Effect of plasmids that confer preservative-resistance

on the performance of bacteria in preservative efficacy tests

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Abstract

The effect of plasmids on the efficacy of pharmaceutical preservatives in simple aqueous preserved systems was assessed. This was done by determining minimum inhibitory concentrations (MICs), susceptibilities of strains to bactericidal concentrations of preservatives, and susceptibilities of strains to preservative challenge using the conditions of the Preservative Challenge Test of the British Pharmacopoeia (1988). The pharmaceutical preservatives used were: benzalkonium chloride (BZC), cetrimide (CTAB), propamidine isethionate (PI), dibromopropamidine isethionate (DBPI), chlorhexidine gluconate (CG) and phenylmercuric nitrate (PMN).

MIC data for all the preservatives tested showed the plasmid-less *Pseudomonas aeruginosa* strain NCIB 8626 to be overall the most resistant organism. This strain recorded MICs (in μg/ml) to BZC = 150, CTAB = 300, PI = 150, DBPI = 75, CG = 5.0 and PMN = 30. However, the plasmid-containing strains *S. aureus* SA1325 (pSK1) and *E. coli* 343/113 (R471-1) paralleled or surpassed the resistance levels of the pseudomonad for certain of the preservatives. MICs for the staphylococcus of 150 μg/ml and for the *E. coli* of 40 μg/ml were recorded against PI and PMN, respectively.

Short-term killing rate experiments compared the sensitivities of plasmid-containing and plasmid-less strains to bactericidal concentration of the preservatives. In contrast to the MIC data, bactericidal activity experiments showed *P. aeruginosa* to be the most sensitive organism to 5 μg/ml CG, 1 and 10 μg/ml PMN, 5 mg/ml PI and 1 mg/ml DBPI. Survival of *S. aureus* strain SA1325 (pSK1) in 5 μg/ml CG was 100-fold greater after 90 min when compared to its isogenic parent SA1439. Results of the bactericidal experiments that correlated with MIC data were obtained for the pseudomonad in 10

μg/ml BZC and in 10 μg/ml CTAB solution in which the organism was highly resistant. However, in most cases the resistance levels of the plasmid-containing *S. aureus* and *E. coli* strains surpassed the resistance levels exhibited by the pseudomonad. It is concluded this was due to the method of preparation of inocula, which may have sensitized the pseudomonads to some of the preservatives.

The efficacies of three different aqueous concentrations of BZC (10, 30 and 100 μg/ml), CTAB (10, 30 and 100 μg/ml), PMN (1, 10 and 20 μg/ml) and CG (5, 20 and 100 μg/ml) were measured using the protocol of the BP Preservative Challenge Test. These experiments were performed to determine if the presence of plasmids in test strains made any difference to the compliance of preservative systems with the test. The results demonstrated increased survival of *S. aureus* SA1325 (pSK1) in 10 μg/ml BZC or CTAB and in 1 μg/ml PMN when compared to the plasmid-less *S. aureus* strains. The pseudomonad produced a pattern of death and regrowth in 10 μg/ml CTAB that was reproducible.

It was concluded that plasmids do provide bacteria with the necessary protection to grow, as for example in MIC experiments, or survive, as in bactericidal tests or BP challenge tests, in unfavourable environments. However, the in-use preservative concentrations tested still provided an acceptable degree of protection against plasmid-containing and persistent organisms.

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Introduction

1 Historical introduction

Ever since man made his appearance on earth, the need to preserve has been apparent and its technology pursued relentlessly. Archaeological evidence supports the utilization of the skill of drying meat or split fish carcasses to preserve them for future consumption (Hugo, 1991). Even nowadays, in places lacking modern facilities, people preserve meat by salting, drying or mixing with spices.

The Book of Numbers gives a written account of the flaming procedure for metal objects (silver, brass, iron, tin and lead), a method still used by modern microbiologists to sterilize loops and glassware. In addition, it provides a step-by-step procedure to prepare the "water of separation" which was used to purify materials that could not withstand fire. This "water of separation" was nothing more than wood and animal ashes (potassium and ammonium carbonates), well known to the ancients for their cleansing properties (Grier, 1937). Studying the Book of Leviticus we find a complete compendium of hygienic rules with elaborate procedures for the disinfection of people, clothing and houses (Holy Bible).

Ancient Egypt also contributed to the art of preservation introducing the process of mummification, which utilized dessication and treatment with natural alkali (natron or sodium carbonate) with successive applications of oils and balsams. The Ebers Papyrus is a written legacy of the vasts skills and knowledge in the art of extracting, preparing and applying medicines.

In the Greaco-Roman period, Aristotle (384-322 B.C.) acquired great fame and knowledge because he was a careful observer. He advised Alexander the Great to boil the troops' water supplies before they drank them and to bury their dung to avoid illness (Block, 1968; Hugo, 1991). Greeks and Egyptians burnt sulphur, generating sulphur

dioxide, as fumigant for premises and food vessels to purify and deodorize (Hugo and

Russell, 1992).

Persian laws instructed the populace to store drinking water in bright copper vessels,

while filtration was employed by the Egyptians as a method for purifying water, unglazed

porcelain filters being mentioned by Aristotle (Sykes, 1958). The use of wine and vinegar

in the dressing of wounds dates back at least to Hippocrates (Harvey, 1985), who

recommended the latter for the treatment of ulcers. Acetic acid (vinegar) is still being

suggested as a useful agent for the treatment of superficial wounds infected with

Pseudomonas aeruginosa (Phillips *et al.*, 1968).

Mercury was known to the Greeks before 200 B.C., but it had been used medicinally

long before that in India and China. Dioscorides (A.D. 60) in his Materia Medica shows

how to prepare mercury (hydrargyros) by subliming cinnabar (red mercuric sulphide) and

charcoal in an iron pot (Grier, 1937). Arabs used mercury for skin diseases, and

Paracelsus (1493-1541) treated syphillis with it. Calomel, corrosive sublimate (mercuric

chloride), silver nitrate, ammonia and borax were preserving substances known to the

Arabs (Grier, 1937; Hugo, 1992a).

Alcohol appears to have been distilled from wines in the thirteenth century, as Groot

Albertus Magnus (1193-1280) describes, intelligently, its concentration by distillation.

The modern name "alcohol" was introduced by Libavius (1540-1616) a physician and

chemist. Paracelsus introduced the use of alcoholic "tinctures", but their popularity really

began in the 18th century, at the end of which they had become a very important class

of preparations, mostly due to their stability. Previously, plant extracts were made with

water (Grier, 1937; Hugo, 1991).

These empiric activities, resulting from careful observation and a degree of curiosity, were the humble beginnings of antimicrobial preservation.

1.1. A history of preservation and disinfection

The premise that malodour and decay were related to disease was a concept that steadily developed. Religion played a leading role in the development of a series of protocols and instructions. Early religious beliefs maintained reasonably high health standards in communities; Romans, Greeks and Egyptians were known for their obsessions with hygiene. The Holy Bible introduced the concept of "quarantine" in which lepers and anyone suffering from a contagious disease was obligued to stay outside the boundaries of the community for seven days.

Moving into the eleventh century leprosarias (institutions for the segregation of lepers) were common and lazarettos, which were plague hospitals, followed the quarantine system. However, sanitation and hygiene were totally neglected. All types of refuse were thrown out into the streets, producing increases in pestilence and disease, which had remained at acceptable levels for many years. These and the religious belief of mortifying the flesh, paved the way for the appearance of the plague, which occurred in the 14th century and devastated Europe, claiming the lives of twenty-five million people (Grier, 1937).

Girolamo Fracastoro, poet, physician and philosopher, was professor of logic at the University of Padua in Renaissance Italy. He conceived the theory of the "seeds of infection" or "seminaria", which transfered disease from inanimate objects or contaminated air into healthy organisms. He thus described contagion and airborne infections long before Pasteur (Grier, 1937; Block, 1968; Hugo, 1992a).

Antonie van Leeuwenhoek (1632-1723) was a draper from Delft (Holland), and another enthusiastic observer of nature. His foremost invention, the microscope, uncovered a new world to the scientific minds of his time. He discovered small living things, designating them "animalcula", in a diversity of habitats such as tooth scrapings, stagnant water and blood; he made meticulous drawings of each. These drawings together with his letters, gained his appointment as a Fellow of the Royal Society, even though he never came to England. van Leeuwenhoek was one of the Society's most valued contributors and his drawings were published in the Philosophical Transactions of the Royal Society in 1677. It is said the greatest magnification he achieved was 300 times, but from his written statements and drawings, it is obvious that he must have observed cocci, bacilli, spirillae and spirochaetae (Grier, 1937). This was a remarkable achievement; until that time, microorganisms had not been seen as single entities, but only as colonies. The Greek historian Siculus (writing about the siege of Tyre in 332 B.C.) declared that bread, which was being distributed to the Macedonians, had a bloody look. Hugo (1992) suggests this was probably due to infestation by *Serratia marcescens* which produces a red or pink pigment at ambient temperature.

van Leeuwenhoek's findings proved the existence of Fracastoro's "seminaria", but their significance was to remain dormant for some years until the theory of spontaneous generation was invalidated. Others who believed that disease could be transmitted by invisible living agents, known as *contagium animatum*, included Kircher in 1658, Lange in 1659, Lancisi in 1728 and Marten in 1720 (Hugo, 1992a).

Redi (1626-1694) demonstrated that life was generated from pre-existing organisms of the same kind with his simple experiment using jars filled with meat and covered with screens to impede the reproduction of maggots. Nonetheless, this demonstration was not

enough for followers of the spontaneous generation theory like the English priest Needham. He claimed that after sterilizing infected broth, new organisms appeared in the sterile broth on standing. The Italian priest, Spallanzani, vigorously opposed this and logically maintained that the broth was not adequately sterilized (Grier, 1937; Hugo, 1992a).

However, it was Louis Pasteur (born 1822) who finally sent the theory of spontaneous generation into oblivion and laid the foundations for a new era in scientific research.

1.1.1 Heat sterilization

The earliest account of an autoclave was by Papin in 1681 entitled "A new digester or engine for softening bones" which was a steam cooker or "digester", capable of maintaining water at temperatures above 100°C. The apparatus had the first known safety valve to control pressure, but was not intended to sterilize (Gaughran and Goidie, 1975).

In 1809 the French government offered a prize to anyone who conceived a method for preserving food during extended sea journeys. Nicholas Appert won the prize with his method of sealing edibles in glass jars and then heating them. Although widely used, this method escaped the attention of prominent scientists for some years (Hugo, 1991).

In 1830 William Henry, a Manchester physician who worked in public health, published "Experiments of the disinfecting power of increased temperature with a view to the suggestion of a substitute for quarantine". Two years later, he studied the effect of heat on contagion by placing contaminated material (fomites), such as clothes worn by sufferers from typhus, smallpox and scarlet fever, in air heated by water sealed in a pressure vessel. He realized that he could achieve temperatures higher than 100°C by using a closed vessel fitted with a proper safety valve. He found that garments so treated

could be worn with impunity by others who did not contract the disease (Gaughran and Goudie, 1975; Hugo, 1991; Hugo, 1992a).

After Pasteur had shown that microorganisms were the direct cause of milk souring, French vintners sought his assistance. He recommended heating new wine at 55°C in the absence of air. This procedure, later known as pasteurization, helped preserve wine without affecting its flavour (Hugo, 1991). Pasteur also used a pressure vessel with safety valve for sterilizing, designed by Lucas Championnière. Unfortunately, in the absence of records, the credit for this device went to a student and collaborator of Pasteur, Charles Chamberland. He later designed the steam sterilizer, which Weisnegg of Paris built it in 1880 for Pasteur's laboratory. It underwent various modifications being later called the Pasteur-Chamberland Autoclave (Gaughran and Goudie, 1975).

Robert Koch, in collaboration with Gustav Wolffhügel, popularized the hot air sterilizer in Germany and evaluated the bactericidal effect of dry heat. Scientifically-based regimens for both dry heat and steam sterilization were proposed by Pasteur in 1876, and by Koch and Wolffhügel in 1881. The fact that moist heat was more effective than dry heat was noted by Pasteur. He raised the temperature of moist heat by immersing sealed containers in a boiling solutions of calcium chloride and found that the exposure to moist heat at 110 to 120°C for 30 min was as effective as exposure to dry heat at 130 to 150°C for one hour (Grier, 1937; Hugo, 1991).

Meanwhile in 1897, Tyndall observed that while vegetative forms of bacteria were destroyed by boiling, the spores of some species were resistant. However, if these spores were allowed to germinate, their vegetative progeny were killed by a repeat of the heating process. He therefore proposed the technique (Tyndallisation) in which material capable of supporting spore germination is heated for an hour on each of three to five successive

days. This became the standard method of sterilizing bacterial culture media until, and even after the introduction of the autoclave (Hugo, 1991).

Another method of sterilization which does not involve the use of heat is filtration. Contaminated waters that percolated through soil or gravel were observed to emerge clear and drinkable. Hence artificial filters were constructed of pebbles. Later, the construction of tubes of unglazed porcelain or compressed kieselguhr, the so-called Chamberland or Berkefeld filters, made their appearance in 1884 and 1891, respectively, and were used to sterilize pharmaceutical products. These filters were a major breakthrough, but presented some disadvantages such as low flow rates, difficulty in cleaning and media migration into the filtrate (Phillips and Halleck, 1985; Hugo, 1991).

1.1.2 Chemical disinfection

Many compounds still in current use were introduced as disinfectant and preservative substances a long time ago. Among these are: mercury (4th century); mercuric chloride (since the Middle Ages); copper sulphate (1767); zinc chloride (1815); sodium permanganate and Condy's crystals (1850s). Lime sulphur, an aqueous suspension of elementary sulphur and calcium hydroxide, was introduced as a horticultural fungicide in 1803. Later the salts, chiefly sodium, potassium or calcium, of sulphurous acid were used in wine and food preservation (Hugo, 1992a).

In 1835, Agostino Bassi, an Italian lawyer, demonstrated that a fungus caused the silkworm disease muscardine and also proposed the fungal origin of certain human diseases. He is thought to be the first person to establish the link between microorganisms and disease, using disinfectants such as, alkalis, acids, chlorine, sulphur and alcohol in their treatment (Rost, 1976; Hugo, 1991, 1992a).

Chlorine was discovered in 1744 and proved very useful in the textile industry as a bleaching agent. Later on, it was realized that gaseous chlorine could be "fixed" by bubbling it through potash solution; this produced the first metal alkali hypochlorite or Eau de Javel. Some years later, Tennant (in 1789), passed chlorine gas though a slurry of slaked lime, producing a bleaching powder. In 1823, Labarraque passed chlorine gas into sodium carbonate solution and suggested its use in wound treatment. Other formulations that emerged were Dakin's solution and Eusol, which were widely used in hospitals (Block, 1968; Hugo, 1991).

Chlorine derivatives mostly displaced the parent compound, but chlorine itself still had some use, both as solution and as a gas. In Europe, chlorine water was used as a gargle for infected sore throats and in 1832 the gas was dispersed in the streets of Paisley to stop a cholera epidemic (Hugo, 1991). In 1830, Oliver Wendell Holmes, an American physician reported success in preventing the transmission of puerperal fever by washing the hands in chlorinated lime after visiting infected patients. Semmelweiss (1847) decreased the incidence of puerperal fever in the obstetrical ward of the Allgemeines Krakenhaus of Vienna from about 10 to 1% by ordering the medical students (who were prone to come directly from the autopsy room to the obstetrical ward) to wash their hands in chlorinated lime before examining patients. "Aetiologie" was published by Semmelweiss in 1861 and contained detailed statistical data, which proved that medical students were responsible for the spreading of the disease; the opposition this aroused was overwhelming. He finally lost his position at the hospital and died insane, but his work endured, stressing the need for proper disinfection procedures. Following the introduction of aseptic surgery by Lister in 1867, the importance of disinfecting the patient's skin

together with the hands of the surgeon, his instruments and the hospital environment was readily appreciated (Block, 1968; Harvey, 1985).

Another halogen used as an antibacterial agent was Iodine. It was suggested as a wound dressing by Davies in 1839 and its use evaluated by Koch and Pasteur. Tincture of Iodine was popular until 1949, the year in which Shelanski solubilized the halogen in an aqueous solution of a nonionic surface active agent, producing iodophores. These had the advantages of being non-stinging and less staining than the previous alcoholic solutions (Whittet *et al.*, 1965; Hugo, 1991).

Phenol or carbolic acid was the first aromatic compound to be used as an antiseptic; it is also the most caustic. It succeded in reducing the mortality in operations and drew coal-tar into attention as a source of disinfectants, especially the fraction containing the cresols. In 1865, Lister demonstrated the sterilization properties of phenol to the extent that the medical profession began to accept the "germ theory of disease" (Rost, 1976). At the beginning only watery solutions of cresols were tried, but they were substituted by saponified emulsions, which were found to give a higher bactericidal efficiency. In 1844, a Frenchman called Bayard made an antiseptic powder of coal tar, plaster, ferrous sulphate and clay. Later on, in 1850, Ferdinand Le Beuf prepared an emulsion of coal tar using quillaia bark which is rich in saponins and hence favours the solubilization of the tar. This preparation became an invaluable liquid disinfectant in the hands of Lemaire, a French surgeon. He published *L'acide phenique* in 1863 in which he reported the use of carbolic acid in surgery. He seems to have been the first in his field to recognize the true nature of infection and the first to use carbolic acid in his field (Kelly, 1901). The *Pharmaceutical Codex* of 1979 still described a coal tar "solution" prepared with quillaia bark, although, in 1988, the *British Pharmacopoeia* replaced it with polysorbate 80. There

were many other preparations of coal tar, but they all worked on the principle of solubilization. Additionally, other liquid disinfectants were prepared by Damman in 1889 and John Jeyes from Northampton in 1887, who patented Jeyes fluid. Lysol was first prepared by Engler and Pieckhoff in Germany in 1897 (Hugo, 1991, 1992a).

In 1889 Fraenkel and Henle drew attention to the fact that the higher homologues of phenol contained in the cresol fractions were the more powerful germicides, and being much more insoluble in water, these were less toxic than phenol. New discoveries helped the evaluation of coal-tar disinfectants, such as the facts that decreased solubility in water and bulky alkyl groups in the benzene ring, gave increased germicidal power. Koch found that thymol had the same antibacterial activity on anthrax bacilli in a 1:80,000 dilution as phenol at 1:1,250. Laubenheimer made experiments with the same concentration (1%) of different preservatives, including phenol, propyl-phenol and iso-propyl-phenol, and observed significant differences in the killing of staphylococci. Bechold and Ehrlich, in 1906, studied the structure-activity relationship of phenols. Scholler and Schrauth showed that the introduction of halogens in the benzene ring notably augemented the disinfectant efficacy of the compound, but only up to a certain limit. Sommerville was able to increase the bactericidal effect up to 60%, by saturation of phenol with chlorine (Grier, 1937; Hugo, 1991).

Hydrogen peroxide, discovered in 1818, was introduced in medicine in 1891. It is now used in conjuction with ultraviolet light for bactericidal purposes and on its own in the disinfection of contact lenses. Another chemical employed was formaldehyde, discovered in 1859 and tested as a bactericide by Loew and Fischer in 1886. It was used as a fumigant as early as the beginning of the 19th century (Hugo, 1991).

In 1887, Rozahegyi reported that certain strains of bacteria did not grow on nutrient agar in the presence of certain dyes. Stilling in 1890 showed aniline dyes to be highly active antibacterials, but although Ehrlich showed in 1891 that methylene blue stained malarial organisms, it had only limited success in killing the parasites (Grier, 1937).

Paul Ehrlich used aromatic dyes to elucidate the distribution of poisons and drugs in the animal body and to throw light on the selective action of cells. It was not until 1904 that he found certain benzidine and triphenylmethane dyes to be powerful antiseptics against bacteria. Some years before, Bechamp in 1859 heated aniline and arsenic trioxide and obtained a compound believed to be the anilide. In 1903, Ehrlich tested this compound and found that it was inactive *in vitro* against trypanosomes, but did not test it *in vivo*. Thomas and Breinl, in 1905, demonstrated its activity *in vivo* and showed it was 40 times less toxic than potassium arsenite. As a result of this, it was named atoxyl and Koch proved it to be effective against African sleeping sickness (Grier, 1937; Rost, 1976). These findings revived Ehrlich's interest in the arsenical compounds and he performed further studies to show the correct structure of atoxyl. In 1909, he demonstrated it had good trypanosomicidal activity *in vitro* when reduced to the trivalent arsenous state. He suggested this reduction was performed by the host cells, and that the trivalent arsenous state was the active form. A year later, Ehrlich introduced salvarsan (arsphenamine or compound 606) a trivalent arsenical for the treatment of syphilis, which was a major breakthrough. Salvarsan was sparingly soluble in water and required large volumes of solvent when treating a patient. Nevertheless, these disadvantages were overcome by the development of neosalvarsan which was a less toxic derivative, also known as neoarsphenamine (Mann, 1984). In the early stages of his work, Ehrlich developed the *chemoreceptor theory* to explain the emergence of resistant strains,

including concepts of reduced affinity and suggesting the possibility of different types of

receptors. He became known as the Father of Chemotherapy (Rost, 1976).

Another breakthrough was the production of an acridine derivative, originally prepared

by Benda in 1912 at the request of Ehrlich, which was found to have a very marked effect

on trypanosome infections, hence it was named trypaflavine. Browning and Gilmore were

the first to observe its powerful action on bacteria. Its preparation was elucidated by

Barger and Ewins, working at the direction of the Medical Research Committee and

arrangements made in 1917 for its large-scale manufacture to fulfill the needs of the

troops in the Great War. It became official in the B.P. 1932 as acriflavine (Grier, 1937;

Hugo, 1965; Rost, 1976).

Hexamethylenetetramine was introduced as a urinary antiseptic by Nicollaier in 1894.

In 1908, Einhorn and Göttler recognized the antibacterial properties of

chloroacetylaminoethanol, a derivative of the previous compound. In 1913, Reychler

described the synthesis and properties of cetyltriethylammonium iodide and two years

later, Jacobs and Heidelberger studied the structure-activity relation of the quaternary

hexamethylenetetramine salts. They tried to increase the antibacterial effect by

introducing alkyl groups in the hexamethylenetetramine nucleus. In 1928, Hartmann and

Kaegi prepared quaternary ammonium compounds that are more closely related to the

present day structures and these showed strong antiseptic activity. However, it was not

until 1935 that the use of benzalkonium chloride as a detergent and germicidal agent was

reported by Domagk. Since then, many other related compounds have emerged (Davis,

1960; Lawrence, 1968; Rost, 1976; Warner and Warner, 1976; Hugo, 1991).

Morgenroth in 1918 investigated the action of some simple homologues of

hydroquinone which were initially prepared by Grimaux and Arnaud in 1891. In these

homologous alkaloids the antipyretic and antimalarial properties of quinine were repressed and the antiseptic and bactericidal properties were developed (Grier, 1937).

In 1930 the esters of *p*-hydroxy-benzoic acid and their corresponding sodium salts were marketed under the names nipagin and nipasol. They showed great activity against microorganisms at concentrations of 0.05% for the methyl ester and 0.01% for the propyl ester and proved efficient preservatives of facial creams, mucilages and a diversity of edibles (Grier, 1937).

Research on the bis-phenolic series of compounds began in 1932 and, as a result, hexachlorophene was patented in 1941 (Rost, 1976).

Fuller (1942) investigated the antibacterial activity of aromatic amidines, compounds initially studied for their antitrypanocidal effect. At present, propamidine and dibromopropamidine are used in semi-solid preparations for topical use and in eye-drops (Hugo, 1991). Diamidines and biguanids emerged from earlies studies on the treatment of malaria with methylene blue. After Ehrlich's limited success with the dye, German researchers studied other ring systems. Later, Schönhofer in 1942 suggested that antimalarial activity in chloroquine was due to the possibility of tautomerism. Then, a series of pyrimidine derivatives were prepared as potential antimalarial agents resulting in the production of a biguanide when the amino-substituted pyrimidine ring was opened (Rost, 1976). The high antibacterial activity of these compounds prompted research on others, leading to the discovery of the antibacterial activity of propamidine by Thrower and Valentine in 1943 (Hugo, 1965). Further studies on antibacterial activity-structure relationships demonstrated dibromopropamidine to be more active than the parent compound (Wien *et al.*, 1948).

The antimicrobial properties of chlorhexidine were extensively studied by Davies *et al* (1954). Microbiological and toxicity tests showed chlorhexidine had the desired properties of high activity at low concentrations, which provided a good margin of safety (Davies *et al.*, 1954).

1.2. Modern preservatives and their mechanisms of action

1.2.1 Phenols

The use of phenolics has endured the passage of time; they are still employed as general disinfectants (either in solution or mixed with slaked lime, for toilets, cesspools, floors, or drains) and as preservatives of manufactured products, including some pharmaceuticals although their use is limited by toxicity (Figure 1).

This group of compounds promotes a concentration-dependent leakage of cell contents from microbial cells (Starr and Judis, 1968; Elek, 1968; Warner and Warner, 1990), becoming general protoplasmic poisons at higher concentrations. The activity of phenols is closely related to their structure, and it is possible to modulate their antibacterial response by structural alterations. For example: (1) although *ortho* or *meta* substitutions are active, the greatest increase in antibacterial activity is produced by introduction of alkyl chains of up to six carbon atoms in the *para* position, (2) the introduction of both a halogen atom and an alkyl group enhances activity, with the greatest effect obtained when the halogen is *para* and the alkyl is *ortho* to the phenolic group, (3) nitration of the molecule increases activity by enabling it to interfere with oxidative phosphorylation and (4) generating bis-phenols by forming a direct bond between the two benzene rings or separating them by $-CH_2-$, $-S-$ or $-O-$ groups (Hugo, 1965).

Other synthetic phenolic derivatives include octylphenol (4-tertiary octylphenol), 2-phenylphenol and 4-hexylresorcinol. Octylphenol is many times as effective in alcoholic solution as phenol against Gram-positive organisms but to a lesser extent against Gram-negative. This compound is fungistatic and has been used as a preservative for products such as glues and non-food gelatines (Hugo and Russell, 1992).

An ingredient in disinfectants of the pine-type is 2-phenylphenol. It is both antibacterial and antifungal and is used as a preservative for cutting oils and as a general agricultural disinfectant. In the paper and cardboard industry it has been particularly useful as a slimicide and fungicide. 4-hexylresorcinol is employed as a skin disinfectant at a concentration of 0.1% in 30% glycerol and in lozenges and medicated sweets for the treatment of throat infections (Anon., 1993).

Chlorophenols are widely used in industry for the preservation of a diversity of materials: cutting oils, wood, textiles (trichlorophenol and pentachlorophenol), glues and paints (chlorocresol). Other chlorophenols are used as topical antiseptics or as ingredients in pine-type disinfectants and in medicated soaps and hand scrubs (chloroxylenol and dichloroxylenol). Nitrophenols are more toxic than halophenols. 4-nitrophenol is the most widely used, at concentrations of 0.1 - 0.5% for the preservation of leather (Anon., 1993).

Hydroxy-halogenated derivatives of bis-phenols (diphenyl methane, diphenyl ether and diphenyl sulphide) are active against bacteria, fungi and algae, but they all have low activity against *P. aeruginosa*. Diphenyl methane derivatives include dichlorophane, which is used as a preservative for toiletries, textiles, cutting oils and as a bacteria growth-preventive in water-cooling systems and humidifying plants. Hexachlorophane, which was used until recently as the active ingredient of deodorants and toothpastes, is

now restricted to a maximum concentration of 0.1% and must not be used for children or in personal hygiene products (Warner and Warner, 1976).

Triclosan is the most widely used derivative of diphenyl ether. Although wide spectrum, it is almost ineffective against pseudomonads, and is found in some medicated soaps and hand cleansing gels (Lynn and Hugo, 1983). Recently, it was shown to be highly active (MIC < 0.12 µg/ml) against methicillin-resistant *Staphylococcus aureus* (MRSA) with low-level chlorhexidine resistance (Cookson *et al.*, 1991a), and equally as active as sodium lauryl sulphate as an antiplaque agent (Jenkins *et al.*, 1991).

Diphenyl sulphide derivatives include fentichlor and its chlorinated analogue. Fentichlor is used for the treatment of dermatophytic conditions. It can cause photosensitization and this has restricted its used as a cosmetic preservative. Fentichlor is used in the treatment of superficial fungal infections and can be found in creams, sprays and dusting powders (Lynn and Hugo, 1983). Its mode of action was described by Hugo and Bloomfield (1971a, b and c). The chlorinated analogue of Fentichlor is almost insoluble in water, but proved to be an effective inhibitor of microbial growth in cutting oil emulsions (Hugo and Russell, 1992).

All these phenolic compounds share the disadvantage of being inactivated by organic matter and non-ionic surfactants.

1.2.2 Organic and inorganic acids

In general, acid preservatives (Figure 2) act as uncoupling agents, uncoupling electron transport from ATP synthesis. They act as proton carriers and provide another pathway (besides that supplied by the ATP synthetase) for the flow of protons across the cytoplasmic membrane. Therefore, the proton-motive force is completely dissipated and
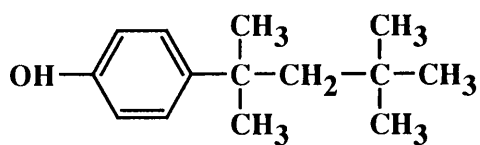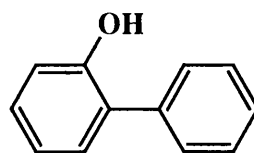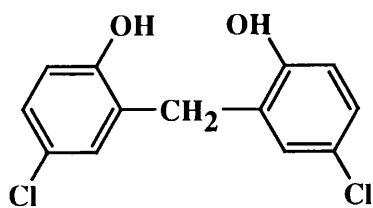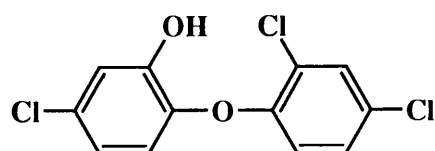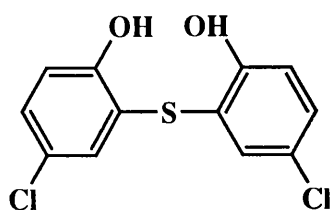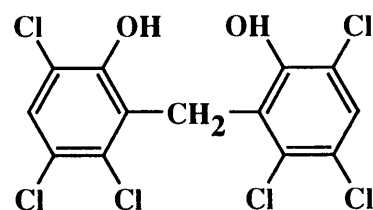
Figure 1. Phenolic compounds.

ATP is no longer produced (Hanstein, 1976). Substrates which depend on the proton-motive force for their entry into the cell, such as sugars and aminoacids, are no longer imported which results in growth inhibition (Freese *et al.*, 1973). The biocidal activity of organic acids is pH- dependent and increases when pH values are lowered, indicating that inhibition is the result of the undissociated acid (Simon and Beavers, 1952).

Vinegar was known for its preservative properties long ago and dilute solutions of it have been recommended as wound dressings in pseudomonal infections (Phillips *et al*, 1968; Harvey, 1990). Other organic acids (aromatic and aliphatic) and some inorganic acids have also found useful application as preservatives. Benzoic acid is used widely as a pharmaceutical preservative and is also used in the topical treatment of fungal infections of the skin, together with salicylic and undecylenic acids. In recent studies, salicylic acid has been shown to decrease the adherence of *Escherichia coli* to silastic catheters, an observation which may be useful for the prevention of urinary tract infections in hospitalized patients (Farber and Wolff, 1993).

Derivatives of benzoic acid, for example esters of *p*-hydroxybenzoic acid, have a wide range of activity against fungi, but have a low effect on bacteria. They are widely used as preservatives in food, toiletries and pharmaceutical products such as creams, emulsions or lotions, but are inadequate for injections and ophthalmic preparations (Lynn and Hugo, 1983).

An approved disinfectant for foot-and-mouth virus is citric acid; because of its chelating properties it is also capable of increasing the outer membrane permeability of Gram-negative bacteria (Shibasaki and Kato, 1978).
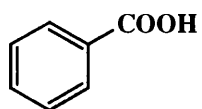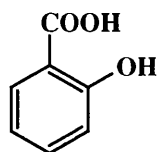
Benzoic acid   Salicylic acid
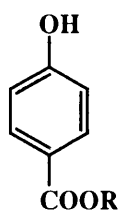
Esters of p-hydroxybenzoic acid
(R= methyl, ethyl, propyl, butyl)
Figure 2. Organic acids and esters.
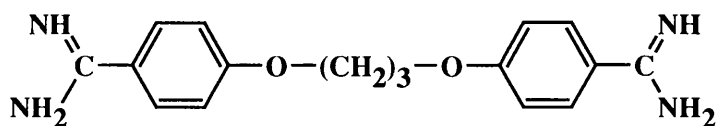
Propamidine
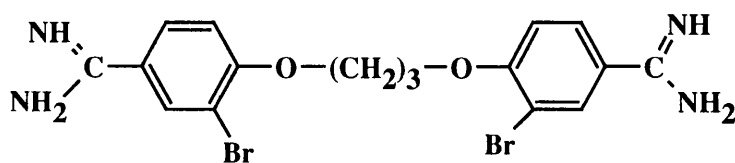
Dibromopropamidine
Figure 3. Diamidines.

1.2.3 Aromatic diamidines

Amidines are chemically related to the biguanids, and because of the presence of the guanidino group, they were used initially in the treatment of protozoal infections such as trypanosomiasis and malaria. These organisms require high concentrations of glucose for reproduction and compounds with the guanidino group such as cycloguanil are powerful hypoglycemic agents. However, when tested for antimicrobial activity (Wein *et al.*, 1948) they were also found to be active against fungi, and Gram-positive and Gram-negative bacteria, although the former organisms were less sensitive (Lynn and Hugo, 1983). Propamidine and dibromopropamidine (Figure 3) are the most important derivatives and the latter was included in the *British Pharmacopoeia* of 1980. The activity of diamidines is reduced by serum, blood and low pH values. Dibromopropamidine isethionate has been reported to enhance the *in vitro* activity of silver sulfadiazine against *P. aeruginosa*, *S. aureus* and *Enterobacter cloacae*, due to its effect on cell permeability, which increases the uptake of the sulphonamide (Richards *et al.*, 1991). Resistance is rapidly acquired to both compounds by serial subculture in increasing doses (Paniagua-Crespo *et al.*, 1989). Propamidine isethionate is currently used in the treatment of keratitis caused by *Acanthamoeba castellani* (John *et al.*, 1990).

1.2.4 Biguanides

Biguanides are potent antimicrobials and include chlorhexidine and alexidine (Figure 4). Chlorhexidine has a wide spectrum of antibacterial activity against Gram-positive and Gram-negative organisms, although there are some bacterial strains, such as *Proteus* and *Providencia* spp., that are highly resistant (Russell, 1986; Baillie, 1987). This antibacterial increases membrane permeability at low concentrations, and at higher concentrations

produces general protein coagulation, which inhibits membrane-bound ATPase (Hugo and Longworth, 1964; Chopra *et al.*, 1987). Although, chlorhexidine is not sporicidal (Russell and Chopra, 1990), it rapidly becomes so at 98-100°C; it is tuberculocidal in ethanolic solution. Alexidine differs from chlorhexidine only in the end groups, ethylhexyl in the former and chloro-phenol in the latter. Their spectra of activity are the same, but cell leakage from *E. coli* is more actively induced by alexidine (Chawner and Gilbert, 1989a,b).

Due to their cationic properties biguanides are incompatible with anionic surfactants and other anionic compounds, forming insoluble salts with borates, bicarbonates, carbonates, chlorides, citrates, phosphates and sulphates. Nonionic surfactants reduce their activity, as do soaps, blood and pus; synergism has been observed with ethanol, benzyl alcohol, phenylpropanol and phenethyl alcohol (Richards, 1971; Anon., 1989).

The main uses of chlorhexidine are as a skin disinfectant (Holloway *et al.*, 1990; Ayliffe *et al.*, 1990; Kjolen and Andersen, 1992), in obstetrics and gynaecology (Burman *et al.*, 1992), odontology (Urbani *et al.*, 1992) and, on account of its low oral toxicity, in throat medication (Hugo and Russell, 1992). Chlorhexidine is the most effective antiplaque agent discovered to date, but there are no commercially available products containing it. Although experimental formulations have produced promising results in terms of oral hygiene, staining of the buccal cavity continues to be an undesirable side effect which is still being investigated (Netuschil *et al*, 1989; Waaler, 1990; Oosterwaal *et al.*, 1991; Joyston-Bechal and Hernaman, 1993).

1.2.5 Surface active agents

Surfactants or surface-active agents have two distinct regions in their molecule; one

is hydrophobic and the other is polar or hydrophilic (Figure 5). Depending on the charge

or lack of it, these agents are classified as cationic, anionic, nonionic or ampholytic

compounds (Eleck, 1968).

Cationic surfactants, such as quaternary ammonium compounds, are strong bactericidal

agents. Their basic structure consists of a nitrogen atom with a valency of five, with four

of the substituents (R^1-R^4) being alkyl or heterocyclic radicals, and the fifth a small anion

(X^-). High antimicrobial activity is found when at least one of the R groups has a chain

of 8-18 carbon atoms (Domagk, 1935).

Benzalkonium chloride is active against bacteria, mostly at alkaline pH values, but

being a cationic compound, is incompatible with soaps and anionic detergents. Calcium

and magnesium ions also antagonize its action. This blocking effect may be due to

competition by the cations for the active site (Brown and Richards, 1965; Richards and

Cavill, 1976). Also, incompatibilities may arise in the presence of non-ionic surfactants

(tweens and lubrols) and phospholipids, in which case, solubilization within surfactant

micelles seems to be the cause of inactivation (Brown and Richards, 1964; Blanchard,

1980; Bartnick, 1992). Synergy has been demonstrated when combined with

ethylenediamine tetraacetic acid (EDTA), benzyl alcohol, 2-phenethylethanol, bronopol,

phenylmercuric derivatives and m-cresol (Brown, 1968; Richards and McBride, 1971;

Denyer et al., 1985; McCarthy and Ferrerira, 1990).

The monoquaternary compounds are the most widely used, but derivatives of 4-

aminoquinaldine (e.g. laurolinium) and the bisquaternary compounds (e.g. hedaquinium

chloride and dequalinium) are also potent antimicrobial agents. Polymeric quaternary

ammonium compounds are used in industry. One such compound is

poly[oxyethylene(dimethylimino)ethylene]dichloride; organosilicon-substituted (silicon-

bonded) quaternary ammonium salts have also recently been introduced (Hugo and Russell, 1992). In general, quaternary ammonium compounds are very useful disinfectants and pharmaceutical and cosmetic preservatives (Bassett, 1971; Kelsey and Maurer, 1972; Quack, 1976; Davies, 1980).

Anionic quaternary ammonium compounds include sodium lauryl sulphate, sulphated fatty acids, amine soaps and alkali-metal and metallic soaps. These compounds have weak antimicrobial properties (Kawabata *et al.*, 1993), but can induce lysis of Gram-negative organisms at high concentrations (Kabara, 1984).

Nonionic surface active agents include cetomacrogols, which have hydrocarbon chains with non-polar water-attracting groups (ethylene oxide units). Tweens, or polysorbates, are also nonionics. Although considered to have no antimicrobial activity at low concentrations, they are able to render Gram-negative cells sensitive to other antibacterial compounds. It is believed they affect the permeability of the outer membrane (Brown and Richards, 1964; Brown, 1975). A recent study showed Tween 80 [polyoxyethylene (20) sorbitan mono-oleate] enhanced the antibacterial activity of methyl-*p*-hydroxybenzoate, phenoxyethanol and chlorocresol against *P. aeruginosa*, at both low concentrations and even above critical micelle concentration (Kurup *et al.*, 1991). In addition, experiments in mycobacteria have demonstrated that polysorbate inhibits nucleic acid synthesis when in aqueous solution containing phosphate (Tsukamura, 1991).

Amphoteric or ampholytic agents combine the detergent properties of anionic with the bactericidal activity of cationic compounds. Their antimicrobial activities remain constant throughout a wide pH range (Barrett, 1969) and are less readily inactivated by proteins than cationic compounds (Clegg, 1970). Compounds included here are dodecyl-β-alanine, dodecyl-β-aminobutyric acid and dodecyl-di(aminoethyl)-glycine, the latter being a Tego®

H3C(CH2)3—C—H2C—NH—C—NH—C—NH—(CH2)6—NH—C—NH—C—NH—CH2—C—(CH2)3CH3

H (top), CH2/CH3 (bottom) on left C; NH (double bond) on C; NH below C; NH (top right), CH2/CH3 on right C

Alexidine

Cl—⟨benzene⟩—NH—C—NH—C—NH—(CH2)6—NH—C—NH—C—NH—⟨benzene⟩—Cl

NH groups on the C atoms
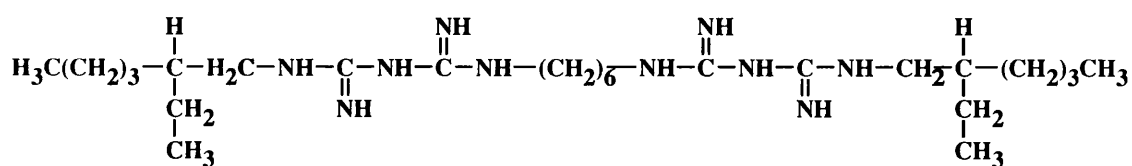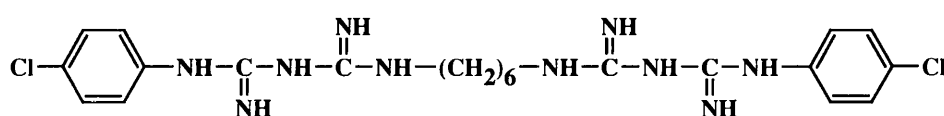
Chlorhexidine

Figure 4. Structures of biguanide preservatives.

$$\begin{bmatrix} R_1 \diagdown \diagup R_3 \\ N \\ R_2 \diagup \diagdown R_4 \end{bmatrix}^{+} \quad X^{-}$$

General structure

⟨benzene⟩—CH2—N^{+}—C_nH_{2n+1} Cl^{-}

with CH3 groups above and below N

(n= 8 to 18)

Benzalkonium chloride

$$\left[O-CH_2-CH_2-\overset{CH_3Cl^{-}}{\underset{CH_3}{N^{+}}}-CH_2CH_2-\overset{CH_3}{\underset{CH_3Cl^{-}}{N^{+}}}-CH_2-CH_2 \right]_{n}$$
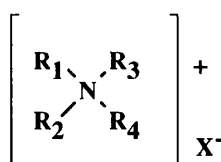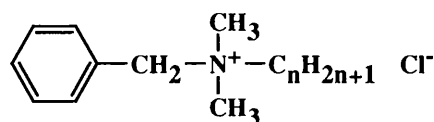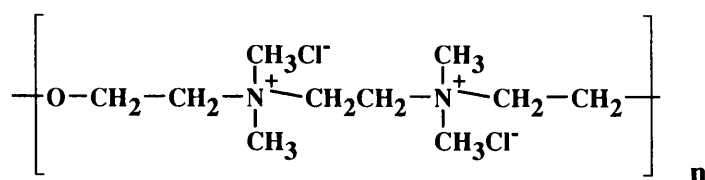
Polymeric QAC

Figure 5. Quaternary ammonium compounds.

compound, used as a disinfectant in the food industry (Kornfeld, 1966).

1.2.6 Aldehydes

Ethanedial, propanedial, butanedial, adipaldehyde, formaldehyde and glutaraldehyde are all included in this category, but only the last two are widely used for disinfection purposes. They all possess some sporicidal action, but members of this family with more than 6 carbon atoms have vitually no sporicidal effect (Pepper and Chandler, 1963).

Glutaraldehyde is a saturated 5-carbon dialdehyde commercially available as a 2, 25 or 50% acidic solution, which has to be made alkaline before use. It is highly active against bacteria and their spores, mycelial and spore forms of fungi and various types of viruses (Borick, 1968; Borick and Pepper, 1970). Even though it has a wide spectrum of activity and is non-corrosive to metals, rubber and lenses, its potential mutagenic and carcinogenic effects (Quinn, 1987) make its use hazardous to personnel. This compound reacts with the ε-amino group of lysine forming internal protein cross-links, impairing the function of membrane transport proteins and porins (Hugo, 1992b).

Formaldehyde solution (formalin) contains 34-38% w/w of methanedial. It interacts with protein molecules by attaching to primary amide and amino groups (Fraenkel-Conrat *et al.*, 1945), and also reacts extensively with amino groups of nucleic acid bases, (Staehelin, 1958). At a concentration of 8%, formaldehyde solutions are capable of destroying a wide range of vegetative organisms, fungi, viruses, and even spores, but its sporicidal action is lower than that of glutaraldehyde. Temperature increases activity, but its penetration power is not very high (Lynn and Hugo, 1983). The primary use of these compounds is in the sterilization of equipment or material that cannot withstand heat.

