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Antibacterial activity of low amperage and low voltage electric current (DC)

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Doctor of Philosophy

The University of Aston in Birmingham

February 1997

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THE UNIVERSITY OF ASTON IN BIRMINGHAM

Antibacterial activity of low amperage and low voltage electric current (DC)

Thesis submitted by Wai-Kin LIU for the degree of Doctor of Philosophy
1997

SUMMARY

Infection is a major clinical problem associated with the use of intravenous catheters. The efficacy of a direct electric current (10 μ A, 9V) via electro-conducting carbon impregnated catheters to prevent colonisation of catheters by micro-organisms was investigated. The range of organisms susceptible to 10 μ A was determined by a zone of inhibition test. The catheters acting as the anode and the cathode were inserted into a nutrient agar plate inoculated with a lawn of bacteria. There was no zone of inhibition observed around the anode. Organisms susceptible to 10 μ A at the cathode were *Staphylococcus aureus* (2 strains), *Staphylococcus epidermidis* (5 strains), *Escherichia coli* and *Klebsiella pneumoniae* (2 strains each), and one strain of the following micro-organisms: *Staphylococcus hominis*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Candida albicans*. The zones ranged from 6 to 16 mm in diameter according to the organisms under test. The zone size was proportional to the amperage (10 -100 μ A) and the number of organisms on the plate. Ten μ A did not prevent adhesion of staphylococci to the cathode nor did it affect their growth in nutrient broth. However, it was bactericidal to adherent bacteria on the cathodal catheter and significantly reduced the number of bacteria on the catheter after 4 to 24 h application of electricity. The mechanisms of the bactericidal activity associated with the cathode were investigated with *S. epidermidis* and *S. aureus*. The inhibition zone was greatly reduced in the presence of catalase. There was no zone around the cathode when the test was carried out under anaerobic conditions. Hydrogen peroxide was produced at the cathode surface under aerobic conditions but not in the absence of oxygen. A salt-bridge apparatus was used to demonstrate further that hydrogen peroxide was produced at the cathode, and chlorine at the anode. The antimicrobial activity of low amperage electric current under anaerobic conditions and in the absence of chloride ions against bacteria attached to the surface of a current carrying electrode was also investigated. Antibacterial activity was reduced under anaerobic conditions, which is compatible with the role of hydrogen peroxide as a primary bactericidal agent of electricity associated with the cathode. A reduction in chloride ions did not significantly reduce the antibacterial activity suggesting chlorine plays only a minor role in the bactericidal activity against organisms attached to anodal electrode surfaces. The bactericidal activity of electric current associated with the cathode and H₂O₂ was greatly reduced in the presence of 50 μ M to 0.5 mM magnesium ions in the test menstrem. Ten μ A applied via the catheters did not prevent the initial biofilm growth by the adherent bacteria but reduced the number of bacteria in the biofilm by 2 log order after 24 h. The results suggested that 10 μ A may prevent the colonisation of catheters by both the extra- and intra-luminal routes. The localised production of hydrogen peroxide and chlorine and the intrinsic activity due to electric current may offer a useful method for the eradication of bacteria from catheter surfaces.

Key Words: bactericidal activity, catheter related infections, cathode, hydrogen peroxide, biofilm

DEDICATION

I dedicated this thesis first and foremost, to my wife, Wai-Lan, who has and is always supportive of all my work and selflessly gave all herself to the family.

I also dedicated this thesis to my father who passed away last year and my mother. They both had little education but gave me all their lives. I know they would be proud of this work.

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ABBREVIATIONS

| | |
|-------|---|
| AC | alternating current |
| ATCC | American Type Culture Collection |
| CFU | colony forming units |
| CRI | catheter-related infection |
| CVC | central venous catheter/s |
| DC | direct current |
| DPD | <i>N,N</i> -diethyl- <i>p</i> -phenylene diamine |
| HPF | high power field |
| HVP | high voltage pulse |
| MBC | minimum bactericidal concentration |
| MIC | minimum inhibitory concentration |
| MRD | maximum recovery diluent |
| NCIMB | National Culture of Industrial and Medical Bacteria |
| OD | optical density |
| PBS | phosphate buffered saline |
| PVC | polyvinyl chloride |
| tRNA | transfer ribonucleic acids |
| rpm | revolution per minute |

PUBLICATIONS

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1. Introduction

Intravascular (IV) catheters are an essential component for the successful treatment of patients with chronic or critical illness, which provide dependable vascular access for the delivery of various types of infusions ranging from nutrition to medication. They are widely used throughout the world, and it is estimated that over 200,000 central venous catheters (CVC) are used annually in the United Kingdom (Elliott, 1993).

1.1 Different types of catheters

1.1.1 Peripheral intravenous catheters

Peripheral IV catheters are composed of plastic tubing which is inserted percutaneously into a peripheral vein. They are used to administer drugs and fluids to patients who require short term treatment. They are unsuitable for prolonged venous access, and it is recommended that they should be changed every 48 to 72 h (Maki, 1989). The requirements for total parenteral nutrition (TPN) and prolonged chemotherapy for cancer treatment involving irritant solutions has led to the development of central venous catheters (Decker & Edwards, 1988).

1.1.2 Central venous catheters

Central venous catheters (CVC) are made of a variety of plastics including polyethylene, polypropylene, polyvinyl, polyurethane, and teflon. They are percutaneously inserted into the jugular or subclavian vein and passed into the right side of the heart. These catheters are available as either single or multiple lumen with triple lumen being the most common in use.

In 1973, Broviac *et al.* (1973) reported the use of a silicone rubber catheter that was inserted into a central vein after being subcutaneously tunnelled. Various modifications of Broviac catheters, for example, Hickman catheters have now become the dominant route for long-term intravascular access. A further development has been the utilisation of a totally implantable venous access system. These include the Port-a-cath, which consists of a

subcutaneous reservoir with a self-sealing silicone septum (Neiderhuber *et al.*, 1982). The resultant system is entirely intracorporeal.

1.2 Complications associated with IV catheters

The extensive use of IV catheters is associated with various problems. The clinical complications can be divided into either early or late onset (Table 1-1). The early onset complications are predominantly linked to localised insertion site trauma including arterial or venous damage with haematoma formation and air embolism. Other complications include haemothorax, pneumothorax and subcutaneous emphysema. The complications associated with IV catheters which may develop at a later stage include thrombosis and infection (Elliott & Faroqui, 1992).

Table 1-1 Some of the clinical problems associated with intravascular catheters.
(Elliott & Faroqui, 1992)

| | Type of device | |
|-------------|------------------------|------------------------|
| | Peripheral | Central |
| Early onset | Haematoma | Haemothorax |
| | Arterial/venous damage | Arterial/venous damage |
| | Air embolism | Air embolism |
| | Localised pain | Pneumothorax |
| | | Subcutaneous emphysema |
| | | Pleural effusion |
| | | Cardiac perforation |
| Late onset | Infection | Infection |
| | Thrombosis | Thrombosis |
| | | Endocardial damage |

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1.3 Infections and intravascular devices

1.3.1 Incidence

Infection is the leading complication associated with IV catheters, and the severity ranges from local insertion site inflammation to life threatening catheter-related septicaemia.

In an international study of bacteraemia in 10,616 surgical patients, 63 % of the patients surveyed had an IV device inserted at some time during their hospital stay (Nyström *et al.*, 1983). The incidence of hospital acquired bacteraemia amongst patients without an IV device was 0.5 cases per 1000, 3.7 for patients with a peripheral IV device and 44.8 with a CVC. Ten percent of patients on IV therapy developed thrombophlebitis, and overall they suffered from bacteraemia more than four times as often as patients who did not receive it. These findings are similar to other reports (Maki *et al.*, 1973), demonstrating the strong association between the use of CVC and bacteraemia.

1.3.2 Infection rate with different types of catheters

The incidence of catheter-related sepsis varies with the type of device (Table 1-2). In a summary of 30 studies there was a four-fold reported range in the infection rate associated with different types of catheters (Hampton & Sherertz, 1988). Subclavian haemodialysis catheters had the highest rate of infection. The duration of the catheter *in situ* (Elliott, 1988), the amount of manipulation to the catheter such as haemodialysis (Hampton & Sherertz, 1988) and the number of lumina (Weightman *et al.*, 1988) are some of the factors that affect the infection rate.

With the introduction of Broviac catheters, CVC can be placed for a relatively long period. To take into account the duration of the catheter use, a more accurate description of infection rate is sepsis rate per days of catheter use. In a summary of 21 studies of 2020 patients (Decker & Edwards, 1988), with 2339 catheters, the sepsis rate was 1.75 infections per 1000 days of catheter use; which translated into 19.5 % of the catheters being related to sepsis at some time during clinical use.

Table 1-2 Infection rates with different types of catheters.

(Data modified from Hampton & Sherertz, 1988)

| Type of catheter | Number of catheters studied | Number of infected catheters ¹ |
|----------------------------|-----------------------------|---|
| Total parenteral nutrition | 1274 | 128 (10.0) ² |
| Subclavian haemodialysis | 314 | 64 (20.4) |
| Short plastic peripheral | 2008 | 83 (4.1) |
| Multilumen | 39 | 5 (12.8) |
| Pulmonary artery | 1110 | 40 (3.6) |
| Arterial | 1196 | 50 (4.2) |
| Central venous | 338 | 38 (11.2) |

1. Infected catheters as determined by quantitative culture
2. Number in parenthesis represents the percentage of catheters that were infected in the study

The totally implantable CVC have the lowest infection rate, ranging from 0 to 1.89 infections per 1000 days of catheter use (Decker & Edwards, 1988). A more recent review of reports on infection associated with long term CVC reported an even lower incidence of infection, as low as 0.0-0.18 infections per 1000 days of catheter use (Mayhall, 1992). In comparison, the rate of infection for Broviac-type catheters ranged from 0.17 to 1.4 infections per 1000 days of catheter use (Mayhall, 1992). This low infection rate associated with the totally implantable CVC is attributed to the fact that these catheters lie completely within body tissue, with no opening to the body surface. The reported infection rate is also related to age with paediatric patients having a six fold higher rate than adults (Decker & Edwards, 1988;

Mayhall, 1992). The difference in the infection rate related to age is partly due to the different frequency of the underlying diseases.

Despite the low infection rate of the totally implantable CVC, they are inappropriate for short term use. Short term CVC are used more extensively, especially in intensive care units. In the United Kingdom, approximately 200,000 CVC are used each year. In 1991, nearly 4000 bacteraemia associated with intravascular catheters were reported to the Communicable Disease Surveillance Centre, U.K. suggesting a bacteraemia rate of 2 % (Elliott, 1993). However, there was an increase of 39 % in the number of reports of catheter-associated bacteraemia from 1989 to 1991, which is indicative of the magnitude of the problem.

1.3.3 Clinical manifestations of infections

1.3.3.1 Local infections

Purulence around the catheter insertion site in the absence of a blood stream infection represents a local infection. Other symptoms include erythema, tenderness, increased warmth or induration. Inflammation is usually suggestive of infection but may represent a sterile mechanical condition (Raad & Bodey, 1992). Tunnel infection is characterised by a spreading cellulitis along the subcutaneous tunnel tract of tunnelled long-term catheters, such as Hickman and Broviac catheters. Pocket infection around the reservoir of totally implantable devices presents with local signs of inflammation, including erythema and necrosis of the skin (Mayhall 1992).

1.3.3.2 Systemic infection

While the catheter itself cannot be infected, it is clear that once it is colonised with organisms, it becomes the source of the organisms causing septicaemia. Septicaemia is the most serious of all the catheter-related infections. The clinical signs and symptoms are pyrexia (commonly low grade), leucocytosis and evidence of localised infection. The fever generally does not resolve despite the use of broad spectrum antibiotics (Elliott & Faroqui, 1992).

1.3.4 Micro-organisms responsible for catheter-related infections

Staphylococcus aureus and coagulase negative staphylococci (CNS) represent more than 50 % of the organisms responsible for catheter-related bacteraemia (Table 1-3). This pattern is similar for all types of catheters (Decker & Edwards, 1988; Hampton & Sherertz, 1988; and Clarke & Raffin 1990). The micro-organisms responsible for these infections are not restricted to staphylococci. The range of organisms is diverse and includes streptococci and enterococci, *Enterobacteriaceae*, *Pseudomonas* species, *Corynebacterium*, *Bacillus* species, *Mycobacterium fortuitum*, yeasts, and the fungi *Malassezia furfur*. Catheter related candida infections have, in particular, been associated with high rates of morbidity (Decker & Edwards, 1988).

Table 1-3 Organisms that are responsible for catheter-related bacteraemia, expressed as a percentage of total number of infections.

| Organisms | Types of catheters | | |
|---------------------------|------------------------|----------------------|--|
| | All types ¹ | Broviac ² | Broviac & totally implantable CVC ³ |
| CNS | 27 | 36 | 31 |
| <i>S. aureus</i> | 26 | 16 | 14 |
| Streptococci | 3 | 6 | 3 |
| Enterococci | 5 | 6 | 6 |
| <i>Enterobacteriaceae</i> | 18 | 14 | 14 |
| Other aerobic GNR | 4 | 13 | 10 |
| Yeasts | 17 | 5 | 7 |
| Others ⁴ | 0 | 4 | 15 |

1. Hampton & Sherertz, 1988

2. Decker & Edwards, 1988

3. Clarke & Raffin, 1990. Percentages added up to >100, because they included polymicrobial infection

4. These include *Bacillus* sp., *Corynebacterium* sp., *Mycobacterium fortuitum*, etc.

1.3.5 Emergence of coagulase negative staphylococci

The emergence of CNS as the most frequent cause of catheter-related bacteraemia is well illustrated in the reports from two British hospitals. In St. Thomas Hospital, London, CNS was responsible for only 10 % of catheter-related septicaemia between 1974-78, and the incidence increased to 30 % between 1979-83 (Eykyn, 1984). Between 1988-1990 in the Queen Elizabeth Hospital, Birmingham, CNS alone is responsible for over half of all the catheter-related bacteraemia (Elliott & Faroqui, 1992). Thus, CNS replaced *S. aureus* as the most frequently isolated organism in these infections.

Due to their ubiquitous nature and relative low virulence, in the past, CNS have been considered to be clinically insignificant contaminants when isolated from clinical specimens. However, in recent years, CNS have been increasingly recognised as important agents of nosocomial infections. Their role as significant pathogens in patients following ophthalmologic, neurologic and thoracic surgery, with compromised immune system, on continuous ambulatory peritoneal dialysis and with prosthetic devices (Pfaller & Herwaldt, 1988; Kloos & Bannerman, 1994) has been extensively reviewed.

The postulated reasons for the current prevalence and clinical importance of these organisms include their predominant number on the skin, their selection as a result of widespread usage of broad-spectrum antibiotics in the hospital (Eykyn, 1984), their ability to adhere to and form biofilms on the surfaces of vascular catheters and other medical devices (Costerton *et al.*, 1987), and their undemanding nutritional requirement (Pfaller & Herwaldt, 1988).

