Growth and survival of *Klebsiella pneumoniae* in the presence of pyrithione

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GROWTH AND SURVIVAL OF KLEBSIELLA PNEUMONIAE IN THE PRESENCE OF PYRITHIONE

A THESIS

Submitted in partial fulfilment of the requirements for the award of the Degree of

DOCTOR OF PHILOSOPHY

by

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SUPERVISORS: Dr W. G. SALT & Dr R. J. STRETTON

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UNIVERSITY OF TECHNOLOGY
LOUGHBOROUGH

DEDICATION

TO ALL THOSE WHO DID AND CONTINUE TO ENRICH MY LIFE
Acknowledgements

I would like to thank Dr W. G. Salt and Dr R. J. Stretton for their guidance and help throughout this project. I am also grateful to all members of staff and research in the Medicinal Chemistry section for their helpful comments and discussions. My thanks also to the technical staff for their assistance.

I am indebted to Diane for her love and support throughout.

Above all, I thank my family, and in particular my brother Ismat, who supported me both financially and morally throughout. Without their support, love and commitment, this thesis would not have materialised.
ABSTRACT

Pyrithione (2-mercaptopyridine-N-oxide; Omadine) showed broad-spectrum antimicrobial activity with Gram-positive bacteria and yeasts being more sensitive than Gram-negative bacteria. This study examines the growth and survival profiles of Klebsiella pneumoniae in the presence of pyrithione. Studies of growth and survival of Bacillus licheniformis and Candida albicans are included for comparative purposes.

In the presence of the drug, Kl. pneumoniae and B. licheniformis exhibited similar growth patterns, namely a drug concentration-dependent lag phase followed by growth at a rate similar to that of untreated cells. In contrast, C. albicans exhibited a drug concentration-dependent rate of growth. Viable counts of Kl. pneumoniae and C. albicans in the presence of pyrithione, mirrored the growth patterns observed by turbidity measurements.

Size analysis of ampicillin-treated (divisionless) cells of Kl. pneumoniae in the presence and absence of pyrithione, suggested that all cells are inhibited from growth during the lag phase, and re-growth is due to recovery of all cells rather than the growth of a resistant population within the culture.

Bacteria grown in the presence of sub-inhibitory concentrations of pyrithione were insensitive to further challenge with the same concentrations of the drug. The resistance of Kl. pneumoniae was maintained during 7 days of subculture in drug-free medium, but fell to a lower level after 4 days of subculture, and cross-resistance to chloramphenicol was observed, but not to 8-hydroxyquinoline. The fatty acid profile of pyrithione-sensitive and pyrithione-resistant Kl. pneumoniae are essentially similar. Cells of B. licheniformis grown in the presence of pyrithione showed no cross-resistance with any of the antimicrobial agents tested.

Pyrithione also inhibited the incorporation of $^{14}$C-glycine, $^{14}$C-thymidine and $^{14}$C-uridine in both Kl. pneumoniae and B. licheniformis. Further investigation showed that in Kl. pneumoniae, only the $^{14}$C-uridine uptake profile resembled the
growth inhibition profile. Pyrithione, at sub-inhibitory concentration, caused 70% inhibition of the activity of purified *Escherichia coli* RNA polymerase *in vitro*. Scanning electron microscopy showed that some cells of *Kl. pneumoniae* underwent elongation upon prolonged incubation (24 hours) in the presence of pyrithione approaching the minimum inhibitory concentration. The morphology of *B. licheniformis* was not significantly affected by pyrithione, but the drug caused some damage to the cell surface in *C. albicans* when at concentrations approaching the minimum inhibitory concentration.

It is concluded that pyrithione has more than one mode of action. Results are discussed in the light of pyrithione having an indirect effect on protein synthesis in *Kl. pneumoniae*, perhaps by interfering with RNA synthesis at the RNA polymerase level.
<table>
<thead>
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<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined medium</td>
</tr>
<tr>
<td>CDMA</td>
<td>Chemically defined medium agar</td>
</tr>
<tr>
<td>CTP</td>
<td>Cytidine-5'-triphosphate</td>
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<tr>
<td>DNA</td>
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</tr>
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</tr>
<tr>
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<td>Fatty acids methyl esters</td>
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<tr>
<td>GLC</td>
<td>Gas Liquid chromatography</td>
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<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
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<tr>
<td>TTP</td>
<td>Thymidine-5'-triphosphate</td>
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<td>Uridine-5'-triphosphate</td>
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CHAPTER ONE

INTRODUCTION
1.1. Targets for Antimicrobial Agents in the Bacterial Cell

1.1.1. The Cell Wall

The bacterial cell wall is a complex structure composed of a range of molecules, many of which are not found elsewhere in nature. The cell wall polymers function as an envelope to protect the protoplast surrounded by its delicate cytoplasmic membrane, and to confer the characteristic shape on the bacterium. They also contain on their outside surface sites for (1) the adsorption of bacteriophages, (2) the recognition and transport of certain substances and (3) cell-cell recognition and interaction (Gale et al., 1981). Bacteria are divided into two main groups according to their reaction to Gram-stain. This division reflects the difference in the chemical composition of the cell wall between Gram-positive and Gram-negative bacteria (Table 1.1).

Table 1.1 Bacterial Cell Wall Polymers (Gale et al., 1981)

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Gram-positive</th>
<th>Gram-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Teichoic and/or teichuronic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lipopolysaccharides</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lipoprotein</td>
<td>-</td>
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<tr>
<td>Polysaccharide</td>
<td>+/-</td>
<td>-</td>
</tr>
</tbody>
</table>
Peptidoglycan is a polymer found in cell walls of both Gram-positive and Gram-negative bacteria. It consists of a macromolecular network made up from linear glycan strands of β-(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid (Fig. 1.1a). The glycan strands are cross-linked by peptide substituents: oligopeptide chains containing a sequence of L- and D-amino acids are present on each N-acetylmuramic acid residue. Some of these peptides are cross-linked, and the nature of the cross-link is a feature distinguishing peptidoglycans from different organisms (Lambert, 1988). In addition to the peptidoglycan layer, the simpler Gram-positive wall contains teichoic acid. The Gram-negative cell wall is more complex and contains outer layers of lipopolysaccharide and lipoprotein (comprising the outer-membrane) in addition to peptidoglycan (Fig. 1.1b). The structure of the bacterial cell wall has been extensively reviewed (Salton, 1962, 1964; Schleifer and Kandler, 1972; Costerton and Cheng, 1975; Schleifer and Stackebrandt, 1983; Lambert, 1983, 1988; Schleifer, 1985).

The biosynthesis of the peptidoglycan layer is the target for many antibiotics and antimicrobial agents, notably β-lactam antibiotics. β-lactam antibiotics have the ability to bind and inhibit a group of proteins, hence known as penicillin binding proteins (PBP), associated with various enzymic activities and functions in peptidoglycan synthesis (Spratt and Pardee, 1975; Spratt, 1977). Other antibiotics and antimicrobial agents acting on peptidoglycan synthesis include cycloserine, vancomycin, and bacitracin. Figure 1.2 shows the various stages of peptidoglycan synthesis and the effects of antibiotics. The mode of action of bacterial cell wall synthesis inhibitors have been reviewed (Salton and Tomasz, 1974; Gale et al., 1981; Franklin and Snow, 1989).
Figure 1.1 (a) Cross-linked peptidoglycan arrangement in *Staphylococcus aureus*.
NAM, N-acetylmuramic acid; NAG, N-acetylglucosamine; L-ala, L-alanine; L-lys, L-lysine; gly, glycine; D-ala, D-alanine; D-glu, D-glutamate.

(Edwards, 1980)

(b) Diagrammatic representation of the (a) Gram-negative and (b) Gram-positive cell envelopes: CW, cell wall; PS periplasm; CM, cytoplasmic membrane; CMP, cytoplasmic membrane protein; OM, outer membrane; G, glycocalyx; OMP, outer-membrane protein; LPS, lipopolysaccharide; PL, phospholipid; LP, lipoprotein; PP, porin protein; TA, teichoic acid; PG, peptidoglycan. (Gilbert, 1988).
Figure 1.2. Peptidoglycan synthesis in *Escherichia coli*, and the effect of antibiotics. NAG, N-acetylglucosamine; PEP, Phosphoenolpyruvate; NAM, N-acetylmuramic acid. (Quesnel and Russell, 1983).
1.1.2. The Cell Membrane

The cytoplasmic membrane is a thin structure that completely surrounds the bacterial cell. It consists of a phospholipid bilayer liberally studded with a wide variety of polypeptides (Hancock and Nicas, 1984). The currently accepted model of the membrane is that suggested by Singer and Nicholson (1972), namely, the fluid mosaic model shown in Figure 1.3.

The cytoplasmic membrane is a dynamic structure containing at least 40% fluid lipids and is a critical permeability barrier vital to the function of the cell (Kabara, 1984). The major functions of the cytoplasmic membrane proteins include energy generation (e.g., electron-transport chain carrier proteins and Ca\(^{2+}\), Mg\(^{2+}\)-stimulated ATPase); active and facilitated transport of nutrients and export of toxic products, and enzymic synthesis of cell wall components (Hancock and Nicas, 1984). The structure and composition of the cytoplasmic membrane have been reviewed (Hughes, 1962; Salton and Owen, 1976; Edwards, 1980; Gale et al., 1981; Hugo, 1983).

Many antimicrobial agents affect the cytoplasmic membrane function and they can be divided into the following groups (Gale et al., 1981):

1. drugs causing major disorganisation of the cytoplasmic membrane such, e.g., tyrocidins, phenols and polymyxins;
2. drugs that produce aqueous pores in membranes thus disturbing the controlled transport of substances across the membrane, e.g., gramicidines and polyene antibiotics (mainly antifungal);
3. drugs causing specific changes in cation permeability (ionophores), e.g., dinitrophenyl and valinomycin;
4. drugs that inhibit membrane-bound enzymes involved in energy transfer, e.g., Chlorhexidine and dicyclocarbodiimide;
5. inhibitors of enzymes involved in the synthesis of essential components of membrane, such as cerulenin which inhibits fatty acid synthesis.
The mode of action of antimicrobial agents against the cytoplasmic membrane has been reviewed (Salton and Tomasz, 1974; Gale et al, 1981; Franklin and Snow, 1989).

Figure 1.3 Fluid mosaic membrane model. The solid bodies represent globular proteins which are immersed in the lipid bilayer (Singer and Nicholson, 1972).
1.1.3. Nucleic Acids

The chromosome of the bacterial cell consists of a single circular molecule of double stranded DNA. In general, drugs which inhibit nucleic acid function are not especially selectively toxic since the mechanisms of nucleotide synthesis, replication and transcription are very similar in both prokaryotic and eukaryotic cells (Edwards, 1980).

Gale et al. (1981) defined three levels at which inhibitors of nucleic acid synthesis may exert their primary effect. The first group include those agents which interfere with nucleotide metabolism. Examples of this group include azaserine which inhibits purine synthesis, 5-fluorouracil which inhibits nucleotide interconversion, cytosine arabinoside inhibiting nucleotide utilisation and 5-iododeoxyuridine which serves as an unnatural substrate that is incorporated into polynucleotides causing distortion to the structure and function of the nucleic acid.

The second group includes agents that impair the template function of the DNA. Classical examples of this group of compounds are the intercalating drugs ethidium and proflavine. Because of their planar ring structure and their width corresponding with that of a base pair (0.34 nm), these drug molecules are inserted into the DNA helix causing frameshift mutagenesis, and subsequent inhibition of the function of DNA.

Compounds that inhibit enzymic processes in nucleic acid synthesis comprise the third and final group in this division. Rifampicin, for example, selectively binds and inhibits RNA polymerase in bacteria, whilst nalidixic acid and its analogues interfere with DNA synthesis by inhibiting the enzyme DNA gyrase (Pedrini, 1979; Cozzarelli, 1977, 1980). The mode of action of nucleic acid biosynthesis inhibitors has been reviewed by Gale et al. (1981) and Franklin and Snow (1989).
1.4. Ribosomes

Ribosomes are the centres of protein synthesis. In bacteria they are composed of RNA and protein in the mass ratio 65:35, respectively, and contain 80-90% of the total cellular RNA (Gale et al., 1981). Ribosomes of prokaryotic cells can be distinguished from those of eukaryotic cells on the basis of their sedimentation coefficients ('S' values). Ribosomes of bacteria and blue-green algae have a sedimentation coefficient of 70S, and each ribosome dissociates to give 30S and 50S particles. In fungi, yeasts, algae and protozoa, 80S ribosomes are synthesized and each is built of one 60S and one 40S particle (Rose, 1968). Aggregation of ribosomal particles is brought about and regulated by varying concentration of Mg$^{2+}$ (Rose, 1968).

Inhibitors of ribosomal function in bacteria are classified on the basis of their site of action, that is, whether they bind primarily to the 30S or 50S ribosomal subunit (Table 1.2). Inhibitors of the smaller 30S unit include tetracyclines which inhibits the binding of aminoacyl-tRNA to the acceptor site on the ribosome, and aminoglycosides such as streptomycin, Neomycin and Gentamycin also bind to the 30S subunit causing misreading of the genetic code (Davis, 1988). Drugs acting at the 50S subunit include chloramphenicol which inhibits peptide bond formation (and therefore peptide elongation) through inhibition of the peptidyltransferase reaction, and macrolides such as erythromycin which act by stimulating the dissociation of peptidyl-tRNA from ribosomes thus interrupting the completion of peptide chains (Franklin and Snow, 1989).

The mode of action of ribosomal function inhibitors has been reviewed (Gale et al., 1981; Franklin and Snow, 1989).
Table 1.2 Classification of protein synthesis inhibitors (Edwards, 1980)

Drugs acting on the 30S ribosomal subunit

The aminoglycoside antibiotics

The tetracyclines

Drugs acting on the 50S ribosomal subunit

Puromycin
chloramphenicol
Erythromycin
Lincomycin
Clindamycin
Fusidic acid

1.1.5 Metabolism

Some antibacterial agents act by specifically inhibiting some enzymic reactions.
Both sulphonamides and trimethoprim belong to this group of compounds and act by inhibiting the biosynthesis of folic acid which is essential to the production of nucleic acid constituents. Sulphonamides interfere with folic acid biosynthesis at the earlier stage by competing with p-aminobenzoic acid (PABA) for the active site on the enzyme dihydropteroic acid synthetase. Trimethoprim acts at a later stage by binding and inhibiting the enzyme dihydrofolate reductase (Franklin and Snow, 1989).
1.2. Bacterial Resistance to Antimicrobial Agents

1.2.1. Introduction

Micro-organisms are capable of establishing resistance towards substances which normally have a selective lethal or inhibitory action against them. Thus resistance can be defined as the temporary or permanent ability of an organism and its progeny to remain viable and multiply under environmental conditions that would destroy or inhibit other cells (Lambert and Hammond, 1983). Bacterial resistance to antimicrobial agents has always attracted intense interest since it poses an obviously serious problem in the treatment of infectious diseases (Moyed, 1964), preservation of pharmaceutical products (Gilbert, 1988) and disinfection (Lambert and Hammond, 1983).

A distinction can be made between 'inherent' and 'acquired' resistance of bacteria to antibacterial agents (Franklin, 1983). Certain bacteria, such as Gram-negative bacteria, are generally more resistant to antimicrobial agents. This 'inherent' resistance of Gram-negative bacteria has been attributed to the impermeability of the complex outer membrane to some drugs which, with periplasmic enzymes, may prevent the attainment of an inhibitory concentration within the cell (Franklin, 1983; Nikaido, 1988). Furthermore, within the Gram-negative group, Pseudomonas aeruginosa and Klebsiella pneumoniae are inherently more resistant to many antimicrobial agents (Brown, 1975).

The acquired ability of bacteria to grow and multiply in the presence of an antimicrobial agent may reflect a difference in the genetic makeup between these and sensitive organisms (Franklin, 1983). When a bacterial population adapts to the presence of an antimicrobial agent, sensitive cells are gradually replaced by resistant cells. Such resistance may be acquired through spontaneous mutations at appropriate genetic loci, and the resulting resistant mutants will eventually overgrow and replace
sensitive cells in the presence of an antimicrobial agent. Alternatively, genetic material conferring resistance may be transferred from one cell to another through processes such as conjugation, transduction or transformation (Franklin, 1983). These mechanisms of acquisition of resistance will not be discussed here, but the biochemical mechanisms of bacterial resistance to antimicrobial agents will be examined more closely using representative examples.

1.2.2. Biochemical mechanisms of resistance

A number of mechanisms have been demonstrated:

1. Conversion of active drug to an inactive derivative by enzyme(s) produced by resistant cells.

The most important mechanism of bacterial resistance to β-lactam antibiotics is through hydrolysis of the β-lactam ring by β-lactamases (Bryan, 1988). β-Lactamases are generally induced in both Gram-negative (Jacoby and Sutton, 1985) and Gram-positive bacteria (Lyon and Skurray, 1987; Boyce et al, 1990) by the presence of minute quantities of β-lactam antibiotics. However, β-lactamases of Gram-positive bacteria act in a different manner from those of Gram-negative organisms (Bryan, 1988). Generally, Gram-positive β-lactamases are released into the medium, whereas those from Gram-negative bacteria, typically remain within the periplasmic space through which the β-lactam must pass during cellular uptake (Bryan, 1988; Franklin and Snow, 1989).

Another important example of bacterial resistance due to antibiotic inactivation is the enzymatic acetylation of chloramphenicol (Sands and Shaw, 1973; Roberts et al, 1985; Martinez-Suarez et al, 1985; Powell et al, 1989). The enzyme responsible, chloramphenicol acetyltransferase (CAT) converts chloramphenicol to the microbiologically inactive 3-acetoxy derivative using acetyl-CoA as an essential cofactor (Franklin and Snow, 1989).
(2) Modification of the drug-sensitive site.

A common example of the modification of a drug-sensitive site resulting in a high level of resistance is the loss of ribosomal sensitivity to streptomycin (Eliopoulos et al., 1984). The change in ribosomal structure has been traced to a single amino acid replacement in either of two positions in the S12 protein of the 30S ribosomal subunit. The modified 30S subunit no longer binds streptomycin and the drug no longer inhibits protein synthesis (Franklin and Snow, 1989).

Similarly, the resistance of Mycobacterium tuberculosis to other protein synthesis inhibitors, such as viomycin and kanamycin, is believed to result from shifts in ribosomal structure (Yamada et al., 1985). Escherichia coli resistance to quinolone antibiotics, such as nalidixic acid, may also be due to an altered DNA gyrase, the usual target enzyme (Piddock and Wise, 1989).

(3) Loss of cell permeability to a drug.

The bacterial cell envelope is a natural barrier to the penetration of antimicrobial agents into Gram-negative and Gram-positive cells (Costenon and Cheng, 1975). The outer-membrane present in Gram-negative bacteria is itself an additional barrier that in part explains the resistance of Gram-negative bacteria to many antimicrobial agents (Nikaido, 1988). However, more specific mechanisms of resistance associated with reduced permeability to certain drugs do exist. For example, bacterial resistance to tetracyclines is the result of an efflux energy-dependent process that specifically pumps tetracyclines out of the cell against a concentration gradient (Durckheimer, 1975; Chopra and Howe, 1978; McMurry et al., 1987; Park et al., 1987).

Chloramphenicol resistance in Gram-negative bacteria such as Haemophilus influenzae and Pseudomonas species has been shown to be mediated by the absence of a porin protein from the outer membranes of these organisms, resulting in reduced uptake of the drug (Burns et al., 1985, 1986, 1989). Similar alterations in the outer membrane proteins are also responsible for the reduced uptake of some β-lactam
antibiotics such as impenem by Enterobacter aerogenes (Hopkins and Towner, 1990) and Pseudomonas aeruginosa (Bellido et al, 1990).

(4) Synthesis of an additional drug-resistant (target) enzyme.

Bacterial resistance to sulphonamides and trimethoprim exemplifies the synthesis of additional drug-resistant forms of the target enzyme. In the case of sulphonamides, drug-resistant variants of the target enzyme (dihydropteroate synthetase) are produced which have lower affinities to the drugs (Swedberg, 1987; Facirelli and Varaldo, 1987). Similarly, the production of drug-resistant variants of the enzyme dihydrofolate reductase confers resistance in bacteria to trimethoprim (Huovinen, 1987; Wylie and Koornhof, 1989; Heikkila et al; 1990). Such mechanism of resistance is also seen as a case where the organism utilises an alternative metabolic pathway that is less sensitive to the antimicrobial agent (Franklin and Snow, 1989).

(5) Increased production of a metabolite that antagonises the inhibitor.

When a drug inhibits microbial growth by the competitive antagonism of a normal metabolite, resistance may be due to increased production of the metabolite. The hyperproduction of p-aminobenzoic acid (the natural substrate for the enzyme dihydropteroate synthetase) with which sulphonamides are believed to compete for the active site on the enzyme, is an example of this mechanism of resistance (Franklin and Snow, 1989). This mechanism, however, is not clinically as significant as the previous mechanisms.

Mechanisms of bacterial resistance have been extensively reviewed (Moyed, 1964; Gale et al, 1981; Franklin, 1983; Bryan, 1988; Williams and Moosdeen, 1986; Nord, 1986; Franklin and Snow, 1989).
1.3. The Role of Metal Ions in the Microbial Cell

1.3.1. Introduction

All forms of life require metal ions for survival and growth. Heavy metals, such as cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), Molybdenum (Mo) and Zinc (Zn) are usually less abundant in living cells than the lighter metals like calcium (Ca), magnesium (Mg), potassium (K) and sodium (Na) (Albert, 1985). The importance of metal ions in microbial nutrition was first acknowledged back in the nineteenth century when Louis Pasteur found that the addition of ash was necessary for the growth of yeasts on a medium containing ammonium salts and sucrose (Knight, 1951). It is now well established that metal ions play a fundamental role in the integrity, growth and metabolism of microbial cells, as well as their tolerance towards environmental factors.