Dilute solutions of formaldehyde are used as a mouthwash and gargle (Lynn and Hugo, 1983).

1.2.7 Antimicrobial dyes

Acridines, triphenylmethane dyes (parafuchsin, crystal violet, brilliant green and malachite green) and quinones are included in this category. In general, antimicrobial dyes require a cationic ionization and a planar molecule to exert their antibacterial effect (Albert, 1966; Elek, 1968). Their activity is relatively slow and they are not sporicidal (Foster and Russell, 1971) or inactivated by serum. Acridines (Figure 6) act as intercalating agents between nucleic acid base pairs; ribonucleic acid polymerase has also been identified as a target (Nicholson and Peacocke, 1965). Recently, doubts about intercalation being the dominant binding effect have arisen, leading to a reassessment of the binding of proflavine to DNA (Benigni et al., 1990; Berman et al., 1992; Herzyk et al., 1992; Schelhorn et al., 1992).

Resistance towards these compounds develops as a result of chromosomal mutation followed by selection of resistant mutants. It is interesting that R plasmid-mediated resistance can be cured (eliminated) by growth in subinhibitory concentrations of acridines (Watanabe, 1963). Recent studies with 9-aminoacridine against Salmonella typhymurium show mutagenesis may be inhibited by the presence of glucose (Kopsidas and MacPhee, 1993), and in studies on the antibacterial activity of benzacridines against E. coli it was concluded that the antimicrobial and antiplasmid activity exhibited by these agents was different from their antitumor, differentiation-inducing and carcinogenic activity (Motohashi et al., 1992). Other reports show that the mutagenic activity of proflavine may differ by up to two orders of magnitude if there are variations in bacterial growth

conditions (Ferguson *et al.*, 1991). At present, proflavine hemisulphate and 9-aminoacridine are used in the treatment of wound infections (Hugo and Russell, 1992).

In contrast to acridine, the triphenylmethane dyes (Figure 7) are liposoluble and penetrate cells easily (Elek, 1968). These dyes are more effective against Gram-positive bacteria, but the presence of serum decreases their activity, which in turn is regulated by their degree of ionization and their equilibrium constants. These compounds have found useful application in the formulation of selective diagnostic media (i.e., crystal violet blood agar and crystal violet lactose broth). Crystal violet binds externally to DNA, without intercalating and interacts with two adjacent adenine-thymine (A-T) base pairs (Wakelin *et al.*, 1981); subsequently DNA replication catalyzed by polymerase I is inhibited (Docampo and Moreno 1990).

Quinones (Figure 8) include, in order of toxicity to bacteria, moulds and yeast, the naphthoquinones, phenanthrenequinones, benzoquinones and anthraquinones. Their activity is increased by halogenation and they are used as agricultural fungicides (chloranil and dichlone). The effects of anthraquinone derivatives on plasmid DNA have been recently studied by Morier-Teissier *et al* (1992). In addition, the potential carcinogenic hazard of anthraquinones has been evaluated by structure-mutagenic activity experiments on *Salmonella* strains (Krivobok *et al.*, 1992). Their virucidal activity has also been assessed, demonstrating that these compounds are capable of disrupting the virus envelope (Sydiskis *et al.*, 1991). However, adenovirus and rhinovirus particles are not inactivated (Sydiskis *et al.*, 1991; Andersen *et al.*, 1991). Napththoquinones have been studied for their synergic effect when combined with antibiotics (Didry *et al.*, 1992), for their antimutagenic activity (Durga *et al.*, 1992) and for their ability to intercept electrons from NADH dehydrogenase (Imlay and Fridovich, 1992).
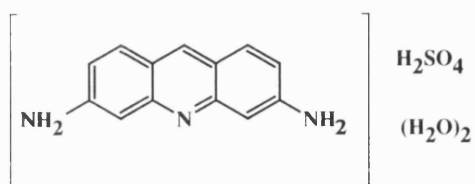
Proflavine hemisulfate

(3,6- Diaminoacridine hemisulfate)
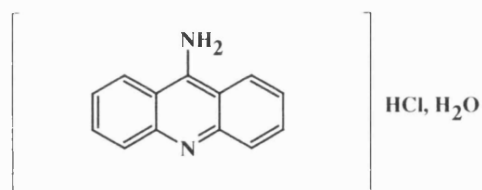
Aminacrine hydrochloride

(9- Aminoacridine hydrochloride)

Figure 6. Acridines.
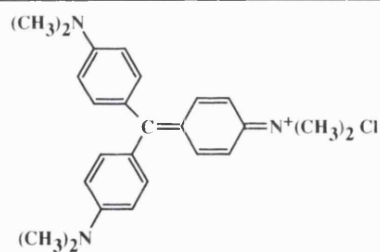
Crystal violet
(Methyl violet; Gentian violet)
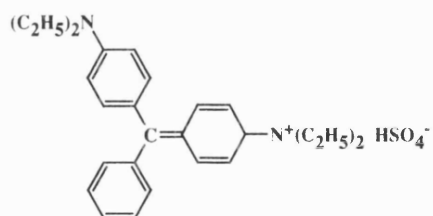
Brilliant green

Figure 7. Antimicrobial dyes.

1.2.8 Halogens

Iodine, chlorine, bromine, fluorine, iodophors, chloroform and chlorine-releasing (Figure 9) compounds are included here. Initially, halogens were thought to be highly reactive, binding readily to organic compounds, but their antibacterial action is now known to be achieved in a more complex way. In aqueous solutions hypohalogenated acids penetrate the bacterial cell wall as neutral molecules and react with the sulfhydryl groups of enzymes (Hugo and Russell, 1992).

Iodine has a rapid lethal effect against bacteria and their spores, yeasts, moulds and viruses. Aqueous or alcoholic solutions of this halogen are commonly used, which at high concentrations is little affected by organic matter. The toxicity of iodine solutions and its unsuitable property of staining fabrics led to the introduction of iodophors. Here, iodine is solubilized by a surface-active agent but the germicidal action is retained (Blatt and Maloney, 1961), depending solely on the concentration of free iodine (Allawala and Riegelman, 1953; Elek, 1968; Williams and Russell, 1991). The efficacy of iodophors as antiseptics or disinfectants depends on concentration; when low they are not sporicidal (Gershenfeld, 1962; Favero, 1985). Iodophores are widely used in the dairy industry and for skin and wound disinfection.

Chlorine is present in hypochlorites and chlorine-releasing N-chloro compounds; their antibacterial spectrum is wide, but activity is said to be slower with the latter. The activity of hypochlorites depends greatly on pH and on the presence of organic matter. Sporicidal activity is enhanced by low concentrations of sodium hydroxide or ammonia (Weber and Levine, 1944; Russell, 1982).

N-chloro compounds comprise a wide list of substances such as chloramine-T, dichloramine-T, halazone and dichloroisocyanuric acid. Chlorine is released by hydrolysis in water and activity can be increased by decreasing pH (Cousins and Allan, 1967).

Uses of chlorine-releasing compounds are confined to certain medical treatments, such as wound disinfection, irrigations of bladder and vaginal infections with, for example, Dakin's Solution or chlorinated lime and boric acid solution. Chlorine gas is used for the disinfection of public water supplies, and sodium hypochlorite in swimming pools and for decontaminating blood spillages containing human immunodeficiency virus or hepatitis B virus.

Chloroform, has been used as a preservative for more than a century; it is bactericidal, but not sporicidal. Its high volatility can result in microbial growth due to a decrease in concentration. It is used in aqueous solutions at a concentration of 0.2% as preservative for aqueous extracts of plant and animal tissues (BPC, 1973) and in mixtures including insoluble powders (Lynch and Wilson, 1977). It has however, been totally banned in certain countries, for example The United States. At present, its use is allowed in the UK in oral pharmaceutical products at concentrations not exceeding 0.5% and in cosmetic products, a maximum of 4%, restricted only to toothpaste (Hugo and Russell, 1992).

1.2.9 Quinoline and isoquinoline derivatives

These include 8-hydroxyquinoline, 4-aminoquinaldinium (Figure 10) and isoquinoline derivatives. The 8-hydroxyquinolines possess more antibacterial activity against Gram-positive than Gram-negative bacteria. Antifungal activity is also present, but the rate at which it develops is slow. These compounds are generally used in topical preparations (Jaffe et al., 1989; Minnich et al., 1991) due to their low water-solubilities.
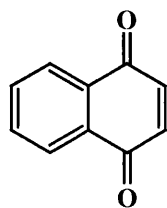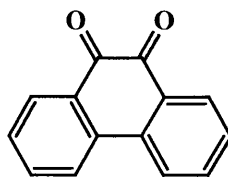
1,4-Naphthoquinone   9,10- phenanthrenequinone
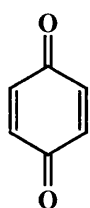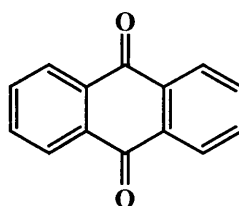
1,4- Benzoquinone   9,10- Anthraquinone

Figure 8. Quinones.
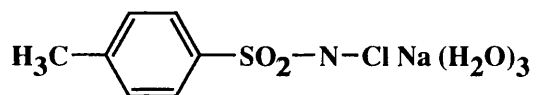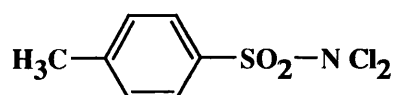
Chloramine T   Dichloramine T
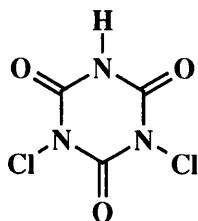
Dichloroisocyanuric acid

Figure 9. Chlorine-releasing compounds.

They have the distinctive characteristic of acting as chelating agents, for example 8-hydroxyquinoline with iron (Elek, 1968).

The 4-aminoquinaldinium derivatives are quaternary ammonium compounds. Important members are laurolinium acetate and dequalinium chloride. These possess activity mainly against Gram-positive bacteria (Cox and D'Arcy, 1962) and also, against many species of yeasts and fungi (Frier, 1971). Dequalinium chloride is widely used in lozenges or in paints for mouth and throat infections.

Hedaquinium chloride is the most important isoquinoline derivative. It possesses antibacterial and antifungal activity and is said to be the most active antifungal quaternary ammonium compound (D'Arcy, 1971).

1.2.10 Alcohols

These compounds are bactericidal to vegetative organisms, including acid-fast bacilli, but almost non-lethal to spores and have poor activity against many viruses. Alcohols (Figure 11) denature proteins and their biocidal activity increases with increasing chain length up to a maximum of 5-8 carbon atoms.

Ethanol, along with all other alcohols, requires the presence of water for activity; its most effective aqueous concentration is 60-70% (Price, 1950; Croshaw, 1977). Solutions of iodine or chlorhexidine in 70% ethanol are employed for pre-operative disinfection of the skin. Methyl alcohol or methanol has poor antibacterial activity and is not sporicidal. Although, mixtures of sodium hypochlorite with this alcohol are highly sporicidal (Coates and Death, 1978).

Isopropanol and n-propyl alcohol are more effective germicides than ethanol (Kelsey and Maurer, 1972), but are not sporicidal. Isopropanol is used in cosmetics as an

alternative to ethanol, either as a solvent or preservative. Benzyl alcohol, apart from its activity against moulds (D'Arcy, 1971), and Gram-positive and Gram-negative bacteria, is also a weak local anaesthetic. Phenylethyl alcohol is an antimicrobial with activity against Gram-negative organisms (Lilley and Brewer, 1953) and its use is recommended in ophthalmic solutions in conjuction with another bactericide (Richards and McBride, 1973a,b; Denyer *et al.*, 1985). It also inhibits initiation of DNA replication at very high concentrations (Lark and Lark, 1966).

Bronopol and phenoxyethanol are effective against *P. aeruginosa*, with the former being able to oxidize thiol groups to disulphides in bacteria (Hugo, 1992). Chlorbutanol is antibacterial and antifungal, but it is not widely used due to its instability. Finally, 2,4-dichlorobenzyl alcohol is soluble in water (up to 1%) and readily soluble in alcohols. It is active over a wide pH range and has a broad spectrum of activity, even though, pseudomonads and *S. aureus* show some resistance (Toler, 1985).

1.2.11 Oxidizing agents

Hydrogen peroxide can be obtained as a 20- or 10-volume solution, the numbers indicating the volume of oxygen evolved from 1 volume of the peroxide solution. This compound is bactericidal and sporicidal, it evolves oxygen when in contact with living tissue and many metals (Russell, 1982, 1990, 1991). It is believed to cause DNA strand breakage due to the generation of free hydroxyl radicals.

Peracetic acid is commercially available as a 15% aqueous solution in which it exists in equilibrium with its decomposition products, acetic acid and hydrogen peroxide. The activity spectrum of this compound is broad, including bacteria and their spores, fungi, moulds, yeasts, algae and viruses. The leading uses of peracetic acid are in the food

industry, for disinfecting sewage sludge (Hugo and Russell, 1992), as a hospital disinfectant (Melichercikova, 1989; Dusart *et al.*, 1992) and currently, as a preventive of surgical infections (Turcic *et al.*, 1989). Recently, combinations of peracetic acid and hydrogen peroxide have been shown to be synergic (Alasri *et al.*, 1992, 1993).

1.2.12 Chelating agents

Chelation is another mode of action of some antibacterial agents. Metal ions which are vital constituents of the cell surface and various enzymes, may be removed by chelating agents, therefore disrupting the normal functions of a microorganism. Chelating agents can be harmless by themselves, but are able to function in two ways: by potentiating the antibacterial effect of other bactericides, for example benzalkonium chloride with EDTA (Richards and Cavill, 1976) or by forming a lethal complex with a bacterial trace metal, i.e. 8-hydroxyquinoline with iron (Elek, 1968).

EDTA is used to potentiate the activity of antibacterial agents against Gram-negative organisms. This enhancement may be related to its chelating properties, as it induces a non-specific increase in permeability (Leive, 1974), which allows better penetration of unrelated compounds. The lytic action and synergistic effect with antibacterial agents involves removal of Ca^{+2} or Mg^{+2} ions, or both, from the cell membrane (Brown and Richards, 1965; Richards and Cavill, 1976). On the other hand, although synergism appears to be the rule, recent reports demonstrate a protective effect on *E. coli* exposed to paraquat (Minakami *et al.*, 1990).

Chelating agents based on EDTA are ethylenedioxybis[ethyliminodi(acetic acid)] (EGTA), N-hydroxyethylethylenediamine-N,N'N'-triacetic acid (HDTA), trans-1,2-diaminocyclohexane-NNN'-tetraacetic acid (CDTA), iminodiacetic acid (IDA) and

NH$_2$

CH$_3$ CH$_3$

Cl$^-$

(CH$_2$)$_{10}$

Dequalinium chloride

NH$_2$

CH$_3$COO$^-$

CH$_3$

(CH$_2$)$_{11}$CH$_3$
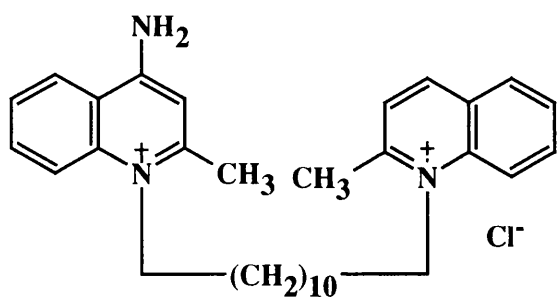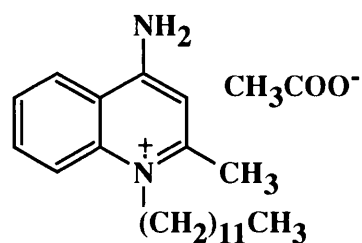
Laurolinium acetate

Figure 10. Derivatives fo 4-aminoquinaldinium.

CH$_2$—CH$_2$—OH

2- Phenylethanol

CH$_2$—OH

Benzyl alcohol

O—CH$_2$—CH$_2$—OH
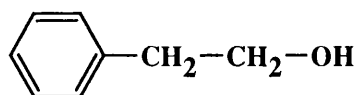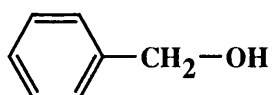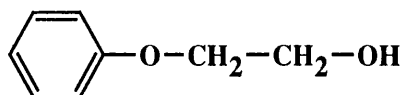
2- Phenoxyethanol

Br

OH—H$_2$C—C—CH$_2$OH

NO$_2$
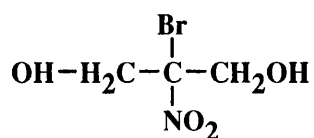
Bronopol

Figure 11. Alcohols.

nitrilotriacetic acid (NTA). These have, in some instances, certain advantages over the parent compound. CDTA has being found to be the most toxic to *P. aeruginosa* and other Gram-negative organisms (Roberts *et al.*, 1970; Haque and Russell, 1976). EGTA forms stronger complexes with calcium, barium and mercury, than does EDTA, but for most other metals, complexation is weaker (West, 1969). However, HDTA is less effective for disinfection purposes than the parent compound as the complexes formed are not stable (Haque and Russell, 1976), and IDA and NTA, although exhibiting little intrinsic activity against *P. aeruginosa*, are able to potentiate the effects of other agents against this organism.

Other chelating agents, apart from EDTA and related compounds, are lactate, citrate, 2-(N,N,N-trialkylammonio)alkyl hydrogen phosphates (Tsubone, 1991) and acetylacetone. Recently, these compounds have been proposed as anticaries agents, due to their ability to prevent sucrose-dependent adhesion of decay-causing organisms (Lu-Lu *et al.*, 1992), a phenomenon which is known to require divalent cations (Kelstrup and Funder-Nielsen, 1974; McCabe *et al.*, 1976).

1.2.13 Permeabilizers

These agents increase the permeability of the outer envelope of Gram-negative cells. Examples of these compounds are chelating agents, polycations, lactoferrin and transferrin. Polycations induce lipopolyssacharide (LPS) release from the outer membrane of Gram-negative bacteria; one such compounds is poly-L-lysine or PLL. Organisms treated with this agent show increased sensitivity to hydrophobic antibiotics (Viljanen, 1987; Vaara, 1990).

Lactoferrin induces partial LPS loss from the outer membrane of Gram-negative bacteria (Ellison *et al.*, 1988) and transferrin is believed to have a similar effect, both acting as chelating agents. Experiments performed to determine the bacteriostatic activity of transferrins on *Legionella spp.* demonstrated that activity depended on the iron-free state of the molecule, and that there was no bactericidal effect at concentrations up to four times the minimal bacteriostatic concentration (Goldoni *et al.*, 1991).

1.2.14 Heavy-metal compounds

Heavy metal derivatives (Figure 12) can exert activity in their own right, or combine with another agent to become active. Included in this category are copper, silver, mercury and tin compounds.

Copper is thought to act by the poisoning effect of the copper (II) ion on thiol enzymes and possibly other thiol groups in microbial cells (Hugo and Russell, 1992). These compounds are used as algicides and fungicides. Copper metal in powder form is added to cements and concretes to inhibit microbial attack. Cotton fabrics have been successfully preserved with copper complexes, for example copper naphthenate and copper-7-hydroxyquinolate. Wood, paint, paper and timber are also preserved with copper compounds. Copper complexes with symmetrical triazine hydrazones are being tested as promising antiviral agents (Tomas *et al.*, 1991; Popescu *et al.*, 1992).

Silver compounds have the disadvantage of precipitating protein molecules, but this has been overcome by incorporating the ion into a high molecular weight polymer such as gelatin or albumen. The water-soluble adduct so produced slowly releases silver, and the astringent effect is eliminated. In filters for water purification, metallic silver is coated onto sand (Katadyn silver), maintaining a sufficient concetration to inhibit

microbial growth (oligodynamic action). Recent experiments showed that electrochemical Ag^+ solutions exhibited better antimicrobial effectiveness against bacteria, yeasts and mould than silver solutions from inorganic salts, indicating that the former could be used successfully in preservative preparations (Simonetti *et al.*, 1992).

Mercury and organomercurials are lethal to living organisms because of their solubility in lipids and their ability to form covalent bonds with sulfhydryl groups present in enzymes and membrane proteins (Lyon and Skurray, 1987). Derivatives of mercury, such as mercurochrome, nitromersol, thiomersal and phenylmercuric nitrate are used as bacteriostatic and fungistatic agents, and also as preservatives and bactericides in injections. Until recently, mercury salts such as stearates and oleates were extensively used as wood, paint, leather and textiles preservatives, but concern about the use of mercury, because of its effects on environmetal pollution, is increasing.

Thiomersal is employed in concentrations of 0.01-0.02% as a preservative of bacterial and viral vaccines (BPC, 1973). Phenylmercuric nitrate is used as a bactericide in multi-dose containers of parenteral injections and as a preservative for eye-drops at 0.002% w/v (Brown and Anderson, 1968).

Tin (IV) oxide was initially used in the treatment of superficial staphylococcal infections. This metal is much less toxic than the previous ones and is used to coat cans and vessels used to prepare and store food, or boil water. Oxidative phosphorylation is affected by organotin compounds (Aldrigde and Threlfall, 1961); they also act as ionophores for anions (Hugo and Russell, 1992). The effect of stannous fluoride on the periodontal microflora was recently tested and proved to be bactericidal, a 99% reduction of the microflora was achieved (Oosterwaal *et al.*, 1991). Tributyltin oxide, triphenyltin acetate and tributyltin fluoride are some examples of organotin compounds which have

been used as bactericides and fungicides and as textile and wood preservatives. Environmental awareness has resulted in the use of tributyltin being restricted in its use as an antifouling agent on ships (Evans and Clarkson, 1993).

Recent studies involving the use of osmium and gold as topical agents against Gram-positive and anaerobic species showed they were effective against methicillin-resistant and methicillin-susceptible *S. aureus* strains, with MICs ranging from 0.5 to 2.0 µg/ml (Chin and Neu, 1992).

1.2.15 Anilides

Anilides comprise two major derivatives: salicylanilide and carbanilide, or diphenylurea (Figure 13). These compounds are bacteriostatic, having the ability to discharge part of the protonmotive force, thereby inhibiting processes dependent upon it, such as energy metabolism and active transport (Terada *et al.*, 1988).

Although salicylanilide is an effective bacteriostatic agent, attempts have been made to increase its activity with the introduction of halogen atoms (Lemaire *et al.*, 1961). Halogenation increased activity, but photosensitization became a problem. Therefore, the use of tribromo and tetrachloro salicylanilides are restricted in any situation in which they may come in contact with skin (Hugo and Russell, 1992).

Carbanilides inhibit the growth of many Gram-positive bacteria, but in contrast to salicylanilide, its activity against fungi is low (Beaver *et al.*, 1957). Although this agent is present as the active ingredient in some disinfectant soaps, its use in other topical preparations is restricted because of photosensitization.

1.2.16 Vapour-phase disinfectants

Gases and vapours are used for sterilizing the atmosphere and for objects that cannot be appropriately sterilized by heat or chemical treatments, such as the metal or plastic surfaces of a variety of equipment, and plastic catheters and syringes (Anon., 1989). In general, only surface sterilization occurs. Many gases have been used for this purpose such as β-propiolactone, methylbromide and propylene oxide, but the most extensively employed are ethylene oxide and formaldehyde.

All types of microorganisms, including spores, are inactivated by ethylene oxide (Phillips, 1977). Its mechanism of action is influenced by concentration, temperature, duration of exposure and humidity (Christensen, 1964; Dadd and Daley, 1980, 1982). Ethylene oxide reacts with proteins and amino acids, and with the nucleic acid guanine, the alkylation of phosphated guanine being the possible reason for its activity (Michael and Stumbo, 1970). Ethylene oxide has two advantages over formaldehyde these are the ability to penetrate complex structures and better performance at ambient temperatures (Christensen and Kristensen, 1992).

Formaldehyde vapour is employed as a disinfectant in poultry houses (Nicholls et al., 1967; Anon., 1970), in the hatchery (Harry, 1963) and in safety cabinets. Factors influencing its activity are the same as those mentioned for ethylene oxide, but with the disadvantage of polymerization at temperatures below 80°C, which makes it less suitable for large-scale sterilization of medical supplies. Other sources of formaldehyde are the formaldehyde releasing agents, such as paraformaldehyde (Tulis, 1973), melamine formaldehyde and urea formaldehyde (Russell, 1976). Formaldehyde is also an alkylating agent (Grossman et al., 1961).
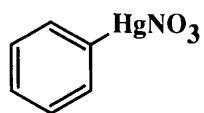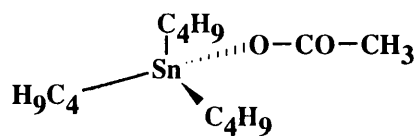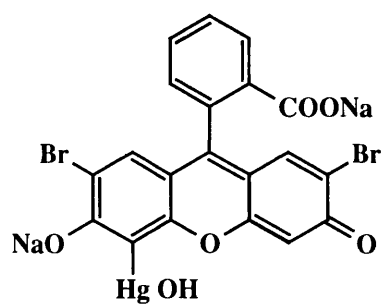
Figure 12. Heavy metal compounds.
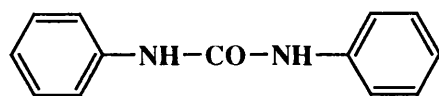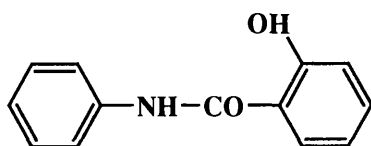
Figure 13. Anilides.

1.2.17 Miscellaneous preservatives

This section includes agents which are relatively new as preservatives and which differ in chemical structure (Figure 14), although derived from some of the compounds discussed previously.

1.2.17.1 Polymeric biguanides.

A polymer of hexamethylene biguanide is used as a cleansing agent in the food industry, under the trade name of Vantocil 1B (Davies *et al.*, 1968). It is capable of inhibiting bacterial growth at between 5 and 25 µg/ml, with *P. aeruginosa* and *P. vulgaris* requiring 10 times these concentrations. This compound damages the bacterial membrane which in turn leads to loss of essential cellular components (Broxton *et al.*, 1984). Polyhexamethylene biguanide is also emerging as a promising agent in the treatment of keratitis caused by *Acanthamoeba keratitis* (Larkin *et al.*, 1992).

1.2.17.2 Derivatives of 1,3-dioxane.

Dioxin is active against a wide range of microorganisms, with inhibitory concentrations varying from 300 to 2500 µg/ml (Anon., 1962). Changes in pH and the presence of nonionic surface-active agents do not alter its efficacy. Its activity is thought to be due to the production of aldehyde by hydrolysis in aqueous solution. Bronidox is a nitrobromo derivative of dioxane, which probably acts by oxidizing -SH to -S-S- groups in essential enzymes. It is active against bacteria and fungi including pseudomonads (Hugo and Russell, 1992), and has been recommended as a preservative for toiletries.

1.2.17.3 Derivatives of imidazole.

Far back in 1946, imidazolines were used as agricultural fungicides; more recently, derivatives carrying the imidazole ring have found application as preservatives. Dantoin [1,3-di(hydroxymethyl)-5,5-dimethylhydantoin] retains activity over a wide range of pH and is compatible with most ingredients used in

cosmetics. Its spectrum of activity against bacteria and fungi (Rai *et al.*, 1992) is wide and its mechanism of action is attributed to its ability to release formaldehyde. The rate of release increases at pH values between 9 and 10.5 (Schanno *et al.*, 1980). Germall 115 (imidazolidinyl urea), is intrinsically more active against bacteria than fungi, and can act synergistically with other preservatives (Berke and Rosen, 1980); most of the microbiological data accumulated for this compound is based on challenge tests in cosmetic formulations (Berke and Rosen, 1978). A new nitroimidazole derivative (5-nitro-1-methyl-imidazolyl-2-hydroxy-3 terbutylphenyl carbinol) has shown moderate activity against Gram-positive and Gram-negative organisms, with a similar inhibitory activity as metronidazole against *Trichomonas vaginalis* (Dubini *et al.*, 1992).

1.2.17.4 Isothiazolones. These compounds are commercially available as suspensions and are used as preservatives of industrial emulsions, adhesives, polishes, glues, paper products and cutting oils. Proxel CRL (1,2-benzisothiazolin-3-one) and Skane M8 (2-n-octyl-4-isothiazolin-3-one) are employed in industry and are not recommended for cosmetic or medicinal use as they show eye and skin irritancy. On the other hand, Kathon 886 MW (a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one), which does not show a pseudomonas gap, has been suggested as a preservative for cosmetic products (Hugo and Russell, 1992).

1.2.17.5 Derivatives of hexamine. Hexamine had been used as a urinary tract disinfectant since 1894. It acts by the slow release of formaldehyde. Chemical modification of this compound resulted in Dowicil 200 (Scott and Wolf, 1962). This is a derivative of hexamine quaternized with cis-1,3-dichloropropene, which is active against bacteria and fungi. It is recommended as a preservative for cosmetic preparations as it

is not inactivated by the majority of ingredients used in cosmetic manufacture (Rossmore and Sondossi, 1988).

1.2.17.6 Triazines. Bactocide® and Grotan® are derived from the condensation of formaldehyde and ethylamine and ethanolamine, respectively. Both have antibacterial and antifungal activity, but, fungal superinfection is likely with Grotan®, making it necessary to include an additional antifungal compound (Rossmore *et al.*, 1972; Paulus, 1976). Sugai *et al* (1991) studied the inhibition of cell separation by a triazine dye in Gram-positive bacteria. They observed elongation of individual cells into filaments.

1.2.17.7 Oxazolo-oxazoles. This series of derivatives is obtained by reacting formaldehyde with tris (hydroxymethyl) methylamine. Its use is recommended for the preservation of cutting oils, water treatment plants, emulsion paints and industrial slurries, among other. As it is a formaldehyde releaser it is irritant to eyes and skin (Hugo and Russell, 1992).

1.2.17.8 Methylene bisthiocyanate. This compound is recommended for the control of slime in paper manufacture (as an alternative to mercurials) and, although a skin and eye irritant, its toxicity is low enough to be used in the manufacture of paper for the packaging of foodstuffs (Hugo and Russell, 1992).

1.2.17.9 Captan. This carboximide is active against Gram-positive and Gram-negative bacteria, yeasts and moulds. It blocks the binding of RNA polymerase to DNA, but does not inhibit the enzyme's activity once it is in the process of polymerization (Luo and Lewis, 1992). It has been used in the treatment of skin infections and also to prevent spoilage of stored fruit (Hugo and Russell, 1992). However its carcinogenic potential is being reassessed, due to findings of increased incidence of malignant tumors in laboratory animals (Quest *et al.*, 1993).

1.2.17.10 Essential oils. Essential oils of various plant species have been tested for antibacterial activity against many microorganisms with promising results (Cruz *et al.*, 1989; Barel *et al.*, 1991; Gundidza *et al.*, 1992; Lopez-García *et al.*, 1992; Hammerschmidt *et al.*, 1993). However, some volatile oils show activity only against Gram-positive bacteria (Langezaal *et al.*, 1992). Antimicrobial effect has unusually, been reported to be enhanced by a decrease in temperature (Moleyar and Narasimham, 1992). Amoebicidal activity has also been observed (DeBlasi *et al.*, 1990). Because of their natural origins, these compounds are now being reassessed for use as preservatives (Deans and Ritchie, 1987).
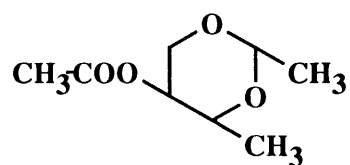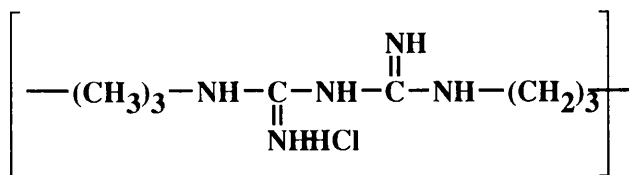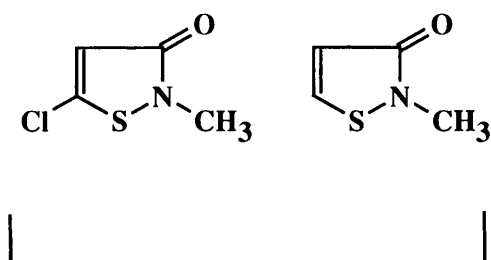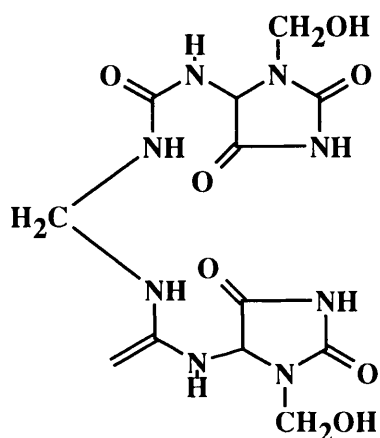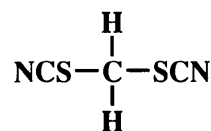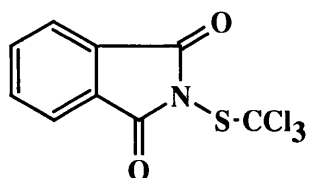
Figure 14. Miscellaneous preservatives.

1.3 Resistance

1.3.1 Inhibition of preservatives by excipients or containers

The manufacturing process of any liquid or semisolid pharmaceutical dosage form may require the incorporation of a preservative to kill contaminating microorganisms. However, the antibacterial activity of these compounds may be hampered by a number of reasons: insufficient concentration of preservative in the aqueous phase of the product, interaction with excipients such as the container, surfactants, colloidal materials or salts, the pH of the formulation, or the presence of resistant bacterial species (Gilbert, 1988).

Before the late 1930s, all cosmetic and pharmaceutical emulsions were stabilized with soaps. The high pH of these formulations made them unfavourable to microbial growth. Microbial spoilage was therefore a rare problem (Wedderburn, 1964). A wider range of emulsifiers and other excipients came into use after the Second World War, which resulted in new preservation difficulties. Incompatibilities arose between the new materials and the conventional preservatives. Reports showed, (1) the inactivation of parabens by surface active agents and vegetable gums, (2) interactions between polysorbate 80 and methyl and propyl parabens, (3) molecular complex formation when parabens were mixed with polyethylene glycol, methylcellulose, polyvinylpirrolidone or gelatin, (4) interaction of sorbic acid with polysorbate, but not with polyethylene glycols, (5) reduced activity of quaternary ammonium compounds when used in conjunction with polysorbate and (6) benzyl alcohol adsorption by certain types of rubber stoppers (Patel and Kostenbauder, 1958; Pisano and Kostenbauder, 1959; Blaug and Ahsan, 1961; Ravin, 1985).

Apart from these incompatibilities, interactions with containers were also observed. Glass is an inert impermeable material capable of interacting with preservatives by surface

adsorption phenomena, although a significant decline in concentration would only be significant if the surface exposed was considerable (Dempsey, 1988). Preservative inactivation has, however been demonstrated with increased bacterial survival being directly proportional to the area of glass surface (Zobell and Anderson, 1936; Zobell, 1943; Gwynn *et al.*, 1981; Hugo *et al.*, 1986; Dempsey, 1988; Lu-Lu *et al.*, 1992). Preservative inactivation by, or even diffusion through, non-glass containers is also well documented in the works of Dempsey (1988), Kurup *et al* (1991,1992), Pons *et al* (1992) and Oehring (1992).

All of the preceeding factors may contribute to lowering the active concentration of preservative present in a pharmaceutical formulation. However, bacterial resistance to preservatives is our major interest here and will be considered in more detail. Although, endless modifications have been carried out to parent preservative nucleii in pursuit of more active structures, the preservatives currently employed in medicines, toiletries and edibles comprise a very narrow list. The continuous exposure of the microbial population to a small number of antimicrobials contributes greatly to the undesirable problem of resistance, which may be of two types, natural (intrinsic) or acquired.

1.3.2 Natural or intrinsic resistance

Natural resistance involves the cell envelope, which protects the organism from change in the environment. Recent studies assessing the mechanism of resistance of *S. marcescens* and *Pseudomonas cepacia* to chlorhexidine, demonstrated that the outer membrane composition plays an important role in resistance mechanisms (Ohta, 1990).

1.3.2.1 Slime layer or capsule (glycocalyx)

The external layers of many bacteria are dispensable extracellular slime layers or capsules (glycocalyx). The presence of these layers, which consist in general of carbohydrates, is subject to the conditions of the culture. With the aid of the glycocalyx, bacterial cells can aggregate as microcolonies and biofilms. In the case of biofilms, the glycocalyx occurs as a diffuse slime surrounding a huge number of microbial cells. This material confers advantages upon the cells with respect to colonization of surfaces and resistance to dessication (Costerton et al., 1978). The glycocalyx forms an additional barrier to the entry of a drug into the microbial cell, functioning as an ion exchange column, and removing strongly-charged molecules from solutions as they pass through it (Wagman et al., 1975; Gilbert, 1988; Brown and Gilbert, 1993).

Biofilms are responsible for contamination in manufacturing plants, allowing organisms to endure in water storage tanks (Dawson, 1973), cooling towers, air conditioners and sinks (Marrie and Costerton, 1984; Nickel et al., 1985) and increasing the level of resistance to antimicrobial agents (Ruseska et al., 1982; Costerton and Lashen, 1984). As an example, pseudomonads isolated as a biofilm in pipework used in the manufacture of povidone-iodine antiseptic (Bond et al., 1983) caused an outburst of bacteraemias after the use of the contaminated product (Craven et al., 1981; Anderson et al., 1983).

1.3.2.2 Cell envelope

The cell envelope is responsible for the existence of the two main divisions of bacterial cells: Gram-positive and Gram-negative. This differential staining is caused by the ability of the cell wall to retain a basic dye (crystal violet) after fixation with iodine

and washing with alcohol (Salton, 1963). Removal of the cell wall after staining, but prior to washing with alcohol, demonstrated that Gram-positive organisms can be decolorized (Beveridge and Davies, 1983). However, the reason why the Gram-positive cell wall blocks the extraction of the dye is still unclear (Anon., 1989). The outer membrane, present in Gram-negative organisms, and the peptidoglycan matrix, present in both groups, function as molecular sieves, preventing the access of large hydrophobic molecules to the cell cytoplasm. These structural differences in the cell wall composition are responsible for the greater involvement of Gram-negative organisms in manufactured product spoilage (Hugo, 1990).

The Gram-positive cell wall consits of a highly cross-linked network of peptidoglycan with teichoic and teichuronic acids incorporated into it (Gerhardt and Judge, 1964). Polysaccharide chains of both acids radiate to the outside and contribute to the net negative charge of the bacterial cell surface. This prevents the access of hydrophobic agents to the cytoplasm and favours both the uptake of cationic nutrients and the binding of cationic antimicrobial agents (Meers and Tempest, 1970).

Thickness and extent of cross-linking are the factors that determine how efficiently the cell wall acts as a molecular sieve. The size exclusion limit of the peptidoglycan matrix is 100,000 daltons which may be significantly decreased by the presence of charged species (Marquis, 1968), as was shown by Nadir and Gilbert (1980) when studying the effect of potassium ions and other cations on the bactericidal activity of chlorhexidine.

Because they lack an outer membrane, Gram-positive organisms can only rely on size exclusion as a natural protection against adverse environmental conditions. Hence, they

are more susceptible to biocidal attack and are less frequently found as contaminants in preserved cosmetics, pharmaceuticals and toiletries (Leech, 1990).

The Gram-negative cell wall is more complex and there are many reviews detailing with the molecular composition of the Gram-negative cell envelope (Costerton, 1977; Nikaido and Nakae, 1979; Lugtenberg and Van Alphen, 1983). It is an asymmetric membrane bilayer, which consists of lipopolysaccharide and phospholipid, attached via lipoproteins and porin proteins to a thin matrix of peptidoglycan (Nikaido and Nakae, 1979; Osborn and Wu, 1980). The lipopolysaccharide layer has the hydrophilic chains oriented outwards into the surrounding medium with the lipophilic region oriented into the phospholipid layer. This packaging restricts dissolution and diffusion of hydrophobic drugs, inwards and outwards (Gilbert, 1988).

Lipoproteins and divalent cation bridges are responsible for maintaining the outer membrane-peptidoglycan complex (De Pamphilus, 1971; Schnaitman, 1971a,b; Costerton *et al.*, 1974). The synergic effects observed when EDTA is used in conjunction with other antibacterial agents is explained by the removal of these cations (Brown and Richards, 1965; Richards and Cavill, 1976).

1.3.3 Acquired resistance

Acquired resistance results from genetic changes in a bacterial cell and originates either by mutation or by the acquisition of genetic material from another cell (Russell and Gould, 1988). When antibiotic resistance was first encountered among bacteria, it was believed to arise exclusively by mutation and selection. In the laboratory, spontaneous bacterial mutants resistant to certain antibiotics may be selected at frequencies of 10^{-6} to 10^{-8} per cell, therefore it was assumed that resistant organisms developed from natural

populations by an analogous event. Indeed, resistance to several therapeutically useful antibiotics, including rifampin, fusidic acid, and novobiocin, is thought to be derived by chromosomal mutation (Richmond, 1972).

However, antibiotic resistant mutants are frequently less virulent. Therefore, the acquisition of new characters, such as resistance, without affecting the ability of the organism to survive in its natural environment, would have taken a prolonged period of time. Hence, the mechanism of mutation did not offer a satisfactory explanation for the rapid emergence of multi-resistant bacteria.

1.3.3.1 Gene transfer

The discovery of gene transfer and the demonstration that bacteria could acquire additional genetic material in the form of extrachromosomal, or plasmid, deoxyribonucleic acid (DNA), explained the relatively minor role played by spontaneous mutations in the clinical appearance of antibiotic resistant microorganisms. The existence of plasmid DNA molecules was suggested by the transfer, at the same time, of several genetic units of resistance between bacterial strains, and the irreversible loss of such units from cells at relatively high frequencies (Lyon and Skurray, 1987). This acquisition of new genetic material was observed to take place by three different mechanisms (1) transfomation, (2) conjugation and (3) transduction.

1.3.3.1.1 Transformation

During transformation a bacterial cell takes up a fragment of naked DNA from its environment. This process was discovered when heat-killed cells of *Streptococcus pneumoniae* were mixed with live cells of a non-virulent strain and these later became

virulent. Transformation requires a competent cell or one that can bind the incoming DNA to its surface. Expression of the genes carried by the transforming DNA is only possible if they are incorporated into the chromosome of the cell. Incorporation is achieved by the process of recombination, which involves the replacement of part of one strand of the recipient's chromosome with part or all of the strand from the incoming DNA (Hotchkiss and Gabor, 1970).

1.3.3.1.2. Conjugation

This process involves the transfer of DNA from one cell to another while both cells are in physical contact (Brinton, 1965). Conjugation has been largely studied in Gram-negative organisms, however it also occurs in Gram-positive bacteria. Plasmids play a major role in this type of gene transfer process.

They encode the production of donor cell characteristics, such as the formation of appendages called sex pili. When donor and recipient cells are mixed, contact is made via the pilus, thus bringing the cells into contact. Then one strand of the double-stranded plasmid DNA is transferred to the recipient, with DNA replication taking place in both donor and recipient cells.

Bacterial plasmids code for the production of toxins, metabolic enzymes or increased resistance to antibacterials. The latter are referred to as R plasmids (R for resistance). Conjugative plasmids, or plasmids that can transfer copies of themselves by conjugation, have been found in many different groups of Gram-negative bacteria, and in some Gram-positive genera, including *Streptococcus*, *Staphylococcus*, *Streptomyces*, *Clostridium* and *Bacillus* (Kimber, 1986; Archer and Scott, 1991).

1.3.3.1.3 Transduction

This process involves the transfer of bacterial genes from one cell to another by means of bacteriophages. There are two types of transduction, generalized and specialized.

In generalized transduction any of the genes present in the bacterium may be transferred to another, due to the fact that during phage assembly double-stranded chromosomal or plasmid DNA may be incorporated into the phage head. Eventually, when the phage progeny is released, the bacterial DNA, and not the phage DNA, is injected into the new recipient.

Specialized transduction involves a temperate phage, such as lambda from *E. coli*. When lysogenic cells are induced, the prophage leaves the chromosome, sometimes carrying with it the *gal* or *bio* genes that flanks its chromosomal integration site. Therefore, a small proportion of progeny phages are assembled with *gal* or *bio* and remaining genes from the phage (Ozeki and Ikeda, 1968). Generalized transduction is thus capable of transferring resistance genes between bacteria, whereas such transfer by specialized transduction depends upon the site of integration of the transducing particle.

1.3.3.2 Plasmid-mediated resistance mechanisms

During the last three decades, evidence has accumulated indicating that bacterial, resistance to antimicrobial agents is due, in many instances, to the presence of plasmids that carry genetic determinants of resistance.

