan as opposed to an LTA [Sutcliffe, 1994a]. Unusually, within the serotypes Ia and III of *S. agalactiae* a PGP-LTA has been isolated. This is particularly interesting from a pathogenesis point-of-view as the majority of infections appear to be serotype III instigated, thus, it is thought that the PGP-LTA may be involved in the virulence of this serotype [Cumming *et al.*, 1983; Teti *et al.*, 1987]. Two other bacteria namely *Granulicatella adiacens* and *Abiotrophia defectiva* (formerly 'nutritionally variant' streptococci [Collins & Lawson, 2000; Kawamura *et al.*, 1995]) have been found to possess another unusual structure of LTA, again distinct from the more common PGP-LTA, these bacteria incorporate a unique type of macroamphiphile described as a lipopolyribitol teichoic acid. In the case of *A. defectiva* the polyribitol core chain is substituted with alanine and galactose [George & Van De Rijn, 1988], but the polyribitol core of *G. adiacens* is substituted with alanine and a trisaccharide composed of *N*-acetylgalactosamine and two galactose molecules [Sieling, Thomas & Van De Rijn, 1992].

1.2.2 Variation in the Hydrophobic Lipid Anchor

1.2.2.1 Lipid Anchor

The hydrophobic portion of LTA most frequently consists of a disaccharide, which is bound to an acylated glycerol molecule (collectively called the lipid anchor). The glycerol molecule is acylated with a range of different fatty acids and it is this portion that provides the overall structure with an amphiphilic nature. The majority of lipid anchor structures have been elucidated. This is carried out by selective hydrolysis of the LTA component, followed by the removal of the fatty acids (using petroleum ether). The resultant fractions are derivatised and finally analysed by GC [Nakano & Fischer, 1978].

Focusing further on the structures of the lipid anchor, Fischer demonstrated the diversity in the structures and also described which bacteria share the same lipid anchors [Fischer, 1988]. The most predominant structural theme appears to be the dihexosyldiacylglycerol lipid anchor, which occurs in many bacteria including *Staphylococcus aureus*, *Macrococcus caseolyticus* (formerly *Staphylococcus caseolyticus* [Kloos *et al.*, 1998]), *Staphylococcus xylosus* and some species of bacilli [Fischer, 1988]. *M. caseolyticus* was initially named *Micrococcus varians* until specific biochemical data was acquired which demonstrated that the classification was incorrect [Schleifer *et al.*, 1982; Sutcliffe & Alderson, 1995]. Together with this biochemical evidence is the presence of an LTA amphiphile as opposed to lipoglycan, which is possessed by other *Micrococcus* spp. and was a major determinant in the reclassification of this bacterium [Schleifer *et al.*, 1982].

Also common, but to a lesser degree are the trihexosyldiacylglycerols lipid anchors. These have been associated with the LTA from *Lactobacillus casei*, *L. plantarium* and *L. helveticus* [Nakano & Fischer, 1978]. A phosphodiester bond also joins the hydrophilic chain of pneumococcal LTA to a trihexosyldiacylglycerol lipid anchor [Behr *et al.*, 1992]. Furthermore, it was also suggested that the glycolipid moiety of the pneumococcal lipid anchor is unique to the macroamphiphile in that it is not be found anywhere else among the free lipids within the cell. This is an unusual feature since in all other species characterised, the glycolipid can be found in the free lipids of the organism [Behr *et al.*, 1992]. There are some species of bacteria that have been found to be absent of a saccharide unit, examples of these species include *Bacillus coagulans*, *B. megaterium* and a mutant strain of *B. licheniformis*. Here, the diacylated glycerol is bound directly to the hydrophilic moiety [Button & Hemmings, 1976; Iwasaki, Shimada & Ito, 1986].

1.2.2.2 Fatty Acid Acylations

Many of the anchors discussed above are acylated with at least two fatty acid residues and may possess a third or even a fourth fatty acid residue acylated to the C-6 of the terminal and pre-terminal saccharide unit respectively [Fischer, 1988]. Bacterial species

possessing three or four fatty acid residues are expected to anchor the LTA structure more firmly, due to the critical micellar concentration (CMC) of the amphiphile decreasing with the corresponding increase in the aliphatic carbon number [Marsh & King, 1986].
The fatty acid content of the LTA can be determined by hydrolysing the molecule and then derivatising the fatty acids to form the corresponding methyl esters. Analysis is then carried out using gas chromatography (GC) with either a mass spectrometer (MS) for detection [Roethlisberger *et al.*, 2000] or using flame ionisation detection (FID) against standards derivatised in the same way [Komagata & Suzuki, 1987]. Studying the collective data [Fischer, 1988] the fatty acid content can be seen to vary considerably between species of bacteria, but generally consists of C12-C19, with C18:1 (cis-Δ11-octadecanoic acid) being the major fatty acid in most species. In many cases the composition of cellular lipids dictates the fatty acid incorporated into LTA [Fischer, 1988]. The fatty acids present can often act as a marker in taxonomic studies in that it is used as a 'fingerprint' for a bacterial species [Wayne-Moss *et al.*, 1969].

1.3 Structure and Diversity among Lipoglycans

It is commonly accepted that the mollicutes and actinomycetes have a G-C ratio of >55% and inherently possess lipoglycans as their major cytoplasmic membrane component [Fischer, 1994a; Fox *et al.*, 1980; Sutcliffe, 1994a]. There has been widespread interest in the structure of certain lipoglycans, mainly due to the species of bacteria that carry the molecule. These bacteria include *Mycobacterium tuberculosis* and *Mycobacterium leprae*, both of which are associated with serious human infections [Meyer, May & Stark, 1998]. The structures of LTA and lipoglycan differ mainly within the hydrophilic chain of the macroamphiphile. Lipoglycans have been shown to posses a polysaccharide backbone, in contrast to LTA which is described as having an alditolphosphate-containing hydrophilic backbone [Fischer, 1994a]. As with LTAs there are many variations in the lipoglycan

structure and this can be between species and strain of bacteria. Furthermore, the fatty acids of lipoglycans also vary between different species of bacteria, again as with LTA, these reflect the distribution of cellular fatty acids within the bacterial cell wall, but generally consists of C12-C19, both branched and non branched-chain fatty acids [Fischer, 1994a]. The different structures of lipoglycan, characterised to date are discussed.

1.3.1 Lipomannans of Micrococcus sp.

The lipoglycan of *Micrococcus luteus*, (formerly *M. lysodeikticus*) was one of the first to be characterised as a component which was distinctly different from LTA [Powell, Duckworth & Baddiley, 1974]. The structure of the lipoglycan was found to be succinylated lipomannan [Pless, Schmit & Lennarz, 1975; Powell, Duckworth & Baddiley, 1975]. These are held by the majority of the *Micrococcus sp.* and are composed of a hydrophilic chain consisting of 50-70 (1-2)-, (1-3)- and (1-6)-linked α-D-mannopyranosyl residues. It is these mannosyl residues that can be substituted with ester-linked succinate groups, which complete the succinylated lipomannan. The lipid moiety in this case is described as a diglycosyldiacylglycerol grouping [Pless *et al.*, 1975; Powell *et al.*, 1975].

The lipomannan was shown to form micelles in an aqueous environment, as does LTA and the succinyl esterifications provide the lipomannan with an overall negative charge, allowing it to readily bind cations such as Mg^{2+} ions, again the same as LTA [Lim & Salton, 1985]. Within the *Micrococcus* spp. there are variations to this theme, such as *Micrococcus agilis* (now referred to as *Arthrobacter agilis* [Koch, Schumann & Stackebrandt, 1995]) which has been shown to possess a similar lipomannan, but lacking the succinyl esterifications. This results in a neutrally charged molecule [Lim & Salton, 1985]. Furthermore, other bacteria have also been shown to share this type of lipomannan, with the same dimannosyl diglyceride glycolipid joining the mannose core chain, this includes *Rothia dentocariosa*, again this amphiphile does not carry the succinyl esters and is also considered to be a neutral lipomannan. *R. dentocariosa* has now been grouped with the genus *Micrococcus* thus, the structural characterisation of LMAPs may be a useful chemotaxonomic marker in assigning

taxonomic relationships [Sutcliffe & Alderson, 1995; Sutcliffe, 1994b]. Furthermore, *Stomatococcus mucilaginosus* was also shown to produce a similar lipomannan and has since been reclassified as *Rothia mucilaginosa* [Collins *et al.*, 2000; Sutcliffe & Old, 1995] and therefore further demonstrating the chemotaxonomic value of LMAP structures.

1.3.2 Lipoglycans of Bifidobacterium

Within the genus *Bifidobacterium*, the amphiphilic chain structure was found to be a lipoglucogalactofuranan with monomeric substitutions of glycerophosphate and was therefore the direct opposite of the situation observed in LTA where carbohydrate substitutions are found bound to the glycerophosphate chain. Further characterisation revealed that, like LTA, the glycerophosphate side chains also had alanine substituted, although intriguingly not D-alanine as in LTA, but L-alanine instead. Subsequent analysis of other strains demonstrated structural and serological similarities in the lipoglycan in all tested, except one, this being *B. angulatum* [Op Den Camp *et al.*, 1985a]. As with LTA, complex hydrolysis methods were employed to prepare the various regions of the lipoglycan for subsequent derivatisation and analysis of the derivatives to determine the structure was carried out using GC-MS [Fischer, 1987; Fischer, Bauer & Fiegel, 1987]. In addition ^{1}H, ^{13}C and ^{31}P-NMR spectroscopy was required to support the structures derived from the chemical analysis above [Fischer *et al.*, 1987].

1.3.3 Lipomannan and Lipoarabinomannan of Mycobacterium sp.

The majority of studies that have examined lipoglycans have focussed on *Mycobacterium* spp. The lipoglycans of *M. tuberculosis* and *M. leprae* were first identified as such in 1986 and were shown to be important immune stimulators [Hunter, Gaylord & Brennan, 1986]. The lipoglycans were found to consist of lipomannan and an arabinose containing lipomannan, which was termed lipoarabinomannan (LAM) [Chatterjee *et al.*, 1992c; Hunter & Brennan, 1990]. However, mycobacterial LAM was originally perceived to be an arabinomannan polysaccharide of the cell wall [Azuma *et al.*, 1968; Chargraff &

Schaefer, 1935; Weber & Gray, 1979]. This was concluded following the analysis of alkali-hydrolysed cell wall extractions and consequently lacking the lipid moiety [Azuma *et al.*, 1968]. This was later revised, after the development and successful application of solvent extraction procedures allowing the purification of lipomannan and LAM in their native acylated states [Chatterjee *et al.*, 1992c; Hunter *et al.*, 1986]. Both lipoglycans were found to possess a hydrophilic polysaccharide backbone comprising of a (1-6)-α-D-mannopyranan with (1-2)-α-D-mannopyranose side chains. Within LAM on the exterior of the chain was identified an O-3-branched (1-3)-α-D-arabinofuranan with non-reducing arabinose residues at the termini [Chatterjee *et al.*, 1992c]. The lipid anchor, common to both the mycobacterial lipomannan and LAM was determined as a phosphatidylinositol unit and thus LAM and lipomannan are thought to be multiglycosylated forms of the phosphatidylinositol mannoside [Hunter *et al.*, 1986; Khoo *et al.*, 1995a].

1.3.3.1 Hydrophilic Chain Structure

Focussing on the structure of LAM, the hydrophilic portion is composed of a mannan backbone of approximately 16 residues with mannose substitutions at the C-2 position, which promote branching. Attached to this core is the arabinan portion, which contains 70-80 arabinose residues [Brennan *et al.*, 1990]. It has been suggested that this portion of the LAM is exposed on the extracellular surface, while the phosphatidylinositol lipid anchor and mannan core are embedded within the cell wall [Brennan *et al.*, 1990]. The arabinofuranosyl terminated LAM's were called AraLAM's and were found to have two predominant types of termini, both of which contained only arabinofuranosyl residues. Analysis of each was carried out using a novel endoarabinase isolated from a species of *Cellulomonas*, which was used to degrade the arabinose molecules in a stepwise manner. The digestion products from the LAM were then analysed using fast atom bombardment-mass spectroscopy (FAB-MS) and shown to be a branched and a linear arabinan termini.

The proposed structure of each is detailed in Figure 1.3 [Khoo *et al.*, 1995b; McNeil *et al.*, 1994].

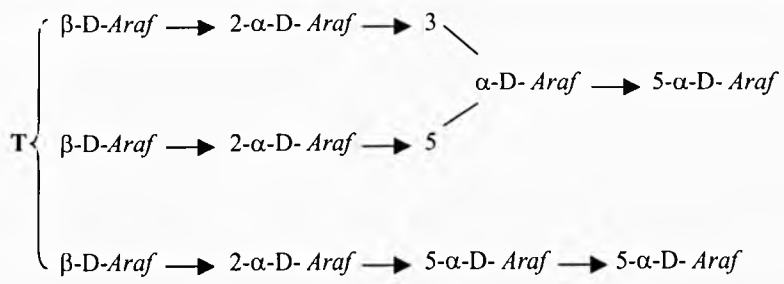


Figure 1.3 The Two Possible Arabinofuranosyl Termini for AraLAM

***Araf*, individual arabinofuranosyl molecule. T, molecular terminals. Taken and adapted from [Chatterjee et al 1992a].**

1.3.3.2 Structural Variation within Lipoarabinomannans

Recent studies, which have used specific enzyme digests and FAB-MS, suggest that in some virulent strains of *Mycobacterium*, the arabinose termini were extensively capped (up to 70%) with mono, di or trimannosyl residues. These mannose-capped LAM's were designated ManLAM's [Chatterjee *et al.*, 1992a]. It was originally thought that the mannose-capping moieties could be responsible for virulence, as they were first identified in virulent strains of *Mycobacterium* [Chatterjee *et al.*, 1992a]. This idea was later rejected with the identification of ManLAM in the attenuated *M. bovis BCG* vaccine strain [Prinzis, Chatterjee & Brennan, 1993].

Other capping motifs have since been identified within *M. smegmatis* and also fast-growing *Mycobacteria* spp., in the form of *myo*-inositol-1-phosphate terminals. These were found within otherwise uncapped AraLAM's and interestingly, these were shown to be potent stimulators of murine macrophages [Khoo *et al.*, 1995b]. Following these findings, AraLAM's are now more commonly referred to as PI-LAM's [Gilleron *et al.*, 1997; Khoo *et al.*, 1995b]. Methods now exist for quick and effective analysis of the capping moiety within LAM. One method used endoarabinases, as described above, for the digestion of intact

LAM, replacing the use of harsh chemicals for degradation, which affect the integrity of the fragile arabinofuranosyl bonds. Analysis of the oligomers produced from the arabinase digestion was carried out using direct high pH anion exchange chromatography coupled to mass spectroscopy. Alternatively, the oligomers can be fluorescently labelled and analysed via HPLC [Khoo, Tang & Chatterjee, 2001].

It is now acknowledged that the mycobacterial LAM is the major antigenic determinant [Prinzis *et al.*, 1993] and therefore the interest in this molecule has increased further still. With the use of one and two-dimensional NMR spectroscopy, details of the finer structural variations are now becoming apparent. An example of this, is the further characterisation of the multiacylated forms of the phosphatidylinositol lipid anchor by 2-D NMR. This allows analysis of the *native* lipid anchor without chemical destruction of the component, which is thought to be critical in understanding the 3-dimensional structure of this region of the LAM [Nigou, Gilleron & Puzo, 1999b]. This has also been particularly important in establishing the binding process to immune receptors such as CD1, which are involved in presentation of LAM's to T-cell receptors [Sieling *et al.*, 1995].

In addition to this structural information, it has also been shown that two types of ManLAM exist within *M. bovis* BCG and these have been termed parietal and cellular ManLAM. These were found to differ in the types and amounts of fatty acid acylated to the phosphatidylinositol lipid anchor. The parietal form was shown to possess a novel fatty acid, previously unidentified in the *Mycobacterium* genus and identified as 12-*O*-methoxypropanoyl-12-hydroxystearic acid and this was the only fatty acid acylated to the C-1 of the glycerol residue [Nigou *et al.*, 1997]. Within the cellular ManLAM, a combination of palmitic and tuberculostearic acids was observed at varying acylation locations on the lipid anchor. Furthermore, the parietal and cellular ManLAM also differed in the percentage of mannose capped LAM's, which were found to be 76% and 40% respectively [Nigou *et al.*, 1997]. Following the separation of these two classes of ManLAM, further structural investigations revealed the presence of succinyl substitutions localised to the arabinose units,

thus suggesting further variation maybe possible. It was suggested that this could account for differences observed in the efficacy of vaccines from various sources.

The parietal and cellular ManLAM's required a novel approach to allow purification of each molecule. Firstly, whole bacterial cells were delipidated and then extracted by refluxing with 50% ethanol/water. The resulting mixture was then centrifuged at low speed and the supernatant was removed. Treatment of the supernatant with Triton X-114, followed by gel filtration allowed the purification of parietal ManLAM. Meanwhile, the pelleted cells were mechanically disrupted and then extracted and purified in a similar manner to that of the parietal ManLAM. This yielded the cellular ManLAM [Nigou *et al.*, 1997]. This novel purification procedure may have implications for the purification of other novel LAM's from different organisms. Recently, the parietal and cellular ManLAM's of *M. tuberculosis* were extracted and purified as above. From the NMR analysis in their native forms, both were shown to share the same mannosyl capping motifs and in the same relative abundance. It was also found that only one acyl-form was observed in the parietal ManLAM purification, while three acyl-forms were found in the cellular ManLAM purification. The significance of this remains unclear [Gilleron *et al.*, 2000].

1.3.4 Lipomannan of Propionibacteria

The lipoglycan of the genus *Propionibacterium* has also been investigated, though not in any way near as much detail as the major amphiphiles of mycobacteria. As part of the analysis into the constituent lipids of *Propionibacterium freudenreichii*, (formerly *P. shermanii* [Cummins & Johnson, 1981]) the capability of *in vitro* synthesis of a phosphatidylinositol mannoside (PIM) was observed [Brennan & Ballou, 1968] suggesting the initial biosynthetic stages of a lipoglycan. Furthermore, from the analysis of acid hydrolysed cellular lipids, both mannose and inositol were observed. These were assumed to be components of the PIM [Laneelle, Asselineau & Trefouel, 1968]. However, soon after this, mass spectrometry was used for the analysis of the cellular lipids extracted from different species of propionibacteria, which included *P. freudenreichii*. The lipids from all

extracts were found to contain significant amounts of a glycolipid composed of fatty acid, inositol and mannose in a 2:1:1 ratio, suggesting a novel diacylinositol mannoside glycolipid, as opposed to a PI anchor [Shaw & Dinglinger, 1969]. It was some years later that the lipoglycan was successfully extracted from *P. freudenreichii* and was partially purified using anion exchange chromatography [Sutcliffe & Shaw, 1989]. The hydrophilic core structure was determined as a mannosyl polymer of, on average, approximately 30 residues. The presence of inositol, glycerol and phosphate within the lipid anchor supported the previously suggested phosphatidylinositol structure and was thought to be similar to that isolated from mycobacteria [Sutcliffe & Shaw, 1989]. In contrast to this, the phospholipids of *P. freudenreichii* were re-examined for the presence of a PIM. This investigation revealed an absence of PIM within the membrane lipids and it is believed that the presence of mannose and inositol in the products released by hydrolysiss observed earlier by Laneelle was due to the diacylinositol mannoside glycolipid, identified by Shaw [Shaw & Dinglinger, 1969; Sutcliffe & Shaw, 1993]. This glycolipid is known to represent >40% of the total lipid within propionibacteria [Brennan & Ballou, 1968]. Currently, the structure of the lipid anchor is still unclear and although a phosphatidylinositol mannoside was not found in the membrane lipids, this does not exclude such a phospholipid being present within the lipomannan.

1.3.5 Lipoglycan of Clostridium sp.

Within *Clostridium* spp. it has been reported that there are two distinct types of LMAP suggesting that the genus *Clostridium* may be divisible into two genera. Within *Clostridium difficile*, a lipoglycan was extracted and purified using sepharose gel chromatography. The structural characterisation of the lipoglycan revealed glucose, glucosamine, phosphate and fatty acids [Poxton & Cartmill, 1982]. When this lipoglycan was subjected to polyacrylamide gel electrophoresis (PAGE) a similar pattern was observed to that observed with Gram-negative lipopolysaccharide [Sharpe & Poxton, 1986]. In contrast to this, following extraction of *C. inoculum,* a polygalactosylglycerophosphate LTA

was identified [Johnston, Goldfine & Fischer, 1994]. Moreover, *C. tetani*, *C. histolyticum*, *C. sporogenes*, and *C. septicum* are all thought to lack a PGP LTA [McCarty, 1959] as each were found to be negative for a PGP antigen, suggesting the possibility of a lipoglycan instead. In support of the chemotaxonomic diversity observed between the distinct species of clostridia, there is also a significant difference in the G-C ratio between the bacteria. The clostridia possessing the lipoglycan have been found to possess a G-C ratio of between 22-34%, while *C. inoculum* has a G-C ratio of 40-55% [Cato, George & Finegold, 1986]. More recently, these differences have been highlighted by the analyses of 16sRNA gene sequences of a number of named and un-named clostridia. The results of these analyses concluded that the genus was extremely heterogeneous, with many species phylogenetically intermixed and therefore the genus was clearly in need of major taxonomic restructuring [Collins *et al.*, 1994].

1.4 Absence of Lipid Macroamphiphiles

In some species of bacteria the extraction and characterisation of any macroamphiphile, be it LTA or lipoglycan has been unsuccessful. This includes *Bacillus polymyxa*, *Bacillus circulans* [Iwasaki *et al.*, 1989] and three species of *Heliobacterium* [Beck, Hegeman & White, 1990]. One possible reason for this maybe due to the occurrence of components with unusual physical properties and therefore failing to extract under the current extraction protocols. In support of this is the extraction of the F antigen of *S. pneumoniae* which does not follow the normal amphiphile extraction procedure using hot aqueous phenol extraction [Fischer, 1991], but requires extraction with methanol and chloroform [Fischer *et al.*, 1993]. Similarly, the purification of the distinctly different ManLAM's (i.e. parietal and cellular ManLAM) required a novel method to achieve this [Nigou *et al.*, 1997]. This would suggest that before it can be categorically concluded that there is an absence of a component in these bacterial species, additional methods should be identified and applied.

1.5 Biosynthesis of Lipid Macroamphiphiles

1.5.1 Cellular Location

The location of LMAPs within bacterial cell walls was demonstrated by using *in situ* immuno-electron microscopy, in which, the LTA was probed with anti-LTA antibodies, containing a ferritin conjugate [Van Driel *et al.*, 1973]. Using whole cells of *L. casei* and *L. fermenti* the LTA was seen to cover the entire cell surface of the Gram-positive bacteria [Van Driel *et al.*, 1973]. Furthermore, by taking cross-sections of the cells and again labelling as before it was shown that LTA is bound to the cell membrane and protrudes through the cell wall exposing the hydrophilic chain on the exterior of the cell [Van Driel *et al.*, 1973]. In *Mycobacterium* spp. the LAM has also been shown to be part of the exterior cell wall and exists as a capsular sheath, surrounding the bacterial cell wall. This was demonstrated with use of immuno-gold staining (antibodies directed to LAM conjugated to gold particles) and electron microscopy [Hunter & Brennan, 1990].

1.5.2 Biosynthesis of Lipoteichoic Acid

1.5.2.1 Hydrophilic Chain

The biosynthesis of the hydrophilic moiety of LTA has only been resolved in PGP LTA with the use of *in vivo*, pulse-chase experiments and analogues of precursor molecules. It was suggested from *in vivo* observations involving *S. aureus*, that the glycerophosphate, which makes up the PGP core of the hydrophilic moiety of LTA, is supplied by phosphatidylglycerol (PG) [Glaser & Lindsay, 1974]. Thus, the following reaction scheme was proposed:-

$$\text{PG} + \text{LTA-poly(glycerophosphate)}_n \longrightarrow \text{LTA-poly(glycerophosphate)}_{n+1} + \text{1,2-diglyceride}$$

This was later confirmed by an *in vitro* study, which used another Gram-positive bacteria, *B. subtilis* and demonstrated the inhibition of phosphatidylglycerol biosynthesis, with the use of 3, 4 dihydroxybutyl-1-phosphonate (DHBP), an analogue of the precursor molecule *sn*-glycero-3-phosphate used in PG biosynthesis. It was already known that DHBP inhibited the synthesis of PG, therefore, if the synthesis of PG was inhibited, then LTA biosynthesis should also be inhibited, in the presence of DHBP. Indeed this appeared to be the case, as inhibition LTA biosynthesis was observed [Deutsch, Engel & Tropp, 1980].

Additionally other potential donors such as cytidine diphosphate glycerol (CDP-glycerol), which is a donor of glycerophosphate used in teichoic acid biosynthesis, was also examined using particulate enzyme fractions (enzymes located on the cell wall, as opposed to within the cytoplasm). These fractions were incubated with, CDP- glycerol, containing ^{3}H-glycerol, and the results demonstrated that CDP-glycerol could not substitute for phosphatidylglycerol in LTA biosynthesis [Pieringer *et al.*, 1981].

The mechanism by which the PGP-LTA is elongated was initially investigated in *Streptococcus faecium* in which the cells were firstly grown in the presence of [^{14}C]glycerol. The cells were then harvested and the [^{14}C]glycerol-LTA was extracted and then further elongated using particulate enzyme preparations and [^{3}H]glycerol. The double-radiolabelled LTA was then degraded in a step-wise fashion from the phosphoglycerol terminus to the lipid moiety using a combination of phosphomonoesterases and phosphodiesterases (from *Aspergillus niger*). From the degradation pattern produced, it was determined that the polymer elongation was distal to the lipid anchor, meaning that the polymeric glycerophosphate chain is synthesised while attached to the final lipid anchor [Cabacungan & Pieringer, 1981].

This was further investigated by growing *L. casei*, labelled with tritiated glycerol to form [2-*glycerol*-^{3}H]-LTA. These cells were then treated with toluene, which inhibits the cells from producing new LTA and teichoic acid and the cells now converted the common substrate of both, phosphatidylglycerol, to cardiolipin [Moses & Richardson, 1970]. The LTA was further elongated by incubating with a second label of [^{14}C]glycerol and this formed [^{14}C

^{3}H]glycero-1-phosphate-LTA. The LTA was then extracted and degraded enzymatically as discussed above. From this and similar studies, it was suggested that the fate of the 1,2-diglyceride was that it is recycled back to PG (via phosphatidic acid) using enzymes such as diglyceride kinase. [Taron, Childs III & Neuhaus, 1983].

The direction of chain extension is therefore similar to that of cell wall teichoic acid [Burger & Glaser, 1964] and the opposite to that of peptidoglycan, also within the bacterial cell wall, which is synthesised by transfer of a growing chain to the lipid moiety [Ward & Perkins, 1973].

1.5.2.2 Hydrophobic Lipid Anchor

Synthesis of the glycolipid portion bound within the cell membrane proceeds with the addition of a 1,2-di-*O*-acyl-*sn*-glycerol, following this, hexosyl groups are transferred from sugar nucleotides [uridine diphosphate (UDP), formed from uridine triphosphate and the hexose sugar to form an 'Activated Sugar'] to the diacylglycerol [Fischer *et al.*, 1978]. The phosphatidyl- and fatty acyl-diglycosyldiacylglycerolipids constitute the lipid portions of LTA within the *Lactobacilli* spp. These same glycolipids are commonly found freely with other membrane lipids. They may therefore function as potential acceptor substrates in the biosynthesis process [Fischer *et al.*, 1978]. Furthermore, *sn*-glycerol-1-phosphate-bearing derivatives of the diglycosyldiacylglycerolipids and phosphatidylglycosyldiacylglycerolipids (PTGDAG-lipids) were also observed, thus, further supporting the hypothesis that these glycolipids are the starting block for LTA biosynthesis [Nakano & Fischer, 1978]. Once again, using particulate membrane enzymes, this time from *Enterococcus faecalis* and radiolabelled PTGDAG-lipids, it was shown that the PTGDAG-lipids were used as an acceptor substrate. The theory above was supported further by the presence of the radiolabel that later appeared in the aqueous phase of a chloroform/methanol/water extraction. The hydrophilic polymer binding the radiolabelled PTGDAG-lipid was responsible for this aqueous partitioning, the length of which seemed to increase over time. This was characterised as a micellar, non-substituted glycerophosphate polymer or nascent LTA, as it

appeared to lack the substituents of LTA extracted from whole cells of *Enterococcus faecalis* [Ganfield & Pieringer, 1980].

Conclusive evidence was obtained from pulse-chase investigations where *S. aureus* fatty acyl residues were labelled with [2-^{3}H]-glycerol and [^{14}C]-acetate. The radioactive lipid amphiphiles were separated by two-dimensional thin layer chromatography (2D-TLC) and hydrophobic binding was used to purify LTA. The results showed that the level of radiolabel increased continuously within the glycolipid portion and thus demonstrating that the glycolipid is not recycled for LTA biosynthesis.

It was also demonstrated that the hydrophilic chain is polymerised on the final lipid anchor as successive elongation of the [^{14}C]-label was observed in the lipid amphiphiles, starting with the diacyglycerol (DAG), followed by glucosyl-DAG, diglucosyl-DAG and finally with LTA. In addition to this glycerophosphodiglucosyl-DAG was also found as an intermediate. The majority of 1,2-diacylglycerol produced by the elongation of the PGP hydrophilic chain was shown to be recycled via phosphatidic acid, with approximately 10% being used for glycolipid synthesis. Following this work the inter-relationship between LTA biosynthesis and membrane lipid metabolism was shown in a schematic representation, shown in Figure 1.4 [Koch, Haas & Fischer, 1984].

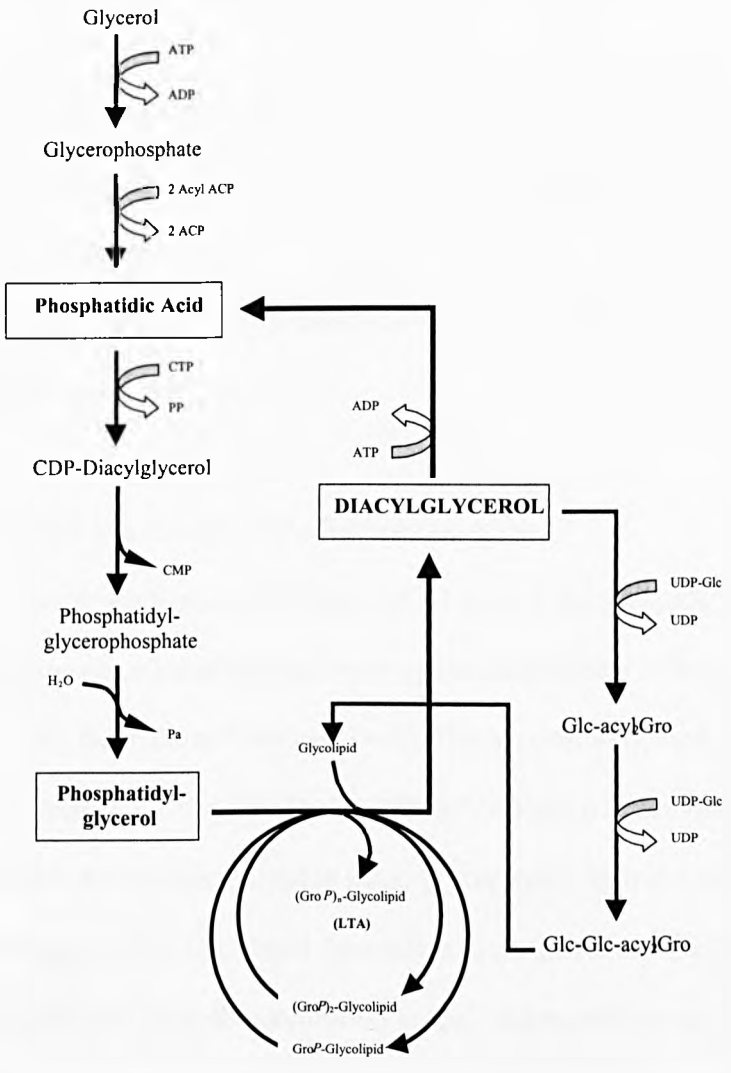


Figure 1.4 Summary of the Inter-relationship between LTA Biosynthesis and Membrane Lipid Metabolism

Gro, glycerol; Gro*P*-Glycolipid, glycerophosphodiglucosyl-DAG intermediate; Glc-Glc-acyl$_2$Gro, diglycosyl-DAG (lipid anchor); Glc-acyl $_2$Gro, glycosyl-DAG; UDP-Glc, Uridine diphosphate-glucose (i.e. activated glucose); ACP, acyl carrier protein; CTP, cytidine triphosphate; Pa, phosphate. Adapted from [Koch et al 1984].

The ability of bacteria to synthesise LTA from the available glycerophosphate and glycolipids requires two different chemical linkages, one to join the glycerophosphate to the glycolipid and another to polymerise the glycerophosphates together within the core hydrophilic region of the LTA molecule [Fischer, 1988]. These observations suggest the

existence of two distinct glycerophosphate transferases. Further speculation on the properties of these putative enzymes was hypothesised from collective data and reviewed by Fischer [Fischer, 1988]. From this data it was suggested that the first enzyme would be highly specific for a particular glycolipid and in all structural analyses to date, it appears to link the glycerophosphate, exclusively to the C-6 of the non-reducing hexose terminal of the glycolipid. Furthermore, the second enzyme would simply bind the glycerophosphates together to form the polyglycerophosphate chain via a 1,3-linkage with the phosphate group and the glycerol [Fischer, 1988].

1.5.2.3 Carbohydrate Substitution of the Hydrophilic Chain

Substitution of the hydrophilic moiety of LTA, with carbohydrate, occurs at the C-2 of the glycerophosphate unit and has been shown to be independent of the PGP synthesis [Fischer *et al.*, 1990; Ganfield & Pieringer, 1980]. This was demonstrated with the *in vitro* incubation of pre-formed LTA in the absence of UDP-Glucose (i.e. activated glucose), in which, elongated LTA was detected, but in a non-glycosylated form (i.e. nascent LTA) [Ganfield & Pieringer, 1980]. In contrast, incubation in the presence of UDP-[^{3}H]-Glucose and subsequent hydrolysis with 40% hydrofluoric acid, radioactive mono, di, tri and tetraglucosylglycerol structures were detected [Cabacungan & Pieringer, 1985]. It is believed that glycosylation of this kind occurs during polymerisation of the glycerophosphate residues during chain elongation. In an *in vivo* investigation with the bacteria *Lactococcus lactis*, on extraction of LTA, an α-D-galactopyranosyl residue was identified, bound to one of the glycerophosphate residues [Laine & Fischer, 1978]. The pathway by which this glycosylation occurs is thought to be a two step process [Mancuso & Chiu, 1982]. The first of these steps was shown using a particulate enzyme preparation from *Streptococcus sanguis* and it was found that UDP-[^{14}C]-glucose was incorporated into a glucosyl lipid, that was soluble in chloroform/methanol. Using NMR and mass spectrometry the structure was determined as glucosyl-1-phosphorylundecaprenol [Mancuso & Chiu, 1982]. In the presence of the particulate enzymes, transfer of the glucosyl residue to a

macromolecule was observed. This macromolecule was identified as LTA [Mancuso & Chiu, 1982]. Further evidence for a two-stage reaction has been provided using membrane preparations of *B. coagulans*, where galactosyl substitutions occur within the LTA. Here, galactosyl phosphorylpolyprenol was identified as the carbohydrate-lipid intermediate. Furthermore, it was also suggested that the two reactions might occur at different locations of the cell as the two enzymes involved were shown to have different pH optima. The optimum pH for the formation of the galactosyl lipid intermediate was 8.4 [Yokoyama, Araki & Ito, 1988]. In contrast, for the transfer of the galactose to LTA the optimum pH was 4.6 [Yokoyama, Araki & Ito, 1988].

1.5.2.4 D-alanine Substitution of the Hydrophilic Chain

Addition of D-alanyl residues as esterified substituents can also occur frequently along the glycerophosphate polymer chain. These substitutions are thought to play a significant role in the physiology of the cell wall [Heptinstall, Archibald & Baddiley, 1970; Hughes, Hancock & Baddiley, 1973; Ntamere, Taron & Neuhaus, 1987]. Using preparations of crude enzyme separations from *Lactobacillus casei* the incorporation of D-alanine was shown to require two reactions. The cell wall associated enzymes of lysed bacteria were separated, via centrifugation, as a pellet (particulate fraction). The resulting supernatant contained all other enzymes that are located in the cytosolic (soluble) fraction. From these preparations it was found that both the particulate and supernatant fractions were required for the successful esterification of the D-alanine residues, together with Mg^{2+} ions and ATP. The following reaction sequence was proposed [Baddiley & Neuhaus, 1960; Linzer & Neuhaus, 1973; Neuhaus, Linzer & Reusch Jr, 1974; Reusch & Neuhaus, 1971].

enzyme + D-alanine + ATP ⟶ enzyme-AMP-D-alanine + PP_i

enzyme-AMP-D-alanine + membrane acceptor ⟶
D-alanyl-membrane acceptor + enzyme + AMP

By following the incorporation of [^{14}C]alanine, the ultimate membrane acceptor was later confirmed to be endogenous PGP-LTA. Moreover, the position of this esterification was determined by conducting a step-wise degradation of the LTA hydrophilic moiety with phosphodiesterase II and phosphatase. The degradation species were then analysed and shown to contain C-2 alanine substituents, therefore it is the C-2 position of the glycerol that is utilised for this alanylation [Childs & Neuhaus, 1980]. In addition, it was also concluded that alanine substitution occurred during or in parallel to growth of the PGP chain. This was reported after *L. casei*, was incubated with D-[^{14}C]-alanine, following extraction of the lipophilic compounds, via aqueous/organic phase partitioning, the radiolabel was detected. These were concluded to be short-chain homologues of LTA, due to their chromatographic properties, which were very similar to that of the glycerophosphoglycolipids also extracted from *L. casei* [Brautigan, Childs III & Neuhaus, 1981].

The enzyme responsible for the second reaction, denoted above, was originally identified as a membrane acceptor ligase, but has more recently been renamed as the D-alanyl carrier protein (Dcp) and was found in the supernatant fraction. This enzyme was characterised as an acidic, heat-labile protein of 8.8kDa. From the amino acid analysis it was deduced that it was similar to a group of enzymes required for fatty acid biosynthesis called the acyl carrier proteins (ACPs). It was suggested that it was this enzyme that carries the activated D-alanine to the next acceptor, thought to be membrane-bound LTA (mLTA) component [Heaton & Neuhaus, 1994].

The other enzyme was also identified from the supernatant fraction and is required for the first of the reactions above. This enzyme is responsible for the binding of AMP and thus, the activation of the D-alanine. Following these observations the enzyme was initially termed the D-alanine-activating enzyme and was shown to be a 56kDa protein, determined with the use of sodium dodecyl-PAGE (SDS-PAGE). As discussed above, the D-alanine-activating enzyme is responsible for the activation of D-alanine and was later more appropriately renamed the D-alanine-Dcp ligase (Dcl), as it firstly binds the D-alanine, activates it with ATP and finally transfers and ligates the D-alanine to the carrier protein, Dcp. Following

this the D-alanine is then esterified to the C-2 of the glycerophosphate residue of the LTA [Heaton & Neuhaus, 1992].

It has been suggested that membrane components are also required for alanine substitution, due to the discovery of mutant strains of *L. casei*, which completely lacked D-alanyl residues, although every other aspect of the LTA was found to be the same. In this investigation, the membrane fragments were unable to esterify the alanine, but in the reverse situation where LTA was incubated with membrane preparations from wild-type strains, alanine substitution occurred [Ntamere *et al.*, 1987]. This was also supported by the identification of an operon in the chromosome of *B. subtilis* [Glaser *et al.*, 1993]. The operon was termed the *dlt* operon and has been shown to contain five genes, *dltA, B, C, D* and *E*, four of which (*dltA*, *dltB*, *dltC*, and *dltD*) if inactivated result in inhibition of alanyl substitution [Perego *et al.*, 1995]. The *dltA* gene has been shown to encode D-alanine-Dcp ligase, Dcl, while *dltC* has been shown to encode the acyl carrier protein, Dcp. *dltB* is thought to encode a putative transport protein, the function of which is still unclear (Neuhaus, 1996). The protein product of *dltD*, has been shown to facilitate the binding of Dcl and Dcp and the ligation of D-alanine to Dcp. This function is further supported by the observation of thioesterase activity for D-alanyl-ACP's, which would be required for the ligation of D-alanine to Dcp [Debabov, Kiriukhin & Neuhaus, 2000]. Recently it has been suggested that D-alanyl-Dcp has a unique binding site that interacts with the PGP of membrane bound LTA and thus, it is this interaction that allows the transfer of the D-alanine. It was also noted that the D-alanine transfer is reversible, thus providing a mechanism for the transacylation of D-alanine between LTA and teichoic acid within the bacterial cell wall [Kiriukhin & Neuhaus, 2001b]. This has been suggested in earlier research, focussing on the turnover of D-alanine in *S. aureus*, the details of which will be discussed in Section 1.5.2.5 [Haas, Hoch & Fischer, 1984]. The reaction scheme for alanyl substitution is illustrated in Figure 1.5.

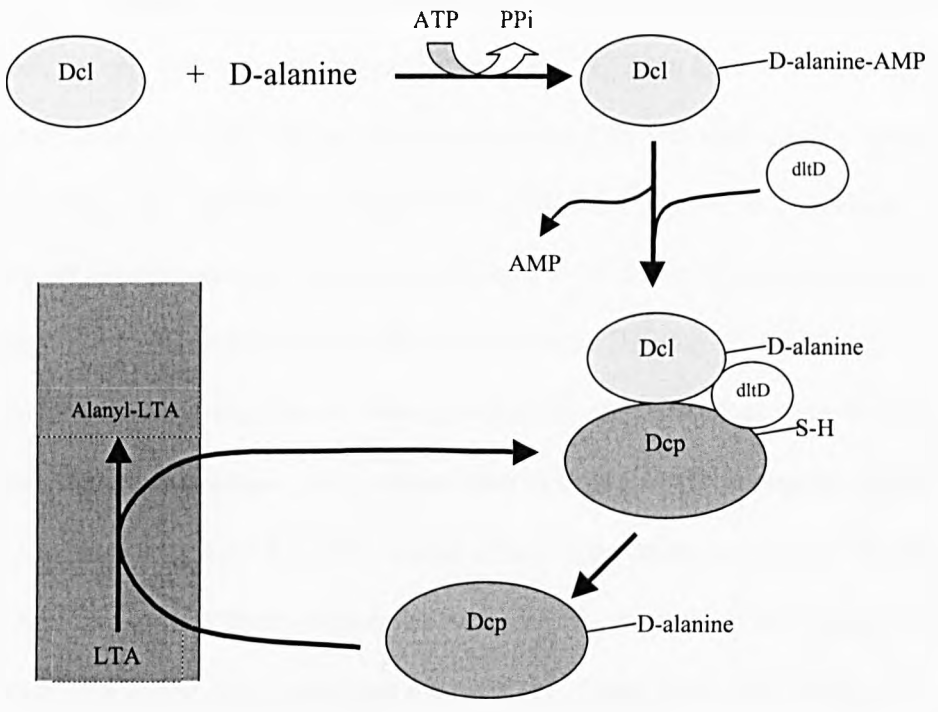


Figure 1.5 D-Alanine Substitution in LTA

The proposed mechanism, by which D-alanine is substituted into the LTA of PGP-LTA, bound to the cell membrane. Dcp, D-alanyl carrier protein; Dcl, D-alanine-Dcp ligase; dltD, protein product of *dltD* and is thought to facilitate the binding of Dcp and Dcl.

