The Mechanisms of Antibacterial Action of Some Nonionic Surfactants

by

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ABSTRACT

Antibacterial agents are composed of a diverse group and many such agents have entered common usage through experience with little information on their mechanism of action. Study of the mechanism of action of an antimicrobial agent provides an insight into resistance mechanisms, toxicological problems and the design and development of new agents or combinations.

The primary target of most antimicrobial agents (excluding antibiotics) is the cytoplasmic membrane and associated enzymes. Membrane-active agents can cause a change in the fluidity and/or permeability of the cytoplasmic membrane. Such changes can be determined by the leakage of cellular constituents such as potassium ions, nucleotides and their constituents and amino acids. The effect of an antibacterial agent on the cytoplasmic membrane can also be determined by elucidating the effect of the antibacterial agent on the activity of membrane-bound enzymes and substrate uptake.

A range of anionic and nonionic surfactants were screened for potential antibacterial activity by a growth inhibition method. From this screening process a homologous series of alcohol ethoxylates were chosen for study. The series contained the same headgroup size (E6) but differed in the number of carbon atoms in their 'tail group' from 10 to 16.

A range of probes of cytoplasmic membrane damage were used to elucidate mechanism of action information for this series. Tetrazolium salts were used as a marker of damage to membrane-bound dehydrogenase enzymes. The two higher members of the homologous series (C14E6 and C16E6) had, in general little effect on enzyme activity compared with the other two members of the series and therefore limited antibacterial properties. Therefore the further studies concentrated on the other two members of the series (C10E6 and C12E6).

Both C10E6 and C12E6 are capable of damaging the cytoplasmic membrane, however, there appear to be subtle differences in the mode of action of the two surfactants. C10E6 has very little effect below its MIC whilst C12E6 exerted its effects at sub-MIC levels; both surfactants are present as monomers at these concentrations. It is proposed that these differences in interaction are due to the relative sizes of the hydrophobic tails of these compounds. The tail group of C12E6 is approximating that of phospholipid and is likely to insert itself into and flip-flop across the membrane. The shorter chain length of C10E6 increases the hydrophilicity of this surfactant which accumulates at the exterior of the bilayer until a saturation point is reached and gross membrane damage occurs. The use of DSC and ESR may provide considerable insight into this proposed interaction.

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Finally, I would like to dedicate this thesis to my brother Andrew.

ABBREVIATIONS USED IN THE TEXT

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ADP	adenosine diphosphate
HLB	hydrophilic-lipophilic balance
HMC	hemimicelle concentration
CF	carboxyfluorescein
CMC	critical micelle concentration
CTAB	cetyltrimethylammonium bromide
C10E6	hexa(ethyleneglycol)-monodecylether
C12E4	buta(ethyleneglycol)-monododecylether
C12E5	penta(ethyleneglycol)-monododecylether
C12E6	hexa(ethyleneglycol)-monododecylether
C12E7	hepta(ethyleneglycol)-monododecylether
C12E8	octa(ethyleneglycol)-monododecylether
C14E6	hexa(ethyleneglycol)-monotetradecylether
C16E6	hexa(ethyleneglycol)-monohexadecylether
DNA	deoxyribose nucleic acid
EO	ethoxylate
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
LPS	lipopolysaccharide
MDO	membrane derived oligosaccharides
MIC	minimum inhibitory concentration
MW	molecular weight
n	alkyl chain length of surfactant
NAD	nicotinamide dinucleotide

NCTC	National Collection of Type Cultures
NMR	nuclear magnetic resonance
OG	octyl glucoside
Omp A	outer membrane protein A
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
POE	polyoxyethylene
PMF	proton motive force
TCA	trichloroacetic acid
TTC	2,3,5-triphenyltetrazolium chloride
UV-IR	ultraviolet-infrared

CHAPTER ONE

Introduction

1.1 The structure of the bacterial cell

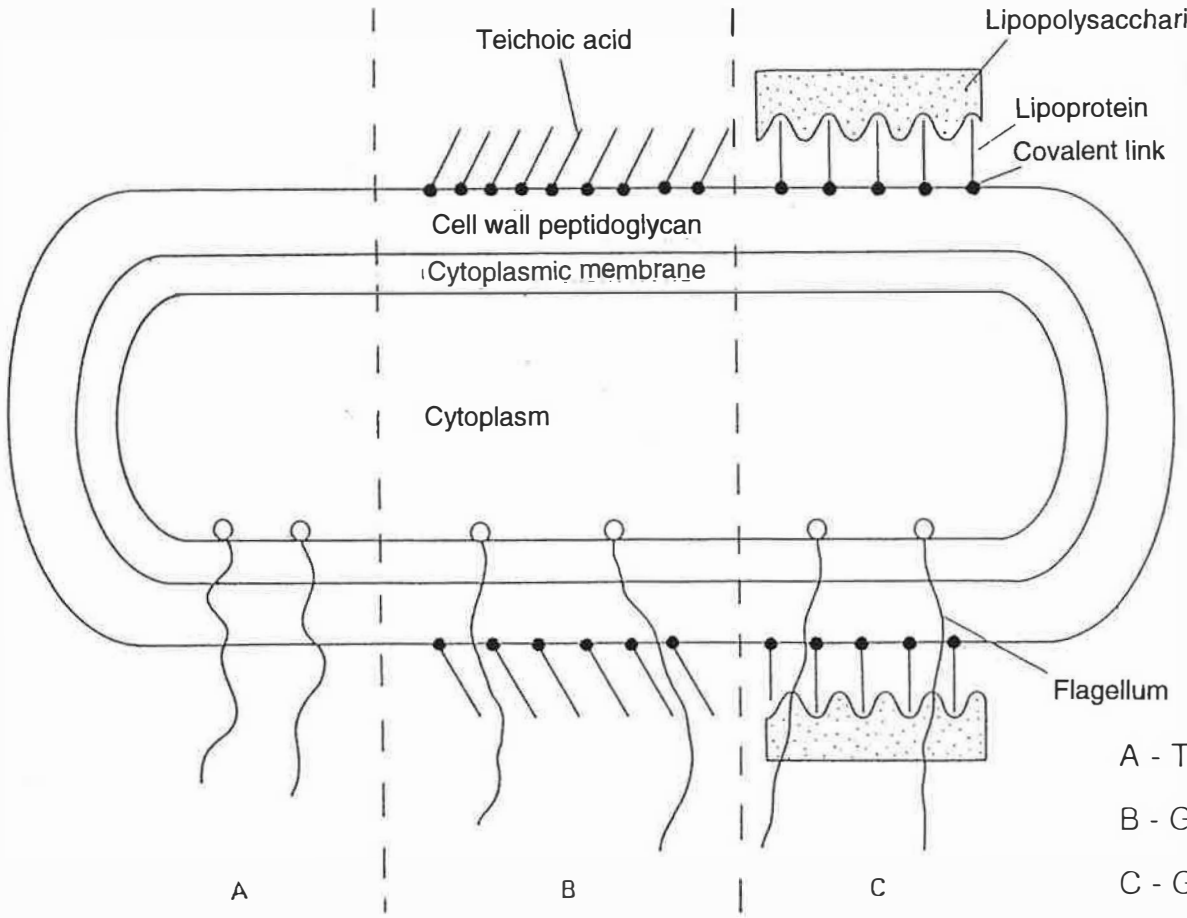
To enable the mechanism of action of any antibacterial agent to be investigated it is essential that the structure and function of the target of the agent, the bacterial cell, is fully understood. Much of this information can be found in any microbiological textbook (Mandelstam *et al*, 1982; Brock & Madigan, 1991; Goss *et al,* 1995; Prescott *et al,* 1996) and many in-depth reviews have provided information on this topic (Fuller & Lovelock, 1976; Salton & Owen, 1976; Lugentenberg & Van Alphen, 1983; Hancock, 1984; Nakaido & Vaara, 1985; Benz, 1988). Therefore only detailed information on structures relevant to the rest of this thesis are included in this section.

The generalised structure of the bacterial cell can be seen in figure 1.1.

The bacterial cell can be divided into three main components: the cell wall; the cytoplasmic membrane; and the cytoplasm.

1.1.1 The Cell Wall

The cell wall comprises about 20% of the dry weight of the bacterial cell. The cell wall is necessary to maintain the shape and integrity of the bacterium and all bacteria, with the exception of mycoplasmas, possess this structure. There is extensive shape and size variation amongst the genera and species of bacteria but the majority can be placed into one of two categories depending on the characteristics of their cell wall: Gram-positive or Gram-negative bacteria, depending on the ability of the cell wall to retain the basic dye crystal violet when washed with ethanol. During the Gram stain reaction an insoluble crystal violet-iodine complex is formed, this



A - The generalised structure of the bacterial cell

B - Gram positive structure

C - Gram negative structure

Figure 1.1 Diagram of the bacterial cell (After Hugo & Russell, 1992)

Figure 1.2 The structure of a Gram-positive bacterium (After Prescott *et al*, 1993)

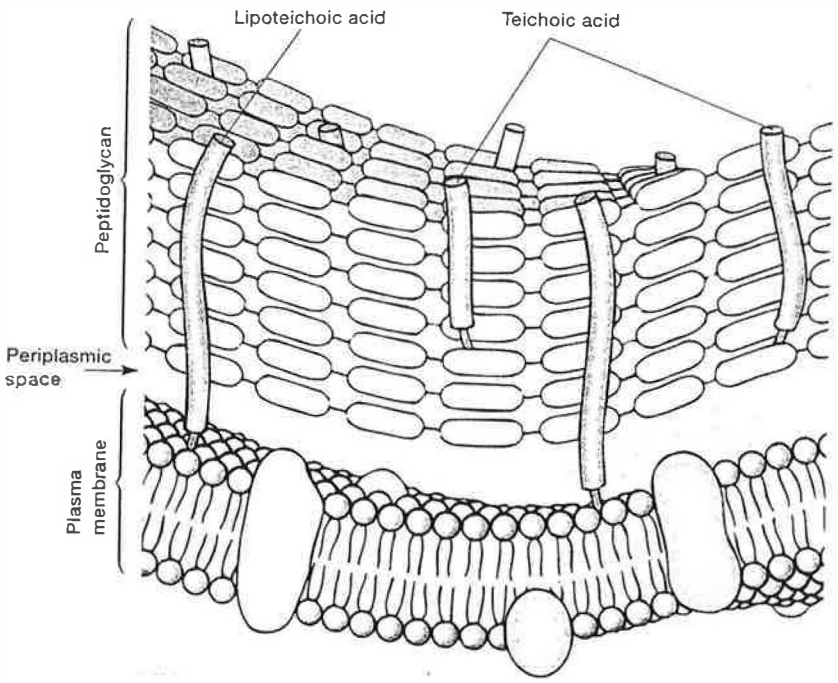
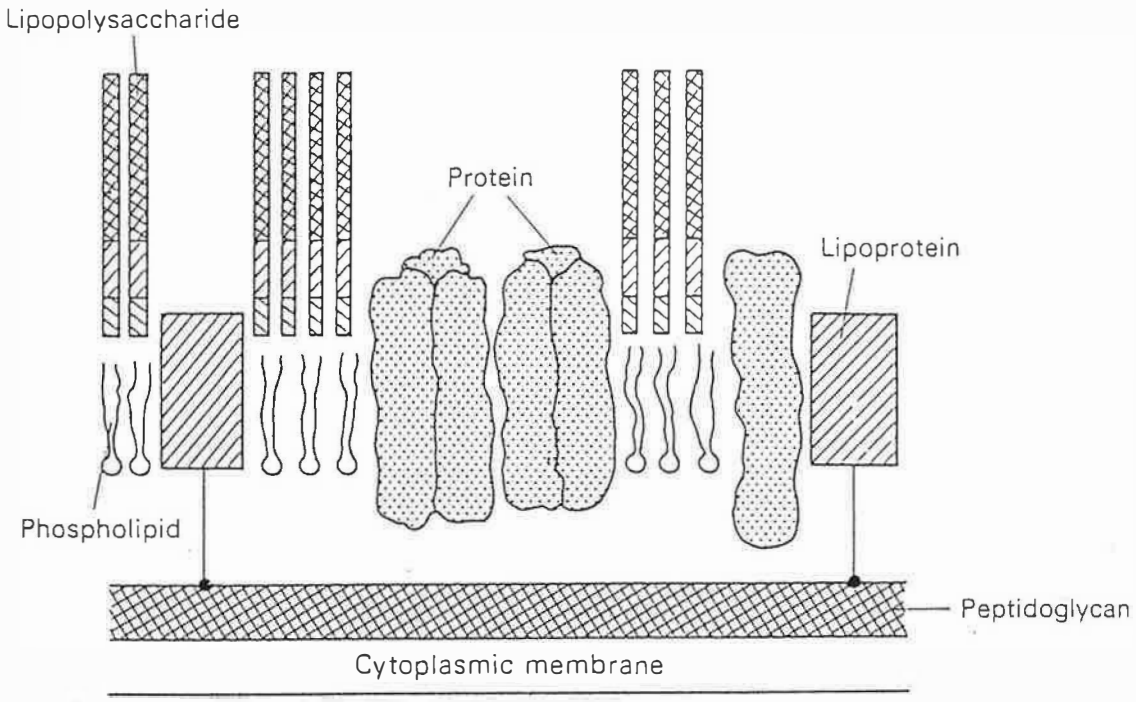


Figure 1.3 The structure of the cell wall of a Gram-negative bacterium (After Hugo & Russell 1992)



complex can be extracted by alcohol from Gram-negative cells but not Gram-positive. Gram-positive bacteria have very thick cell walls composed of several layers of peptidoglycan, these become dehydrated by alcohol causing pores in the walls to close, thus preventing the insoluble complex from escaping. In Gram-negative bacteria the solvent readily penetrates the outer layer and the solvent passage is not prevented by the thin peptidoglycan layer.

The wall of a Gram-positive bacterium is a structure between 15 and 80 nm thick. This structure has little lipid, a high amino sugar content, (15-20% of the dry weight) but a limited range of amino acids. The peptidoglycan content can comprise 50-80% of dry weight of the cell walls in most Gram-positive bacteria.

Walls of Gram-negative bacteria tend to have a high lipid content (10-20% of the dry weight) in comparison to Gram-positive cell walls which contain little extractable lipid, and a low amino acid content (2-5% of dry weight). The peptidoglycan content ranges from 1-10% of dry weight of the cell wall in Gram-negative bacteria. A large proportion of the lipid present in Gram-negative bacteria is due to the presence of the lipid A of lipopolysaccharides, diglyceride and fatty acid residues attached to the *N*-terminal cysteine of the lipoprotein, and phospholipids. These lipids are distributed in different regions of the membrane; lipid A tends to be located in the outer half of the outer membrane whilst the lipoprotein and phospholipid can be found in the inner half.

The Gram-negative cell envelope is composed of two membranes: the cytoplasmic (or inner membrane) and the outer membrane. These

membranes are separated by a layer of peptidoglycan and a cellular compartment called the periplasmic space. These structures will be discussed in more detail in sections 1.1.4 and 1.1.5.

1.1.2 The periplasmic space

The periplasmic space is an additional cell compartment occupying between 5 and 20% of the total volume of the cell and is an important physiological feature of the cell. This compartment may be filled with a loose network of peptidoglycan, suggesting it is more of a gel rather than fluid filled (Prescott *et al*, 1996). It contains a variety of enzymes, some functioning as scavenger or processing enzymes for the conversion of non-transportable metabolites to transport substrates and binding proteins for solutes including maltose, phosphate and ß-lactam.

The periplasmic space is almost iso-osmotic to the cytoplasm and hence osmotic pressure is maintained across the outer membrane and not across the inner membrane (Benz, 1988). The periplasmic space is strongly anionic in nature in comparison to the external medium, this is due to the presence of anionic groups attached to the outer membrane and the presence of anionic membrane derived oligosaccharides (MDO) which maintain the osmolarity of the periplasm. The protein content of the periplasmic space is approximately 4% of the total cell protein, these proteins can be subdivided into three classes by virtue of their function: proteins with catabolic functions, for example alkaline phosphatase; binding proteins with an affinity for nutrients such as sugars, ions and amino acids; and proteins included in the degradation or modification of harmful components, for example heavy metals and antibiotics (Lugtenberg & Van

Alphen, 1983).

1.1.3 The cytoplasm

The cytoplasm comprises the intracellular contents surrounded by the cytoplasmic membrane. It does not possess the array of specialised membrane systems that are found in eucaryotic cells, but it has a high overall density due to the existence of large numbers of ribosomes which are the site of protein synthesis. In addition to ribosomes other features are also present, including granules of reserve material such as polyhydroxybutyric acid or phosphate, or volutin granules. A range of enzymes, macromolecular precursors and low molecular weight metabolites are also contained in this compartment (Russell & Chopra, 1996).

The bacterial chromosome is a single circular molecule of double stranded DNA not associated with histones. It is not surrounded by a nuclear membrane but is present in the cytoplasm in a highly condensed state. The bacterial cytoplasm also contains other chromosomal material which may be present as either episomes or plasmids. Episomes are portions of the main chromosome that have become detached from it and plasmids are small pieces of DNA which can carry a limited amount of genetic information. Plasmids are often associated with conferring resistance against antibacterial agents or enhancing overall virulence.

1.1.4 The outer membrane

The outer membrane is located on the outside of the peptidoglycan. Thin sections of bacteria viewed using electron microscopy show this feature as a

trilamellar structure (Nikaido & Vaara, 1985; Beveridge & Graham, 1991). The majority of soluble proteins present in the cell are situated in the cytosol and some of these proteins are located in the region between the two membranes known as the periplasmic space. The two membranes are interconnected by 'zones of adhesion' or 'zones of fusion' (Lugtenberg & Van Alphen, 1983). These areas appear to be regions at which the inner and outer membranes are joined together. This allows the movement of newly synthesised membrane components such as lipids and lipopolysaccharides from the cytoplasmic membrane to the outer membrane, thus explaining the similar lipid composition of both these membranes.

Table 1.1 Principal components of bacterial cell walls (After Mandelstam *et al*, 1982)

Component	**Gram positive cell wall**	**Gram negative cell envelope**
Peptidoglycan	+	+
Teichoic acid and/or Teichuronic acid	+	-
Polysaccharide	+	+
Protein	± (not all)	+
Lipid	-	+
Lipopolysaccharide	-	+
Lipoprotein	-	+

Teichoic acids are associated with the cell wall and/or the cell membrane of Gram-positive bacteria and can comprise up to half the weight of the cell wall. They are not, however, present in all species of Gram-positive bacteria, their inclusion being dependent on growth conditions. It is thought that it is essential for the bacteria to include a negatively charged polymer in the cell wall to enable the binding of cations, such as Mg^{2+}, hence providing a cationic environment at the cell membrane.

Phosphatidylethanolamine, a zwitterionic lipid, is the major lipid component of the cell wall of the enteric bacteria *Escherichia coli* and *Salmonella typhimurium*. The outer membrane also contains phosphatidylglycerol and cardiolipin which are negatively charged. The inner monolayer of the outer membrane of enteric bacteria exclusively contain phospholipids where they cover approximately half of the surface, the other half of the surface being covered by proteins (Benz, 1988).

The outer surface of enteric bacteria is composed of lipopolysaccharide and protein in the ratio of approximately 40:60 with the result that the cell surface is very hydrophilic in nature. Lipopolysaccharides are ampipathic in character and hence have a structural similarity to lipids. The lipid A moiety, which is hydrophobic, is found in all lipopolysaccharides although the hydrophilic polysaccharide unit may vary within a single species. The basic structure of lipid A is that of a D-glucosaminyl-ß-D-glucosamine backbone linked to between 5 and 7 side chains via ester and amine bonds. These side chains are saturated fatty acid residues between 14 and 16 carbon atoms in length. The fatty acid residues often contain hydroxyl groups at the third carbon in the chain which can be utilised for the binding of additional

fatty acid residues via ester bonds.

The lipopolysaccharides form a barrier to the diffusion of hydrophobic molecules through the outer membrane partly due to the long oligosaccharides side chains attached to lipid A and also the ionic bridges between charged groups in the polysaccharide unit. 'Rough' mutants possess significantly shorter oligosaccharides and have an increased permeability to hydrophobic compounds thus illustrating their barrier function.

Electron micrographs, using freeze-fracture, have shown structural discontinuities in the outer membrane as particles and pits on opposing fracture faces of the outer membranes (Hancock, 1984). The particles are between 4 and 8 nm in diameter and are thought to contain both proteins and lipopolysaccharide and as many as 60,000 of these particles can be present in one cell.

The outer membrane has, by virtue of the habitat of many Gram-negative bacteria, developed into an effective barrier conferring protection to cells. It acts as a permeability membrane in enteric and other Gram-negative bacteria and is very efficient in this function (Nikaido & Vaara, 1985; Gilbert & Wright, 1987). Antibiotics effective against other bacteria, even if able to diffuse through the outer membrane, are slowed down. This enables the bacteria to inactivate the small quantities of penetrating antibiotic as opposed to the large amount which would be present in the external medium. This leads to high levels of resistance which is often found in Gram-negative bacteria. The outer membrane is also responsible for conferring the surface of the bacterial cell with a hydrophilic character. This

assists the bacteria to evade phagocytosis, whilst confering some complement resistance and an ability to avoid specific immune attack by alteration of the constitution of surface antigens.

Isolated envelope preparations of Gram-negative bacteria include the protein rich cytoplasmic membrane. The lipid composition of the outer membrane is very similar to that seen in the cytoplasmic membrane. The inner and outer membranes can be separated and analysis of the outer membrane has shown it to contain protein in addition to phospholipid and lipopolysaccharide. The proteins associated with the outer membrane interact with phospholipids and peptidoglycan as well as with each other producing comprehensive protein-protein interactions.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the outer membrane shows a limited number of bands representing five major proteins and roughly thirty minor proteins. The major proteins include porins, murein-lipoprotein and Omp A (outer membrane protein A); the latter is Braun's lipoprotein with a molecular weight of 7,200 in *E. coli*. Omp A and lipoprotein have no pore function (Benz, 1988).

Of the major proteins which are present in the outer membrane of *E. coli*, all can act as phage adsorption sites illustrating that they are present on the outer surface of the membrane. Some of the proteins also span the entire depth of the membrane and thus interact with the peptidoglycan. A substantial number of the minor proteins appear to have many functions. They have a structural role in the architecture of the membrane and are involved in the transport of vitamin B_{12} and high molecular weight ferric iron complexes. Some of these proteins are inducible and when induced will

appear as major bands on SDS-PAGE.

Approximately one third of the lipoprotein is bound covalently to peptidoglycan, binding the rigid cell wall to the outer membrane, in the ratio of one molecule of lipoprotein to every ten disaccharide units present in the peptidoglycan. The remaining two thirds of lipoprotein which is not bound to the peptidoglycan is present in the outer layer in the free form.

Porins, which have also been called variously 'peptidoglycan associated proteins', 'peptidoglycan associated general diffusion pore proteins' or 'matrix proteins', are very important for Gram-negative bacteria as they provide a route of entry to the bacteria for all hydrophilic nutrients. In the outer membrane most porins are closely associated with the peptidoglycan layer, the term 'matrix protein' is misleading as the real matrix proteins, i.e. proteins essential for the integrity of the outer membrane, are Omp A and lipoproteins. The porins may, however, have a function in further stabilising the outer membrane. The outer membrane of Gram-negative bacteria contains approximately 10^5 copies of each of the porins per cell. The proteins have molecular weights in the region of 30 to 50 kD and most porins are arranged in the outer membranes as trimers of 3 identical proteins.

1.1.5 The cytoplasmic membrane

The innermost, or cytoplasmic, membrane is usually composed of phospholipid and protein in roughly equal proportions. Gram-positive bacterial membranes are composed of a number of different phospholipids, however, Gram-negative bacteria contain mainly phosphatidylethanolamine.

The types and amount of phospholipid present in a particular bacterium will depend on the bacterial species and the growth conditions, particularly the pH of the medium. Some of the phospholipids found in Gram-positive bacteria include: phosphatidylglycerol; cardiolipin; phosphatidylinositol; phosphatidylserine; glycolipids; and glycophospholipids together with minor components such as carotenoids and ubiquinones, which function in the respiratory chain.

Peptidoglycan is present in the majority of bacterial cell walls (exceptions including halobacteria and mycoplasma) though the amount present varies between Gram-negative and Gram-positive cell walls. The semi-permeable cytoplasmic membrane which is positioned directly behind the cell wall is a very delicate structure and needs to be protected from the considerable osmotic force exerted by the protoplast. Such pressure can vary between 5 atmospheres in a Gram-negative bacillus and 15 atmospheres in a Gram-positive coccus, therefore the cell wall must be a very strong rigid structure. The presence of peptidoglycan in the cell wall confers the shape and osmotic stability to a cell.

Measurements taken from electron micrographs of Gram-positive and Gram-negative cell walls support the proposition that the Gram-positive cell wall is made up of many layers of peptidoglycan whilst the Gram-negative cell envelope is composed of much fewer layers (Beveridge, 1993).

Gram-negative and Gram-positive bacteria also have significant differences in the fatty acid composition of membrane lipids. Gram-positive bacteria contain branched chain fatty acids, almost all of these being unsaturated. This is in contrast to those found in Gram-negative bacteria which contain

both saturated and unsaturated fatty acids which tend to have chain lengths of between 14 and 16 carbon atoms. The saturated to unsaturated ratio of fatty acids is influenced by growth temperature (Benz, 1988).

The bacterial cytoplasmic membrane has a central role in the functioning of the cell since, unlike eucaryotic organisms, bacteria do not possess specialised membrane organelles such as mitochondria, endoplasmic reticulum and chloroplasts. The cytoplasmic membrane is multi-functional, the chemiosmotic hypothesis proposing a transmembrane proton motive force powering osmotic, chemical and mechanical functions by the vectorial movement of protons across the membrane.

1.1.6 The structure of the cytoplasmic membrane

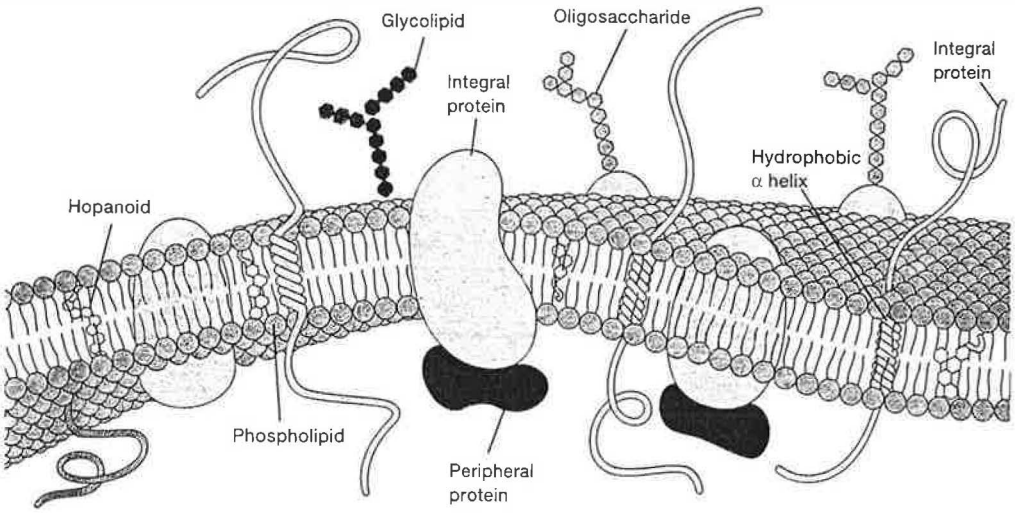
The polar groups of the phospholipids are orientated to the outside of the layer and are electrostatically linked to such charged groups as proteins or glycoproteins; these are thought to appear as the electron dense regions observed on the micrographs. It was also proposed that the proteins present existed in their ß-configuration. This model explained many properties of the cytoplasmic membranes, such as impermeability to substances including amino acids, sugars and ions but did not fit with subsequent data (Salton & Owen, 1976).

Studies of the interactions of membrane proteins and lipids have suggested that proteins may be involved in the actual structure of the membrane by interactions with other protein molecules and also with phospholipids. This leads to the idea of lipoprotein subunits which have a structural and functional role. However no such exclusively structural proteins have been

found and hence there is no firm evidence for this hypothesis.

Data from techniques such as differential scanning calorimetry and electron spin resonance have led to a different interpretation of the unit membrane hypothesis. It is suggested that the cytoplasmic membrane consists mainly of a lipid bilayer containing fatty acid side chains which are in constant motion as opposed to a semi-rigid manner; this lipid bilayer covers between 60 to 80% of the cytoplasmic membrane. Proteins are thought to penetrate this fluid bilayer to different degrees causing only minor disturbance to the fatty acid side chains of the phospholipids and with mainly hydrophobic interactions with other molecules. The position of a particular protein in the membrane is determined by the amino acid sequence of that particular protein as well as the location of the particular phospholipids with which it can associate. Proteins may therefore penetrate the membrane to different extents, pass completely through the bilayer or be located within the hydrophobic core of the bilayer. This model is known as the 'Fluid Mosaic Model' and was first proposed by Singer and Nicholson in 1972. Movement of different molecules across the cytoplasmic membrane suggests that the proteins or phospholipids are arranged symmetrically and freeze-fracture electron micrographs of these membranes support the suggestion of the location of proteins in the hydrophobic region of the bilayer. The phospholipid molecules adjacent to globular molecules are relatively fixed in position; those more distant are increasingly mobile. A diagram of the structure of the plasma membrane can be seen in figure 1.4.

Figure 1.4 The structure of the plasma membrane (After Prescott *et al.* 1996)



1.2 Biochemical pathways in bacteria

1.2.1 Embden-Meyerhof-Parnas or glycolytic pathway

Microorganisms utilise several metabolic pathways for the breakdown of glucose and other sugars. The Embden-Meyerhof-Parnas or glycolytic pathway is present in all major classes of bacteria and operates under anaerobic and aerobic conditions. Miller & Wolin (1996) showed that the Embden-Meyerhof-Parnas pathway was a major route of glucose catabolism for the colonic microflora. The pathway can be divided into two parts (see figure 1.5). The first stage - the six carbon stage, involves phosphorylating glucose twice which is then converted into fructose 1,6-bisphosphate; other sugars can enter the pathway via conversion to glucose 6-phosphate or fructose 6-phosphate. No energy is yielded at this stage, in fact two molecules of ATP are expended by adding phosphates to each end of the sugar molecule. The three carbon or second stage starts with the cleavage of fructose 1,6-bisphosphate into two, each half of the molecule retaining a phosphate group. These two molecules are then oxidised using NAD^+ as an electron acceptor and a phosphate is incorporated simultaneously giving 1,3-bisphosphoglycerate, a high energy molecule. The phosphate is donated to ADP to give ATP, a process known as substrate level phosphorylation. A second molecule of ATP is produced by a similar process.

1.2.2 Pentose Phosphate pathway

The pentose phosphate pathway (see figure 1.6) can function aerobically or anaerobically and can operate at the same time as the glycolytic pathway or

Figure 1.5 Embden-Meyerhof- Parnas or glycolytic pathway

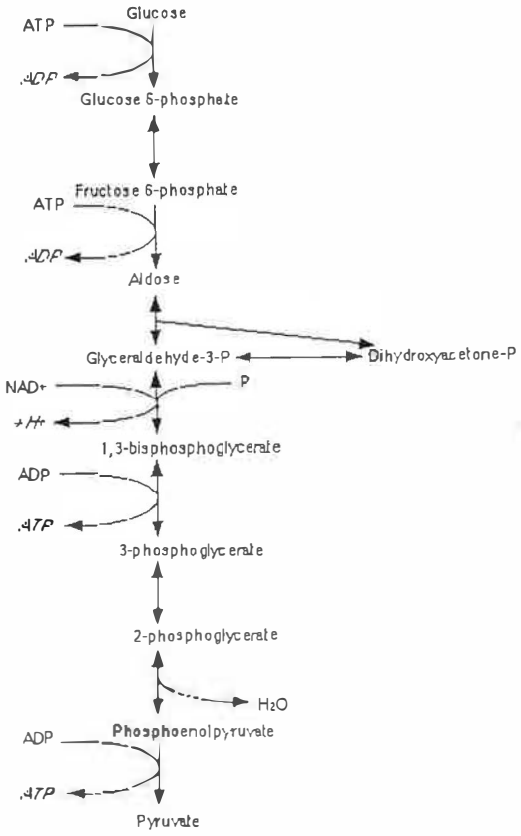


Figure 1.6 Pentose Phosphate pathway

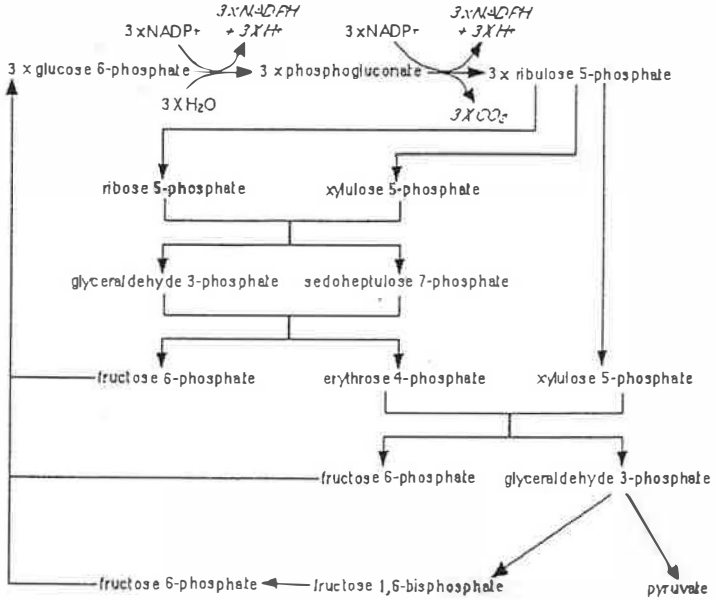
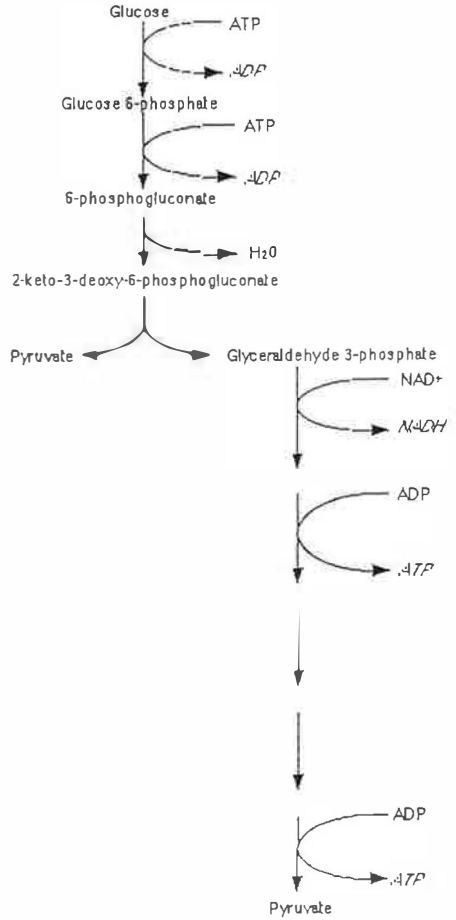


Figure 1.7 Entner-Doudoroff pathway



the Entner-Doudoroff pathway. The pentose phosphate pathway can be used for the production of ATP as follows: glyceraldehyde-3-phosphate can enter the three carbon stage of the glycolytic pathway resulting in the production of pyruvate in addition to ATP, pyruvate can then enter the tricarboxylic acid cycle. NADPH is also produced in the pathway and is used as an electron source for the reduction of molecules in biosynthesis (Sprenger, 1995).

The pathway synthesises four and five carbon sugars which are utilised by the bacteria for the synthesis of a range of compounds such as nucleic acids and aromatic amino acids. Pentoses can also be catabolised in this pathway due to the presence of pentose intermediates in the pathway. This pathway is often of more importance in biosynthesis despite being a major energy source in many microorganisms.

1.2.3 Entner-Doudoroff pathway

This pathway (figure 1.7) is found in *Pseudomonas, Rhizobium, Azobacter, Agrobacterium* and a few other Gram-negative genera. Fliege *et al* (1992) demonstrated the Entner-Doudoroff pathway could be induced for oxidative glucose metabolism when *Escherichia coli* was provided with the glucose dehydrogenase cofactor pyrroloquinoline quinone. The Entner-Doudoroff pathway is not, however, commonly found in Gram-positive bacteria. Glucose is converted to pyruvate beginning with the same reactions as the pentose phosphate pathway, however 6-phosphogluconate is dehydrated to form 2-keto-3-deoxy-6-phosphogluconate which is cleaved to produce pyruvate and glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate is

catalysed sequentially to pyruvate using enzymes common to the glycolytic pathway.

1.2.4 Tricarboxylic Acid Cycle

The degradation of glucose to pyruvate does yield some energy as described in the preceding sections (sections 1.2.1-1.2.3) however; the aerobic breakdown of pyruvate to CO_2 produces a superior yield (see figure 1.8). Pyruvate is degraded by pyruvate dehydrogenase (a multi-enzyme system) to acetyl-CoA which then enters the TCA cycle. The TCA cycle functions in many aerobic bacteria, fungi and algae and is a major source of energy. The facultative anaerobe *E. coli* does not utilise the full cycle whilst in anaerobic conditions, or when glucose concentrations are high but at all other times the complete cycle is in use.

1.2.5 Electron transport chain and oxidative phosphorylation

The cytoplasmic membrane contains the various components of the respiratory chain including cytochromes, flavins and ubiquinones, several enzymes feeding reducing equivalents into it (for example succinate, malate and NADH dehydrogenase) in addition to the associated ATPase on the inner surface of the membrane (Thony-Meyer, 1997). In this respect the bacterial cytoplasm is analogous to the inner membrane of mitochondria.

The electron transport chain is a series of electron carriers which reside within the bacterial cytoplasmic membrane. These carriers transfer electrons from donors including NADH and $FADH_2$, to acceptors, for example O_2. The Chemiosmotic theory proposed by Mitchell (1961, 1966)

Figure 1.8 Tricarboxylic Acid Cycle

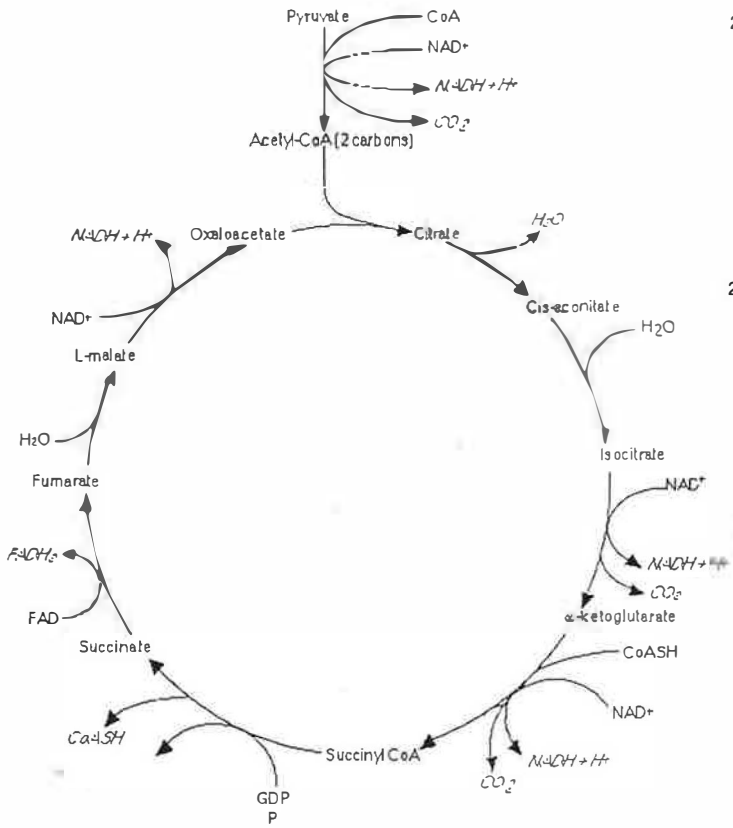


Figure 1.9 Generation of a proton motive force

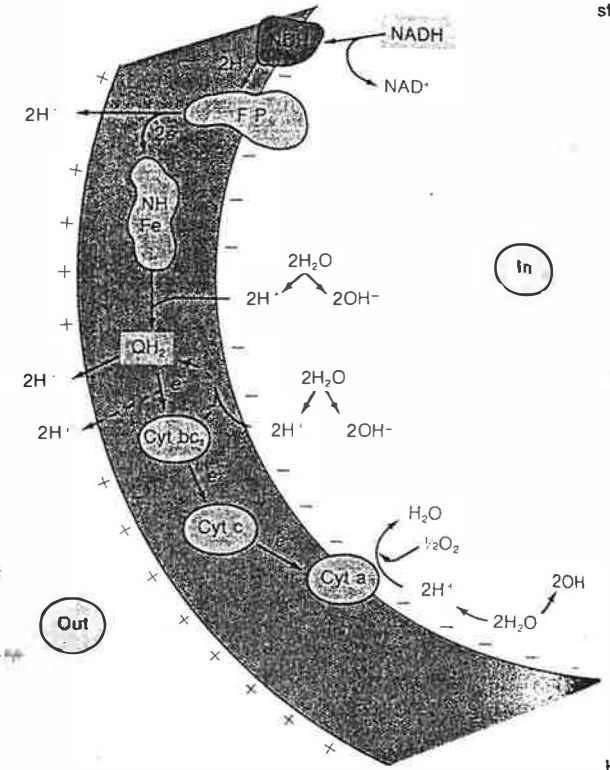
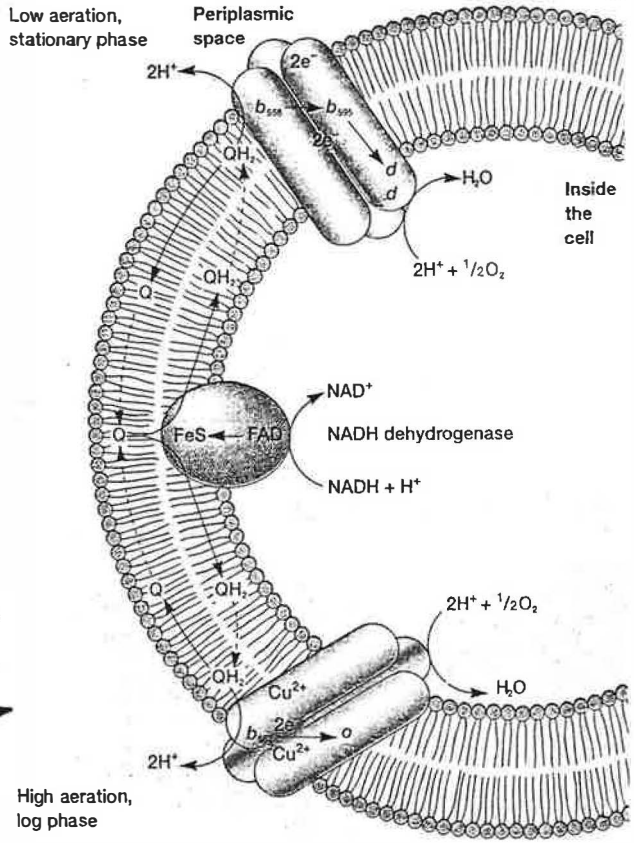


Figure 1.10 Electron transport chain of *E. coli*



hypothesised that the electron transport chain is organised so that hydrogen atoms which are removed from their carriers, such as NADH, on the interior of the membrane are separated into protons and electrons. Specific carriers transport electrons to the inside of the cell and electron flow causes the movement of protons to the outside of the membrane. This movement results in a pH and electrical gradient across the membrane: the inside of the membrane has a net negative charge and is alkaline whilst the outside of the membrane is electropositively charged and acidic (see figure 1.9) generating a proton motive force (PMF). When protons diffuse back across the membrane the proton motive force drives flagellar rotation, ion transport and ATP synthesis. This process of using energy from electron transport to generate ATP is known as oxidative phosphorylation.

Bacteria, however, display a great deal of variation in their electron transport chains. They tend to have more than one oxidase, several dehydrogenases can feed electrons into the chain, and two or three dehydrogenases which are able to reduce molecular oxygen to produce water. One of these oxidases may predominate depending on growth conditions. In conditions of high aeration the cytochrome *o* terminal oxidase complex of *E. coli* predominates whilst in low oxygen tension the cytochrome *d* complex is predominant (Poole, 1983) as shown in figure 1.10. *E. coli* also possesses two oxidases for the oxidisation of ubiquinol (Anraku & Gennis, 1987). This does not appear necessary at first glance as genetic studies have determined that the elimination of either of these oxidases does not cause a deficiency in growth under laboratory conditions (Green & Gennis, 1983, Au *et al*, 1985). It has been hypothesised that there must be a selective advantage to retaining both oxidases, possibly related to the higher affinity of the cytochrome oxidase *d* complex for oxygen or the difference in

sensitivity to respiratory inhibitors (Ingledew & Poole, 1984).

The membrane possesses some enzymes and carriers involved in the synthesis of extracellular polymers, which are used in the construction of bacterial cell walls and capsules. It is involved in the biosynthesis of teichoic acids, teichuronic acids, lipopolysaccharides and polysaccharides and in the connection of growing polymers to itself via polyisoprenoid alcohol or lipoteichoic acid. The membrane is also the site of biosynthesis of phospholipids and glycolipids. Due to the impermeability of the cytoplasmic membrane, transport mechanisms are essential to the membrane and are sited within the hydrophobic region of the membrane. Transport is especially important to bacteria living in an aqueous environment and it is estimated that transport processes can require as much as 50% of the total energy production of the cell. The cytoplasmic membrane is also involved in cell division and serves as an anchor for DNA, at least during replication.

The major functions of the cytoplasmic membrane proteins are energy generation, active and facilitated transport of nutrients and export of toxic by-products, enzymatic synthesis and macromolecule assembly, and transport of cell envelope components. The membrane functions as a barrier for principally hydrophilic or charged molecules as it is generally accepted that at growth temperatures moderately hydrophobic molecules can enter and cross the lipid bilayer.

1.3 Antibacterial Agents

1.3.1 Mechanisms of action of antibacterial agents

Chemical antibacterial agents are composed of a diverse group used extensively as protection against spoilation for a wide range of products including foods, beverages, cosmetics, pharmaceutical formulations, paints and textiles in addition to being used as antiseptics and disinfectants. Many such agents have entered common usage through a history of experience with little information on their mechanism of action. Study of the mechanism of action of an antimicrobial agent provides an insight into resistance mechanisms, toxicological problems and the design and development of new agents or combinations (Denyer, 1995).

In order to exert an antimicrobial effect the agent in question must achieve an effective concentration at the biochemical site of action (Gilbert & Wright, 1987). The vast majority of agents do not affect the outer surface of the bacteria, notable exceptions being antibiotics, which are not considered in the scope of this review. Therefore the agent must be capable of penetrating this to produce any damage. Therefore there exists a range of sensitivities of individual bacterial species to antibacterial agents. Gram-positive cells lacking the additional lipopolysaccharide layer of Gram-negative bacteria are more sensitive to many antibacterial agents. *Escherichia coli* and *Staphylococcus aureus* spheroplasts are equally sensitive to tetrachlorosalicylanilide (TCS); however, the sensitivities of the intact cells to this agent differ by a factor of 200, with the Gram-positive *S. aureus* being more sensitive (Gilbert & Wright, 1987).

The target areas for antibacterial agents can be conveniently sub-divided into the following groups:-

(1) The cell wall

(2) The cytoplasm and

(3) The cytoplasmic membrane - damage at this site can be divided into two effects: changes in membrane structure and function; and disturbance of the homeostasis and biochemistry of the cell affecting, for example, transport processes, intracellular substrate reservoirs, charge gradients, co-factor concentrations, ion gradients and nutrient concentration.

These categories are for convenience only and are not intended to suggest that interactions at these regions are mutually exclusive; indeed, the converse is often true for many antibacterial agents, for example cetyltrimethylammonium bromide (CTAB) interacts with both the cell wall (Salton, 1957) and cytoplasmic membrane (Rosenthal & Buchanan, 1974). It also has to be remembered that these structures are not distinct in terms of cellular functions.

The main targets for non-antibiotic antibacterial agents can be seen in figure 1.11.

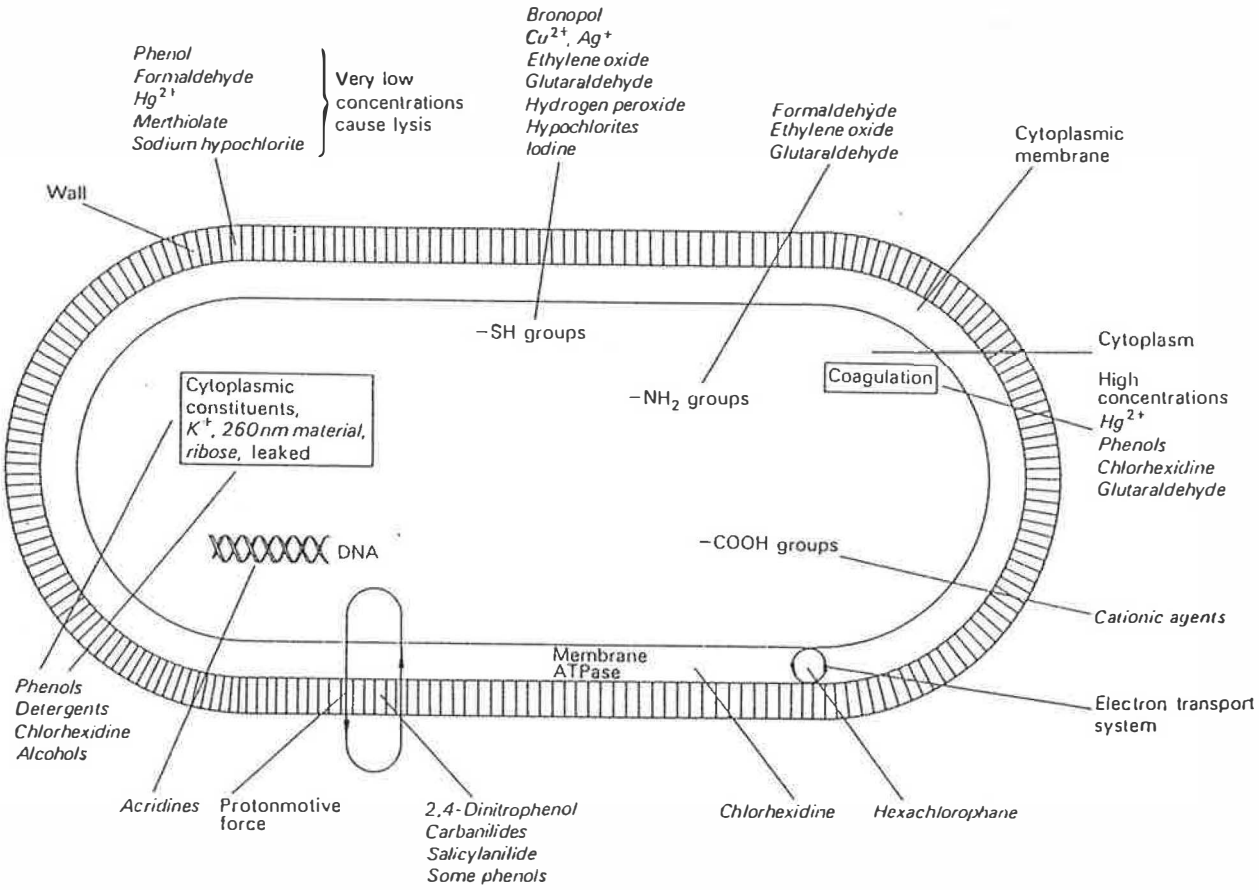
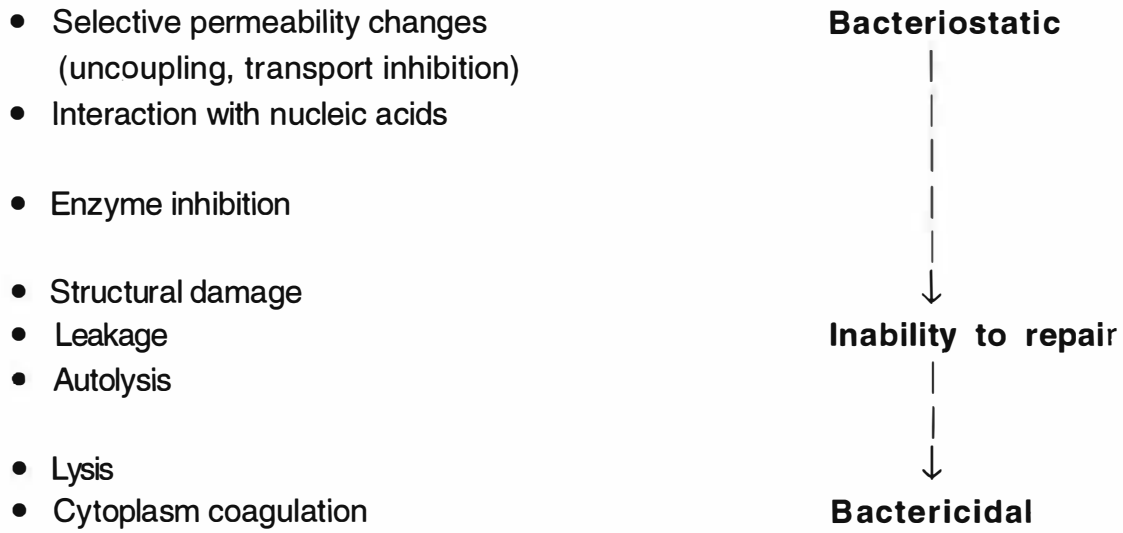


Figure 1.11 The main targets for non-antibiotic antibacterial agents (After Hugo & Russell, 1992)

Antibacterial agents can elicit both bacteriostatic and bactericidal effects, the mechanism of action for each need not be the same. Bacteriostatic injury is generally categorised as being reversible following the removal or neutralisation of the antibacterial agent (Fitzgerald *et al*, 1989), while bactericidal action arises through irreversible or irreparable damage to a vital cell structure or function (Denyer, 1995). However, it must be noted that even though a particular injury is repairable, unless the antibacterial agent is neutralised and cell recovery initiated the appropriate repair mechanisms will not be activated and cell death may still occur. It is important that this is recognised when studying the mechanism of action of an antibacterial agent since the bacteriostatic and bactericidal events may not be the same.

Figure 1.12 The consequences of antibacterial agent-induced damage (After Denyer, 1990)

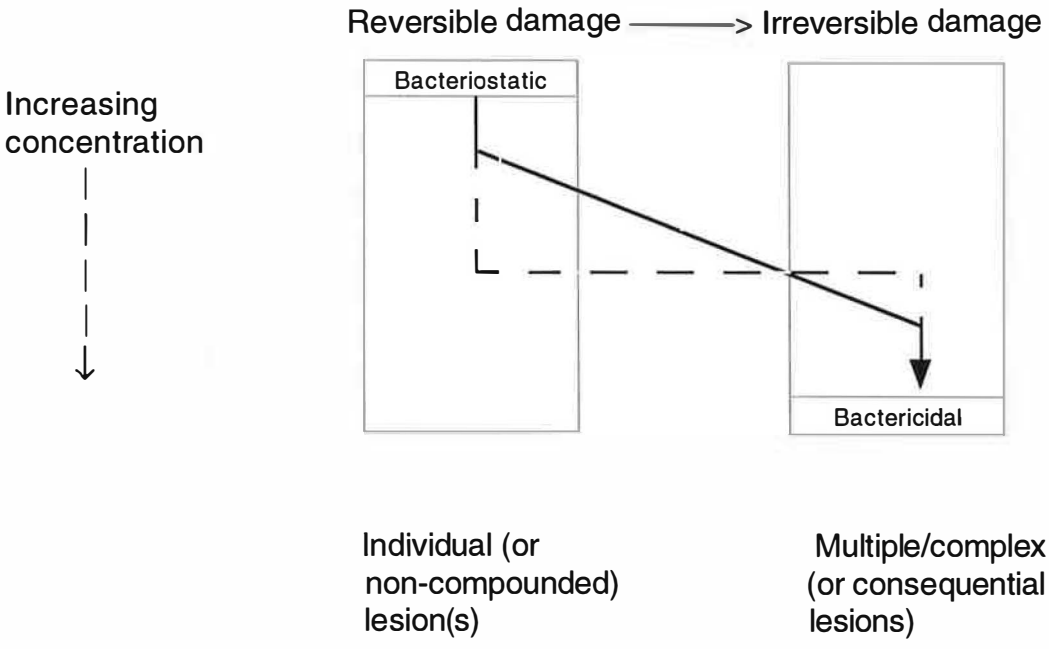


The interaction of antibacterial agent with a bacterium takes place in several stages: antibacterial agent uptake by the bacterial cell; partition/passage of the antibacterial agent to the target(s); antibacterial agent concentration at

the target(s); and damage to the target(s) (Denyer, 1995).

The degree of uptake and the kinetics of this process are described by a range of sorption isotherms and these are dependent on the nature of both the cell and the antibacterial agent. These steps are not immediate and the time taken to complete each of these steps may vary for different classes of antibacterial agents, even if the final target is the same. The result of this time-dependency is that, in theory, the effect of any antibacterial agent can be reversed if it is neutralised in time. This however, does not necessarily work in practice as some injuries occur so quickly or the damage is so great that it is not possible to reverse it. Damage can also be compounded by increasing the concentration of antibacterial agent.

Figure 1.13 Relationship between bacteriostatic and bactericidal effects (After Denyer, 1995)



——— = progressive damage, e.g. organic acid
--------- = catastrophic damage, e.g. cationic agent

Bacteria display a wide range of responses against antibacterial agents. Such responses are dependent upon several factors including: the nature of the antimicrobial agent; the type of organism; the pH of the environment; the temperature at which the contact is effected and the presence of any organic (or other binding) material. Of the environmental factors, pH is the most important as it can modify the practical application of the agent (Russell, 1991).

Gram-positive bacteria have no specific receptor molecules or permeases which assist the passage of antibacterial agents into the cell. However, the exclusion limit of the cell wall of vegetative cells of *B megaterium* was calculated by Lambert (1983) to be at least 30,000. Therefore antibacterial agents can easily penetrate these cells and hence resistance to these agents is low.