1.3.2. Role of metal ions in the integrity of the microbial cell

Although metal ions that are required by micro-organisms serve mainly as activators or active groups of a variety of enzymes (Snell, 1957), some play an essential role in the integrity of cellular structures. The bulk of bacterial magnesium (Mg^{2+}) is associated with ribosomes and is needed for the preservation of the structure of ribosomes (Allen and Wong, 1986). In addition, Mg^{2+} stabilises spheroplasts, suggesting its involvement in the integrity of the cellular membrane (Jasper and Silver, 1977). Calcium (Ca^{2+}) is also involved in the integrity of bacterial cells as it stabilises cell wall structure by bridging carboxyl groups in peptidoglycan chains (Silver, 1977). Metal ions also play a significant role in the stability and integrity of nucleic acids. Wacker and Vallee (1959) showed that several metals including chromium (Cr), manganese (Mn), nickel (Ni), iron (Fe) and zinc (Zn), were associated with RNA.
from various biological sources. Their findings suggested that metals may play a role in the maintenance of the configuration of the RNA molecule by linking purine and/or pyrimidine bases through covalent bonds. Various mono- as well as divalent metal ions are also associated with bacterial DNA (Sigee and El-Masry, 1987), and their role in the integrity and conformation of the DNA has also been studied (Subirana, 1987).

1.3.3. Role of metal ions in enzymes

A metallo-enzyme is that enzyme which retains stoichiometric, tightly bound, functional metal ions upon purification, in contrast to activated enzymes which require the addition of metal ions for activity (Boyer et al, 1964). The list of metalloenzymes is probably large; some of these enzymes and their metallic components are listed in Table 1.3.

Table 1.3 Some known metallo-enzymes.

<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>Metal ion(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase</td>
<td>Mg, Ca</td>
<td>Jasper and Silver (1977)</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>Ca</td>
<td>Silver (1977)</td>
</tr>
<tr>
<td>RNA and DNA polymerases</td>
<td>Zn</td>
<td>Wu and Wu (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wu and Chatterji (1983)</td>
</tr>
<tr>
<td>Nitrogenase</td>
<td>Fe</td>
<td>Byers and Arceneaux (1977)</td>
</tr>
<tr>
<td>Cytochromes</td>
<td>Fe</td>
<td>Coughlan (1971)</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>Mo</td>
<td>Rajagopalan (1987)</td>
</tr>
<tr>
<td>Microbial Ureases</td>
<td>Ni</td>
<td>Hausinger (1987)</td>
</tr>
</tbody>
</table>
1.3.4. Regulation of secondary metabolism by metal ions

In addition to their functions as coenzymes and stabilisers of cellular structures, some metals play a critical role in the regulation of biosynthesis of microbial metabolites such as toxins, vitamins and antibiotics. The quantity of zinc, generally, is critical for secondary metabolic processes in fungi and actinomycetes, whilst the concentration of manganese is important in species of *Bacillus* and the amount of iron is influential in other bacteria including the *actinomycetes* (Weinberg, 1982).

Many examples of the effects of iron on the biosynthesis of microbial metabolites are available as iron's role in microbial metabolism has received intensive attention over the years. Toxin production by *Corynebacterium diphtheriae*, for example, is greatly enhanced by low levels of iron in the medium (Coughlan, 1971). Similarly, iron-deficiency in *Candida* species (Goodwin, 1959) or *Clostridium acetobutylicum* (Hickey, 1945) results in increased production of riboflavin. The production of antibiotics, such as actinorubin by actinomycetes, chloramphenicol by *Streptomyces venezuelae* and penicillin by *Penicillium chrysogenum* is greatly enhanced by concentrations of iron greater than required for optimal growth (Weinberg, 1962). The production of other antibiotics, such as chlorotetracycline by *Streptomyces aureofaciens*, oxytetracycline by *Streptomyces remosus* and neomycin by *Streptomyces fradiae* is, however, reduced by higher concentrations of iron (Weinberg, 1962; Coughlan, 1971).

1.3.5. Bacterial response to iron-deficiency

Iron is so essential for the viability of some micro-organisms that in iron-restricted environments, they produce substances, known as siderophores, that have high affinity for iron (Fe$^{3+}$) and which facilitates active iron uptake. A siderophore is defined as a low-molecular-weight (500 - 1000 daltons) virtually ferric specific
ligand, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell (Byers and Arceneaux, 1977; Neilands, 1981; Bagg and Neilands, 1987; Brown and Williams, 1985; Ankenbauer et al., 1988; Crosa, 1989; Martinez et al., 1990). Although the majority of siderophores that have been isolated and identified are of bacterial origin, they are also produced by fungi under iron-deficiency conditions (Holzberg and Artis, 1983). Table 1.4 lists some micro-organisms and the siderophores they produce.

In general, siderophores may be broadly classed, according to their chemical structure as either hydroxamates [-CO-N(O-)–] or phenolates - catecholates (Neilands, 1981):

![Chemical structure of siderophores]

Table 1.4 Some siderophores and their microbial origin (Neilands, 1981).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Siderophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteric species</td>
<td>Enterobactin, aerobactin</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>Agrobactin</td>
</tr>
<tr>
<td>Pseudomonas species</td>
<td>Pyochelin, pyoverdin, pseudobactin, ferribactin</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>Schizokinen</td>
</tr>
<tr>
<td>Mycobacteria</td>
<td>Mycobactins</td>
</tr>
<tr>
<td>Actinomyces species</td>
<td>Ferrioxamines</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td>Penicillia, AspegiIIi,</td>
<td></td>
</tr>
<tr>
<td>Neurospora</td>
<td>Ferrichromes, coprogen</td>
</tr>
<tr>
<td>Rhodotorula species</td>
<td>Rhodotorulic acid</td>
</tr>
</tbody>
</table>
In addition to the production of siderophores, bacteria growing under iron-restriction derepress the synthesis of several outer-membrane proteins, some of which serve as receptors for ferric-siderophores (Brown and Williams, 1985; Chart et al., 1988).

The significance of siderophores as microbial virulence factors stems from the knowledge that the level of freely available iron in the healthy mammalian host is severely restricted (Chart et al., 1988). It follows that siderophores, with their exceptionally high affinity to iron, scavenge the minute amount of free iron available in the host during infection in order to maintain growth and viability. Outer-membrane proteins profiles of Pseudomonas aeruginosa isolated, without subculturing, from the lungs of Cystic Fibrosis patients, were similar to the outer-membrane proteins profiles of the same organism grown in the iron-limited medium under laboratory conditions (Brown et al., 1984). This provided evidence that, in vivo, bacteria grew under iron-restricted conditions, and was further supported by similar observations on Klebsiella pneumoniae isolated from the urinary tract of infected patients (Shand et al., 1985). Such changes in the outer-membrane may also contribute to the resistance of Gram-negative bacteria to antibiotics (and hence to chemotherapy), since the outer-membrane serves as a barrier to permeation of antibiotics (Nikaido, 1988). Indeed, the resistance of Pseudomonas aeruginosa to penicillin G, of Staphylococcus aureus to phagocytosis and of Neisseria gonorrhoeae to the bactericidal action of serum have all been enhanced by changes in outer-membrane- or surface-associated proteins provoked by iron-limitation (Johnson, 1985; Domingue et al., 1989).

This microbial response to iron-restriction has potential therapeutic applications. Iron-regulated outer-membrane proteins may serve as possible vaccine components where the antibodies produced in response to such a vaccine would bind specifically to the outer-membrane proteins of the respective pathogen. Siderophores and their
semi-derivatives may prove useful as antibacterial agents probably acting by depriving bacteria of iron (Brown, 1985).

Such is the significance of metal ions in the survival and growth of microorganisms, that organic compounds that are able to bind strongly to metal ions forming stable complexes exhibit antimicrobial action against many micro-organisms. The antimicrobial action of such compounds, known generally as chelating agents, is discussed in the next section.
1.4. The Antimicrobial Activity of Chelating Agents

1.4.1. The Process of Chelation

Chelating agents are metal-binding substances or 'ligands'. When a metal is gripped, in a ligand, between any of the elements nitrogen (N), oxygen (O), or sulphur (S), a 'chelate' is formed in which the metal is more tightly bound than when it is not part of the ring (Albert, 1985). The term 'chelate' was first used to describe this phenomenon by Morgan and Drew (1920), and is derived from the Greek word 'chely', the great claw.

Chelating agents may be classified according to the number of donor atoms capable of combining with a metal atom. Thus, a chelating agent can be bidentate (only two donor atoms available), e.g. 8-hydroxyquinoline; tridentate, e.g. terpyridine; quadridentate, e.g. triethylenetetramine; quinquedentate, e.g. tetraethylenepentamine; or even sexadentate, e.g. ethylenediaminetetraacetic acid (EDTA) (Mellor, 1964).

1.4.2. Stability of Metal-Chelates Complexes

The stability of metal-chelates complexes depends on a variety of factors that have been discussed by Mellor (1964), namely:

(1) The size of the ring formed; 5-membered ring chelates are generally more stable than 4- or 6-membered ring chelates.

(2) Number of rings formed; chelating agents forming the greater number of chelate rings with a given metal, form the more stable complex compared to similar chelating agents.

(3) For a series of closely related chelating agents, the greater the basic strength (pKₐ), the greater the stability of the metal chelate.

(4) The steric hindrance arising from the process of chelation.
Chelating agents with different donor atoms may form more stable complexes than if they had similar donor atoms.

Influence of the central metal atom; chelating agents have the following general preference for metal atoms (Albert, 1985):

<table>
<thead>
<tr>
<th>Metal</th>
<th>Stability Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe³⁺ , Hg²⁺</td>
<td>greatest avidity</td>
</tr>
<tr>
<td>Cu²⁺ , Al³⁺</td>
<td></td>
</tr>
<tr>
<td>Ni²⁺ , Pb²⁺</td>
<td></td>
</tr>
<tr>
<td>Co²⁺ , Zn²⁺</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺ , Cd²⁺</td>
<td></td>
</tr>
<tr>
<td>Mn²⁺</td>
<td></td>
</tr>
<tr>
<td>Mg²⁺</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td></td>
</tr>
<tr>
<td>Li⁺</td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td></td>
</tr>
<tr>
<td>K⁺</td>
<td>least avidity</td>
</tr>
</tbody>
</table>

To quantify the stability of metal-chelates, stability constants (K) are used. These are constants governing the mass-action equilibrium between the ligand(s) and one ion of the metal (Albert, 1985). Since one ion of the metal may bind with more than one ligand, more than one stability constant can be measured, thus, $K_1, K_2, \ldots, K_n$ are stability constants for a $1:1$, $1:2$, $\ldots$, $1:n$ (metal : ligand) chelates. The product of the individual constants is the cumulative stability constant $\beta_n$, where $(n)$ is the number of individual constants multiplied to give $\beta$. The determination of stability constants has been discussed in detail by Albert and Serjeant (1984).

1.4.3. The Antimicrobial Activity of Chelating Agents

Albert (1985) suggested two main mechanisms for the antimicrobial action of
chelating agents. A chelating agent may bind and remove an essential metal from the cell thus causing deficiency in this particular element or the inactivation of metal-containing structures within the cell (e.g. enzymes). Alternatively, a chelating agent may cause the imposition on the cell of metals in greater quantity (or at a higher oxidation potential) than normal, and the complex of the chelating agent and the metal would then cause damage to various cell components. Two examples of these mechanisms, namely EDTA and 8-hydroxyquinoline, respectively, are discussed in the next two subsections.

1.4.4. The Antimicrobial Action of Ethylenediaminetetra-acetic acid (EDTA)

The antimicrobial value of the EDTA has been reviewed (Russell, 1971; Wilkinson, 1975; Hart, 1984). Due to its low cost and toxicity, EDTA and other related compounds (Fig.1.4) have been used in many pharmaceutical preparations (Hart, 1984). EDTA is not normally considered as being an antibacterial compound in its own right (Russell, 1971), since it lacks broad-spectrum antibacterial activity and does not achieve total bacterial kill (Hart, 1984). However, it has a considerable value in microbiology since: (1) EDTA has a specific and potent antibacterial (lytic) activity against *Pseudomonas aeruginosa* and (2) EDTA potentiates the antibacterial activity of a wide range of compounds against a wide range of micro-organisms, and in particular Gram-negative bacteria. These two characteristics of EDTA are discussed below.

1.4.4.1. The Antibacterial Action of EDTA against *Pseudomonas aeruginosa*

Whilst investigating the lytic effects of the combination of lysozyme and EDTA against Gram-negative bacteria, Repaske (1958) noted that EDTA alone caused a significant lysis of cells of *Pseudomonas aeruginosa* in Tris (tris(hydroxymethyl)aminoethane) buffer. Other organisms such as *Escherichia coli* and *Azotobacter vinelandii* were not lysed by EDTA alone (Repaske, 1958). These observations
Figure 1.4  
(a) Ethylenediaminetetraacetic Acid (EDTA)  
(b) Hydroxyethylethylenediaminetriacetic Acid (HEEDTA)  
(c) Diethylenetriaminepentaacetic Acid (DTPA)
were supported by Eagon and Carson (1965), who also found that the metal ions Ca\(^{2+}\), Mg\(^{2+}\) and Zn\(^{2+}\) but not Mn\(^{2+}\) were chelated from the cells of *Pseudomonas aeruginosa* treated with EDTA. Ca\(^{2+}\) was the metal ion chelated in the greatest quantity from the cells and Mg\(^{2+}\) the least. Eagon and Carson (1965) concluded that divalent ions, and in particular calcium, are essential for the integrity of the cell walls of *Pseudomonas aeruginosa*, and that they may function to form cross-linkages between mucopeptide and non-mucopeptide components of the cell wall. EDTA, therefore, through its chelating action, extracts sufficient of such metal ions from the cell wall to cause the loss of cellular integrity and subsequently cell lysis. Other workers, however, have suggested that Mg\(^{2+}\) is the main target for chelation by EDTA in *Pseudomonas aeruginosa*, since equivalent concentrations of Mg\(^{2+}\) prevent EDTA action (Leive, 1968). Indeed, cells of *Pseudomonas aeruginosa* whose cell walls are richest in Mg\(^{2+}\) compared to other strains of the same organism and which contain high quantities of Ca\(^{2+}\) (0.15%) and Mg\(^{2+}\) (0.2%) of the total ash content (7% of dry weight, Eagon *et al*, 1965), were most sensitive to the lytic action of EDTA according to Haque and Russell (1974, 1976). It is probable that both Mg\(^{2+}\) and Ca\(^{2+}\) play a significant role in the integrity of the cell wall. Cells of *Pseudomonas aeruginosa* grown in minimal media deficient in either Mg\(^{2+}\) or Ca\(^{2+}\) were more resistant to EDTA than when grown in minimal media supplemented with these cations (Boggis *et al*, 1979; and Kenward *et al*, 1979).

In addition to its chelation of metal ions in the cell envelope of *Pseudomonas aeruginosa*, EDTA causes the release of 30 - 50% of lipopolysaccharides from the outer-membrane, leading to further disintegration of the outer-membrane and cell envelope (Stinnett *et al*, 1973; Spicer and Spooner, 1974; Haque and Russell, 1976; Matsushita *et al*, 1978; Rogers *et al*, 1969; Russell *et al*, 1987; Russell and Furr, 1987). Furthermore, it has been suggested that the lysis of cells of *Pseudomonas aeruginosa* by EDTA is due to the potentiation of autolysins, specific to
this organism (Leive, 1968). The various effects of EDTA on 
*Pseudomonas aeruginosa* and other micro-organisms are summarized in Table 1.5.

Table 1.5 Summary of the Antimicrobial activity of EDTA (Russell, 1971).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Effect of EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ps. aeruginosa</em> and other</td>
<td>1- Leakage of intracellular material, lysis</td>
</tr>
<tr>
<td>EDTA-sensitive Pseudomonads</td>
<td>(in sucrose, no leakage, spheroplast formation).</td>
</tr>
<tr>
<td></td>
<td>2- Release of lipopolysaccharides (LPS).</td>
</tr>
<tr>
<td></td>
<td>3- Increased sensitivity to other antibacterial agents.</td>
</tr>
<tr>
<td>EDTA-resistant Pseudomonads</td>
<td>1- No leakage, lysis or release of LPS.</td>
</tr>
<tr>
<td>Coliforms</td>
<td>1- Release of surface-bound enzymes.</td>
</tr>
<tr>
<td></td>
<td>2- No lysis. Some release of LPS.</td>
</tr>
<tr>
<td></td>
<td>3- Increased sensitivity to other antibacterial agents.</td>
</tr>
<tr>
<td>Gram-positive bacteria, e.g. <em>Staphylococcus aureus</em>, <em>Staphylococcus albus</em> and <em>M. lysodeikticus</em></td>
<td>1- No apparent effect. No increased sensitivity to other antibacterial agents.</td>
</tr>
</tbody>
</table>

1.4.4.2. The Potentiation of other Antibacterial agents by EDTA

MacGregor and Elliker (1958) reported that cells of *Pseudomonas aeruginosa*, resistant to alkyldimethylethylbenzylammonium chloride, became sensitive following treatment with EDTA. It was concluded that quaternary ammonium germicide resistant strains of *Pseudomonas aeruginosa* owe their resistance to their impermeability to this
class of compound, and that EDTA enhances cell permeability by changing the outer-
membrane integrity by metal removal.

Many examples of this synergy between EDTA and antibacterial agents followed. EDTA enhanced the activity of polymyxin B sulphate and chlorhexidine diacetate against *Pseudomonas aeruginosa* (Brown and Richards, 1965). This synergy was blocked by the presence of Mg$^{2+}$ or Ca$^{2+}$ in the medium, supporting the hypothesis that the potentiation action of EDTA is due to its chelation of these metal ions from the cell envelope. Synergy between EDTA and each of the antibiotics penicillin, ampicillin, tetracycline and chloramphenicol against *Pseudomonas aeruginosa*, *Escherichia coli* and *Proteus mirabilis* has also been observed (Weiser et al, 1968). EDTA potentiates the action of many other antibacterial agents, and some of these are summarized in Table 1.6.

In summary, this action of EDTA is generally believed to be the result of the compound 'stripping' away a portion of the outer-membrane and cell wall of Gram-negative organisms (by chelating Mg$^{2+}$ and Ca$^{2+}$ ions normally responsible for outer-membrane stability). This exposes the underlying cytoplasmic membrane and cell interior to attack by the other agent in the combination (Denyer et al, 1985).

The most useful application of EDTA has undoubtedly been in the area of preservation of pharmaceutical products against Gram-negative organisms, and in particular *Pseudomonas aeruginosa* (Hart, 1983, 1984). The use of EDTA with preservatives such as quaternary ammonium compounds, chlorhexidine, phenolics and esters of $p$-hydroxybenzoic acid is highly recommended and already applied (Hart, 1984). EDTA is also used to potentiate general-purpose antiseptics and disinfectants, and combinations of EDTA and antibiotics have already been suggested for the treatment of surface infections (Wilkinson, 1975).

At an appropriate concentration (20mg/ml), EDTA is an effective anticoagulant, and it has been suggested as a replacement for heparin to maintain the patency of the
lumen in central venous catheters. EDTA has the advantage over heparin of having inherent antibacterial activity against some problematic organisms in this area such as *Staphylococcus epidermidis* (Root et al., 1988).

Table 1.6 Antimicrobial agents that show synergistic interaction with EDTA against bacteria.

<table>
<thead>
<tr>
<th>Antimicrobial Agents</th>
<th>Organisms</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quaternary Ammonium Compounds</td>
<td><em>Ps. aeruginosa</em></td>
<td>MacGregor and Elliker (1958)</td>
</tr>
<tr>
<td>Polymyxin B, Benzalkonium chloride, Chlorhexidine</td>
<td><em>Ps. aeruginosa</em></td>
<td>Brown and Richards (1965)</td>
</tr>
<tr>
<td>Penicillin, Ampicillin, Tetracycline and Chloramphenicol</td>
<td><em>E. coli</em> and <em>Pr. mirabilis</em></td>
<td>Weiser et al (1968)</td>
</tr>
<tr>
<td>Vancomycin</td>
<td><em>E. coli</em></td>
<td>Russell (1967)</td>
</tr>
<tr>
<td>Chloroxylenol</td>
<td><em>E. coli</em> and <em>Ps. aeruginosa</em></td>
<td>Russell and Furr (1977)</td>
</tr>
</tbody>
</table>
1.4.5. The Antimicrobial Mode of Action of Oxine (8-hydroxyquinoline)

Oxine (Fig.1.5a) exhibits broad spectrum antimicrobial activity, though Gram-positive bacteria are more sensitive than fungi which are more sensitive than Gram-negative bacteria (Frier, 1971). The compound has found use as a preservative in topical cosmetic preparations, as a disinfectant, and as an antiseptic (Wallhausser, 1984).

The mode of action of oxine against micro-organisms has been the subject of investigation for many years. The earliest report linking the chelating properties of oxine to its antimicrobial activity is that by Zentmyer (1943), who suggested that the antifungal action of oxine was due to its ability to chelate metal ions from enzymes and vitamins in microbial cells. This was based on the finding that oxine was inactive at low pH values at which the metal chelates dissolve and ionise.