Conjugative R plasmids were discovered in Japan in 1957 (Watanabe, 1972) during an outbreak of dysentery due to *Shigella* spp. Antibiotics were available without prescription and indiscriminately consumed by the population. This focused the attention

of researchers who observed that at the early stages of the outbreak the *Shigella* population was sensitive to antibiotics, but resistant towards the end (Richmond, 1972). Eventually, the multiply resistant strain of *Shigella* became the predominant type in Japan. Further studies revealed that multiply resistant strains of *E. coli* were sometimes isolated from patients excreting resistant *Shigella*, which lead Akiba and Ochiai in 1959 to suggest resistance had been transferred between the two species by conjugation (Kimber, 1986).

Subsequently, R plasmids were found in different strains around the world, occuring in Gram-positive and Gram-negative organisms and in almost all pathogenic species. Plasmids carried by these organisms may increase resistance towards a given antibacterial agent by (1) altering the target site, (2) producing structural modifications leading to inactivation of the compound, (3) producing an increased efflux of the toxic substance or (4) specifying an enzyme which is a substitute of the one targeted by the antibacterial (Foster, 1983).

1.3.3.2.1 Alteration of target sites

Resistance to erythromycin and lincomycin is brought about by a modification of the target site. Plasmids code for enzymes which produce an N^6-dimethylation of an adenine residue in the 23S RNA of the ribosome, preventing the binding of erythromycin and lincomycin to their target (Lai *et al.*, 1973). *Streptomyces erythreus*, the organism which produces erythromycin, has dimethylated 23S RNA to protect itself from its own toxic product.

1.3.3.2.2 Structural modifications leading to inactivation of antibacterial compounds

There are many plasmid-mediated resistance mechanisms which rely on structural

modifications of the toxic substance to hinder its lethal effect and included here are resistances to chloramphenicol, β-lactam antibiotics, such as penicillins and cephalosporins, organomercurials and aminoglycosides.

1.3.3.2.2.1 Resistance to chloramphenicol. Chloramphenicol binds to the 50S ribosomal subunit and inhibits the transpeptidation step in protein synthesis (Shaw, 1983). In Gram-positive and Gram-negative bacteria plasmids encode for the production of chloramphenicol acetyltransferase which catalyses the formation of inactive 3-acetoxychloramphenicol. This enzyme is intracellular and is expressed constitutively in *E. coli*, but is inducible in *S. aureus*. However, plasmid-mediated mechanisms of decreased permeability to chloramphenicol have also been reported (Burns *et al.*, 1989).

1.3.3.2.2.2 Resistance to β-lactam antibiotics. β-lactam antibiotics, such as penicillin, ampicillin, carbenicillin, methicillin or c ephalosporins, inhibit the membrane-bound enzymes responsible for catalyzing vital stages in cell wall biosynthesis (Reynolds, 1984). These compounds covalently bind to and inhibit the penicillin-sensitive enzymes known as penicillin-binding proteins present in the cell wall (Waxman and Strominger, 1983). They are inactivated by the β-lactamases enzymes in Gram-negative and Gram-positive bacteria. These enzymes catalyse the hydrolysis of the β-lactam ring; they may be encoded chromosomally or by plasmids. In Gram-negative bacteria penicillinases are synthesized constitutively and accumulate in the periplasmic space. However, in Gram-positive bacteria they are usually inducible and extracellular (Richmond, 1965).

There are two more mechanisms of resistance to β-lactam antibiotics which are not plasmid-mediated. These are (1) an intrinsic resistance involving reduced affinity of, or

the lowering of the amount of, penicillin-binding proteins and (2) tolerance (Tuomanen *et al.*, 1986). Bacteriophages have been implicated in this last mechanism (Bradley *et al.*, 1980).

Methicillin, a semi-synthetic penicillin, was introduced in Britain in 1960. This antibiotic was active against all clinical strains of *S. aureus* (Rolinson *et al.*, 1960). However, after its introduction the population of methicillin-resistant *S. aureus* (MRSA) strains began to increase, with the organisms showing concomitant resistance to other antimicrobial agents, such as inorganic ions (Novick and Roth, 1968), organomercurial compounds (Summers, 1978), antiseptics, disinfectants, dyes and ethidium bromide (Johnston and Dyke, 1969), quaternary ammonium compounds (Lyon *et al.*, 1984), erythromycin (Mitsuhashi *et al.*, 1965), fusidic acid (Lacey and Grinsted, 1972) and aminoglycosides (Gillespie and Skurray, 1986).

Plasmids are present in almost all MRSA strains (Coia *et al.*, 1988), however most of the genes involved in resistance mechanisms, which were originally located on them, have now been incorporated into the chromosome (Carroll *et al.*, 1989). Studies on MRSA strains have revealed that all have probably emerged from a single clone, characterized by ribosomal resistance to streptomycin, plasmid-mediated resistance to tetracycline, and production of orange pigment (Cundliffe, 1984; Rouch *et al.*, 1990; Paulsen and Skurray, 1993).

There is no evidence that MRSA are more or less virulent than the average *S. aureus* (Keane and Cafferkey, 1984), however several hospital units have reported significant mortatlity (Cookson and Phillips, 1990). These MRSA have in some instances been referred to as epidemic because they behave as opportunists in the hospital environment and cause infectious outbreaks. Generally, MRSA are selected in patients treated with

antibiotics or who have their skin treated with antiseptics (Law and Gill, 1988). In addition, antibiotics such as gentamicin and vancomycin, which are commonly used in the hospital environment, stimulate plasmid transfer (Al-Masaudi *et al.*, 1991). The mortality and epidemic potential associated with these *S. aureus* strains result from a combination of factors of which the most important are the patients and the hospital personnel (Locksley *et al.*, 1982; Humphreys *et al.*, 1990).

1.3.3.2.2.3 Mercury resistance. Plasmids commonly encode resistance to mercurials. This resistance may have been selected and spread in pathogenic bacteria as a result of the use of mercury compounds in clinical environments (Porter *et al*, 1982) or as was recently demonstrated by Summers *et al* (1993), by the exposure of gut and mouth organisms to mercury released by dental amalgams.

Plasmids which confer resistance to mercurials are of two types (Schottel *et al.*, 1974) (1) "narrow spectrum" which involves reduction of Hg^{2+} to metallic mercury Hg^0 and provides resistance to some inorganic mercurials, and "broad spectrum" which provides resistance to all mercurials by enzymatic hydrolysis of organomercurials, via an organomercuric lyase, to inorganic mercury and the latter's subsequent reduction as in the previous mechanism (Silver and Misra, 1988). Mercuric reductase and lyase are both coordinately induced by Hg^{2+}, phenylmercuric acetate and other organomercurials, which suggested that the genes for mercury (*merA*) and organomercurial (*merB*) resistance are under joint regulatory control of a *merR* gene (Weiss *et al.*, 1977; Summers, 1986). The mercuric reductase gene product is an inducible NADPH-dependent enzyme found in the cytosol and is encoded on transposons Tn*501* and Tn*21*.

The cytosolic nature of the mercuric reductase suggested the existance of a transport mechanism that delivered the ion to the reductase. Plasmids conferring mercury resistance specify a mechanism for the uptake of mercuric ions (Summers and Sugarman, 1974). The genes for the reductase and for transport are part of an operon which is inducible by Hg^{2+}. Small amounts of the mercuric reductase enzyme appear to partition into the cytoplasmic membrane (Jackson et al., 1982) and this suggested that it interacts directly with membrane-bound elements of the Hg^{2+} uptake system. This was later confirmed by the finding of two inner-membrane polypeptides, MerT and MerC, and one periplasmic polypeptide, MerP, within the operon encoded by Tn21 (Jackson et al., 1982).

MerP is responsible for Hg^{2+} binding, however the precise function of MerT and MerC are not yet known. It has been postulated that these proteins may form a joint structure (MerT-MerC) involved in the uptake and extrusion of mercury or operate separately with distinct functions (Summers, 1986). Recent studies on the Gram-negative transposon Tn21 have shown that *merT* and *merP* are sufficient to specify mercury transport, however the role of *merC* remains obscure (Hamlett et al., 1992).

1.3.3.2.2.4 Resistance to aminoglycosides. Aminoglycosides are taken up by bacterial cells in three stages (1) binding to the outer surface, (2) energy-dependent transport across the cytoplasmic membrane and (3) a more rapid energy-dependent uptake with ribosome binding (Bryan and Kwan, 1983). Mutations in genes encoding ribosomal proteins or changes in cellular permeability may result in bacterial resistance to these compounds. However the modification of the antibacterial by plasmid-encoded enzymes is the most widespread type of resistance (Lyon and Skurray, 1987).

Plasmid-mediated modification of aminoglycosides prevents the uptake of the antibacterial. The plasmid-encoded enzymes are found in Gram-negative and Gram-positive bacteria and may bring about N-acetylation, O-nucleotidylation or O-phosphorylation. Acetylating enzymes require acetylcoenzyme A as a co-factor and the other two require nucleotides and ATP as substrates, respectively. Aminoglycosides modified at their amino or hydroxyl groups no longer bind to ribosomes and are therefore unable to inhibit protein synthesis (Yamada *et al.*, 1968).

1.3.3.2.3 Increased efflux of the toxic substance.

Early studies suggested that some resistance mechanisms resulted from a diminished uptake of antibacterial into the cell. However it has now been established in most of them that an efflux system, which prevents intracellular accumulation of the toxic substance is the mechanism that prevails.

1.3.3.2.3.1 Cadmium. Two genes encode cadmium resistance *cadA* and *cadB* (Smith and Novick, 1972); they also confer resistance to zinc ions (Perry and Silver, 1982). Cadmium inhibits respiratory mechanisms in all bacteria, although plasmid-mediated resistance to this ion has only been demonstrated in *S. aureus* (Novick and Roth, 1968; Kondo *et al.*, 1974). Cadmium sensitive strains take up the ion in response to membrane potential via a specific energy-dependent Mn^{2+} transport system. Uptake results in marked inhibition of respiration and growth, even at very low Cd^{2+} levels (Tynecka *et al.*, 1981a). Plasmid-containing $cadA^+$ strains possess an energy-dependent efflux system mediated by the *cadA* determinant, which prevents internal accumulation of Cd^{2+} (Weiss *et al.*, 1978; Tynecka *et al.*, 1981b).

Some carrier proteins transport a single solute from one side of the membrane to the other; these are hence known as uniports. Others function as coupled transporters, in which the transfer of one solute depends on the simultaneous transfer of a second solute, either in the same direction, symport, or in the opposite direction, antiport (Thauer *et al.*, 1977). Chemiosmotic criteria (Hamilton, 1975; Harold, 1977) suggests the cadmium efflux system may be an antiporter that ejects Cd^{2+} in exchange for protons (Tynecka *et al.*, 1981b). The *cadB* gene encodes for a mechanism which does not involve efflux of cadmium ions and produces a lower level of resistance. The *cadB* gene product may be an inducible cadmium and zinc ion binder, located in the cell membrene or cytoplasm (Perry and Silver, 1982).

1.3.3.2.3.2 Arsenate, arsenite and antimony (III). In *E. coli*, and presumably in *S. aureus*, an indiscriminate phosphate transport system delivers arsenate ions into the cell (Silver *et al.*, 1981), where it inhibits enzymes such as kinases, and interrupts energy transfer during glycolysis (Summers and Silver, 1978; Summers, 1984). Although, mutation of chromosomal genes responsible for transport may result in arsenate resistance, it is more likely to be plasmid-mediated and due to both reduced uptake of arsenate ions, and accelerated efflux (Silver *et al.*, 1981).

The *ars* operon of *E. coli* plasmid R773 (Chen *et al.*, 1986; San Francisco *et al.*, 1990; Kaur and Rosen, 1992) contains four determinants *ars*R, *ars*A, *ars*B and *ars*C. The *ars*A gene product exhibits an arsenite and antimonite-dependent ATPase activity (Rosen *et al.*, 1988) with the arsenite and arsenate efflux system being energized by ATP hydrolysis (Mobley and Rosen, 1982; Rosen and Borbolla, 1984). However, staphylococcal arsenical resistance operons lack the ATPase gene (Kaur and Rosen, 1992).

The *ars*B gene product has been identified as an integral inner membrane protein, and there is evidence that it functions as the channel of an arsenite pump (San Francisco *et al.*, 1989; Tisa and Rosen, 1990). The ArsC protein changes the specificity of the extrussion pump from arsenite to arsenate (Kaur and Rosen, 1992). ArsR acts as a repressor protein, which is inactivated by arsenate, arsenite and antimonite (Rosenstein *et al.*, 1992)

Recent studies on ArsR indicate that arsenate is not a true inducer; it must first be reduced to arsenite *in vivo* (Wu and Rosen, 1993). Finally, a question that remains is how arsenical efflux is energized by staphylococci, since they lack *ars*A. It is probable that this is brought about by hydrolysis of adenosine triphosphate rather than via the proton motive force (Silver and Keach, 1982). Similarly, although arsenite and antimony (III) bind to the cysteine residues of proteins (Summers, 1984), and the genetic determinants for arsenite and antimony (III) resistance are linked to the plasmid-borne *ars*R determinant, the precise mechanisms of resistance to these ions are still poorly understood (Novick and Roth, 1968; Novick *et al.*, 1979; Silver *et al.*, 1981).

1.3.3.2.3.3 Resistance to acriflavine, ethidium bromide and surface active agents

Plasmid-mediated resistance mechanisms in Gram-negative organisms may sometimes involve surface changes which may alter their response to disinfectants, antiseptics and preservatives (Russell and Gould, 1988; Morimyo *et al.*, 1992). Ethidium bromide efflux mechanisms have been reported, however in some instances, the genes involved show no hybridisation with similar genes found in staphylococci (Purewal *et al.*, 1990).

Staphylococcal resistance to ethidium bromide and acriflavine was associated with the presence of β-lactamase plasmids more than twenty years ago (Ericson, 1969; Johnston

and Dyke, 1969). More recently, gentamicin resistance has been observed to be coincident with resistance to ethidium bromide (McDonnell *et al.*, 1983; Asch *et al.*, 1984; Coleman *et al.*, 1985). Concomitant resistance to related compounds such as quaternary ammonium compounds, propamidine isethionate and diamidinodiphenylamine dihydrochloride has been reported (Emslie *et al.*, 1985a), as well as to chlorhexidine (Brumfitt *et al.*, 1985; Coleman *et al.*, 1985).

Initially it was thought the mechanism of ethidium bromide resistance involved diminished uptake (Johnston and Dyke, 1969; McDonnell *et al.*, 1983), but recent reports have established an efflux system is responsible for the effect (Purewal *et al.*, 1990; Sasatsu *et al.*, 1992). The frequent used of agents, such as acriflavine and cetrimide, as antiseptics and disinfectants, has been proposed to be the selective pressure which accelerated the emergence of resistance (Tennent *et al.*, 1985).

The resistance mechanism to ethidium bromide and quaternary ammonium compounds involves at least three distinct determinants, *qacA*, *qacB* and *qacC* (Emslie *et al.*, 1985a,b; Gillespie and Skurray, 1986). The *S. aureus* plasmid pSK1 contains the *qacA* determinant, which is a 3.4-kb segment of DNA encoding resistance to acridine, ethidium bromide, quaternary ammonium compounds, propamidine isethionate and diamidinodiphenylamine dihydrochloride, *qacA* is expressed when cloned into *E. coli* (Tennent *et al.*, 1985). The *qacA* gene is also present on some β-lactamase/heavy-metal resistance plasmids (Gillespie *et al.*, 1986) and shows common ancestry with tetracycline- and sugar-transport proteins (Rouch *et al.*, 1990). Work by Tennent *et al* (1989), Rouch *et al* (1990) and Littlejohn *et al* (1992) demonstrated that *qac* genes encode efflux systems relying on proton-motive force.

The *qacB* determinant mediates only resistance to acridines, ethidium bromide and quaternary ammonium compounds; it was found on the *S. aureus* plasmid pSK23 by Gillespie and Skurray (1986). The third determinant, *qacC*, mediates resistance to quaternary ammonium compounds and low-level ethidium bromide resistance; it does not demonstrate homology with *qacA* or *qacB* (Lyon and Skurray, 1987).

The widespread use of quaternary ammonium compounds as disinfectants probably explains the emergence of resistant organisms (Tennent *et al.*, 1985).

1.3.3.2.3.4 Resistance to tetracycline. Tetracycline is transported into the bacterial cell by a process energized by proton-motive force (McMurry *et al.*, 1981). The antibacterial then attaches to the 30S ribosomal subunit and interferes with the binding of aminoacyl-transfer RNA, hence inhibiting protein synthesis. Plasmid-mediated tetracycline resistance is inducible (Izaki *et al.*, 1966), with concomitant synthesis of a plasmid-encoded inner membrane protein (Levy and McMurry, 1974). Early studies proposed a reduced uptake of tetracycline by resistant cells (Levy and McMurry, 1978). However it has now been established that tetracycline is excreted from the cell by enzymes called antiporters coupled with proton influx (Kaneko *et al.*, 1985). Recent studies have demonstrated that transposon Tn*10* encodes a metal-tetracycline-proton antiporter responsible for the efflux of a tetracycline-metal complex out of the cells (Yamaguchi *et al.*, 1990, 1991, 1992).

1.3.3.2.4 Specifying a new enzyme.

1.3.3.2.4.1 Resistance to sulfonamides. The synthesis of dihydrofolate requires the enzyme dihydropteroate synthetase, which is competitively inhibited by sulfonamides. Plasmids found in Gram-negative bacteria code for an enzyme that is 1000-fold less

sensitive to sulfonamide and is synthesized constitutively (Swedberg and Skold, 1980).

In staphylococci, only one plasmid confering resistance to sulfonamide is known, however

its mechanism of action has not been described (Courvalin and Fiandt, 1980).

1.3.3.2.4.2 Resistance to trimethoprim. This compound inhibits the dihydrofolate

reductase responsible for the reduction of dihydrofolic acid to the activated

tetrahydrofolate form (Foster, 1983). In Gram-negative bacteria resistance may result

from an o verproduction of the normal enzyme. However, resistance is usually plasmid-

mediated, with the production of a resistant dihydrofolate reductase (Smith and Amyes,

1984). In Gram-positive bacteria resistance may also be intrinsic (Lyon *et al.*, 1983) or

plasmid-mediated (Archer *et al.*, 1986).

1.4 Compendial Tests for Preservative Efficacy

Preservatives are added to pharmaceutical preparations to kill contaminating

microorganisms and therefore avoid consequent spoilage of the product, or pathogenicity

to the patient (Swinyard and Lowenthal, 1990). Microbial contamination during

pharmaceutical manufacture results from equipment, the atmosphere, processing

techniques, raw materials and personnel (Phillips, 1975). It represents, apart from a health

hazard, a potential economic loss in terms of product spoilage, as demonstrated by the

degradation of shampoos, sun-screens and facial lotions (Tenenbaum, 1967). In addition,

Gram-negative organisms oxidize fatty acids and produce lipases (Saltzer, 1961; Alford

and Pierce, 1963) making them capable of turning a challenging environment into their

favour.

In complex pharmaceutical formulations there is a risk of preservative inactivation by other components of the formulation, which will prevent the desired preservative effect from being achieved. Inactivation requires the addition of excess antibacterial to compensate for that which is complexed, adsorbed or degraded. Consequently, not only must the production of pharmaceutical preparations be strictly controlled to limit contamination, but also the effectiveness of preservation must be demonstrated (Wedderburn, 1964).

The first quantification of chemical preservation was published by Joseph Pringle in 1775, when trying to evaluate preservation by salting. 130 years later, Rideal and Walker developed their method of disinfectant testing using phenol as control (Hugo, 1992a). Other methods were developed by Koch, Krönig and Paul, and Chick and Martin, and these became the basis for the modern preservative tests (Sykes, 1958).

Guidelines attempting to evaluate preservative efficacy abound in the literature (Rdzok *et al.*, 1955; Anderson and Crompton, 1967; Brown, 1968). However, the first serious attempt was introduced in the *United States Pharmacopoeia* (USP) XVIII in 1970. It gathered together the preservative efficacy protocols that had emerged from the works of industrial scientists, such as Eisman *et al* (1963) and Kenney *et al* (1964). In 1973, the *British Pharmacopoeia* (BP) suggested that for parenteral preparations, the incorporated preservative should be capable of "sterilizing an injection within three hours of inoculation with one million vegetative bacterial cells per ml" (BP, 1973; Allwood, 1978). Seven years later, the BP (1980) introduced a complete set of guidelines for the testing of parenteral, ophthalmic, topical and oral liquid preparations. Recently, and after many reviews of the matter, the *European Pharmacopoeia* (EP) has finally incorporated a preservative efficacy test (1992). This EP test is to supercede the BP (1988) protocol

when the new BP (1993) comes into effect on 1st December 1993. The work reported and discussed in this thesis was carried out during the BP (1988) protocol.

1.4.1 Preservative efficacy tests

The ultimate objective of a challenge test is to predict the performance of the preservative during manufacture and use (Leak and Leech, 1988). These tests are defined by several parameters, which will be discussed subsequently.

1.4.1.1 Challenge organisms

Organisms used in these tests are representative of the types of resistant contaminants which are likely to spoil a product during manufacture, arise from the raw materials, show to be well-adapted to growth in the product or represent a health hazard if they grow in the product (Parker, 1988). Yeasts, moulds and bacteria are used, with single-strain challenges being prefered over mixed-cultures (Davison, 1988). *S. aureus* and *P. aeruginosa* are the two bacterial strains used. The former is reputed to cause almost 90% of eye infections resulting from contamination of ophthalmic solutions (Davies, 1978), while the latter is included due to its high level of resistance to antibacterial agents and its survival and growth in aqueous environments (Akers and Taylor, 1990). Yeasts and moulds are also found as contaminants of manufactured products, hence their inclusion in the preservative challenge tests (Spooner and Corbett, 1985).

One drawback is that these tests may not reflect the true manufacturing or in-use conditions of a given product, as the challenge organisms are grown under laboratory conditions and may not be as aggressive as those found in practice (Yablonsky, 1972; Moore and Taylor, 1976; Al-Hiti and Gilbert, 1980; Leak, 1983). However, the use of

isolates from contaminated products, raw materials, or the manufacturing environment are

approved by the BP and USP. As an example, oral liquid formulations must be

challenged with an appropriate strain of *E. coli*, such as ATCC 8739, and products with

a high sugar content with *Zygosaccharomyces rouxii* strain NCYC 381 (BP, 1988; USP,

1990; EP, 1992).

Type of organism	Organism	Reference
Bacteria	*Staphylococcus aureus*	ATCC 6538 (NCTC 10788)
	Pseudomonas aeruginosa	ATCC 9027 (NCIB 8626)
Moulds	*Aspergillus niger*	ATCC 16404 (IMI 149007)
Yeasts	*Candida albicans*	ATCC 10231 (NCPF 3179)
	Zygosaccharomyces rouxii	- (NCYC 381)

Table 1. Organisms specified for preservative efficacy tests. BP and EP culture collection numbers appear in brackets.

1.4.1.2 Protocol

The protocols of the BP, USP and EP are very similar. They state that the product

should be inoculated with the challenge organisms at approximately 10^6 viable cells per

milliliter or gram. Viable counts of the inoculated products are then obtained immediately

and at intervals up to 28 days by plate count methods. Subsequently, the number of viable cells remaining at each specified interval is determined.

1.4.1.3 Preparation of inoculum

Stock cultures of bacteria are grown on soybean casein digest agar or tryptone soya agar (TSA) slopes, and stocks of yeasts and moulds on Sabouraud agar slopes. These may be stored for up to four weeks. Bacteria are subcultured on TSA, 18 to 24 h before the test and incubated at 30 to 35°C. Fungi such as *A. niger* are subcultured on Sabouraud agar and incubated for 7 days before the test at 20 to 25°C to allow growth and sporulation. Yeasts are subcultured on the same medium and grown at the same temperature as the *Aspergillus*, but only for 48 h before the test. Bacterial and yeast cells are harvested in sterile 0.1% peptone water, while fungi require the addition of 0.05% Tween 80 to the peptone water. The counts of the suspension are then reduced to about 10^8/ml by diluting with 0.1% peptone water. The preparation of the inoculum is basically similar for the three Compendia, however the EP allows an extra incubation time for *A. niger* until good sporulation occurs.

1.4.1.4 Temperature

Microorganisms are capable of growing over a wide range of temperatures, however optimal growth occurs generally within 30 to 40°C for bacteria and yeasts or between 20 to 25°C for moulds (Akers and Taylor, 1990). In the BP, USP and EP preservative efficacy tests, the inoculated product is maintained at 20 to 25°C. This temperature range is suitable as it is the most likely for product storage and permits growth of all the test organisms (Akers and Taylor, 1990). This temperature range also permits recovery of

organisms surviving exposure to a preservative, which is known to occur at temperatures below the optimum for undamaged cells (Harris, 1963).

1.4.1.5 Duration of Test

Testing procedures normally extend over 28 days. However, sometimes it is necessary to test for longer periods; in some instances a period of 3 months is suggested (BP, 1988). This suggestion probably arose due to criticism of the 28 days period, which some believe may be too short for slow-growing organisms (Allwood, 1986) and others argue that regrowth may not be observed (Leak, 1983).

In addition, it is advisable to test the product at the end of its shelf-life to determine that storage conditions have not impaired the preservative efficacy (BP, 1988).

1.4.1.6 Testing in the product container

If at all possible, it is recommended by the three Compendial tests, that the product be tested in its original container. However, in the case of ampoules, samples of product may be removed from their original containers, pooled, and placed in sterile vials. The container must be carefully chosen, as the preservative may adsorb to, or permeate through, the closure.

1.4.1.7 Inactivation of the preservative system

Recovered viable cells may still be inhibited from cell division and growth by the presence of residual preservative. A preservative inactivator is therefore often required in the diluting medium, such as thioglycollate for mercurials or Tween 80 (Collins *et al.*, 1989) for most other preservatives. If no suitable inactivator is available for the

preservative under test, then filtration through a 0.45 μm membrane filter may be performed. Samples are diluted, filtered and the filters rinsed with the specified diluent.

1.4.2 Comparison between Compendial Tests

Under the 1964 Convention on the Elaboration of a European Pharmacopoeia, the standards of the EP are required to take precedence over those of national pharmacopoeias of contracting countries. Therefore, the EP test is now mandatory for the countries party to the Convention, including Austria, Belgium, Cyprus, Denmark, Finland, France, Germany, the Netherlands, Norway, Spain, Sweden and Switzerland, with Portugal expected to join in the near future. The preservative efficacy tests of the USP XXII and the EP section VIII.14 are compared in Table 2, with the test included in the BP 1988 Appendix XVI C, as it was the latter protocol that was followed during the work presented in this thesis.

The USP XXII presents guidelines for testing parenteral, otic, nasal and ophthalmic preparations, but not topical or oral. Five microorganisms are specified, *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *C. albicans* ATCC 10231 and *A. niger* ATCC 16404. The product is inoculated with 10^5-10^6 organisms/ml, incubated at 20-25°C and sampled at 0, 7, 14, 21 and 28 days. Acceptance of the product is attained if, in the case of bacteria, the initial viable count is reduced to not more than 0.1% and, for moulds and yeasts, the viable count falls or remains unchanged during the first 14 days. Furthermore, viable counts of the test organisms must remain at or below the designated levels until 28 days.

The BP presents a much more detailed guideline including tests for parenteral and ophthalmic, topical and oral liquid preparations. Only four organisms are included, *S.*

aureus ATCC 6538, *P. aeruginosa* ATCC 9027, *C. albicans* ATCC 10231 and *A. niger* ATCC 16404. However, *E. coli* ATCC 8739 and *Z. rouxii* NCYC 381 are allowed for the challenge of oral liquid preparations and syrups, respectively. The product is inoculated with 10^6 organisms/ml or g, incubated at 20-25°C and sampled at 0, 6, 24 and 48 h and 7, 14 and 28 days. Acceptance criteria are determined by the type of product. Parenteral and ophthalmic preparations require, for bacteria, a 10^3 reduction of the initial count within 6 h and no recovery of viable organisms from 1 ml of product after 24 h and thereafter. For moulds and yeasts a 10^2 reduction of the initial count is required within 7 days, with no increase thereafter. Topical preparations require, for bacteria, a 10^3 reduction of the initial count within 48 h and no recovery from 1 ml or g at 7 days and thereafter; for yeasts and moulds a 10^2 reduction is required within 14 days with no increase thereafter. Finally, for oral liquid preparations, a 10^2 reduction of the initial viable counts of bacteria are required within 7 days, with no increase thereafter, and for yeasts and moulds, there must be no increase in counts recovered per ml within 14 days and thereafter.

The EP guidelines introduced in 1992 have a lot in common with the BP protocol. Test organisms are *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *C. albicans* ATCC 10231 and *A. niger* ATCC 16404. *E. coli* ATCC 8739 and *Z. rouxii* NCYC 381 are also included for the challenge of oral liquid preparations and syrups, respectively. The product is in oculated with 10^5-10^6 organisms/ml or g and incubated at 20-25°C. Sampling intervals depend on the type of product, parenteral and ophthalmic preparations are sampled at 0, 6, 24 h and 7, 14 and 28 days, while oral liquid preparations are sampled at 14 and 28 days. Acceptance criteria also depend on the type of product and are presented in terms of logarithmic reductions in viable counts. Parenteral and

ophthalmic preparations include two criteria of acceptance for each type of

microorganism; for bacteria criterion A requires a log reduction of 2 at 6 h, 3 at 24 h and

no recovery from 1 ml at 28 days. There are no log reductions established for 7 and 14

days. If by any justifiable reason criterion A cannot be met, then criterion B should be

satisfied: it requires a log reduction of 1 at 24 h, 3 at 7 days, nothing established at 14

days and no increase at 28 days. Yeasts and moulds should comply with the following:

criterion A requires a log reduction of 2 at 7 days and no increase at 28 days. There are

no log reduction values established for 6 and 24 h and 14 days. Criterion B requires a

log reduction of 1 at 14 days and no increase at 28 days, with no established requirements

at the initial sampling intervals. Oral preparations have more definite requirements,

bacteria should present a log reduction of 3 at 14 days with no increase of viable

organisms thereafter, and yeasts and moulds require a log reduction of 1 at 14 days with

no increase of viable organisms thereafter.

The methodology of the three protocols is similar and there seems to be a consensus

on the test organisms, the inoculum size, incubation temperature, the method of subculture

of the test organisms and, to some extent, the media. However, *E. coli* ATCC 8739 is

included in the protocol of the USP XXII for challenging parenteral and ophthalmic

preparations. The BP and EP allow the inclusion of this organism when it is a probable

contaminant of the manufacturing process, but only for challenging oral preparations.

Nevertheless, the test is best undertaken with *S. aureus* and *P. aeruginosa* due to the fact

that these organisms are more likely to contaminate a product during manufacture. *S.*

aureus is released from personnel, and its presence in a product indicates contamination

from a human source, for example hands or skin (Hugo, 1990). *P. aeruginosa* is highly

resistant to biocides and is a free-living opportunist able to proliferate in distilled water,

which is used in considerable amounts in the pharmaceutical industry. Therefore it needs to be included. *Z. rouxii* NCYC is allowed by two   Compendia as a challenge organism for syrups.

The sampling intervals of the BP and EP are quite similar. However, in the USP XXII the gap between the 0 h and 7 days sampling interval would not detect a "death and regrowth" pattern at early stages of the test.

The compliance criteria for parenteral and ophthalmic preparations for bacteria are more stringent in the BP guidelines (Table 3). The USP requires a survival level of less than 0.1% by the 14th day, and the count must remain at or below this until the remainder of the test. This implies an exceedingly low rate of kill. Viable organisms are maintained for long periods, which could prompt the emergence of resistant mutants. Criterion A of the EP guideline requires log reductions of 2 at 6 h and 3 at 24 h. Again, the required rate of kill is low and this becomes lower with criterion B, which permits the presence of viable organisms at the final sampling stage.

Acceptance criterion for yeasts and moulds show a consensus between the BP and specification A of the EP. However, the USP XXII again allows a low rate of kill. The viable count could just be reduced by one log cycle, and the product accepted. Criterion B of the EP resembles that of the USP XXII, therefore the same applies.

Topical preparations (Table 4) are only considered by the BP guidelines. They do not require such a high rate of kill as that for parenteral or ophthalmic preparations.

Protocol	USP XXII (1990)	BP (1988)	EP (1992)
Test organisms	*S. aureus* *E. coli* *P. aeruginosa* *C. albicans* *A. niger*	*S. aureus* *P. aeruginosa* *C. albicans* *A. niger*	

Media
    Bacteria
 	
    Yeasts and
    Moulds
	Soybean casein
	digest agar
		Soybean casein digest agar
		
		Sabouraud agar	

Inoculum, incubation and cell density	Bacteria: 30 - 35°C, 18 - 24 h *C. albicans*: 20 - 25°C, 48 h *A. niger*: 20 - 25°C, 7 days 10^8/ml		
Test procedures	Test in original container. Otherwise transfer 20 ml to each of 5 sterile tubes.	Test in original container. If not possible choose an appropriate one.	Test in original container.
Inoculum	10^5 - 10^6 organisms/ml or gram	10^6 organisms/ml or gram	10^5 - 10^6 organisms/ml or gram
Incubation of inoculated product	20 - 25°C		

Sampling
intervals
	0, 7, 14, 21 and 28
	days.
		0, 6, 24 and 48 h
		7, 14 and 28 days.
			Parenteral and
			ophthalmic:
			  0, 6 and 24 h, 7,
			14 and 28 days.
			
			Oral:
			  14 and 28 days.

Table 2. Comparison of protocols for preservative efficcay tests of the USP XXII, the BP 1988 and the recently incorporated guidelines of the EP 1992.

The acceptance criteria for oral liquid preparations are more flexible in the EP guidelines than in the BP; the USP XXII does not consider this type of preparation (Table 5). Although, the criteria of the EP and the BP appear similar, the EP allows a longer time to satisfy the 3 log reduction, therefore permitting higher counts than the BP at early time intervals. The criterion for yeasts and moulds are, if anything, more stringent in the EP than in the BP.

It is interesting to observe that the acceptance criteria for yeasts and moulds of the BP for oral liquid preparations resemble those of the USP XXII for parenteral and ophthalmic preparations. Oral liquid preparations, in general, should require less stringent guidelines due to their route of administration. Parenteral and ophthalmic preparations are used in more delicate situations, where the chances of a contaminating organism causing disease are greater, However this seems to have been overlooked by the USP XXII criteria.

There are no recommendations for topical preparations in the USP XXII and the EP, and no recommendations for oral liquid preparations in the USP XXII. Therefore the most complete set of guidelines is that provided by the BP 1988; these are also the most stringent. Preservation in the BP protocol for ophthalmic and parenteral preparations requires absence of viable organisms from samples taken 24 h after challenge and thereafter. This, to our view, is translated into better protection for the public and a safer product. Criteria of acceptance for ophthalmic and parenteral preparations in the USP XXII and EP (Table 3) are more flexible, permiting the presence of low counts of viable organisms, which could cause a particular problem in susceptible groups of patients. For example, there are reports of products that complied with the acceptance criteria of the USPXIX protocol, but were subsequently found to be inadequately preserved (Moore,

1978; Leak and Leech, 1988). Since there have not been many changes from this version

of the protocol to the present one, problems may still arise.

Therefore, the BP preservative efficacy test is the most stringent and ensures a better-

protected and safer pharmaceutical product.

Organism	USP XXII (1990)	BP (1988)	EP (1992)		
			Criterion A		B
			t†	log reduction	
Bacteria	≤0.1% by the 14th day and at or below that level thereafter.	10³ reduction of initial count within 6 h and no recovery from 1 ml at 24 h and thereafter.	6 h 24 h 7 d 14 d 28 d	2 3 - - NR	- 1 3 - NI
Yeasts and Moulds	≤ of initial concentration during the first 14 days and at or below this level thereafter.	10² reduction within 7 days with no increase thereafter.	6 h 24 h 7 d 14 d 28 d	- - 2 - NI	- - - 1 NI

NR = No recovery
NI = No increase
† = Time of sampling in hours (h) and days (d).

Table 3. Acceptance criteria for preservative efficacy tests of parenteral (multidose) and ophthalmic preparations.

Organism	USP XXII (1990)	BP (1988)	EP (1992)
Bacteria	No recommendations	10^3 reduction of initial count within 48 h with no recovery from 1 ml at 7 days and thereafter.	No recommendations
Yeasts and moulds		10^2 reduction within 14 days with no increase thereafter.	

Table 4. Acceptance criteria for the BP (1988) preservative efficacy test for topical preparations.

Organism	USP XXII (1990)	BP (1988)	EP (1992)	
			t	log reduction
Bacteria	No recommendations	10^2 reduction within 7 days with no increase thereafter.	14 d 28 d	3 NI
Yeasts and Moulds		There is no increase in the number of microorganisms recovered within 14 days and thereafter.	14 d 28 d	1 NI

NR = No recovery.
NI = No increase.

Table 5. Acceptance criteria for preservative efficacy tests of oral liquid preparations.

1.5 Aim of the present work

This introduction has outlined how resistance of microorganisms to unfavourable conditions can be natural, with bacterial cells surviving because of their own intrinsic resources such as a slime layer, or acquired, in which case resistance plasmids may play a major role. Pharmaceutical preparations, as with any other perishable product, are liable to bacterial attack. Some are also used by patients in ways that penetrate natural barriers to infection, or are applied to damaged tissue. Antimicrobial preservatives must therefore be included in pharmaceutical formulations.

Plasmids code for the production of enzymes, capable of promoting resistance to such pharmaceutical preservatives. The aim of this work was, therefore, to determine the effect of plasmids on the efficacy of pharmaceutical preservatives in simple aqueous preserved systems. This was done by determinating minimum inhibitory concentrations (MICs), by assessing susceptibility of strains to bactericidal concentration of preservatives, and by assessing the susceptibility of plasmid-containing strains to conditions of Compendial Preservative Challenge Tests. The pharmaceutical preservatives used in this work were: cetrimide, chlorhexidine gluconate, benzalkonium chloride, phenylmercuric nitrate, dibromopropamidine isethionate and propamidine isethionate. The first four are widely-used in eye-drop and topical formulations as preservatives, while the remaining two are included as active ingredients of eye-drops and eye-ointments.

MIC data for all the preservatives were collected against a plasmid-less strain of *P. aeruginosa* as a representative preservative-resistant, slime producing organism and against plasmid-carrying and plasmid-less strains of *S. aureus* and *E. coli*. Subsequently, concentrations above MIC values were tested in ranging experiments to obtain suitable concentrations to investigate bactericidal activity. Short-term killing rate experiments

were then performed, comparing plasmid-containing and plasmid-less strains to evaluate whether survival levels of the different strains were enhanced by the plasmids.

Finally, the efficacies of three different aqueous concentrations of benzalkonium chloride, cetrimide, phenylmercuric nitrate and chlorhexidine were measured using the protocol of the British Pharmacopoeia Preservative Challenge Test, to show if the presence of plasmids made any difference to the survival of bacteria under such conditions, and therefore to the interpretation of test results.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Strains and Plasmids

The bacterial strains used are listed in Tables 6 and 7. They were supplied by Dr. R. J. Pinney, Microbiology Section, Department of Pharmaceutics, the School of Pharmacy, University of London. *S. aureus* A.D. was obtained from Dr. A. Davison, Pharmaceutical Microbiology Laboratory, North East Thames Regional Health Authority, St. Bartholomews Hospital, London EC1A 7BE.

Plasmid-carrying strains used during the development of this work were *S. aureus* SA1325 which is actually strain SA1439 carrying plasmid pSK1, and *E. coli* 343/113 (R471-1). The *S. aureus* plasmid pSK1 is a 28.4 kbase plasmid conferring resistance to trimethoprim, kanamicin/tobramycin, gentamicin, diamidines, quaternary ammonium compounds, acriflavin and ethidium bromide (Lyon and Skurray, 1987). The *E. coli* R plasmid R471-1 is a deletion mutant of R471 (Hedges *et al.*, 1975), and confers resistance to ampicillin and phenylmercuric nitrate. These plasmids were chosen for the development of this work because they encode resistance mechanisms against commonly-used pharmaceutical preservatives such as quaternary ammonium compounds, biguanides and phenylmercuric nitrate (Table 8). Plasmid-containing and isogenic plasmid-less strains provided a reliable means for comparison: the only difference between them was the presence of a plasmid.

S. aureus strain A.D. was of special interest since it was isolated as a resistant strain from an ophthalmic solution preserved with benzalkonium chloride; the inclusion of such contaminants is recommended by the protocol of the BP Preservative Challenge Test. In addition, it was of interest to test its resistance to other preservatives in comparison to the other strains, especially *S. aureus* strain SA1325(pSK1).

S. aureus NCTC 10788 is used in the BP Preservative Challenge Test and was included here to compare its sensitivity with the plasmid-less strain *S. aureus* SA1439 and determine if it could be used as a control for the *S. aureus* strain isolated from the ophthalmic solution.

S. aureus NCTC 6571 is a plasmid-less strain, known to be ultra-sensitive to most antibacterials. It was included here to compare its sensitivity to the rest of the plasmid-less *S. aureus* strains.

P. aeruginosa NCIB 8626 is also used in the BP Preservative Challenge Test. It was included because it is a plasmid-less strain, which is naturally resistant to a wide variety of preservatives, able to proliferate in water and adapt to different substrates.

E. coli strain 343/113 was not only included to facilitate the comparison between a plasmid-containing and a plasmid-less strain. It was also interesting to compare its sensitivity to the different preservatives tested, with those of the BP test strains, *P. aeruginosa* strain NCIB 8626 and *S. aureus* NCTC 10788 (Table 7).

Organism	Strain Designation	Phenotype	Culture Collection No.
Staphylococcus aureus	NCTC 6571 (SA39)	S	DP1210
Staphylococcus aureus	SA1439	S	DP1211
Staphylococcus aureus	SA1325 (pSK1)	R	DP1212
Staphylococcus aureus	A.D.	(R)	DP1241
Escherichia coli	343/113	S	DP956
Escherichia coli	343/113 (R471-1)	R	DP968

S = Plasmid-less sensitive strain.

(R)= Resistant strain isolated from eye drops preserved with benzalkonium
chloride. Plasmid status unknown.

R = Plasmid-carrying resistant strain.

Table 6. Laboratory strains used.

Organism	Reference	Culture Collection No.
Staphylococcus aureus	NCTC 10788 (ATCC 6538)	DP1243
Pseudomonas aeruginosa	NCIB 8626 (ATCC 9027)	DP1244

Table 7. BP preservative challenge test control organisms.

Plasmid	Resistance to	Size (kb)	Reference
pSK1	Gm,Tm, Km, Ac, Eb, Qa, Pi, Dd, Tp	28.4	Lyon *et al.* (1983, 1986)
R471-1	Ap, Om	78.52	Hedges *et al.* (1975)

Abbreviations used: Ac, acriflavine; Ap, ampicillin; Dd, diamidinodiphenylamine dihydrochloride; Eb, ethidium bromide; Gm, gentamicin; Km, kanamycin; Om, organomercurial; Pi, propamidine isethionate; Qa, quaternary ammonium compounds; Sm, streptomycin; Tm, tobramycin; Tp, trimethoprim.

Table 8. Plasmids.

2.1.2 Solid media

Nutrient broth Oxoid No. 2 (Code CM67), Peptone Water (Code CM9), Isosensitest Agar Oxoid (Code CM471) and Tryptone Soya Broth (Code CM129) were from Unipath Limited, Basingstoke, Hampshire, England. Nutrient Broth and Tryptone Soya Broth were solidified with 1.5 percent Lab M agar No. 1 (Microbiological Supply Company, P.O. Box 23, Toddington, Beds) to make nutrient agar and tryptone soya agar, respectively. Each medium was made according to the manufacturer's instructions.

2.1.3 Reagents

Polyoxyethylene (20) sorbitan mono-oleate (tween 80) was obtained from two sources: BDH Limited, Poole, England) (lot no. 5599550A) and Koch-Light Laboratories Ltd. (Colnbrook Bucks, England) (lot no. 80566).

Thioglycollic acid was lot no. 279095 from Fluka Chemika (Fluka Chemie AG, Gillingham, Dorset, England).

2.1.4 Preservatives

The preservatives used were: chlorhexidine gluconate 5% w/v solution from ICI Limited (Alderley Park, Macclesfield, Cheshire, England); benzalkonium chloride 50% w/v solution from E. Merck (BDH Limited, Poole, Dorset, England); phenylmercuric nitrate (batch no.

0372060L) from BDH Limited, Poole, England; dibromopropamidine isethionate (substance M&B 1270A, batch no. 890001) and propamidine isethionate (substance 782A, batch no. X301), both from Rhone-Poulenc (Rainham Road South, Dagenham, Essex, England); and cetrimide lot no. 118F0048 (M-7635) from Sigma Chemical Co. Limited

(Fancy Road, Poole, Dorset, England).