1.4 Pathogenesis

1.4.1 Source of infection

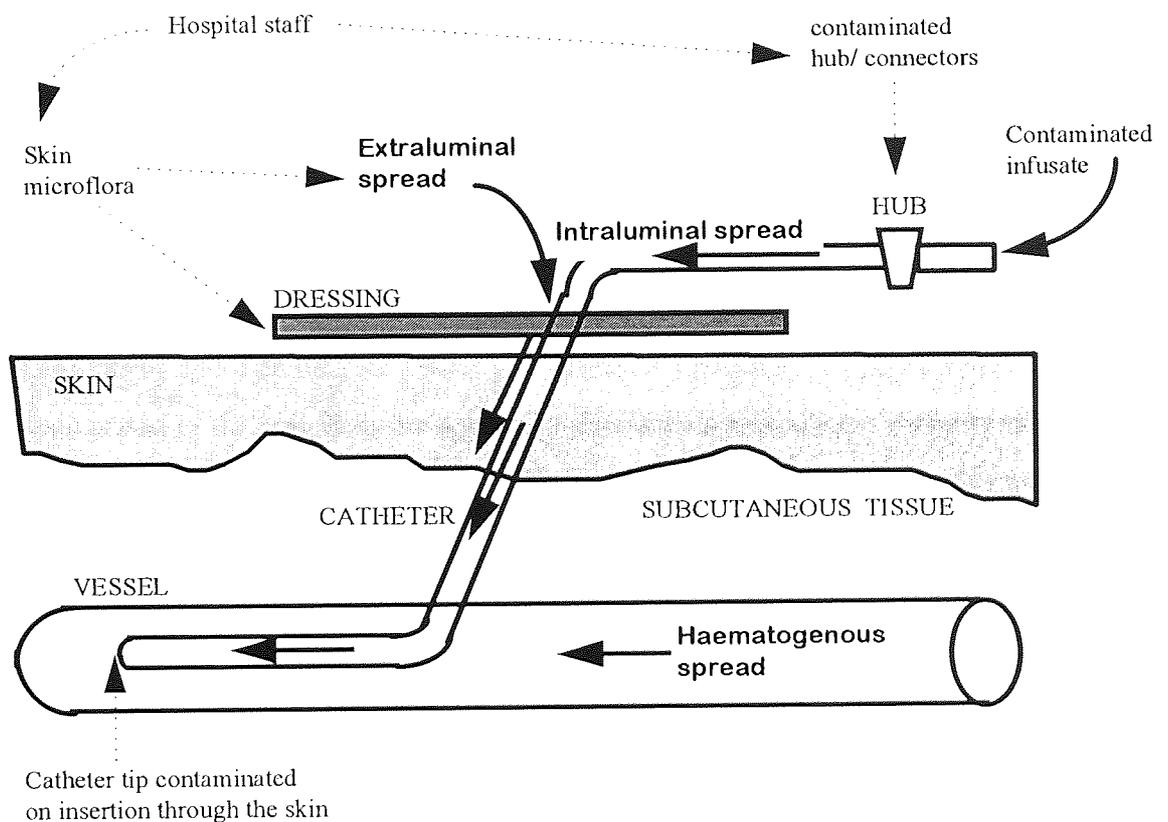
The most common source of organisms which cause catheter-related infections is the skin commensal. *S. epidermidis* are the predominant skin commensal which are associated with these infections. There is a strong correlation between high levels of colonisation at the skin insertion site (Egebo *et al.*, 1996), external catheter colonisation, and catheter-related sepsis (Bjornson *et al.*, 1982). The successful reduction of infection rates by lowering bacterial load supports this concept (Raad & Bodey, 1992). This included the use of antibiotic ointment around the insertion site (Hill *et al.*, 1990) and skin disinfection before catheter placement (Maki *et al.*, 1991). In contrast, factors that increase the bacterial load, such as occlusive dressings which promote bacterial growth due to moisture, resulted in higher rate of infections (Conly *et al.*, 1989; Hoffmann *et al.*, 1992).

Skin acts as a physical barrier that prevents the entry of micro-organisms into the body. Whilst the catheter is *in situ*, it breaches this protective barrier. There are differences in the degree of tissue disruption with the various CVC insertion techniques. For example, insertion of CVC by the cut-down procedure resulted in a higher level of skin and tissue disruption which has been shown to increase the risk of infection and is therefore no longer recommended (Moran *et al.*, 1965).

1.4.2 Routes of infection

Organisms gain access to the catheter via four major routes: extraluminal, intraluminal, haematogenous seeding or via contaminated infusates (Figure 1-1).

Figure 1-1 Source and routes of access of organisms to catheter.



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1.4.2.1 Extraluminal

Organisms from the patient's skin may be impacted onto the distal tip of the catheter surface during insertion (Elliott *et al.*, 1995). After the catheter is *in situ*, organisms may also migrate from the skin surface and along the interface of the catheter and the tissue, eventually

reaching the distal tip of the catheter (Maki, 1989). The migration of the organisms may be aided by capillary action between the tissue and the catheter surface (Cooper *et al.*, 1988). Mermel *et al.* (1991) studied the source and route of infection of 297 Swan-Ganz pulmonary artery (PA) catheters by culturing the skin, the introducer, the PA catheter tip, all hubs, infusates from each lumen and the extravascular portion of the PA catheter prospectively. Eighty percent of infected Swan-Ganz catheters showed concordance with organisms cultured from skin at the insertion site as demonstrated by the plasmid profile of causative *S. epidermidis*. They concluded that patient's skin is the single most important source of organisms of these infections.

1.4.2.2 Intraluminal

Organisms from the operator's skin may also gain access to the catheter via the hub. They are transferred to the hub during manipulation and then migrate or are flushed towards the distal tip of the catheter. Linares *et al.* (1985) showed that significant numbers of bacteria were isolated from the hub of 16 out of 20 infected catheters. They proposed that the contamination of the hub from the operator's skin flora is a significant pathway that leads to its colonisation. A more recent study of 113 Broviac catheters in neonates also supports this hypothesis. Fifty four percent of episodes of catheter related infection were preceded by or coincided with contamination of the hub (Salzman *et al.*, 1993).

1.4.2.3 Significance of extraluminal and intraluminal spread

Migration of organisms from the patient's skin along the catheter-tissue interface and hub contamination from the operator's hand are thought to be the two most significant pathways of pathogenesis of catheter related infection. In a study of CVC for parenteral nutrition (De Cicco *et al.*, 1989), CVC which were inserted by traditional subclavian catheterisation were found to be more commonly colonised around the intravascular segment (31 % of all catheters studied). Hub contamination was more common among the catheters that were tunnelled (42 % of all catheters studied). The organisms isolated from 6 out of the 9 colonised hubs were also isolated from the nurses who attended the patients. They therefore concluded that both routes namely intraluminal and extraluminal are equally important in the pathogenesis of catheter related infections.

Raad and Bodey (1992) have further demonstrated that both the internal and external surfaces of catheters that have been *in situ* for less than fifteen days were colonised to more or less the same extent. However, for the catheters that have been placed for a longer time (mean duration 109 days), the degree of colonisation and biofilm formation on the internal surface was more extensive than the external surface. They proposed that prolonged use of the CVC and hence, the increased manipulation of the catheter would result in a high degree of internal surface colonisation as compared to with organisms originating from the insertion skin site. It therefore, seems that both routes of infections are probably not exclusive to each other, and factors such as duration of catheterisation and the extent of manipulations will favour the spread of organisms by one particular route.

1.4.2.4 Haematogenous spread

The third possible route of infection is by haematogenous spread from a distant site. For example, urinary tract infections may produce a bacteraemia with subsequent seeding of organisms that leads to colonisation of a CVC. It has been suggested that many catheter related infections caused by yeasts (Bjornson *et al.*, 1982) and enteric organisms such as enterococci, *Escherichia coli* and *Klebsiella* are the results of haematogenous spread (Pettigrew *et al.*, 1985). In the study of 297 Swan-Ganz catheters by Mermel *et al.* (1991), 32 catheters were exposed to blood stream infections from other sites. Only two catheters were colonised with the same organisms. This route of infection is therefore thought to be uncommon.

1.4.2.5 Contamination of infusates

Contaminated infusate is another source of organisms causing catheter related infection. Contamination of infusate during manufacturing has resulted in epidemics (Maki *et al.*, 1976), although developments in manufacturing process means that this source of organisms is now rare. However, inappropriate handling of infusate in use may lead to contamination. The causative organisms are those that are able to flourish in the infusate, and include Gram negative bacilli, such as *Enterobacter*, *Pseudomonas*, *Citrobacter* and *Serratia* species. Parenteral nutrition solutions and lipid emulsion can promote the growth of many bacteria

and fungi such as *Candida parapsilosis* and *Malassezia furfur* (Goldmann *et al.*, 1973; Goldmann & Pier, 1993). Although many epidemics of nosocomial bacteraemia have been caused by contaminated infusate, the contribution of such a source to nosocomial primary bacteraemia is very low.

1.4.3 Mechanism of infection

1.4.3.1 Transport of organisms to the catheter surface

Microbial colonisation of catheter surfaces is thought to occur in the following sequence. The first stage is transport of organisms to the catheter surface. Organisms may be impacted onto the catheter surface during insertion (Elliott *et al.*, 1995). Dealler *et al.* (1988) measured the potential gradient across the human skin and found that the dermal tissue was electrically positive relative to the skin surface. They proposed that CNS being negatively charged would have been attracted into the skin catheter interface by the potential gradient. Organisms may also be directly transferred from the operator's skin to the components of the catheters such as the hub, during manipulation. These organisms could then pass to the catheter by direct spread.

1.4.3.2 Adhesion

The next stage in catheter colonisation is initial adhesion, which is mainly a physicochemical process. It may be reversible or irreversible. Van Loosdrecht *et al.* (1990) proposed that the process could be attributed to the colloidal chemistry theory. The total interaction Gibbs energy obtained from the summation of the van der Waals and the electrostatic interaction may attract organisms and facilitate adhesion. The van der Waals interaction is usually attractive and the electrostatic interaction between bacteria and catheter is usually repulsive due to the negative charges of both bacteria and catheter surfaces. When a bacterium is approximately 10 nm from the catheter surface, Van der Waals force effectively positions the bacterium near the surface. At closer ranges repulsion is at a maximum until the distance is 2-3 nm when the van der Waals force is at a maximum and attraction occurs. The magnitude of the van der Waals and electrostatic forces will be influenced by cell size, surface charge and surface hydrophobicity, the latter being reflection of surface composition and ionic

environment (Gilbert *et al.*, 1993). Hydrophobic forces are exerted at distances as great as 15 nm and at 8 to 10 nm are 10 to 100 times greater than the van der Waals force. Attractive hydrophobic interactions tend to overcome repulsion and position bacteria at the primary minimum. At this distance, cellular appendages such as fimbriae and pili effectively bridge the cell and the surface (Korber *et al.*, 1995). Moreover, accumulation of exopolymers produced by the cells can act as 'cement' and reinforce the adhesion.

Polymers are physicochemically active and their structure bears direct effect on bacterial adhesion. In general, they show the highest degree of adherence to polyvinyl chloride (PVC) and the least to polyurethane (Sheth *et al.*, 1983; Kristinnsson, 1989). However, there is strain to strain variation among the *S. epidermidis*. Moreover, some bacteria have a hydrophilic coating which may reduce bacterial adhesion (Kristinnsson, 1989). When the distance between the bacterium and the catheter surface is less than 1nm, chemical bonding may occur. Some interaction between the PVC catheter surface and the bacterial cell wall may also involve aminosugars (Franson *et al.*, 1984).

After a catheter is inserted into a blood vessel, a catheter may become coated by blood protein. Within 24 h of insertion, the catheter is usually coated with a fibrin sleeve (Hoshal *et al.*, 1971). Platelets can also be deposited onto the catheter surface. Organisms are then adhered to these contact-activated platelets via fibrinogen or fibrin (Herrmann *et al.*, 1993), though the adherence to fibrinogen is heterogenous among different strains of *S. epidermidis* (Wang *et al.*, 1993).

1.4.3.3 Extracellular polymer and its role in colonisation

After the initial attachment, firm adhesion takes place. On the catheter surface, *S. epidermidis* then multiply and produce an extracellular substance described as glycocalyx or slime. The nature of this slime substance has not been fully characterised, and it appears that both the quantity and components are dependent on growth conditions such as carbon dioxide level, and the type of available nutrients (Hussain *et al.*, 1992). However, it is well recognised that the slime layer plays a significant role in the colonisation of catheters by *S. epidermidis*.

Slime interferes with granulocyte functions which include reduction of organisms phagocytosis, increase degranulation of polymorphonuclear neutrophils and interference of chemotaxis (Johnson *et al.*, 1986). Slime also drastically reduces the proliferative response of mononuclear phagocytosis and reduces the cell viability (Gray *et al.*, 1984). In addition to interfering with the host immune system, it interferes with the activity of antimicrobial agents. Extracts of crude slime increase the MIC of *S. epidermidis* to vancomycin and teicoplanin and reverse the synergism between vancomycin and gentamicin (Farber *et al.*, 1990). Organisms with slime were also significantly resistant to phenolic antiseptics (Kolawole, 1985). Costerton *et al.* (1987) suggested that slime acts as an ion-exchange resin in binding charged antibiotic molecules and that it limits their penetration into the biofilm, protecting the innermost cells from the action of these agents.

Biofilm of micro-organisms therefore becomes a nidus for further colonisation of the catheter surface. The creation of a micro-environment that is favourable to growth, the resistance to the host defence mechanism and to the activity of antimicrobial agents all help to explain the difficulties in successfully treating these infections without the removal of the catheter.

1.5 Treatment

1.5.1 Local infection

Insertion site infection is the least serious complication related to catheters. They can be treated with antibiotics and insertion site care without removal of the catheter. Tunnel infections are often serious and are best managed by removing the CVC and administering intravenous antibiotics (Raad & Bodey, 1992).

1.5.2 Systemic infection

Catheter-related septicaemia with or without local site infection are usually treated by removal of the catheter and administration of antibiotics. However, there are recent reports of successful treatment with antibiotics alone, without removal of the catheter (Webster *et al.*, 1987; Benezra *et al.*, 1989). The antibiotics of choice are the glycopeptides, such as

vancomycin and teicoplanin (Elliott 1993). However, if the CVC is not removed, there is a 20 % chance that bacteraemia will recur, compared with only a 3 % risk of recurrence if the CVC is removed (Raad & Bodey 1992).

1.6 Prevention

A summary of the approaches to the prevention of catheter-related infections is in Table 1-4 & 1-5. The strict aseptic technique in catheterisation and catheter care by an expert infusion therapy team of workers are considered to be the most important in the prevention of infections (Johnson & Oppenheim, 1992; Elliott, 1993). However, it is clear that the mere presence of foreign implant material itself predisposes it to infection (Ward *et al.*, 1992). Therefore, developments to modify the catheter so as to minimise colonisation is a logical approach to prevent catheter related infection.