1.5.2.5 Turnover of Cell Wall D-alanine

The D-alanine substitution that occurs within LTA is susceptible to base-catalysed hydrolysis, due to the adjacent phosphate and hydroxyl groups present within the LTA structure. This can be determined by making comparisons of the D-alanine ester linkages in model compounds such as hydroxylamine [Archibald & Baddiley, 1966]. As a result of pulse-chase experiments, using pre-labelled [^{3}H]glycerol and D[^{14}C]-alanine LTA, it is thought that an enzyme-catalysed hydrolysis may also occur. After the chase, the ^{14}C of the D-alanine was seen to decrease significantly in the LTA extract, while the ^{3}H of the LTA remained constant, thus, indicating the loss of the D-alanine. Furthermore, the velocity of the D-alanine turnover was 20-times greater than expected for a base-catalysed hydrolysis and therefore suggesting the intervention of an enzyme [Haas *et al.*, 1984]. In the study, it was

also concluded that the hydrolysed D-alanyl residues from LTA are transferred to teichoic acids within the cell wall, as correlating amounts of the ^{14}C, from the pre-labelled D[^{14}C]-alanine, was found in the cell wall as wall-linked alanyl. This was supported by the analysis of the hydrofluoric acid hydrolysis products of both the LTA and teichoic acid from post-chase cells, which demonstrated the loss of radioactivity from the D[^{14}C]-alanylglycerol and a subsequent rise in the radioactivity of the D-alanylribitol [Haas *et al.*, 1984].

Further studies examined whether the sites of D-alanine ester hydrolysis were re-esterified with further D-alanine residues. Using toluene-treated cells (i.e. preventing the biosynthesis of *de novo* teichoic acid and LTA) of *S. aureus* which were incubated with D[^{14}C]-alanine, ATP and Mg^{2+} ions it was shown that the cells retained the capacity to incorporate D-alanine into existing LTA and teichoic acid components [Koch, Döker & Fischer, 1985].

Furthermore, it was shown that by increasing the pH of the environment, the presence of D[^{14}C]-alanine increased in teichoic acid and was found to be reduced in LTA. In addition to this, re-esterification was apparent and this increased with an increasing pH. Therefore, the D-alanine/glycerol ratio was constant. In the same study, an increase in the label was observed within the wall-associated teichoic acids and as teichoic acid synthesis was inhibited transfer of D-alanine must have occurred to the completed wall-linked teichoic acids, thus, re-esterification was evident. In summary, it was deduced that the cell wall teichoic acid is the final acceptor of the D-alanine and thus, LTA, might acts as a carrier between the free pool of D-alanine and teichoic acid within the bacterial cell wall [Koch *et al.*, 1985].

Lastly, the growth conditions to which the bacteria are exposed to had been found to have a considerable effect on the substitution of D-alanine. This was shown with *S. aureus* and a varying concentration of sodium chloride. By increasing the salt concentration the degree of alanyl substitution decreases. The mechanism of this control has been suggested to be associated with the activity of the enzymes, involved in alanyl substitution and the effect was non specific in that NaCl, KCl and Na_2SO_4 all demonstrated similar activities [Koch *et al.*, 1985].

1.5.3 Biosynthesis of Lipoglycans

1.5.3.1 Hydrophobic Lipid Anchor

The biosynthesis of lipoglycans has been studied in detail using mainly *Mycobacteria* spp. As discussed in Section 1.3.3, the lipoglycans identified within *M. tuberculosis* are lipomannan (LM) and lipoarabinomannan (LAM) [Hunter *et al.*, 1986]. Biosynthesis of both LM and LAM initiates with the addition of the lipid anchor to a specific phospholipid within the cell membrane. In mycobacteria, the membrane phospholipid, phosphatidylinositol (PI) has been identified as the lipid anchor acceptor. This lipid anchor accepts mannose residues from GDP-mannose (guanine diphosphate) donors to form a phosphatidylinositol dimannoside (PIM_2). Three PIM_2 have been identified that differ only in the number of acylated fatty acids they contain. These can be two, three or four fatty acids per phosphate, suggesting that acylation occurs on the inositol and/or mannose residues, as the phosphatidyl residue can only hold a maximum of two fatty acids [Brennan & Ballou, 1967]. The enzyme responsible for the transfer of mannose from GDP-mannose to the PIM, to produce PIM_2, has been identified through the screening of the *M. tuberculosis* genomic library and expressing the various genes of interest in *E. coli*. The gene was identified as *pimB* and the protein product of *pimB* was overexpressed in *E. coli* and partially purified. This was then shown to catalyse the mannose transfer described above *in vitro* [Schaeffer *et al.*, 1999]. Recently, a related mannosyl transferase has been identified and designated PimC (expressed from the gene *pimC*) [Kremer *et al.*, 2002]. This enzyme was found to catalyse the formation of triacyl-PIM_3. However, deletion of the *pimC* gene did not effect the synthesis of more complex PIM's nor LAM, thus suggesting the existence of redundant genes [Kremer *et al.*, 2002]. Furthermore, on analysis of clinical isolates of *M. tuberculosis* for *pimC*, only 22% of the isolates tested positive for the gene [Kremer *et al.*, 2002] and therefore supporting the suggestion of gene redundancy.

Until recently the biosynthesis of PI has remained elusive, but with the aid of *M. smegmatis*, the PI biosynthetic pathway has finally been characterised and is now a potential target for chemotherapeutic agents against *M. tuberculosis* [Salman *et al.*, 1999a].

PI and the mannosides of PI, have been shown to constitute 56% of the total phospholipids of the mycobacterial cell wall and 37% of those in the cytoplasmic membrane [Goren, 1984]. The fatty acid content of PI, PIM, lipomannan and LAM have all been routinely shown to possess 10-methyloctadecanoic acid (tuberculostearic acid) esterified to the C-1 position of the of the glycerol and also palmitic acid esterified to the C-2 [Lee, Brennan & Besra, 1996]. By incubating different mycobacterial cell fractions with [^{3}H]-inositol, only the cell wall fraction had the capacity to synthesise PI, suggesting that PI biosynthesis is localised [Salman *et al.*, 1999a]. Interestingly, the identification of PI in this fraction accounted for >90% of the total inositol containing products. The enzyme responsible for the synthesis of PI was identified as PI synthase, [Salman *et al.*, 1999a]. The pathway for PI biosynthesis has been characterised in mammalian systems, the substrates for which, have been identified as inositol and cytidine diphosphate diacylglycerol (CDP-DAG), although CDP-DAG synthetic precursors have also been shown to be acceptable as substrate [Paulus & Kennedy, 1960]. However, it has been shown that the PI synthase of mycobacteria had marked differences with respect to substrate specificity when compared to that of mammalian PI synthase. This was demonstrated with the use of cytidine diphosphate-dipalmitoyl-diacylglycerol (CDP-DP-DAG) and cytidine diphosphate-1,2-[oleyl,(*N*-(nitrobenzo-2-oxa-1,3-diazole)aminocaproyl)]diacylglycerol (CDP-NBD-DAG), both identified as synthetic precursor substrates for mammalian PI synthase. *In vivo* addition of these substrates resulted in an increase in the yield of PI within the microsomes of rats and also *Saccharomyces cerevisiae*, but when incubated with mycobacterial preparations an increase in PI was not observed. PI synthase of mycobacteria was therefore shown to be specific for endogenous CDP-DAG only [Salman *et al.*, 1999]. These results demonstrated that there was strict substrate specificity within the PI synthase of mycobacteria for the endogenous substrate. Furthermore, as there is significant amino acid similarity (95%) between rat and human PI synthases (concluded using Basic Local Alignment Search Tool), it was suggested that human PI synthase would differ to the PI synthase possessed by mycobacteria, thus, a potential target for anti-tuberculosis drugs [Salman *et al.*, 1999a].

More recently, PI has been shown to be an essential phospholipid for the survival of mycobacteria, as deactivation of a gene responsible for PI synthesis (*pgsA*) results in a loss of mycobacterial cell viability [Jackson, Crick & Brennan, 2000]. Furthermore, the enzymes required for the initial mycobacterial *de novo* biosynthesis of inositol for utilisation in PI production and the mannosides there of, are now being investigated, focussing on the regulation of inositol monophosphatase (IMP). This enzyme is responsible for the dephosphorylation of inositol-1-phosphate (produced by the action of inositol-1-phosphate synthase on glucose-1-phosphate) to yield inositol [Nigou & Besra, 2002]. In this investigation it was shown that IMP is Mg^{2+} ion-dependent and inhibited by univalent cations such as Na^{+} and Li^{+} resulting in the inhibition of mycobacterial growth [Nigou & Besra, 2002].

1.5.3.2 Lipoglycan Hydrophilic Chain

On completion of the PIM_2 anchor the bulk of the mannan core is then synthesised, by extending the linear α1-6 mannose linked chain to approximately 12 residues [Yokoyama & Ballou, 1989]. Addition of these mannose residues is not thought to occur directly from GDP-mannose as with PIM_2, but through lipid-soluble intermediates. This was first suggested by incubating membrane fractions of *M. smegmatis* with GDP-[^{3}H]mannose. Here it was shown that the mannose was firstly incorporated into mannooligosaccharide intermediates, shown to contain phospholipid [Yokoyama & Ballou, 1989]. These mannosyl phospholipid intermediates were determined to be two types of polyprenylphosphomannoses, more specifically, heptaprenol-phosphomannose (C35-P-Man) and decaprenol-phosphomannose (C50-P-Man). To investigate this biosynthetic progression, a complicated *in situ* chase experiment was carried out using cell-free incubations containing GDP-[^{14}C]Mannose-labelled polyprenylphosphomannoses and mycobacterial PIM's. Re-isolation of these compounds revealed a depletion of the polyprenylphosphomannoses and significant amounts of lipomannan. Further information was gained from the addition of the antibiotic, amphomycin, a lipopeptide compound that specifically inhibits polyprenyl-P-

requiring transferases. In the presence of this antibiotic, biosynthesis was inhibited [Besra *et al.*, 1997]. Evidence now suggests that the principal donor of mannose for incorporation into lipomannan and LAM is C50-P-Man, as the C35-P-Man has been found to be associated with the transfer of mycolic acid to arabinogalactan (AG). These form large polysaccharides that are bound to peptidoglycan, via a linkage unit that is composed of a disaccharide containing phosphorylated forms of rhamnose and *N*-acetylglucosamine. These complexes constitute a major component of the mycobacterial cell wall and are referred to as the mycolyl arabinogalactan-peptidoglycan complex (mAGP) [Besra *et al.*, 1995]. Furthermore, the C35-P-Man was also found to be the most abundant polyprenylphosphomannose in mycobacterial cells. This together with the identification of the mycolyl-C35-P-Man intermediate, would suggest two distinct biochemical pathways, with polyprenylphosphomannosyl transferases that can distinguish between C50-P-Man and C35-P-Man [Wolucka & de Hoffmann, 1998].

At this stage in the biosynthesis, the mannan core can have further additions of mannose at the C-2 position of the mannose residues within the mannan core backbone. These additions give rise to branching and result in a mannan core averaging 26 mannose residues in total. The structure is such that it allows considerable heterogeneity with respect to the length and degree of branching within the mannan core. This heterogeneity can be further enhanced by the presence of phosphorylation along the chain [Chatterjee *et al.*, 1992c].

Biosynthesis of the arabinan portion of LAM was previously thought to share the same enzymes as the biosynthesis of AG, but with the use of an anti-tuberculosis drug, ethambutol it was shown that ethambutol inhibited AG and LAM synthesis in different ways. By following the incorporation of [^{14}C]-glucose into arabinose residues of AG, it was found that AG synthesis was inhibited immediately, while inhibition of LAM biosynthesis did not occur until 1 hour after exposure [Deng *et al.*, 1995; Mikusová *et al.*, 1995]. In addition to these observations, within *M. smegmatis*, ethambutol mutants were derived by consecutively passaging the culture with increased concentrations of the drug. On analysis of the cell wall components AG was found to be normal while LAM was found to be truncate [Mikusová *et*

al., 1995]. Following this, it was shown that the truncation had primarily arisen from the inhibition of a linear $(arabinan)_4$ terminal motif (see Figure 1.3 in Section 1.3.3.1). This terminal arabinan motif was not found in AG. It can therefore be concluded that the arabinan moiety of LAM has a distinctly different biosynthetic pathway to the AG polysaccharides and it is this that is targeted in some way by ethambutol [Khoo *et al.*, 1996]. Moreover, it was shown that by chemically synthesising [^{14}C]decaprenol-phospho-arabinose ([^{14}C]C50-P-Ara) and incubating this with within a membrane preparation of *M. smegmatis* the radiolabel can be later found in the bacterial cell wall. The C50-P-Ara was suggested as the donor molecule for the synthesis of the arabinan portion of LAM and AG. By adding ethambutol to the incubation again, it was seen to inhibit polymerisation of the arabinan chain and also this inhibition lead to an accumulation of the precursor [^{14}C]C50-P-Ara. From these observations it was suggested that arabinotransferases were targeted by the action of ethambutol [Lee *et al.*, 1995].

The products of C50-P-Ara-arabinan transfer have been investigated to identify the final acceptor components of the arabinose residues. The resulting polymers were degraded with a mixture of arabinases, isolated from a *Cellulomonas* soil organism [McNeil *et al.*, 1994; Xin *et al.*, 1997]. The results from this work demonstrated that the major structural motifs of both AG and LAM were present, thus, C50-P-Ara was thought to be the sole donor of arabinosyl residues in mycobacteria [Xin *et al.*, 1997]. In contrast to this were the observations made by Wolucka in the identification of heptaprenol-phosphoarabinose (C35-P-Ara) [Wolucka *et al.*, 1994] and the previous observations of the transfer of mannosyl residues to AG utilising C35-P-Man [Wolucka & de Hoffmann, 1998]. It was suggested that C35-P-Ara might be involved in the transfer of arabinose residues to AG and therefore the primary role of C50-P-Ara, being the donor for LAM. Although to date, the mycolyl-C35-P-Ara derivative, which would support this hypothesis has not yet been identified.

In the past, the LAM of many strains of mycobacteria have been shown to have a capping motif consisting of an oligomannoside or in some cases of rapidly growing mycobacteria, an inositol-1-phosphate residue [Chatterjee *et al.*, 1993]. To date, the biosynthesis of these

capping motifs has yet to be investigated, although attempts are being made to synthesise these capping motifs and related structures in a bid to use them as potential inhibitors of LAM biosynthesis [Désiré & Prandi, 1999].

1.6 The Role of Lipid Macroamphiphiles Within Bacteria

The physiological role of LMAP within bacteria is still under debate. In the case of LTA at least three possible functions have been hypothesised, and all of which can also be applied to lipoglycans, thus, bolstering the statement that these molecules replace each other functionally between Gram-positive bacteria [Fischer, 1994a; Fox *et al.*, 1980; Sutcliffe, 1994a].

1.6.1 Carrier Molecules for Teichoic Acid Biosynthesis

Originally, it was proposed that the function of large amphiphilic components within the bacterial cell membrane is that they acted as carrier molecules, which facilitated the synthesis of other molecules such as teichoic acids. It was suggested that an amphiphilic carrier molecule was required for the synthesis of teichoic acid in *S. aureus*. This carrier molecule would have to contain a non-specific lipid moiety and a tetra(glycerophosphate) sequence at the terminal which would allow the enzyme poly(ribitol phosphate) polymerase to bind [Fischer *et al.*, 1980a]. Both of these criteria were met by LTA, thus, it was suggested that LTA could be the carrier molecule involved in teichoic acid biosynthesis. Furthermore, using LTA *in vitro*, poly(ribitol phosphate) polymerase activity was observed [Fischer *et al.*, 1980a]. A short time later, this hypothesis was discredited after LTA was extracted from a range of bacteria using a lower pH extraction buffer. The newly extracted LTA was shown to possess a significant amount of alanine substitution along the PGP chain [Fischer *et al.*, 1980b]. The presence of alanine anywhere along the terminal penta(glycerophosphate) blocked the binding of the poly(ribitol phosphate) polymerase

enzyme and therefore, LTA could not be involved in teichoic acid biosynthesis in this manner [Fischer *et al.*, 1980b; Koch, Fischer & Fiedler, 1982]. To support this further, when alanine-substituted LTA was used in the *in vitro* assays it could not be utilised by poly(ribitol phosphate) polymerase [Fischer *et al.*, 1980b]. It was proposed that in previous extractions of LTA the alanine esterification was lost due excessively high pH and therefore accounted for the poly(ribitol phosphate) polymerase activity initially observed [Fischer *et al.*, 1980b; Koch *et al.*, 1982].

It has now been shown that the poly(ribitol phosphate) is synthesised directly at the glycerophosphate terminal of the teichoic acid linkage unit and that these reactions (teichoic acid biosynthesis and alanyl substitution of teichoic acid and LTA) must be spatially separated for the process to occur. To further support these 'biosynthetic rules' it was found that alanylation occurs on the outer layer of the membrane and the teichoic acid only arrives at this location once in its completed form [Hancock & Baddiley, 1985]. Thus, on the evidence presented to date, the theory that LTA and lipoglycan are involved in teichoic acid biosynthesis can be dismissed.

1.6.2 Ion-scavenging Function

One other function suggested for both LTA and lipoglycan is that the components may act as ion-scavenging molecules for divalent cations, such as Mg^{2+} ions. The theory being that the LMAP, along with teichoic acid and teichuronic acid on the surface of the bacterial membrane forms a polyanionic network between the membrane and the surface of the cell. This network lures and then traps the cations and thus, creates a reservoir of ions, which would then be available for transport pathways and other membrane associated enzymes [Heptinstall *et al.*, 1970; Lambert, Hancock & Baddiley, 1975; Lambert, Hancock & Baddiley, 1977]. Extending this theory further, the positively charged alanine substitutions along the PGP chain and similarly the negatively charged succinate residues along the lipomannan chain (*Micrococcus* spp.), can be added or subtracted and therefore used to alter the anionic charge along the chain structure, thereby allowing regulation of the

cation reservoir [Lambert *et al.*, 1975]. However, conflicting observations have been made, primarily the identification and characterisation of lipoglycans that do not carry anionic substitutions along the hydrophilic chain. Examples include *R. dentocariosa* and *A. agilis* [Lim & Salton, 1985; Sutcliffe & Alderson, 1995; Sutcliffe, 1994b]. These lipoglycans would therefore be neutral and would not perform the ion-scavenging role hypothesised above. Further contrasting data was obtained from ^{31}P NMR spectroscopy on PGP-LTA where it was concluded that there was little or no detectable effect of alanine-esterifications, along the PGP chain, on the association constant of Mg^{2+} ions with the LTA macroamphiphile. Furthermore, it was argued that the mobile counter-ions present would eliminate any intramolecular electrostatic discrepancy [Batley *et al.*, 1987]. This argument is supported from studies using *B. subtilis*, in which insertional inactivation was used to disable the genes responsible for alanyl substitution. These genes are located on the *dlt* operon and are thought to control the expression of Dcp (refer to Section 1.5.2.4. describing D-alanine substitution of the hydrophilic chain). In preventing alanyl substitution it renders the PGP-LTA completely anionic. These bacterial cells were then stained with cationized ferritin granules and viewed under an electron microscope. From this analysis, no differences were observed between the mutant bacterial cells and parent *B. subtilis* [Wecke, Perego & Fischer, 1996]. However, in contrast to these findings, using cytochrome *c* instead of cationized ferritin, a significant difference between mutant *B. subtilis* and wild-type PGP-LTA was demonstrated and an overall increase in the negative charge of the cell wall was observed in the mutant strain of *B. subtilis* that lacked alanine substitution. Cytochrome *c* was primarily used as it had the advantage of being a lot smaller in size (13kDa as opposed to 50,000kDa) and could also be measured spectrophotometrically [Wecke, Madela & Fischer, 1997]. More recently, it has been shown that an increase in the negative charge of the cell wall, due to the absence of D-alanine, caused an increase in the rate of post-translocational folding in the proteins exported from the bacterial cell [Hyyrylainen *et al.*, 2000]. However the precise physiological purpose of the D-alanylation and consequent

charge modulation are yet to be clarified, thus the ion-scavenging role of LMAP remains a possibility.

1.6.3 Regulation of Autolytic Enzymes

Another possible role and probably the most plausible function of LTA and lipoglycans is the ability to inhibit or regulate autolytic enzymes at the cell wall. It has been shown using PGP-LTA that LTA has an inhibitory effect on autologous autolysins. This effect was also observed with heterologous autolysins, providing they were from bacteria that also contained PGP-LTA [Cleveland *et al.*, 1976a; Cleveland *et al.*, 1976b]. This inhibitory effect was further defined as a sequence of negatively charged glycerophosphate residues, as negatively charged phospholipids such as cardiolipin were also found to exert the autolysin inhibitory response whereas neutral glycolipids did not [Fischer, Rosel & Koch, 1981]. In further experiments, it was shown that by removing the lipid portion of the LTA molecule or adding a detergent such as Triton X-100 the inhibitory action was destroyed [Cleveland *et al.*, 1976a; Cleveland *et al.*, 1976b]. This suggested that the micellar properties of the amphiphiles might be responsible for the earlier observations. The theory being, the formation of micelles exposes a high density of negative charge and it was this that the autolysin was interacting with. In a cellular context, the amphiphiles are present in the membrane and are therefore separated by membrane lipids, thus, this micellar effect would not be observed [Fischer, 1988; Gutberlet *et al.*, 1991]

With regards to the substituents of the hydrophilic chains, again it has been shown with LTA, that with the absence of alanine, autolysis is enhanced. This acceleration in autolysis was shown by measuring the OD_{578}. Furthermore, it was also noted that non-alanine substituted PGP-LTA containing bacteria were more susceptible to methicillin and that the re-growth of the bacteria in an antibiotic-free media was greatly reduced after methicillin exposure. Therefore the alanine substitution may have prevented methicillin-induced autolysis. Curiously, addition of Mg^{2+} ions with methicillin was found to prevent autolysis in both wild-type and mutant strains of *B. subtilis* [Wecke *et al.*, 1997]. More recently, further

information regarding the above observations was provided by research focusing on the susceptibility of *S. aureus* to vancomycin. It was shown that in mutants that lacked D-alanine within the LTA and wall teichoic acid, through inactivation of the *dlt*ABCD operon, were more susceptible to vancomycin. In addition to this, these mutants were also found to have reduced autolytic activity. This implies that any autolytic inhibition observed in earlier studies may be a legitimate effect, as these LTAs were *in situ* and therefore, no micellar effect would be present. [Peschel *et al.*, 2000]. Thus, it appears that it is the substitution of LTA that regulates the autolytic enzymes at the cell wall and may well be a primary responsibility shared between LMAPs and wall teichoic acids.

1.6.4 An Adhesin Virulence Factor

It remains possible that LMAPs maybe important for the adhesion of bacterial cells to host cells in the preliminary stages of infection and therefore function as an adhesin virulence factor [Henderson, Poole & Wilson, 1996]. In support of this hypothesis was the recent observations made in *Listeria monocytogenes*, in which mutants lacking the *dltA* gene required for D-alanine substitution of LTA, demonstrated a significantly reduced adherence to hepatocyte, macrophage and epithelial cell lines [Abachin *et al.*, 2002]. It was suggested that the increased electronegativity caused by the absence of the D-alanine was responsible for altering the binding activity of the LTA component and therefore D-alanyl LTA was required for bacterial pathogenecity [Abachin *et al.*, 2002]. The potential role of LMAP as a virulence factor is supported by the extensively categorised immune responses observed between both LTA and lipoglycan on interaction with the mammalian immune system. The details of which are discussed in Section 1.7.

1.7 Effects of Lipid Macroamphiphiles on Mammalian Systems

LPS, present on the cell surface of Gram-negative bacteria, has been the focus of interest into the physiological purpose and the mechanism by which it exerts its metabolic and immune effects within mammalian cells. Recently, it appears that this interest has shifted towards the molecules of Gram-positive bacteria, mainly due to the realisation that many of these components may be responsible for a whole range of mammalian effects. For this reason, an increasing degree of recent research has focused on the lipids, proteins and carbohydrates of Gram-positive bacteria. The biological roles of this group of bacterial immune-stimulating components remain unclear. One overarching hypothesis is that bacteria use these components as pathogenecity factors or virulence factors, by which the bacteria synthesise a range of cell surface molecules primarily to promote their ability to invade the host and in doing so, elicit an immunostimulatory response [Henderson *et al.*, 1996]. These molecules have been collectively defined as modulins in which all LMAPs discussed have been included [Henderson *et al.*, 1996].

1.7.1 Immunological Effects of Lipoteichoic Acids

1.7.1.1 Induction of Monocytes and the Cytokine Network

There is currently a vast array of documented effects that have now been characterised with lipoteichoic acid, many of which are related to Gram-positive sepsis. It was thought that sepsis was associated only with Gram-negative bacteria, but recently it has been suggested that up to 50% of sepsis or septic shock cases arise as the result of Gram-positive bacterial infection [Kieft *et al.*, 1993]. The onset of sepsis is centred around the activation of inflammatory pathways which involves cytokines, such as tumour necrosis factor-α (TNF-α), interleukins (IL) and interferon's (IFN). All of which are released by monocytes and macrophages [Van Der Poll & Van Derventer, 1999].

In one study, cultures of human monocytes were exposed to different LTAs extracted from *S. aureus*, *Streptococcus pyrogenes*, *Enterococcus faecalis*, *Streptococcus pneumoniae* and

Listeria monocytogenes [Bhakdi *et al.*, 1991]. The induction of three cytokines, IL-1β, IL-6 and TNF-α was monitored and it was shown that at concentrations of less than 5.0μg ml^{-1} both *S. aureus* and *S. pneumoniae* LTA failed to induce any of the three cytokines. However, in parallel *E. faecalis* induced all three factors [Bhakdi *et al.*, 1991]. The study also demonstrated that the activation was independent of complement components as the activation of the monocytes also occurred when incubated with foetal calf serum as opposed to human serum that contains the autologous complement proteins and glycoproteins [Bhakdi *et al.*, 1991]. Furthermore, deacylation of LTA caused a complete loss in the monocyte stimulatory effect, but deacylated LTA did not inhibit cytokine induction by intact LTA. From this observation it was suggested that primary binding does not involve a simple membrane-receptor interaction by the hydrophilic portion of LTA [Bhakdi *et al.*, 1991]. In contrast, an earlier study suggested that production of IL-1, was stimulated in response to as little as 1.0 pg ml^{-1} of pneumococcal LTA. Although, it is difficult to substantiate this effect as details of the LTA preparation were not provided, thus, it is impossible to assess the purity of the LTA and eliminate endotoxin contamination [Reisenfeld-Orn *et al.*, 1989]. Evidence was later found that the well-characterised endotoxin (LPS) receptor, CD14, a 55 kDa, glycosylphosphatidylinositol-anchored protein (GPI-protein) located on monocytes is involved in the binding of LTA epitopes. Stimulating monocytes in the presence monoclonal antibodies directed towards CD14 resulted in a complete block of TNF-α production [Cuzzola *et al.*, 2000; Gupta *et al.*, 1996].

More recently it has been found that CD14 is only the binding receptor for LPS and LTA and not responsible for signal transduction. As discussed earlier, CD14 is a GPI protein and thus, lacks a transmembrane portion [Means *et al.*, 1999b] and for this reason a signal transduction receptor was predicted. This transduction receptor was later identified and has been called the Toll-like Receptor (TLR) because it resembles that of a similar receptor found in the immune system of the insect *Drosphilia* that is designated as a Toll receptor [Medzhitov, Preston-Hurlburt & Janeway Jr, 1997]. Recently, the tenth member of the

TLR's has been identified [Chuang & Ulevitch, 2001]. Of the ten TLR members, TLR2 and TLR4 have been the focus of much research, due to their likely involvement in the signal transduction activated by LPS [Takeuchi *et al.*, 1999; Yang *et al.*, 1998]. It was originally suggested that TLR2 was induced by LPS [Yang *et al.*, 1998], but later it was found to activate TLR4. This was shown in TLR2 deficient mice which had been injected with a lethal dose of LPS and later died. Furthermore, the cytokine levels were shown to be of a similar level to that of the wild-type mice, thus indicating a TLR other than TLR2 was responsible for signal transduction [Takeuchi *et al.*, 1999]. To support this further, TLR4 transfected cells (i.e. only TLR4 present) were shown to be responsive to LPS. The contrasting results observed previously were concluded to be due to 'endotoxin protein' as after re-purification of the LPS, the TLR2 induction was eliminated [Hirschfeld *et al.*, 2000]. This was shown by monitoring the production of nuclear factor κB (NF-κB), a transcription factor, activated and translocated to the nucleus where it promotes the transcription of cytokine genes [Ghosh *et al.*, 1998]. The corresponding gene for NF-κB can be labelled with a luciferase reporter plasmid and detected by utilising the luciferin-luciferase reaction [Hirschfeld *et al.*, 2000]. Using mice deficient in TLR4 and administering injections of LTA, the mice were shown to be unresponsive and thus LTA was suggested to be an activator of TLR4 also [Takeuchi *et al.*, 1999]. Recently, LTA extracted from *S. aureus* and *B. subtilis* was shown to induce TLR2, again by monitoring an increase in the production of NF-κB in cell lines which were deficient of TLR4. The major difference in the methodology of this study was the use of highly purified LTA [Opitz *et al.*, 2001]. This highly purified LTA was prepared by a recently devised method where the phenol was substituted for a less aggressive solvent, *n*-butanol during the extraction protocol of LTA and this produced LTA of a more stimulatory nature [Morath, Geyer & Hartung, 2001]. Therefore, both LPS and LTA bind CD14 and it could be suggested that both utilise the same signal transduction pathway involving TLR4. However, as discussed, 'high purity' LTA was shown to stimulate TLR2 and thus, the induction observed previously with TLR4 [Takeuchi *et al.*, 1999], may

be due to inadequately purified preparation of LTA sample material (i.e. containing protein contamination), as was originally shown with LPS [Hirschfeld *et al.*, 2000].

Other IL's were also investigated to determine whether they too, were activated in the presence of LTA. One such study examined the secretion of cytokines within whole blood and then deduced the origins of each. From this study, it was found that IL-6 and IL-10 were also produced, along with the IL's previously mentioned. Attempts were made to deduce the origins of these IL's by purifying the monocytes, T-cells, B-cells and granulocytes via immunomagnetic separation. These were analysed for the mRNA transcripts via reverse transcriptase-polymerase chain reaction (RT-PCR) and specific primers for each mRNA. If the transcript being probed for was present then the specific primers could be utilised within the RT-PCR and thus form multiple copies of a larger mRNA species. These are then separated using agarose electrophoresis and detected by ethidium bromide staining [Solberg *et al.*, 1998; Wang *et al.*, 2000]. The induction of IL-8 was observed in cultured human monocytes in response to LTA from *B. subtilis*, but when exposed to the LTA of *Streptococcus sanguis* or *Streptococcus mutans*, no inducing effect was seen. Furthermore, the LTA from *S. sanguis* and *S. mutans* was shown to actually inhibit the IL-8 induction by *B. subtilis* LTA and also LPS in a similar manner to that observed with anti-CD14 monoclonal antibodies. These results suggested that LTA could act as an agonist and antagonist, both of which are CD14-dependant and is probably due to the structural heterogeneity among LTAs from different bacteria [Sugawara *et al.*, 1999].

To determine which part of the LTA component was responsible for the cytokine induction observed above, LTA was taken from *Enterococcus hirae* and separated further into two discrete fractions using ion-exchange chromatography (IEC). These two fractions were designated high-anionic fraction (major component, >90%) and low-anionic fraction (minor component, <5%), based on where each fraction eluted from the anion exchange column. When these fractions were tested for their ability to induce TNF-α, only the minor, low-anionic fraction was found to be active. Following this work, synthetic compounds that 'partially' mimicked specific moieties of LTA were produced and tested for their ability to

induce TNF-α. These studies demonstrate that none of the synthetic compounds were active. Importantly, these synthetic compounds lacked both glucosyl- and alanyl substitution, contained saturated fatty acids, as opposed to unsaturated fatty acids and only consisted of a four glycerophosphate residues within the PGP, as opposed to between 9 and 40 in natural LTA. Therefore there are two possibilities, either, the lack of cytokine-inducing activity is due to the differences between the synthetic and natural LTA moieties discussed above or the activity is due to previously unidentified components within native LTA (cited in Suda, 1995 #729].

The minor, low-anionic fraction discussed above was further fractionated, again using IEC This produced five separate fractions, of these the fourth fraction was shown to the most active in terms of TNF-α production. This fraction was considered to be a novel glycolipid within the LTA preparation and was named GL4. After hydrofluoric acid hydrolysis to remove the acylated lipid anchor, the structure of the hydrophilic portion of GL4 was determined to be a highly branched, mannose-rich polysaccharide bound to PGP via a phosphodiester bond. From this it was suggested that it is this structure within the LTA preparations that induces cytokines [Hashimoto *et al.*, 1999].

Recently, it has become apparent that the D-alanine substitutions within the PGP of LTA are of significant importance for the ability to stimulate monocytes in producing cytokines. LTA from *S. aureus* was extracted using a novel extraction and purification procedure. Using NMR and MS, over 50% of the D-alanine substitution was lost by conventional means of LTA preparation. LTA from each of the preparations was incubated with whole blood and the TNF-α levels were monitored using an enzyme-linked immunosorbent assay (ELISA). From these experiments it was found that the highly D-alanyl-substituted LTA preparations had a 50% increased stimulatory effect in comparison to the conventionally purified LTA. In the same study, D-alanine was deliberately hydrolysed from natural LTA and in doing this, it was found that loss of D-alanine paralleled a loss of TNF-α inducting ability, further supporting the role of D-alanine in monocyte activation [Morath *et al.*, 2001]. These

observations are contrary to the findings of Suda and Hashimoto above, who concluded that the LTA was not responsible for monocyte activation and that a specific glycolipid was responsible [Hashimoto *et al.*, 1999; Suda *et al.*, 1995]. This also discredits the lack of cytokine induction observed with the synthetic compounds used to mimic specific moieties within the LTA molecule and therefore suggests that the whole LTA component is required for activation. Although the more recent information provided by Morath strongly favours the first of the possibilities proposed by Suda, that certain structural features are required for effective cytokine activation, it does not disparage the results obtained from GL4 glycolipid prepared by further ion-exchange. Thus, the structure of the amphiphile responsible for cytokine induction is still under debate.

Specific groups of cytokines known to be associated with the monocyte activation pathway are the chemokines. These are protein mediators that have been shown to influence neutrophil (circulatory, short-lived polymorphonuclear leukocytes) function and activation of macrophages (tissue monocytes) [Matsushima & Oppenheim, 1989]. A specific chemokine known as macrophage inflammatory protein-1α (MIP-1α) has been shown to be involved in the inflammatory response to LTA, as expression of MIP-1α mRNA was found to be dose-dependent [Danforth *et al.*, 1995]. Also identified was the monocyte chemotactic protein-1 (MCP-1), which was found to be secreted by endothelial cells in response to *S. aureus*, treated with β-lactam antibiotics [Van Langevelde *et al.*, 1999].

1.7.1.2 Induction of Nitric Oxide Synthesis

Nitric oxide (NO) is an important mediator of the antimicrobial and tumourcidal activities of macrophages and its synthesis is stimulated by Gram-negative endotoxin [Stuehr & Marletta, 1985]. In addition to these observations, it was also shown that increases in the levels of NO occur when LTA was incubated with murine macrophages. Furthermore, increases in the level of expression of inducible NO synthase (iNO synthase), the enzyme that produces NO via the oxidation of terminal guanidino nitrogen atom from L-arginine,

were also observed in the presence of LTA. This was further supported by the observation made using a specific inhibitor of NO synthase, L-N-iminoethyl-ornithine, which is an analogue of arginine, as this increase in NO synthesis was inhibited [Cunha *et al.*, 1993]. This effect on NO levels may also be associated with a common pathway that leads to the vascular failure commonly occurring in septic shock caused by endotoxin and possibly LTA [Natanson *et al.*, 1989]. In addition to this, LTA was also found to induce the synthesis of tetrahydrobiopterin an essential co-factor of iNO synthase. This was demonstrated using cultured rat vascular smooth muscle cells and a selective inhibitor for GTP cyclohydrolase I, the enzyme responsible for tetrahydrobiopterin synthesis within vascular smooth muscle [Hattori *et al.*, 1998]. In studying the mechanism of Gram-positive shock it was shown that on its own LTA stimulated NO formation, but if the LTA was incubated in addition to peptidoglycan (PG), also from the cell wall of Gram-positive bacteria, a synergistic increase in NO formation was observed. In the same study it was found that incubation of the LTA from *S. aureus* with the PG from *B. subtilis* also displayed the synergistic effect, but the reverse of this, i.e. *B. subtilis* LTA with PG from either bacteria was found to have no effect on NO formation. From these observations it was concluded that it is the structure of the LTA that determines whether the bacteria can cause septic shock via NO formation and the PG acts merely to amplify the LTA response [Kengatharan *et al.*, 1998].

It has been suggested that the endothelial injury observed in sepsis may be due to antiplatelet activity, possessed by LTA and thus, a possible explanation for bleeding in Gram-positive septicaemia [Sheu *et al.*, 2000a]. This was demonstrated by incubating washed platelets with LTA purified from *S. aureus* and it was found that the inhibitory activity was directly proportional to both the amount of LTA used and also to the length of time of incubation [Sheu *et al.*, 2000a]. In support of this, similar observations were obtained from studies that used Wistar rats, although greater amounts of LTA were required to elicit a response. The response to LTA also included a significant increase in TNF-α and a biphasic fall in mean arterial blood pressure [Kengatharan, De Kimpe & Thiemermann, 1996]. Further investigation into the mechanism of the inhibition of antiplatelet activity by LTA

demonstrated that LTA dose-dependently increased the levels of cyclic adenosine monophosphate (cAMP) this could in turn inhibit the mobilisation of Ca^{2+} that are required for protein kinase C activity. Protein kinase C has been shown to be a key enzyme for the role of regulating platelet aggregation and therefore the increase in cAMP levels may ultimately be responsible for the antiplatelet effect observed following incubation of LTA with washed platelets [Sheu *et al.*, 2000b; Walter *et al.*, 1993].

1.7.2 Immunological Effects of Lipoglycans

1.7.2.1 Cytokine Activation

As initially stated the majority of the effects determined for lipoglycans come from the observations made with mycobacterial LAM. This is primarily due to the large amount of research that is focussed on the pathogenesis of *M. tuberculosis* and *M. leprae*.
LAM from mycobacteria possess cytokine inductive activity [Moreno *et al.*, 1989] and the first cytokine to be characterised was TNF-α. Furthermore, this was shown to be in similar amounts to that observed with LPS addition, using both human macrophages *in vitro* and with *in vivo* studies on murine macrophages [Moreno *et al.*, 1989]. Following this, a number of different interleukins were identified as LAM-inducible within monocytes, these included IL-1a, IL-1b, IL-6, IL-8, IL-10 and granulocyte-macrophage-colony stimulating factor [Barnes *et al.*, 1992]. The lipomannan and PIM were also assayed for their cytokine inducing abilities and found to also be active for the same cytokines, thus, it was concluded that the cytokine induction was derived from the phosphatidylinositol moiety of the lipoglycans [Barnes *et al.*, 1992]. At about the same time, differences in the LAM hydrophilic termini were shown to induce TNF-α to different amounts. The AraLAM was found to be a 100-fold more potent stimulator of TNF-α than that of ManLAM [Chatterjee *et al.*, 1992a; Chatterjee *et al.*, 1992b]. More recently, IL-12 has also been identified as another proinflammatory interleukin secreted by macrophages in response to LAM. IL-12 has been shown to facilitate the differentiation of naive T-cells into becoming T-helper type 1 cells

[Yoshida *et al.*, 1995]. Again, the terminal capping residues have been implicated in determining the capacity of this activation as it was shown that only the arabinose-terminated LAM was capable of inducing the IL-12 expression in significant amounts [Yoshida & Koide, 1997]. Furthermore, using dendritic cells (antigen-presenting cells found in lymphoidal tissue) primed with LPS to induce IL-12, ManLAM was shown to actually inhibit the production of IL-12 [Nigou *et al.*, 2001]. This difference in signalling may be due to the receptor that each binds [Means *et al.*, 1999b]. With AraLAM, induction of macrophages was shown to be mediated via the toll-like receptor, TLR2, thus, CD14-dependent [Means *et al.*, 1999b]. Conversely, ManLAM was shown to bind a mannose receptor (MR) within the dendritic cells. This was shown using antibodies directed to the MR, which blocked their activity and suppressed the inhibitory effect of ManLAM [Means *et al.*, 1999a; Nigou *et al.*, 2001].

1.7.2.2 T-cell Recognition and Responses

Previously, it has been shown that the LAM from both *M. leprae* and *M. tuberculosis* can suppress T-cell proliferation, via suppresser T-lymphocytes [Kaplan *et al.*, 1987]. More recently it was also shown that LAM was capable of inserting into the membranes of murine and human lymphomonocytic cells (monocytes derived from lymphoidal tissue) [Llangumaran *et al.*, 1995]. Interestingly, the LAM appeared to insert through a GPI-linked anchor (GLA), as only in GLA-rich regions was this observed. In the same study, it was also demonstrated that the mannan core was required for specificity of integration, as using PI from Soya bean did not inhibit insertion while PIM_6 did [Llangumaran *et al.*, 1995]. Furthermore, the acyl chains of the LAM were required for actual insertion to occur as deacylated LAM was not integrated. This suggested that the insertion was directly due to PI of LAM and is not receptor-based, thus may play an important role in influencing the functions of T-lymphocytes [Llangumaran *et al.*, 1995]. The ManLAM and MR have been implicated in the binding and phagocytosis of *M. tuberculosis*. Complement receptors were also shown to play an important role in this

phagocytic process [Schlesinger, 1993]. During early infection, macrophages and dendritic cells phagocytose the bacterial cells [Schlesinger *et al.*, 1990]. This is a host defence mechanism that normally allows destruction of the infecting bacteria. However, in the case of *M. tuberculosis*, once within the endosome, the bacteria can resist destruction and multiply freely, without being bound by complement components [Schlesinger, 1993; Schlesinger *et al.*, 1990].

Once internalised the LAM is also thought to be responsible for the transport of bacterial cells to the endosome prior to preparation for T-cell recognition [Prigozy *et al.*, 1997]. In addition, it was shown that LAM could bind CD1b, a major histocompatibility complex (MHC) class I-like molecule. This differs to MHC class I in that these molecules are non-polymorphic. The binding of LAM by CD1b is thought to be via the hydrophobic acyl chains and is then transported to the cell surface and presented for T-cell recognition [Ernst *et al.*, 1998; Sieling *et al.*, 1995]. Once at the cell surface CD1b/LAM complexes are then recognised by specific T-cell receptors, termed αβ, which have been shown to require both the mannoside and the GPI moiety. Once recognition has been established the T-cells secrete interferon-γ which is cytolytic. This is a novel method of antigen presentation and also the first non-peptide antigen presentation characterised [Sieling *et al.*, 1995].