2.1.6 Laboratory Equipment

The following laboratory equipment was used:

Multipoint Inoculator from Denley Instruments Limited (Billingshurst, Sussex, England).

Peristaltic pump dispensor from Camlab Limited (Nuffield Road, Cambridge, England).

Water bath from Grant Instruments Limited (Barrington, Cambridge, England).

2.2 Methods

2.2.1 Growth and sub-culture of strains

Strains were stored in liquid nitrogen until required as frozen over-night nutrient broth

cultures. When in use the plasmid-less strains were kept on nutrient agar plates and

plasmid-containing strains were grown on nutrient agar plates containing a preservative

or antibiotic relevant to the plasmid. Strains were subcultured every month. Two plates

of each strain, marked A and B, were inoculated and incubated over-night at 37°C. The

following day, the plates were transferred to and stored in the fridge. Plate A was sealed

with tape and kept as the stock, and B was used for laboratory subcultivation. After a

month, organisms were taken from plate A and the process repeated. This procedure was

followed to subculture as infrequently as possible to avoid the possible selection of

mutants (Collins *et al.*, 1989). Plates A of the stock cultures were kept in plastic bags to

avoid drying up of the agar.

E. coli 343/113(R471-1) was maintained together with its isogenic plasmid-less

derivative, on nutrient agar, and on nutrient agar containing phenylmercuric nitrate (10

μg/ml) or ampicillin (10 μg/ml) to confirm the presence of the plasmid. These strains were renewed every month using the procedure stated above.

Staphylococcal strain SA1325 (pSK1) was subcultured monthly on nutrient agar, and to confirm the presence of plasmid pSK1, on isosensitest agar containing trimethoprim (10 μg/ml), and nutrient agar containing cetrimide (3 μg/ml), benzalkonium chloride (2 μg/ml), dibromopropamidine isethionate (20 μg/ml) or propamidine isethionate (50 μg/ml). The other *S. aureus* strains were maintained on nutrient agar.

P. aeruginosa NCIB 8626 was kept on nutrient agar plates.

2.2.2 Determination of MICs in solid nutrient agar

Nutrient agar (20 ml) plates containing benzalkonium chloride, cetrimide, chlorhexidine gluconate, phenylmercuric nitrate, dibromopropamidine isethionate or propamidine isethionate were prepared by dissolving appropriate volumes of concentrated solutions of the preservatives in molten, double strength nutrient agar and making up to volume with sterile distilled water. Concentrations in the plates ranged from 300 to 0.1 μg/ml in decimal stages. Subsequently, the plates were over-dried at 37°C for 45 min.

Over-night cultures of the different strains grown in nutrient broth were diluted 10^{-2} and 10^{-4} with nutrient broth. 1 μl volumes of these dilutions and of the undiluted cultures were then applied to the plates using a Denley multipoint inoculator A400. After over-night incubation at 37°C, the plates were observed to determine the concentrations at which growth had been inhibited. Plates were incubated a minimum of two days to observe for any further growth. MICs were recorded as the lowest concentration tested that inhibited visible growth of the 10^{-4} dilutions, which gave between 10 and 50 isolated colonies when plated on drug-free medium.

These wide-range experiments gave an indication of MIC. Narrower ranges of concentrations were then used to provide more accurate estimates of MIC.

2.2.3 Comparative bactericidal activities determined by short-term tests

The procedure to compare bactericidal activities was as follows: 4.5 ml cultures were grown at 37°C over-night in nutrient broth. 20 ml of sterile water were then added and the suspensions spun down at 3,600 rev/min for 15 min in an MSE centrifuge, model WR2204. The supernatant was decanted and the cells resuspended in 20 ml of sterile water and spun again. This was done to remove traces of nutrient broth from the pellets. These were then finally resuspended in 4.5 ml of sterile water. At this point, a viable count was performed on the washed culture by diluting in nutrient broth and plating on over-dried nutrient agar plates, as described below.

1.0 ml of each washed suspension was then added to 9.0 ml of an aqueous preservative-containing solution, maintained at 25°C in a water bath (Grant Instruments Limited). Immediately, and at each sampling interval, 0.5 ml were removed into 4.5 ml, and 0.1 ml into 9.9 ml of nutrient broth containing a suitable preservative inactivator to give 10^{-1} and 10^{-2} dilutions, respectively. The 10^{-2} dilution was further diluted to 10^{-3} and 10^{-4} by the same procedure; finally a 10^{-5} dilution was prepared from the 10^{-4} dilution. The bottoms of agar plates were divided in four sections, marked as -2, -3, -4 or -5 using a felt-ink pen. Then, three drops of each dilution were delivered with a pasteur pipette to their corresponding section on the plate beginning always with the highest (10^{-5}) dilution and ending with the lowest one (10^{-2}). The pipettes delivered between 48 to 52 drops/ml. The same pipette was used to deliver all dilutions of a sample. The lids were replaced on the nutrient agar plates, and the drops allowed to dry into the agar. Finally,

the plates were inverted and incubated over-night at 37°C. Colonies that grew after over-night incubation were counted, and the plates were then incubated 2 more days to observe for further growth.

Benzalkonium chloride, cetrimide and chlorhexidine gluconate were tested in the range of 5 to 40 μg/ml. Dibromopropamidine isethionate and propamidine isethionate were tested for bactericidal activity in concentrations ranging from 80 μg/ml to 6 mg/ml. Phenylmercuric nitrate was used at 1 and 10 μg/ml.

2.2.4 BP Preservative Challenge Test

The guidelines for the BP preservative challenge test (Anon., 1988) were followed. The protocol states that the volume of the inoculum should not exceed 1% of the total volume of the sample. Therefore, as the final volume was 10 ml, the volume of inoculum used was 0.1 ml.

4.5 ml cultures were grown over-night in tryptone soya broth at 30°C. 20 ml of 0.1% peptone water was then added and the suspensions spun down at 3,600 rev/min for 15 - 20 minutes. The supernatant was decanted and the cells resuspended in 20 ml of 0.1% peptone water and spun down again. The pellets were finally resuspended in 10 ml of 0.1% peptone water to lower the count to approximately 10^8/ml. At this stage, a viable count was performed on the washed culture by diluting in 0.1% peptone water and plating on over-dried tryptone soya agar plates, as described in the previous section.

0.1 ml of each washed suspension was then added to 9.9 ml of an aqueous preservative solution, maintained at 25°C in a water bath. Immediately, and at each sampling interval, 0.1 ml was removed into 9.9 ml of 0.1% peptone water containing a suitable preservative inactivator. This gave a 10^{-2} dilution, which was further diluted to

10^{-3} and 10^{-4}. This was done by removing from the 10^{-2} dilution, 0.5 ml into 4.5 ml, and 0.1 into 9.9 ml of 0.1% peptone water. Then, from the 10^{-4} dilution a 10^{-5} dilution was prepared by removing 0.5 ml into 4.5 ml of 0.1% peptone water.

Finally, as for short-term bactericidal tests, the bottom of tryptone soya agar plates were divided in four sections and each was marked as -2, -3, -4 and -5. Then, three drops of each dilution were delivered with a pasteur pipette to their corresponding section on the plate. Plates were inverted and left in the incubator over-night at 30°C. Colonies were counted and the plates were then incubated for another 2 days to observe for further growth.

Sampling intervals were those described in the BP challenge test protocol, 0, 6, 24, 48 hours and 7, 14 and 28 days. After the 6 h sampling interval the inoculated preservative solutions were stored in a 25°C incubator.

Benzalkonium chloride and cetrimide were tested at concentrations of 10, 30 and 100 µg/ml, chlorhexidine gluconate was tested at 5, 20 and 100 µg/ml and phenylmercuric nitrate at 1, 10 and 20 µg/ml.

Propamidine and dibromopropamidine isethionates were not included in the preservative challenge test because these compounds are used as active ingredients of pharmaceutical formulations and not as preservatives.

RESULTS

3.1 MICs in solid nutrient agar

Minimum inhibitory concentrations (MICs) for single cell inocula on nutrient agar

gave the simplest assessment of the sensitivities of the various strains to the antimicrobial

agents. Wide-ranging experiments were performed first, which enabled the range of test

concentrations to be narrowed to give the results quoted in Table 9.

Strains	MIC in µg/ml					
	BZC	CTAB	PI	DBPI	CG	PMN
S. aureus NCTC 6571	0.75	0.75	10	1.0	0.75	0.04
S. aureus SA1439	1.5	1.5	15	1.5	0.75	0.05
S. aureus SA1325 (pSK1)	4.0	3.0	150	40	1.0	0.05
S. aureus NCTC 10788	1.5	0.75	5.0	0.75	0.75	0.075
S. aureus A.D.	5.0	3.0	200	40	1.0	0.75
E. coli 343/113	15	50	50	1.5	1.0	1.5
E. coli 343/113 (R471-1)	15	50	50	1.5	1.0	40
P. aeruginosa NCIB 8626	150	300	150	75	5.0	30

Table 9. MICs in solid nutrient agar for *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* strains. Abbreviations used: BZC, benzalkonium chloride; CTAB, cetrimide; PI, propamidine isethionate; DBPI, dibromopropamidine isethionate; CG, chlorhexidine gluconate; PMN, phenylmercuric nitrate.

Undoubtedly, *P. aeruginosa* strain NCIB 8626 was the least sensitive of the known

plasmid-less organisms to the antibacterials tested (Table 10). *S. aureus* strain A.D. has

been excluded from Table 10, because its plasmid status was unknown. The resistance

of *P. aeruginosa* was expected because this Gram-negative organism is exceedingly

resistant to a wide variety of antibacterial compounds (Al-Hiti and Gilbert, 1980).

E. coli strain 343/113 was not as resistant as *P. aeruginosa* NCIB 8626, however it was more resistant than all of the plasmid-less *S. aureus* strains (Table 10). The composition of the cell wall, as discussed in the Introduction, plays an important role in natural resistance mechanisms. Gram-negative organisms have a more complex set of layers, which protects them far more efficiently from chemical insult than those in Gram-positive organisms. Results obtained showing the difference in preservative sensitivities between Gram-negative and Gram-positive organisms supports this fact (Russell and Gould, 1988).

The plasmid-less *S. aureus* strains NCTC 10788, which is used in the BP preservative test, and SA1439, the isogenic parent of *S. aureus* SA1325 (pSK1), had very similar sensitivities (Table 10). It is also interesting to observe that all three plasmid-less *S. aureus* strains were equally sensitive to chlorhexidine gluconate.

Strains	MIC in µg/ml					
	BZC	CTAB	PI	DBPI	CG	PMN
S. aureus NCTC 6571	0.75	0.75	10	1.0	0.75	0.04
S. aureus SA1439	1.5	1.5	15	1.5	0.75	0.05
S. aureus NCTC 10788	1.5	0.75	5.0	0.75	0.75	0.075
E. coli 343/113	15	50	50	1.5	1.0	1.5
P. aeruginosa NCIB 8626	150	300	150	75	5.0	30

Table 10. Sensitivities of plasmid-less *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* strains.

Results for the two *E. coli* strains accurately demonstrated the difference in sensitivity

brought about by the presence of plasmid R471-1; resistance to phenylmercuric nitrate

was enhanced dramatically by a factor of 27-fold by the presence of this plasmid.

However the sensitivities of the plasmid-containing and the plasmid-less control strain to

the rest of preservatives were the same (Table 11). This was to be expected since plasmid

R471-1 confers resistance only to mercury and not to any of the other compounds tested

(Hedges *et al.*, 1975).

Strains	MIC in µg/ml					
	BZC	CTAB	PI	DBPI	CG	PMN
E. coli 343/113	15	50	50	1.5	1.0	1.5
E. coli 343/113 (R471-1)	15	50	50	1.5	1.0	40

Table 11. Sensitivities of plasmid-less *Escherichia coli* strain 343/113
and plasmid-containing strain 343/113 (R471-1).

The comparative sensitivities of the *S. aureus* strains showed that plasmid pSK1 does

indeed increase resistances to all the preservatives tested except phenylmercuric nitrate

(Table 12). This was expected since pSK1 has been reported to confer resistance to

quaternary ammonium compounds, diamidines and, to some extent, chlorhexidine (Lyon

and Skurray, 1987), but not to organomercurial compounds. Therefore, as pSK1 does not

confer resistance to phenylmercuric nitrate, sensitivities obtained for the plasmid-

containing *S. aureus* strain SA1325 (pSK1) and its isogenic plasmid-less derivative strain

SA1439 were similar.

It is interesting to observe that sensitivities of *S. aureus* strain A.D. were very similar to those obtained for *S. aureus* strain SA1325 (pSK1) (Table 12). *S. aureus* A.D. was obtained from an ophthalmic solution preserved with benzalkonium chloride. Therefore, although this strain was initially selected as only benzalkonium chloride resistant, it also demonstrated resistance to cetrimide, propamidine isethionate, dibromopropamidine isethionate, chlorhexidine and phenylmercuric nitrate. The presence of all these resistances in the A.D. strain suggested at least some might be plasmid-mediated.

Strains	MIC in µg/ml					
	BZC	CTAB	PI	DBPI	CG	PMN
S. aureus SA1439	1.5	1.5	15	1.5	0.75	0.05
S. aureus SA1325 (pSK1)	4.0	3.0	150	40	1.0	0.05
S. aureus A.D.	5.0	3.0	200	40	1.0	0.75

Table 12. Sensitivities of plasmid-less *Staphylococcus aureus* strain SA1439 and its plasmid-containing derivative SA1325 (pSK1). Observe the increase in resistances due to the presence of the plamid and the similar levels of resistance of strain SA1325 (pSK1) and *S. aureus* A.D.

The ratios of the MICs of the *S. aureus* strains (Table 13) show that plasmid pSK1 increased resistance to benzalkonium chloride and cetrimide by 5.3- and 2.0-fold, respectively. This is considered by some authors as trivial (Lacey *et al.*, 1986), since in-use concentrations of preservatives and antiseptics are higher than the MIC's demonstrated by plasmid-carrying organisms. However, in the hospital environment these

"insignificant" resistances will still function as a selection force for the survival of clinically important organisms (Gillespie *et al.*, 1986).

Resistances to propamidine and dibromopropamidine isethionate were greatly increased by the presence of pSK1, with the plasmid appearing to increase resistance to the halogenated diamidine by a greater extent than to the parent compound (ratio C/B, Table 13). As mentioned before, it is interesting to observe the similarity in MIC ratios obtained for benzalkonium chloride, propamidine isethionate, dibromopropamidine isethionate, cetrimide and chlorhexidine when *S. aureus* strains SA1325 (pSK1) and *S. aureus* A.D. are compared with strain NCTC 10788 (ratios C/A and D/A in Table 13).

Ratio	MIC ratios					
	BZC	CTAB	PI	DBPI	CG	PMN
A/B	1.0	1.5	0.3	0.5	0.5	1.5
C/B	2.7	2.0	10	27	1.3	1.0
C/A	2.7	4.0	30	53	1.3	0.7
D/A	3.3	4.0	40	53	1.3	10

Table 13. Comparative sensitivities of *Staphylococcus aureus* strains to preservatives tested. BP test strain NCTC 10788 (A), SA1439 (B), SA1325 (pSK1) (C) and *S. aureus* A.D. (D).

The results presented in Table 14 show that the plasmid-containing strain SA1325 (pSK1) and the highly resistant *S. aureus* strain A.D. are capable of surviving when exposed to concentrations of pharmaceutical preservatives that kill sensitive plasmid-less

S. aureus. However, Table 14 shows that plasmid-less *P. aeruginosa* strain NCIB 8626 was, in general, more resistant than the plasmid-containing *S. aureus* and *E. coli* strains and the resistant *S. aureus* strain A.D. There were some exceptions. *S. aureus* A.D., the strain isolated from the ophthalmic solution, was more resistant than *P. aeruginosa* NCIB 8626 to propamidine isethionate, and *E. coli* 343/113 (R471-1) was more resistant to phenylmercuric nitrate. Also, *P. aeruginosa* NCIB 8626 was as sensitive to propamidine isethionate as the plasmid-containing *S. aureus* strain SA1325 (pSK1).

Ratio	MIC ratios					
	BZC	CTAB	PI	DBPI	CG	PMN
E/C	38	100	1.0	1.9	5.0	600
E/D	30	100	0.75	1.9	5.0	40
E/F	10	6.0	3.0	50	5.0	0.75

Table 14. Ratios of the MICs of the plasmid-less *Pseudomonas aeruginosa* strain NCIB 8626 (E) and plasmid-containing *Staphylococcus aureus* SA1325 (pSK1) (C) and *Escherichia coli* strain (R471-1) (F) and *S. aureus* strain A.D. (D).

Summary

Using the criterion of MICs obtained on nutrient agar, Gram-negative organisms were shown to be far more resistant to the preservatives tested than Gram-positive organisms. *P. aeruginosa* strain NCIB 8626 was the most resistant strain tested to chlorhexidine gluconate, benzalkonium chloride, cetrimide, propamidine isethionate and dibromopropamidine isethionate. *E. coli* 343/113 (R471-1) displayed the highest resistance to phenylmercuric nitrate with an MIC 27-fold greater than its isogenic plasmid-less parent *E. coli* 343/113.

Gram-positive organisms were more susceptible to the preservatives tested, but the plasmid-carrying *S. aureus* strain SA1325 (pSK1) and *S. aureus* strain A.D. were more resistant to cetrimide, benzalkonium chloride and chlorhexidine gluconate than the control plasmid-less *S. aureus* strain. Indeed, the resistance levels of SA1325 (pSK1) and strain A.D. to propamidine and dibromopropamidine isethionates, approached or even surpassed those of the plasmid-less *P. aeruginosa* strain NCIB 8626.

These results substantiate the fact that plasmids do provide bacteria with the necessary protection to grow in unfavourable environments.

3.2 Comparative bactericidal activities determined by short-term tests

Bactericidal activities were determined using inocula that had been grown over-night in nutrient broth at 37°C. These over-night cultures were washed and resuspended in sterile distilled water. Viable counts of the suspensions were performed to obtain the initial number of organisms/ml. Then, 1 ml of the over-night, washed suspension was added to aqueous solutions of each of the different preservatives held at 25°C in a water bath. Viable counts were performed immediately after inoculation of the preservative solution, and it was sampled again at 10, 20, 30, 60, 90, 120, 150 and 180 min. The inoculated preservative solutions were maintained at 25°C until the end of the experiment. Samples were diluted in nutrient broth, containing a preservative inactivator and organisms plated on nutrient agar. Plates were incubated over-night at 37°C, counted and then incubated a further 2 days to observe any additional growth.

3.2.1 The use of inactivator in the diluting media

Since there are numerous reports of inhibition of growth by preservative carried over into the diluting medium, Tween 80 was tested at 1% and 10%, as recommended in the BP (1988) as inactivator in experiments using various strains of *S. aureus*.

3.2.1.1. Benzalkonium chloride

Figure 15 shows the effect of Tween 80 on the recovery of *S. aureus* SA1439 after exposure to 20 µg/ml benzalkonium chloride in sterile water. Viable counts obtained after diluting in nutrient broth were lower than those obtained after diluting in nutrient broth containing Tween 80 at either concentration. Hence, dilution in nutrient broth alone did not eliminate the effect of the preservative completely. Increasing the concentration of
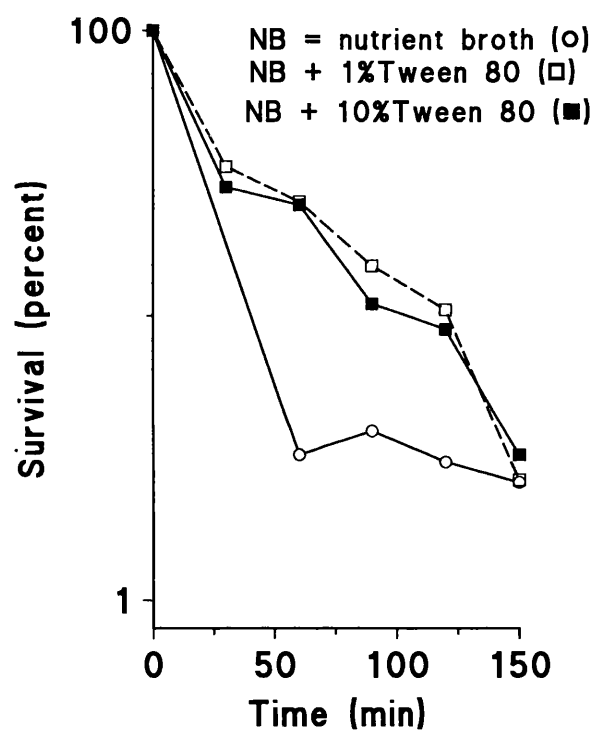
Figure 15. Effect of the addition of Tween 80 to the nutrient broth (NB) diluting medium on the recovery of *Staphylococcus aureus* strain SA1439 after exposure to 20 µg/ml benzalkonium chloride.

the Tween from 1 to 10% had no observable effect, recovery was similar from both concentrations.

Recovery of other *S. aureus* strains showed the same trend. Figure 16A shows the recovery of plasmid-containing *S. aureus* strain SA1325 (pSK1) exposed to 20 µg/ml benzalkonium chloride in sterile water. Once more, viable counts obtained after diluting in nutrient broth alone were lower than those obtained after diluting in nutrient broth containing either of the Tween concentration. Similar results were observed with the plasmid-less *S. aureus* strain NCTC 10788 exposed to 30 µg/ml benzalkonium chloride in sterile water (Figure 16B). Once again, the differences in Tween concentration had no observable effect, and recovery was similar from either concentration. Hence, 1% Tween 80 in nutrient broth was used as inactivator for benzalkonium chloride.

3.2.1.2 Chlorhexidine gluconate

Figure 17 shows the effect of Tween 80 on the recovery of *S. aureus* strain SA1439 from 5 µg/ml chlorhexidine gluconate in sterile water. Again, viable counts obtained after diluting in nutrient broth containing Tween 80 at either concentration were higher than when dilution was performed nutrient broth alone. Once again, variations in Tween concentration had no observable effect, recovery was similar from either. An interesting result was obtained with the *S. aureus* plasmid-containing strain SA1325 (pSK1), which is a derivative of strain SA1439. After exposure to 5 µg/ml chlorhexidine gluconate in sterile water, it was found that the addition of Tween 80 to the nutrient broth diluting medium made no difference to the recovery of this plasmid-containing *S. aureus* (Figure 18A). Viable counts were similar after dilution in nutrient broth alone or in nutrient broth containing Tween at either concentration. In contrast, the recovery of the plasmid-less

A

100
NB = nutrient broth (o)
NB + 1%Tween 80 (□)
NB + 10%Tween 80 (■)

Survival (percent)

1.0

SA1325 (pSK1)

0.01

40   120   200
Time (min)

B

100
NB = nutrient broth (o)
NB + 1%Tween 80 (□)
NB + 10%Tween 80 (■)

Survival (percent)

1.0

NCTC
10788

0.01

0.0001
0   20   40   60
Time (min)
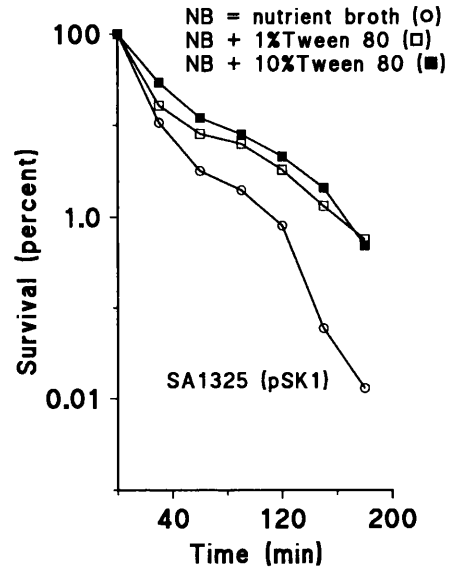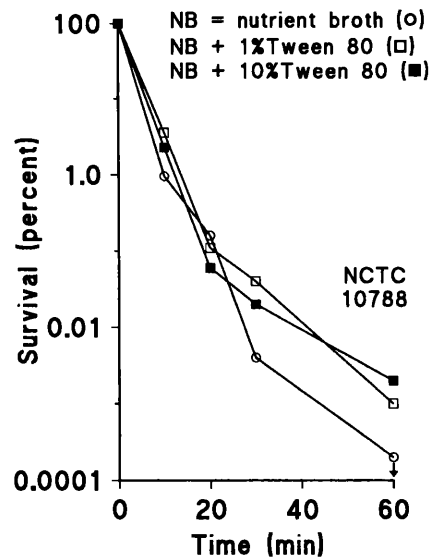
Figure 16. Effect of the addition of Tween 80 to the nutrient broth diluting medium on recovery of (A) plasmid-containing *Staphylococcus aureus* strain SA1325 (pSK1) and (B) plasmid-less NCTC 10788 exposed to 20 µg/ml or 30 µg/ml benzalkonium chloride, respectively.
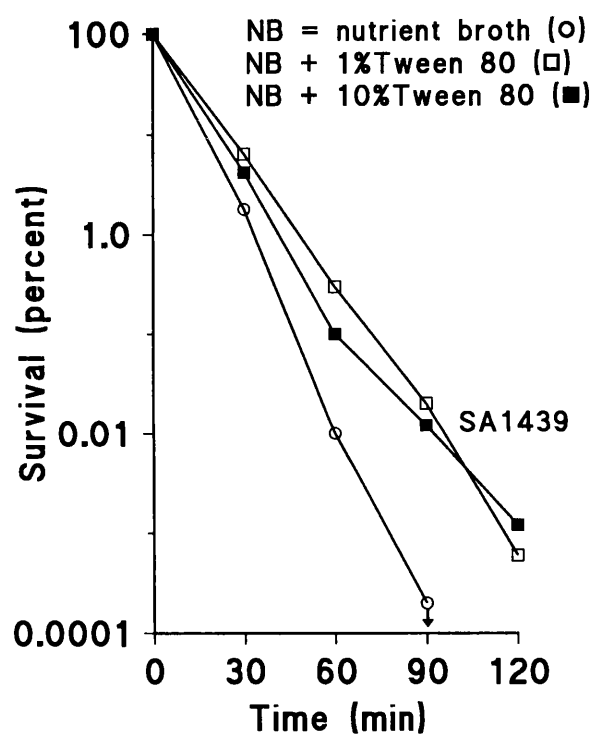
Figure 17. Effect of the additon of Tween 80 to the nutrient broth diluting medium on the recovery of *Staphylococcus aureus* strain SA1439 after exposure to 5 µg/ml chlorhexidine gluconate.

strain NCTC 10788 (Figure 18B) was higher when nutrient broth containing Tween at either concentration was used as diluent. Hence, 1% Tween 80 in nutrient broth was used as the inactivator in diluents used for chlorhexidine gluconate-exposed cells.

3.2.1.3 Cetrimide

Cetrimide showed a different trend, in that the addition of 10% Tween 80 to the diluting medium actually appeared to sensitize the cells to higher concentrations of this preservative. Viable counts were lower than those obtained using nutrient broth containing 1% Tween 80 as diluent. Figure 19 shows the effect of the addition of Tween 80 on the recovery of *S. aureus* strain SA1439 after exposure to 2.5 µg/ml (A), 5 µg/ml (B), 10 µg/ml (C) or 20 µg/ml (D) cetrimide in sterile water. At low concentrations of cetrimide, similar viable counts were obtained using nutrient broth alone or nutrient broth containing Tween 80 at either concentration. However, at 10 and 20 µg/ml cetrimide, the viable counts obtained after dilution in nutrient broth containing 1% Tween 80 were similar to those obtained using nutrient broth alone, whereas counts obtained after dilution in nutrient broth containing 10% Tween 80 were significantly lower. Therefore, since the inclusion of 1% Tween 80 made little difference to the recovery of strain SA1439, this concentration was included in the nutrient broth for the inactivation of cetrimide. The same diluent could then be used for benzalkonium chloride, chlorhexidine and cetrimide.

3.2.1.3 Diamidines

Dibromopropamidine isethionate was tested as a representative diamidine to observe the effect of the addition of Tween 80 to the diluting medium on the apparent bactericidal activity of concentrations ranging from 80 µg/ml to 6 mg/ml. Figure 20A shows the

A
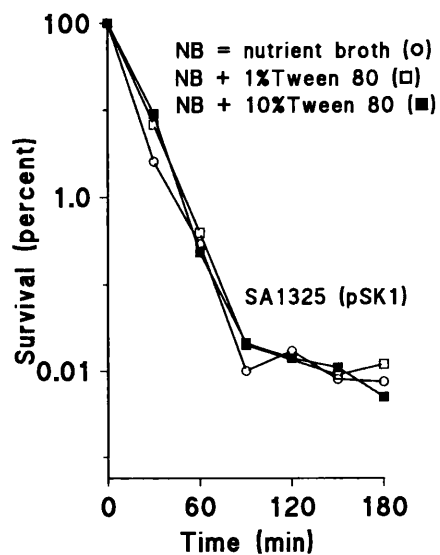
B
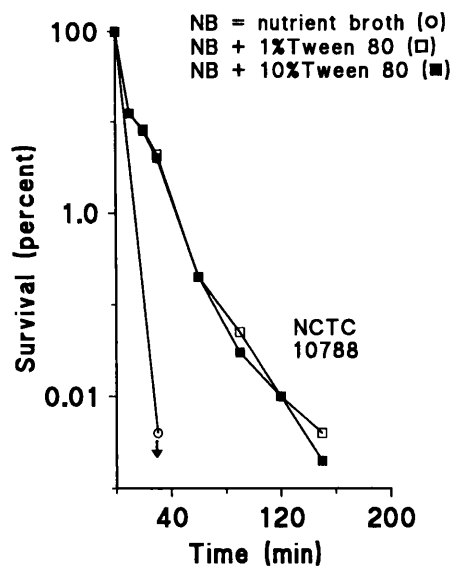
Figure 18. Effect of the addition of Tween 80 to the nutrient broth diluting medium on the recovery of (A) the plasmid-containing *Staphylococcus aureus* strain SA1325 (pSK1), and (B) the plasmid-less NCTC 10788 after treatment with 5 µg/ml chlorhexidine gluconate.

A
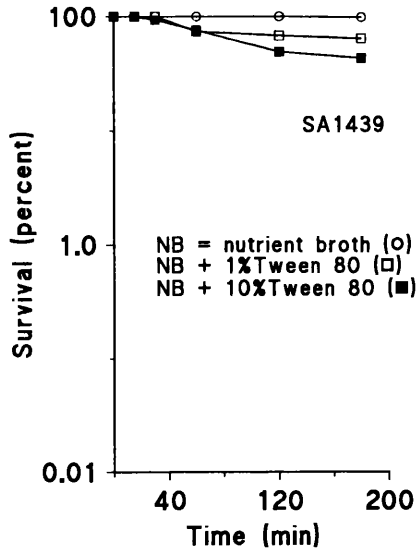
B
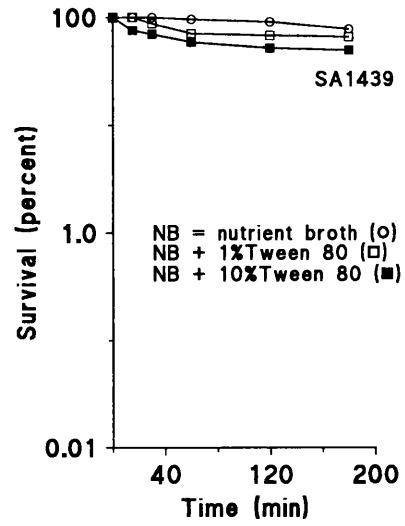
C
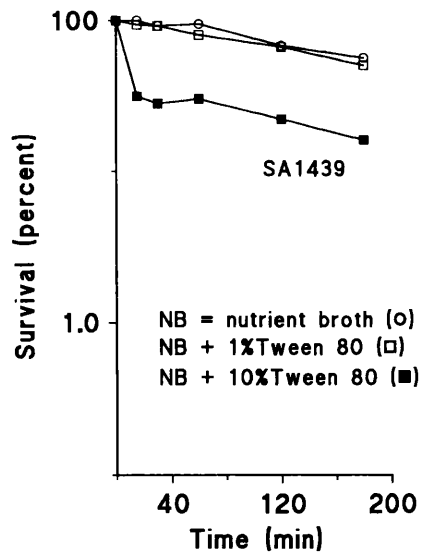
D
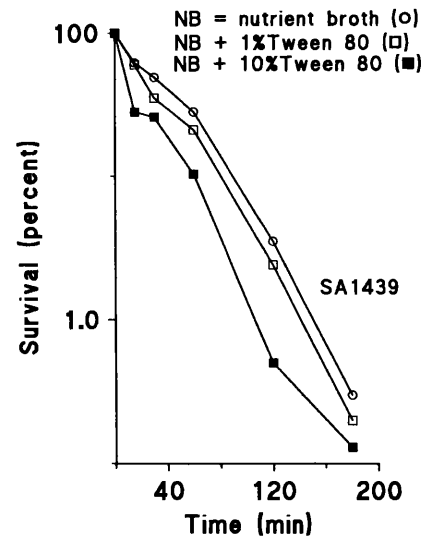
Figure 19. Effect of the addition of Tween 80 to the nutrient broth diluting medium on the recovery of *Staphylococcus aureus* strain SA1439 after exposure to (A) 2.5, (B) 5, (C) 10 or (D) 20 µg/ml cetrimide.

A
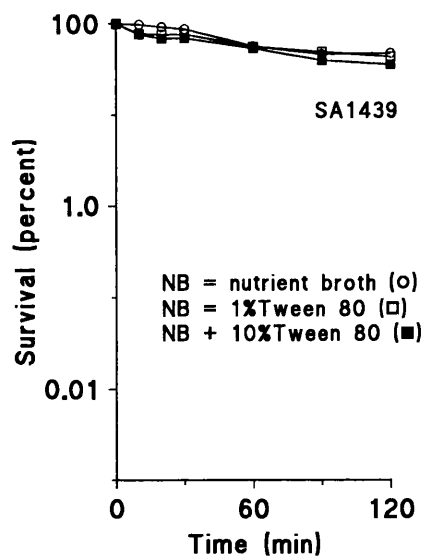
B
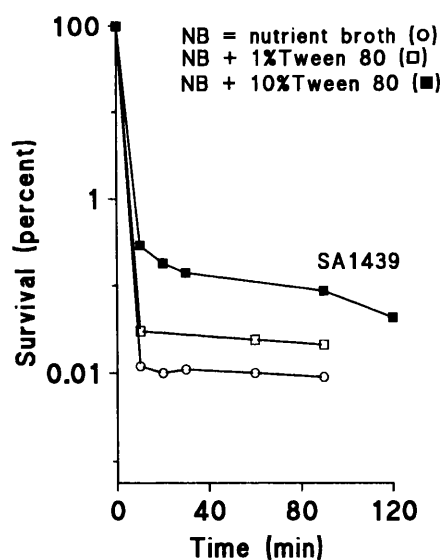
Figure 20. Effect of the addition of Tween 80 to the nutrient broth diluting medium on recovery of *Staphylococcus aureus* strain SA1439 exposed to (A) 80 and (B) 800 µg/ml of dibromopropamidine isethionate in sterile water.

effect of the addition of Tween 80 to the nutrient broth diluting medium on the recovery

of $S.$ $aureus$ strain SA1439 after exposure to 80 µg/ml dibromopropamidine isethionate

in sterile water. The addition of Tween made no difference to recovery from the

bactericidal effect of this low concentration. However, after plating cells exposed to

higher dibromopropamidine concentrations, colonies were obtained from 10^{-4} dilutions in

nutrient broth, but not from 10^{-2} dilutions, even though extrapolation of death curves

predicted there should be some viable cells present. It was likely these viable cells were

being inhibited from growth by carry-over of dibromopropamidine onto the nutrient agar

plate. Therefore, Tween 80 was tested as inactivator. It was found that at high

concentrations of dibromopropamidine, the higher concentration of nonionic surfactant

was required to achieve good inactivation. Figure 20B shows the effect of increasing the

concentration of Tween 80 on the recovery of $S.$ $aureus$ strain SA1439 exposed to 800

µg/ml of dibromopropamidine isethionate. The viable counts obtained were lower after

dilution in nutrient broth containing 1% Tween 80. Other $S.$ $aureus$ strains gave similar

results (Figure 21), but since in these experiments strain A.D. and NCTC 10788 appeared

to be more resistant than strain SA1439 to the effects of 800 µg/ml dibromopropamidine,

the viable counts recorded in Figures 21A and 21B were achieved with high dilutions of

test suspensions. Because of these high dilutions, the effect of Tween 80 is less

pronounced than is seen in Figure 20B. Nevertheless, to maintain the preservative

concentration below the MIC value in all dilutions plated, and to allow good recovery of

the organism, nutrient broth containing 10% Tween 80 was used to inactivate diamidines.

3.2.1.5 Phenylmercuric nitrate

There are many recommendations in the literature on the use of thioglycollate

A
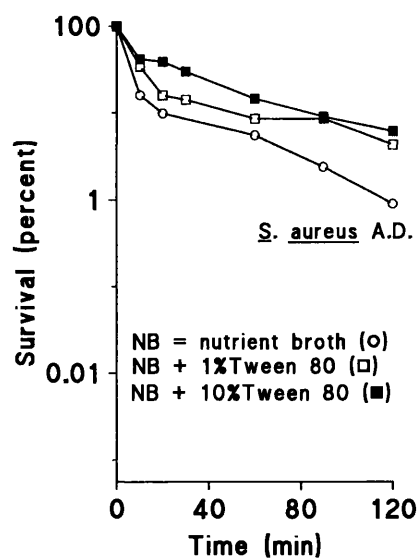
B
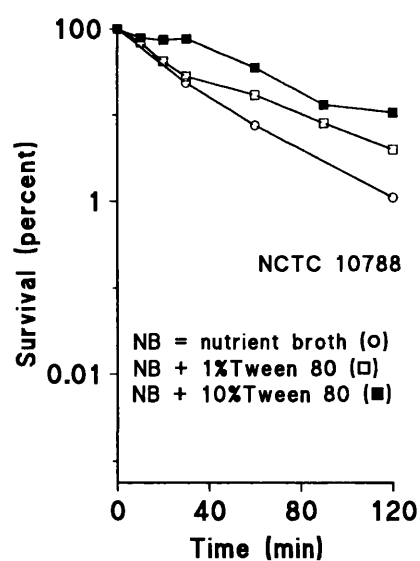
Figure 21. Effect of the addition of Tween 80 to the nutrient broth diluting medium on the recovery of *Staphylococcus aureus* strains exposed to 800 μg/ml dibromopropamidine isethionate in sterile water.

containing medium for the inactivation of mercurials (Hart, 1973; Russell, 1979). The

concentration recommended for inactivation of organomercurials is 0.1%. Therefore this

concentration was used to inactivate phenylmercuric nitrate in the nutrient broth diluting

medium. Confirmation of the need for thioglycollate was shown by the fact that viable

counts were lower after dilution in nutrient broth than in nutrient broth containing 0.1%

thioglycollic acid. This was true for both of the *S. aureus* strains tested (Figure 22). The

plasmid-less *S. aureus* strain SA1439 showed higher viable counts after dilution in

nutrient broth containing thioglycollic acid compared with dilution in nutrient broth alone

(Figure 22A). Similar results were observed for the plasmid-containing strain SA1325

(pSK1) (Figure 22B). Therefore, 0.1% thioglycollic acid was included in nutrient broth

diluents as inactivator for phenylmercuric nitrate.
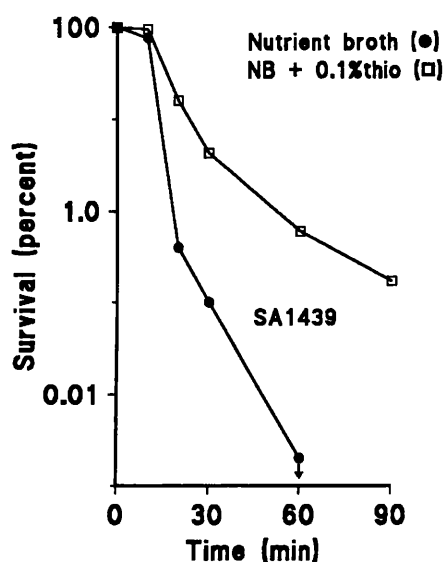
A   B
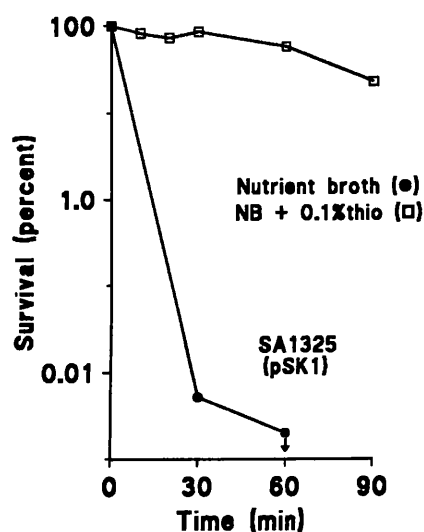
Figure 22. Effect on the addition of thioglycollic acid (thio) to the nutrient
broth (NB) diluting medium on *Staphylococcus aureus* strains exposed to
1 µg/ml phenylmercuric nitrate in sterile water.

3.2.2 The effect of increasing concentration of the preservative on bactericidal activity

Short-term bactericidal experiments were performed with various concentrations of each of the preservatives to obtain suitable working concentrations for future strain sensitivity comparisons. The concentrations required were those that produced moderate bactericidal rates, so as to permit accurate observation of the declining counts.

3.2.2.1 Benzalkonium chloride

The effect of increasing concentrations of benzalkonium chloride on the viability of *S. aureus* strain SA1439 can be seen in Figure 23A. The concentration chosen for performing the short-term bactericidal tests was 10 µg/ml.

3.2.2.2 Chlorhexidine gluconate

This preservative was tested at concentrations ranging from 5 to 40 µg/ml, however results were obtained only for 5 and 10 µg/ml, higher concentrations were too active to obtain viable counts with the sampling times used. The concentration chosen to perform comparative bactericidal activity experiments was 5 µg/ml (Figure 23B).

3.2.2.3 Cetrimide

Cetrimide was tested at concentrations ranging from 2.5 to 40 µg/ml, however the latter concentration produced an excessive rate of kill and viable counts were not obtained. For further comparative experiments 20 µg/ml was the concentration chosen (Figure 24A).

A
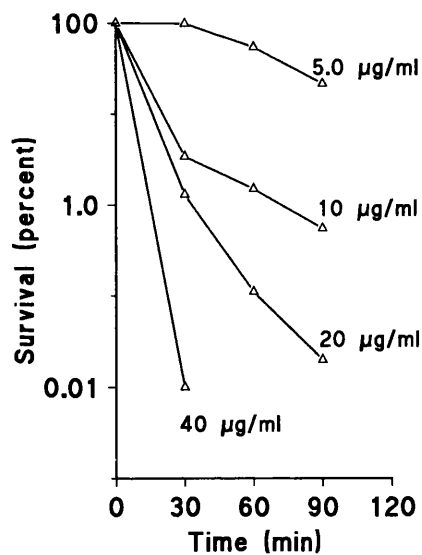
B
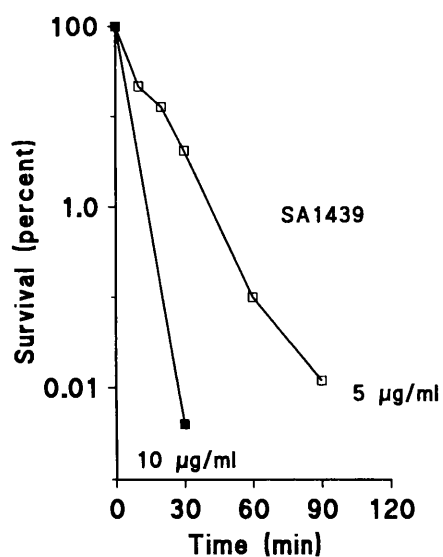
Figure 23. Effect of increasing the preservative concentration on the viability of *Staphylococcus aureus* SA1439 exposed to (A) benzalkonium chloride and (B) chlorhexidine gluconate. Inactivator used, 1% Tween 80 in nutrient broth.