Table 1-4 Prevention of catheter-related sepsis: catheter design.

| Approach | Effect |
|---|--|
| Smooth topography | Discourage thrombus deposition, microbial adherence, and subsequent colonisation |
| Fashion tapered tip with bevelled edge needle | Reduces damage at the insertion site |
| Specialised coatings | Reduces ability of organisms to attach to surface |
| Antimicrobial polymers | Prevents microbial colonisation |
| Electrical charge | Prevents microbial colonisation |

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Table 1-5 Prevention of catheter-related sepsis: care of the insertion site.

| Approach | Effect |
|--|---|
| Skin preparation | Reduces the numbers of organisms present on the skin and minimises the possibility of contamination of the catheter |
| Use of scalpel | Facilitates insertion and reduces local damage |
| Application of antimicrobials (e.g. alcohol) around insertion site | Reduces the skin bacterial load |
| Application of antiseptics (e.g. alcohol) around the insertion site | Reduces the number of organisms |
| Use of an antimicrobial cuff around the insertion site | Prevents organisms migrating down the external surface of the catheter |
| Use of non-occlusive dressings at the insertion site | Reduces the collection of moisture and microbial multiplication |
| Regular application of antiseptics to the catheter hub | Reduces hub contamination |

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1.6.1 Modification of polymer surface

By radiation or glow discharge treatment of polymeric materials new functional chemical groups are introduced into the polymer surface (Jansen & Peters, 1991). This modification of the polymeric surface leads to altered physicochemical surface properties and thus to altered interactions with proteins and cells. By radiation modification of polyurethane with the hydrophilic substance 2-hydroethylmethacrylate, the polyurethane surface is rendered hydrophilic. Adhesion of several *S. epidermidis* strains to such a hydrophilic surface is

strongly reduced in comparison with the original more hydrophobic polyurethane surface (Jansen & Peters, 1991).

1.6.2 Incorporation of antimicrobials into catheter

Antimicrobial substances may be superficially bound to polymer surfaces or they can be incorporated into the polymer. If such devices are brought into contact with an aqueous solution, a gradual release of the drug, and thus a high local concentration is observed (Jansen & Peters, 1991). This results in inhibition of colonisation of micro-organisms and a rapid elimination of already adherent organisms. A summary of antimicrobial substances that have been incorporated into polymer aiming at the prevention of colonisation of catheter is in Table 1-6.

Of all these catheters with antimicrobial substances, only the chlorhexidine-silver sulphadiazine (Maki *et al.*, 1991) and the cefazolin bonded catheters (Kamal *et al.*, 1991) have reached clinical trials. All the others were investigated either by in-vitro experiments or animal model. However, Mermel *et al.* (1993) in reviewing clinical reports of uncontrolled studies, they noted that the infection rate of Swan-Ganz pulmonary artery catheter which has benzalkonium chloride as the bonding agent, showed a three-fold lower infection rate than those catheters without benzalkonium chloride. They attributed the low infection rate associated with these catheter to the activity of benzalkonium chloride, since it is known that heparin itself has no antibacterial activity. This indirect evidence suggests that the novel benzalkonium chloride catheter has a great promise in the prevention of catheter-related infections (Tebbs & Elliott, 1993).

Table 1-6 Incorporation of antimicrobial substances into catheter material for the prevention of catheter colonisation.

| Antiseptic | Catheter material | Reference |
|--|-----------------------------|-------------------------------|
| Chlorhexidine-silver sulphadiazine | polyurethane | Maki <i>et al.</i> , 1991 |
| Irgasan | polyurethane, polypropylene | Kingston <i>et al.</i> , 1992 |
| Iodine | Hydrocath ^R | Jansen <i>et al.</i> , 1992 |
| Benzalkonium chloride | Hydromer ^R | Tebbs & Elliott, 1993 |
| Chlorhexidine | not stated | Sherertz <i>et al.</i> , 1993 |
| Antibiotics | Catheter material | Reference |
| Penicillin | polyethylene | Trooskin <i>et al.</i> , 1985 |
| Dicloxacillin | polyurethane | Sherertz <i>et al.</i> , 1989 |
| Clindamicin, flucloxacillin | not stated | Jansen <i>et al.</i> , 1989 |
| Cefazolin | not stated | Kamal <i>et al.</i> , 1991 |
| Teicoplanin | Hydrocath ^R | Jansen <i>et al.</i> , 1992 |
| Dicloxacillin, clindamicin, fusidic acid, ciprofloxacin, cefuroxime, cefotaxime | polyurethane | Sherertz <i>et al.</i> , 1993 |
| Minocycline & rifampin | polyurethane | Raad <i>et al.</i> , 1996 |

Note: Hydromer^R and Hydrocath^R: both are polyurethane with hydrophilic coating

Catheters incorporated with antiseptics are preferable to antibiotics because the use of antibiotics will increase the selective pressure of resistant organisms (Goldmann, 1993). Moreover, multiple antibiotic resistant strains of *S. epidermidis* are not uncommon in the hospital environment, and thus catheters with antibiotics may not be able to prevent the colonisation of such organisms.

1.6.3 Electrical charge

Among the various attempts to prevent the colonisation of catheters, Elliott *et al.* (1990) reported a novel approach. They applied electric current (DC) to a carbon impregnated catheter made of polyurethane and connected the catheter to the negative terminal. The resultant negatively charged catheter was able to repel micro-organisms. As the initial stage of bacterial adhesion to the catheter is dependent on charge, this seems to be a logical approach to the problem. Further work showed that a negatively charged catheter was also capable of preventing both internal and external migration of micro-organisms along the catheter surfaces (Crocker *et al.*, 1992). These findings suggested that a negatively charged catheter is able to disrupt the important initial stages of colonisation of catheters.

1.7 Electric current and microbes

Electricity has various effects on bacteria. Electricity was reported to be antibacterial as early as 1919 (Anderson & Findelstein, 1919). Since then electricity in different forms such as high voltage pulses (Gilliland & Speck, 1967a), low frequency alternating current (Pareilleux & Sicard, 1970) and direct current (Sato *et al.*, 1989) have been applied to bacteria suspended in various menstrua and found to be bactericidal (Gilliland & Speck, 1967a; Pareilleux & Sicard, 1970). However, this bactericidal effect has not been confirmed in other reports. Rowley (1972) reported that alternating current (AC) has only a small effect on the growth rate of *E. coli* in Davis' medium. Other authors similarly reported that electric current *per se* had only minor effects on bacteria, but enhanced the action of antimicrobial agents (Blenkisopp *et al.*, 1992; Khoury *et al.*, 1992).

Direct effects which depend on the nature *per se* of the electrical charge have also been described. An anodic carbon fibres adsorbed bacteria in fluid and resulted in total removal of bacteria from a liquid suspension (Golub *et al.*, 1987), but a cathodic carbon-containing catheter repelled bacteria (Elliott *et al.*, 1990). The diversity of phenomena reported is partly due to the diversity of experimental procedures and apparatus.

1.7.1 Electric current and test system

Various methods have been used to apply electric fields and currents to fluids containing bacteria. When applied in a high voltage form, electricity was stored in one or more capacitors. The current was then discharged across a gap in which the bacteria were suspended. The discharge was in the form of pulses instead of continuous current (up to 30 microseconds per pulse) and up to 30 pulses. Voltage in the range of 5 to 20 kilovolt (kV) was achieved in these pulses. However, the application of high energy electric current required special equipment to control the form of energy discharged and to prevent arcing (Gilliland & Speck, 1967a).

It soon became apparent that low voltage electricity was easier and simpler to utilise than high voltage pulse electricity. In this case, the voltage is in the range of a few hundred millivolts and the amperage from several hundred milliampere (mA) to tens of microampere (μA) (Parielleux & Sicard, 1970). Power sources of the lower voltage electric current were also simpler; they were either a constant or variable current generator (Shimada & Shimahara, 1982; Davis *et al.*, 1989), or battery (Elliott *et al.*, 1990; Bolton *et al.*, 1980).

The electrodes were usually metal, such as titanium (Patermakakis & Foutoukidis, 1990), gold, silver, copper, nickel (Davis *et al.*, 1989), stainless steel (Stoner *et al.*, 1982) or platinum with 10% iridium (Rowley, 1972). Carbon or graphite in various forms including incorporation into polymers have also been utilised (Elliott *et al.*, 1990; Golub *et al.*, 1987; Matsunaga *et al.*, 1993). In the simplest form, electrodes were metal wires (Davis *et al.*, 1989) or flat plates (Patermakakis & Fountoukidis, 1990). Electric current has also been connected to a dialysis chamber (Golub *et al.*, 1989), carbon impregnated polyurethane extruded as intravenous catheters (Elliott *et al.*, 1990; Crocker *et al.*, 1992), or silver

impregnated fabric (Chu *et al.*, 1987). Corrosion limited the life span of metal electrodes (Davis *et al.*, 1991). The nature of the electrode also influenced the antibacterial activity. For example, cupric ions could be released from copper electrodes which are themselves bactericidal (Gilliland & Speck, 1967). Even relatively inert metals such as platinum were inhibitory to cell growth (Mortensen & Bojsen, 1982). Similarly the bactericidal effect of silver was enhanced by weak DC electric current (Falcone & Spadaro 1986; Chu *et al.*, 1987). Electrodes made of carbon do not have the problem of corrosion nor enhancement of activity of metallic ions but were less bactericidal than metal when the current was applied (Davis *et al.*, 1991).

1.7.2 Attraction and repulsion effects of electric current

The adsorption of bacteria onto solid surfaces is driven partly by electrostatic attractive forces between the charged groups existing on the bacterial cell wall and on the adsorbent of opposite charge. Overall, the surface of bacteria has a negative charge. Golub *et al.* (1987) fitted fibrous carbon and graphite as filters. Carbon in fibrous form has both large pores (10-50 μm in diameter) which is a result of interstitial spaces and small pores existing in the fibres, and the surface area can be as high as 500-600 m^2/g . They connected fibrous carbon disc in anode-cathode pairs and arranged in series to form a multistage electro-filter through which a bacterial suspension passed in a flow-through manner. Without applying electric current to the series of carbon disc filters, over 95% of *E. coli* and *Salmonella typhimurium* were removed from the saline suspension. When 1.5V of electric current was applied to the series of the filters, the remaining 5% of bacterial population was removed from the suspension. The chlorine content of the effluent was below 0.1 ppm by the ortho-tolidine test. When the electric current was disconnected, the bacterial count of the effluent increased to the original level without the electric current. It was suggested that the total removal of bacteria from the suspension was due to the reversible adsorption of bacteria onto the anodic surface. However, they did not investigate the viability of bacteria after adsorption onto the carbon electrodes.

Similarly, Matsunaga *et al.* (1992) showed that *E. coli*, *S. aureus* and *B. subtilis* and *Saccharomyces cerevisiae* were adsorbed onto granular carbon without electric current.

They then applied + 0.7 V electric current to the granular carbon, which affected the surface anodic. After an hour exposure, the viable count of bacteria in the fluid decreased even further and those that adsorbed onto the granular carbon decreased to less than 1% of the original concentration (Matsunaga *et al.*, 1992).

While these authors aimed to remove bacteria from suspension by attraction of the cells onto an anodic surface, Elliott *et al.* (1990) investigated the repulsion of bacteria by negative charge from a cathodic surface. When 20 μ A of DC was applied to a carbon impregnated polyurethane catheter with it as the cathode, bacteria were repelled from the surface. They suggested that such a cathodic catheter would be able to prevent bacterial adhesion and colonisation of catheter. Crocker *et al.* (1992) further demonstrated that a cathodic catheter prevented bacterial migration along both the internal and external surfaces of a catheter to a wide range of bacteria including *P. mirabilis*, *S. aureus* and *P. aeruginosa*. Therefore, the cathodic catheter with a continuously driven electric current may provide continuous protection of catheters from colonisation by bacteria.

1.7.3 Effect of low voltage electric current on bacteria in nutritious media

Reports of the effects of electric current on bacteria tested in nutritious media have been varied. Rowley (1972) reported that 40 mA AC has no deleterious effect on the growth of *E. coli*. In contrast, Davis *et al.* (1990) showed a remarkable bactericidal effect on urinary tract pathogens with 400 mA DC, which is only one hundredth of what Rowley (1972) tested. Other effects included repulsion of bacteria (Elliott *et al.*, 1990).

Electric current (AC) up to 40 mA applied to bacteria suspended in 89 ml minimal Davis medium and incubated at 37 °C, did not affect the growth rate of *E. coli*. When the current was increased to 140 mA (DC), however, the generation time of *E. coli* was increased by 40%. The number of bacteria in the final stationary phase remained unchanged (Rowley, 1972). Therefore, it seemed that electric field did not inhibit bacterial growth.

Blenkinsopp *et al.* (1992) similarly reported that electricity alone did not inhibit colonisation of metal surface by bacteria. They passed M56 medium with *P. aeruginosa* in the medium

over the surfaces of steel studs in their continuous flow system and applied electric current to the steel studs. Application of 2.1 mA AC from time zero did not inhibit colonisation or biofilm formation of *P. aeruginosa* on the surface, but it reduced the population of an established (24 h) biofilm from 10^5 CFU/cm² to 10^4 CFU/cm² after 8 h of application of electric current. Khoury *et al.* (1992), with the same test system, showed that 15-400 mA AC did not inhibit colonisation or biofilm formation of the surfaces by *E. coli*, *S. epidermidis* and *C. albicans*. Moreover, the current had no effect on established biofilms. However, they demonstrated synergism between electric current and antimicrobials including biocides and antibiotics.

In contrast, Davis *et al.* (1989), reported bactericidal effects of electric current (DC) on micro-organisms growing in synthetic urine. When *E. coli*, *P. mirabilis*, *S. saprophyticus* and *C. albicans* in 10 ml of synthetic urine were exposed to electric current of 20 to 400 mA (DC), the viable counts were reduced from 10^9 CFU/ml to $<10^2$ CFU/ml after an 8 h exposure. The only exception was *K. pneumoniae* where the viable count was only reduced to 1×10^6 CFU/ml. However, *K. pneumoniae* required the addition of tryptone soya broth to the synthetic urine for growth which may affect the effectiveness of the electric current. The nature of the electrode also affected the result, with gold-gold as cathode-anode being the most effective and carbon-carbon the least in terms of time required to achieve reduction of bacterial population. The apparent better efficacy of the metal electrodes may be due to the enhanced release of metallic ions from electrolysis.

Instead of a bactericidal effect, Crocker *et al.* (1992) reported inhibition of bacterial growth in a zone of inhibition test. They seeded nutrient agar plates with *S. epidermidis* and *S. aureus*, and then applied two carbon impregnated catheters into each agar plate. Electric current of 10 to 50 μ A (DC) was then discharged via the carbon impregnated catheters for 16 hours with the plates incubated at 37 °C. After incubation, a circular zone of inhibition around the cathodal catheter was observed with a diameter 10 to 14 mm when tested with *S. epidermidis* and 8 mm with *S. aureus*, but no zone of inhibition was observed around the anode. They did not examine the presence and viability of the bacteria within the zone of inhibition, but suggested that the formation of the inhibition zone was due to the repulsion of bacteria by the negative charges on the surface of the cathode.