The significance of chelation in the antibacterial action of oxine was investigated more thoroughly by Albert and colleagues. Of seven possible isomeric mono-hydroxyquinolones, only oxine is capable of chelation and was shown to possess antibacterial activity (Albet et al., 1947). Using the Gram-positive organism, *Staphylococcus aureus*, it was shown that oxine *per se* has no antibacterial activity and that its toxicity is evident only in the presence of iron or copper, with iron being the more important for antibacterial action (Rubbo et al., 1950). The molar ratio of iron to oxine in the medium is also critical for the antibacterial action of that compound. In the presence of excess oxine, a 2:1 oxine-iron complex (Fig.1.5b) is formed in place of the 1:1 complex (Fig.1.5c). The 2:1 oxine-iron complex is more lipophilic than the 1:1 complex and therefore penetrates the bacterial cell envelope more rapidly. Albert et al. (1953), however, suggested that the former complex is actually devoid of antibacterial activity against *Staphylococcus aureus*, whilst the latter 1:1 complex is the antibacterial species. They suggested that the 2:1 oxine-iron complex will readily penetrate the bacterial cell but would then dissociate inside the cell to give the toxic 1:1
The actual mechanism of action of oxine at the molecular level (specific targets etc.) remains unclear, but would appear to reside within the cytoplasmic membrane (Albert, 1985). The unsaturated 1:1 oxine-iron complex is believed to catalyse the oxidation, by atmospheric oxygen, of several vital cell constituents, particularly mercapto-compounds such as dihydrolipoic acid. Such reactions are greatly enhanced in the presence of traces of iron or copper and may lead to the production of highly damaging peroxide radicals ($'O^-_2$). These would initiate chain reactions causing widespread destruction (Albert, 1985).

This co-operative effect between oxine and iron is antagonised strongly by trace amounts of cobalt (Rubbo et al, 1950; Albert et al, 1953). This antagonism could not be simply explained on the basis of competition between cobalt and iron for the chelating site in oxine, since the stability constant of the oxine-iron complex is higher than that of the oxine-cobalt complex (Albert, 1985). Instead, cobalt is believed to act as a chain breaker of the oxidative chain reaction induced by the oxine-iron complex (Dainton, 1966; Albert, 1985).

The antifungal action of oxine has also been investigated. Both oxine and the oxine-copper complex are fungicidal with the latter being more toxic (Powell, 1946; Sijpsteijn et al, 1957). This is in contrast to the observations on the action of oxine and its iron complex against Gram-positive organisms discussed above (Albert et al, 1947; Rubbo et al, 1950; Albert, 1953). Copper 8-quinolinolate is also more toxic to fungi than other metal-oxine chelates such as magnesium-, iron- and zinc 8-quinolinolates (Mason, 1948). Zentmyer et al (1960) observed that amino acids such as cysteine, histidine, tryptophan and casamino acids reversed oxine toxicity against spores of Aspergillus niger. They suggested that oxine enters the cell as the copper-oxine complex, and that amino acids of the cell remove copper from the complex releasing oxine in situ. Whilst free copper poisons amino acids, proteins and

29
enzymes, free oxine sequesters prosthetic trace metals such as iron and zinc from enzymes and systems that require them for biological activity.

In more recent studies, aliphatic thiol-containing compounds (such as cysteine, glutathione, dithioerythritol and dithiothreitol) and DL-α-lipoic acid were found to reverse the fungitoxic activity of oxine but not that of the copper chelate (Gershon et al., 1975). It has been suggested that oxine itself interferes with the biosynthesis of lipoic acid (a cofactor for oxidative enzymes such as pyruvate oxidase; Guirard and Snell, 1962), and that the mechanism of fungitoxicity of the oxine-copper chelate is different from that of free oxine (Gershon and Shanks, 1981; Gershon et al., 1985).

The antimicrobial action of oxine has been reviewed (Weinberg, 1957; Albert, 1982, 1985).
Figure 1.5

(a) 8-Hydroxyquinoline (oxine)

(b) 2:1 oxine : Fe complex

(c) 1:1 oxine : Fe complex
1.5. Sodium and Zinc Pyrithione

1.5.1. History

Pyrithione (2-mercaptopyridine-N-oxide), also known as omadine, was first synthesized by Shaw et al (1950). In the preparation, 2-bromopyridine was converted to its N-oxide by oxidation with m-chloroperoxybenzoic acid or peracetic acid. Treatment of the N-oxide with sodium sulphide or sodium hydrosulphide under mild conditions gave pyrithione (Fig.1.6a) or sodium pyrithione, respectively. Zinc pyrithione (Fig1.6b) is prepared from sodium pyrithione using a water soluble zinc salt. Pyrithione was prepared during the search for analogues of the naturally occurring antibiotic, aspergillic acid (Lott and Shaw, 1949; Shaw et al, 1950).

The first patent, covering the composition and manufacture of pyrithione and its sodium salt, was granted in 1954 (Shaw and Bernstein, 1954). A second patent followed, which covered the heavy metal derivatives of pyrithione (Bernstein and Losee, 1957). The antidandruff properties of zinc pyrithione were discovered in the 1960's and patents were issued covering its use in antidandruff shampoos and preparations (Karsten et al, 1966; Karsten and Taylor, 1968).

1.5.2. General properties

1.5.2.1 Physical properties

The physical and chemical properties of sodium and zinc pyrithion have been reviewed previously (Olin Corporation, 1983; Nelson and Hyde, 1981; Hyde and Nelson, 1984).

Sodium pyrithione is a salt and hence is highly soluble in water (53% w/w at 25°C), whereas zinc pyrithione is a lipophillic chelate barely soluble in water (0.0015% w/w at 25°C). Both compounds absorb ultraviolet light; typically zinc pyrithione absorbs at 244, 275 and 320nm in methanol and 239, 268 and 320nm in water. Sodium
Figure 1.6
(a) 2-Mercaptopyridine-N-oxide, sodium salt (pyrithione)
(b) Zinc pyrithione

Figure 1.7: Tautomeric forms of pyrithione
pyrithione absorbs at 248, 290 and 345nm in methanol and at 243, 281 and 322nm in water (Olin Corporation, 1983).

1.5.2.2 Chemical properties

Pyrithione is a weak acid with a pKa value of 4.7 (Chandler and Segel, 1978). In solution, pyrithione exists in two tautomeric forms, the thiol and the thione as shown in Figure 1.7 (Shaw, 1949; Shaw et al, 1950; Jones and Katritzky, 1960). The thione form prevails at or below pH 3, the thiol form appears as the pH is raised with the equivalence point being around 7.6.

Pyrithione is oxidised by peroxides to the disulphide which under alkaline conditions produces sulphinic acid, further oxidation yields sulphonic acid. In the presence of reducing agents, pyrithione is converted to 2-mercaptopyridine. Both sulphinic and sulphonic acids are microbiologically inactive and 2-mercaptopyridine is less active than pyrithione itself.

One of the most important properties of pyrithione is its ability to chelate metal ions. Pyrithione forms complexes with almost all metal ions under almost any conditions, with many of these complexes being coloured. Transchelation will also occur, for example, iron, copper, mercury and silver will replace zinc in zinc pyrithione because the stability constant of zinc pyrithione is less than that of the other four metal-pyrithione complexes (Olin Corporation, 1983).

1.5.2.3 Stability

Sodium and zinc pyrithione are generally stable over the range pH 4.5 to 9.5, though the recommended pH range for the use of sodium pyrithione is 7 to 10. Both compounds are stable at 100°C for at least 120 h and there is no loss of biological activity of aqueous solutions of sodium pyrithione after storage at 40°C for 3 months (Nelson and Hyde, 1981).

Pyrithione undergoes rapid photodegeneration (Evans et al, 1975; Neihof et
After 5 to 10 hours exposure to light of an aqueous solution at 100ppm, ten fold increases in bioassay concentrations were necessary to equal toxicities of the unirradiated solution. Wave lengths of 320 to 355nm were most effective in producing photodegradation.

1.5.3 Analytical Methods

Several techniques have been developed for the assay of pyrithione and its metal complexes, including polarography (Krivis et al, 1963; Krivis and Gazda, 1969), and thin-layer chromatography (TLC; chloroform : methanol (49:1); Seymour and Bailey, 1981). Polarographic analysis of pyrithiones is specific and widely used, whereas TLC analysis requires special conditions to minimise photodecomposition and chemical interaction between the pyrithione and the materials in the thin-layer plate. A simple colorimetric method has been developed and involves reacting pyrithione with ferric ammonium sulphate under acidic conditions to yield the pyrithione-Fe (3:1) complex. This complex absorbs light at 610nm.

1.5.4 Antimicrobial and Mode of Action of Pyrithione

The antimicrobial activity of pyrithione was observed when it was first synthesized (Shaw et al, 1950). Pyrithione inhibited the growth of both Gram-positive and Gram-negative bacteria with the latter being comparatively less sensitive to the drug. In addition, sodium pyrithione showed powerful anti-fungal activity (Pansy et al, 1953). In spite of this, and the wide use of pyrithione as an antidandruff agent as well as a general preservative, only a few attempts have been made since its discovery to fully understand the mode of action of this compound.

Albert et al (1956) suggested that pyrithione had an identical mode of action to that of oxine (8-hydroxyquinoline), since the former is rapidly bactericidal, inactive in the absence of traces of iron, inactive in the presence of iron if cobalt was added, and
inactive in concentrated solution. The 1:1 complex with iron would thus be the toxic species whilst the 2:1 complex would be more lipophilic and may penetrate bacterial cells easier, releasing the 1:1 complex intracellularly. The 1:1 complex is highly reactive producing, within cells, various peroxide and superoxide radicals causing damage and cell death (Albert, 1985). The antifungal activity of pyrithione, however, was attributed to the copper complex rather than the iron complex, following the observation that both the 1:1 and the 1:2 copper-pyrithione complexes are equally toxic to the fungus Aspergillus niger (Sijpesteijn et al., 1957). The bactericidal activity of zinc pyrithione is quenched by sodium thioglycollate, suggesting that pyrithione acts by oxidising free thiol groups (-SH) in proteins within the cell (Elkhouly, 1974). Although the concentration of sodium thioglycollate used was 25-50 (w/v) times the minimum inhibitory concentration of zinc pyrithione against Staphylococcus aureus in the study, such a mechanism of action cannot be ruled out. In a study of the relationship of structure to antimicrobial activity of a series of pyrithione-N-oxides, the most active compounds were those in which the 2 position on the pyridine ring was substituted by a sulphur-containing group wherein the sulphur atom is attached directly to the ring (Leonard et al., 1956).

The structural similarity between pyrithione and, nicotinic acid and vitamin B₆, suggests the possibility that pyrithione acts as an antimetabolite (Cooney, 1969). However, no experimental evidence has been provided to support this hypothesis. Another possibility is that pyrithione chelates metal cofactors of various enzymes. Indeed, pyrithione does inhibit the zinc-requiring enzyme alcohol dehydrogenase (Cotton, 1963).

Pyrithione has been shown to inhibit membrane transport processes in fungi (Chandler and Segel, 1978). Investigations into the mode of action of pyrithione against Penicillium crysogenum and P. notatum suggest that pyrithione acts as a proton conductor whereby the unionised molecule diffuses across the cell membrane and ionises intracellularly thereby collapsing a transmembrane pH driving force. This
also excludes the possibility that the main sites of action of pyrithione are the disulphide bonds within proteins. Pyrithione also inhibited \textit{in vivo} protein synthesis and caused a reduction in intracellular ATP levels in \textit{Penicillium}; the thiol and N-oxide groups in the molecule were again found necessary for membrane activity.

\textbf{1.5.5. Toxicity of Pyrithione}

The toxicity of pyrithione has been reviewed (Black and Howes, 1978). The data on acute toxicity of some pyrithiones suggests that the metal complexes are more toxic than pyrithione itself, which in turn is more toxic than the sodium salt, when they are given orally or intraperitoneally.

The effects of pyrithione on mammalian cells have been investigated both \textit{in vivo} and \textit{in vitro}, and various results have been reported. Imokawa and Okamoto (1983) suggested that zinc pyrithione acted primarily by reversibly inhibiting DNA synthesis in human skin cells \textit{in vitro}, whilst RNA and protein synthesis were uninhibited. In comparison Gibson and colleagues (1985a) showed that zinc pyrithione had a clear effect on human cells, and that DNA, RNA and protein synthesis were all affected. The relatively low toxicity of zinc pyrithione when applied \textit{in vivo} to the skin may be due to its inability to pass through the epidermis in high concentrations (Gibson \textit{et al}, 1985b). Studies using human leukaemic myeloid cell lines have also shown pyrithione to have cytotoxic effects, including inhibition of DNA and protein synthesis, leading to cell death (Kontoghiorghes \textit{et al}, 1986a and b). Such DNA inhibitory effects were also reported by Forsbeck \textit{et al} (1987) and were found to be partially reversed in the presence of iron.

\textbf{1.5.6. The Pharmaceutical Value of Pyrithiones}

Pyrithione, as the zinc complex, is used worldwide as a very successful anti-dandruff agent. The mechanism by which zinc pyrithione reduces dandruff was and
still is a matter of debate. Dandruff is excessive, clinically non-inflammatory scaling of the scalp (Kligman et al, 1976). In the great majority of cases diagnosis can be made almost instantly by simply inspecting and scratching the scalp surface. The idea that micro-organisms in general and *Pityrosporum ovale* in particular, incite or contribute to the production of dandruff dates back to the last century (Leyden et al, 1976). The hypothesis explaining the microbial involvement in dandruff is that *P. ovale* can lyse sebum triglycerides to release fatty acids, some of which may irritate and produce scaling (Van Abbé, 1964). Supporters of this theory rely on the evidence the counts of *P. ovale* in subjects with dandruff are higher than those in healthy subjects (Kligman et al, 1976). The suppression of dandruff by zinc pyrithione is also accompanied by a reduction in *P. ovale* counts (Imokawa et al, 1982) as well as yeast micro-organisms in general (Marks et al, 1985).

In contrast, other workers believe that the higher incidence of *P. ovale* in the scalp of people with dandruff is due to the increase in surface area and nutrients for growth provided by the scaling process (Kligman et al, 1976). They suggest that the increased number of scalp micro-organisms found in dandruff occurs as a secondary event, and that micro-organisms play no primary role in the pathogenesis of dandruff (Leyden et al, 1976).

Antidandruff agents such as zinc pyrithione and selinium sulphide may thus act cytotastically, slowing down the epidermal proliferative activity which leads to the scaling and dandruff (Plewig and Ligman, 1969). However Pearse and co-workers (1985) found that zinc pyrithione had no effect on epidermal renewal in normal human skin *in vivo*.

The question of microbial involvement in dandruff remains, therefore, unresolved (Van Abbé, 1986). The importance of micro-organisms and particularly *P. ovale* in dandruff is, however, so deeply entrenched in theories of causation that the search for new antidandruff agents often utilizes an *in vivo* screen against *P. ovale* (Kligman et
Zinc pyrithione apparently entered the channels of commerce via this route, and patents covering formulations of antidandruff shampoos containing zinc pyrithione are still being granted.

### 1.6. Aims of Project

To investigate the effects of pyrithione on the growth and survival of the Gram-negative organism *Klebsiella pneumoniae*, as well as this organism's ability to develop resistance to pyrithione. The project also aims at examining the effects of pyrithione on some essential metabolic processes in *Kl. pneumoniae*. Other organisms are included in the study for comparative purposes. It is hoped that this study will improve our understanding of the nature of drug-cell interactions in aid of the continuous search for improved antimicrobial agents.
CHAPTER TWO

MATERIALS AND METHODS
2.1. General

2.1.1. Micro-organisms

The following micro-organisms were used in this study:


2.1.2. Growth Media

(1) Nutrient Broth (NB) containing:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/Litre</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab-Lemco (Oxoid, Code L29)</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Neutralised bacteriological peptone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Oxoid, Code L34)</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

(2) Chemically defined medium (CDM) containing:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams/Litre</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Chemicals Defined Medium (continued)</td>
<td>grams / litre</td>
<td>group</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>--------------</td>
<td>-------</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>KNO₃</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.05</td>
<td>4</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>0.005</td>
<td>5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.0025</td>
<td>5</td>
</tr>
<tr>
<td>MnCl₂·4H₂O</td>
<td>0.005</td>
<td>5</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>DL-aspartic acid</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>L-proline</td>
<td>0.5</td>
<td>6</td>
</tr>
<tr>
<td>biotin</td>
<td>0.0002</td>
<td>7</td>
</tr>
</tbody>
</table>

Group 1 nutrients (Nutrient Broth) were dissolved in distilled water (final volume 1 litre) and sterilised by autoclaving (121°C, 15 lb per square inch) for 15 minutes. Groups 2, 3 and 4 chemicals were dissolved in 700, 100 and 100 ml of distilled water respectively, and sterilised by autoclaving. Group 6 chemicals were dissolved in 100 ml of distilled water and sterilised by filtration through Whatman membrane filters (pore size 0.45 μm). Groups 2, 3, 4 and 6 were then mixed aseptically. Concentrated solutions of groups 5 (FeCl₃, 1.25%; MnCl₂·4H₂O, 1.25%; CaCl₂, 0.625% w/v) and 7 (0.2% w/v) were prepared in distilled water and sterilised by membrane filtration as above, and small volumes (0.4 ml from group 5; 1 ml from group 7) from each concentrate were added to the medium to give the required concentrations.

When required, agar no. 1 (Oxoid, Code No. L11) was added to nutrient broth (NB) at 1.5% (w/v) before sterilisation to give nutrient agar. Agar was added at
1.5% (w/v) to group 2 before sterilisation to give chemically defined medium agar (CDMA). The final pH of either media was adjusted to 7.00± 0.2.

2.1.3. Antimicrobial agents and related compounds

The following compounds were used:

1) Sodium pyrithione (2-mercaptopyridine -N-oxide; sodium salt), Sigma Chemical Company LTD., Poole, Dorset, UK.

2) Diaminoethanetetraacetic acid, sodium salt (EDTA), Fisons Pharmaceuticals, Loughborough, U.K.

3) 8-Hydroxquinoline (Fisons).

4) Ampicillin, sodium salt (Sigma).

5) 2- Mercaptopyridine (MP; Sigma).

6) 2- Mercaptopirimidine (MPY; Sigma).

7) 2- Mercaptotiacetic acid (MNA; Sigma).

8) 3- Hydroxy -2- mercaptopypyridine (HMP; Sigma).

9) Rifampicin; Sigma.

2.1.4. Radiolabelled compounds

The following radiolabelled compounds were purchased from Amersham International, Cardiff, U.K.:

1) (1-14C) Glycine (specific activity 58 mCi/mmole).

2) (2-14C) Thymidine (specific activity 53 mCi/mmole).

3) (2-14C) Uridine (specific activity 52 mCi/mmole).

4) [α-32P] UTP (specific activity 800 Ci/mmole).
2.1.5. Nucleotides and other chemicals

(1) ATP, lithium salt (100 mmole/l; pH 7; Boehringer Mannheim).
(2) CTP, lithium salt (100 mmole/l; pH 7; Boehringer Mannheim).
(3) GTP, lithium salt (100 mmole/l; pH 7; Boehringer Mannheim).
(4) UTP, lithium salt (100 mmole/l; pH 7; Boehringer Mannheim).
(5) Salmon sperm DNA (10 mg/ml; Sigma).
(6) Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol); Molecular Biology Grade; Boehringer Mannheim.
(7) tRNA from *Escherichia coli* MRE600 (RNAse negative; Boehringer Mannheim).
(8) β-Mercaptoethanol (Sigma).

2.1.6. Enzymes

(1) RNA Polymerase (EC 2.7.7.6.; 2500 units/ml) from *Escherichia coli* MRE600; Boehringer Mannheim.

All other chemicals were of general laboratory grade, unless stated otherwise.
2.2. Experimental

2.2.1. Sterilisation methods

(1) Autoclave, at 121°C and 15 pounds per square inch for 15 minutes.

(2) Membrane-filtration, using Whatman cellulose nitrate membrane filters with pore size of 0.45μm.

(3) Dry sterilisation in an oven at 160°C for 1 hour.

2.2.2. Maintenance of cultures

All master cultures were maintained on both NA and CDMA slopes at 4°C in the dark. Organisms for routine experimental use were maintained by daily subculture on NA or CDMA.

2.2.3. Preparation of standard cell suspension

Isolated colonies (CDMA) of test organisms were grown in 50 ml of fresh CDM in a Gallenkamp orbital incubator (37°C, 90 rpm). After 12 hours, cells were harvested by centrifugation (Mistral 6L centrifuge) at 4000 rpm for 15 minutes, and resuspended in 50 ml fresh CDM (37°C). This suspension was then used for further experimental work. In general, all suspensions for experimental use were used within 30 minutes of preparation.

2.2.4. Determination of the minimum inhibitory concentrations (MICs)

The tube dilution method was used throughout. Serial two-fold dilutions of the antimicrobial agent (or related compound) were prepared in 5 ml volumes of liquid medium (NB) in universal bottles. These were inoculated with 30 μl of an overnight (12-16 hours, 37°C, NB) culture for bacteria and C. albicans, 48 hours culture for S. cerevisiae (30°C) and 48 hours culture for D. hansenii (20°C). All tubes were then
incubated at the appropriate temperature. The MIC was recorded as the lowest concentration of the test compound that inhibited growth (visible turbidity) of the organism after 24 hr incubation.

The MICs of sodium pyrithione were determined against all organisms in nutrient broth and additionally against *Kl. pneumoniae*, *B. licheniformis* NCIB 6346, *Ps. aeruginosa* NCTC 6749, *E. coli* NCTC 9001 and *C. albicans*NCYC 597 in CDM. The MICs of other antimicrobial compounds were determined against these latter organisms were determined in CDM only.

2.2.5. Influence of sodium pyrithione on the growth rate of micro-organisms

The effect of pyrithione on microbial growth was studied using *Kl. pneumoniae*, *B. licheniformis* NCIB 6346 and *C. albicans* NCYC 597. Standard cell suspensions of each organism were prepared as described in section 2.2.3, and used to inoculate fresh 100ml batches of CDM containing various concentrations of pyrithione in 250ml conical flasks. Cultures were incubated (37°C, 90 rpm) in a Gallenkamp orbital incubator and the optical densities measured using a Pye-Unicam SP500, series 2 spectrophotometer (650 nm, 1 cm path) at various times.