This is the reverse of Gram-negative bacteria, whose outer membrane limits the entry of antibacterial agents into the cell (Nikaido & Vaara, 1985). Smooth, wild-type Gram-negative bacteria possess a cell surface that is hydrophilic, while deep rough mutants have a more hydrophobic character due to the presence of phospholipid patches on the surface of the cell. In wild type bacteria aqueous porins transport hydrophilic molecules of a low molecular weight (<*ca* 600-650) but hydrophobic preservatives are prevented from entering the cell by the lipopolysaccharide molecules. Several studies have been carried out on the role of the outer membrane as a barrier to the penetration of antimicrobial agents. The entry of long-chain fatty acids into Gram-negative cells is prevented by lipopolysaccharides (Sheu & Freese, 1973; Freese & Chernov-Levin, 1978; Kabara, 1983, 1984). The effect of the outer membrane was also determined with mutants of *E.*

coli and *Sal. typhimurium* for a homologous series of esters - the parabens (Russell *et al*, 1985, 1987; Russell & Furr, 1986). The series increases in hydrophobicity from methyl to butyl and activity increases against the smooth strains as the homologous series is extended.

Certain antimicrobial agents are thought to enter the Gram-negative bacterial cell by damaging the outer membrane and therefore promoting their own uptake, for example, chlorhexidine and quaternary ammonium compounds (Hancock, 1984). Studies have shown that quaternary ammonium compounds have a reduced activity against wild-type bacteria compared to deep-rough strains, but chlorhexidine has a similar activity against both strains. This suggests therefore that lipopolysaccharide is acting as a barrier against quaternary ammonium compounds but not against chlorhexidine.

1.3.2 The effect of antibacterial agents on the cytoplasmic membrane

The primary target of most antimicrobial agents (with the exception of antibiotics) is the cytoplasmic membrane and associated enzymes with the mode of action usually influenced by concentration. The cytoplasmic membrane is an important structural and functional component of the bacterial cell, it also has a large surface area which increases the chances of antibacterial agent interaction, and also is in close vicinity of the external aqueous environment.

1.3.2.1 General membrane permeability effects

Membrane-active agents such as CTAB (Salton, 1951) can cause a change in the fluidity and/or permeability of the cytoplasmic membrane. Such changes can be determined by the leakage of cellular constituents (Denyer & Hugo, 1991). One of the earliest and most sensitive indicators of damage to the cell membrane is the leakage of potassium ions (Lambert & Hammond, 1973). Broxton *et al* (1984) assessed sensitivity towards biocides as induced potassium ion loss from intracellular pools.

Leakage has been shown to occur very rapidly after contact with membrane-active antimicrobial agents, such examples include phenol and *Serratia marcescens* (Kroll & Anagnostopoulos, 1981), alexidine and *E. coli* (Chawner and Gilbert, 1989) and 1,1-dialkyl-piperidinium bromides and *E.coli* (Mlynarcik *et al,* 1992).

Other cell constituents that are released due to increases in the permeability of the cytoplasmic membrane include nucleotides and their constituents (pyrimidines, purines, pentoses and inorganic phosphate) and amino acids. Hugo and Longworth (1964) reported loss of cellular constituents from *E. coli* and *S. aureus* after treatment with chlorhexidine and Broxton *et al* (1983) reported increased leakage from *E. coli* ATCC 8739 as polyhexamethylene bisguanide concentration increased.

1.3.2.2 Cytoplasmic membrane bound enzymes

The effect of an antibacterial agent on the cytoplasmic membrane can also be inferred by its influence on the activity of membrane-bound enzymes

(Gilbert *et al*, 1977b). Using this information and that obtained from leakage studies it may also be possible to ascertain the degree of damage to the cytoplasmic membrane, that is whether the antibacterial agent is interacting with a particular protein or is causing indiscriminate damage to the cell membrane.

Tetrazolium salts are often used as markers of oxidative enzyme activity. On mild reduction the colourless salts form their corresponding formazan which is coloured (Altman, 1970) and thus a quantitative measure of enzyme activity can be determined by measuring the amount of coloured formazan produced. A selection of tetrazolium salts have been synthesised and are used for enzyme activity studies, some examples of these can be seen in table 1.2.

Table 1.2 Tetrazolium salts used in enzyme activity studies

CTC	5-cyano-2,3-ditolyl tetrazolium chloride	Smith & McFeters (1996)
INT	2-(4-iodophenyl)-3-(4-nitro-phenyl) -5-phenyl tetrazolium chloride	Kopecka-Leitmanova*et al* (1989) Smith & McFeters (1996)
TTB	2,3,5-triphenyl tetrazolium bromide	Hugo 1954 Hugo & Longworth 1966
MTT	(3-(4,5-dimethyl thiazolyl-2)-2,4-diphenyl tetrazolium bromide	Altman, 1970

Damage to the cytoplasmic membrane can be probed by investigating the activity of membrane-bound dehydrogenases using tetrazolium salts.

Succinate dehydrogenase has been shown to be an integral part of *E. coli*. The activity of this enzyme has been shown to be almost completely inhibited at concentrations which are germicidal to the suspension of bacteria under test (Harding, 1984). Tetrazolium salts have been used to determine the activity of lactate, succinic, isocitrate and malate dehydrogenase activity in *E. coli* (Gilbert *et al*, 1977b; Vadehra & Wahi, 1983) demonstrating the range of dehydrogenase enzymes that can be investigated using this method.

ATPase is also found associated with the cytoplasmic membrane. Thus one way in which damage to the cytoplasmic membrane may be probed is to monitor the yield of ATP in the presence of bacterial cells in the presence of the antibacterial agent under test (Mlynarcik *et al*, 1981; Denyer *et al*, 1986). ATP levels have been shown to be reduced by chlorhexidine and D-arabino-2,3,4-tris(4-chlorobenzyl)pentane-1,5-diamine (Barrett-Bee *et al*, 1996) and thymol (Shappiro & Guggenheim, 1995).

1.4 Surfactants

1.4.1 Definitions, classes and general properties of surfactants

Surfactants or surface active agents have been defined as substances which in solution, in particular aqueous solution, have a tendency to congregate at interfaces, rather than in the bulk of the solution, thus reducing interfacial tensions (Cullum, 1993). This is in contrast to other solutes which, by maximising solvent/solute interaction, are concentrated in the bulk of the solution away from surfaces. Surfactants may have a limited solubility in

water and those that do have high solubility may exist in bulk phase aggregates or micelles. Clint (1992) further described surfactants as materials that not only tend to accumulate at surfaces but which, by their presence, can alter the properties of those surfaces.

These properties of surfactants are due to the molecular structure of the surfactant which is amphiphilic (from the Greek, amphi meaning 'on both sides' and philein, meaning 'to love'). The molecule has two distinct parts:- a non-polar, hydrophobic tail group, usually an alkyl chain composed of at least 10 carbon atoms which is joined to a polar, hydrophilic head group. Surfactant self-association occurs due to the preferred association of water, excluding the hydrophobic alkyl chains which then aggregate. Such aggregates are dynamic structures - surfactant monomer is constantly leaving and joining any particular aggregate in a matter of milliseconds with the result that a single aggregate has a very short lifetime.

Surfactants can be sub-divided on the basis of the charge on the head group into the following: cationic; anionic; nonionic and amphoteric surfactants. Table 1.3 lists the surfactants in this section and their head group class. They can also be classified by the composition of their tail groups (table 1.4).

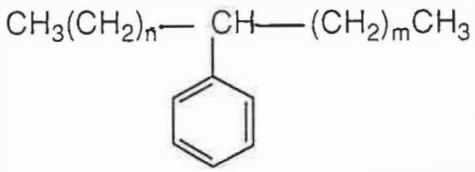
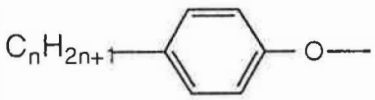
Table 1.3 Some surfactants and their head group class

Surfactant	**Head group class**
Quaternary ammonium salts	cationic
Aerosol OT (dioctyl sodium sulphosuccinate)	anionic
Aerosol OS	anionic
Aerosol DGA	anionic
β-D-glucopyranosyl-1-alkyl phosphate	anionic
Dreft (alkyl sulphate)	anionic
Sodium cholate	anionic
Sodium dodecyl sulphate	anionic
Sulphonated oils	anionic
Sodium 2-lauroyloxypropionate	anionic
Sodium alkyl sulphates	anionic
Triton No. 720 (sulphonated ether)	anionic
Ag-98 (80% octyl phenoxy-polyethoxyethanol)	nonionic
Alkylethoxylate	nonionic
Alkylphenolethoxylates	nonionic
Amine oxides	nonionic
Brij series e.g. Brij 58 (Polyoxyethylene-23-lauryl ether)	nonionic
Linear alkyl ethoxylates	nonionic
Lissapol NX	nonionic
Nonyl phenyl ethylene oxide	nonionic
Octa(ethylene glycol)-mono-dodecylether ($C_{12}E_8$)	nonionic
Octyl glucoside (OG)	nonionic

Table 1.3 continued

Surfactant	Head group class
Pluronic F68 (polyoxyethylene-polyoxypropylene block co-polymer)	nonionic
Polyethylene esters of fatty acids	nonionic
Polyethylene glycols	nonionic
Polyethylene mercaptans	nonionic
Polyoxyethylated 1-dodecanol	nonionic
Polyoxyethylene n alkyl ester	nonionic
Polyoxyethylene n monosterate	nonionic
Substituted alcohols	nonionic
Tergitols	nonionic
Tributyl phenyl ethylene oxide	nonionic
Triton X-100 (polyoxyethylene glycol octyl phenol)	nonionic
Triton X 305 (polyoxyethylene glycol octyl phenol)	nonionic
Tween series e.g. Tween 80 (polyoxyethylene sorbitol esters)	nonionic

Table 1.4 Classification of surfactants by tail group composition

Group	Structure
Alkyl	$CH_3(CH_2)_n$
Linear alkyl benzene	
Alkylayrl e.g. alkyl phenol	
α-Olefin	$CH_3(CH_2)_n-CH=CH$
	$CH_3(CH_2)_m-CH(OH)-(CH_2)_pCH(CH_2)_rCH_3$
Polypropylene oxide	$-[CH_2CH(CH_3)O]_n-$

The net effect of the hydrophobic (lipophilic) tail and the hydrophilic head group of the molecule have been described empirically by a value termed the hydrophile-lipophile balance (HLB). The extent of surface activity and the type of application for which a surfactant may be suited correlate with their HLB value. For example, detergents have an HLB in the range of 13 - 15 compared to water-in-oil emulsifiers which have HLB values in the range of 7 - 9. Nonionic surfactants exhibit a wide range of HLB values and in the vast majority of cases possess very low toxicity thus making them suitable for a wide range of applications such as detergency (laundering, dishwashing, hard surface cleaning) and cosmetics and pharmaceuticals (emulsifying agents) (Cross, 1992).

Surfactants are soluble amphiphiles which, above a certain concentration and temperature, form micelles in a variety of shapes and sizes (Lictenberg*et al*, 1983). Surfactants form unstable monolayers i.e. the surfactant molecules in the monolayer are in equilibrium with the bulk solution. Once a critical concentration (the Critical Micelle Concentration, or CMC) and temperature is reached the further addition of surfactant causes the formation of micelles or aggregates.

When water is added to surfactants, three types of behaviour can occur (Tiddy, 1980):

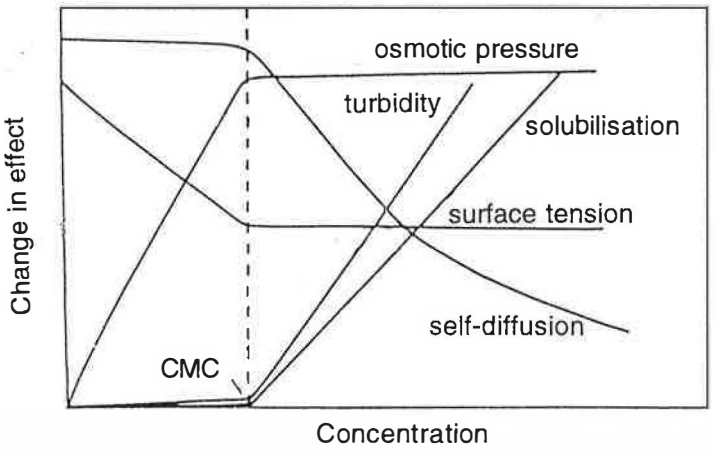
(1) the surfactant is almost entirely insoluble and will remain as a solid crystal and an aqueous solution of surfactant monomers;

(2) some surfactant dissolves to form an aqueous micellar solution above a certain concentration;

(3) formation of lyotrophic liquid crystals which may dissolve in more water to produce an aqueous micellar solution.

Liquid crystals occur at least as often as micelles and will be one of the four major phase structures known: hexagonal; lamellar; cubic; and reversed hexagonal. Surfactants can be subdivided into those which form liquid crystal structures at a high concentration (e.g. most anionic, cationic and non-ionic detergents) and those which do not, probably due to bulky cyclic and aromatic moieties (e.g. bile salts) (Helenius & Simons, 1975). Insoluble amphiphiles will form a stable monolayer at the air/water interface and include long chain fatty acids, cholesterol and phospholipids.

Figure 1.14 Changes in concentration dependency of a wide variety of physico-chemical properties around the critical micelle concentration (Modified from Lindman, 1984)



The use of surfactants is not exclusive to detergency, other industrial processes also rely heavily on the use of surfactants. Examples of such applications can be seen in table 1.5.

Table 1.5 Examples of the application of surfactants in industrial processes (Modified from Cross, 1992)

Field of Application	Function
Detergency	Laundering, dishwashing, hard surface cleaners
Textile processing	Aid to all stages including bleaching, and dyeing
Agriculture	Emulsifiers and wetting agents for insecticides and herbicides
Leather processing	Aid to all stages including degreasing and tanning
Polymers	Emulsion stabilisers, plasticisers
Cosmetics and Pharmaceuticals	Emulsifying agent
Food	Fat emusifier for bakery products, ice cream, margarine etc.

1.4.2 Chemical composition of surfactants

The majority of surfactants possess a tail which is a simple hydrocarbon group of between 12 and 18 carbon atoms. The tail group may also be branched or contain an aromatic ring. However, surfactants are much more commonly classified on the basis of the charge of their head group.

Cationic surfactants have a positively charged head group. This group is mainly composed of quaternary ammonium, imidazolinium and alkyl pyridium compounds. Their positive charge makes them ideal for use in hair and fabric conditioners as they are attracted to negatively charged biological

material such as cotton and hair.

Anionic surfactants have a negatively charged head group and the counter ion is usually Na^+. This group contains the traditional soaps (the carboxylates) and the first synthetic detergents: the sulphates and the sulphonates. These compounds are still an important feature of modern detergents (Schmitt, 1992). The sulphates and sulphonates have an advantage over the carboxylates in that they can be used in hard water as they have a greater tolerance of divalent metal ions.

Zwitterionic and amphoteric surfactants are often used in toiletries and baby care products due to their reduced skin irritation and low ocular irritation characteristics when compared to anionics. Examples of such compounds include betaines, sulphobetaines and lecithins or phosphatidyl cholines.

Nonionic surfactants are used in a wide range of industries, due to their wide range of HLB values and their very low toxicity, including detergency, food, cosmetics and pharmaceuticals. Examples of this class of surfactant include: fatty alcohols and alcohol ethoxylates; polyethylene glycol esters (ethoxylated fatty acids) and block copolymers of ethylene and propylene oxides. This class also includes a group of compounds known as semi-polar compounds, for example, amine oxides. Amine oxides are cationic only in acid solution and are nonionic in neutral or alkaline solution (Cullum, 1993).

1.4.3 Surfactant adsorption

The sorption of alcohol ethoxylates (nonionic surfactants) to various fabrics has been followed by the use of a radiotracer (Gordon & Shebs, 1968). They found that washing fabric with these nonionic surfactants resulted in the selective sorption of those with shorter EO chains, i.e. the size of the head group has an influence in the sorption of these surfactants. It was also shown that by exchange washing with unlabelled surfactant the bulk of the surfactant was reversibly sorbed. This finding was in agreement with that of Schott (1967) who had carried out similar experiments with polyoxylated 1-dodecanol and cotton. He described the resultant adsorption isotherm as levelling off close to the CMC of the surfactant and suggested a close packed monolayer of surfactant molecules lying flat against the surface of the cotton as the level of adsorbed surfactant was of the order required to cover the cotton completely.

The nature of the surface can affect surfactant adsorption (Clint, 1992). Surfaces can be differentiated by the properties most likely to have an influence on the intramolecular forces between surface and surfactant:

(1) hydrophobic or hydrophilic;

(2) charged or uncharged.

Surfactant adsorption is described by an adsorption isotherm which displays the amount of material adsorbed as a function of equilibrium concentration, the most common form for such isotherms is the Langmuir isotherm.

When the concentration of surfactant increases beyond very low concentrations (area AB on the graph) the isotherm shows a fast upturn. This tends to occur at surfactant concentrations an order of magnitude below

the CMC of the surfactant. This is caused by the start of attractive interactions between adjacent surfactant molecules. It is at this concentration that two dimensional aggregates, or 'hemimicelles', are formed. This is known as the hemimicelle concentration or HMC (Clint, 1992). The hemimicelles are aggregates which have polar groups orientated to the solid surface and present a hydrophobic patch consisting of closely packed alkyl groups to the solution. This situation is, however, dependent on the surface charge relative to that of the surfactant so the reverse situation also occurs (area BC on the graph).

The size and number of hemimicelle aggregates increases as the surfactant concentration increases beyond the HMC and the adsorption isotherm rises rapidly, almost reaching linearity. The slope of the isotherm eventually starts to decrease (area CD on the graph), and reaches an adsorption plateau (point D) which is close to the CMC of the surfactant. The position of the plateau correlates with maximum adsorption of either one or two monolayers on a solid surface. Which of these two scenarios occurs is dependent on the chemical character of the surface, in particular whether it is hydrophobic or hydrophilic in nature (Clint, 1992).

Figure 1.15 A Typical Langmuir Adsorption Isotherm (After Clint, 1992)

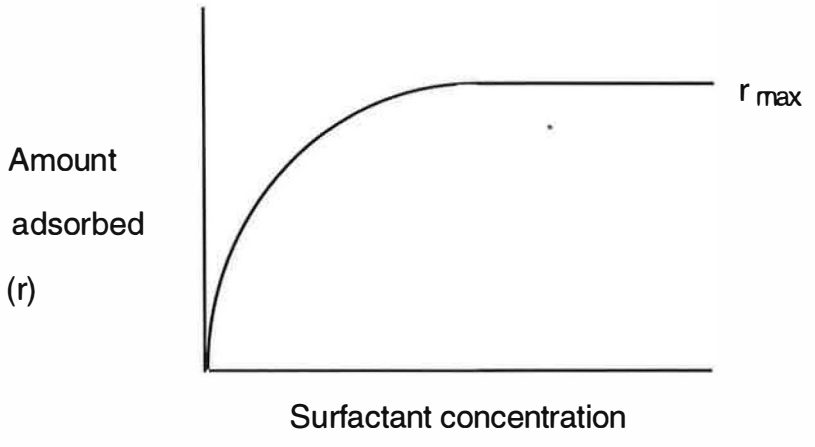


Figure 1.16 A generalised adsorption isotherm showing the types of surfactant aggregation encountered on the solid surface in the various regions (After Clint, 1992)

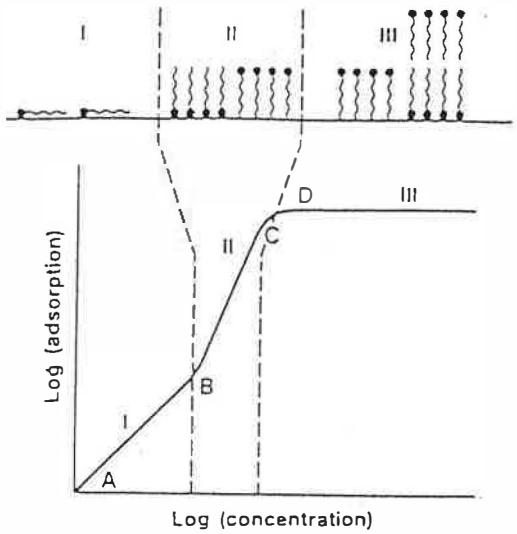
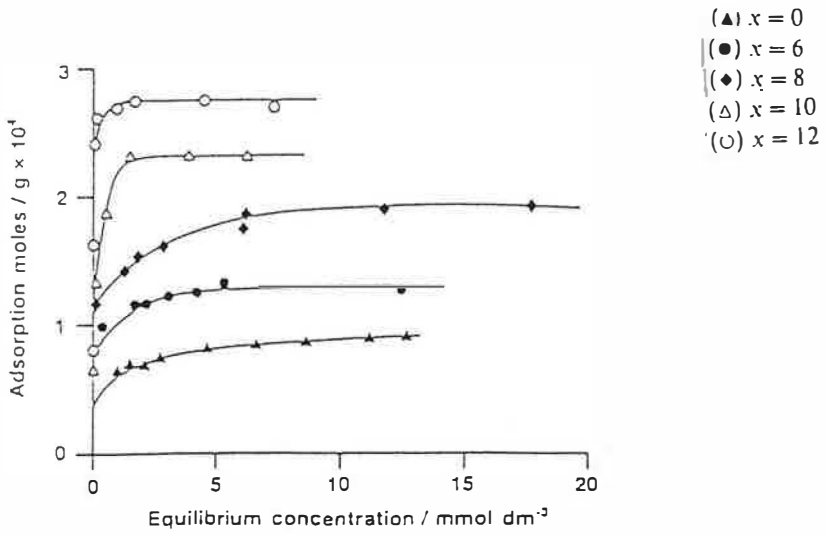


Figure 1.17 Adsorption of non-ionic surfactants C_xE_6 on Graphon (After Corkhill *et al*, 1966)

C_x - no. of carbon atoms in the alkyl chain of the alcohol ethoxylates.

E_6 -no. of ethoxylate groups in the head group, i.e. 6.



The adsorption of nonionic surfactants onto a hydrophobic surface tends to be Langmuirian in nature indicating a moderate affinity of the surfactant molecule for the solid surface. Corkhill and co-workers (1966) showed that the adsorption of linear alkyl ethoxylates on graphitised carbon black produced a Langmuir isotherm.

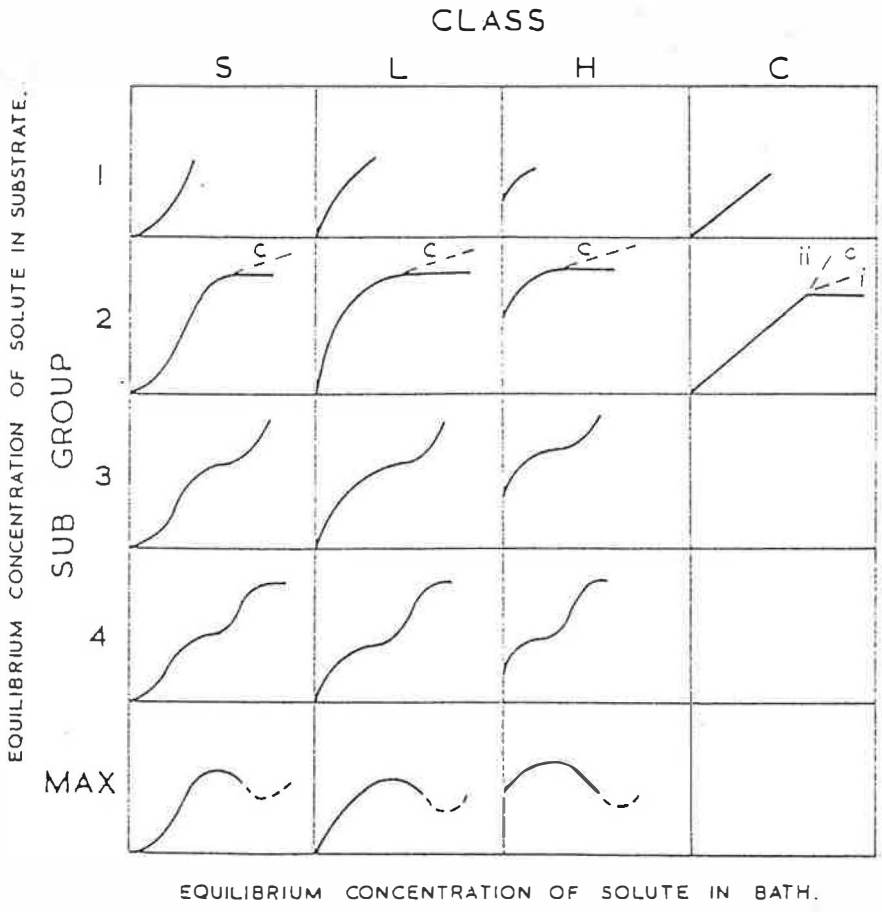
As can be seen from figure 1.17, as the alkyl chain length increases for a fixed size head group, the plateau adsorption level increases by roughly the same proportion. The head group itself shows a tendency to adsorb to Graphon (i.e. when X=0). The area of this head group molecule is 1.5 nm^2 which implies a model of adsorption where the molecules lie flat against the surface of the Graphon, this is the same model as proposed by Schott (1967) for polyoxyethylated 1-dodecanol.

The adsorption of nonionics to a hydrophilic surface is similar in some respects to that seen for hydrophobic surfaces. The general behaviour is determined by the solution properties of the particular surfactant. Surfactants of the general form C_xE_y reach an adsorption plateau level which increases by increasing x, decreasing y or increasing temperature.

The monolayer pattern of adsorption has been seen for the uptake of Tergitols (nonionic surfactants) onto polyester fibre (Gum & Goddard, 1981). Adsorption studies showed Langmuirian adsorption to polyester with the adsorption plateau occurring at the CMC of the surfactant. The data fitted a Langmuirian monolayer adsorption model and this was further reinforced when sorption increased proportionally when a higher surface polyester was used.

Many surfactants initially appear to obey a Langmuirian adsorption pattern, but on closer inspection this is not the true picture. Giles *et al* (1974) proposed a classification system for solid solute adsorption (SSA) isotherms. Such isotherms are divided by their initial slope into four main types: S (co-operative sorption); L (Langmuirian); H (high affinity) and C (constant partition) and further into several sub-groups: 1,2,3,4 etc or max. by variations as shown in figure 1.8. This classification system is often used for categorising biocide adsorption (Denyer, 1990; Russell & Chopra, 1996) although the subgroup classifications are rarely used.

Figure 1.18 Isotherm classifications for solid solute adsorption (After Giles *et al*, 1974)



The subgroups of the adsorption isotherms are described as follows: in subgroup 1 the absorbate monolayer is not yet completed; in subgroups 2 and higher a plateau can be identified at the end of the turning point with the completion of the first monolayer. A subsequent rise represents the development of a second layer which is completed at subgroup 4. Subgroups beyond 4 do occur but are extremely rare.

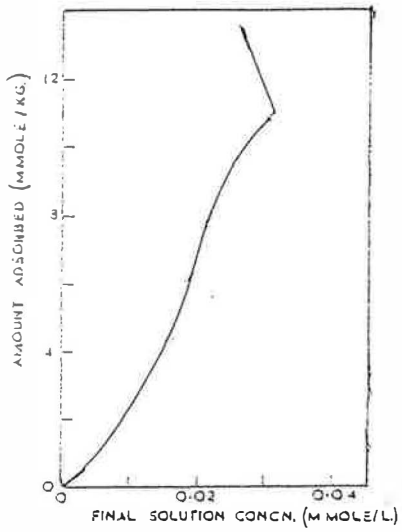
In the known cases of subgroup "max", a curve with a maximum occurs in aqueous solutions of solutes which associate in solution, for example detergents. It appears that with an increase in concentration of the solution a point is reached where solute Van der Waals interactions overcome solute-substrate interactions so that some solute will desorb from the surface and become incorporated into solvated micelles.

The C-type absorption isotherm subgroup 2 occurs in a few cases where the curve at first is a normal L shaped isotherm. At the turning point the curve continues to rise but linearly, this indicates microporosity in the substrate.

Giles *et al* (1974) also identified a further adsorption isotherm with a shape very distinct from those already identified, this was described as a Z curve (see figure 1.19)

The main characteristics of the five adsorption isotherms and examples of antimicrobial agents producing these isotherms can be seen in Table 1.6.

Figure 1.19 Z type adsorption isotherm (After Giles *et al*, 1974)



There is evidence to suggest, however, that some membrane active antimicrobial agents produce a 'Z' type adsorption isotherm (Gilbert *et al*, 1978). Substituted alcohols have been shown to exert an antimicrobial effect on the cytoplasmic membrane (Gilbert *et al*, 1977a, b). The adsorption of these alcohols by *E. coli* NCTC 5933, *Ps. aeruginosa* NCTC 6750 and *Candida lipolytica* NYC 376 increased initially with increasing applied drug concentration ('C' type). However, in the majority of cases, at the CMC a marked inflection in the isotherm occurred resulting in a pronounced 'Z' curve. Very little leakage of cellular constituents, an indicator of damage to the cytoplasmic membrane, was seen at concentrations below the CMC. However at concentrations in excess of the CMC significant leakage of intracellular material occurred and electron microscopy of the treated bacteria indicated gross damage to the membrane and outer layer of the cell envelope. It is suggested, therefore that these agents induce structural disorganisation resulting in a rise in the interactive surface for drug penetration from the outer layer but particularly the cytoplasmic membrane which is a major lipophilic barrier to drugs (Gilbert *et al*, 1978). This is not typical of other antimicrobials whose mechanism of action is gross

Table 1.6 Types and examples of adsorption isotherms

Adsorption Isotherm pattern	Explanation	Example(s)
S (co-operative sorption)	Moderate intermolecular attraction, orientate vertically; solvent molecules compete strongly for substrate sites	Some phenolic compounds (Bean & Das, 1966)
L (Langmuirian)	As more sites on cell surface are filled, becomes increasingly difficult for biocide to find vacant site; no tendency for multi-layer formation	Phenol (Hugo, 1976) QACs
H (high affinity)	Absorbate-substrate affinity especially high, biocide almost completely adsorbed	Iodine (Hugo & Newton, 1964)
C (constant partition)	Biocide penetrates more readily into adsorbate than does the solvent, surface available for adsorption expands proportionally with amount of solute adsorbed	Isothiazolone biocides (Collier*et al* ,1990) Chlorhexidine, alexidine (Chawner & Gilbert, 1989)
Z (enhanced uptake)	Sharp break in isotherm followed by an increasing uptake: biocide promotes breakdown in structure of adsorbate, thereby generating new adsorbing sites	2-phenoxyethanol, 2-phenoxypropanol (Gilbert*et al* 1978)

disorganisation of the cytoplasmic membrane. Such agents usually produce a Langmuirian adsorption isotherm, for example, phenol (Hugo, 1976), 'S' type or 'S' type becoming 'C' type at higher concentrations.

1.4.4 The interaction of surfactants with membranes

Surfactants are useful for the solubilisation of phospholipids and form micelles themselves in aqueous solution. Nonionic surfactants are particularly useful for membrane solubilisation as the polar headgroup for this class of compound is usually much larger than the hydrophobic tail and therefore its solution properties are different from ionics (from which classical micellar behaviour and structure are derived); this provides many advantages for membrane solubilisation.

The interactions of soluble and insoluble amphiphiles are complex. With low concentrations of soluble amphiphiles the surfactant associated with the phospholipid bilayer causes little loss of the bilayer structure (Seeman, 1972). Above certain concentrations some surfactants lead to increased permeability of membranes prior to having any effect on the bilayer structure. As the concentration of the surfactants in the bilayer increases above the critical lamellar/micellar transition concentration, mixed surfactants and phospholipid micelles result.

If an aqueous solution of surfactants is added to phospholipid bilayers the detergent distributes between the bilayers and the solution. Studies with sodium dodecyl sulphate, sodium taurocholate and octyl glucoside (Jackson *et al,* 1982) and their interaction with PC bilayers indicate that the

concentration of free surfactants remains below that of pure surfactants. Thermodynamic considerations suggest the concentration of monomeric surfactant in equilibrium with phospholipid/surfactant mixtures remains below the CMC of the pure detergent.

When unilamellar vesicles or membranes are exposed to a surfactant an equilibrium state can be approached fairly rapidly. This state depends on the lipid, surfactant and their relative concentrations. The transformation of all vesicles into mixed micelles occurs when the effective ratio of surfactant to lipid exceeds a critical level - Re_{sol}, this value depends on the surfactant and lipid in question but was in the molar ratio range of 1.84 to 4.20 for a series of octylphenol polyethoxylated surfactants and egg phosphatidyl-choline liposomes (de la Maza & Parra, 1994).

There is evidence to imply that the first stage in membrane solubilisation by surfactants does not include membrane rupture but solubilisation of some proteins, which are likely to be peripheral membrane-associated extrinsic proteins (Helenius & Simons, 1975). These proteins are solubilised as surfactant-protein aggregates and the extraction of these membrane proteins is followed, at higher surfactant concentrations, by the complete solubilisation of the membrane. These membrane proteins may be extracted prior to the complete solubilisation of the lipid; preferential extraction of the proteins may be due to a specific surfactant having a higher affinity for the extracted protein.

When surfactant is added to a suspension of phospholipid liposomes, part of the surfactant interacts with the bilayer lipids and part remains free in solution. The free concentration remains below the CMC of the pure

surfactant and decreases with decreasing surfactant/lipid ratio.

Small amounts of surfactant can be incorporated into liposomes without disrupting them. When the bilayers become saturated with surfactant, additional surfactant induces the formation of mixed micelles. These micelles will have the highest possible phospholipid content, i.e. they will be saturated with phospholipid. At intermediate surfactant/lipid ratios bilayers saturated with surfactant and micelles saturated with phospholipids coexist in different proportions. All the phospholipid will be converted to mixed micellar form when sufficient surfactant is added so that the phase transition limit is reached. The addition of further detergent will cause a gradual increase in the surfactant/phospholipid ratio in the mixed micelle.

The sequence of events when increasing amounts of surfactant are added to a suspension of pure phospholipids can be summarised as follows (Helenius & Simons, 1975):

Stage I - Surfactant binding. The surfactant is incorporated into the bilayer and brings about changes in the physical properties of the bilayer;

Stage II - Lamellar-micellar phase transition. Once the bilayer becomes saturated with surfactant mixed micelles begin to form, eventually resulting in complete phase transition;

Stage III Size decrease of mixed micelles. Once the phase transition has been completed the surfactant/phospholipid ratio in the mixed micelles increases and their size decreases.

This sequence of events involves systems in equilibrium, the kinetic aspects have not been taken into consideration. The formation and dissolution of micelles are however fast having rate constants higher than $10s^{-1}$.

The formation of mixed micelles and lamellar has been verified by Klose and Hollerbuhl (1981) using ^{31}P-NMR. Studies with ionic phosphorous containing surfactants and egg yolk lecithin concluded that intercalation of lecithin into detergent molecules as well as intercalation of surfactant molecules into lecithin take place.

The binding of water soluble proteins by a number of surfactants occurs when the surfactant is in the monomeric form as opposed to the micellar form when both the surfactant and protein are present in low concentrations (Makino *et al,* 1973; Nozaki *et al,* 1974). It is therefore the concentration of free monomer which determines the amount of surfactant that is bound to the protein. If this free concentration is increased above the CMC very little further binding is seen, therefore the binding of surfactant to protein is in competition with the self association of surfactant monomers to form micelles.

The binding of surfactants to membrane-bound proteins is different from that seen for water soluble proteins, this is due to the differences in structure of the two types of protein (Helenius & Simmons, 1975). Proteins associated with the membrane can be roughly divided into two categories: peripheral (extrinsic or membrane associated) and integral (intrinsic). Peripheral proteins can be dislodged with relative ease. Integral proteins are tightly bound as they are only able to be removed by the disruption of the membrane by detergents or organic solvents.

Biological membranes are complex mixtures of lipids, proteins, ions etc. Despite this complexity the general principles of surfactant action shown

with studies using model lipid bilayers and isolated proteins can be applied to membranes too. However, the presence of a carbohydrate coat, the glycocalyx (Rahmbourg, 1971), highly charged proteins or glycoproteins (Bretsher, 1973) or a dense protein network (Franklin, 1971) on the surface of a membrane may influence the penetration and the effects of a surfactant on the membrane.

Surfactants and other soluble amphiphiles appear to bind to a membrane even at very low concentrations. However, the result of surfactant binding on membrane properties is, in the main, unknown. They may involve subtle alterations in the permeability of the membrane and at higher concentrations membrane lysis and fusion. Binding isotherms show that lysis of membranes correlates to an enormous increase in surfactant binding to the membrane.

The lytic activity of surfactants was first studied using red blood cells as the process can be followed quantitively by the release of haemoglobin. The lytic process can be divided into five stages (Reman *et al*, 1969):

(1) the surfactant monomers adsorb to and;

(2) penetrate into the membrane;

(3) they induce changes in the molecular organisation of the membrane;

(4) leading to alteration in the permeability of the membrane and the osmotic equilibrium;

(5) finally haemoglobin is released.

Stages 2,3 and 4 are rate limiting.

Zaslavsky *et al* (1979) investigated the haemolytic and membrane perturbing action of a homologous series of β–D-glucopyranosyl 1-

alkylphosphates, with alkyl chain lengths of 10, 13, 15 and 16 carbon atoms. It was concluded that the affinity for the erythrocyte membrane is a strictly linear function of the alkyl chain length. C_{lys} - the concentration of agent at which complete lysis occurs - decreases as chain length of the surfactant increases. Kellermayer *et al* (1994) proposed following the treatment of erythrocytes with a range of nonionic surfactants (Triton X-100 and Brij 35, 56, 58, 78 and 99) that the membranolytic and cytoplasmic protein destabilising action of nonionic detergents corresponded to their HLB values.

Ohnishi and Sagitani (1993) investigated the haemolytic activity of a range of polyoxyethylene (POE) type nonionic surfactants. In common with Zaslavsky *et al* (1979) they concluded for the polyoxyethylene-n-oleyl ether and polyoxyethylene-n-monostearate series that haemolytic activity decreased as POE chain length increased. Haemolytic activity was also decreased by branching of the hydrophobic tail. The molecular weight of surfactants was also found to have an influence on haemolysis. As molecular weight increases, so the haemolytic activity of these nonionic surfactants decreases.

The lytic activity of the surfactants was also investigated on lecithin liposomes incorporating carboxyfluorescein (CF) (de la Maza *et al*, 1992). Surfactant induced lysis was followed by the leakage of CF. A good relationship between percentage haemolysis and CF leakage was found to occur, CF leakage and percentage haemolysis both decreasing with increasing POE chain length for the linear alkyl nonionic surfactant series, haemolysis being related to the interaction of lipids in erythrocyte membranes and nonionic surfactants.

It is thought that lysis of membranes occurs due to the interaction between surfactants and lipids of the membrane (Haydon & Taylor, 1963). Surfactants appear to act as 'wedges' thus destroying the orientation of the lipid bilayer. Low surfactant concentrations affect membrane-bound enzymic activities (Kagawa, 1972; Coleman, 1973), variously activating, inhibiting or modifying function. At very low concentrations phosphorylation in mitochondria and chloroplasts is uncoupled, diffusion is facilitated and carrier mediated transport is affected.

Low concentrations of surfactants alter the majority of membrane-bound enzymes either by activating, modifing or inhibiting them. However, it is difficult to ascertain whether this is merely a consequence of the altered permeability of the membrane to substrates and products or the modification of the substrate by the surfactant in some way. A biphasic effect of surfactant on membrane-bound enzymes can sometimes be observed. At low surfactant concentration activation can be seen and at higher concentrations this becomes inhibition. Optimum activation tends to be seen when the enzyme is still membrane-bound (Helenius & Simons, 1975).

Direct evidence (Bont *et al*, 1969; Deamer, 1973; Helenius & Soderland, 1973; Becker *et al*, 1975) shows that as, the concentration of surfactant is raised, increasing amounts of detergent are bound. Saturation point is reached and the gradual disintegration of the membrane starts. It is primarily the monomer form of surfactant that binds to membranes, as with surfactant binding to proteins, not the micellar form (Tanford, 1972).

Jackson *et al* (1982) followed the solubilisation of large unilamellar egg

phosphatidylcholine vesicles by the nonionic surfactant octyl glucoside (OG) using a number of techniques including NMR, electron microscopy and turbidity measurements. A solubilisation process was put forward which had previously been proposed for other surfactants (Helenius & Simmons, 1975). Stage one involves OG partitioning between the membrane bilayer and aqueous phase. The result of this is to cause a small 'fluidising' effect on the membrane. In the latter stages of this rearrangement large mixed bilayers are formed. In stage two surfactant saturated bilayers are converted into mixed bilayers. Stage three is characterised when all the phophatidylcholine is present as mixed micelles.

This model for the solubilisation of membranes by OG has been proposed by other workers (Paternostre *et al*, 1988). They also found that this mechanism was true for the solubilisation of large unilamellar phosphatidylcholine liposomes by Triton X-100 and sodium cholate. Permeability changes in the liposomes were also observed at sub-solubilising concentrations of the surfactants. This study also supports the assertion that for membrane solubilisation to occur the concentration of free surfactant must be at the CMC (Lictenberg, 1985). The nonionic surfactant octa(ethyleneglycol)-monododecylether ($C_{12}E_8$) is incorporated into Ca^{2+}-ATPase containing membranes at low concentrations without causing solubilisation. Solubilisation as with Triton X-100, OG and sodium cholate also occurs at concentrations close to the CMC of the free surfactant. Electron-spin resonance studies reveal that $C_{12}E_8$ increases the fluidity of the membrane in the same way as temperature. However, unlike the effect of a rise in temperature, $C_{12}E_8$ alters the functional properties of the Ca^{2+}-ATPase; it was concluded that ATPase turnover is disturbed by the

interaction of surfactant with the enzyme directly as opposed to indirect means such as by protein aggregation or changes in the lipid phase (Andersen *et al*, 1983).

Studies investigating the binding of the $C_{12}E_8$ to unilamellar and multilamellar liposomes and sarcoplasmic reticulum vesicles show that $C_{12}E_8$ is quickly distributed through the leaflets even if there are 10-20 bilayers to be crossed (le Maire *et al,* 1987), challenging the theory that the lipid bilayer acts as a barrier to the entry of nonionic surfactants. Edwards and Almgren (1991) investigated the addition of $C_{12}E_8$ to small unilamellar lecithin vesicles by light scattering methods and cryo-transmission electron microscopy. A slight swelling of the liposomes was seen at low surfactant concentration, due to the incorporation of surfactant monomers into the liposomal membrane. As the surfactant concentration was increased large unilamellar vesicles were seen, increasing in size with increasing surfactant concentration. When the surfactant concentration reached a critical concentration (40 mol%) the onset of liposome solubilisation occurred. This corresponds with the formation of lipid/surfactant mixed micelles. Complete lipid solubilisation was achieved at concentrations exceeding 70 mol%.

Further studies involving the interaction of betaine type surfactants (de la Maza & Parra, 1993a, b) and nonionic surfactants (de la Maza & Parra, 1994) with unilamellar liposomes have been carried out. Three parameters were used to characterise the surfactant /lipid molar ratios (R_e) at which the surfactant (i) saturated the liposomes (Re_{sat}); (ii) caused a 50% solubilisation of the liposomes ($Re_{50\%}$); and (iii) completely solubilised the vesicles (Re_{sol}). These determinations of membrane solubilisation were

found to be inversely related to the CMC of the surfactant/lipid system, suggesting that the CMC of the systems is associated with mixed micelle formation (de la Maza & Parra, 1993a). A mixed system of amphoteric and anionic surfactant also show an ability to alter the permeability of unilamellar liposomes. A positive correlation between the partition coefficient of the mixed surfactant systems and alteration in the permeability of the liposomes was determined (de la Maza & Parra, 1993b). For the nonionic surfactants, octyl phenols, Re_{sat} was inversely proportional to the CMC of the surfactants but $Re_{50\%}$ and Re_{sol} does not depend on the CMC (de la Maza & Parra, 1994).

Nonionic surfactants show a correlation between structure and solubilising potency. The vast majority of effective solubilisers have an HLB value in the range of 12.5 to 14 (Hengstenberg, 1970; Umbreit & Strominger, 1973). It is not just the HLB value of a surfactant that determines its potency as a membrane solubiliser, the chemical structure of the hydrophilic and hydrophobic groups have also been shown to be important (Stromberg, 1971). In general terms nonionic surfactants are effective in solubilising the cytoplasmic membranes of bacteria (de Phamphilis & Adler, 1971; Schnaitman, 1971a, b). However, Gram-negative bacteria possess an outer membrane which is very resistant to detergent action.

1.4.5 Surfactants as antimicrobial agents

Surfactants have long been known to have a considerable antimicrobial activity. However, the knowledge draws mainly from experience with the cationic surfactants, most notably the quaternary ammonium salts or 'quats'. These compounds were shown to have an antimicrobial effect as long ago

as 1916 when Jacobs and Heidelberger published a number of papers detailing the antimicrobial activity of a range of such compounds previously synthesised by them (Petrocci, 1977).

Quaternary ammonium salts, and cationic surfactants in general were considered to be excellent antimicrobial agents but had very little detergent effect, whilst anionic and nonionic surfactants had excellent detergent properties but were considered to have little or no antibacterial action (Davis, 1960). This view, however, is at odds with the literature which reported the antibacterial potential of anionic and nonionic surfactants as far back as the 1930's. Cowles (1938) studied the bacteriostatic properties of a series of sodium alkyl sulphates with differing carbon chain lengths against a range of Gram-negative and Gram-positive bacteria. It was found that in general Gram-positive bacteria were inhibited by the concentrations of alkyl sulphates used whereas the Gram-negative bacteria (with the exception of two species) were not. In fact, *Escherichia coli* was able to withstand much higher concentrations of two of the surfactants when compared to other Gram-negative bacteria. Cowles also concluded that there existed a relationship between chain length and inhibition against *Staphylococcus aureus* for these straight chain alkyl sulphates, the optimum chain length being between 12 and 16 carbon atoms.

Similar findings were published by Birkeland & Steinhaus (1939) and Kabara (1978). Birkeland & Steinhaus (1939) investigated the growth inhibitory properties of sodium lauryl sulphate (sodium dodecyl sulphate) and a commercial product, "Dreft" (containing large amounts of this alkyl sulphate), against a wide range of Gram-positive and Gram-negative bacteria, in addition to some moulds. It was concluded that sodium lauryl

sulphate and "Dreft" inhibited the growth of Gram-positive bacteria and moulds whilst almost all of the Gram-negative bacteria grew freely.

The bactericidal properties of a selection of anionic detergents were compared with a series of cationics in a study by Baker *et al* (1941). In common with other studies the detergents were tested against a range of Gram-positive and Gram-negative bacteria. It was concluded that the anionics were only effective against the Gram-positive bacteria and even then much less effective than the cationics.

The benefit of anionic detergents in use is their stability and their lack of corrosive action. They also have wetting qualities which result in a uniform film forming over a surface to be disinfected thus producing a complete disinfecting action, this makes such agents more appropriate and efficient than traditional sterilising solutions such as chlorine. Scales and Kemp (1941) investigated the germicidal properties of a range of anionic surfactants, including Triton No. 720; Aerosol OS; Aerosol OT; Aerosol DGA; and various sulphonated oils. They concluded that solutions of such commercial surfactants possessed excellent germicidal properties, particularly when the pH of the solution was acidic. They found that solutions of the surfactants with a pH of 4.0 possessed a germicidal action greater than that seen with sodium hypochlorite. At this pH they also found no difference in action of these anionic surfactants against Gram-positive and Gram-negative bacteria.

The synergistic effect of Aerosol OT (dioctyl sodium sulphosuccinate) on certain germicides was investigated by James (1948). He found it to have a potentiating effect on the germicidal activity of phenol and cresol; however,

he reported it to have no germicidal activity when used alone which is at odds with the findings of Scales and Kemp (1941).

Armstrong (1957) investigated the effect of sodium dodecyl sulphate, an anionic detergent, on the aerobic fermentation of glucose by brewer's yeast. He concluded that inhibition of yeast metabolism occurs only when the detergent is above its CMC, i.e. it is present as micellar aggregates.

Studies have shown the cytoplasmic membrane to be the likely target of ionic detergents (Salton, 1957, Pethica 1958). Gilby and Few (1957) investigated the action of anionic and cationic detergents containing the C_{12}-alkyl chain on the protoplasts of *Micrococcus lysodeikticus*, concluding that the site of action for the detergents was the phospholipid component of the protoplast membrane. Further work (Gilby & Few, 1960) determined the lytic action of sodium dodecyl sulphate (SDS), on isolated protoplast membranes using spectrophotometric methods. An initial rapid decrease in optical density was observed for membranes treated with SDS. The following 12 hours saw a further very slow decrease in optical density, demonstrating disintegration of the protoplast membrane. Gilby and Few suggested that this could be taking place through surfactant interaction with the membrane protein moiety.

A review of the mechanism of action of surfactants was published by Newton in 1960 summarising the hypotheses up to that date. He surmised that treatment of bacteria with particular anionic or cationic surfactants rapidly disrupts the organisation of the cytoplasmic membrane which controls the osmotic equilibrium of the bacterial cells; a close correlation exists between the bactericidal concentration and the concentration which causes

alterations in cell permeability.

The detergents polyethylene glycol 300, polyethylene glycol 1000 and Lissapol NX were found to cause leakage of cellular material from bacteria, thus demonstrating their ability to alter the permeability of the cell membrane (Davies & Field, 1969). Schnaitman (1971a) published a study demonstrating the effects of Triton X-100, a nonionic surfactant, on *Escherichia coli* which show a specific solubilising effect on the cell membrane. It is quite specific in that only proteins in the cytoplasmic membrane but not the cell wall are solubilised. Lima (1980) found that Triton X-100 increased the dissolution of the membrane of *B. subtilis* and ribosome disorganisation. After 15 minutes contact extensive cell lysis was observed. This compared with sodium dodecyl sulphate which at low concentrations reduced growth of *B. subtilis* and caused some slight lysis of the cell population. At higher levels membrane dissolution and cytoplasmic coagulation was seen. Tween 80, another nonionic surfactant, also has membrane disruptive properties (Young *et al*, 1983). Lipid vesicles incubated with Tween 80 were found to have increased permeability.

Antimicrobials are used in a range of products in the cosmetic industry to control unpleasant body odours. Many of these antimicrobials are toxic, for example quaternary ammonium compounds which are irritating to the skin and to the eyes, and thus limit the usable dose and the range of products they can be used for (Gucklhorn, 1969). Studies undertaken to find suitable alternatives (Pandey *et al,* 1985) used fatty acid esters, namely sodium 2-lauroyloxy propionate which was found to be germicidal against skin microflora (99% of which are Gram-positive) in addition to having a far lower toxicity.

Nonionic surfactants have also been shown to possess antifungal properties (Spotts & Cervantes, 1987). The nonionic surfactant Ag-98, which is 80% octyl phenoxypolyethoxyethanol, inhibited the spore germination, germ tube growth and mycelial growth of *Botrytis cinerea, Mucor piriformis* and *Penicillium expansium*. It was also observed that Ag-98 had a potentiating effect on the antifungal activity of chlorine. The effect of nonionic surfactants on green algae, *Chlamydomonas,* has also been demonstrated (Ernst *et al*, 1983). A homologous series of alcohol ethoxylates was used and it was shown that growth of *Chlamydomonas* decreased as the hydrophilicity of the surfactant increased. The most lipophilic alcohol ethoxylate demonstrated growth promoting effects. This contrasts with sodium alkylsulphates which did not significantly inhibit growth; sulphobetaines were growth inhibitory when the lipophile contained twelve or more carbon atoms and only at micellar concentrations.

Pluronic F68 (polyoxyethylene-polyoxypropylene block co-polymer) has been shown to have an effect on membrane permeabilisation and the enzyme activity of a batch culture of *Saccharomyces cerevisiae.* (Laouar*et al*, 1996) This was illustrated by a reduction in *in situ* alcohol dehydrogenase activity and the enhanced growth inhibitory effects of sub-lethal doses of antibiotics. Similar results were also seen with Triton X-100, but this also inhibited the log phase and slowed the cell culture kinetics of the batch culture. All of these effects occurred at concentrations in excess of the CMC of the surfactants, no measurable effect was seen below these concentrations.

The respiratory state of an organism appears to influence the effectiveness

of surfactant to solubilise membranes (Lanyi, 1973). The UV-IR spectrum of the bacteriorubin pigment of *Halobacterium cutirubrum* is altered in the presence of Triton X-100 in the lipid region of the membrane and thus can be utilised as an indicator for Triton binding. Little surfactant is bound to the membrane of respiring cells, and thus the membrane is not solubilised. The situation is reversed when the cells are not respiring, binding is observed and the membrane is completely solubilised. The molecular reason for this is not known.

1.4.6 Structure-function relationships of surfactants

Tiehm (1994) investigated the effect of alkylethoxylate and alkylphenolethoxylate type nonionic surfactants, with an average chain length of between 9 and 12 carbon atoms on *Mycobacterium* sp. He found that the growth of *Mycobacterium* sp. was inhibited due to the toxic effect of these surfactants. A trend in structure and activity was also noted. Tiehm found that the surfactants with the highest lipophilicity/hydrophobicity were the most inhibitory against *Mycobacterium* sp. This supported data from other studies which found that HLB values are related to the toxicity of surfactants against several soil bacteria (Cserhati *et al*, 1991), and nonionic surfactants with hydrophobic tails of a similar structure to those in the Tiehm study were most toxic when the ethylene groups were between 6 and 13 per molecule (Cserhati *et al*, 1982).

The correlation between the physical characteristics of surfactants and their antimicrobial properties has been observed by other workers (some examples of these structure function relationships are summarised in Table 1.7). For instance the length of the tail group has a profound effect on the

antimicrobial properties of surfactants (Cowles, 1938; Birkeland & Steinhaus 1939; Kabara, 1978; Mlynarcik *et al* 1979a, b; Devinsky *et al* 1982). The optimum length of the alkyl chain constituting the tail group has been found to be between 9 and 16 carbon atoms. This variation appears to arise by the virtue of the type of surfactant under study. The optimum chain length for alkyl sulphates has been found to be between 12 and 16 carbon atoms (Cowles, 1938; Birkeland & Steinhaus 1939) whereas for amine oxides the optimum is lower, between 9 and 13 (Mlynarcik *et al* 1979a; Devinsky *et al* 1982).

The relationship between chain length and antimicrobial activity is complex, the correlation is not linear and is determined to some extent by the organism under test and the nature of the polar group (Kabara, 1978). Szogyi *et al* (1981) observed increased permeability of dipalmitoyl-phosphatidylcholine model membranes in the presence of various nonionic surfactants (nonyl phenyl ethylene oxide and tributyl phenyl ethylene oxide polymers) in ion-free and different ionic environments. They observed that the effectiveness of the surfactants depended on the number of ethylene oxide groups that the surfactant contained. It was proposed that the hydrophilic ethylene oxide chain interacts with the polar head groups of the membrane phospholipid therefore controlling the penetration of the lipophilic alkyl chains.

The asymmetry of the surfactant molecule also appears to play a part in the effectiveness of the surfactant in terms of antimicrobial activity (Devinsky*et al,* 1982). A degree of asymmetry in a surfactant molecule brought about a marked decrease in antimicrobial activity.

Table 1.7 Structure function relationships for some surfactants

Reference	Parameter	Results	Surfactant tested
Cowles (1938)	Alkyl chain length (n)	Optimum chain length is n=12-16	Alkyl sulphates
Birkeland & Steinhaus (1939)	Alkyl chain length (n)	Optimum chain length is n=12-16	Alkyl sulphates
Kabara (1978)	Alkyl chain length (n)	Relationship between n and antimicrobial activity, complex. Correlation not linear, determined to some extent by test organism and nature of polar group	
Galbraith *et al* (1971)	Alkyl chain length (n)	n=12, most active saturated fatty acid, but activity less than that of n=18 unsaturated fatty acids	Long chain fatty acids
Zaslavsky *et al* (1979)	Alkyl chain length (n)	As n increases, concentration at which haemolysis occurs decreases	β-D-glucopyranosyl-1-alkyl phosphates
Mlynarcik *et al* (1979a)	Alkyl chain length (n)	Optimum chain length is n=9-13	Amine oxides
Mlynarcik *et al* (1979b)	Alkyl chain length (n)	Chain length is n=9-13 for antimicrobial activity, further elongation reduces activity due to reduced water solubility. Optimum n=11-12	Bis-quaternary ammonium salts
Kourai *et al* (1980)	Alkyl chain length (n)	Growth inhibition reaches a maximum when alkyl chain length is lauryl	Quaternary ammonium salts

Table 1.7 continued

Reference	Parameter	Results	Surfactant tested
Devinsky *et al* (1982)	Alkyl chain length (n)	Optimum chain length is n=9-13	Amine oxides
Lamikanra & Sholola (1984)	Alkyl chain length (n)	Activity is of the following order: 14>12>16>18	Saturated fatty acids
Devinsky *et al* (1985)	Alkyl chain length (n)	Related to CMC and MIC, CMC must be in certain range for optimal activity	Non-aromatic quats amine oxides
Miyazawa *et al* (1984)	Alkyl chain length (n)	As n increases, so does the irritation properties of the surfactants	Anionic, cationic, amphoteric
Devinsky *et al* (1990)	Alkyl chain length (n)	As n increases, activity increases to a maximum when n=14-16, then decreases	Morpholium bromide, pyrrolidinium, piperidinium types
Ohnishi & Sagitani (1993)	Alkyl chain length (n)	As n increases, concentration at which haemolysis occurs decreases	Polyoxyethyleneε - surfactants
Miyazawa *et al* (1984)	Branching of chain	Branching increases the irritation properties of the surfactant	Saturated soaps
Kabara (1978)	Saturation of chain	n<12 - unsaturated compounds less active n=14-16 - saturated compounds less active point of saturation influences activity	Fatty acids

Table 1.7 continued

Reference	Parameter	Results	Surfactant tested
Szogyi *et al* (1981)	Ethylene oxide(EO) groups	As the number of EO goups increase, so does activity	Nonyl phenyl EO, tributyl phenyl EO
Kabara (1978)	Head group size	Head group size does not affect optimum chain length for particular surfactant series but appears to direct action to specific microorganisms	Fatty acids
Mlynarcik *et al* (1978)	Heterocycle size	Activity most effective when ring enlarged to 7 or 8 members, presence of oxygen decreases activity	Piperidine and pyrrolide derivatives
Devinsky *et al* (1990)	Heterocycle size	Presence of an oxygen atom in ring causes moderate decrease in activity (due to increased hydrophilicity by replacing CH_2 group with oxygen atom)	Morpholium bromide, pyrrolidinium, piperidinium types
Devinsky *et al* (1982)	Asymmetry	Asymmetry reduces surfactant activty	Amine oxides
Zaslavsky *et al* (1978b)	Lipophilicity	Increased lipophilicity reduces activty	Ionics and non-ionics
Devinsky *et al* (1991)	Lipophilicity (C_k)	Most active compounds have a C_k of *ca* 1 mmol.dm^{-3}	Quaternary ammonium amphilies

Table 1.7 continued

Reference	Parameter	Results	Surfactant tested
De la Maza*et al* (1992)	Partition coefficient	Positive correlation between partition coefficient and activity	Octyl phenols
Zaslavsky *et al* (1978a)	HLB	No correlation (but tail and head group contributions influence activity)	Polyethylene esters of fatty acids, tweens, triton, polyethylene mercaptans
De la Maza & Parra (1993a)	HLB	As HLB increases so does activity	Octyl phenols
Kellermayer *et al* (1994)	HLB	Postive correlation between HLB and activity	Triton X-100 Brij series
Kitasuji *et al* (1996)	HLB	HLB of 11-15 lyse bacteria effectively	Polyoxyethylene alkyl alcohol ethers
Ikeda *et al* (1986)	MW	Antibacterial activity strongly dependent on MW of poly cation, optimal MW range for biocidal activity is 5-10 x 10^4	Polycationic bioicides
Ohnishi & Sagitani (1993)	MW	As MW increases, % haemolysis decreases	Polyoxyethylene surfactants

Table 1.7 continued

Reference	Parameter	Results	Surfactant tested
Kanazawa *et al* (1993a,b)	MW	Antibacterial activity increases as MW increases	Polymeric phosphonium salts
Galbraith *et al* (1971)	CMC	Max. activity when CMC *ca* 1 mmol.dm^{-3}	Pyridinium bromide quats
Devinsky *et al* (1990)	CMC	CMC determines activity, if CMC=1x10^{3}M surfactant active regardless of microorganism	Morpholium bromide, pyrrolidinium, piperidinium type
Kanazawa *et al* (1993a)	Counter ion structure	Low antibacterial activity when counter and phosphonium ions form tight ion pair, high activity if dissociate into free ions	Polymeric phosphonium salts

Devinsky and coworkers (1985) also illustrated the relationship between alkyl chain length, antimicrobial properties (measured by the minimum inhibitory concentration) and critical micelle concentration, for a range of non-aromatic quaternary ammonium compounds and amine oxides. It was concluded the length of the alkyl chain of a surfactant influenced its CMC and MIC values and therefore a correlation between MIC and CMC as a function of the alkyl chain group length was made. It was concluded that only surfactants with a CMC within a certain range showed optimal antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans.*

It is not only the chain length of the hydrophobic tail group of a surfactant molecule that has an effect on its biological activity although this is recognised as the most important influence (Kabara, 1978). The size of the polar head group does not appear to affect the optimum chain length for a particular series of surfactants but it does appear to direct action towards specific microorganisms.