As briefly discussed above, *M. tuberculosis* has the ability to survive and multiply freely within the endosomes of phagocytic cells and thus, avoid being digested and displayed for T-cell recognition. How the bacterium avoids this series of events has been extensively researched. It is currently thought that the bacterium prevents the maturation of the early endosome to a late endosome by inhibiting the acidification process. This would therefore inhibit the binding of a lysosome and thus, the destruction of the bacteria [Clemens & Horwitz, 1995]. From these observations LAM can be seen to play a significant role in the phagocytosis and internal processing pathways within phagocytes, but whether it is LAM that mediates this acidification inhibition is still unclear. A graphical representation of the suggested pathway for T-cell presentation is detailed in Figure 1.6.

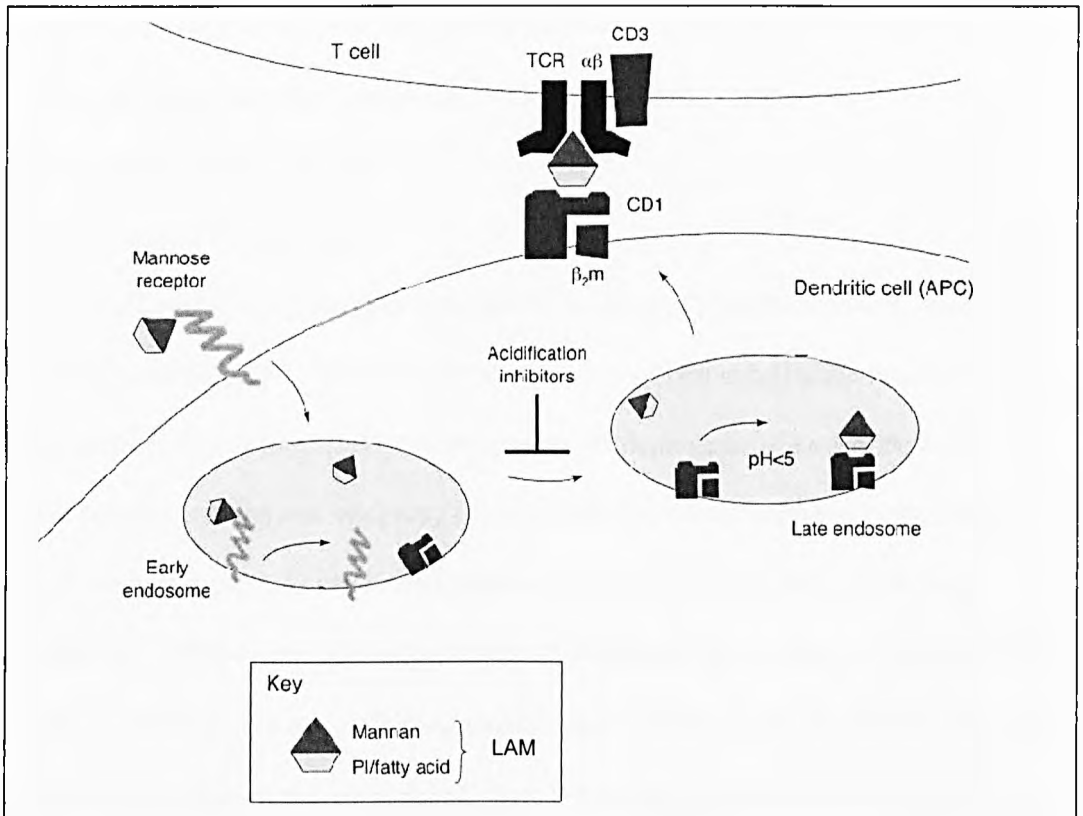


Figure 1.6 Inhibition of Presentation and Survival of LAM within Phagocytes

The proposed pathway for the presentation of LAM by CD1 molecules and the suggested LAM-induced inhibition of the acidification of the early endosome. This inhibition would prevent presentation and allow survival within the phagocyte [Clemens & Horwitz 1995] . Adapted from the review by [Prigozy & Kronenberg 1998] . TCR, αβ containing T-cell receptor ; β_2m, β_2-microglobulin, a polypeptide which constitutes part of some membrane proteins including the Class 1 MHC molecules and is non-covalently bound to CD1b [Ernst *et al* 1998].

LAM has also been shown to induce a chemotactic response in human, peripheral blood, T-lymphocytes. This was suggested from the observations of culture supernatants from human alveolar macrophages infected *in vitro* with *M. tuberculosis*. The chemotaxis of these lymphocytes was later inhibited when antibodies directed to LAM were added [Berman *et al.*, 1996]

LAM has been shown to increase the production of nitric oxide (NO) within murine macrophages *in vitro*, in a dose-dependent manner. γ-interferon was found to synergistically increase this NO production further, but it has also been shown that LAM inhibits the

production of γ-interferon. Thus, the precise pathway and reasons for this induction are still poorly understood [Schuller-Levis *et al.*, 1994; Sibley *et al.*, 1988].

1.7.3 Metabolic Effects of LMAP

1.7.3.1 Effects of Lipoglycan

ManLAM specifically, has been shown to induce the expression of a tyrosine phosphatase called SHP-1. This enzyme was shown to down regulate the response of the human monocytic cell line, THP-1 by promoting the dephosphorylation of tyrosine on several cellular proteins and inhibiting their activity. This may therefore, be an additional method used by bacteria to evade host responses [Knutson *et al.*, 1998]. Other reported activities for LAM have also been suggested, these include the blocking of many actions of interferon-γ, such as, the tumour killing ability once LAM has been internalised into the cytoplasmic vacuoles of the macrophage. Again, this may also be a method employed to evade the host defence [Sibley, Adams & Krahenbuhl, 1990; Sibley *et al.*, 1988]. Inhibition of protein kinase C (PKC), has also been shown which in turn can inhibit the activation of macrophages [Chan *et al.*, 1991]. This was supported by the observations made with peripheral blood mononuclear cells, *in vitro*, where LAM was shown to inhibit PKC in a dose-dependent manner and in turn, inhibit the phosphorylation of several endogenous proteins thought to be important for maintaining cellular function. Furthermore, the generation of superoxide anions was also inhibited and this too is thought to be PKC dependent. Inhibition of PKC and thus, inhibition of superoxide anion synthesis would therefore assist in evasion of intracellular killing [Ghosh *et al.*, 1998].

1.7.3.2 Effects of Lipoteichoic Acid

Many of the effects of LTA have been investigated primarily for comparison to the effects of LPS. With the effects of LTA on lipid metabolism this is also the case.
LPS has been shown to induce rapid *in vivo* change in the metabolism of lipids. This was characterised as an increase in serum triacylglycerols (TAG) following injection of LPS,

leading to hypertriglyceridemia [Feingold *et al.*, 1992]. It was also noted in this study that a low LPS dose (10ng/100g body weight) gave rise to hepatic *de novo* fatty acid biosynthesis and adipose tissue lipolysis which provided the substrates required for hepatic TAG synthesis and secretion. If larger LPS doses (50μg/100g body weight) were administered, there was a decrease in the activity of lipoprotein lipase (LPL) which is responsible for the lipid clearance. This was determined by the observations that serum TAG was increased without increases in adipose tissue lipolysis, hepatic *de novo* fatty acid biosynthesis was not increased and no increased secretion of TAG was evident [Feingold *et al.*, 1992]. Furthermore, using antibodies directed towards TNF-α and IL-1, it was shown that in the presence of these cytokine inhibitors hypertriglyceridemia still occurred [Feingold *et al.*, 1992] and therefore removing the possibility of these immune effectors causing the change in LPL activity. However, this does not rule out the potential for other secondary components influencing this metabolic flux.

Similar metabolic changes in rats have also been shown for LTA, the induction of hypertriglyceridemia appeared to be dose-dependent from 0.1-300μg/200g of body weight. This increase in serum TAG was shown to originate from hepatic *de novo* fatty acid biosynthesis and adipose tissue lipolysis followed by hepatic TAG secretion, but no reduction in the activity of LPL was observed. It was also suggested that α-adrenergic receptors might mediate these changes. This hypothesis was tested using an inhibitor to α-adrenergic receptors and it was shown that suppression of the LTA-induced hypertriglyceridemia did occur. This LTA-induced hypertriglyceridemia suppression was not observed when β-adrenergic inhibitors were used, thus, as with LPS, LTA-induced hypertriglyceridemia is mediated by endogenous catecholamines via α-adrenergic receptors [Nonogaki *et al.*, 1995]. As with LPS-induced hypertriglyceridemia the effect of LTA-induced hypertriglyceridemia was shown *in vivo* and therefore it is not possible to establish whether the effect is due to the LTA *per se*, or if it is secondary to other effectors. It is likely that the latter possibility is the more probable since TNF-α, IL-1 and IL-6 can all mediate

changes in lipid metabolism in rats *in vivo* [Grunfeld & Feingold, 1991; Nonogaki *et al.*, 1994]. However, to date, there are no published reports detailing an *in vitro* investigation, therefore the effect of LTA and other LMAP structures on lipid metabolism remain unclear. It has therefore been suggested that changes in lipid metabolism are part of an acute host defence against infection [Grunfeld & Feingold, 1992]. This is supported by evidence that suggests that lipoproteins can inhibit the LPS-induction of monocytes [Feingold *et al.*, 1995; Flegel *et al.*, 1989]. A more recent observation also demonstrated that lipoproteins could inhibit the activation of TNF-α secretion by macrophages in response to LTA, although other factors were also required. One additional factor required was identified as the lipopolysaccharide-binding protein (LBP) which is also used, as its name suggests, to bind LPS. This LBP is therefore thought to facilitate the binding of both LPS and LTA to lipoproteins [Grunfeld *et al.*, 1999; Tobias, Soldau & Ulevitch, 1989]. Further support for the role of LBP was acquired using an antibody directed to LBP, as when this was incubated with LBP prior to LTA administration it was shown to block the ability of LBP and lipoprotein to inhibit the LTA induction of macrophages [Grunfeld *et al.*, 1999].

1.8 Extraction and Purification of LMAP

1.8.1 Current Methodology

To enable the study of the structure and functionality of LMAP components, efficient extraction and purification protocols have been devised. Coley, Duckworth and Baddiley first reported this in 1975, in which they utilised hot 80%(w/v) aqueous phenol extraction to remove the LTA from the other cellular lipids from *S. aureus* and other Gram-positive bacteria [Coley, Duckworth & Baddiley, 1975]. The aqueous phase extract was then applied to a 6B sepharose, gel-filtration column and the elution of LTA was detected by monitoring the fractions for phosphorus. This produced a preparation that was relatively free of nucleic acid and protein, but did contain large amounts of teichoic acid [Coley *et al.*,

1975]. In 1983, Fischer considerably improved upon the procedure for purification of LTA [Fischer, Koch & Haas, 1983]. The bacteria were again extracted using hot 80%(w/v) aqueous phenol, but the bacterial cells were mechanically disrupted prior to extraction, which improved the overall yield of component. Furthermore, the pH of the extraction buffer was reduced to pH 4.7 to prevent the loss of D-alanine substitution. The crude extract was purified using hydrophobic interaction chromatography (HIC) with an octyl sepharose medium. This chromatographic technique utilises the binding interaction of the acylated fatty acid residues attached to both lipoglycans and LTA. The nucleic acid, protein and neutral polysaccharide (including teichoic acid) is not retained by the column and elutes immediately. Elution of the retained LMAP is achieved by applying a propan-1-ol gradient, within the low pH buffer system of between 15 and 60%(v/v) [Fischer *et al.*, 1983]. Following the success of this method, it was also applied to the lipoglycan of *Micrococcus luteus* and the lipopolysaccharide of *Salmonella typhimurium* and found to yield equally pure LMAP in all cases [Fischer, 1991]. In addition to this, the method also allowed the separation and consequent identification of LTA containing mono, di, tri and tetra-acylated molecular species [Fischer, 1993; Fischer, 1996]. Other methods have also been utilised for the purification of LTA these include the extraction from *Streptococcus mutans* which was achieved using the anionic detergent Triton X-114, followed by anion exchange chromatography [Sutcliffe & Hogg, 1993]. Triton X-114 has also been used to isolate LAM from *M. bovis*, prior to purification using gel filtration [Severn *et al.*, 1997]. LAM has more recently been extracted using 80%(w/v) aqueous phenol [Hamasur, Källenius & Svenson, 1999]. A solvent extraction system was employed, involving chloroform and methanol, allowing more specific extraction of LAM. Finally, LAM was purified on a sephacryl-100, gel exclusion column and found to be >98% pure. Purity was determined with the use of a novel ELISA assay [Hamasur *et al.*, 1999].

Up until recently, the method of Fischer [Fischer, 1991] appears to be the more commonly employed procedure for both the purification of LTA and lipoglycan. However, this method now appears to have been superseded by a less destructive method that exchanges the hot

phenol extraction step for *n*-butanol at room temperature [Morath *et al.*, 2001]. NMR spectroscopy was applied to compare the structures purified using both methods. From this analysis it was found that while the use of low pH buffers reduced the loss of D-alanine esterifications, there was still a marked reduction in the D-alanine lost in the extraction with phenol, when compared to that of *n*-butanol method [Morath *et al.*, 2001]. Quantitative analysis of the amount of D-alanine present in each preparation demonstrated that in using the phenol extraction method only 30% of the LTA molecule was D-alanine-esterified. This was compared to 70% D-alanine-esterification when the *n*-butanol extraction method was employed [Morath *et al.*, 2001]. Using this information it appears that the *n*-butanol extraction protocol is a better method for the initial extraction of LTA, but currently there is no data validating this method for the extraction of lipoglycans.

1.8.2 Application to Propionibacterium acnes

Propionibacterium acnes is the bacterium implicated in the pathogenesis of the inflammatory condition, acne vulgaris [Cunliffe, 1998]. This common skin condition is described as a multifactorial disease of the pilosebaceous follicles of the face and upper trunk [Cunliffe & Eady, 1992]. Three etiological factors have been identified in the development of acne lesions. These are the presence of the *P. acnes* bacteria on the skin surface, an increased sebum production (lipid secretion from the sebaceous follicle) and hypercornification of the pilosebaceous follicles (blocking of the follicle by proliferation of the ductal epidermis) [Cunliffe & Eady, 1992]. It is well documented that sebum secretion is considerably increased in acne patients. Furthermore, reduced amounts of linoleic acid have also been observed in the sebum of acne patients and therefore representing a compositional change in the sebum in comparison to healthy individuals [Morello, Downing & Strauss, 1976]. This would suggest a shift in the lipid metabolism in those patients suffering from acne. However, the mechanisms underlying this shift are not understood. Additionally, it has been proposed that the hypercornification of the pilosebaceous follicle is a consequence of

the changes in sebum composition and thus further accentuating the importance of these changes in lipid metabolism in the presence of *P. acnes* [Downing *et al.*, 1986].

In addition to the pathogenic roles in acne, *P. acnes* has also been observed in the manifestation of sepsis [Martínez, Collazos & Mayo, 1998] together with significant study as an immunomodulator in various animal models of cancer and inflammatory disease, much of which has been extensively reviewed by Ingham [Ingham, 1999]. However, the nature of the immunostimulatory components of *P. acnes* remains relatively poorly defined.

Propionibacteria have been shown to possess a G-C ratio of between 53-67% and are actinomycete bacteria [Cummins & Johnson, 1986]. Moreover, as discussed previously in Section 1.3.4, *P. freudenreichii* has been shown to produce an inositol-containing lipomannan [Sutcliffe & Shaw, 1989]. With this in mind, *P. acnes* are expected to contain a lipoglycan. To date, the LMAP of *P. acnes* has not been purified. However, attempts to characterise the LMAP structure have been made [Kokeguchi *et al.*, 1985]. The extraction of the amphiphile utilised the hot aqueous phenol procedure discussed above followed by gel filtration. The amphiphile fractions were assayed for carbohydrate and found to contain large amounts of ribose, mannose and glucose [Kokeguchi *et al.*, 1985]. The ribose probably originated from nucleic acid impurity which has been shown to co-extract with LMAP in previous extractions [Fischer, 1991]. In addition to this, focussing on the resulting gel filtration profile, poor separation was observed between the phenol-extracted molecular species [Kokeguchi *et al.*, 1985]. These preparations were found to possess a coagulation effect on sheep red blood cells incubated with anti-*P. acnes* serum [Kokeguchi *et al.*, 1985].

Other crude preparations have also been shown to possess immune-stimulating properties. Again, using various fractions obtained from hot aqueous phenol extractions of *P. acnes* cells, anti-tumour activity (now known as TNF-activity) was observed [Cantrell & Wheat, 1979]. This activity was associated with a carbohydrate-containing component present within the aqueous phase of the phenol-extracted material [Cantrell & Wheat, 1979]. These findings were supported by observations made within cell wall fractions, where activation of the alternative complement cascade (ACC) was observed [Webster, Nilsson & McArthur,

1981]. This ACC activity was resistant to lipid extraction, protease, nuclease, formamide and lysozyme treatment and therefore it was concluded that the activity was based on the, carbohydrate containing components of the cell wall [Webster *et al.*, 1981]. Furthermore, the treated cell wall fraction was applied to a gel filtration column and the carbohydrate was determined within the ACC-activating fractions and found to contain glucose, mannose and galactose [Webster & McArthur, 1982].

In using these crude extractions for the characterisation of the structure and the physiological effects has left many questions unanswered and thus, the molecular pathogenecity of the bacterium is poorly understood. Consequently, there is a need to purify and characterise the LMAP of *P. acnes*, as this may be a virulence or antigenic determinant of this organism.

1.9 Aims and Objectives of the Present Study

To address these structural details and the pathogenic roles of the LMAP of *P. acnes*, this study initially focused on the current methods for extraction and purification of LMAPs, essentially using the methods of Fischer [Fischer, 1991; Fischer *et al.*, 1983]. This methodology was applied to the purification of the well-characterised LTA of *S. aureus*. Following the success in the validation of these procedures, a similar protocol will be applied to *P. acnes* to allow for effective purification of the LMAP. Once purification of the LMAP components has been accomplished, the chemical moieties will then be structurally characterised to allow comparisons to be made to previously characterised LMAP structures. Finally, as it has been shown that a change in the composition of lipids within pilosebaceous follicles is an important etiological factor in the pathogenesis of acne [Downing *et al.*, 1986; Morello *et al.*, 1976], the effects of *P. acnes* LMAP components on the *in vitro* metabolism of lipids will be investigated to determine whether the LMAP structures can influence the cellular metabolism of lipids and therefore evaluate their importance in the pathogenecity of *P. acnes* in disease.

CHAPTER 2

Materials and General Methods

2.1 Materials

2.1.1 Reagents

Microbiological reagents including brain heart infusion (BHI) broth, nutrient broth and agar, bacteriological agar and phosphate buffered saline (PBS) were purchased from Oxoid, UK. Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM), penicillin/streptomycin, foetal calf serum (FCS), non-essential amino acids (NEAA) were purchased from Labtech International, UK. L-glutamine was purchased from Lancaster, UK. All other reagents used were of analytical grade and purchased from Sigma, UK. Purified water used was deionised (18.2mΩ cm) and filtered though a 0.2μm membrane via a Milli-Q system, Millipore UK. Gases used throughout were purchased from BOC, UK.

2.1.2 Consumables

Polystyrene and polypropylene universals and centrifuge tubes were obtained from Bibby Sterilin, UK. Cryovials were purchased from Nalgene, UK. All cell culture flasks, dishes and cellulose acetate syringe filters were obtained from Iwaki, Japan. Hepa-vents and all other filtration devices were purchased from Whatman, UK. All other consumables were purchased from Fisher Scientific, UK.

2.1.3 Facilities, Equipment and Instruments

Due to the nature of this study, there was a requirement for both bacterial and mammalian cell culture. To limit the possibility of infection of the mammalian cell cultures, separate laboratories were used for each type of culture. Furthermore, bacterial culture procedures were not carried out prior to mammalian cell culture procedures.

All microtitre plate spectrometric analyses were performed using a Dynex Revelation plate reader (Dynex, UK). Larger sample volumes were determined using WPA Lightwave spectrophotometer, (WPA,UK). For all tissue culture procedures a class II Laminar flow hood was used (Microflow, UK) and incubations performed in a Forma 310 direct-heat CO_2

incubator (Forma Scientific Inc, USA). For anaerobic growth of microorganisms a variable atmosphere incubator (VAIN) was used (Don Whitley Scientific, UK). All low speed centrifugation was performed on a PK121R (ALC, USA) with the exception of mammalian cell centrifugation procedures, which were performed on Sigma 2-4 bench centrifuge (Sigma Laboratories, Germany). All weights <100g were determined using a AC100 analytical balance (Mettler Toledo, UK), while weights >100g were determined on 1216MP top-pan balance (Sartorius, USA). Homogeneous aerobic and anaerobic bacterial cultures were maintained using orbital shakers (Stuart Scientific, UK) or for larger cultures a G25 controlled environment shaking incubator (New Brunswick Scientific, USA) was used. To support aerobic growth in large cultures a Hi-Tech 7500 air pump (Maika, UK) was used.

2.1.4 Bacterial and Mammalian Cells

Propionibacterium acnes, DSM 1897 (NCTC 0737) was purchased from the German culture collection (DSMZ, Germany) as a lyophilised culture. *Staphylococcus aureus*, NCTC 8532, was purchased from the Public Health Laboratory (PHLS, UK) also as a lyophilised culture. All mammalian cell lines were obtained from European cell culture collection (ECCAC, UK)

2.2 Analytical Methods

2.2.1 Determination of Protein

Analysis of the amount of protein present was required to determine the percentage protein impurity when purifying LMAP from various sources. Protein has been shown to be a significant extraction contaminant [Fischer, Koch & Haas, 1983] and therefore required monitoring throughout the purification procedures to ensure negligible amounts were present in final preparations. Initially, the method of Bradford [Bradford, 1976] was utilised, but in the presence of certain LMAP components an interference was observed, in which the

method was found to over-estimate the amount of protein present (discussed in Section 3.4.4). On this evidence the bicinchoninic acid (BCA) method [Smith *et al.*, 1985] was used and was shown to report the amount of protein present more accurately in this particular chemical environment (Section 3.4.4) and therefore the BCA method will be used for all determinations involving LMAP.

In this method the protein binds copper under alkaline conditions, forming a copper-protein complex. The BCA reduces this complex and this results in an intense purple colour with an absorbance maximum of 562nm. The method was adapted to increase the sensitivity of the assay to enable determinations in the range of 0.5-10μg protein. This increase in sensitivity was achieved by increasing the concentrations of the initial stock reagents by 4-fold [Smith *et al.*, 1985] and determining the absorbance with a microtitre plate-reader.

Reagent A:

8%(w/v) anhydrous sodium carbonate, 1.6%(w/v) sodium hydroxide, 1.5%(w/v) sodium tartrate, made to pH 11.25 with solid sodium bicarbonate. This was stored indefinitely at room temperature.

Reagent B:

4%(w/v) BCA ($C_{20}H_{10}N_2O_4Na$) prepared in water. This was stored indefinitely at room temperature.

Reagent C:

This reagent was prepared immediately prior to use and required the addition of 4ml of 4%(w/v) copper sulphate ($CuSO_4 \cdot 5H_2O$) to 100ml of reagent B (1:25 ratio).

Assay Procedure:

Prior to each assay, reagent A was added to reagent C in a 1:1 ratio to produce reagent D. To 100μl of sample and all subsequent dilutions of the sample (to ensure the resulting

absorbance was within the range of the prepared standards), was added 100µl of reagent D. The samples were incubated for 60 minutes at 60°C, cooled and the absorbance was determined at 560nm. All samples and standards were determined in triplicate.

This method was applied to standard protein solutions ranging from 0.5-10.0µg ml^{-1} and prepared using the same procedure as the samples. The absorbance of these was used to obtain a standard curve of r^2>0.99. A typical standard curve is shown in Figure 2.1.

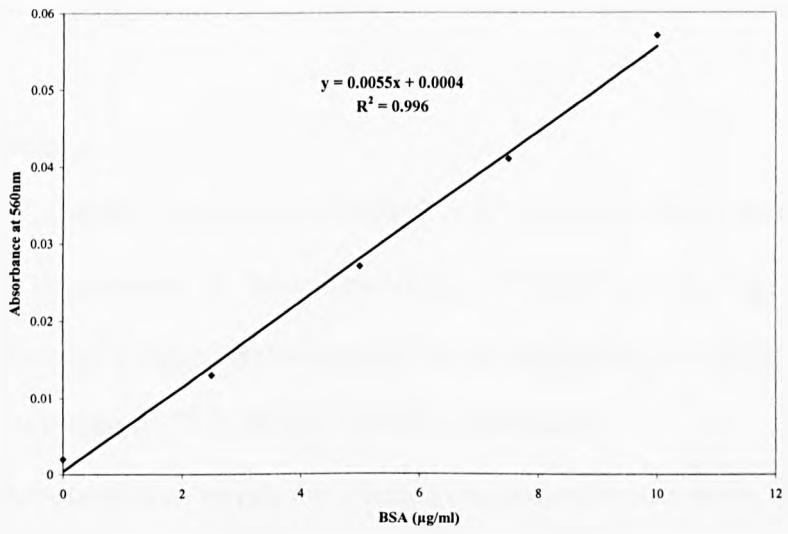


Figure 2.1 Protein Standard Curve using the BCA Method

A typical standard curve demonstrating the absorbance at 560nm against standard concentrations of BSA in µg ml^{-1}.

2.2.2 Determination of DNA (nucleic acid)

Analysis of the amount of DNA present was required to determine the percentage nucleic acid impurity when purifying LMAP. DNA has been reported to be the major contaminant in the initial stages of LMAP extraction [Fischer *et al.*, 1983] and was therefore monitored throughout. It was assumed that due to the instability of RNA the nucleic acid material present will be in the form of DNA [Sambrook, Fritsch & Maniatis, 1989]. Using this assumption, quantitation of the amount of DNA within the LMAP purification was considered to represent the total amount of nucleic acid as impurity. The method used was based on the diphenylamine assay [Burton, 1956], but scaled down to allow for the photometric determination using a microtitre plate reader. The method relies on the binding

of diphenylamine to deoxyribose and was therefore specific for DNA, producing a blue colour that could be determined photometrically at 595nm.

Diphenylamine Reagent:

1.5%(v/v) sulphuric acid was prepared in glacial acetic acid. Using this, a 1.5%(w/v) diphenylamine solution was prepared. This reagent was stable for one month at room temperature.

Assay Procedure:

A 16mg ml^{-1} aqueous solution of acetaldehyde was prepared by mass, due to the high volatility of the acetaldehyde. Prior to each assay, 20.0ml of working reagent was produced by taking 100µl of 16mg ml^{-1} aqueous acetaldehyde and making to volume with diphenylamine reagent. This solution was used immediately.

50µl of either standard or sample was pipetted into microtitre plate wells, in triplicate. To each of these was added 10µl of 25%(v/v) perchloric acid to give a final concentration of approximately 0.5M. 120µl of working reagent was added to each well and the microtitre plate was incubated at 30°C for 18 hours. After such time the microtitre plate was read at 595nm.

Standards were produced using a 0.5mg ml^{-1} aqueous stock standard solution. This was prepared using DNA purified from salmon testes. Standards ranged between 10-200µg ml^{-1}. The absorbance of these was used to obtain a standard curve of $r^2>0.99$. A typical standard curve is shown in Figure 2.2.

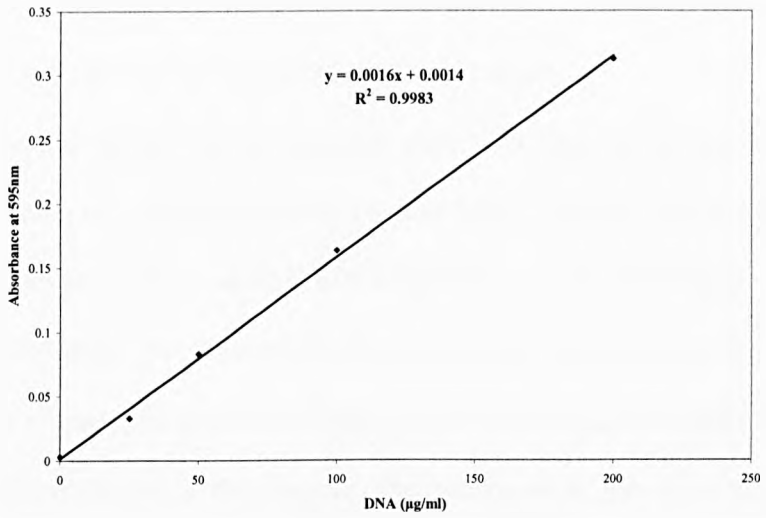


Figure 2.2 DNA Standard Curve using the Diphenylamine Method

A typical standard curve demonstrating the absorbance at 595nm against standard concentrations of DNA in µg ml^{-1}.

2.2.3 Determination of Total Carbohydrate

In order to follow the extraction and purification of lipoglycan the amount of total carbohydrate present was determined. Carbohydrate was used as a marker for the presence of lipoglycan, as the majority of the molecule was a polysaccharide (see Section 1.3 for structural details). This was followed using a microtitre plate-adapted method of the phenol/sulphuric acid method [Fox & Robyt, 1991]. The method relies on a condensation reaction between phenol and pentose/hexose sugars, and was initiated by the dehydration of the sugars, by sulphuric acid at 80°C, to furfural and hydroxymethyl furfural respectively. The resulting furfural compounds subsequently react with the phenol to form furfural derivatives, which were orange/brown in colour. The intensity of this colour was directly proportional to the amount of carbohydrate present and could be determined photometrically at 490nm.

This method was modified to reduce the error associated with the time taken to add the sulphuric acid to the sample wells. In the original method, the microtitre plate was cooled to 4°C, prior to the addition of sulphuric acid. In doing this, the reaction of the sulphuric acid

was only slowed, and as the reaction was time-dependent, there was a significant amount of error observed between the first sample well and the last.

It was found that by freezing the microtitre plate, containing the sample and phenol, to -30°C and then adding the sulphuric acid; the reaction of the sulphuric acid could be completely inhibited. This allowed the addition of the sulphuric acid to each sample well without the reaction proceeding. On addition of the sulphuric acid, the sample melts due to the exothermic reaction between the sulphuric acid with the aqueous sample and therefore allows sufficient mixing of the reagents. The reaction could then be initiated for all of the samples, at the same time, by increasing the temperature to 80°C.

Assay Procedure:

A standard concentration series with a range between 10-200μg ml^{-1} was prepared using mannose in DI water. Mannose was used as this reflected the majority of carbohydrate expected within the samples (see Section 1.3). 25μl of either sample or standard was added, in triplicate, to a microtitre plate. To each well was added 25μl of 5%(w/v) aqueous phenol and the plate was gently shaken for 30 seconds and frozen to -30°C. Following freezing, 125μl of concentrated H_2SO_4 at 4°C was quickly added to each well and the plate was gently shaken for 30 seconds. The plate was then incubated in a waterbath at 80°C for 30 minutes. After such time the plate was cooled to room temperature and sonicated in a sonication bath to remove any bubbles within the microtitre plate wells. The absorbance was determined at 490nm.

Using the absorbance recorded for each of the standard concentrations a standard curve of $r^2>0.99$ was generated as shown in Figure 2.3.

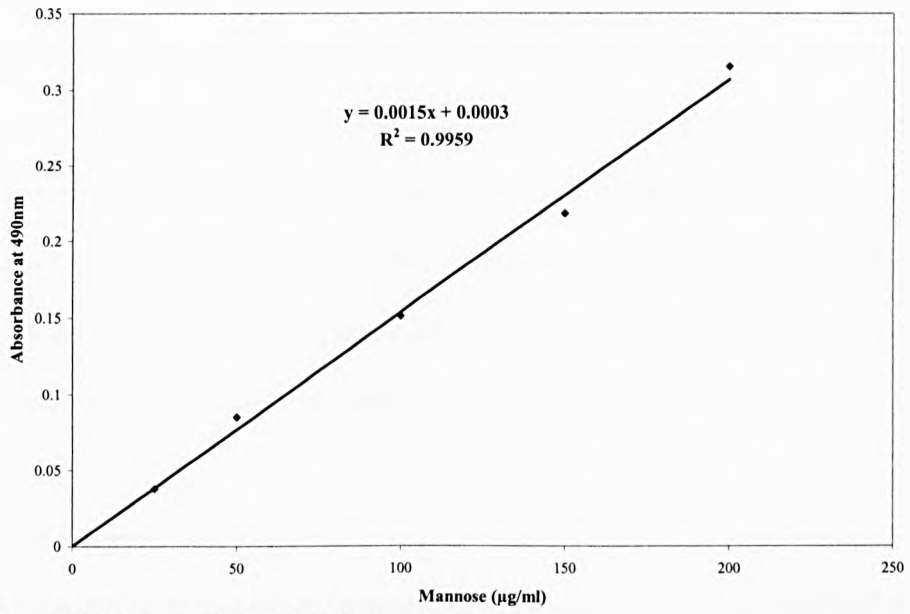


Figure 2.3 Carbohydrate Standard Curve

A typical standard curve demonstrating the absorbance at 490nm against standard concentrations of mannose in µg ml^{-1}.

2.2.4 Estimation of Inorganic Phosphorus

In order to follow the extraction and purification process of LTA the amount of inorganic phosphorus present was determined. Phosphorus was used as a convenient marker for the presence of LTA, as many bacteria possess polyglycerophosphate hydrophilic moieties within their LTA and were therefore abundant in phosphorus (see Section 1.2.2 for structural review). Once the nucleic acid and protein had been removed the identification of a large amount of phosphorus indicated the presence of LTA. To permit the quantitation of phosphorus a modified version of the method of Chen *et al.* [Chen, Toribara & Warner, 1956] was employed. All glassware used was washed overnight with 6M nitric acid and then flushed with DI water at least three times to remove traces of phosphorus. The samples initially required digestion to free the phosphorus from the LMAP polymers. This was achieved by heating the samples with magnesium nitrate to induce oxidation. The original method suggested the use of a heating block for this oxidation procedure. However, using these conditions it was found that the sample-to-sample reproducibility was poor, as total

oxidation was often not achieved. To overcome this, oxidation was accomplished over a Bunsen flame, initially using moderate heating to carefully evaporate the ethanol and continuous and vigorous heating followed this until no further reaction occurred. This digestion step allowed the cleavage of covalently bound elements within the polymers producing a phosphate ion. Following this, ammonium molybdate was added which bound the phosphate ions. The resulting phosphomolybdic acid was then reduced to give molybdenum blue and this was measured photometrically at 820nm [Chen *et al.*, 1956].

Molybdate Reagent:

2%(w/v) ascorbic acid was freshly prepared in 0.6M sulphuric acid containing 0.5%(w/v) ammonium molybdate. This was stable for one hour after preparation.

Assay Procedure:

To 100µl aqueous samples, 200µl of 10%(w/v) magnesium nitrate ($MgNO_3$) dissolved in absolute alcohol was added. This was heated in a Pyrex tube over a Bunsen flame (as described above) until the brown nitrogen dioxide gas ceased to evolve. To the tube was added 300µl of 0.05M HCl and the heating procedure was repeated. After cooling, 3.9ml of DI water was added and the tube was sonicated in a sonication bath for 10 minutes. 4.0ml of molybdate reagent was added and the tube was incubated at 37°C for 90 minutes. After this time the absorbance was measured at 820nm. Standards were prepared between 10-100µg ml^{-1} using potassium orthophosphate and also followed the method above. The absorbance of these was used to obtain a standard curve. A typical standard curve is shown in Figure 2.4.

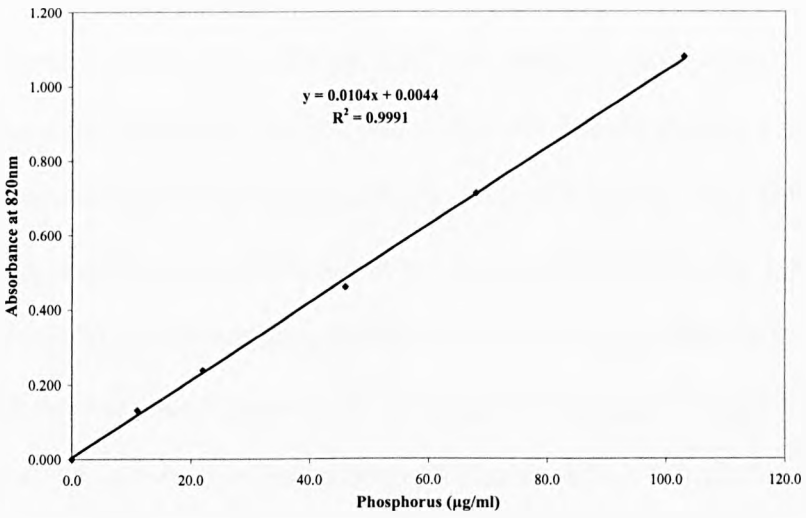


Figure 2.4 Phosphorus Standard Curve

A typical standard curve demonstrating the absorbance at 820nm against standard concentrations of phosphorus in µg ml^{-1}.

2.3 Bacterial Cell Maintenance

Staphylococcus aureus and *Propionibacterium acnes* were maintained on agar plates for between 5 and 10 days. The conditions were similar to those described in Bergey's Manual [Cummins & Johnson, 1981; Kloos & Schleifer, 1986]. Growing bacterial cultures were sub-cultured a maximum of 20 times, after which a new culture was resuscitated from cryo-storage. In order to provide a constant source of bacteria and to preserve the strain characteristics, each bacterium was cryo-preserved in quantities suitable to last the duration of the study.

2.3.1 Reconstitution from Lyophilised Cells

Resuscitation of lyophilised cells followed the instructions obtained on receipt of the microorganism from the Public Health Laboratory (*Staphylococcus aureus*) or the German culture collection (*Propionibacterium acnes*).

2.3.1.1 *Staphylococcus aureus*

Staphylococcus aureus, NCTC 8532 was aseptically resuspended in 1ml of nutrient broth prepared by dissolving 13g in 1 litre of DI water and autoclaving at 121°C/15psi for 20 minutes. Nutrient agar was prepared with 28g of nutrient agar in 1 litre of DI water and autoclaving as with nutrient broth. *S. aureus* was grown aerobically, on nutrient agar at 37°C. After 36 hours, the resulting bacterial colonies were examined for their colony and cell morphology to ensure bacterial cell authenticity. These were compared to the description of *S. aureus* provided in Bergey's Manual [Kloos & Schleifer, 1986], where the colony morphology was described as circular in shape, approximately 6mm in diameter after 48-72 hours and golden in colour [Kloos & Schleifer, 1986]. Furthermore, the cells were also examined microscopically and were expected to be Gram-positive cells, as determined using the method of Gram [Collins & Lyne, 1976], spherical in shape and between 0.5 and 1.0μm in diameter. Only if all of these criteria were met was the culture used.

2.3.1.2 *Propionibacterium acnes*

Propionibacterium acnes, DSM 1897 (NCTC 0737) was aseptically resuspended in 1ml of brain heart infusion (BHI) broth, prepared by dissolving 37g in 1 litre of DI water and autoclaving at 121°C/15psi for 20 minutes. BHI agar plates were also prepared as with the BHI broth, but with 1%(w/v) bacteriological agar. A BHI agar plate was streaked with the reconstituted suspension and this was incubated in a variable atmosphere incubator (VAIN) at 37°C, 5% CO_2 and 95%N_2. After 72 hours the resulting bacterial colonies were examined for their colony and cell morphology to ensure bacterial cell authenticity. These were compared to the description of *P. acnes* provided in Bergey's Manual [Cummins & Johnson, 1986], where the colony morphology was described as circular in shape, approximately 0.4mm in diameter after 48-72 hours of growth and white to tan in colour [Cummins & Johnson, 1986]. Furthermore, the cells were also examined microscopically and were expected to be Gram-positive, as determined using the method of Gram [Collins &

Lyne, 1976], pleomorphic, rod-like in shape and of approximately 1.5µm in length. Only if all of these criteria were met was the culture used.

2.3.2 Cryo-preservation of Bacterial Cells

2.3.2.1 *Staphylococcus aureus*

A colony of *S. aureus*, aerobically grown on nutrient agar for 48 hours at 37°C was aseptically removed from the surface and used to inoculate a 10ml nutrient broth. This was incubated aerobically at 37°C in an orbital shaker at 200rpm. After 48 hours of growth, 1ml of homogeneous culture was removed and added to 9ml of sterile nutrient broth containing 10%(v/v) glycerol. The resulting 10ml culture was gently shaken and dispensed into 10 cryovials as 1ml aliquots. These were placed into a polystyrene tube holder and frozen slowly in a -80°C freezer, where they were stored indefinitely [Collins & Lyne, 1976].

2.3.2.2 *Propionibacterium acnes*

A colony of *P. acnes* grown on BHI agar for 5-days at 37°C, 5% CO_2 and 95%N_2 was aseptically removed from the surface and used to inoculate a 10ml BHI broth. This culture was incubated at 37°C, 5% CO_2, 95%N_2 and shaken at 200rpm on an orbital shaker. After 72 hours of growth, 1ml of homogeneous culture was removed and added to 9ml of sterile BHI broth containing 10%(v/v) glycerol. The resulting 10ml culture was gently shaken and dispensed into 10 cryovials as 1ml aliquots. These were placed into a polystyrene tube holder and frozen slowly in a -80°C freezer, where they were stored indefinitely [Collins & Lyne, 1976].

2.3.3 Resuscitation of Cryopreserved Bacterial Cells

On removal of a cryopreserved bacterial culture from -80°C storage, the cryovial was immediately immersed into a waterbath at 37°C, and just as the last of the ice had melted the vial was removed [Collins & Lyne, 1976]. An agar plate was streaked with the

resuscitated suspension and incubated using the appropriate conditions for each bacterium as described in Section 2.3.1.

From the streaked agar plate, the bacterium was sub-cultured on a regular basis to maintain a viable culture.

2.3.4 Large-scale Production of Bacterial Cultures

In order to produce sufficient quantities of bacterial material for extraction, culture volumes were scaled-up from a 1 litre culture to 12 litres. For each bacterium this process was different, due the growth rates and nutrient requirements of each.

2.3.4.1 Autoclave Procedure of a 12 Litre Broth

To ensure that culture broths were sterile prior to inoculation, extensive autoclave times were required. To prevent the damage of the nutrients within the broth, 11 litres of water was firstly brought to 100°C within the culture vessel (I.D x H 289mm x 429mm). To this, was aseptically added 1 litre of 12 x concentrated nutrient or BHI broth, based on the preparations described in Section 2.3.1.1 and 2.3.1.2 respectively. The 12 litre broth was free-steamed for 20 minutes and then autoclaved at 121°C/15psi for 45 minutes. The autoclave procedure was checked to ensure that the broth had autoclaved successfully using a glass indicator vial.

2.3.4.2 Scale-up Requirements for a 12 Litre Culture of *S. aureus*

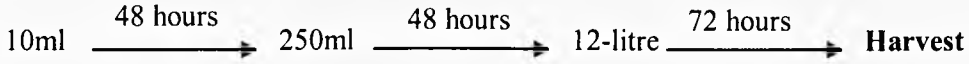
From the inoculation of a 10ml nutrient broth as in Section 2.3.2.1, the following inoculation train was adhered to:

10ml $\xrightarrow{36\text{ hours}}$ 250ml $\xrightarrow{36\text{ hours}}$ 12-litre $\xrightarrow{60\text{ hours}}$ **Harvest**

The 10ml and 250ml culture volumes were incubated at 37°C in an orbital shaker at 200rpm. The 12 litre culture vessel was also incubated at 37°C, but in an orbital shaker at 140rpm. This culture was sparged with air at a rate of 5l $hour^{-1}$ from the bottom of the vessel using a 5ml glass pipette. The air pump was connected to the culture vessel via an in-line Hepa-vent to maintain an axenic culture. At all inoculation stages an aliquot of the inoculum was streaked onto a nutrient agar plate to ensure that the *S. aureus* culture was axenic. Throughout the incubation of the 12 litre culture, turbidity measurements were made at 650nm. After 60 hours of growth, no further increase in the turbidity was observed, indicating that the culture had entered stationary phase [Pirt, 1975].