2.2.6. Effects of rechallenge on the growth rate of micro-organisms

Preliminary experiments indicated that bacterial culture re-growth could occur at low pyrithione concentrations. This effect was investigated further as follows: Standard cell suspensions of either *Kl. pneumoniae* or *B. licheniformis* NCIB 6346 were used to inoculate 6 x 100 ml batches of CDM containing various concentrations of pyrithione in 250 ml conical flasks. These were incubated in the orbital incubator as described above, and measurements of optical density were taken at set time intervals. When bacterial cultures had reached an optical density of approximately 0.4, 0.1-0.5 ml of a drug concentrate in CDM was added to re-establish the drug concentration at levels at least equivalent to that present initially. Optical density
readings were recommenced after the re-addition of pyrithione. *C. albicans* NCYC 597 cultures were treated similarly, but pyrithione was re-added after a fixed time interval to cultures instead of at a fixed optical density.

In addition, to observe the effects of cell density on pyrithione effectiveness, duplicate cultures of all three organisms were grown as previously described but in the absence of pyrithione. When each culture was in (approximately) the mid-exponential phase, pyrithione was added using a drug-concentrate, and incubation continued with optical density monitoring as before.

2.2.7. Influence of pyrithione on culture viability

Standard cell suspensions of either *Kl. pneumoniae* or *C. albicans* NCYC 597 were used to inoculate fresh 100 ml batches of CDM in 250 ml conical flasks containing various concentrations of pyrithione. Cultures were incubated as before (37°C, 90 rpm), and at set time intervals, 1 ml samples were withdrawn and diluted into a series of 9 ml volumes of sterile physiological saline (0.85% (w/v) NaCl). The viability of the cells was assessed using the method of Miles and Misra (1938), where 20 μl aliquots were transferred from suitable dilutions onto the surface of surface dried nutrient agar plates. Plates were incubated at 37°C for 12-18 hr and colonies counted.

2.2.8. Cell size distribution of division-inhibited (ampicillin) cells of *Kl. pneumoniae* growing in the presence of pyrithione

A method based on that devised by Rye and Wiseman (1968a) was used. Preliminary experimentation had established that *Kl. pneumoniae* would grow at an uninhibited rate, but without dividing, in CDM containing 50 μg/ml ampicillin. Growing batch cultures (100 ml CDM) of *Kl. pneumoniae* were prepared as described above containing 50 μg/ml ampicillin and, in addition, 2.0 μg/ml pyrithione representing approximately 40% of the minimum inhibitory concentration (MIC) as determined by the tube dilution. Control cultures containing cells in CDM alone and in
CDM containing 50µg/ml ampicillin were also prepared. All cultures incubated at 37°C and 90 rpm and Samples (1-5 ml) were removed from each culture at various times. Cells were collected from each sample by membrane filtration (0.45µm Whatman cellulose nitrate membrane filters), and resuspended in Isoton containing 0.2% formaldehyde. These suspensions were then analysed for size distribution using a coulter counter (Model TA II) fitted with a 30µm size tube.

2.2.9. Interaction of EDTA with pyrithione

2.2.9.1. Agar diffusion

The method of Maccacaro (1961) was used. Whatman No.1 filter paper strips (1x4 cm) were prepared and dry-sterilised as described in section 2.2.1. Strips were soaked in EDTA solution (0.1% or 1.0% (w/v)) for 2-3 hours. A strip soaked with pyrithione (0.001% or 0.1% (w/v)) was placed on the surface of a CDMa (15ml) plate seeded with *Kl. pneumoniae*, *B. licheniformis* NCIB 6346 or *C. albicans*NCYC 597. Another strip soaked in EDTA was placed at right angles to the first strip, with the ends overlapping. Plates were incubated at 37°C for 24-48 hours.

2.2.9.2. Influence of EDTA on pyrithione induced changes in growth rate

Test organisms *Kl. pneumoniae*, *B. licheniformis* NCIB 6346 and *C. albicans* NCYC 597 were grown, as before, in the presence and absence of pyrithione and/or EDTA. In each case, the concentrations were selected to be sub-inhibitory. Growth was monitored by optical density measurements (650 nm, 1 cm path) at various times.
2.2.10. Interaction of pyrithione with metal ions and 8-hydroxyquinoline

The agar diffusion method was used as described above (section 2.2.9.1.). Solutions (0.01% (w/v)) of metal ions were prepared using the following water-soluble salts: MgSO_4·7H_2O, CaCl_2, FeCl_3, MnCl_2·4H_2O and ZnCl_2. All three organisms *Kl. pneumoniae*, *B. licheniformis* NCIB 6346 and *C. albicans*NCYC 597, and both CDMA and nutrient agar seeded plates were used. The same method was used to assess the interaction between 8-hydroxyquinoline and pyrithione against all three organisms in both NA and CDMA.

2.2.11. Influence of 8-hydroxyquinoline on the growth of micro-organisms

The effect of 8-hydroxyquinoline on the growth of *Kl. pneumoniae*, *B. licheniformis* NCIB 6346 and *C. albicans*NCYC 597 was investigated in CDM. The growth of *B. licheniformis* and *C. albicans* was followed by measuring the optical density (650 nm, 1 cm path), while the growth of *Kl. pneumoniae* was assessed using the viable count method described in section 2.2.6. since precipitation interfered with optical density measurements.

2.2.12. Influence of pyrithione on the uptake of radiolabelled substrates by

*Kl. pneumoniae* and *B. licheniformis* NCIB 6346

A method based on that outlined by Douglas and Freer (1982 a,b) was used. Batch cultures (4x50 ml CDM) of the test organism were prepared and shake incubated (90 rpm in a Gallenkamp orbital shaker at 37°C) for 60 minutes. Approximately 0.2 μCi of either ^14^C-thymidine, ^14^C-uridine or ^14^C-glycine (specific activity approximately 5mCi/mmol) was added to each flask, and incubation continued. After 3-4 minutes, pyrithione was added, as a concentrated solution, to three flasks to give final concentrations of 1, 2.5 and 5.0 μg/ml in the case of *Kl. pneumoniae* and 0.01, 0.025 and 0.05 μg/ml in the case of *B. licheniformis*. An equal volume of sterile distilled water was added to the fourth (control) flask, and incubation re-commenced. Samples (1 ml) were taken appropriate time intervals up to 30 or 60 minutes after the addition of
the radiolabelled substrates. The samples were added to 5 ml volumes of ice-cold 10% trichloroacetic acid (TCA), and mixed vigorously. Such mixtures were maintained at 4°C for 30 minutes and then filtered through 0.45μm Whatman cellulose nitrate membrane filters. Filters were washed twice with cold TCA before transfer to scintillation vials containing 10 ml of liquid scintillant (Ecosint). The radioactivity present in each vial was measured using a Philips P4700 scintillation counter.

In addition, the incorporation of radiolabelled thymidine, uridine and glycine was followed in a culture of *Kl. pneumoniae* incubated for a long period of time (in the presence of pyrithione (2.5μg/ml)), which allowed the re-establishment of logarithmic growth.

2.2.13. Effect of pyrithione on RNA polymerase activity *in vitro*.

The method used was based on that of Chamberlin and Berg (1962). A standard assay mix (SAM) was prepared containing: 1.17ml H₂O; 0.4 ml 1M Tris.HCl (pH 7.8); 0.1 ml 1M MgCl₂; 0.08 ml of each of 50mM ATP, CTP, GTP and UTP; 12μl β-mercaptoethanol. Three reaction mixtures (RM) were prepared (at 4°C) in a 1.5ml microfuge tube as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>RM1</th>
<th>RM2</th>
<th>RM3</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>DNA (salmon sperm; 10mg/ml)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>40μCi [α-32P] UTP</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pyrithione (250μg/ml)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rifampicin (1mg/ml)</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>RNA polymerase (2500 units/ml)</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Total volume (μl)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
The tubes were incubated at 37°C for 20 minutes followed by storage on ice (4°C) for 30 minutes. To assay the incorporation of [α-32P]UTP, 2 μl from each reaction mixture was added to ice-cold solution containing 47 μl H₂O, 12 μl of 50% (w/v) TCA and 2 μl of 20 mg/ml (in H₂O) tRNA. The mixture was stored at 4°C for 15 minutes, after which they were filtered through Whatman GF/C glass microfibre filters and washed once with 10 ml of ice-cold 10% TCA and once with 10 ml of ice-cold ethanol. Filters were transferred into scintillation vials and counted in a Philips P4700 scintillation counter.

2.2.14. Some aspects of the adaptation of *Kl. pneumoniae* to pyrithione

2.2.14.1. Determination of the MIC of pyrithione against control and pyrithione-treated cells

Batch culture (100 ml CDM) of *Kl. pneumoniae* was prepared and shaken incubated (37°C, 90 rev. per minute) for 24 hours. The MIC of pyrithione was assessed against this culture in CDM as described above (section 2.2.4.), using the tube dilution method; the culture was used to inoculate 2x100 ml CDM batches to be labelled A and B respectively. Culture A contained no pyrithione whilst culture B contained 5.0 μg/ml pyrithione. A and B were incubated as described above. After 24 hours, the MIC of pyrithione was assessed against samples of cells taken from A and B. A was then subcultured into drug-free CDM (100 ml), whereas B was subcultured into both pyrithione-free (labelled C) and pyrithione-containing (5.0 μg/ml; labelled D) CDM.

Cultures A, C and D were incubated and repeatedly subcultured into their respective media (with or without pyrithione), every 24 hours for 7 days. The MIC of pyrithione was assessed and recorded for sample organisms from each culture every 24 hours prior to sub-culturing.
2.2.14.2. Influence of Pyrithione on the growth of Pyrithione-treated cells of *Kl. pneumoniae*

Using a standard cell suspension of *Kl. pneumoniae* (section 2.2.3.), a 100ml batch of CDM containing 5.0μg/ml pyrithione was inoculated (1ml) and incubated (37°C, 90 rpm) for 24h. This culture was then used to inoculate (to give an O.D.₆₅₀nm of approximately 0.1) 6x100ml batches of fresh CDM containing 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 μg/ml pyrithione. Incubation followed (37°C, 90 rpm) and growth was monitored using optical density measurements (650nm, 1cm path).

2.2.14.3. Cross-resistance of pyrithione-treated cells of *Kl. pneumoniae* and *B. licheniformis* NCIB 6346

Inocula from either organism were grown in 2x100 ml of fresh CDM containing 0.0 and 5.0 μg/ml pyrithione in the case of *Kl. pneumoniae* and 0.0 and 0.05μg/ml of the drug in the case of *B. licheniformis* for 24 hours (37°C, 90 rpm). These cultures were used to prepare nutrient agar seeded plates (15 ml of nutrient agar per plate). To examine cross-resistance, Oxoid multodisks (Code 30-44K) containing 8 antibiotics disks were used. Multodisks were placed on the surface of seeded plates, and these were incubated overnight at 37°C, and zones of inhibition were observed.

Cross-resistance of pyrithione-treated cells of *Kl. pneumoniae* to chloramphenicol and 8-hydroxyquinoline was also examined in CDM. The MICs of chloramphenicol, 8-hydroxyquinoline and pyrithione against the control and pyrithione-treated cultures of *Kl. pneumoniae* were determined in CDM as described above in section 2.2.4.

2.2.14.4. Fatty acid profiles of control and pyrithione-treated cells of *Kl. pneumoniae*

2.2.14.4.1. Preparation of fatty acid methyl esters

Batch cultures (2x100 ml CDM) of *Kl. pneumoniae* were prepared containing 0.0 and 5.0 μg/ml pyrithione, and incubated for 24 hours (37°C, 90 rpm). Cells were harvested by centrifugation at 4000 rpm for 15 minutes, washed three times with sterile distilled water and frozen at -70°C. Cells were then freeze-dried in a Taddington
freeze-drier. To esterify and extract fatty acids from the freeze-dried cells, a method based on that outlined by Stretton and Dart (1976) was used. Freeze-dried cells were placed in a quick-fit (100ml) round flask containing 15 ml of re-distilled methanol with 1-2 drops of concentrated sulphuric acid, and incubated at 37°C for 48 hours. Mixtures were filtered through 0.45μm Whatman GF/F glass fibre filters, and the filtrates were extracted twice with equal volumes of re-distilled hexane. The hexane extract was then reduced in volume to about 0.5-1.0 ml under vacuum using a rotary evaporator. This was then ready for analysis by gas-liquid chromatography (GLC, see below).

2.2.14.4.2. GLC of fatty acids methyl esters (FAME)

(a) Column:

A 1.5m x 4mm glass column was packed under vacuum with 10% diethylglycolsuccinate (DEGS) on 100/120 mesh Chromosorb W. The column was then conditioned by placing it in a nitrogen flow near its maximum operating temperature (170°C) for a day without being connected to the detector.

(b) Chromatograph:

Pye Series 104.

(c) Detector:

Flame ionisation detector.

(d) Syringes:

SGE syringes of 1.0μl and 5.0μl capacities were used.

(e) Conditions:

Nitrogen flow (40 ml/minute), temperature (170°C).

(f) Standards:

Methyl esters of the following fatty acids were purchased from Sigma: capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and arachidic acid (C20:0) methyl esters.
2.2.15. The influence of pyrithione on the morphology of micro-organisms

*Kl. pneumoniae*, *B. licheniformis* NCIB 6346 and *C. albicans* NCYC 597 were used in this study. Cells were grown in 100 ml batches of CDM containing various concentrations of pyrithione. To prepare samples for analysis by scanning electron microscopy, the method of Ghannoum and Al-khars (1984) was used. Glutaraldehyde was added to cultures to give a final concentration of 1.5% (w/v). After 2 minutes contact, the cells were removed by centrifugation at 4000 rpm (4°C, 15 minutes) and resuspended in 2 ml of 5% (w/v) glutaraldehyde solution at 4°C for 16 hours. Cells were then collected by centrifugation, washed three times with distilled water and resuspended in distilled water to give the required density (faint opalescence). One drop (approximately 30μl) from each suspension was allowed to air-dry on clean circular glass cover slip and then dehydrated over CaCl₂ (anhydrous) under partial vacuum. Samples were then coated with gold-palladium in a high vacuum coating unit, until a coating of approximately 10nm thickness was achieved. Samples were then examined in an ISI-SS40 scanning electron microscope at an accelerating voltage of 10 kV.
CHAPTER THREE

RESULTS
3.1. MIC Determinations of pyrithione and related compounds (tube dilution)

Pyrithione inhibited the growth of all 15 species (20 strains) examined. In nutrient broth, MICs ranged from 0.03 to 80μg/ml; *S. cerevisiae* was the most sensitive (0.03μg/ml) whilst *P. aeruginosa* showed the highest resistance amongst the organisms tested (80μg/ml; Table 3.1). In addition, it was observed that *Kl. pneumoniae*, *Salm. abony*, *S. marcescens* and *P. vulgaris* always showed turbidity in some tubes containing the higher concentrations of the drug well above the MIC range. Microscopic examination and viability testing of loopful samples onto nutrient agar, showed growth in these tubes.

Table 3.2 shows the MICs of pyrithione determined in CDM against five selected organisms. *Ps. aeruginosa*, *B. licheniformis* and *C. albicans* were more sensitive in CDM than in nutrient broth, although *Ps. aeruginosa* remained relatively resistant. *E. coli* and *Kl. pneumoniae* did not show considerable variations in their sensitivity compared with that obtained in nutrient broth.

Table 3.3 shows the MIC values of pyrithione-related compounds against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*. None of the four compounds: 2-mercaptopyridine (MP), 2-mercaptopyrimidine (MPY), 2-mercaptonicotinic acid (MNA) and 3-hydroxy-2-mercaptopyridine (HMP) showed any apparent antimicrobial activity against *Kl. pneumoniae* at concentrations up to 100μg/ml. All four compounds inhibited the growth of *B. licheniformis* with MIC values of 0.625μg/ml (HMP), 2.5μg/ml (MPY), 5.0μg/ml (MP) and 50μg/ml (MNA). MNA was inactive against *C. albicans* (MIC > 100μg/ml), whilst MP, MPY and HMP were equally active against *C. albicans* with MIC value of 12.5μg/ml each.
Table 3.1: Minimum inhibitory concentrations (MIC) of pyrithione against micro-organisms in nutrient broth.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em> NCTC 9001</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> NCIB 8267</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em> NCIB 8067</td>
<td>5.0</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> NCTC 6749</td>
<td>80.0</td>
</tr>
<tr>
<td><em>Salmonella abony</em> NCTC 6017</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Serratia marcescens</em> NCTC 1377</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>Gram-positive bacteria</strong></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em> LUT 11755</td>
<td>0.6</td>
</tr>
<tr>
<td><em>B. licheniformis</em> NCIB 6346</td>
<td>0.6</td>
</tr>
<tr>
<td><em>B. licheniformis</em> NCTC 1097</td>
<td>0.6</td>
</tr>
<tr>
<td><em>B. megaterium</em> NCIB 8291</td>
<td>0.6</td>
</tr>
<tr>
<td><em>B. subtilis</em> NCIB 3610</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Mycobacterium phlei</em> NCTC 8151</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> NCIB 8625</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Yeasts</strong></td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em> NCYC 597</td>
<td>0.15</td>
</tr>
<tr>
<td><em>C. albicans</em> NCYC 1363</td>
<td>1.25</td>
</tr>
<tr>
<td><em>C. albicans</em> NCYC 1466</td>
<td>0.15</td>
</tr>
<tr>
<td><em>C. albicans</em> A39</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Debaryomyces hansenii</em> NCYC 8</td>
<td>0.2</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> NCYC 345</td>
<td>0.03</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> NCYC 975</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Table 3.2: Minimum inhibitory concentrations (MIC) of pyrithione against microorganisms in chemically defined medium.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em> NCTC 6749</td>
<td>20.0</td>
</tr>
<tr>
<td><em>Escherichia coli</em> NCTC 9001</td>
<td>2.5</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> NCIB 8267</td>
<td>5.0</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> NCIB 6346</td>
<td>0.05</td>
</tr>
<tr>
<td><em>Candida albicans</em>NCYC 597</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Table 3.3: Minimum inhibitory concentrations (MIC) of pyrithione-related compounds against micro-organisms in chemically defined medium.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MP*</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>&gt;100</td>
</tr>
<tr>
<td>NCIB 8267</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>5.0</td>
</tr>
<tr>
<td>NCIB 6346</td>
<td></td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>12.5</td>
</tr>
<tr>
<td>NCYC 597</td>
<td></td>
</tr>
</tbody>
</table>

* : MP, 2-mercaptopyridine; MPY, 2-mercaptopyrimidine; MNA, 2-mercaptonicotinic acid; HMP, 3-hydroxy-2-mercaptopyridine
3.2. Effect of pyrithione on the growth of micro-organisms

Figures 3.1, 3.2 and 3.3 show the effect of pyrithione on the growth of *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*, respectively. Both bacteria showed an overall similarity of growth curves in the presence of increasing sub-inhibitory concentrations of pyrithione, with an extended lag phase, the length of which depended on the concentration of the drug (Figs 3.1 and 3.2). After this lag phase, both organisms grew at a rate similar to that of the control (untreated) culture.

For *Kl. pneumoniae*, 5 and 6 μg/ml pyrithione induced lags of greater than 600 minutes after which recovery was occasionally observed. *C. albicans*, however, showed a different response to pyrithione. The drug reduced the overall growth rate of the organism, the effect on growth increasing with drug concentration and did not induce an obvious lag phase. At high concentrations, pyrithione inhibited the growth of all three organisms.

The effect of the re-addition (re-challenge) of pyrithione on cultures of *Kl. pneumoniae* and *B. licheniformis* initially grown in the presence of pyrithione is illustrated in Figs 3.4 and 3.5. Cultures showed the established (Figs 3.1 and 3.2) pattern of an extended lag phase in the presence of initial levels of pyrithione. Once growth was established, however, further additions of pyrithione had little or no effect on overall growth rate, which in each case was similar to that of the control (untreated) culture. For *C. albicans* (Fig 3.6), growth rate after re-addition of pyrithione was slightly reduced.

Figs 3.7 and 3.8 show the effect of adding concentrations of pyrithione to mid-log phase (O.D. 0.4) cultures of *Kl. pneumoniae* and *B. licheniformis*, respectively. A drug concentration-dependent lag phase was observed but the length of the lag phase was reduced compared with that observed with the same concentration of pyrithione.
present from the commencement of incubation (Figs 3.1 and 3.2). Adding pyrithione to mid-exponential phase cultures of \textit{C. albicans} (Fig 3.9) reduced growth rate in a drug concentration-dependent manner as before (Fig 3.3).

3.3 Effect of pyrithione on the viability of \textit{Kl. pneumoniae} and \textit{C. albicans}.

The effects of pyrithione on the optical density and viability of growing cultures of \textit{Kl. pneumoniae} are shown in Figure 3.10. The sets of curves for optical density (Fig 3.10a) and viability (Fig 3.10b) are essentially similar. Both show, in the presence of pyrithione, a first stage (lag) during which optical density (Fig 3.10a) and viable count (Fig 3.10b) remain virtually constant for a time dependent on pyrithione concentration. This is followed by a phase during which both optical density and viability increase at a rate similar to that of untreated controls. In the case of \textit{C. albicans}, the drug concentration-dependent reduction in growth rate as monitored by optical density (Fig 3.11a) is also mirror-imaged with viability curves (Fig 3.11b).
Figure 3.1: The effect of various concentrations of pyrithione on the growth (CDM, 37°C, 90rpm) of *Klebsiella pneumoniae*.