Saturation of the alkyl chain also has some influence on antimicrobial action. Unsaturated fatty acids were less active than those which were saturated below chain lengths of 12 carbon atoms. The converse of this was true for alkyl chains containing between 14 and 16 carbon atoms with the position of unsaturation also entering into the equation.

Chain length is also an important factor in toxicological studies. As surfactants are used to a great extent in the cosmetics industry, skin irritation (contact dermatitis) must be an important factor for a potential antimicrobial agent. The shorter chain length (C_8 and C_{10}) saturated soaps cause

significant irritation, this irritation factor decreases as chain length increases with C_{12} being less irritating and C_{14} to C_{18} having a very low toxicity. Branching also increases the irritation power of a surfactant.

The polar head group also has an influence on the toxicity of the surfactant. In general, the US Food and Drug Administration have found the ocular irritation properties of different charged head groups to be as follows, in descending order of irritation: cationics > anionics > nonionics. Protein denaturation used as an *in vitro* method to evaluate the irritation potency of a range of surfactants on human skin gave similar findings, showing nonionic surfactants to have the lowest irritation properties (Miyazawa *et al*, 1984). The low toxicity of nonionics in addition to their antimicrobial properties makes them highly suitable for use as preservatives in the food, cosmetics and toiletry industries.

Zaslavsky *et al* (1978a) also examined structure-function relationships in relation to nonionic surfactants and their action on membranes. The aim of their study was to determine whether the HLB of a surfactant was one of the most important parameters in determining membrane activity. The nonionic surfactants under study included Tweens, Triton X-305 and polyethylene esters of fatty acids and polyethylene mercaptans of differing headgroup and chain length size. The surfactants were investigated for their ability to haemolyse red blood cells. It was noted that the HLB value of a surfactant did not correlate with membrane activity for the surfactants tested but the contributions of both the hydrophobic tail and hydrophilic head group did.

The same finding was obtained when the haemolytic activity of ionic and nonionic surfactants were investigated (Zaslavsky *et al*, 1978b). Haemolytic

activity is the result of two distinct processes, first, the take up of the surfactant from the solution by the cells (the affinity of the surfactant for the cell membrane) and, secondly, the uptake needed to bring about lysis. The former occurs even at low concentrations due to the surfactants affinity for the membranes. It was found that membrane affinity decreased as the lipophilicity of the compounds decreased.

1.5 Aims and objectives

Antibacterial agents in common usage today have become established by experience with little information known about their mechanism of action. A knowledge of the mechanism of action of an antibacterial agent provides an insight into toxicological and resistance problems and allows synergistic combination of agents to be developed, thereby increasing the spectrum of activity and reducing the effective dose required.

A range of nonionic and anionic surfactants will be screened for antibacterial activity using a growth inhibition method. From this initial group, surfactants demonstrating suitable antibacterial activity will be chosen for further investigation.

Literature suggests the cytoplasmic membrane to be the most likely site of action and a series of biochemical probes will be used to investigate this hypothesis. Probes will look at damage to the cytoplasmic membrane by studying leakage profiles, substrate uptake and enzyme activity. This information will then be used to comment on a possible site and mode of action for the surfactants.

CHAPTER TWO

Antimicrobial screening studies of a range of non-ionic surfactants

2.1 Introduction

Antibacterial agents can elicit both bacteriostatic and bactericidal effects: bacteriostatic injury has been categorised by Fitzgerald *et al* (1989) as reversible damage following biocide removal or neutralisation, whilst bactericidal action was described by Denyer (1995) as irreversible or irreparable damage to a vital cell structure or function.

Potential antibacterial agents can be assayed for efficacy by a number of different methods (Bloomfield, 1991; Hugo & Russell, 1992). Agar diffusion tests involve filling cups/troughs in a nutrient agar plate inoculated with microorganisms with antibacterial solution, or placing filter paper discs or strips impregnated with antibacterial agent on the agar plate. The plates are stored for a short time to allow diffusion of the antibacterial agent through the agar and the plates then incubated. The radius of the zone of inhibition is then measured. This assay however, requires the antibacterial agent in question to be able to diffuse through the agar. The determination of minimum inhibitory concentration (MIC) is an important parameter for defining the bacteriostatic effect of a biocide in solution. Serial dilutions of antibacterial agent in broth are inoculated with the test organism and incubated, the lowest concentration at which growth fails to occur is defined as the bacteriostatic concentration or the MIC. The size of inoculum used must be defined as this will influence the MIC obtained (Hugo & Russell, 1992). Inoculum history must also be taken into account for biocide testing as any variation in the biocide target site could influence sensitivity for the agent under test (Al-Hiti & Gilbert, 1980; Brown *et al*, 1990).

Determination of bactericidal activity comprises of adding a bacterial

suspension to a solution of antibacterial agent. At measured time intervals samples of the suspension are withdrawn, neutralised and transferred to nutrient medium and the viability determined. Bactericidal action can be quantified in several ways: MDT (mean death time) - time taken to kill an inoculum using a given concentration of antibacterial agent, MBC (minimum bactericidal concentration) - minimum concentration of antibacterial agent to kill an inoculum during a given time and MSST (mean single survivor time) - time taken to reduce number of surviving organisms to one using a given concentration of antibacterial agent.

2.2 Chemicals used

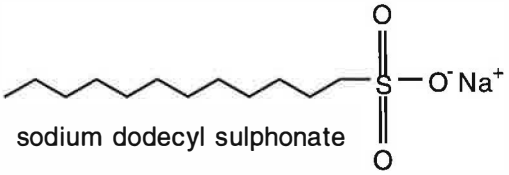
2.2.1 Surfactants

The anionic and nonionic surfactants used in this study were supplied by Unilever Research, Port Sunlight. They are listed, with their structures in figure 2.1.

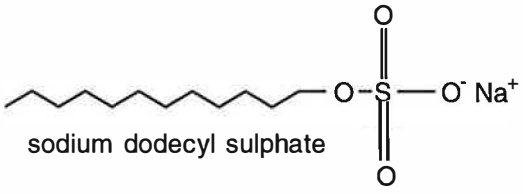
2.2.2 Culture Media

Synthetic broth AOAC (Difco Ltd.) was prepared in 100 mL quantities by mixing 1.7g of the powder with 100mL of distilled water and boiling for 2 minutes. This was sterilised by autoclaving at 121°C for 15 minutes. Prior to inoculation, 1 mL of a 10% w/v glucose solution (previously sterilised by autoclaving for 15 minutes at 115°C) was added aseptically. Nutrient broth no.2 and nutrient agar (Oxoid Ltd.) were prepared in 100 mL quantities and sterilised at 121°C for 15 minutes; nutrient agar was poured aseptically into petri dishes to prepare agar plates. To produce agar slopes 10 mL aliquots

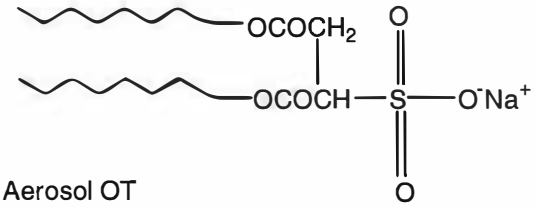
Figure 2.1 Anionic and non-ionic surfactants tested for bacteriostatic activity



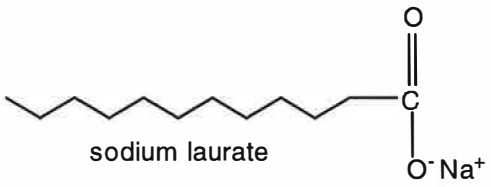
sodium dodecyl sulphonate



sodium dodecyl sulphate



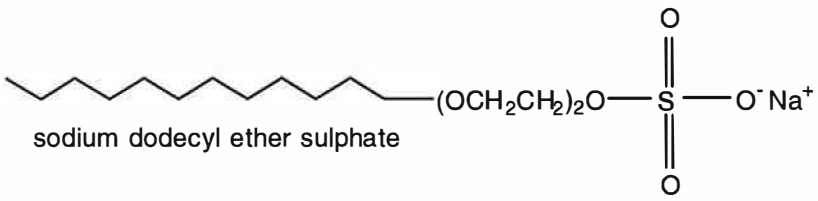
Aerosol OT
(di-octyl sodium sulphosuccinate)



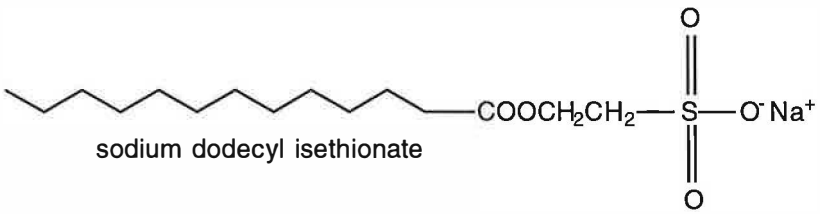
sodium laurate

Alcohol ethoxylate, C12En

e.g. hexa(ethyleneglycol)-monododecylether when n=6



sodium dodecyl ether sulphate



sodium dodecyl isethionate

of agar were placed in universal bottles, autoclaved as before and left to set at an angle of 15°.

Roux slopes were prepared by mixing 8.5g of synthetic broth AOAC powder with 500 mL with distilled water and to this, 1.5g w/v agar technical (no. 3) was added to solidify the broth. This was sterilised by autoclaving at 121° for 20 minutes. While the agar was still molten 5 mL of a 10% w/v glucose solution (sterilised as described above) was added aseptically and the slopes were then left to set.

2.3 Washing of Glassware

All glassware used was soaked overnight in a 0.1%v/v solution of Decon (Decon Laboratories Ltd.) in distilled water, rinsed and soaked for 1 hour in distilled water to which hydrochloric acid was added (to give a final concentration of approximately 0.1M) and then thoroughly rinsed with distilled water. This method has been previously shown to remove contaminating detergents employed in laboratory glassware washing.

2.4 Growth and Maintenance of Microorganisms

Escherichia coli NCTC 8196 and *Staphylococcus aureus* NCTC 4163 were both obtained from the Central Public Health Laboratory Service, National Collection of Type Cultures, Colindale, London, as freeze-dried cultures. These were chosen as they are recommended disinfectant assay strains (British Standard 6905).

The cultures were reconstituted in nutrient broth no. 2 (Oxoid Ltd.), incubated

by shaking overnight at 37˚C, then subcultured into nutrient broth no. 2 to obtain exponentially growing cultures. To these dense suspensions 10% of sterile glycerol was added and 1 mL aliquots of the cultures placed in ampoules and stored at -70˚C, the stocks produced being sufficient to last the duration of the research project.

When a sample of bacteria was required from the stock an ampoule was thawed and incubated by shaking overnight in 100mL of nutrient broth, at 37˚C. The suspension was then streaked onto 10 ml nutrient agar slopes (Oxoid Ltd.), incubated at 37˚C for 18 hours and then stored at 4˚C until required. A slope was used to produce a starter culture in 10 mL of synthetic broth AOAC (Difco Ltd.) which was then used to inoculate liquid or solid media. A new ampoule was opened every two months.

Each time a new frozen culture was used its identity was confirmed by the use of API Staph and API 20E (Biomerieux) to determine the biochemical characteristics of *S.aureus* NCTC 4163 and *E. coli* NCTC 8196, respectively. Microscopical examination and Gram-staining were also employed as an additional check on the identity of the bacteria. Typical biochemical profiles for *E. coli* NCTC 8196 and *S. aureus* NCTC 4163 are shown respectively in table 2.1 and table 2.2.

2.4.1 Production of Cell Suspension Calibration Curves

100mL of nutrient broth was inoculated with 1mL of a starter culture of bacteria and incubated with shaking, at 37°C for 18 hours. The cultures were then harvested by centrifugation at 3,300g for 20 minutes in a Sorvall RC-5B Refrigerated Superspeed Centrifuge, washed twice with 20 mL

Table 2.1 Biochemical Profile of *Escherichia coli* NCTC 8196

SUBSTRATE	REACTION/ENZYMES	RESULTS
Ortho-nitro-phenyl-galactosidase	Beta-Galactosidase	+
Arginine	Arginine Dehydrogenase	-
Lysine	Lysine Decarboxylase	+
Ornithine	Ornithine Decarboxylase	-
Sodium Citrate	Citrate Utilisation	-
Sodium Thiosulphate	H2S Production	-
Urea	Urease	-
Tryptophane	Tryptophane Desaminase	-
Trytophane	Indole Production	+
Sodium Pyruvate	Acetonin Production	-
Kohn's Gelatin	Gelatinase	-
Glucose	Fermentation/Oxidation	+
Mannitol	Fermentation/Oxidation	+
Inositol	Fermentation/Oxidation	-
Sorbitol	Fermentation/Oxidation	+
Rhamnose	Fermentation/Oxidation	-
Sucrose	Fermentation/Oxidation	-
Melibiose	Fermentation/Oxidation	+
Amygdalin	Fermentation/Oxidation	-
Arabinose	Fermentation/Oxidation	+

Table 2.2 Biochemical Profile of *Staphylococcus aureus* NCTC 4163

SUBSTRATE	REACTION/ENZYMES	RESULTS
D-Fructose	Acidification due to Carbohydrate Utilization	+
D-Mannose	Acidification due to Carbohydrate Utilization	+
Maltose	Acidification due to Carbohydrate Utilization	+
Lactose	Acidification due to Carbohydrate Utilization	+
D-Trehalose	Acidification due to Carbohydrate Utilization	+
D-Mannitol	Acidification due to Carbohydrate Utilization	+
Xylitol	Acidification due to Carbohydrate Utilization	-
D-Melibiose	Acidification due to Carbohydrate Utilization	-
Potassium Nitrate	Reduction of Nitrate to Nitrite	+
ß-Naphthyl-Acid Phosphate	Alkaline Phosphatase	+
Sodium Pyruvate	Production of Acetyl-Methyl Carbinol	+
Raffinose	Acidification due to Carbohydrate Utilisation	-
Xylose	Acidification due to Carbohydrate Utilisation	-
Saccharose	Acidification due to Carbohydrate Utilisation	+
∂-Methyl-D-Glucoside	Acidification due to Carbohydrate Utilisation	-
Arginine	Arginine Dehydrogenase	+

McIlvaine's buffer and resuspended in a known volume of the same solution. Dilutions of this stock solution were then prepared using McIlvaine's buffer as the diluent and their optical densities measured using a Pharmacia LKB Novaspec II Spectrophotometer at 420 nm.

Viable cell counts and total cell counts were performed on the bacterial suspensions. For the viable count, ten-fold dilutions of the stock suspension in McIlvaine's buffer were prepared and 0.2 mL of appropriate bacterial suspensions were spread over the surface of an over-dried Nutrient Agar plate. The plates were then incubated overnight at 37°C and the colonies counted. Each dilution was plated out in triplicate. Total counts were obtained by placing a diluted bacterial suspension on a counting chamber and counting the cells under phase contrast microscopy at x40 magnification.

These data enabled a plot of cell number against optical density at 420 nm to be constructed for each bacterium (figures 2.2 and 2.3). At a wavelength of 420 nm a linear relationship exists between optical density and cell numbers for optical density values less than 0.4.

2.4.2 Production of Growth Curves

Growth profiles were determined for both *E. coli* NCTC 8196 and *S. aureus* NCTC 4163. 1 mL of a starter culture was placed into each of five flasks containing 100 mL of synthetic broth AOAC and the flasks were incubated at 37°C, shaking at approximately 100 rpm on an orbital (reciprocating) shaker. 1 ml samples were removed from each flask every 20 minutes for *E. coli* NCTC 8196 and every 30 minutes for *S. aureus* NCTC 4163. The optical

Figure 2.2 Relationship between optical density and cell numbers for *E. coli* NCTC 8196
(n=5, mean±sd)

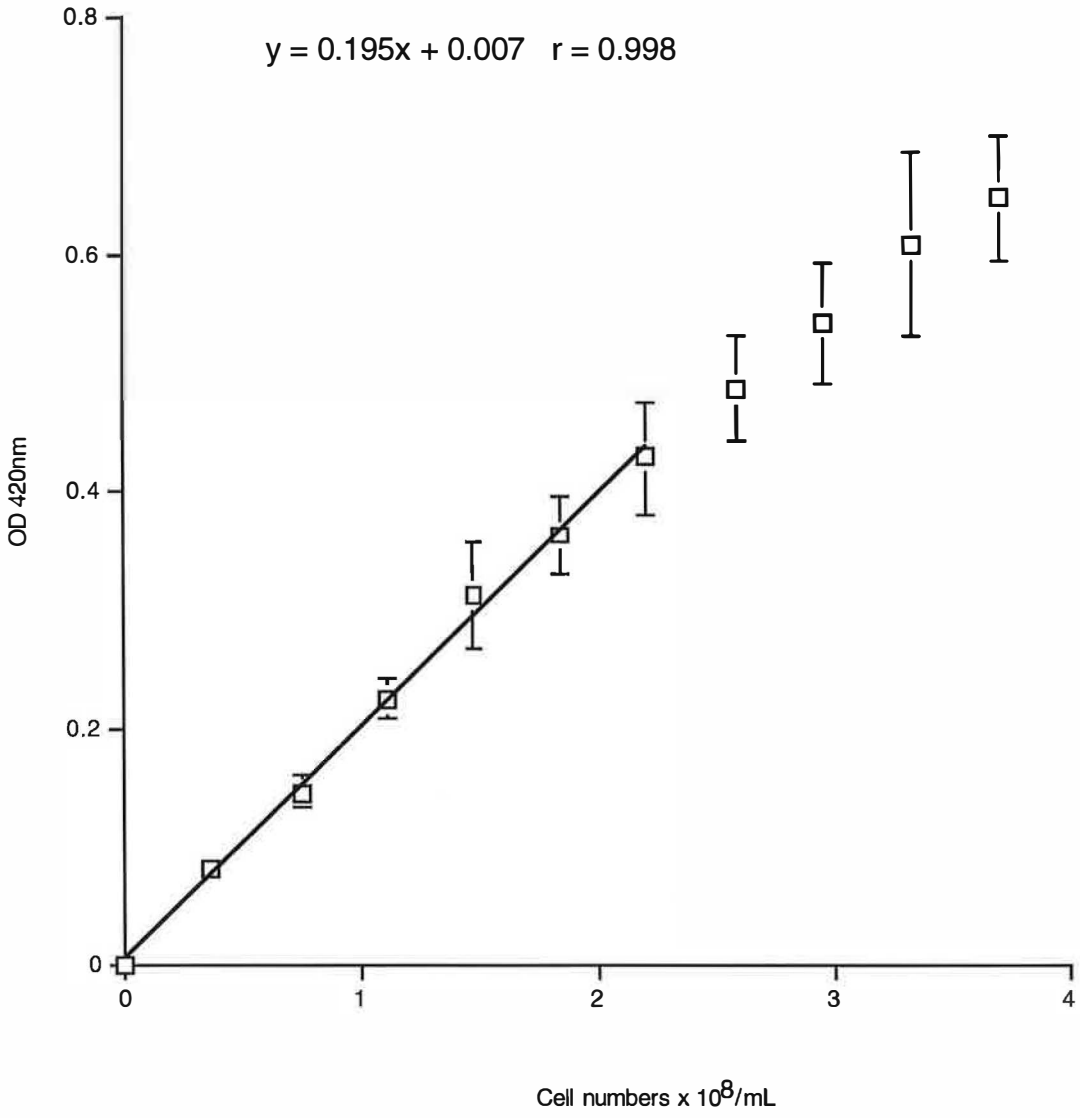
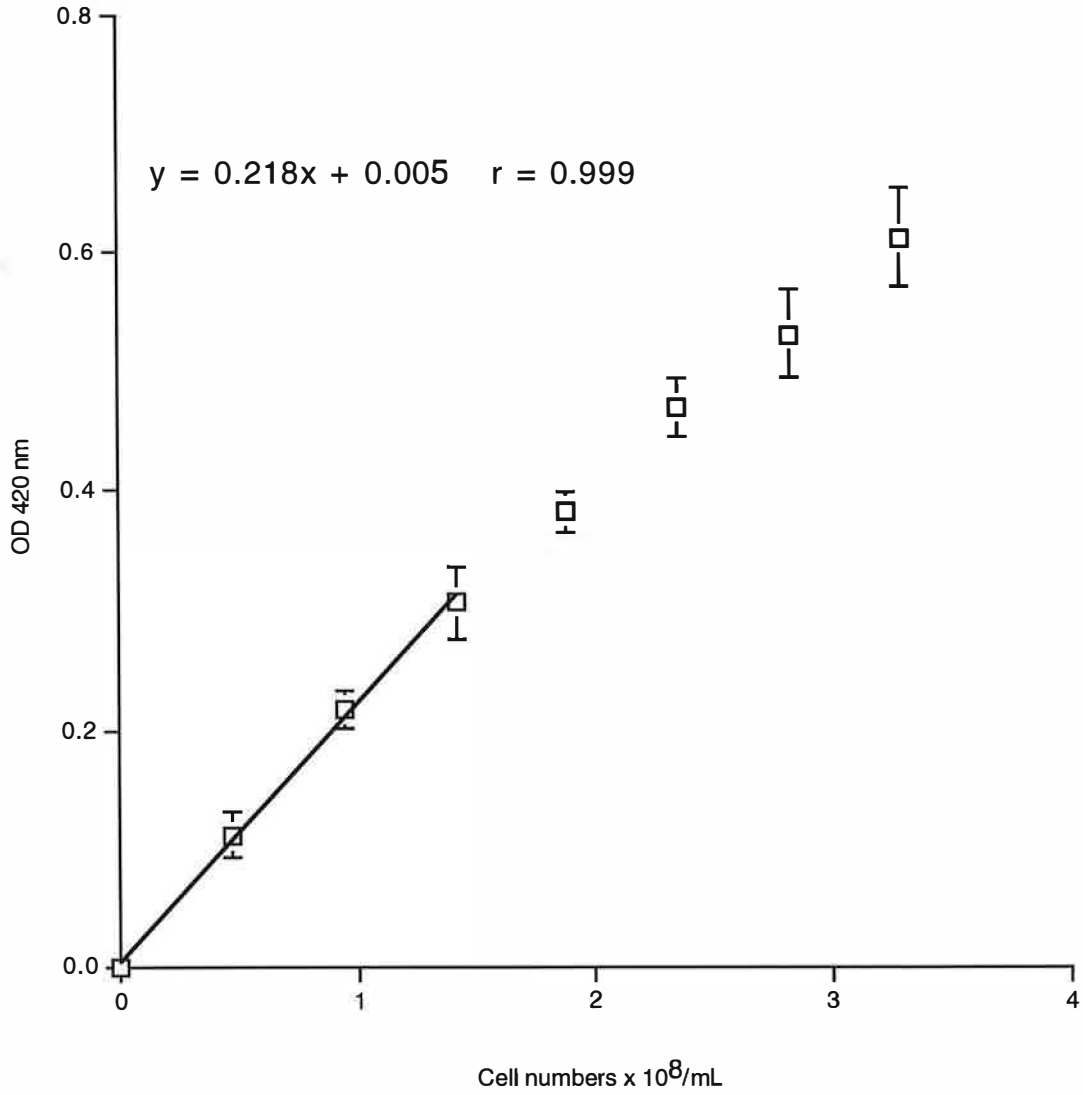


Figure 2.3 Relationship between optical density and cell numbers for *S.aureus* NCTC 4163 (n=5, mean±sd)



density of these 1 mL samples were measured at 420 nm with a Pharmacia LKB Novaspec II Spectrophotometer against a blank of synthetic broth AOAC. Figures 2.2 and 2.3 demonstrated that a linear relationship between optical density and cell numbers existed up to an optical density of 0.4, therefore samples with an optical density exceeding 0.4 were diluted to give readings within this linear range.

2.4.3 Results

The growth curves of *E. coli* NCTC 8196 and *S. aureus* NCTC 4163 can be seen in figures 2.4 and 2.5. The final cell densities for both growth curves were approximately $1x10^9$ cells/mL. The growth curve of *E. coli* NCTC 8196 approached stationary phase at approximately 5 hours and exceeded 10 hours for *S. aureus* NCTC 4163. To eliminate any differences in biocide susceptibility in the mechanism of action studies, the growth phase and conditions of bacteria used were standardised reducing variation in the biocide target site (Gilbert & Wright, 1987; Stewart & Olson, 1992). All bacteria used in the various growth inhibition and biochemical experiments were grown in synthetic broth AOAC for 15 h, shaking at 37˚C.

Figure 2.4 A growth curve of *E.coli* NCTC 8196, incubated in synthetic broth AOAC at 37˚C (n=5, mean±sd)

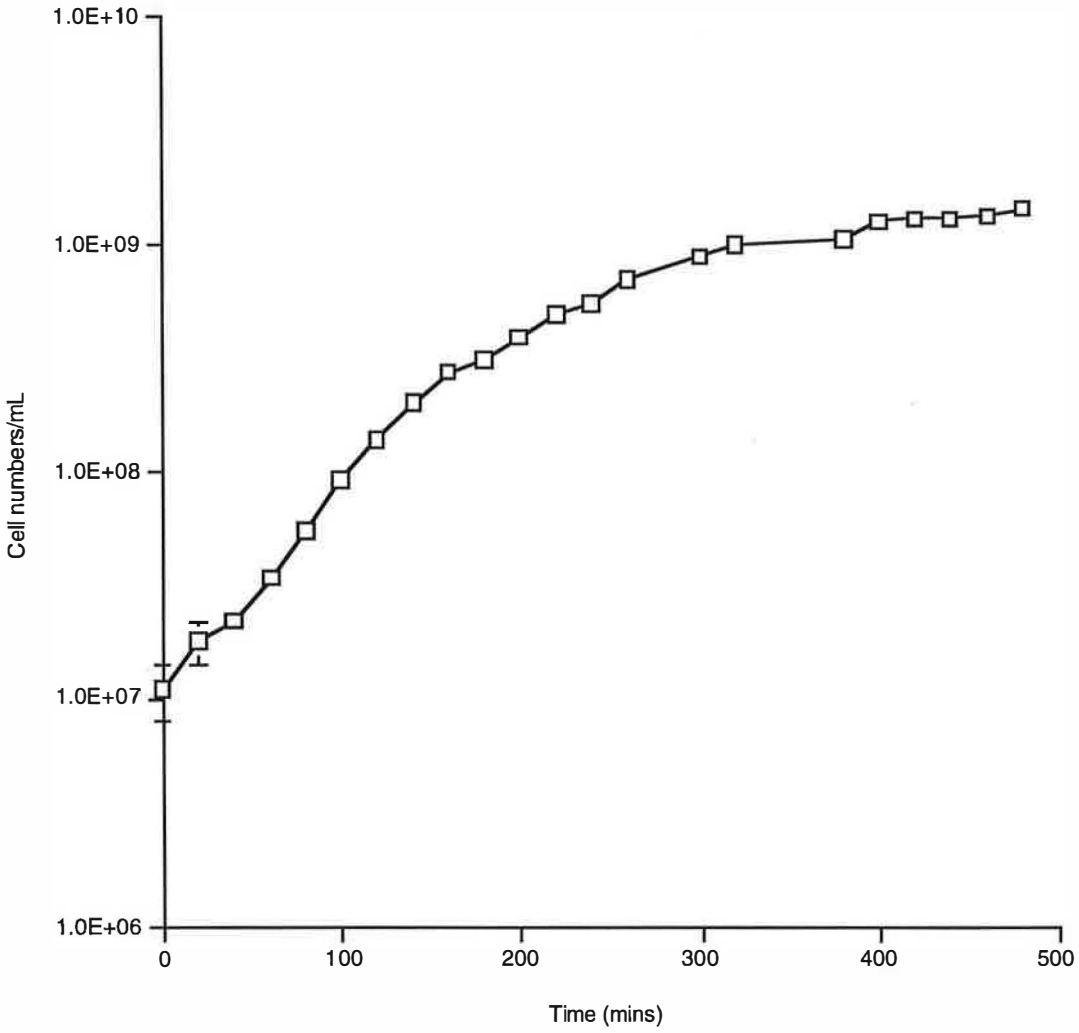
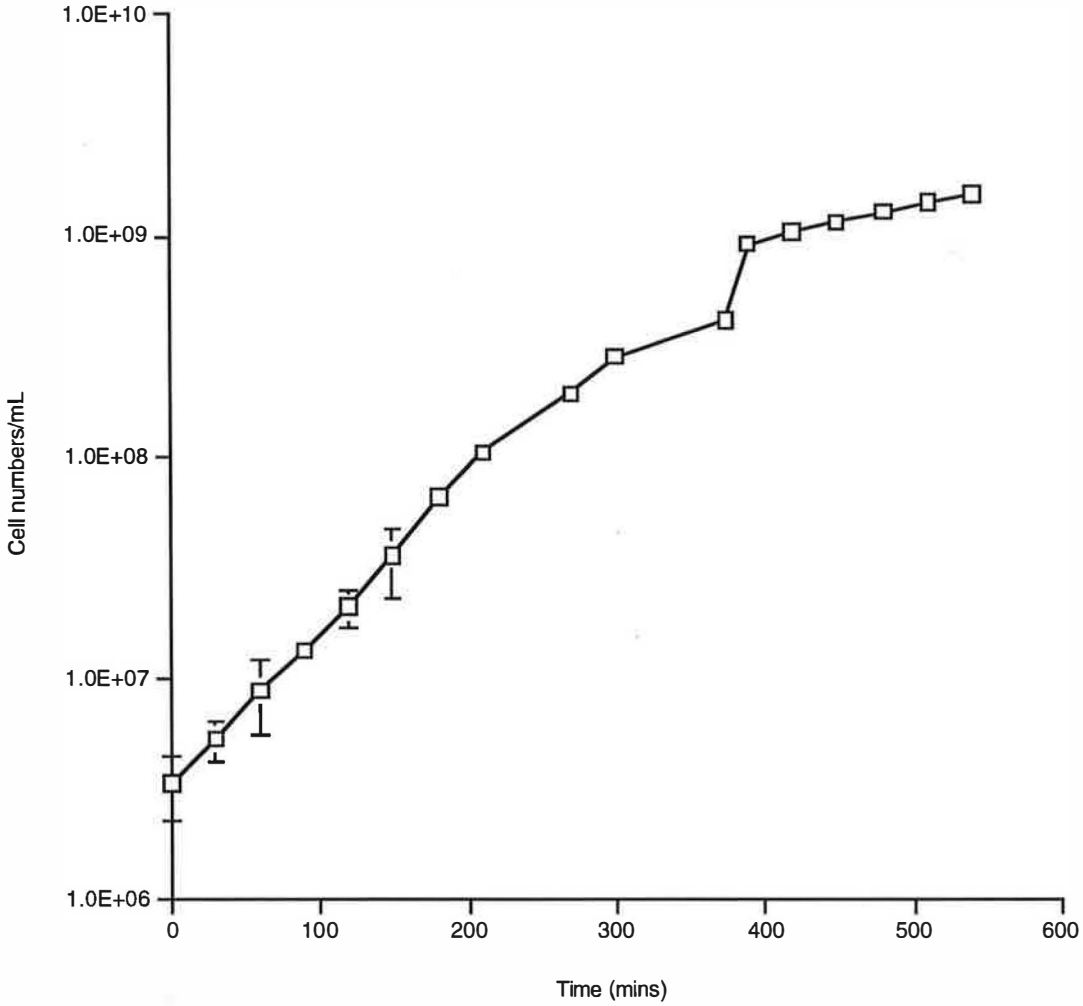


Figure 2.5 A growth curve of *S. aureus* NCTC 4163, incubated in synthetic broth AOAC at 37°C (n=5, mean±sd)



2.5 Choice of Buffer

To enable potassium ion leakage studies to be determined (section 5.1) it was necessary to employ a buffer which did not contain potassium. McIlvaine's buffer (McIlvaine, 1921) was chosen for the biochemical and biocidal studies; however, it was necessary to determine whether the citrate in the buffer could be utilised by the bacteria. Use of an API 20E strip enabled a biochemical profile to be compiled for *Escherichia coli* NCTC 8196, from this it was seen that *E. coli* NCTC 8196 does not utilise citrate. To determine whether *Staphylococcus aureus* NCTC 4163 utilises citrate, loopfuls of *S.aureus* NCTC 4163 were streaked on Simmons citrate agar (Oxoid Ltd.). *E. coli* NCTC 8196 was used as a control. The slopes were incubated for 24 hours. No colour change was seen with any of the slopes, indicating *S. aureus* NCTC 4163 does not utilise citrate.

2.5.1 Preparation and sterilisation of McIlvaine's buffer

100 mL McIlvaine's buffer was prepared by the addition of 18.15 mL of 0.1 M citric acid (BDH) to 81.85mL 0.2 M di-sodium hydrogen orthophosphate 2-hydrate (BDH) and adjusted to pH 7.0 with hydrochloric acid or sodium hydroxide. This was sterilised by autoclaving at 121˚C for 15 minutes.

2.6 Antimicrobial properties of a range of surfactants

2.6.1 Determination of Minimum Inhibitory Concentrations (MIC)

The Minimum Inhibitory Concentration (MIC) of the anionic and nonionic surfactants (Unilever Research, Port Sunlight) were determined for *S.*

aureus NCTC 4163 and *E. coli* NCTC 8196. MIC values were determined after 6 hours and 24 hours contact time.

Stock solutions of the surfactants were prepared in distilled water and then filter-sterilised using a 0.2μm cellulose nitrate membrane. Filtration was used to sterilise the surfactant solutions since their stability on autoclaving was not known.

To overcome any possible effects caused by sorption of the surfactants to the syringe and filter, the syringe was washed out with the surfactant solution and the first 5 mL of surfactant solution filtered was discarded.

Doubling dilutions of the surfactant solutions (from 0.01-10mM, depending on the surfactant tested) were prepared in synthetic broth AOAC. 200μL of each concentration of surfactant solution was placed in the wells of a 96 well microtitre plate each containing 50μL of a suspension of $5x10^6$ cells/mL bacteria (grown and prepared in synthetic broth AOAC), giving a final cell concentration of $1x10^6$ cells/mL. Surfactant dilutions without bacteria and bacteria in the absence of surfactant were also prepared as negative and positive controls, respectively. The microtitre plate was then incubated at 37˚C. Wells were examined for turbidity (as an indication of growth) at 6 and 24 hours. The Minimum Inhibitory Concentration is defined as the lowest concentration at which there is no visible turbidity.

2 6.2 Results

All of the surfactants exhibited antibacterial action against at least one of the bacteria. In general the surfactants were more effective i.e. had a lower MIC,

Table 2.3 Minimum inhibitory concentrations for a range of surfactants

Surfactant	MIC mg/mL E. coli (6h)	MIC mM E. coli (6h)	MIC mg/mL E. coli (24h)	MIC mM E. coli (24h)	MIC mg/mL S. aureus (6h)	MIC mM S. aureus (6h)	MIC mg/mL S. aureus (24h)	MIC mM S. aureus (24h)
sodium dodecyl sulphate	0.08	0.28	0.16	0.55	0.08	0.28	0.16	0.55
sodium laurate	5	22.5	5	22.5	<0.04	<0.18	0.08	0.36
sodium dodecyl sulphonate	0.16	0.59	0.31	1.14	<0.01	<0.04	0.63	2.31
sodium dodecyl ether sulphate	0.16	0.42	0.31	0.82	<0.01	<0.03	0.63	1.67
sodium dodecyl isethionate	0.16	0.47	0.31	0.9	<0.04	<0.12	0.08	0.23
aerosol OT	>10	>22.5	>10	>22.5	<0.01	<0.02	0.31	0.7
C12E4	0.04	0.11	>0.31	>0.86	<0.01	<0.03	0.04	0.11
C12E5	0.04	0.1	>0.31	>0.76	<0.01	<0.02	0.04	0.1
C12E6	0.04	0.09	>0.31	>0.69	<0.01	<0.02	0.04	0.09
C12E7	0.04	0.08	>0.31	>0.63	<0.01	<0.02	0.04	0.08
C12E8	0.16	0.3	>0.31	>0.58	<0.01	<0.02	0.08	0.15

against the Gram-positive bacterium *S. aureus* NCTC 4163 than the Gram-negative bacterium *E. coli* NCTC 8196. Of the surfactants tested the nonionic alcohol ethoxylates were the most effective antibacterial agents against both bacteria at 6 hours.

Each determination was performed twice with the exception of sodium dodecyl sulphate and *E. coli* at 24 hours; this experiment was performed six times to determine the reproducibility of the method (the MIC was unchanged for each replicate). The results are presented in table 2.3.

One of the problems encountered when determining the MIC of some of the surfactants was their intrinsic turbidity at high concentrations due to the presence of micelles in the solution. This was a particular problem with, for example, aerosol OT. To determine whether the turbidity seen was due to bacterial growth, the test and control wells were diluted to 25% with synthetic broth AOAC. This dilution was sufficient to eliminate the turbidity due to the surfactant, and any residual turbidity could be attributed to bacterial growth.

The growth inhibition studies suggested that the nonionic alcohol ethoxylates possessed greatest antimicrobial activity at the 6 hour time point irrespective of challenge organism; this group of surfactants were selected to form the focus of subsequent detailed studies. The Gram-negative bacterium *E. coli*, demonstrated greater resistance to these surfactants and was considered to offer the best opportunity for discriminating between agents within a series. This organism was therefore selected as the target organism for further studies.

2.7 Antimicrobial properties of a homologous series of alcohol ethoxylates

The head group size of the alcohol ethoxylates, with the exception of C12E8, appeared to have little influence on antimicrobial activity (table 2.3). To determine whether varying the alkyl chain length of the alcohol ethoxylates may have a significant effect, an homologous series sharing the E6 head group and with alkyl chains from 10 to 16 carbon atoms was investigated for antimicrobial activity .

To provide greater precision, the MICs were derived not from doubling dilutions but by dilutions prepared on an arithmetic scale over the range 0.05 to 10 mM. Six hours was chosen as the time point for the determination for MICs for the homologous series. As seen in the growth curve, *E. coli* had reached stationary phase at this time. A shorter antibacterial agent contact time period was felt to be representative of an in-use situation and was closer to the timescales employed in the biochemical experiments.

2.7.1 Results

The results from the MIC determinations for the homologous series of alcohol ethoxylates are given in table 2.4.

These results show C10E6 and C12E6 to have much greater growth inhibitory properties than C14E6 and C16E6 with the dodecyl derivative offering the greatest efficacy.

Table 2.4 Minimum inhibitory concentrations for the alcohol ethoxylate series C10E6 - C16E6

ALCOHOL ETHOXYLATE	MIC mM / E. coli NCTC 8196 (6h)
C10E6	0.2
C12E6	0.12
C14E6	5
C16E6	5

An attempt was made to determine the minimum bactericidal activity of the homologous series of alcohol ethoxylates. However, the concentration of surfactant required to determine bactericidal activity for the timescale employed (24 hours) was considerably in excess of the critical micelle concentration. Therefore, due to the influence of the cloud point concentrated surfactant solutions could not be prepared.

The cloud point and critical micelle concentration of the surfactants is discussed in more detail in chapter 3.

2.8 Discussion

Bacteriostatic studies demonstrated that all of the surfactants tested had some degree of bacteriostatic action against at least one of *E. coli* and *S. aureus*. All of the surfactants were more effective against the Gram-positive *S. aureus* than against the Gram-negative *E. coli*, with the exception of sodium dodecyl sulphate which required the same concentration to inhibit the growth of both organisms. Cowles (1938) and Baker (1941) reported differing susceptibilities of Gram-positive and Gram-negative organisms to

anionic surfactants, demonstrating Gram-positive bacteria to be inhibited by the surfactants whilst the vast majority of Gram-negative bacteria tested were not. This may be attributable to the cell wall differences between Gram-negative and Gram-positive bacteria. Kramer *et al* (1980, 1984a, b) and Aspendon and Nickerson (1993) have reported sodium dodecyl sulphate resistance in *Enterobacter cloacae*, the outer membrane being proposed as a barrier against the surfactant.

A parabolic relationship between MIC and chain length is observed for the homologous series of alcohol ethoxylates, as described by Gilbert (1985) and this is discussed in more detail in sections 3.1 and 3.5.

An important aspect of this study is to correlate the effect of surfactant concentration on activity. A sub-MIC of surfactant may produce a different response to that of a supra-MIC. This could yield information concerning the mode of action of the surfactants.

CHAPTER THREE

Some physical properties of an homologous series of alcohol ethoxylates

3.1 Introduction

The physicochemical properties of compounds have a marked influence on their biological activity (Devinsky *et al*, 1985). The critical micelle concentration of a compound has been shown to be correlated with antibacterial properties. Cella *et al* (1955) demonstrated that decreasing CMC values correlated with germicidal activity, whilst Lien & Perrin (1976) reported increased protein binding ability with decreasing CMC values.

Log P, a measure of the lipophilicity of a compound also displays a relationship with biological activity. Gilbert (1985) described a parabolic relationship when log P was plotted against biological activity for a homologous series of compounds. The optimum log P value for a Gram-negative cell has been determined as 4.0 and 6.0 for Gram-positive cells (Lien *et al*, 1968).

Micellar solutions of nonionic surfactants become cloudy at a defined temperature, classified as the cloud point and this value is dependent on the concentration of the surfactant and the solvent in use, as well as electrolyte present. This is due to the separation of two liquid phases as the temperature is raised (Clint, 1992). The cloud point temperature must therefore be taken into consideration for studies determining the effect of concentration on biological activity as above the cloud point temperature there is no effective increase in the amount of solute in solution.

3.2 Determination of Critical Micelle Concentrations (CMC)

Critical micelle concentrations for the homologous series of alcohol ethoxylates described in section 2.7 were determined by a dye solubilisation method (McBain & Green, 1946; Merrill &. Getty, 1948, Kolthoff & Stricks, 1948, 1949; Rigg & Liu, 1953; Anacker *et al*, 1977; Vulliez-Le Normand & Eisele, 1993). The method is based on the observation that solubilisation of a hydrophobic dye in a detergent solution only occurs if micelles are present. Orange OT was employed as the hydrophobic dye as it demonstrated improved reproducibility (±4.9%) compared with dimethylaminoazobenzene (±6.2%) for the CMC determination of sodium laurate (Ginn *et al*, 1959).

300µL aliquots of 0.1% w/v Orange OT in ethanol were pipetted into each well of a 48 well microtitre plate. The ethanol was allowed to evaporate leaving a film of dye on the bottom of the wells. 1.2mL aliquots of increasing concentrations of surfactant (0.002 - 10 mM) in both synthetic broth AOAC and McIlvaine's Buffer (pH 7.0) were then added to the wells and the plate agitated for one minute on an orbital shaker. The plate was then incubated for 18 hours at 37˚C. After this period of incubation the samples were transferred to a fresh plate for subsequent spectrophotometric analysis.

The samples were examined using a Perkin Elmer Lambda 2 spectrophotometer at 492 nm against a blank of either synthetic broth AOAC or McIlvaine's buffer (pH 7.0) previously incubated with the dye under the same conditions as the samples.

Data were plotted as surfactant concentration against absorbance (see

figures 3.1 to 3.8) giving a clear discontinuity in the relationship. Linear regression analysis was used to accurately determine the intersection point between the two lines of best fit; this inflection point was taken as the CMC of the surfactant. The resultant values are summarised in table 3.1.

Table 3.1 Critical Micelle Concentrations for the homologous series of alcohol ethoxylates in both McIlvaine's buffer and synthetic broth AOAC

ALCOHOL ETHOXYLATE	CMC mM BUFFER, 37°C	CMC mM BROTH, 37°C	*CMC mM WATER, 25°C
C10E6	0.653	0.908	0.9
C12E6	0.189	0.344	0.087
C14E6	0.17	0.258	0.01
C16E6	0.082	0.222	0.0017

* Determined by surface tension measurements (Mukerjee & Mysels, 1971)

The CMC values in broth and buffer can be compared with those measured in water, at 25°C (Mukerjee & Mysels, 1971). It can be seen from the values above that as the length of the alkyl chain increases the CMC decreases.

It was thought that the indistinct CMC values obtained for the surfactants in synthetic broth AOAC could be due to the glucose content of the broth. Therefore the CMC of C12E6 was obtained in McIlvaine's buffer and McIlvaine's buffer with the addition of 10 mM of glucose (the same concentration of glucose as was present in synthetic broth AOAC). Figure 3.9 illustrates that the addition of glucose gives a less distinct CMC by broadening the range of surfactant concentration at which dye solubilisation is first visualised.

Figure 3.1 CMC of C10E6 in McIlvaine's buffer pH 7.0 (n=6, mean±sd)

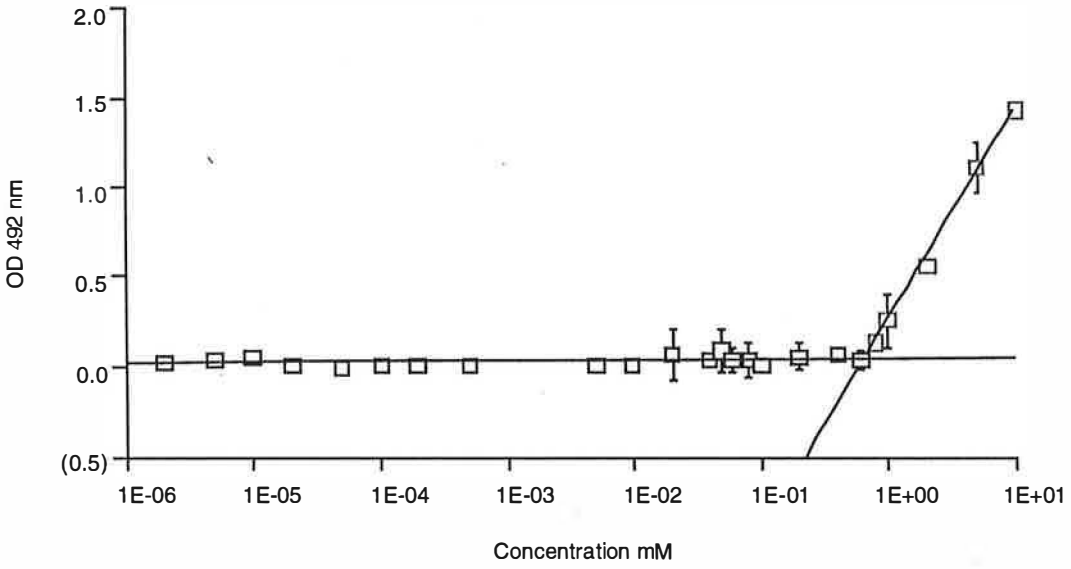


Figure 3.2 CMC of C10E6 in synthetic broth AOAC (n=6, mean±sd)

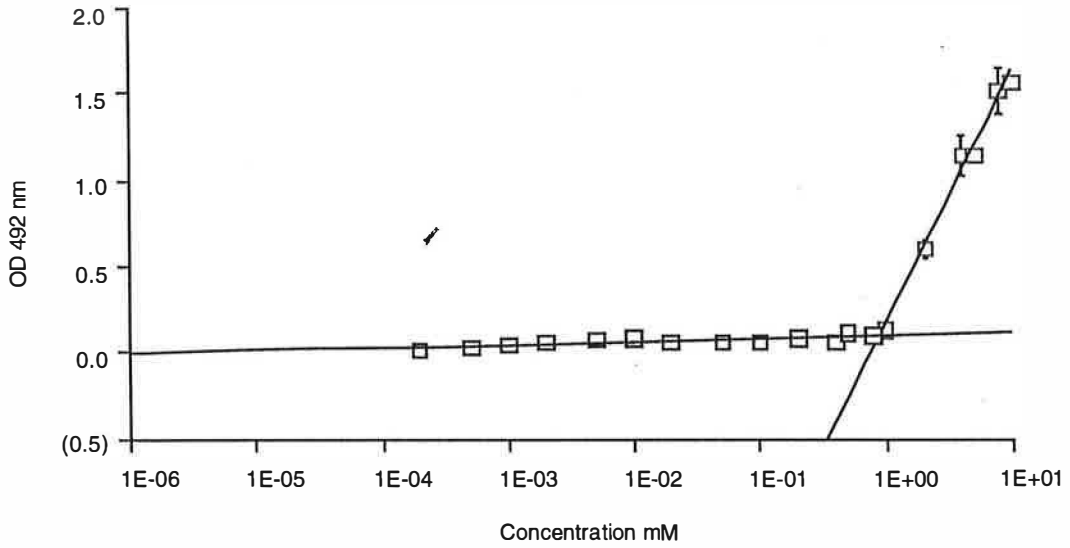


Figure 3.3 CMC of C12E6 in McIlvaine's buffer, pH 7.0 (n=6, mean±sd)

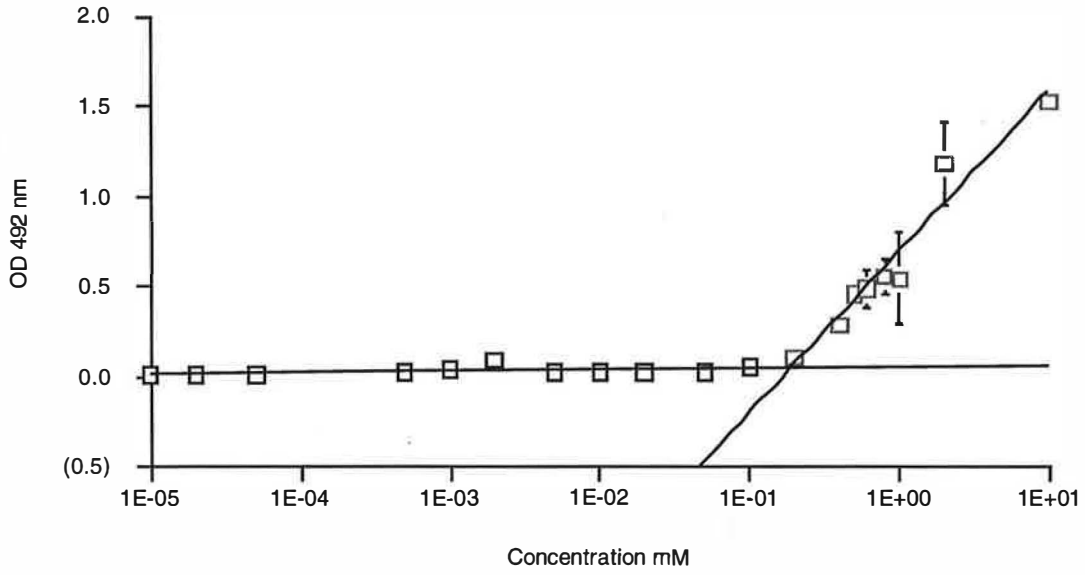


Figure 3.4 CMC of C12E6 in synthetic broth AOAC (n=6, mean±sd)

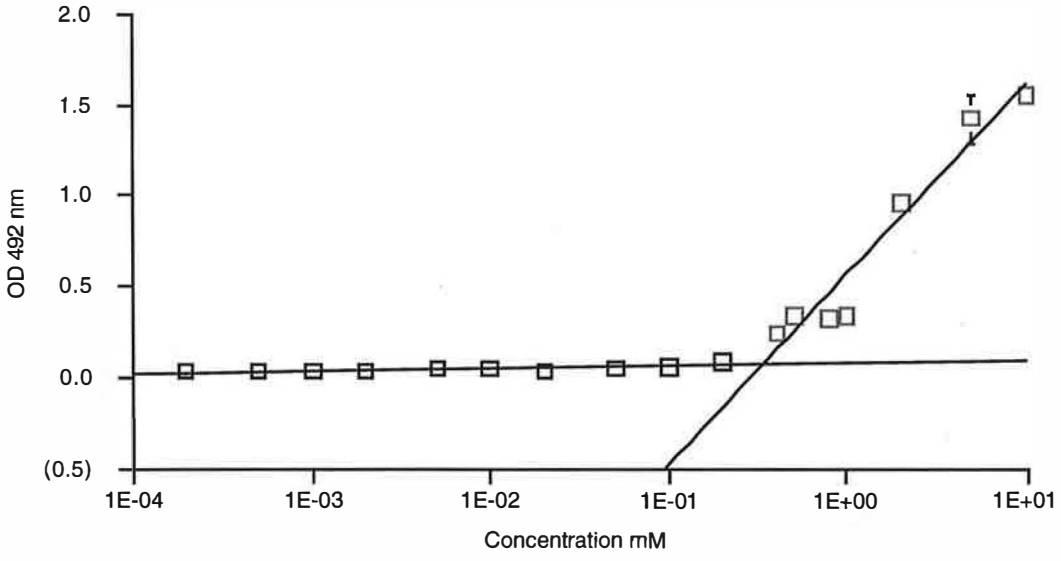


Figure 3.5 CMC of C14E6 in McIlvaine's buffer, pH 7.0 (n=6, mean±sd)

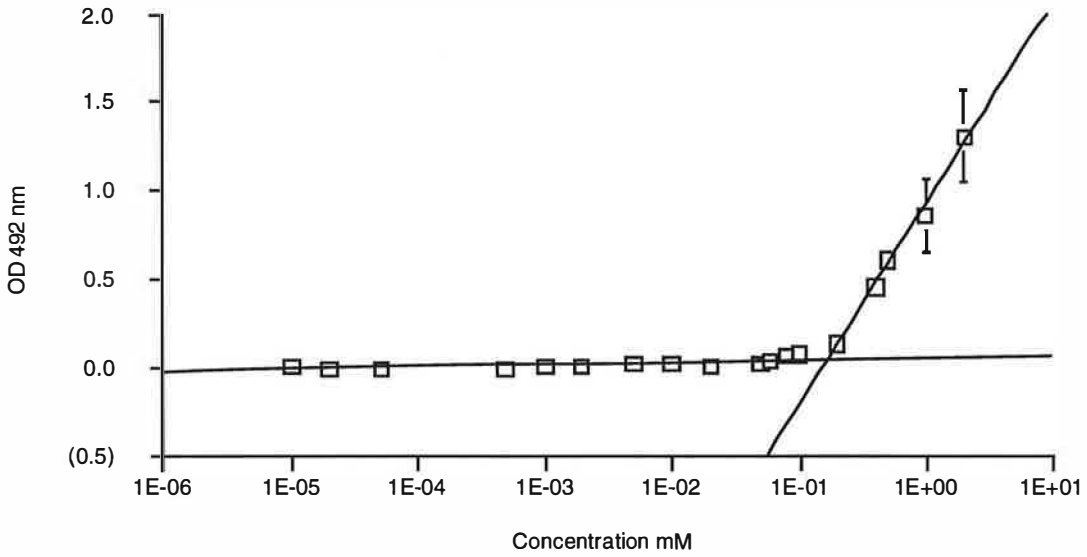


Figure 3.6 CMC of C14E6 in synthetic broth AOAC (n=6, mean±sd)

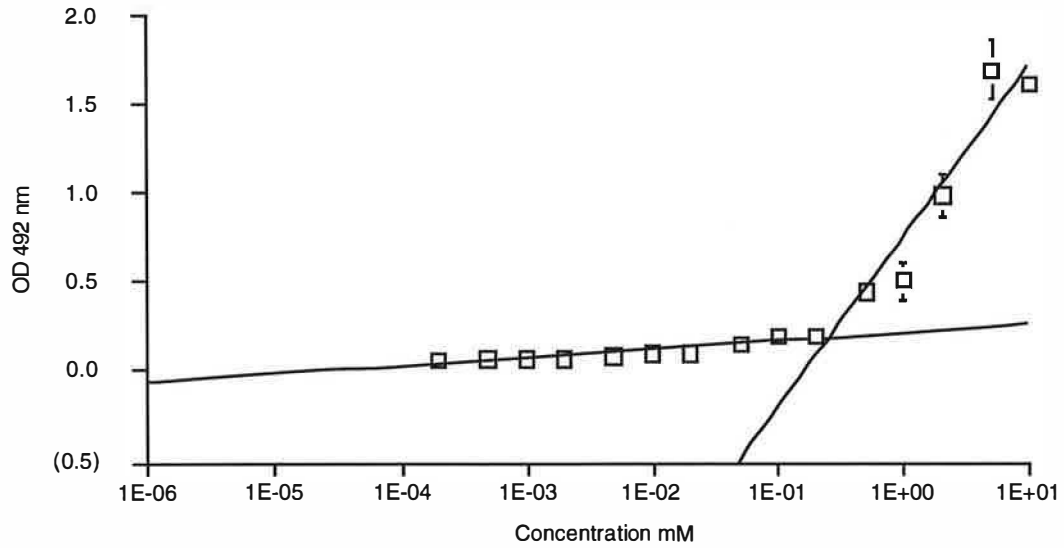


Figure 3.7 CMC of C16E6 in McIlvaine's buffer pH 7.0 (n=6, mean±sd)

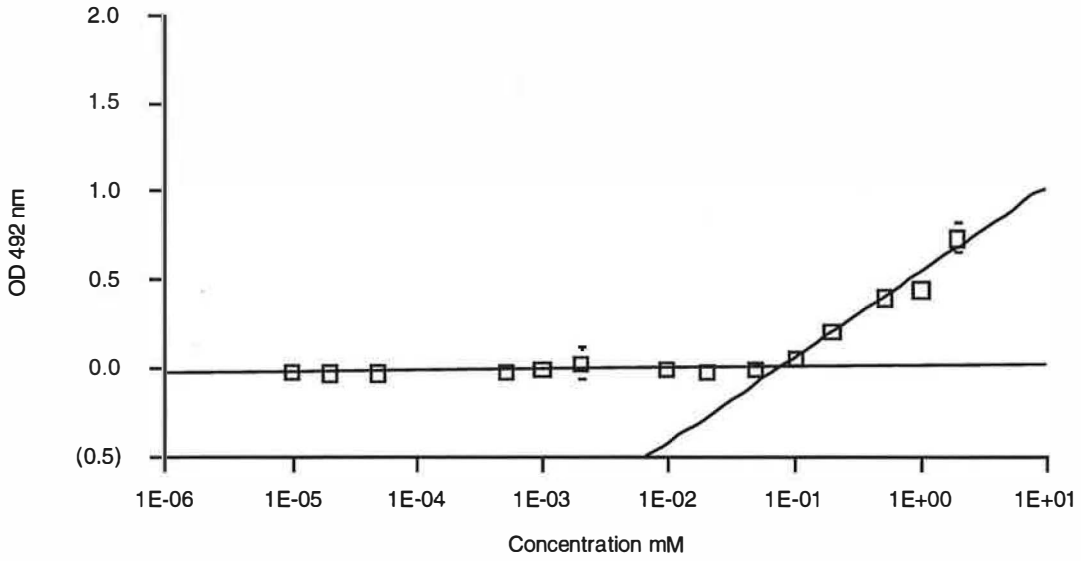


Figure 3.8 CMC of C16E6 in synthetic broth AOAC (n=6, mean±sd)

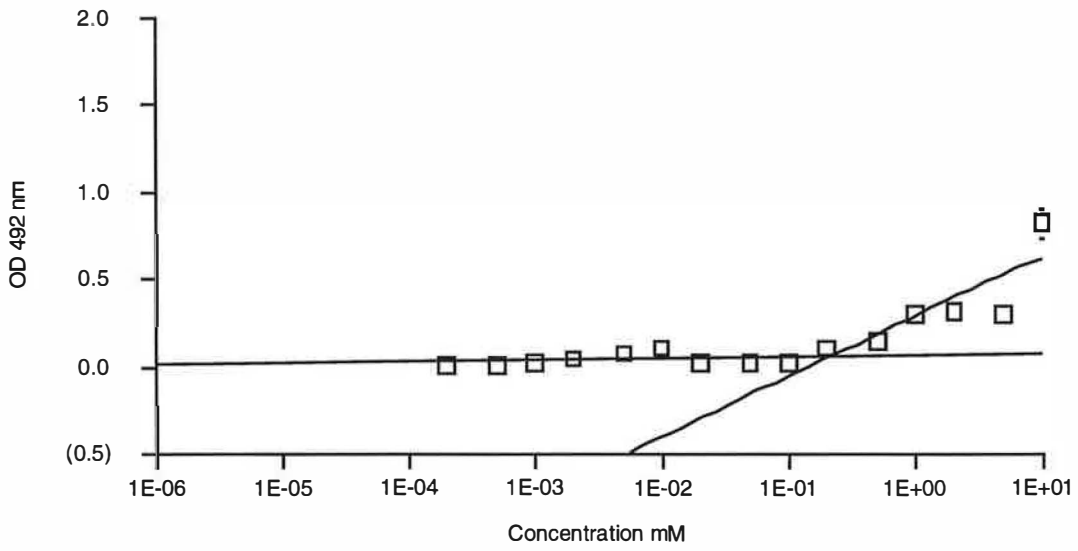
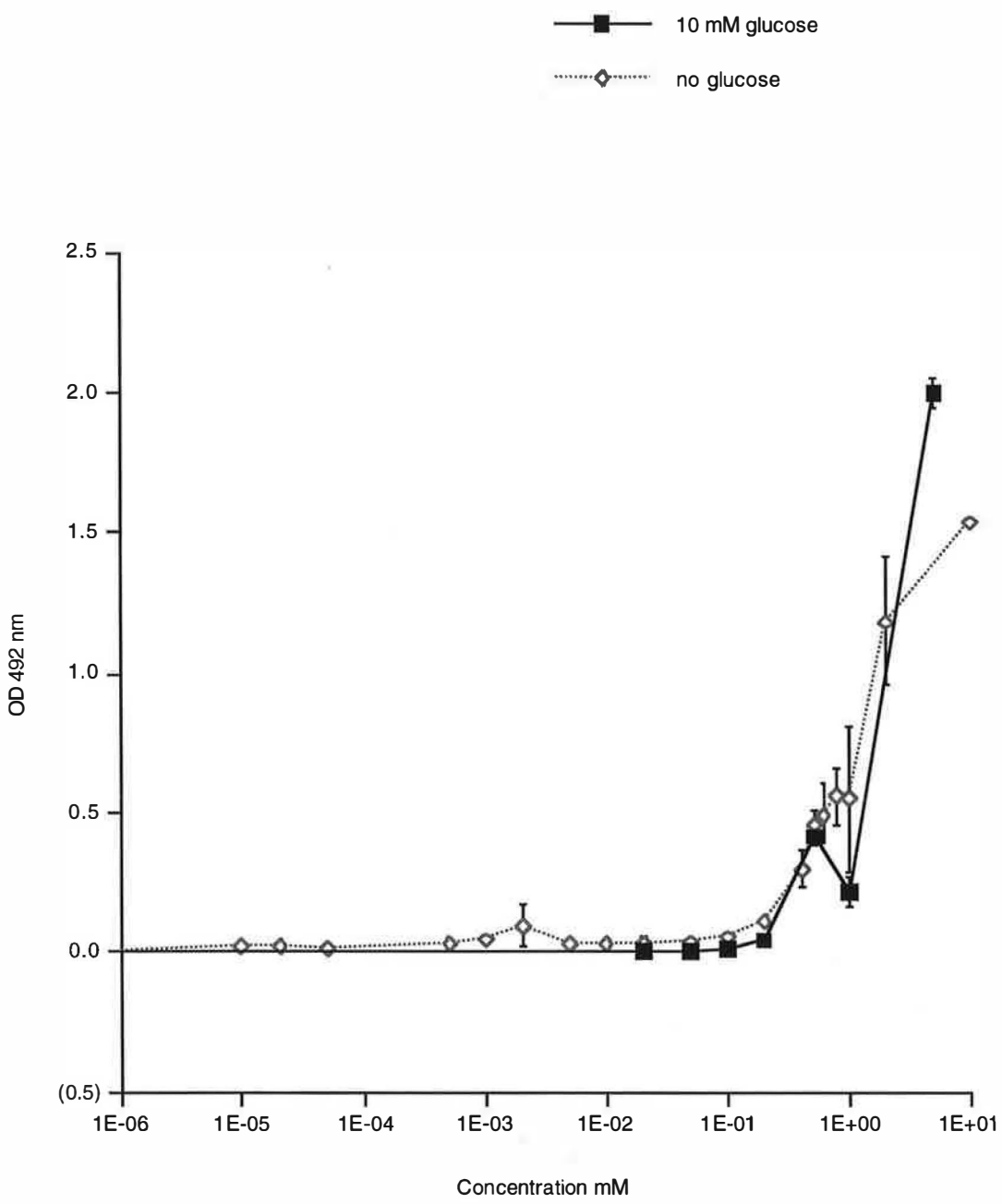


Figure 3.9 The effect of glucose on the CMC of C12E6 in McIlvaine's buffer pH 7.0
(n=6, mean±sd)



3.3 Log P determination of the surfactants

The log P values of the homologous series of surfactants were calculated using the method of Hansch and Leo (1979). These values are the log of P, where P is the octanol/water partition coefficient and therefore illustrate the relative hydrophilicity and lipophilicity of the surfactants.