Bolton *et al.* (1980) reported a reverse effect: there was no bactericidal effect around the cathode but was around the anode. They inoculated different strains of *S. epidermidis* into 0.4 ml of peptone or sheep blood within a rayon fabric which was placed underneath a polyvinyl chloride disc impregnated with carbon (PVC-carbon). This PVC-carbon disc was laid on the skin of human subjects. When 100 μ A of DC was applied to the PVC-carbon discs as an anode for 24 h, the 10^6 CFU of *S. epidermidis* were reduced to <5 CFU. But, no bactericidal effect was observed when the disc was connected as the cathode. They suggested that the bactericidal effect is due to electrolysis of the constituents in the fluids.

The conflicting results of these reports are probably due to many factors. The volume of the test media in which the micro-organisms was suspended and then exposed to electric current varied enormously, ranging from 0.4 ml to 89 ml. Any toxic substances possibly produced as a result of electrolysis would reach high concentrations in the tests of the smaller volume. Moreover, if contact between the electrode surface and the organisms is significant in the bactericidal activity, then in the larger volume tests, the contact time between the micro-organisms and the electrodes would be very brief in comparison to the tests of smaller volume. The constituents of the test media in which the test were carried out may also neutralise any toxic substances produced, as cysteine and albumin were found to be protective against the bactericidal activity (Pareilleux & Sicard, 1970). When the microbes were exposed to the electric current (DC) in the near vicinity of the electrodes, there seemed to be a difference between the cathode and anode in the antibacterial effect. Bolton *et al.* (1980) reported bactericidal effect beneath the anodal carbon pad only, while Crocker *et al.* (1992) reported no inhibitory effect around the anode. These apparent conflicting results may be due to emphasis on different effects. Bolton *et al.*'s (1980) experiment found no bactericidal effect around the cathode, but the experiment would not be able to detect the repulsion of bacteria. Conversely, Crocker *et al.* (1992) did not investigate the presence or viability of bacteria within the inhibition zone. Lastly, the nature of the electrodes may affect the bactericidal activity.

1.7.4 Bactericidal effect of low voltage electric current

In the reports indicating that electric current alone is bactericidal, the main aim was to look for an alternative to water sterilisation by chlorination. Hence the test media have usually been non-nutritious fluids such as salt solutions (Stoner *et al.*, 1982), phosphate buffered solution (Shimada & Shimahara, 1982), sterile water (Matsunaga *et al.*, 1992) or sea water (Nakasono *et al.*, 1993). The volume of medium in which the bacteria were suspended in these experiments varied considerably, ranging from 0.75 ml (Pareilleux & Sicard, 1970) to 350 ml (Patermarakis & Fountoukidis, 1990). Most experiments were carried out in a chamber and bacteria were exposed to current for a wide range of period, from 10 s (Pareilleux & Sicard, 1972) to 5 h (Shimada & Shimahara, 1982). Others adopted a flow system and so the exposure time of bacteria to electric current is only brief, ranging from 4 to 10 s (Matsunaga *et al.*, 1992; Stoner *et al.*, 1982). The amperage of the electric current applied ranged from 10-600 mA, which is a few hundred times higher than the current applied in those reports with nutritious media as test media. Despite the diverse experimental conditions among these reports, results were similar. These included residual toxicity in the test media which have been treated with electricity, reduced bactericidal activity when the test were carried out in anaerobic conditions and the general agreement on electricity being a bactericidal agent. However, such diverse experimental conditions made it difficult to compare results to determine the optimal conditions of the bactericidal activity of the low voltage electricity.

1.7.4.1 Effect observed with alternating current

Pareilleux & Sicard (1970) placed *E. coli* in 0.75 ml of minimal salt solution and then exposed the bacteria to a current of 10 to 200 mA AC for 10 sec. Twenty five mA was shown to be the minimum effective current to have a bactericidal effect. The larger the current, the more effective was the bactericidal action. Interestingly, after exposure to electric current, the test medium retained toxicity to bacteria and any delay in subculturing resulted in a significantly reduced colony count of bacteria. Chloride ions in the medium were essential to the bactericidal action of the electric current, since its omission in the test medium did not reduce the colony count. However, albumin and cysteine offered protection to the bacteria. The remarkable bactericidal effect observed in terms of time required to sterilise the solution (10

s) is probably due to a combination of small volume of the test media and the large flow (200 mA) of electric current. The very small volume of the test fluid would mean that any toxic substances produced would be in relatively high concentration.

But the results from Patermarakis & Fountoukidis (1990) suggested that with a larger volume of test medium, a longer exposure time is all that is required to demonstrate bactericidal activity. They treated 350 ml of natural water contaminated with coliform and *Enterococcus faecalis* (*Ent. faecalis*) with a similar level of electric current (125-250 mA AC). A pair of titanium plates of area 25 cm² were placed in the sample at a distance of 4 cm. After exposure to electric current, the water was examined for the presence of coliforms and *Ent. faecalis* by standard membrane filtration technique. Sterilisation was achieved after a 15 min application of electric current. They also noted the residual toxicity of the treated water.

Electric current also had bactericidal activity in medium without chloride ions. Shimada & Shimahara (1982) examined the effect of 100-300 mA/cm² of electric current (AC) on 10⁵ CFU/ml of *E. coli* suspended in 8.5 ml of phosphate buffer solution. After a 5 h exposure, the viable count was reduced to 10² CFU/ml. Increasing the bacterial cell number also reduced the rate of killing. When oxygen was removed from the medium, the disinfection rate decreased, and this suggested that oxygen is also essential for the activity.

Stoner *et al.* (1982) examined the effect of a lower current (8.5 mA AC). They suspended *E. coli* (1 to 5 × 10⁵ CFU/ml) in 0.85 % aq. sodium chloride (NaCl) or sodium bromide (NaBr) and passed this bacterial suspension through a graphite epoxy chamber. The contact time of the bacteria with the electric current was only 4 sec. Sterilisation was observed when the current density was 7.5 mA/cm² with the corresponding voltage 5.5 V. The disinfection rate was optimal at pH 4 to 5 with no reduction in viable count occurring at pH 7. Removal of oxygen also reduced the efficiency of disinfection, which is similar to the reports of Shimada and Shimahara (1982). They also demonstrated that NaBr was more effective than NaCl, but suggested another electrolysis reaction. As the dissociation of HClO or HBrO is pH dependent, they suggested that HClO or HBrO was produced as a result of electrolysis.

1.7.4.2 Effect observed with direct current

Sato *et al.* (1989) connected 0.81 A/dm² of DC (14V) to an electro dialysis apparatus. The apparatus consisted of five chambers with an anion and cation exchange membrane. Various electrolyte solutions containing *E. coli* (10⁸ CFU/ml) were passed through the desalting chamber at a flow rate of 3 ml/min. The viable count decreased with increasing current. When the current was greater than 1.35 A/dm², the effluent became sterile after 7 min of application of electric current. The effectiveness of electric current was dependent on the constituents, namely chloride ions of the fluids in the dialysis chamber, and did not require direct contact of bacteria to the membrane.

In all the reports mentioned above, the micro-organisms were suspended in fluids and thus exposed to the field effect and any electrolysis products from the electric current. Matsunaga *et al.* (1985) however, studied the effect of low energy DC (up to 10 μ A) to *Sacc. cerevisiae*, *B. subtilis* and *E. coli*, that had already been attached to a graphite electrode by cyclic voltammogram. As the microbial cells had already attached to the electrode surface, their exposure to the electric current is continuous and direct electron exchange may occur. The respiratory activity of the microbial cells measured by oxygen electrode, decreased with a simultaneous decrease in viable counts. For the optimal bactericidal effect to take place, the voltage of the graphite electrode was + 0.7 V versus a standard reference electrode, and this rendered the graphite electrode anodic. This voltage was also investigated for the sterilisation of water containing 10² cells of *E. coli*, *S. aureus*, *B. subtilis* and *Sacc. cerevisiae*. After the microbial cells were allowed to adhere to granular activated carbon, +0.7 V of electric current was applied for 5 h to the granular carbon which became the anode. The viable count of all organisms was reduced to less than 1% of the original inoculum (Matsunaga *et al.*, 1992).

The granular carbon was subsequently modified to carbon cloth and adapted to a flow device. Water containing 10² CFU/ml of *E. coli* was sterilised by this technique (Matsunaga *et al.*, 1992). In contrast to the findings of Sato *et al.* (1989), no bactericidal effect was observed if the carbon cloth was separated from the water by a dialysis membrane, so they concluded that direct contact with the electrode surface is essential for the action. Addition of cysteine, catalase or albumin did not neutralise the bactericidal effect. A further application was the modification of the electrode to a carbon-chloroprene sheet (Nakasono *et al.*, 1993). After

adhesion of *Vibrio alginolyticus* to the chloroprene sheet, +1.2 V versus a standard reference electrode was applied to the carbon chloroprene for 20 min., and 67% of adsorbed cells was killed. In a one year field experiment (Nakasono *et al.*, 1993), a carbon chloroprene pipe was left in sea, which resulted in a reduction of invertebrate attached to the pipe with electric current applied. The electric potential applied may have reduced the adhesion and biofilm formation of marine bacteria and thereby reduced the subsequent colonisation by invertebrates.

Similarly, Bolton *et al.* (1980) investigated the effect of electric current (DC) on bacteria on intact skin. A polyvinyl chloride disc impregnated with carbon (PVC-carbon) of 10 cm² circle was used as the electrode and electricity was supplied by a 6 V or 9 V battery. Different strains of *S. epidermidis* (10⁶ CFU/ml) which have been isolated from the skin of the test human subjects, were inoculated into 0.4 ml of water. A piece of rayon fabric was then saturated with this bacterial suspension and laid on the skin of the human subject, and the PVC-carbon disc was placed on top of the rayon. Twenty five to 100 µA DC was then applied with the carbon disc as the anode. After application of electric current, viable count by scrub culture of the skin demonstrated bactericidal activity for the duration of 20 h. Effects were similar when the bacteria was suspended either in saline, peptone or sheep blood. However, with saline as the suspension medium, sterilisation was observed after 4 h of application of electric current, as compared to 24 h with peptone or sheep blood. No bactericidal effect was observed when the PVC-carbon disc was connected as the cathode. It was suggested that the bactericidal action was due to electrochemical reaction products under the anode as the killing effect was diminished by separating the skin from the electrode with a dialysis membrane, thus separating the organisms from the electrochemical products but not the electric current *per se*. This is in contrast to the report of Matsunaga *et al.* (1985) that the bacteria kept in close contact with the electrode for the whole duration of exposure was essential for the bactericidal activity. Although both reports stated anode was the active electrode, different mechanisms of the bactericidal activity were suggested.

1.7.5 Mechanism of the bactericidal action of electric current

Mechanisms of the bactericidal effects of electric current have not been fully established. A summary of experimental data in investigating the mechanism is in Table 1-7. Phenomena

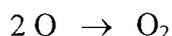
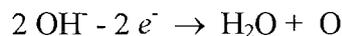
observed may be due to a combination of physical forces and chemical reactions. Toxic products from electrolysis have been investigated as the major bactericidal agent (Stoner *et al.*, 1982). Many ionic species produced during electrolysis are unstable, and may be difficult to detect their presence, but are implicated from the electrochemical equations. Different reports indicated different products as the major bactericidal agents, such as HOCl (Stoner *et al.*, 1982), OH⁻ and H⁺ (Bolton *et al.*, 1980), and H₂O₂ (Shimada & Shimahara, 1982). However, factors such as the constituents of the test medium, the amperage applied and the resultant voltage will influence which substances become more prominent in the bactericidal activity. The damage caused by electric current *per se* may be difficult to separate from the damage caused by the toxic products from electrolysis.

Table 1-7 Mechanism of low voltage electricity on microbe.

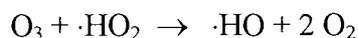
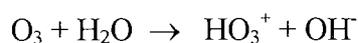
| Mechanisms investigated | Method of detection | AC/DC | Amperage | Reference |
|-----------------------------------|--|-------|------------------------|--------------------------------|
| HClO, HBrO production | O-tolidine | AC | 8.5 mA | Stoner <i>et al.</i> , 1982 |
| Chlorine based compounds | <i>N,N</i> -diethyl- <i>p</i> -phenylene diamine | DC | 400 μ A | Davis <i>et al.</i> , 1994 |
| Hydrogen peroxide production | colorimetric | AC | 300 mA/cm ² | Shimada & Shimahara, 1982 |
| Increased negative surface charge | flocculation of cationic molecules, electrophoretic mobility | AC | 600 mA/cm ² | Shimada & Shimahara, 1985a |
| Reduced respiratory rate | oxygen uptake | AC | 600 mA/cm ² | |
| Leakage of DNA-related material | O.D. at 260 nm | AC | 600 mA/cm ² | Shimada & Shimahara, 1985b |
| Leakage of cellular material | ninhydrin reaction | AC | 600 mA/cm ² | |
| Reduced respiratory rate | ion selective electrode | DC | 10 mA | Matsunaga <i>et al.</i> , 1985 |
| Dimerisation of enzyme CoA | cyclic voltammetry | DC | 10 mA | |
| Membrane disruption | electron microscopy | DC | 1 mA | Capella <i>et al.</i> , 1985 |

1.7.5.1 Indirect effect due to electrolysis

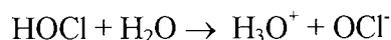
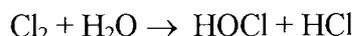
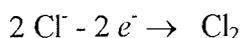
Many authors thought that the bactericidal action of electric current is due to the electrolysis of water and the chemical constituents in the test medium (Patermaralis & Fountoukids 1990; Stoner *et al.*, 1982). At the anode, the following reactions may occur:



As a result, active but unstable oxygen will be produced. In water containing some electrolytes, ozone may be produced at the anode, and probably participates in the following reactions:



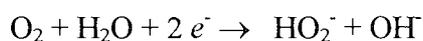
The free radicals $\cdot\text{HO}_2$ and $\cdot\text{HO}$ exert a strong disinfective action for a short time. The chloride ions, when present in the medium are oxidised at the anode which immediately reacts with water.



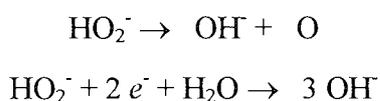
The Cl_2 , HOCl and OCl^- ions are powerful bactericidal agents and their half-life in the fluid medium will be much longer than the oxygen radicals. This will also result in their dispersal in the whole bulk of the medium. Pareilleus & Sicard (1970) reported that chloride in the medium was essential for the bactericidal activity provided indirect evidence of these reactions occurring. Later, Stoner *et al.* (1982) detected 0.2-0.3 ppm Cl_2 or Br_2 in the test media when 8.5 mA was applied. Therefore, it is conclusive that electrolysis products were

involved in the bactericidal activity at the mA level. The carbonate and sulphate ions, if present in the water could be oxidised at the anode to form percarbonate or persulphate which are strong oxidising agents.