2.3.4.3 Scale-up Requirements for a 12 Litre Culture of *P. acnes*

From the inoculation of a 10ml BHI broth as in Section 2.3.2.2, the following inoculation train was adhered to:



The 10ml and 250ml culture volumes were incubated at 37°C in an orbital shaker at 200rpm, within the VAIN at 5% CO_2 and 95%N_2. The 12 litre culture was also incubated at 37°C, but in an orbital shaker at 140rpm. This culture was sparged with 95%/5% N_2/CO_2 mixed gas supply at a flow rate of 5l $hour^{-1}$ from the bottom of the vessel using a 5ml glass pipette. The gas cylinder was connected to the culture vessel via an in-line Hepa-vent to maintain an axenic culture. At all inoculation stages an aliquot of the inoculum was streaked onto a BHI agar plate to ensure that the *P. acnes* culture was axenic. Throughout the incubation of the 12 litre culture, turbidity measurements were made at 650nm. After 72 hours of growth, no further increase in the turbidity was observed, indicating that the culture had entered stationary phase [Pirt, 1975].

2.4 Mammalian Cell Maintenance

Hepatocytes and adipocytes were maintained in various media described below. All glassware used in these procedures was autoclaved at 121°C/15psi for 20 minutes. Wherever possible, sterile, disposable plastic utensils were used. Strict aseptic technique was followed throughout and 70%(v/v) ethanol in water was used to sterilise all non-autoclavable surfaces. All cell lines were cultured in a CO_2 incubator at 37°C, 5% CO_2 and 100% relative humidity. All reagents used in tissue culture procedures were of cell culture grade and filtered using 0.2µm filters. Only purified water was used as described in Section 2.1.1.

2.4.1 Resuscitation of Cryopreserved Cells

Cell lines were shipped as 1ml frozen ampoules and were resuscitated as described [Freshney, 1994]. Upon arrival these were immediately immersed into liquid nitrogen and stored at -196°C until required. To resuscitate the cells, a vial was removed from liquid nitrogen and vented in the laminar flow hood before warming rapidly in a waterbath at 37°C. The cryoprotectant was removed by suspending the contents of the vial to 10ml of culture medium, appropriate for the type of cell line being resuscitated and centrifuging for 3 minutes at 1000 x g. The supernatant was decanted and the cell pellet was resuspended in 5ml of fresh culture medium, again appropriate to the cell line. The cell suspension was then transferred to a 25cm^2 culture flask and placed in the CO_2 incubator to facilitate attachment and subsequent growth. Cell growth was monitored microscopically and the medium was exchanged on a daily basis.

2.4.2 Cell Line Description and Growth Requirements

All cell lines were obtained via the ECACC and were grown in culture medium recommended by the ECACC guidelines. These were specific for each type of cell line.

2.4.2.1 Adipocytes (3T3L1, ECACC No. 86052701)

3T3L1 was selected to study the effect of purified bacterial LMAP on lipid metabolism as changes in lipid metabolism have been shown to take place as part of the immune response to infection [Grunfeld & Feingold, 1991; Grunfeld & Feingold, 1992]. After differentiation this cell line has been shown to behave in a manner that parallels the development of adipose tissue in that the cells accumulate triacylglycerols from lipid droplets [Green & Kehinde, 1976]. Furthermore, the regulation of these cells has been shown to be effected by hormones and other effectors [Rosen *et al.*, 1978; Rosen *et al.*, 1979]. This makes this cell line a good model for the study of lipid metabolism.
The culture was obtained via ECACC and was described as a fibroblast-like adipocyte cell line, originating from a mouse embryo. The cell line was a continuous strain of 3T3 developed through clonal selection and was not contact inhibited.

Culture Medium:

The cells were maintained in DMEM supplemented with 25mM glucose, 25mM Hepes and 45mM sodium bicarbonate at pH7.4. To this medium was added 2mM L-glutamine, 50μg ml^{-1} penicillin, 50μg ml^{-1} streptomycin (purchased as 100 x stock solution and added accordingly) and 10%(v/v) foetal calf serum. All of the above were certified endotoxin-free and were filter-sterilised using a sterile 0.2μm cellulose acetate syringe filter prior to the addition to DMEM.

2.4.2.2 Hepatocytes (HepG2, ECACC No. 85011430)

It has been shown in previous *in vivo* experiments that LPS caused hypertriglyceridemia [Feingold *et al.*, 1992; Grunfeld & Feingold, 1991]. In addition to this, LTA has also been shown to stimulate lipolysis and hepatic triglyceride secretion [Nonogaki *et al.*, 1995]. It has not been specified whether this was a primary effect of the LTA or if it involved a secondary mechanism. Therefore, a hepatocyte cell line was selected to investigate the effect of purified bacterial LMAP on lipid metabolism *in vitro* and thus,

determine whether the previously observed effects on the liver were of a primary or secondary nature.

HepG2 was derived from a human liver biopsy from a patient with a well-differentiated hepatocellular carcinoma and was therefore a continuous hepatocyte cell line.

Culture Medium:

The cells were maintained in Minimum Essential Medium (MEM). This contained Earle's Balanced Salts, 25mM Hepes and 25mM sodium bicarbonate to pH 7.4. To the MEM was added 2mM L-glutamine, 1 x non-essential amino acids (purchased as 100 x stock solution and added accordingly), 50μg ml^{-1} penicillin, 50μg ml^{-1} streptomycin (purchased as 100 x stock solution and added accordingly) and 10%(v/v) foetal calf serum. All of the above were certified endotoxin-free and were filter-sterilised using a sterile 0.2μm cellulose acetate syringe filter prior to the addition to MEM.

2.4.3 Sub-cultivation of Cell Lines

Both cell lines were routinely sub-cultured, the frequency of which was dependent on the rate of growth. The method used was described by Freshney [Freshney, 1994]. Firstly, a 25cm^2 flask was decanted of culture medium and washed twice with 3ml PBS, at pH7.4. To the washed cells was added 1ml of pre-warmed, 0.05%(w/v) trypsin, supplemented with 0.53mM EDTA and phenol red indicator and the flask was incubated at 37°C for 3 minutes (adipocytes) or 8 minutes (hepatocytes). After incubation, the side of the flask was gently tapped to dislodge the adhered cells from the flask surface. The cells were examined microscopically to ensure complete detachment and the cell suspension was transferred to a 30ml universal. The flask was washed twice with 2ml of culture medium, supplemented as in Section 2.4.2.1 (adipocytes) or 2.4.2.2 (hepatocytes) to inhibit the action of the trypsin and this was added to the trypsin-cell suspension. The suspension was centrifuged at 1000 x g for 3 minutes. Following centrifugation, the supernatant was aspirated, thus, removing the trypsin and the cell pellet was gently resuspended using an

appropriate volume of new culture medium. The number of cells and the required final cell density determined the volume of culture medium used for resuspension. Adipose cells were generally resuspended in 20ml of culture medium and this was split between 4 x 25cm^2 flasks (i.e. 1/4), resulting in a seeding density of 7 x 10^5. Hepatocytes were resuspended in 10ml of culture medium and split between 2 x 25cm^2 flasks (1/2), resulting in a seeding density of 1.4 x 10^6.

Following sub-cultivation the culture medium was exchanged every 24 hours. With all cell lines, the cells were not sub-cultured more than 20-times, before which, a new cryopreserved ampoule was resuscitated as described in Section 2.4.1.

2.4.4 Cryopreservation of Cells

To provide a constant source of mammalian cells and to preserve the cell line characteristics, the cell lines were cryo-preserved in quantities suitable to last the duration of the project (~20 vials). This method used was described by Freshney [Freshney, 1994].

Six 80-90% confluent, 25cm^2 flasks were prepared as in Section 2.4.3. The resulting cell pellet was resuspended in 10ml of culture medium supplemented as in Section 2.4.2.1 (adipocytes) or 2.4.2.2 (hepatocytes) and containing 8%(v/v) cell culture-grade dimethyl sulphoxide as a cryoprotectant. 1ml aliquots of the cell suspension were transferred to sterile cryovials and these were placed in a -30°C freezer for 24 hours, following this, the vials were transferred to a -80°C freezer for a further 24 hours. Finally, the cryovials were plunged into liquid nitrogen where they were stored until required.

CHAPTER 3

Extraction and Purification

of Bacterial Lipid Macroamphiphiles

3.1 Introduction

To determine the effects of bacterial cell surface LMAPs, on mammalian lipid metabolism and due to the lack of commercially available material, LMAP components were extracted using a similar method to the procedure detailed by Fischer [Fischer, Koch & Haas, 1983]. This approach was used for the extraction and purification of LTA. A similar method was later reported for the generic extraction and purification of both LTA and lipoglycan [Fischer, 1991]. The method firstly involved the lysis of the bacterial cells to expose both sides of the cell membrane. This required a mechanical process in which a bacterial cell paste was vigorously stirred with small glass beads and the collisions between the beads and cells caused the breakage of the cells. The procedure was carried out at low pH and at 4°C, in order to inhibit the action of intracellular lipases and thus, prevent autolytic digestion of the LMAP components. Following cell disruption, the cells were extracted using hot aqueous phenol. The pH was raised slightly to allow for efficient separation of the proteins and lipids from LMAP components. The protein, membrane phospholipids and other cellular lipid partition into the phenol phase, while the LMAP, along with neutral polysaccharides and some nucleic acid partition into the aqueous phase. The aqueous phase was collected and was then prepared for HIC. It has frequently been reported that octyl Sepharose allowed the separation of both LTA and lipoglycans from crude extracts of LMAP [Fischer, 1991; Fischer, 1993; Fischer, 1996; Fischer *et al.*, 1983; Gao *et al.*, 2001; Leopold & Fischer, 1992; Morath, Geyer & Hartung, 2001]. However, there has been no report of the performance of other HIC matrices (i.e. phenyl or butyl Sepharose). Consequently, the efficiency of each matrix was determined using small disposable columns, prior to the scale-up to a preparative-size HIC purification procedure. To monitor the elution of LMAP from HIC, different assays were employed. These assays were dependent on the type of LMAP eluting from the column. For the preparation of LTA, phosphorus was measured in each fraction. This was because LTA from *S. aureus* contains a polyglycerophosphate hydrophilic chain and therefore LTA was rich in phosphorus [Fischer,

1994b]. Within *P. acnes*, a lipoglycan structure was suspected, based on the high guanine-cytosine % molar ratio found in *P. acnes* [Cummins & Johnson, 1981; Fischer, 1994a; Fox *et al.*, 1980] and the analysis of other propionibacteria [Sutcliffe & Shaw, 1989]. Consequently, to allow the elution of LMAP extracted from *P. acnes* to be followed the amount of carbohydrate was determined in each fraction. Furthermore, to determine whether the *P. acnes* LMAP had been successfully purified via HIC, sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) was used in conjunction with a silver-based staining procedure adapted from Tsai and Frasch [Tsai & Frasch, 1982]. This detection method stains carbohydrate material as well as protein, but to varying degrees. A single band on the SDS-PAGE denoted a successful purification procedure.

3.2 Materials

3.2.1 Reagents

Acrylamide/ bisacrylamide was purchased from Anachem, UK as a premixed solution. Octyl Sepharose HIC medium was purchased as an 80% slurry in 20% aqueous ethanol from Amersham Phamacia Biotech, UK. All organic solvents were obtained from Fisher Scientific, UK. Glycerol, β-mercaptoethanol and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from ICN Biomedicals, Germany. Lipoteichoic acid from *S. aureus* was obtained from Sigma, UK. All other reagents were of analytical grade and were also purchased from Sigma, UK. Purified water was deionised (18mΩ.cm) and filtered through a 0.2μm membrane via a Milli-Q system, Millipore, UK. Gases purchased from BOC, UK were used throughout.

3.2.2 Consumables

Polystyrene and polypropylene universals and centrifuge tubes were obtained from Bibby Sterilin, UK. 3kDa MWCO ultrafiltration membranes were purchased from Millipore, UK. All other filtration membranes and filters were obtained from Whatman, UK. Dialysis tubing was purchased from Spectrum Laboratories Inc, USA. Electro-dialysis membrane caps required for electroelution were supplied by Bio-Rad, UK. All other consumables were purchased from Fisher Scientific, UK.

3.2.3 Equipment and Instruments

Centrifugation of large volumes (>200ml) was performed using a 4237R refrigerated centrifuge (ALC, UK). High speed centrifugation (>10,000 x g) was accomplished using an Optima L preparative ultracentrifuge (Beckman, UK). All other centrifugation was carried out using a PK121R bench centrifuge (ALC, UK). All HIC chromatography was performed on a Fast Protein Liquid Chromatography (FPLC) system, comprising two P500 pumps, P1 peristaltic pump, LCC500 controller, V-7 valve, mixing chamber, 10ml superloop and a Frac-100 fraction collector (Amersham Phamacia Biotech, UK). HIC screening columns and a XK26/40 preparative column (Amersham Phamacia Biotech, UK) were used in conjunction with the FPLC system. Bacterial cells were disrupted using a Bead-Beater (Bio-Spec Products, USA) and Ballotini No.13 glass beads (Jencons-PLS, UK). Ultrafiltation was achieved using a solvent-resistant stirred-cell (Millipore, UK). Samples were freeze-dried using a Modulyo freeze-drier and RV5 pump (Edwards, UK). Analytical-scale electrophoresis and electro-elution were performed on a Mini-Protean II and 422 Electro-Eluter respectively (Bio-Rad, UK). Preparative-scale electrophoresis required the use of a V20-CDC electrophoresis kit (Scie-Plas Ltd, UK).

3.3 Experimental Methods

3.3.1 Preparation of Bacteria for Extraction

3.3.1.1 Harvest of Bacterial Cells

A 12 litre bacterial culture of either *S. aureus* or *P. acnes* was prepared as described in Section 2.3.4. From this, 500ml of culture was aliquotted into four 600ml polypropylene centrifuge buckets and the mass recorded. These were centrifuged at 2000 x g for 45 minutes at 4°C. The supernatant was then decanted and to each was carefully added another 500ml of culture and the centrifugation procedure was repeated. Once all 12 litres of the culture had been harvested, the content of each was weighed to determine the total wet cell weight of harvested bacteria.

3.3.1.2 Resuspension of Bacterial Cells

S. aureus was resuspended by gently swirling, in a small amount 0.1M sodium citrate buffer at pH3.0 to prevent clumps of cells forming. Once homogenous, the paste was made to a approximately 30%(w/v) cell suspension with 0.1M sodium citrate buffer at pH3.0.

P. acnes was resuspended by gently swirling, in a small amount 0.1M sodium acetate buffer at pH4.7 and then made to a approximately 30%(w/v) cell suspension with 0.1M sodium acetate at pH4.7.

3.3.1.3 Mechanical Disruption of Bacterial Cells

Disruption of the re-suspended bacterial cell pastes prepared as described in Section 3.3.1.2 was achieved using a Bead-beater. The re-suspended bacterial cells were added to the bead-beater sample vessel, containing an equivalent volume of 0.1mm-diameter glass pre-cooled to 4°C. The bead-beater sample vessel was cooled with an ice jacket to maintain a low temperature and was used twice at high-speed for 2 minutes. Between the pulses the disruptate was allowed to cool for 3 minutes.

Following disruption, the glass beads were removed using a No.2 sintered glass filter funnel and the beads were washed with approximately 100ml of 0.1M sodium acetate buffer at pH4.7 and at 4°C.

3.3.2 Extraction of Bacterial Macroamphiphiles

S. aureus disruptate was adjusted to pH4.7 with 2M NaOH, while, *P. acnes* was adjusted to pH6.0 again with 2M NaOH. 80%(w/v) phenol was prepared in DI water and this was warmed to 68°C in a water bath. To each disruptate was added an equivalent volume of the hot aqueous phenol, equivalent to approximately 4g of phenol to each gram of wet biomass. The extraction mixture was incubated at 68°C and stirred with a 4cm diameter impellor with an overhead stirrer at 500rpm for 90 minutes.

The extraction mixture was cooled to 4°C and dispensed into 50ml polypropylene centrifuge tubes and centrifuged for 12 hours at 2000 x g and at 4°C. After centrifugation the upper aqueous phase was removed and retained. To each centrifuge tube was added 20ml of 0.1M sodium acetate buffer at pH4.7 to further wash the lower phenol phase of LMAP material. The tubes were shaken for 2 minutes and centrifuged as before. Following the second centrifugation the upper phase was again removed and this was added to the first aqueous collection. The aqueous extract was extensively dialysed to remove the traces of phenol, using 6-8kDa MWCO Spectrapor 2 dialysis tubing in a 10:1 ratio with 0.1M sodium acetate buffer at pH4.7 with continuous stirring and at 4°C. The buffer was exchanged at 6, 12 and 24 hours.

The extract was removed from dialysis and concentrated by ultrafiltration using 3kDa MWCO, regenerated cellulose ultrafiltration membranes. The extract was subjected to 90psi with oxygen-free nitrogen and the volume of the extract was reduced to <1ml. To wash the extract, 100ml of 0.1M sodium acetate buffer at pH4.7 was added and the ultrafiltration procedure was repeated. This washing step was again repeated and finally the volume was reduced to 20ml.

The filtrate was then centrifuged at 20,000 x g for 6 hours and the supernatant was decanted leaving a nucleic acid-rich gel pellet at the bottom of the centrifuge tube. The LMAP-rich supernatant was made to 15%(v/v) propan-1-ol in 0.1M sodium acetate buffer at pH4.7 with the addition of an appropriate volume of propan-1-ol. This was then used for HIC purification.

3.3.3 Purification of Bacterial Macroamphiphiles

3.3.3.1 Screening of HIC Matrices

To determine the resolving properties of the range of HIC media available, different HIC columns were tested for their ability to elute a commercial preparation of LTA from *S. aureus* prepared according to the method of Fischer [Fischer, 1991]. Elution from the column required the use of a decreasing salt gradient, followed by an increase in the propan-1-ol concentration within the buffer. Finally the columns were washed with detergent to ensure that no LTA remained bound to the column. The column showing the most efficient elution profile was then scaled-up to allow the preparation of larger amounts of LMAP
The HIC columns were 1ml Hi-Trap columns containing phenyl, butyl or octyl Sepharose. These were equilibrated using 0.1M sodium acetate buffer at pH4.7, containing 1.0M NaCl for 5 minutes at a flow rate of 1ml min^{-1}. The flow rate was controlled with a P-1, peristaltic pump.

Preparation of LTA Sample

1.0mg of LTA from *S. aureus* was re-suspended in 1ml of 0.1M sodium acetate buffer at pH4.7. From this stock solution, 500µl was taken and adjusted to 0.5 mg ml^{-1} with 0.1M sodium acetate buffer at pH4.7, containing 2.0M NaCl (1.0M NaCl final concentration). 1.0ml of the LTA sample was loaded onto the column at 1ml min^{-1} using a 1ml polypropylene syringe. Following loading, the column was eluted at a rate of 1ml min^{-1}, using the buffers described in Table 3.1. The columns were eluted for 1ml of each buffer, from A to J. From this elution system 1ml fractions were collected and labelled, as described

in Table 3.1. The column eluent obtained from the loading of the sample onto the column was also retained and labelled 1.

Table 3.1 Buffer System Utilised for the Elution of LTA from Different HIC Matrices

Buffer	Buffer Composition †	Elution Volume (ml)
A	1.0M NaCl	2
B	0.75M NaCl	3
C	0.50M NaCl	4
D	0.25M NaCl	5
E	No NaCl	6
F	15% Propan-1-ol	7
G	30% Propan-1-ol	8
H	45% Propan-1-ol	9
I	60% Propan-1-ol	10
J	0.1% Triton X100	11

† All prepared in 0.1M sodium acetate buffer at pH4.7.

The total amount of phosphorus was determined in each fraction, using the method described in Section 2.2.4, as an indication of the amount of LTA present.

3.3.3.2 Additional Screening of HIC Matrices

Following the initial column screening, the phenyl and octyl Sepharose columns were further optimised and their ability to bind and elute LTA was again compared. The flow rate during sample loading was reduced to 0.25ml min^{-1} and the overall flow rate was also decreased to 0.5ml min^{-1} to improve the initial binding and retention of the LTA respectively. The buffer system for elution was also amended as shown in Table 3.2. Each buffer was used in-turn from A to D for 5 minutes for each buffer. The resulting column eluent was collected as 1ml volumes and labelled accordingly with the corresponding elution volume identification. The column eluent obtained from the loading of the sample onto the column was also retained and labelled 1.

Table 3.2 Buffer System Utilised for the Elution of LTA from Phenyl and Octyl Sepharose HIC Matrices

Buffer	Buffer Composition	Elution Volume (ml)
A	4.0M NaCl†	2-6
B	No NaCl†	7-11
C	45% Propan-1-ol†	12-16
D	70% Ethanol‡	17-18

† Prepared in 0.1M sodium acetate buffer at pH4.7.

‡ 2ml applied, thus, only two fractions collected.

The total amount of phosphorus was determined in each fraction, using the method described in Section 2.2.4, as an indication of the amount of LTA present.

3.3.3.3 Determination of the Elution Profile for LTA on Octyl Sepharose

To characterise the elution of LTA from octyl Sepharose prior to scaling-up the procedure to a preparative size column, an elution profile was generated. A commercial preparation of LTA was used as in Section 3.3.3.1, but the 1.0 mg ml^{-1} sample was prepared in 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol. Sample loading was carried out using 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol, as the presence of the propan-1-ol prevented the binding of hydrophilic molecules such as nucleic acid, neutral polysaccharide and proteins, thus, increasing the binding capacity of the HIC medium for LTA. The rate of sample loading was 0.25ml min^{-1}. Elution of the column was carried out at 0.5ml min^{-1}. Protein and DNA were also determined for each fraction, to ensure effective purification. The buffer system for elution was also amended as shown in Table 3.3. Each buffer was used in-turn from A to D to elute the HIC column for 5 minutes for each buffer. The resulting column eluent was collected as 1ml volumes and labelled accordingly with the corresponding elution volume identification. The column eluent obtained from the loading of the sample onto the column was also retained and labelled 1.

Table 3.3 Buffer System Utilised for the Elution of LTA from Octyl Sepharose HIC Matrix

Buffer	Buffer Composition	Elution Volume (ml)
A	15% Propan-1-ol†	2-6
B	30% Propan-1-ol†	7-11
C	45% Propan-1-ol†	12-16
D	60% Propan-1-ol†	17-21
E	70% Ethanol	22

† Prepared in 0.1M sodium acetate buffer at pH4.7.

The total amount of phosphorus was determined for each of the collected fractions, using the method described in Section 2.2.4. Protein and DNA (nucleic acid) were estimated at 280nm and 260nm respectively for each fraction and for which, no sample preparation was required.

3.3.3.4 Purification of LTA from a Crude Extract from *S. aureus*

S. aureus was cultured as described in Section 2.3.4. 40g wet cell weight of *S. aureus* bacterial cells were prepared and disrupted (as detailed in Section 3.3.1) and finally extracted as described in Section 3.3.2. From this extract 1.7ml was taken and made to 2ml with propan-1-ol. This resulted in a 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol. This was loaded at a rate of 0.25ml min^{-1} and the column was eluted as illustrated in Section 3.3.3.3.

The total amount of phosphorus was determined for each of the collected fractions, using the method described in Section 2.2.4. Protein and DNA (nucleic acid) were estimated spectroscopically at 280nm and 260nm respectively for each fraction, for which, no sample preparation was required.

3.3.3.5 Column Preparation for the Scale-up of HIC

In order to produce sufficient amounts of LTA for further investigations, the HIC purification process was scaled-up to allow the purification of ≤100mg of LTA. This initially required the efficient column packing of a preparative HIC column with the octyl Sepharose medium.

An XK 26/40 empty column of dimensions 2.6 (I.D) x 40cm (L) was filled with an 80%(w/v) aqueous slurry of octyl Sepharose. The octyl Sepharose-filled column was then packed at a rate of 10ml min^{-1} with DI water, using an FPLC system. The DI water had been extensively degassed by vacuum and filtered through a 0.45µm nitrocellulose filter prior to use. Once the Sepharose bed height had reached a constant height the majority of the liquid was removed and the column adapter was lowered to 0.5cm from the bed surface. Packing was continued, as before, until the Sepharose bed height was again constant. Once again the liquid was removed. This process was repeated until the Sepharose bed height was a constant 0.5cm below the adapter, indicating that packing was complete. The final column volume was 125ml.

Now that the column was successfully packed, it was equilibrated using 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol. Equilibration of the column was carried out at 2.5ml min^{-1} for three column volumes (2.5 hours). The propan-1-ol in the buffer was maintained using the two FPLC pumps. Pump A delivered 0.1M sodium acetate buffer at pH4.7, while Pump B delivered 0.1M sodium acetate buffer at pH4.7, containing 60%(v/v) propan-1-ol. Both buffer solutions were extensively degassed under vacuum prior to use, as gases dissolved readily within the aqueous buffer and were consequently released on mixing with propan-1-ol, damaging the packing of the Sepharose within the column.

3.3.3.6 Scale-up Elution of LTA from *S. aureus*

After equilibration of the HIC column 22mg of LTA from *S. aureus* was made to 20ml with 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol. This was centrifuged at 3000 x g for 20 minutes to pellet any insoluble material and to remove any dissolved gas. The LTA sample was injected onto the column in 2 x 10ml aliquots, one directly after the other using a 10ml super-loop and V7 valve at 1ml min^{-1}. The loop was washed with 2ml of 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol, directly onto the column. Elution of LTA from the column followed the elution profile depicted in Figure 3.1 at a continuous rate of 2.5ml min^{-1}, in which a stepped gradient was

applied. The resulting column eluent was collected as 5ml volumes throughout elution, using a fraction collector. The column eluent obtained from the loading of the sample onto the column was also retained for later analysis. Following elution, the total amount of phosphorus was determined for each of the collected fractions using the method described in Section 2.2.4. Protein and DNA (nucleic acid) was estimated for each fraction, by measuring the absorbance at 280nm and 260nm respectively for each fraction, for which, no sample preparation was required.

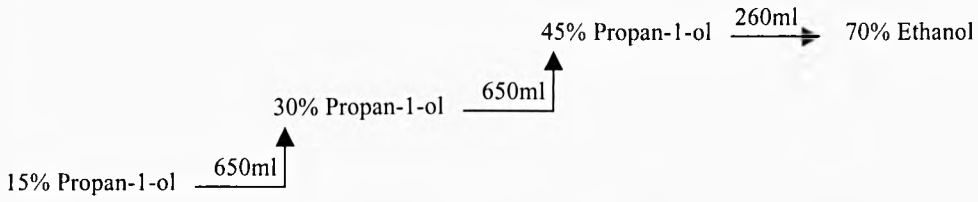


Figure 3.1 Elution Profile for the Scale-up Purification of LTA.

Elution followed the above profile prepared in 0.1M sodium acetate buffer at pH4.7 with a continuous flow rate of 2.5ml min^{-1}.

3.3.3.7 Scale-up Purification of LTA from a Crude Extract from *S. aureus*

12.75 ml of extract prepared as described in Sections 3.3.1 and 3.3.2 was made to 15% propan-1-ol with the addition of 2.25ml propan-1-ol. This was centrifuged at 3000 x g for 20 minutes and loaded onto the preparative HIC column as described in Section 3.3.3.6 as two consecutive 7.5ml fractions. The sample loop was washed with an additional 2ml of 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol, directly onto the column. Elution of LTA from the column followed the elution profile shown in Figure 3.2, with a linear increase in the propan-1-ol gradient from 15%(v/v) to 60%(v/v). The flow rate was maintained at a continuous rate of 2.5ml min^{-1}. During the loading of the column and throughout elution of the LTA, 5ml fractions were collected using a fraction collector. Following elution, the total amount of phosphorus, protein and DNA (nucleic acid) was

determined for each of the collected fractions, using the methods described in Section 2.2. The LTA-containing fractions were pooled, desalted and freeze-dried as described below.

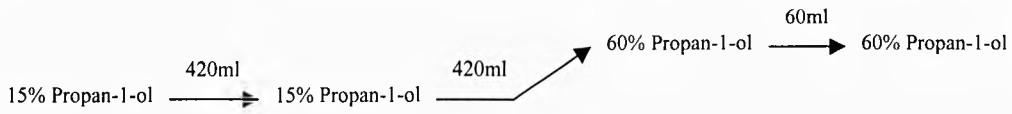


Figure 3.2 Elution Profile for the Purification of LTA from a Crude Extract Obtained from *S. aureus*.

Elution followed the above profile prepared in 0.1M sodium acetate buffer at pH4.7 with a continuous flow rate of 2.5ml min^{-1}.

3.3.3.8 Desalting and Freeze-drying of HIC-purified LTA

The HIC-eluted material contained sodium acetate and propan-1-ol. To remove these buffer constituents, the suspected LTA-containing fractions were pooled and ultrafiltrated using a 3kDa MWCO regenerated cellulose membrane. Once the volume had reduced to <1ml, 100ml of purified water was added. The volume was again reduced to <1ml and a further 100ml of purified water was added. This was repeated a final time, after which the volume was reduced to 5ml and the LTA material was removed from the stirred cell. The stirred cell and the ultrafiltration membrane were washed three times with 5ml of purified water. The washes were collected, added to the original 5ml and freeze-dried. The freeze-dried LTA was stored at -30°C in a desiccated tube until required.

3.3.3.9 HIC-elution of LMAP from a Crude Extract from *P. acnes*

17.0 ml of extract prepared as described above in Sections 3.3.1 and 3.3.2 was made to 15%(v/v) propan-1-ol with the addition of 3.0ml propan-1-ol. This was centrifuged at 3000 x g for 20 minutes and then loaded onto the preparative HIC column as detailed in Section 3.3.3.6 as two consecutive 10ml fractions. The sample loop was washed with an

additional 2ml of 0.1M sodium acetate buffer at pH4.7, containing 15%(v/v) propan-1-ol, directly onto the column. Elution of LMAP from the column followed the elution profile described in Figure 3.3 at a continuous rate of 2.5ml min^{-1}. During the loading of the column and throughout elution of the LTA, 5ml fractions were collected using a fraction collector. Following elution, the total amount of carbohydrate, protein and DNA (nucleic acid) were determined for each of the collected fractions, using the methods described in Section 2.2. The LMAP-containing fractions were pooled, desalted and freeze-dried as described in Section 3.3.3.8.

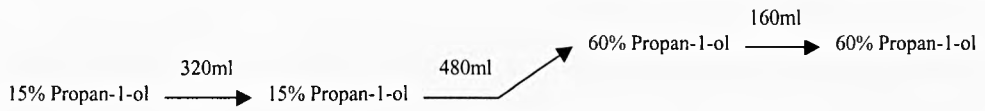


Figure 3.3 Profile for the HIC-elution of LMAP from a Crude Extract Obtained from *P. acnes*. Elution followed the above profile prepared in 0.1M sodium acetate buffer at pH4.7 with a continuous flow rate of 2.5ml min^{-1}.

3.3.4 Analysis of HIC-eluted Lipid Macroamphiphiles from P. acnes

In order to determine the purity of the HIC-eluted material, the component was de-salted, freeze-dried and prepared for analysis using SDS-PAGE.

3.3.4.1 Preparation of HIC-purified LMAP for SDS-PAGE

Following desalting and freeze-drying of the LMAP material, 1.3mg of LMAP was taken and made to 500µl (2.6mg ml^{-1} stock) with purified water. Double-strength solubilisation solution was prepared containing 20%(w/v) glycerol, 10%(w/v) SDS, 20%(v/v) β-mercaptoethanol, 20%(w/v) of 1mg ml^{-1} aqueous bromophenol blue, 12.5%(v/v) 1.0M Tris-HCl buffer at pH6.8 in an aqueous solution. This solution was stored at -30°C for up to 3 months. Using the 2.6mg ml^{-1} LMAP stock solution, dilutions were prepared ranging from 0.325 to 1.3µg $µl^{-1}$, in purified water and an equivalent volume of double-strength

solubilisation solution. All samples were prepared in 0.5ml Eppendorf tubes, boiled for 3 minutes in a waterbath, cooled and then centrifuged at 5000 x g for 2 minutes. Standard protein molecular weight marker mixtures were prepared from a commercial source and contained molecular weights ranging between 14.2 to 66kDa. These were prepared to a total protein concentration of 2.33μg μl^{-1} using a single strength solubilisation solution (double strength solubilisation solution diluted with an equivalent amount of purified water).

3.3.4.2 Preparation of Polyacrylamide Gels for SDS-PAGE

Resolving Gel (15%):

10ml of 15% tris-glycine resolving gel was prepared using 5.0ml of 30%(w/v) acrylamide/bisacrylamide (37.5/1.0) stock solution. To this was added 2.3ml of purified water, 2.5ml of 1.5M tris buffer at pH8.8, 100μl of 10%(w/v) aqueous SDS, 100μl of 10%(w/v) aqueous ammonium persulphate, followed by 4.0μl of TEMED to polymerise the acrylamide. After addition of the TEMED the solution was immediately poured into a Mini-protean II gel cassette and layered with 500μl of iso-butanol to prevent oxidation of the polymerising acrylamide solution. This was left for 45 minutes to polymerise.

Stacking Gel (5%):

The resolving gel was extensively washed with purified water to remove the iso-butanol and then blotted dry. 2ml of 5% tris-glycine stacking gel was prepared using 333μl of 30%(w/v) acrylamide/bisacrylamide (37.5/1.0) stock solution. To this was added 1.4ml of purified water, 250μl of 1.0M tris buffer at pH6.8, 20μl of 10%(w/v) aqueous SDS, 20μl of 10%(w/v) aqueous ammonium persulphate, followed by 2.0μl of TEMED, to polymerise the acrylamide. The 5% acrylamide solution was immediately poured onto resolving gel and a sample well comb was placed at the top of the cassette. This was left to polymerise for 45 minutes.

3.3.4.3 Separation of HIC-eluted LMAP by SDS-PAGE

Reservoir buffer was prepared with the addition of 1.51g of tris base, 14.4g of glycine and 5.0ml of 10%(w/v) aqueous SDS. This was adjusted to 500ml with purified water and used to fill the electrophoresis tank. Using the polyacrylamide gel prepared above, 5.0µl standards and samples were loaded into the pre-formed wells and the gel was run at 200V constant voltage for 50 minutes. The gel was then removed from the tank and gel cassette and then stained as described in Section 3.3.4.4.

3.3.4.4 Silver Staining of HIC-eluted LMAP Separated by SDS-PAGE

The LMAP was detected on the resolved polyacrylamide gel using an adapted method of Tsai and Frasch [Tsai & Frasch, 1982], previously used for the detection of lipopolysaccharide in polyacrylamide gels. The method was adapted to reduce the time scale of the procedure and to take into consideration the reduced thickness of the polyacrylamide gel. This involved the elimination of the overnight fixing procedure and amending the staining times and wash times accordingly.

The gel was fixed by soaking in an aqueous solution containing 40%(v/v) ethanol and 5%(v/v) glacial acetic acid (fixing solution) for 90 minutes. Following this, the gel was oxidised using 0.7%(w/v) periodic acid prepared in fixing solution for 10 minutes. The gel was then washed three times in purified water for 10 minutes at a time. Immediately prior to use, 3.0ml of 200mg ml^{-1} aqueous silver nitrate solution was added to 16.8ml of 0.1M NaOH, containing 1.2ml of 35% ammonia solution. This solution was made to 90ml with purified water and the gel was soaked in this for 20 minutes. The gel was washed three times in purified water for 10 minutes and soaked in 100ml of 5mg ml^{-1} aqueous citric acid containing 50µl of formaldehyde. Finally, the gel was developed until the yellow/brown colour of the separated standard protein markers were evident. The gel was then removed from the developing solution, washed in purified water and scanned using a flatbed scanner.

3.3.5 Development of Optimal conditions for the Solublisation of LMAP for SDS-PAGE

In order to effectively analyse the HIC-eluted LMAP material by SDS-PAGE, the solution used for the solubilisation of the samples required optimising. A third band frequently appeared on the gel when the HIC-eluted material was loaded in relatively high concentrations. It was suspected that this was due to aggregation of the LMAP, thus the concentration of the compounds within the solubilisation solution was modified to correct this effect.

The double-strength solublisation solution was prepared as described in Section 3.3.4.1, but in a separate solution the concentration of SDS was increased by two-fold (2xSDS) to give a final sample concentration of 20%(w/v). In another solublisation solution preparation, the β-mercaptoethanol was increased by two-fold (2xME) to give a final concentration of 20%(v/v) when mixed with the sample. Both of the above solublisation solutions were diluted two-fold with purified water to make single-strength solutions. Freeze-dried, HIC-eluted material was separately made to 1.5μg μl^{-1} with each of the single strength solubilisation solutions above. Half of each of the sample volumes was taken and sonicated for 60 seconds. All samples were run on SDS-PAGE as detailed in Sections 3.3.4.2 and 3.3.4.3. The resulting gel was then silver-stained as described in Section 3.3.4.4.

3.3.6 Purification of the Lipid Macroamphiphiles of P. acnes

In order to purify the individual LMAP components from the HIC-eluted material, preparative SDS-PAGE was utilised. Previously, analytical SDS-PAGE was shown to be an effective method of separation of the unresolved HIC components, thus this analytical-scale method was scaled-up to allow the purification of milligram quantities of each component. Following preparative SDS-PAGE, the separated components were removed from the polyacrylamide matrix by electro-elution and the purification of each component was assessed using analytical SDS-PAGE. Furthermore, the purified LMAP components were compared to specific HIC fractions eluting immediately before and also immediately after the elution of the main LMAP peak.

3.3.6.1 Separation of HIC-eluted LMAP using Preparative SDS-PAGE

Equivalent proportions of acrylamide/bisacrylamide, tris buffer, SDS, ammonium persulphate and TEMED were used to produce 50ml and 20ml of 15% resolving gel and 5% stacking gel respectively as described in Section 3.3.4.2. A 20cm x 20cm-gel cassette was used to form the gels with 2mm spacers and a 15cm x 2cm x 2mm single well, teflon sample comb. The polymerised gel cassette was loaded into a Maxi V20-CDC gel tank and to the upper and lower chambers was added 1 litre and 3.5 litres of reservoir buffer respectively, prepared as described in Section 3.3.4.3.

The HIC-eluted LMAP was prepared in single-strength solubilisation solution as detailed in Section 3.3.5 to a final concentration of 5mg ml^{-1}. From this, 500µl was loaded into the single well of the gel cassette and the electrophoresis was ran at 35V constant voltage, for 48 hours, at 10°C while continually stirring the reservoir buffer to dissipate any heat generated. After this time, a vertical gel slice of width 1.5cm was removed and silver-stained as described in Section 3.3.4.4. The staining method was further modified to take into account the extra thickness of the gel and thus the gel was initially fixed for 4 hours and all wash stages were increased to five washes with purified water for 15 minutes each.

3.3.6.2 Electro-elution of Separated LMAP Components Following Preparative SDS-PAGE

The silver-stained gel slice prepared above was lined-up with the original gel slab and the stained bands were used to determine where on the gel the separated components were located. This area was then excised with a wheel-cutter. Each piece of polyacrylamide gel was separately cut into small cubes using a scalpel and placed into individual electro-elution tubes fitted with 3.5kDa MWCO membrane caps. Reservoir buffer was prepared by dissolving tris base (25mM), glycine (192mM) and 0.1%(w/v) SDS in 1 litre of purified water and this was added to both the upper (200ml) and lower chambers (500ml). Electro-elution was carried out at 10mA constant current/elution tube for 8 hours with vigorous stirring with a magnetic stirring bar. After this time, the buffer was removed from the lower chamber and replaced with aqueous tris-glycine buffer at 25mM and 192mM respectively.

Electro-elution was continued at 8mA constant current/elution tube for 12 hours to remove the SDS from the samples via electro-dialysis.

Following electro-elution, the samples were removed from the membrane caps and dialysed in Spectropor dialysis tubing of 3.5kDa MWCO against 100 volumes of purified water. The purified water was exchanged every 24 hours for 4 days at 4°C with continuous stirring throughout. Each sample was freeze-dried and a small amount was solubilised in single strength solubilisation solution (as detailed in Section 3.3.5) to a concentration of approximately 5µg μl^{-1}. Each component was analysed on analytical-scale SDS-PAGE as described in Section 3.3.4.

3.3.6.3 Analysis of Fractions Before and After LMAP Elution from HIC

Following elution of the LMAP from HIC, three fractions immediately before elution (585-595ml) and three fractions immediately after elution (655-665ml) of the major LMAP-containing peak were collected and each set of samples was pooled. These fractions were dialysed against purified water at 100:1 using Spectropor dialysis tubing of 3.5kDa MWCO. The purified water was exchanged every 24 hours for 4 days at 4°C with continuous stirring throughout. Each sample was freeze-dried and solubilised in single strength solubilisation solution (as in Section 3.3.5) to a concentration of approximately 5µg μl^{-1}. Samples were then analysed using analytical-scale SDS-PAGE as described in Section 3.3.4 and the relative mobility of each was compared to the LMAP components purified using the procedures illustrated in Sections 3.3.6.1 and 3.3.6.2.

3.4 Results and Discussion

3.4.1 Screening of HIC matrices

To determine the resolving properties of the range of HIC media available, different HIC columns were tested for their ability to bind and elute LTA from *S. aureus*. The amount of phosphorus determined in each of the eluting fractions was plotted against the elution volume (Figure 3.4). The more hydrophobic the matrices, the further up the elution gradient the LTA appeared thus, the octyl Sepharose provided the better elution profile, with the LTA eluting when 45%(v/v) propan-1-ol was applied. This allowed the elution of the contaminants in the flow-through, while the LTA was tightly bound to the column. The 15%(v/v) propan-1-ol mobile phase eluted the LTA on the phenyl Sepharose column, while the LTA eluted using the buffer containing 0.50M NaCl with the butyl Sepharose column. This data suggested that octyl Sepharose was the more efficient matrix for LMAP separation as it retained a greater amount of LTA and for longer than the other matrices whilst allowing a progressively more concentrated propan-1-ol gradient, which was beneficial for the elution of the less hydrophobic impurities.

Large amounts of phosphorus were observed at the start of elution in the fraction collected during sample loading. This indicated that the LTA was not binding the column matrix efficiently. In order to increase the binding the flow rate was reduced during sample loading from 1.0 to 0.25ml min^{-1} to allow the LTA more time to bind the matrices. Furthermore, the ionic strength of the loading buffer was increased to maximise the binding of the LTA. Lastly, the overall flow rate during elution was also reduced from 1.0 to 0.5ml min^{-1}.