Figure 3.10 demonstrates that log P and hence lipophilicity increase with increasing alkyl chain length whilst CMC decreases with an increase in the chain length.

3.4 Cloud point determinations of the alcohol ethoxylates

The cloud points for the alcohol ethoxylates were determined in McIlvaine's buffer at three concentrations: the CMC for the particular surfactant, half the CMC, and twice the CMC.

5 mL of each surfactant solution was placed in a test tube and the test tubes incubated in a water bath. The samples were then heated and the temperature at which the solutions became visibly cloudy was noted.

The cloud points and their relationship with CMC is presented graphically in figure 3.11. It can be seen from this figure that as chain length and concentration increase the cloud point decreases.

Figure 3.10 Relationship between Log P and CMC in McIlvaine's buffer

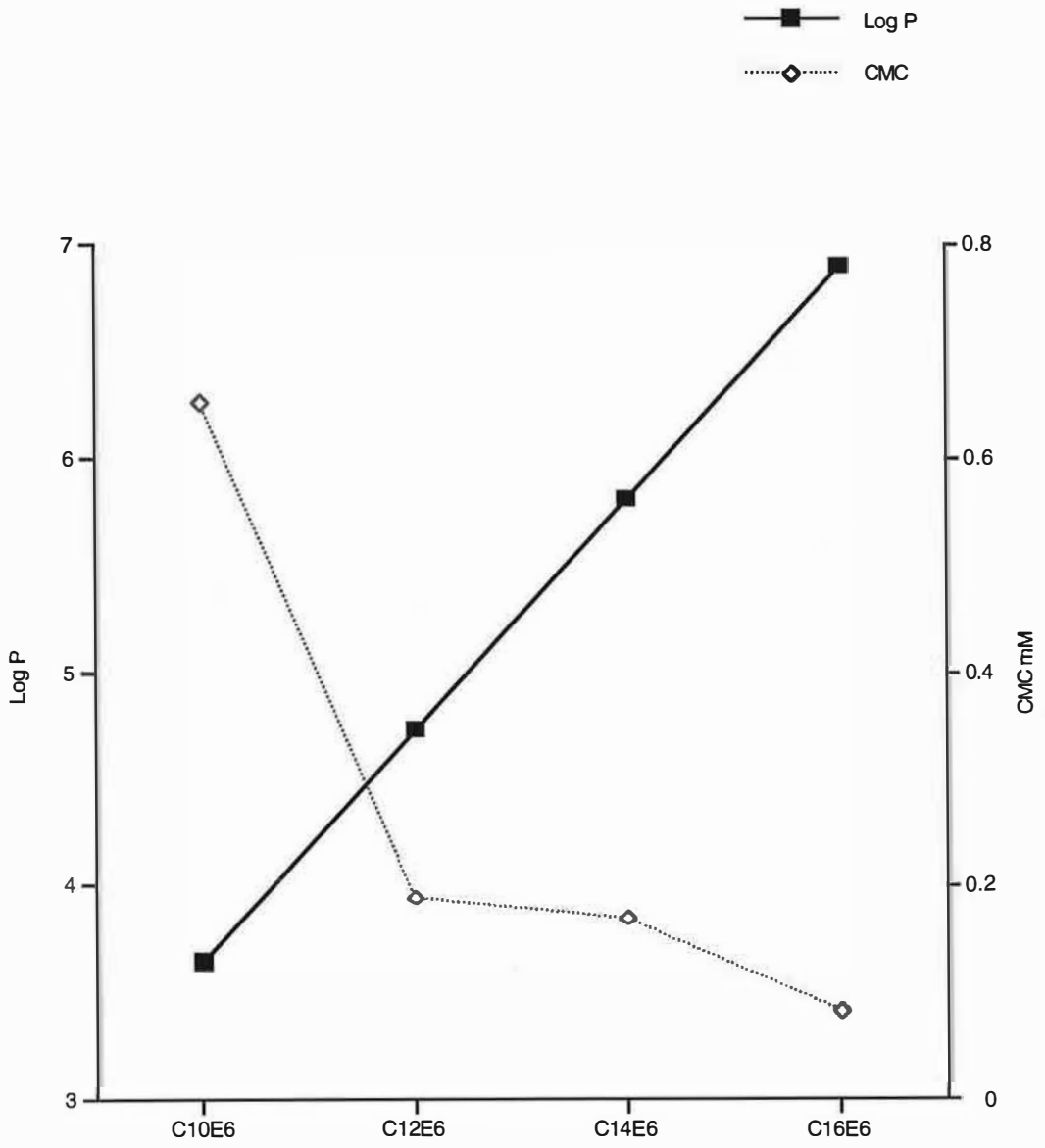
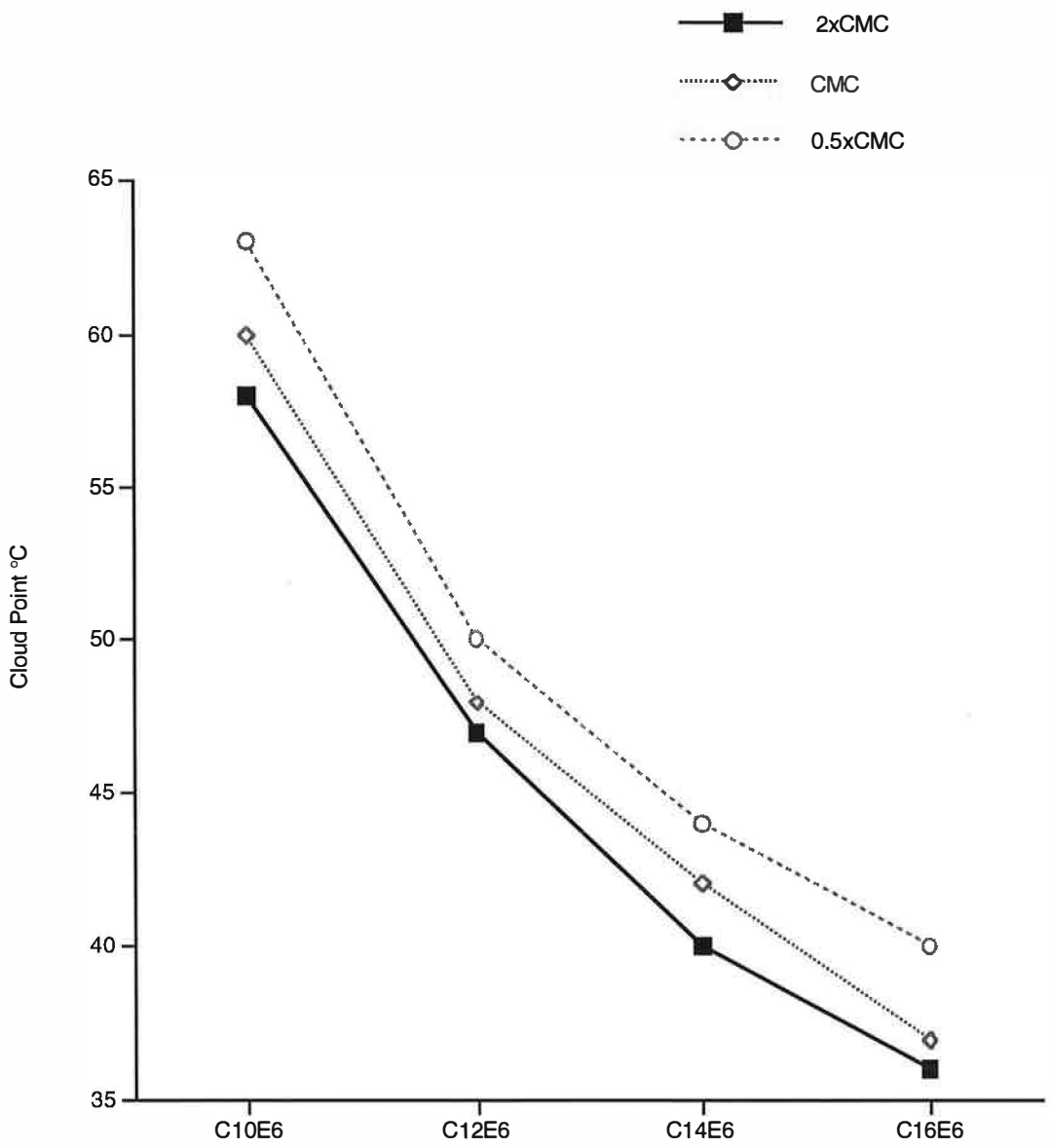


Figure 3.11 The effect of carbon chain length and concentration on cloud point for alcohol ethoxylates in McIlvaines buffer pH 7.0



3.5 Discussion

The CMC values obtained for the alcohol ethoxylates decreased as the alkyl chain length increased as has been reported previously (Elworthy *et al*, 1968). There is some correlation between the MIC and CMC of the homologous series, the MIC is inversely proportional to the CMC value obtained with the exception of C12E6 which possesses a lower MIC than that for C10E6. With the exception of C12E6 these results are in broad agreement with those obtained by Lien and Perrin (1976) who demonstrated that as CMC decreased for a homologous series the germicidal activity increased.

Using MIC as a measure of biological activity it can be seen that C10E6 and C12E6 have a much higher biological activity than C14E6 and C16E6. These data plotted as 1/MIC against log P approximated to a parabola (Gilbert 1985). However, values for C8E6 would be needed to confirm this. The increased biological activity of C10E6 and C12E6 may be due to the fact that their log P values (3.65 and 4.73 respectively) are close to the optimum value of 4.0 for Gram-negative bacterium (Lien *et al*, 1968).

The cloud point has an important influence on further studies. Above the cloud point temperature there is no effective increase in the amount of solute in solution. As can be seen from the experimental data (figure 3.11) both C14E6 and C16E6 are approaching their cloud point temperature at 37˚C (the temperature used for the previous experiments and to be used for all subsequent experiments), depending on the concentration of surfactant used. Therefore the cloud point must be taken into account when looking at concentration-dependent effects as an increase in the concentration of a

surfactant solution may not result in an actual increase in concentration, depending on the alcohol ethoxylate and the concentration at which it will be used.

CHAPTER FOUR

The effect of alcohol ethoxylates on the cellular metabolism of *Escherichia coli* NCTC 8196

4.1 Oxygen Uptake Studies

The cytoplasmic membrane contains the various components of the respiratory chain including cytochromes, flavins and ubiquinones, several enzymes providing reducing equivalents to it, for example succinate, malate and NADH dehydrogenase, in addition to the associated ATPase on the inner surface of the membrane. In this respect the bacterial cytoplasmic membrane is analogous to the inner membrane of mitochondria.

Bacteria, however, display a great deal of variation in their electron transport chains. They tend to have more than one oxidase, several dehydrogenases can feed electrons into the chain and two or three dehydrogenases which are able to reduce molecular oxygen to produce water. One of these oxidases may predominate depending on growth conditions. This has been demonstrated with *E. coli* in conditions of high aeration where the cytochrome *o* terminal oxidase complex predominates whilst in low oxygen tension the cytochrome *d* complex is predominant (Anraku & Gennis, 1987).

To examine the nature of respiratory activity in *E. coli* and the potential sensitivity of such processes to surfactants a Clark oxygen electrode was used to determine the effect of C10E6 on oxygen uptake by *E. coli* NCTC 8196 *in situ*.

4.1.1 Calibration of the Clark oxygen electrode

McIlvaine's buffer (pH 7.0) and McIlvaine's buffer containing glucose (0.02 M) were allowed to equilibrate at 37˚C and then vigorously shaken to aerate the solutions. Samples were then added to the cell compartment of the

Clark oxygen electrode, the chart recorder was allowed to stabilise and then set to 100%. Once a stable 100% reading was obtained, 0.1g sodium sulphite was added to the solution in the cell compartment to effect the complete removal of dissolved oxygen and the chart recorder was set to 0%. This process was repeated until the 100% and 0% values were obtained, without further adjustment.

4.1.2 The rate of oxygen uptake by *E. coli* NCTC 8196

Cultures of *E. coli* NCTC 8196 were grown for 15 hours at 37˚C on Roux slopes prepared from synthetic broth AOAC (Difco Laboratories) solidified with 1.5%w/v agar technical (Oxoid). Bacterial cells were harvested by washing the surface of the slope with 40mL McIlvaine's buffer. The cell suspension was centrifuged at 2,100g for 20 minutes and then washed twice with 25mL McIlvaine's buffer.

McIlvaine's buffer, McIlvaine's buffer containing glucose (to give a final concentration in the electrode of 0.02 M) and the washed bacterial suspension were incubated separately at 37˚C to equilibrate to temperature. To determine the most appropriate cell concentration for use in subsequent experiments the oxygen uptake for $1x10^7$, $1x10^8$ and $1x10^9$ cells/mL was determined.

The Clark oxygen electrode was calibrated as described in section 4.1.1. Bacterial cells were suspended in McIlvaine's buffer at final concentrations of $1x10^7$, $1x10^8$ and $1x10^9$ cells/mL and samples were added to the cell compartment of the oxygen electrode. Glucose in McIlvaine's buffer was added to the cell compartment to give a final concentration of 0.02M and the

rate of oxygen uptake followed with time via the chart recorder. $1x10^8$ cells/mL produced an oxygen uptake trace in 10 minutes (see figure 4.1a) and was therefore used in the following experiments.

4.1.3 The rate of oxygen uptake by *E. coli* NCTC 8196 in the presence of C10E6

Cultures of *E. coli* NCTC 8196 were grown and harvested as described in section 4.1.2.

The Clark oxygen electrode was calibrated as described in section 4.1.1. McIlvaine's buffer, C10E6 in McIlvaine's buffer, glucose in McIlvaine's buffer and the washed bacterial suspension were incubated at 37°C to equilibrate to temperature.

McIlvaine's buffer containing C10E6 at final concentrations of either 0.05, 0.2 or 0.3mM and bacteria to a final concentration of $1x10^8$ cells/mL were added to the cell compartment of the oxygen electrode. Glucose in McIlvaine's buffer was then added to the cell compartment (to give a final concentration of 0.02M) and the rate of oxygen uptake followed with time via a trace on the chart recorder. A typical oxygen uptake trace by *E. coli* NCTC 8196 in the presence of 0.3mM C10E6 can be seen in figure 4.1b.

Two controls containing McIlvaine's buffer and glucose were run at the start of a set of experiments to ensure the rate of oxygen uptake was constant; controls were then run periodically throughout the course of the experiment to ensure this rate did not vary. Four replicates were carried out for each concentration of C10E6. The rate of oxygen uptake was determined from

Figure 4.1a A typical oxygen uptake trace for *E.coli* NCTC 8196

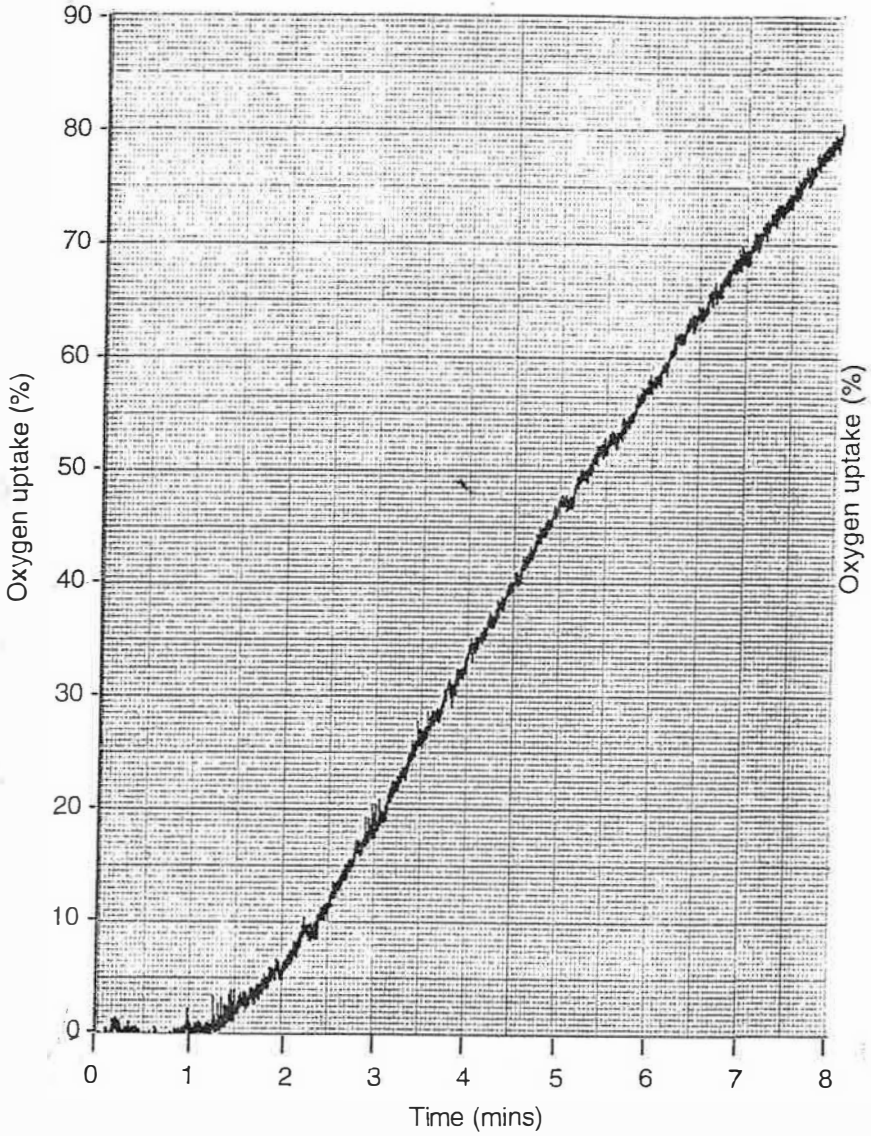
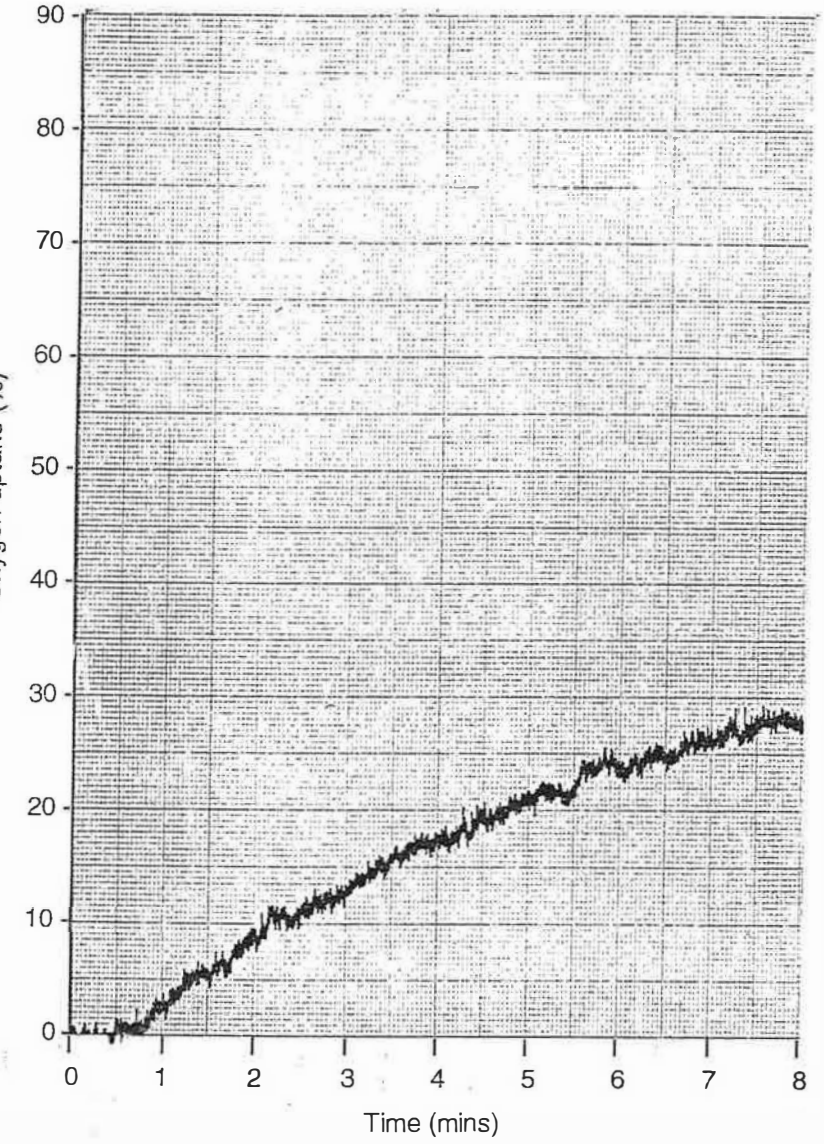


Figure 4.1b A typical oxygen uptake trace for *E.coli* NCTC 8196 in the presence of 0.3mM C10E6



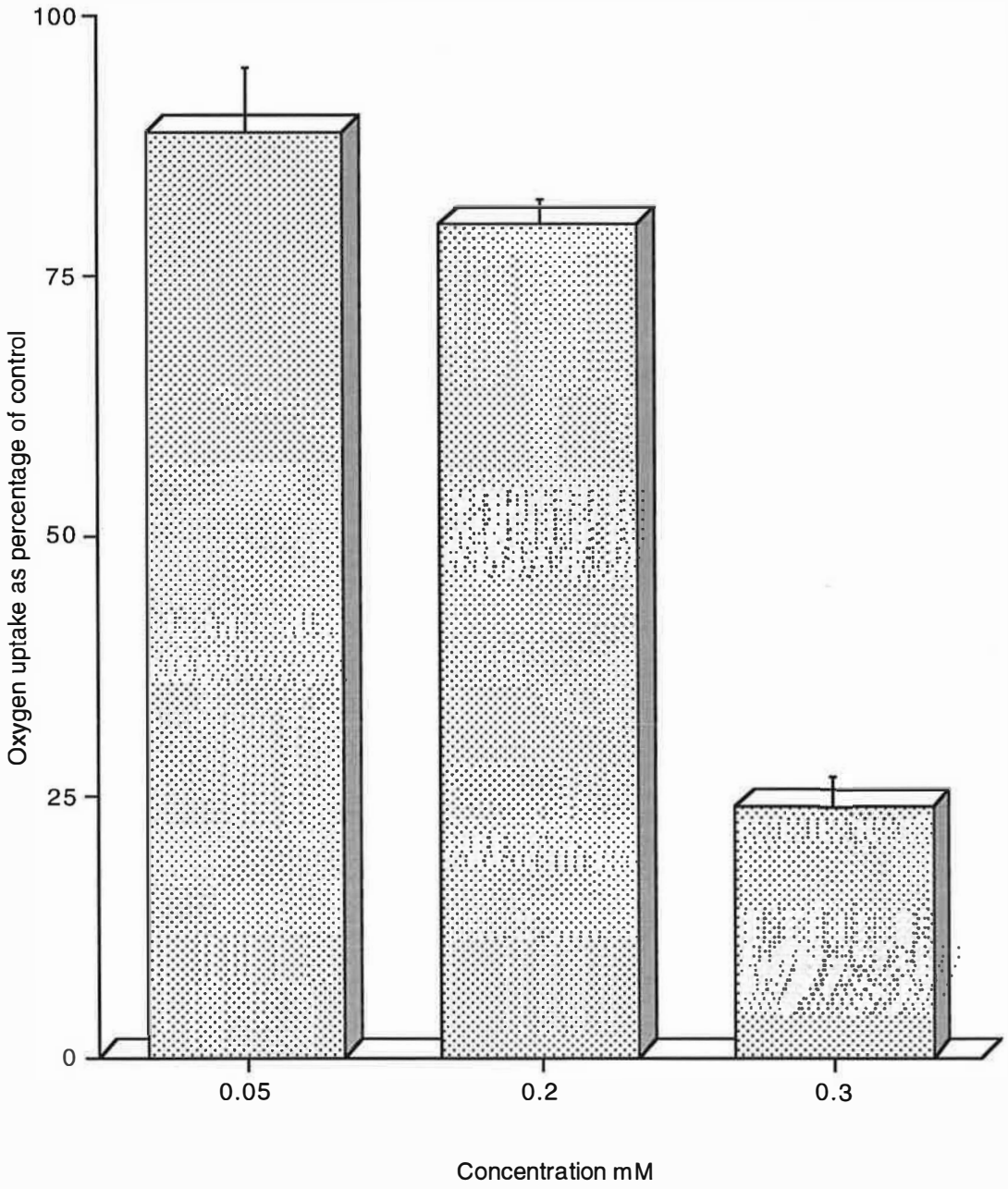
the chart recorder traces, the mean of these rates were expressed as a percentage of the control rate and can be seen in figure 4.2.

4.1.4 Results

The rate of oxygen uptake was reduced in the presence of C10E6 in a concentration-dependent manner. At 0.05mM the rate of oxygen uptake was reduced to approximately 90% of the control; however, this result is not significant (Mann Whitney, p=0.205). At 0.2mM (the MIC), the rate of oxygen uptake was further reduced compared to that of the control (Mann Whitney, p=0.016) to about 80%. Above the MIC at 0.3mM the rate of oxygen uptake was dramatically reduced (Mann Whitney, p=0.007) to approximately one quarter of that of the control.

The Clark oxygen electrode consists of an almost sealed chamber and as such *E. coli* NCTC 8196 is present in increasingly anoxic conditions. It is likely therefore that the cytochrome *d* complex is predominant under these conditions (Anraku & Gennis, 1987). The effects on oxygen uptake in the presence of C10E6 will be influenced by the activity of the respiratory chain and monitoring dehydrogenase activity offers the opportunity to probe the effect on the respiratory chain more precisely.

Figure 4.2 The effect of increasing concentrations of C10E6 on oxygen uptake by *E. coli* NCTC 8196 (n=4, mean±sd)



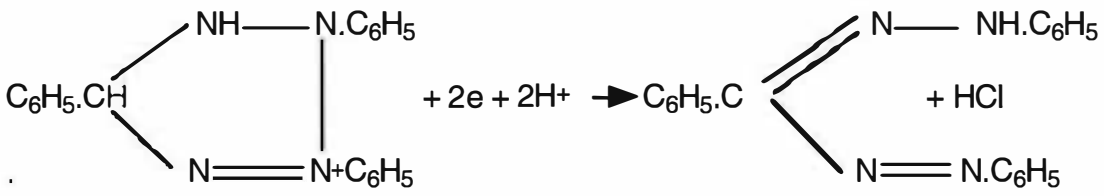
4.2 The use of 2,3,5-triphenyltetrazolium chloride as an indicator of dehydrogenase activity

Damage to the cytoplasmic membrane can be probed by investigating the activity of membrane-bound dehydrogenases using tetrazolium salts. Tetrazolium salts are often used as quantitative markers of oxidative enzyme activity (Altman, 1970). Succinate dehydrogenase has been shown to be an integral part of the cell surface of *E. coli*, the activity of this enzyme has been shown to be almost completely inhibited at concentrations which are germicidal to the suspension of bacteria under test (Harding, 1984). 2,3,5-triphenyl-tetrazolium bromide has been used to determine the activity of succinate, glucose, manntiol, glycerol and lactate dehydrogenase activity in *E. coli* (Hugo, 1954, Gilbert *et al*, 1977b) demonstrating the range of dehydrogenase enzymes that can be investigated using this method.

The results from the Clark oxygen electrode experiments demonstrated that the surfactant C10E6 inhibited the respiratory activity of *E. coli*. Techniques employing either the Clark oxygen electrode or the tetrazolium salts are comparable in that oxygen is limited in both systems (the Clark oxygen electrode containing an almost sealed chamber while the tetrazolium salt experiments are conducted under static conditions). The oxygen uptake experiments therefore demonstrate that the respiratory chain is fully operational in the presence of 0.02 M glucose and increasingly anoxic conditions.

Tetrazolium salts undergo a colour change on mild reduction and have been established as quantitative markers of oxidative enzyme activity as discussed in a review by Altman (1972). 2,3,5-triphenyltetrazolium chloride

(TTC) is such a compound with a redox potential of +490 mV. When reduced the corresponding formazan, which is red in colour, is produced:



triphenyltetrazolium chloride (colourless) triphenylformazan (red)

4.2.1 Development of experimental conditions

4.2.1.1 Determination of appropriate wavelength at which to measure formazan

In daylight the extracted formazan in ethyl acetate is converted from red in colour to yellow, the coloured extracts representing two forms of formazan (Nineham, 1955). Subsequent UV/visible scanning of extracts of these two coloured products revealed a different λ_{max} for each extract (figures 4.3 and 4.4). The presence of two isobestic points, one at 345 nm (UV region) and the other at 450 nm (visible region), were seen after scanning red, yellow and intermediate formazan extracts in ethyl acetate (figure 4.5). 450 nm was chosen as the wavelength at which to measure the extracted formazan as λ_{max} was greater than at 345 nm and is in the visible region thus removing the likelihood of interference from the absorbance spectra of other species present. Therefore all extracted formazan samples were assayed

spectrophotometrically at 450 nm to eliminate any errors which may have occurred due to exposure to light. The stability of the extracted triphenylformazan over time at this wavelength can been seen in figure 4.6 demonstrating that the extract was stable for at least120 minutes.

4.2.1.2 The effect of aeration on formazan production

It has been reported that the degree of aeration of *E. coli* cultures has a marked effect on the amount of red triphenylformazan produced, oxygen being a competitive antagonist of the reaction (Jones & Prasad, 1964; Altman, 1970; Hurwitz & McCarthy,1986). Experiments were, therefore, designed to investigate the effect of aeration on the production of formazan under the conditions employed.

Cultures of *E. coli* NCTC 8196 were grown for 15 hours at 37˚C on a Roux slope composed of synthetic broth AOAC (Difco Laboratories) solidified with 1.5%w/v agar technical (Oxoid). The bacteria were harvested by washing the surface of the slope with 40mL McIlvaine's buffer (pH 7.0). The cell suspension was centrifuged at 2,100g for 20 minutes and then washed twice with 25mL McIlvaine's buffer. The washed cells were standardised to give a cell concentration of 1x 10^9 cells/mL.

Two 20mL bottles were prepared each containing: 2mL 0.02M glucose in McIlvaine's buffer, 1mL 0.05% 2, 3, 5-triphenyltetrazolium chloride in McIlvaine's buffer, and 5mL McIlvaine's buffer. The bottles were incubated at 37˚C prior to the start of the experiment, one shaking and the other static, to enable the temperature of the solutions to equilibrate. Each container

Figure 4.3 Red solution of formazan extracted in ethyl acetate

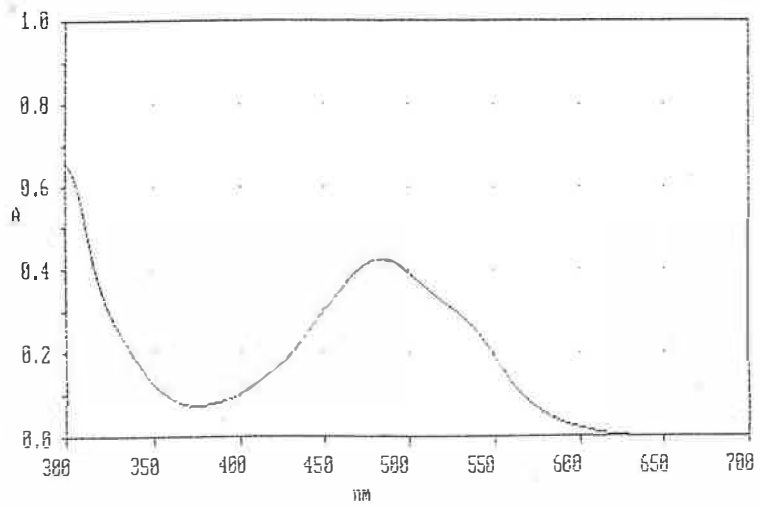


Figure 4.4 Yellow solution of formazan extracted in ethyl acetate

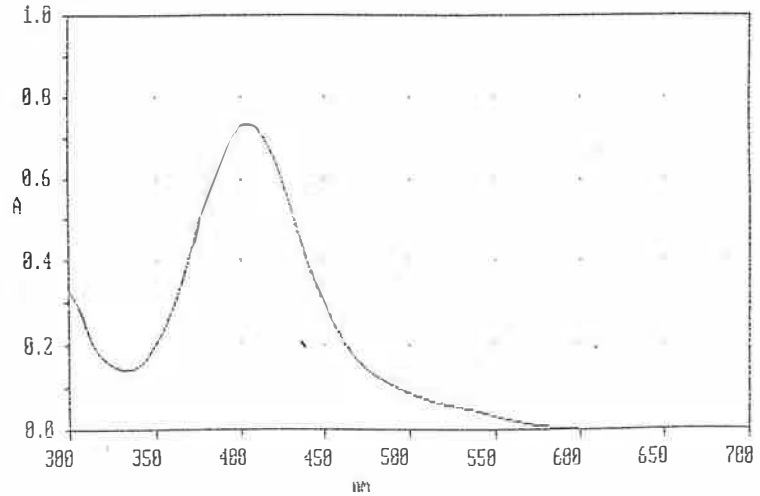
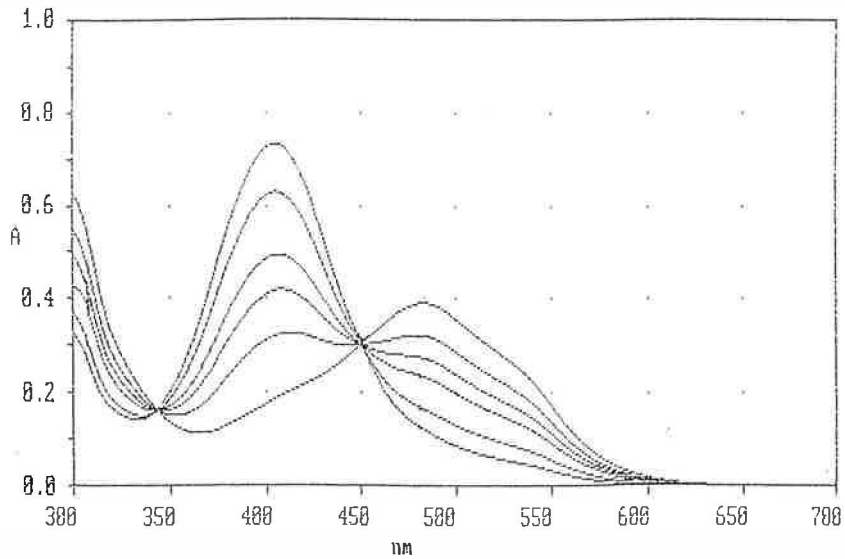


Figure 4.5 Red, yellow and intermediate solutions of formazan extracted in ethyl acetate



was covered with silver foil since TTC is light sensitive. The experiment was initiated by the addition of 1mL $1X10^{10}$ cells/mL (to give a final concentration of $1X10^{9}$ cells/mL), inverting the bottle to ensure even distribution of the bacteria. At 10 minute intervals over a period of 50 minutes a 1.25ml sample was removed and placed in a centrifuge tube containing 1.25mL ethyl acetate and 0.25mL 1M NaOH. The tube was stoppered and then vortexed to extract the formazan into the ethyl acetate: this process was checked visually. The samples were then centrifuged for 5 mins at 2,100g (Sorvall bench top centrifuge) to reduce the emulsion formed during the extraction procedure. The organic layer was removed for subsequent spectrophotometric analysis using a Perkin Elmer Lambda 2 spectrophotometer. The samples were measured against an ethyl acetate extract of bacteria, glucose and McIlvaine's buffer using the same method as for the samples. The results from this experiment can be seen in figure 4.7. The graph demonstrates that TTC was only reduced to produce the corresponding formazan when the sample was incubated statically. This result is in agreement with the results of Jones & Prasad (1964), Altman (1970) and Hurwitz & McCarthy (1986).

The effect of bottle size and formazan production was also determined, the bottle size determines the amount of oxygen available to the culture of *E. coli* during the course of the reaction.

A series of bijou bottles were set up containing:

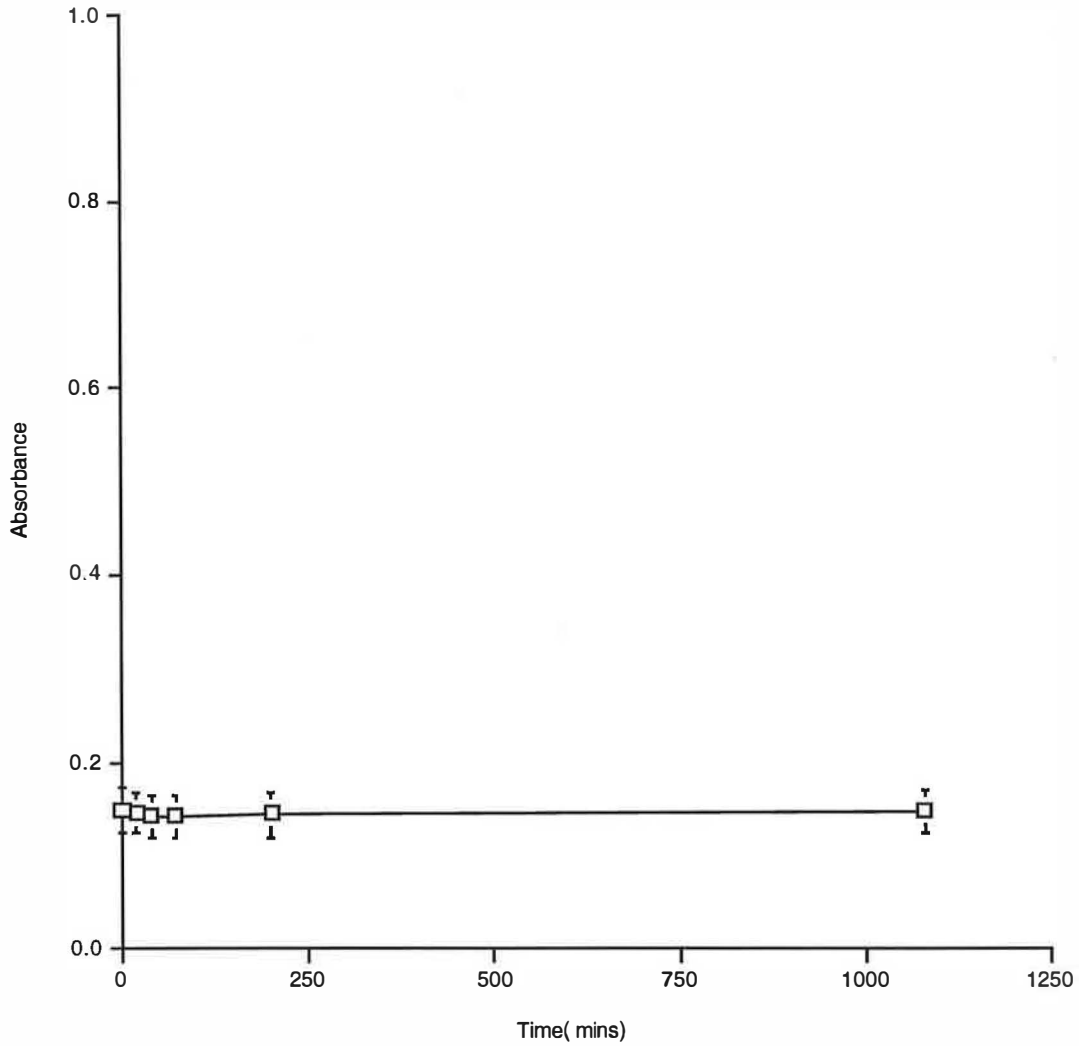
1mL 0.02M glucose in McIlvaine's buffer

1mL 0.05% TTC in McIlvaine's buffer

2.5mL McIlvaine's buffer

Each bottle represented a single time point for the experiment, samples were incubated at 37˚C, statically and shaking over a 50 minute period. The

Figure 4.6 The stability of formazan extracted in ethyl acetate with time (n=6, mean±sd)



experiment was started by the addition of 0.5mL 1X10^{10} cells/mL (to give a final concentration of 1X10^{9} cells/mL), inverting the bottle to ensure even distribution of the bacteria. At 10 minute intervals the contents of the bijou bottles were placed in a centrifuge tube containing 5mL ethyl acetate and 1mL 1M NaOH , stoppered and vortexed to extract the formazan into the ethyl acetate and examined spectroscopically as described above. The results of this experiment can be seen in figure 4.8.

As can be seen from the graph, reducing the air space in the reaction vessel allowed some reduction of TTC to formazan under shaking conditions; however, formazan production was not at the levels seen when the reaction mixture was incubated statically.

From the results of both these experiments it was decided to carry out the experiment with 5mL of reaction solution in a 5mL bijou bottle and incubate statically to eliminate the effect of aeration on formazan production.

4.2.1.3 The effect of cell concentration on formazan production

The effect of cell concentration on triphenylformazan production was also determined. The experiment was set up as described above except that the final cell concentrations in the experiment ranged from 1x10^{7} to 1x10^{9} cells/mL and the samples were incubated for 50 minutes before extraction. The results from this experiment can be seen in figure 4.9 indicating high concentrations of cells are required to obtain sufficient formazan over the timescale employed in the course of the experiment. Therefore a cell concentration of 1x10^{9} cells/mL was employed in future experiments.

Figure 4.7 Effect of aeration on formazan production by *E. coli* NCTC 8196 (10mL volume)

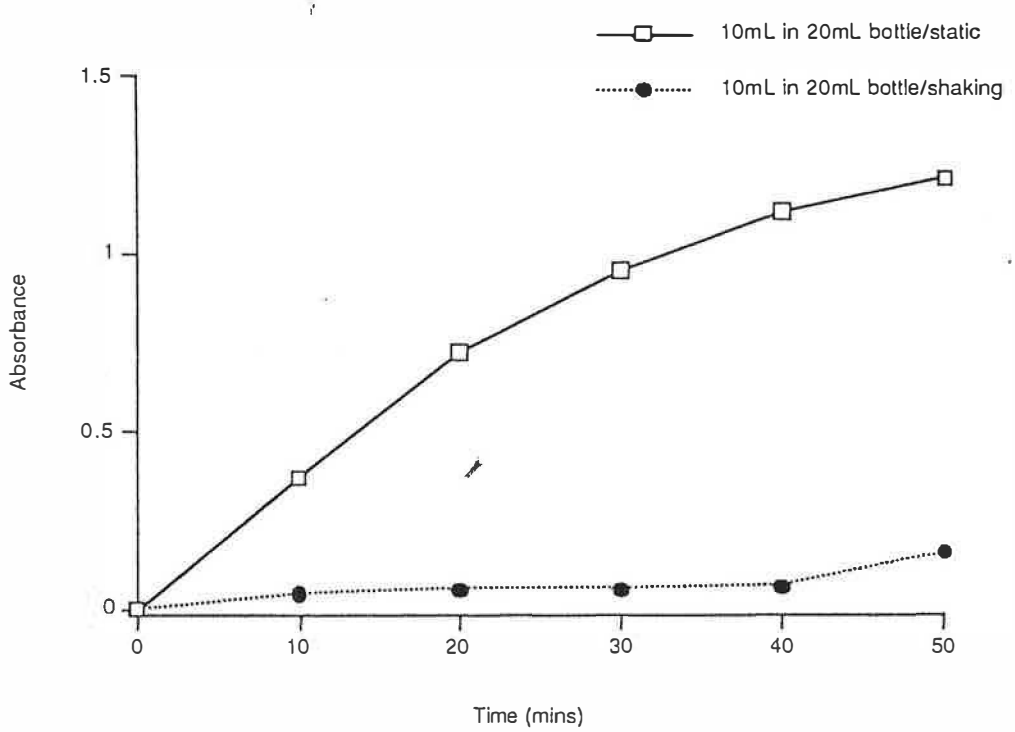


Figure 4.8 Effect of aeration on formazan production by *E. coli* NCTC 8196 (5mL volume)

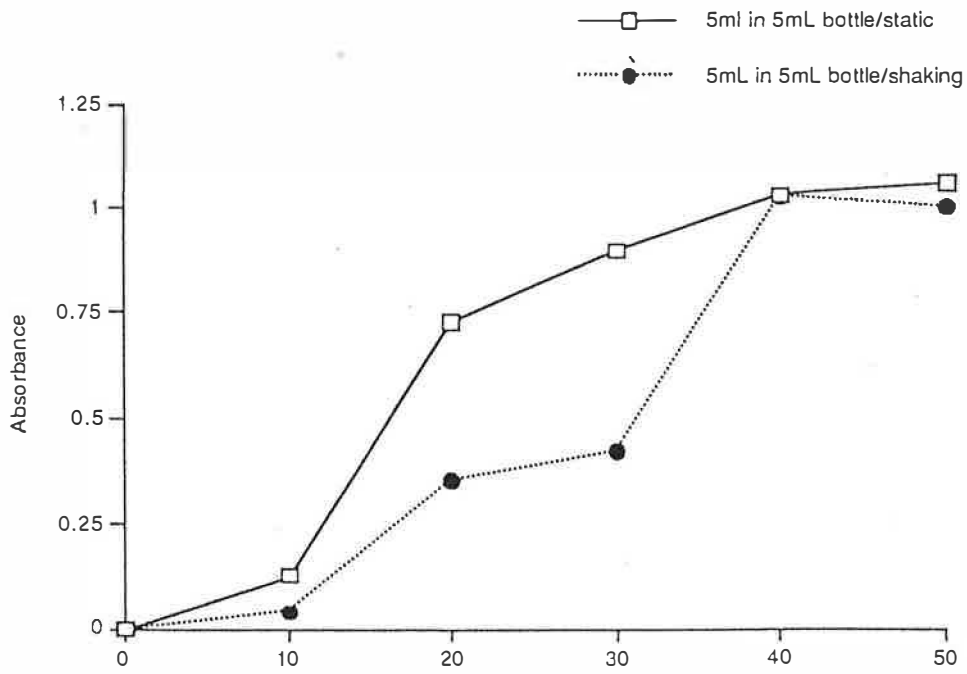
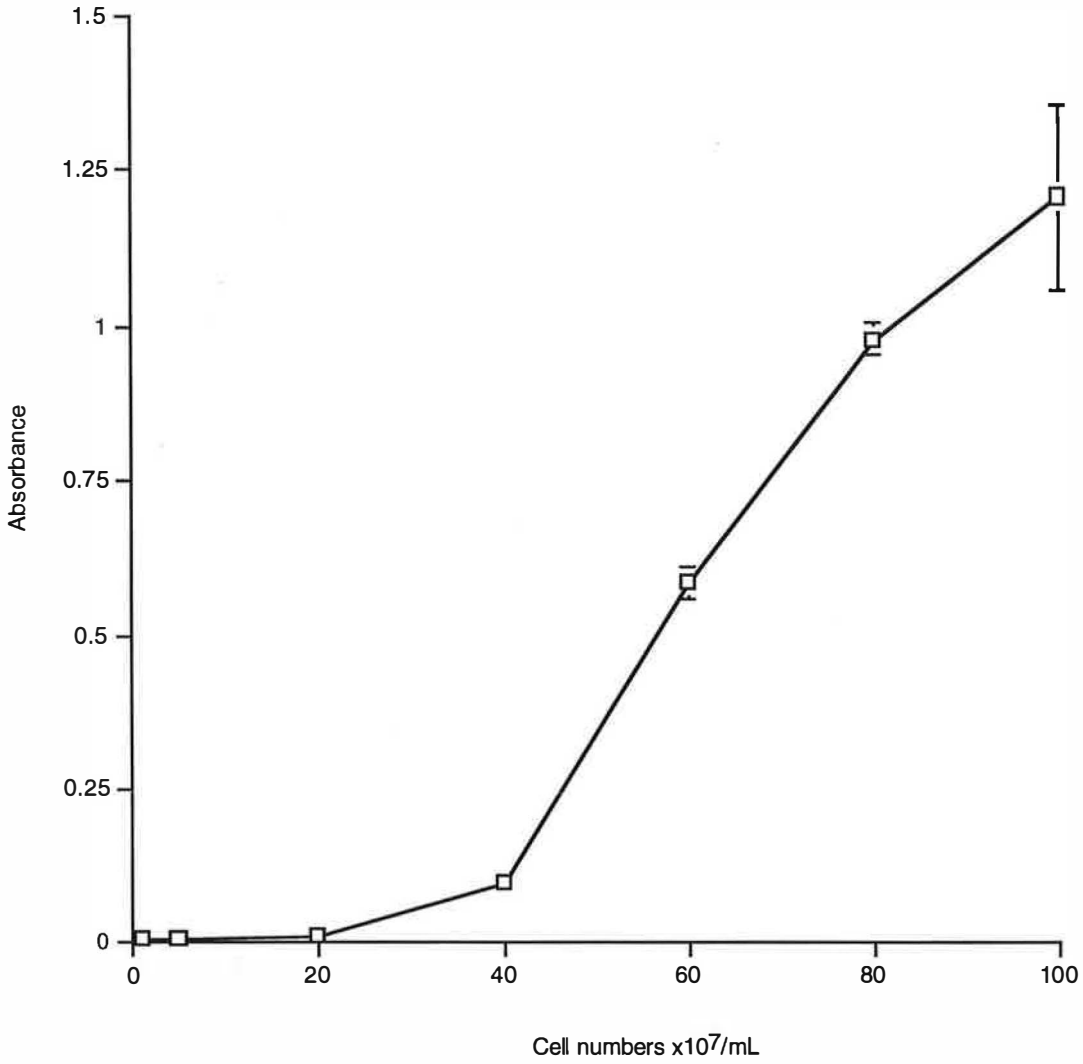


Figure 4.9 The effect of cell concentration of *E. coli* NCTC 8196 on formazan production after 50 minutes incubation (n=6, mean±sd)



4.2.2 The effect of alcohol ethoxylates on formazan production

The purpose of this study was to ascertain the effect of the homologous series of alcohol ethoxylates on the dehydrogenase activity of *E. coli* NCTC 8196. Two different substrates were used:- glucose and succinate. The reason for this was to provide information about the possible site(s) of damage in the respiratory chain as glucose and succinate enter the respiratory chain at different points.