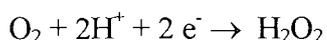
Lethal ion species can also be produced at the cathode by the reaction of molecular oxygen, which is transported by diffusion to the cathode with water. Hydroperoxide ions are produced according to the following reaction:



They are very active disinfective agents but unstable. These ions can decompose to form active oxygen or undergo reduction to give alkaline OH⁻ ions according to the following reaction:



Another strong oxidising agent, hydrogen peroxide may also be produced at the cathode:



When AC is applied, the production of these lethal ions will be alternating between the electrodes. All these reactions may not occur at the same time and which ones to take place will depend on the electrode potential of the reaction, the amount of electric energy applied and other competing reactions.

Shimada & Shimahara (1982, 1985a,b, 1987) have by far the most comprehensive experimental data on investigating the mechanisms of the bactericidal activity of low voltage AC. They showed that hydrogen peroxide is the major component of the bactericidal action of electric current. When 200 mA of AC was applied to 3 ml of phosphate buffered solution for 5 h, up to 0.5 mM hydrogen peroxide per hour was detected. The survival rate of *E. coli*

was inversely proportional to the amount of hydrogen peroxide produced. Addition of catalase to the solution and deoxygenation of the medium increased the survival rate of *E. coli*. The test medium in which *E. coli* were suspended contained no chloride ions and therefore, chlorine would not be produced. Further experiments demonstrated that the surface charge of *E. coli* after exposure to 600mA/sq cm AC became more negative, and the respiratory rate of *E. coli* as determined by oxygen uptake with manometric method decreased with increased exposure to AC (Shimada & Shimahara, 1985a). When the supernatant of the bacterial suspension which had been exposed to AC (600mA/cm²) was investigated by spectrophotometry, there was an increase in absorbance at 260 nm which indicated the presence of DNA-related substances and also substances reacted with ninhydrin which implied the presence of cytoplasmic constituents (Shimada & Shimahara, 1985b). Leakage of small molecules suggested an increase in the membrane permeability. It was concluded that a few hundred mA AC affected *E. coli* by increasing the membrane permeability and decreasing the respiratory rate, and the presence of an oxidising agent such as hydrogen peroxide eventually led to cell death.

1.7.5.2 Direct effect of electric current

Matsunaga *et al.* (1985) also investigated the effect of electric current on the respiratory activity of yeast cells. The respiratory rate of whole cells of *Sacc. cerevisiae*, *B. subtilis* and *E. coli* decreased to 25% of the initial activity after 7 min when the electrode potential was controlled at +0.74 V versus a standard reference electrode. The electron transport of the microbial cells was studied by cyclic voltammogram and the content of the functional enzyme CoA as determined by phospho-tranacetylene method was reduced from 45 nmol/10⁸ cells to 10 nmol. At that voltage, chlorine could not be detected in seawater nor was there a change of pH (Nakasano *et al.*, 1993). Cells died after 20 min exposure to the current but there was no change in cell morphology by scanning electron microscopy (Kitajima *et al.*, 1988). It was concluded that CoA existing in the cell was electrochemically oxidised to dimeric CoA and as a result, the respiration of the cells was inhibited, which eventually led to cell death.

1.7.5.3 Effect of electric current on micro-organisms as studied by microscopy

Studies by microscopy on the effect of electric current on micro-organisms have not been conclusive. There is no observable change in morphology of *E. coli* that had been exposed to 600 mA/cm² of AC for 5 h, as studied by light microscopy at ×1000 magnification. Similarly, the Gram stain reaction was not altered, though the bacteria took up the stain less readily (Shimada & Shimahara, 1985). Electron photomicrographs of cells exposed to electric current showed an electron dense area in the cytoplasm and was described as "nucleus-like" area and was thought to be DNA aggregation. However, there was no change to the morphology of the cell wall or the membrane (Shimada & Shimahara, 1985).

In contrast, Capella *et al.* (1991) reported morphological damage to the bacterial cell membrane with only 1/600th of the current and 1/300th of the exposure time of what Shimada and Shimahara reported (1985b). Cells of *E. coli* which have been exposed to 1.0 mA DC alone and resulted in 10 % survival, were examined under electron microscope by negative staining with phosphotungstic acid and uranyl acetate and thin section technique. There was a stronger penetration of the stain and zones of disrupted membranes as well as blisters in the membrane of the exposed cells compared to the control cells. They concluded that electric current alone induced modifications in the cellular membrane. However, they did not consider the possibility that the damage may be due to the toxic products of electrolysis.

With a much lower current, Kitajima *et al.*, (1988) examined cells of *Sacc. cerevisiae* that have been exposed to 10 µA DC for 20 min, and became non-viable. Not surprisingly, there was no visible damage to the cell wall of non-viable cells as examined by scanning electron microscopy.

1.7.6 Synergism between antimicrobial agents and electric current

Electricity enhanced the activity of both chemical and physical antimicrobial agents. Sparado *et al.* (1974) first reported the enhancement effect of 75 mA DC on the bactericidal activity of silver. The minimum inhibitory concentration (MIC) to electrically generated silver was 10 to 100 times lower than that for silver sulphadiazine (Berger *et al.*, 1976). Chu *et al.* (1987)

applied 0.4 to 400 mA DC to a silver impregnated suture line as an anode in a zone inhibition test against *S. aureus*, and seven strains of *Enterobacteriaceae*. A significantly larger zone of inhibition was observed around the anodal suture line than the silver suture line without the electric current. It was speculated that the enhancement is due to increased mobility of the silver ion.

Biocides and antibiotics also carry charges and will be affected by electric current. Khoury *et al.* (1992) and Blenkinsopp *et al.* (1992) investigated the effect of electric current on biofilm formation and MIC of the bacteria to antimicrobial agents in a biofilm. Electric current (2.1 mA AC) was applied to steel studs from time zero. A minimal salt growth medium, M56 medium containing *P. aeruginosa* was passed over the surface of the steel studs in a flow-through manner. Electric current alone did not inhibit colonisation of the surface of the stainless steel stud by *P. aeruginosa* (Blenkinsopp *et al.*, 1992), but reduced the number of bacteria in an established biofilm by 10 fold, and this lower level of bacterial population was maintained for 24 h. At 15 to 400 mA, colonisation or biofilm formation by *S. aureus*, *E. coli*, *Candida albicans* and *P. aeruginosa* were not prevented, nor the number of bacteria in the biofilm reduced (Khoury *et al.*, 1992). Bacteria in a mature biofilm are more resistant to antibiotics or biocides than bacteria in a suspension and the MIC can very often be 500 to 5000 times higher. When electric current as low as 15 mA/cm² was applied to a biofilm, the MIC of the antimicrobial agents required to sterilise the surface was only 0.1 to 5.0 MIC after a 12 h exposure. It was suggested that the electric current drive the charged molecules including antimicrobials through the exopolysaccharide matrix which is thought to be a physical barrier to antimicrobial agents, resulting in a lower MIC (Blenkinsopp *et al.*, 1992).

Electric current was also shown to be synergistic with ionising radiation (Capella *et al.*, 1991). Electric current of 1.0 mA DC which had very little effect on the viable count of *E. coli* suspended in 3 ml of nutrient medium, was shown to act synergistically with both electromagnetic radiation (X-ray) and charged particles (β -radiation). Electric current or X-ray (dose: 0.15 Gy/s) alone resulted in limited bactericidal effect, but when they were applied simultaneously, the bactericidal effect became more prominent. After an 8 min exposure, the viable count was reduced from 10⁸ to 10⁴ CFU/ml. Synergism was not observed when electricity was applied immediately after X-ray or *vice versa*. Of β -radiation emitted from

radionuclides and electricity, synergism was observed only with ^3H or ^{14}C but not ^{35}S , there was no synergism between electricity and UV light (254nm). The synergism observed is thought to be due to the production of charged particles as a result of radiation, and electric current directs the charged particles to the opposite polarity and so reduces the collisions and neutralisation, hence increasing the damage caused by the charged particles (Capella *et al.*, 1991).

Table 1-8 Synergism between electricity and other antimicrobial agents

| Agents | Electricity | Amperage | Reference |
|---|-------------|-------------------------------------|--|
| Silver compounds | DC | 75 μA | Spadaro <i>et al.</i> , 1974 Chu <i>et al.</i> , 1987 |
| Antibiotics: tobramycin ciprofloxacin cycloheximide | AC | 15-400 $\mu\text{A}/\text{cm}^2$ | Khoury <i>et al.</i> , 1992 |
| Biocides: isothiazalone dimethyl ammonium chloride glutaraldehyde | AC | 2.1 mA/cm^2 | Blenkinsopp <i>et al.</i> , 1992 |
| X-ray, β -particle irradiation (^3H , ^{14}C) | DC | 1.0 mA | Capella <i>et al.</i> , 1991 |

1.7.7 High voltage electric current

Instead of applying electrical energy to bacterial suspension as a continuous flow, another form of releasing the energy is high voltage pulses (HVP). High voltage (10-20 kV) electricity stored in one or more capacitors (1-6 mF each) is rapidly discharged as pulses across an electrode gap beneath the surface of fluid. The duration of the pulses ranges from ten to a few hundred micro-seconds, and 10-30 pulses have been applied (Hülshger & Neimann, 1980). Up to 40 kJoule of electric energy can be discharged into the fluid and heat will also be generated. The degree to which the temperature of the fluid is raised depends on the volume of fluid in which the bacteria were suspended.

1.7.7.1 Bactericidal effect of HVP

Gilliland & Speck (1967a) reported that HVP reduced the viable count of *E. coli*, *Ent. faecalis*, vegetative cells and spores of *B. subtilis* and bacteriophage specific for *S. cremoris*. Ten discharges resulted in the killing of more than 95 % of the organisms in a one litre suspension containing 10^{10} CFU of micro-organisms. The bacteriophage was the most susceptible micro-organism, and *Micrococcus radiodurans* and the spores of *B. subtilis* were the most resistant. The rate of bacterial death was proportional to the amount and the form of electrical energy discharged. Water previously exposed to high voltage current had residual toxicity and they attributed this to the release of copper ions from the copper electrodes since no such toxicity effect was observed when iron or aluminum was used as an electrode. Addition of protein into the water reduced the effectiveness of the treatment.

Further work from Hülshger *et al.* (1987) confirmed the findings of Gilliland & Speck (1967b) and showed that the relative rate of killed bacteria was mainly governed by the field strength and the treatment time which is the product of the number of pulses and decay constant. Below 2 kV, there was no bactericidal effect. Other parameters that influenced the action of HVP were electrolytes containing chloride ion which resulted in residual toxicity in the water, while phosphate and sulphate solutions did not. Moreover, magnesium and calcium ions in the medium reduced the susceptibility of treated cells. Increasing the starting temperature of treated suspensions leads to higher killing rates. Although the temperature of the suspension increases with each pulse as a result of applied electric energy, the temperature

rise was less than 10 °C. Furthermore, no effect from varying the pH value was observed in the range of pH 5 to 9. Cells from log growth phase were killed in significantly higher percentages than cells harvested from the stationary growth phase, and Gram-positive bacteria and yeast are less sensitive than Gram-negative bacteria.

1.7.7.2 Mechanism of high voltage pulse electric current

The bactericidal action of HVP is not due to the heat produced, despite the large quantity of energy released, as the temperature of the suspension did not increase by more than 0.5 °C (Gilliland & Speck, 1967a). The effect is throughout the suspension fluid even when the discharge takes place in a large volume, i.e. 1 litre. The principal action is probably due to the oxidation of compounds important in cellular metabolism (Gilliland & Speck, 1967b). Of the compounds investigated, HVP oxidised NADH₂, the free sulphhydryl groups from glutathione, and destroyed ATP but not DNA. However, if the suspension medium contains chloride ion, chlorine produced as a result of electrolysis becomes an additional bactericidal agent. Nucleotides such as ADP, AMP, NAD and NADH were released from bacteria and yeast cells after pulse treatment (Hülshager *et al.*, 1983), hence HVP altered the cell membrane permeability. This is in agreement with the observation that the presence of magnesium and calcium ions in the treated suspension increased the survival rate, since these bivalent ions are known to be essential in membrane function. Moreover, the success of bacterial genetic electrotransformation by HVP provided more indirect evidence.

1.7.7.3 Bacteria genetic transformation by electroporation

Another application of high voltage electric energy to the study of micro-organism concentrates not on its bactericidal action, but the phenomenon that it transiently increases the membrane permeability of bacterial cells. Though nucleotide and other co-enzymes are damaged by HVP, DNA molecules were unaffected (Gilliland & Speck, 1967b). To achieve genetic transformation, the bacterial cells are mixed with plasmid DNA and then exposed to high voltage electric discharge for a few micro seconds. During this short time, pores are formed in the membrane and through them cellular contents can leach out and the macromolecules such as DNA can enter. Within seconds, the pores of some cells reseal

spontaneously and viable cells can be recovered, while other cells experience irreversible damage and die. This method of transforming bacterial genetic component is rapidly gaining ground as a simple and efficient technique, as many microbes of interest are recalcitrant to the conventional techniques of calcium chloride treatment or enzymatic digestion of cell wall (Solioz & Bienz, 1990).

1.8 Aim of the study

Most studies of electricity as an antimicrobial agent had been focused on inanimate objects such as milk, water, plastic pipe and the amperage used was in tens or hundreds of mA. Such high amperage of electricity precludes its application on human bodies. The maximum current allowed in a human body is 15 μA (British Standard, BS 5724, 1989), as higher electric current will affect cardiac functions. The few studies with applications to clinical situations included bactericidal effect on micro-organisms in synthetic urine (Davis *et al.*, 1992) and on intact skin (Bolton *et al.*, 1980), the synergism with silver compounds against bacteria causing wound infections (Chu *et al.*, 1987), and the prevention of migration of bacteria from the catheter surfaces (Elliott *et al.*, 1990; Crocker *et al.*, 1992). A common feature of these studies is the low level of current ($<100 \mu\text{A}$) applied. Electricity DC of $<20 \mu\text{A}$ alone is able to repel bacteria and prevent migration along the catheter surfaces (Elliott *et al.*, 1990; Crocker *et al.*, 1992), and if there are other antibacterial activities then it will be a major advance in the prevention of catheter-related infections.

Therefore, the aims of this study are:

1. to investigate further the activity and the mechanisms of electrically charged catheters in the zone of inhibition test. This will provide a basis for investigation of its effect on bacterial adhesion and colonisation of catheter.
2. to study the effectiveness of an electrically charged catheter in the prevention of bacterial adhesion.
3. to examine the effect of electric current on bacteria that have already attached to a catheter surface.
4. to determine the mechanisms of any antibacterial activity observed.