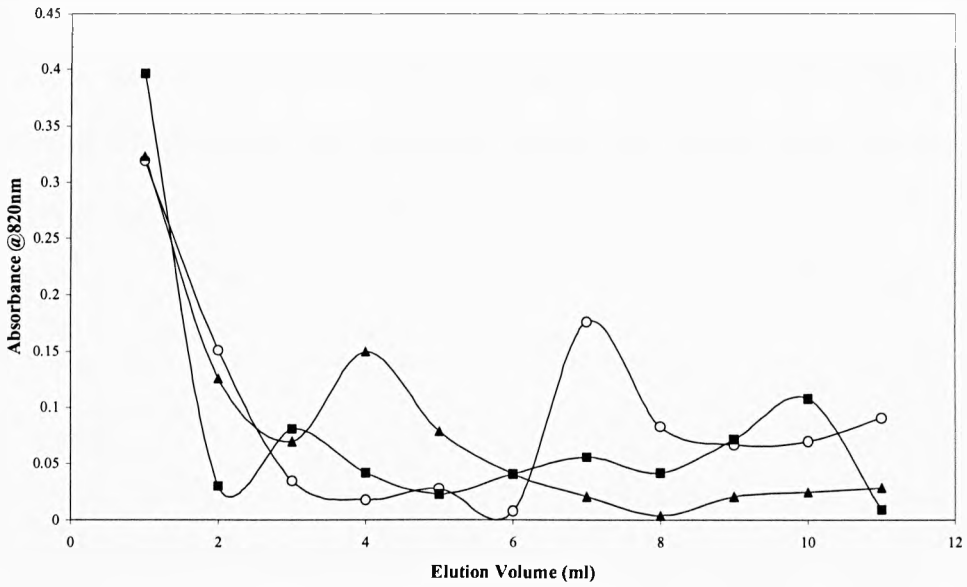


Figure 3.4 Screening of HIC matrices

Determination of the amount of phosphorus (absorbance at 820nm), indicative of the amount of LTA eluting with a progressively hydrophobic elution volume (ml) from butyl Sepharose (▲), phenyl Sepharose (○) and octyl Sepharose (■). For the elution conditions see Table 3.1.

3.4.2 Additional Screening of HIC matrices

Following the initial screening studies, the experiments were repeated concentrating on the octyl Sepharose and phenyl Sepharose HIC matrices, as these appeared to show better retention of the LTA than that of the butyl Sepharose. The reduction in flow rate from 1.0 to 0.25ml min^{-1} and the increased salt concentration during sample loading resulted in a 10-fold reduction in the amount of phosphorus observed in the fraction that eluted during sample loading (Figure 3.5). Therefore, these modifications significantly reduced the amount of LTA eluting during the loading process on both columns. Furthermore, an increase in the amount of phosphorus was also recorded in the major LTA peak, thus providing further evidence for a more efficient binding of LTA to the column. The elution profile of both of the matrices suggested that the octyl Sepharose performed better than the phenyl Sepharose in that the main LTA peak was separate from the smaller peak that elutes just before the LTA. This smaller peak was suspected of being an impurity. Furthermore, more LTA bound the octyl Sepharose matrix than the phenyl Sepharose, as a larger amount of phosphorus was

determined for the octyl Sepharose, than on the phenyl Sepharose. It was also noteworthy that the peak shape was much improved for the elution of LTA from both columns, thus indicative of efficient elution from the column and therefore suggesting that the elution flow rate has been optimised.

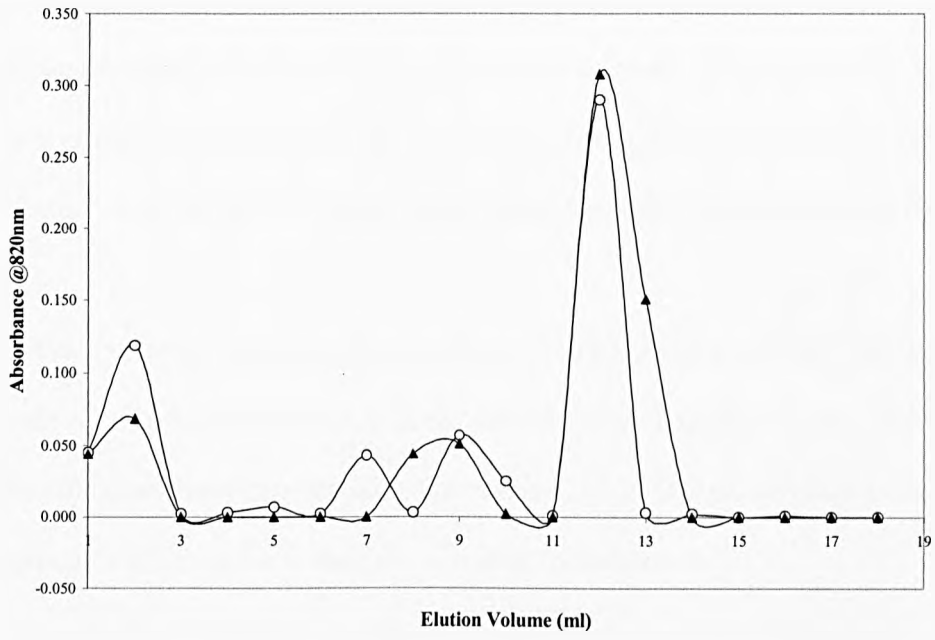


Figure 3.5 Additional Screening of HIC matrices

Determination of the amount of phosphorus (absorbance at 820nm), indicative of the amount of LTA eluting with a progressively hydrophobic elution volume (ml) from phenyl Sepharose (o) and octyl Sepharose (▲). For the elution conditions see Table 3.2.

3.4.3 Determination of the Elution Profile for Octyl Sepharose

To characterise the elution of LTA from octyl Sepharose prior to scaling-up the procedure to a preparative size column, an elution profile was generated and is shown in Figure 3.6. This profile clearly shows a large increase in the amount of total phosphorus, suggesting the elution of LTA at fraction 7 (7ml) with 30%(v/v) propan-1-ol. Also in the elution profile a significant amount of phosphorus was detected within the sample loading fractions collected, suggesting once again, inefficient binding of LTA as observed in Section 3.4.1. On further analysis of these fractions (Figure 3.7), it was shown that these fractions

contained significant amounts of both nucleic acid and protein. This was not expected as the sample used was a commercial preparation of LTA and therefore would be expected to be reasonably pure. In support of these observations it has recently been reported that such commercial preparations were relatively impure and have been found to contain large amounts of LPS [Gao *et al.*, 2001]. Also within this investigation, significant amounts of phosphorus-containing hydrophilic components were also detected, although these were not analysed for the presence of protein or nucleic acid [Gao *et al.*, 2001]. Furthermore, it has recently been reported that commercial preparations also suffered from decomposition of the LTA structure, characterised by loss of glycerophosphate and D-alanine residues [Morath *et al.*, 2002].

In light of these results, commercial preparations of all LMAP material were not used to analyse the cellular responses to LTA, as the presence of such impurities might have profound effects on the results obtained. Furthermore, all LMAP preparations were analysed for the presence of LPS prior to their use in further investigations.

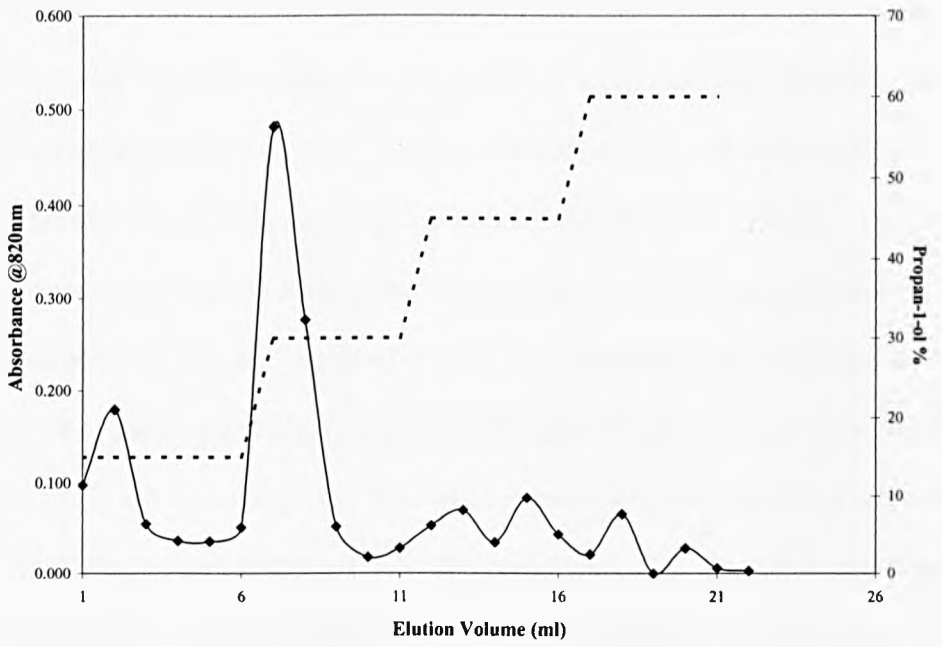


Figure 3.6 Determination of the Elution Profile for LTA on Octyl Sepharose

Analysis of 1ml fractions to determine the amount of phosphorus using the absorbance at 820nm (◆). This is indicative of the amount of LTA eluting from the octyl sepharose, following the increase in propan-1-ol gradient (---).

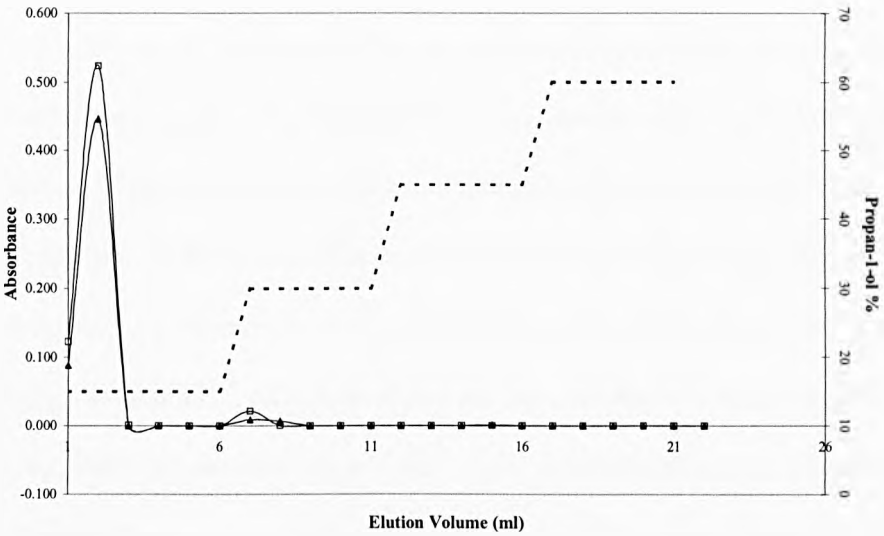


Figure 3.7 The Elution of Impurities within Commercial LTA Using Octyl Sepharose

Suspected impurities within the LTA standard solution separated using octyl sepharose is shown, following the increase in propan-1-ol gradient. These were determined by measuring the absorbance at 260nm for DNA (□) and the absorbance at 280nm for protein (▲). These were determined for each sequential elution fraction.

3.4.4 Purification of LTA from a Crude Extract of S. aureus *using Octyl Sepharose HIC*

Following the characterisation of the elution of a commercial preparation of LTA on octyl Sepharose, a crude extract from *S. aureus* was applied. The resulting profile demonstrates that the octyl Sepharose HIC column could efficiently separate LTA from a crude extract obtained from *S. aureus*, as a similar elution profile to the separation of the standard commercial LTA was produced (Figure 3.8). The amount of protein present within the pooled LTA-containing fractions was originally quantified using the Bradford method [Bradford, 1976] and found to be 5%. This was compared to the determination using the BCA method [Smith *et al.*, 1985] and only <1% was found. This variation in precision suggested the possibility that the presence of LTA was interfering with the protein determination or the Bradford reagent was prone to interference by the buffer constituents. To investigate this further, the HIC-separated LTA was passed through a cation exchange column at pH 4.7, which binds protein, but does not retain the LTA, thus allowing it to elute immediately. A bovine serum albumin (BSA) control sample of similar concentration to that

suggested within the LTA sample was also passed through the column to ensure the column was functioning correctly. The eluted LTA was tested again using both protein assays and was found to be unchanged, while the eluted fractions from the BSA control did not contain protein. This confirmed that the earlier abundance of protein found using the Bradford method was likely to be due to the LTA or buffer constituents interfering with the determinations and not attributable to the presence of protein. From this investigation it was concluded that the eluted LTA was free of protein impurity. Nucleic acid was also quantified, by measuring the amounts of DNA within the HIC-eluted LTA and was found to be negligible (<0.1%).

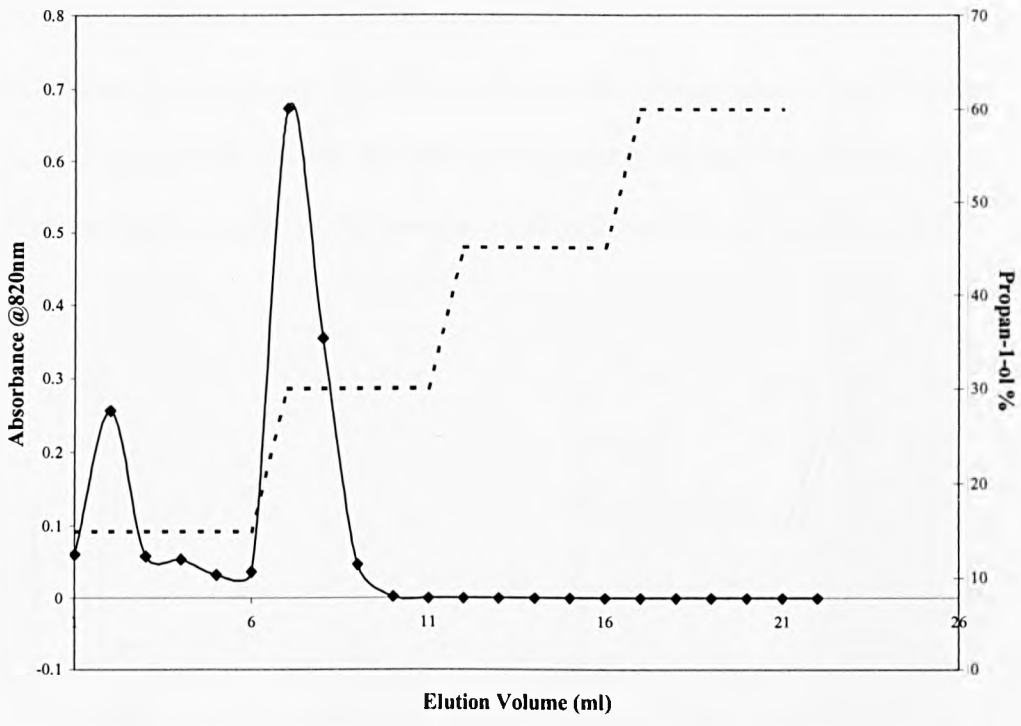


Figure 3.8 Elution Profile for the Separation of LTA from a Crude Extract from *S. aureus* Analysis of 1ml fractions to determine the amount of phosphorus using the absorbance at 820nm (♦). This is indicative of the amount of LTA eluting from the octyl Sepharose, following the increase in propan-1-ol gradient (---).

3.4.5 Scale-up Elution of LTA from S. aureus

The purification of LTA from *S. aureus* was scaled-up to allow larger quantities of LTA to be produced. The scale-up of the chromatography was successful with a large phosphorus peak eluting between 505-540ml, after the addition of the 30%(v/v) propan-1-ol buffer (Figure 3.9). From the observations made with previous LTA elution profiles, this was where the LTA was expected to elute. These fractions were tested for protein using the BCA method (as described in Section 2.2.1) and found to be <1%. The nucleic acid was estimated by measuring the absorbance at 260nm and was found to be <0.1%. A significantly large amount of material also eluted between 100-165ml. These fractions were assayed for protein and found to contain <0.1%. Consequently, these fractions were also estimated for nucleic acid, again by determining the absorbance at 260nm, and found to contain between 5 and 25µg ml^{-1}, i.e. up to 25-fold greater than measured in the LTA fraction. This data further support the findings discussed in Section 3.4.3 which suggested significant amounts of impurity was contained within the commercially obtained LTA.

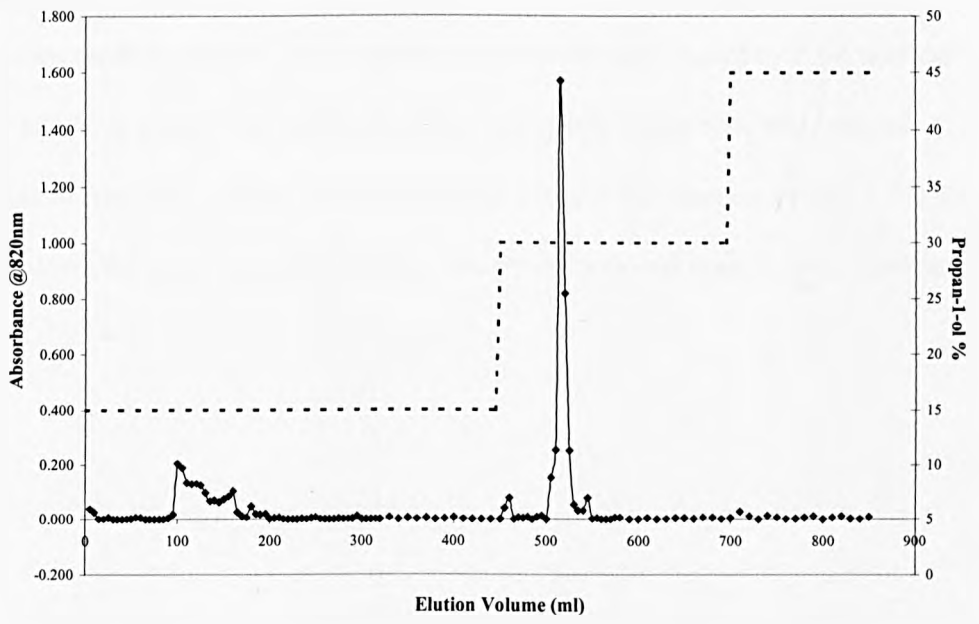


Figure 3.9 Elution Profile for the Scale-up Elution of an LTA Standard from *S. aureus*
The eluting fractions were analysed to determine the amount of phosphorus in each using the absorbance at 820nm (◆). This is indicative of the amount of LTA standard eluting from the octyl sepharose, following the increase in propan-1-ol gradient (---).

3.4.6 Scale-up Purification of LTA from a Crude Extract Obtained from S. aureus

The scaled-up chromatography procedure was applied to a crude extract obtained from *S. aureus* to allow the purification of LTA. Figure 3.10 demonstrates that the separation of the LTA from the nucleic acid and protein impurities was successful with fractions containing a large amount of phosphorus eluting at approximately 30%(v/v) propan-1-ol which agrees with the elution of the standard LTA in Section 3.4.5. The amount of protein was estimated using the BCA method described in Section 2.2.1 and found to be negligible in all fractions except the LTA-containing fractions where again protein was <1%. This also included the fractions at the start of elution, which were known to be associated with elution of the impurities (120-150ml). Therefore, suggesting that it was the presence of the LTA that was responsible for the previously observed interference as opposed to the buffer constituents. In contrast to the low amounts of protein found in the initial elution fractions, using the diphenylamine assay described in Section 2.2.2, the amount of nucleic acid (DNA) present was >90μg ml^{-1} in some fractions, suggesting that nucleic acid was the major co-extracting impurity within the crude extract. This was also supportive of the conclusions made in Section 3.4.5, regarding the interference caused by LTA within the protein assays. If protein was absent from the hydrophilic impurities, which eluted immediately from HIC column, then it would be unlikely that the amphiphilic LTA fractions would contain the amount of protein suggested by the previous protein determinations.

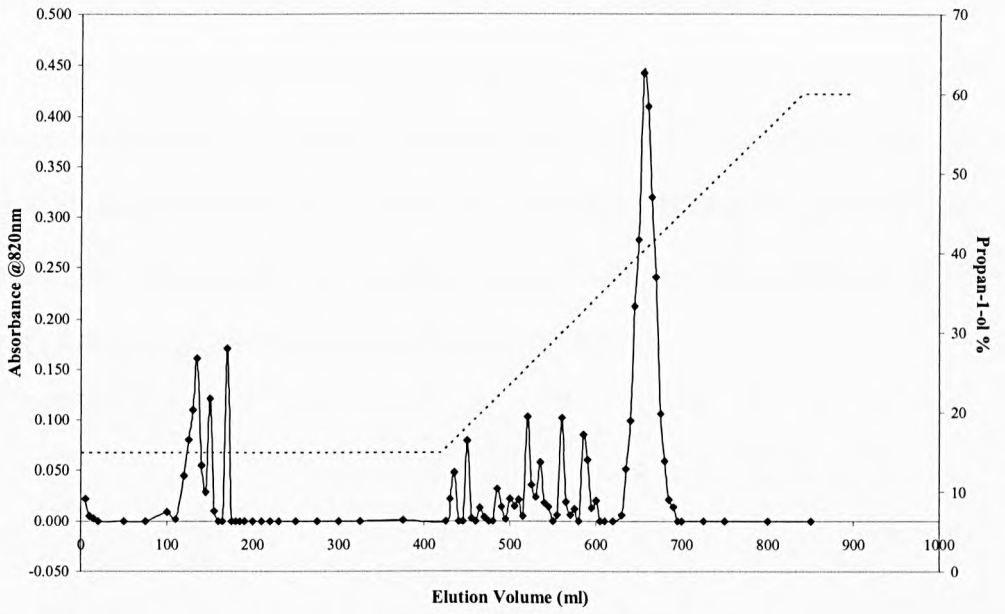


Figure 3.10 Scale-up Purification of LTA from a Crude Extract from *S. aureus*

The elution profile for the separation of LTA was determined by measuring the total amount of phosphorus present using the absorbance at 820nm (♦) within each of the eluting 5ml fractions. Elution was controlled using an increase in the propan-1-ol gradient (--).

3.4.7 HIC-elution of LMAP from a Crude Extract Obtained from P. acnes

The HIC method was applied to the suspected lipoglycan from a crude extract from *P. acnes* and each fraction was analysed for total carbohydrate, as described in Section 2.2.3. This enabled separation and elution of the LMAP extracted from *P. acnes* to be followed. The resulting elution profile (Figure 3.11) was shown to be similar to that of *S. aureus*, with the hydrophilic impurities eluting almost immediately after sample loading (100-200ml) and at approximately 40% propan-1-ol (560-675ml) the amphiphilic component began to elute from the column. Although good separation was achieved between the hydrophilic impurities and the amphiphilic components, a more detailed examination of the elution profile suggested the possibility of two LMAP components, as two peaks were present that elute very close to each other, resulting in a 'split peak' appearance (Figure 3.11). Therefore, suggesting that the two components share similar amphiphilic properties. Alternatively, the double peak might have been caused by cracks or air bubbles within the octyl Sepharose gel,

thus allowing two routes for elution. The quicker route being via the void caused by the crack or air bubble. However, after a thorough visual inspection of the column this did not appear to be the case. The LMAP-containing fractions were analysed for protein and nucleic acid where both were found to be absent therefore, the possibility of a protein and nucleic acid impurity was ruled out. To determine whether two components were present the HIC-eluted LMAP component was analysed using SDS-PAGE.

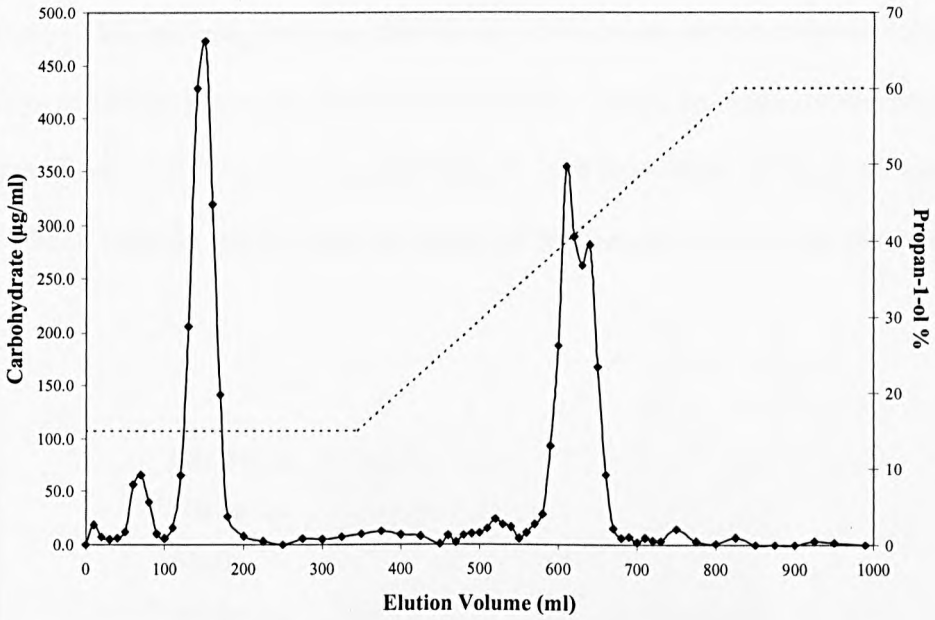


Figure 3.11 Purification of LMAP from a Crude Extract from *P. acnes*

The elution profile for the purification of LMAP from *P. acnes* was determined by measuring the total amount of carbohydrate present (♦) within the eluting 5ml fractions. Elution was controlled using an increase in the propan-1-ol gradient (--).

3.4.8 Separation of HIC-eluted LMAP by SDS-PAGE

After running the HIC-eluted LMAP material obtained from *P. acnes* on SDS-PAGE and consequent visualisation of the separated components via the silver staining procedure, the results demonstrated two distinct bands (Figure 3.12). This confirmed that rather than a column artefact, two discrete components were present that shared similar amphiphilic characters to each other. This suggested the existence of two components within *P. acnes* both of which, due to their elution on HIC, were suspected of being LMAP

components and will therefore be referred to as LMAP components from here after. By plotting the standards (distance travelled against $\log_{10}$ molecular weight) surrounding elution of the two components a curve of $R^2 = 0.982$ was produced ($y = -114.64x + 198.51$). The average distance of the two components was measured and the molecular weight of each was calculated to be 27.8kDa for the upper component and 17.5kDa for the lower component (assuming that the LMAP components run in a similar fashion to the protein standard markers). A third, faint band was also observed in the higher concentration LMAP samples. This band migrated less than the above bands and had a corresponding molecular weight of 36kDa. Due to the micellar nature of the LMAP, previously investigated in LTA [Gutberlet *et al.*, 1991], it was possible that the faint band might be due to the aggregation of the LMAP material and therefore an artefact of the sample solubilisation process.

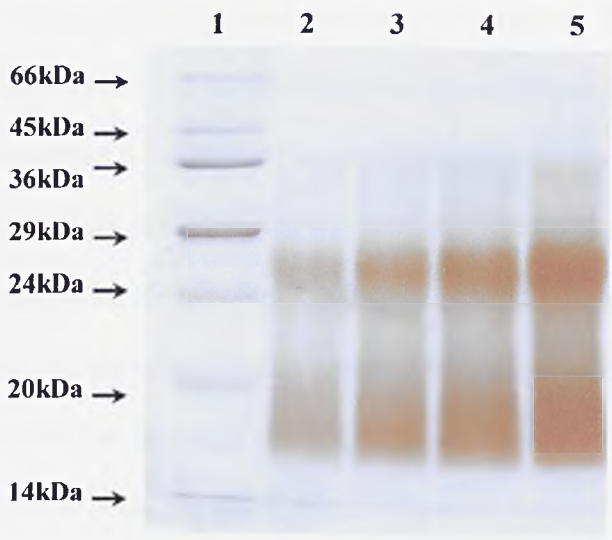


Figure 3.12 Separation of HIC-eluted LMAP by SDS-PAGE

Lane 1, SDS-7 standard markers (66kDa, bovine albumin; 45kDa, egg albumin; 36kDa, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle; 29kDa, bovine carbonic anhydrase; 24kDa, bovine pancreatic trypsinogen; 20kDa, soybean trypsin inhibitor; 14kDa, bovine milk α-lactalbumin); Lane 2, HIC-eluted LMAP (1.63μg); Lane 3, HIC-eluted LMAP (3.25g); Lane 4, HIC-eluted LMAP (4.88μg); Lane 5, HIC-eluted LMAP (6.50μg). All loaded as 5μl samples.

3.4.9 Development of Optimal Solublisation Conditions of LMAP for SDS-PAGE

In order to test the efficacy of the solubilisation solution (as discussed in Section 3.4.8) different solubilisation solutions were prepared with varying amounts of SDS and β-mercaptoethanol in attempt to disrupt possible aggregations. In addition to this, each of the samples was also subjected to sonication to ensure the maximum interaction of the LMAP components and the solubilisation solution. Following electrophoresis and detection with silver stain, significant differences could be seen between the different samples. The suspected aggregate that had a mobility corresponding to a molecular weight of 36kDa was present within the regular solubilisation solution (prepared in Section 3.3.4.1) and to a lesser extent within the 2xME sample, both with and without sonication. However, the samples solublised with the 2xSDS-sample solubilisation solution, without sonication, greatly reduced the presence of the 36kDa band and in the lane containing the 2xSDS solubilisation solution with sonication, the 36kDa band was completely absent. This demonstrated that a higher concentration of SDS was required for complete solublisation of the LMAP components and therefore for all future SDS-PAGE analyses of the LMAP components the final concentration of SDS in the samples was increased from 10%(w/v) to 20%(w/v). This observation supported the hypothesis that the faint band was an aggregation of the LMAP components and thus the SDS detergent had the ability to disrupt the aggregate. Furthermore, the molecular weight of this aggregate suggested that it was the lower component of 17.5kDa, binding a similar sized component (producing a 36kDa aggregate) and therefore responsible for the formation of the aggregate. However, during attempts to purify the aggregate from the polyacrylamide gel, via electroelution, the subsequent analysis on SDS-PAGE (using methods described in Section 3.3.6.1 and 3.3.6.2) often yielded only the upper band. This suggested that the aggregate was composed of the upper component (28kDa) and a smaller, unidentified component of approximately 10kDa, again assuming that the LMAP components run in a similar fashion to the protein standard molecular weight markers. The identity of this component would likely be hydrophobic in nature and therefore

could potentially be the lipid portion of LMAP, which fails to stain with the silver stain, due to lack of material and/or carbohydrate.

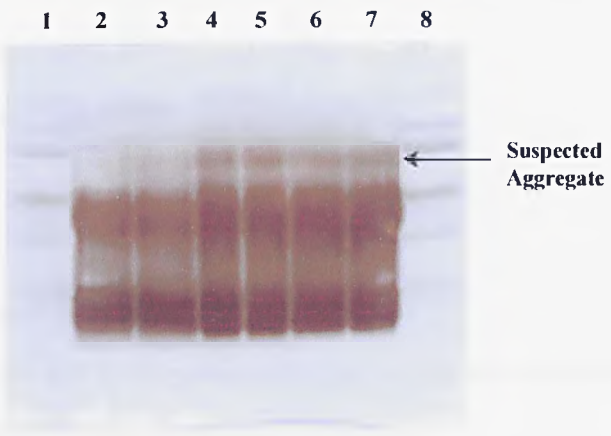


Figure 3.13 SDS-PAGE Analysis of LMAP from *P. acnes* using Different Sample Solublisation Solutions

Lane 1, SDS-7 standard markers (as in Figure 3.12); Lane 2, 7.5µg µl^{-1} LMAP in 2xSDS solublisation solution and sonicated; Lane 3, 7.5µg µl^{-1} LMAP in 2xSDS solublisation solution without sonication Lane 4, 7.5µg µl^{-1} LMAP in regular solublisation solution and sonicated; Lane 5, 7.5µg µl^{-1} LMAP in regular solublisation solution without sonication; Lane 6, 7.5µg µl^{-1} LMAP in 2xME solublisation solution and sonicated; Lane 7, 7.5µg µl^{-1} LMAP in 2xME solublisation solution without sonication; Lane 8, SDS-7 standard markers (as in Figure 3.12). All samples loaded as 5µl volumes.

3.4.10 Separation of P. acnes *LMAP Via Preparative SDS-PAGE*

In order to separate the two LMAP components for later purification, preparative SDS-PAGE was used. Following preparative electrophoresis and subsequent detection within the silver-stained gel slice, good separation of the two components was evident (Figure 3.14) and the gel image was similar to that observed within the analytical-scale SDS-PAGE, in terms of relative mobility and the abundance of each component. In addition to providing a means of assessing the separation, the staining of the gel slice also allowed the determination of the location of each of the bands on the whole gel, and thus the subsequent removal of the polyacrylamide, containing each of the separated components.

Figure 3.14 Preparative SDS-PAGE of *P. acnes* LMAP

Separation of the two LMAP components detected using a silver-stain within a 1.5cm width gel slice following preparative SDS-PAGE.

3.4.11 Removal of LMAP Components from Polyacrylamide using Electro-elution

To allow purification and subsequent analysis of LMAP components each component was separately removed from the polyacrylamide gel as described in Section 3.3.6.2, desalted and freeze-dried. By determining the mass of each of the purified components, the percentage recovery was estimated to be >70%. The majority of this loss in recovery was suspected to be due to the electro-elution stages and attributable to the high percentage of acrylamide in the gels. To assess the purity of the components each component was analysed using analytical-scale SDS-PAGE (Figure 3.15). From this gel, it could be seen that each LMAP has been successfully purified from the HIC eluted component mixture. Each component has a molecular weight corresponding to the equivalent component within the HIC-eluted material and therefore it could be concluded that the components had been successfully removed from the polyacrylamide gel without significant degradation. Sufficient purity has also been attained denoted by the absence of any other components within the silver-stained gel. Following the successful purification of the upper and lower LMAP components these were designated LMAP1 and LMAP2 respectively.

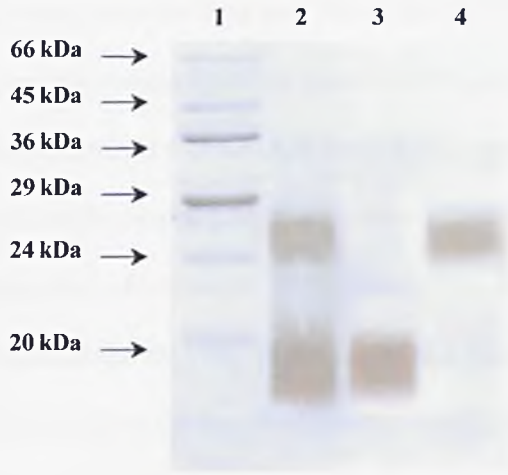


Figure 3.15 Analysis of LMAP Components via SDS-PAGE following Electro-elution Determination of the purity of LMAP components separated by preparative SDS-PAGE and electro-elution, by analytical SDS-PAGE. Lane 1, SDS-7 standard markers (as in Figure 12); Lane 2, HIC-eluted LMAP (5µg); Lane 3, lower LMAP component (~5µg); Lane 4, upper LMAP component (~5µg). 5µl sample volumes were applied to all wells.

3.4.12 Analysis of Fractions Before and After LMAP Elution from HIC

To determine the order in which the components eluted from the HIC column, three fractions eluting immediately before and immediately after the major LMAP peak (585-595ml and 655-665ml respectively from Figure 3.11) were collected and prepared for SDS-PAGE. After freeze-drying, small amounts of each sample were recovered (<0.5mg). This was expected, as only a small amount of carbohydrate was detected in these fractions. Each of the samples was made to approximately 5µg μl^{-1} in single strength solubilisation solution and run against HIC-eluted LMAP (obtained over a range of fractions i.e. 600-650ml from Figure 3.11). After silver staining, the results demonstrated that the fractions eluting prior to the major LMAP peak were composed of mainly of LMAP1. Conversely, the fractions eluting after the major LMAP peak contained mainly LMAP2 (Figure 3.16). The purity of each component was shown to be relatively poor (cf. Figure 3.15), as only in the more

concentrated samples could a visible amount of silver-stained material be seen. Increasing the amount of component recovered required the collection of fractions eluting closer to the major LMAP peak and this would therefore jeopardise the purity of each of the LMAP components obtained as a mixture of LMAP1 and LMAP2 would be observed.

This analysis provides further support for the hypothesis that within the elution profile the LMAP peak was composed of two peaks. These results also suggest that the larger 28kDa LMAP1 was more hydrophilic than that of the 17.5kDa, LMAP2. This difference in size therefore might be due to either the length of the hydrophilic chain being greater in LMAP1 or the hydrophilic chain in LMAP2 might possess more substitution along the chain. Both of these scenarios would result in a larger and more hydrophilic amphiphile eluting before LMAP2 on HIC and migrating a lesser distance on SDS-PAGE. This hypothesis was supported by the observations made by Leopold and Fischer in which it was reported that HIC has the ability to separate amphiphiles on the basis of the length of the hydrophilic moiety [Leopold & Fischer, 1992].

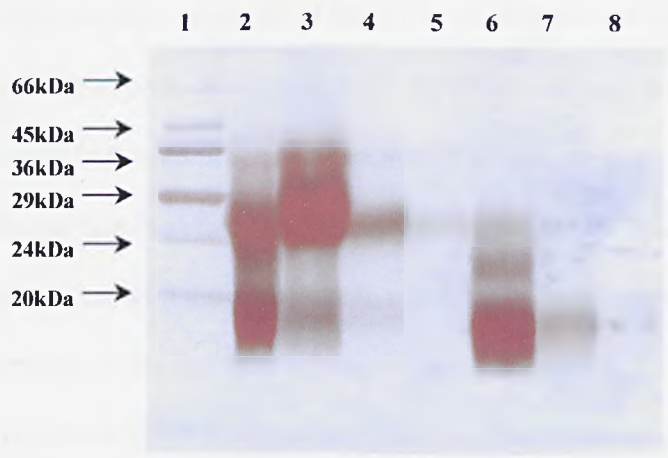


Figure 3.16 SDS-PAGE Analysis of Fractions Before and After LMAP Elution from HIC
Analytical SDS-PAGE analysis of the fractions 585-595ml and 655-665ml (see Figure 3.11). Lane 1, SDS-7 standard markers (as in Figure 3.12); Lane 2, HIC-eluted LMAP (5µg); Lane 3, fractions 117-119, before elution of major LMAP containing peak (~5µg); Lane 4, as in lane 3, diluted 5-fold (~1µg); Lane 5, as in lane 3, diluted 10-fold (~0.5µg); Lane 6, fractions 131-133, after elution of major LMAP containing peak (~5µg) Lane 7, as in lane 6, diluted 5-fold (~1µg); Lane 8, as in lane 6, diluted 10-fold (~0.5µg). 5µl samples were applied to each well.

3.5 Conclusions

In summary of these results it has been shown that octyl Sepharose was the most effective HIC medium for the separation of LTA from *S. aureus* crude extracts. Furthermore, commercially available LTA from *S. aureus* contains significant amounts of protein and nucleic acid impurity. This is supported by earlier studies that have reported similar impurities together with considerable amounts of endotoxin [Gao *et al.*, 2001]. Separation of crude cell extracts from *P. acnes* fails to yield a single LMAP peak on octyl Sepharose HIC and instead results in two closely eluting components producing a 'split-peak' appearance on the HIC elution profile. Analysis of the HIC-eluted LMAP 'split-peak' by SDS-PAGE revealed two distinct components possessing different degrees of migration. Application of the HIC-eluted material to preparative SDS-PAGE and consequent electro-elution to remove each component from the polyacrylamide allowed purification of two components. These two components were found to elecrophoretically migrate in the 28kDa region and 17.5kDa region of the SDS-PAGE gel. Due to the elution characteristics from HIC, both components were suspected of being LMAP components and were designated as LMAP1 and LMAP2 respectively. Finally analysis of the start and end fractions surrounding the elution of the LMAP 'split peak' material from HIC demonstrated that LMAP1 eluted prior to LMAP2 and was therefore less hydrophobic than LMAP2. This difference in hydrophobicity together with the difference in migration on application to SDS-PAGE suggested a difference in the structure of each LMAP component.

Following the successful purification of the LMAP components from *P. acnes*, sufficient amounts of each were purified to allow the further characterisation of the chemical and molecular properties of each LMAP component.

CHAPTER 4

Chemical & Immunochemical Characterisation of the Amphiphiles of *Propionibacterium acnes*

4.1 Introduction

SDS-PAGE analysis in Section 3.4.8 revealed the existence of two discrete LMAP components within *P. acnes*. The molecular weight of these components was estimated to be approximately 28kDa for the upper component (LMAP1) and approximately 17.5kDa for the lower component (LMAP2). These findings were similar to the observation that mycobacterial lipomannan also typically runs in the approximately 14-20kDa region whereas the larger LAM (with additional arabinan units) runs in the approximately 36kDa region [Chatterjee *et al.*, 1992b]. Moreover, when fractions eluting either directly before or after the bulk of the HIC-eluted material were selected and subsequently analysed by SDS-PAGE they were found to contain predominantly LMAP1 and LMAP2, respectively. Elution from HIC yields progressively more hydrophobic components, but where the hydrophobic moiety was similar, fractionation based on the size of the hydrophilic component was also possible [Fischer, 1996], with larger amphiphiles eluting first. Collectively, this data suggested that LMAP2 possessed a shorter glycan chain in comparison to LMAP1. Whether there was any direct relationship (e.g. at the biosynthetic level) between these two components remains to be determined, although again it was notable that both LAM and lipomannan were both simultaneously present in the membranes of mycobacteria [Chatterjee & Khoo, 1998].

Following the extraction and purification of these components by preparative SDS-PAGE, the structures of each were investigated to characterise the chemical moieties constituting the two components. This initially involved the identification of the carbohydrate possessed by both, followed by analysis of the fatty acids and amino groups present. The immunochemical properties were also examined to enable a structural comparison, with other previously identified LMAP components and therefore facilitating chemotaxonomic comparisons to be made.

4.2 Materials

4.2.1 Reagents

Mannose-capped lipoarabinomannan (ManLAM), isolated from *M. tuberculosis* strain H37Rv and two monoclonal antibodies, CS-40 (α-ManLAM) and CS-35 (α-LAM) were kindly provided by Dr John T. Beslisle (Colorado State University, Fort Collins, CO), through the National Institutes of Health, NIAID, Contract NO1-AI-75320, entitled "Tuberculosis Research Materials and Vaccine Testing". Alditol acetate and fatty acid methyl ester (FAME) standards were obtained from Supelco, UK. All organic solvents and acids were of analytical grade and were purchased from Fisher Scientific, UK. Lectin, secondary antibodies and all other reagents were of analytical grade and purchased from Sigma, UK. Purified water was deionised (18mΩ cm) and filtered through a 0.2μm membrane via a Milli-Q system, Millipore, UK. Gases purchased from BOC, UK were used throughout.

4.2.2 Consumables

Nitrocellulose membranes were purchased from Bio-Rad, UK. Polystyrene and polypropylene universals and centrifuge tubes were obtained from Bibby Sterilin, UK. All filtration membranes and filters were purchased from Whatman, UK. All other consumables were supplied by Fisher Scientific, UK.

4.2.3 Equipment and Instruments

Gas chromatography with FID was performed on a Hewlett Packard 5860 using either a DB225 or DB5 capillary column produced by J&W Scientific, USA. Mass spectrometry was accomplished using a Hewlett Packard 5971A . Electroblotting of polyacrylamide gels was achieved using a Mini Protean II Trans-blot (Bio-Rad, UK). Spectrometric analyses were performed using a Dynex Revelation plate reader, (Dynex,

UK). Centrifugation of samples was achieved using a PK121R bench centrifuge (ALC, UK). All temperature-controlled incubations were carried out using either a static incubator (Gallenkamp, Germany) or an orbital shaker (Stuart Scientific, UK) with the exception of sample hydrolysis procedures used a heating block (Grant, UK). Procedures which required temperatures above 60°C which used an EAF furnace (Carbolite, UK). All weights were determined using a AC100 analytical balance (Mettler Toledo, UK).