4.2.2.1 The effect of alcohol ethoxylates on formazan production using glucose as substrate

Cultures of *E. coli* NCTC 8196 were grown and harvested as described in section 4.2.1.

A series of bijou bottles were prepared containing:

1mL 0.02M glucose in McIlvaine's buffer

1mL 0.05% 2, 3, 5-triphenyltetrazolium chloride in McIlvaine's buffer

2.5mL alcohol ethoxylates giving final concentrations from 0.005 to 5mM, in McIlvaine's buffer.

Each bottle represented a single time point for the experiment. Samples were incubated at 37°C, statically over a 60 minute period. The bottles were incubated at 37°C prior to the start of the experiment to enable the temperature of the solutions to equilibrate and were covered to prevent contact with light. The experiment was started by the addition of 0.5mL $1x10^{10}$ cells/mL (to give a final concentration of $1x10^{9}$ cells/mL), inverting the bottle to ensure even distribution of the bacteria. Control samples were prepared with the surfactant/buffer solution replaced by buffer.

At appropriate time intervals the contents of the bijou bottles were placed in a centrifuge tube containing 5mL ethyl acetate and 1mL 1M NaOH , stoppered and vortexed to extract the formazan into the ethyl acetate, as described previously (section 4.2.1.1). The samples of extracted formazan in ethyl acetate were then measured at 450 nm with a Perkin Elmer Lambda 2 spectrophotometer against a blank prepared as described in Section 4.2.1.1.

The results from these experiments can be seen in figures 4.10, 4.12, 4.14 and 4.16.

4.2.2.2 The effect of alcohol ethoxylates on formazan production using succinate as substrate

Cultures of *E. coli* NCTC 8196 were grown and harvested as described in Section 4.2.1.1

A series of bijou bottles were set up as described in section 4.2.2.1 except that glucose was replaced with sodium succinate at the same concentration.

The experiment was set up and carried out as described in Section 4.2.2.1.

The results from these experiments can be seen in figures 4.11, 4.13, 4.15 and 4.17.

Figure 4.10 Formazan production by*E.coli* NCTC 8196 with glucose substrate, in the presence of varying concentrations of C10E6 (n=6, mean±sd)

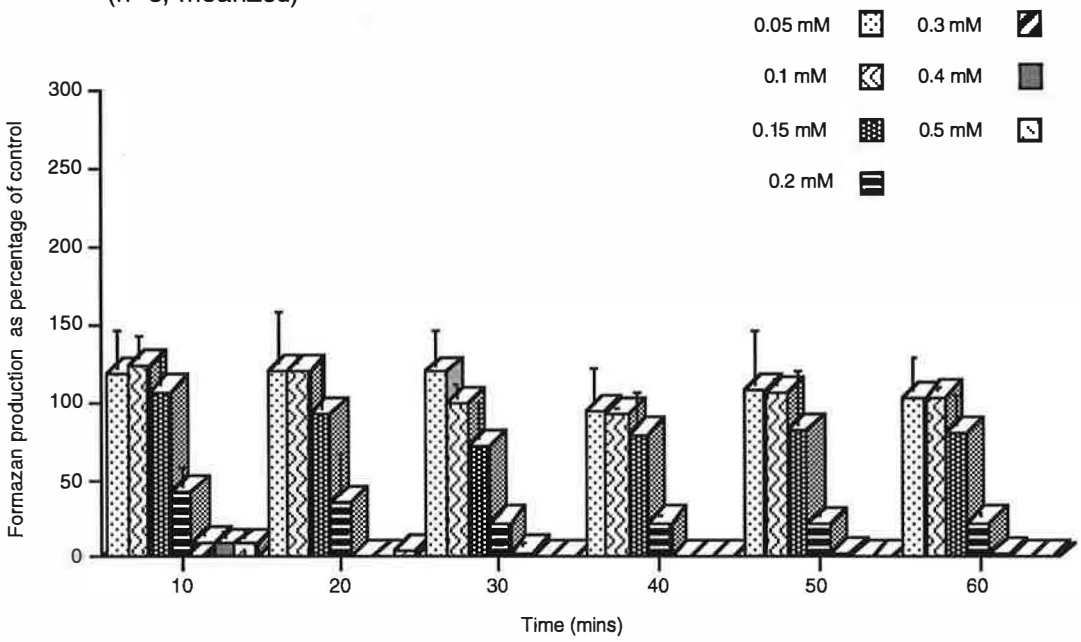


Figure 4.11 Formazan production by*E.coli* NCTC 8196 with succinate substrate, in the presence of varying concentrations of C10E6 (n=3, mean±sd)

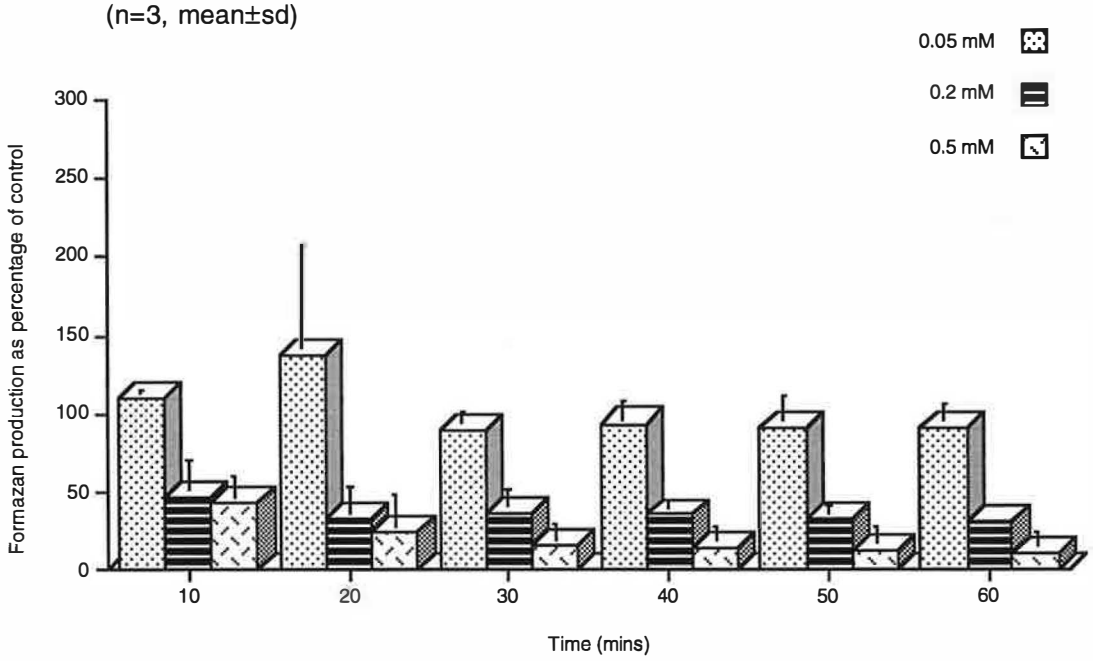


Figure 4.12 Formazan production by*E.coli* NCTC 8196 with glucose substrate, in the presence of varying concentrations of C12E6 (n=6, mean±sd)

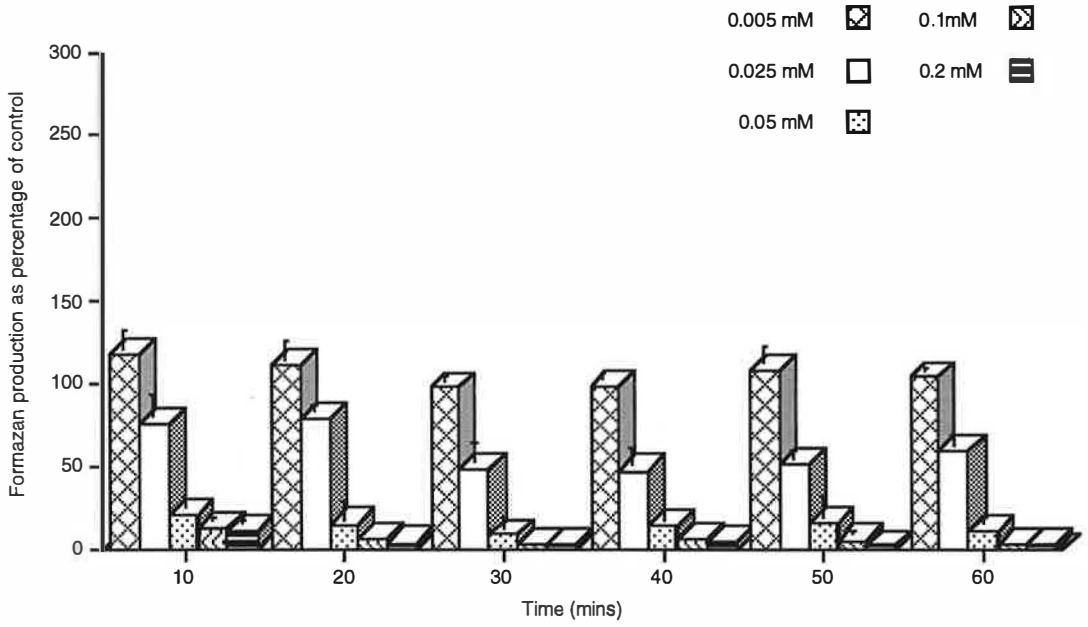


Figure 4.13 Formazan production by*E.coli* NCTC 8196 with succinate substrate, in the presence of varying concentrations of C12E6 (n=3, mean±sd)

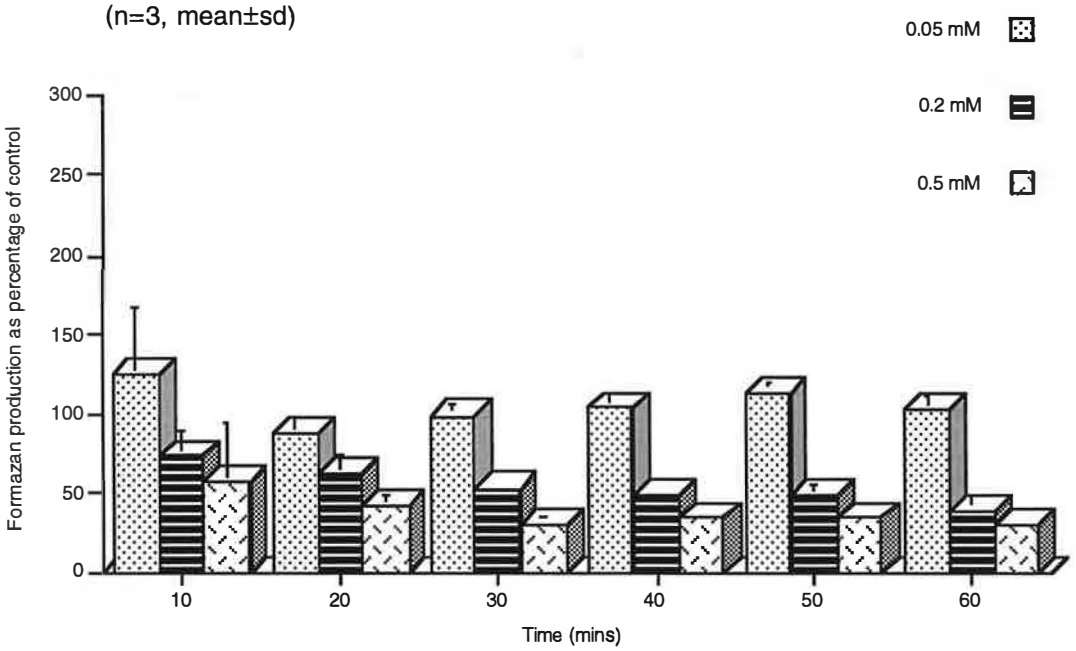


Figure 4.14 Formazan Production by*E. coli* NCTC 8196 with glucose substrate, in the presence of varying concentrations of C14E6 (n=6, mean±sd)

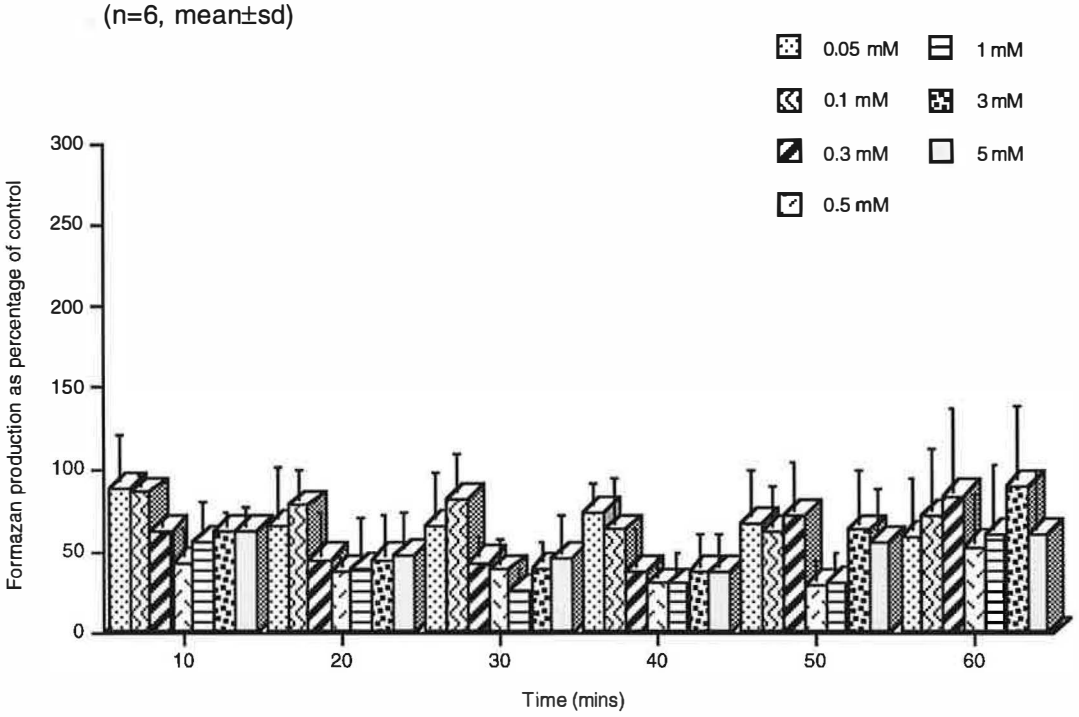


Figure 4.15 Formazan Production by*E. coli* NCTC 8196 with succinate substrate, in the presence of varying concentrations of C14E6 (n=3, mean±sd)

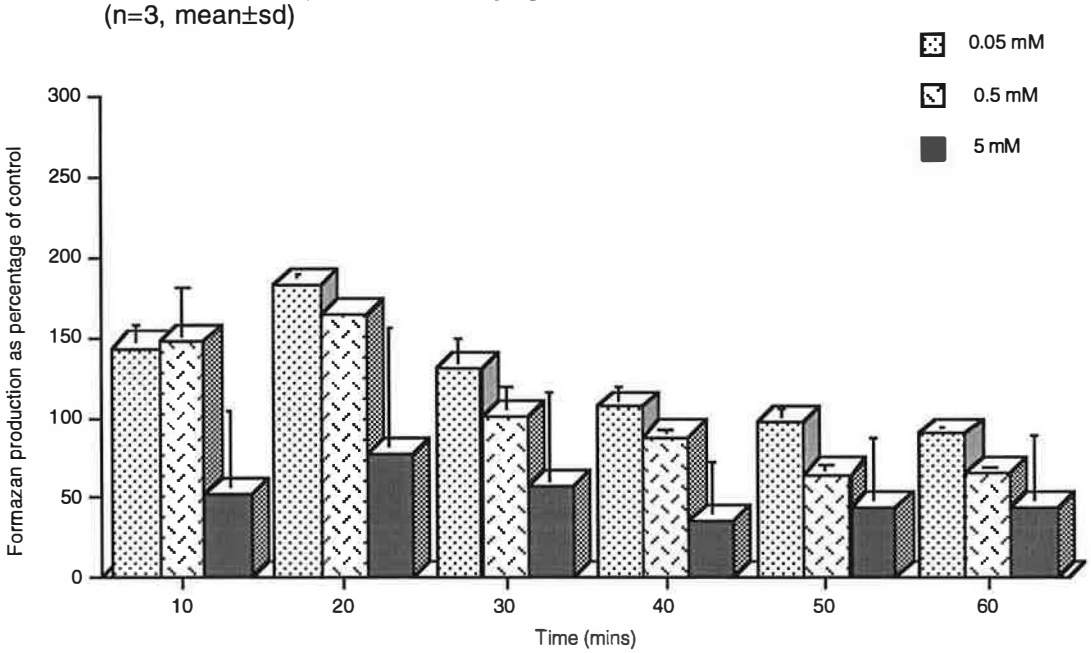


Figure 4.16 Formazan production by*E. coli* NCTC 8196 with glucose substrate in the presence of varying concentrations of C16E6 (n=6, mean±sd)

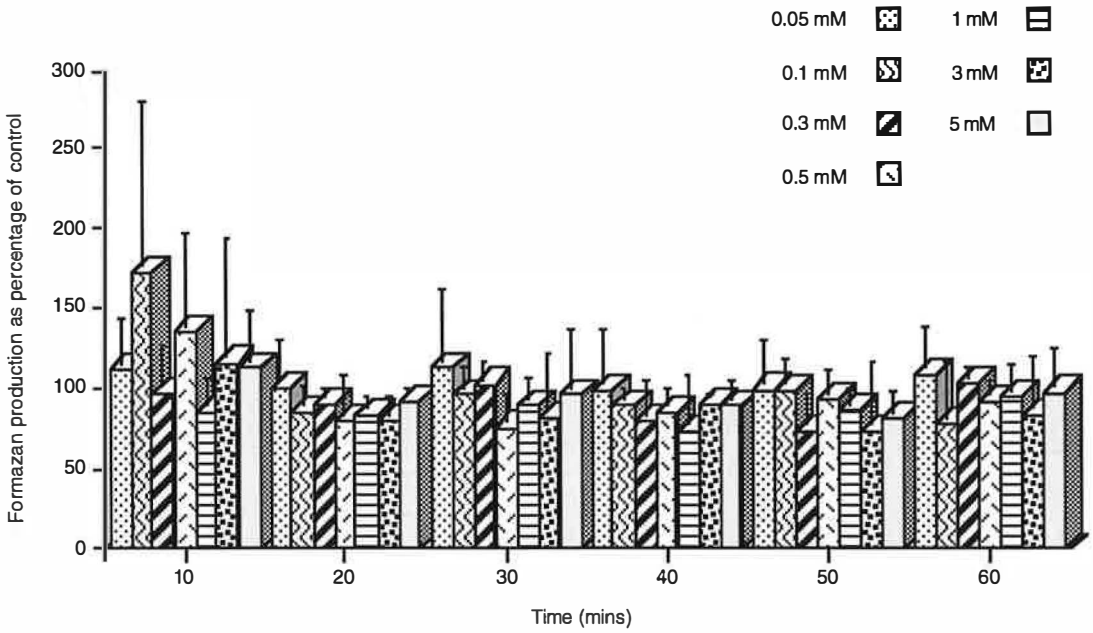
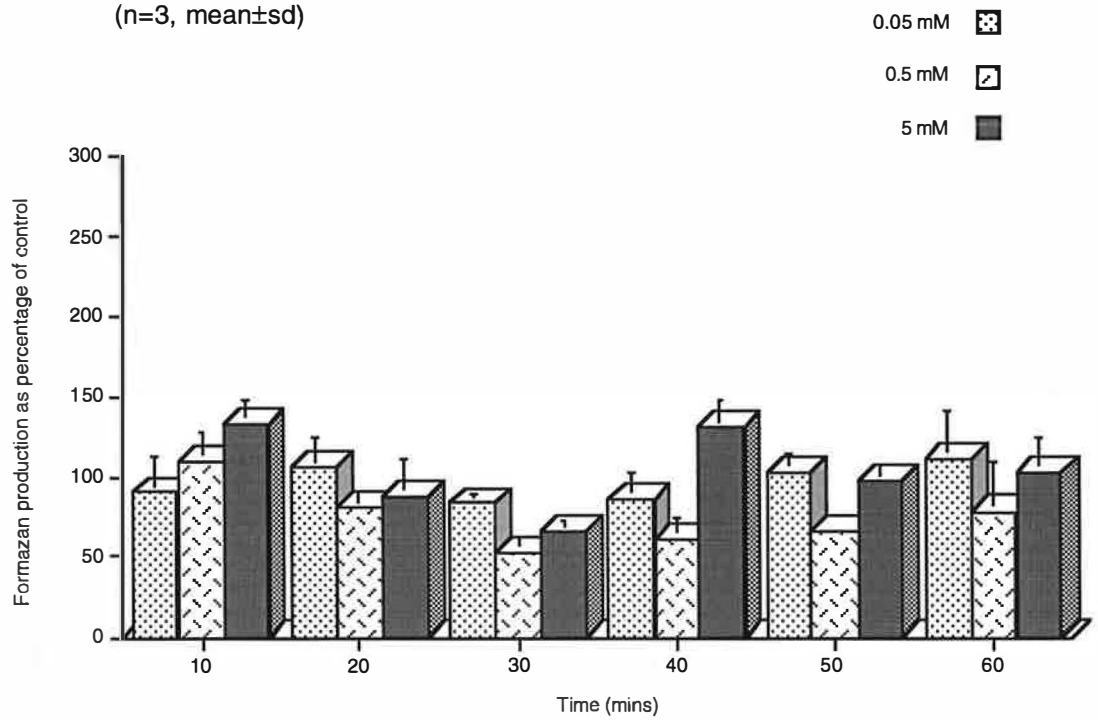


Figure 4.17 Formazan production by*E. coli* NCTC 8196 with succinate substrate in the presence of varying concentrations of C16E6 (n=3, mean±sd)



4.2.5 Results

Figures 4.10 to 4.17 show the results of this study for each surfactant with either glucose or succinate as substrates. Figures 4.18 to 4.21 compare formazan production with time in the presence of the four surfactants with glucose and succinate substrates.

Figures 4.10 and 4.11 show the results of the assay with respect to C10E6. Inhibition occurs at the MIC (0.20mM) with both the glucose and succinate substrates ($p<0.05$, Mann Whitney) which is well below the CMC of C10E6 (0.653mM) in McIlvaine's buffer. Figures 4.12 and 4.13 show the results of the assay with respect to C12E6. Inhibition in this case occurs below the MIC (0.12mM) and CMC of C12E6 (0.189mM) in McIlvaine's buffer with the glucose substrate. Inhibition also occurs below the MIC and CMC with the succinate substrate but not to such a large degree. Figures 4.14 and 4.15 show the results of the assay with respect to C14E6. Some inhibition can be seen with all the concentrations used which span above and below the CMC of C14E6 (0.17mM) in McIlvaine's buffer with the glucose substrate, however, there is not the concentration dependent effect seen with C10E6 and C12E6. All the concentrations used were below or at the MIC (5mM). There is substantial inhibition seen at 5mM C14E6 with the succinate substrate but the experimental variation at this concentration reduces the significance of this result. The standard deviation seen in this assay is much increased in comparison to that seen with C10E6 and C12E6. Figures 4.16 and 4.17 show the results of the assay with respect to C16E6. As with C14E6, C16E6 displays the greatest inhibition at the 5mM concentration with succinate as substrate. All the concentrations used were below the MIC (5mM). The variation seen is also large in comparison to that obtained for

C10E6 and C12E6.

Figure 4.18 Comparison of formazan production by*E. coli* NCTC 8196 in the presence of C10E6 with glucose and succinate substrates

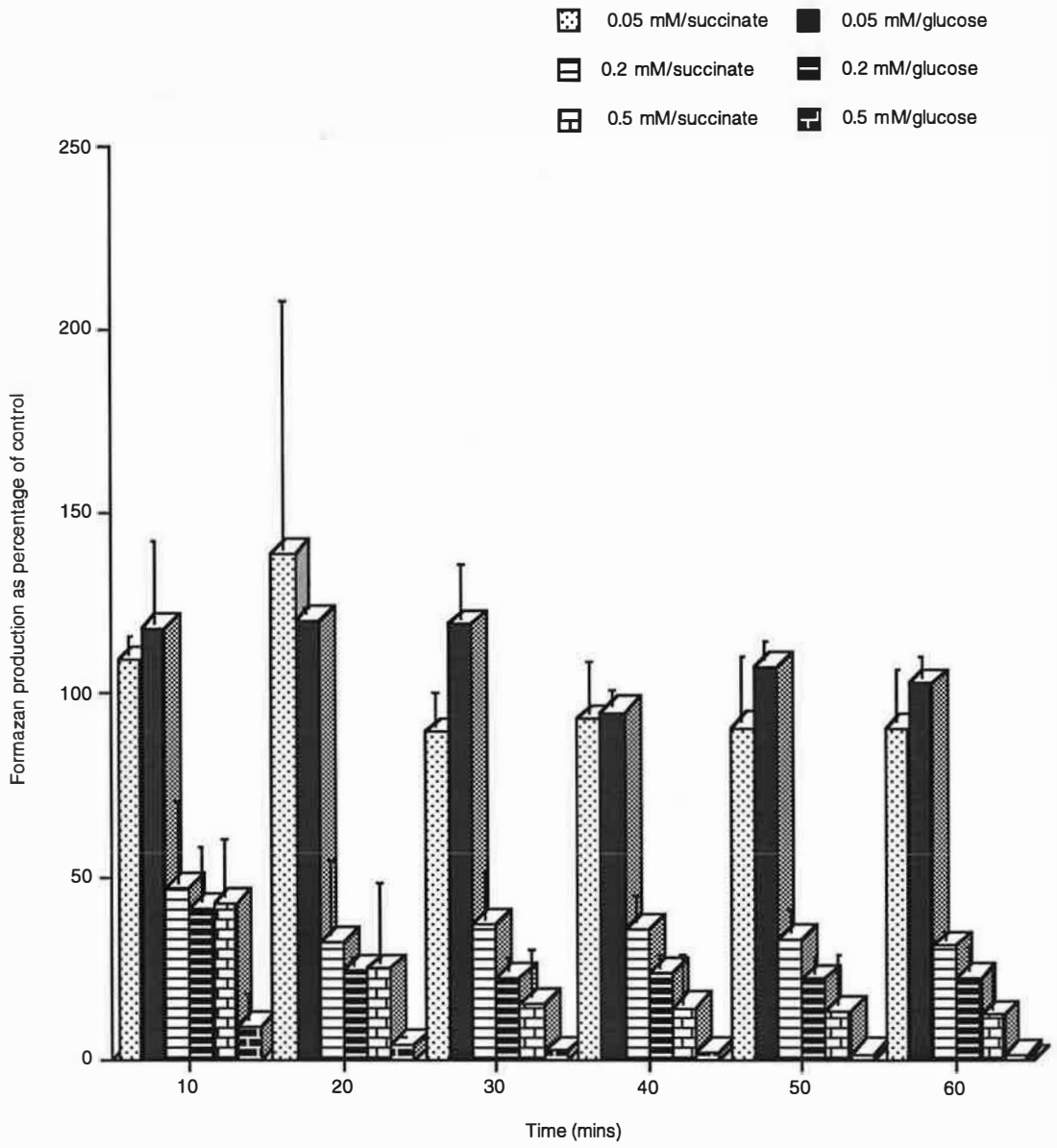


Figure 4.19 Comparision of formazan production by *E. coli* NCTC 8196 in the presence of C12E6 with glucose and succinate substrates

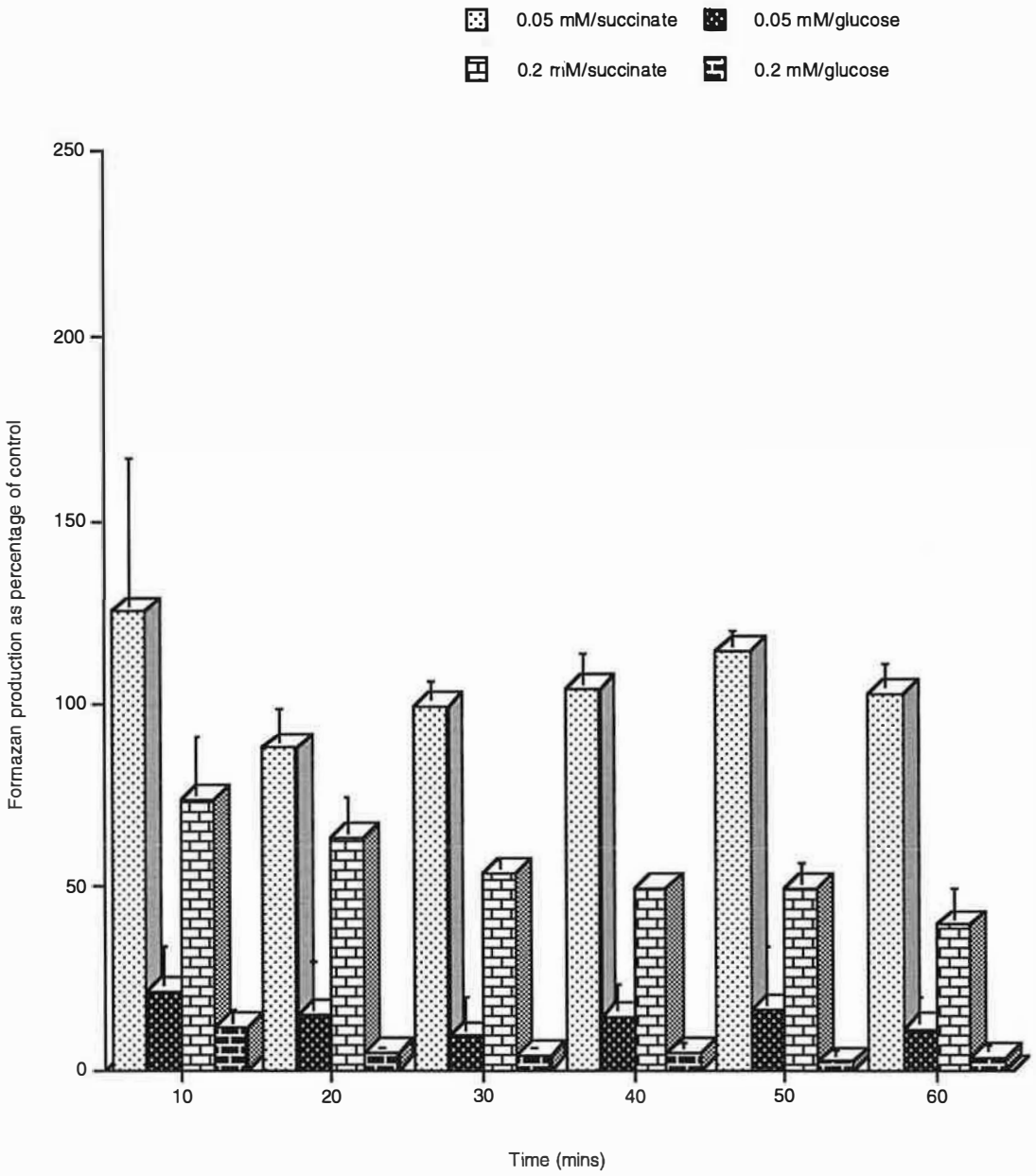


Figure 4.20 Comparision of formazan production by*E. coli* NCTC 8196 in the presence of C14E6 with glucose and succinate substrates

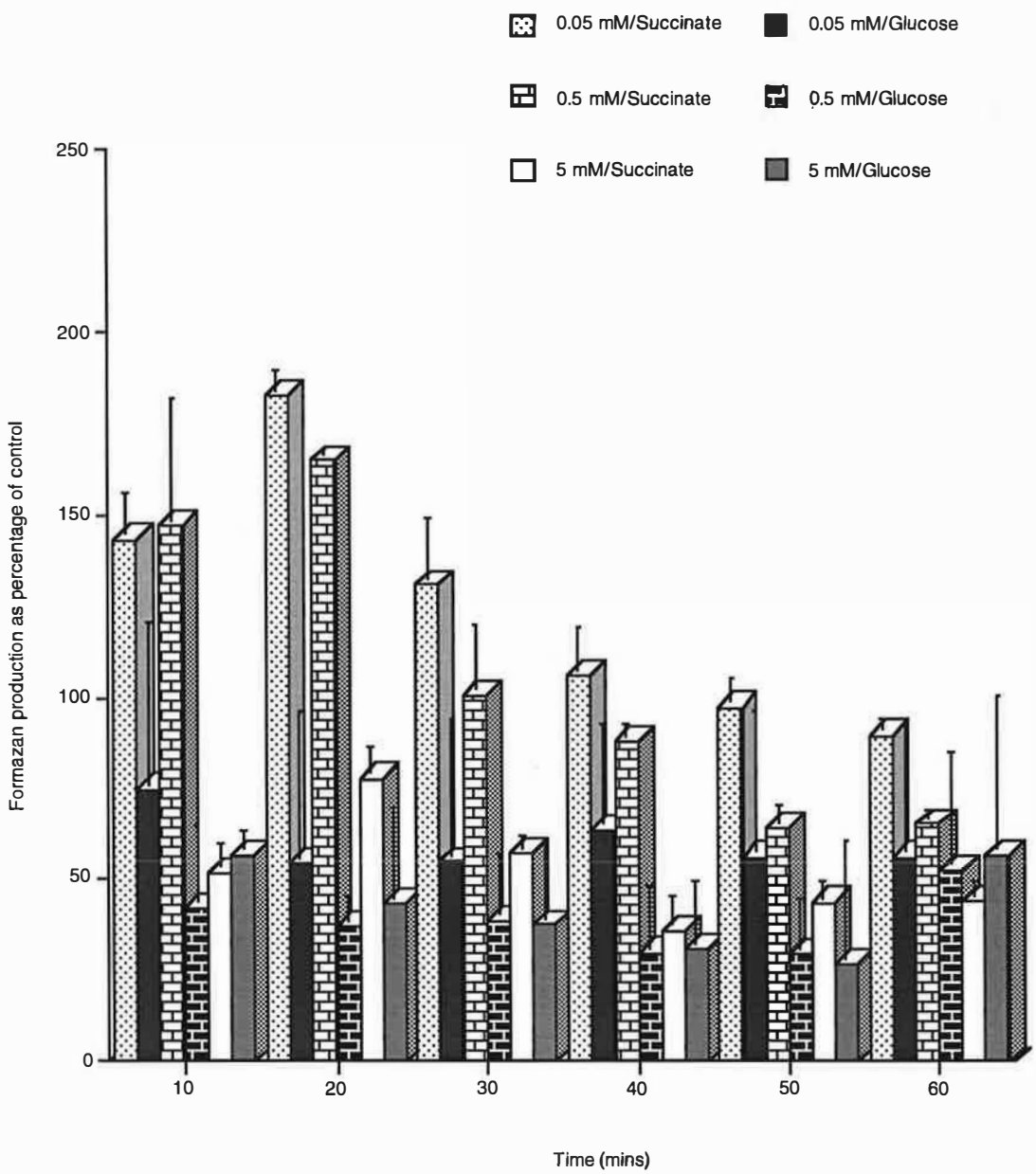
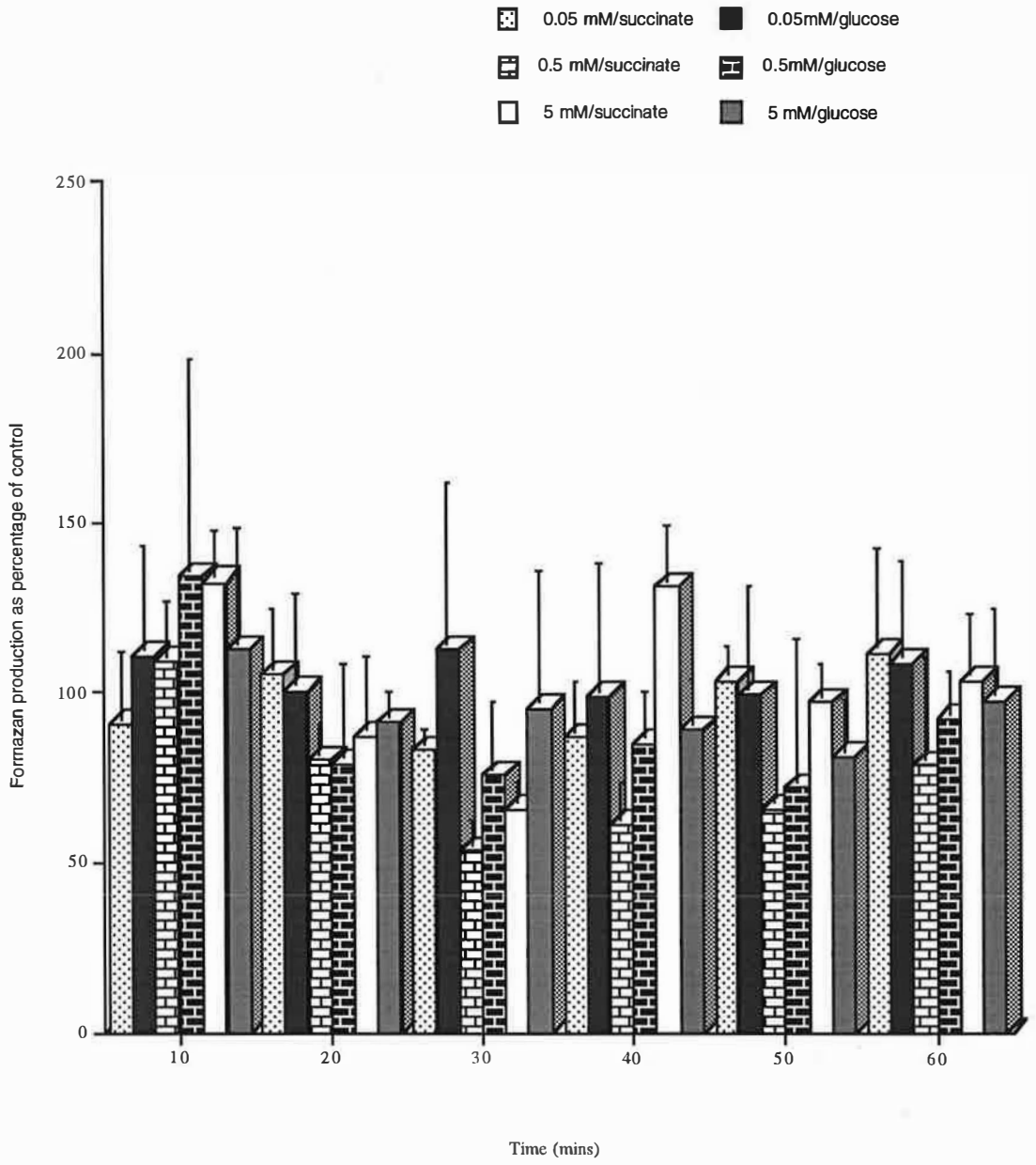


Figure 4.21 Comparison of formazan production by *E. coli* NCTC 8196 in the presence of C16E6 with glucose and succinate substrates



4.3 Bioluminescent ATP Assay

The process of using energy from electron transport to generate ATP is known as oxidative phosphorylation and uses the membrane-bound ATPase. There is evidence from the dehydrogenase experiments of mild uncoupling; intracellular ATP levels may be used to follow this. Further, since the ATPase is membrane-bound cytoplasmic membrane damage may be probed by following the yield of ATP in bacterial cells in the presence of the antibacterial agent under test.

The effect of surfactants C10E6 and C12E6 on the ATP production of *E. coli* was determined using a bioluminescent assay (BioOrbit). (This assay of ATP and subsequent assays were not carried out with C14E6 and C16E6 as results in section 4.2 illustrated their minimal antimicrobial activity when compared with the other two members of the homologous series). The ATP assay measures a stable level of light produced by an enzyme reaction catalysed by firefly luciferase. The equation for light production is :

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow{\text{luciferase}} \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

Trichloroacetic acid (TCA) is used as an ATP extraction reagent and on addition to living cells it will disrupt cell membranes. This results in the release of cell contents including the adenine nucleotides; it also irreversibly inactivates the enzymes which catalyse adenine nucleotide converting reactions. The TCA method of extraction has been found to most closely reflect actual levels of adenine nucleotides in the intact cell (Lundin & Thore, 1975). ATP is assayed using an enzymic reaction and chemical

modification before the assay, perhaps by reversibly inactivated enzymes, can interfere with the ATP determination. This is a particular problem with the firefly luciferase assay as Mg^{2+} is necessary for the reaction and yet is also a co-factor for other ATP-converting reactions.

4.3.1 Determination of Extraction Conditions

Cultures of *E. coli* NCTC 8196 were grown for 15 hours at 37˚C on a Roux slope composed of synthetic broth AOAC (Difco Laboratories) solidified with 1.5%w/v Agar technical (Oxoid). The bacteria were harvested by washing the surface of the slope with 40mL McIlvaine's buffer (pH 7.0). The cell suspension was centrifuged at 2,100g for 20 minutes and then washed twice with 25mL McIlvaine's buffer. The washed cells were standardised to give a cell concentration of 1x 10^9 cells/mL.

1 mL aliquots of the cell suspensions were placed in eppendorf tubes and harvested by centrifugation (2,500g for 5 mins). The supernatant liquid was discarded and the cells resuspended in 500µL of 0, 0.2, 0.5, 1, 1.5, 2 and 2.5% trichloroacetic acid (TCA) containing 2mM EDTA and 0.0015% xylenol blue. (Note between pH 1.2 and 2.8 this indicator changes colour from red to orange. During ATP extraction the indicator must be red to allow for maximal extraction).

The samples were left in contact with the TCA for 30 minutes and the reaction stopped by dilution of the samples with ice cold tris-acetate buffer (pH 7.75). The samples were diluted to a final concentration of 0.1% TCA as concentrations greater than this in the bioluminescent assay will inhibit the

firefly luciferase. The samples were then left on ice until the assay was performed.

ATP determination was carried out by first pipetting 40µL aliquots of monitoring reagent (which contains the firefly luciferase) into a 96 well microtitre plate and the light output measured with an Amersham Amerlite luminometer, this gave the background light reading (B). 150µL of sample was then placed in the wells and the light output recorded; this represented the extracted ATP in the sample (I). Finally 50µL of a known concentration of ATP standard (chosen to be of similar magnitude to that expected from the sample) was added to the wells to calibrate the system and the light reading of this standard (S) obtained. The light readings were converted to ATP yield by the use of the formula in appendix 1.

From this experiment it could be seen that maximum ATP extraction was obtained after contact with 0.5% w/v TCA (Figure 4.22.). The experiment was then repeated using 0.5% w/v TCA in 2mM EDTA as lysis medium in contact with cells for between 0 and 30 minutes with samples taken at 5 minute intervals. The data from this experiment can be seen in figure 4.23 and demonstrate that maximum ATP extraction is obtained at 5 minutes and does not increase with time. For all future experiments the cells were lysed for 10 minutes with 0.5% w/v TCA in 2mM EDTA.

Figure 4.22 The effect of TCA extractant concentration on ATP yield from *E. coli* NCTC 8196 (n=6, mean±sd)

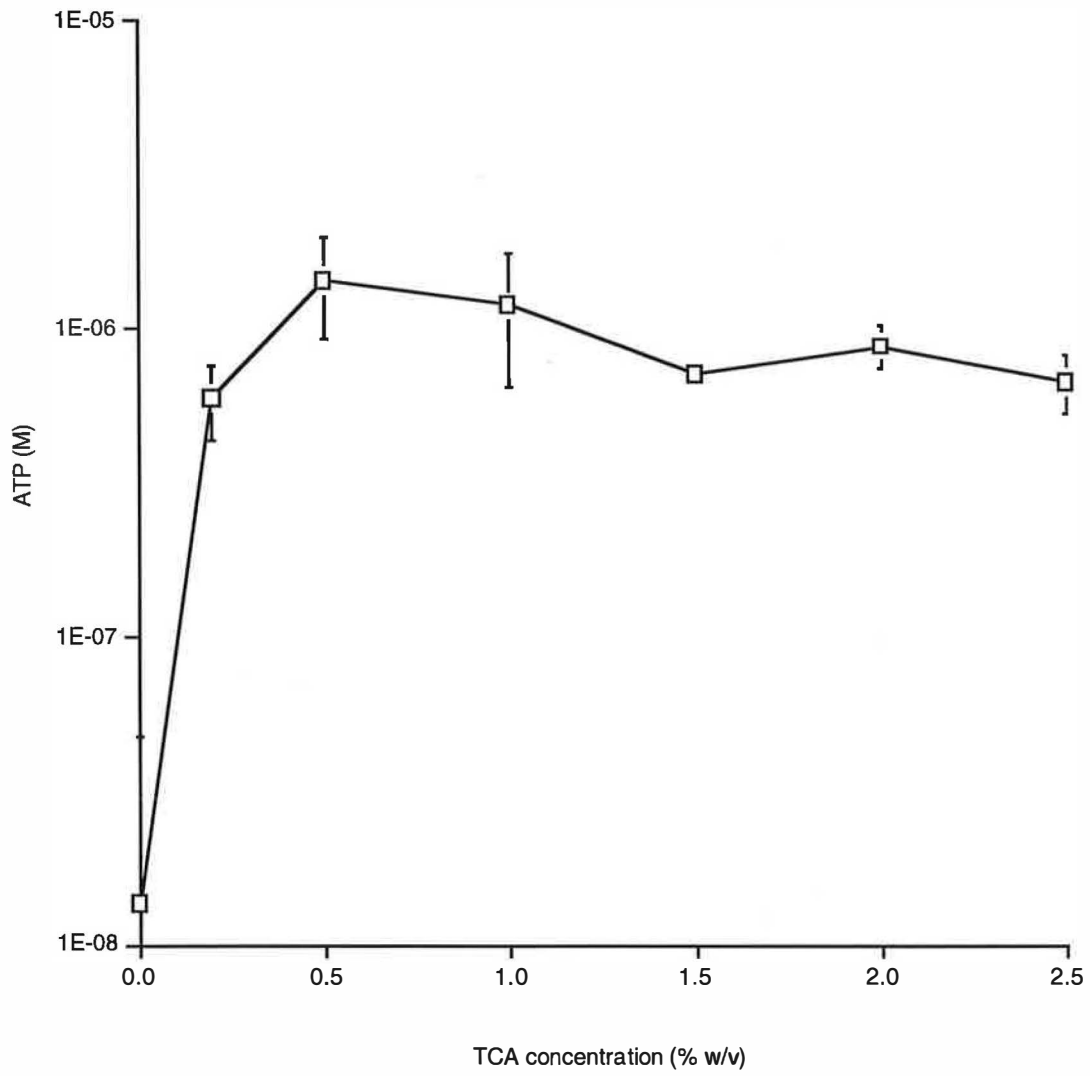
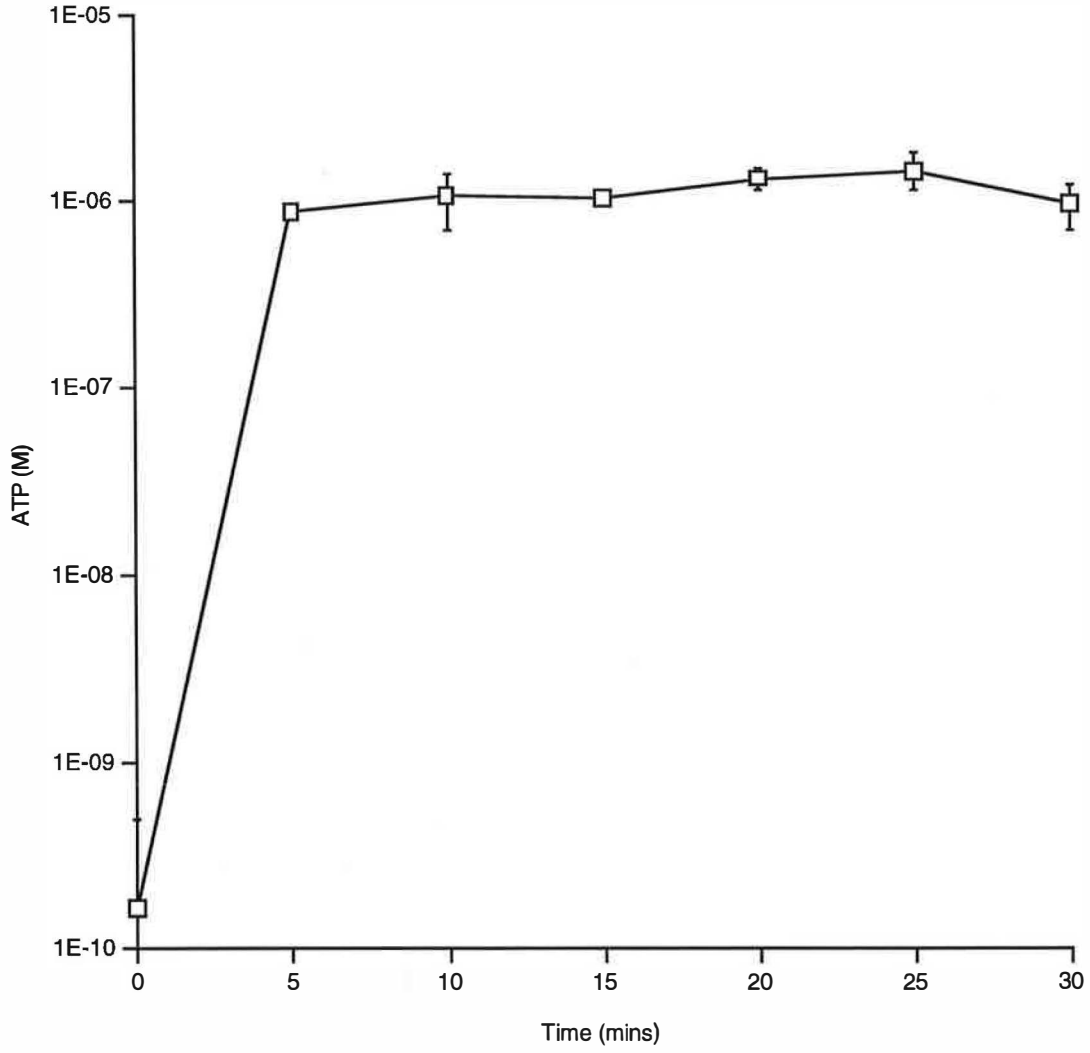


Figure 4.23 The effect of time on ATP extraction from *E.coli* NCTC 8196 using 0.5% TCA (n=6, mean±sd)



4.3.2 Validation of the ATP assay

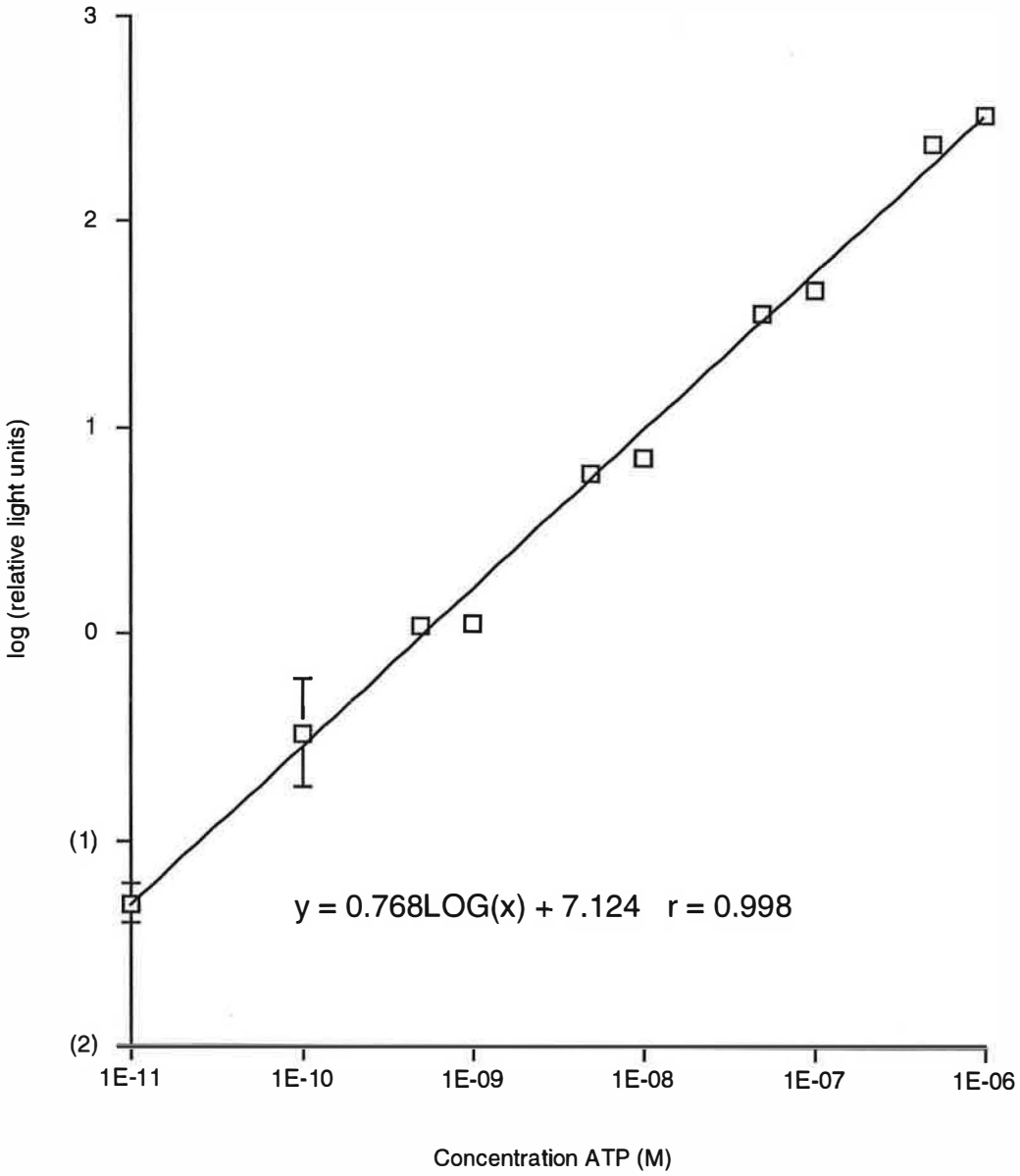
4.3.2.1 The effect of cell debris on the assay

During the extraction procedure it was noticed that visible amounts of cell debris were being produced by the action of TCA on the bacterial cells. It was considered that this might interfere with the assay since excessive amounts of cell debris will cause light scattering in the luminometer. 20 samples were lysed with TCA as described in section 4.3.1. Half of the samples were assayed as before and the other half centrifuged to pellet the cell debris and the supernatant liquid subsequently analysed. The results show that the cell debris does not affect the values obtained ($p \geq 0.05$, Mann Whitney).

4 3.2.2 Determination of the ATP concentration range of the assay

The concentration range at which there exists a linear relationship between light output and ATP concentration was established by using known levels of ATP in place of samples in the assay; these represented the range of normal values that are likely to be obtained in an assay. A series of ATP solutions were prepared in tris-acetate buffer containing $1x10^{-6}$ to $1x10^{-11}$M ATP. The assay was carried out as described in section 4.3.1 using the series of ATP solutions as samples. The resultant graph can be seen in figure 4.24, this shows the working range of the assay to be from $1x10^{-6}$ to $1x10^{-11}$M ATP.

Figure 4.24 ATP calibration plot (n=6, mean±sd)



4.3.2.3 Determination of the stability of the light output

As there is a small time delay between the addition of both sample and standard to the first well and last well of a microtitre plate the stability of light output in the assay, with time, was of paramount importance. Repeated scans of the samples from section 4.3.2.2 (a range of known concentrations of ATP) were carried out at various time intervals during a 30 minute period. The decay rate was found to be <1%/min (see figure 4.25).

4.3.2.4 Variation between batches of monitoring reagent

The variation between different batches of ATP monitoring reagent was investigated by comparing the light output reading from a known ATP standard using monitoring reagent taken from two different batches. The data showed that there was no significant difference ($p \geq 0.05$, Mann Whitney) in light output reading from the two different batches of monitoring reagent but, as a precautionary measure, when more than one batch of monitoring reagent had to be used for a particular experiment the batches were mixed together before use.

4.3.2.5 The effect of surfactants on luciferase

Data from earlier experiments (section 4.2) demonstrated that the alcohol ethoxylates could have a profound effect on membrane-bound enzyme activity. As the bioluminescent ATP assay is based on an enzymic reaction a knowledge of the effect of the surfactants on the enzyme luciferase was needed.