2. Materials and Methods

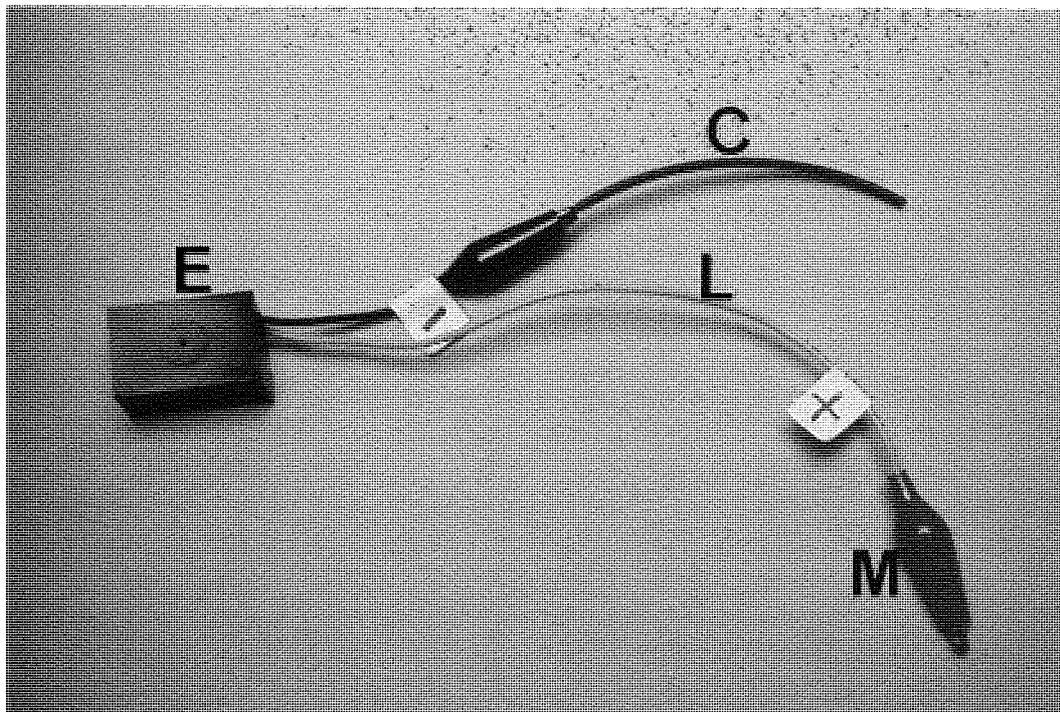
2.1 *Materials*

2.1.1 *Catheters*

Intravascular catheters made of 15 % carbon-impregnated polyurethane with an external diameter of 2.3 mm and which conducted electricity, were kindly supplied by Viggo-Spectramed (Swindon, UK). Initial batches were steam-treated at 100 °C for 30 min before use. Later batches of catheters could be sterilised by autoclaving at 121 °C for 15 min without altering the conductivity of the catheter and therefore were autoclaved before use.

2.1.2 *Electrical Device*

The electrical devices were constant direct current (DC) generators. Each was constructed with either a miniature 12V alkaline battery (Duracell MN21; Farnell components LS1 22TU), or a 9 V alkaline battery (PP9, Ever Ready, UK) in series with a 0.5 % high stability carbon film resistor (RS Components, NN17 9RS). By incorporating resistors of different ohmage, the device generated current from 2 to 100 μA (Crocker *et al.*, 1992). The resistor and the miniature battery were enclosed with a general-purpose epoxy resin in a plastic container of a dimension 28 mm \times 18 mm \times 14 mm and weighed 10 g (Figure 2-1). Flexible external electrical leads were used to attach the device to the catheters. All experiments were carried out with the electrical device generating 10 μA , unless otherwise specified. In all experiments, flow of electric current was confirmed by placing an ammeter in series in the circuit.

Figure 2-1 The electrical device with carbon catheters.

Key: Electrical device (E) was connected to two flexible external leads (L) which had metal clip (M) at the end and connected to a carbon catheter (C).

2.2 Media and chemicals

All chemicals were obtained from Sigma Chemical Co. Ltd, UK. Culture media and diluents such as maximum recovery diluent (MRD) and phosphate buffered saline (PBS, BR 149) were from Unipath Ltd (Basingstoke, UK) unless otherwise specified. Nutrient agar was prepared by adding Agar No.1, 1.5 % w/v (L11) to nutrient broth No.2 (CM67). In the investigation of the effect of electric current on bacteria growing on catheter surfaces in nutrient agar, triphenyl tetrazolium salt (BDH Chemical Ltd, Poole, UK) was added to the nutrient agar at a final concentration of 0.01 % . The salt is reduced by bacteria to form a red metabolite, formazan, which facilitated observation of bacterial growth (Cowan, 1977).

Dilution of bacterial suspensions were prepared in MRD. Culture, including enumeration, of bacteria was carried out on Columbia agar (CM331) supplemented with 7 % sterile defibrinated horse blood (Tissue Culture Service, Buckingham, UK). In the investigation of the bactericidal effect of electric current on bacteria attached to a catheter surface, the catheters were suspended in a modified Stuart's medium (Medium A) which maintains the viability of fastidious organisms for over 48 h (The Oxoid Manual, 1990). It consisted of sodium glycerophosphate 10.0 g/l, sodium thioglycolate 0.5 g/l, cysteine hydrochloride 0.5 g/l, and calcium chloride 0.1 g/l. Experiments to study the activity of electric current on bacteria suspended in fluid or attached to catheter surfaces were performed in the continuous culture broth described by Duguid *et al.* (1992) or its modifications as specified. The medium (Medium B) was a peptone-supplemented minimal-salts medium containing glycerol, 10 mM; (NH₄)₂SO₄, 6 mM; MgSO₄, 0.5 mM; KCl, 13.5 mM; KH₂PO₄, 28 mM; Na₂HPO₄, 72 mM; thiamine, 1 mg/l; biotin, 0.5 mg/l; peptone, 0.5 g/l, and the medium buffered to pH 7.4. All media were sterilised by autoclaving at 121 °C for 15 min.

2.3 Culture identification and storage

Thirteen strains of bacteria and one strain of *Candida albicans* were used in the investigation of the activity of electric current by the zone inhibition test. These included reference strains and fresh clinical isolates obtained from patients at the Queen Elizabeth Hospital, Birmingham, UK. Reference strains included *Staphylococcus aureus* NCIMB 6571, *S. aureus* ATCC 6538, *Staphylococcus epidermidis* NCIMB 12721, *Escherichia coli* NCIMB 8879, and *Klebsiella pneumoniae* ATCC 4352. Clinical isolates were *S. epidermidis* 023 and 462 (non-slime producers), *S. epidermidis* 811 and 983 (slime producers), one strain of *E. coli*, *K. pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *C. albicans*. All clinical isolates were identified by standard microbiological techniques and api STAPH, 20E, and 20 NE (bioMérieux sa, Marcy l'Etoile, France), and the slime production test was carried out according to the method of Christensen *et al.* (1982). In subsequent experiments, only the staphylococci were tested, as they are the most common bacteria associated with catheter-related infections.

Reference strains received in the freeze-dried form were initially cultured in nutrient broth. Then both the clinical and reference strains were plated out on blood agar. After overnight incubation at 37 °C, the culture was harvested and stored in beads in cryopreservative broth (Microbank™, Pro-Lab Diagnostics, Ontario, Canada) according to the manufacturer's instructions. The beads were then kept at -70 °C. Every month, a fresh bead was removed and plated out on to blood agar and incubated. It was then subcultured to nutrient agar slope. After overnight incubation at 37 °C, the slope was stored at 4 °C and subsequently subcultured for daily use. The slope was discarded after a month.

2.4 Epifluorescence microscopy: acridine orange stain to observe bacteria on the catheter surface in situ

Epifluorescence microscopy is particularly suitable for the examination of bacterial colonisation, both the extent of colonisation and enumeration of organisms, *in situ* on the external surface of catheter since the incident and fluorescent light come on the same direction. Catheters were fixed in 5 % formalin for 5 min and then immersed in freshly prepared 0.02 % acridine orange in Tris buffer (0.1 M, pH 7.6) for 2 min. After removing excess stain, the catheters were air dried and examined by epifluorescence microscopy (Yu & McFeters, 1994). A Zeiss Photomicroscope III (Carl Zeiss Ltd., Oberkochen, Germany) with an epifluorescence illumination system was used, which consisted of an exciter filter (BP450-490), a beam splitter (FT 510), a barrier filter (LP 520) and a 50 W mercury lamp.

2.5 Enumeration of bacteria in fluid suspension

2.5.1 Colony counts of bacteria in fluid suspension

The total number of viable bacteria in fluid suspension was determined by the Spiral System™ (Spiral System, Inc., Cincinnati, Ohio, USA) according to the instruction manual. The Spiral System™, using the Spiral Plater Model D enabled the viable count

of a bacterial suspension which contained 1×10^2 to 1.1×10^5 organisms/ml to be determined without any dilution. The coefficient of variation of the System is 15 %, which is comparable with conventional dilution methods (User's Manual, Spiral System™).

The Model D Spiral Plater is a specialised dispenser which distributes 50 μ l of the sample on the surface of a rotating 9 cm agar plate from the centre to the edge in an ever-decreasing amount. Every sample is deposited in the form of an Archimedes spiral in such a manner that the volume of any portion of the plate is known and always the same. After incubation, colonies appear on the lines of the spiral. The bacteria density is determined by counting the colonies on an appropriate countable portion of the plate and dividing this number by the volume of the sample contained in the areas counted. A 1 in 10^2 or 1 in 10^4 dilution of bacterial suspension was always prepared and processed if the viable count of the suspension was thought to possibly exceed the upper counting limit of the system.

The procedure in brief was as follows: a well mixed bacterial suspension was taken up via the stylus of the Spiral Plater Model D. A blood agar plate was placed on the turntable with the lid removed and then the stylus was lowered onto the surface of the agar. On starting the machine, the turntable rotated and the stylus moved across the agar plate centripedally and at the same time delivered a total of 50 μ l of the bacterial suspension. After the absorption of the fluid into the agar, the plate was incubated at 37 °C for 24 h, in air. To determine the viable count, a counting grid was placed over the agar plate and the number of colonies within the set segments was counted and the corresponding viable count was obtained from the conversion table provided with the manual.

2.5.2 Estimation of the number of bacteria in fluid by turbidity

A well mixed overnight broth culture of *S. epidermidis* NCIMB 12721 was serially diluted in nutrient broth. The turbidity of the dilution was determined by placing 0.2 ml

of the suspension in a well of a microtitre plate. The OD at 570 nm was measured with a microplate scanner, Multiskan[®] MK II (Labsystem Oy, Helsinki, Finland). The colony count of the dilution was determined by the Spiral System[™]. The test was done in triplicate and then the mean OD were plotted against the mean colony counts. The straight line graph thus obtained was used to estimate the number of bacteria in broth cultures. An appropriate dilution was then made to obtain the desired number of bacteria.

2.6 Colonisation of carbon catheters with bacteria

Apart from attraction by physicochemical forces to catheter surfaces, microorganisms may also impact onto the catheter surfaces during insertion via the skin (Elliott *et al.*, 1995). Once adhered to the catheter surface, bacteria may grow and eventually form microcolonies. It is, therefore essential to study the potential activity of electric current against bacteria that have already attached to the surface of catheters. To simulate this initial stage of colonisation, catheters were colonised with bacteria before the application of electric current. Sterile catheters were incubated in bacterial culture broth with continuous shaking or stirring to allow maximum contact between bacteria and the catheter surface. After colonisation, the catheters were then removed and rinsed to remove any loosely attached bacteria (section 2.7) and the number of bacteria adhered to the surface enumerated (section 2.8).

2.6.1 Colonisation of catheters in nutrient broth in a shaker-incubator

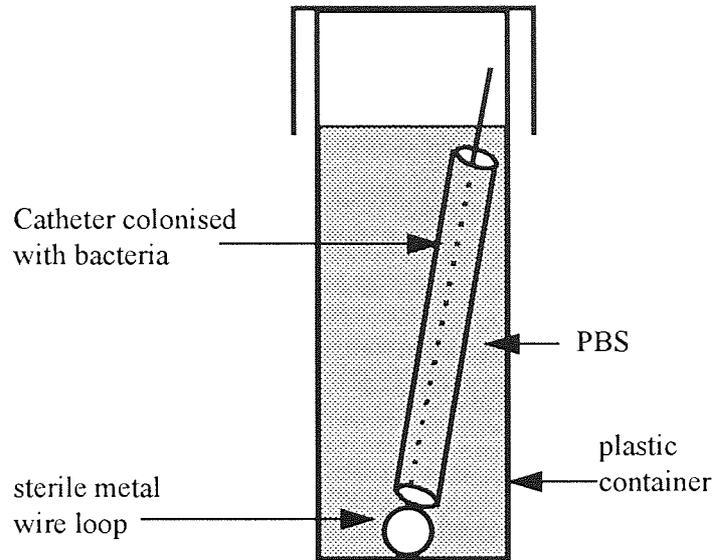
Sterile carbon catheters were placed in 100 ml of bacterial suspension which was prepared by diluting an overnight broth culture 200 times in fresh nutrient broth. They were then incubated at 37 °C for 2.5 h in a shaker-incubator (Unipath Ltd., Basingstoke, UK).

2.6.2 Colonisation of catheters in nutrient broth with continuous stirring

One colony of staphylococci was inoculated into 5 ml of nutrient broth and incubated at 37 °C in air for 2 to 3 h. The total number of bacteria in the suspension was estimated (section 2.5.2) and then an appropriate volume was added to 100 ml of fresh nutrient broth to give a final concentration of ca. 1×10^5 CFU/ml. Segments of catheters were then added to the broth and incubated at 37 °C for 2.5, 16, or 24 h with continuous stirring to allow the catheters to be colonised. After incubation, the catheters were removed from the suspensions and the loosely attached bacteria removed by one of the rinsing procedures (see section 2.7.2).

To determine the variance of this colonisation method, 10 segments of catheters were colonised for 2.5 h. A sterile metal wire loop was then threaded through the lumen of each catheter. The catheter on the loop was then placed in a plastic universal container containing 25 ml PBS, with the loop end at the bottom (Figure 2-2). The bottle with the catheter was centrifuged at 660 g for 5 min. After centrifugation, the catheters were removed and then cultured on blood agar by the roll plate method (section 2.8.1). After incubation of the agar plates at 37 °C for 24 h, the number of colonies were counted and the mean with the *SD* then calculated.

Another 10 segments of catheters were similarly colonised but for 24 h. After colonisation, each catheter was immersed in 30 ml of PBS. Each catheter was then removed and placed in 10 ml MRD. To remove the bacteria that were still attached to the catheter surface, the MRD with the catheter was sonicated at 11 W for 1.0 min (section 2.8.2). The viable count of the resulting bacterial suspensions was determined (section 2.5.2) and the mean number (± 1 *SD*) of bacteria colonised the catheter was calculated.

Figure 2-2 Colonised catheter in container for rinsing by centrifugation.

2.7 Rinsing of colonised catheters to remove loosely attached bacteria

During the incubation of the bacterial culture broth with the catheters, the number of bacteria in the broth also increased. When the catheters were removed from the broth at the end of the incubation period, a film of culture broth containing high number of bacteria would also adhere to the catheter due to the surface tension. Therefore, it was essential to rinse the loosely attached organisms from the catheter surface. Three different rinsing procedures and their efficiency in the removal of loosely attached bacteria were studied.