3.2.2.4 Diamidines

Experiments with a range of concentrations of each of these preservatives showed that high concentrations were needed to achieve bactericidal activity; these were higher for propamidine (Figure 24B) than for dibromopropamidine isethionate (Figure 25A). For future work, concentrations of 1 mg/ml and 5 mg/ml were chosen for dibromopropamidine and propamidine isethionates, respectively.
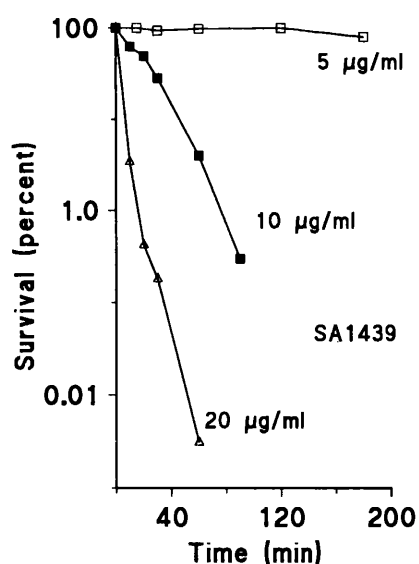
A   B
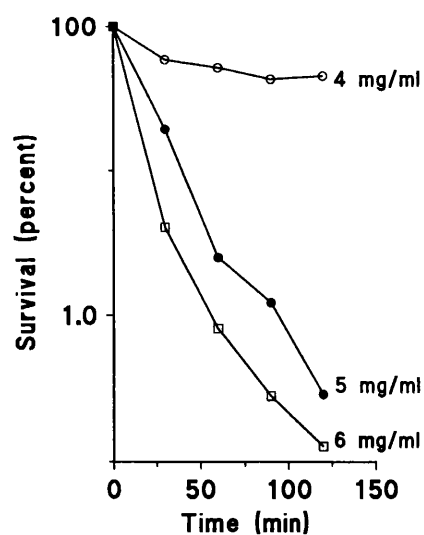
Figure 24. Effect of increasing the preservative concentration on recovery of *Staphylococcus aureus* SA1439 exposed to (A) cetrimide and (B) propamidine isethionate.

3.2.2.5 Phenylmercuric nitrate

Concentrations of 1 and 10 µg/ml of this compound were tested in sterile water (Figure 25B). Inactivation of phenylmercuric nitrate was achieved by including 0.1% thioglycollic acid in the nutrient broth used for dilution. Both concentrations will be used throughout this work.
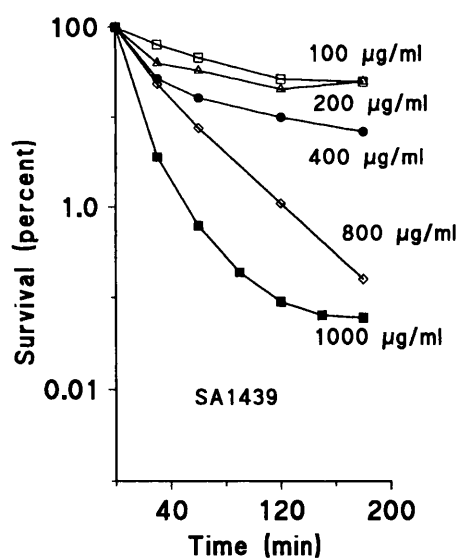
A   B
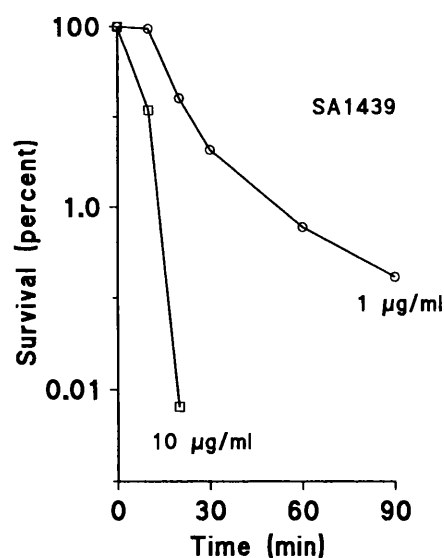
Figure 25. Effect of increasing preservative concentration on recovery of *Staphylococcus aureus* strain SA1439 exposed to (A) dibromopropamidine isethionate and (B) phenylmercuric nitrate.

3.2.3 Comparison of strain sensitivities using short-term bactericidal activity tests

3.2.3.1 Benzalkonium chloride

Results of experiments in which the different strains were exposed to 10 μg/ml benzalkonium chloride in sterile water can be seen in Figure 26. The plasmid-less *P. aeruginosa* strain NCIB 8626 was highly resistant to this quaternary ammonium compound, which explains why this species is frequently found as a contaminant of hospital disinfecting solutions (Russell *et al.*, 1986). Calculations based on survival levels after 180 min of exposure showed NCIB 8626 to be 100-fold more resistant than the plasmid-carrying *S. aureus* strain SA1325 (pSK1). The MIC data (Table 9) had revealed similar sensitivities of the plasmid-less *S. aureus* strains NCTC 10788 and SA1439 to benzalkonium chloride. This was not paralleled by similar sensitivities to the bactericidal concentration of 10 μg/ml (Figure 26). Similarly, the MIC of *S. aureus* strain SA1325 (pSK1) to benzalkonium chloride was greater than its plasmid-less parent strain SA1439 (Table 9). However, when tested in the bactericidal concentration of 10 μg/ml the survival of the plasmid-containing strain was surprisingly less than that observed for its plasmid-less parent strain SA1439 after 60 min. This difference in survival began to decrease rapidly towards the end of the experiment (Figure 26). Once again the MIC data (Table 9) did not correlate with sensitivities observed in bactericidal concentrations.

Another surprise was that *S. aureus* strain A.D. was not resistant to the bactericidal concentration of benzalkonium chloride (Figure 26). This was predicted because it had been isolated from a contaminated ophthalmic solution preserved with this same compound. It had also shown high resistance levels in the MIC experiments (Table 9).

Because the results obtained with strains SA1325 (pSK1) and A.D. were so
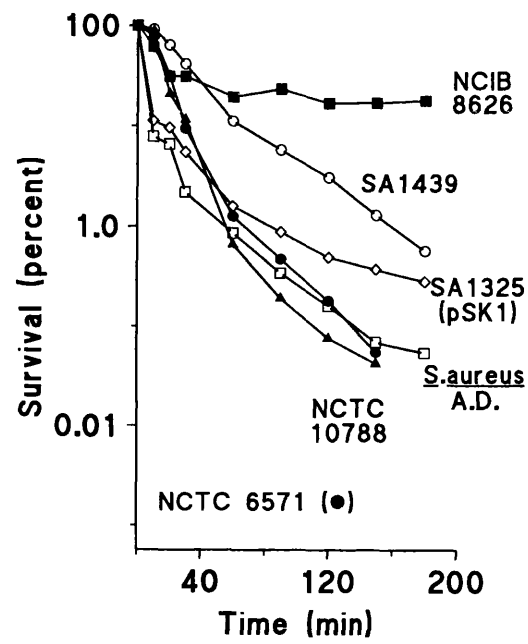
Figure 26. Survival of *Staphylococcus aureus* strains and
Pseudomonas aeruginosa NCIB 8626 in aqueous 10 μg/ml
benzalkonium chloride solution at 25°C.

unexpected, the experiments were repeated at least five times using cultures grown from single colony isolates of *S. aureus* strains A.D. and SA1325 (pSK1) as inocula. However, in every instance, the pattern was repeated, strain SA1439 was always more resistant than either SA1325 (pSK1) or A.D. in short term bactericidal activity experiments.

3.2.3.2 Chlorhexidine gluconate

Results of short-term bactericidal activity experiments with 5 µg/ml chlorhexidine gluconate are shown in Figure 27. *P. aeruginosa* NCIB 8626, which demonstrated the highest resistance level towards chlorhexidine gluconate in the MIC experiments (Table 9), was highly sensitive to the bactericidal concentration of this preservative in sterile water. No viable organisms could be recovered after 30 min of exposure.

The plasmid-less *S. aureus* strains NCTC 6571, NCTC 10788 and SA1439, were all susceptible to chlorhexidine gluconate, with NCTC 6571 being the most susceptible; no viable organisms were recovered after 60 min of exposure from an initial inoculum of 1.22×10^8/ml. These plasmid-less *S. aureus* strains showed a similar order of bactericidal sensitivities as observed in the MIC tests (Table 10).

S. aureus strain SA1325 (pSK1) and *S. aureus* A.D. gave similar, high resistance levels to the bactericidal concentration of chlorhexidine gluconate (Figure 13). This paralleled their high MIC's. Comparing *S. aureus* strain SA1325 (pSK1) and its isogenic plasmid-less parent SA1439, it is evident that the plasmid increased survival by almost 100-fold after 90 min and subsequent periods of exposure.

The results obtained with the *S. aureus* strains in the bactericidal concentration of chlorhexidine therefore correlate with their MIC data and, demonstrate the increase in survival brought about by the presence of the plasmid. However, the sensitivity of *P.*
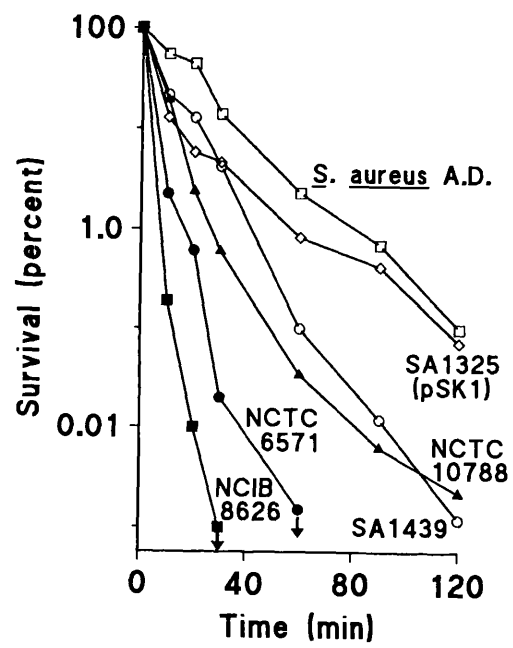
Figure 27. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous 5 µg/ml chlorhexidine gluconate solution at 25°C.

aeruginosa was unexpected. It had exhibited the highest resistance level in the MIC experiments.

3.2.3.3 Cetrimide

Details of the sensitivities of the different strains to 20 µg/ml cetrimide in sterile water can be seen in Figure 28. Here the results were much more as expected because this resistances paralleled the MIC data (Table 9). *P. aeruginosa* NCIB 8626 and *S. aureus* strain SA1325 (pSK1) were highly resistant to this preservative, with viable counts remaining above 10% survival until the end of the test. In fact, the viable counts of *P. aeruginosa* appeared to increase after 90 min, however this is not too surprising as this organism is known to grow in nutrient agar containing 0.1% cetrimide (Brown and Lowbury, 1965). In fact, cetrimide agar is used as a selective medium for pesudomonads (Collins *et al.*, 1989).

After 60 min of exposure the survival of the plasmid-carrying *S. aureus* strain SA1325 (pSK1) was more than 1000-fold greater than its isogenic parent strain SA1439. No viable cells of SA1439 could be recovered after 90 min, whereas the survival of SA1325 was maintained at about 10% even after 180 min. Although, more sensitive than the plasmid-containing strain, *S. aureus* A.D. was more resistant to cetrimide than the plasmid-less strains NCTC 10788, SA1439 and NCTC 6571; the latter three strains were all susceptible to cetrimide, with no viable organisms being recovered after 120 min from the initial inoculum of 10^8/ml.

Results obtained with cetrimide correlated well with the MIC data previously obtained. However the survival of *S. aureus* A.D. might have been expected to be more similar to that of strain SA1325 (pSK1), as they exhibited similar MICs to cetrimide
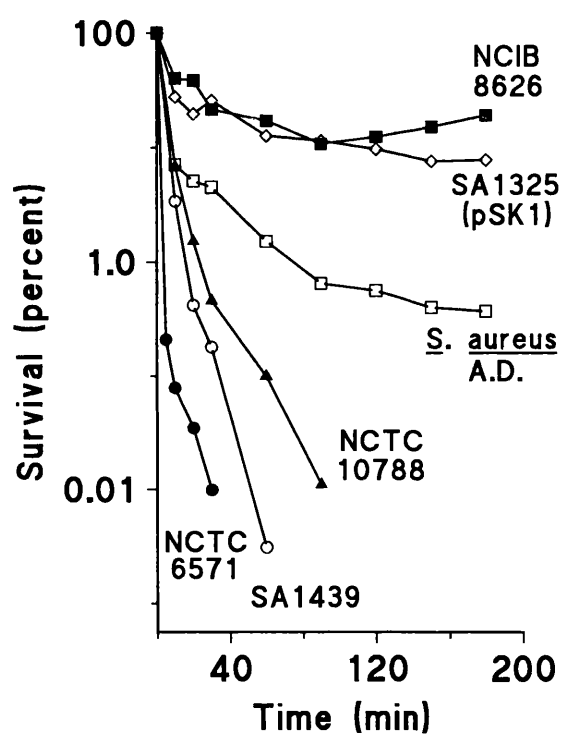
Figure 28. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous 20 µg/ml cetrimide solution at 25°C.

(Table 12). Once more, the plasmid presence makes a significant difference to survival in aqueous solutions of pharmaceutical preservatives.

3.2.3.4 Diamidines

3.2.3.4.1 Dibromopropamidine isethionate.

Results of short-term bactericidal activity tests with 1 mg/ml dibromopropamidine isethionate in sterile water are shown in Figure 29. Surprisingly, *P. aeruginosa* NCIB 8626 was the most sensitive organism to this preservative, although it was highly resistant to it in the MIC experiments. No viable cells of this strain could be recovered after 30 min from an initial inoculum of 1.25 x 10^8/ml. However, as with chlorhexidine, it should be stressed that the MIC studies were performed on nutrient agar. All necessary nutritional requirements were therefore present, which were not available in the sterile water used for bactericidal experiments. The plasmid-less *S. aureus* strains SA1439 and NCTC 10788 were more resistant than the pseudomonad to the bactericidal activity of dibromopropamidine isethionate. However, no viable cells of strains NCTC 6571 and SA1439 were recovered after 60 and 120 min of exposure from initial inocula of 1.69 and 4.72 x 10^8/ml, respectively.

The plasmid-carrying *S. aureus* strain SA1325 (pSK1) was at least 10-fold more resistant than its isogenic plasmid-less parent strain SA1439 at sampling times, and whereas viable SA1325 (pSK1) cells were recovered after 180 min exposure, no viable SA1439 were found after 120 min.

The most resistant organism towards dibromopropamidine isethionate was the *S. aureus* A.D. strain obtained from a contaminated ophthalmic solution. Survival of this organism was 1000-fold greater than the plasmid-less strain SA1439 after 120 min. The
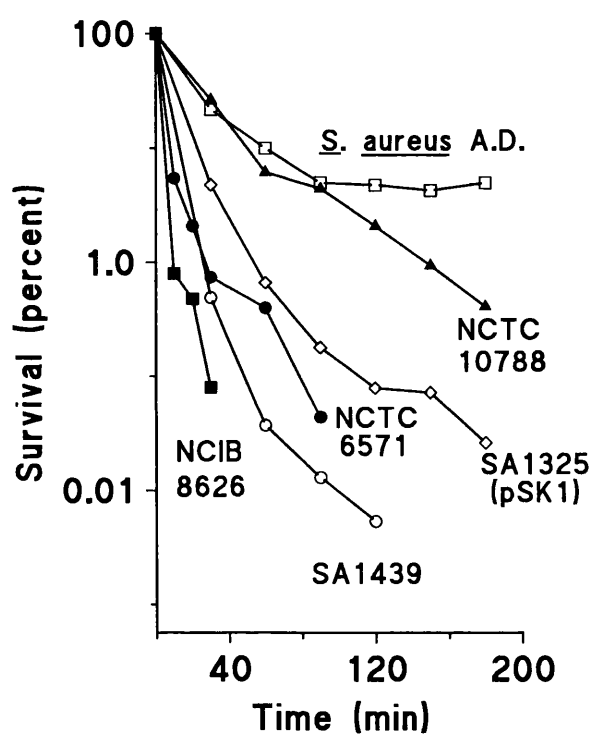
Figure 29. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous dibromopropamidine isethionate 1 mg/ml solution at 25°C.

resistance of *S. aureus* strain NCTC 10788 was an unexpected result. It did not correlate

with the order of resistances found for the *S. aureus* strains in the MIC experiments

(Table 9).

3.2.3.4.2 Propamidine isethionate

The bactericidal activities of aqueous 5 mg/ml propamidine isethionate can be seen

in Figure 30. An interesting observation is that the shape of the killing rate curves

obtained for propamidine and dibromopropamidine isethionate are very similar (compare

Figures 29 and 30). *P. aeruginosa* NCIB 8626 was the most susceptible organism to

propamidine, with no viable cells being recovered after 120 min. This finding does not

correlate with the resistance to propamidine shown in the MIC experiments on nutrient

agar (Table 9).

S. aureus SA1325 (pSK1) was more resistant than its isogenic plasmid-less parent

SA1439, with the presence of the plasmid increased survival in propamidine by about

100-fold after 90 min of exposure. As with dibromopropamidine, it is interesting and

unexpected that the plasmid-less *S. aureus* strain NCTC 10788 was more resistant than

the plasmid-containing strain SA1325 (pSK1).

S. aureus A.D. was the most resistant organism to propamidine (Figure 30), as it was

to dibromopropamidine (Figure 29). It gave approximately 1000-fold and 10-fold more

survivors after 300 min of contact than the plasmid-less *S. aureus* strains SA1439 and

NCTC 10788, respectively.
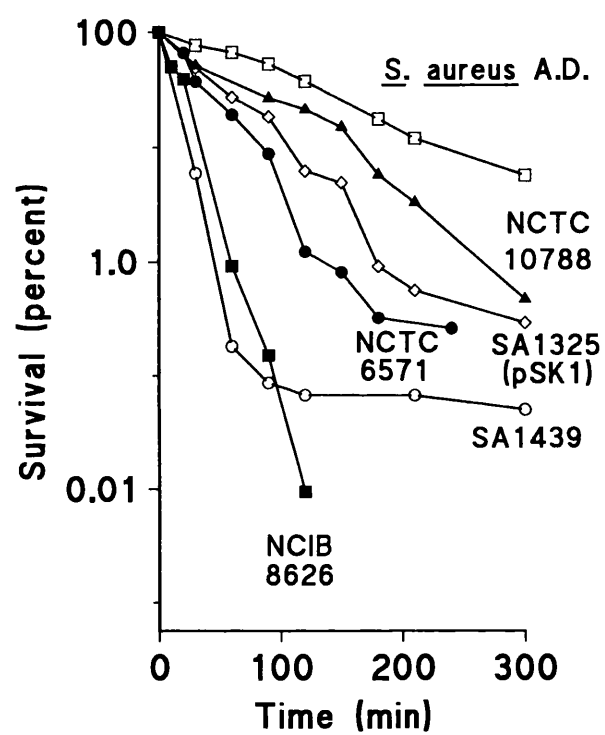
Figure 30. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous 5 mg/ml propamidine isethionate solution at 25°C.

3.2.3.5 phenylmercuric nitrate

3.2.3.5.1 *E. coli* strains.

The only plasmid tested in this work that was known to confer mercury resistance was the *E. coli* plasmid R471-1. Therefore to show its effects on survival in mercury-preserved systems, plasmid-containing and plasmid-less strains of *E. coli* were exposed to 1 and 10 µg/ml phenylmercuric nitrate in sterile distilled water.

MIC results demonstrated similar sensitivities for *E. coli* strains 343/113 and 343/113 (R471-1) to a wide range of non-mercury preservatives (Table 9), but a significant difference was observed when tested with phenylmercuric nitrate (Table 11). This difference in susceptibility was also observed in the bactericidal activity experiments. Figure 31 shows the survival of *E. coli* strains 343/113 and 343/113 (R471-1) exposed to phenylmercuric nitrate in sterile water. At both concentrations of the preservative, the survival of strain 343/113 was improved by the presence of the plasmid. At 1 µg/ml phenylmercuric nitrate in sterile water, the survival of the plasmid-containing strain was 10-fold higher than that of the isogenic plasmid-less strain after 180 min exposure; 10 µg/ml, survival was almost 100-fold greater after the same time. Here again is demonstrated, without doubt, the importance of plasmids to the survival mechanisms of bacteria.

3.2.3.5.2 *S. aureus* strains and *P. aeruginosa* NCIB 8626

Phenylmercuric nitrate was also tested against the plasmid-carrying and plasmid-less *S. aureus* strains and against *P. aeruginosa*. The MIC obtained with the latter organism was much higher than MICs for any of the *S. aureus* strains (Table 9), which made some of the results obtained with bactericidal concentrations unexpected (Figure 32 and 33).

At a phenylmercuric nitrate concentration of 1 µg/ml the plasmid-less *P. aeruginosa*
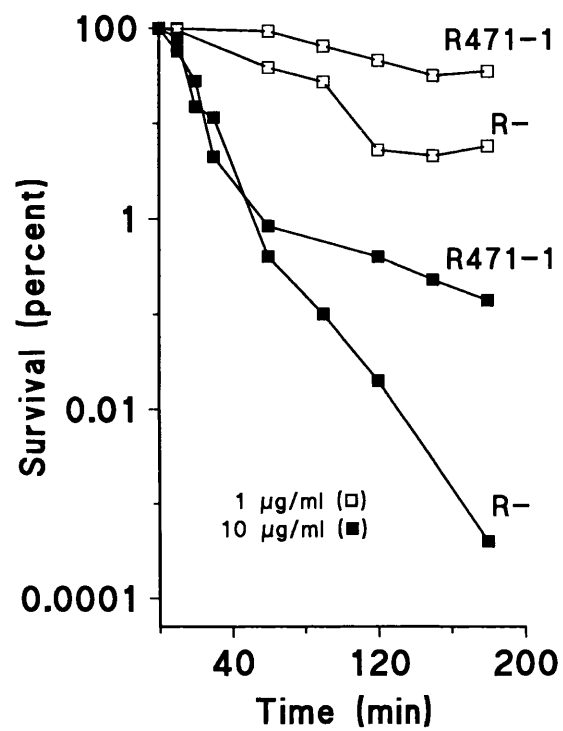
Figure 31. Survival of plasmid-less *Escherichia coli* strain 343/113 (R⁻) and plasmid-containing strain 343/113 (R471-1) in aqueous phenylmercuric nitrate 1 and 10 µg/ml solution at 25°C.

strain NCIB 8626 showed similar sensitivity as the resistant *S. aureus* strain A.D. during the first 60 min of exposure, after which time, the count of the pseudomonad began to decline more rapidly than that of the staphylococcus (Figure 32). These comparative sensitivities to the bactericidal activity of phenylmercuric nitrate did not therefore correlate with the MIC experiments. Better correlation between MICs and sensitivity to the bactericidal effects of 1 μg/ml phenylmercuric nitrate in water were obtained for *S. aureus* strains NCTC 6571, A.D., NCTC 10788 and SA1439 (compare Figure 32 with Table 9). Viable cells of all *S. aureus* strains were recovered after 180 min, with the exception of NCTC 6571 the highly sensitive strain, of which no viable cells could be recovered after 150 min. It is interesting that the survival of the plasmid-containing *S. aureus* strain SA1325 (pSK1) was more than 10-fold greater than the plasmid-less isogenic parent SA1439. However pSK1 is not claimed to confer resistance to mercurials (Lyon and Skurray, 1987).

When, all strains were exposed to 10 μg/ml phenylmercuric nitrate in sterile water their survival decreased dramatically (Figure 33). No viable cells of *P. aeruginosa* NCIB 8626 could be recovered after 10 min of exposure, even though the MIC studies with this organism showed it to be highly resistant on nutrient agar (Table 9). The *S. aureus* strains were slightly more resistant than the pseudomonad. However, after 60 min of exposure none could be recovered with the exception of *S. aureus* A.D. Although possessing an MIC lower than that by *P. aeruginosa* NCIB 8626 when tested in nutrient agar, *S. aureus* A.D. was highly resistant to the bactericidal activity of this organomercurial in an aqueous environment. Survival of this strain was 10,000-fold greater than the rest of the strains tested and viability was maintained at greater than 1% until the end of the experiment (Figure 33).
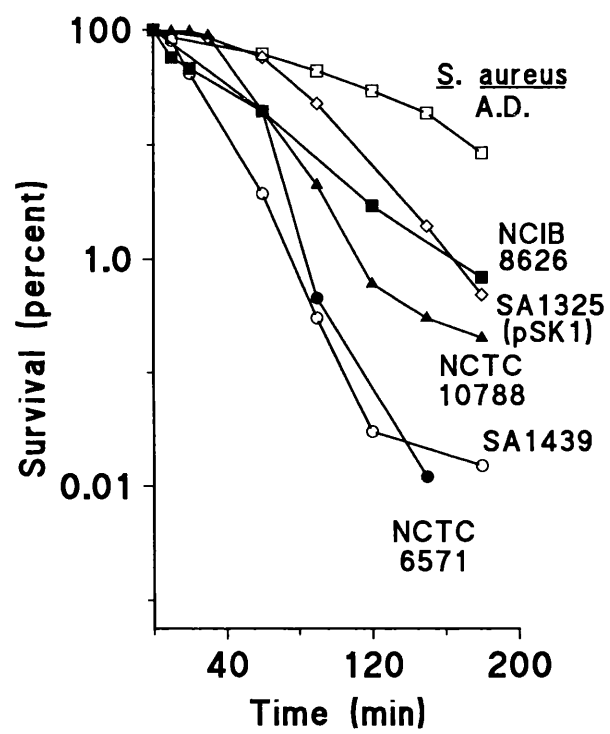
Figure 32. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous phenylmercuric nitrate 1 μg/ml solution at 25°C.
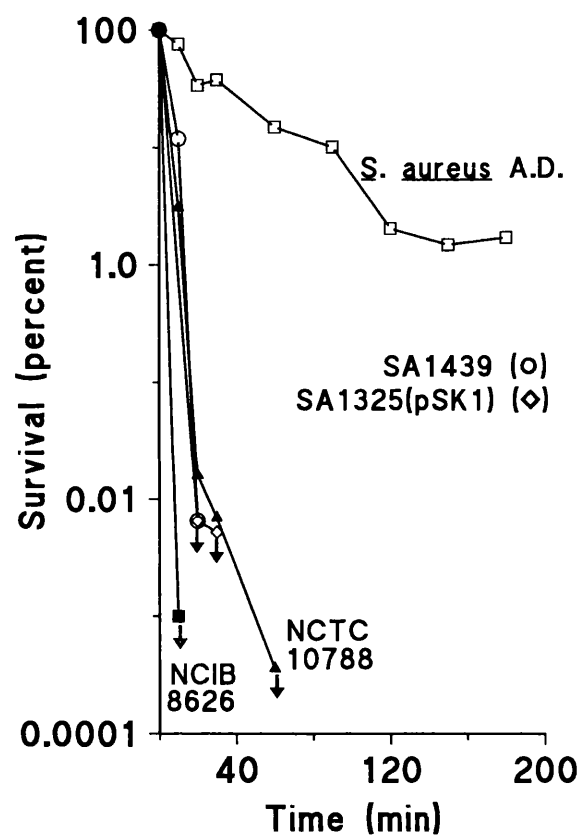
Figure 33. Survival of *Staphylococcus aureus* strains and *Pseudomonas aeruginosa* NCIB 8626 in aqueous phenylmercuric nitrate 10 µg/ml solution at 25°C.

3.2.4 Comparative short-term bactericidal activity tests using the plasmid-less strains

S. aureus SA1439, P. aeruginosa NCIB 8626 and E. coli 343/113.

The previous set of bactericidal activity experiments were undertaken to compare the sensitivities of the *S. aureus* strains and the highly resistant *P. aeruginosa* strain NCIB 8626 to a wide range of preservatives. However, *E. coli* 343/113 was only tested against phenylmercuric nitrate. Therefore, a series of experiments were performed to compare the sensitivities of the plasmid-less *E. coli* 343/113 to those of a plasmid-less *S. aureus* strain and the pseudomonad.

3.2.4.1 Benzalkonium chloride. Bactericidal activity experiments with 10 μg/ml benzalkonium chloride (Figure 34A) showed *E. coli* 343/113 was the most resistant organism. Survival was maintained at 100% until the end of the test. After 180 min the survival of this organism was almost 10-fold, and more than 100-fold greater respectively, than that observed for *P. aeruginosa* NCIB 8626 and *S. aureus* SA1439. The viable count of *P. aeruginosa* NCIB 8626 fell rapidly during the first 20 min of exposure and then remained constant with counts just above 90% survival. These bactericidal results do not correlate with the results obtained in the MIC experiments. The MIC of the pseudomonad was 10-fold higher than that of *E. coli* 343/113 (Table 10). *S. aureus* strain SA1439 was the least resistant organism in both MIC (Table 10) and bactericidal experiments (Figure 34A).

3.2.4.2 Chlorhexidine gluconate. *E. coli* 343/113 showed survival levels 10,000-fold and 100,000-fold greater than *P. aeruginosa* strain NCIB 8626 and *S. aureus* SA1439, respectively in chlorhexidine gluconate 5 μg/ml (Figure 34B). Once again this did not

correlate with the MIC experiments which showed the pseudomonad to be the most resistant organism, with the *E. coli* and the *S. aureus* having similar sensitivities (Table 10).

3.2.4.3 Cetrimide.

P. aeruginosa NCIB 8626 was the most resistant organism when exposed to 20 μg/ml cetrimide (Figure 34C). Its viable count remained above 90% survival until the end of the test. *E. coli* 343/113 exhibited a sharp initial reduction in viable count, but after 10 min the count remained constant until the end of the experiment. *S. aureus* strain SA1439 was highly sensitive; after 60 min, no viable cells could be recovered. These bactericidal activities correlated with the results of the MIC experiments (compare Figure 34C with data in Table 10).

3.2.4.4. Diamidines.

3.2.4.4.1 Dibromopropamidine isethionate.

P. aeruginosa NCIB 8626 was the least resistant organism to the antibacterial effect of dibromopropamidine isethionate. No viable organisms could be recovered after 30 min of exposure. *S. aureus* strain SA1439 was slightly more resistant than the pseudomonad, with no viable organism recovered after 120 min. *E. coli* 343/113 remained viable until the end of the test (Figure 34D). MIC studies showed *P. aeruginosa* strain NCIB 8626 was highly resistant to this compound, with *S. aureus* strain SA1439 and *E. coli* strain 343/113 having identical MIC's (Table 10). The bactericidal activity tests did not therefore give the same order of resistance (Table 10).

3.2.4.4.2 Propamidine isethionate.

Once again, *P. aeruginosa* NCIB 8626 was the least resistant organism, no viable cells being recovered after 120 min. The *S. aureus* was as

sensitive as the pseudomonad initially. However after 120 min the viable count of the staphylococcus remained constant. The survival level of *E. coli* 343/113 was 100-fold and 10-fold greater than *P. aeruginosa* NCIB 8626 and *S. aureus* SA1439 respectively, after 120 min. These results are therefore similar to the data obtained for dibromopropamidine and do not correlate with strain sensitivities as determined by MIC tests.

3.2.4.5 Phenylmercuric nitrate. At a concentration of 1 µg/ml phenylmercuric nitrate, the sensitivities of *P. aeruginosa* NCIB 8626 and *E. coli* 343/113 were initially similar, but after 180 min the *E. coli* was 10-fold more resistant than the pseudomonad (Figure 34F). This order of sensitivities does not correlate with the MIC data (Table 10). As expected, *S. aureus* strain SA1439 was the least resistant organism (Figure 34F, Table 10).

Exposure at a concentration of 10 µg/ml phenylmercuric nitrate resulted in only *E. coli* 343/113 cells being recovered at the end of the test. No viable *P. aeruginosa* NCIB 8626 or *S. aureus* SA1439 could be recovered after 10 and 20 min exposure, respectively (Figure 35A). Once again the bactericidal results with phenylmercuric nitrate do not correlate with the MIC data (Table 10).
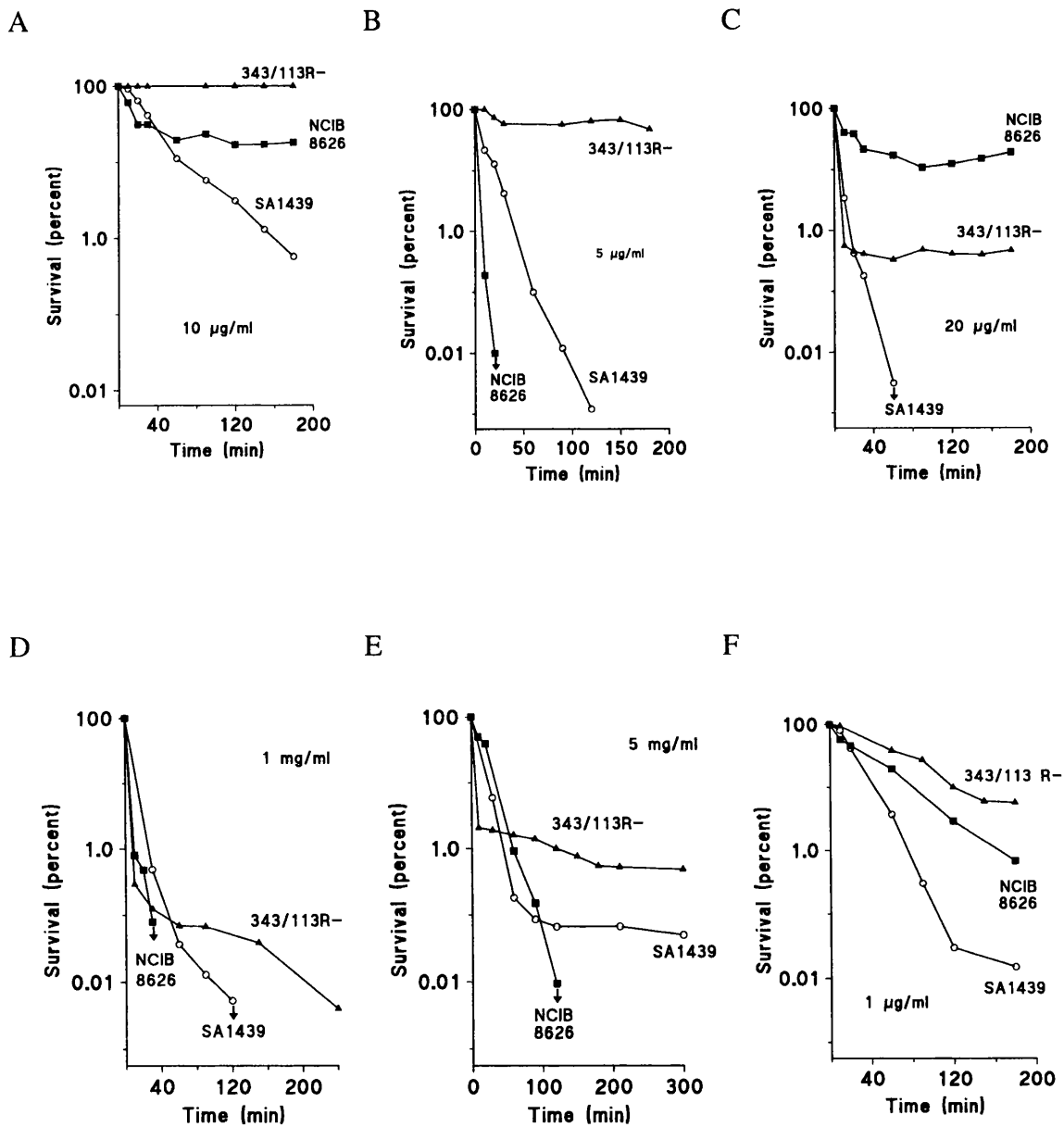
Figure 34. Survival of plasmid-less *Staphylococcus aureus* SA1439, *Escherichia coli* 343/113 and *Pseudomonas aeruginosa* NCIB 8626 in aqueous (A) benzalkonium chloride, (B) chlorhexidine gluconate, (C) cetrimide, (D) dibromopropamidine isethionate, (E) propamidine isethionate and (F) phenylmercuric nitrate.

C
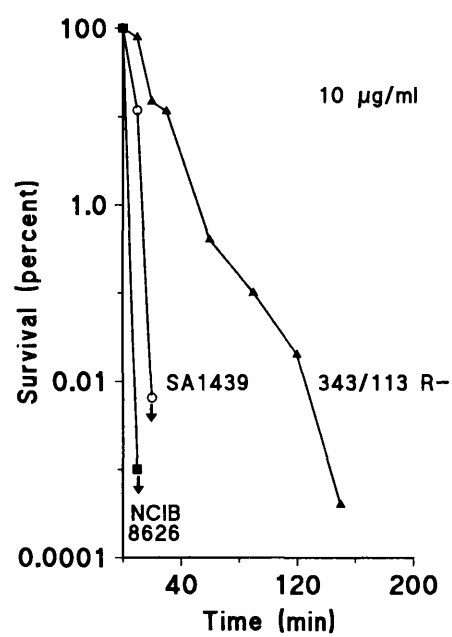
Figure 35. Survival of plasmid-less *Staphylococcus aureus* SA1439, *Escherichia coli* 343/113 and *Pseudomonas aeruginosa* NCIB 8626 in aqueous 10 μg/ml phenylmercuric nitrate.

Summary

Tween 80 was tested as preservative inactivator in the nutrient broth diluting medium, as suggested by the BP 1988, appendix XVI C. Suitable inactivation was produced at a concentration of 1% Tween in nutrient broth for 10 μg/ml benzalkonium chloride, 5 μg/ml chlorhexidine gluconate and 20 μg/ml cetrimide. Propamidine and dibromopropamidine isethionates, at 1 and 5 mg/ml respectively, required 10% Tween 80. Phenylmercuric nitrate at 1 and 10 μg/ml was inactivated by the inclusion of 0.1% thioglycollic acid in the nutrient broth diluting medium.

Some of the short-term bactericidal activity experiments in sterile water showed the preservative sensitivities of *P. aeruginosa* NCIB 8626 did not correlate with MICs determined on nutrient agar. For example, short-term bactericidal activity experiments in chlorhexidine gluconate, phenylmercuric nitrate, propamidine or dibromopropamidine isethionate showed *P. aeruginosa* strain NCIB 8626 to be the most sensitive organism. Correlation with MIC data for the pseudomonad was only obtained for benzalkonium chloride and cetrimide.

Results of bactericidal activity experiments in chlorhexidine gluconate, cetrimide, propamidine or dibromopropamidine using the plasmid-containing *S. aureus* strain SA1325 (pSK1) paralleled those obtained in MIC tests. The plasmid-containing strain showed higher survival levels than its isogenic plasmid-less parent. MIC data did not correlate with bactericidal activity tests in the case of benzalkonium chloride in which the plasmid-less SA1439 exhibited a higher survival than the plasmid-containing SA1325. In addition, the plasmid-containing *E. coli* strain 343/113 demonstrated the efficacy of (R471-1) in enhancing survival of the organism in a phenylmercuric nitrate preservative solution.

The resistant *S. aureus* strain A.D., although having MIC's lower than those exhibited

by *P. aeruginosa* strain NCIB 8626, was more resistant to propamidine, dibromopropamidine, cetrimide and phenylmercuric nitrate than the rest of the plasmid-less strains. Resistance of this *S. aureus* strain was also exceedingly high to phenylmecuric nitrate, even higher than that observed for *E. coli* 343/113 (R471-1). Once more, MIC data did not correlate with results of bactericidal activity experiments.

3.3 Effect of plasmids on performance of strains under *British Pharmacopoeia* Preservative Challenge Test conditions

Minimum inhibitory concentration tests (Chapter III, section 3.1) and short-term bactericidal activity tests (Chapter III, section 3.2) have illustrated the variation in susceptibility of the various strains to the range of preservatives used. The short-term bactericidal tests were not performed by an official compendial method, and being short term they did not show how persistant or resistant organisms would survive in preserved systems under long term conditions. The BP Challenge Test procedure (1988) was therefore employed to assess the performance of strains under such conditions. In addition, it was of interest to observe if survival of the *S. aureus* and *E. coli* plasmid-containing strains exceeded or paralleled that exhibited by the highly resistant, but plasmid-less, *P. aeruginosa* NCIB 8626 test strain.

The inoculated preservative solutions were simple aqueous systems, such as those encountered in injections and ophthalmic solutions. Since some of the preservatives, such as benzalkonium chloride and chlorhexidine gluconate, are used in eye-drops but not in injections, the criterion of acceptance of the BP Challenge Test Appendix XVI C, for ophthalmic solutions was used as the end point for compliance. For this type of product, compliance requires a 10^3 (or 3 log cycle) reduction of the initial viable count of 10^6 organisms per ml after 6 hours of challenge, and no viable cells recovered from 1 ml of sample after 24 hours and thereafter, up to a period of 28 days.

Test organisms were grown in tryptone soya broth or soybean-casein broth overnight at 30°C. The following day the overnight cultures were washed with sterile normal saline and resuspended in 0.1% peptone water. Enough diluent was then added to reduce the counts to 10^8/ml. This procedure differed from that used in the preparation of inocula for

the short-term bactericidal tests; in the latter, sterile distilled water had been used for washing and resuspending organisms. A viable count was performed on the washed suspension and 0.1 ml of it was added to 9.9 ml of preservative solution at 25°C. Immediately after inoculation, a viable count was performed to obtain the initial number of viable organisms per ml, diluting in 0.1% peptone water. Comparison of the control count obtained from the washed and diluted overnight culture and the zero time count from the preservative suspension showed that the method used to inactivate the preservative was satisfactory, at least when viable counts were high, and dilution therefore played a part in preservative inactivation.

Experiments to test preservative inactivation in the presence of low numbers of viable organisms were performed following the British Standards Institution (1960) methodology, with an overnight washed culture of *S. aureus* strain SA1439. This was resuspended in 4.5 ml sterile distilled water and diluted further in sterile distilled water to obtain 10^3 organisms/ml. From this, 1.0 ml was taken and added to 9.0 ml of 3% Tween 80 solutions containing 100 µg/ml benzalkonium chloride, cetrimide or chlorhexidine gluconate. Control suspensions in sterile distilled water and 3% Tween 80 were also prepared. 0.1 ml samples were plated on nutrient agar at time 0, and at 30 min intervals for up to 2 h. After incubation at 37°C, it was found that viable counts of controls and preservative-treated organisms were similar during the first hour of the experiment. After 1 h, viable counts of preservative-treated cells began to decline (Table 15).

The results in Table 15 show that a concentration of 3% Tween 80 was suitable for the recovery of low numbers of viable organisms from preservative solutions. Cells could be maintained in preservative solutions containing 3% Tween 80 for up to an hour with little difference observed between these counts and those of water or 3% Tween 80

controls. This gave an added safety margin, because in the experiments reported in this

thesis, cells were plated immediately they were sampled from preservative solutions.

Therefore, 3% Tween 80 was used as the inactivator.

Sampling intervals (min)	sterile distilled water	3% Tween 80	100 µg/ml BZC in 3% Tween 80	100 µg/ml CTAB in 3% Tween 80	100 µg/ml CG in 3% Tween 80
0	100	100	100	100	100
30	100	95	84	91	97
60	100	100	85	100	95
120	83	100	45	56	47

Table 15. Viable counts of *Staphylococcus aureus* strain SA1439 in water, 3% Tween 80 and in solutions of 3% Tween 80 containing different preservatives. Counts are expressed as percentages of the initial viable count (10^2/ml) of the suspension in distilled water.

Preservative solutions tested by the BP (1988) protocol were sampled at 0, 6, 24 and

48 h and at 7, 14 and 28 days. After the 6 h sample they were stored in a 25°C incubator

and removed just for sampling. Viable counts were obtained by serial dilution in 0.1%

peptone water, with 3% Tween 80 present as inactivator in the first dilution for all

preservatives apart from phenylmercuric nitrate, where 0.1% thioglycollic acid was used.

Dilutions were plated on tryptone soya agar using the Miles and Misra (1938) method.

The plates were incubated at 30°C overnight, counted and then re-incubated for 2 more

days to allow any further growth to occur.

3.3.1 Benzalkonium chloride

The two bacterial strains that are recommended for use in the BP Preservative Efficacy Test are *S. aureus* NCTC 10788 and *P. aeruginosa* NCIB 8626.

BP Preservative Efficacy experiments in 10 μg/ml benzalkonium chloride produced interesting results (Table 16). After 6 h of challenge, there was a log reduction in viable count of more than 10^3 for *S. aureus* NCTC 6571. However this was not observed for the rest of the strains. The viable counts of the other *S. aureus* strains, *E. coli* and *P. aeruginosa* were slightly lowered after 6 h, but non reached a reduction factor of 10^3. At 24 h no viable cells were recovered of *S. aureus* strains SA1439, NCTC 10788 and NCTC 6571, but there were still 5.8×10^3/ml and 2.7×10^4/ml viable cells of the plasmid-containing strain SA1325 (pSK1) and the resistant strain A.D., respectively. However, no viable cells of strains SA1325 (pSK1) or *S. aureus* A.D. could be recovered after 48 h of challenge or at each subsequent interval up to 28 days.