- ●: 0.0μg/ml
- ○: 1.0μg/ml
- ■: 2.0μg/ml
- □: 3.0μg/ml
- △: 4.0μg/ml
- +: 5.0μg/ml
- ×: 6.0μg/ml
Figure 3.2: The effect of various concentrations of pyrithione on the growth (CDM, 37°C, 90 rpm) of *B. licheniformis*;

- •: 0.0μg/ml
- ○: 0.002μg/ml
- ■: 0.004μg/ml
- □: 0.006μg/ml
- △: 0.008μg/ml
- +: 0.01μg/ml
Figure 3.3: The effect of various concentrations of pyrithione on the growth (CDM, 37°C, 90 rpm) of Candida albicans;

- : 0.0μg/ml
○ : 0.002μg/ml
■ : 0.004μg/ml
□ : 0.006μg/ml
△ : 0.008μg/ml
+ : 0.01μg/ml
X : 0.02μg/ml
Figure 3.4: The effect of re-challenge* (arrows) with pyrithione on the growth (CDM, 37°C, 90 rpm) of Klebsiella pneumoniae:

- : 0.0µg/ml (final conc. 0.0µg/ml)
○ : 1.0µg/ml (final conc. 2.0µg/ml)
■ : 2.0µg/ml (final conc. 4.0µg/ml)
□ : 3.0µg/ml (final conc. 6.0µg/ml)
△ : 4.0µg/ml (final conc. 8.0µg/ml)
➕ : 5.0 µg/ml (final conc. 10.0µg/ml)

*concentration after re-challenge was in each case at least that initially present.
Figure 3.5: The effect of re-challenge* (arrows) with pyrithione on the growth (CDM, 37°, 90 rpm) of *Bacillus licheniformis*:

- ●: 0.0μg/ml (final conc. 0.0μg/ml)
- ○: 0.002μg/ml (final conc. 0.004μg/ml)
- ■: 0.004μg/ml (final conc. 0.008μg/ml)
- □: 0.006μg/ml (final conc. 0.012μg/ml)
- △: 0.008μg/ml (final conc. 0.016μg/ml)
- †: 0.01μg/ml (final conc. 0.02μg/ml)
- ✖: 0.05μg/ml (final conc. 0.1μg/ml)
- ◇: 0.1μg/ml (final conc. 0.2μg/ml)

*concentration after re-challenge was in each case at least that initially present.*
OPTICAL DENSITY (650 nm.)

TIME (MINUTES)
Figure 3.6: The effect of re-challenge* (arrows) with pyrithione on the growth (CDM, 37°C, 90 rpm) of Candida albicans;

- : 0.0µg/ml (final conc. 0.0µg/ml)
O : 0.002µg/ml (final conc. 0.004µg/ml)
■ : 0.004µg/ml (final conc. 0.008µg/ml)
□ : 0.006µg/ml (final conc. 0.012µg/ml)
△ : 0.008µg/ml (final conc. 0.016µg/ml)
➕ : 0.01µg/ml (final conc. 0.02µg/ml)
✖ : 0.05µg/ml (final conc. 0.1µg/ml)
◇ : 0.1µg/ml (final conc. 0.2µg/ml)

* concentration after re-challenge was in each case at least that initially present.
Figure 3.7: The effect of adding (arrows) pyrithione to exponentially growing (CDM, 37°C, 90 rpm) cells of *Klebsiella pneumoniae*; final pyrithione concentrations:

- •: 0.0μg/ml
- ○: 1.0μg/ml
- ■: 2.0μg/ml
- □: 3.0μg/ml
- △: 4.0μg/ml
- +: 5.0μg/ml

...
Figure 3.8: The effect of adding (arrows) pyrithione to exponentially growing (CDM, 37°C, 90 rpm) cells of *Bacillus licheniformis*; final pyrithione concentrations:

- ●: 0.0μg/ml
- ○: 0.002μg/ml
- ■: 0.004μg/ml
- □: 0.006μg/ml
- △: 0.008μg/ml
- +: 0.01μg/ml
- ×: 0.05μg/ml
- ◇: 0.1μg/ml
Figure 3.9: The effect of adding (arrows) pyrithione to exponentially growing (CDM, 37°C, 90 rpm) cells of *Candida albicans*;

final pyrithione concentrations:

- $\bullet$: 0.0µg/ml
- $\bigcirc$: 0.002µg/ml
- $\blacksquare$: 0.004µg/ml
- $\square$: 0.006µg/ml
- $\vartriangle$: 0.008µg/ml
- $\oplus$: 0.01µg/ml
- $\times$: 0.05µg/ml
- $\lozenge$: 0.1µg/ml
Figure 3.10: The effect of pyrithione on the growth and viability of *Kl. pneumoniae* (CDM, 37°C, 90 rpm); pyrithione concentrations:

- ○: 0.0µg/ml
- □: 1.0µg/ml
- ■: 2.0µg/ml
- □: 4.0µg/ml
- △: 5.0µg/ml

(a) optical density

(b) viable count
Figure 3.11: The effect of various concentrations of pyrithione on the growth and viability of *C. albicans* (CDM, 37°C, 90 rpm);

- ●: 0.0µg/ml
- ○: 0.002µg/ml
- ■: 0.004µg/ml
- □: 0.006µg/ml
- △: 0.008µg/ml
- ‡: 0.01µg/ml
- ◯: 0.05µg/ml

(a) optical density

(b) viable count
3.4. Cell size distribution of division-inhibited (ampicillin) cells of *K. pneumoniae* in the presence of pyrithione.

The MIC of ampicillin against *B. licheniformis* in CDM is 1.6\(\mu\)g/ml, whereas the growth of *K. pneumoniae* was not inhibited at 100\(\mu\)g/ml or less (Table 3.4). Preliminary experiments showed that in CDM (37°C, 90 rpm) containing 50\(\mu\)g/ml ampicillin, cells of *K. pneumoniae* grew (increased in mass/optical density) without division, at an uninhibited rate (equivalent to control rate). This was confirmed by both microscopy (in which filamentous growth was observed), cell count and assessment of cell size distribution at various times.

The analysis of the cell size distribution of cultures of *K. pneumoniae* incubated in CDM and in CDM containing 2\(\mu\)g/ml pyrithione or 50\(\mu\)g/ml ampicillin is shown in Figs 3.12 and 3.13, respectively. Control (untreated) cultures have unimodal cells size (equivalent spheres) profiles with a mean size of 1.23\(\mu\)m\(^3\) that are essentially unchanged following incubation (Fig 3.12). Cultures incubated in the presence of 50\(\mu\)g/ml ampicillin show unimodal profiles of increased mean size (1.96\(\mu\)m\(^3\)) after 120 minutes (Fig 3.13). Cultures incubated in the presence of both ampicillin (50\(\mu\)g/ml) and pyrithione (2.0\(\mu\)g/ml) for up to 120 minutes had size profiles that were unimodal and similar to that of the drug-free control (cf. Figs 3.12 and 3.14). Cultures incubated further (240 - 360 minutes; Fig 3.14) demonstrated bimodal size distributions following the appearance of a population of increased mean size. Prolonged incubation (480 minutes) produced cultures with a shift to a unimodal population of increased mean size (2.47\(\mu\)m\(^3\)) with concurrent loss of the population of smaller mean size (1.23\(\mu\)m\(^3\); Fig 3.14).
Table 3.4: Minimum inhibitory concentrations (MIC; tube dilution) of ampicillin against micro-organisms in chemically defined medium.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Kl. pneumoniae</em> NCIB 8267</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>B. licheniformis</em> NCIB 6346</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Figure 3.12: Cell size (equivalent spheres) distribution of *Kl. pneumoniae* grown in CDM (37°C, 90 rpm) after:

- • : 0.0 minutes
- O : 120 minutes
Figure 3.13: Cell size (equivalent spheres) distribution of *Kl. pneumoniae* grown in CDM (37°C, 90 rpm) after:

- : 0.0 minutes (50μg/ml ampicillin or 2.0μg/ml pyrithione)
- : 120 minutes (2.0μg/ml pyrithione)
- : 120 minutes (50μg/ml ampicillin)
Figure 3.14: Cell size (equivalent spheres) distribution of 

*Kl. pneumoniae* grown in CDM (37°C, 90 rpm) containing 50μg/ml ampicillin and 2.0μg/ml pyrithione, after:

- : 0.0 minutes
- : 120 minutes
- : 240 minutes
- : 360 minutes
- : 480 minutes
3.5. Interaction of pyrithione with EDTA

3.5.1. Agar diffusion

EDTA interacted antagonistically with pyrithione against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans* (Fig 3.15 a, b and c, respectively). The antagonism was characterised by reduction of pyrithione's inhibition zone size around the area where the two paper strips overlap. The antagonism was most evident with *B. licheniformis* (Fig 3.15b) followed by *C. albicans* (Fig 3.15c) and *Kl. pneumoniae* (Fig 3.15a).

3.5.2. Influence of EDTA on pyrithione-induced changes in growth rate

Table 3.5 shows the MIC values of EDTA against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*. The concentration of EDTA up to 200μg/ml did not inhibit the growth of either *Kl. pneumoniae* or *B. licheniformis*. The MIC of EDTA against *C. albicans* was 50μg/ml.

In the case of *Kl. pneumoniae* (Fig 3.16), EDTA (50μg/ml) showed some antagonism shortening the observed lag phase induced by pyrithione (3.0μg/ml). EDTA (50μg/ml) completely antagonised the action of pyrithione (0.002μg/ml) against *B. licheniformis* (Fig 3.17). This supports the strong antagonism observed on solid medium previously described (Fig 3.15b). With *C. albicans* (Fig 3.18), EDTA (10μg/ml) reduced the effectiveness of pyrithione (0.01μg/ml) though the new growth rate was less than that of the control (untreated) culture.
Figure 3.15: Interaction between pyrithione and EDTA (CDMA, 37°C);

(a) *Klebsiella pneumoniae*; pyrithione 0.1%, strip P;
   EDTA 1.0%, strip E.

(b) *Bacillus licheniformis*; pyrithione 0.01%, strip P;
   EDTA 1.0%, strip E.

(c) *Candida albicans*; pyrithione 0.01%, strip P;
   EDTA 1.0%, strip E.
Table 3.5: Minimum inhibitory concentrations of EDTA against micro-organisms in chemically defined medium.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Klebsiella pneumoniae</em> NCIB 8267</td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> NCIB 6346</td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>Candida albicans</em> NCYC 597</td>
<td>50.0</td>
</tr>
</tbody>
</table>
Figure 3.16: Influence of EDTA on the growth (CDM, 37°C, 90rpm) of pyrithione-treated cells of *Klebsiella pneumoniae*;

- ●: control (untreated)
- ○: 3.0 μg/ml pyrithione
- ■: 50μg/ml EDTA
- □: 3.0μg/ml pyrithione plus 50μg/ml EDTA
Figure 3.17: Influence of EDTA on the growth (CDM, 37°C, 90 rpm) of pyrithione-treated cells of *Bacillus licheniformis*;

- ■: control (untreated)
- ●: 0.002 µg/ml pyrithione
- □: 50 µg/ml EDTA
- ○: 0.002 µg/ml pyrithione plus 50 µg/ml EDTA
Figure 3.18: Influence of EDTA on the growth (CDM, 37°C, 90 rpm) of pyrithione-treated cells of Candida albicans;

- : control (untreated)
- : 0.01μg/ml pyrithione
- : 10μg/ml EDTA
- : 0.01μg/ml pyrithione plus 10μg/ml EDTA
3.6. Interaction of metal ions with pyrithione

3.6.1. In nutrient agar

Figures 3.19, 3.20 and 3.21 show the interaction of pyrithione with some metal ions in nutrient agar against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*, respectively. Synergy was observed between pyrithione and Zn$^{2+}$ against *Kl. pneumoniae* (Fig 3.19a), but no significant interaction was observed between pyrithione and any of the other metal ions: Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Fe$^{3+}$ (Fig 3.19b, c, d and e, respectively).

Against *B. licheniformis* (Fig 3.20), slight synergy was observed between pyrithione and either Mg$^{2+}$ (Fig 3.20b), or Ca$^{2+}$ (Fig 3.20d) or Fe$^{3+}$ (Fig 3.20e), but no apparent interaction between pyrithione and either Zn$^{2+}$ (Fig 3.20a) or Mn$^{2+}$ (Fig 3.20c) was observed. Synergy was apparent between pyrithione and all the five metal ions against *C. albicans* (Fig 3.21).

3.6.2. In CDMA

Figures 3.22, 3.23 and 3.24 show the interaction between pyrithione and metal ions in CDMA against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*, respectively. In the case of *Kl. pneumoniae*, slight synergy was observed between pyrithione and either Zn$^{2+}$ (Fig 3.22a) or Fe$^{3+}$ (Fig 3.22e), but no significant interaction was observed between pyrithione and either Mg$^{2+}$, Mn$^{2+}$ or Ca$^{2+}$ (Fig 3.22b, c and d, respectively).

Against *B. licheniformis* (Fig 3.23), the action of pyrithione was slightly potentiated by Zn$^{2+}$ (Fig 3.23a), Mn$^{2+}$ (Fig 3.23c) and Fe$^{3+}$ (Fig 3.23e). No interaction was observed between pyrithione and either Mg$^{2+}$ (Fig 3.23b) or Ca$^{2+}$.
In the case of *C. albicans* (Fig 3.24), the action of pyrithione was slightly potentiated by Zn$^{2+}$ (Fig 3.24a) but not by any of the other ions: Mg$^{2+}$ (Fig 3.24b), Mn$^{2+}$ (Fig 3.24c), Ca$^{2+}$ (Fig 3.24d) and Fe$^{3+}$ (Fig 3.24e).

### 3.7. Interaction between pyrithione and 8-hydroxyquinoline against micro-organisms

No apparent interaction was observed between pyrithione and 8-hydroxyquinoline in either nutrient agar (Fig 3.25) or CDMA (Fig 3.26) against *Kl. pneumoniae*, *B. licheniformis* or *C. albicans*.

### 3.8. The antimicrobial action of 8-hydroxyquinoline

#### 3.8.1. MIC determinations

Table (3.6) shows the MIC values for 8-hydroxyquinoline in CDM against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*. *Kl. pneumoniae* was the most resistant (MIC 50μg/ml) whereas *C. albicans* was the most sensitive (MIC 0.15μg/ml). *B. licheniformis* was also more sensitive than *Kl. pneumoniae* with an MIC value of 0.625μg/ml.

#### 3.8.2. Effect on growth

Figure 3.27 shows the effect of 8-hydroxyquinoline on the viability of *Kl. pneumoniae*. An initial phase of highly reduced growth rate (lag phase, the length of which depended on the concentration of the drug) was observed. This was followed, in the presence of sub-inhibitory concentrations of 8-hydroxyquinoline, by
an increase in viability at a rate approaching that of the control (untreated) culture (Fig 3.27). In the case of B. licheniformis (Fig 3.28) and C. albicans (Fig 3.29), optical density measurements were used to monitor growth. In the presence of sub-inhibitory concentrations of 8-hydroxyquinoline, B. licheniformis showed an initial lag phase, the length of which depended on the drug concentration. This was followed by growth at a rate similar to that of the control (untreated) culture (Fig 3.28). The effect of 8-hydroxyquinoline on the growth of C. albicans was characterised by drug concentration dependent reduction of the overall growth rate of the organism, but no obvious lag phase was induced (Fig 3.29).

Table 3.6: Minimum inhibitory concentrations (MIC) of 8-hydroxyquinoline against micro-organisms in chemically defined medium.

<table>
<thead>
<tr>
<th>Micro-organism</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Klebsiella pneumoniae NCIB 8267</td>
<td>50.0</td>
</tr>
<tr>
<td>Bacillus licheniformis NCIB 6346</td>
<td>0.625</td>
</tr>
<tr>
<td>Candida albicans NCYC 579</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Figure 3.19: Interaction between pyrithione and metal ions against *Kl. pneumoniae* (Nutrient Agar, 37°C);

(a) Pyrithione 0.1%, strip P; ZnCl₂ 0.01%, strip Zn.

(b) Pyrithione 0.1%, strip P; MgSO₄·7H₂O 0.01%, strip Mg.

(c) Pyrithione 0.1%, strip P; MnCl₂·4H₂O 0.01%, strip Mn.

(d) Pyrithione 0.1%, strip P; CaCl₂ 0.01%, strip Ca.

(e) Pyrithione 0.1%, strip P; FeCl₃ 0.01%, strip Fe.
Figure 3.20: Interaction between pyrithione and metal ions against *B. licheniformis* (Nutrient Agar, 37°C);

(a) Pyrithione 0.01%, strip P; ZnCl₂ 0.01%, strip Zn.

(b) Pyrithione 0.01%, strip P; MgSO₄·7H₂O 0.01%, strip Mg.

(c) Pyrithione 0.01%, strip P; MnCl₂·4H₂O 0.01%, strip Mn.

(d) Pyrithione 0.01%, strip P; CaCl₂ 0.01%, strip Ca.

(e) Pyrithione 0.01%, strip P; FeCl₃ 0.01%, strip Fe.
Figure 3.21: Interaction between pyrithione and metal ions against

*C. albicans* (Nutrient Agar, 37°C);

(a) Pyrithione 0.01%, strip P; ZnCl₂ 0.01%, strip Zn.

(b) Pyrithione 0.01%, strip P; MgSO₄·7H₂O 0.01%, strip Mg.

(c) Pyrithione 0.01%, strip P; MnCl₂·4H₂O 0.01%, strip Mn.

(d) Pyrithione 0.01%, strip P; CaCl₂ 0.01%, strip Ca.

(e) Pyrithione 0.01%, strip P; FeCl₃ 0.01%, strip Fe.
Figure 3.22: Interaction between pyrithione and metal ions against *Kl. pneumoniae* (CDMA, 37°C);

(a) Pyrithione 0.1%, strip P; ZnCl₂ 0.01%, strip Zn.
(b) Pyrithione 0.1%, strip P; MgSO₄·7H₂O 0.01%, strip Mg.
(c) Pyrithione 0.1%, strip P; MnCl₂·4H₂O 0.01%, strip Mn.
(d) Pyrithione 0.1%, strip P; CaCl₂ 0.01%, strip Ca.
(e) Pyrithione 0.1%, strip P; FeCl₃ 0.01%, strip Fe.
Figure 3.23: Interaction between pyrithione and metal ions against *B. licheniformis* (CDMA, 37°C);

(a) Pyrithione 0.001%, strip P; ZnCl\textsubscript{2} 0.01%, strip Zn.

(b) Pyrithione 0.001%, strip P; MgSO\textsubscript{4}.7H\textsubscript{2}O 0.01%, strip Mg.

(c) Pyrithione 0.001%, strip P; MnCl\textsubscript{2}.4H\textsubscript{2}O 0.01%, strip Mn.

(d) Pyrithione 0.001%, strip P; CaCl\textsubscript{2} 0.01%, strip Ca.

(e) Pyrithione 0.001%, strip P; FeCl\textsubscript{3} 0.01%, strip Fe.
Figure 3.24: Interaction between pyrithione and metal ions against *C. albicans* (CDMA, 37°C);

(a) Pyrithione 0.001%, strip P; ZnCl₂ 0.01%, strip Zn.
(b) Pyrithione 0.001%, strip P; MgSO₄·7H₂O 0.01%, strip Mg.
(c) Pyrithione 0.001%, strip P; MnCl₂·4H₂O 0.01%, strip Mn.
(d) Pyrithione 0.001%, strip P; CaCl₂ 0.01%, strip Ca.
(e) Pyrithione 0.001%, strip P; FeCl₃ 0.01%, strip Fe.
Figure 3.25: Interaction between pyrithione and 8-hydroxyquinoline (Nutrient Agar, 37°C) against:

(a) *Klebsiella pneumoniae*; pyrithione 0.1%, strip P; 8-hydroxyquinoline 0.1%, strip HQ.

(b) *Bacillus licheniformis*; pyrithione 0.01%, strip P; 8-hydroxyquinoline 0.1%, strip HQ.

(c) *Candida albicans*; pyrithione 0.01%, strip P; 8-hydroxyquinoline 0.1%, strip HQ.
Figure 3.26: Interaction between pyrithione and 8-hydroxyquinoline
( CDMA, 37°C ) against:

(a) *Klebsiella pneumoniae*; pyrithione 0.1% , strip P;
8-hydroxyquinoline 0.1% , strip HQ.

(b) *Bacillus licheniformis*; pyrithione 0.001% , strip P;
8-hydroxyquinoline 0.01% , strip HQ.

(c) *Candida albicans*; pyrithione 0.001% , strip P;
8-hydroxyquinoline 0.01% , strip HQ.
Figure 3.27: The effect of various concentrations of 8-hydroxyquinoline on the viability (CDM, 37°C, 90 rpm) of Klebsiella pneumoniae.

- ●: 0.0μg/ml
- ○: 10μg/ml
- ■: 20μg/ml
- □: 30μg/ml
- △: 40μg/ml
- + : 50μg/ml
Figure 3.28: The effect of various concentrations of 8-hydroxyquinoline on the growth (CDM, 37°C, 90 rpm) of *Bacillus licheniformis*;

- **○**: 0.0μg/ml
- **○**: 0.025μg/ml
- **■**: 0.05μg/ml
- **□**: 0.075μg/ml
- **△**: 0.1μg/ml
- **+**: 0.2μg/ml
- **×**: 0.3μg/ml
Figure 3.29: The effect of various concentrations of 8-hydroxyquinoline on the growth (CDM, 37°C, 90 rpm) of *Candida albicans*;

- ●: 0.0μg/ml
- ○: 0.025μg/ml
- ■: 0.05μg/ml
- □: 0.075μg/ml
- △: 0.1μg/ml
- +: 0.2μg/ml
- X: 0.3μg/ml
3.9. Effect of pyrithione on the incorporation of radiolabelled substrates by 
*Kl. pneumoniae* and *B. licheniformis*

3.9.1. *Kl. pneumoniae*

The incorporation of $^{14}$C-glycine by *Kl. pneumoniae* was rapidly inhibited by pyrithione at 1.0, 2.5 and 5.0μg/ml (Fig 3.30). Glycine incorporation ceased within 2 minutes of the addition of pyrithione. However, prolonged incubation of the cells (420 minutes, allowing re-establishment of growth approximately 120 minutes after the addition of pyrithione) in the presence of pyrithione (2.5μg/ml) showed that glycine incorporation continued at a reduced rate compared with control (untreated) cells (Fig 3.31).