4.3 Experimental Methods

4.3.1 Analyses of HIC-eluted LMAP for Carbohydrate and Phosphorus

The amounts of carbohydrate and phosphorus were determined within the HIC-eluted LMAP component to allow the molar ratio to be calculated. LTA's tend to have a lower carbohydrate/phosphorus ratio (<1) due to the large amount of phosphorus present within the hydrophilic moiety [Sutcliffe, 1994b]. Lipoglycans however, were expected to possess much larger ratios (approx. 30) due to the large polysaccharide hydrophilic moiety and the relatively limited amount of phosphorus [Sutcliffe, 1994b]. Obtaining the carbohydrate/phosphorus ratio was therefore indicative of whether the LMAP was either LTA or lipoglycan and was an initial step in the process of chemical characterisation. Carbohydrate and phosphorus was determined as described in Sections 2.2.3 and 2.2.4, respectively. Using the data from each analysis the molar ratio was calculated. For the calculation of molar amount of carbohydrate the molecular weight of glucose was used.

4.3.2 Preparation of LMAP by Acid Hydrolysis

For analysis of the carbohydrates and amino groups suspected within the HIC-eluted LMAP components, the LMAP was hydrolysed under acidic conditions to degrade the polymers and allowed the release of the individual compounds. There were many methods described in the literature to enable hydrolysis. These include treatment with 2M

trifluoroacetic acid (TFA) for 14 hours at 100°C [Bergstrom *et al.*, 2002], 4M hydrochloric acid for 2 hours at 100°C [Campo *et al.*, 2001] and 2M TFA for 2 hours at 120°C [Linker, Evans & Impallomeni, 2001]. For the present study 2M hydrochloric acid was used for 4 hours at 120°C as described by Sutcliffe [Sutcliffe, 1995] as this method has been reported for lipoglycans from a variety of other bacteria [Sutcliffe, 1994a; Sutcliffe, 1995].

Freeze-dried LMAP component (1mg) was dissolved in 250µl of 2M HCl which was then sealed in a glass Pasteur pipette by constricting both ends over a hot Bunsen flame. The LMAP solution was incubated at 120°C in a heating block for 4 hours.

Following hydrolysis the tube was cooled to room temperature and the top of the tube was removed by vigorously heating the upper end of the hydrolysis tube. To remove the HCl from the hydrolysed material, the sample was placed in a desicator containing silica-gel desiccant and NaOH pellets i*n vacuo* at 50°C for 12 hours. The hydrolysed sample was then resuspended in 200µl of purified water and dried as before. Finally, the hydrolysed sample was resuspended in 100µl and transferred to a 2ml glass vial. This was freeze-dried and stored desiccated, at -80°C until required.

4.3.3 Examination of LMAP Carbohydrates by Gas Chromatography

It was predicted that the LMAP components of *P. acnes* would be lipoglycans as opposed to LTA due to the high guanine-cytosine % molar ratio determined in *P. acnes* [Cummins & Johnson, 1981; Fischer, 1994a; Fox *et al.*, 1980]. This was supported by the analysis of total carbohydrate in post-HIC fractions obtained from a crude extract of *P. acnes*, in which the amphiphilic components were shown to contain significant amounts of carbohydrate (discussed in Section 3.4.7). The structural characterisation of other lipoglycans (Section 1.3) suggested that the hydrophilic portion of the component would be the major carbohydrate-containing moiety. To allow the comparison of *P. acnes* LMAP with other lipoglycan structures the individual carbohydrate units were determined. To facilitate this, LMAP components required hydrolysis to break down polymeric structures. The resulting monomeric carbohydrate units were derivatised to form corresponding alditol

acetates and these were separated and identified using gas chromatography (GC). The method used was similar to the method described by Saddler [Saddler *et al.*, 1991], but the amount of material initially used was only 1mg compared to between 50-100mg of whole cell biomass [Saddler *et al.*, 1991]. Using the derivatised samples of LMAP1 and LMAP2, the carbohydrates were identified using GC [Saddler *et al.*, 1991]. In this procedure the temperature profile was modified to maximise resolution of the carbohydrate peaks. Separation of the alditol acetate species used a GC column consisting of 50% cyanopropylphenylsiloxane and 50% dimethylpolysiloxane as a stationary phase. This phase was considered to be of mid-to-high polarity. The carbohydrate derivatives elute from the column in relation to number of carbon atoms, therefore, glycerol (C3) was the first to elute and the hexoses were the last (C6). These conditions also permitted the separation of similar sugars such as mannose, glucose and galactose; these carbohydrate structures differing only in the relative position of the hydroxyl groups. Detection of the separated alditol acetates after chromatography relied on a flame ionisation detector (FID) and the resulting retention times were compared to a mixture of carbohydrate standards derivatised in the same way.

4.3.3.1 Derivatisation Procedure

1mg of LMAP1, LMAP2 and HIC-eluted LMAP (i.e. mixture of both components) were prepared as described in Section 4.3.2. The freeze-dried hydrolysed samples were each resuspended in 150µl of 3.6M ammonium hydroxide (prepared by mass) and transferred to a 12ml screw-capped Pyrex tube. To each sample was added 15µl of 100mg ml^{-1} sodium borohydride prepared in 3.6M ammonium hydroxide. The tubes were incubated at 37°C for 1 hour. After this time, 50µl of glacial acetic acid was added to decompose residual borohydride and the solution cooled to 4°C. To each sample 300µl of 1-methylimidazole was slowly added, followed by 2ml of acetic anhydride. The addition of these reagents initiates a highly exothermic reaction thus, the sample solutions were maintained on ice while these reagents were added to ensure the temperature of the reaction mixture did not increase significantly. After addition, the samples were shaken vigorously and left to stand

for 15 minutes at room temperature to allow complete acetylation of the sample carbohydrate. Purified water (5ml) was added to each sample to decompose excess acetic anhydride. This was followed by the addition of 2ml of dichloromethane and the tubes were shaken vigorously for 3 minutes. The tubes were then centrifuged at 500 x g for 30 minutes at room temperature to separate the aqueous and organic phases. The lower organic phase was aspirated and transferred to a 4ml vial. The derivatised sample was concentrated to dryness under a stream of oxygen-free nitrogen and stored at -80°C for subsequent analysis.

4.3.3.2 Analysis of Alditol Acetate Derivatives via Gas Chromatography

Both the injection and detection ports were set at 280°C. A DB225 column was used of dimensions 30m x 0.25mm internal diameter and film thickness of 0.15µm. Helium was used as the carrier gas and was set to 34cm s^{-1} linear velocity (1ml min^{-1} column flow) with a 1/50 split ratio. Hydrogen and air flow rates were set to 20 and 400ml min^{-1} respectively for the FID. The samples prepared in Section 4.3.3.1 were resuspended in 10µl of dichloromethane and from this 0.25µl was injected onto the column using a 0.50µl Hamilton syringe. Following elution of all samples, 0.25µl of standard alditol acetate mixture was injected, again using a 0.50µl Hamilton syringe. The temperature profile used for the chromatography is shown in Figure 4.1

Figure 4.1 Temperature Profile for the Separation of Alditol Acetates by GC

The oven temperature followed the above profile to allow the separation of alditol acetates produced by the derivatisation of carbohydrates from *P. acnes* LMAP.

The retention times of each of the carbohydrate constituents separated within the LMAP samples were compared to the retention times provided by the standard alditol acetate mixture and the identities were assigned, together with the relative abundance of each carbohydrate in both samples.

4.3.3.3 Examination of LMAP Carbohydrates by Gas Chromatography-Mass Spectrometry

To allow a more detailed examination of the eluting carbohydrates from the GC column, a mass spectrometer was used for detection. Due to the GC-MS configuration, the GC column used was less polar than the column described in Section 4.3.3.2 and for this reason, the method was amended to achieve adequate resolution.

- Procedure:

Both the injection and detection ports were set at 280°C. A DB5 column was used of dimensions 30m x 0.25mm internal diameter and film thickness of 0.15µm. Helium was used as the carrier gas and was set to 34cm s^{-1} linear velocity (1ml min^{-1} column flow) and the flow was splitless. The HIC-eluted LMAP hydrolysate was prepared as described in Section 4.3.3.1 and was resuspended in 10µl of dichloromethane. From this solution 0.1µl was injected onto the column using a 0.50µl Hamilton syringe. Glucose, galactose, mannose and *myo*-inositol standards were derivatised as described in Section 4.3.3.1 and following elution of all samples, 0.10µl of each of these standards was injected using a 0.50µl Hamilton syringe. The temperature profile used for the chromatography is shown in Figure 4.2. The acquisition mode of the MS detector was set to 'Scan' to allow detection of fragments with molecular weights ranging between 50 and 550. The electron gun was set to an electromagnetic voltage of 2140V and the resulting mass spectrum was recorded for the inositol-like material within the sample and compared to the inositol standard. The retention times of other carbohydrate peaks were compared to the derivatised carbohydrate standards described above in order to confirm their identities within the GC-MS spectrum as described with the GC-FID analysis in Section 4.3.3.2.

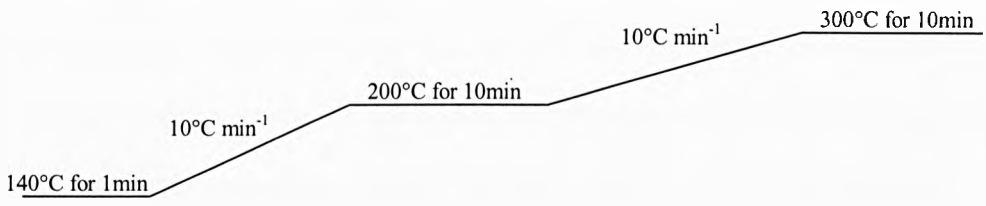


Figure 4.2 Temperature Profile for the Separation and Detection of Alditol Acetates by GC-MS

The oven temperature followed the above profile to allow the separation of alditol acetates produced by the derivatisation of carbohydrates from *P. acnes* LMAP and consequent detection by MS.

4.3.4 Examination of LMAP Fatty Acids by Gas Chromatography

For the analysis of fatty acids within whole *P. acnes* cells and the purified LMAP components gas chromatography was employed. The separation of fatty acids by GC required a derivatisation step, in which the fatty acids were converted to their corresponding fatty acid methyl esters (FAME's). The method was devised by Komagata [Komagata & Suzuki, 1987], but has been improved by exchanging the methanolic hydrochloric acid for methanolic sulphuric acid during the methylation procedure [Christie, 1989]. The FAME's were consequently extracted using phase separation with hexane and analysed by GC.

4.3.4.1 Derivatisation Procedure

To ≤1mg of each purified, freeze-dried LMAP component or 20mg of whole *P. acnes* cells (prepared as described in Section 2.3.4.3, washed twice with purified water and freeze-dried) was added 2ml of 1.5%(v/v) concentrated sulphuric acid in anhydrous methanol which was prepared by adding 1g of anhydrous sodium sulphate to 100ml methanol and filtering through a 0.45µm PTFE syringe filter. The samples were incubated for 12 hours at 50°C to allow trans-esterification. Following incubation the solution was cooled to room temperature and 1ml of purified water was added, together with 3ml of

hexane. The tubes were stoppered, shaken vigorously for 3 minutes and left to stand to allow the phases to separate. Once the phases were clear, the upper hexane layer was transferred to a clean tube and a further 3ml of hexane was added to the aqueous esterification solution. The tubes were shaken vigorously as before and once again left to allow the phases to separate. The hexane layer was removed and pooled with the initial hexane extract. This was repeated, resulting in 9ml total hexane extract. To the hexane extract was added an equivalent volume of purified water to back-wash the extract. This was shaken vigorously for 3 minutes and the phases were left to stand. Once the phases had separated the hexane fraction was transferred to a clean tube containing 0.5g of anhydrous Na_2SO_4 to remove traces of water. The extract was concentrated under a stream of oxygen-free nitrogen to 1ml and then transferred to a 3ml glass vial. The extraction tube was washed with 0.5ml of hexane and this was added to the vial. The extract was concentrated, as before, to a final volume of approximately 25µl, capped and stored at -80°C until required.

4.3.4.2 Analysis of FAME Derivatives by via GC

Both the injection and detection ports were set at 280°C. The column used was a DB225 of dimensions 30m x 0.25mm internal diameter and film thickness of 0.15µm. Helium was used as the carrier gas and was set to 1ml min^{-1}, with a 1/50 split ratio. Hydrogen and air flow rates were set to 20 and 400ml min^{-1} respectively for the FID. Using the samples prepared in Section 4.3.4.1, 0.5µl was injected onto the column using a 0.5µl Hamilton syringe. The temperature profile used for the chromatography is shown in Figure 4.3.

Figure 4.3 Temperature Profile for the Separation of FAME's by GC

The oven temperature followed the above profile to allow the separation of FAME's produced by the derivatisation of fatty acids from whole cells of *P. acnes* and purified LMAP components.

Following the elution of all samples, 0.5µl of a FAME standard was injected, containing an array of FAME's, representative of the FAME's expected within the samples.

The retention times of each of the FAME's separated within LMAP samples, together with the whole cell preparations were compared to the retention times provided by the standard FAME mixture. From these retention times the identities of each peak was assigned, together with the relative abundance of each fatty acid within the samples.

4.3.5 Examination of LMAP Components for Amino Sugars

4.3.6.1 Analysis of LMAP Components using Ninhydrin-Collidine Reagent

LMAP components were hydrolysed as described in Section 4.3.2 and resuspended in purified water to a concentration of 1mg ml^{-1} (calculated as a pre-hydrolysis mass). From this solution a 1/5 dilution (200µg ml^{-1}) and a 1/10 dilution (100µg ml^{-1}) were prepared using purified water. A glycine standard was also prepared to 10mg ml^{-1} in purified water and used as a positive control. From this standard solution a 10-fold dilution series was prepared ranging between 10mg ml^{-1} to 1µg ml^{-1}, diluting with purified water. Each sample and standard was vortexed for 30 seconds to aid dissolution and from each solution 15µl was spotted onto an amino acid-free sheet of Whatman No.1 filter paper. 15µl of purified water was also spotted and used as a negative control. The filter paper was dried at 40°C for 15 minutes. Once dry, the filter paper was evenly sprayed with ninhydrin-collidine reagent, prepared by dissolving 50mg of ninhydrin in 50ml of 95%(v/v) ethanol in an aqueous

solution containing 20%(v/v) glacial acetic acid and 2%(v/v) 2,4,6-collidine. The filter paper was developed in an oven at 90°C for precisely 3 minutes, after which the filter paper was removed and observations were made immediately, as the colour begins to degrade after approximately 5 minutes.

Comparisons were made of the colour and intensity of each of the LMAP samples to the range of positive (glycine) and negative (purified water) controls.

4.3.5.2 Estimation of Aminohexuronic Acid within HIC-eluted LMAP

The amount of amino acid present within the LMAP material was estimated using the Morgon-Elson reaction which utilises dimethylaminobenzaldehyde (DMAB). This reagent binds *N*-acetylglucosamine and therefore any amino sugars available within the LMAP material initially required *N*-acetylation. 2mg of HIC-eluted LMAP was hydrolysed as described in Section 4.3.2 and resuspended in 500µl of purified water. From this solution 100µl was removed and adjusted to 500µl with purified water (5-fold dilution). This was used to prepare a further three, 5-fold, serial dilutions. An aqueous glucosamine standard solution was prepared to a concentration of 100nmol using glucosamine hydrochloride. 400µl of both the sample dilutions and the glucosamine standard were added to separate 9ml screw-cap tubes. To each was added with 50µl of 1.5%(v/v) acetic anhydride prepared fresh in dry acetone (prepared by adding 1g of anhydrous sodium sulphate to 100ml acetone and filtering through a 0.45µm PTFE syringe filter). This was incubated for 5 minutes at room temperature to allow acetylation. To the acetylated derivatives was added 150µl of 0.7M potassium tetraborate solution at pH9.2. The tubes were sealed and placed in a boiling waterbath for 3 minutes. After such time the tubes were removed from the waterbath and cooled to room temperature. To the samples and standards was added 300µl of 10%(w/v) DMAB, prepared in glacial acetic acid containing 5%(v/v) concentrated sulphuric acid. This was followed by 2.7ml of glacial acetic acid. The tubes were vortexed for 60 seconds and incubated at 37°C for 20 minutes. Following incubation, the absorbance each was determined at 595nm and compared to the 100nmol standard solution of glucosamine which

has been previously reported to produce an absorbance of 0.5 at 595nm [Hancock & Poxton, 1988].

4.3.6 Structural and Immunochemical Analyses of LMAP Components

To allow further characterisation of the LMAP components the structural and immunochemical properties of each of the components were examined. To facilitate this, SDS-PAGE was performed to separate the LMAP components within the HIC-eluted LMAP. The separated components were then transferred to nitrocellulose via electro-blotting. Once bound to nitrocellulose the components were probed with Concanavalin A. In addition, both monoclonal and polyclonal antibodies directed towards LAM were also used to establish the immunochemical properties of the two LMAP components and allowed comparisons to be made between the LMAP of *P. acnes* and LAM of mycobacteria.

4.3.6.1 Transfer of SDS-PAGE-Separated LMAP to Nitrocellulose via Electroblotting

To enable the structural and immunochemical properties to be investigated, the LMAP components were prepared as described in Section 3.3.4.1 and separated on SDS-PAGE (Section 3.3.4.3). The gel was then electroblotted onto nitrocellulose. Electroblotting allowed the electrophoretic transfer of the separated components onto a solid support using a method originally described by Towbin [Towbin, Staehelin & Gordon, 1979]. The method was modified to maximise the transfer efficiency to the nitrocellulose. This involved the reduction of the voltage applied and an increase in the electroblotting-time. The transfer buffer was also modified to further increase transfer efficiency.

- Procedure:

Following SDS-PAGE separation (Section 3.3.4.3) the gel was separated from the gel cassette and the glass plates were carefully removed. The bottom, right corner of the gel was clipped to allow the orientation of the gel to be assigned. Transfer buffer was prepared which consisted of 25mM Tris base, 192mM glycine and 20%(v/v) methanol, the resulting

pH was pH8.3. A 0.45µm-nitrocellulose membrane and two pieces of Whatman No.1 filter paper were cut to the size of the gel. These were soaked in transfer buffer, together with two fibre pads for 30 minutes. The polyacrylamide gel was placed facedown onto the pre-soaked nitrocellulose membrane, with the clipped corner maintained on the bottom, right-hand-side. To both the gel and the nitrocellulose membrane, the pre-soaked filter paper was positioned, followed by the fibre pads. The air bubbles were removed from all surfaces and completely wetted with transfer buffer. The resulting gel-membrane stack was tightly sandwiched between a transfer cassette and the cassette was placed into the electrophoresis tank, with the gel-side of the cassette facing the cathode. The electrophoresis tank was half-filled with transfer buffer and a frozen heat-sink was positioned in the tank. Transfer buffer was added to completely submerge the transfer cassette and a stirring bar was placed at the bottom of the electrophoresis tank.

The electrophoretic transfer was performed at 30V constant voltage for 16 hours with continuous stirring throughout.

After the electrophoretic transfer was complete, the transfer cassette was dismantled. The polyacrylamide gel was fixed and silver-stained as described in Section 3.3.4.4 to ensure transfer of LMAP components was non-discriminant. The nitrocellulose membrane was washed in a PBS-Tween solution prepared using phosphate buffered saline (PBS) at pH7.4 (Dulbecco's A) containing 0.05%(w/v) Tween 80. The nitrocellulose membrane was stained with 0.1%(w/v) Ponceau S aqueous solution containing 5%(v/v) glacial acetic acid to detect the protein standard markers. Once the standard markers had been identified, the location of each band was marked with a soft pencil and the Ponceau S solution was removed by washing with three changes of PBS-Tween for 10 minutes each. A blocking solution was prepared by dissolving 0.05%(v/v) Tween 80 in phosphate buffered saline at pH7.4 (Dulbecco's A) and 5%(w/v) skimmed milk powder. The nitrocellulose membrane was washed in the blocking solution five times for 15 minutes each, to block the non-specific binding sites on the nitrocellulose membrane. The blot was then ready for use within immunochemical investigations.

4.3.6.2 Structural Characterisation of LMAP using Concanavalin A

Concanavalin A (Con A) is a 55kDa lectin extracted from the Jack Bean plant, which preferentially binds terminal mannose residues (and to lesser extent glucose) [Barondes, 1981]. The lectin requires the presence of both Mn^{2+} and Ca^{2+} for transformation of the lectin into a 3-dimensional structure leading to the formation of a mannosyl binding site [Angal & Dean, 1995]. To determine whether the LMAP components possessed this structural feature, LMAP components were electroblotted onto nitrocellulose and probed with this Con A.

- Procedure:

HIC-eluted LMAP was prepared as detailed in Section 3.3.4.1 to a final concentration of 1.3μg μl^{-1}. Mycobacterial LAM was also prepared as described in Section 3.3.4.1 to a final concentration of 2μg μl^{-1}. Protein standard molecular weight markers were prepared as described in Section 3.3.4.1. Two polyacrylamide gels were prepared as detailed in Section 3.3.4.2 and each pre-formed well was loaded with 5μl samples. Both gels were loaded identically and were run as described in Section 3.3.4.3. After electrophoresis the first gel was fixed and silver-stained as detailed in Section 3.3.4.4. The second gel was electroblotted as detailed in Section 4.3.6.1. Following identification of the standard protein markers and removal of the Ponceau S stain, the nitrocellulose membrane was blocked with blocking solution. The membrane was rinsed, twice, for 5 minutes with PBS-Tween and incubated for 2 hours with Con A-biotin conjugate, prepared as a 20μg ml^{-1} solution in PBS-Tween, containing 0.1mM $CaCl_2$ and 0.1mM $MnCl_2$. This incubation was performed at room temperature on an orbital shaker set to 50rpm. After such time, the Con A-biotin conjugate was removed and washed five times for 15 minutes each with PBS-Tween to remove non-bound lectin. Following washing, the membrane was incubated with Extravidin (streptavidin-alkaline phosphatase conjugate) prepared as a 1/70,000 dilution in PBS-Tween, for 1.5 hours at room temperature and shaking as before. After incubation the Extravidin was removed and the membrane was washed five times for 10 minutes each with PBS-Tween. Immediately prior to use, a 10ml solution of SigmaFast, 5-bromo-4-chloro-3-indolyl

phosphate/nitroblue tetrazolium (BCIP/NBT) alkaline phosphatase substrate was prepared with the addition of 1 tablet to 10ml of purified water. Once completely dissolved this was added to the nitrocellulose membrane and incubated at room temperature until sufficient colour had developed, but before excessive background staining occurred. The blot was dried on tissue and scanned on a flatbed scanner.

4.3.6.3 Investigation into the Immunochemical Properties of LMAP using Antibodies Directed Towards LAM

Three different antibodies were chosen to investigate the structural similarities of LMAP components in relation to mycobacterial LAM. These antibodies consisted of a polyclonal antibody raised against mycobacterial LAM (A193), a monoclonal antibody raised against all types of mycobacterial LAM (CS-35) and lastly, a monoclonal antibody specific for mycobacterial mannose-capped LAM (CS-40) the structural differences between AraLAM and ManLAM are described in Section 1.3.3. All LAM primary antibodies were obtained as discussed in Section 4.2.1 and used to probe the separated and electroblotted LMAP components on nitrocellulose (prepared as described in Section 4.3.6.1). The polyclonal antibody was raised in rabbit and therefore required an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G secondary antibody where as the monoclonal antibodies used an alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G secondary antibody for the detection of successfully binding primary antibodies.

- Procedure:

HIC-eluted LMAP was prepared as detailed in Section 3.3.4.1 to a final concentration of 1.9μg μl^{-1}. Mycobacterial LAM was also prepared as described in Section 3.3.4.1 to a final concentration of 2μg μl^{-1}. Standard protein molecular weight markers were prepared as described in Section 3.3.4.1. The polyacrylamide gel was prepared as detailed in Section 3.3.4.2 and loaded with 5μl samples in each pre-formed well. One half of the gel was loaded with the required samples and standards and this was repeated in the second half of the gel. This resulted in both sides of the gel identically loaded. The gel electrophoresis was run as

described in Section 3.3.4.3. After electrophoresis the first half of the gel was fixed and silver-stained as described in Section 3.3.4.4. The second half of the gel was electroblotted as detailed in Section 4.3.6.1 and the spent polyacrylamide gel used for the electroblotting procedure was silver-stained to ensure all components had been successfully transferred the nitrocellulose membrane. After identification of the standard protein markers and removal of the Ponceau S stain, the nitrocellulose membrane was blocked with blocking solution (also outlined in Section 4.3.6.1). Electroblotted membranes were incubated under the following conditions:

- Polyclonal rabbit anti-LAM (A193)

Prepared as a 1/1,200 dilution in blocking solution and incubated for 2 hours at 10°C while shaking on an orbital shaker at 50rpm. The non-bound antibody was removed by washing with five times, for 10 minutes each with blocking solution. The nitrocellulose blots were incubated for 2 hours with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G secondary antibody prepared as a 1/30,000 dilution in blocking solution.

- Monoclonal mouse anti-LAM (CS-35) and anti-ManLAM (CS-40)

Both monoclonal antibodies were prepared as 1/1,000 dilutions in blocking solution and incubated for 16 hours at room temperature while shaking on an orbital shaker at 50rpm. The non-bound antibody was removed by washing with five times, for 10 minutes each with blocking solution. The nitrocellulose blots were incubated for 2 hours with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin G secondary antibody prepared as a 1/10,000 dilution in blocking solution.

Following the above polyclonal and monoclonal antibody incubations, the non-bound antibody was removed by washing five times, for 10 minutes each with blocking solution. The nitrocellulose blots were incubated with a 10ml solution of SigmaFast BCIP/NBT (as described in Section 4.3.6.2) at room temperature until sufficient colour had developed, but without excessive background staining. The blot was dried on tissue and scanned on a flatbed scanner.

4.4 Results and Discussion

4.4.1 Analyses of HIC-eluted LMAP for Carbohydrate and Phosphorus

The amounts of both carbohydrate and phosphorus were determined in three samples of *P. acnes* LMAP following HIC-elution. The results of each assay were converted to average molar amounts and using these amounts the molar ratio was calculated. The molar amounts of each were calculated to be 0.16µmol of phosphorus and 2.74µmol of carbohydrate (using the molecular weight of glucose for the determination) per mg of freeze-dried HIC-eluted LMAP material. From these calculations, the carbohydrate to phosphorus ratio was 17:1. This strongly suggested the identity of the LMAP to be a lipoglycan as opposed to LTA, as LTA would be expected to possess a ratio of 1:1 or less [Sutcliffe, 1994b]. This value represented an average ratio, as it has been shown in the analysis by SDS-PAGE (detailed in Section 3.4.8) that a range of molecular weights existed within the HIC-eluted LMAP. It was suspected that this range in molecular weight was governed by the length of the hydrophilic chain, which has been shown to vary considerably within purified samples prepared using HIC [Fischer, 1996; Leopold & Fischer, 1992].

4.4.2 Examination of LMAP Components for Carbohydrates by Gas Chromatography

From the analysis of the carbohydrate derivatives good chromatographic profiles were produced in all samples analysed with clear resolution between the intended analytes (Figure 4.4).

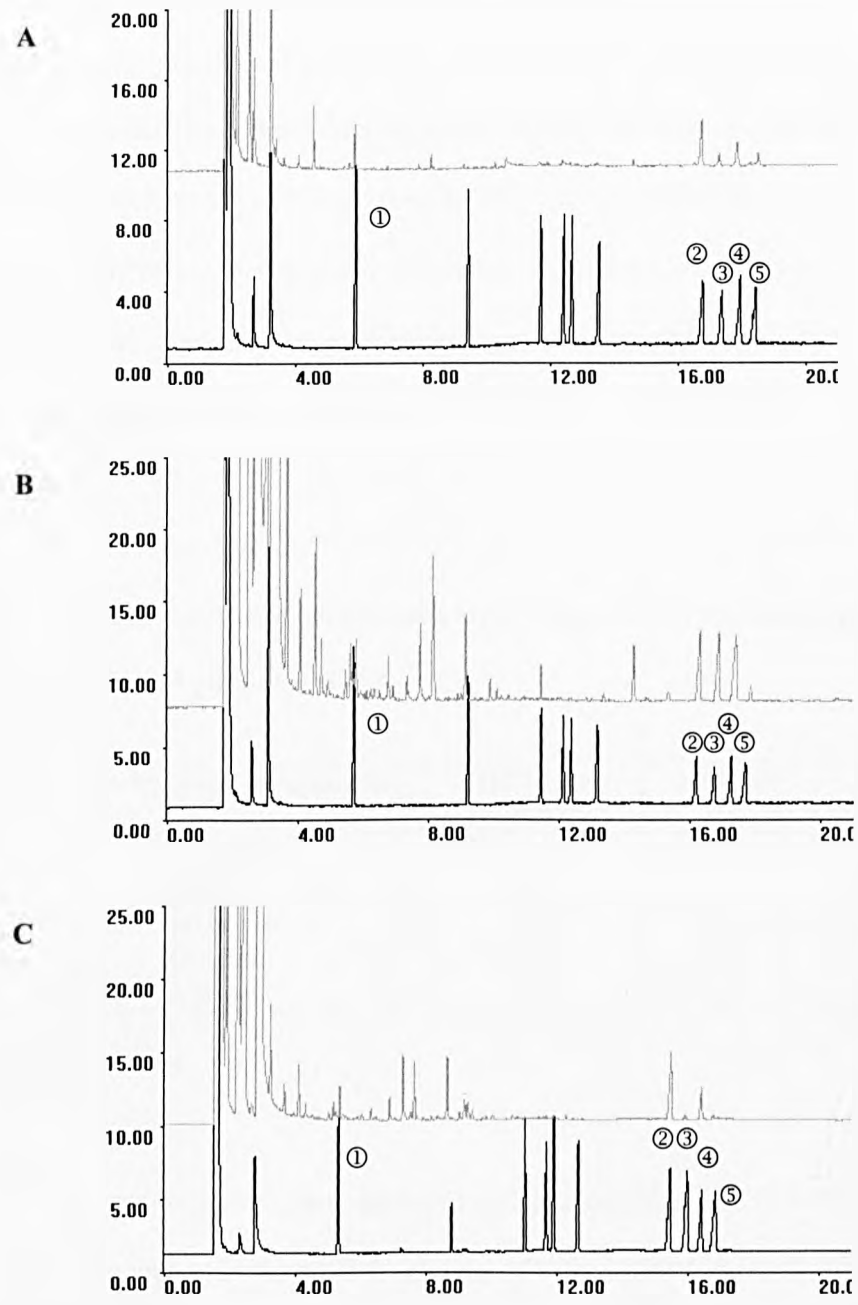


Figure 4.4 Analysis of the LMAP Components for Carbohydrates by GC

The carbohydrates of each LMAP component were analysed by derivatisation to the corresponding alditol acetates. These were separated and detected using GC-FID and the chromatograms produced illustrate the FID response against the retention time (min). In all chromatograms, the lower trace represents the standard alditol acetate mixture, where ① to ⑤ were glycerol, mannose, galactose, glucose and inositol respectively. The upper trace is the LMAP component. HIC-eluted LMAP (A), LMAP1 (B) and LMAP2 (C).

From the analysis of HIC-eluted LMAP, significant amounts of glycerol, mannose, galactose and glucose were observed. Furthermore, a peak was also apparent eluting with a retention time similar to inositol, although the retention time for this analyte was marginally greater than the inositol within the standard derivatisation. In both of the purified LMAP components, there was also a significant amount of glycerol, mannose and glucose, with smaller amounts of galactose and the peak suspected of being inositol. A difference in the relative abundance of galactose and glucose was observed between the two components (see Table 4.1).

Table 1. Comparison of the relative carbohydrate composition of HIC-eluted component, purified LMAP1 and purified LMAP2.

Carbohydrate Assignment	HIC-eluted LMAP (%)	LMAP1 (%)	LMAP2 (%)
Glycerol	6.7	0.9	8.1
Mannose	45.5	30.6	66.5
Galactose	18.6	29.1	Trace
Glucose	22.8	34.9	25.4
Inositol	6.4[†]	4.4[†]	Trace[†]

[†] Suspected as inositol.

LMAP1 appeared to possess more galactose and glucose relative to LMAP2 and this might have ultimately increased the relative amounts of both glycerol and mannose. The difference in the amount of galactose and glucose could have been due to a decrease in the amount of substitution within the hydrophilic moiety and therefore would reduce the overall size of LMAP2. In support of this was the increased migration of LMAP2 on SDS-PAGE demonstrated in Section 3.4.8, which suggested that LMAP2 was a smaller component in relation to LMAP1. The interaction between the SDS and LMAP has not been investigated and therefore it was not known whether the migration of LMAP components on SDS-PAGE was dependent on size only (as with proteins) and not influenced by charge differences between the components. However, the SDS-PAGE analysis of the fractions eluting

immediately prior to the HIC-eluted material were found to contain mainly LMAP1, where as the fractions eluting directly after the bulk of the HIC-eluted material was composed of only LMAP2 (detailed in Section 3.4.12). As elution from HIC yields progressively more hydrophobic constituents, it can be concluded that LMAP2 was more hydrophobic than LMAP1. This difference in hydrophobicity can be explained by a difference in substitution of the hydrophilic moiety by galactose and glucose, as increasing the degree of substitution has been shown to decrease the overall hydrophobicity of the amphiphile [Fischer, 1996; Leopold & Fischer, 1992]. In addition to these differences discussed above, rhamnose was also identified as a minor constituent (2% of the total material) in LMAP1, eluting with a retention time of approximately 11 minutes. From examination of the chromatogram of LMAP2, rhamnose was distinctly absent. This monosaccharide could be another substitution component of the hydrophilic chain as suggested for glucose and galactose. Interestingly, rhamnose has long been established as an integral component of the mycobacterial cell wall [Hunter, Fujiwara & Brennan, 1982] and lipopolysaccharide [Gupta *et al.*, 1992]. Recently, it has been suggested that some LTA's might also possess rhamnose as a constituent [Tateno *et al.*, 2002].

The presence of inositol, together with glycerol and phosphorus, within the LMAP components were indicative of a phosphatidylinositol (PI) lipid anchor [Sutcliffe, 2000], but due to the shift in retention time within the chromatogram, observed within HIC-eluted LMAP and LMAP1, the presence of inositol peak could not be concluded. This shift might be due to the difference between the sample and standard matrices as within the sample there were many more components present. These components were absent from the standard preparations and this might have had an effect in increasing the retention times of the inositol within the sample. Although why inositol only appears to be present as trace amounts within LMAP2 was unclear. To further investigate the possible existence of inositol within the LMAP components a structural analysis was required.

4.4.3 Examination of LMAP Carbohydrates by Gas Chromatography-Mass Spectrometry

In order to confirm the identity of an inositol-like peak eluting from the GC analysis of the HIC-eluted components in Section 4.3.3.2, the GC procedure was repeated using mass spectrometry (MS) for detection and structural analysis. The resulting retention times of eluting peaks were compared to standard solutions prepared in the same way.

The inositol-like peak observed within the chromatogram possessed a retention time that was similar to the inositol within the standard mixture. The retention times were not an exact match and therefore it was not possible to confirm whether this peak was inositol. The presence of inositol would be significant, as it would indicate the possibility of the existence of a phosphatidylinositol (PI) lipid anchor within the LMAP components of *P. acnes*. Interestingly, a PI lipid anchor has previously been identified in the LMAP of other bacteria which includes mycobacteria and therefore a structural similarity might exist between these LMAP of these bacteria [Hunter & Brennan, 1990; Sutcliffe, 1995]. It was for this reason, that more conclusive data was required.

MS analysis of the separated carbohydrate derivatives allowed a structural comparison of the inositol-like material to be made by examination of the fragmentation pattern produced by the sample and the authentic inositol standard. These fragmentation patterns were also compared to a database of patterns and from this information a likely identification was assigned. The fragments were produced from the bombardment of electrons and the consequent breakage of the molecular structure [Holst, 1999].

The resulting chromatogram (Figure 4.5) demonstrated that there was a difference in the elution order, with inositol eluting first (17.77 minutes), followed by mannose (18.33 minutes), glucose (18.59 minutes) and finally galactose (18.79 minutes). This difference in elution was due to the difference in the stationary phase of the column used for the analysis. The inositol eluted at 17.7 minutes and was compared to the derivatised inositol standard, which eluted at 18.0 minutes. This small difference in the retention times was found to be due to the amount of inositol aplied to the column as when the standard inositol was applied it eluted between 17.67 and 18.10 minutes. In the LMAP sample the suspected inositol

eluted between 17.68 and 17.85 minutes, therefore elution of the inositol started at the same point for both the standard and sample. The only difference between the two peaks was a broader peak for the standard inositol due to the larger sample application (abundance of 10^6 in the standard compared to $<10^5$ in the sample). This had an impact on the peak shape which ultimately had an effect on the mean retention time reported. This suggested that the previously unconfirmed peak was inositol.

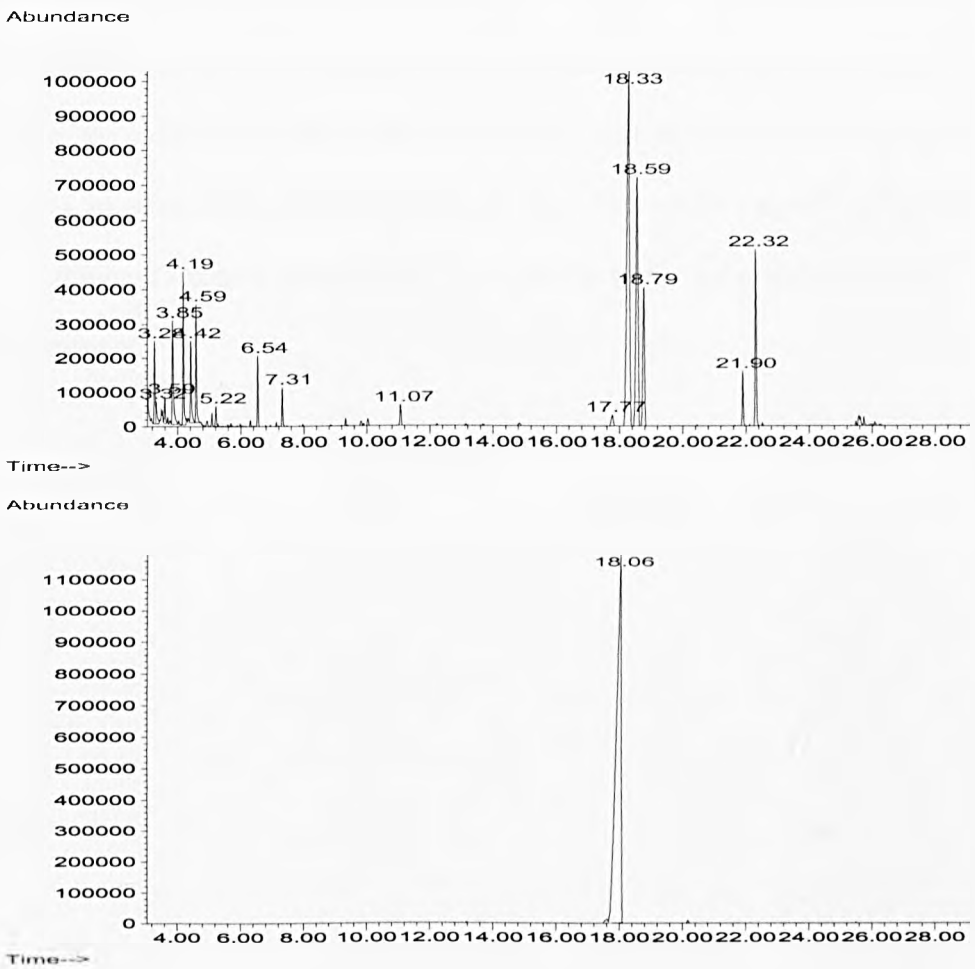


Figure 4.5 Analysis of LMAP Components for Inositol by GC-MS (Chromatograms)

The retention times of the suspected inositol (top chromatogram) and the inositol standard (bottom chromatogram) following separation and detection by GC-MS.

Analysis of the fragmentation pattern generated from the suspected inositol peak showed significant fragments with mass/charge ratios of 210,168, 157, 126 and 115. This was

compared to the database fragmentation pattern, which possessed 210,168, 126 and 115, thus lacking 157 (see Figure 4.6). The percentage match according to the fragmentation database was 78%. A further comparison was made to the fragmentation pattern of the standard inositol in which the significant fragments had mass/charge ratios of 210,168, 157, 126 and 115. Therefore, the fragment of mass/charge ratio 157 was likely to be a derivatisation artefact. This information was conclusive of the presence of inositol within the purified LMAP components and thus with the recent identification of both glycerol and phosphorus above, the data was suggestive of a PI anchor. In further support of this, the calculation of the molar ratio of the mannose to inositol calculated from the relative areas under each of the separated peaks was found to be 40:1. This would suggest one lipid anchor to every 40 mannose residues and is therefore in a similar range to that expected for an LMAP component.

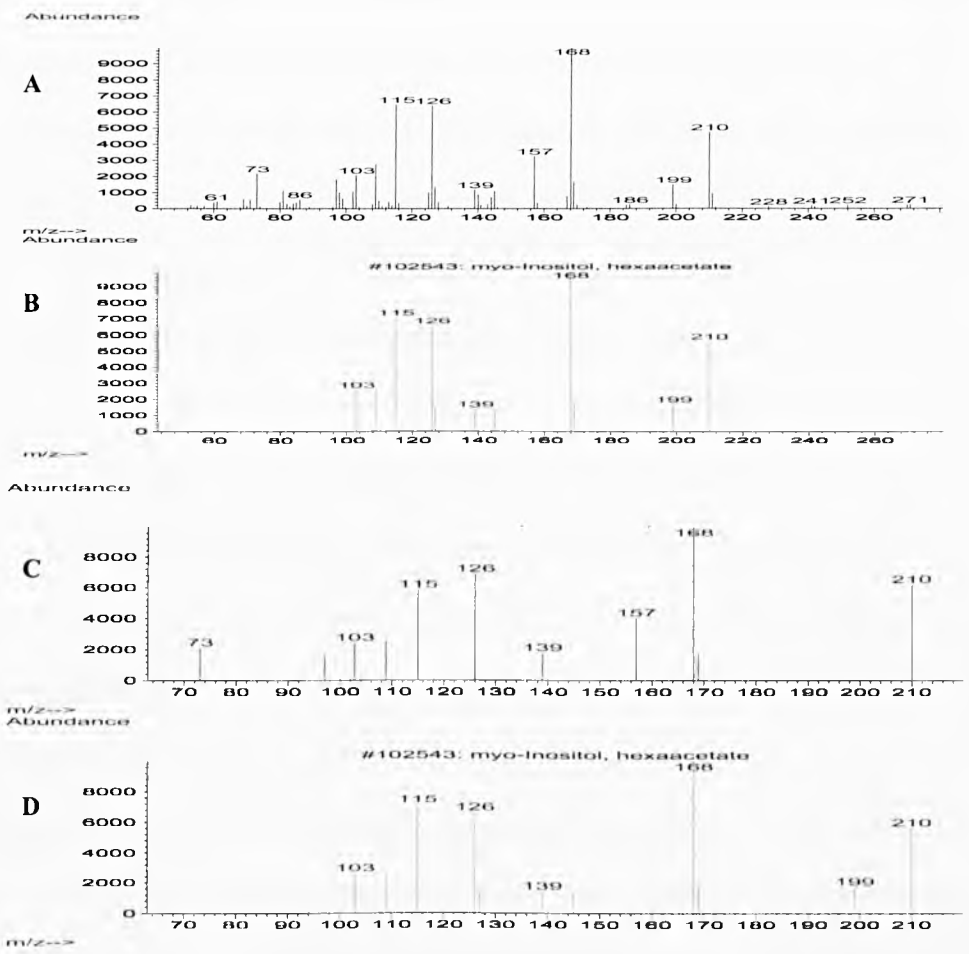


Figure 4.6 Analysis of LMAP Components for Inositol by GC-MS (Fragmentation Patterns) The comparison of the fragmentation patterns produced by the MS analysis of the suspected inositol (A), the database fragmentation pattern (B), the standard inositol (C) and again the database fragmentation pattern (D).