2.7.1 Effect of repeat rinsing to the number of bacteria attached to catheter surface

After colonisation for 24 h (section 2.6.2), one catheter was removed from the broth and then excess fluid was shaken off and placed in 10 ml MRD. The remaining catheters were rinsed by inversion. A colonised catheter was placed in a bottle containing 25 ml PBS. The bottle was then screw-capped and inverted upside down 10 times and the PBS discarded. For further rinsing, 25 ml fresh PBS was replaced after every 10 inversions. Colonised catheters were rinsed by inverting 10, 50 or 100 times. After the rinsing

procedures, each catheter was placed in 10 ml MRD and then all the catheters including the catheters which had not been rinsed were sonicated at 11 W for 1.5 min (section 2.8.2) to remove bacteria still attached to the catheters. The total number of bacteria in the suspensions was then determined using the Spiral System™ (section 2.5.1). The test was carried out in triplicate.

2.7.2 Comparison of three rinsing procedures to remove loosely attached bacteria

After colonisation for 24 h (section 2.6.2), the catheters were divided into three sets. The loosely attached bacteria to the surface of each set of catheters were removed by either one of the following rinsing procedures: immersion, centrifugation, and inversion. Rinsing by immersion was carried out by placing the colonised catheter in 30 ml PBS. The bottle was left standing without shaking or inversion for 5 s. The catheter was then removed and the excess fluid shaken off. For centrifugation, a sterile metal wire loop was threaded through the central lumen of each colonised catheter. The catheter on the loop was placed in a plastic universal container containing 25 ml PBS, with the loop end at the bottom so that the catheter would be above the base of the container (Figure 2-2). This was then centrifuged at 660 *g* for 5 min and then the catheter taken out without disturbing the deposit. The last set of catheters were rinsed by inversion 10 times (section 2.7.1). After rinsing, two catheters were stained by the acridine orange method and examined for the extent of colonisation by epifluorescence microscopy (section 2.4). Each of the remaining catheter was then placed in 10 ml MRD and then sonicated for 1.5 min with the power set at 11 W (section 2.8.2). The total number of viable bacteria of the suspension was then determined (section 2.5.1).

2.8 Enumeration of bacteria attached to catheter surface

In order to enumerate viable bacteria attached to the catheter surface, physical force is commonly used to remove them and then the number determined. The simplest method is the rolling of catheter tip on agar plate (Maki *et al.*, 1977). Imprints are formed on the agar surface during rolling and the bacteria detached from the surface. After incubation

of the agar plate, the number of colonies formed is counted. Bacteria were also removed from catheter surfaces by sonication in the diagnosis of catheter-related infections (Kristinsson *et al.*, 1989; Sheretz *et al.*, 1990). Khoury *et al.* (1992) scraped biofilm bacteria from a metal surface into diluent and then used a combination of vortex mixing and sonication to disperse organisms before serial dilution of the suspension was made and the viable count determined. Other methods include flushing of the internal lumen of catheter with culture broth and then determining the viable count of the organisms in the culture broth (Kristinsson *et al.*, 1989).

The efficacy of the roll plate culture method (Maki *et al.*, 1977), vortex mixing and sonication for the removal of bacteria from surface was studied. The aim was to investigate the efficiency of removal of bacteria from the catheter surface and if appropriate, the ability to disperse clumps and microcolonies of bacteria after detachment from the catheter surface.

2.8.1 Roll plate culture method and its efficiency in the removal of bacteria attached to the external surface

The number of viable bacteria on the external surface of the catheter was determined by a modification of the procedure described by Maki *et al.* (1977). A 2.5 cm length of catheter was gently rolled with slight pressure on part of the surface of a blood agar plate and at the same time pushed across the plate. It was then taken up and rolled on the uninoculated part of the agar plate as before and repeated once more. As a result, there were rectangular sections of rolled imprints on the agar surface. The plate was then incubated at 37 °C, in air, for 16-18 h and the number of colonies formed counted.

To determine the efficiency of this method to remove bacteria from the surface, 20 catheters were colonised for 2.5 h and rinsed by centrifugation (section 2.7.2). Half of the catheters were then cultured by the roll plate method on blood agar. Then all the catheters were stained by the acridine orange method and examined by epifluorescence

microscopy (section 2.4). The total number of bacteria on the whole length of catheter surface on one observation plane was counted.

2.8.2 Comparison of vortex mixing and sonication in the enumeration of bacteria in biofilm

Catheters were colonised for 16 h with *S. epidermidis* NCIMB 12721 (section 2.6.2). After colonisation, the catheters were removed and then rinsed by inversion for 100 times (section 2.7.1). After the final rinse, each catheter was placed in 10 ml MRD. The staphylococci which remained attached to the catheter were then removed by either vortex mixing or sonication. For vortex mixing, the universal container containing the catheter was placed on the vortex mixer (Miximatic, Jencons [Scientific] Ltd., Leighton Buzzard. UK) and mixed at full speed for 0.5, 1.5 or 2.5 min. For sonication, the colonised catheter was placed in a plastic universal container containing 10 ml PBS and then the sonicator probe (Ultrasonic Processor. Model GE50. Jencons [Scientific] Ltd., Leighton Buzzard. UK) was immersed in the MRD. The catheter was then sonicated with the power level set at 5 or 11 W for 0.5 to 2.5 min. The colony count of the resulting bacterial suspension was then determined (section 2.5.1).

2.8.3 Effect of sonication on the viability and dispersal of staphylococci in a suspension

To determine the effect of sonication on the viability and dispersal of bacteria, 0.1 ml of the nutrient broth culture in which the catheters had been colonised for 16 h was added to 10 ml of MRD and sonicated at 11 W for 0.5 to 2.5 min. Colony counts of the sonicated and non-sonicated bacterial suspensions in MRD were then determined by the Spiral System™ (section 2.5.1). Ten µl of each of the bacterial suspensions which had been sonicated was then placed on a glass slide. After drying, the slide was fixed by heat and then Gram stained. At least 5 fields of the stained films were examined under the microscope at × 1000 magnification. Each single cell, a pair or a clump of staphylococci

was counted as one unit. This was because each unit would grow as one colony after the bacterial suspension was plated out on agar and incubated. The percentages of staphylococcal units in singles, pairs and clumps were calculated.

2.8.4 Enumeration of bacteria in a 26 h old biofilm by sonication

Catheters were colonised with *S. epidermidis* NCIMB 12721 for 24 h (section 2.6.2) and then rinsed by immersion (section 2.7.2). Three catheters were stained by the acridine orange stain method and examined by epifluorescence microscopy (section 2.4). Each of the remaining catheters was then placed in 10 ml MRD and sonicated for 0.5 to 2.5 min with sonication power level set at 5 or 11 W. Colony counts of the sonicated bacterial suspensions were determined by the Spiral System™ (section 2.5.1). After sonication, the catheters were removed and stained by the acridine orange method (section 2.4) to examine the extent of the removal of biofilms from the catheter surface. Fifty µl of the resultant bacterial suspension obtained after sonication at 11 W for 0.5 min was also spread onto a glass slide. After air drying, the smear was also Gram stained and the clump size of the staphylococci were counted in 10 fields with × 1000 magnification.

3. Evaluation of basic methods

3.1 Estimation of the number of bacteria in fluid by turbidity

A well mixed overnight broth culture of *S. epidermidis* NCIMB 12721 was serially diluted with nutrient broth. The OD at 570 nm of the dilution was measured with the Multiskan® MK II (section 2.5.2) and the bacterial count was determined by the Spiral System™ (section 2.5.1). The OD was directly proportional to the colony count when the bacterial count was between 1.0×10^7 and 1.5×10^8 CFU/ml. This standard curve was used to estimate the number of bacteria in a 3 to 4 h broth culture before an appropriate volume was added to fresh broth to obtain the desired number of bacteria.

3.2 Colonisation of carbon catheters with bacteria

Sterile carbon catheters were placed in a nutrient broth culture of bacteria and placed in a shaker-incubator which was maintained at 37 °C (section 2.6.1). After a 2.5 h incubation in the shaker-incubator, the surfaces of the catheters were colonised evenly with bacteria. This method of colonisation was found to be adequate for the investigation of the effect of electric current on the growth of adherent bacteria embedded in nutrient agar described in section 6.

Another method of colonisation of catheters was the placing of the catheters in a broth culture of staphylococci in a 37 °C incubator and the broth continually stirred with a magnetic stirrer (section 2.6.2). This colonisation method was used for all the studies of quantification of the effect of electric current to bacteria adhered to catheter surfaces. After 2.5 h incubation, the organisms were in early log phase of growth and so this short period of colonisation was to simulate the initial stage of colonisation of catheters *in vivo*. Examination of the catheter surfaces by epifluorescence microscopy (section 2.4) showed bacterial cells were either single, pairs or clumps of four cells and so a biofilm had not yet formed. The mean (± 1 SD, $n = 10$) number of bacteria on the external surface of the catheter as determined by the roll plate method was 899 (± 235) CFU/cm

length of catheter and the *CV* was 26.1 %. Therefore, this colonisation procedure was reproducible and allowed a useful number of bacteria to be attached to the catheter surface, which was easily countable with the roll plate culture method (section 2.8.1).

This project also included a study of the activity of electric current against the formation of biofilm. Therefore, a colonisation period of 24 h was carried out to represent a relatively young biofilm and the parameters of rinsing and removal of bacteria from colonised surface could be investigated. After 24 h colonisation, the biofilm was removed and then dispersed by sonication. The mean (± 1 *SD*, $n = 8$) number of bacteria colonising both external and internal surfaces of a 2.5 cm length catheter, as determined by the Spiral System™ (section 2.5.1) was 9.3×10^4 ($\pm 3.4 \times 10^4$) CFU/cm length of catheter and the *CV* was 37 %.

3.3 Rinsing of colonised catheters to remove loosely attached bacteria

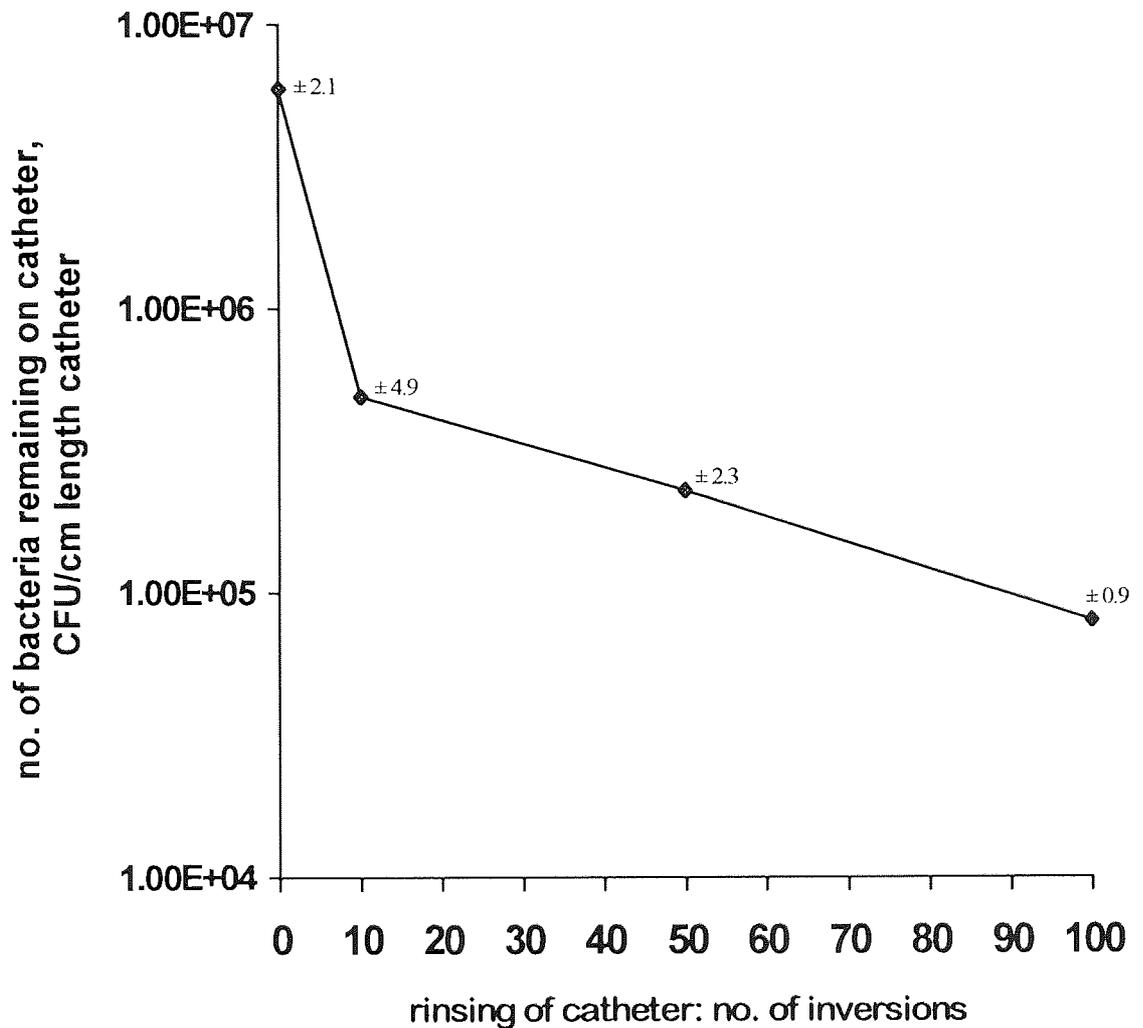
3.3.1 Effect of repeat rinsing to the number of bacteria attached to catheter surface

After colonisation in nutrient broth for 24 h with *S. epidermidis* NCIMB 12721, catheters were rinsed by inversion (section 2.7.1). Each catheter was placed in 25 ml PBS in a screwed capped bottle and then gently inverted once. The number of bacteria on the colonised catheters that had not been rinsed was higher than the catheters that had had a rinse of 10 inversions. This higher than expected colony count suggested that the number of bacteria on a colonised catheter without any rinsing consisted of bacteria attached to the catheter surface during colonisation and an additional number of bacteria. This additional number of bacteria was probably from the broth which contained a high cell density. When the catheters were removed from the bacterial culture broth, a film of the broth culture would adhere to the catheter surface due to surface tension. This population of bacteria was removed by rinsing. However, the number of bacteria remaining on the catheter surface decreased with repeat rinsing and was inversely proportional to number of inversion (Figure 3-1). By extrapolating the plotted line, the

number of bacteria actually attached to the catheter, but excluding the bacteria adhered due to the surface tension film was estimated to be 2.9×10^5 CFU/cm length of catheter.

As each rinse by inversion removed a proportion of bacteria attached to the catheter surface, there may be a range of firmness in bacterial attachment to the catheter rather than two populations of 'loosely' and 'firmly' attached bacteria. Instead, the number of bacteria remaining on the catheter surface after each rinsing procedure represented a proportion of the number of bacteria originally attached to the catheter surface. The rinsing procedure selected for all the experiments had to be followed in a consistent manner for a valid comparison of bacterial counts.