Both *E. coli* strains were resistant to 10 μg/ml benzalkonium chloride. Viable counts of *E. coli* strains 343/113 and 343/113 (R471-1) were not reduced to the level required by the BP criterion after 6 h of challenge, and viable cells of both strains were recoverable throughout the sampling period. During the course of the test the viable counts of both *E. coli* strains decreased, with the plasmid-containing strain giving slightly higher viable counts than the plasmid-less strain (Table 16). In the previously-reported short-term bactericidal activity experiments (Figure 34A) a higher inoculum of 10^8/ml was used. The results in Table 16 of the BP Challenge Test were produced using an inoculum of 10^6/ml. However, in both experiments the survival curve of *E. coli* 343/113 showed little death at the early stages of the challenge (compare Figure 34A with 36A). The survival of *E. coli* 343/113 in the benzalkonium chloride-preserved solution did not,

Preservative: Benzalkonium chloride				Concentration: 10 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	2.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	4.8×10^6	1.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	4.6×10^6	9.5×10^5	5.8×10^3	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	6.2×10^6	2.1×10^6	$<3.9 \times 10^3$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	6.0×10^6	5.4×10^6	2.7×10^4	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	1.5×10^6	6.4×10^5	5.2×10^5	2.9×10^5	3.1×10^5	5.4×10^4	7.6×10^3
343/113 (R471-1)	1.5×10^6	1.1×10^6	5.4×10^5	6.2×10^5	4.2×10^5	1.3×10^5	1.5×10^4
NCIB 8626	5.0×10^6	1.6×10^6	4.1×10^6	3.8×10^6	1.4×10^6	6.0×10^6	5.1×10^6

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.4	<2.6	>3.8
SA1439	6.7	6.1	0.6
SA1325 (pSK1)	6.7	6.0	0.7
NCTC 10788	6.8	6.3	0.5
A.D.	6.8	6.7	0.1
343/113 R⁻	6.2	5.8	0.4
343/113 (R471-1)	6.2	6.0	0.2
NCIB 8626	6.7	6.2	0.5

Table 16. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 10 µg/ml benzalkonium chloride solution at 25°C.

therefore, come as a surprise. It was similar to the results obtained in short-term bactericidal activity experiments (Results, section 3.2.4).

P. aeruginosa NCIB 8626 was highly resistant to 10 µg/ml benzalkonium chloride, with the viability of challenged cells remaining within a log 0.5 reduction of the initial viable count (Table 16 and Figure 36C). After 24 h the viable count of a control suspension of *P. aeruginosa* NCIB 8626 in sterile water was higher than the count from the preservative solution. However, after 14 days the count in the preserved suspension increased and remained higher than the control until the end of the test (Figure 36C).

All organisms and strains tested complied with BP preservative test criteria for ophthalmic solutions in concentrations of 30 and 100 µg/ml benzalkonium chloride (Table 17 and 18). 10^3 log reductions of initial viable counts were readily achieved for all organisms tested after 6 h and no viable cells of any strain were recovered after 24 h and at each subsequent sampling interval.

3.3.2 Chlorhexidine gluconate

This biguanide was tested at 5, 20 and 100 µg/ml. All concentrations produced compliance with the Challenge Test with all bacterial species and strains tested (Tables 19, 20 and 21). The data for the 5 µg/ml solution showed *P. aeruginosa* NCIB 8626 to be the most resistant strain. The viable count was lowered by the required amount ($>10^3$) after 6 h of challenge, but viable cells could still be recovered after this time (Table 19). This contrasts with the results obtained with all *E. coli* or *S. aureus* strains, where no viable cells were recovered after 6 h or subsequently.

Concentrations of 20 and 100 µg/ml chlorhexidine demonstrated highly efficient preservative activity. A greater than 10^3 reduction in viable count was achieved after 6

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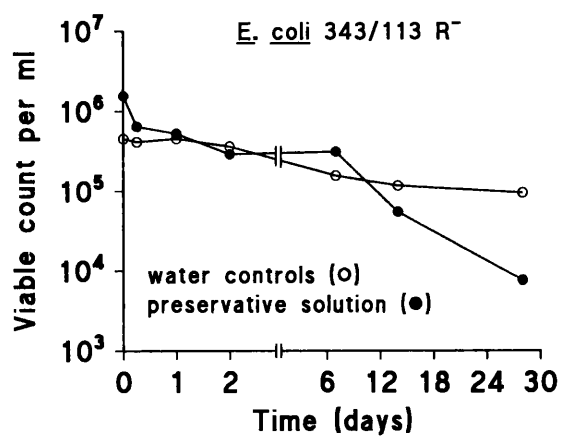
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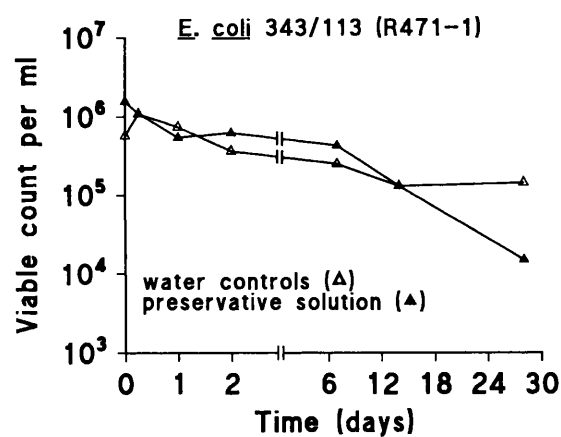
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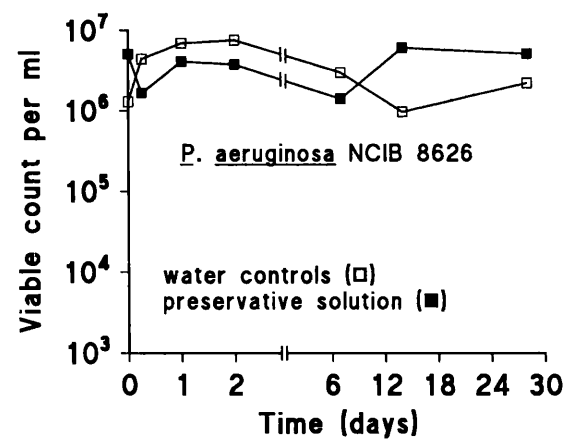
Figure 36. BP Challenge Tests on 10 µg/ml benzalkonium chloride solutions.

Preservative: Benzalkonium chloride				Concentration: 30 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	1.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	5.4×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	4.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC	8.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	6.6×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	2.3×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	1.5×10^7	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.1	<2.6	>3.5
SA1439	6.7	<2.6	>4.1
SA1325 (pSK1)	6.6	<2.6	>4.0
NCTC 10788	6.9	<2.6	>4.3
A.D.	6.8	<2.6	>4.2
343/113 R⁻	6.4	<2.6	>3.8
343/113 (R471-1)	6.1	<2.6	>3.5
NCIB 8626	7.2	<2.6	>4.6

Table 17. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 30 µg/ml benzalkonium chloride solution at 25°C.

Preservative: Benzalkonium chloride				Concentration: 100 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	3.5×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	1.4×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	2.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	1.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	7.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	1.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	4.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	5.5	<2.6	>3.0
SA1439	6.2	<2.6	>3.6
SA1325 (pSK1)	6.3	<2.6	>3.7
NCTC 10788	6.0	<2.6	>3.4
A.D.	6.9	<2.6	>4.3
343/113 R⁻	6.0	<2.6	>3.4
343/113 (R471-1)	6.1	<2.6	>3.5
NCIB 8626	6.7	<2.6	>4.1

Table 18. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 100 µg/ml benzalkonium chloride solution at 25°C.

Preservative: Chlorhexidine gluconate				Concentration: 5 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	1.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	1.3×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	1.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	5.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	4.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	1.4×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.9×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	7.6×10^6	1.36×10^3	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strains	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.2	<2.6	>3.6
SA1439	6.1	<2.6	>3.5
SA1325 (pSK1)	6.2	<2.6	>3.6
NCTC 10788	6.8	<2.6	>4.2
A.D.	6.7	<2.6	>4.1
343/113 R⁻	6.1	<2.6	>3.5
343/113 (R471-1)	6.3	<2.6	>3.7
NCIB 8626	6.9	3.1	3.8

Table 19. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 5 µg/ml chlorhexidine gluconate solution at 25°C.

h for all organisms tested, and no viable organisms were recovered thereafter at each time of sampling (Tables 20 and 21).

3. Cetrimide

Cetrimide was tested in aqueous solutions of 10, 30 and 100 µg/ml. At 10 µg/ml the initial compendial requirement of a 10^3 reduction in viable count after 6 h of challenge was achieved with *S. aureus* strain NCTC 6571, the two *E. coli* strains and the pseudomonad (Table 22). The remaining *S. aureus* strains were much more resistant. The viability of *S. aureus* strains NCTC 10788, A.D. and SA1439 were reduced by about 1 log cycle after 6 h of challenge, but for *S. aureus* SA1325 (pSK1) this reduction was even less. This was expected from the results observed in the short-term bactericidal activity experiments (Chapter III, section 3.2.3.3), where the plasmid-containing *S. aureus* strain was shown to be highly resistant to cetrimide. After the 24 h and at subsequent sampling times, the challenge test criteria were met for all the *S. aureus* strains. No viable cells were recovered from 1 ml at each time of sampling (Table 22).

P. aeruginosa NCIB 8626 produced an interesting profile of death and re-growth in 10 µg/ml cetrimide. Initial viable counts were decreased by the required level (10^3) after 6 h, but at 24 h there was only a further 4-fold reduction in viable count. At each subsequent sampling interval, viable counts increased until they reached 10^6/ml at 7 days and stayed at that level for the remaining period of challenge (Figure 37).

To determine if this re-growth was due to the emergence of resistant mutants in the 10 µg/ml cetrimide solution, a loopful of the organisms present in the preservative solution after 28 days was streaked onto a nutrient agar plate containing 10 µg/ml cetrimide. An

Preservative: Chlorhexidine gluconate				Concentration: 20 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	1.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	1.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	2.0×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	1.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	2.0×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	2.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	4.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.2	<2.6	>3.6
SA1439	6.3	<2.6	>3.7
SA1325	6.3	<2.6	>3.7
NCTC 10788	6.2	<2.6	>3.6
A.D.	6.3	<2.6	>3.7
343/113 R⁻	6.3	<2.6	>3.7
343/113 (R471-1)	6.2	<2.6	>3.6
NCIB 8626	6.6	<2.6	>4.0

Table 20. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 20 µg/ml chlorhexidine gluconate solution at 25°C.

Preservative: Chlorhexidine gluconate				Concentration: 100 µg/ml		
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	1.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	2.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	7.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	4.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	2.0×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	3.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	4.3×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	3.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.2	<2.6	>3.6
SA1439	6.4	<2.6	>3.8
SA1325 (pSK1)	6.9	<2.6	>4.3
NCTC 10788	6.6	<2.6	>4.0
A.D.	6.3	<2.6	>3.7
343/113 R⁻	6.6	<2.6	>4.0
343/113 (R471-1)	6.6	<2.6	>4.0
NCIB 8626	6.5	<2.6	>3.9

Table 21. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 100 µg/ml chlorhexidine gluconate solution at 25°C.

Preservative: Cetrimide				Concentration: 10 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	9.2×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	5.0×10^6	4.0×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	6.5×10^6	4.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	4.0×10^6	4.8×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	7.5×10^6	1.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	6.4×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	7.0×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	1.6×10^7	4.1×10^3	9.8×10^2	4.1×10^3	1.7×10^6	1.3×10^6	3.3×10^6

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	5.9	<2.6	>3.3
SA1439	6.7	5.6	1.1
SA1325 (pSK1)	6.8	6.6	0.2
NCTC 10788	6.6	5.7	0.9
A.D.	6.9	6.1	0.8
343/113 R⁻	5.8	<2.6	>3.2
343/113 (R471-1)	5.8	<2.6	>3.2
NCIB 8626	7.2	3.6	3.6

Table 22. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 10 µg/ml cetrimide solution at 25°C.
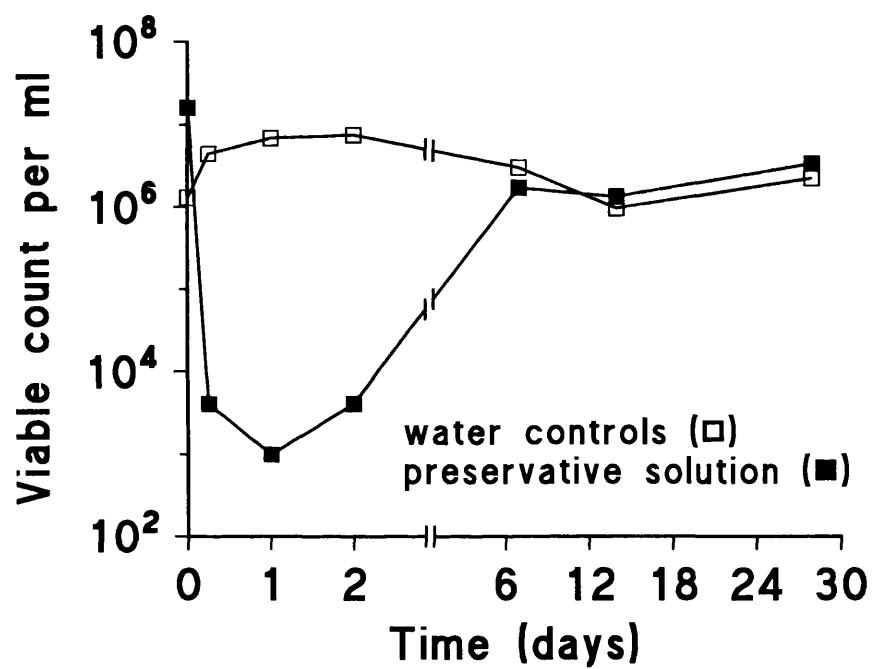
Figure 37. Survival of *Pseudomonas aeruginosa* strain NCIB 8626 in sterile water and in cetrimide 10 μg/ml. Observe death and re-growth pattern of *P. aeruginosa* in the cetrimide solution.

isolated colony was removed from this plate and subcultured in tryptone soya broth overnight at 30°C. The following day the culture was washed, resuspended and tested as before. This experiment was performed in triplicate and, although there were variations between counts obtained at 6 and 24 h, the overall shape of the survivor curves (Figure 38) remained unchanged from that obtained with the *P. aeruginosa* inoculum that had not been exposed to cetrimide (Figure 37). These results indicate that death and re-growth is not due to the emergence of resistant mutants. This pattern of survival is typical of *P. aeruginosa* in inadequately preserved formulations. Viable counts may decrease below the detection limits, but be followed by adaptive growth with an increase to 10^6/ml or more in 7 to 28 days (Davison, 1988).

No viable cells of *E. coli* 343/113 (R471-1) or its isogenic plasmid-less parent *E. coli* 343/113 were recovered after 6 h of challenge and thereafter from any of the cetrimide solutions (Tables 22, 23 and 24). In the short-term bactericidal experiments with 20 μg/ml cetrimide, the plasmid-less *E. coli* 343/113 strain was shown to be less resistant than the pseudomonad, but viable cells were recovered throughout the sampling period (Figure 34C). However, bactericidal activity experiments with 20 μg/ml cetrimide used an inoculum of 10^8/ml, while the inoculum size in Challenge Tests was 10^6/ml. Therefore, as the starting inoculum was lower for the BP Challenge Tests, it is likely that the *E. coli* viable count had fallen under the limits of detection after 6 h of challenge (compare Figure 34C with data in Table 22).

Preservative challenge tests performed with cetrimide at 10 μg/ml, therefore showed this concentration to be unsuitable for preservation. However, data for the survival of organisms in cetrimide concentrations of 30 and 100 μg/ml exhibited compliance with the Challenge Test at all time intervals. This was true even for *P. aeruginosa* strain NCIB
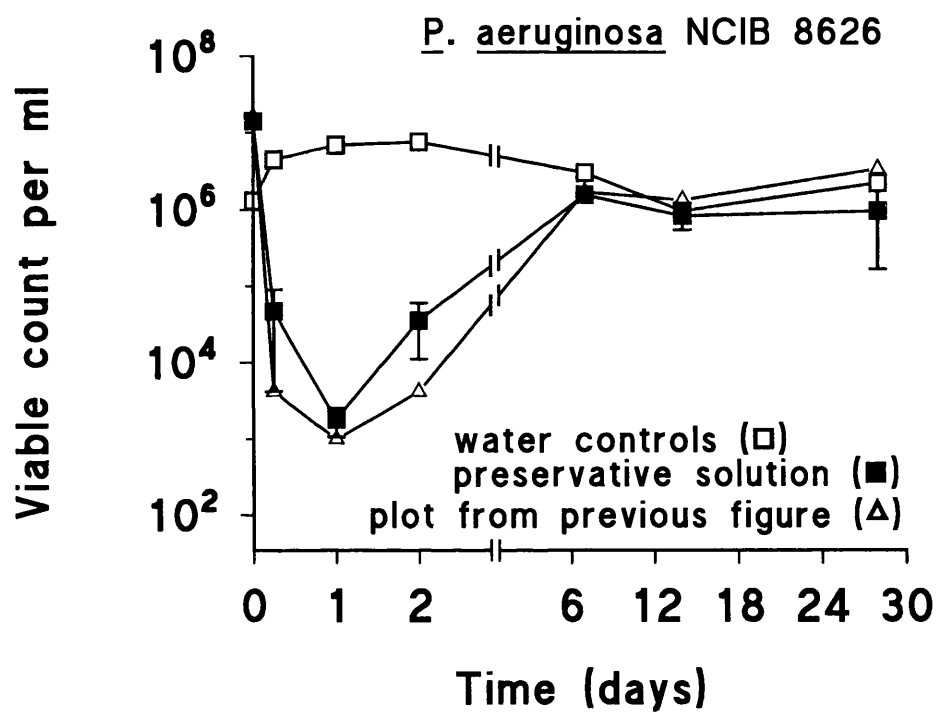
Figure 38. *Pseudomonas aeruginosa* NCIB 8626 cells harvested from 10 µg/ml cetrimide solution and re-tested against cetrimide 10 µg/ml. Repeat experiments show death and re-growth pattern is reproducible.

Preservative: Cetrimide			Concentration: 30 µg/ml				
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	1.0×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	4.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^3$
SA1325 (pSK1)	6.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	1.0×10^7	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	7.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	8.3×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	5.4×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	2.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.0	<2.6	>3.4
SA1439	6.6	<2.6	>4.1
SA1325 (pSK1)	6.8	<2.6	>4.2
NCTC 10788	7.0	<2.6	>4.4
A.D.	6.9	<2.6	>4.3
343/113 R⁻	5.9	<2.6	>3.3
343/113 (R471-1)	5.7	<2.6	>3.1
NCIB 8626	6.5	<2.6	>3.9

Table 23. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 30 µg/ml cetrimide solution at 25°C.

Preservative: Cetrimide				Concentration: 100 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	6.0×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	4.1×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325	1.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	6.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	6.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	1.4×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	3.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strains	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	5.8	<2.6	>3.2
SA1439	6.6	<2.6	>4.0
SA1325	6.2	<2.6	>3.6
NCTC 10788	6.8	<2.6	>4.2
A.D.	6.8	<2.6	>4.2
343/113 R⁻	6.1	<2.6	>3.5
343/113 (R471-1)	6.2	<2.6	>3.6
NCIB 8626	6.5	<2.6	>3.9

Table 24. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 100 µg/ml cetrimide solution at 25°C.

8626, where no "death and re-growth" pattern was observed. No viable cells of any of the plasmid-containing or plasmid-less *S. aureus* and *E. coli* strains were recovered from samples after 6 h of challenge or thereafter (Table 23 and 24).

4. Phenylmercuric nitrate

Challenge tests with this organomercurial produced interesting results. There was compliance with criteria at a concentration of 1 µg/ml for *S. aureus* strains NCTC 6571, SA1439 and NCTC 10788, for *E. coli* strains 343/113 (R471-1) and 343/113, and for *P. aeruginosa* NCIB 8626. Whereas with *S. aureus* strains SA1325 (pSK1) and A.D., the required reduction of initial count was not achieved in 6 h. After this time, survival was still of the order of 10^4/ml for both these *S. aureus* strains (Table 25). Plasmid pSK1 is not claimed to confer resistance to phenylmercuric nitrate (Lyon and Skurray, 1987). However the results in Table 25 correlate with those obtained in the short-term bactericidal activity tests using the same phenylmercuric nitrate concentration (Figure 32), in which strain SA1325 (pSK1) was also shown to be the most resistant staphylococcus. After 24 h and at subsequent sampling times, the data for all strains complied with the test criteria; no viable cell of any strain was recovered (Table 25).

After 6 h in 10 µg/ml phenylmercuric nitrate solution the required initial 10^3 reduction in viable count was achieved with all strains (Table 26). Viable cells of *S. aureus* A.D. were still recoverable after 6 h, but no viable cells of this nor any other strain were recovered after 24 h and at each following interval.

Results obtained using 20 µg/ml phenylmercuric nitrate solution were similar to those obtained with 10 µg/ml. *S. aureus* strains NCTC 6571, SA1439, NCTC 10788, SA1325 (pSK1), *E. coli* strains 343/113 (R471-1) and 343/113 and *P. aeruginosa* NCIB 8626

Preservative: Phenylmercuric nitrate				Concentration: 1 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	3.6×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	9.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	4.7×10^6	1.4×10^4	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	5.5×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	5.4×10^6	6.6×10^4	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	2.2×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	1.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	1.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.6	<2.6	>4.0
SA1439	6.9	<2.6	>4.3
SA1325 (pSK1)	6.7	4.1	2.6
NCTC 10788	6.7	<2.6	>4.1
A.D.	6.7	4.8	1.9
343/113 R⁻	6.4	<2.6	>3.8
343/113 (R471-1)	6.3	<2.6	>3.7
NCIB 8626	6.3	<2.6	>3.7

Table 25. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 1 µg/ml phenylmercuric nitrate solution at 25°C.

Preservative: Phenylmercuric nitrate				Concentration: 10 µg/ml		
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	3.6×10^6	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
SA1439	1.2×10^6	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
SA1325 (pSK1)	7.8×10^6	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
NCTC 10788	7.8×10^6	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
A.D.	3.5×10^6	1.95×10^3	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
343/113 R⁻	6.0×10^5	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
343/113 (R471-1)	6.0×10^5	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$
NCIB 8626	9.9×10^6	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$	$<3.9\times10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	6.6	<2.6	>4.0
SA1439	6.1	<2.6	>3.5
SA1325 (pSK1)	6.9	<2.6	>4.3
NCTC 10788	6.9	<2.6	>4.3
A.D.	6.5	3.3	3.2
343/113 R⁻	5.8	<2.6	>3.2
343/113 (R471-1)	5.8	<2.6	>3.2
NCIB 8626	7.0	<2.6	>4.4

Table 26. Viable counts and log reductions of the BP preservative test strains Staphylococcus aureus NCTC 10788 and Pseudomonas aeruginosa NCIB 8626 compared to the other Staphylococcus aureus and Escherichia coli strains in aqueous 10 µg/ml phenylmercuric nitrate solution at 25°C.

Preservative: Phenylmercuric nitrate				Concentration: 20 µg/ml			
Strain	t_{0h}	t_{6h}	t_{24h}	t_{48h}	t_{7days}	t_{14days}	t_{28days}
NCTC 6571	9.1×10^5	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1439	5.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
SA1325 (pSK1)	9.4×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCTC 10788	1.2×10^7	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
A.D.	1.6×10^7	1.9×10^3	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 R⁻	5.3×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
343/113 (R471-1)	4.7×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$
NCIB 8626	9.8×10^6	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$	$<3.9 \times 10^2$

Strain	log viable count t_{0h}	log viable count t_{6h}	log reduction after 6 h
NCTC 6571	5.9	<2.6	>3.3
SA1439	6.8	<2.6	>4.2
SA1325 (pSK1)	7.0	<2.6	>4.4
NCTC 10788	7.1	<2.6	>4.5
A.D.	7.2	3.3	3.9
343/113 R⁻	6.7	<2.6	>4.1
343/113 (R471-1)	6.7	<2.6	>4.1
NCIB 8626	7.0	<2.6	>4.4

Table 27. Viable counts and log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 20 µg/ml phenylmercuric nitrate solution at 25°C.

showed compliance with the test at all sampling times. *S. aureus* A.D. also complied, but its viable count after 6 h was still of the order of 10^3/ml. Nevertheless, no viable cells of this nor any other strain were recovered after 24 h and thereafter (Table 27).

Summary

The protocol of the BP (1988) Test for Preservative Efficacy demonstrated that the survival of strains differed in low concentrations of benzalkonium chloride and cetrimide. With phenylmercuric nitrate, *S. aureus* strain A.D. was shown to be the most resistant organism at all concentrations tested.

P. aeruginosa NCIB 8626 was highly resistant to 10 µg/ml benzalkonium chloride and 10 µg/ml cetrimide. In both instances this organism showed a pattern of "death and re-growth", which was more pronounced in cetrimide. *E. coli* strains were also resistant to 10 µg/ml benzalkonium chloride, but their viable counts were lower than those observed for the pseudomonad. It may be concluded that *P. aeruginosa* NCIB 8626 was the most resistant of the plasmid-less organisms to quaternary ammonium compounds.

The plasmid-containing *S. aureus* strain SA1325 (pSK1) persisted longer in benzalkonium chloride, cetrimide and phenylmercuric nitrate solutions than the plasmid-less *S. aureus* strains. Viable cells of SA1325 (pSK1) could still be recovered 24 h after challenge with 10 µg/ml benzalkonium chloride. None of the plasmid-less *S. aureus* strains could be recovered from 1 ml of sample after this time. The resistant *S. aureus* strain A.D. showed an increased survival in phenylmercuric nitrate and in 10 µg/ml benzalkonium chloride compared to the plasmid-less *S. aureus* strains.

The importance of the correct selection of organisms for use in the Challenge Test was underlined by the results obtained with *S. aureus* strain NCTC 6571. This strain

provided compliance with all concentrations of all preservatives tested. Being a very

sensitive organism, it was rapidly killed even by the lowest preservative concentrations.

This emphasizes the need to select an appropriate organism with a moderate resistance

profile to ensure the required level of stringency in preservative efficacy testing.

DISCUSSION

The work reported in this thesis was performed to determine the effect of plasmids on the antibacterial efficacy of pharmaceutical preservatives in simple aqueous systems. This was done by determining MICs on solid nutrient agar and by assessing susceptibilities to bactericidal concentrations of preservatives. Susceptibilities observed were compared with *P. aeruginosa* strain NCIB 8626, which was used as a control plasmid-less resistant organism. In addition, Compendial Preservative Challenge Tests were performed to assess the susceptibility of strains under these conditions. This enabled the survival of plasmid-containing strains and their plasmid-free parents to be compared with the BP test strains *S. aureus* NCTC 10788 and *P. aeruginosa* NCIB 8626.

While performing short-term bactericidal activity experiments and BP Challenge Tests, the efficiency of Tween 80 as inactivator for benzalkonium chloride, chlorhexidine gluconate, cetrimide and diamidines, was tested.

4.1 Effect of plasmids on MICs in solid nutrient agar

The MIC results summarized in Table 9 showed Gram-negative organisms to be less susceptible than Gram-positive organisms to the inhibitory activity of benzalkonium chloride, chlorhexidine gluconate, cetrimide and propamidine and dibromopropamidine isethionates in nutrient agar. This greater resistance of Gram-negatives is widely recognized (Baird-Parker and Holbrook, 1971), and is believed to be due to the barriers provided by the three different layers present in the cell envelope, compared with the less complex outer structure of Gram-positive organisms.

The plasmid-less *P. aeruginosa* strain NCIB 8626 was the most resistant organism tested, capable of growing in nutrient agar containing exceedingly high concentrations of each of the preservatives. *E. coli* strain 343/113, although more sensitive than the

pseudomonad, was more resistant than the plasmid-containing and plasmid-less *S. aureus* strains. *E. coli* strain 343/113 (R471-1) displayed the highest MIC to phenylmercuric nitrate in nutrient agar (Table 9), with R plasmid R471-1 increasing the resistance of the strain by nearly 30-fold (Table 11).

The plasmid-less *S. aureus* strains were less resistant than both the pseudomonad and the *E. coli* strains to the inhibitory effects of preservatives (Table 9). The plasmid-containing *S. aureus* strain SA1325 (pSK1) showed higher resistance to cetrimide, benzalkonium chloride, chlorhexidine gluconate, propamidine and dibromopropamidine isethionate, than its plasmid-less isogenic parent strain SA1439, confirming the resistance phenotype conferred by plasmid pSK1 (Lyon and Skurray, 1987). However, with phenylmercuric nitrate *S. aureus* strain SA1325 (pSK1) was as sensitive as SA1439 due to the fact that pSK1 does not confer resistance to this compound (Lyon and Skurray, 1987).

The resistant *S. aureus* strain A.D. showed similar MICs to benzalkonium chloride, cetrimide, chlorhexidine gluconate, propamidine and dibromopropamidine isethionates, as strain SA1325 (pSK1) (Table 12). Compared to the control, sensitive strain SA1439, the resistance of these two *S. aureus* strains to diamidines was many times higher than to quaternary ammonium compounds. However, only strain A.D. demonstrated increased resistance to phenylmercuric nitrate (Table 9).

Comparison of the MIC ratios of *P. aeruginosa* strain NCIB 8626 with the plasmid-containing *E. coli* and *S. aureus* strains (Table 14) showed that even with a resistance plasmid present in the latter species, the pseudomonad was far more resistant to most of the preservatives tested. There were, however, some exceptions. *S. aureus* strain A.D. was more resistant to propamidine isethionate and *E. coli* strain 343/113 (R471-1) to

phenylmercuric nitrate than *P. aeruginosa* NCIB 8626. While strain SA1325 (pSK1) was as sensitive to propamidine as the pseudomonad. Therefore, it was clearly demonstrated that, in certain cases, the presence of a plasmid in *E. coli* or *S. aureus* could increase preservative resistance levels to those exhibited by the naturally-resistant pseudomonad.

4.2 The use of Tween 80 as inactivator in the diluting medium

4.2.1 Short-term bactericidal activity experiments

MIC experiments were performed on nutrient agar, with unwashed over-night cultures and using single cell inocula. The short-term bactericidal tests were performed with washed overnight cultures, using high inocula (10^8/ml) and with cells suspended in aqueous preservative solutions. It was, therefore, necessary to investigate the inactivation of the preservatives during dilution of test samples before plating on nutrient agar for viable counting.

Testing Tween 80 as inactivator for 5 µg/ml chlorhexidine gluconate solution provided more evidence of the effect of plasmid pSK1. Addition of Tween 80 at 1 or 10% in nutrient broth, made no difference to the recovery of *S. aureus* strain SA1325 (pSK1). Viable counts were similar in nutrient broth alone or with Tween 80 at either concentration (Figure 18A). However, recovery of the plasmid-less *S. aureus* strains SA1439 (Figure 17) and NCTC 10788 (Figure 18B) benefited from the addition of the nonionic surfactant. Viable counts were higher for these organisms when nutrient broth containing Tween 80 at either concentration was used as diluent.

Nutrient broth containing 1% Tween 80 proved to be a suitable inactivator for benzalkonium chloride (Figures 15, 16A and B), chlorhexidine gluconate (Figure 17, 18A and B) and cetrimide (Figures 19A and B). However, with respect to 10 and 20 µg/ml

cetrimide, increasing the Tween concentration produced a fall in the viable count of strain SA1439 (Figures 19C and D). This was an unexpected result. However, as discussed earlier in the Introduction of this work, nonionic surfactants may enhance the antimicrobial activity of preservatives, by increasing the permeability of the bacterial membrane to preservative molecules (Kurup *et al.*, 1991).

The need for an increase Tween 80 concentration as the concentration of preservative increased was observed with diamidines (Figure 20B). At low concentrations of diamidines, which produced low toxicity, preservative activity could be inhibited by a one in a hundred dilution in nutrient broth. This was demonstrated by the recovery of *S. aureus* strain SA1439 from 80 µg/ml dibromopropamidine isethionate in sterile water (Figure 20A). However, higher concentrations of dibromopropamidine isethionate, required nutrient broth containing 10% Tween 80 for inactivation (Figure 20B). It was observed that viable counts of *S. aureus* strain SA1439 recovered from 800 µg/ml dibromopropamidine isethionate were higher when 10%, rather than 1% Tween 80 in nutrient broth was used as diluent.

4.2.2 BP Preservative Efficacy Tests

The preservative concentrations used in the BP Preservative Efficacy Test experiments were higher than those employed in short-term bactericidal tests. As detection of low numbers of organisms was required, the inactivator concentration was tested to assess its suitability. Table 15 summarizes the results of the British Standards Institution (1960) test for the suitability of inactivators. 3% Tween 80 produced suitable inactivation of the antibacterial effects of high concentrations of benzalkonium chloride, chlorhexidine gluconate and cetrimide over a 1 h period. Since dilutions were performed and plated immediately, this ensured optimal recovery of viable counts.

4.3 Effect of plasmids on preservative sensitivities in short-term bactericidal tests

Short-term bactericidal activity experiments with chlorhexidine gluconate (Figure 27), dibromopropamidine (Figure 29), propamidine (Figure 30) and phenylmercuric nitrate (Figure 33) showed *P. aeruginosa* NCIB 8626 to be the most sensitive organism tested. This contradicted with the MIC experiments, which showed the pseudomonad to be highly resistant to the activity of these preservatives (Table 9). It was suspected that the different sensitivities exhibited by the pseudomonad in the two types of experiments were due to the preparation of the inocula. In the short-term bactericidal tests, the pseudomonad was washed and resuspended in water. This would possibly wash away the slime layer that surrounds the organism (Introduction, section 1.3.2.1), and thus render it susceptible to antibacterials (Kolawole, 1984; Costerton and Lashen, 1984; LeChevalier *et al.*, 1988).

Pseudomonads are especially sensitive to sudden changes in temperature, pH or tonicity (Brown and Winsley, 1969), which again suggests that the water-washing procedure was responsible for the outcome of the results. Studies with unwashed and water-washed inocula of different *P. aeruginosa* strains grown in nutrient broth (Brown, 1968) demonstrated that the unwashed organisms were able to survive longer than water-washed organisms in preserved aqueous solutions. Water-washed inocula rapidly lost viability, illustrating the damaging effect of water upon the cells. In addition, it is necessary to stress the importance of the growth medium to preservative sensitivity (Brown, 1968). In experiments using nutrient broth it was observed that the volume of broth added to preservative solutions as part of the cell inoculum, even if minimal, contributed to the survival of pseudomonads.

4.3.1 Benzalkonium chloride

P. aeruginosa NCIB 8626 was highly resistant to 10 µg/ml benzalkonium chloride

solution in short-term bactericidal tests (Figure 26). This organism is known to proliferate in solutions of quaternary ammonium compounds, especially benzalkonium chloride (Russell *et al.*, 1986) and to utilize such preservatives as carbon sources (Bloomfield, 1988). Benzalkonium chloride resistance has been ascribed to an increase in cell wall contents of phospho- and other lipids, which decrease the adsorption of the cationic compound (Sakagami *et al.*, 1989). Pseudomonads have also been shown to grow in chlorhexidine solutions (Burdon and Whitby, 1967), in soaps containing hexachlorophane (Cooke *et al.*, 1970) and in a wide variety of disinfectants. However, considering the effect of water-washing on the sensitivities of *P. aeruginosa* strain NCIB 8626 to the rest of the preservatives, its increased survival in 10 μg/ml benzalkonium chloride was surprising.

The plasmid-less *S. aureus* strain SA1439 showed a 10-fold higher survival than the plasmid-containing strain SA1325 (pSK1) in 10 μg/ml benzalkonium chloride. However, after 60 min this difference in survival levels began to reduce rapidly (Figure 26). The MIC data (Table 9) showed the plasmid-containing *S. aureus* strain to be highly resistant to benzalkonium chloride; this was not observed in short-term bactericidal activity experiments (Figure 26).

4.3.2 Chlorhexidine

The bactericidal activity of chlorhexidine gluconate solution correlated with the data obtained in the MIC experiments, with the exception of *P. aeruginosa* (compare Figure 27 and Table 9). Plasmid-containing *S. aureus* strains SA1325 (pSK1) and *S. aureus* A.D. were more resistant than the pseudomonad and almost 100-fold more resistant than the plasmid-less *S. aureus* strains NCTC 10788 and SA1439. *S. aureus* strain NCTC 6571 was the least resistant of the *S. aureus* strains and after 30 min in 5 μg/ml chlorhexidine

no viable cells could be recovered. Although, some reports of the bactericidal activities

of chlorhexidine-based products (Haley *et al.*, 1985; Cookson *et al.*, 1991b) have failed

to show a difference between killing rates of *S. aureus* strains that differ in MICs, my

results did demonstrate such a difference. After 180 min of exposure, the viable counts

of the plasmid-containing *S. aureus* strain SA1325 (pSK1) and the resistant strain A.D.

were approximately 10^4/ml. No viable cells of the plasmid-less strains, including *P.*

aeruginosa, could be recovered after the same time (Figure 27).

4.3.3 Cetrimide

Experiments with 20 µg/ml cetrimide solution showed the plasmid-containing *S.*

aureus strain SA1325 (pSK1) to be as resistant as the pseudomonad at the initial stages

of the test. *S. aureus* A.D. also showed resistance to this preservative, but no viable cells

of the rest of the plasmid-less *S. aureus* strains could be recovered after 90 min of

exposure (Figure 28). The resistances demonstrated by *P. aeruginosa* strain NCIB 8626

and *S. aureus* strain SA1325 (pSK1) in 20 µg/ml cetrimide paralleled those obtained in

the MIC experiments (compare Figure 28 and Table 9).

4.3.4 Diamidines

4.3.4.1 Dibromopropamidine isethionate

Short-term bactericidal activity tests with 1 mg/ml dibromopropamidine isethionate

demonstrated the plasmid-containing *S. aureus* strain SA1325 (pSK1) to be 10-fold more

resistant than its isogenic parent SA1439 (Figure 29). *S. aureus* A.D. was also highly

resistant to this preservative, giving 1000-fold greater survival compared with strain

SA1439 after 120 min.

4.3.4.2 Propamidine isethionate

Results for propamidine isethionate provided killing rate curves with similar shapes

to those obtained with dibromopropamidine isethionate (compare Figures 29 and 30).

However the concentration used was 5 times that of the halogenated compound. Plasmid-

less *P. aeruginosa* NCIB 8626 survived longer in propamidine than in

dibromopropamidine isethionate, but after 120 min no viable cells could be recovered.

Once more, *S. aureus* A.D. was highly resistant, and the plasmid-containing *S. aureus*

strain SA1325 (pSK1) showed a 10-fold increase in survival compared to its isogenic

plasmid-less parent SA1439 (Figure 30). The MIC data for the diamidines (Table 9) did

not correlate with results obtained in bactericidal activity experiments. The *P. aeruginosa*

which was resistant according to the MIC data, was highly sensitive (Figures 29 and 30)

in aqueous diamidine solution. In contrast, *S. aureus* strain NCTC 10788, which was the

most sensitive organism in the MIC experiments (Table 9) exhibited increased survival

on exposure to bactericidal concentrations of these preservatives (Figures 29 and 30).

4.3.5 Phenylmercuric nitrate

Bactericidal tests with 1 and 10 µg/ml phenylmercuric nitrate solution demonstrated

the increase in survival brought about by the presence of the *E. coli* plasmid R471-1

(Figure 31). Survival levels for *E. coli* 343/113 (R471-1) compared with the plasmid-less

parent strain increased approximately 10-fold and more than 100-fold, respectively, for

E. coli 343/113 (R471-1) compared with the plasmid-less parent strain. A previous study

with the same plasmid, but a different *E. coli* strain showed that at 10 µg/ml there was

a 10-fold increase in resistance to phenylmercuric nitrate after 3 h exposure (Pinney,

1978).

Although plasmid pSK1 is not claimed to confer mercury resistance (Lyon and

Skurray, 1987), strain SA1325 (pSK1) was significantly more resistant than the plasmid-

less SA1439 strain to 1 µg/ml phenylmercuric nitrate (Figure 32). However, this

difference in sensitivities was not observed in 10 µg/ml phenylmercuric nitrate (Figure 33). In the latter concentration *S. aureus* A.D. was shown to be highly resistant, but it was the only strain surviving until the end of the test (Figure 33).

4.3.5 Conclusions

The results of the short-term bactericidal activity tests demonstrated that plasmid-containing *E. coli* or *S. aureus* are able to surpass or parallel the survival of highly resistant strains like *P. aeruginosa* NCIB 8626. Although the water-washed pseudomonad appeared more sensitive in the bactericidal tests with 5 µg/ml chlorhexidine gluconate, 1 mg/ml dibromopropamidine isethionate, 5 mg/ml propamidine isethionate or 1 and 10 µg/ml phenylmercuric nitrate, it is important to stress that its survival in cetrimide, which was extremely high, was paralleled by *S. aureus* SA1325 (pSK1) (Figure 28) and surpassed by the same *S. aureus* strain in 5 µg/ml chlorhexidine gluconate (Figure 27). Therefore, once more it was demonstrated that plasmids in *S. aureus* and *E. coli* strains can increase the survival of these organisms in bactericidal concentrations of preservatives to significantly high levels.

4.4 BP Preservative Efficacy Tests

BP preservative efficacy tests (Anon., 1988) aim to assess if formulated products are adequately protected from microbial contamination during manufacture, storage and use. In these tests a product is challenged with 10^6 organisms/ml of the test strains *S. aureus* NCTC 10788 and *P. aeruginosa* NCIB 8626. The inoculated product is incubated at 20 to 25°C for up to 28 days and sampled at specified intervals. These compendial tests therefore assess the performance of preserved systems under long term conditions. It was, therefore, of interest to observe if survival of the *E. coli* and *S. aureus* plasmid-containing

strains surpassed or paralleled that exhibited by the highly resistant *P. aeruginosa* strain NCIB 8626 using the BP protocol.

4.4.1 Benzalkonium chloride

The results of the BP preservative efficacy tests using *S. aureus* strains A.D. and SA1325 (pSK1) in 10 µg/ml benzalkonium chloride solution disagree with those obtained with the same strains in the short-term bactericidal tests. Short-term bactericidal tests (Results, section 3.2.3.1) showed the survival of these strains was lower than that of the plasmid-less control strain SA1439 (Figure 26), although the opposite was observed in MIC experiments (Table 9). However, in the BP preservative efficacy test strains A.D. and SA1325 (pSK1) survived better than the rest of the plasmid-less *S. aureus* strains (Table 16).

In short-term bactericidal activity experiments the difference in survival between SA1325 (pSK1) and its isogenic plasmid-less parent SA1439 became narrower towards the end of the test (Figure 26). This suggests that if the sampling times were extended, a cross-over of the survival curves might have occurred, making the plasmid-containing strain more resistant. This was not observed in the short-term bactericidal activity tests due to their short duration. In addition, it is interesting to observe that the lowest log reduction after 6 h of challenge in the BP test was that for *S. aureus* strain A.D. (Table 15). This resistant strain was isolated from an ophthalmic solution preserved with benzalkonium chloride, but because of the results obtained in short-term bactericidal activity experiments (Figure 26), it was thought that strain A.D. might have lost its ability to persist in the quaternary compound. It has been reported that organisms isolated as contaminants of preserved pharmaceutical products often loose their ability to proliferate in the environment from which they were originally taken, when grown in laboratory

media (Gilbert *et al.*, 1980; Leak and Leech, 1988). However the results of the BP Challenge Test, which was performed subsequent to the short-term bactericidal activity experiments, showed A.D. to be highly resistant, and therefore confirmed that the cross-over of survival curves was the most plausible explanation for the differences in survival observed between the two resistant strains SA1325 (pSK1) or A.D. and the plasmid-less SA1439 in the various experiments.

Benzalkonium chloride concentrations of 30 and 100 µg/ml gave compliance with the BP criteria for all organisms tested at all time intervals (Tables 17 and 18). The presence or absence of plasmids conferring preservative resistance made no difference to the interpretation of test data.

4.4.2 Chlorhexidine gluconate

Chlorhexidine gluconate complied with the Challenge Test at all concentrations tested and at all sampling intervals up to 28 days. Viable cells of *P. aeruginosa* strain NCIB 8626 were still present, after 6 h in the 5 µg/ml solution, but none could be recovered after 24 h, or subsequently (Table 19). The presence of viable *P. aeruginosa* after 6 h of challenge was unexpected, because the short-term bactericidal activity experiments had shown it to be highly sensitive to this preservative (Figure 27). The differences in results between bactericidal activity experiments and the BP preservative efficacy tests are probably due, once again, to the washing procedures employed to prepare the inocula. The BP preservative efficacy test uses sterile saline, which does not produce the sensitizing effect of the water wash used in the bactericidal activity experiments.

In contrast to the results obtained with the pseudomonad, the plasmid-less *E. coli* 343/113 strain, which was more resistant than *P. aeruginosa* strain NCIB 8626 in short-term tests on 5 µg/ml chlorhexidine gluconate (Figure 34B), was not recovered at any

stage in the BP test. The washing procedure may also play a role in this reversal of sensitivities. *E. coli* is susceptible to washing with saline solution, but not with distilled water, the opposite of that observed for pseudomonads (King and Hurst, 1963).