The incorporation of $^{14}$C-thymidine was also inhibited by pyrithione within 2 minutes of the addition of the drug (Fig 3.32), and did not recommence in the presence of 2.5 μg/ml of the drug during prolonged (420 minutes) incubation (Fig 3.33).

The incorporation of $^{14}$C-uridine was also inhibited within 2 minutes of the addition of pyrithione (Fig 3.34). However, $^{14}$C-uridine incorporation recommenced in the presence of 2.5μg/ml of pyrithione at a rate similar to that of the control during prolonged incubation (300 minutes) which allowed, as before, recommencement of the growth approximately 120 minutes after the addition of the drug (Fig 3.35).

3.9.2. *B. licheniformis*

The incorporation of $^{14}$C-glycine (Fig 3.36), $^{14}$C-thymidine (Fig 3.37) and $^{14}$C-uridine (Fig 3.38) was completely inhibited within 3-5 minutes of the addition of pyrithione at 0.01, 0.025 and 0.05μg/ml.
Figure 3.30: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-glycine (0.2μCi) by *Klebsiella pneumoniae* (CDM, 37°C, 90 rpm);

- ●: 0.0μg/ml
- ○: 1.0μg/ml
- ■: 2.5μg/ml
- □: 5.0μg/ml
Figure 3.31: The effect of pyrithione concentration (arrow) on
the incorporation of $^{14}$C-glycine (0.2μCi) by Klebsiella
pneumoniae (CDM, 37°C, 90 rpm), during prolonged
incubation;

● : 0.0μg/ml

○ : 2.5μg/ml
Figure 3.32: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-thymidine (0.2μCi) by *Klebsiella pneumoniae* (CDM, 37°C, 90 rpm);

- : 0.0μg/ml
- : 1.0μg/ml
- : 2.5μg/ml
- : 5.0μg/ml
Figure 3.33: The effect of pyrithione concentration (arrow) on the incorporation of $^{14}$C-thymidine (0.2μCi) by *Klebsiella pneumoniae* (CDM, 37°C, 90 rpm), during prolonged incubation;

- ●: 0.0μg/ml
- ○: 2.5μg/ml
Figure 3.34: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-uridine (0.2μCi) by Klebsiella pneumoniae (CDM, 37°C, 90 rpm);

- ●: 0.0μg/ml
- ○: 1.0μg/ml
- ■: 2.5μg/ml
- □: 5.0μg/ml
Figure 3.35: The effect of pyrithione concentration (arrow) on the incorporation of $^{14}$C-uridine (0.2µCi) by *Klebsiella pneumoniae* (CDM, 37°C, 90 rpm), during prolonged incubation;

- : 0.0µg/ml
- : 2.5µg/ml
Figure 3.36: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-glycine (0.2µCi) by Bacillus licheniformis (CDM, 37°C, 90 rpm);

- : 0.0µg/ml
- : 0.01µg/ml
- : 0.025µg/ml
- : 0.05µg/ml
Figure 3.37: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-thymidine (0.2μCi) by *Bacillus licheniformis* (CDM, 37°C, 90 rpm);

- ●: 0.0μg/ml
- ○: 0.01μg/ml
- ■: 0.025μg/ml
- □: 0.05μg/ml
Figure 3.38: The effect of pyrithione (arrow) at various concentrations on the incorporation of $^{14}$C-uridine (0.2µCi) by *Bacillus licheniformis* (CDM, 37°C, 90 rpm);

- ●: 0.0µg/ml
- ○: 0.01µg/ml
- ■: 0.025µg/ml
- □: 0.05µg/ml
3.10. Aspects of the resistance of *Kl. pneumonias* to pyrithione

3.10.1. The MIC of pyrithione against control and pyrithione-treated cells

Table 3.7 shows the MIC values for pyrithione against control and pyrithione treated cells of *Kl. pneumonias*. The MIC of pyrithione against untreated cells (grown for 24 hours in CDM, 37°C, 90 rpm) was 5.0μg/ml (culture A). The 24 hourly subculturing of these untreated cells in fresh CDM over a period of 7 days did not affect the MIC value, which was assessed every 24 hours immediately before subculturing. Cells of *Kl. pneumonias* that were incubated for 24 hours in CDM containing 5.0μg/ml pyrithione were more resistant than untreated cells (culture A) with an MIC value of 40μg/ml (culture B), and this MIC value was not affected by 24 hourly subculturing in pyrithione (5.0μg/ml)-containing CDM. Cells that were initially incubated in CDM containing pyrithione (5.0μg/ml) for 24 hours, then subcultured into drug-free CDM every 24 hours over a period of 7 days responded differently (culture C). The MIC of pyrithione against these cells was 40μg/ml over the period of 4 days but was reduced to 20μg/ml over the remaining period of 3 days.

3.10.2. The effect of pyrithione on the growth curve of pyrithione-treated cells of *Kl. pneumonias*

The effect of pyrithione on the growth of pyrithione-treated (grown in CDM containing 5.0μg/ml pyrithione at 37°C, 90 rpm for 24 hours) cells of *Kl. pneumonias* is shown in Figure 3.39. Pyrithione induced a lag phase the length of which (approximately 60 minutes) was independent of the concentration (1.0, 2.0, 3.0, 4.0 and 5.0μg/ml) of the drug. The lag phase was followed by growth at a rate similar to that of the control (pyrithione-treated cells grown in drug-free CDM).
Table 3.7: Minimum inhibitory concentrations of pyrithione against control and pyrithione-treated cells of *Klebsiella pneumoniae* (CDM, 37°C).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture A*</td>
</tr>
<tr>
<td>0</td>
<td>5.0</td>
</tr>
<tr>
<td>1</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>5.0</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
</tr>
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</tr>
<tr>
<td>6</td>
<td>5.0</td>
</tr>
<tr>
<td>7</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Culture A: cells of *Klebsiella pneumoniae* subcultured in drug-free CDM every 24 hours.

Culture B: cells of *Klebsiella pneumoniae* subcultured in CDM containing 5.0µg/ml pyrithione every 24 hours.

Culture C: cells of *Klebsiella pneumoniae* initially grown in CDM containing 5.0µg/ml pyrithione for 24 hours, then subcultured in drug-free CDM every 24 hours.
Figure 3.39: The effect of pyrithione at various concentrations on the growth (CDM, 37°C, 90 rpm) of cells of Klebsiella pneumoniae previously grown for 24 hours in CDM (37°C, 90 rpm) containing 5.0μg/ml pyrithione:

- • : 0.0μg/ml
- ○ : 1.0μg/ml
- ■ : 2.0μg/ml
- □ : 3.0μg/ml
- △ : 4.0μg/ml
- + : 5.0μg/ml
3.11. Cross-resistance of control and pyrithione-treated cells of *Kl. pneumoniae* and *B. licheniformis* to other antimicrobial agents

3.11.1. Agar diffusion

Figure 3.40 shows the zones of growth inhibition of control and pyrithione-treated cells of *Kl. pneumoniae* induced by a group of antibiotics. Control cells of *Kl. pneumoniae* were inhibited by chloramphenicol, colistin sulphate, nitrofurantoin, kanamycin, streptomycin and tetracycline, whilst ampicillin and sulphafurazole had little or no effect (Fig 40a). Pyrithione-treated (resistant) cells showed a similar pattern to that of the control (untreated) cells with the exception that the inhibition zone induced by chloramphenicol has almost completely disappeared (Fig 40b). Inhibition zones of other drugs remained essentially unchanged.

Figure 3.41 shows the zones of inhibition of control and pyrithione-treated cells of *B. licheniformis* by the same group of antibiotics. Control cells were inhibited by chloramphenicol, nitrofurantoin, sulphafurazole, kanamycin, ampicillin, streptomycin and tetracycline, but to a lesser degree by colistin sulphate (Fig 41a). Pyrithione-treated cells were inhibited in a similar fashion and to the same extent (Fig 41b).

3.11.2. MIC determinations

The MIC values of pyrithione, 8-hydroxyquinoline and chloramphenicol (tube dilution) against *Kl. pneumoniae* are 5.0, 50 and 12.5µg/ml, respectively (Table 3.8). Pyrithione-treated cells of *Kl. pneumoniae* were less sensitive than control (untreated) cells to pyrithione (MIC 40µg/ml) and chloramphenicol (MIC 100µg/ml) but as sensitive as control (untreated) cells to 8-hydroxyquinoline (MIC 50µg/ml), as shown in Table 3.8.
3.12. Fatty acid content of control and pyrithione-treated cells of *Kl. pneumoniae*

Fatty acid contents (GLC of methyl esters of chloroform/methanol extracts) of control (untreated) and pyrithione-treated cells of *Kl. pneumoniae* were essentially similar (Table 3.9). The major fatty acids were: myristic (C14:0), palmitic (C16:0), stearic (C18:0) and arachidic (C20:0). Palmitic acid (C16:0) was the most abundant (48.6-50% of total fatty acid content) whilst arachidic acid was the least (9.3-12.8%, Table 3.9).
Figure 3.40: Agar diffusion sensitivity screen (Nutrient Agar, 37°C) of the following antibiotics:

C: Chloramphenicol, 50μg,
CT: Colistin sulphate, 10μg,
F: Nitrofurantoin, 200μg,
SF: Sulphafurazole, 500μg,
K: Kanamycin, 30μg,
AMP: Ampicillin, 25μg,
S: Streptomycin, 25μg,
TE: Tetracycline, 50μg,

against:

(a) control cells of *Klebsiella pneumoniae*.

(b) pyrithione-treated (grown in CDM containing 5.0μg/ml pyrithione at 37°C and 90 rpm for 24 hours) cells of *Klebsiella pneumoniae*. 
Figure 3.41: Agar diffusion sensitivity screen (Nutrient Agar, 37°C) of the following antibiotics:

- C : Chloramphenicol, 50μg,
- CT : Colistin sulphate, 10μg,
- F : Nitrofurantoin, 200μg,
- SF : Sulphafurazole, 500μg,
- K : Kanamycin, 30μg,
- AMP : Ampicillin, 25μg,
- S : Streptomycin, 25μg,
- TE : Tetracycline, 50μg,

against:

(a) control cells of *Bacillus licheniformis*

(b) pyrithione-treated (grown in CDM containing 0.05μg/ml pyrithione at 37°C and 90 rpm for 24 hours) cells of *Bacillus licheniformis*. 
Table 3.8: Minimum inhibitory concentrations (MIC; tube dilution; CDM) of pyrithione, 8-hydroxyquinoline and chloramphenicol against control (untreated) and pyrithione-treated cells of *Kl. pneumoniae*.

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>MIC (µg/ml) control</th>
<th>pyrithione-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrithione</td>
<td>5.0</td>
<td>40.0</td>
</tr>
<tr>
<td>8-hydroxyquinoline</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>12.5</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 3.9: Fatty acids (GLC of fatty acid methyl esters) composition of control (untreated) and pyrithione-treated (5.0µg/ml, 37°C, 90 rpm, 24 hr) cells of *Kl. pneumoniae*.

<table>
<thead>
<tr>
<th>% of total fatty acids</th>
<th>Myristic (C14:0)</th>
<th>Palmitic (C16:0)</th>
<th>Stearic (C18:0)</th>
<th>Arachidic (C20:0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated) cells</td>
<td>15.4</td>
<td>50.0</td>
<td>21.8</td>
<td>12.8</td>
</tr>
<tr>
<td>Pyrithione-treated cells</td>
<td>17.8</td>
<td>48.6</td>
<td>24.3</td>
<td>9.30</td>
</tr>
</tbody>
</table>
3.13 The effect of pyrithione on the morphology of micro-organisms

Figures 3.42, 3.43 and 3.44 show the scanning electron micrographs of control and pyrithione-treated cells of *Kl. pneumoniae*, *B. licheniformis* and *C. albicans*, respectively. Control (untreated) cells of *Kl. pneumoniae* are typically short rods (3-4 μm; Fig 3.42a). When grown in the presence of pyrithione, some cells of *Kl. pneumoniae* showed elongation. Elongated cells (10-20 μm in length) were observed in the presence of 2.5 and 5.0 μg/ml of pyrithione (Fig 3.42b and c), but the longest cells were observed at 10 μg/ml pyrithione (Fig 3.42d).

Control cells of *B. licheniformis* are typically rod shaped (4-6 μm; Fig 43a), but longer than cells of *Kl. pneumoniae* (cf. Figs 42a and 43a). Pyrithione at 0.025, 0.05 and 0.1 μg/ml (Fig 43b, c and d, respectively) had no apparent effect on the morphology of cells of *B. licheniformis*.

Control cells of *C. albicans* are typically oval-shaped with smooth surfaces (Fig 44a). Cells grown in the presence of 0.025 μg/ml pyrithione are similar to control cells and show no apparent signs of morphological alterations (Fig 3.44b). Cells grown in the presence of 0.05 or 0.1 μg/ml of the drug show signs of cell surface damage and leakage of cytoplasmic material, with these effects more evident at 0.1 μg/ml pyrithione (Fig 3.44c) than at 0.05 μg/ml of the drug (Fig 3.44d).
Figure 3.42: Scanning electron micrographs of cells of *Kl. pneumoniae* grown (CDM, 37°C, 90 rpm, 24 hr) in the presence of pyrithione at the following concentrations:

(a) 0.0μg/ml; mag. 1kx.
(b) 2.5μg/ml; mag. 1kx.
(c) 5.0μg/ml; mag. 1kx.
(d) 10.0μg/ml; mag. 1kx.
Figure 3.43: Scanning electron micrographs of cells of *B. licheniformis* grown (CDM, 37°C, 90 rpm, 24 hr) in the presence of pyrithione at the following concentrations:

(a) 0.0μg/ml; mag. 1kx.

(b) 0.025μg/ml; mag. 1kx.

(c) 0.05μg/ml; mag. 3.3kx.

(d) 0.1μg/ml; mag. 3.3kx.
Figure 3.44: Scanning electron micrographs of cells of *C. albicans* grown (CDM, 37°C, 90 rpm, 24 hr) in the presence of pyrithione at the following concentrations:

(a) 0.01 µg/ml; mag. 2.8kx.
(b) 0.025 µg/ml; mag. 2.8kx.
(c) 0.05 µg/ml; mag. 2.8kx.
(d) 0.1 µg/ml; mag. 0.6kx.

Pyrithione, at 2.5µg/ml, caused a 70% reduction in the level of [α-32P]UTP incorporation into RNA transcribed by purified *E. coli* RNA polymerase *in vitro* compared to the control (no drug added; Table 3.10). For comparison the effect of rifampicin (10µg/ml) on the incorporation of radiolabelled UTP was assessed; rifampicin caused a 93% reduction under the same conditions compared with the control (Table 3.10).

Table 3.10: Effects of rifampicin and pyrithione on the activity of purified *E. coli* RNA polymerase *in vitro* (37°C, 20 min., pH 7.8).

<table>
<thead>
<tr>
<th>Antimicrobial Agent</th>
<th>Counts per minute of incorporated [α-32P]UTP</th>
<th>Percentage inhibition of incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4142</td>
<td>0.0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>248</td>
<td>93</td>
</tr>
<tr>
<td>Pyrithione</td>
<td>1235</td>
<td>70</td>
</tr>
</tbody>
</table>
Pyrithione inhibited the growth of a wide range of micro-organisms including both Gram-negative and Gram-positive bacteria, and yeast (Table 3.1). In general, Gram-positive bacteria were more sensitive than Gram-negative bacteria. This is in agreement with earlier observations on the antimicrobial spectrum of pyrithione (Shaw et al., 1950; Pansy et al., 1953). *Ps. aeruginosa* was particularly resistant (MIC 80µg/ml, table 3.1), and with some organisms the level of sensitivity was medium dependent (Tables 3.1 and 3.2). For example, the MIC of pyrithione against *Ps. aeruginosa* in nutrient broth (80µg/ml, table 3.1) is four times the MIC in chemically-defined medium (20µg/ml, table 3.2). Similarly, the MIC of pyrithione against *B. licheniformis* in nutrient broth (0.6µg/ml, table 3.1) is approximately ten times that in chemically-defined medium (0.05µg/ml, table 3.2).

The fact that pyrithione is active against a wide range of micro-organisms suggests that it may act at a site(s) common to a variety of micro-organisms, or that pyrithione has more than one mode of action, thus affecting different micro-organisms in different ways. Of particular interest is the observed variability in the activity of pyrithione against some Gram-negative but not Gram-positive bacteria or yeast. As mentioned previously (section 3.1.), when determining the MIC of pyrithione in nutrient broth against *Kl. pneumoniae*, *Salm. abony*, *S. marcescens* and *P. vulgaris* using tube dilution method, growth (turbidity) was always detected in some tubes containing concentrations of the drug well above the MIC range. This, however, was not observed when a chemically-defined medium was used to determine the MIC of pyrithione against *Kl. pneumoniae*.

This concentration dependent reduced-enhanced activity (paradoxical effect) of some antibacterial agents, also sometimes known as the 'Eagle Effect' (Eagle and Musselman, 1948; Eagle, 1951) has been reported mainly with two classes of compounds: chelating agents and β-lactam antibiotics. Feeney et al (1957) reported that in agar diffusion assays of chelating agents as oxine and 5-methyl-1,10-phenanthroline against *Micrococcus pyogenes*, concentric zones (rings) of growth and inhibition were observed around filter
paper discs saturated with the chelating agent. Similar zones were observed around filter paper discs saturated with metal ions such as copper, iron or cobalt placed on agar medium containing the chelating agent. Feeney et al (1957) suggested that various metal-chelate complexes with different ratios of metal to ligand formed as either the chelating agent diffused through the agar containing metal ions, or as the metal ion diffused through an agar medium containing the chelating agent. Hence a ring of no growth would be obtained in areas where the particular mixture was inhibitory, and a ring of growth obtained in areas where a different mixture was formed which was not inhibitory.

A similar explanation has been offered for the paradoxical activity of oxine against S. aureus. A 1:1 oxine:Fe complex is toxic to S. aureus whereas a 2:1 oxine:Fe complex is not (Albert et al, 1947; Rubbo et al, 1950; Albert et al, 1953). Hence too high or too low concentrations of oxine, in relation to iron concentration, would fail to inhibit the growth of S. aureus which can only be achieved if the ratio of ligand to iron is 1:1. Albert et al (1956) reported similar results with pyrithione against S. aureus. This suggests that in order for pyrithione to exhibit its antibacterial activity it has to form a 1:1 complex with iron. The mode of action of pyrithione would thus be identical to oxine (Albert, 1985).

Although such a selective and concentration dependent activity of pyrithione has been observed with some Gram-negative bacteria, it was not detected 'in this study' with any of the Gram-positive strains, including S. aureus. However, the activity of pyrithione against S. aureus as reported by Albert et al (1956) was observed using a simple system in which the medium used was glass-distilled water to which pyrithione, iron and bacterial cells were added, whereas, in the present study, the assessment of the activity of pyrithione against S. aureus was carried out in a complex medium (nutrient broth). This highlights the role of the medium in the activity of pyrithione.

Furthermore, the fact that this type of activity is exhibited by pyrithione against some, but not other, micro-organisms lends support to the possibility that pyrithione acts in
different ways against different micro-organisms. Indeed, Husseini and Stretton (1980) reported that the chelating agent phanquone (4,7-phenanthroline-5,1-quinone) showed concentration dependent, reduced/enhanced activity against *S. aureus* but not *E. coli*. This lead to the conclusion that phanquone, although acting mainly through chelation, acted against *S. aureus* through the formation of a lethal 1:1 complex with iron. Against *E. coli*, however, its action would depend on chelating (other) metal ions that are essential for the activity of metal-containing systems (e.g. metallo-enzymes) within the cell. Hence, phanquone may have a similar mode of action to that of oxine (Husseini and Stretton, 1980). It is, therefore, reasonable to suggest that pyrithione may have at least two modes of action, where against organisms like *Kl. pneumoniae*, *Salm. abony*, *S. marcescens* and *P. vulgaris*, the ratio of pyrithione to some metal species in the medium is of significance, but not against other organisms in this study.

The concentration dependent effect discussed above is not limited to chelating agents. β-lactam antibiotics have recently been shown to produce such effect which may have clinical implications. Goldstein and Rosdahl (1981) reported the banded zone phenomenon around ampicillin discs tested against 76 strains of *E. coli*. Similar results were obtained with agar diffusion assays of imipenem against *S. haemolyticus* (Traub, Kohl and Sphor, 1988). Ikeda *et al* (1987), using both broth dilution and disc diffusion assays, reported that cefmenoxime, a broad spectrum cephalosporin, exhibited paradoxical activity against *P. vulgaris*. Compared to other cephalosporins that did not behave similarly, cefmenoxime at high concentrations induced higher levels of β-lactamase to which it is highly sensitive (Ikeda and Nishino, 1988). Hence, cefmenoxime shows diminished activity at high concentrations against *P. vulgaris*. In an experimental infection model in mice using *P. vulgaris*, this phenomenon was also demonstrated (Ikeda *et al*, 1990). Such findings may have implications in the chemotherapy of infections where attention has to be paid so that drug concentration at the site of infection does not approach a level high enough that could elicit such a response. The reduction of the *in vitro* activity of high
concentrations of penicillin against *Enterococcus faecalis* was, however, attributed to the reduced activity of autolytic enzymes at higher drug concentrations (Fontana *et al.*, 1990). Whatever the reasons for the paradoxical activity of some antimicrobial agents, it is certainly a phenomenon that occurs with a wide range of bacteria including Gram-positive and Gram-negative strains.