Figure 4.25 The stability of light ouput from the bioluminescent ATP assay, with time (n=6, mean±sd)

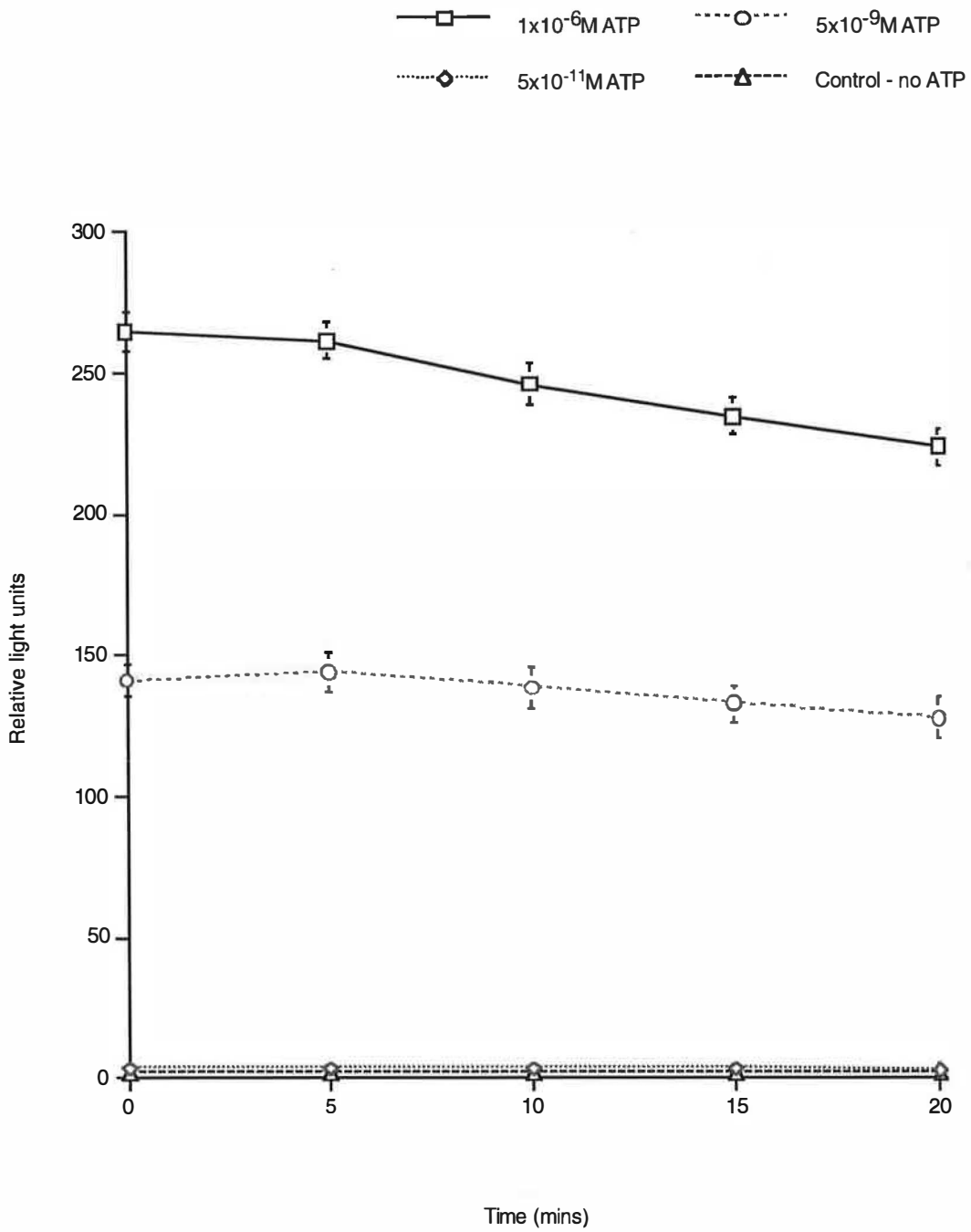


Figure 4.26 The effect of increasing concentrations of C10E6 on luciferase activity
(n=6, mean±sd)

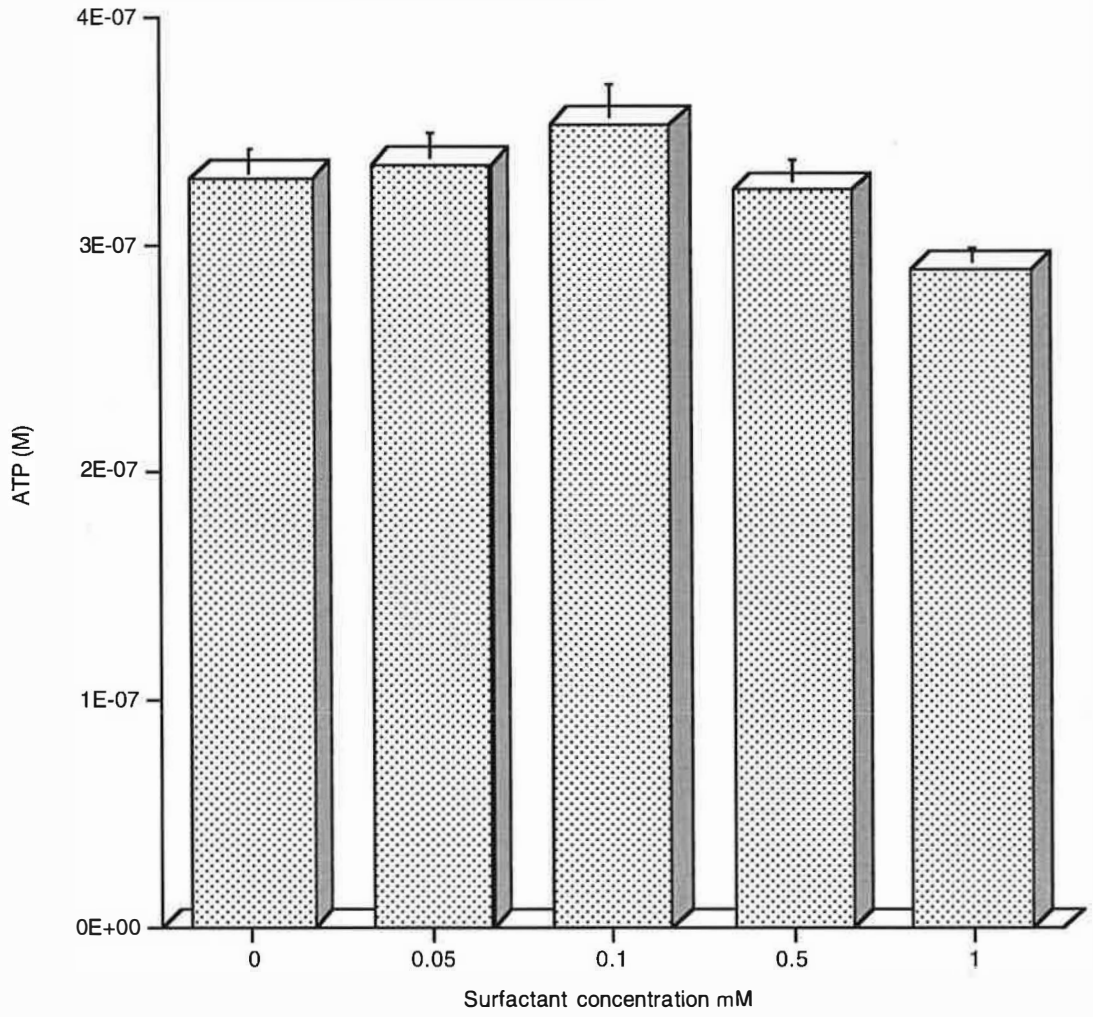
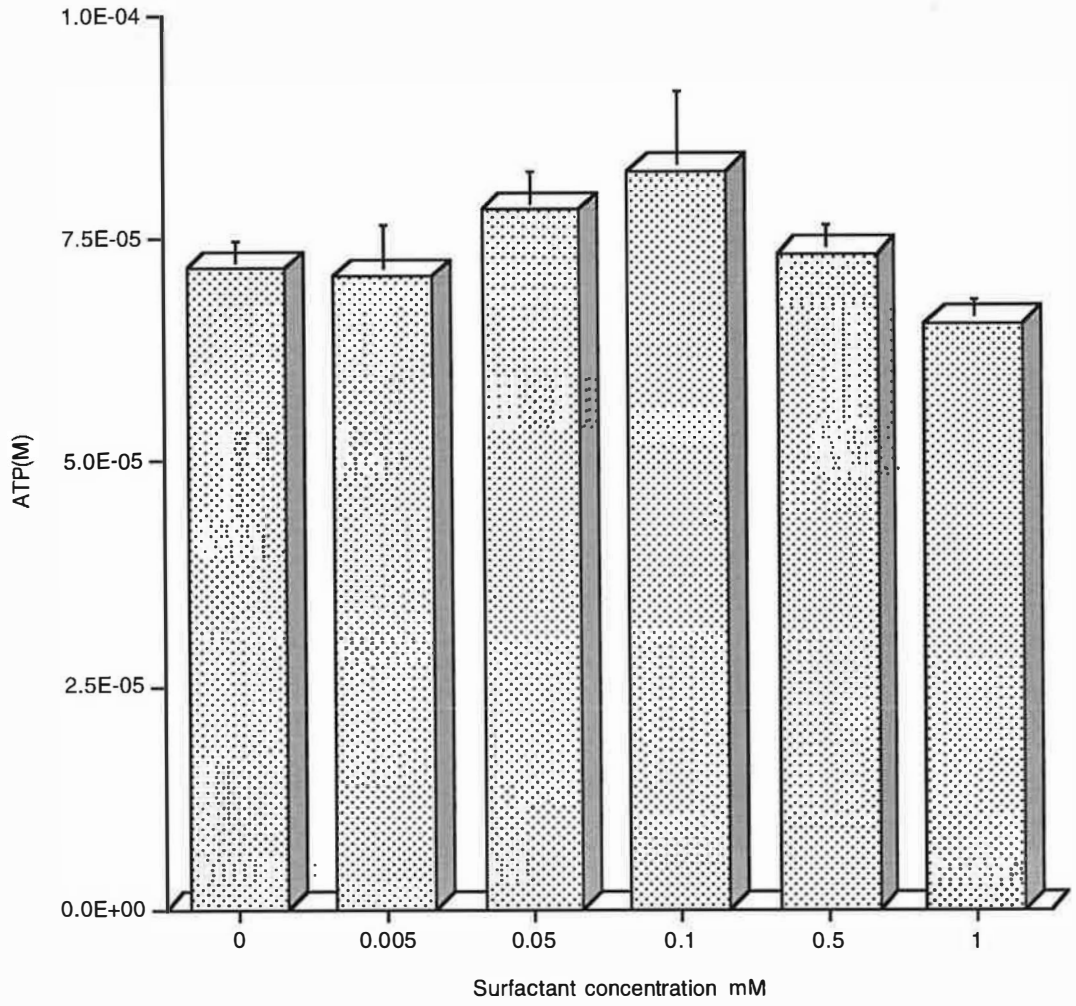


Figure 4.27 The effect of increasing concentrations of C12E6 on luciferase activity
(n=6, mean±sd)



A series of $1x10^{-4}$M solutions of ATP were prepared containing a final concentration of 0, 0.05, 0.1, 0.5 and 1 mM of C10E6 and a second series containing the same concentrations of C12E6. The assay was carried out as described in section 4.2.1 using the solutions of ATP as the sample.

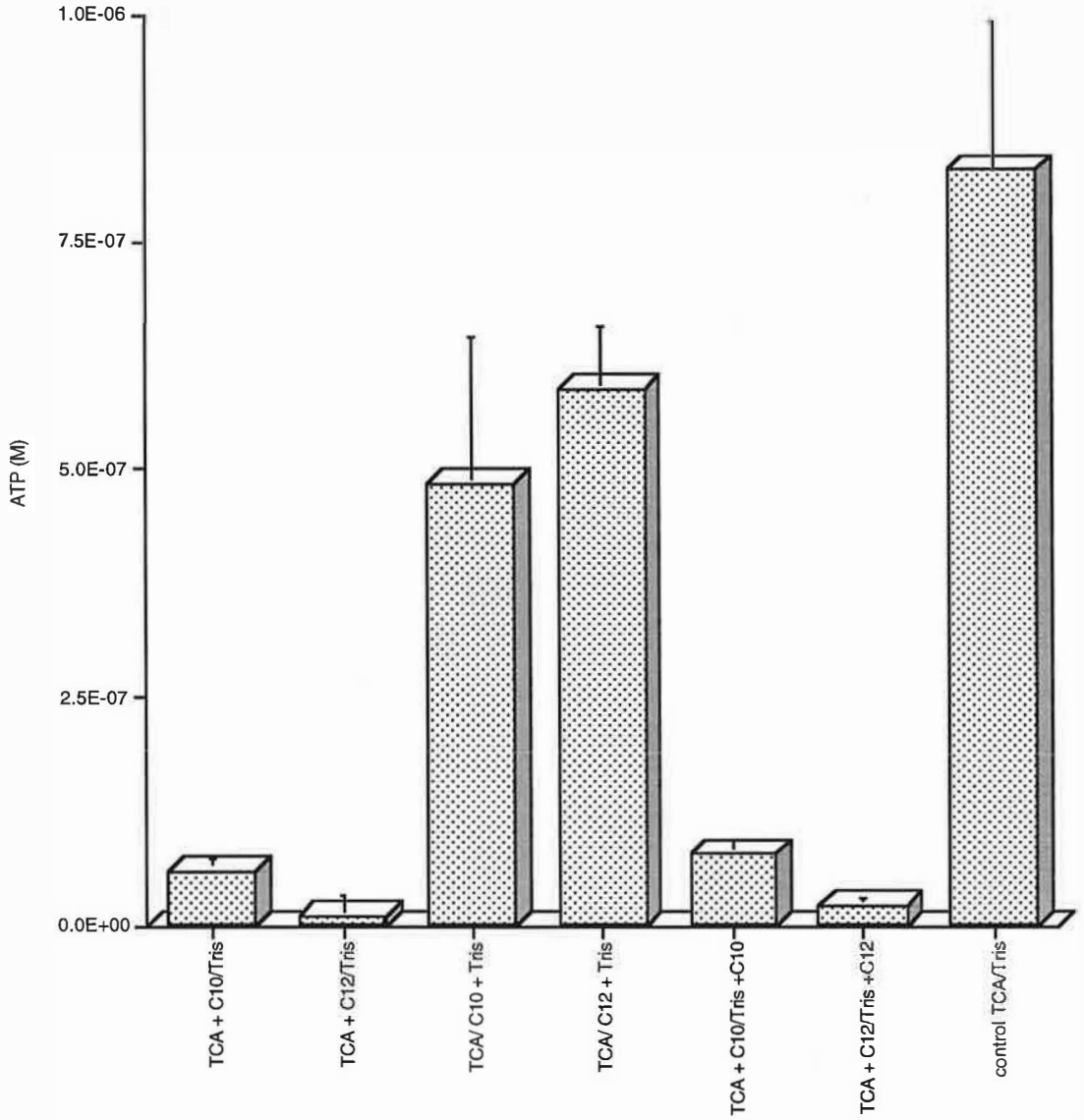
The data were plotted as ATP (M) after carrying out the calculation in appendix 1 (figures 4.26 and 4.27).

Figures 4.26 and 4.27 show that the inclusion of surfactant in the ATP solution affected the light output from the luciferase to a limited degree.

4.3.2.6 The effect of surfactants on the extraction of ATP

To determine whether the presence of surfactant in the sample for analysis would interfere with the extraction and neutralisation procedures these processes were carried out in the presence of 1mM C10E6 (which represents the highest concentration of that surfactant used). Tris-acetate buffer and TCA were prepared to contain 1mM C10E6 and cell samples were extracted using a combination of the reagents both with and without the surfactant. It can be seen in figure 4.28 that the surfactant in the tris-acetate buffer reduces the ATP yield compared to the control. However the effect of the C10E6 in TCA reduces ATP yield to a much greater degree than in tris-acetate buffer compared to the control. The same experiment was carried out using 0.5mM C12E6 in place of C10E6 producing the same pattern of results as seen with 1mM C10E6.

Figure 4.28 The effect of surfactant on the extraction of ATP from*E. coli* NCTC 8196
(n=6, mean±sd)



In the light of these results and those in Section 4.3.2.5, for all future experiments cell samples were centrifuged and the supernatant liquid removed, thus removing surfactant which would interfere with ATP extraction and assay. The resultant cell pellet was then resuspended in the extraction medium to release ATP from the cells. Control cells treated by this method produced a comparable ATP yield to that seen for the control sample in figure 4.28.

4.3.3.1 ATP yield from *E.coli* NCTC 8196 in the absence of surfactants

E. coli NCTC 8196 was grown and harvested as described in Section 4.3.1. A series of universal bottles were prepared containing 9mL of McIlvaine's buffer. The bottles were placed in a water bath at 37˚C prior to the start of the experiment to allow the contents to equilibrate. The reaction was started by the addition of 1mL of a $1x10^{10}$ cells/mL suspension of *E. coli* NCTC 8196 to give a final cell concentration of $1x10^{9}$ cells/mL. The bottles were incubated at 37˚C in a shaking water bath (approximately 100 rpm). 1mL samples were removed at 0, 10, 20, 30, 45 and 60 minutes. The samples were placed in eppendorf tubes and centrifuged at 2,500g for 5 minutes. The supernatant liquid was removed and the cell pellet resuspended in 500µL 0.5% w/v TCA in 2mM EDTA and left to lyse for 10 minutes. A diluted sample for subsequent analysis was then obtained by removing 50µL of the cell pellet resuspended in TCA and diluting it with the addition of 200µL of ice-cold tris-acetate buffer.

The samples were analysed as before (section 4.3.1) using $2x10^{-6}$ M ATP as the standard.

4.3.3.2 ATP yield from *E. coli* NCTC 8196 in the presence of surfactants

E. coli NCTC 8196 was grown and harvested as described in Section 4.3.1.

A series of universal bottles were prepared containing 9mL of a solution of either C10E6 or C12E6 in McIlvaine's buffer to give final concentrations of: 0.025mm; 0.05mM; 0.2mM; 0.5mM or 1mM C10E6 and 0.005mM; 0.05mM;

0.2mM; or 0.5mM C12E6. A set of control bottles were also set up containing McIlvaine's buffer. The bottles were placed in a water bath at 37˚C prior to the start of the experiment to allow the contents to equilibrate to temperature. The reaction was initiated by the addition of 1mL of a $1x10^{10}$ cells/mL suspension of *E. coli* NCTC 8196 to give a final cell concentration of $1x10^{9}$ cells/mL. The bottles were incubated at 37˚C in a shaking water bath (approximately 100 rpm). 1mL samples were removed at 10, 20, 30, 45 and 60 minutes. The samples were centrifuged, lysed and assayed as described in section 4.3.3.1.

The results, expressed as percentage of the control can be seen in figures 4.29 and 4.30.

Figure 4.29 ATP yield of *E. coli* NCTC 8196 in the presence of increasing concentrations of C10E6 (n=6, mean±sem)

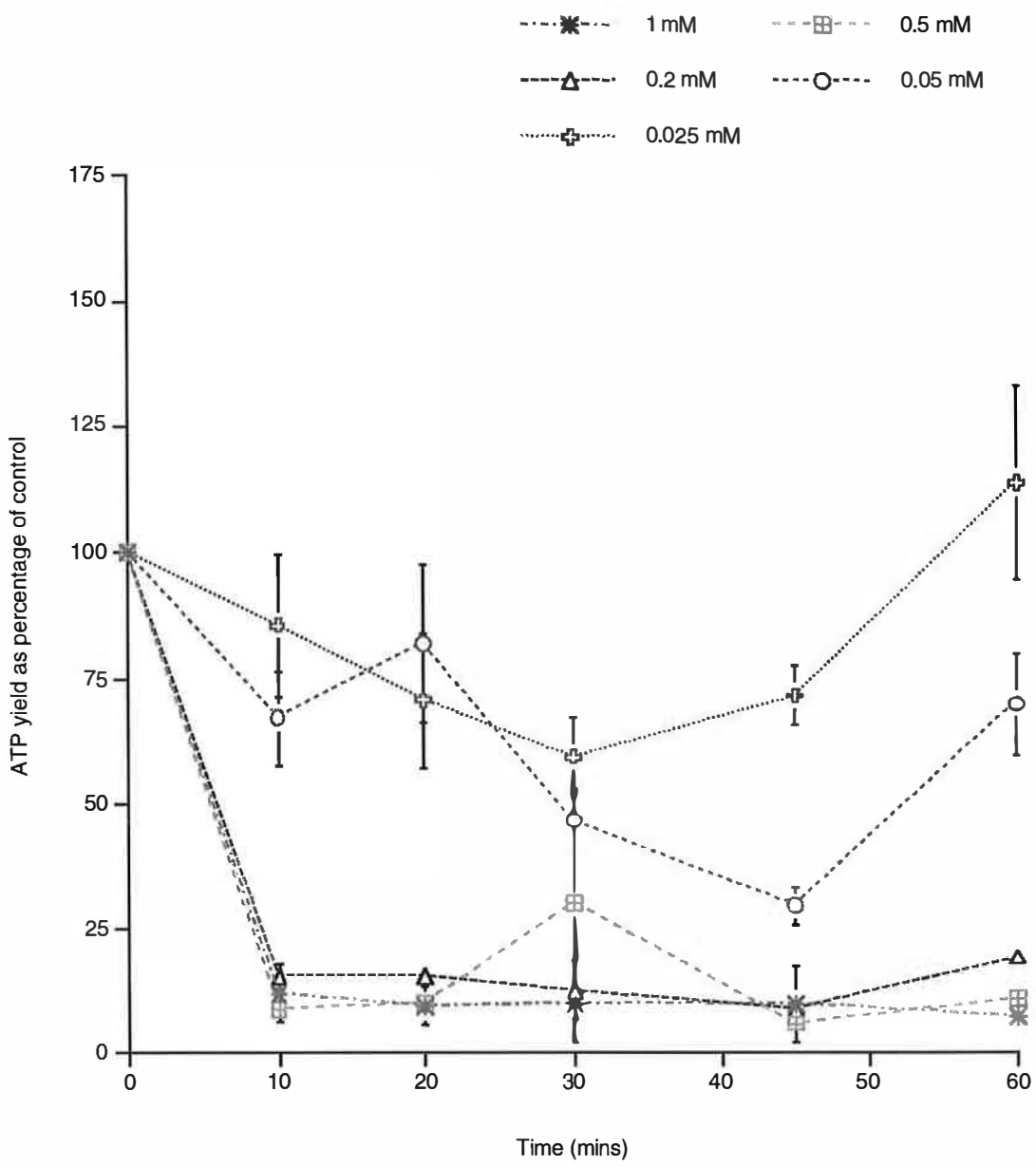
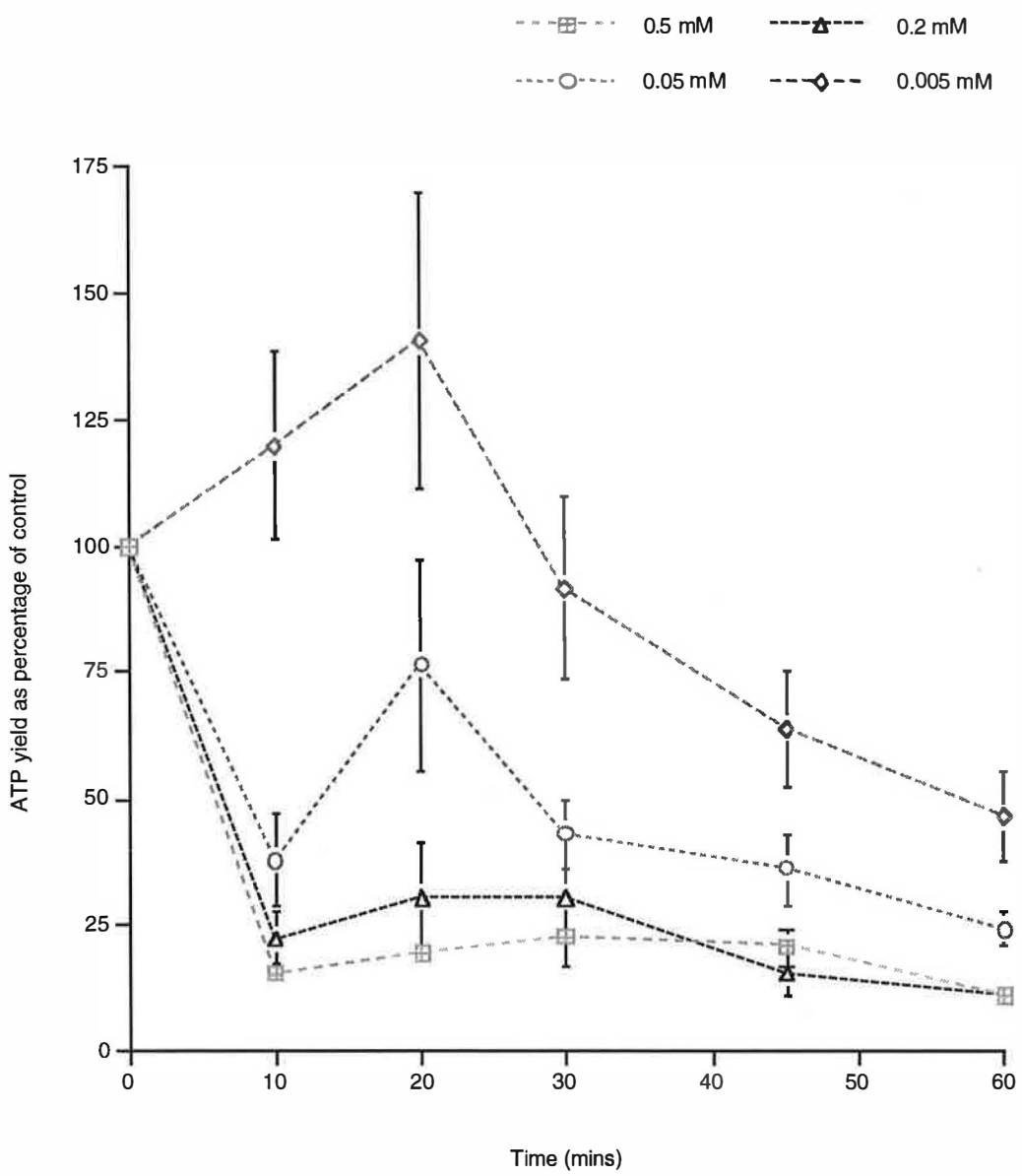


Figure 4.30 ATP yield of *E. coli* 8196 in the presence of increasing concentrations of C12E6
(n=6, mean±sem)



4.3.4 Results

Figure 4.29 shows ATP yield, as a percentage of control, in the presence of C10E6. At the highest concentrations of C10E6: 1, 0.5 and 0.2mM ATP yield is between 10 and 15% of the control values. Therefore significant reduction in ATP yield is occurring at and above the MIC of C10E6 (0.20mM) ($p<0.05$, Mann Whitney). Below the MIC, at 0.05mM there is some reduction of ATP yield, particularly at the later time points. At 0.025mM there is a small reduction in ATP yield but after 30 minutes the yield increases with time.

In figure 4.30 ATP yield reduced as the concentration of C12E6 increased and reduced in most cases with time. Reduction in ATP yield, below about 40%, was seen with all concentrations of C12E6, with the exception of the lowest concentration - 0.005mM. Thus reduction in ATP yield occurs both above and below the MIC of C12E6 (0.12mM) which is in contrast to the data seen with C10E6 in which significant ATP reduction occurs at and above the MIC of C10E6. At the lowest concentration of C12E6 used i.e. 0.005mM there is an increased ATP yield compared to the control in the first 20 minutes of the experiment after which ATP yield reduces with time below the values seen with the control.

4.4 Glucose uptake studies

Microorganisms utilise several metabolic pathways for the breakdown of glucose and other sugars. Of these the Embden-Meyerhof or glycolytic pathway is the most common pathway for the breakdown of glucose to pyruvate. It operates under aerobic and anaerobic conditions generating a net yield of 2 molecules of ATP and is present in all major classes of bacteria.

The pentose phosphate pathway can function aerobically or anerobically and can operate at the same time as the glycolytic pathway or the Entner-Doudoroff pathway. The pentose phosphate pathway can be used for the production of ATP. The pathway synthesises four and five carbon sugars which are utilised by the bacteria for a range of functions such as synthesising nucleic acids and aromatic amino acids.

The alcohol ethoxylates C10E6 and C12E6 have been shown to inhibit bacterial respiration (sections 4.1 and 4.3). This could be as a result of inhibition of glucose transport which would limit the available substrate.

4.4.1 Phenol-sulphuric acid assay for glucose determination

4.4.1.1 Glucose uptake by *E. coli* NCTC 8196 using the phenol-sulphuric acid assay

Glucose uptake by *E. coli* NCTC 8196 was measured in this study, as disappearance of glucose from the medium over time. Cultures of *E. coli* NCTC 8196 were grown for 15 h at 37˚C in on a Roux slope composed of synthetic broth AOAC (Difco Laboratories) solidified with 1.5%^{w}/v Agar technical (Oxoid). The bacteria were harvested by washing the surface of the slope with 40mL McIlvaine's buffer (pH 7.0). The cell suspension was centrifuged at 2,100g for 20 minutes and then washed twice with 25mL McIlvaine's buffer. The washed cells were standardised to give a cell concentration of $1x10^{10}$ cells/mL.

18mL of McIlvaine's buffer containing glucose at a final concentration of 0.5 mM was placed into a universal bottle and allowed to equilibrate to 37˚C. 2mL of the washed and standardised cell suspension was added to the McIlvaine's buffer to give a final concentration of 1×10^{9} cells/mL. The suspensions were then incubated at 37˚C and at 0, 10, 20, 40, 60, 80, 100 and 120 minutes 2mL of the reaction mixture was removed with a syringe and filtered through a 0.2µm cellulose nitrate filter membrane. The samples were then frozen until analysis.

Surfactant has been shown to affect enzymic activity (see sections 4.2 and 4.3.2.5); a non-enzymic assay was initially sought for glucose determination The phenol-sulphuric acid assay (Chaplin, 1986) was used which measures

total carbohydrate, this was suitable for detecting glucose in the range of 0.1-0.5mM. 1mL of sample (previously diluted 1:1 with buffer) or standard glucose solution was mixed with 1mL of 5% v/v phenol solution in a boiling tube. 5mL of concentrated sulphuric acid was then added directly to the solution surface (without touching the sides of the tube) and thoroughly mixed. The reaction mixtures were left to stand for 25 minutes, thoroughly mixed again and their absorbance measured at 490nm using the Perkin-Elmer Lambda 12 spectrophotometer, against a buffer blank similarly treated with phenol and sulphuric acid. The calibration curve and the results from this assay can be seen in figures 4.31 and 4.32 respectively.

4.4.1.2 The effect of intracellular material on the phenol sulphuric acid assay

Data obtained from the investigation into the release of 260 nm absorbing material (Chapter 5, section 2) demonstrate that there is significant leakage of cytoplasmic contents from the cell following exposure to surfactant. To determine whether these cell exudates affect the assay a series of glucose standards were prepared to include intracellular material (as indicated by nucleotides and nucleosides absorbing at 260 nm). These samples were assayed for glucose and compared with a set of glucose standards without the intracellular material. The results can be seen in figure 4.33 and show that cell contents cause an apparent elevation of glucose levels when compared to standard solutions and an apparent loss of linearity. The phenol-sulphuric acid assay determines total carbohydrate, the apparent elevation in glucose levels in the presence of the intracellular material is likely to be due to the presence of hydrolysed cell carbohydrates This method was considered unsuitable for future use.

Figure 4.31 Calibration curve for glucose using the phenol-sulphuric acid assay
(n=5, mean±sd)

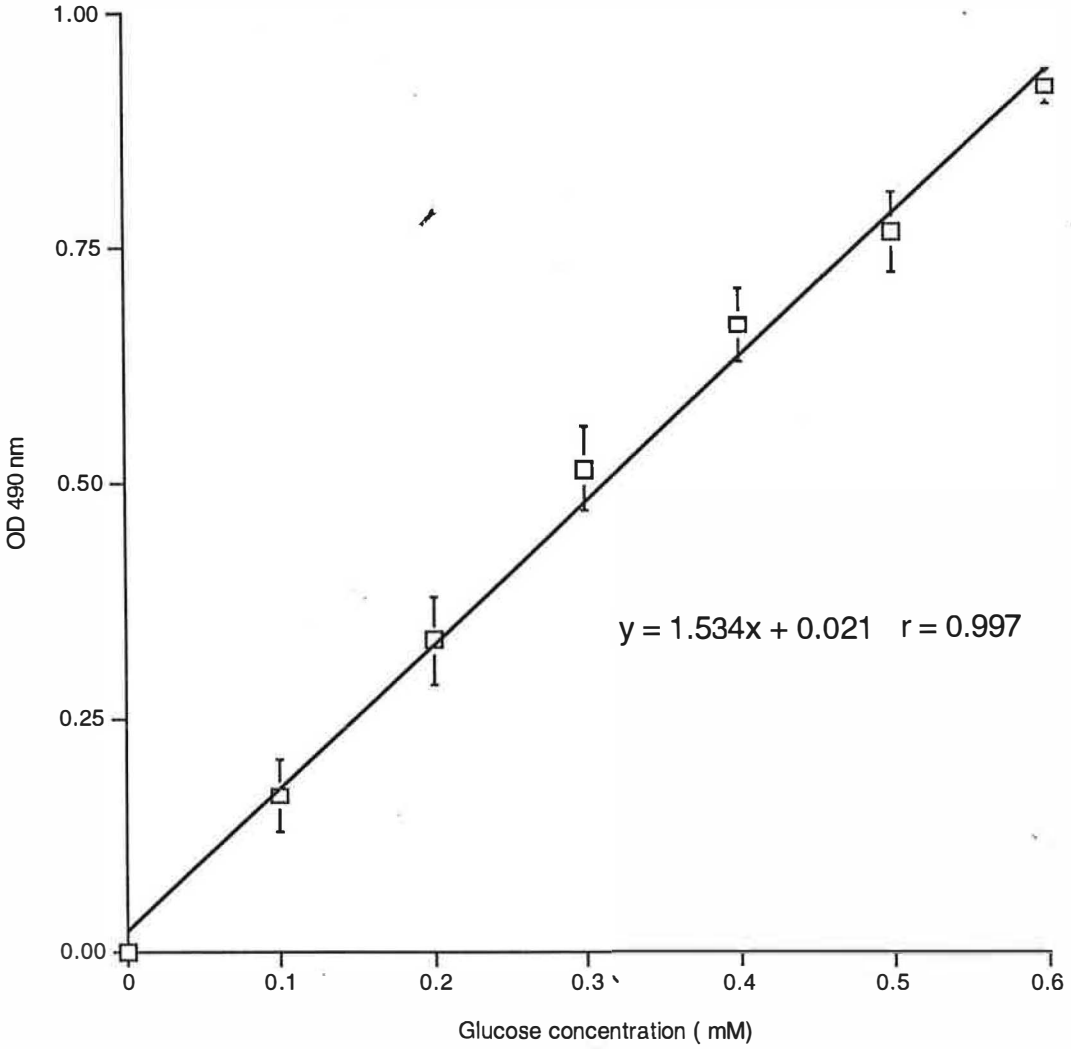


Figure 4.32 Glucose uptake by *E. coli* NCTC 8196 measured with the phenol-sulphuric acid assay

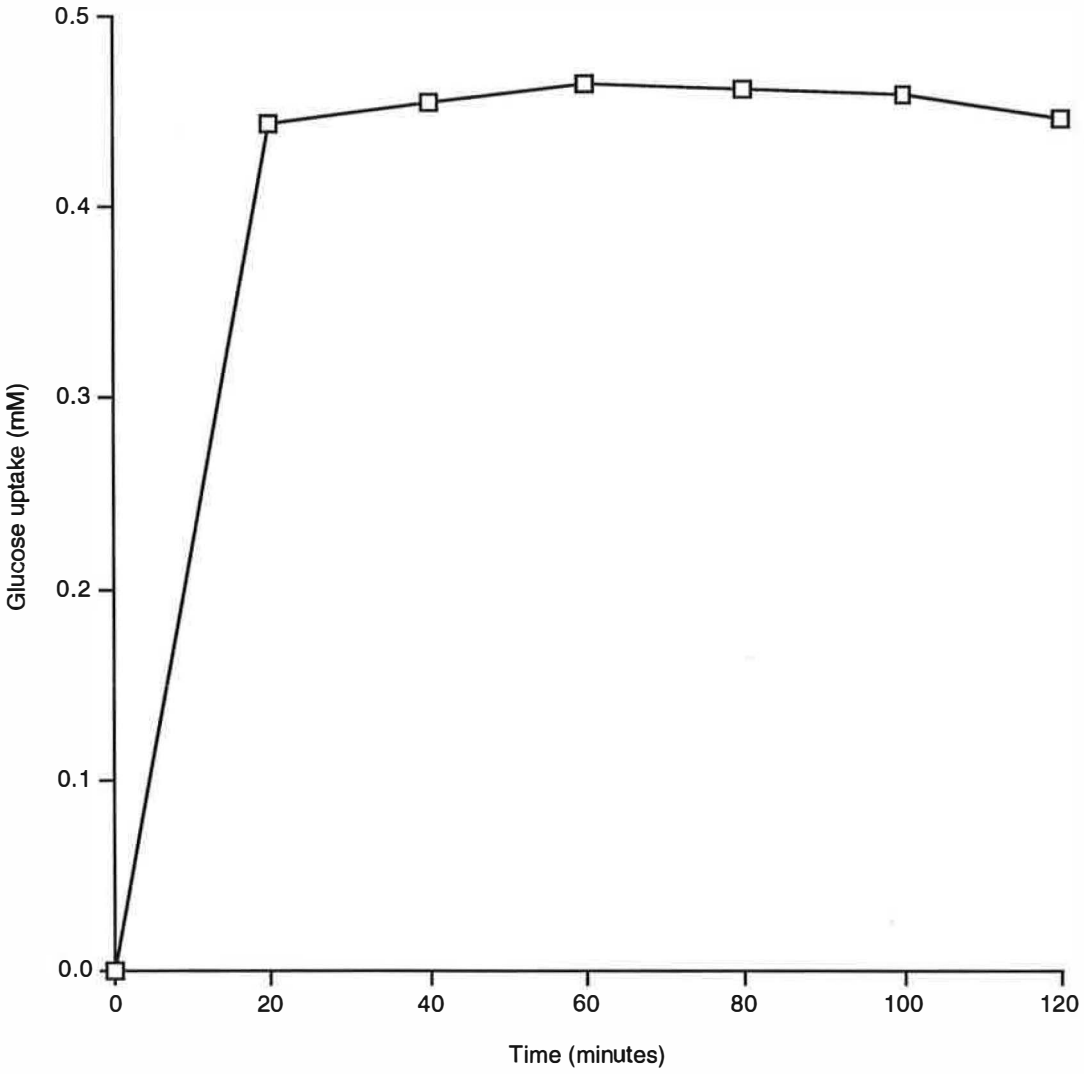
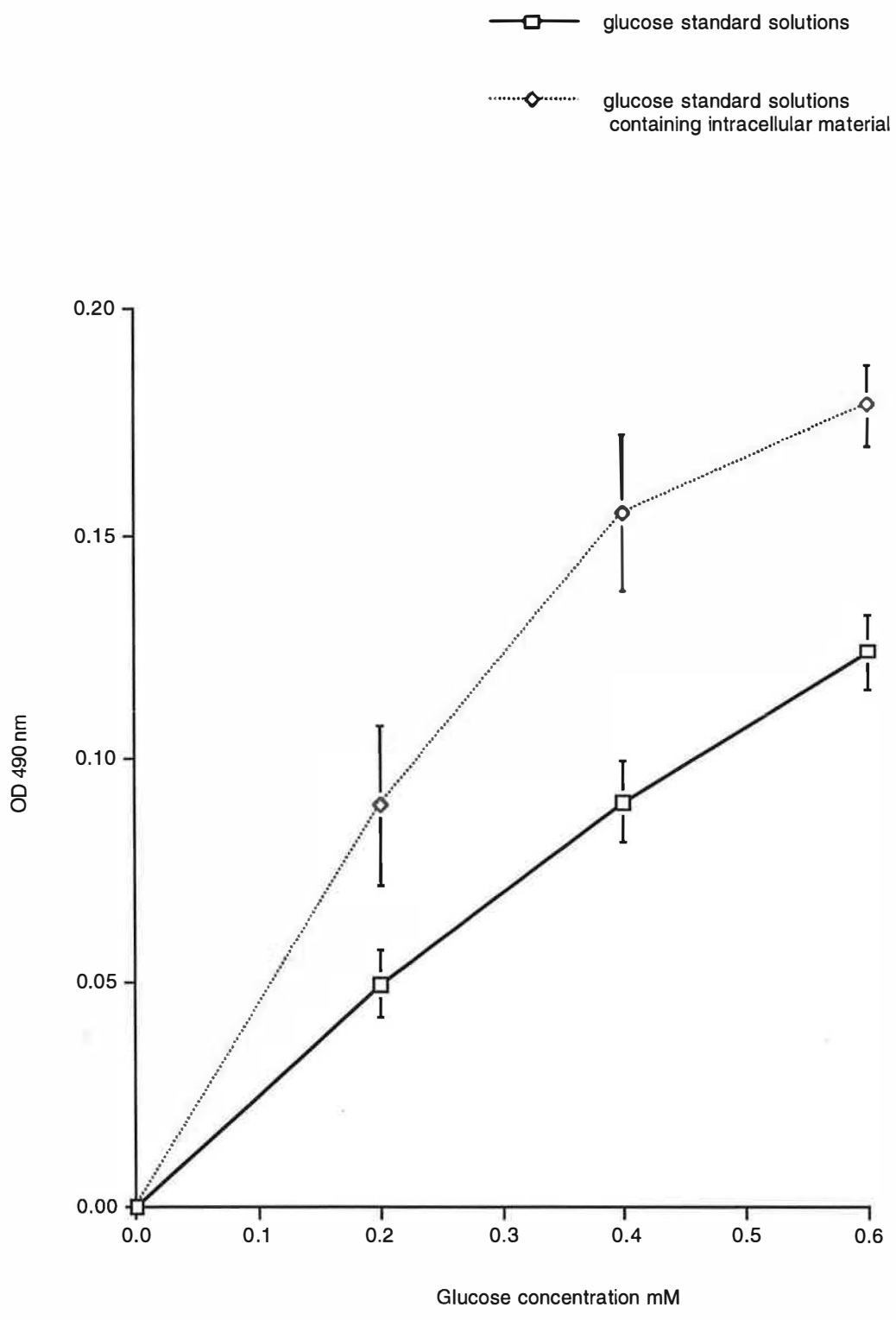


Figure 4.33 The effect of cytoplasmic material leaked from *E. coli* NCTC 8196on the phenol sulphuric acid assay for glucose (n=6, mean±sd)



4.4.2 Glucose oxidase assay

In light of the non-specific nature of the phenol sulphuric acid assay, an assay with greater specificity was sought. The glucose oxidase assay uses the enzyme-coupled glucose oxidase and peroxidase reaction with a chromogenic oxygen acceptor, o-dianisidine (Raabo & Terkildsen, 1960). The reactions involved can be seen below:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow{\text{Peroxidase}} \text{Oxidised o-Dianisidine}$$

colourless → brown

The intensity of the brown colour is then measured spectrophotometrically at 450 nm and is proportional to glucose concentration.

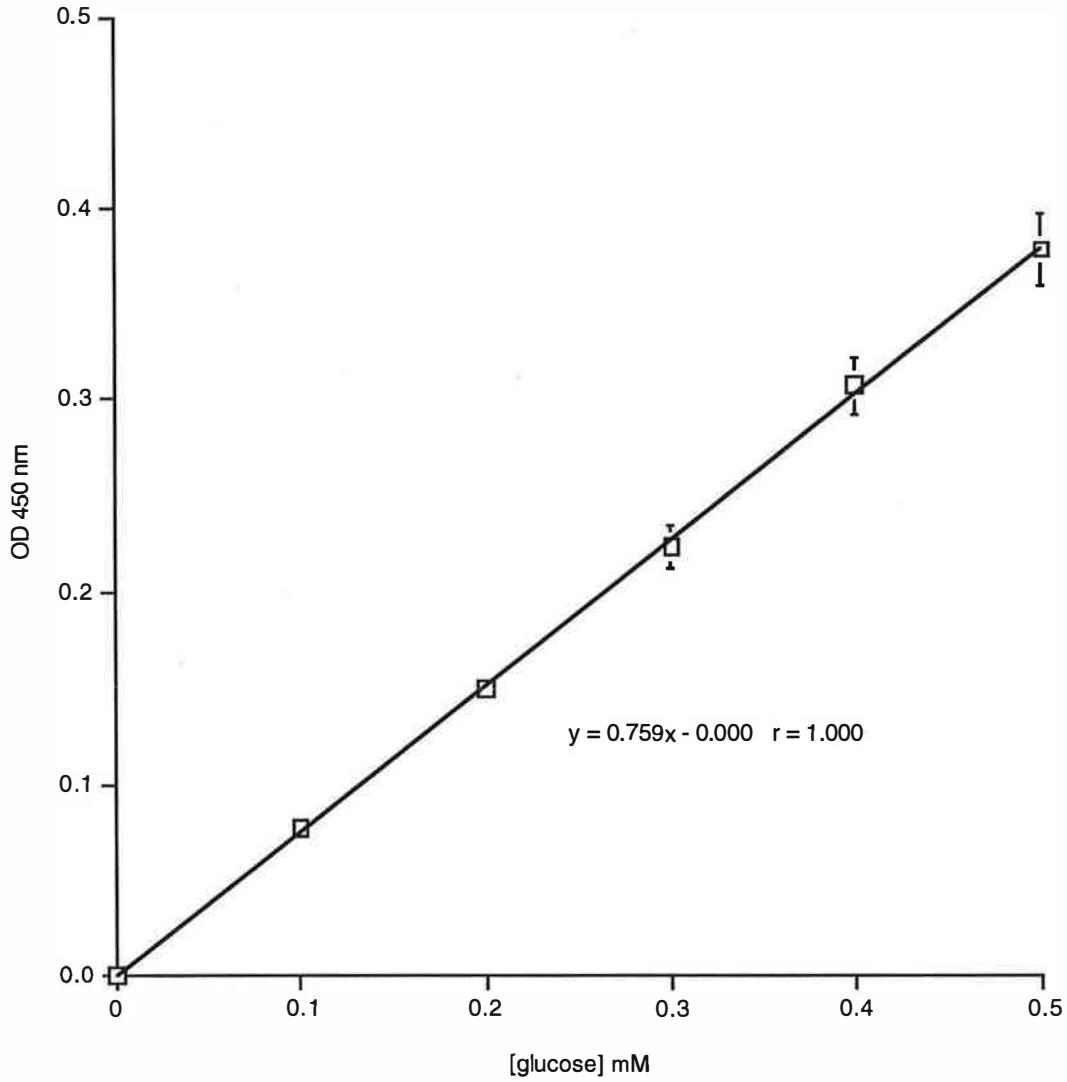
4.4.2.1 Assay validation

4.4.2.1.1 Preparation of calibration curves

Standard glucose solutions over the range 0.1 - 0.5 mM were prepared in McIlvaine's buffer. 5mL of combined enzyme colour reagent, which contained glucose oxidase, peroxidase, o-dianisidine hydrochloride and buffer salts (Sigma Diagnostics) were added to 0.5mL of each standard solution. The solutions were then thoroughly mixed and incubated at 37°C for 30 minutes. At the end of the incubation period the absorbance of the solutions at 450 nm was measured against a buffer blank treated in the same way as the standard solutions. Six replicates were carried out for

each standard solution. The mean absorbance of the replicates were plotted on the abscissa against solution concentration on the ordinate. The line through these points was linear and passed through the origin (see figure 4.34). The concentration of glucose present in an unknown solution could then, therefore be determined from the equation of the straight line of the calibration curve.

Figure 4.34 Calibration curve for glucose (n=6, mean±sd)



As the glucose assay utilises an enzyme the effect of the surfactants under test on the assay was determined. Standard glucose solutions of the following concentrations: 0.1, 0.2, 0.3, 0.4 and 0.5mM were prepared in McIlvaine's buffer, containing the highest concentrations of each of the surfactants under test. The samples were assayed and measured as described earlier. As can be seen from figures 4.35 and 4.36 the surfactants have an effect on the linear relationship between glucose concentration and absorbance. It was therefore decided to produce a separate calibration curve and linear regression equation for each concentration of the two surfactants used.

4.4.2.1.2 Stability of oxidised o-dianisidine

The stability of the oxidised o-dianisidine, at room temperature, over the course of 120 minutes was determined. 120 minutes was chosen as this would be the maximum time a sample would be left prior to measurement. 5 mL aliquots of combined enzyme-colour reagent were added to six 0.5 mL samples of standard glucose solutions and incubated as described earlier. The absorbance of the samples at 450 nm was measured against a buffer blank after incubation and at intervals for 120 minutes. The absorbance of oxidised o-dianisidine was stable for the time period tested.

4.4.2.2 Glucose uptake by *E. coli* NCTC 8196 using the glucose oxidase assay

18mL of McIlvaine's buffer containing glucose at a final concentration of 0.5 mM were placed in a universal bottle, incubated at 37˚C and allowed to equilibrate. 2mL of the washed and standardised cell suspension was

Figure 4.35 The effect of 1mM C10E6 on the calibration curve for glucose (n=6, mean±sd)

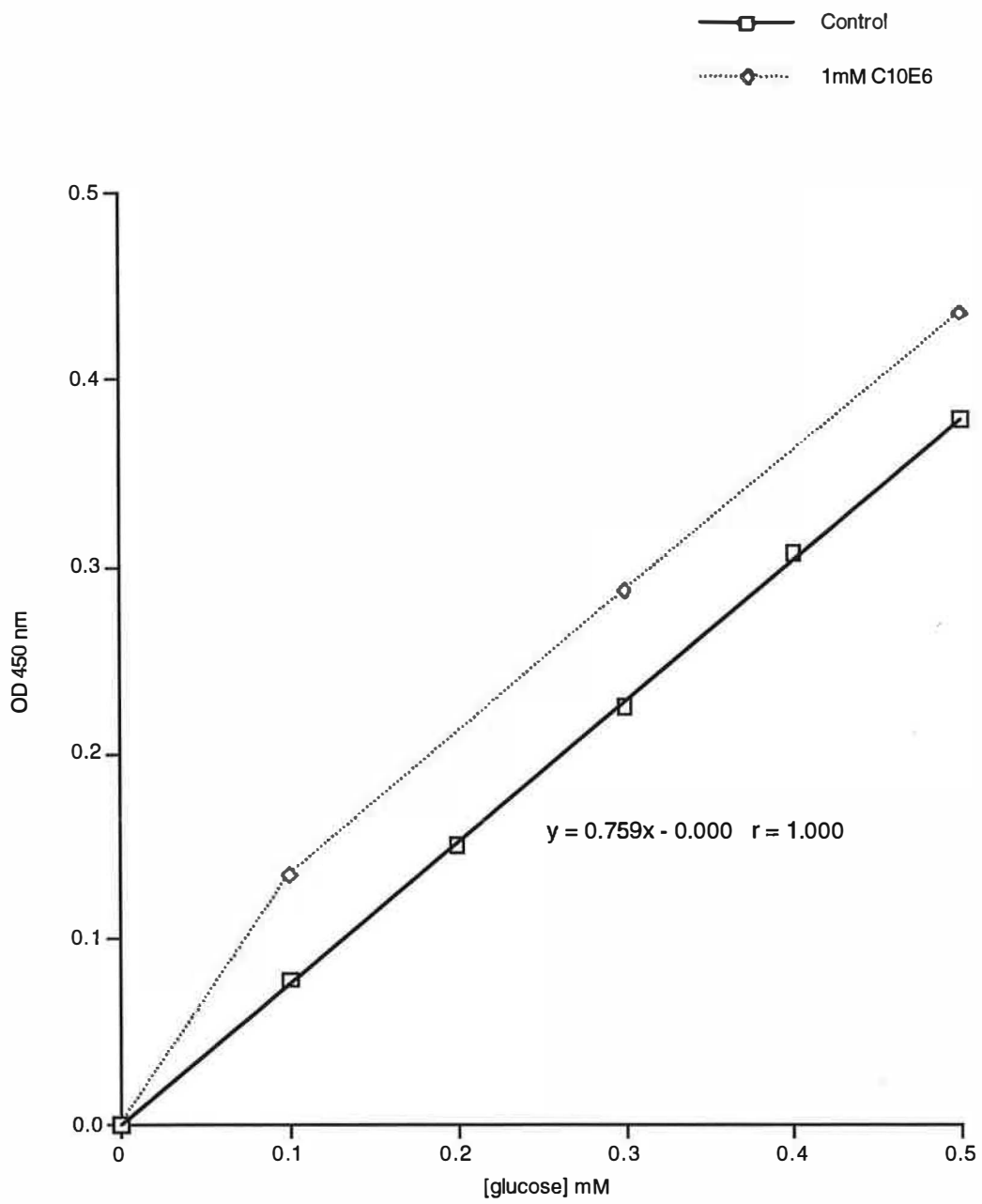
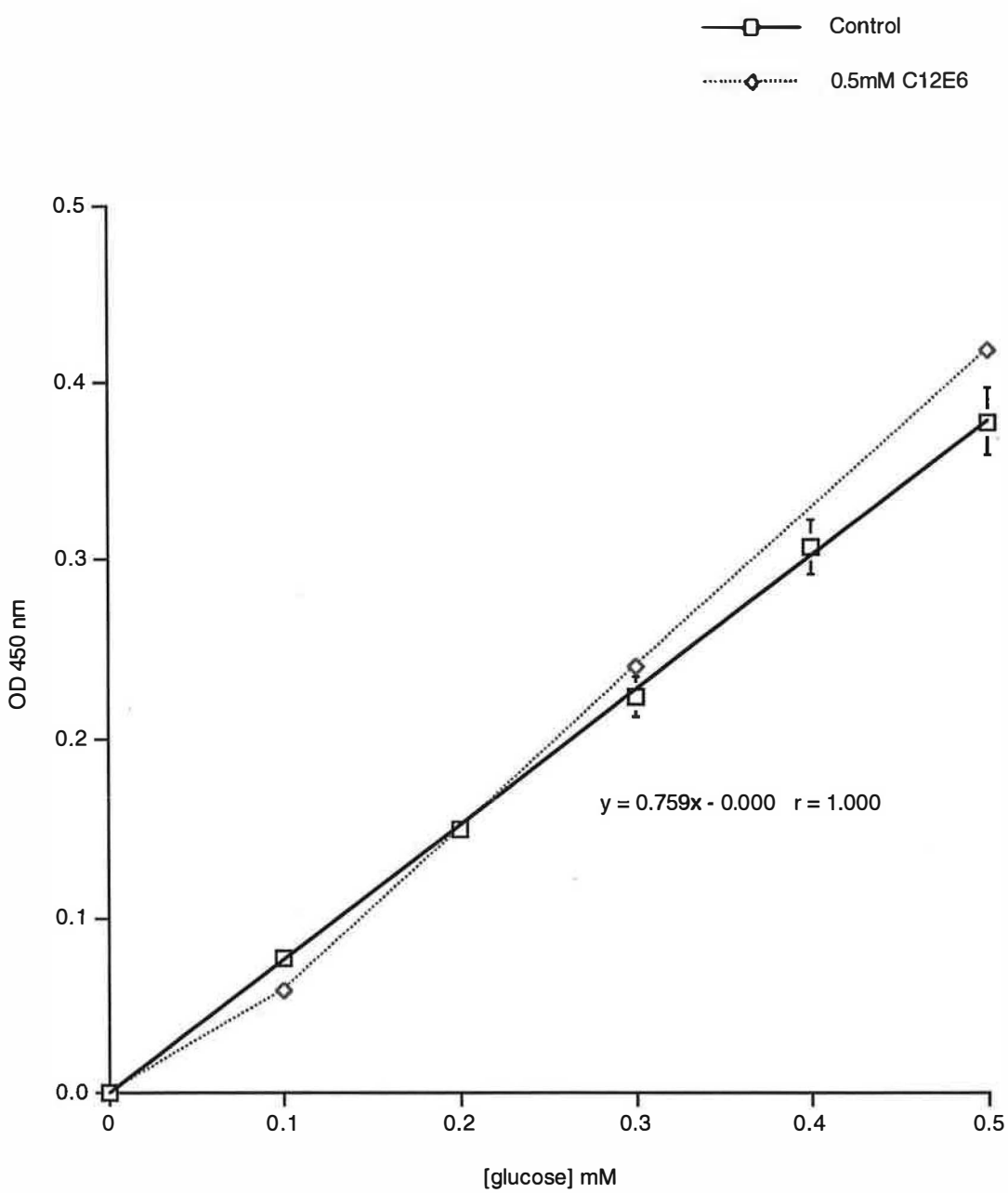


Figure 4.36 The effect of 0.5mM C12E6 on the calibration curve for glucose (n=6, mean±sd)



added to the McIlvaine's buffer to give a final concentration of $1x10^9$ cells/mL. The suspensions were then incubated at 37˚C and at 0, 5, 10, 20, 30, 45, and 60 minutes 2mL of the reaction mixture was removed with a syringe and filtered through a 0.2µm cellulose nitrate filter membrane. Six replicates performed out for each time point.

The concentration of glucose present in the filtrate was determined by adding 5mL of combined enzyme-colour reagent to 0.5mL of filtrate. The solutions were then incubated and the absorbances measured against a buffer blank as described in section 4.4.2.1 and the concentration of glucose determined from these values. Glucose uptake was then determined by difference. The results of these control samples can be seen in figures 4.37 and 4.38.

4.4.2.3 Glucose uptake by *E. coli* NCTC 8196 in the presence of alcohol ethoxylates

18mL of McIlvaine's buffer with glucose at a final concentration of 0.5 mM containing surfactants to give final concentrations of either 0.025; 0.05; 0.2; 0.5 or 1mM C10E6 or 0.005; 0.05; 0.2 or 0.5mM C12E6 were placed in a universal bottle.

The bottles and their contents were incubated at 37˚C and allowed to equilibrate. 2mL of the washed and standardised cell suspension were added to the reaction mixture to give a final concentration of 1×10^9 cells/mL. The suspensions were then incubated at 37˚C and 2mL of the reaction mixture were removed with a syringe and filtered through a 0.2µm cellulose nitrate membrane filter at 0, 5, 10, 20, 30, 45, and 60 minutes .

The concentration of glucose present in the filtrate was determined as described in section 4.2.1 and the absorbance measured against a blank of buffer and the appropriate concentration of either C10E6 or C12E6. Glucose uptake was measured as described in section 4.4.2.2. Six replicates were carried out for each time point. The results from these experiments can be seen in figures 4.37 and 4.38.

4.4.2.4 Results

Glucose uptake by *E. coli* NCTC 8196 occurred within the first 10 to 20 minutes of the course of the experiment and then reached a plateau. This plateau was at 0.5mM for the control cells, the maximum concentration of glucose available to the bacteria.

0.025 and 0.05mM C10E6 had very little effect on glucose uptake compared to the control. Some reduction in glucose uptake was seen with 0.2mM C10E6. Above the MIC of 0.2mM, at 0.5mM, approximately 30% reduction in glucose uptake was seen.

The pattern of glucose uptake in figure 4.38 was similar to that in figure 4.37. The plateau of uptake was seen within the same time scale in the presence of C12E6 as was seen with C10E6. At the lower concentrations of C12E6, i.e. 0.005mM and 0.05mM there was also very little change in glucose uptake compared to the control. 0.5mM C12E6, above the MIC of 0.12mM, reduced glucose uptake by approximately 50%.