4.4.2 Cetrimide

BP Preservative Challenge Tests on a cetrimide solution of 10 µg/ml did not achieve the required 10^3 reduction in viable count for *S. aureus* strains SA1439, SA1325(pSK1), NCTC 10788 or *S. aureus* A.D. after the first 6 h sampling period (Table 21). In addition, this concentration was not effective against *P. aeruginosa* NCIB 8626, which exhibited a death and re-growth pattern that was reproducible and not due to the emergence of resistant mutants (Figures 37 and 38). This death and re-growth pattern is often observed and is believed due to the ability of the organism to adapt its metabolic processes to the needs of its environment (Davison, 1988). Pseudomonads are also able to utilize cetrimide as a carbon source (Results, section 3.2.3.3).

Effective preservation with 10 µg/ml cetrimide was achieved only with *S. aureus* strain NCTC 6571 and *E. coli* strains 343/113 (R471-1) and 343/113. However, cetrimide concentrations of 30 and 100 µg/ml proved to satisfy the criteria of the BP preservative efficacy test with all organisms tested (Tables 23 and 24).

4.4.3 Phenylmercuric nitrate

Phenylmercuric nitrate at a concentration of 1 µg/ml complied with criteria, apart from two exceptions. The viable count of *S. aureus* strains SA1325 (pSK1) and A.D. were not reduced by the required factor of 10^3 after 6 h (Table 25). At concentrations of 10 and 20 µg/ml phenylmercuric nitrate, both strains complied with the test. The required reductions of 10^3 after the 6 h sampling were achieved and no viable cells were recovered after 24 h (Tables 26 and 27).

4.5 The new EP guidelines

The new guidelines of the European Pharmacopoeia for preservative efficacy testing (1993) which are incorporated in the BP (1993) and which will come into effect in December 1993, contain two acceptance criteria (section 1.5). These are designated A and B. Criterion B, which is the less stringent, should be satisfied if for justifiable reasons criterion A cannot be achieved. For example, when there is an increased risk of adverse reactions due to high concentration of antimicrobial agents.

Parenteral and ophthalmic preparations include two criteria of acceptance for each type of microorganism; for bacteria criterion A requires a log reduction of 2 at 6 h, 3 at 24 h and no recovery from 1 ml at 28 days. Viable counting is not required at 7 or 14 days. If, for any justifiable reason criterion A cannot be met, then criterion B should be satisfied: it requires a log reduction of 1 at 24 h, 3 at 7 days, with no increase at 28 days. There is no mention of a 14 day count for criterion B.

Comparing results obtained using the BP (1988) protocol with the new BP 1993 criterion A for ophthalmic and parenteral preparations demonstrates, once again, that concentrations of 10 µg/ml benzalkonium chloride or cetrimide are not suitable for preservation.

4.5.1 Benzalkonium chloride

In all but one of the S. aureus strains tested, 10 µg/ml benzalkonium chloride (Table 28) did not produce the 2 log reduction in viable count after 6 h, as required by criterion A. At 24 h only the plasmid-less S. aureus strains NCTC 6571, NCTC 10788 and SA1439 exhibited reductions higher than the log 3 value required. S. aureus strains SA1325 (pSK1) and A.D. gave borderline log reductions after 24 h of 2.9 and 2.4, respectively.

The results in Table 28 also show, the lack of stringency of criterion B for ophthalmic solutions. In contrast to the absence of compliance with criterion A, all plasmid-containing and plasmid-less *S. aureus* strains produced compliance with criterion B at all sampling intervals. However this was not so for *P. aeruginosa* NCIB 8626 and the *E. coli* strains, which all failed to meet both criterion A and criterion B (Table 28).

Strain	log viable count t_{0h}	log reduction after 6 h	log reduction after 24 h	log reduction after 7 d	log reduction after 28 d
NCTC 6571	6.4	>3.8	>3.8	>3.8	>3.8
SA1439	6.7	0.6	>4.1	>4.1	>4.1
SA1325 (pSK1)	6.7	0.7	2.9	>4.1	>4.1
NCTC 10788	6.8	0.5	>4.2	>4.2	>4.2
A.D.	6.8	0.1	2.4	>4.2	>4.2
343/113 R⁻	6.2	0.4	0.5	0.7	2.3
343/113 (R471-1)	6.2	0.2	0.5	0.6	2.0
NCIB 8626	6.7	0.5	0.09	0.6	0

Table 28. Log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 10 µg/ml benzalkonium chloride solution at 25°C.

4.5.2 Chlorhexidine gluconate

In respect to 5 µg/ml chlorhexidine gluconate, there was compliance with all organisms tested using either criterion A or B (Table 19).

4.5.3 Cetrimide

Cetrimide at a concentration of 10 µg/ml showed compliance with criterion A only with *S. aureus* NCTC 6571 and both *E. coli* strains and the *P. aeruginosa* strain NCIB 8626. These three organisms gave the required reductions of higher than log 2 after 6 h (Table 29), whereas the plasmid-containing *S. aureus* strain SA1325 (pSK1) and the resistant strain A.D. gave exceedingly low reductions after 6 h. Acceptance criterion A requires no recovery after 28 days of challenge, which was achieved for all organisms apart from the pseudomonad. The regrowth observed with strain NCIB 8626 meant that even though it satisfied criterion A after 6 h, it failed the efficacy test overall. Criterion B requires a log 1 reduction after 24 h, which was achieved with all organisms tested. However, after 7 days this criterion requires a log 3 reduction which was not achieved with *P. aeruginosa* strain NCIB 8626 because of its regrowth. However, all the staphylococci and *E. coli* strains produced compliance.

4.5.4 Phenylmercuric nitrate

BP challenge tests on 1 µg/ml phenylmercuric nitrate produced compliance with criterion A for all organisms tested, although it was borderline with *S. aureus* strain A.D. (Table 25). As expected, all strains complied with criterion B.

It was said in the Introduction of this work that the compliance criteria for bacterial challenge of preserved parenteral and ophthalmic preparations are more stringent in the BP (1988) guidelines than in the EP. The results obtained in this thesis according to the BP (1988) procedure have been compared with the new guidelines (EP, 1993a). It appears that these new acceptance criteria could indeed produce problems with compliance in cases of borderline preservation. However, the same preservative solutions that did not comply with the acceptance criteria of the BP (1988), did not comply with the new

acceptance guidelines of the EP and the BP 1993.

Strain	log viable count t_{0h}	log reduction after 6 h	log reduction after 24 h	log reduction after 7 d	log reduction after 28 d
NCTC 6571	5.9	>3.3	>3.3	>3.3	>3.3
SA1439	6.7	1.1	>4.1	>4.1	>4.1
SA1325 (pSK1)	6.8	0.2	>4.2	>4.2	>4.2
NCTC 10788	6.6	0.9	>4.0	>4.0	>4.0
A.D.	6.9	0.8	>4.3	>4.3	>4.3
343/113 R⁻	5.8	>3.2	>3.2	>3.2	>3.2
343/113 (R471-1)	5.8	>3.2	>3.2	>3.2	>3.2
NCIB 8626	7.2	3.6	4.2	1.0	0.7

Table 29. Log reductions of the BP preservative test strains *Staphylococcus aureus* NCTC 10788 and *Pseudomonas aeruginosa* NCIB 8626 compared to the other *Staphylococcus aureus* and *Escherichia coli* strains in aqueous 10 3µg/ml cetrimide solution at 25°C.

4.6 Conclusions

The importance of selecting a suitable challenge organism for use in preservative efficacy tests was demonstrated by the results obtained in this thesis using the ultra-sensitive *S. aureus* strain NCTC 6571, which provided compliance at all concentrations of preservatives tested. Even marginally preserved systems, such as the 10 µg/ml solutions of benzalkonium chloride and cetrimide (Tables 16 and 22), satisfied the acceptance criteria of the BP and the EP when challenged with strain NCTC 6571. However, when

challenged by the BP test organism strain NCTC 10788, not to mention *P. aeruginosa*

strain NCIB 8626, it was obvious that 10 µg/ml solution of benzalkonium chloride or

cetrimide were inadequately preserved. The presence of the plasmid in strain SA1325

(pSK1) underlined the importance of testing preservative systems against a resistant

organism. The resistant *S. aureus* strain A.D., which was isolated as a contaminant of an

ophthalmic solution preserved with benzalkonium chloride, could still be recovered after

24 h of exposure to 10 µg/ml benzalkonium chloride (Table 16). The BP Preservative

Challenge Test protocol allows the use of isolates from contaminated products, and the

results obtained with *S. aureus* strain A.D. amply justify this. However, the more resistant

the test strain, the more stringent the test, and if preservative testing is made too stringent,

high preservatives concentrations would be needed, which may produce toxic effects.

There must therefore be a balance between what is pragmatically achievable and desirable

in respect to preservation, without compromising the production of a safe, non-toxic

formulation.

One major parameter that influences the effectiveness of an antibacterial agent is

concentration (Denyer and Wallhaeusser, 1990). This was demonstrated in detail during

the BP Challenge Tests. Increase in survival of plasmid-containing organisms was

observed at low preservative concentrations for benzalkonium chloride, chlorhexidine and

cetrimide (Tables 16, 19 and 22). This underlines the dangers of preservative loss from

formulations due to adsorption to the container, inactivation or many factors already

discussed in the Introduction of this work (section 1.3) Unless extreme care is taken

microorganisms may overcome the preservative barrier to become a health hazard.

Finally, the aim of this work was to determine the effect of plasmids on the efficacy

of pharmaceutical preservatives in simple aqueous-preserved systems. MIC experiments

(section 3.1), short-term bactericidal activity tests (section 3.2.3) and BP Preservative

Efficacy Tests (section 3.3) showed that plasmids can increase the resistance of bacteria

to a wide variety of antibacterial preservatives. Furthermore, this increased level of

resistance may parallel that exhibited by highly resistant organisms like *P. aeruginosa*.

In addition, the link between antibiotic and preservative resistance is well documented

(Coleman *et al.*, 1985; Sivaji *et al.*, 1986) and in a situation in which antibiotic and

preservative resistances are determined by the same plasmid, a mechanism for selection

of antibiotic resistant organisms in preserved systems is provided. However, the in-use

preservative concentrations tested (Tables 18, 20, 24 and 27) still provided an acceptable

degree of protection against persistant and resistant plasmid-containing organisms, and the

data obtained with such strains still complied with compendial preservative efficacy test

criteria.

References

Akers, M.J. and Taylor, C.J. (1990) Official methods of preservative evaluation and testing. In *Guide to Microbiological Control in Pharmaceuticals*. ed. Denyer, S. and Baird, R. pp. 292-303. London: Ellis Horwood Limited.

Al-Hiti, M.M.A. and Gilbert, P. (1980) Changes in preservative sensitivity of the USP antimicrobial agent effectiveness test micro-organisms. *Journal of Applied Bacteriology* **49**, 119-126.

Al-Masaudi, S.B., Day, M.J. and Russell, A.D. (1991) Effect of some antibiotics and biocides on plasmid transfer in *Staphylococcus aureus*. *Journal of Applied Bacteriology* **71**, 239-243.

Alasri, A., Roques, C., Michel, G., Cabassud, C. and Aptel, P. (1992) Bactericidal properties of peracetic acid and hydrogen peroxide, alone and in combination, and chlorine and formaldehyde against bacterial water strains. *Canadian Journal of Microbiology* **38**, 635-642.

Alasri, A., Valverde, M., Roques, C., Michel, G., Cabassud, C. and Aptel, P. (1993) Sporocidal properties of peracetic acid and hydrogen peroxide, alone and in combination, in comparison with chlorine and formaldehyde for ultrafiltration membrane disinfection. *Canadian Journal of Microbiology* **39**, 52-60.

Albert, A. (1966) *The Acridines: Their Preparation, Properties and Uses*. 2nd edn. London: Edward Arnold.

Aldrigde, W.N. and Threlfall, C.J. (1961) Trialkyl tins and oxidative phosphorylation. *Biochemical Journal* **79**, 214-219.

Alford, J.A. and Pierce, D.A. (1963) Production of lipase by *Pseudomonas fragi* in a synthetic medium. *Journal of Bacteriology* **86**, 24-9.

Allawala, N.A. and Riegelman, S. (1953) The properties of iodine in solutions of surface-active agents. *Journal of the American Pharmaceutical Association, Scientific Edition* **42**, 396-401.

Allwood, M.C. (1978) Antimicrobial agents in single- and multi-dose injections. *Journal of Applied Bacteriology* **44**, Svii-Sxvii.

Allwood, M.C. (1986) Preservative efficacy testing of pharmaceuticals. *Pharma International* **7**, 172-175.

Andersen, D.O., Weber, N.D., Wood, S.G., Hughes, B.G., Murray, B.K. and North, J.A. (1991) *In vitro* virucical activity of selected anthraquinones and anthraquinone derivatives. *Antiviral Research* **16**, 185-196.

Anderson, R.L., Berkelman, R.L. and Holland, B.W. (1983) Microbiologic investigations with iodophor solutions. *Proceedings of the International Symposium on Povidone*.

pp. 146-157. Kentucky: University of Kentycky Press.

Anderson, K. and Crompton, D. (1967) A test for the bactericidal activity of eye-drops. *Lancet* ii, 968-970.

Anon. (1962) Dimethoxane, a new preservative effective with non-ionic agents. *American Perfumer and Cosmetics* 77, 32-38.

Anon. (1970) *The Disinfection and Disinfestation of Poultry Houses.* Ministry of Agriculture, Fisheries and Food: Advisory Leaflet 514, revised 1970. London: HMSO.

Anon. (1989) *Martindale. The Extra Pharmacopoeia.* ed. Reynolds, J.E.F. 29th edn. London: The Pharmaceutical Press.

Anon. (1993) *Martindale. The Extra Pharmacopoeia.* ed. Reynolds, J.E.F. 30th edn. London: The Pharmaceutical Press.

Archer, G.L. and Scott, J. (1991) Conjugative transfer genes in Staphylococcal isolates from the United States. *Antimicrobial Agents and Chemotherapy* 35, 2500-2504.

Archer, G.L., Coughter, J.P. and Johnston, J.L. (1986) Plasmid-encoded trimethoprim resistance in staphylococci. *Antimicrobial Agents and Chemotherapy* 29, 733-740.

Asch, D.K., Goering, R.V. and Ruff, E.A. (1984) Isolation and preliminary characterization of a plasmid mutant derepressed for conjugal transfer in *Staphylococcus aureus. Plasmid* 12, 197-202.

Ayliffe, G.A., Babb, J.R., Davies, J.G., Newsom, S.W., Rowland, C., Platt, J.H. and Mason, B. (1990) Hygienic hand disinfection tests in three laboratories. *Journal of Hospital Infection* 16, 141-149.

Baillie, L. (1987) Chlorhexidine resistance among bacteria isolated from urine of catheterized patients. *Journal of Hospital Infection* 10, 83-86.

Baird-Parker, A.C. and Holbrook, R. (1971) The inhibition and destruction of cocci. In *Inhibition and Destruction of the Microbial Cell.* ed. Hugo, W.B. pp. 369-397. London: Academic Press.

Barel, S., Segal, R. and Yashphe, J. (1991) The antimicrobial activity of the essential oil from *Achillea fragrantissima. Journal of Ethnopharmacology* 33, 187-191.

Barrett, M. (1969) Biocides for food plant. *Process Biochemistry* 4, 23-24.

Bartnick, F.G. (1992) Interaction of anionic surfactants with proteins, enzymes and membranes. In *Anionic Surfactants. Biochemistry, Toxicology, Dermatology.* ed. Gloxhuber, C. and Künstler, K. 2nd edn. pp. 2-42. New York: Marcel Decker,

Inc.

Bassett, D.C.J. (1971) The effect of pH on the multiplication of a pseudomonad in chlorhexidine and cetrimide. *Journal of Clinical Pathology* **24**, 708-711.

Beaver, D.J., Roman, D.P. and Stoffel, P.J. (1957) The preparation and bacteriostatic activity of substituted ureas. *Journal of the American Chemical Society* **79**, 1236-1245.

Benigni, R., Calcagnile, A., Giuliani, A. and Leopardi, P. (1990) Inhibition of replicative DNA synthesis and induction of DNA repair in human fibroblasts by the intercalating drugs proflavine and 9-aminoacridine. *Journal of Toxicology and Environmental Health* **31**, 117-124.

Berke, P.A. and Rosen, W.E. (1978) Imidazolidinyl urea activity against *Pseudomonas*. *Journal of the Society of Cosmetic Chemists* **29**, 757-766.

Berke, P.A. and Rosen, W.E. (1980) Are cosmetic emulsions adequately preserved against *Pseudomonas*? *Journal of the Society of Cosmetic Chemists* **31**, 37-40.

Berman, H.M., Sussman, J.L., Joshua-Tor, L., Revich, G.G. and Ripley, L.S. (1992) A structural model for sequence-specific proflavin-DNA interactions during *in vitro* frameshift mutagenesis. *Journal of Biomolecular Structure and Dynamics* **10**, 317-331.

Bernhard, K., Schrempf, H. and Goebel, W. (1978) Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. *Journal of Bacteriology* **133**, 897-903.

Beveridge, T.J. and Davies, J.A. (1983) Cellular responses of *Bacillus subtilis* and *Escherichia coli* to the Gram stain. *Journal of Bacteriology* **156**, 846-858.

Blanchard, J. (1980) Effect of polyols on interaction of paraben preservatives with polysorbate 80. *Journal of Pharmaceutical Sciences* **69**, 169-172.

Blatt, R. and Maloney, J.V. (1961) An evaluation of the iodophor compounds as surgical germicides. *Surgery, Gynaecology and Obstetrics* **113**, 699-704.

Blaugh, S.M. and Ahsan, S.S. (1961) Interaction of parabens with non-ionic macromolecules. *Journal of Pharmaceutical Sciences* **50**, 441-443.

Block, S.S. (1968) Historical review. In *Disinfection, Sterilization and Preservation*. eds. Lawrence, C.A. and Block, S.S. pp. 3-8. U.S.A.: Lea and Febiger.

Bloomfield, S.F. (1988) Biodeterioration and disinfectants. In *Biodeterioration*. ed. Houghton, P.R., Smith, R.N. and Egins, H.O.W. Vol. 7 pp. 135-145. London: Elsevier Applied Science.

Bond, W.W., Favero, M.S., Peterson, N.J. and Cook, E.J. (1983) Observations on the intrinsic bacterial contamination of iodophor germicides. *Proceedings of the Annual Meeting of the American Society for Microbiology*, Q133.

Borick, P.M. (1968) Chemical sterilizers (Chemosterilizers). *Advances in Applied Microbiology* 10, 291-312.

Borick, P.M. and Pepper, R.E. (1970) The spore problem. In *Disinfection*. ed. Benarde, M. pp. 85-102. New York: Marcel Dekker.

Bradley, H.E., Wetmur, J.G. and Hodes, D.S. (1980) Tolerance in *Stapylococcus aureus*: evidence for bacteriophage role. *Journal of Infectious Diseases* 141, 233-237.

Brinton, C.C. (1965) The structure, function, synthesis and genetic control of bacterial pili and a molecular mode for DNA and RNA transport in Gram-negative bactria. *Transactions of the New York Academy of Science* 27, 1003.

British Pharmaceutical Codex (1973) London: HMSO.

British Pharmacopoeia (1973) Injections. Addition of Bactericides. p. 241. London: HMSO.

British Pharmacopoeia (1980) Efficacy of antimicrobial preservatives in pharmaceutical products. Appendix XVI C, A192-A194. London: HMSO.

British Pharmacopoeia (1988) Efficacy of antimicrobial preservatives in pharmaceutical products. Appendix XVI C, A109. London: HMSO.

British Pharmacopoeia (1993) Efficacy of antimicrobial preservation. Appendix XVI C, A191-A192. London: HMSO.

British Standards Institution (1960) Method for laboratory evaluation of disinfectant activity of quaternary ammonium compounds by suspension test procedure. BS3286. British Standards Institution, London.

Brown, M.R.W. (1968) Survival of *P. aeruginosa* in fluorescein solution. Preservative action of PMN and EDTA. *Journal of Pharmaceutical Sciences* 57, 389-392.

Brown, M.R.W. (1975) The role of cell envelope in resistance. In *Resistance of Pseudomonas aeruginosa* (ed. Brown, M.R.W.) pp. 71-107. London: John Wiley and Sons.

Brown, M.R.W. and Anderson, R.A. (1968) The bacterial effect of silver ions on *Pseudomonas aeruginosa*. *Journal of Pharmacy and Pharmacology* 20, 1S-3S.

Brown, M.R.W. and Gilbert, P. (1993) Sensitivity of biofilms to antimicrobial agents. *Journal of Applied Bacteriology* 74 (Symposium Series No. 22), 87S-97S.

Brown, M.R.W. and Richards, R.M.E. (1964) Effect of polysorbate (tween 80) on the resistance of *Pseudomonas aeruginosa* to chemical inactivation. *Journal of Pharmacy and Pharmacology* **16** (Supplement), 51T-55T.

Brown, M.R.W. and Richards, R.M.E. (1965) Effect of ethylenediamino tetraacetate on the resistance of *Pseudomonas aeruginosa* to antibacterial agents. *Nature* **207**, 1391-1393.

Brown, M.R.W. and Winsley, B.E. (1969) Effect of polysorbate 80 on cell leakage and viability of *P. aeruginosa* exposed to rapid changes of pH, temperature and tonicity. *Journal of General Microbiology* **56**, 99-107.

Brown, V.I. and Lowbury, E.J.L. (1965) Use of an improved agar medium and other culture methods for *Pseudomonas aeruginosa*. *Journal of Clinical Pathology* **18**, 752-756.

Broxton, P., Woodcock, P.M. and Gilbert, P. (1984) Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. *Journal of Applied Bacteriology* **57**, 115-124.

Brumfitt, W., Dixson, S. and Hamilton-Miller, J.M.T. (1985) Resistance to antiseptics in methicillin and gentamicin resistant *Staphylococcus aureus*. *Lancet* **i**, 1442-1443.

Bryan, L.E. and Kwan, S. (1983) Roles of ribosomal binding, membrane potential, and electron transport in bacterial uptake of streptomycin and gentamicin. *Antimicrobial Agents and Chemotherapy* **23**, 835-845.

Burdon, D.W. and Whitby, J.L. (1967) Contamination of hospital disinfectants with *Pseudomonas* species. *British Journal of Medicine* **2**, 153-155.

Burman, L.G., Christensen, P., Christensen, K., Gryklund, B., Helgesson, A.M. Svenningsen, N.W. and Tullus, K. (1992) Prevention of excess neonatal morbidity associated with group B streptococci by vaginal chlorhexidine disinfection during labour. *Lancet* **340**, 65-69.

Burns, J.L., Hedin, L.A. and Lieu, D.M. (1989) Chloramphenicol resistance in *Pseudomonas cepacia* because of decrease permeability. *Antimicrobial Agents and Chemotherapy* **33**, 136-141.

Carroll, J.D., Pomeroy, H.M., Russell, R.J., Arbuthnott, J.P., Keane, C.T., McCormick, O.M. and Coleman, D.C. (1989) A new methicillin- and gentamicin-resistant *Staphylococcus aureus* in Dublin: molecular genetic analysis. *Journal of Medical Microbiology* **28**, 15-23.

Chawner, J.A. and Gilbert, P. (1989a) A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine. *Journal of Applied Bacteriology* **66**, 243-252.

Chawner, J.A. and Gilbert, P. (1989b) Interaction of the bisbiguanides chlorhexidine and alexidine with phospholipid vesicles: evidence for separate modes of action. *Journal of Applied Bacteriology* **66**, 253-258.

Chen, C.M., Misra, T.K., Silver, S. and Rosen, B.P. (1986) Nucleotide sequence of the structural genes for an anion pump. The plasmid encoded arsenical resistance operon. *Journal of Biological Chemistry* **4**, 165-170.

Chin, N and Neu, H.C. (1992) The activity of metal compounds against aerobic and anaerobic bacteria. *Diagnostics Microbiology and Infectious Disease* **15**, 549-552.

Chopra, I., Johnson, S.C. and Bennett, P.M. (1987) Inhibition of *Providencia stuartii* cell envelope enzymes by chlorhexidine. *Journal of Antimicrobial Chemotherapy* **19**, 743-751.

Christensen, E.A. (1964) Newer methods of sterilization. *Ugeskrift for Laeger* **126**, 330-339.

Christensen, E.A. and Kristensen, H. (1992) Gaseous sterilization. In *Principles and Practice of Disinfection, Preservation and Sterilization* ed. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J., 2nd edn. p. 557-572. Oxford: Blackwell Scientific Publications.

Clegg, L.F.L. (1970) Disinfection in the dairy industry. In *Disinfection*. ed. Bernarde, M.A. pp. 311-375. New York: Marcel Dekker.

Coates, D. and Death, J.E. (1978) Sporocidal activity of mixtures of alcohol and hypochlorite. *Journal of Clinical Pathology* **31**, 148-152.

Coia, J., Noor-Hussain, I. and Platt, D.J. (1988) Plasmid profiles and restriction enzyme fragmentation patterns of plasmids of methicillin-sensitive and methicillin-resistant isolates of *Staphylococcus aureus* from hospital and community. *Journal of Medical Microbiology* **27**, 271-276.

Coleman, D.C., Pomeroy, H., Estridge, J.K., Keane, C.T., Cafferkey, M.T., Hone, R. and Foster, T.J. (1985) Susceptibility to antimicrobial agents and analysis of plasmids in gentamicin- and methicillin- resistant *Staphylococcus aureus* from Dublin hospitals. *Journal of Medical Microbiology* **20**, 157-167.

Collins, C.H., Lyne, P.M. and Grange, J.M. (1989) *Microbiological Methods.* 6h ed. London: Butterworths.

Cooke, E.M., Shooter, R.A., O'Farrell, S.M. and Martin, D.R. (1970) Faecal carriage of *Pseudomonas aeruginosa* by newborn babies. *Lancet* **ii**, 1045-1046.

Cookson, B. and Phillips, I. (1990) Methicillin-resistant staphylococci. *Journal of Applied Bacteriology* **19** (Symposium Supplement), 55S-70S.

Cookson, B.D., Bolton, M.C. and Platt, J.H. (1991b) Chlorhexidine resistance in methicillin-resistant *Staphylococcus aureus* or just an elevated MIC? An *in vitro* and *in vivo* assessment. *Antimicrobial Agents and Chemotherapy* **35**, 1997-2002.

Cookson, B.D., Farrely, H., Stapleton, P., Garvey, R.P. and Price, M.R. (1991a) Transferable resistance to triclosan in MRSA. *Lancet* **337**, 1548-1549.

Costerton, J.W. (1977) Cell-envelope as a barrier to antibiotics. In *Microbiology*. American Society for Microbiology, pp. 151-157. Washington, DC.

Costerton, J.W. and Lashen, E.S. (1984) Influence of biofilm on efficacy of biocides upon corrosion causing bacteria. *Materials Performance* **23**, 34-37.

Costerton, J.W., Ingram, J.M. and Cheng, K.-J. (1974) Structure and function of the cell envelope of Gram-negative bacteria. *Bacteriological Reviews* **38**, 87-110.

Costerton, J.W., Ingram, J.M. and Cheng, K.-Y. (1978) How bacteria stick. *Scientific American* **238**, 86-95.

Courvalin, P. and Fiandt, M. (1980) Aminoglycoside-modifying enzymes of *Staphylococcus aureus*: expression in *Escherichia coli*. *Gene* **9**, 247-269.

Cousins, C.M. and Allan, C.D. (1967) Sporicidal properties of some halogens. *Journal of Applied Bacteriology* **30**, 168-174.

Cox, W.A. and D'Arcy, P.F. (1962) A new cationic antimicrobial agent, N-dodecyl-4-amino quinaldinium acetate (Laurolinium acetate). *Journal of Pharmacy and Pharmacology* **15**, 129-137.

Craven, D.E., Moody, B., Connoly, M.G., Kollisch, N.R., Stottmeier, K.D. and McCabe, W.R. (1981) Pseudobacteraemia caused by povidone-iodine antiseptics contaminated by *Pseudomonas aeruginosa*. *New England Journal of Medicine* **305**, 621-623.

Croshaw, B. (1977) The destruction of mycobacteria. In *Inhibition and Destruction of the Microbial Cell* (ed. Hugo, W.B.) pp. 419-449. London: Academic Press.

Cruz, T., Cabo, M.P., Cabo, M.M., Jiménez, J, Cabo, J. and Ruiz, C. (1989) *In vitro* antibacterial effect of the essential oil of *Thymus longiflorus* Boiss. *Microbios* **60**, 59-61.

Cundliffe, E. (1984) Self defence in antibiotic-producing organisms. *British Medical Bulletin* **40**, 61-67.

D'Arcy, P.F. (1971) Inhibition and destruction of moulds and yeasts. In *Inhibition and Destruction of the Microbial Cell* (ed. Hugo, W.B.) pp. 613-686. London: Academic Press.

Dadd, A.H. and Daley, G.M. (1980) Resistance of microorganisms to inactivation by gaseous ethylene oxide. *Journal of Applied Bacteriology* **48**, 89-101.

Dadd, A.H. and Daley, G.M. (1982) Role of the coat in resistance of bacterial spores to inactivation by ethylene oxide. *Journal of Applied Bacteriology* **53**, 109-116.

Davies, A., Bentley, M. and Field, B.S. (1968) Comparison of the action of Vantocil, cetrimide and chlorhexidine on *Escherichia coli* and the protoplasts of Gram-positive bacteria. *Journal of Applied Bacteriology* **31**, 448-461.

Davies, D.J.G. (1978) Agents as preservatives in eye-drops and contact lens solutions. *Journal of Applied Bacteriology* **44**, Sxix-Sxxviii.

Davies, D.J.G. (1980) Manufacture and supply of contact lens products. I. An academic's view. *Pharmaceutical Journal* **225**, 343-345.

Davies, G.E., Francis, J., Martin, A.R., Rose, F.L. and Swain, G. (1954) 1:6-Di-4'-chlorophenyldiguanidohexane ("Hibitane"). Laboratory investigation of a new antibacterial agent of high potency. *British Journal of Pharmacology* (formerly *Journal of Pharmacology and Chemotherapy*) **9**, 192-196.

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

Davison, A.L. (1988) Preservative efficacy testing of non-sterile pharmaceuticals, cosmetics and toiletries and its limitations. In *Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries*. eds. Bloomfield, S.F., Baird, R., Leak, R.E. and Leech, R. pp. 119-128. London: Ellis Horwood Limited.

Dawson, F. (1973) Some techniques for microbial control in manufacturing plants. *Journal of the Society of Cosmetic Chemists* **24**, 655-662.

De Pamphilus, M.C. (1971) Dissociation and reassembly of *Escherichia coli* outer membrane and of lipopolysaccharide and their reassembly onto flagellar basal bodies. *Journal of Bacteriology* **105**, 1184-1199.

Deans, S.G. and Ritchie, G. (1987) Antibacterial properties of plant essential oils. *International Journal of Food Microbiology* **5**, 165-180.

DeBlasi, V., Debrot, S., Menoud, P.A., Gendre, L. and Schowing, J. (1990) Amoebicidal effect of essential oils *in vitro*. *Journal De Toxicologie Clinique Et Experimentale* **10**, 361-373.

DeLuca, P.P. and Kostenbauder, H.B. (1960) Interaction of preservatives with macromolecules. IV. Binding of quaternary ammonium compounds by non-ionic agents. *Journal of the American Pharmaceutical Association, Scientific Edition* **49**,

430-437.

Dempsey, G. (1988) The effect of container materials and multiple-phase formulation components on the activity of antimicrobial agents. In *Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries*. eds. Bloomfield, S.F., Baird, R., Leak, R.E. and Leech, R. pp. 95-103. London: Ellis Horwood Limited.

Denyer, S.P. and Wallhaeusser, K.-H. (1990) Antimicrobial preservatives and their properties. In *Guide to Microbiological Control in Pharmaceuticals*. ed. Denyer, S. and Baird, R. pp. 251-273. London: Ellis Horwood.

Denyer, S.P., Hugo, W.B. and Harding, V.D. (1985) Synergy in preservative combinations. *International Journal of Pharmaceutics* **25**, 245-253.

Didry, N., Duberuil, L. and Pinkas, M. (1992) Antimicrobial activity of naphtoquinones and *Allium* extracts combined with antibiotics. *Pharmaceutica Acta Helvetica* **67**, 48-51.

Docampo, R. and Moreno, S.N.J. (1990) The metabolism and mode of action of Gentian Violet. *Drug Metabolism Reviews* **22**, 161-178.

Domagk, G. (1935) Eine neue Klasse von Disinfektionsmitteln. *Deutsche Medizinische Wochenschrift* **61**, 829-932.

Dubini, R., Riviera, L., Cocuzza, C. and Bellotti, M.G. (1992) Antibacterial, antimycotic and trichomonicidal activity of a new nitroimidazole (EU 11100). *Journal of Chemotherapy* **4**, 342-346.

Durga, R., Sridhar, P. and Polasa, H. (1992) Antimutagenic activity of plumbagin in Ames *Salmonella typhimurium* test. *Indian Journal of Medical Research* **96**, 143-145.

Dusart, G., Zuccarelli, M., Ossia-Ongagna, Y. and Simeon de Buochberg, M. (1992) Kinetics of bactericidal and sporicidal effects of a disinfectant against bacteria isolated from hospital units. *Pathologie Biologie* **40**, 523-528.

Eisman, P.C., Jaconia, D. and Lazarus, J. (1963) A proposed microbiological method for studying the stability of preservatives in parenteral solutions. *Bulletin of the Parenteral Drug Association* **17**, 10-17.

Elek, S.D. (1968) Principles of antimicrobial activity. In *Disinfection, Sterilization and Preservation*. eds. Lawrence, C.A. and Block, S.S. pp. 15-63. U.S.A.: Lea and Febiger.

Ellison, R.T., Gichl, T.J. and LaForce, F.M. (1988) Damage of the outer membrane of enteric Gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity* **56**, 2774-2781.

Emslie, K.R., Townsend, D.E. and Grubb, W.B. (1985a) A resistant determinant to nucleic acid-binding compounds in methicillin-resistant *Staphylococcus aureus*. *Journal of Medical Microbiology* **20**, 139-145.

Emslie, K.R., Townsend, D.E., Bolton, S. and Grubb, W.B. (1985b) Two distinct resistance determinants to nucleic acid-binding compounds in *Staphylococcus aureus*? *FEMS Microbiological Letters* **27**, 61-64.

Ericson, C. (1969) Resistance to acriflavine and cadmium, and changed phage reactions-markers of a new staphylococcal penicillinase plasmid. *Acta Pathologica Microbiologica Scandinavica* **76**, 333.

European Pharmacopoeia (1992) Test for efficacy of antimicrobial preservation. Maison Neuve, Sainte Ruffine.

Evans, C.V. and Clarkson, N. (1993) Antifouling strategies in the marine environment. *Journal of Applied Bacteriology* **74** (Symposium Series No. 22), 119S-124S.

Farber, B.F. and Wolff, A.G. (1993) The use of salicylic acid to prevent the adherence of *Escherichia coli*. *Journal of Urology* **149**, 667-670.

Favero, M.S. (1985) Sterilization, disinfection and antisepsis in the hospital. In *Manual of Clinical Microbiology*. ed. Lennette, E.H., Balows, A., Hausler, W.J., Jr. and Shadomy, H.J. 4th edn. pp. 129-137. Washington, D.C.: American Society for Microbiology.

Ferguson, L.R., Pogai, H. and Turner, P.M. (1991) Variation in the mutagenic potential of acridines in *Salmonella typhimurium*, with stage of the culture. *Mutation Research* **262**, 227-231.

Foster, J.H.S. and Russell, A.D. (1971) Antibacterial dyes and nitrofurans. In *Inhibition and Destruction of the Microbial Cell*. ed. Hugo, W.B. pp. 185-208. London: Academic Press.

Foster, T.J. (1983) Plasmid determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiological Reviews* **47**, 361-409.

Fraenkel-Conrat, H., Cooper, M. and Alcott, H.S. (1945) The reaction of formaldehyde with proteins. *Journal of the American Chemical Society* **67**, 950-954.

Freese, E., Sheu, W. and Galliers, E. (1973) Function of lipophilic acids as antimicrobial food additives. *Nature* (London) **241**, 321-325.

Frier, M. (1971) Derivatives of 4-amino-quinaldinium and 8-hydroxyquinoline. In *Inhibition and Destruction of the Microbial Cell* (ed. Hugo, W.B.) pp. 107-120. London: Academic Press.

Fuller, A.T. (1942) Antibacterial action and chemical constitution in long-chain aliphatic bases. *Biochemical Journal* **36**, 548-558.

Gaughran, E.R.L. and Goudie, A.J. (1975) The heat sterilization methods. *Acta Pharmaceutica Suecica* **12** (Supplement), 15-25.

Gerhardt, P. and Judge, J.A. (1964) Porosity of isolated cell walls of *Saccharomyces cerevisiae* and *Bacillus megaterium. Journal of Bacteriology* **87**, 945-995.

Gershenfeld, L. (1962) Povidone-iodine as a sporocide. *American Journal of Pharmacy* **134**, 78-81.

Gilbert, P. (1988) Microbial resistance to preservative systems. In *Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries*. eds. Bloomfield, S.F., Baird, R., Leak, R.E. and Leech, R. pp 171-194. London: Ellis Horwood Limited.

Gilbert, P., Al-Hiti, M.M. and Beveridge, E.G. (1980) Rational approach towards improving the reproducibility and predictive effectiveness of "official" antimicrobial preservative challenge tests. *Journal of Pharmacy and Pharmacology* **32** (Supplement), 14P.

Gillespie, M.T. and Skurray, R.A. (1986) Plasmids in multi-resistant *Staphylococcus aureus. Microbiological Science* **3**, 53-58.

Gillespie, M.T., May, J.W. and Skurray, R.A. (1986) Plasmid-encoded resistance to acriflavine and quaternary ammonium compounds in methicillin-resistant *Staphylococcus aureus. FEMS Microbiologial Letters* **34**, 47-51.

Goldoni, P., Visca, P.l., Pastoris, M.C., Valenti, P. and Orsi, N. (1991) Growth of *Legionella spp.* under conditions of iron restriction. *Journal of Medical Microbiology*, **34**, 113-118.

Grier, J. (1937) *A History of Pharmacy.* London: The Pharmaceutical Press.

Grossman, L., Levine, S.S. and Allison, W.S. (1961) The reaction of formaldehyde with nucleotides and T2 bacteriophage DNA. *Journal of Molecular Biology* **3**, 47-60.

Gundidza, G.M., Deans, S.G., Svoboda, K.P. and Mavi, S. (1992) Antimicrobial activity of essential oil from *Hoslundia opposita. Central African Journal of Medicine* **38**, 290-293.

Gwynn, M.N., Webb, L.T. and Rolinson, G.N. (1981) Regrowth of *Pseudomonas aeruginosa* and other bacteria after the bactericidal action of carbenicillin and other β-lactam antibiotics. *Journal of Infectious Diseases* **144**, 263-269.

Haley, C.E., Marling-Cason, M., Smith, J.W., Luby, J.P. and Mackowiak, P.A. (1985) Bactericidal activity of antiseptics against methicillin-resistant *Staphylococcus*

aureus. Journal of Clinical Microbiology **21**, 991-992.

Hamilton, W.A. (1971) Membrane active antibacterial compounds. In *Inhibition and Destruction of the Microbial Cell*. ed. Hugo, W.B. 1st edn. pp. 77-93. Great Britain: Butter and Tanner Ltd.

Hamlett, N.V., Landale, E.C., Davis, B.H. and Summers, A.O. (1992) Roles of the *Tn21*, *merT*, *merP*, and *merC* gene products in mercury resistance and mercury binding. *Journal of Bacteriology* **174**, 6377-6385.

Hammerschmidt, F.J., Clark, A.M., Soliman, F.M., et-Kashoury, E.S., Abd el-Kawy, M.M. and el-Fishawy, A.M. (1993) Chemnical composition and antimicrobial activity of essential oils of *Jasonia candicans* and *J. montana. Planta Medica* **59**, 68-70.

Hanstein, W.G. (1976) Uncoupling of oxidative phosphorylation. *Trends in Biochemical Sciences* **1**, 65-67.

Haque, H. and Russell, A.D. (1976) Cell envelopes of Gram-negative bacteria: composition, response to chelating agents and sensitivity of whole cells to antibacterial agents. *Journal of Applied Bacteriology* **40**, 89-99.

Harold, F.M. (1977) Ion currents and physiological functions in microorganisms. *Annual Review of Microbiology* **31**, 181-203.

Harris, N.D. (1963) The influence of recovery medium and incubation temperature on the survival of damaged bacteria. *Journal of Applied Bacteriology* **26**, 387-397.

Harry, E.G. (1963) The relationship between egg spoilage and the environment of the egg when laid. *British Poultry Science* **4**, 91-100.

Hart, A. (1973) Antibacterial activity of phenylmercuric nitrate in Zinc sulphate and Adrenaline eye drops. *Journal of Pharmacy and Pharmacology* **25**, 507-508.

Harvey, C.S. (1985) Antiseptics and disinfectants; fungicides; ecto-parasiticides. In *The Pharmacological Basis of Therapeutics*. eds. Goodman Gillman, A., Goodman, L.S., Rall, T.W. and Murad, F. 7th edn. p. 959. New York: Macmillan Publishing Company.

Harvey, C.S. (1990) Antimicrobial Drugs. In *Remington's Pharmaceutical Sciences*. ed. Gennaro, A.lR., Chase, G.D., Marderosian, A.D., Harvey, S.C. and Hussar, D.A. pp. 1163-1241. Pennsylvania: Mack Publishing Company.

Hedges, R.W., Rodríguez-Lemoine, V., Datta, N. (1975) R factors from *Serratia marcescens. Journal of General Microbiology* **86**, 88-92.

Herzyk, P., Neidle, S. and Goodfellow, J.M. (1992) Conformation and dynamics of drug-DNA intercalation. *Journal of Biomolecular Structure and Dynamics* **10**, 97-139.

Holloway, P.M., Platt, J.H., Reybrouck, G., Lilly, H.A., Mehtar, S. and Drabu, Y. (1990) A multi-centre evaluation of two chlorhexidine-containing formulations for surgical hand disinfection. *Journal of Hospital Infection* **16**, 151-159.

Holy Bible. England: Eyre and Spottiswoode Limited.

Hotchkiss, R.D. and Gabor, M. (1970) Bacterial transformation with specific reference to recombination. *Annual Review of Genetics* **4**, 193- .

Hugo, W.B. (1965) Disinfection. In *Sterilization and Disinfection*. eds. Whittet, T.D., Hugo, W.B. and Wilkinson, G.R. pp. 188-264. London: William Heinemann Medical Brooks Ltd.

Hugo, W.B. (1990) Introduction to Microbiology. In *Guide to Microbiological Control in Pharmaceuticals*. ed. Denyer, S. and Baird, R. pp. 9-28. London: Ellis Horwood Limited.

Hugo, W.B. (1991) A brief history of heat and chemical preservation and disinfection. *Journal of Applied Bacteriology* **71**, 9-18.

Hugo, W.B. (1992a) Historical introduction. In *Principles and Practice of Disinfection, Preservation and Sterilization*. eds. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. 2nd edn. pp. 3-6. Oxford: Blackwell Scientific Publications.

Hugo, W.B. (1992b) Disinfection Mechanisms. In *Principles and Practice of Disinfection, Preservation and Sterilization*. eds. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. 2nd edn. pp. 187-210. Oxford: Blackwell Scientific Publications.

Hugo, W.B. and Bloomfield, S.F. (1971a) Studies on the mode of action of the phenolic antimicrobial agent Fentichlor against *Staphylococcus aureus* and *Escherichia coli*. The effects of Fentichlor on the bacterial membrane and the cytoplasmic constituents of the cell. *Journal of Applied Bacteriology* **34**, 569-578.

Hugo, W.B. and Bloomfield, S.F. (1971b) Studies on the mode of action of the phenolic antibacterial agent Fentichlor against *Staphylococcus aureus* and *Escherichia coli*. I. The adsorption of Fentichlor by the bacterial cell and its antibacterial activity. *Journal of Applied Bacteriology*, **34** 557-567.

Hugo, W.B. and Bloomfield, S.F. (1971c) Studies on the mode of action of the phenolic antibacterial agent Fentichlor against *Staphylococcus aureus* and *Escherichia coli*. III The effect of Fentichlor on the metabolic activities of *Staphylococcus aureus* and *Escherichia coli*. *Journal of Applied Bacteriology* **34**, 579-591.