The MIC's of four compounds, structurally related to pyrithione, against *Kl. pneumoniae*, *B. licheniformis* and *C. albicans* are shown in Table 3.3. The structures of these compounds are shown in Figure 4.1. It is clear that none of these compounds showed any detectable activity against *Kl. pneumoniae* (MIC >100 μg/ml; Table 3.3.). Whilst neither MP nor MPY are able to chelate metal ions, both HMP and MNA are able to form 5- and 6-membered chelate rings, respectively. Although chelation is believed to be essential for the antimicrobial activity of pyrithione (*Albert et al.*, 1956), our results suggest that pyrithione's activity against *Kl. pneumoniae* may not be solely due to this feature. This is indeed substantiated by the finding that all four compounds inhibit the growth of *B. licheniformis* (Table 3.3.) albeit to a lesser extent than that achieved by pyrithione under similar conditions (Table 3.2.). Furthermore, MP, MPY and HMP all inhibited the growth of *C. albicans* similarly (MIC 12.5 μg/ml; Table 3.3.) but still to a lesser extent than pyrithione under similar conditions (Table 3.2.). MNA, although able to chelate metal ions, showed no apparent inhibition of the growth of *C. albicans* (MIC >100 μg/ml; Table 3.3.). Hence, although chelation may play a significant role in the antimicrobial activity of pyrithione, other modes of action cannot be excluded. Further work was therefore undertaken to try to understand the nature of the interaction between pyrithione and *Kl. pneumoniae*, with *B. licheniformis* and *C. albicans* included for comparison.
Figure 4.1:  
(a) 2-mercapto pyridine  
(b) 2-mercapto pyrimidine  
(c) 2-mercaptonicotinic acid  
(d) 3-hydroxy-2-mercapto pyridine
Two different growth patterns were observed in the presence of pyrithione. Bacterial cells of *Kl. pneumoniae* (Figure 3.1) and *B. licheniformis* (Figure 3.2) suspended in the chemically-defined medium containing pyrithione at various concentrations exhibited growth curves with distinct, extended lag phase, the extent of which was dependent on pyrithione concentration. This was followed by resumption of growth at a rate similar to that of control (untreated) cells. Similar growth curves have been reported for cultures of *Salmonella typhimurium* treated with hydrogen peroxide (Watson and Schubert, 1969). This was explained on the basis of the short term instability of peroxides and the ability of *S. typhimurium* to breakdown H$_2$O$_2$ with the enzyme catalase. Although there have been reports of its photodegradation on exposure to ultra-violet light (Neihof et al., 1979), and loss of toxicity over a 4 hour period in activated sludge (Hyde and Nelson, 1984), solutions of pyrithione are still active after three months at 40°C (Nelson and Hyde, 1981). Thus, such an instability seems unlikely for pyrithione within the experimental times in this study. Indeed, compound instability is further excluded as a cause of the growth pattern observed with bacterial cultures in the presence of pyrithione by observations of the different growth patterns exhibited by cells of *C. albicans* (Figure 3.3) growing in the presence of pyrithione in the same medium under similar conditions to those used for bacteria. The overall effectiveness of pyrithione against *C. albicans* did not deviate markedly over the same time period (cf. Figure 3.3 and Figs. 3.1 and 3.2). Such yeast cells showed a drug-dependent fall in growth rate without extended lag.

Bacterial cells are, therefore, able to survive pyrithione challenge, at low drug concentrations, and re-establish exponential growth at an uninhibited rate. Furthermore, cells of *Kl. pneumoniae* and *B. licheniformis* that have survived pyrithione treatment are certainly more resistant to its effects on re-challenge than cultures of similar cell density grown in its absence (cf. Figs. 3.4 and 3.5 with 3.7 and 3.8, respectively). Again, *C.albicans* cells responded differently and rechallenging growing cultures with fresh pyrithione induced, in general, an additional fall in growth rate (Figure 3.6). Adding
pyrithione in mid-exponential phase to a previously untreated yeast culture inhibited growth (Figure 3.9). Only a slight loss of sensitivity was apparent with both bacteria and yeast in these higher density cultures (cf. Figs 3.7, 3.8 and 3.9 with Figs 3.1, 3.2 and 3.3). The overall pyrithione-cell ratio thus appears of slight significance and results indicate a shift in bacterial resistance during growth in the presence of the drug.

Bacterial cultures are, therefore, able to 'adapt' rapidly to the presence of pyrithione whilst yeast cultures remain sensitive to the drug at the drug levels employed in the experiment. This lends further support to the suggestion that pyrithione may have more than one mode of action against micro-organisms as discussed above. Bacterial adaptation or resistance to pyrithione may be considered in the light of two alternatives:

1. induced changes in the majority of cells, or
2. selection of resistant cells within an initially heterogeneous population (McCarthy and Hinshelwood, 1958). Thus, bacterial cells are either adapting to the presence of pyrithione, perhaps by creating a by-pass for an essential metabolic step, or that the growth curves are reflecting the overgrowth of a small, inherent population of cells initially resistant to pyrithione and whose presence finally dominates the culture.

Partially inhibited cultures have been studied by Rye and Wiseman (1968) who investigated the effects of combinations of ampicillin and other antimicrobial agents. Culture absorbance (assuming no lysis) may then follow equation of the type:

\[ A_t = A_o e^{\mu t} + A'_o \]

in which \( A_o \) is the initial absorbance of uninhibited cells, \( A'_o \) is the absorbance of non-growing cells and \( A_t \) the absorbance of the overall culture at time \( t \); \( \mu \) is the exponential growth rate constant. It can be shown that cultures in which more than 99% of the population are unable to grow and in which the remaining cells grow at an uninhibited rate, would give rise to curves similar to those shown in Figures 3.1 and 3.2.

In order to assess whether pyrithione caused growth inhibition of the whole of the bacterial population of *Kl. pneumoniae* during the initial lag phase or whether it inhibited
the growth of part of the population whilst allowing the uninhibited growth of an inherently resistant fraction, viability studies were undertaken using *Kl. pneumoniae* and *C. albicans* for comparison of the two distinctly different growth patterns. Figure 3.10 shows the effect of pyrithione on the optical density (a) and viability (b) of growing cultures of *Kl. pneumoniae*. The sets of curves for viability (Figure 3.10b) and optical density (Figure 3.10a) are essentially similar. Both show, in the presence of pyrithione, a lag phase during which optical density and viable count remain virtually constant for a time dependent on pyrithione concentration. This is followed by a phase during which both optical density and viability increase at a rate similar to that of the untreated cells. These results suggest that pyrithione inhibits the growth of the whole population of *Kl. pneumoniae* for the duration of the lag phase, and that the re-establishment of growth is due to the adaptation of the whole population rather than the overgrowth of a fraction of the population initially resistant to pyrithione.

In comparison, Figure 3.11 shows the effect of pyrithione on the optical density (a) and viability (b) of growing culture of *C. albicans*. The reduction in the overall growth rate and the absence of a lag phase in cultures of *C. albicans* as measured by the optical density (Figure 3.11a) is mirror-imaged in viable counts (Figure 3.11b). Hence, at sub-inhibitory levels, pyrithione does not cause complete inhibition of either growth or division of the yeast culture, but partial inhibition. These results support the assumption that pyrithione may, therefore, act through different mechanisms against bacteria and yeast.

Further studies on the behaviour of a bacterial population of *Kl. pneumoniae* in the presence of pyrithione were conducted using a method based on that devised by Rye and Wiseman (1967, 1968). At low sub-inhibitory levels of ampicillin, bacterial cells are able to grow (increase in mass) logarithmically without dividing. Thus, under these conditions cells undergo an increase in size (filamentation) and optical density (mass), but the viable counts remain virtually unchanged for the duration of the incubation. By measuring the cell size distribution of bacterial cells such as *E. coli* in the presence of
sufficient ampicillin to suppress cellular division plus an antibacterial agent at various concentrations, it is possible to monitor population behaviour and relate to the presence of the antibacterial agent (Rye and Wiseman, 1968).

Hence cells incubated in the presence of ampicillin alone would exhibit a unimodal size distribution with time-dependent shift in the mean size. An antibacterial compound with a uniform action against all cells would thus be expected to cause a time-dependent shift in the mean size of division-less cells. This was observed with cultures of *E. coli* treated with phenol and tetracycline (Rye and Wiseman, 1968). Similar responses were obtained with isopropyl or benzyl alcohols, and with *Ps. aeruginosa* (carbenicillin-treated) in the presence of gentamycin (Wiseman, 1974). Under similar experimental conditions, phenylmercuric acetate caused a gradual widening of the cell size distribution and the continual but slow decrease in the number of cells in the lower size ranges, suggesting that all the cells are growing but at widely different rates (Rye and Wiseman, 1968). Thus, phenylmercuric acetate appears to inhibit the individual cells in a culture to different extents either by causing a non-uniform decrease in their rates of growth, or by imposing a lag period on the cells the duration of which varies from cell to cells (Rye and Wiseman, 1968).

A third possible mechanism of growth inhibition was illustrated by the action of membrane active agents such as cetyltrimethylammonium bromide (Rye and Wiseman, 1968), decyl- and dodecyltrimethylammonium bromides, benzalkonium chloride, chlorhexidine and polymyxin B against *E. coli* (Wiseman, 1974). In these cases, a bimodal distribution of cell sizes developed in the presence of the antibacterial agent showing the presence of two types of cells. The smaller cells which retain the size of untreated cells and were not growing, and the larger cells that appeared to be growing at an uninhibited rate (Rye and Wiseman, 1968). Hence the partial inhibition of growth caused by these agents resulted from completely arresting the growth of some of the cells whilst allowing the remainder to grow at an uninhibited rate (Wiseman, 1974).

In the present study, preliminary studies showed that the MIC of ampicillin against
*Kl. pneumoniae* was higher than 100 µg/ml (Table 3.4). However, cells grew at an apparently uninhibited rate in CDM in the presence of 50 µg/ml of ampicillin and under these conditions, a complete suppression of cell division was achieved as confirmed by both (light) microscopy and viable counts. Filamentous growth was observed and the viable count of such cultures remained virtually unchanged over a period of three hours; no lysis was observed.

Figure 3.12 shows the size distribution of growing cells of *Kl. pneumoniae* (CDM, 37°C, 90 rpm) at 0.0 and 120 min. times of incubation. Cells show a unimodal distribution with a mean size 1.23 µm³. An increase in mean size from 1.23 to 1.96 µm³ (Figure 3.13) resulted from the inhibition of cell division during incubation of the cells in the presence of ampicillin. The effects of pyrithione on (ampicillin induced) division-inhibited cells of *Kl. pneumoniae* are shown in Figure 3.14. During the initial lag phase (0.0 - 120 min.) caused by pyrithione (2 µg/ml), the cells give rise to a unimodal distribution similar to that of control (untreated) cells (Figure 3.12), with a mean size of 1.23 µm³. This suggests that all the cells are unable to grow during this phase, and thus the action of pyrithione is exerted on the whole of the population of individual cells. Further incubation (up to 480 min.) of the cells allows growth to be re-established and this phase is characterised by the appearance of a bimodal size distribution (Figure 3.14) with a shift to a unimodal population with a mean size of 2.47 µm³. It can be concluded, therefore, that the effects of low concentrations of pyrithione are short term affecting the whole population of bacterial cells, and resulting in an initial lag phase. Prolonged incubation, thus, allows the recovery of all previously inhibited cells, and that the growth and viability profiles of *Kl. pneumoniae* in the presence of pyrithione are not due to the overgrowth of a small unaffected population of initially resistant cells.

The effects of oxine on microbial growth were examined for comparison with pyrithione. Oxine, like pyrithione, showed strong activity against *B. licheniformis* and *C. albicans* (MIC's 0.625 and 0.15 µg/ml, respectively; Table 3.6), but was less active.
against *Kl. pneumoniae* (MIC 50μg/ml; Table 3.6). Oxine also showed two distinctly different effects on microbial growth patterns. Against *Kl. pneumoniae* (Figure 3.27) and *B. licheniformis* (Figure 3.28), oxine induced an initial lag phase, the length of which was proportional to drug concentration. This was followed by exponential growth at a rate similar to that of control (untreated) cells. Like pyrithione, oxine induced an overall reduction in the growth rate of *C. albicans* (Figure 3.29), the extent of which was proportional to drug concentration. No lag phase was observed. The growth curves of both bacteria (Figs. 3.27 and 3.28) also suggest that *Kl. pneumoniae* and *B. licheniformis* are able to rapidly overcome the action of oxine and re-establish exponential growth.

Hence, pyrithione and oxine induce similar responses in the growth patterns of the micro-organisms tested, perhaps lending support to the assumption that these two compounds have similar modes of action (Albert *et al.*, 1956).

As has been demonstrated so far, cells of *Kl. pneumoniae* are able to overcome the growth inhibitory effects of sub-inhibitory concentrations of pyrithione, and resume exponential growth. This is achieved, apparently, through the adaptation of the whole of the population rather than the selection of a sub-population that is inherently resistant to pyrithione. This is further supported by observations on the stability of this, essentially, pyrithione-induced resistance. Cells of *Kl. pneumoniae* that have been grown in the presence of pyrithione (5μg/ml) for 24 hours show a much reduced lag phase (approximately 60 minutes) upon fresh challenge with pyrithione (at 1, 2, 3, 4 and 5μg/ml) in CDM (Figure 3.39). The length of the lag phase is, under these conditions, irrespective of the concentration of pyrithione and is followed by exponential growth. The MIC of pyrithione against pyrithione-trained (24 hours; 5μg/ml) cells of *Kl. pneumoniae* is 40μg/ml (Table 3.7). Daily subculture of such cells in pyrithione (5μg/ml)-containing CDM over a period of 7 days showed that the MIC value remained at 40μg/ml (Table 3.7; culture B). However, the MIC of pyrithione against the same pyrithione-trained cells,
dropped from 40µg/ml to 20µg/ml after 4 daily subcultures in pyrithione-free CDM (Table 3.7; culture C). The MIC of pyrithione against control cells, subcultured daily in drug-free medium, remained at 5.0µg/ml (Table 3.7; culture A).

The rapidity with which cells of *Kl. pneumoniae* adapt to the presence of pyrithione, and the relative ease with which such resistance is lost on growth in the absence of the drug, suggests that this pattern of behaviour consists of relatively unstable physiological adaptation of the cells to resist the action of the drug; similar observations on the adaptation of *Aerobacter aerogenes* to barbitone (Dean and Moss, 1967), and of *Bact. lactis aerogenes* to 2,4-dinitrophenol (McCarthy and Hinshelwood, 1958) have been reported allowing similar interpretations.

Of particular interest, and perhaps relevance to the possible mechanism of *Kl. pneumoniae* to pyrithione, are the results on the cross-resistance of pyrithione-trained cells of *Kl. pneumoniae* to other antimicrobial agents (Figure 3.40). Pyrithione-trained cells of *Kl. pneumoniae* are significantly more resistant to chloramphenicol (Figure 3.40a) than control cells (Figure 3.40b), as is clearly shown by comparing the sizes of the two respective zones of inhibition around the chloramphenicol disc (cf. Figs. 3.40a and b). This was further confirmed by MIC determinations where pyrithione had an MIC of 40µg/ml against pyrithione-trained cells of *Kl. pneumoniae* (compared to 5µg/ml against control cells), and chloramphenicol had an MIC of 100µg/ml against pyrithione-trained cells (compared to 12.5µg/ml against control cells; Table 3.8). Pyrithione-trained cells of *Kl. pneumoniae*, interestingly, showed no apparent cross-resistance to any of the other antimicrobial agents tested (cf. Figs. 3.40a and b).

To examine the relationship between the resistance of pyrithione-trained cells of *Kl. pneumoniae* to pyrithione and chloramphenicol, consideration of the commonly known mechanisms of bacterial resistance to chloramphenicol is essential. Bacterial
resistance to chloramphenicol may be achieved by plasmid-encoded enzyme, chloramphenicol acetyl transferase (CAT) that catalyses acetylation of chloramphenicol with acetyl coenzyme A, the product of which is non-inhibitory to ribosomal protein synthesis (Sands and Shaw, 1973; Martinez-Suarez et al., 1985; Roberts et al., 1985; Powell et al., 1989). Although CAT-mediated chloramphenicol resistance is the most frequently reported mechanism in Gram-negative bacteria (Burns et al., 1985), three other mechanisms have been reported. The first involves a permeability barrier, possibly through the lack of one or more of the outer-membrane proteins (Burns et al., 1985, 1986, 1989). The second mechanism involves the reduction of the nitro (–NO₂) group, essential for antimicrobial action in chloramphenicol (Smith and Worrel, 1953; Merkel and Steers, 1953; Herman and Steers, 1953; O'Brien and Morris, 1971). Thirdly, mutants of Bacillus subtilis have been isolated that have decreased ribosomal sensitivity to chloramphenicol (Osawa et al., 1973).

In trying to understand how pyrithione-resistant cells of Kl. pneumoniae become more resistant to chloramphenicol, it is reasonable to suggest that the mechanism of resistance (adaptation) in this case is: (1) inducible over a short period of time, (2) unstable and may be lost on successive incubations in pyrithione-free medium and (3) involves a physiological adaptation rather than a specific genetic alteration. These are the characteristics so far concluded for the adaptation of Kl. pneumoniae to pyrithione and consequently may apply to the observed adaptation to chloramphenicol.

Chloramphenicol resistance mediated by CAT, reduced ribosomal sensitivity or reduced permeability, result from genetic alterations (acquisition of genetic material such as the gene coding for CAT, or mutations in the other two cases), that occur at low frequency and are stable. On the other hand, the ability of E. coli to reduce the nitro group in chloramphenicol, and thus become resistant to the drug, is achieved through training of the cells by exposure to gradually increasing concentrations of chloramphenicol (Merkel and Steers, 1953). In addition, such cells lose their resistance rapidly when
subcultured into chloramphenicol-free medium (Herrmann and Steers, 1953).

Furthermore, cells adapted to chloramphenicol in this manner, are able to reduce a wide range of nitro-aryl compounds, some of which are derivatives of chloramphenicol (Smith and Worrel, 1953), whilst others are not (O'Brien and Morris, 1971). Egami et al. (1951) reported that inorganic nitrite interferes with the enzymatic reduction of chloramphenicol (and vice versa) by bacterial cell-free extracts of Streptococcus haemolyticus. They indicated that chloramphenicol and nitrite were probably reduced by the same enzyme system, although Saz and Slie (1954), working with E. coli extracts, concluded that the two enzyme systems were different.

Since the N-oxide group is essential for the antimicrobial activity of pyrithione, it is possible that the adaptation of Kl. pneumoniae is mediated by an inducible enzyme system(s) able to reduce the N-oxide group and thus account for the rapid recovery of the cells. The same system(s) may be able to reduce the nitro group of chloramphenicol, hence resulting in the observed cross-resistance. In this context, the length of the lag phase observed with cultures of Kl. pneumoniae in the presence of pyrithione (Figure 3.1) would reflect the time required to reduce the concentration of active pyrithione below inhibitory levels, which would then allow the re-establishment of exponential growth.

Although cells of B. licheniformis exhibit a similar growth pattern in the presence of pyrithione (Figure 3.2) to that of Kl. pneumoniae (Figure 3.1), pyrithione-trained cells of B. licheniformis do not show any apparent cross-resistance to any of the antimicrobial agents tested, including chloramphenicol (cf. Figs. 3.4a and b). The mechanism of resistance (adaptation) of B. licheniformis to pyrithione does not, therefore, result in cross-resistance to any of the antimicrobial agents tested.

Pyrithione-trained cells of Kl. pneumoniae are as sensitive to oxine as control cells (Table 3.8). Oxine has an MIC of 40 μg/ml against both types of cells. Although it has been suggested that oxine has a similar mode of action to pyrithione (Albert et al., 1956), pyrithione differs structurally from oxine. If the mechanism of resistance (adaptation) to
pyrithione involves the reduction of the N-oxide group in the molecule, as has been suggested above, then it is unlikely to result in resistance to oxine, since the latter does not have a similar functional group that might be reduced via the same enzyme system(s). Indeed, the inability of pyrithione-trained cells of *Kl. pneumoniae* to exhibit increased resistance to oxine lends further support to the possibility that such resistance to pyrithione results from the modification of the drug rather than a possible alteration of the site(s) of action.

Analysis and comparison of fatty acid profiles of both control and pyrithione-treated cells of *Kl. pneumoniae* showed, essentially, no differences (Table 3.9). The main fatty acids in both cells were myristic, palmitic, stearic and arachidic acids. Variations in fatty acids profiles have been associated with the adaptation of bacteria to antimicrobial agents such as in the case of adaptation of *Ps. aeuginosa* to polymyxin B (Conrad and Galanos, 1989), were such alterations result in reduced permeability of the cell envelope and hence exclusion of the drug. In the case of pyrithione initial analysis showed no significant alterations in fatty acid profiles between pyrithione-sensitive and pyrithione-treated cells of *Kl. pneumoniae*. That the adaptation of *Kl. pneumoniae* to pyrithione could be due to structural alterations in the cell envelope leading to reduced uptake of the drug is, therefore, unlikely. The adaptation of *Kl. pneumoniae* to pyrithione is most likely the result of an inducible biochemical event rather than any major structural change.

The effects of pyrithione on both bacteria and yeast were antagonised by the chelating agent EDTA (Figure 3.15). The antagonism was most detectable with *B. licheniformis* (Figure 3.15b) and *C. albicans* (Figure 3.15c), and less so with *Kl. pneumoniae* (Figure 3.15a). Further confirmation of this antagonism was demonstrated in growth studies in which EDTA shortened the pyrithione-induced lag phase in bacterial cultures of *Kl. pneumoniae* (Figure 3.16) and *B. licheniformis* (Figure 3.17), and increased the growth rate of pyrithione-treated cells of *C. albicans* (Figure 3.18). EDTA itself had no
apparent inhibitory action against either *Kl. pneumoniae* or *B. licheniformis* (MIC > 200\(\mu\)g/ml; Table 3.5), but cells of *C. albicans* were inhibited by EDTA (MIC 50\(\mu\)g/ml; Table 3.5).