Figure 4.37 Glucose uptake by *E. coli* NCTC 8196 in the presence of increasing concentrations of C10E6 (n=6, mean±sem)

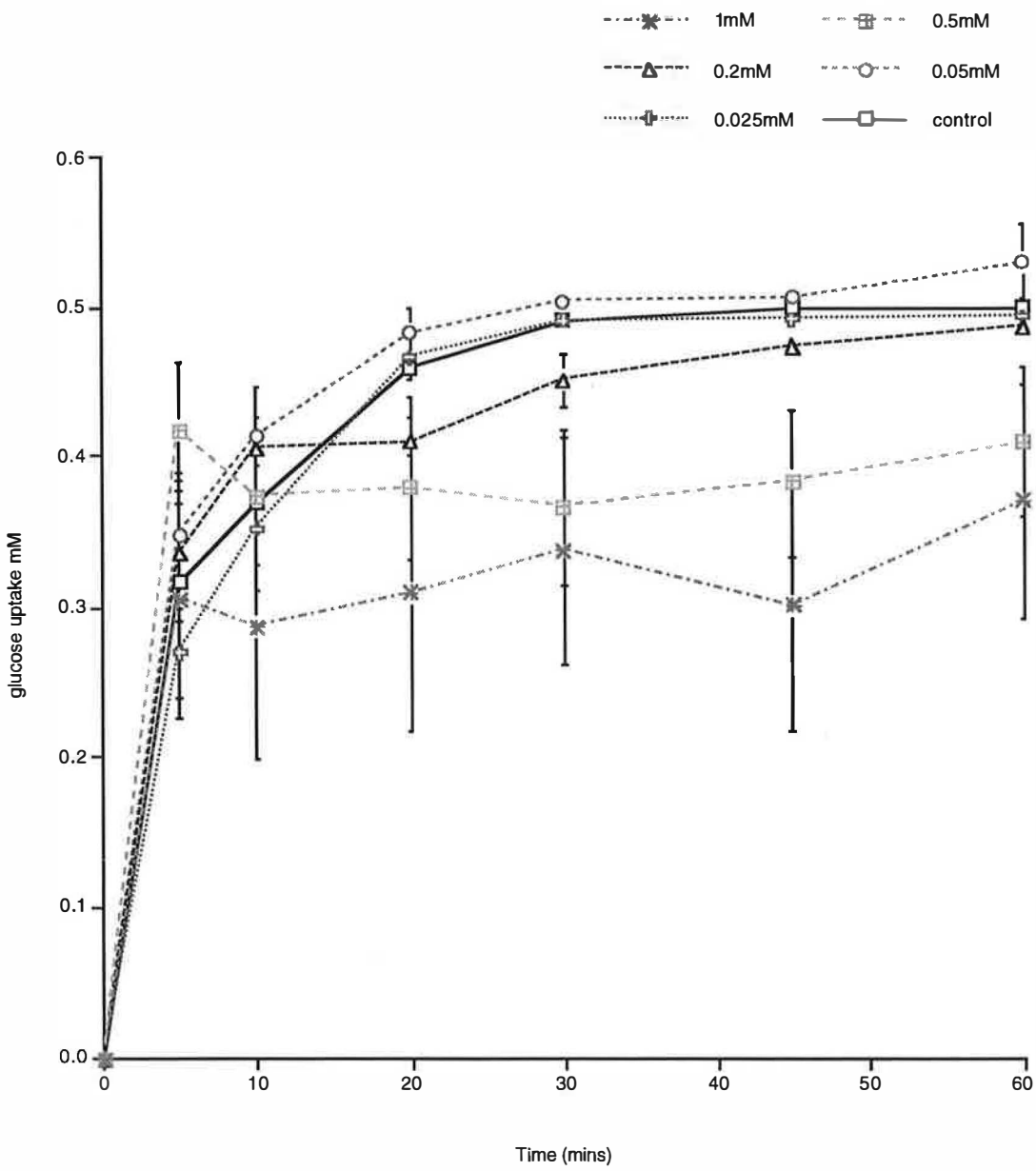
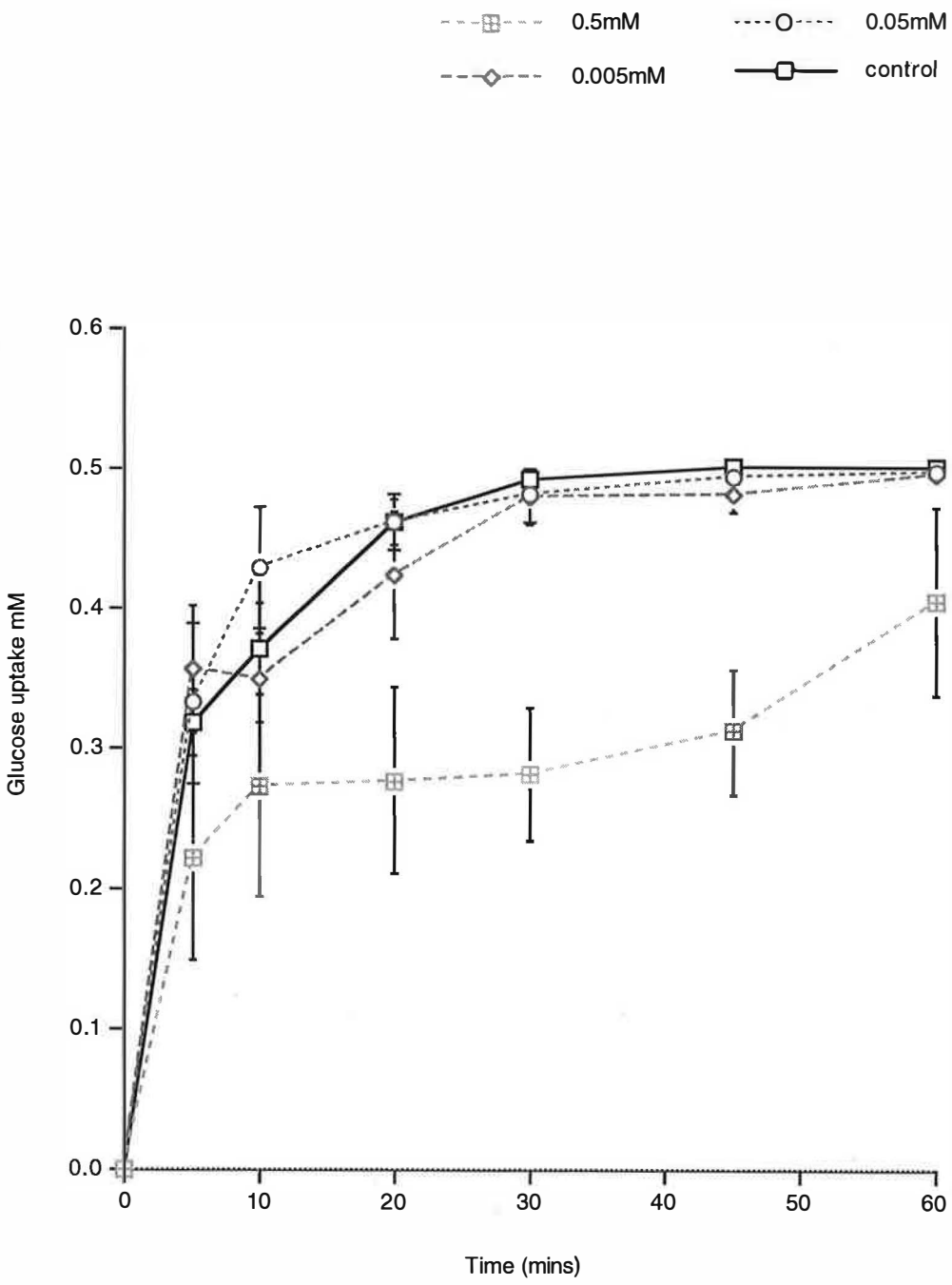


Figure 4.38 Glucose uptake by *E. coli* NCTC 8196 in the presence of increasing concentrations of C12E6 (n=6, mean±sem)



4.5 Discussion

Data from the oxygen electrode work demonstrated that C10E6 significantly inhibited the respiratory activity of *E. coli* NCTC 8196 at and below its MIC (0.20mM). The oxygen uptake experiments also demonstrated that the respiratory chain of *E. coli* is fully operational in the presence of 0.02 M glucose and depleting oxygen levels; these conditions are those employed in the tetrazolium salt studies.

The tetrazolium salt studies demonstrated that when using both glucose and succinate as substrates there was inhibition of dehydrogenase activity. However, at the early time points for almost every experiment, a stimulation of enzyme activity was seen. This has also been reported by Hugo and Longworth (1966) and Gilbert *et al* (1977b) with chlorhexidine and phenoxyethanol respectively. This stimulation has been suggested to be an indirect effect of the compounds uncoupling oxidative phosphorylation (Gilbert *et al*, 1976). At the later time points, significant inhibition is seen with higher surfactant concentration, particularly in the presence of C10E6 and C12E6. This suggests there is damage to membrane-bound dehydrogenase enzyme function and therefore disturbance of the respiratory chain. The pattern of inhibition with glucose and succinate is not the same for both surfactants. C10E6 shows similar inhibition in the presence of either substrate whilst C12E6 demonstrates greatly reduced formazan production in the presence of glucose compared to that seen with succinate. This suggests there could be a subtle difference in the effect of these two surfactants on the respiratory chain.

The inhibiton of glucose uptake in the presence of C10E6 and C12E6 is

minimal indicating that the effect on respiratory chain activity is not caused by glucose uptake.

Damage to the cytoplasmic membrane allows leakage of protons and other molecules which makes it increasingly difficult for the bacterial cell to maintain a proton motive force and hence respiratory activity. Therefore ATP hydrolysis occurs more readily in an attempt to restore the proton gradient. ATP yield in the presence of C10E6 and C12E6 was also much reduced, compared to the control, at the higher surfactant concentrations. It is possible that this reduction could be due in part to leakage of ATP and substrates and cofactors required for ATP synthesis from the cell in addition to a direct effect on ATPase itself (Shapiro & Guggenheim, 1995). It has been demonstrated that there is significant leakage from *E. coli* NCTC 8196 at the concentrations at which intracellular ATP levels are reduced (Chapter 5).

CHAPTER FIVE

The effect of alcohol ethoxylates on the permeability of the cytoplasmic membrane of *Escherichia coli* NCTC 8196

5.1 The effect of alcohol ethoxylates on potassium ion leakage from *E. coli* NCTC 8196

The bacterial cytoplasmic membrane is a major site of action for many anti-bacterial compounds. Membrane-active agents can result in a change in the fluidity and/or permeability of the membrane. These changes can be followed by the leakage of cellular constituents such as potassium ions and/or pentose sugars. The leakage of intracellular potassium ions has been used as an indicator of damage to the membrane integrity of the bacterial cell following addition of biocide (Lambert and Hammond, 1973) and therefore enables assessment of biocidal capability. Potassium leakage occurs very rapidly after contact with an antibacterial agent and therefore is an effective marker of subtle alterations to the permeability of the cytoplasmic membrane. The study of potassium ion leakage may therefore provide an insight into the mode of action of the surfactants under investigation.

5.1.1 Method development

Cultures of *E. coli* NCTC 8196 were grown for 15 hours at 37˚C on a Roux slope composed of synthetic broth AOAC (Difco Laboratories) solidified with 1.5%w/v Agar (Oxoid) supplemented with 0.1%w/v potassium chloride to encourage enhanced intracellular potassium ion concentration. The bacteria were harvested by washing the surface of the slope with 100mL 0.1M magnesium chloride (AnalaR, BDH) solution. The magnesium enables the bacteria to resist various stresses (Tempest and Strange 1966) thus helping to maintain the intracellular potassium ion pool by reducing cell damage during the harvesting and washing process. The cell suspension

was centrifuged at 2,100g for 20 minutes and the pellet washed twice with 25mL magnesium chloride solution. The washed cell pellet was standardised to give a cell concentration of 1x 10^{10} cells/mL in McIlvaine's buffer.

I8mL aliquots of McIlvaine's buffer were pipetted into universal bottles and placed in a water bath at 37°C for the temperature to equilibrate. 2mL of 1 x10^{10} cells/mL suspension of *E. coli* NCTC 8196 was added, mixed thoroughly and a 1 mL sample removed immediately for analysis. Further samples were removed at various time intervals over the next 60 minutes. The samples were centrifuged at 12,000rpm (Denley eppendorf centrifuge) for 2 minutes. The supernatant liquid was removed, diluted 1 in 5 with McIlvaine's buffer and analysed for potassium ions.

Flame Photometry (Corning 410 Flame Photometer) was initially used to analyse the supernatant liquid (Allwood & Hugo, 1971; Bernheim, 1984). This technique was found not to be sensitive enough to analyse the samples for potassium ion concentration and a Perkin Elmer M1100 Atomic Absorption Spectrophotometer in the Flame Emission mode was used instead.

Analysis of these samples showed that potassium ion leakage was occurring within 5 minutes of the initiation of the experiment and therefore it was necessary to take samples at very short time intervals after addition of biocide. The initial step in processing the sample was to centrifuge to remove the cells, however, as this process took at least 2 minutes it was not possible to take samples that accurately reflected the potassium ion leakage

at the early time points. This processing time was reduced by removing the cells from the sample by filtration. A 2mL sample was removed from the reaction vessel with a syringe and passed through a 0.2µm cellulose nitrate filter membrane (Nalgene) and the filtrate retained for subsequent analysis.

5.1.2 Method Validation

5.1.2.1 Calibration of the Perkin Elmer M110 Atomic Absorption Spectrophotometer

Perkin Elmer M1100 Atomic Absorption Spectrophotometer in the Flame Emission mode was used to assay the supernatant samples for potassium ion content. Prior to analysing the samples the instrument was calibrated using the following series of standard solutions of potassium chloride (analytical grade) in McIlvaine's buffer (prepared with analytical grade reagents): 3; 6; 9; 15; 21; 27; and 30µM. A linear relationship between potassium concentration and emission was seen (appendix 3).

A standard solution of potassium chloride in McIlvaine's buffer was measured at frequent intervals during analysis of samples for potassium ion content. This ensured that any problems with the aspiration of the sample would be detected therefore reducing the risk of any systematic errors.

To determine the reproducibility of measurements obtained from the instrument a solution of known concentration of potassium chloride in McIlvaine's buffer was measured six times. There was found to be no significant difference between measurements obtained from the same solution.

5.1.2.2 The effect of filtration on the measured potassium ion concentration

To determine the effect of filtration on the potassium ion concentration within the supernatant liquid samples, six 2mL aliquots of McIlvaine's buffer were filtered and then diluted prior to analysis as described earlier (section 5.1.1). The samples were analysed for potassium ion content as were six aliquots of unfiltered buffer. There was no significant difference in the filtered samples compared to the unfiltered samples ($p>0.05$, Mann Whitney).

5.1.2.3 The effect of alcohol ethoxylates on the measured potassium ion concentration

To determine the effect of the alcohol ethoxylates and cetrimide (to be used as a known membrane disruptor releasing the potassium ion pool) on potassium ion concentration measurement, potassium chloride solutions of known concentration in McIlvaine's buffer were prepared containing either cetrimide, 1mM C10E6 or 0.5mM C12E6 (the highest concentrations of the alcohol ethoxylates that would be used during the course of the experiments) or no surfactant. These samples were then diluted and analysed for potassium ion content as before and compared against corresponding samples containing potassium ions alone. The samples containing either cetrimide or alcohol ethoxylate did not have a significantly different potassium ion content to those without ($p>0.05$, Mann Whitney).

5.1.3.1 Leakage of potassium ions from *E. coli* NCTC 8196

E. coli NCTC 8196 was grown and harvested as described in section 5.1.1.

To minimise the leakage of potassium ions into buffer prior to the start of the experiment 2mL aliquots of washed and standardised bacterial cell suspension were placed into tubes and centrifuged at 2,100g for 5 minutes. The supernatant was discarded and the cell pellets reconstituted with 2mL McIlvaine's buffer (pH 7.0) immediately prior to use.

To determine potassium ion leakage the reconstituted cell pellets were added to 18mL McIlvaine's buffer prewarmed to 37°C as described previously (section 5.1.1). 2mL samples were removed at 0, 2·5, 5, 10, 20, 30, 45 and 60 minutes and filtered through a 0.2µm cellulose nitrate membrane filter to remove the bacteria; the filtrate was frozen prior to analysis for potassium ion content. For analysis the samples were diluted 1 in 5 with McIlvaine's buffer and examined for potassium ion content using the Perkin Elmer M1100 Atomic Absorption Spectrophotometer in the Flame Emission mode.

5.1.3.2 Determination of the intracellular potassium ion pool of *E. coli* NCTC 8196

To determine the intracellular potassium ion pool the cells were reconstituted with 2mL McIlvaine's buffer (pH 7.0), added to 18mL McIlvaine's buffer containing 1 mM cetrimide (prewarmed to 37°C), mixed and incubated at 37°C for 10 minutes, the action of the cetrimide causing

the release of the intracellular potassium pool. Six 2mL samples were then withdrawn and filtered through a 0.2 μm cellulose nitrate membrane filter to remove the bacteria and the filtrate was frozen until analysis for potassium ion content.

5.1.3.3 Leakage of potassium ions from *E. coli* NCTC 8196 in the presence of alcohol ethoxylates

To determine potassium ion leakage induced by surfactant the cell pellets were reconstituted with 2mL McIlvaine's buffer and added to 18mL McIlvaine's buffer containing either C10E6 (to give final concentrations of 0.025, 0.05, 0.2, 0.5 and 1 mM) or C12E6 (to give final concentrations of 0.005, 0.05, 0.2 and 0.5 mM). 2mL samples were removed at 2·5, 5, 10, 20, 30, 45 and 60 minutes and filtered through a 0.2μm cellulose nitrate membrane filter to remove the bacteria and the filtrate frozen until analysis for potassium ion content. The results can be seen in figures 5.2 and 5.3.

5 1.4 Results

Potassium ion leakage was seen in the presence of both C10E6 and C12E6 (figures 5.2 and 5.3); however, the leakage from control cells was higher than was expected. It was thought that this was a consequence of the harvesting and washing procedure despite the use of magnesium chloride which helps to protect cells from various stresses (Tempest and Strange, 1966). However, significant leakage over and above that seen with the controls was induced in the presence of the higher concentrations of alcohol ethoxylates.

Figure 5.1 Potassium ion leakage from *E. coli* NCTC 8196 in the presence of increasing concentrations of C10E6 (n=6,mean±sem)

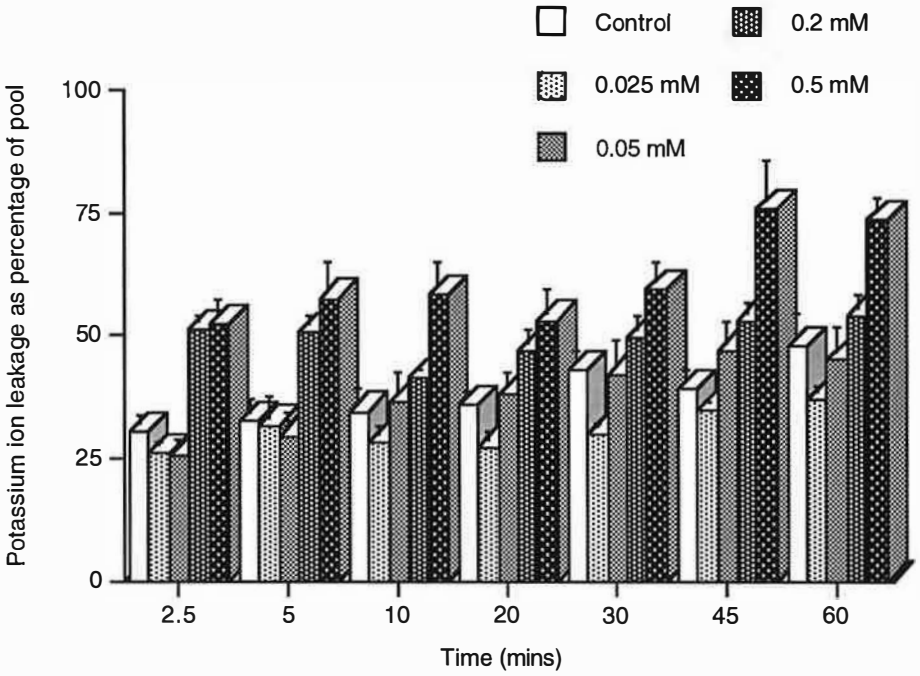
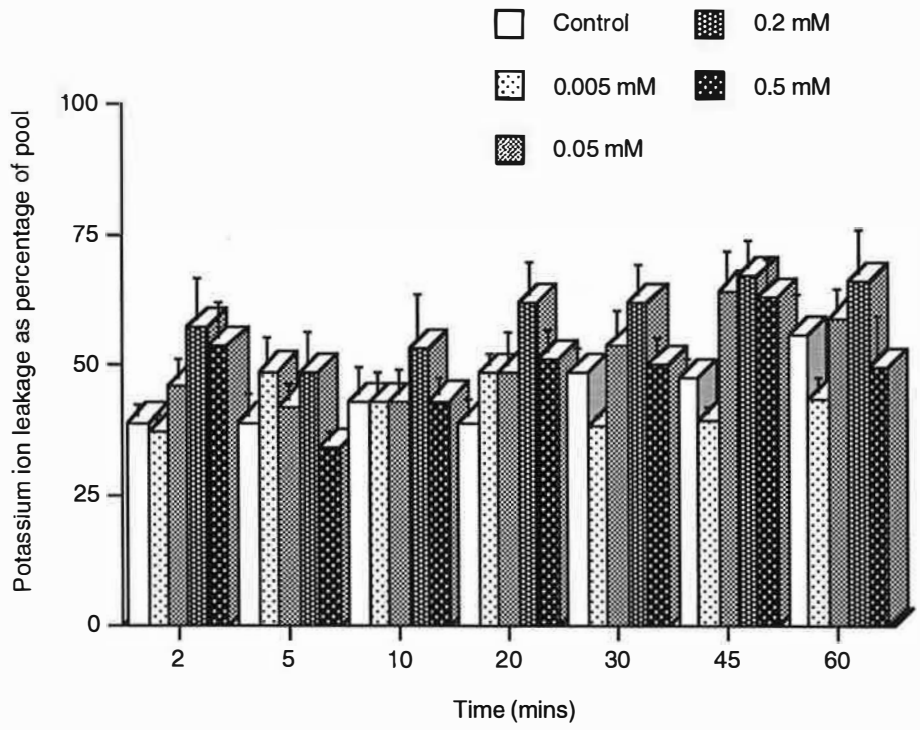


Figure 5.2 Potassium ion leakage from *E. coli* NCTC 8196 in the presence of increasing concentrations of C12E6 (n=6, mean±sem)



At the lowest concentration of C10E6 (0.025mM) potassium ion leakage was lower than that seen with the control. This was also the case with the lowest concentration of C12E6 (0.005mM). This could be due to a 'sealing-in' effect where there is a reduction in leakage (Hugo & Bowen, 1973). In this situation low concentrations of surfactant protect the membrane from potassium ion leakage; this may be due to surfactant monomers introducing themselves into the cytoplasmic membrane thus reducing the fluidity of the membrane and therefore leakage. As the concentration of surfactant in the membrane increases so too would disruption to the membrane structure and therefore the cytoplasmic constituents would begin to leak out.

Potassium ion leakage occurs in a concentration-dependent manner in the presence of C10E6. Significant potassium leakage occurs at the MIC of 0.20mM ($p=0.0136$, Mann Whitney) and above: no leakage in excess of the control is seen below this concentration of C10E6. With C12E6 the pattern is quite different. Potassium ion leakage is seen at all the concentrations above 0.005mM, with the exception of 0.5mM which actually produces less leakage than 0.2mM. Leakage occurs both above and below the MIC of 0.12mM in the presence of C12E6. Leakage in all cases is very rapid, within the first few minutes, and there is no further significant increase in potassium ion leakage with time.

The results are also illustrated in response surface graphs with concentration and leakage plotted with time (figures 5.3 and 5.4).

Figure 5.3 Response surface graph of potassium ion leakage, with time from *E. coli* NCTC 8196 in the presence of increasing concentrations of C10E6

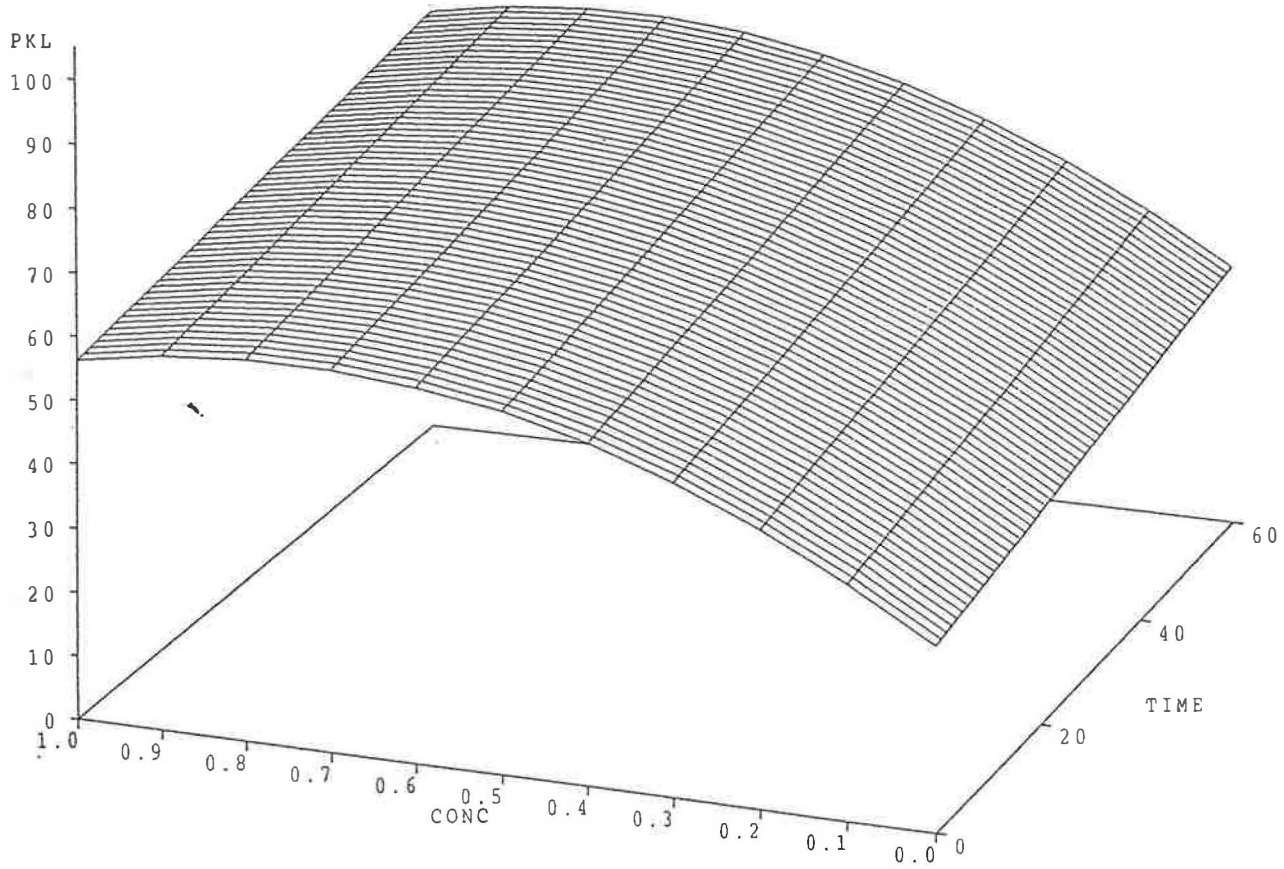
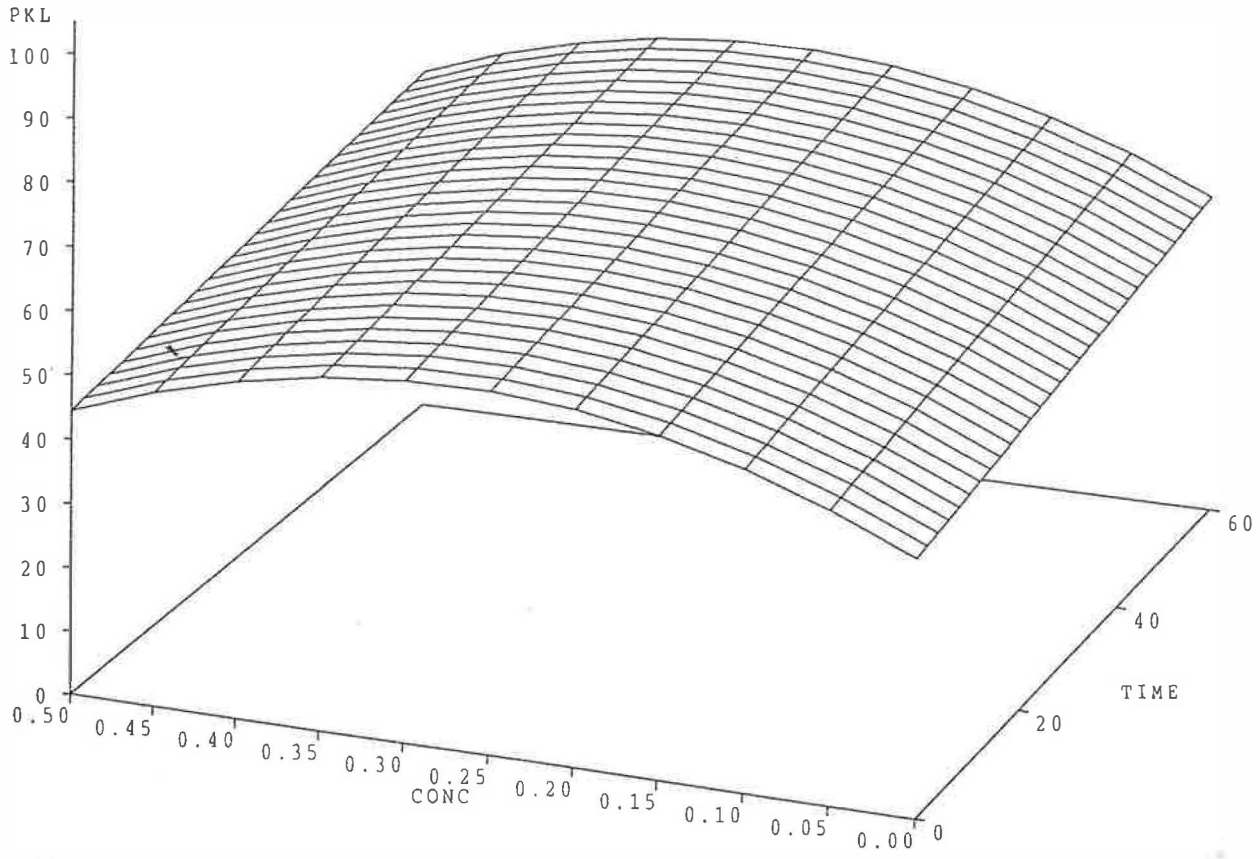


Figure 5.4 Response surface graph of potassium ion leakage, with time from *E. coli* NCTC 8196 in the presence of increasing concentrations of C12E6



5.2 The effect of alcohol ethoxylates on the leakage of 260 nm absorbing material from *E. coli* NCTC 8196

The leakage of nucleotides and their constituents (pyrimidines and purines) in the presence of membrane-active compounds can be observed by absorbance measurements (at 260 nm) of cell-free exudates. Such leakage is dependent not only on biocide concentration but also on the organism itself, its metabolic pool, and the conditions under which leakage is being studied.

At physiological temperatures (35-40˚C) a rapid release of 260 nm absorbing material occurs which is followed by a more gradual leakage (Salton, 1951; Hugo and Longworth, 1964; Hugo and Frier, 1969; and Hugo and Bloomfield, 1971b). It has been proposed that this secondary leakage is due to the activation of latent ribonucleases which break down ribosomal RNA (Lambert and Smith, 1976).

5.2.1 Determination of the metabolic pool of 260 nm absorbing material from *E. coli* NCTC 8196

Cultures of *E. coli* NCTC 8196 were grown for 15 hours at 37˚C on a Roux slope composed of synthetic broth AOAC (Difco Laboratories) solidified with 1.5%w/v Agar (Oxoid). The bacteria were harvested by washing the surface of the slope with 40mL McIlvaine's buffer (pH 7.0). The cell suspension was centrifuged at 2,100g for 20 minutes and then washed twice with 25mL McIlvaine's buffer.

To determine the nature of the 260 nm absorbing material, i.e. soluble

material constituting the metabolic pool and insoluble material from the autolysis of nucleic acids, the trichloroacetic acid (TCA) method of Gale and Folkes (1953) was used. 10 mL of a 1 x 10^9 cells/mL suspension of *E. coli* NCTC 8196 in 5%w/v TCA was incubated for 2 hours at 20˚C. This extracts the soluble metabolic pool, the cell suspension was then centrifuged for 15 minutes at 2,100g (Denley Centrifuge) and the supernatant liquid filtered through a 0.2µm cellulose nitrate membrane filter. The remaining cell pellet was then extracted a further three times with 5%w/v TCA at 100˚C for 10 minutes and filtered as described previously. Three extractions with hot TCA was found by Gale and Folkes to be sufficient to release all the 260 nm absorbing material from the cells. The absorbance of all the extracts was measured at 260 nm against a 5%w/v TCA blank. The results from this experiment can be seen in table 5.1.

Table 5.1 Characterisation of the 260 nm absorbing material from *E. colli* NCTC 8196

EXTRACTION METHOD	NATURE OF EXTRACTED MATERIAL	OD 260 nm
Cold TCA	Metabolic pool	0.676
Hot TCA	Structural material	2.087
Total TCA soluble material	Total nucleic acid material	2.763

5.2.2 Method Validation

To determine whether the alcohol ethoxylates absorbed at 260 nm and if so to what extent, the following alcohol ethoxylate solutions in McIlvaine's buffer were prepared: 0.025, 0.05, 0.2, 0.5 and 1 mM solutions of C10E6; and 0.005, 0.05, 0.2 and 0.5 mM solutions of C12E6. The solutions were scanned from 225 to 325 nm using a Perkin Elmer Lambda 12 Spectrophotometer. The alcohol ethoxylates absorb very weakly in this range, with an absorbance of less than 0.05; therefore it was decided to take this into account by measuring the samples against a blank solution comprising McIlvaine's buffer and the appropriate concentration of alcohol ethoxylate.

5.2.3 Leakage of 260 nm absorbing material from *E. coli* NCTC 8196

E. coli NCTC 8196 was grown and harvested as described in section 5.2.1. The cell suspension was standardised to give a concentration of 1 x 10^{10}cells/mL. 18mL of McIlvaine's buffer was placed in a universal bottle and allowed to equilibrate at 37˚C. 2mL of the cell suspension were added to the McIlvaine's buffer to give a final concentration of 1 x 10^9 cells/mL. The suspensions were then incubated at 37˚C. 2mL samples were removed at 0, 5, 10, 20, 30, 45, 60, 120 and 240 minutes and filtered through a 0.2µm cellulose nitrate membrane filter. The samples were diluted 1 in 5 with McIlvaine's buffer and measured at 260 nm against a blank of McIlvaine's buffer.

5.2.4 Leakage of 260 nm absorbing material from *E. coli* NCTC 8196 in the presence of alcohol ethoxylates

E. coli NCTC 8196 was grown and harvested as described in section 5.2.1. The cell suspension was standardised to give a concentration of 1 x 10^{10} cells/mL. 18mL of McIlvaine's buffer containing either C10E6 (to give final concentrations of 0.025, 0.05, 0.2, 0.5 and 1 mM) or C12E6 (to give final concentrations of 0.005, 0.05, 0.2 and 0.5 mM) were placed in universal bottles and allowed to equilibrate at 37°C. 2mL of the cell suspension were then added to the solutions to give a final concentration of 1 x 10^9 cells/mL. The suspensions were then incubated at 37°C and at 5, 10, 20, 30, 45, 60, 120 and 240 minutes 2mL of the reaction mixture removed with a syringe and filtered through a 0.2µm cellulose nitrate membrane filter.

The samples were frozen until subsequent dilution and analysis at 260 nm. The samples were measured against a blank solution containing the appropriate concentration of C10E6 or C12E6 and diluted with McIlvaine's buffer to the same degree as the samples. The results from this experiment can be seen in figures 5.4 and 5.5.

5 2.5 Results

The patterns of release of 260 nm absorbing material were similar to those seen for potassium ion leakage. The presence of low concentrations of both C10E6 and C12E6 caused a reduced level of leakage compared to that seen with the control. This is likely to be a consequence of the 'sealing-in' effect, as described in section 5.1.4.

Figure 5.5 260 nm leakage from *E. coli* NCTC 8196 in the presence of increasing concentrations of C10E6 (n=6, mean±sem)

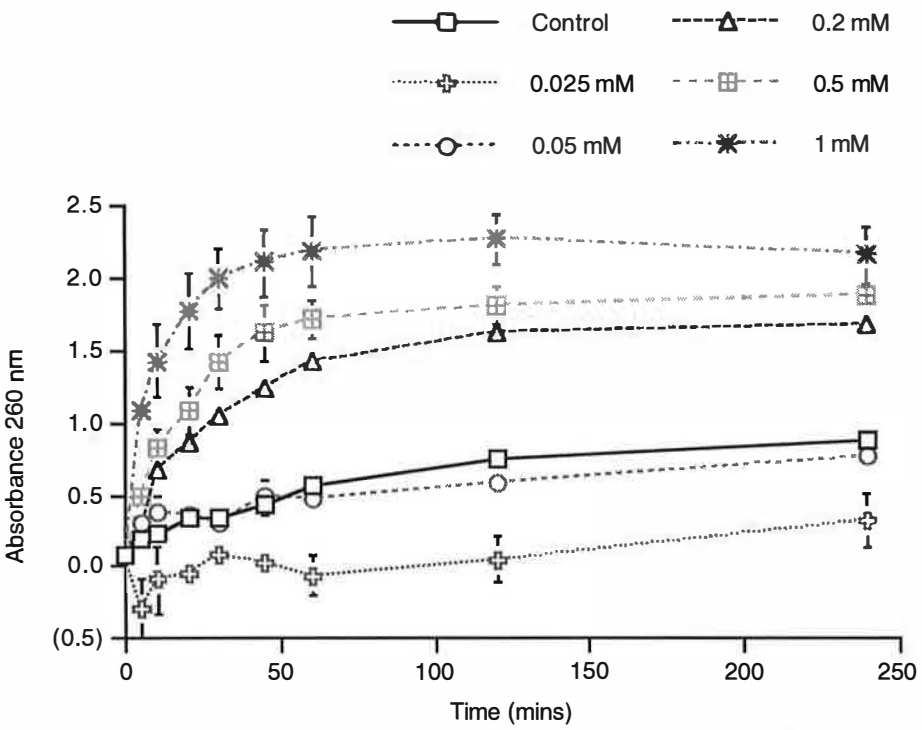
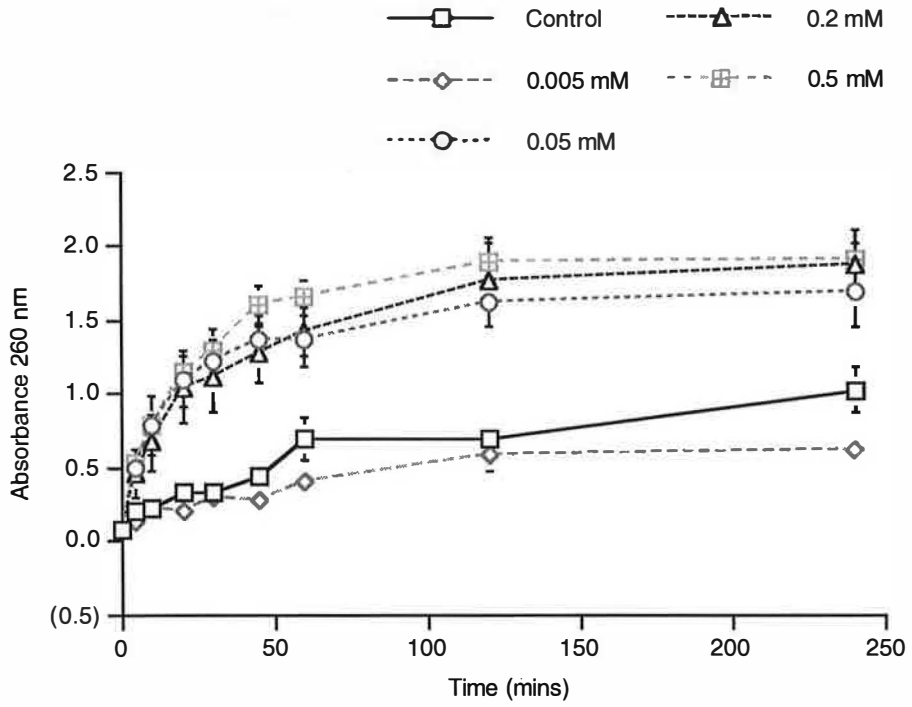


Figure 5.6 260nm leakage from *E. coli* NCTC 8196 in the presence of increasing concentrations of C12E6 (n=6, mean±sem)



There is a concentration dependent relationship with 260 nm absorbing material release for both C10E6 and C12E6. As the concentration of alcohol ethoxylate increased, so did the amount of 260 nm material released. As with the potassium ion leakage, 260 nm absorbing material was not released until the MIC (0.20mM) was reached for C10E6. In contrast, for C12E6, release of 260 nm absorbing material was seen at all but the lowest concentration.

Where significant release occurred, this was in excess of the metabolic pool indicating that nucleic acids were being broken down and therefore autolysis was occurring. At these concentrations release was rapid during the first 10 to 20 minutes, after which the rate of release decreased and reached a plateau at 60 minutes. Comparing like concentrations of C10E6 and C12E6 both compounds appeared to have been equally effective at causing the release of 260 nm absorbing material.

5.3 Discussion

Both C10E6 and C12E6 altered the permeability of the cytoplasmic membrane of *E. coli* NCTC 8196 causing leakage of cytoplasmic constituents. Low levels of both surfactants appeared to protect the cytoplasmic membrane from damage as leakage of both 260nm absorbing material and potassium was less than that seen from the control. This could be due to a 'sealing-in' effect; in this situation surfactant monomers may fit into spaces within the cytoplasmic membrane thus reducing the fluidity of the membrane and therefore leakage.

Potassium ion and 260nm material leakage from *E. coli* NCTC 8196 in the

presence of C10E6 occurred in a concentration-dependent manner as seen by Lamikanra (1982) who found that treatment of *S. aureus* and *E. coli* with butyl hydroxyanisole (BHA) caused leakage of intracellular materials, leakage increasing as biocide concentration increased. The data show that approximately 50% of the potassium ions are released from the cells within the first five minutes of contact. This early leakage has been seen by other workers (Kroll & Anagnostopoulos, 1981; Chawner and Gilbert, 1989; Mlynarcik *et al,* 1992). As contact time with the surfactants lengthens 260nm material is increasingly released. This could be due to progressive membrane damage, early leakage consists of small potassium ions and as the contact time increases the discontinuities in the membrane increases allowing leakage of the larger 260 nm material. High levels of 260nm material, in excess of that from the metabolic pool are due to the autolytic breakdown of insoluble nucleic acid (Hugo & Bloomfield, 1971b).

The chemiosmotic theory (Mitchell, 1961, 1966) proposed that the production of ATP by the cell was a consequence of the proton gradient across the membrane of the cell, as are some transport processes. If this gradient is disturbed as it must be when the permeability of the membrane is disrupted sufficiently to allow leakage of the cellular constituents then these processes will be affected. Therefore the effect of C10E6 and C12E6 on the permeability of the cytoplasmic membrane may cause the inhibition of ATP-ase and glucose uptake illustrated in Chapter 4.

CHAPTER SIX

Discussion

6 Discussion

6.1 Growth inhibition tests

When conducting growth inhibition studies it is important to have an understanding of those parameters which may influence the outcome of tests. Inoculum size is an important parameter in MIC determinations since the inoculum size used in determining the MIC for a particular compound can greatly affect the value obtained (Al-Hiti and Gilbert, 1980). In addition to inoculum size, pH, temperature and medium composition have also been shown to affect MIC determinations. In this thesis the original screen for antibacterial activity was carried out in synthetic broth AOAC, a nutrient growth media, using $1x10^7$ cells/mL. The biochemical tests used to probe the mechanism of action of the homologous series of alcohol ethoxylates on the other hand were carried out in non-growth media, McIlvaine's buffer using $1x10^9$ cells/mL. Growth supporting media may enable repair for drug induced damage and therefore organisms may not be as susceptible to the drug in nutrient media as they would be in buffer. However, the use of $1x10^7$ cells/mL and synthetic broth were chosen for a number of methodological reasons. The method employed (section 2.6) required the determination of the presence or absence of growth by examining bacterial growth by visible turbidity, therefore the use of a higher inoculum would have its own inherent turbidity. To enable the inoculum to grow in the presence of non-inhibiting concentrations of surfactant, a growth supporting media was required and therefore synthetic broth AOAC, not McIlvaine's buffer was used. The surfactants were generally believed to exert bacteriostatic rather than bactericidal activity and it was felt it would be easier to distinguish between

growth and inhibition of growth if the inoculum size was smaller, thus the organic load would be smaller. The use of the smaller inoculum may also prevent higher concentrations of surfactants being required and therefore preventing any solubility problems which may arise for some of the surfactants under test. An inoculum size of $1x10^9$ cells/mL would also require significant oxygenation during the course of the experiment which would not be necessary for a smaller inoculum size. Therefore the use of the smaller inoculum would prevent problems in later experiments, for example the tetrazolium salt experiments, as oxygen is a competitive antagonist for formazan production. The determination of MICs for the surfactants was used as a screening process to rank the surfactants for antibacterial property.

Earlier work has shown many anionic and nonionic detergents to have bacteriostatic activity but a general absence of bactericidal activity (Cowles, 1938; Birkeland & Steinhaus, 1939; Baker *et al,* 1941, Scales and Kemp, 1941; James, 1948; Armstrong, 1957 and Kabara, 1978). The anionic and nonionic surfactants studied in the initial screen for antibacterial activity were all growth inhibitory against $1x10^7$ cells/mL *E.coli* NCTC 8196 and *S. aureus* NCTC 4163 after 6 and 24 hours incubation in an aerated nutrient growth medium, synthetic broth AOAC. This screen was used to rank the antibacterial action with a view to selecting those surfactants for mechanism of action studies. The alcohol ethoxylates had the greatest bacteriostatic effect against both the Gram-negative and Gram-positive organisms and hence it was decided to investigate the properties of this group of surfactants further. Preliminary studies confirmed the poor bactericidal effect of these agents; reduction in viability of between 10 and 90% only could be achieved at supra-MIC surfactant levels. Attempts to raise the concentration further

encountered solubility problems. Such reductions in viability were not satisfactory for classical MBC determinations but could account for the levels of 260nm leakage which would normally be thought to reflect loss of viability.

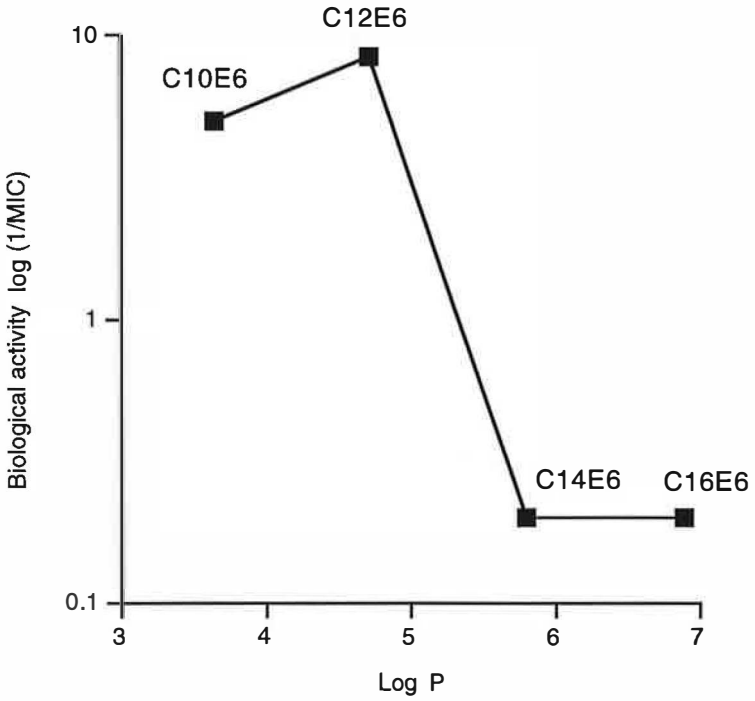
S. aureus NCTC 4163 required lower concentrations of surfactant to inhibit growth than that for *E. coli* NCTC 8196. This finding has been reported by other workers including Cowles (1938) and Baker (1941) who investigated the antibacterial properties of anionic surfactants; it is likely to be attributable to differences in the cell walls of Gram-positive and Gram-negative bacteria. Gilbert and Wright (1987) demonstrated the increased resistance of Gram-negative bacteria compared to Gram-positive bacteria. Spheroplasts of *E. coli* and *S. aureus* were equally sensitive to tetrachlorosalicylanilide whilst intact cells differed in sensitivity by a factor of 200. The outer membrane of Gram-negative bacteria is composed of closely packed LPS which is orientated so that the hydrophilic polysaccharide portion of LPS envelopes the cell surface and confers upon it a hydrophilic character. This, in addition to the lack of membrane fluidity caused by lipid A greatly reduces the dissolution and diffusion of hydrophobic molecules through the outer membrane. Hydrophilic molecules therefore enter the membrane by specific transport proteins and porins with a molecular weight exclusion of *ca* 600 Daltons (Chopra & Ball, 1982). The Gram-positive cell wall is a complex of peptidoglycan incorporating teichoic and teichuronic acids. This network functions as a molecular sieve with the peptidoglycan able to exclude only large polymeric molecules (100,000 Daltons).

6.2 Structure-function relationships

The relationship between biological activity and various physicochemical characteristics can be complex. A relationship between chain length and activity has been established by several workers including Zaslavsky *et al* (1979) and Ohnishi and Sagitani (1993) who concluded that as chain length increases, activity decreases. This study, however, found that using log 1/MIC as a measure of biological effect (as described by Witham, 1983) C12E6 showed maximum activity (figure 6.1) indicating this relationship is more parabolic in nature; an optimum value has been described by Kabara (1978) and Devinsky (1990). Ohnishi & Sagitani (1993) found that for polyoxyethylene type nonionic surfactants the relationship between molecular weight and membrane activity was of the same form as that determined by Zaslavsky *et al* (1979), i.e. the higher the molecular weight the less active the compound. De la Maza *et al* (1992) concluded the same relationship existed for partition coefficient and activity for octyl phenols. Witham (1983) determined a relationship between chain length and biological activity and concluded that a linear correlation was seen for members of the series up to hexylphenol, after which there was a general trend away from linearity.

For the homologous series of alcohol ethoxylates tested in this thesis molecular weight and calculated partition coefficient increase linearly with chain length. Therefore a non-linear relationship is also seen between 1/MIC and molecular weight, and 1/MIC and partition coefficient differing from the relationships described by Ohnishi & Sagitani (1993) and de la Maza *et al* (1992).

Figure 6.1 The relationship between biological activity and log P for a homologous series of alcohol ethoxylates



6.3 Surfactant adsorption

Membrane-active agents usually produce a Langmuirian adsorption isotherm, for example, phenol (Hugo, 1976), 'S' type or 'S' type becoming 'C' type at higher concentrations, whilst Corkhill (1966) showed that the adsorption of C12E6 to Graphon (graphitised carbon black), a hydrophobic surface, produced a Langmuir isotherm. This was also the case for other alcohol ethoxylates with the same C12 carbon chain but varying sizes of ethoxylate head group. Uptake to a hydrophilic surface is in general similar so a Langmuirian isotherm would also be observed.

On first inspection the 'S' and 'L' isotherms have a very similar shape; however, the 'S' type isotherm usually occurs when the following three criteria are met (Salt & Wiseman, 1991): (1) the solute molecule has a relatively large hydrophobic portion (in excess of 5 carbon atoms); (2) the solute molecule has moderate intermolecular action, causing it to pack vertically in a regular array in the absorbed layer; and (3) the solute molecule meets strong competition, for substrate sites form molecules of the solvent or of another adsorbed species. The 'L' type adsorption isotherm therefore occurs in the absence of strong competition from the solvent or where the absorbed molecules are not vertically orientated.

Studies by Kennedy (1996) using radiolabelled C12E6 have shown the adsorption isotherm for C12E6 to *E. coli* ATCC 11229 to be a 'C' type adsorption isotherm as described by Giles *et al* (1974), i.e there is a constant partition of C12E6 to the bacterial cells. This type of adsorption was also seen with chlorhexidine and alexidine and is commonly observed with biocides (Chawner & Gilbert, 1989). It is possible that if other alcohol

ethoxylates do not demonstrate a 'C' type adsorption isotherm then the 'S' type may be seen due to the large hydrophobic portion of the molecules.

6.4 Damage to cytoplasmic membrane

Previous work determining the effect of a range of anionic and nonionic surfactants against bacteria suggested the site of damage for these surfactants to be the cytoplasmic membrane (Salton, 1957; Pethica, 1958; Gilbey and Few, 1957; Gilbey & Few, 1960; Davies & Field, 1969; Schnaitman, 1971a; Lima, 1980). As a result of this the cytoplasmic membrane was examined as the site of damage for surfactants tested in this study and hence a variety of biochemical approaches were used to determine this including leakage of cell constituents, substrate uptake and membrane-bound.enzyme activity.

The outer membrane contains pores which allow the diffusion of certain molecules across it. These porins have been calculated to have a exclusion molecular weight minimum of 600 Daltons (Chopra & Ball, 1982) and possess an aqueous environment inevitably selecting for the passage of hydrophilic molecules. As described previously (section 4.4.2), the two longer chain alcohol ethoxylates C14E6 and C16E6 had limited antimicrobial activity against *E. coli* NCTC 8196. The nature of the porins may at least partly explain why the activity of C10E6 and C12E6 was significantly higher than that seen for C14E6 and C16E6. Although all members of the homologous series had a molecular weight of less than 600 daltons, there was significant variation in the hydrophilicity of the four compounds. The two shorter chain length members of the series were relatively hydrophilic in character (log P values are 3.65 and 4.73,

respectively) whilst C14E6 (log P=5.81) and C16E6 (log P=6.89) were more lipophilic and therefore much less likely to enter the cell via the porins. C14E6 and C16E6 are also more prone to micelle formation at the low concentrations employed aggravating their passage through the porin. Lien *et al* (1968) concluded that the optimum value of log P for biological activity against a Gram-negative cell was 4.0 and from the log P values of the surfactants it can be seen that C10E6 and C12E6 were closest to this value. This suggested that C14E6 and C16E6 were less likely to cross the outer membrane and elicit an effect on the cytoplasmic membrane and therefore it was decided to focus the study on the mechanism of action of the remaining two alcohol ethoxylates in the homologous series, C10E6 and C12E6. This was determined by using various biochemical probes of membrane damage at a range of concentrations both above and below the MIC obtained for the surfactants.

Results from the membrane probes all show a similar pattern in terms of the concentration at which damage first occurs. By looking at the concentration profiles for each probe of membrane damage it can be seen that damage to the cytoplasmic membrane of *E. coli* occurs when the concentration of C10E6 used is at the MIC. This pattern was also seen for the uptake of propidium iodide in the presence of alcohol ethoxylates (Mitchell, 1997). This compound is a fluorescent marker and can only be taken up by cells when damage to the membrane has occurred enabling the compound to enter the cell. Therefore, it can be seen that the amount of fluorescence detected can be related to the amount of damage to the cell.

This study was carried out using a wide range of alcohol ethoxylates, and the uptake of propidium iodide by *E. coli* ATCC 11229 was determined in the

presence of a range of concentrations of these surfactants. At the lower concentrations of surfactant studied, approximately 0.01 to 0.5 mM, the uptake of propidium iodide was minimal, therefore there was little cell damage. Above this concentration, uptake increased rapidly demonstrating substantial membrane damage. This concentration was above the MIC of C10E6 (0.20 mM).

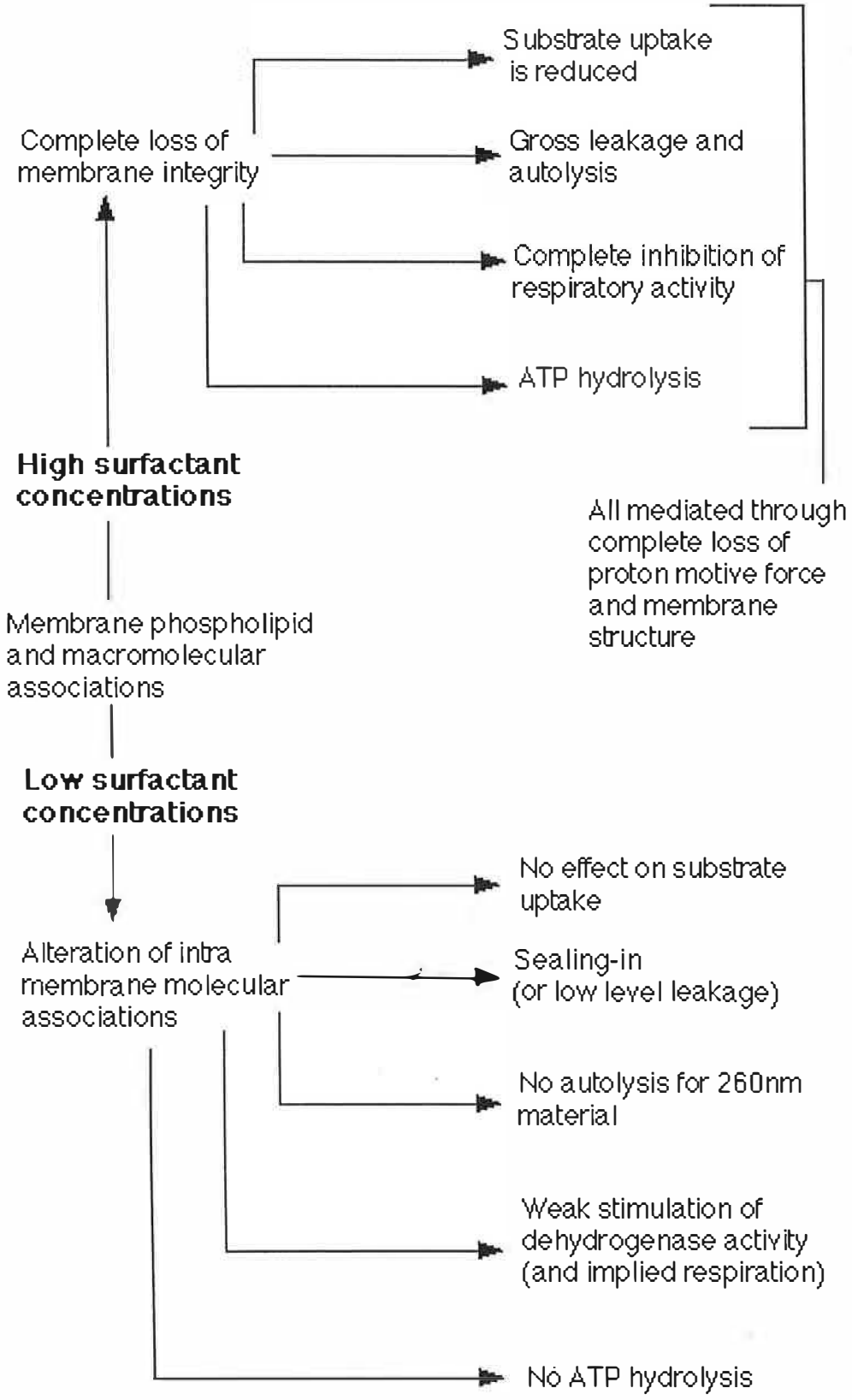
The results obtained for the same tests using a range of concentrations for C12E6 show a different pattern from that seen for C10E6. For this surfactant, membrane damage is occurring at concentrations below that of the MIC (0.12 mM) for all of the biochemical probes used. Again, propidium iodide data (Mitchell, 1997) agree with these results. From 0.01 to approximately 0.05 mM propidium iodide uptake is minimal and occurs to the same extent as that seen in the presence of C10E6. However, up to approximately 0.5 mM C12E6 is taken up to a much greater degree than that seen with C10E6 and hence greater membrane damage has been caused by the presence of C12E6 on an eqimolar basis. These concentrations include those below the MIC of C12E6 of 0.12 mM. Above 0.5 mM propidium uptake by C12E6 is in excess of that seen for C10E6.