Hugo, W.B. and Longworth, A.R. (1964) Some aspects of the mode of action of chlorhexidine. *Journal of Pharmacy and Pharmacology* **16**, 655-662.

Hugo, W.B. and Russell, A.D. (1992) Types of antimicrobial agents. In *Principles and*

Practice of Disinfection, Preservation and Sterilization. eds. Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J. 2nd edn. pp. 7-88. Oxford: Blackwell Scientific Publications.

Hugo, W.B., Pallent, L.J., Grant, D.J.W., Denyer, S.P. and Davies, A. (1986) Factors contibuting to the survival of astrain of *Pseudomonas cepacia* in chlorhexidine solutions. *Letters in Applied Bacteriology* **2**, 37-42.

Humphreys, H., Carroll, J.D., Keane, C.T., Cafferkey, M.T., Pomeroy, H.M. and Coleman, D.C. (1990) Importation of methicillin-resistant *Staphylococcus aureus* from Baghdad to Dublin and subsequent nosocomial spread. *Journal of Hospital Infection* **15**, 127-135.

Imlay, J. and Fridovich, I. (1992) Exogenous quinones directly inhibit the respiratory NADH dehydrogenase in *Escherichia coli*. *Archives of Biochemistry and Biophysics* **296**, 337-346.

Izaki, K., Kiuchi, K. and Arima, K. (1966) Specificity and mechanism of tetracycline resistance in a multiple drug resistant strain of *Escherichia coli*. *Journal of Bacteriology* **91**, 628-633.

Jackson, W.J. and Summers, A.O. (1982) Biochemical characterization of $HgCl_2$ induced polypeptides encoded by the *mer* operon of plasmid R100. *Journal of Bacteriology* **151**, 962-970.

Jaffe, G.V., Grnshaw, J.J. and Constad, D. (1989) Benzoyl peroxide in the treatment of acne vulgaris: a doble-blind, multi-centre comparative study of "Quinoderm" cream and "Quinoderm" cream with hydrocortisone versus their base vehicle alone and a benzoyl peroxide only gel preparation. *Current Medical Research and Opinion* **11**, 453-462.

Jawetz, E., Melnick, J.L., Adelberg, E.A., Brooks, G.F., Butel, J.S. and Ornston, L.N. (eds.) (1989) *Medical Microbiology*. 18th ed. U.S.A.: Prentice-Hall International Inc.

Jenkins, S., Addy, M. and Newcome, R. (1991) Triclosan and sodium lauryl sulphate mouthrinses. Effects of a 4-day plaque regrowth. *Journal of Clinical Periodontology* **18**, 145-148.

John, T., Lin, J. and Sahm, D.F. (1990) *Acanthamoeba keratitis* successfully treated with prolonged propamidine isethionate and neomycin-polymyxin-gramicidin. *Annals of Ophthalmology* **22**, 20-23.

Johnston, L.H. and Dyke, K.G.H. (1969) Ethidium bromide resistance, a new marker on the staphylococcal penicillinase plasmid. *Journal of Bacteriology* **100**, 1413-1414.

Joyston-Bechal, S. and Hernaman, N. (1993) The effect of a mouthrinse containing

chlorhexidine and fluoride on plaque and gingival bleeding. *Journal of Clinical Periodontology* **20**, 49-53.

Kabara, J.J. (1984) Medium chain fatty acids and esters as antimicrobial agents. In *Cosmetics and Drug Preservation: Principles and Practice*. ed. Kabara, J.J. pp. 275-304. New York: Marcel Dekker.

Kaneko, (1985) Energetics of tetracycline efflux system encoded by *Tn10* in *Escherichia coli*. *FEBS Letters* **193**, 194-198.

Kaur, P. and Rosen, B.P. (1992) Plasmid-encoded resistance to arsenic and antimony. *Plasmid* **27**, 49-40.

Kawabata, S., Torii, M., Minami, T., Fujiwara, T. and Hamada, S. (1993) Effects of selected surfactants on purified glucosyltransferases form mutans streptococci and cellular adherence to smooth surfaces. *Journal of Medical Microbiology* **38**, 54-60.

Keane, C.T. and Cafferkey, M.T. (1984) Re-emergence of methicillin-resistant *Staphylococcus aureus* causing severe infection. *Journal of Infection* **9**, 6-16.

Kelly, H.A. (1901) Jules Lemaire, the first to recognize the true nature of wound infection and inflammation and the first to use carbolic acid in surgery. *Journal of the American Medical Association* **36**, 1083-1088.

Kelsey, J.C. and Maurer, I.M. (1972) *The Use of Chemical Disinfectants in Hospitals*. Public Health Laboratory Service Monograph Series No. 2. London: HMSO.

Kelstrup, J. and Funder-Nielsen, T.D. (1974) *Journal of General Microbiology* **81**, 485-489.

Kenney, D.S., Grudny, W.E. and Otto, R.H. (1964) Spoilage and preservative tests as applied to pharmaceuticals. *Bulletin of the Parenteral Drug Association* **18**, 10-19.

Kimber, H. (1986) Bacterial Plasmids. 2nd edn. England: Van Nostrand Reinhold (UK) Co. Ltd. pp. 23-80.

King, W.L. and Hurst, A. (1963) A note on survival of some bacteria in different diluents. *Journal of Applied Bacteriology* **26**, 504-506.

Kjolen, H. and Andersen, B.M. (1992) Handwashing and disinfection of heavily contaminated hands--effective or ineffective? *Journal of Hospital Infection* **21**, 61-71.

Kolawole, D.O. (1984) Resistance mechanisms of mucoid-grown *Staphylococcus aureus* to the antibacterial action of some disinfectants and antiseptics. *FEMS Microbiology Letters* **25**, 205-209.

Kondo, I., Ishikawa, T. and Nakahara, H. (1974) Mercury and cadmium resistances mediated by the penicillinase plasmid in *Staphylococcus aureus. Journal of Bacteriology* **117**, 1-7.

Kopsidas, G. and McPhee, D.G. (1993) Glucose inhibition of mutagenesis by 9-aminoacridine in *Salmonella typhimurium. Mutation Research* **285**, 101-108.

Kornfeld, F. (1966) Properties and techniques of application of biocidal ampholytic surfactants. *Food Manufacture* **41**, 39-46.

Krivobok, S., Seigle-Murandi, F. Steiman, R., Marzin, D.R. and Betina, V. (1992) Mutagenicity of substituted anthraquinones in the Ames/*Salmonella* microsome system. *Mutation Research* **279**, 1-8.

Kurup, T.R., Wan, L.S., Chan, L.W. (1991) Effect of surfactants on the antibacterial activity of preservatives. *Pharmaceutica Acta Helvetica* **66**, 274-280.

Kurup, T.R.R., Wan, L.S.C., and Chan, L.W. (1992) Preservative requirements in emulsions. *Pharmaceutica Acta Helvetica* **67**, 204-208.

Lacey, R.W. and Grinsted, J. (1972) Linkage of fusidic acid resistance to the penicillinase plasmid in *Staphylococcus aureus. Journal of General Microbiology* **73**, 501-508.

Lacey, R.W., Barr, K.W., Barr, V.E. and Inglis, T.J. (1986) Properties of methicillin-resistant *Staphylococcus aureus* colonizing patients in a burns unit. *Journal of Hospital Infection* **7**, 137-148.

Lai, C.J., Dahlberg, J.E. and Weisblum, B. (1973) Structure of an inducibly methylatable nucleotide sequence in 23S ribosomal ribonucleic acid from erythromycin-resistant *Staphylococcus aureus. Biochemistry* **12**, 457-460.

Langezaal, C.R., Chandra, A. and Scheffer, J.J. (1992) Antimicrobial screening of essentil oils and extracts of some *Humulus lupulus L.* cultivars. *Pharmaceutisch Weekblad -Scientific Edition* **14**, 353-356.

Lark, K.G. and Lark, C. (1966) Regulation of chromosome replication in *Escherichia coli* a comparison of the effects of phenylethyl alcohol treatment with those of amino acid starvation. *Journal of Molecular Biology* **20**, 9-19.

Larkin, D.F., Kilvington, S. and Dart, J.K. (1992) Treatment of *Acanthamoeba keratitis* with polyhexamethylene biguanide. *Ophthalmology* **99**, 185-191.

Law, M.R. and Gill, O.N. (1988) Hospital-acquired infection with methicillin-resistant and methicillin-sensitive staphylococci. *Epidemiology and Infection* **101**, 623-629.

Lawrence, C.A. (1968) Quaternary ammonium surface-active disinfectants. In *Disinfection, Sterilization and Preservation*. eds. Lawrence, C.A. and Block, S.S.

pp. 430-452. U.S.A.: Lea and Febiger.

Leak, R.E. (1983) *Some factors affecting the preservative testing of aqueous systems.* Ph.D. Thesis. University of London.

Leak, R.E. and Leech, R. (1988) Challenge tests and their predictive ability. In *Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries.* eds Bloomfield, S.F., Baird,R., Leak, R.E. and Leech, R. pp. 133-146. London: Ellis Horwood Limited.

LeChevalier, M.W., Cawthorn, C.D. and Lee, R.G. (1988) Inactivation of biofilm bacteria. *Applied and Environmental Microbiology* **54**, 2492-2499.

Leech, R. (1990) New methodology for microbiological quality assurance. In *Guide to Microbiological Control in Pharmaceuticals.* ed. Denyer, S. and Baird, R. pp. 195-216. London: Ellis Horwood.

Leive, L. (1974) The barrier function of the Gram-negative envelope. *Annals of the New York Academy of Sciences* **235**, 109-127.

Lemaire, H.C., Schramm, C.H. and Cahn, A. (1961) Synthesis and germicidal activity of halogenated salicylanilides and related compounds. *Journal of Pharmaceutical Sciences* **50**, 831-837.

Levy, S.B. and McMurry, L. (1974) Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. *Biochemical and Biophysical Research Communications* **56**, 1060-1068.

Levy, S.B. and McMurry, L. (1978) Plasmid-determined tetracycline resistance involves new transport system for tetracycline. *Nature* **276**, 90-92.

Lilley, B.D. and Brewer, J.H. (1953) The selective antibacterial activity of phenylethyl alcohol. *Journal of the American Pharmaceutical Association, Scientific Edition,* **42**, 6-8.

Littlejohn, T.G., Paulsen, I.T., Gillespie, M.T., Tennent, J.M., Midgley, M., Jones, I.G., Purewal, A.S. and Skurray, R.A. (1992) Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus. FEMS Microbiology Letters* **74**, 259-265.

Locksley, R.M., Cohen, M.L., Quinn, T.C., Tompkins, L.S., Coyle, M.B., Kirihara, J.M. and Counts, G.W. (1982) Multiply antibiotic-resistant *Staphylococcus aureus*: introduction, transmission and evolution of nosocomial infection. *Annals of Internal Medicine* **97**, 317-324.

Lopez-García, R.E., Hernández-Perez, M., Rabanal, R.M., Darias, V., Martín-Herrera, D., Arias, A. and Sanz, J. (1992) Essential oils and antimicrobial activity of two varieties of *Cedronella canariensis* (L.) W. et B. *Journal of Ethnopharmacology* **36**, 207-211.

Lu-Lu, Singh, J.S., Galperin, M.Y., Drake, D., Taylor, K.G. and Doyle, R.J. (1992) Chelating agents inhibit activity and prevent expression of streptococcal glucan-binding lectins. *Infection and Immunity* **60**, 3807-3813.

Lugtenberg, B. and Van Alphen, L. (1983) Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other Gram-negative bacteria. *Biochimica Biophysica Acta* **737**, 51-115.

Luo, G. and Lewis, R.A. (1992) Inhibition of RNA polymerase by captan at both DNA and substrate binding sites. *Biochemical Pharmacology* **44**, 2251-2258.

Lynch, M., Lund, W. and Wilson, D.A. (1977) Chloroform as a preservative in aqueous systems. Losses under "in use" conditions and antimicrobial effectiveness. *Pharmaceutical Journal* **219**, 507-510.

Lynn, B. and Hugo, W.B. (1983) Chemical disinfectants, antiseptics and preservatives. In *Pharmaceutical Microbiology.* ed. Hugo, W.B. and Russell, A.D. 3rd edn. pp. 210-236. London: Blackwell Scientific Publications.

Lyon, B.R. and Skurray, R. (1987) Antimicrobial resistance of *Staphylococcus aureus*: genetic basis. *Microbiological Reviews* **51**, 88-134.

Lyon, B.R., May, J.W. and Skurray, R.A. (1983) Analysis of plasmids in nosocomial strains of multiple-antibiotic resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **23**, 817-826.

Lyon, B.R., May, J.W. and Skurray, R.A. (1984) *Tn4001*: a gentamicin and kanamycin resistance transposon in *Staphylococcus aureus*. *Molecular and General Genetics* **193**, 554-556.

Mann, R.D. (1984) *Modern Drug Use. An Enquiry on Historical Principles.* pp. 507-596. Lancaster: MTP Press Limited.

Marquis, R.E. (1968) Salt-induced contractions of bacterial cell walls. *Journal of Bacteriology* **95**, 775-781.

Marrie, T.J. and Costerton, J.W. (1984) Scanning and transmission electron microscopy of *in-situ* bacterial colonisation of intravenous and intraarterial catheters. *Journal of Clinical Microbiology* **19**, 687-693.

Mobley, H.L.T. and Rosen, B.P. (1982) Energetics of plasmid-mediated arsenate resistance in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 6119-6122.

McCabe, M.M., Haynes, A.V. and Hamelik, R.M. (1976) *Microbial Aspects of Dental Caries* eds. Stiles, H.M., Loesche, W.J. and O'brien, T.C. pp. 413-424.

McCarthy, T.J. and Ferreira, J.H. (1990) Attempted measurement of the activity of

selected preservative combinations. *Journal of Clinical Pharmacy and Therapeutics* **15**, 123-129.

McDonnell, R.W., Sweeney, H.M. and Cohen, S. (1983) Conjugational transfer of gentamicin resistance plasmids intra- and interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy* **23**, 151-160.

McMurry, L.M., Cullinane, J.C., Petrucci Jr., R.E. and Levy, S.B. (1981) Active uptake of tetracycline by membrane vesicles from susceptible *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **20**, 307-313.

Meers, J.L. and Tempest, D.W. (1970) The influence of growth limiting substrate and medium NaCl concentration on the synthesis of magnesium binding sites in the walls of *Bacillus subtilis var niger*. *Journal of General? Microbiology* **63**, 325-331.

Melichercikova, V. (1989) Disinfectant effect of Persteril in combination with detergents. *Journal of Hygiene, Epidemiology, Microbiology and Immunology* **33**, 19-28.

Michael, G.I. and Stumbo, C.R. (1970) Ethylene oxide sterilization of *Salmonella senftenberg* and *Escherichia coli*: death kinetics and mode of action. *Journal of Food Science* **35**, 631-634

Miles, A.A. and Misra, S.S. (1938) The estimation of the bactericidal power of the blood. *Journal of Hygiene* (Cambridge) **38**, 732-749.

Minakami, H., Kitzler, J.W., Fridovich, I. (1990) Effects of pH, glucose, and chelating agents on lethality of paraquat to *Escherichia coli*. *Journal of Bacteriology*, **172**, 691-695.

Minnich, S.M., Moeschberger, M. and Anderson, J. (1991) Effect of oxyquinoline ointment on diaper dermatitis. *Dermatology Nursing* **3**, 25-28.

Mitsuhashi, S., Hashimoto, H., Kono, M. and Morimura, M. (1972) Drug resistance of staphylococci. II. Joint elimination and joint transduction of the determinants of penicillinase production and resistance to macrolide antibiotics. *Journal of Bacteriology* **89**, 988-992.

Moleyar, V. and Narasimham, P. (1992) Antibacterial activity of essential oil components. *International Journal of Food Microbiology* **16**, 337-342.

Moore, K.E. (1978) Evaluating preservative efficacy by challenge testing during the development stage of pharmaceutical products. *Journal of Applied Bacteriology* **44**, Sxliii-Slv.

Moore, K.E. and Taylor, J.E. (1976) Microbiological standards for nasal solutions. *Journal of Applied Bacteriology* **41**, 379-387.

Morier-Teissier, E., Bernier, J.L., Coulaud, D., Henichart, J.P. and Delain, E. (1992) Electron microscopic observations of the effects of anthraquinone derivatives on plasmid DNA. *Journal of Biomolecular Structure and Dynamics* **9**, 653-666.

Morimyo, M., Hongo, E., Hama-Inaba, H. and Machida, I. (1992) Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. *Nucleic Acids Research* **20**, 3159-3165.

Motohashi, N., Sakagami, H., Kurihara, T., Ferenczy, L., Csuri, K. and Molnar, J. (1992) Antimicrobial activity of phenothiazines, benzo[a]phenothiazines and benz[c]acridines. *Anticancer Research* **12**, 1207-1210.

Nadir, M.T. and Gilbert, P. (1980) Influence of potassium chloride upon the binding and antibacterial activity of chlorhexidine diacetate and (ethoxy)₅octyl phenol on *Bacillus megaterium*. *Microbios* **26**, 51-63.

Netuschil, L., Reich, E. and Brecx, M. (1989) Direct measurement of the bactericidal effect of chlorhexidine on human dental plaque. *Journal of Clinical Periodontology* **16**, 484-488.

Nicholls, A.A., Leaver, C.W.E. and Panes, J.J. (1967) Hatchery hygiene evaluation as measured by microbiological examination of samples of fluff. *British Poultry Science* **8**, 297.

Nicholson, B.H. and Peacocke, A.R. (1965) The inhibition of ribonucleic acid polymerase by acridines. *Biochemical Journal* **100**, 50-58.

Nickel, J.C., Ruseska, I., Wright, J.B. and Costerton, J.W. (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary tract catheter materials. *Antimicrobial Agents and Chemotherapy* **27**, 619-624.

Nikaido, H. and Nakae, T. (1979) The outer-membrane of Gram-negative bacteria. *Advances in Microbial Physiology* **20**, 163-250.

Novick, R.P. and Roth, C. (1968) Plasmid-linked resistance to inorganic salts in *Stapylococcus aureus*. *Journal of Bacteriology* **95**, 1335-1342.

Novick, R.P., Murphy, E., Gryczan, T.J., Baron, E. and Edelman, I. (1979) Penicillinase plasmids of *Staphylococcus aureus*: restriction-deletion maps. *Plasmids* **2**, 109-129.

Oehring, H., Beleites, E., Straube, E., Volker, U. and Muller, R. (1992) The effect of biomaterials and other industrial materials on the growth of several aerobic

bacterial species *in vitro*. *Zentralblatt fur Hygiene und Umweltmedizin* **192**, 462-472.

Ohta, S. (1990) Studies on resistant mechanisms in the resistant bacteria to chlorhexidine. II. Chemical components of the cell membrane and the electron microscopical observation of cell surface structure of chlorhexidine-resistant bacteria. *Yakugaku-Zasshi* **110**, 414-425.

Oosterwaal, P.J., Mikx, F.H., van't Hof, M.A. and Renggli, H.H. (1991) Short-term bactericidal activity of chlorhexidine gel, stannous fluoride gel and amine fluoride gel tested in periodontal pockets. *Journal of Clinical Periodontology* **18**, 97-100.

Osborn, M.J. and Wu, H.C.P. (1980) Proteins of the outer-membrane of Gram-negative bacteria. *Annual Review of Microbiology* **34**, 369-422.

Ozeki, H. and Ikeda, H. (1968) Transduction mechanisms. *Annual Review of Genetics* **2**, 245-278.

Paniagua-Crespo, E., Hasle, D.P., Aouizerate, F., Volant, A. and Simitris-Le Flohic, A.M. (1989) In vitro study of propamidine isethionate: value in the follow-up of *Acanthamoeba* amebic keratitis. *Journal Francais D Ophtalmologie* **12**, 489-491.

Parker, M.S. (1988) Microbial contamination and preservation of pharmaceutical preparations. In *Pharamceutics: The Science of Dosage Form Design*. ed. Aulton, M.E. pp. 502-504. London: Churchill Livingstone.

Patel, W.K. and Kostenbauder, H.B. (1958) Binding of *p*-hydroxybenzoic acid esters by polyoxyethylene 21 sorbitan mono-oleate. *Journal of the American Pharmaceutical Association* **47**, 289-293.

Paulsen, I.T. and Skurray, R.A. (1993) Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes--an analysis. *Gene* **124**, 1-11.

Paulus, W. (1976) Problems encountered with formaldehyde-releasing compounds used as preservatives in aqueous systems, especially lubricoolants--possible solutions to the problems. In *Proceedings of the 3rd International Biodegradation Symposium*. ed. Shaply, J.M. and Kaplan, A.M. pp. 1075-1082. London: Applied Science Publishers.

Pepper, R.E. and Chandler, V.L. (1963) Sporicidal activity of alkaline alcoholic saturated dialdehyde solutions. *Applied Microbiology* **11**, 384-388.

Perry, R.D. and Silver, S. (1982) Cadmium and manganese transport in *Staphylococcus aureus* membrane vesicles. *Journal of Bacteriology* **150**, 973-976.

Phillips, C.R. (1977) Gaseous sterilization. In *Disinfection, Sterilization and Preservation*.

ed. Block, S.S. 2nd ed. pp. 592-611. Philadelphia: Lea and Febiger.

Phillips, G.B. (1975) Contamination control during the manufacture of medical products. *Acta Pharmaceutica Suecica* 12 (Supplement), 6-14.

Phillips, G.B. and Halleck, F.E. (1985) Sterilization. In *Remington's Pharmaceutical Sciences*. ed. Gennaro, A. 17th edn. p. 1445. Pennsylvania: Mack Publishing Company.

Phillips, I., Lobo, A.Z., Fernándes, R. and Gundara, N.S. (1968) Acetic acid in the treatment of superficial wounds infected by *Pseudomonas aeruginosa*. *Lancet* i, 11-12.

Pinney, R.J. (1978) Survival of plasmid-containing strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in phenylmercuric nitrate and thiomersal. *Journal of Pharmacy and Pharmacology* 30, 228-232.

Pisano, F.D. and Kostenbauder, H.B. (1959) Correlation of binding data with required preservative concentrations of *p*-hydroxybenzoates in the presence of Tween 80. *Journal of the American Pharmaceutical Association* 48, 310-314.

Pons, J.L., Bonnaveiro, N., Chevalier, J. and Cremieux, A. (1992) Evaluation of antimicrobial interactions between chlorhexidine, quaternary ammonium compounds, preservatives and excipients. *Journal of Applied Bacteriology* 73, 395-400.

Popescu, A., Jucu, V., Tomas, E., Zuiwertz, A., Cristescu, C. and Tomas, S. (1992) Antiviral activity of some copper complexes of as-triazines. *Revue Roumaine de Virologie* 43, 125-126.

Porter, F.D., Silver, S., Ong, C., Nakahara, H. (1982) Selection for mercurial resistance in hospital settings. *Antimicrobial Agents and Chemotherapy* 22, 852-858.

Price, P.B. (1950) Re-evaluation of ethyl alcohol as a germicide. *Archives of Surgery* 60, 492-502.

Purewal, A.S., Jones, I.G. and Midgley, M. (1990) Cloning of the ethidium efflux gene from *Escherichia coli*. *FEMS Microbiology Letters* 56, 73-76.

Quack, J.M. (1976) Quaternary ammonium compounds in cosmetics. *Cosmetics and Toiletries* 91, 35-52.

Quest, J.A., Fenner-Crisp, P.A., Burnam, W., Copley, M., Dearfield, K.L., Hamernik, K.L., Saunders, D.S., Whiting, R.J. and Enler, R. (1993) Evaluation of the carcinogenic potential of pesticides. 4-Chloroalkylthiodicarboximide compounds with fungicidal activity. *Regulatory Toxicology and Pharmacology* 17, 19-34.

Quinn, P.J. (1987) Evaluation of veterinary disinfectants and disinfection processes. In *Disinfection in Veterinary and Farm Animal Practice*. eds. Linton, A.H., Hugo, W.B. and Russell, A.D. pp. 66-116. Oxford: Blackwell Scientific Publications.

Rai, M.K., Upadhyay, S.K. and Agrawal, S.C. (1992) *In vitro* evaluation of three topical antimycotics against ring-worm fungi singly and in combination. *Hindustan Antibiotics Bulletin* **34**, 104-107.

Ravin, L.J. (1985) Preformulation. In *Remington's Pharmaceutical Sciences*. ed. Gennaro, A.R. 17th edn. p. 1422. Pennsylvania: Mack Publishing Company.

Rdzok, E.J., Grundy, W.E., Kirchmeyer, F.J. and Sylvester, J.C. (1955) Determining the efficacy of preservatives in pharmaceutical products. *Journal of the American Pharmaceutical Association, Scientific Edition* **44**, 613-616.

Reynolds, P.E. (1984) Resistance of the antibiotic target site. *British Medical Bulletin* **40**, 3-10.

Richards, R.M., Taylor, R.B. and Xing, D.K. (1991) An evaluation of the antibacterial activities of combinations of sulfonamides, trimethoprim, dibromopropamidine, and silver nitrate compared with their uptakes by selected bacteria. *Journal of Pharmaceutical Sciences* **80**, 861-867.

Richards, R.M.E. (1971) Inactivation of resistant *Pseudomonas aeruginosa* by antibacterial combinations. *Journal of Pharmacy and Pharmacology* **23** (Supplement), 136S-140S.

Richards, R.M.E. and Cavill, R.H. (1976) Electron microscope study of effect of benzalkonium chloride and edetate disodium on cell envelope of *Pseudomonas aeruginosa*. *Journal of Pharmaceutical Sciences* **65**, 76-80.

Richards, R.M.E. and McBride, R.J. (1971) Phenylethanol enhancement of preservatives in ophthalmic solutions. *Journal of Pharmacy and Pharmacology* **23** (Supplement), 141S-146S.

Richards, R.M.E. and McBride, R.J. (1973a) Effect of 3-phenylpropan-1-ol, 2-phenylethanol and benzyl alcohol on *Pseudomonas aeruginosa*. *Journal of Pharmaceutical Sciences* **62**, 585-587.

Richards, R.M.E. and McBride, R.J. (1973b) Enhancement of benzalkonium chloride and chlorhexidine acetate activity against *Pseudomonas aeruginosa* by aromatic alcohols. *Journal of Pharmaceutical Sciences* **62**, 2035-2037.

Richmond, M.H. (1965) Dominance of the inducible state in strains of *Staphylococcus aureus* containing two distinct penicillinase plasmids. *Journal of Bacteriology* **90**, 370-374.

Richmond, M.H. (1972) Some environmental consequences of the use of antibiotics: or "what goes up must come down." *Journal of Applied Bacteriology* **35**, 155-176.

Roberts, N.A., Gray, G.W. and Wilkinson, S.G. (1970) The bactericidal action of ethylenediamine tetraacetic acid on *Pseudomonas aeruginosa. Microbios* **2**, 189-208.

Rolinson, G.N., Stevens, S., Batchelor, F.R., Wood, S.C. and Chain, E.B. (1960) Bacteriological studies on a new penicillin-BRL 1241. *Lancet* **ii**, 564-567.

Rosen, B.P. and Borbolla, M.G. (1984) A plasmid-encoded arsenite pump produces aresenite resistance in *Escherichia coli. Biochemical and Biophysical Research Communications* **124**, 760-765.

Rosenstein, R., Peschel, A., Wieland, B. and Gotz, F. (1992) Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *Journal of Bacteriology* **174**, 3676-3683.

Rossmore, H.W. and Sondossi, M. (1988) Applications and mode of action of formaldehyde condensate biocides. *Advances in Applied Microbiology* **33**, 223-277.

Rossmore, H.W., DeMare, J. and Smith, T.H.F. (1972) Anti- and pro-microbial activity of hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine in cutting fluid emulsion. In *Biodeterioration of Materials*. ed. Walters, A.H. and Hueck-van der Plas, E.H. Vol. 2, pp. 266-293. London: Applied Science Publishers.

Rost, W.J. (1976) Chemotherapy: an introduction. In *Principles of Medicinal Chemistry*. ed. Foye, W.O. 1st edn. pp. 660-668. Philadelphia: Lea and Febiger.

Rouch, C.A., Cram, D.S., DiBerardino, D., Littlejohn, T.G. and Skurray, R.A. (1990) Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. *Molecular Microbiology* **4**, 2051-2062.

Ruseska, I., Robbins, J. and Costerton, J.W. (1982) Biocide testing against corrosion-causing oil-field bacteria helps control plugging. *Oil Gas Journal* **CS-457**, 3-82.

Russell, A.D. (1976) Inactivation of non-sporing bacteria by gases. *Society for Applied Bacteriology Symposium No. 5: Inactivation of Vegetative Micro-organisms*. ed. Skinner, F.A. and Hugo, W.B. pp. 61-88. London: Academic Press.

Russell, A.D. (1982) *The Destruction of Bacterial Spores*. London: Academic Press. Russell, A.D. (1986) Chlorhexidine: antibacterial action and bacterial resistance. *Infection* **14**,212-215.

Russell, A.D. (1990) The bacterial spore and chemical sporicides. *Clinical Microbiology Reviews* **3**, 99-119.

Russell, A.D. (1991) Mechanisms of bacterial resistance to non-antibiotics: food preservatives and food and pharmaceutical preservatives. *Journal of Applied Bacteriology*, **71**, 191-201.

Russell, A.D., Ahonkhai, I. and Rogers, D.T. (1979) A Review. Microbiological applications of the inactivation of antibiotics and other antimicrobial agents. *Journal of Applied Bacteriology* **46**, 207-245.

Russell, A.D. and Chopra, I. (1990) *Understanding Antibacterial Action and Resistance*. Chichester: Ellis Horwood.

Russell, A.D. and Gould, G.W. (1988) Resistance of Enterobacteriaceae to preservatives and disinfectants. *Journal of Applied Bacteriology (Symposium Supplement)*, 167S-195S.

Russell, A.D., Hammond, S.A. and Morgan, J.R. (1986) Bacterial resistance to antiseptics and disinfectants. *Journal of Hospital Infection* **7**, 213-225.

Sakagami, Y., Yokoyama, H., Nishimura, H., Ose, Y., and Tashima, T. (1989) Mechanism of resistance to benzalkonium chloride by *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **55**, 2036-2040.

Salton, M.R.J. (1963) The relationship between the nature of cell wall and the Gram stain. *Journal of General Microbiology* **30**, 223-235.

Saltzer, B.M. (1961) Oxidative activity of psychrophilic and mesophilic bacteria on saturated fatty acids. *Journal of Bacteriology* **82**, 492-497.

San Francisco, M.J.D., Hope, C.L., Owolabi, J.B., Tisa, L.S. and Rosen, B.P. (1990) Identification of the metalloregulatory element of the plasmid encoded arsenical resistance operon. *Nucleic Acids Research* **18**, 619-624.

San Francisco, M.J.D., Tisa, L.S. and Rosen, B.P. (1989) Identification of the membrane component of the anion pump encoded by the arsenical resistance operon of R-factor R773. *Molecular Microbiology* **3**, 15-21.

Schanno, R.J., Westlund, J.R. and Foelsch, D.H. (1980) Evaluation of 1,3-dimethylol-5,5-dimethylhydantoin as a cosmetic preservative. *Journal of the Society of Cosmetic Chemists* **31**, 85-96.

Schelhorn, T., Kretz, S. and Zimmermann, H.W. (1992) Reinvestigation of the binding of proflavine to DNA. Is intercalation the dominant binding effect? *Cellular and Molecular Biology* **38**, 345-365.

Schnaitman, C.A. (1971a) Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X45. *Journal of Bacteriology* **108**, 545-552.

Schnaitman, C.A. (1971b) Effect of ethylenediaminetetraacetic acid, Triton X100 and lysozyme on the chemical compositon and morphology of isolated cell walls. *Journal of Bacteriology* **108**, 533-544.

Schottel, J.L., Mandal, A., Clark, D., Silver, S., Hedges, W. (1974) Volatilization of mercury and organomercurials determined by inducible R-factor systems in enteric bacteria. *Nature* **251**, 335-337.

Scott, C.R. and Wolf, P.A. (1962) The antibacterial activity of a series of quatenaries prepared from hexamethylenetetramine and halohydrocarbons. *Applied Microbiology* **10**, 211-216.

Shaw, W.V. (1983) Chloramphenicol acetyltransferase: enzymology and molecular biology. *Crit Review Biochem* **14**, 1-46.

Shibasaki, I. and Kato, N. (1978) Combined effects on antibacterial activity of fatty acids and their esters against Gram-negative bacteria. In *The Pharmacological Effects of Lipids*. ed. Kabara, J.J. pp. 15-24. Illinois: The American Oil Chemists' Society.

Silver, S. and Keach, D. (1982) Energy-dependent arsenate efflux: the machanisms of plasmid mediated resistance. *Proceedings of the National Academy of Science of the United States of America* **79**, 6114-6118.

Silver, S. and Misra, S. (1988) Plasmid-mediated heavy metal resistances. *Annual Review of Microbiology* **42**, 717-743.

Silver, S., Budd, K., Leahy, K.M., Shaw, W.V., Hammond, D., Novick, R.P., Willsky, G.R., Malamy, M.H. and Rosenberg, H. (1981) Inducible plasmid-determined resistance to arsenate, arsenite, and antimony (III) in *Escherichia coli* and *Staphylococcus aureus*. *Journal of Bacteriology* **146**, 983-996.

Simon, E.W. and Beavers, H. (1952) The effect of pH on the biological activities of weak acids and bases. II. Other relationships between pH and activity. *New Phytologist* **51**, 191-197.

Simonetti, N., Simonetti, G., Bougnol, F. and Scalzo, M. (1992) Electrochemical Ag^+ for preservative use. *Applied and Environmental Microbiology* **58**, 3834-3836.

Sivaji, Y., Mondal, A. and Agaswal, D.S. (1986) Disinfectant induced changes in the antibiotic sensitivity and phage-typing pattern in *Staphylococcus aureus*. *Journal of Hospital Infection* **7**, 236-243.

Smith, J.T. and Amyes, S.G.B. (1984) Bacterial resistance to antifolate chemotherapeutic agents mediated by plasmids. *British Medical Bulletin* **40**, 42-46.

Spooner, D.F. and Corbett, R.J. (1985) The rational use of preservatives in cosmetics and

toiletries. *Symposium on Formulating Better Cosmetics*, University of Nottingham.

Staehelin, M. (1958) Reaction of tobacco mosaic virus nucleic acid with formaldehyde. *Biochimica et Biophysica Acta* **29**, 410-417.

Starr, J.E. and Judis, J. (1968) Mechanism of action of phenolic disinfectants. Association of phenolic disinfectants with proteins. *Journal of Pharmaceutical Sciences* **57**, 768-773.

Sugai, M., Akiyama, T., Komatsuzawa, H., Miyake Y. and Suginaka, H. (1991) Cibacron blue 3G-A inhibits cell separation of gram-positive bacteria. *Archives of Microbiology* **155**, 199-201.

Summers, A.O. (1978) Microbial transformations of metals. *Annual Review of Microbiology* **32**, 637-672.

Summers, A.O. (1984) Bacterial metal ion resistances. In *Antimicrobial Drug Resistance*. ed. Bryan, L.E. pp. 345-367. Orlando: Academic Press, Inc.

Summers, A.O. (1986) Organization, expression, and evolution of genes for mercury resistance. *Annual Review of Microbiology* **40**, 607-634.

Summers, A.O. and Silver, S. (1978) Microbial transformations of metals. *Annual Review of Microbiology* **32**, 637-672.

Summers, A.O. and Sugarman, L.I. (1974) Cell-free mercury(II)-reducing activity in a plasmid-bearing strain of *Escherichia coli*. *Journal of Bacteriology* **119**, 242-249.

Summers, A.O., Wireman, J, Vimy, M.J., Lorscheider, F.L., Marshall, B., Levy, S.B., Bennett, S. and Billard, L. (1993) Mercury released from dental "silver" fillings provokes an increase in mercury- and antibiotic-resistant bacteria in oral and intestinal floras of primates. *Antimicrobial Agents and Chemotherapy* **37**, 825-834.

Swedberg, G. and Skold, O. (1980) Characterization of different plasmid-borne dihydropteroate synthases mediating bacterial resistance to sulfonamides. *Journal of Bacteriology* **142**, 1-7.

Swinyard, E.A. and Lowenthal, W. (1990) Pharmaceutical necessities. In *Remington's Pharmaceutical Sciences*. ed. Gennaro, A.R. 18th edn. p. 1286. Pennsylvania: Mack Publishing Company.

Sydiskis, R.J., Owen, D.G., Lohr, J.L., Rosler, K.H. and Blomster, R.N. (1991) Inactivation of enveloped viruses by anthraquinones extracted from plants. *Antimicrobial Agents and Chemotherapy* **35**, 2463-2466.

Sykes, G. (1958) *Disinfection and Sterilization*. London: E. and F.N. Spon Ltd.

Tenenbaum, S. (1967) Pseudomonads in cosmetics. *Journal of the Society of Cosmetic Chemists* **18**, 797-807.

Tennent, J.M., Lyon, B.R., Gillespie, M.T., May, J.W. and Skurray, R.A. (1985) Cloning and expression of *Staphylococcus aureus* plasmid-mediated quaternary ammonium resistance in *Escherichia coli. Antimicrobial Agents and Chemotherapy* **27**, 79-83.

Tennent, J.M., Lyon, B.R., Midgley, M., Jones, I.G., Purewal, A.S. and Skurray, R.A. (1989) Physical and biochemical characterization of the *qacA* gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus. Journal of General Microbiology* **135**, 1-10.

Thauer, R., Jungermannn, K. and Decker, K. (1977) Energy conservation in chemothrophic anaerobic bacteria. *Bacteriological Reviews* **41**, 100-180.

Tisa, L.S. and Rosen, B.P. (1990) Molecular characterization of an anion pump: ArsB protein is the membrane anchor for the ArsA protein. *Journal of Biological Chemistry* **265**, 190-194.

Toler, J.C. (1985) Preservative stability and preservative systems. *International Journal of Cosmetic Sciences* **7**, 157-164.

Tomas, S.T., Titire, A., Popescu, A., Tomas, E. and Cajal, N. (1991) Potential antiviral activity of copper complexes, derived from symmetrical triazines hydrazones. *Revue Roumaine de Virologie* **42**, 71-75.

Tsubone, K. (1991) Correlation between antimicrobial activity and chelating ability of 2-(N,N,N-trialkylammonio)alkyl hydrogen phosphates. *Journal of Pharmaceutical Sciences* **80**, 1051-1054.

Tsukamura, M. (1991) Some observations on the mechanism of bactericidal activity of Tween 80 on mycobacteria. *Kekkaku* **66**, 75-79.

Tulis, J.J. (1973) Formaldehyde gas as a sterilant. In *Industrial Sterilization: International Symposium, Amsterdam, 1972.* ed. Phillips, G.B. and Miller, W.S. pp. 209-238.

Tuomanen, E., Durack, D.T. and Tomasz, A. (1986) Antibiotic tolerance among clinical isolates of bacteria. *Antimicrobial Agents and Chemotherapy* **30**, 521-527.

Turcic, J., Hencevic, J. and Bojic-Turcic, V. (1989) The preventive and therapeutic effect of peractic acid on the development of surgical infection. *Chirurgia Maxillofacialis Et Plastica* **19**, 49-54.

Tynecka, Z., Gos, Z. and Zajac, J. (1981a) Reduced cadmium transport determined by a resistance plasmid in *Staphylococcus aureus. Journal of Bacteriology* **147**, 305-302.

Tynecka, Z., Gos, Z. and Zajac, J. (1981b) Energy-dependent efflux of cadmium coded by a plasmid resistance determinant in *Staphylococcus aureus*. *Journal of Bacteriology* **147**, 313-319.

United States Pharmacopoeia (XXII) (1990) Antimicrobial agents--effectiveness. Easton, PA: Mack Publishing Company.

Urbani, G., Filippini, P., Lombardo, G., Consolo, U., Cuzzolin, L. and Benoni, G. (1992) *In vivo* and *in vitro* experimentation with the effects of chlorhexidine in patients who have undergone a periodontal intervention. *Minerva Stomatologica* **41**, 435-443.

Vaara, M. (1990) The effect of oligolysines Lys-3, Lys-4, and Lys-5 on the outer membrane permeability of *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **55**, 15-19.

Viljanen, P. (1987) Polycations which disorganize the outer membrane inhibit conjugation in *Escherichia coli*. *Journal of Anibiotics* **40**, 882-886.

Waaler, S.M. (1990) Further *in vivo* studies on the plaque-inhibiting effect of chlorhexidine and its binding mechanisms. *Scandinavian Journal of Dental Research* **98**, 422-427.

Wagman, G.H., Bailey, J.V. and Weinstein, M.J. (1975) Binding of aminoglycoside antibiotics to filtration materials. *Antimicrobial Agents and Chemotherapy* **7**, 316-319.

Wakelin, L.P.G., Adams, A., Hunter, C. and Waring, M.J. (1981) Interaction of Crystal Violet with nucleic acids. *Biochemistry* **20**, 5779-5787.

Warner, A.M. and Warner, V.D. (1976) Antiseptics and disinfectants. In *Principles of Medicinal Chemistry*. ed. Foye, W.O. pp. 702-714. Philadelphia: Lea and Febiger.

Warner, A.M. and Warner, V.D. (1990) Antiseptics and disinfectants. In *Principles of Medicinal Chemistry*. ed. Foye, W.O. pp. 661-668. London: Lea and Febiger.

Watanabe, T. (1963) Infective heredity of multiple drug resistance in bacteria. *Bacteriological Reviews* **27**, 87-115.

Watanabe, T. (1972) Further outlooks of antibiotics in the shadows of resistance factors. In *Bacterial Plasmids and Antibiotic Resistance. First International Symposium Infectious Antibiotic Resistance*. Czechoslovakia 1971, Prague: Czechoslovak Medical Press. pp. 9-12. eds. Krčméry, V., Rosival, L. and Watanabe, T.

Waxman, D.J. and Strominger, J.L. (1983) Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. *Annual Review of Biochemistry* **52**, 825-869.

Weber, G.R. and Levine, M. (1944) Factors affecting germicidal efficiency of chlorine and chloramine. *American Journal of Public Health* **32**, 719-728.

Wedderburn, D.L. (1964) Preservation of emulsions against microbial attack. In *Advances in Pharmaceutical Sciences*. ed. Bean, H.S., Beckett, A.H. and Carless, J.E. pp. 195-264. London: Academic Press.

Weiss, A.A., Murphy, S.D. and Silver, S. (1977) Mercury and organomercurial resistances determined by plasmids in *Staphylococcus aureus*. *Journal of Bacteriology* **132**, 197-208.

West, T.S. (1969) *Complexometry with EDTA and Related Agents*. 3rd ed. Poole: BDH Chemicals Ltd.

Whittet, T.D., Hugo, W.B. and Wilkinson, G.R. (1965) *Sterilization and Disinfection*. ed. Stenlake, J.B. p. 204. London: Wiliam Heinemann Medical Books Limited.

Wien, R., Harrison, J. and Freeman, W.A. (1948) Diamidines as antibacterial compounds. *British Journal of Pharmacology* (formerly *Journal of Pharmacology and Chemotherapy*) **3**, 211-218.

Williams, N.D. and Russell, A.D. (1991) The effects of some halogen-containing compounds on *Bacillus subtilis* endospores. *Journal of Applied Bacteriology* **70**, 427-436.

Wu, J. and Rosen, B.P. (1993) Metalloregulated expression of the ars operon. *Journal of Biological Chemistry* **268**, 52-58.

Yablonski, J.I. (1972) Fundamental concepts of preservation. *Bulletin of the Parenteral Drug Association* **26**, 220-227.

Yamada, T., Tipper, D. and Davies, J. (1968) Enzymatic inactivation of streptomycin by R-factor resistant *Escherichia coli*. *Nature (London)* **219**, 288-291.

Yamaguchi, A., Adachi, K., Akasaka, T., Ono, N. and Sawai, T. (1991) Metal-tetracycline/H$^+$ antiporter of *Escherichia coli* encoded by a transposon Tn10. Histidine 257 plays an essential role in H$^+$ translocation. *Journal of Biological Chemistry* **266**, 6045-6051.

Yamaguchi, A., Someya, Y. and Sawai, T. (1992) Metal-tetracycline/H$^+$ of *Escherichia coli* encoded by transposon Tn10. The role of a conserved sequence motif, GXXXXRXGRR, in a putative cytoplasmic loop between helices 2 and 3. *Journal of Biological Chemistry* **267**, 19155-19162.

Yamaguchi, A., Udagawa, T. and Sawai, T. (1990) Transport of divalent cations with tetracycline as mediated by the transposon Tn10-encoded tetracycline resistance protein. *Journal of Biological Chemistry* **265**, 4809-4813.

Zobell, C.E. (1943) The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology* **46**, 39-56.

Zobell, C.E. and Anderson, D.Q. (1936) Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces. *Biological Bulletin* **71**, 324-342.