Although EDTA exhibits strong antibacterial (lytic) activity against *Pseudomonas* species (Eagon and Carson, 1964), its main antimicrobial value and use has been the potentiation of the action of other antimicrobial agents (Hart, 1984), through chelation of Mg\(^{2+}\) and Ca\(^{2+}\) ions normally responsible for outer-membrane stability in Gram-negative organisms (Denyer, 1985). However, Neu and Winshell (1970) reported that EDTA did not enhance the activity of ampicillin, chloramphenicol or tetracycline in strains of *Enterobacteriaceae*. In the case of pyrithione, EDTA positively antagonised the actions of pyrithione against both bacteria and yeast (Figures 3.15, 3.16, 3.17 and 3.18). This suggests a significant role for divalent cations present in the medium in the action of pyrithione (Albert, 1956) and for which pyrithione and EDTA could compete. This is to some extent supported by observations of the growth of some Gram-negative bacteria such as *Kl. pneumoniae*, *Salm. abony*, *S. marcescens* and *P. vulgaris* in the presence of 'high' pyrithione concentrations that should have been inhibitory (section 3.1.), perhaps due to variation in the pyrithione/cation ratio as discussed above. The strong antagonism between EDTA and pyrithione would, therefore, limit the possibility of using both these agents, in combination, for preservation of industrial products or for therapy such as in anti-dandruff formulations.

The possible effects of some metal ions on the antimicrobial activity of pyrithione were tested in a similar manner as with EDTA using the agar-diffusion method (Maccacaro, 1961). A general observation on the results of these experiments is that none of the five metal ions tested (*Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+}*, and Ca\(^{2+}\)) antagonised the action of pyrithione against any of the three micro-organisms tested (*Kl. pneumoniae*, *B. licheniformis* and *C. albicans*) in either nutrient agar or CDMA (Figs. 3.19 to 3.24). Using the strip method (Maccacaro, 1961), Husseini and Stretton (1980) showed
antagonism between each of Co^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Zn^{2+} and phanquone against *E. coli* as well as antagonism between each of Co^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Ni^{2+} and phanquone against *S. aureus*. Metal ions, therefore, probably rendered phanquone inactive either by filling its complexing sites, hence becoming unable to complex with metals in bacterial metal-containing systems (e.g. metallo-enzymes), and/or by rendering phanquone impermeable through the bacterial cell wall or membrane, thus becoming unable to reach its site of action (Husseini, 1978; Husseini and Stretton, 1980).

The lack of antagonism between pyrithione and the metal ions in this study, however, does not necessarily lead to the conclusion that metal ions play no essential role in the activity of pyrithione, since EDTA antagonises the action of pyrithione and the paradoxical activity of pyrithione against some gram-negative bacteria is consistent with observations with other antimicrobial chelators (Albert *et al.*, 1953; Feeney *et al.*, 1957; Husseini and Stretton, 1980). It must be emphasized that the strip method is a crude method to measure possible interactions between various substances against micro-organisms. And whilst clear results are obtained between pyrithione and EDTA, the interaction between pyrithione and metal ions is, undoubtedly, influenced by the presence of a variety of metal ions in the medium itself.

The influence of the medium composition on the interaction of pyrithione with various metal ions is clearly illustrated by comparing the results obtained using nutrient agar with those using CDMA. Whilst only Zn^{2+} (Figure 3.19a) enhanced the activity of pyrithione against *Kl. pneumoniae* in nutrient agar, both Zn^{2+} (Figure 3.22a) and Fe^{3+} (Figure 3.22e) enhanced the activity of pyrithione against the same organism in CDMA. Both Mg^{2+} (Figure 3.20b) and Ca^{2+} (Figure 3.20d) slightly enhanced the activity of pyrithione against *B. licheniformis* in nutrient agar but not in CDMA (Mg^{2+}:Figure 3.23b; Ca^{2+}:Figure 3.23d). Fe^{3+} slightly enhanced the action of pyrithione against *B. licheniformis* in both nutrient agar (Figure 3.20e) and CDMA (Figure 3.23e), whilst
Zn$^{2+}$ and Mn$^{2+}$ showed some potentiation of pyrithione's action in COMA (Figs. 3.23a and c, respectively) but not in nutrient agar (Figs. 3.20a and c, respectively).

Furthermore, only Zn$^{2+}$ (Figure 3.24a) enhanced the action of pyrithione against *C. albicans* in COMA, whereas pyrithione's activity was enhanced by all of the metal ions tested: Zn$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Fe$^{3+}$ (Figs. 3.21a, b, c, d and e, respectively) against *C. albicans* in nutrient agar.

The results highlight the non-specific nature of chelation (Dwyer and Mellor, 1964), in that a given chelating agent will combine with metal ions in general. In a metal-rich environment, such as growth media, a variety of chelates may be formed, and some of these chelates in the case of pyrithione, do enhance its antimicrobial activity as seen in Figures 3.19 to 3.24. The role of Fe$^{3+}$ in the antimicrobial activity of pyrithione (Albert et al., 1956) is not, therefore, necessarily specific or peculiar to this metal ion, since the co-operative effect between Fe$^{3+}$ and pyrithione was demonstrated in a medium (theoretically) devoid of any other metal ions (glass-distilled water). It is reasonable to suggest that through chelation of metal ions in the medium, pyrithione permeates the cell as a chelate and exerts its toxic action within the cell. The potentiation of pyrithione by some metal ions may, therefore, reflect the improved permeability of the microbial cell to such complexes compared to others.

The morphology of *C. albicans* was more obviously affected by pyrithione. Control cells of *C. albicans* were oval-shaped with smooth appearance (Figure 3.44a).

Incubation in the presence of pyrithione (CDM, 37°C, 24hr), caused some structural damage characterised by the collapse of cells accompanied with obvious damage to the cell exterior and leakage of cytoplasmic material. These effects were more apparent at higher concentrations of the drug (0.025, 0.05 and 0.1μg/ml; Figs. 3.44b, c and d, respectively). Pyrithione may, therefore, cause structural damage to the yeast cell envelope, particularly since Chandler and Segel (1978) suggested that pyrithione's site of action in fungi is the cytoplasmic membrane. Short term incubation of bacteria with levels
of pyrithione equal to or greater than the MIC did give rise to filaments with *Kl. pneumoniae* (cf. Figs 3.42a with 3.42b, c and d), though this was not observed with *B. licheniformis* (Figs 3.43a, b, c and d). Since no seen evidence of structural damage was detected, it is likely that pyrithione exerts its antibacterial effect by more subtle influences on cellular biochemistry.

Some evidence was obtained, during this study, as to the likely site of antibacterial activity of pyrithione. The effects of pyrithione on the synthesis of DNA, RNA and proteins were assessed by following the incorporation of \(^{14}\text{C}\)-thymidine, \(^{14}\text{C}\)-uridine and \(^{14}\text{C}\)-glycine, respectively, in cells of *Kl. pneumoniae* in the absence and presence of the drug. Short term studies (up to 30 minutes) showed that pyrithione blocked further incorporation of glycine (Figure 3.30), thymidine (Figure 3.32) and uridine (Figure 3.34) in *Kl. pneumoniae* within 2-3 minutes of the addition of the drug at concentrations (1, 2.5 and 5\(\mu\)g/ml) that are growth-inhibitory (Figure 3.1). It appeared, therefore, that concentrations of pyrithione that induce a lag phase also inhibit the uptake and/or incorporation of glycine, thymidine and uridine in proteins, DNA and RNA, respectively. However, since pyrithione-treated cells of *Kl. pneumoniae* are able to re-establish growth at rates similar to that of untreated cells (Figure 3.1), it was essential to monitor the incorporation of radiolabelled substrates by *Kl. pneumoniae* once the cells have re-established growth in the presence of pyrithione.

Prolonged incubation (300-400 min.) of *Kl. pneumoniae* in the presence of 2.5\(\mu\)g/ml of pyrithione allows the re-establishment of exponential growth (Figure 3.1). Under these conditions, the incorporation of \(^{14}\text{C}\)-thymidine remained inhibited (Figure 3.33), and the incorporation of \(^{14}\text{C}\)-glycine resumed but at a reduced rate compared with control (untreated) cells (Figure 3.31). Interestingly, the incorporation of \(^{14}\text{C}\)-uridine resumed (as the cells started to grow after the initial lag phase) at a rate similar to that of control (untreated) cells (Figure 3.35).

Similar irreversible inhibition of thymidine uptake by pyrithione has been reported for human leukaemic cells (Kontoghioghes *et al.*, 1986; Forsbeck *et al.*, 1987), and
interpreted as indicating a direct effect on DNA synthesis. However, in the case of \textit{Kl. pneumoniae}, culture survival does not seem affected by this inhibition since the onset of the exponential phase of growth occurs at a time when inhibition of thymidine incorporation is still demonstrable (cf. Figs 3.1 and 3.33). The explanation for this may be found by examining the pathway of the incorporation of external thymidine in the DNA of the cell. Before being incorporated into bacterial DNA, external thymidine has to undergo phosphorylation to yield thymidine triphosphate (TTP) by the so-called 'salvage' pathway (Adams \textit{et al}, 1981):

\[
\text{Thymidine} \rightarrow \text{dTMP} \rightarrow \text{dTDP} \rightarrow \text{dTTP},
\]

where dTMP, dTDP and dTTP are deoxythymidine monophosphate, -diphosphate and -triphosphate, respectively. The salvage pathway exists in bacteria (Nelson and Carter, 1969; Black and Hruby, 1991), yeast (Jong and Campbell, 1984) and mammalian cells (Grav and Smellie, 1963; 1965). The first step in this pathway, the production of dTMP from thymidine and ATP, is catalysed by the enzyme thymidine kinase (Black and Hruby, 1991). The sequential production of dTDP and dTTP is catalysed by thymidylate kinase (Nelson and Carter, 1969). Both enzymes require Mg\textsuperscript{2+} as a cofactor, although it could be replaced partially by either Mn\textsuperscript{2+} or Co\textsuperscript{2+} in the case of thymidylate kinase (Nelson and Carter, 1969). It is possible that pyrithione, through chelation of Mg\textsuperscript{2+}, could inhibit either or both enzymes and thus completely block the incorporation of \textsuperscript{14}C-thymidine in \textit{Kl. pneumoniae}.

Such inhibition, however, was not detrimental to the survival of cells of \textit{Kl. pneumoniae} since bacteria are able to produce dTTP, essential for DNA biosynthesis through the following steps: (1) reduction of uridine diphosphate (UDP) to deoxyuridine diphosphate (dUDP) by the enzyme ribonucleotide reductase, (2) phosphorylation of dUDP to deoxyuridine triphosphate (dUTP) followed by rapid hydrolysis by the enzyme dUTPase to yield dUMP, (3) methylation of dUMP to dTMP by thymidylate synthetase, and finally (4) phosphorylation of dTMP to produce dTTP, which is then incorporated into
DNA (Adams et al., 1981). Whilst the enzyme thymidylate kinase catalyses the essential metabolic step dTMP → dTTP, the phosphorylation of thymidine to give dTMP is probably not an essential step for the survival of bacterial cells. This is supported by the isolation of a mutant of *E. coli* (strain KY608), deficient in thymidine kinase activity, that is able to grow normally in general medium (Higara et al., 1967; Igarashi et al., 1967; Black and Hruby, 1991).

The irreversible inhibition by pyrithione of the incorporation of extracellular 14C-thymidine in *Kl. pneumoniae* (Figure 3.33) during the initial lag phase and the subsequent phase characterised by normal growth and division (cf. Figs. 3.33 and 3.1), could be the result of an irreversible inhibition of the enzyme thymidine kinase, perhaps through chelation of the cofactor Mg2+. Any possible inhibition of thymidylate kinase would have to be reversible in order for the cells to recover from the growth inhibitory effects of pyrithione.

Just as the inhibition of thymidine incorporation into cells of *Kl. pneumoniae* could not account for the growth inhibition by pyrithione, so could not the partial inhibition of 14C-glycine incorporation by pyrithione (Figs. 3.30 and 3.31). Short term experiments (up to 30 min.) showed complete inhibition of glycine incorporation by pyrithione (Figure 3.30). However, glycine incorporation continued at a reduced rate (compared to untreated cells) throughout the initial lag phase and the subsequent exponential phase of growth once the cells have recovered from the effects of pyrithione (cf. Figs. 3.1 and 3.31). The inhibition of glycine uptake could be due to an inhibition of a membrane transport mechanism similar to that shown in yeast in the presence of pyrithione (Chandler and Segel, 1978). Such an inhibition is both partial and long term (persisting after the recovery and resumption of growth) in *Kl. pneumoniae*, but could not account for the growth inhibitory action of pyrithione, since, as mentioned above, cells are able to grow and divide at a rate similar to that of untreated cells, whilst the inhibition of glycine incorporation is still demonstrable (cf. 3.1 and 3.31).
The inhibition of $^{14}$C-uridine incorporation in the RNA of *Kl. pneumoniae* by pyrithione is, probably, the most interesting and relevant to the possible mechanism(s) of growth inhibition. Short term (up to 30 min.) experiments showed that pyrithione (at 1, 2.5 and 5µg/ml) completely inhibited the incorporation of uridine in *Kl. pneumoniae* (Figure 3.34). However, prolonged incubation (300 min.) of the cells with pyrithione (2.5µg/ml) (allowing recommencement of exponential growth) showed the resumption of uridine incorporation at a rate similar to that of untreated cells (Figure 3.35).

RNA synthesis is mediated by the RNA polymerase, the activity of which depends on the dissociable cations Mn$^{2+}$ and Mg$^{2+}$, and on Zn$^{2+}$ which is tightly bound to the enzyme (Scrutton *et al*., 1971; Valenzuela *et al*., 1973). It is conceivable that pyrithione, through chelation of any one or more of these ions, inhibits RNA polymerase leading to cessation of uridine incorporation and growth. Indeed, pyrithione (2.5µg/ml) caused a 70% inhibition of DNA-directed synthesis of RNA by purified *E. coli* RNA polymerase in vitro, compared with a 93% inhibition of the enzyme by rifampicin (10µg/ml; Table 3.10).

Further support for this comes from studies of two antimicrobial agents, namely, 8-hydroxyquinoline (oxine) and diallyl thiosulfinate (allicin). Fraser and Creanor (1974) showed that oxine caused rapid and selective inhibition of RNA synthesis in *Schizosaccharomyces pombe* as measured by the incorporation of radiolabelled uridine. Further in vitro experiments using isolated RNA polymerase from *E. coli* showed that this inhibition can be reversed by the addition of extra Mg$^{2+}$ and Mn$^{2+}$ to reactions inhibited by oxine (Ronald *et al*., 1975). Oxine also inhibits RNA synthesis in plant cells presumably through chelation of Mg$^{2+}$ and Mn$^{2+}$ associated with RNA polymerase (Ferrero and De La Tore, 1986). Since pyrithione is thought to have a similar mode of action to that of oxine (Albert, 1985), a selective but reversible inhibition of RNA polymerase, possibly through chelation of Mn$^{2+}$ or/and Mg$^{2+}$, may explain the pattern of inhibition and resumption of uridine uptake in *Kl. pneumoniae*.
The antimicrobial action of allicin (H₂C=CH—CH₂—SO—S—CH₂—CH₂=CH₂) against *Salmonella typhimurium*, may also be comparable to that of pyrithione against *K. pneumoniae*. Feldberg *et al* (1988) showed that, in cells of *Salm. typhimurium*, allicin induced a lag phase whose duration was proportional to allicin concentration. This was followed by resumption of growth at a slightly lower rate than control (untreated) cells. Their studies on the incorporation of radiolabelled leucine, thymidine and uridine showed that the incorporation of uridine was inhibited in a way which mirrored the growth inhibition pattern of allicin against *Salm. typhimurium*. The incorporation of radiolabelled leucine and thymidine were only partially inhibited by allicin and were restored to normal level even before the full restoration of uridine incorporation to control levels.

Feldberg *et al* (1988) concluded that allicin may selectively inhibit RNA synthesis by inhibiting RNA polymerase. The ability of cells of *Salm. typhimurium* to recover from the effects of allicin was attributed to their ability to metabolise allicin to a non-inhibitory compound or/and to titration of allicin with 'noncritical' targets, thus decreasing its interaction with critical targets, especially since the duration of the lag phase was inversely proportional to culture density (Feldberg *et al*, 1988).

Pyrithione may inhibit the growth of *K. pneumoniae* by inhibiting RNA polymerase through the chelation of its metal cofactors. The recovery of *K. pneumoniae* may be due to the cell's ability to metabolise the drug. The irreversible inhibition of extracellular thymidine incorporation and the partial inhibition of glycine incorporation by pyrithione represent other effects and possibly partial damage to certain targets in the cell, but do not reflect accurately, in this case, the ability of cells to recover from the growth inhibitory action of the drug. Although allicin and pyrithione are not structurally related, both have an oxide group (N—O, pyrithione; S—O, allicin) which is essential for the antibacterial properties of the compound (Small *et al*, 1947; Leonard *et al*, 1956).

The short term effects of pyrithione on the incorporation of radiolabelled substrates by cells of *B. licheniformis* were also examined for comparison. Again, as with
*Kl. pneumoniae*, pyrithione inhibited further incorporation of $^{14}$C-glycine (Figure 3.36), $^{14}$C-thymidine (Figure 3.37) and $^{14}$C-uridine (Figure 3.38) by cells of *B. licheniformis* within 2-5 minutes of the addition of the drug. Based on these short-term effects, pyrithione appears to have similar effects on both *B. licheniformis* and *Kl. pneumoniae* in line with the similar growth patterns exhibited by these two organisms (Figs. 3.2 and 3.1, respectively) in the presence of pyrithione. It remains to be seen whether the recovery of *B. licheniformis* from the initial pyrithione-induced lag phase is reflected by a resumption of incorporation of any of the three radiolabelled substrates, in particular, uridine.
Conclusions

Pyrithione exhibits a mainly biostatic activity against a wide range of micro-organisms, with Gram-positive bacteria and yeast being more sensitive to the drug than Gram-negative bacteria. Pyrithione, however, may have more than one mode of action against different organisms.

Pyrithione exhibits the phenomenon of 'concentration quenching' against some Gram-negative bacteria but not against Gram-positive bacteria or yeast. At high concentrations, pyrithione is ineffective against *Kl. pneumoniae*, *Salm. abony*, *S. marcescens* and *P. vulgaris*; a phenomenon observed with other chelating agents such as 8-hydroxyquinoline and believed to reflect the importance of drug : metal ion(s) ratio (in the medium) in the activity of the drug. Metal ions play an important role in the antimicrobial activity of pyrithione, as the activity of the drug is reversed by ethylenediaminetetraacetic acid (EDTA).

The responses of bacteria and yeast to pyrithione are distinctly different. Sub-inhibitory concentrations of pyrithione induce a lag phase in bacterial growth (*Kl. pneumoniae* and *B. licheniformis*), the length of which depends on the drug concentration. Pyrithione-induced lag phase in *Kl. pneumoniae* results from inhibition of all cell growth and division, and the resumption of growth which follows signals the ability of the whole population to overcome the effects of the drug. In contrast, yeast cells of *C. albicans* exhibit a drug-concentration-dependent reduction in growth rate, in the presence of pyrithione, with no apparent lag phase.

Once exponential growth is re-established in bacterial cultures treated with pyrithione, such cultures become resistant to further challenge by pyrithione at levels equivalent to those initially present. Yeast cells do not develop such tolerance.
Pyrithione has been shown to interfere in the processes of uptake and/or incorporation of substrates into DNA, RNA and proteins in both *Kl. pneumoniae* and *B. licheniformis*. However, only the uptake/incorporation profiles of uridine into RNA of *Kl. pneumoniae* reflected accurately the growth inhibition pattern of an initial lag phase followed by exponential growth. *In vitro* assay of purified *E. coli* RNA polymerase activity showed that pyrithione inhibited this enzyme by 70% compared to the control.

It is concluded that the main site of pyrithione's action against bacteria is protein synthesis, at the level of RNA synthesis by inhibition of RNA polymerase, possibly through chelation of metal ions (such as Mn$^{2+}$, Mg$^{2+}$ or Zn$^{2+}$) essential for the activity of this enzyme.

Furthermore, cells of *Kl. pneumoniae* that are pyrithione-trained exhibit increased and significant tolerance towards chloramphenicol, but not 8-hydroxyquinoline. The resistance of *Kl. pneumoniae* to pyrithione can be lost after successive sub-culturing in drug-free medium. In addition, pyrithione-treated cells of *Kl. pneumoniae* do not differ in their fatty acids profiles from control (untreated) cultures. The ability of *Kl. pneumoniae* to overcome the action of pyrithione may be due to the cells' ability to metabolise the drug into a non-toxic species, possibly by reduction of the N-oxide group essential for the activity of pyrithione.

Pyrithione's effects on the morphology of *Kl. pneumoniae* are limited to filamentation of some cells at high concentrations of the drug. Pyrithione causes no apparent change in the morphology of *B. licheniformis*, but some damage to cells of *C. albicans* is observed at higher concentrations of pyrithione.

The observations on the interaction of pyrithione with *Kl. pneumoniae* are consistent with it acting at a biochemical rather than structural level. Protein synthesis, and in particular RNA synthesis, appears to be the major target.
Proposals for further work

Further work on the antimicrobial action of pyrithione could focus on aspects of the acquired tolerance of Gram-positive bacteria, such as *B. licheniformis*, to pyrithione. In particular the profiles of uptake/incorporation of radiolabelled substrates (e.g. uridine) into sensitive as well as tolerant cells. This would be comparable to the work on *Kl. pneumoniae* in this study and would show whether the response of Gram-positive bacteria is similar to that of Gram-negative bacteria. In addition, analysis of protein profiles (e.g. gel electrophoresis) of bacterial cells such as *Kl. pneumoniae*, before, during and after pyrithione-induced lag phase may show significant differences that would aid in understanding the mechanisms of inhibition and recovery observed in this study.

The use of radiolabelled pyrithione would be very beneficial in following the uptake of the drug into bacterial cells, as well as its possible breakdown. The rate of uptake, and the site at which pyrithione may be localised within the cell may be determined and would enhance our understanding of how pyrithione and similar substances exert their action against micro-organisms.


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