The immediate and substantial leakage of potassium ions from *E.coli* cells upon treatment of these antibacterial agents is evidence of early modification of membrane structure (and potentially function) by direct physiochemical interactions. Increasing the surfactant concentration above the MIC causes 260 nm leakage to take place. The time-dependent leakage of high molecular weight cytoplasmic constituents after membrane damage as a rule leads to total cell destruction.

The action of a bisquaternary ammonium salt, an amine oxide and an alkoxyphenylcarbamic acid on the metabolic functions of *S. aureus* were determined by Mlynarcik *et al* (1981). All the compounds interfered with the cytoplasmic membrane as determined by potassium ion leakage, ATP synthesis and the effect on respiration. The chemiosmotic theory defines the cytoplasmic membrane as an energy transducer and offers the linking between these events. Protons are generated by the electron transport system which reside in the membrane or by membrane bound ATP, this proton gradient across the membrane creating the proton motive force. Mlynarcik *et al* (1981) argue that membrane perturbation by a drug inserting itself into the fluid mosaic structure may seriously affect energy transduction.

In a homologous series of surfactants their biological activities increase progressively with increasing chain length until a critical point is reached beyond which the compounds cease to be active (Balgavy & Devinsky, 1996). This cut-off effect is seen in almost every surfactant series tested; often not only the biological activity but the mode of action changes at some critical chain length. Seeman (1972) reviewed the concentration dependencies of the biological activities of surfactants. Low surfactant concentrations have been shown to protect cells from osmotic, mechanical or acid lysis, while high concentrations of surfactants potentiate or directly cause lysis. These effects were seen with C10E6 and C12E6, where low concentrations of these surfactants reduced leakage below that seen with the control whilst high levels cause significant leakage of potassium ions and 260nm material. This differing effect of low and high concentrations is not only seen with membrane integrity but also with membrane-bound enzyme activity such as with dehydrogenases (Kopecka-Leitmanova *et al*, 1989) and ATPase (Mlynarcik *et al*, 1981). These enzymes are stimulated at

Figure 6.2 The effect of low and high surfactant concentrations on cellular respiration and cytoplasmic membrane leakage



high concentrations and inhibited at low concentrations. The concentration dependent responses elicited by the alcohol ethoxylates can be seen in figure 6.2. Results from the dehydrogenase assays with *E. coli* NCTC 8196 in the presence of the homologous series of alcohol ethoxylates (Chapter 4) also demonstrated this pattern. Kopecka-Leitmanova *et al* (1989) determined the effect of dehydrogenase activity in the presence of amine oxides and quaternary ammonium salts. The amount of INT reduced increases in the following order, depending on the substrate, glucose ≤ succinate < pyruvate. The respiration of glucose is affected to the smallest extent by increasing the concentration. With pyruvate and succinate the respiration is more sensitive. Kopecka-Leitmanova and co-workers (1989) further proposed that the surfactants do not specifically affect the enzymes involved in these processes but are a secondary consequence of primary physical damage. Tetrazolium salt studies (section 4.2) were carried out using glucose and succinate as substrates in this thesis. The results obtained in the presence of C10E6 are similar whether glucose or succinate is used as a substrate; this is in agreement with the results of Kopecka-Leitmanova *et al* (1989). However, formazan production in the presence of C12E6 is greatly reduced in the presence of glucose compared to that seen for succinate. The difference in these results could suggest a subtle difference in the modes of actions of these two surfactants Succinate dehydrogenase is closely associated with the cytoplasmic membrane which is the site of surfactant action, whereas glycolysis proceeds in the cytoplasm.

Both C10E6 and C12E6 are capable of damaging the cytoplasmic membrane; however, there appear to be subtle differences in the mode of action of the two surfactants. C10E6 has very little effect below its MIC (0.20mM) and as such may require an accumulation of surfactant at the site

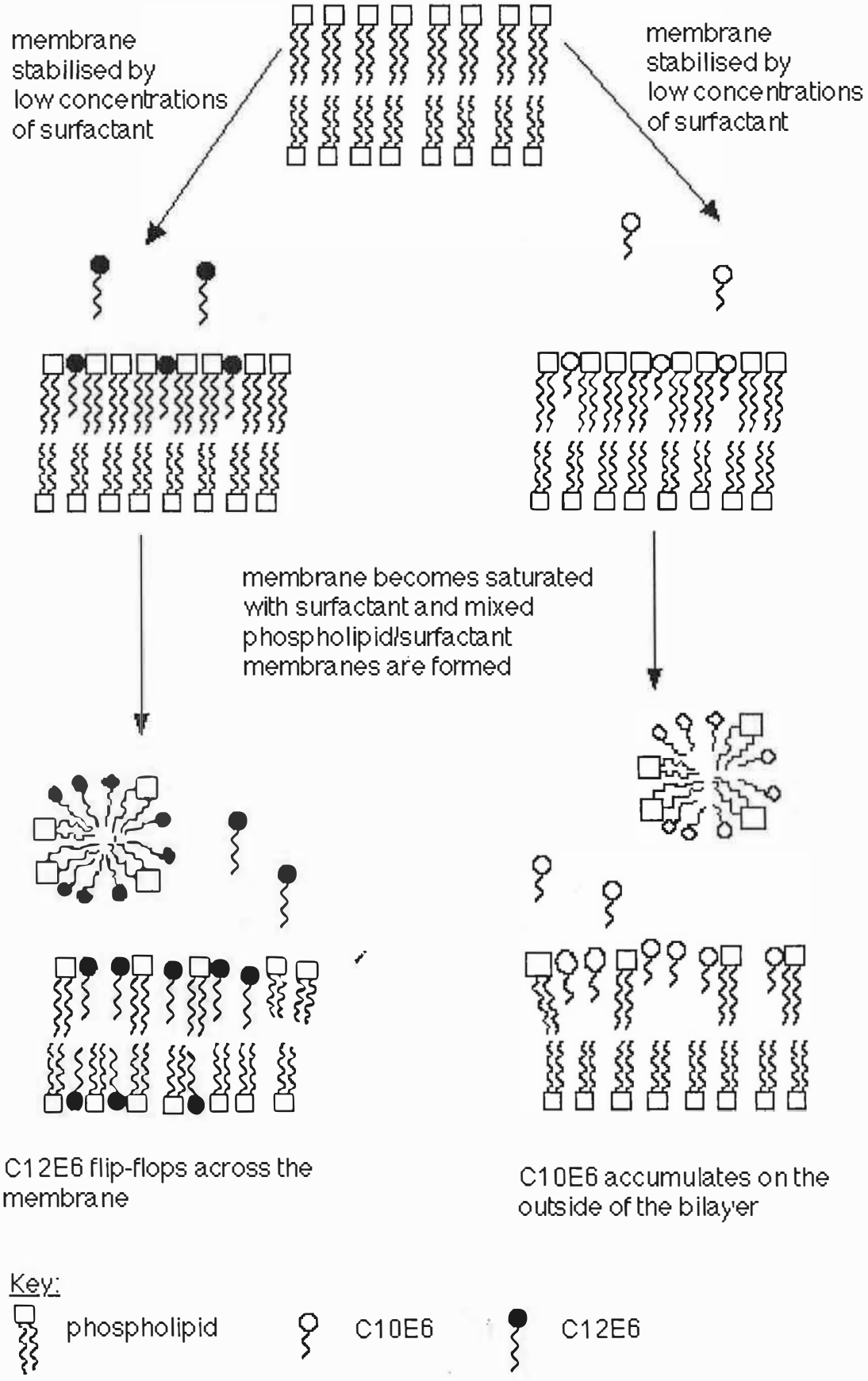
of action. A threshold concentration must then be reached for damage to occur. In contrast, C12E6 shows activity below its MIC (0.12mM), suggesting progressive damage rather than catastrophic injury. Therefore the surfactant appears to partition almost immediately through the membrane. This is consistent with the 'C' type adsorption isotherm obtained by Kennedy (1996) indicating a constant partition of surfactant to the bacterial cells.

These effects may be due to differences in the partitioning of the two surfactants between the lipid bilayer and the aqueous phase. The log P values of the two surfactants are 3.65 for C10E6 and 4.73 for C12E6, these values span the optimum log P value of 4.0 for Gram-negative bacteria (Lien *et al*, 1968). A similar amount of each surfactant, however, will be present in the bacterial membrane for each surfactant as there is a large partition from water.

The differences in the interaction of the two surfactants may be due to the size of the hydrophobic tail. Membrane phospholipids typically have an alkyl chain length of between 16 and 18. Of the two surfactants studied in detail C12E6 has a tail group approaching this length. Both surfactants exhibit a sealing-in effect. The inclusion of low concentrations of surfactants can be incorporated into the lipid bilayer without loss of bilayer structure (Seeman, 1972) and exert a protective effect on the membrane. At higher concentrations the surfactants disrupt the membrane. Results from the markers of membrane damage demonstrate that C12E6 causes changes in membrane intergrity below the MIC. By implication the consequences of these changes are balanced by the metabolic activity of growing cells until the concentration is raised further. It is proposed that as the tail of this surfactant monomer is approximating that of the phospholipids it is more

likely to insert into and flip-flop across the membrane. Le Maire and co-workers (1987) have demonstrated the ability of C12E8 to flip-flop across a variety of intact membranes within milliseconds and this is consistent with the constant partition of C12E6 to the bacterial cells (Kennedy, 1996). It is further proposed that, due to the shorter chain length of C10E6, the head group will have a larger contribution to the hydrophilicity of the surfactant (as shown by the HLB value compared to that of C12E6). The increased hydrophilicity of this surfactant may cause it to accumulate on the exterior of the bilayer rather than flip-flop through as proposed with C12E6. Once the bilayer becomes saturated with surfactant, mixed phospholipid/surfactant micelles form resulting in complete phase transition (Helenius & Simmons, 1975); this appears to occur at concentrations close to the MIC. It is at this stage that the gross membrane damage for both surfactants is likely to occur. The proposed mechanisms for the interaction of C10E6 and C12E6 with lipid bilayers can be seen in figure 6.3. The interaction of the surfactants with the membrane appears to occur when the surfactant is in the monomeric form as opposed to the micellar form (Tanford, 1972) as significant damage is caused to the bacterial cells before the CMC is reached for either surfactant.

Figure 6.3 Interaction between alcohol ethoxylates and membrane



6.5 Further work

The homologous series of alcohol ethoxylates varied with the length of their alkyl chain whilst the head group size was not varied. The effect of the surfactant head group can have an influence on the activity of the surfactant. Edwards and Almgren (1992) have reported increased leakage rates from lecithin vesicles when the headgroup size of polyethylene glycol n-dodecylmonoethers is increased. Therefore the antimicrobial activity of the most active members of the homologous series C10E6 and C12E6 could be optimised by altering the head group size.

The results in this thesis demonstrate that there are subtle differences in the mode of action of C10E6 and C12E6. It has been proposed that this is due to variations in the interaction of these surfactants with lipid bilayers. Differential scanning calorimetry and electron spin resonance may be able to provide more information on the nature of these interactions. Differential scanning calorimetry was used to investigate the interaction between these surfactants and *E. coli* NCTC 8196; however, these studies presented significant experimental challenges which there was no time to resolve. Once problems are overcome, these methods would offer considerable insight into the phospholipid/surfactant interactions which appear fundamental to the mechanism of antibacterial activity.

One of the aims of determining the mechanism of action of a surfactant is so that it can be used advisedly in combination with another agent; occasionally combinations of agents are synergistic providing enhanced activity at lower individual agent concentrations (Denyer, *et al,* 1985). This thesis confirms the cytoplasmic membrane as the site of action for the

nonionic alcohol ethoxylates. Therefore future work could concentrate on the combination of known biocidal agents with surfactants. The alcohol ethoxylates, possessing membrane permeabilising activity would potentiate the entry of other biocidal agents into the interior of the bacterial cell where they may then act at a target within the cell. This approach could also be used to facilitate the incorporation of another membrane-active agent. which could enable further insight to be gained on the action of both agents.

APPENDICES

Appendix 1

The formula used for converting light readings from the bioluminescent ATP assay into ATP yields (M) (section 4.3) was as follows:

$$\frac{(S-B)}{I - (S \times 190/240)} \times (50/240 \times \text{Concentration of S in M}) \times 190/150)$$

where:

B = background light output i.e. light output from monitoring reagent

I = light output from sample and monitoring reagent

S = light output from ATP standard, sample and monitoring reagent

and:

50 = volume of ATP standard added (µL)

150 = volume of sample added (µL)

190 = combined volume of monitoring reagent and sample (µL) i.e. 40+150

240 = combined volume of monitoring reagent, sample and standard (µL)
i.e. 40+150+50

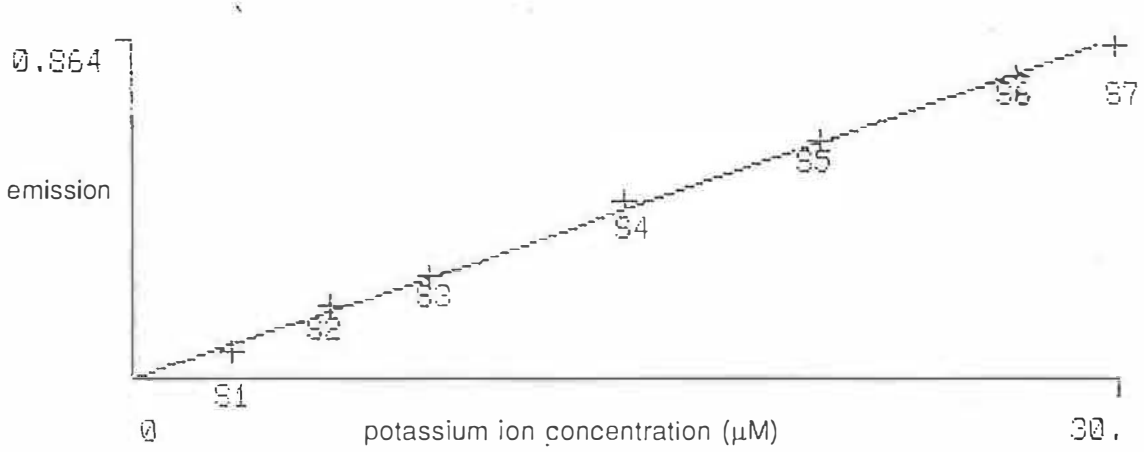
Appendix 2

Some physiochemical data for the homologous series of alcohol ethoxylates

Surfactant	Molecular weight (MW)	Hydrophile-lipophile balance (HLB)	Log P
C10E6	422.6	12.5	3.65
C12E6	450.7	11.8	4.73
C14E6	478.7	11.1	5.81
C16E6	506.8	10.5	6.89

Appendix 3

Linear relationship between potassium ion concentration and emission



REFERENCES

References

ALLWOOD, M.C. and HUGO, W. B. (1971):The leakage of cations and amino acids from *Staphylococcus aureus* exposed to moist heat, phenol and dinitrophenol. *Journal of Applied Bacteriology* 34, 369-375.

ALTMAN, F.P. (1970): On the oxygen-sensitivity of various tetrazolium salts. *Histochemie* 22, 256-261.

ALTMAN, F.P.(1972): *"An introduction to the use of tetrazolium salts in quantitiative enzyme cytochemistry"*, Koch-Light Laboratories, Colnbrook, Bucks., England.

AL-HITI, M.M.A. and GILBERT, P. (1980): Changes in preservative sensitivity of the USP antimicrobial agents effectiveness test micro-organisms. *Journal of Applied Bacteriology* 49, 119-126.

ANACKER, E.W., GERRY, H.E., JACOBS, P.T. and PETRARIU, I. (1977): Group VA effects on surfactant aggregation number and dye solubilisation efficiency. *Journal of Colloid and Interface Science* 60, 514-518.

ANDERSEN, J.P., LE MAIRE, M., KRAGH-HANSEN, U., CHAMPEIL,P. and MOLLER, J.V. (1983): Perturbation of the structure and function of a membranous Ca^{2+}-ATPase by non-solubilizing concentrations of a non-ionic detergent. *European Journal of Biochemistry* 134, 205-214.

ANRAKU, Y. and GENNIS, R.B. (1987): The aerobic respiratory chain of *Escherichia coli*. *Trends in Biochemical Sciences* 12, 262-266.

ARMSTRONG, W.M. (1957): Enhancement of the biological activity of sodium dodecyl sulphate by inorganic cations. *Nature* 179, 780-781.

ASPEDON, A. and NICKERSON, K.W. (1993): The energy dependence of detergent resistance in *Enterobacter cloacae:* a likely requirement for ATP rather than a proton gradient or a membrane potential. *Canadian Journal of Microbiology* 40, 184-191.

AU, D., LORENCE, R.M. and GENNIS, R.B. (1985): Isolation and characterisation of an *Escherichia coli* mutant lacking the cytochrome *o* terminal oxidase. *Journal of Bacteriology* 161, 123-127.

BAKER, Z., HARRISON, R.W. and MILLER, B.F. (1941): The bactericidal action of synthetic detergents. *Journal of Experimental Medicine* 74, 611-620.

BALGAVY, P. and DEVINSKY, F. (1996): Cut-off effects in biological activites of surfactants. *Advances in Colloid and Interface Science* 66, 23-63.

BARRETT-BEE, K., NEWBOULT, L. and STAWPERT, J. (1996): The mode of antibacterial action of the novel agent ZM240304 (D-arabino-2,3,4-tris94-chlorobenzyl)pentane-1,5-di-amine) *Journal of Antimicrobial Chemotherapy* 38, 605-614.

BEAN, H.S. and DAS, A. (1966): The adsorption by *E. coli* of some phenols and their bactericidal activity. *Journal of Pharmacy and Pharmacology* 11,367-373.

BECKER, R., HELENIUS, A. and SIMONS, K. (1975): Solubilization of the Semliki forest virus membrane with sodium dodecyl sulfate. *Biochemistry* 14, 1835-1841.

BENZ, R. (1988): Structure and function of porins from Gram-negative bacteria. *Annual Review of Microbiology* 42, 359-393.

BERNHEIM, F. (1984): The effect of surfactants on potassium efflux from *Pseudomonas aeruginosa* caused by proteins. *Microbios Letters* 25, 17-22.

BEVERIDGE, T.J. (1993): Current trends and future prospects in prokaryotic envelope research: a microscopist's view. *Journal of Applied Bacteriology Symposium Supplement* 74, 143S-153S.

BEVERIDGE, T.J. and GRAHAM, L.L. (1991): Surface layers of bacteria. *Microbiological Reviews* 55, 684-705.

BIRKELAND, J.M. and STEINHAUS, E.A. (1939): Selective bacteriostatic action of sodium lauryl sulfate and of "Dreft". *Proceedings of the Society of Experimental Biological Medicine* 40, 86-88.

BLOOMFIELD, S. (1991): "Methods for assessing antimicrobial activity" in *Mechanisms of Action of Chemical Agents - Their Study and Exploitation* ed DENYER, S.P. and HUGO, W.B., Society for Applied Bacteriology, Blackwell Science. pp1-22

BONT, W.S., EMMELOT, P. and VAZ DIAZ, H. (1969): Studies on plasma membranes VIII The effects of sodium deoxycholate and dodecyl sulfate on isolated rat-liver plasma membranes. *Biochimica et Biophysica Acta* 173, 389-408.

BRITISH STANDARD 6905 (1987): British Standard Method for estimation of concentration of disinfectants used in 'dirty' conditions in hospitals by the modified Kelsey-Sykes test.

BRETSHER, M.S. (1973): Membrane structure: some general principles. *Science* 181, 622-629.

BROCK, T.D. and MADIGAN, M.T. (1991): *"Biology of microorganisms",* 16th ed., Prentice Hall, New Jersey.

BROWN, M.R.W., COLLIER, P.J. and GILBERT, P. (1990): Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. *Antimicrobial Agents and Chemotherapy* 34, 1623-1628.

BROXTON, P., WOODCOCK, P.M. and GILBERT, P. (1983): A study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. *Journal of Applied Bacteriology* 54, 345-353.

BROXTON, P., WOODCOCK, P.M. and GILBERT, P. (1984): Injury and recovery of *Escherichia* coli ATCC 8739 from treatment with some polyhexamethylene biguanides. *Microbios* 40, 187-193.

CELLA, J.A., HARRIMAN, L.A., EGGENBERGER, D.N. and HARWOOD, H.J. (1955): the relationship of charge density, antibacterial activity and micelle formation of quaternary ammonium salts. *Journal of the American Chemical Society* 77, 4264-4266.

CHAPLIN, M.F. (1986): "Monosaccharides" in *Carbohydrate Analysis - a practical approach* ed CHAPLIN, M,.F. and KENNEDY, J.F., IRL Press, Oxford.

CHAWNER, J.A. and GILBERT, P. (1989): Adsorption of alexidine and chlorhexidine to *Escherichia coli* and membrane components. *International Journal of Pharmaceutics* 55, 209-215.

CHOPRA, I. and BALL, P. (1982): Transport of antibiotics into bacteria. *Advances in Microbial Physiology* 23, 183-240.

CLINT, J.H. (1992): *Surfactant Aggregation*, Blackie.

COLEMAN, R. (1973): Membrane-bound enzymes and membrane ultrastructure. *Biochimica et Biophysica Acta* 300, 1-30.

COLLIER, P.J., AUSTIN, P. and GILBERT, P. (1990): Uptake and distribution of some isothiazolone biocides into *Escherichia coli* ATCC 8739 and *Schizosaccharomyces pombe* NCYC 1354. *International Journal of Pharmaceutics* 66, 201-206.

CORKHILL, J.M., GOODMAN, J.F. and TATE, J.R. (1966): Adsorption of non-ionic surface-active agents at the Graphon/solution interface. *Transcripts of the Faraday Society* 62, 979.

COWLES, P.B. (1938): Alkyl sulfates: their selective bacteriostatic action. *Yale Journal of Biological Medicine* 11, 33-38.

CROSS, .J. (1992) "Nonionic surfactants: characteristics and general properties" in *Non-ionic Surfactants, Chemical Analysis*, Marcel Dekker Inc., New York and Basel. pp4-28.

CSERHATI, T., ILLES, Z. and NEMES, I. (1991): Effects of non-ionic tensides on the growth of some soil bacteria. *Applied Microbiology and Biotechnology.* 35, 115-118.

CSERHATI, T., SZOGYI, M. and BORDAS, B. (1982): QSAR study on the biological activity of nonyl-phenyl-ethylene-oxide polymers. *General Physiology and Biophysics.* 1, 225-231.

CULLUM, D.C. (1993): "Surfactant types; classification, identification, separation" in *Introduction to surfactant analysis,* Blackie Academic and Professional. pp15-25

DAVIES, A. and FIELD, B.S. (1969): Action of biguanides, phenols and detergents on *Escherichia coli* and its spheroplasts. *Journal of Applied Bacteriology* 32, 233-243.

DAVIS, J.G. (1960): Methods for the evaluation of the antibacterial activity of surface active compounds: technical aspects of the problem. *Journal of Applied Bacteriology* 23, 318-344.

DEAMER, D.W. (1973): Isolation and characterization of a lysolecithin-adenosine triphosphatase complex from lobster muscle microsomes. *Journal of Biological Chemistry* 248, 5477-5485.

DE LA MAZA, A. and PARRA, J.L. (1993a): Solubilization of unilamellar liposomes by amphoteric alkyl betaine surfactants. *Colloids and Surfaces A: Physiochemical and Engineering Aspects* 70, 189-197.

DE LA MAZA, A. and PARRA, J.L. (1993b): Permeability alterations in unilamellar liposomes as due to betaine-type zwitterionic and anionic surfactant mixed systems. *Journal of the American Oil Chemists Society* 70, 685-691

DE LA MAZA, A. and PARRA JUEZ, J.L. (1993c): Solubilization of phospholipid bilayer caused by surfactants. *Journal of the American Oil Chemists Society* 70, 699-706.

DE LA MAZA, A. and PARRA, J.L. (1994): Solubilization of unilamellar phòspholipid bilayers by nonionic surfactants. *Colloid & Polymer Science* 272, 721-730.

DE LA MAZA, A., PARRA, J.L., GARCIA, M.T., RIBOSA, I. and SANCHEZ LEAL, J. (1992): Permeability changes in the phospholipid bilayer caused by nonionic surfactants. *Journal of Colloid and Interface Sciences* 148, 310-316.

DENYER, S.P. (1990): Mechanisms of action of biocides. *International Biodeterioration* 26, 89-100.

DENYER, S.P. (1995): Mechanisms of action of antibacterial biocides. *International Biodeterioration and Biodegradation* 36 227-245.

DENYER, S.P. and HUGO, W.B. (1991): Mechanisms of antibacterial action - a summary in *"Mechanisms of action of chemical biocides - their study and exploitation".* ed. DENYER, S.P. and HUGO, W.B., Society for Applied Microbiology.

DENYER, S.P., HUGO, W.B. and HARDING, V.D. (1985): Synergy in preservative combinations. *International Journal of Pharmaceutics* 25, 245-253.

DENYER, S.P., HUGO, W.B. and HARDING, V.D. (1986): The biochemical basis of synergy between the antibacterial agents, chlorocresol and 2-phenylethanol. *International Journal of Pharmaceutics* 29, 29-36.

DE PHAMPHILIS, M.L. & ADLER, J. (1971): Attachment of flagellar basal bodies to the cell envelope: specific attachment to the Outer, Lipopolysaccharide Membrane and the Cytoplasmic Membrane. *Journal of Bacteriology* 105, 396-404.

DEVINSKY, F., MLYNARCIK, D., LACKO, I. and KRASNEC, L. (1982): Antimicrobial efficacy of some N,N'-bis(alkylmethyl)-1,2-ethanediamine dioxides and N,N'-bis(alkylmethyl)-1,2-propanediamine dioxides. *Folia Microbiology* 27, 272-274.

DEVINSKY, F., LACKO, I., MLYNARCIK, D., RACANSKY, V. and KRASNEC, L. (1985): Relationship between critical micelle concentrations and minimum inhibitory concentrations for some non-aromatic quaternary ammonium salts and amine oxides. *Tenside Detergents* 22, 10-15.

DEVINSKY, F., LACKO, I., MLYNARCIK, D., SVAJDLENKA, E. and BOROVSKA, V. (1990): Quaternary ammonium salts XXXIII. QSAR of antimicrobially active niketamide derivatives. *Chemistry Papers* 44, 159-170.

DEVINSKY, F., LACKO, I., MLYNARCIK, D., SVAJDLENKA, E. and MASAROVA, L. (1990): QSAR of monoquaternary surface active antimicrobials: the parabolic and bilinear case. *Acta Facultatis Pharmaceuticae* XLIX 127-142

DEVINSKY, F., MASAROVA, L., LACKO, I. and MLYNARCIK, D. (1991): Structure-activity relationships of "soft" quaternary amphiphiles. *Journal of Biopharmaceutical Sciences* 2, 1-10.

EDWARDS, K. and ALMGREN, M. (1991): Solubilization of lecithin vesicles by $C_{12}E_8$ - structural transitions and temperature effects. *Journal of Colloid and Interface Science* 147 1-21.

EDWARDS, K. and ALMGREN, M. (1992): Surfactant induced leakage and structural change of lecithin vesicles: effect of surfactant headgroup size. *Langmuir* 8, 824-832.

ELWORTHY, P.H., FLORENCE, A.T. and MACFARLANE, C.B. (1968): "Solubilization" in *Solubilization by surface-active agents and its applications in Chemistry and the Biological Sciences,* Chapman and Hall, London. pp61-116.

ERNST, R.E., GONZALES, C.J. and ARDITTI, J. (1983): Biological effects of surfactants: Part 6 - Effects of anionic, non-ionic and amphoteric surfactants on a green alga (*Chlamydomonas*). *Environmental Pollution (Series A)* 31, 159-175.

FITZGERALD, K.A., DAVIES, A and RUSSELL, A.D. (1989): Uptake of ^{14}C-chlorhexidine diactate to *Escherichia coli* and *Pseudomonas aeruginosa* and its release by azolectin. *FEMS Microbiology Letters* 60, 327-332.

FLIEGE, R., TONG, S., SHIBATA, A., NICKERSON, K.W. and CONWAY, T. (1992): The Entner-Doudoroff pathway in *Escherichia coli* is induced for oxidative glucose metabolism via pyrroloquinoline quinone-dependent glucose dehydrogenase. *Applied and Environmental Microbiology* 58, 3826-3829.

FRANKLIN, R.M. (1971): *"Current topics in microbiology and immunology"*, Springer, Berlin.

FREESE, E. and CHERNOV-LEVIN, B. (1978): Action mechanisms of preservatives and antiseptics. *Developments in Industrial Microbiology* 19, 207-227.

FULLER, and LOVELOCK (1976): *" Microbial Ultrastructure - the use of the electron microscope"* SAB Technical Series No. 10, Academic Press, London.

GALBRAITH, H., MILLER, T.B., PATON, A.M. and THOMPSON, J.K. (1971): Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol and cholesterol. *Journal of Applied Bacteriology* 34, 803-813.

GALE, E.F. and FOLKES, J.P. (1953): The assimilation of amino-acids by bacteria - nucleic acid and protein synthesis in *Staphylococcus aureus*. *Biochemistry Journal* 53, 483-492.

GILBERT, P. (1985): Non-plasmidic resistance of micro-organisms to chemical inactivation. *Pharmacy International* 209-212.

GILBERT, P., BEVERIDGE, E.G. and CRONE, P.B. (1976): The effect of some glycolmonophenethers on the ionic permeability of *Escherichia coli*. *Proceedings of the Society of General Microbiology* 11, 89.

GILBERT, P., BEVERIDGE, E.G. and CRONE, P.B. (1977a): The effect of 2-phenoxyethanol on the permeability of *Escherichia coli* NCTC 5933 to inorganic ions. *Microbios* 19, 17-25.

GILBERT, P., BEVERIDGE, E.G. and CRONE, P.B. (1977b): Inhibition of some respiration and dehydrogenase enzyme systems in *Escherichia coli* NCTC 5933. *Microbios* 20, 29-37.

GILBERT, P., BEVERIDGE, E.G. and CRONE, P.B. (1980): Effect of 2-phenoxyethanol upon RNA, DNA and protein biosynthesis in *Escherichia coli* NCTC 5933. *Microbios* 28, 7-17.

GILBERT, P., BEVERIDGE, E.G. and SISSONS, I. (1978): The uptake of some membrane-active drugs by bacteria and yeast: possible microbiological examples of 'Z'-curve adsorption. *Journal of Colloid and Interface Science* 64, 377-379.
585-592.

GILBERT, P. and WRIGHT, N. (1987): Non-plasmidic resistance towards preservatives of pharmaceutical products in *"Preservatives in the food, pharmaceutical and environmental industries"*, Society for Applied Bacteriology.

GILBY, A.R. and FEW, A.V (1957): Surface chemical studies on the protoplast membrane of *Micrococcus lysodeikticus. Surface Activity: Solid/Liquid interface and Cell/Water interface, Proceedings of the 2nd International Conference of Surface Activity* 4, 262-270.

GILBY, A.R. and FEW, A.V. (1960): Lysis of protoplasts of *Micrococcus lysodeikticus* by ionic detergents. *Journal of General Microbiology* 23, 19-26.

GILES, G.H., SMITH, D. and HUITSON, A. (1974): A general treatment and classification of the solute adsorption isotherm 1. Theoretical. *Journal of Colloid and Interface Science* 47, 755-765.

GINN, M.E., KINNEY, F.B. and HARRIS, J.C. (1959): Detergent builder effect on the critical micelle concentration of surface active agents. *Journal of the American Oil Chemists Society* 36, 332-337.

GORDON, B. E., and SHEBS, W. T. (1968): Sorption and rinsability of nonionic surfactant under realistic laundering conditions - a radiotracer approach. *Proceedings Vth International Congress of Surface Active Agents, Barcelona* 3, 155.

GOSS, T., FAULL, J., KETTERIDGE, S. and SPRINGHAM, D. (1995): *"Introductory Microbiology",* Chapman and Hall, London

GREEN, G.N. and GENNIS, R.B. (1983): Isolation and characterization of an *Escherichia coli* mutant lacking cytochrome *d* terminal oxidase. *Journal of Bacteriology*. 154, 1269-1275.

GUCKLHORN, I.R. (1969): Antimicrobials in cosmetics. *Manufacturing Chemist and Aerosol News* 40, 23-30.

GUM, M.L., and GODDARD, E.D. (1981): Adsorption study of nonionic surfactants on polyester fiber. *American Oil Chemists Society Annual Meeting, New Orleans*.

HARDING, V.D. (1984): Studies in the mode of action of preservatives and preservative combinations. *PhD Thesis, University of Nottingham.*

HANCOCK, R.E.W. (1984): Alterations in outer membrane permeability. *Annual Review in Microbiology* 38, 237-264.

HANSCH, C. and LEO, A.J. (1979): Substituent Constants for Correlation Analysis in Chemistry and Biology, John Wiley and Sons, Chichester.

HAYDON, D.A. and TAYLOR, J. (1963): The stability and properties of bimolecular lipid leaflets in aqueous solution. *Journal of Theoretical Biology* 4, 281-296.

HELENIUS, A. and SIMONS, K. (1975): Solubilization of membranes by detergents. *Biochimica et Biophysica Acta* 415, 29-79.

HELENIUS, A. and SODERLAND, H (1973): Stepwise dissociation of the Semliki Forest virus membrane with Triton X-100. *Biochimica et Biophysica Acta* 307, 287-300.

HENGSTENBERG, W (1970): Solubilization of the membrane bound lactose specific component of the Staphylococci PEP dependent phospho-transferase system. *FEBS Letters* 8, 277-280.

HUGO, W.B. (1954): The use of 2,3,5-Triphenyltetrazolium bromide in determining the dehydrogenase activity of *Bact. coli.* *Journal of Applied Bacteriology* 17, 31-37.

HUGO, W.B. (1976): Survival of microbes exposed to chemical stress. *Society for General Microbiology Symposium* 26, 383-413.

HUGO, W.B. and BLOOMFIELD, S.F. (1971a): Studies on the mode of action of the phenolic antibacterial agent fentichlor against *Staphlycoccus aureus* and *Escherichia coli* I. The adsorption of fentichlor by the bacterial cell and its antibacterial activity. *Journal of Applied Bacteriology* 34, 557-567.

HUGO, W.B. and BLOOMFIELD, S.F. (1971b): Studies on the mode of action of the phenolic antibacterial agent fentichlor against *Staphlycoccus aureus* and *Escherichia coli* II. The effects of fentichlor on the bacterial membrane and the cytoplasmic constituents of the cell. *Journal of Applied Bacteriology* 34, 569-578.

HUGO, W.B. and BLOOMFIELD, S.F. (1971c): Studies on the mode of action of the phenolic antibacterial agent fentichlor against *Staphlycoccus aureus* and *Escherichia coli* III. The effects of fentichlor on the metabolic activities of *Staphlycoccus aureus* and *Escherichia coli*. *Journal of Applied Bacteriology* 34, 579-591.

HUGO, W.B. and BOWEN, J.G. (1973): Studies on the mode of action of 4-ethylphenol on *Escherichia coli.* *Microbios* 8, 189-197.

HUGO, W.B.and FRIER, M. (1969): Mode of action of the antibacterial compound dequalinium acetate. *Applied Microbiology* 17, 118-127.

HUGO, W.B. and LONGWORTH, A.R. (1964): some aspects of the mode of action of chlorhexidine. *Journal of Pharmacy and Pharmacology* 16, 655-662.

HUGO, W.B. and LONGWORTH, A.R. (1966): The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constiuents, dehydrogenase activity and cell walls of *Escheriichia coli*. *Journal of Pharmacy and Pharmacology* 18, 569-578.

HUGO, W.B. and NEWTON, J.M. (1964):The adsorption of iodine from solution bymicro-organisms and by serum. *Journal of Pharmacy and Pharmacology* 16, 49-55.

HUGO, W.B. and RUSSELL, A.D. (1992): *"Pharmaceutical microbiology"* 5th Ed., Blackwell Scientific Publications, Oxford.

HURWITZ, S.J. and McCARTHY, T.J. (1986): 2,3,5-Triphenyltetrazolium chloride as a novel tool in germicide dynamics. *Journal Pharmaceutical Sciences* 75, 912-916.

IKEDA, T., HIRAYAMA, H., YAMAGUCHI, H., TAZUKE, S. and WATANABE, M. (1986): Polycationic biocides with pendant active groups: molecular weight dependence of antibacterial activity. *Antibacterial Agents and Chemotherapy* 30, 132-136.

INGLEDEW, W.J. and POOLE, R.K. (1984): The respiratory chains of *Escherichia coli*. *Microbiological Reviews* 48, 222-271.

JACKSON, M.L., SCHMIDT C.F., LICTENBERG, D., LITMAN, B.J. and ALBERT, A.D. (1982): Solubilization of phosphatidylcholine bilayers by octyl glucoside. *Biochemistry* 21, 4576-4582.

JAMES, G.V. (1948): The synergistic effect of Aerosol OT on certain germicides. *Journal of the Society of Chemical Industry* 67, 336.

JONES, P.H. and PRASAD, D. (1964): The use of tetrazolium salts as a measure of sludge activity. *Water Pollution Control Federation Journal* 41, R441-R449.

KABARA, J.J. (1978): Structure-function relationships of surfactants as antimicrobial agents. *Journal of the Society of Cosmetic Chemists* 29, 733-741.

KABARA, J.J. (1983): Inhibition of *Staphylococcus aureus* in a model sausage system by monoglycerides. *Journal of the American Oil Chemists Society* 60, 715.

KABARA, J.J. (1984): Antimicrobial agents derived from fatty acids. *Journal of the American Oil Chemists Society* 61, 397-403.

KAGAWA, Y. (1972): Reconstitution of oxidative phosphorylation. *Biochimica et Biophysica Acta* 265, 297-338.

KANAZAWA, A., IKEDA, T. and ENDO, T. (1993): Novel polycationic biocides: synthesis and antibacterial activity of polymeric phosphonium salts. *Journal of Polymer Science: Part A: Polymer Chemistry* 31, 335-343.

KANAZAWA, A., IKEDA, T. and ENDO, T. (1993a): Polymeric phosphonium salts as a novel class of cationic biocides. II. Effects of counter anion and molecular weight on antibacterial activity of polymeric with phosphonium salts. *Journal of Polymer Science: Part A: Polymer Chemistry* 31, 1441-1447.

KANAZAWA, A., IKEDA, T. and ENDO, T. (1993b): Polymeric phosphonium salts as a novel class of cationic biocides. IV. Synthesis and antibacterial activity of polymers with phosphonium salts in the main chain. *Journal of Polymer Science: Part A: Polymer Chemistry* 31, 3031-3038.

KELLERMAYER, M., LUDANY, A., MISETA, A., KOSZEGI, T., BERTA, G., BOGNER, P., HAZELWOOD, C.F., CAMERON, I.L. and WHEATLEY, D.N. (1994): Release of potassium, lipids, and proteins from nonionic detergent treated chicken red blood cells. *Journal of Cellular Physiology* 159, 197-204.

KENNEDY, D. (1997): C12E6 uptake studies (Unilever Research, Port Sunlight)

KITATSUJI, K., MIYATA, H. and FUKASE, T. (1996): Lysis of filamentous bacteria by surfactants. *Water Science and technology* 34, 145-153.

KLOSE, G. and HOLLERBUHL, TH. (1981): Intercalation studies in three-component systems of egg yolk lecithin, detergent and water by ^{31}P-NMR. *Studia Biophysica* 83, 35-40.

KOLTHOFF, I.M. and STRICKS, W. (1948): Solubilisation of dimethylaminoazo-benzene in solutions of detergents I. *Journal Physical and Colloid Chemistry* 52, 915-941.

KOLTHOFF, I.M. and STRICKS, W. (1949): Solubilisation of dimethylaminoazo-benzene in solutions of detergents II *Journal Physical and Colloid Chemistry*. 53, 424-454.

KOPECKA-LEITMANOVA, A., DEVINSKY, F., MLYNARCIK, D. AND LACKO, I. (1989): Interaction of amine oxides and quaternary ammonium salts with membranes and membrane-associated processes in *E. coli* cells: mode of action. *Drug Metabolism and Drug Interactions* 7, 29-51.

KOURAI, H., SATO, T., HORIE, T., TAKEICHI, K. and SHIBASAKI, I. (1980): The antimicrobial characteristics of quaternary ammonium salts. *Journal of antibacterial and antifungal agents* 5, 1-7.

KRAMER, V.C., CALABRESE, D.M. and NICKERSON, K.W. (1980): Growth of *Enterobacter cloacae* in the presence of 25% sodium dodecyl sulfate. *Applied and Environmental Microbiology* 40, 973-976.

KRAMER, V.C. and NICKERSON, K.W. (1984a): A transport-dependent energy burden imposed by growth of *Enterobacter cloacae* in the presence of 10% sodium dodecyl sulfate. *Canadian Journal of Microbiology* 30, 699-702.

KRAMER, V.C., NICKERSON, K.W. and HAMLETT, N.V. (1984b): Prevalence of extreme detergent resistance among Enterobacteriaceae. *Canadian Journal of Microbiology* 30, 711-713.

KROLL, R.G. and ANAGNOSTOPOULOS, G.D. (1981): Potassium leakage as a lethality index of phenol and the effect of solute and water activity. *Journal of Applied Bacteriology* 50, 139-147.

LAMBERT, P.A. (1983): "The bacterial cell surface and drug resistance" in *Role of the envelope in the survival of bacterial in infections* , *Medical Microbiology* ed EASMON, C.S.F., JELJASZEWICZ, J., BROWN, M.R.W. and LAMBERT, P. , Academic Press, London. 2, 1-29.

LAMBERT, P.A. and HAMMOND, S.M. (1973): Potassium fluxes, first indications of membrane damage in micro-organisms. *Biochemical and Biophysical Research Communications* 54, 796-799.

LAMBERT, P.A. and SMITH, A.R. (1976): Antimicrobial action of dodecyldiethanolamine: activation of ribonuclease I in *Escherichia coli*. *Microbios* 17, 35-49.

LAMIKANRA, A. (1982): Effects of butyl hydroanisole (BHA) on the leakage of cytoplasmic materials from *Staphylococcus aureus* and *Escherichia coli*. *Journal of Applied Bacteriology* 53, R16.

LAMIKANRA, A. and ALLWOOD, M.C. (1977): Effects of polyethoxyalkyl phenols on the leakage of intracellular material from *Staphylococcus aureus*. *Journal of Applied Bacteriology* 42, 379-385.

LAMIKANRA, A. and SHOLOLA, O.A. (1984): A study of the activity of soft soaps prepared from a homologous series of saturated fatty acids against cells of *Escherichia coli* NCTC 9001. *Microbios* 41, 139-147.

LANYI, J.K. (1973): Influence of electron transport on the interaction between membrane lipids and Triton X-100 in *Halobacterium cutirubrum. Biochemistry* 12, 1433-1438.

LAOUAR, L., LOWE, K.C. and MULLIGAN, B.J. (1996): Yeast responses to nonionic surfactants. *Enzyme and Microbial Technology* 18, 433-438.

LEIN, E.J., HANSCH, C. and ANDERSON, S.M. (1968): Structure-Activity correlations for antibacterial agents on Gram-positive and Gram-negative cells. *Journal of Medicinal Chemistry* 11, 430-441.

LEIN, E.J. and PERRIN, J.H. (1976): Effect of chain length on critical micelle formation and protein binding of quaternary ammonium compounds. *Journal of Medicinal Chemistry* 19, 849-850.

LE MAIRE, M., MOLLER, J.V. and CHAMPEIL, P. (1987): Binding of a nonionic detergent to membranes: flip-flop rate and location of the bilayer. *Biochemistry* 26, 4803-4810.

LICTENBERG, D. (1985): Characterization of the solubilization of lipid bilayers by surfactants. *Biochimica et Biophysica Acta* 821, 470-478.

LICTENBERG, D., ROBSON, R.J. and DENNIS, E.A. (1983): Solubilization of phospholipids by detergents. *Biochimica et Biophysica Acta* 737, 285-304.

LIMA, R.G., MACEDO, P.M., GONCALVES, A.F.S. and SILVA, M.T. (1980): !. Effects of detergents on bacterial cells. 1- Alterations induced in *Bacillus subtilis* by triton X-100 and sodium dodecyl sulphate. *Cienc. Biol. Mol. Cell Biol.* 5, 25a.

LINDMAN, B. (1984): Structural aspects of surfactant micellar systems" in *Surfactants* ed. Tadros,Th.F., Academic Press, London p83.

LUGENTENBERG, B. and VAN ALPHEN, L. (1983): Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria. *Biochimica et Biophysica Acta* 737, 51-115.

LUNDIN, A. and THORE, A. (1975): Comparison of methods for extraction of bacterial adenine nucleotides determined by firefly assay. *Applied Microbiology* 30, 713-721.

MANDELSTAM, J., MCQUILLEN, K. and DAWES, I. (1982): *"Biochemistry of bacterial growth"*. 3rd Ed. Blackwell Scientific Publication, Oxford.

MAKINO, S., REYNOLDS, J.A. and TANFORD, C. (1973): The binding of deoxycholate and Triton X-100 to proteins. *Journal of Biological Chemistry* 248, 4926-4932.

McBAIN, J.W. and GREEN, A.A. (1946): Solubilisation of water-insoluble dye in soap solutions: effects of added salts. *Journal of the American Chemical Society* 68, 1731-1736.

McILVAINE, T.C. (1921): Buffer solution for colorimetric comparison. *Journal of Biological Chemistry*. 49, 183-186.

MERRILL, R.C. and GETTY, R. (1948): The effect of alkaline electrolytes on micelle formation in soap solutions. *Journal Physical and Colloid Chemistry*. 52, 774-787.

MILLER, T.L. and WOLIN, M.J. (1996): Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora. *Applied and Environmental Microbiology* 62, 1589-1592.

MITCHELL, A. (1997): Uptake of propidium iodide (Unilever Research, Port Sunlight)

MITCHELL, P. (1961): Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature* 191, 144-148.

MITCHELL, P. (1966): Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biological Reviews* 41, 445-502.

MIYAZAWA,K., OGAWA, M. and MITSUI, T. (1984): The physico-chemical properties and protein denaturation potential of surfactant mixtures. *International Journal of Cosmetic Science* 6, 33-46.

MLYNARCIK, D., CUPKOVA, V., DEVINSKY, F. and LACKO, I. (1978): Antimicrobial efficiency of saturated heterocyclic amine oxides. *Folia Microbiology* 23, 493-495.

MLYNARCIK, D., DEVINSKY, F. and LACKO, I. (1979a): Antimicrobial efficacy of some N,N'-dimethyl-1,6-hexadiamine dioxides. *Folia Microbiology* 24, 188-190.

MLYNARCIK, D., DEVINSKY, F., LEITMANOVA, A. and LACKO, I. (1985): Antimicrobial efficiency of a new iodophore prepared on the basis of amine oxide. *Cs. epidemiologie, mikrobiologie, immunologie* 34, 295-303.

MLYNARCIK, D., DENYER, S.P. and HUGO, W.B. (1981): A study of the action of a bisquaternary ammonium salt, an amine oxide and an alkoxy phenylcarbamic acid ester on some metabolic functions in *Staphylococcus aureus. Microbios* 30, 27-35.

MLYNARCIK, D., LACKO, I and DEVINSKY, F. (1979b): Antimicrobial effect of bis-quaternary ammonium salts derived from 1,3-propanediamine. *Experientia* 35, 1044.

MLYNARCIK, D., SIROTKOVA, L., DEVINSKY, F., MASAROVA, L., PIKULIKOVA, A. and LACKO, I. (1992): Potassium leakage from *Escherichia coli* cells treated by organic ammonium salts. *Journal of Basic Microbiology* 32, 43-47.

MUKERJEE, P. and MYSELS, K.J. (1971): Critical micelle concentrations in aqueous surfactant systems. *National Bureau of Standards, NSR DS-NBS 36, Washington.*

NEWTON, B.A. (1960): The mechanism of the bacterial action of surface active compounds: a summary. *Journal of General Microbiology* 12, 226-236.

NIKAIDO, H. and VAARA, M. (1985): Molecular basis of bacterial outer membrane permeability. *Microbiological Reviews* 49, 1-32.

NINEHAM, A.W. (1955): The chemistry of formazans and tetrazolium salts. *Chemical Reviews* 55, 355-475.

NOZAKI, Y., REYNOLDS, J.A. and TANFORD, C (1974): The interaction of a cationic detergent with bovine serum albumin and other proteins. *Journal of Biological Chemistry* 249, 4452-4459.

OHNISHI, M. and SAGITANI, H. (1993): The effect of nonionic surfactant structure on hemolysis. *Journal of the American Oil Chemists Society* 70, 679-684.

PANDEY, N.K., NATRAJ, C.V., KALLE, G.P. and NAMBUDIRY, M.E.N. (1983): Antibacterial properties of soap containing some fatty acid esters. *International Journal of Cosmetic Science* 7, 9-14.

PATERNOSTRE, M.-T., ROUX, M. and RIGAUD, J.-L. (1988): Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by triton X-100, octyl glucoside, and sodium cholate. *Biochemistry* 27, 2668-2677.

PETHICA, B.A. (1958): Lysis by physical and chemical methods. *Journal of General Microbiology* 18, 473.

PETROCCI, A.N. (1977): "Quaternary ammonium compounds" in *Disinfection, sterlization, and preservation*. ed BLOCK,S.S., Lea and Feiber. pp325-347

POOLE, R.K. (1983): Bacterial cytochrome oxidases. A structurally and functionally diverse group of electron transfer proteins. *Biochimica et Biophysica Acta* 726, 205-243.

PRESCOTT, L.M., HARLEY, J.P. and KLEIN, D.A. (1993): *"Microbiology"* 3rd Ed., Wm.C. Brown Publishers, Duduque.

RAADO, E. and TERKILDSEN, T.C. (1960): On the enzymatic determination of blood glucose. *Scandinavian Journal of Clinical and Laboratory Investigation* 12, 402-407.

RAHMBOURG, A. (1971): Morphological and histochemical aspects of glycoproteins at the surface of animal cells. *International Review of Cytology* 31, 57-114.

REMAN, F.C., DEMEL, R.A., DEGIER, J., VAN DEENEN, L.L.M., EIBL, H. and WESTPHAL, O. (1969): *Chemistry and Physics of Lipids* 3, 221-233.

RIGG, M.W. and LIU, F.W. (1953): Solubilisation of Orange OT and Dimethylaminobenzene. *Journal of the American Oil Chemists Society* 30, 14-17.

ROSENTHAL, S.L. and BUCHANAN, A.M. (1974): Influenences of cationic bactericidal agents on membrane ATPase of *Bacillus subtilis. Biochimica et Biophysica Acta* 363, 141-150.

RUSSELL, A.D. (1991): Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology* 71, 1991-201.

RUSSELL, A.D. and CHOPRA, I. (1996): *"Understanding antibacterial action and resistance"* 2nd Ed., Ellis Horwood, London.

RUSSELL, A.D. and FURR, J.R. (1986): The effects of antiseptics, disinfectants and preservatives on smooth, rough and deep rough strains of *Salmonella typhimurium. International Journal of Pharmaceutics* 34, 115-123.

RUSSELL, A.D., FURR, J.R. and PUGH, W.J. (1985): Susceptibilty of porin-deficient and lipopolysaccharide-deficient mutants of *Escherichia coli* to a homologous series of esters of para-hydroxybenzoic acid. *International Journal of Pharmaceutics* 27, 163-173.

RUSSELL, A.D., FURR, J.R. and PUGH, W.J. (1987): Sequential loss of outer-membrane lipopolysaccharides and sensitivity of *Escherichia coli* to antibacterial agents. *International Journal of Pharmaceutics* 35, 227-233.

SALT, W.G. and WISEMAN, D. (1991): "Biocide uptake by bacteria" in *Mechanisms of action of chemical biocides - their study and exploitation* ed. HUGO, W.B. and DENYER, S.P. , Society for Applied Bacteriology, Blackwell Science. pp65-86

SALTON, M.R.J. (1951): The properties of lysozyme and its action on microorganisms. *Bacteriological Reviews* 21, 82-99.

SALTON, M.R.J. (1957): The action of lytic agents on the surface structures of the bacterial cell in *Proceedings of the Second International Congress on surface activity*, ed SCHULMAN, J.H., Butterworth, London. pp245-253.

SALTON, M.R.J. and OWEN, P. (1976): Bacterial membrane structure. *Annual Review of Microbiology* 30, 451-482.

SCALES, F.M. and KEMP, M. (1941): A new group of sterilizing agents for the food industries and a treatment of chronic mastitis. *International Association of Milk Dealers*, 491-519.

SCHMITT, T.M. (1992): "Characterisation of anionic surfactants" in *Analysis of surfactants,* Marcel Dekker.Inc., New York and Basel. pp1-52.

SCHNAITMAN, C.A. (1971a): Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *Journal of Bacteriology* 108, 545-552.

SCHNAITMAN, C.A. (1971b): Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli. Journal of Bacteriology* 108, 553-563.

SCHOTT, H. (1967): Adsorption of a nonionic surfactant by cotton. *Journal of Colloid and Interface Science* 23, 46-51.

SEEMAN, P. (1972): The membrane actions of anesthetics and tranquilizers. *Pharmacological Reviews* 24, 583-655.

SHAPIRO, S. and GUGGENHEIM, B. (1995): The action of thymol on oral bacteria. *Oral Microbiology and Immunology* 10, 241-246.

SHEU, C.W. and FREESE, E. (1973): Lipopolysaccharide layer protection of Gram-negative bacteria against inhibition by long chain fatty acids. *Journal of Bacteriology* 115, 169-875.

SIGMA DIAGNOSTICS GLUCOSE PROCEDURE NO. 510 (Aug. 1995) Sigma Chemical Co., St. Louis, Missouri, USA.

SMITH, J.J. and MCFEETERS, G.A. (1996):Effects of substrates and phosphate on INT (2-(4iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride and CTC (5-cyano-2,3-ditolyl tetrazolium choride) reduction in *Escherichia coli*. *Journal of Applied Bacteriology* 80, 209-215.

SPRENGER, G.A. (1995): Genetics of pentose-phosphate pathway enzymes of *Escherichia coli* K-12. *Archives of Microbiology* 164, 324-330.

SPOTTS, R.A. and CERVANTES, L.A. (1987): Effects of the nonionic surfactant Ag-98 on three decay pear of Anjou pear. *Plant Disease* 71, 240-242.

STEWART, M.H. and OLSON, B.H. (1992): Impact of growth conditions on resistance of *Klebsiella pneumoniae* to Chloramines. *Applied and Environmental Microbiology* 58, 2649-2653.

STROMBERG, K. (1971): Surface-active agents for isolation of the core component of Avian Myeloblastosis virus. *Journal of Virology* 9, 684-697.

SZOGYI, M., TOLGYESI, F. and CSERHATI, T. (1981): Membrane damaging effects of nonionic surfactants. *Acta Biochimica et Biophysica Academiae Scientiarum Hungaricae* 16, 245.

TANFORD, C. (1972): Hydrophobic free energy, micelle formation and the association of proteins with amphiphiles. *Journal of Molecular Biology* 67, 59-74.

TEMPEST, D.W. and STRANGE, R.E. (1966): Variation in content and distribution of magnesium, and its influence on survival, in *Aerobacter aerogenes* grown in a chemostat. *Journal of General Microbiology* 44, 273-279.

THONY-MEYER, L. (1997): Biogenesis of respiratory cytochromes in bacteria. *Microbiology and Molecular Biology Reviews* 61, 337-376.

TIDDY, G.J.T. (1980): Surfactant-water liquid crystal phases. *Physics Reports* 57, 1-46.

TIEHM, A. (1994): Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Applied and Environmental Microbiology* 60, 258-263.

UMBREIT, J.N. and STROMINGER, J.L. (1973): Relation of detergent HLB number to solubilization and stabilization of D-alanine carboxypeptidase from *Bacillus subtilis* membranes. *Proceedings of the National Academy of Sciences of the United States of America* 70, 2997-3001.

VADEHRA, D.V. and WAHI, V. (1983): Comparison of antibacterial properties of Lauricidin and BHA against antibiotic-resistant and sensitive *Staphylococcus aureus* and *Pseudomonas aeruginosa. Journal of the American Oil Chemists Society* 60, 715.

VULLIEZ-LE NORMAND, B. and EISELE, J-L. (1993): Determination of detergent critical micelle concentration by solubilization of a colored dye. *Analytical Biochemistry* 208, 241-243.

WITHAM, R.F., (1983): A study of the antimicrobial activity of a homologous series of phenols. *PhD thesis, University of Nottingham*

YOUNG, M., DINDA, M. and SINGER, M. (1983): Effect of tween 80 on lipid vesicle permeability. *Biochimica et Biophysica Acta* 735, 429-432.

ZASLAVSKY, B.Y., OSSIPOV, N.N., KRIVICH, V.S., BAHOLDINA, L.P. and ROGOZHIN, S.V. (1978a): Action of surface-active substances on biological membranes. II Hemolytic activity of nonionic surfactants. *Biochimica et Biophysica Acta* 507, 1-7.

ZASLAVSKY, B.Y., OSSIPOV, N.N. and ROGOZHIN, S.V. (1978b): Action of surface-active substances on biological membranes. III Comparison of hemolytic activity of ionic and nonionic surfactants. *Biochimica et Biophysica Acta* 510, 151-159.

ZASLAVSKY, B.Y., OSSIPOV, N.N., ROGOZHIN, S.V, SEBYAKIN, Y.L., VOLKOVA, L.V. and EVSTIGNEEVA, R.P. (1979): Action of surface-active substances on biological membranes. IV Hemolytic and membrane-perturbing action of homologous series of β–D-glucopyranosyl-1-alkylphosphates. *Biochimica et Biophysica Acta* 556, 314-321.