4.4.4 Examination of LMAP Components for Fatty Acids by Gas Chromatography

Analysis of the fatty acids present in each of the LMAP components was investigated firstly, to establish whether fatty acids were present within the components. The presence of mannose, galactose and glucose in relatively large amounts (discussed in Section 4.4.2) suggested the presence of a polysaccharide structure similar to the cell wall polysaccharide previously characterised [Nagaoka *et al.*, 1985]. Therefore it was feasible, at this stage that the material purified was an amphiphilic polysaccharide and not membrane

bound LMAP as expected. The presence of fatty acids is a pre-requisite for an authentic LMAP structure and allowed the possibility of the purified components being polysaccharide cell wall constituents to be eliminated. Secondly, the analysis enabled a comparison of the fatty acid profile in each of the suspected LMAP components and identified whether certain fatty acids were more prominent in one LMAP component to another. This permitted the speculation of possible relationships between the two components. Analysis of the whole cell fatty acids was also conducted for comparison to the previously documented fatty acid profile of *P. acnes*, as the fatty acid profiles have been used as a authenticity check on the identity of the bacteria [Wayne-Moss *et al.*, 1969]. However, because cellular fatty acid content is affected by the culture conditions such as the media used, temperature and cultivation period [Suzuki & Komagata, 1983] only the most prominent fatty acids were used for this validation purpose.

Both purified LMAP components were found to contain significant amounts of fatty acid that were comparable to the fatty acid profile found in the whole cell. Profiles illustrating the resolution between fatty acid derivatives were produced for each sample (Figure 4.7). The identities of the majority of the fatty acids present within *P. acnes* whole cell and the purified LMAP's, LMAP1 and LMAP2 were assigned and the relative abundance of each determined (Table 4.2).

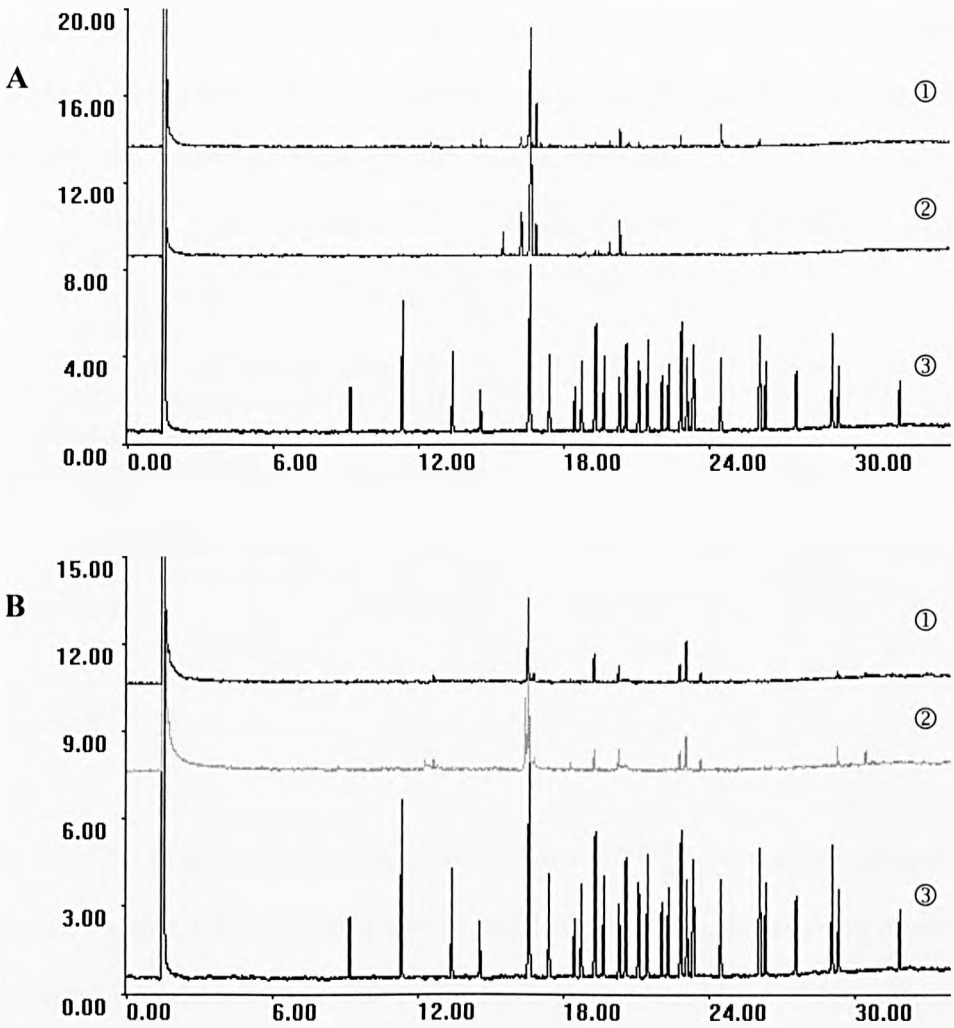


Figure 4.7 Analysis of Derivatised Fatty Acids (FAME's) by GC

The fatty acid profiles were produced from the analysis of the FAME derivatives by GC and illustrate the FID response against the retention time (min). These were compared to a FAME standard mixture ③ in both A and B. The samples analysed were from *P. acnes* whole cells (A) obtained from two separate sources, the University of Sunderland ① and Procter & Gamble UK ②. The second profile (B) shows the analysis of purified LMAP components, LMAP1 ① and LMAP2 ② derived from the Procter & Gamble *P. acnes* strain.

Table 4.2 Analysis of Derivatised Fatty Acids by GC

The analysis of the fatty acids present within the whole cells of *P. acnes*, together with LMAP1 and LMAP2 purification's (derived from the same batch of *P. acnes* culture), indicating both the identity and relative abundance of each fatty acid present.

Fatty Acid Assignment	Whole Cell (%)	LMAP1 (%)	LMAP2 (%)
12-Methyltridecanoate (iC14:0)	1.9	-	-
Unidentified	3.7	-	-
13-Methyltetradecanoate (iC15:0)	63.6	48.5	48.1
12-Methyltetradecanoate (aiC15:0)	15.6	trace	trace
14-Methylpentadecanoate (iC16:0)	-	1.9	trace
Hexadecanoate (C16:0)	trace	6.9	13.6
14-Methylhexadecanoate (iC17:0)	4.6	4.8	7.0
Octadecanoate (C18:0)	2.1	6.2	6.5
9-Octadecenoate (C18:1)	-	17.2	21.0
Unidentified	6.8	2.8	3.5
Eicosanoate (C20:0)	1.5	-	-
13-Docosaenoate (C22:1)	-	6.2	trace
Unidentified	-	4.1	trace

Analysis of the whole cell fatty acids from two sources (University of Sunderland and Procter & Gamble, UK) revealed a similar profile to the previously reported profile for *P. acnes* with 13-methyltetradecanoate being the most prominent fatty acid present and was characteristic of propionibacteria in general [Wayne-Moss *et al.*, 1967]. Within the purified LMAP components 13-methyltetradecanoate (iC15:0) was again the most pronounced fatty acid present and in similar amounts in both LMAP1 and LMAP2. 12-methyltetradecanoate (aiC15:0) appears to have been disregarded within the purified LMAP components, while 9-octadecenoate (C18:1) appears to have been positively selected for, as significant amounts were observed within both of the purified LMAP components, but was not present in detectable amounts within the whole cell analysis. This might have been due to a masking effect imposed by the more abundant fatty acids within the whole cell analysis. Not all of the sample peaks were assigned identities using the standard FAME's shown in Figure 4.7 and therefore additional standard FAME mixtures were applied to establish these fatty acid identities. A good example of this was 12-methyltetradecanoate (aiC15:0) which has been reported as a characteristic fatty acid within propionibacteria and thus confirmation of this

fatty acid was essential [Wayne-Moss *et al.*, 1967]. An anomalous peak eluting at 16.40 minutes was observed in the LMAP2 purification that did not match the retention time of any of the applied FAME standards. After derivatisation of further LMAP2 preparations, this peak was absent from each profile and for this reason was considered to be an artefact of derivatisation procedure.

The presence of a significant amount of fatty acid associated with both purified LMAP components supports the hypothesis that both were likely to be membrane-linked and were therefore both authentic lipid macroamphiphiles.

4.4.5 Examination of LMAP Components for Amino Sugars

Each of the purified LMAP components from *P. acnes* was examined for the presence of amino sugars. This was investigated to evaluate the similarity of the LMAP components to cell wall polysaccharide, as significant amounts of a specific amino sugar have been previously reported [Cummins & White, 1983; Nagaoka *et al.*, 1985]. This amino sugar was initially identified as 2,3-diamino-2,3-dideoxyglucuronic acid [Cummins & White, 1983], but using ^{13}C-NMR spectroscopy it was later established to be 2,3-diacetamido-2,3-dideoxymanuronic acid [Nagaoka *et al.*, 1985]. Interestingly, this amino sugar has also been found in LPS of Gram-negative bacteria [Yoneyama, Araki & Ito, 1984]. It was therefore plausible that this amino sugar might also be a constituent of the LMAP of Gram-positive bacteria such as *P. acnes*.

4.4.5.1 Analysis of the LMAP Components using Ninhydrin-collidine Reagent

To conduct an initial investigation into this possibility, the presence of amino groups within hydrolysed samples of each LMAP component was examined using ninhydrin (triketohydrindene hydrate). Ninhydrin is routinely used to determine amino acids in hydrolysed protein samples, where it forms a purple-coloured complex with the amino group [Holme & Peck, 1993]. Diaminohexuronic acids such as 2,3-diacetamido-2,3-

dideoxymanuronic acid also contain this amino group and have been shown to produce a characteristic blue colour in the presence ninhydrin, when 2% 2,4,6-collidine was added to the reagent [Cummins, 1985]. Addition of ninhydrin-collidine to hydrolysed LMAP preparation resulted in a significant colour development for the 1mg ml^{-1} sample solution and also the 5-fold (200µg ml^{-1}) dilution for both of the purified LMAP components. The 10-fold dilution (100µg ml^{-1}) produced only a faint colour change. Within all samples tested the colour was a distinctive sky blue colour, although this colour degraded into a deep purple colour after 1 hour. The glycine standards also gave a colour response to as little as 100µg ml^{-1} within the standard dilution series, after which, the colour was comparable to that of the amino acid blank (purified water). All glycine standards produced a deep purple colour change immediately and therefore significantly different in colour to the LMAP samples. The results are illustrated in Figure 4.8.

Figure 4.8 Analysis of LMAP Components using a Ninhydrin-Collidine Reagent

The resulting colour development followed the incubation with ninhydrin-collidine reagent. S_1 - S_5 , Serial dilutions (10-fold) of glycine ranging between 10mg ml^{-1} to 1µg ml^{-1}; Bl, negative control (purified water); U_1, 1mg ml^{-1} LMAP1; U_2, 0.2mg ml^{-1} LMAP1; U_3, 0.1mg ml^{-1} LMAP1; L_1, LMAP2; L_2, 0.2mg ml^{-1} LMAP2; L_3, 0.1mg ml^{-1} LMAP2.

The distinctive sky blue colour observed on reaction of hydrolysed LMAP amino sugars with the ninhydrin-collidine reagent has been documented previously [Cummins & White, 1983]. This investigation was focused on the cell wall polysaccharide of *P. acnes* and also observed the sky blue colour under similar reaction conditions. Analysis of the material responsible for producing this distinctive colour identified 2,3-diamino-2,3-dideoxyglucuronic acid. However, a later investigation using NMR revealed that the amino sugar was in fact a 2,3-diacetamido-2,3-dideoxymannuronic acid [Nagaoka *et al.*, 1985]. Regardless of the exact structure of the molecule, all previous publications have attributed this amino sugar to the cell wall polysaccharide of *P. acnes*. With this in mind, together with

the positive determination of fatty acids in Section 4.4.4, it would strongly suggest that the amino sugar previously identified as cell wall polysaccharide was in fact a constituent of the *P. acnes* LMAP or a membrane-anchored polysaccharide, which was very similar to the cell wall polysaccharide. In support of this hypothesis, within the previous studies that focussed on the cell wall polysaccharide of *P. acnes* there was an absence of any examination for fatty acid in the pre-hydrolysed cell wall extractions. Consequently, the fatty acid elements were more than likely lost during acid hydrolysis, prior to the analysis of the fatty acids.

This scenario was analogous to that of mycobacterial LAM, which was originally perceived to be an arabinomannan polysaccharide of the cell wall [Azuma *et al.*, 1968; Chargraff & Schaefer, 1935; Weber & Gray, 1979]. Following the development and successful application of solvent extraction procedures allowing the purification of LAM in their native acylated states this was later revised [Chatterjee *et al.*, 1992c; Hunter & Brennan, 1990]. A similar oversight might have occurred in the case of *P. acnes*.

Comparison of the colour intensity to the glycine standards provided an indication of the amount of amino group present within the LMAP preparations. However, this suggested an equivalent amino group concentration of approximately 1mg ml^{-1}, as the colour intensity resulting from the 1mg ml^{-1} sample was comparable to the 1mg ml^{-1} glycine standard. This estimate is unlikely to be correct as it would imply that ~100% of the LMAP components were comprised of amino moieties. Furthermore, the sky blue colour produced on reaction with the LMAP samples was significantly different to the purple colour observed with the glycine standards. This difference in colour may be due to the difference in the amino group present on reaction with the ninhydrin. It was therefore possible that the colour intensity might also be different because of the difference in the amino group present. It is noteworthy that within the glycine standard solution a solitary amino group was responsible for the colour development upon reaction with the ninhydrin. Therefore, if more than one amino group exists on the amino sugar of the LMAP constituents, a similar concentration of material might result in a two- or three-fold increase in the colour intensity produced. On this basis, colour intensity would be highly dependent on the number of amino groups

present in one molecule of amino sugar. Consequently, if the amino sugar observed was 2,3-diacetamido-2,3-dideoxymannuronic acid, then this amino sugar contains two amino groups and might be expected to produce a colour intensity two-fold greater than that of glycine, which only contains one amino group.

4.4.5.2 Estimation of Amino Sugar within HIC-eluted LMAP

To further investigate the possibility of the presence of an amino sugar within the LMAP, the amount of amino sugar was estimated using a method originally developed for the analysis of hexosamines [Hancock & Poxton, 1988]. This method utilises the Morgon-Elson reaction between *N*-acetylhexosamines and dimethylaminobenzaldehyde (DMAB), however, it has also been found to detect *N*-acetylaminohexuronic acids, though with a lower sensitivity [Hancock & Poxton, 1988]. Following hydrolysis of LMAP, the components required *N*-acetylation, prior to the assay with DMAB. Finally, the reaction with the DMAB reagent results in the formation of an intense red colour that was measured spectrophotometrically at 595nm.

Using a 5-fold dilution of the hydrolysed HIC-eluted component (40μg ml^{-1}) an absorbance of 0.254 was recorded at 595nm. Using the response of glucosamine under similar conditions (Abs_{595} = 0.486 ≡ 100nmol) the amount of amino sugar was calculated to be 0.25μmol. This was equivalent to 3.1% of the total LMAP initially hydrolysed. Therefore further supporting the proposition discussed in Section 4.4.5.1 for the existence of an amino sugar, but in considerably lower quantities than that suggested by the ninhydrin-collidine assay. It has been reported previously that the DMAB was less sensitive to aminohexuronic acids than it was to glucosamine [Hancock & Poxton, 1988]. Therefore, the assay might be significantly under-reporting the amount of amino sugar present. Consequently, the actual amount of aminohexuronic acid present might be significantly greater, but without a commercially available aminohexuronic acid standard this cannot be confirmed.

Additionally, the cell wall polysaccharide has also been reported to contain galactosamine [Cummins & Johnson, 1986; Cummins & White, 1983; Nagaoka *et al.*, 1985]. Moreover, the

DMAB assay employed in the present study would not necessarily distinguish this component, thus diaminohexuronic acid and possibly other amino sugars (notably galactosamine) might be present as significant constituents of *P. acnes* LMAP.

4.4.6 Structural Analysis of LMAP Components

4.4.6.1 Analysis of Nitrocellulose Electroblots using Concanavalin A

Many lipoglycans have been shown to possess significant amounts of mannose within the hydrophilic polysaccharide portion of the components, the best examples being lipomannan and LAM within mycobacteria (see Section 1.3). Con A was commercially purchased as a biotin-labelled conjugate and used to probe the separated LMAP components electroblotted onto nitrocellulose, as described in Section 4.3.6.1. These nitrocellulose blots were probed with Con A to determine whether the purified components contained mannosyl termini and therefore allowed further molecular characterisation of each of the *P. acnes* LMAP components. Significant amounts of bound lectin were visualised using an alkaline phosphatase-bound streptavidin conjugate, which binds the biotin of the bound lectin. This in turn was detected using a substrate that when metabolised by the alkaline phosphatase, results in the development of a purple colour. A positive result was therefore denoted by a purple colour observed on the nitrocellulose membrane. LAM from mycobacteria was included in the original polyacrylamide gel as it was known to contain significant amounts of mannose terminated groups and was therefore a suitable positive control (see Section 1.3.3 for structural references). After development with BCIP/NBT significant mannosyl terminated components were identified. However, many of the components present within the protein standard molecular weight markers also gave a positive reaction. The results are shown in Figure 4.9.

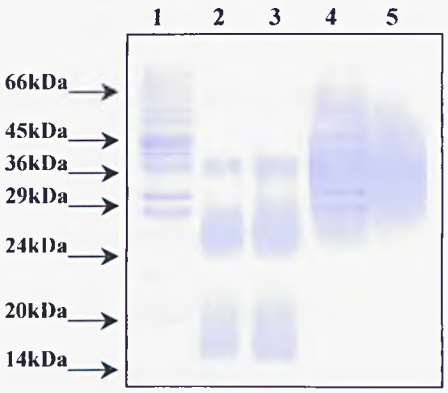


Figure 4.9 Analysis of LMAP Components on Nitrocellulose Membrane using Concanavalin A

LMAP components were probed with Con A to allow the detection of mannosyl termini. Lane 1, protein molecular weight standards (described in Figure 3.12); lane 2, HIC-eluted LMAP at 0.65μg μl^{-1}; lane 3, HIC-eluted LMAP at 0.975μg μl^{-1}; lane 4, mycobacterial LAM at 2.0μg μl^{-1}; lane 5, mycobacterial LAM at 1.0μg μl^{-1}. 5μl samples were loaded in each case.

The protein standard markers were of bacterial origin and probably contain reasonable amounts of impurity such as glycolipid and glycoprotein, thus it was likely to be these that were being bound by the Con A and therefore responsible for the bands visible in lane 1. As it can be seen in lanes 2 and 3 both LMAP1 and LMAP2 have both bound Con A, suggesting that each contain mannose-terminated regions that probably exist within the hydrophilic moiety. Furthermore, there was also a band visible at 36kDa, which has been shown previously to be an aggregate of the LMAP components (discussed in Section 3.4.9). No other bands were observed within the HIC-eluted LMAP, thus demonstrating the effectiveness of the HIC procedure for the preparation of LMAP components. The mycobacterial LAM gave a positive result as expected, and therefore demonstrates that there was some degree of similarity between the LMAP components of *P. acnes* and the LAM of mycobacteria in that both contain significant amounts of mannose termini.

4.4.6.2 Analysis of Nitrocellulose Electroblots using Anti-LAM Polyclonal Antibody

Nitrocellulose blots prepared following the transfer of electrophoretically-separated LMAP components were probed with polyclonal antibody directed towards LAM to investigate any immunochemical similarities between LMAP components from *P. acnes* and the LAM from mycobacteria. It was anticipated that the polyclonal antibody would not be as specific for LAM as a monoclonal antibody and therefore LMAP's possessing structural similarities to LAM might show some degree of cross-reaction, where as a monoclonal antibody would provide confirmation of a definite LAM structure. The primary anti-LAM antibody was probed using the secondary antibodies, described in Section 4.3.6.3, conjugated to alkaline phosphatase. The bound secondary antibody-alkaline phosphatase conjugate was detected visually using a BCIP/NBT alkaline phosphatase substrate that resulted in a purple colour when metabolised. A positive result was therefore denoted by a purple colour observed on the nitrocellulose membrane. After the 30 minutes incubation with the BCIP/NBT little or no reaction was apparent with the LMAP components. In contrast, the reaction with mycobacterial LAM was positive, although weak. The blot was left for a further 12 hours in the presence of the BCIP/NBT. The increase in the BCIP/NBT incubation resulted in the image shown in Figure 4.10.

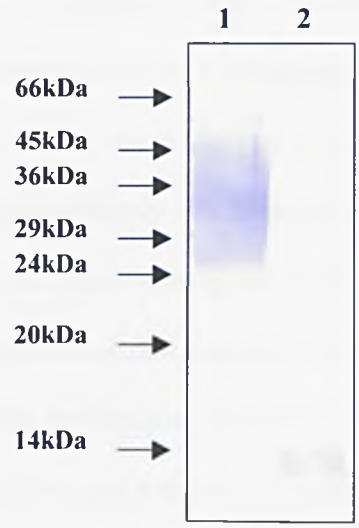


Figure 4.10 Analysis of LMAP Components on Nitrocellulose Membrane using Polyclonal Anti-LAM Antibody

LMAP components were probed with a polyclonal antibody directed towards mycobacterial LAM to allow for structural comparison. Lane 1, mycobacterial LAM at 1.0μg μl^{-1}; lane 2, HIC-eluted LMAP at 2.0μg μl^{-1}. 5μl samples were loaded in each case.

All components were shown to have been transferred via the electroblotting procedure, as no material was detected following silver staining of the spent polyacrylamide gel used for the transfer. The mycobacterial LAM in lane 1 demonstrated a strong colour development in the region where it was expected to migrate on the SDS-PAGE. This was after the extensive incubation period in the presence of BCIP/NBT, but was also present after 30 minutes incubation, although not as prominent. In lane 2, only a small amount of material was found to bind the polyclonal anti-LAM antibody and this appeared to possess a molecular weight of <14kDa as the material was shown to migrate further than the lowest molecular weight protein standard (visualised prior to probing with Ponceau S prior to antibody incubation). Interestingly, this material was not seen on the second half of the polyacrylamide gel, which was identically loaded and silver-stained as detailed in Section 4.3.6.2. This suggested that this component was not a protein and did not contain a significant amount of carbohydrate; otherwise it would have been detected using the silver-stain procedure. It remains possible that this material could be the lipid portion of the LMAP material, either an aggregate of

fatty acids or more likely, the fatty acids with the lipid anchor attached and therefore LMAP that lacks the hydrophilic polysaccharide moiety. The presence of this material was previously suggested in Section 3.4.9, due to the appearance of a 36kDa aggregate, in which a hydrophobic component of approximately 10kDa was proposed. Furthermore, in earlier attempts to investigate the LMAP structure using the polyclonal anti-LAM antibody a cross-reaction with the 36kDa aggregate was often observed. These collective observations suggested that it was the ~10kDa, hydrophobic component responsible for both the aggregation on SDS-PAGE and the cross-reaction with polyclonal anti-LAM antibody. In addition to this, it was significant that the anti-LAM polyclonal cross-reacted with this material, as it suggested that it might possess an epitope similar to a constituent of LAM and therefore supports the possibility of a PI lipid anchor which has previously been suggested in Section 4.4.3. The reasoning being that if the material was the lipid anchor of the *P. acnes* LMAP and anti-LAM antibody bound this material, it would have to be structurally similar to the lipid anchor in LAM which has been described in detail and identified as PI anchor [Hunter & Brennan, 1990]. Whether this PI lipid anchor of the LMAP was a biosynthetic intermediary or a degradation product was unclear.

4.4.6.3 Analysis of Nitrocellulose Electroblots using Anti-LAM Monoclonal Antibodies

Following the polyclonal antibody analysis of LMAP, similar nitrocellulose blots were probed with monoclonal antibodies directed towards both mycobacterial LAM (CS-35) and mycobacterial ManLAM (CS-40) to determine whether the LMAP structures were more closely related to LAM than the polyclonal antibodies suggested. The primary anti-LAM antibodies of both of the monoclonal antibodies were probed using the secondary antibodies described in Section 4.3.6.3 and both of which were conjugated to alkaline phosphatase. The bound secondary antibody-alkaline phosphatase conjugate was detected visually using a BCIP/NBT alkaline phosphatase substrate that resulted in a purple colour when metabolised. A positive result was therefore denoted by a purple colour observed on the nitrocellulose membrane. The results to these analyses are shown in Figure 4.11.

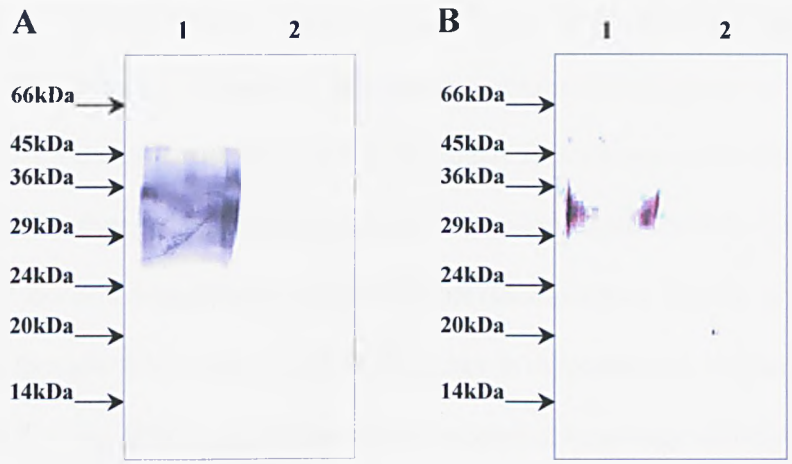


Figure 4.11 Analysis of LMAP Components on Nitrocellulose Membrane using Monoclonal Anti-LAM Antibodies

LMAP components were probed with monoclonal antibodies directed towards mycobacterial LAM (A) and ManLAM (B) to determine whether there was structural homology between the components from each bacterium. The sample loading was the same in both blots and 5µl samples were loaded in each well. Lane 1, mycobacterial LAM at 1.0µg µl^{-1}; lane 2, HIC-eluted LMAP at 2.0µg µl^{-1}.

In both blots, the mycobacterial LAM bound significant amounts of both antibodies. In the analysis of LMAP components no antibody was bound suggesting that neither of the two LMAP components of *P. acnes* were similar to LAM of mycobacteria. This supports the carbohydrate profile observed in Section 4.4.2, as no arabinose was detected in the analysis of either LMAP component.

4.5 Conclusions

In summary, the characterisation of the two components separated on SDS-PAGE has shown that both possess similar fatty acids and were both authentic LMAP components. Both of these LMAP components contain significant amounts of mannose, glucose and galactose and it has been suggested that it was the relative amounts of these carbohydrates that allowed the two discrete components to be distinguished. Both LMAP components also possess glycerol, phosphorus and inositol and these molecular constituents have been suggested to be associated with the existence of a PI lipid anchor, similar to PI lipid anchor

in mycobacteria. Within each component, a significant amount of amino sugar was identified. This amino sugar was shown to possess certain characteristics of 2,3-diacetamido-2,3-dideoxymannuronic acid, previously identified as a cell wall polysaccharide in *P. acnes*. However, it appeared that this specific amino sugar was associated with the LMAP of *P. acnes*, a possibility overlooked in previous analyses. Finally, although the lipid anchor was thought to be similar to the lipid anchor in mycobacteria, neither of the two LMAP's of *P. acnes* shared any further immunochemical homology with LAM and were therefore structurally unrelated.

CHAPTER 5

Effects of Lipid Macroamphiphiles on Mammalian Lipid Metabolism

5.1 Introduction

It has been suggested that the lipid macroamphiphiles of Gram-positive bacteria stimulate the mammalian immune system both *in vivo* and *in vitro*. Such stimulation can lead to a range of effects within the monokine network, together with some more specific repercussions on metabolism, many of which have been discussed (Section 1.7). In contrast to the continuing advancement in the understanding of the immunostimulatory nature of LMAP components, effects on the cellular metabolism have been largely neglected, with only preliminary work to date and the majority of which has been performed *in vivo* [Feingold *et al.*, 1992; Nonogaki *et al.*, 1995; Renzi & Lee, 1995; Van Amersfoort, Van Berkel & Kuiper, 1999]. However, it has been shown that LPS purified from a variety of sources induced rapid *in vivo* changes in the metabolism of lipids. Typically this involves an increase in serum triacylglycerols (TAG) following injection of LPS and consequent hypertriglyceridemia [Feingold *et al.*, 1992]. It was also suggested that LPS gave rise to hepatic *de novo* fatty acid biosynthesis and adipose tissue lipolysis which provided the substrates required for hepatic TAG synthesis and secretion [Feingold *et al.*, 1992]. Larger LPS doses produced a decrease in the activity of adipose and skeletal lipoprotein lipase (LPL) which was responsible for serum lipid clearance. These observations were reported with *in vivo* studies and therefore it was not established whether a primary or secondary response was influencing these metabolic changes [Feingold *et al.*, 1992].

Similar metabolic changes in rats have also been shown for LTA, which also resulted in the induction of hypertriglyceridemia. Again, the increase in serum TAG was shown to originate from hepatic *de novo* fatty acid biosynthesis and adipose tissue lipolysis followed by hepatic TAG secretion, but no reduction in the activity of LPL was observed [Nonogaki *et al.*, 1995]. As with LPS-induced hypertriglyceridemia the effect of LTA-induced hypertriglyceridemia was shown *in vivo* and therefore again, it was not possible to establish whether the effect was due to the LTA *per se*, or whether it was secondary to other effectors. To date, there are no published reports detailing an *in vitro* investigation, therefore the effect of LTA and other LMAP structures on lipid metabolism remains unclear.

Interestingly, it has been reported that changes in the composition of lipids on the skin are frequently associated with a*cne vulgaris*, a skin condition in which *P. acnes* is the likely pathogen [Downing *et al.*, 1986; Morello, Downing & Strauss, 1976]. This compositional change in the lipid species suggested a shift in the lipid metabolism in those patients suffering from acne. However, the mechanisms underlying this shift are not understood. Not surprisingly, the effects of the lipoglycans of *P. acnes* have not yet been established and thus it remains possible that these components may influence this shift in metabolism. Therefore, using the recently purified lipoglycan from *P. acnes* the effects of these components on lipid metabolism were investigated and compared to the effects of other LMAP components. From these comparisons it was then determined whether these lipoglycans could influence the cellular metabolism of lipids and therefore allowed a critical evaluation of the importance of these components in the pathogenicity of *P. acnes* in disease.

5.2 Materials

5.2.1 Reagents

All cell line details together with the culture reagents were described in Section 2.4.2. Pyrogent-Plus limulus amebocyte lysate (LAL) gel-clot, endotoxin testing kits and endotoxin-free water were obtained from BioWhittaker, UK. [1-^{14}C]oleate was supplied by Amersham Biosciences, UK. Lipid-free bovine serum albumin was obtained from ICN Biomedicals Inc, USA. Organic solvents and acids were of analytical grade and supplied by Fisher Scientific, UK. NADH and pyruvate were obtained from Roche, UK. Scintillation cocktail was supplied by Perkin Elmer Life Sciences, UK. Folin-Lowry protein assay kit and all other reagents used were of cell culture grade and purchased from Sigma, UK. Purified water used was deionised (18.2mΩ cm) and filtered though a 0.2μm membrane via a Milli-Q system, Millipore UK.

5.2.2 Consumables

Silica gel 60 thin layer chromatography plates were supplied by Merck, UK. Pre-poured polymixin B affinity (Detoxi-gel) columns were supplied by Pierce, UK. Polystyrene and polypropylene universals and centrifuge tubes were obtained from Bibby Sterilin, UK. All cell culture flasks, dishes and cellulose acetate syringe filters were obtained from Iwaki, Japan. All other filtration devices were purchased from Whatman, UK. All other consumables were purchased from Fisher Scientific, UK.

5.2.3 Equipment and Instruments

Scintillation counting was performed using a Wallac Winspectral 1414 liquid scintillation counter (Perkin Elmer Life Sciences, UK). All microtitre plate spectrophotometric analyses were performed using a Dynex Revelation plate reader (Dynex, UK). Larger sample volumes were determined using WPA Lightwave spectrophotometer, (WPA,UK). For all tissue culture procedures a class II laminar flow hood was used (Microflow, UK) and incubations were performed in a Forma 310 direct-heat CO_2 incubator (Forma Scientific Inc, USA). All centrifugation was performed on a Sigma 2-4 bench centrifuge (Sigma Laboratories, Germany). All weights were determined using a AC100 analytical balance (Mettler Toledo, UK)

5.3 Experimental Methods

5.3.1 Analysis of Endotoxin in LMAP Preparations

To allow the utilisation of the LTA and LMAP in studies involving the responses of cell lines, the different preparations were analysed to determine levels of endotoxin present. If these levels were found to be unacceptably high, an endotoxin reduction step was applied to reduce the amount of endotoxin to a satisfactory level. It has been shown on many occasions that endotoxin has a stimulatory effect on a range of cell types [Blease *et al.*,

1998; Feingold *et al.*, 1992; Jungi, Valetin-Weigrand & Brcic, 1999; Renzi & Lee, 1995] and this has been extensively reviewed by Mamat [Mamat *et al.*, 1999]. Furthermore, the biological activity of endotoxin within these studies was typically reported to be active at concentrations as low as 1ng ml^{-1}. For this reason, the presence of significant amounts of endotoxin within LMAP preparations might lead to either false-positive observations or possibly inhibition of the cell function. This concern was supported by the observations made using a commercial preparation of LTA, which was used to investigate the effects on mouse macrophages [Gao *et al.*, 2001]. On analysis of the LTA, a considerable amount of endotoxin was detected. Furthermore, the endotoxin was found to substantially contribute to the effect of the LTA, thus exaggerating the observed effect of LTA [Gao *et al.*, 2001].

To be confident that any effect observed on incubation of LMAP preparations with cell lines was due to the intended LMAP component and not an endotoxin impurity, the level of endotoxin was determined in preparations prior to use within cell studies. The assay employed an enzyme that was highly specific for endotoxin. This enzyme was present in a lysate prepared from the circulating amebocytes of the horseshoe crab, *Limulus polyphemus*, and was therefore termed the limulus amebocyte lysate (LAL) reagent. On reaction of the LAL reagent with endotoxin, a gel was formed and it was this that indicated a positive result [Levin & Bang, 1968; Solum, 1970]. Using a LAL test kit designed for the quantitation of endotoxin the amounts of endotoxin were determined in both the LTA preparations from *S. aureus* and the HIC-eluted LMAP from *P. acnes*. After which, attempts were made to reduce the amount of endotoxin detected using a strategy based on an affinity chromatography technique. Polymixin B, a fatty acid-acylated cyclopeptide antibiotic has been shown to bind the lipid A portion of endotoxin [Issekutz, 1983]. This polymixin B ligand has been immobilised onto an agarose support and was purchased as a convenient pre-poured 1ml column. This affinity column was therefore employed to reduce the levels of endotoxin detected in LMAP preparations.

5.3.1.1 Determination of the Amount of Endotoxin within LMAP Preparations

The amount of endotoxin was determined in LTA prepared from *S. aureus* as described in Section 3.3.3.7 and the HIC-eluted LMAP prepared from *P. acnes* as described in Section 3.3.3.9. From the freeze-dried preparations, 1.0mg ml^{-1} stock solutions were prepared in 0.1M ammonium bicarbonate at pH7.8 prepared using endotoxin-free water [<0.005 Endotoxin Units (EU) ml^{-1}]. From these stock solutions, serial 10-fold dilutions were prepared ranging between 10µg ml^{-1} and 10ng ml^{-1} using endotoxin-free water (EF-water). All pipette tips, polystyrene universals and bijoux used for these preparations were certified endotoxin-free. Endotoxin standards were prepared from 10ng of lyophilised *Escherichia coli* endotoxin equivalent to 9.0EU ng^{-1} (as certified by the supplier). This was resuspended in 5.0ml EF-water and from this five standards were prepared ranging between 0.5 and 0.03EU ml^{-1} with EF-water. To individual 10 x 75mm glass tubes (incubated at 180°C for 8 hours to remove possible endotoxin contamination) was added 100µl of each standard and sample dilution. To one tube 100µl of 0.1M ammonium bicarbonate at pH7.8 prepared using EF-water, was added and this was used a negative control. To each tube 100µl of LAL reagent was added and the tube was immediately swirled and incubated in a waterbath at 37°C for 1 hour (±2 minutes). The LAL reagent was sensitive to endotoxin of ≥0.125EU ml^{-1} in which the liquid formed a gel. Therefore a positive result was indicated by the formation of a firm gel, that on inversion remained momentarily intact. For preparations found to contain >9EU ml^{-1}, equivalent to >1ng ml^{-1} of endotoxin (i.e. 100-fold dilution), endotoxin reduction was required.

5.3.1.2 Endotoxin Reduction using Polymixin B Affinity Chromatography

For reduction of the endotoxin concentration within LMAP preparations to acceptable levels, 1ml pre-poured affinity columns were used. 1mg ml^{-1} samples of both LTA and LMAP were prepared in EF-water. The columns were prepared by adding 5ml of 1%(w/v) sodium deoxycholate and eluted by gravity-flow. The detergent was washed from

the column with 10ml of EF-water. Samples were applied to the column and eluted by gravity-flow. The column was washed 5ml of EF-water and this was added to the eluted sample. The column was cleaned prior to the use with another sample to remove bound endotoxin. This was achieved by washing with 5ml of 1%(w/v) sodium deoxycholate, followed by 10ml of water. The eluted sample was freeze-dried and resuspended in a volume of EF-water to the required concentration for later use. To enable the percentage recovery to be assessed, in both the pre-eluted and post-eluted samples the amount of phosphorus was quantified within the LTA preparation (as described in Section 2.2.4) and for the lipoglycan preparation the carbohydrate was determined (as described in Section 2.2.3). The amount of endotoxin was also determined in both the pre-eluted and post-eluted samples, to enable the success of the endotoxin removal to be assessed.

5.3.2 Uptake of Radiolabelled Fatty Acid by Mammalian Cells In Vitro

To enable the study of the effects of different LMAP components on mammalian lipid metabolism, adipocytes and hepatocytes were pre-labelled with fatty acid containing a radioisotope. In doing this, the radiolabelled fatty acid was taken up by the cells and stored as TAG, thus resulting in a radiolabelled lipid pool. This allowed the lipids to be quantitatively monitored after the addition of an LMAP component. In order to maintain the fatty acid in an aqueous solution and thus available for uptake by the cells, the fatty acid was non-covalently bound to delipidated BSA to a concentration between 0.1-0.6mM, which was equivalent to mammalian serum levels [Kather & Wieland, 1985]. Equilibrium is established between the free fatty acid pool and the BSA-bound fatty acid. It has been reported that the free fatty acid pool constituted <0.1% [Laurell & Tibbling, 1967] and it was this free fatty acid that was taken up by mammalian cells. As uptake occurs BSA-bound fatty acid dissociates to restore the equilibrium [Spector, 1968].

In order to effectively pre-label the lipid contents of the cells for use in metabolic analyses, but to ensure minimal damage occurred during this pre-treatment, the uptake procedure was monitored and the structural integrity of the cells was determined throughout the uptake

incubation period. To monitor the uptake of fatty acid by the cells, cultures were sacrificed at successive time intervals following the incubation with a radiolabel. The resulting radiolabelled intracellular lipid was extracted from the cells using a modification of the method of Bligh and Dyer [Bligh & Dyer, 1959], in which the lipid was extracted from each culture dish *in situ*. Following extraction the amount of lipid present was determined by scintillation counting. To allow the structural integrity of the cells to be assessed following incubation with the radiolabelled fatty acid, the extracellular medium was monitored for lactate dehydrogenase (LDH) activity. As LDH is a cytosolic enzyme it was used as an indicator of integral damage. The higher the LDH activity within the extracellular medium, the greater the cellular damage [Griffiths, 2000]. Furthermore, the total amount of protein was also recorded, as this would ensure that the amount of fatty acid uptake was increasing per cell and not due to an increase in cell numbers.

5.3.2.1 Preparation of 0.1mM ^{14}C-Oleate Incubation Medium

A 10%(w/v) aqueous BSA solution was initially prepared using delipidated BSA in purified endotoxin-free water. This solution was kept warm at 50°C in a waterbath. 8.5mg of oleate was added to a 100ml Duran bottle and to this was added 50µl of [1-^{14}C]-oleate at a specific activity of 2.11GBq mmol^{-1}, prepared in toluene. The toluene was removed under a stream of oxygen-free nitrogen and the Duran was warmed to 50°C. To the Duran bottle was added 30ml of 10%(w/v) aqueous BSA solution and the solution was stirred at 50°C for 30 minutes until the solution had clarified. The final specific activity of the resulting 1mM oleate solution was 6.17kBq µmol^{-1}. The 1mM oleate solution was transferred to a laminar flow hood and filtered using a 0.2µm cellulose acetate filter syringe to sterilise. Finally, 3ml of 1mM labelled oleate was taken and made to 30ml with MEM (as described for hepatocyte maintenance, Section 2.4.2.2) or with DMEM (as described for adipocyte maintenance, Section 2.4.2.1). Foetal calf serum was not added to either of the incubation media. This 0.1mM oleate media was used immediately for incubation.

5.3.2.2 Preparation of Hepatocytes (HepG2)

Each cell line was prepared differently, depending on the requirements the cells. hepatocytes were grown to 80% confluence in 150cm^2 culture flask. These were sub-cultured as described in Section 2.4.3 and resuspended in 40ml of culture medium (described in Section 2.4.2.2). 1.5ml of the cell suspension was added to 24 x 40mm^2 culture dishes and these were incubated as detailed in Section 2.4.2. After 24 hours the culture dishes were checked for cell adherence and the medium was exchanged. The media exchange was repeated until the cells were between 60 and 80% confluent, after which the cells were used for analyses.

5.3.2.3 Preparation of Adipocytes (3T3L1)

The adipocytes required derivatisation prior to utilisation within metabolic studies. The cells were initially grown to 80% confluence in 150cm^2 culture flask, sub-cultured as described in Section 2.4.3 and resuspended in 40ml of culture medium (described in Section 2.4.2.1). 1.5ml of cell suspension was added to 24 x 40mm^2 culture dishes and these were incubated as detailed in Section 2.4.2. After 24 hours the dishes were checked for cell adherence and the medium was exchanged. This was repeated until the cells were between 60 and 80% confluent. Once the cells were >80% confluent the incubation medium was removed by careful aspiration and to each culture dish 1.5ml of differentiation medium was added. The differentiation medium consisted of the culture medium described in Section 2.4.2.1, supplemented with 1.7μM porcine insulin, 0.5mM isobutyl methyl xanthine and 0.25μM dexamethasone [Green & Kehinde, 1976]. The cells were incubated with differentiation medium for 48 hours, after which the cells were used for analyses.

5.3.2.4 Incubation of Cells with 0.1mM ^{14}C-Oleate

To either the hepatocytes prepared as described in Section 5.3.2.2 or the adipocytes prepared as described in Section 5.3.2.3, 1.5ml of 0.1mM ^{14}C-oleate medium with a specific activity of 6.17kBq μmol^{-1} prepared in Section 5.3.2.1 was added to each 40mm^2 culture dish. These were incubated as described in Section 2.4.2. Two 40mm^2 culture dishes containing 1.5ml of culture medium that was deficient of ^{14}C-oleate were used as negative controls and these were incubated under the same conditions.

5.3.2.5 Determination of Fatty Acid Uptake

At appropriate time points, two of the 40mm^2 culture dishes were removed from the incubator and the spent medium was aspirated into an eppendorf tube. The culture dishes were washed twice with PBS at pH7.4, containing 0.1%(w/v) BSA and stored upside-down at -30°C until required for extraction. The spent medium was centrifuged at 14,000 x g for 30 seconds to sediment any cell debris or detached cells. From the supernatant, 200μl was removed and this was stored at 4°C until required for LDH activity determination. Lastly, the two negative control dishes (no ^{14}C-oleate) were removed from incubation and the spent medium was aspirated into an eppendorf tube and immediately frozen to -30°C and stored for later protein analysis, while the medium was centrifuged as before. 200μl was removed and the spent medium was stored at 4°C until required for LDH activity determination.
To each of the culture dishes the adhered cells were extracted with the addition of 1ml of methanol and the surface of the dish was scraped with a cell scraper. Once all of the cells had been lifted the dish contents was aspirated into a 9ml screw-cap tube. The dish and cell scraper were washed twice with 500μl of methanol and this was pooled with the original extraction volume. To each of the tubes, 2ml of chloroform was added and the tube contents was vortexed for 30 seconds. This was followed by the addition of 1.8ml of water to each extraction tube resulting in a final solvent ratio of 1.0:1.0:0.9 (methanol:chloroform:water). Each tube was vortexed for 30 seconds and centrifuged at 500 x g for 30 minutes to break the emulsion [Bligh & Dyer, 1959].

The bulk of the aqueous upper phase was aspirated to a waste bottle and using a glass pipette, the majority of the lower organic phase was transferred to a clean screw-cap tube. From the organic phase 1ml was volumetrically removed and transferred to a 4ml scintillation tube. This was air-dried until no solvent remained. To each scintillation tube, 4ml of scintillation cocktail was added. The tubes were capped, briefly shaken and the disintegration per min was determined for each sample.

5.3.2.6 Determination of Lactate Dehydrogenase Activity

Lactate dehydrogenase (LDH) activity was used to assess the relative amount of LDH present within the spent medium following incubation with ^{14}C-oleate medium. The method relies on the reaction of pyruvate and NADH, resulting in the production of lactate and the oxidation of NADH to NAD. As NADH absorbs strongly at 340nm, a reduction in the absorbance against time was used to measure the LDH activity and therefore the relative amounts of cell lysis/damage occurring during incubation with ^{14}C-oleate medium [Griffiths, 2000]. The method used for this analysis of LDH activity was taken from Bergmeyer [Vassault, 1985].

81mM aqueous tris buffer was prepared, containing 200mM NaCl, at pH7.2 (adjusted using 5M HCl) and stored at 4°C until required. Following the incubation with ^{14}C-oleate, any cellular material was removed from the spent medium by centrifugation as described in Section 5.3.2.5 and then warmed to room temperature. From this cell-free medium 20µl was pipetted into the well of a microtitre plate in triplicate. To each well was added 230µl of 0.25mM NADH prepared in the tris/NaCl buffer described above. This was shaken for 30 seconds. Finally to each well, 50µl of 10mM pyruvate prepared using tris/NaCl above was quickly added. The microtitre plate was shaken for 30 seconds and the absorbance was determined at 340nm. The absorbance at 340nm was recorded a further three times at 5 minute intervals. From these absorbance readings, the average rate of decrease in absorbance was determined per minute for each sample in triplicate. The results were compared to a positive control in which the two culture dishes were prepared for incubation and incubated

with ^{14}C-oleate for 24 hour as described in Section 5.3.2.4. Following incubation, a cell suspension was prepared from each with the original incubation medium. The cell suspension was then sonicated using a sonication probe with a tip diameter of 2mm set to 200W cm^{-1} for 3 minutes with a 2 second-pulse cycle The temperature of the cell suspension was maintained at 4°C over an ice bath. This positive control was assumed to be representative of total LDH activity (ie ~100% cell lysis) and all incubation time points were compared to this. The LDH assay of the positive control was performed in triplicate as described for the above samples.

5.3.2.7 Determination of Total Protein

Total protein was determined in duplicate 40mm^2 dishes before and during the incubation with ^{14}C-oleate. This was determined to ensure that the amount of fatty acid uptake was increasing per cell and not primarily due to an increase in cell numbers. A protein test kit was employed which utilised the Folin-Lowry method (Sigma, Procedure No. 5656) and was adapted for use with a microtitre plate. In this method, under alkaline conditions the nitrogen of the protein binds copper, forming a copper-protein complex. Phosphomolybdicphosphotungstic acid is reduced to heteropolymolybdenum blue by the copper complex. The resulting blue colour is proportional to the amount of protein present and can be measured spectrophotometrically at 595nm [Harris & Angal, 1995].
Using 40mm^2 culture dishes prepared as described in Sections 5.3.2.2 (hepatocytes) and 5.3.2.3 (adipocytes) a culture dish was taken before the incubation with ^{14}C-oleate, together with time points throughout the incubation period. The culture dishes were washed twice with phosphate buffered saline at pH7.4 and to each was added 1ml of 0.1M NaOH. The surface of each of the culture dishes was scraped using a cell scraper and the cell extract was transferred to a 7ml polystyrene bijoux. The culture dishes were washed twice with 500µl of 0.1M NaOH and this was added to the previously retained extract. From these extracts, 100µl was transferred to a microtitre plate well in triplicate. Standards were prepared ranging between 50 and 400µg ml^{-1} using a 400µg ml^{-1} BSA stock solution and from these

100µl of each was pipetted into separate wells within the microtitre plate, in triplicate. To each well was added 100µl of Lowry reagent. The plate was shaken for 30 seconds and left to stand for 20 minutes at room temperature. With rapid and immediate mixing, 50µl of Folin-Ciocalteu's reagent was added to each well. The plate was shaken for 30 seconds and left to stand for 30 minutes at room temperature. The absorbance was determined at 595nm for all standards and samples. From the absorbance of the standards a standard curve was generated, from which the amount of protein present in the sample solutions was derived.

5.3.3 Influence of LMAP Components on Lipid Metabolism

The effects of the LPS (*E. coli* serotype 0111:B4), LTA (*S. aureus*) and lipoglycan (*P. acnes*) were investigated *in vitro* using hepatocytes and adipocyte cell lines. These cell lines were incubated with each of the LMAP components in the presence of ^{14}C-oleate. Following incubation, the amount of fatty acid uptake was determined by extraction of the intracellular ^{14}C-lipid as described in Section 5.3.2.5. Following this, the radiolabel was removed and the cell lines were incubated with the LMAP components alone. These values were then compared to incubations in the absence of an LMAP effector (negative controls). Firstly, to ensure that each of the cell lines was responsive to external stimuli, cells were incubated in the presence of insulin as it is widely accepted that insulin increases the amount of fatty acid taken up and converted into triglycerides in both hepatocytes and adipocytes [Sherwood, 1993]. This control ensured that both cell lines were functioning correctly.

5.3.3.1 Effect of Insulin

A human insulin stock solution was prepared to a concentration of 1.0µM in 3.0mM HCl (prepared using EF-water). From this stock solution, a 200nM dilution was prepared in medium containing 0.1mM BSA-complexed ^{14}C-oleate (produced from a 1.0mM solution prepared as described in Section 5.3.2.1). Additional dilutions were prepared with final insulin concentrations of 100nM and 10nM, by diluting the 1.0µM stock solution 2-fold and 20-fold respectively with EF-water and then making a further 5-fold dilution with medium

containing 0.1mM BSA-complexed ^{14}C-oleate. Each concentration was filter-sterilised using a 0.2µm syringe filter prior to use.

To two culture dishes, 1.5ml of each concentration of insulin-containing medium described above was carefully pipetted down the inside wall of the culture dish. Additionally, two culture dishes were also incubated with medium that did not contain insulin and these culture dishes were used as negative controls. All culture dishes were incubated at 37°C, 5% CO_2 and 100% relative humidity for 5 hours. Following incubation, the medium was removed and the cells were washed twice with 1ml of PBS containing 0.1%(w/v) BSA at 37°C. To each culture dish the medium was replaced with 1.5ml of an equivalent concentration of insulin-containing medium, but in the absence of ^{14}C-oleate. These were incubated for a further 12 hour under the same environmental conditions as above. After incubation the medium was removed and the cells were carefully washed twice with 1ml of PBS containing 0.1%(w/v) BSA and frozen upside-down to -30°C. The dishes were removed and used for extraction of the intracellular ^{14}C-lipid using the method described in Section 5.3.2.5. The amount of radiolabel present in each extraction was determined via scintillation counting.

5.3.3.2 Preparation of LMAP-containing Medium for Cell Incubation

Dilutions of the LMAP components discussed in Section 5.3.3 were prepared using either DMEM (adipocytes) or MEM (hepatocytes) depending on the cell line used. Both of these culture media were prepared with and without 0.1mM ^{14}C-oleate as described in Section 5.3.2.1.

- Lipopolysaccharide from *Escherichia coli*

LPS from *E. coli* was obtained commercially and prepared as a 5µg ml^{-1} in EF-water. From this stock solution, a 100ng ml^{-1} dilution (1/50) was prepared in medium containing 0.1mM BSA-complexed ^{14}C-oleate (produced from a 1.0mM solution prepared as described in Section 5.3.2.1). This procedure was repeated to produce a 100ng ml^{-1} dilution of LPS in

medium without ^{14}C-oleate. After preparation, both solutions were filter-sterilised using a 0.2μm syringe filter prior to use.

- Lipoteichoic Acid from *S. aureus*

LTA was purified as described in Sections 3.3.3.7 and 3.3.3.8. Prior to use, the endotoxin concentration was reduced as described in Section 5.3.1.2. The LTA was freeze-dried and then resuspended in EF-water to produce a stock solution of 5mg ml^{-1}. From this stock solution, a 100μg ml^{-1} dilution (1/50) was prepared in medium containing 0.1mM BSA-complexed ^{14}C-oleate. This procedure was repeated to produce a 100μg ml^{-1} dilution of LTA in medium without ^{14}C-oleate. After preparation, both solutions were filter-sterilised using a 0.2μm syringe filter prior to use.

- HIC-eluted Lipoglycan from *P. acnes*

HIC-eluted lipoglycan was prepared as described in Sections 3.3.3.9. Prior to use, significant amounts of endotoxin were decreased as described in Section 5.3.1.2. The lipoglycan was freeze-dried and then resuspended in EF-water to produce a stock solution of 5mg ml^{-1}. From this stock solution, a 100μg ml^{-1} dilution (1/50) was prepared in medium containing 0.1mM BSA-complexed ^{14}C-oleate. This was filter-sterilised usinga 0.2μm syringe filter prior to use. This procedure was repeated to produce a 100μg ml^{-1} dilution of lipoglycan in medium without ^{14}C-oleate. After preparation, both solutions were filter-sterilised using a 0.2μm syringe filter prior to use.

- Negative Control

A negative control was also prepared for each of the cell lines, in which EF-water was diluted 1/50 with either DMEM or MEM containing 0.1mM BSA-complexed ^{14}C-oleate. This was filter-sterilised using a 0.2μm syringe filter prior to use. This procedure was repeated to produce a negative control in medium without ^{14}C-oleate. After preparation, both solutions were filter-sterilised using a 0.2μm syringe filter prior to use.

5.3.3.3 Incubation of Cells with LMAP Preparations

The experimental set-up was designed to enable the effects on both the uptake of fatty acid, together with the secretion of ^{14}C-lipid to be determined simultaneously. 40mm^2 culture dishes were prepared as described in Section 5.3.2.2 (hepatocytes) and 5.3.2.3 (adipocytes). Protein was determined in a culture dish as described in Section 5.3.2.7 and was representative of the amount of protein in each culture dish used. To ten culture dishes, 1.5ml of negative control medium containing 0.1mM ^{14}C-oleate (described in Section 5.3.3.2) was carefully pipetted down the inside wall of each culture dish. Additionally, 1.5ml of 100ng ml^{-1} LPS medium containing 0.1mM ^{14}C-oleate, prepared as described in Section 5.3.3.2 was added to four culture dishes. This was repeated for the LTA and lipoglycan media, again containing 0.1mM ^{14}C-oleate, also described in Section 5.3.3.2. All culture dishes were incubated at 37°C, 5% CO_2 and 100% relative humidity for 5 hours. Following incubation, the medium was removed and retained for later analyses. All culture dishes were washed twice with 1ml of PBS containing 0.1%(w/v) BSA. To the ten negative control culture dishes, two dishes were frozen upside-down to -30°C and analysed for intracellular ^{14}C-lipid as described in Section 5.3.2.5. To the remaining eight dishes, 1.5ml of media was added in duplicate, containing either LPS, LTA, lipoglycan or negative control media prepared as described in Section 5.3.3.2 without ^{14}C-oleate. Additionally, using the dishes containing the LMAP media, two culture dishes from each LMAP component incubation were frozen upside-down to -30°C and analysed for intracellular ^{14}C-lipid as described in Section 5.3.2.5. The remaining two dishes were incubated with media that did not contain ^{14}C-oleate. All culture dishes were incubated at 37°C, 5% CO_2 and 100% relative humidity for 16 hours. The incubations are summarised in Table 5.1.

Table 5.1 Experimental Set-up for the Incubation of Cells with LMAP Preparations

LMAP Component (5 hours)	LMAP Component (16 hours)
Medium with ^{14}C-Oleate Radiolabelled	Medium without Radiolabel
Negative Control	*
Negative Control	Negative Control
Negative Control	LTA
Negative Control	LPS
Negative Control	Lipoglycan
LTA	*
LTA	LTA
LPS	*
LPS	LPS
Lipoglycan	*
Lipoglycan	Lipoglycan

* Indicates the culture dish was removed and frozen for intracellular ^{14}C-lipid analysis.

Following incubation, the medium of each was removed and retained for later analyses and the culture dishes were washed twice with 1ml of PBS containing 0.1%(w/v) BSA. These were frozen upside-down to -30°C and extracted for intracellular ^{14}C-lipid as described in Section 5.3.2.5. The amount of radiolabel present from each extraction was determined via scintillation counting.

5.3.3.4 Determination of Secreted TAG and Fatty Acid Following Incubation with LMAP Components

To determine whether the hepatocytes were secreting TAG or if the adipocytes were secreting fatty acid in response to either of the LMAP components prepared in Section 5.3.3.2, the spent LMAP-containing medium collected from the LMAP incubations in Section 5.3.3.3 was analysed. Each spent LMAP-containing media was centrifuged at 14,000 x g for 30 seconds to sediment any cell debris or detached cells. From the each of the different media supernatants, 900µl was removed from each duplicate and transferred to a 9ml screw-cap tube (1.8ml final volume). A further 200µl was removed from each and used to assess the structural integrity of the cells using the LDH assay described in Section 5.3.2.6 where by comparisons were made to the to the negative control. To the 1.8ml volume of

supernatant, 2ml of methanol was added followed by 2ml of chloroform. This resulted in a final solvent ratio of 1.0:1.0:0.9 (methanol:chloroform:water). The extraction tubes were vortexed for 30 seconds and centrifuged at 500 x g for 30 minutes to break the emulsion. The bulk of the aqueous upper phase was aspirated to a waste bottle and using a glass pipette, ≥1.5ml of the lower organic phase was transferred to a clean screw-cap tube. From the organic phase 1.5ml was volumetrically removed and transferred to a 2ml glass vial and dried overnight in a fume cupboard to remove the chloroform. Each extract was then resuspended in 10µl of chloroform. A 10 x 10cm silica gel 60 thin layer chromatography (TLC) plate was prepared by marking an origin with a soft pencil, 1cm from the bottom of the concentration zone. The plate was divided into 1cm-width vertical lanes by scoring the silica layer with a scalpel. From each vial the 10µl sample was removed and spotted onto a lane prepared on the TLC plate at the origin. This was spotted in approximately 0.5µl aliquots, allowing each aliquot to dry between additions. The sample vial was washed with 10µl of chloroform and this was also loaded onto the original sample spot. For analysis of secreted TAG from the hepatocytes, a triolein standard was prepared to 100µg ml^{-1} and 10µl was loaded onto the last lane of the TLC plate. For analysis of secreted fatty acid from adipocytes, an oleate standard was also prepared to 100µg ml^{-1} and 10µl was spotted onto the last lane of the TLC plate. A mobile phase was prepared consisting of 80%(v/v) hexane, 20%(v/v) diethyl ether and 5%(v/v) glacial acetic acid. This was added to a TLC tank and the spotted TLC plate was run until the solvent front was 5mm from the top of the plate. Following chromatography the plate was dried in a fume cupboard and then developed in a separate tank containing iodine vapour. Once the colour had sufficiently developed within the standard, the plate was removed from the tank and the visual components were marked with a soft pencil. The area equivalent to the position of the standard was identified on each of the sample lanes and removed by scraping each into a scintillation tube. To each scintillation tube, 4ml of scintillation cocktail was added. The tubes were capped, briefly shaken and the disintegrations per min were determined for each sample.

5.4 Results and Discussion

5.4.1 Analysis of Endotoxin in LMAP Preparations

The amount of endotoxin was determined in dilutions of both LTA and lipoglycan using a LAL gel-clot assay, sensitive to an endotoxin concentration of ≥0.125EU, (equivalent to 14pg ml^{-1}). In both LTA and lipoglycan preparations, gel-clots were observed in the ≥1/10,000 dilution, which equated to 1250EU or 140ng mg^{-1} of LMAP. Both LMAP components were intended for use at a concentration of 100µg ml^{-1}. This was equivalent to 14ng ml^{-1} of endotoxin. It has been reported that endotoxin concentrations as low as 1ng ml^{-1} produced a measurable response within both human and bovine monocytes [Jungi *et al.*, 1999; Lindemann, Economou & Rothermel, 1988], therefore this level of endotoxin was unacceptable. Additionally, endotoxin contamination has been reported to be responsible for contribution to the effects of LMAP components and therefore providing false-positive results or exaggerated effects for the LMAP constituents used [Gao *et al.*, 2001]. For this reason, it was necessary to reduce the concentration of the endotoxin within intended preparations to acceptable levels (<1ng ml^{-1}). Both LMAP samples were separately applied to a 1ml polymixin B affinity column. Following elution and freeze-drying, the preparations were tested using the LAL gel-clot assay as before. From this assay, both LTA and lipoglycan were found to form gel-clots at dilutions of 100-fold or less. This was equivalent to ≤12.5EU (1.4ng mg^{-1} of LMAP). As both LMAP preparations were for utilisation in incubations at a concentration of 100µg ml^{-1}, this equated to a final endotoxin concentration of ≤140pg ml^{-1} and therefore acceptable. Analysis of the phosphorus within the LTA sample, both before and after elution from the polymixin B affinity column revealed a 40% reduction in the post-elution samples. This represented a 40% loss in recovery, which was suspected to have been caused by non-specific binding via either hydrophobic or ionic interactions. Elution of the LTA from the column was modified by varying the buffer concentrations used in an attempt to increase the percentage recovery. However, by increasing the percentage

recovery a negative effect on the endotoxin binding capabilities was observed, therefore development was abandoned and the losses accepted. Analysis of the carbohydrate within the lipoglycan sample, both before and after elution from the column, resulted in a 98% recovery and therefore both the decrease in endotoxin amount and the percentage recovery were acceptable.

5.4.2 Uptake of Radiolabelled Fatty Acid

5.4.2.1 Hepatocytes

The amount of fatty acid taken up by the hepatocytes was determined by measuring the amount of intracellular ^{14}C-lipid against time and calculated as the mean ±SEM. This data was plotted and a polynomial fit was applied (Figure 5.1). From this plot a reduction in the rate of uptake was observed following the 6 hour incubation. Using the equation for a polynomial line ($y = -0.048x^2 + 53.789x - 75.823$) it was calculated that the curve showed a plateau at 9 hours, after which the amount of intracellular ^{14}C-lipid was expected to decrease. This decrease in uptake was due to a reduction in the rate of uptake of fatty acid coupled with cell death caused by the toxic effects of the radiolabelled oleate. In support of this, the amount of intracellular ^{14}C-lipid within cells following a 24 hour incubation was determined and measured 1952 ±187dpm. This represented >85% reduction in the amount of intracellular ^{14}C-lipid in comparison to the amount of fatty acid uptake within cells incubated for 6 hours (12959 ±146dpm). The incubation medium was assayed for LDH activity and compared to disrupted cells (total LDH activity). After 6 hours there was a 5% increase in the LDH activity in comparison to the total LDH activity. After 24 hour the LDH activity had increased to 18% and therefore suggested that the structural integrity of the cells had been compromised following the extensive incubation period. The LDH activity was minimal after 6 hours in comparison to the total LDH activity and it was therefore assumed that the cells were structurally intact at that time. Furthermore, the amount of protein before and after 6 hours was equal (0.701mg total protein), indicating no change in the cell number

in response to the incubation conditions, therefore, fatty acid was increasing per cell and not due to an increase in cell numbers. Balancing the uptake of fatty acid with the LDH activity, a 6 hour incubation period with ^{14}C-oleate appeared to provide both a sufficiently radiolabelled intracellular ^{14}C-lipid pool and without significant loss of cellular integrity. On the basis of this data, pre-labelling of the hepatocytes was performed using 0.1mM ^{14}C-oleate for ≤6 hours.

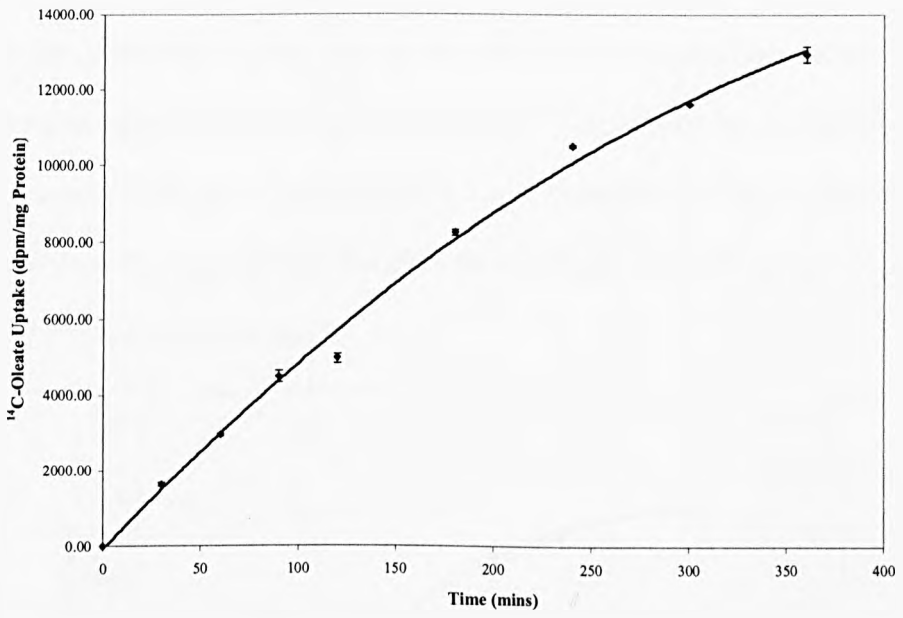


Figure 5.1 Uptake of Radiolabelled Fatty Acid by HepG2 Cells

A graphical representation to show the amount of ^{14}C-oleate taken up (dpm/mg protein) by a hepatocyte cell line over time (min). The results are presented as mean ± SEM for duplicate incubations.

5.4.2.2 Adipocytes

The amount of fatty acid taken up by the adipose cells was determined by measuring the amount of intracellular ^{14}C-lipid against time and calculated as the mean ±SEM. This data was plotted and a polynomial fit was applied (Figure 5.2). From this plot, a reduction in the rate of uptake was observed between 5 and 8 hour incubation. The polynomial fit ($y = -0.0223x^2 + 20.684x + 6.7825$) suggested a plateau time of 7 hours. After determination of the LDH activity in each of the spent extracellular incubation media, a large increase was

observed after 8 hours of incubation. This suggested that the structural integrity of the cells was being compromised and therefore rendered the cells unusable for subsequent studies. After the 5 hour incubation the LDH activity was similar to the LDH activity of the control and therefore cell damage was considered negligible after such time. Furthermore, the total amount of protein (0.331mg total protein) present in both the control and 5 hour incubation was comparable indicating that the fatty acid was increasing per cell and not due to an increase in cell numbers. This collectively suggested that the cells were intact and remained adhered to the culture dish surface. As with the hepatocytes, a balance was sought that enabled the generation of a sufficiently radiolabelled ^{14}C-lipid pool, but did not affect the structural integrity of the cells. On this basis, a 5 hour incubation period was considered to meet both of these requirements and therefore pre-labelling of the adipocytes was performed using 0.1mM ^{14}C-oleate for ≤5 hours.

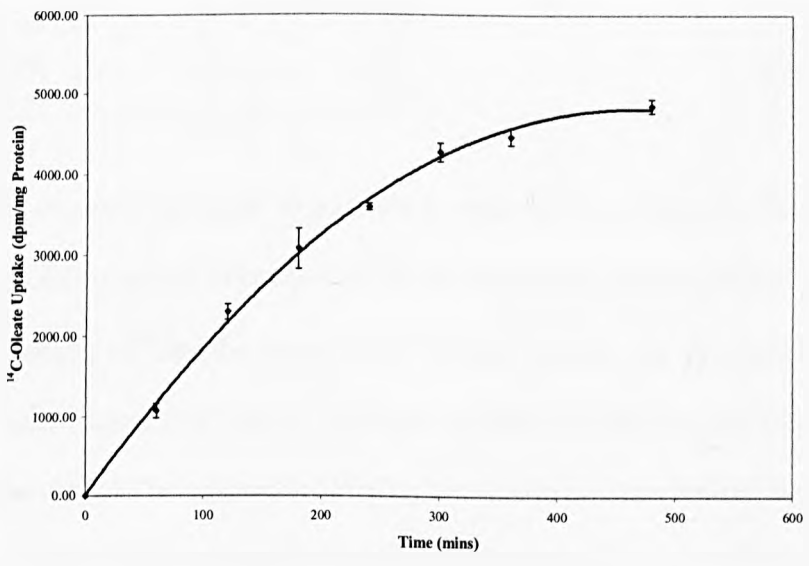


Figure 5.2 Uptake of Radiolabelled Fatty Acid by 3T3L1 Cells

A graphical representation to show the amount of ^{14}C-oleate taken up (dpm/mg protein) by an adipocyte cell line over time (min). The results are presented as mean ± SEM for duplicate incubations.

5.4.3 Influence of LMAP Components on Lipid Metabolism

5.4.3.1 Effect of Insulin

It is widely accepted that insulin increases the amount of fatty acid taken up and converted into TAG in both hepatocytes and adipocytes [Sherwood, 1993]. For this reason the effect of insulin on each of the cell lines was determined, as an increase in the uptake of fatty acid in response to insulin ensured that the cells were responsive to an external stimuli. Following incubation with three concentrations of insulin the amount of fatty acid taken up by the cells was determined by measuring the intracellular ^{14}C-lipid in each case. These values were compared to a negative control. The results are shown in Table 5.2

Table 5.2 The Effect of Insulin on the Uptake of Fatty Acid in Hepatocytes and Adipocytes

Insulin Concentration / nM	Intracellular ^{14}C-lipid (dpm/mg Protein)	
	Hepatocytes	Adipocytes
Negative Control	12473	4595
10	13757	6091
100	14097	6289
200	14177	7149

In the presence of insulin, between 10 and 14% increase in the intracellular ^{14}C-lipid within the hepatocytes was observed in comparison to the negative control incubation. However, within the adipocytes cell line the intracellular ^{14}C-lipid increase was shown to be between 34 and 56% in the presence of insulin. These results demonstrated that both cell lines were positively responding to the presence of insulin. This confirmed that both of the cell lines were functioning correctly to external stimuli and were applicable for use in subsequent incubations.

5.4.3.2 Effect of LMAP components on the Metabolism of Fatty Acid within hepatocytes

The activity of LDH was determined in each of the spent incubation media following the incubation periods. Comparing the LDH activity of the LMAP containing media to that of the control incubation media, no increase was observed following the 5 hour radiolabelling incubations for all conditions, indicating that the structural integrity of the

hepatocytes had not been breached during the radiolabelling procedure. After the 16 hour incubation period the LDH activities for both LPS and lipoglycan were comparable to the LDH activity of the 16 hour control incubation. However, for both 16 hour LTA incubations (i.e. with and without LTA during radiolabelling) a 3-fold increase in the LDH activity in the culture media was observed, suggesting that the structural and functional integrity had been lost in a significant proportion of the incubated cells. This was therefore considered when comparing the effect of the LMAP components to the controls when determining the amount of secretion. Additionally, as an increase in the LDH activity indicated an increase in the amount of cellular damage it suggested that LTA had a specific cytotoxic property that was not possessed by either the LPS or the lipoglycan.

By determining the amount of intracellular ^{14}C-lipid, following incubation with each of the LMAP components during the 5 hour radiolabelling incubation, the effect of each of the LMAP components on the uptake of fatty acid was assessed (Figure 5.3A). The data demonstrated that there was a considerable decrease in the uptake of fatty acid following incubation with all LMAP components, when compared to the 5 hour control incubation. This suggests that all LMAP components have an inhibitory effect on the uptake of fatty acid.

The effect of LMAP components on the metabolism of lipid within HepG2 was also determined following removal of the fatty acid from the incubation media. By subtracting the amount of intracellular ^{14}C-lipid present after the 16 hour incubation period with each LMAP, from the initial intracellular ^{14}C-lipid following the 5 hour fatty acid uptake period, the amount of secreted lipid was calculated (Figure 5.3B and C). Within all LMAP component incubations, a reduction in the amount of secretion was observed in comparison to the control incubations, despite the variability associated with the duplicates. This reduction in secretion could be associated with the decreased amount of fatty acid uptake during the 5 hour incubation whilst in the presence of the LMAP components (Figure 5.3C). However, a similar decrease in secretion was also observed within cells incubated in the absence of LMAP during uptake of fatty acid (Figure 5.3B), where the initial intracellular

^{14}C-lipid pool would have been the same as the control incubations. Analysis of the actual secreted lipid revealed small amounts of secreted ^{14}C-TAG within the control, but only trace amounts for each of the LMAP incubations, thus further validating the values calculated for ^{14}C-lipid secretion and suggesting that the secreted lipid was probably TAG. The values calculated for the secretion of ^{14}C-lipid following the 16 hour incubation with LTA were considerably higher than those calculated for LPS and lipoglycan. Furthermore, the LTA values might be expected to be higher still, if the increased LDH activity was considered, as the increased LDH activity suggested that a proportion of the cells had lysed and this would result in a decrease in the total intracellular ^{14}C-lipid value observed. Consequently, the effect of LTA on the secretion of ^{14}C-lipid would be more comparable to that of the control.

CHAPTER 6

General Conclusions

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6.1 Extraction and Purification of LMAP from *P. acnes*

Following successful replication of the phenol-water extraction and consequent HIC purification of LTA from *S. aureus*, this generic method for purification of LMAP components was applied to *P. acnes* [Fischer, 1991]. Analysis of the carbohydrate enabled the generation of an elution profile for the extracted components and this was suggestive of a lipoglycan-type structure. Due to the suspected elution of two overlapping amphiphilic carbohydrate-containing peaks, the HIC-eluted material was subjected to SDS-PAGE for analysis. Initially, three components were observed and using protein molecular weight marker the migration of these bands was described. The upper-most band ran in the 35-38kDa region, below this, a second component ran in the 24-29kDa region and lastly, the lowest component ran in the 14-20kDa region. The 35-38kDa component was later confirmed as an aggregate of one or more of the LMAP components. Analysis of this aggregate suggested that it was composed of the 24-29kDa component and an unidentified constituent, which was calculated to be approximately 10kDa. After development of an effective sample solublisation process the SDS-PAGE analysis confirmed the presence of two discrete amphiphilic components. These were described as the upper component (24-29kDa region) and the lower component (14-20kDa region). These findings were similar to the observation that mycobacterial lipomannan also runs in the 14-20kDa region whereas the larger LAM (containing the additional arabinan units) runs in the 36kDa region [Chatterjee *et al.*, 1992b]. Additionally it was shown that when fractions eluting either directly before or promptly after the bulk of the HIC-eluted material were selected and analysed by SDS-PAGE they were found to contain predominantly lower and upper components respectively. Using this information together with the observed migration on SDS-PAGE it was deduced that as elution from HIC yields progressively more hydrophobic components the lower component was a smaller and more hydrophobic amphiphile in comparison to the upper component. Attempts were made to separate the two components by modifying the gradient conditions used for the HIC procedure, although this was unsuccessful. Therefore, to enable

purification of the two LMAP components, the analytical-scale SDS-PAGE procedure was scaled-up to preparative-scale SDS-PAGE. This allowed the application of 3mg of HIC-eluted material per gel and resulted in successful separation of the upper and lower components. Using electro-elution approximately 80% of the material was recovered from the gel slab. Both components were desalted and the purity of the components was confirmed by applying a small amount of each component to analytical-scale SDS-PAGE. On the basis of the HIC-elution characteristics, together with the large size of each component, both were suspected as being lipoglycan-like LMAP structures. Following purification of the upper and lower components these were designated LMAP1 and LMAP2 respectively.

6.2 Chemical and Immunochemical Characterisation of LMAP Components from *P. acnes*

To investigate whether the HIC-eluted LMAP was a lipoglycan or LTA the molar ratio of carbohydrate to phosphorus was determined. From this data, a ratio of 17:1 was calculated and thus supported a lipoglycan structure [Sutcliffe, 1994b]. Analysis of the fatty acids within LMAP1 and LMAP2 established that both possessed fatty acids and therefore confirmed that both components were authentic LMAP's as opposed to polysaccharide structures of the cell wall. Furthermore, the fatty acid composition of each of the purified LMAP components was similar and comparable to the profile produced for whole cell fatty acid. This was expected as the composition of the cellular fatty acid often dictates the fatty acids incorporated into the LMAP component [Fischer, 1988].

The successful extraction and purification of practical amounts (~1mg) of both LMAP1 and LMAP2 allowed the chemical analysis of the carbohydrates using GC-FID. On analysis, large amounts of both mannose and glucose were observed together with variable amounts of galactose and glycerol. Furthermore, a significant amount of an inositol-like constituent

was identified within LMAP1 together with trace amounts within LMAP2. Using GC-FID, it was not possible to confirm the presence of inositol, due to disparity in the retention times of the sample material peak and the inositol standard peak. However, using GC-MS the presence of inositol was substantiated by analysis of the fragmentation patterns generated by the sample peak in question and the standard inositol. With the recent identification of both glycerol and phosphorus above, this data was suggestive of a phosphatidylinositol (PI) anchor. In this context it was notable that the lipomannan LMAP of *Propionibacterium freudenreichii* also contained minor amounts of inositol and had a carbohydrate:phosphorus ratio of approximately 33:1 [Sutcliffe & Shaw, 1989], again leading to the suggestion of an anchor unit based on PI. Moreover, an apparently low molecular weight mannose-containing amphiphile, which cross-reacts with a monoclonal anti-phosphatidylinositol mannoside serum, has been observed in some extracts of *P. acnes* (Sutcliffe, personal communication). The occurrence of a PI anchor would be comparable to the anchor of mycobacteria which has also been identified as a PI anchor [Hunter & Brennan, 1990] and therefore suggested the possibility of a common structural theme within the actinomycetes.

Comparison of the relative abundance of the hexoses within each of the purified LMAP components revealed a significant difference in the amounts of both glucose and galactose, as LMAP2 contained relatively less glucose and only a trace amount of galactose in comparison to LMAP1. Using this information, together with the increased migration of LMAP2 on SDS-PAGE it further supported the proposition that LMAP2 was a smaller amphiphile relative to LMAP1. These observations also suggested that the difference in size might reflect the reduction in the galactose and glucose content of the LMAP2 component. Furthermore, it was suspected that the deficiency in glucose and galactose within LMAP2 was likely to be due to a decrease in substitution along the polysaccharide chain, which resulted in the increased hydrophobicity of LMAP2, as observed during the HIC elution of the two components. Whether there was any direct relationship (e.g. at the biosynthetic level) between these two components remains to be determined.

It was apparent that the carbohydrate profile of each of the LMAP components bore a significant resemblance to the constituents previously identified as components of the *P. acnes* cell wall polysaccharide [Cummins & Hall, 1986]. Previous studies [Cummins & White, 1983; Nagaoka *et al.*, 1985] have also shown the presence of diaminohexuronic acid within the cell wall polysaccharide. To investigate whether these characteristic amino sugars were also represented within the LMAP, each of the components was hydrolysed and analysed for the presence of amino constituents. Using a ninhydrin-collidine reagent, an equivalent abundance of an amino compound was observed within the hydrosylates of both LMAP components. Furthermore, a distinctive blue colour was observed which has been previously associated with the presence of diaminohexuronic acid [Cummins, 1985; Cummins & White, 1983]. Attempts were made to define the amounts of amino sugar present more accurately using a dimethylaminobenzaldehyde (DMAB) reagent, which was more commonly utilised for estimation of acetyl-hexosamines. The results confirmed the presence of an amino sugar, but in considerably lower quantities than that intimated by the ninhydrin-collidine assay. However, it has been reported previously that the DMAB-based assay was less sensitive to aminohexuronic acids and therefore the assay might have been under reporting the actual amount present [Hancock & Poxton, 1988]. In summary, this information suggested that LMAP was very similar in composition to the cell wall polysaccharide, although LMAP was clearly distinguished by the presence of fatty acids. It was conceivable that a relationship might exist between the LMAP and cell wall polysaccharide structures of *P. acnes*. This situation was similar to the scenario within *Streptococcus pneumoniae* which was found to produce a structurally complex cell wall teichoic acid which has an identical chain structure to the glycolipid-anchored LTA of this organism [Fischer, 1997].

Using samples that had been electrophoretically transferred to a nitrocellulose membrane support LMAP1 and LMAP2 were probed using Con A and both components were found to possess significant amounts of mannosyl termini. These observations were comparable to the results shown with mycobacterial LAM and therefore suggested a degree of structural

homology between the two LMAP components. A consequent immunochemical analysis with both polyclonal and monoclonal antibody directed to LAM was performed to investigate the possibility of further similarities between the LMAP components and mycobacterial LAM. Both components failed to bind either antibody and it was therefore concluded that neither LMAP component shared mannose-containing epitopes with significant structural similarities with those in mycobacterial LAM. However, a small amount of material was found to bind the polyclonal anti-LAM antibody and possessed a molecular weight of <14kDa. Interestingly, this material was not observed on the polyacrylamide gel visualised by silver-staining which suggested that this component was not protein in nature and did not contain a significant amount of carbohydrate. In earlier immuno-characterisation attempts using the polyclonal antibody, a cross-reaction was observed in the 36kDa region and thus similar to the material previously confirmed as an aggregate. Consequently, this small cross-reacting component of <14kDa might have been involved in the formation of the 36kDa aggregate observed on SDS-PAGE. This was previously proposed following the SDS-PAGE analysis of the aggregate and therefore supported a hydrophobic component of approximately 10kDa. Due to the hydrophobic nature of the component, deduced through its ability to form the 36kDa aggregate in an aqueous environment, it was suggested that this component could be the hydrophobic anchor portion of either of the two LMAP components. Furthermore, as this component cross-reacted with LAM antibody it must share a structural epitope with LAM. If this was the case, the anchor in *P. acnes* was similar to the anchor structure in mycobacterial LAM. It is well established that the anchor within LAM is a PI lipid anchor [Hunter & Brennan, 1990], therefore further supported the earlier proposition of a PI lipid anchor within the LMAP of *P. acnes*.

6.3 Effects of LMAP on Mammalian Lipid Metabolism

Following characterisation of the structure of the lipoglycan components from *P. acnes* the biological activity was investigated in terms of the effects on mammalian lipid metabolism. This response was compared to the effects of other LMAP components namely LPS and LTA using the HepG2 and 3T3L1 cell lines. Prior to the incubation of LTA and lipoglycan with each cell line, the amount of endotoxin was decreased within the LTA and lipoglycan preparations to <1ng ml^{-1}. This was achieved by application of the samples to a polymixin B affinity chromatography column. Successful reduction in the endotoxin concentration was consequently determined using a LAL assay.

Following incubation of HepG2 cells with the LMAP components a reduction in the amount in the uptake of fatty acid was observed in comparison to the control incubations. Furthermore, a sizeable decrease in the secretion of ^{14}C-lipid was also found, although this effect was less prominent in the presence of LTA. However, LTA appeared to possess a cytotoxic element, as considerable amount of cell lysis was recorded. These *in vitro* observations were in contrast to previous *in vivo* observations, which suggested that both LPS and LTA significantly increased TAG secretion [Feingold *et al.*, 1992; Nonogaki *et al.*, 1995] and therefore suggested that the increase in TAG secretion determined *in vivo* might involve a secondary effector in addition to the LMAP components.

In contrast to the effects observed in HepG2, within the 3T3L1 cells both LPS and lipoglycan appeared to increase the uptake of fatty acid. This increase in uptake was coupled to an increase in secretion of ^{14}C-lipid in response to these LMAP components. In the presence of LTA, a similar increase in fatty acid uptake was not apparent, although an increase in ^{14}C-lipid secretion was observed. These increases in ^{14}C-lipid secretion support the previous *in vivo* observations, which demonstrated an increase in adipose lipolysis in response to both LPS and LTA [Feingold *et al.*, 1992; Nonogaki *et al.*, 1995]. Additionally, this *in vitro* analysis would suggest that these effects were primary in nature and therefore do not require the involvement of a secondary effector. Collectively, these observations

suggested that the mechanisms by which adipose lipolysis was induced were similar for both LPS and lipoglycan, but differ considerably for LTA. From these observations, it was concluded that mammalian lipid metabolism was influenced by the lipoglycan of *P. acnes* in a similar manner to that of LPS. Furthermore, as the lipoglycans of *P. acnes* have the ability to alter the metabolism of lipids within certain cells, it was also possible that it could also influence the composition of lipids on the skin. As changes in the lipid composition have been associated with the onset of *acne vulgaris* [Morello, Downing & Strauss, 1976], it was reasonable to speculate that these lipoglycans could therefore play a significant role in the pathogenecity of *P. acnes* and thus in the development of disease.

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