Figure 3-1 Effect of repeated rinsing on the number of *S. epidermidis* NCIMB 12721 remaining attached to a catheter surface following 24 h colonisation (mean \pm 1 SEM, $n = 3$).



Notes:

1. Attached bacteria were removed by sonication at 11 W for 1.5 min and then the colony count determined by the Spiral System™.
2. Number of bacteria remaining on the catheter was on a log scale and $1.00E+N = 1.0 \times 10^N$ CFU/cm length catheter.

3.3.2 Comparison of three rinsing procedures to remove loosely attached bacteria

After 24 h colonisation in nutrient broth containing *S. epidermidis* NCIMB 12721, catheters were rinsed by either one of the following procedures: immersion, centrifugation or inversion 10 times (section 2.7.2). Rinsing by immersion removed the least number of bacteria from the catheter, while 10 inversions removed the most (Table 3-1). Rinsing by centrifugation retained a higher number of bacteria than rinsing by inversion, despite a much greater force being used in centrifugation. Furthermore, it was found in later experiments (section 6.2.1), that the number of bacteria in the rinsing fluid was also the least when the catheter was rinsed by centrifugation. This was probably due to the concentration of bacteria from the rinsing fluid by centrifugal force. Therefore, centrifugation and immersion were the two rinsing methods chosen for the project.

Table 3-1 Comparison of three rinsing procedures on the number of bacteria remaining on the catheter surface.

| Rinsing procedures | Number of bacteria attached to catheter after rinsing, CFU/cm length catheter, (mean \pm 1 SEM, n =3) |
|---------------------------|---|
| None | $6.37 \times 10^6 \pm 2.36 \times 10^6$ |
| Immersion | $2.07 \times 10^6 \pm 0.37 \times 10^6$ |
| Centrifugation | $1.20 \times 10^6 \pm 0.48 \times 10^6$ |
| Inversion 10 times | $6.00 \times 10^5 \pm 1.50 \times 10^5$ |

3.4 Enumeration of bacteria adhered to catheter surface

3.4.1 Roll plate culture method and its efficiency in the removal of bacteria attached to the catheter surface

After the catheters were colonised with *S. epidermidis* NCIMB 12721 for 2.5 h and then rinsed by centrifugation, half of the catheters were cultured by the roll plate culture method (section 2.8.1). The surfaces of all the catheters were examined by epifluorescence microscopy. The staphylococci adhered to the catheters that had not been cultured by the roll plate method were mostly single and sometimes in pairs, but rarely in clumps of four cells. Hence, micro-colonies had not started to form. The mean count was 158 bacteria per cm length of catheter at one observational plane (1 *SEM* = 59.0, median = 62.8, range: 28 to > 500, *n* = 9). After the catheters were rolled on the agar plate (section 2.8.1), the mean count as observed by epifluorescence microscopy was 12 bacteria per cm length of catheter (1 *SEM* = 4.5, median = 9.2, range: 0 to 42, *n* = 9). Therefore, the roll plate culture method removed more than 90 % of the bacteria attached on the external surface of the catheter that had 2.5 h colonisation time.

The advantage of the method is its simplicity, since it required the least amount of preparation time. In contrast to vortex mixing or sonication, bacteria in the roll plate method were subjected to minimal physical force to detach them from the catheter surface. However, the roll plate culture method may not always separate cells in pairs or in clumps and thus underestimate the total bacterial count on the external surface. When the bacterial growth in the initial segment of roll prints was confluent, it became difficult to count individual bacterial colonies. Moreover, bacteria on the internal surface of the catheter could not be examined by the roll plate culture. Therefore, this method was unsuitable for enumeration of bacteria in a biofilm.

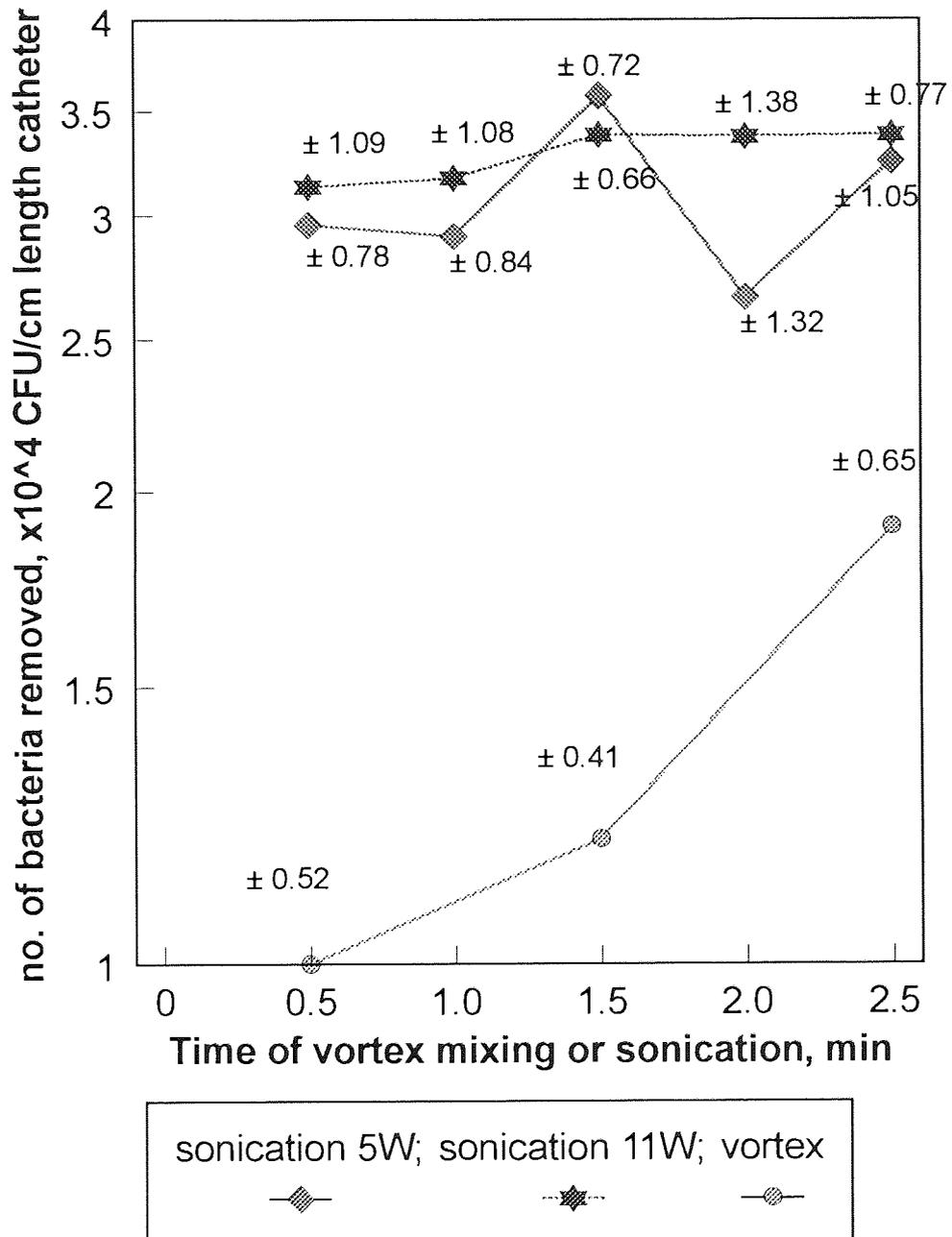
3.4.2 Comparison of vortex mixing and sonication in the enumeration of bacteria in biofilm

Catheters which had been colonised with *S. epidermidis* NCIMB 12721 for 16 h were rinsed by inverting 100 times. The extent of bacterial colonisation of these catheters after rinsing was studied by staining with acridine orange and examining by epifluorescence microscopy. After repeat rinsing (inversion 100 times) of catheters, bacteria that still adhered to the surface were either single or in pairs. Over the entire length of the catheter on one observational plane there were less than three micro-colonies that consisted of 25 or more cells. So, this comparative study of vortex mixing and sonication (section 2.8.2) in the removal of adherent bacteria from the catheter involved the single or bacteria in pairs rather than the micro-colonies attached to the catheter surface.

The bacteria attached to the catheters were removed by placing each catheter in 10 ml MRD and then they were either vortex mixed or sonicated. Both procedures removed the adherent bacteria from the catheter surface, but vortex mixing gave significantly lower mean colony counts than sonication at 5 or 11 W (Mann-Whitney U test, $p < 0.01$, $n = 4$). The mean colony count gradually increased with the time of vortex mixing (Figure 3-2). Vortex mixing for more than 2.5 min may further increase the viable count but the time required would be impracticable. The lower mean bacterial count after vortex mixing was probably due to its inability to remove all the organisms from the catheters or breaking up of paired cells. Therefore, it would be even more unlikely that it sufficiently removed and dispersed bacteria in biofilm.

The colony count of the bacterial suspension after the sonication of the catheter did not change significantly over time, though the general trend was an increase with time of sonication for the first 1.5 min. There was also no significant difference in the colony count whether the catheter was sonicated at 11 or 5 W for any of the duration except at 2.0 min. This was probably due to single or paired staphylococci being easily removed from the catheter surface by sonication. Therefore, sonication of colonised catheters at 5 or 11 W was investigated further.

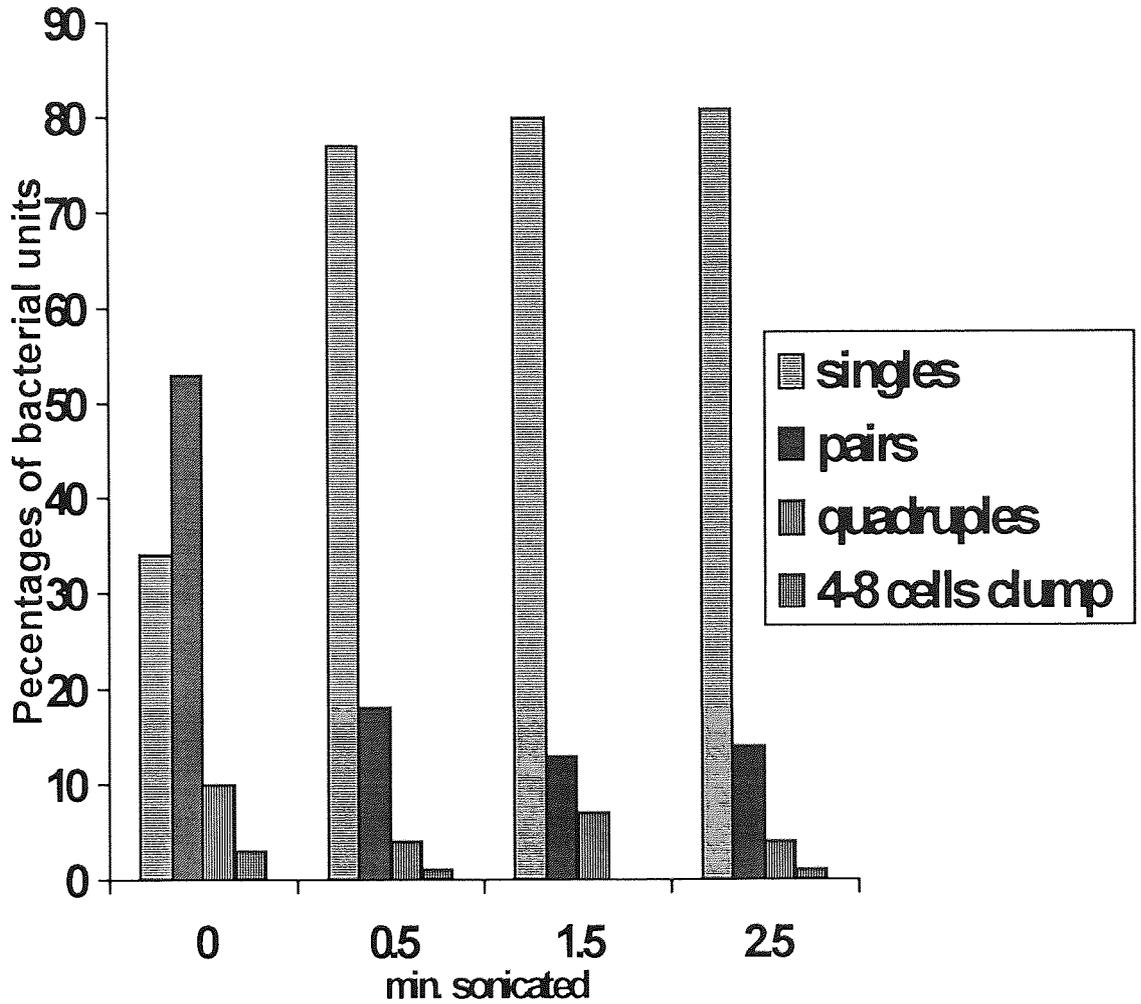
Figure 3-2 Comparison of vortex mixing and sonication in removing *S. epidermidis* NCIMB 12721 attached to catheters after 16 h colonisation (mean \pm 1 SEM, $n = 4$).



3.4.3 Effect of sonication to the colony count and clump size of a staphylococcal suspension

The effect of sonication on bacteria in the nutrient broth in which the catheters had been colonised for 16 h was investigated (section 2.8.3). Examination of the cells in the suspension may give some indications of the effect that sonication may have on the bacteria attached to the catheter surface. The mean colony count of the 16 h nutrient broth culture of *S. epidermidis* NCIMB 12721 decreased after sonication at 11 W for 0.5 min or more (Figure 3-3). However, this decrease in the colony count was not significantly different from the colony count of the broth culture that had not been sonicated (Mann Whitely U test, $n = 4$, 95 % CL). During sonication, clumps of bacteria were dispersed into smaller colony forming units which included single cells. A Gram stain of the nutrient broth culture before and after sonication showed a significant increase of single cells (χ^2 test, $d.f. = 1$, $p < 0.01$). In the broth culture which was not sonicated, 66 % of the bacterial units observed consisted of pairs or clumps of 4 to 8 cells (Figure 3-3). If they were all broken up to give individual bacteria, there would have been a 100 % increase in the colony count, assuming all the cells could do so. Instead, there was no significant changes in the colony count of the sonicated bacterial suspension. Thus, sonication appeared to affect the viability of bacterial cells depending on the power and duration of sonication. Hence, the increase of bacterial count as a result of breaking up of clumps and pairs of cells was offset by the decrease in viability of the cells and resulted in no significant changes in colony count. This became even more apparent when the biofilm bacteria was sonicated and is described in the following section.

Figure 3-3 Effect of sonication at 11 W on the colony count and clump size of bacteria of a 16 h nutrient broth culture of *S. epidermidis* NCIMB 12721.



| Time sonicated, min | 0.0 | 0.5 | 1.5 | 2.5 |
|--|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| Colony count, CFU/ml (mean ± 1 SEM, n = 4) | $6.5 \times 10^6 \pm 3.1 \times 10^6$ | $3.8 \times 10^6 \pm 1.1 \times 10^6$ | $4.0 \times 10^6 \pm 1.0 \times 10^6$ | $3.9 \times 10^6 \pm 0.3 \times 10^6$ |

3.4.4 Enumeration of biofilm bacteria after sonication

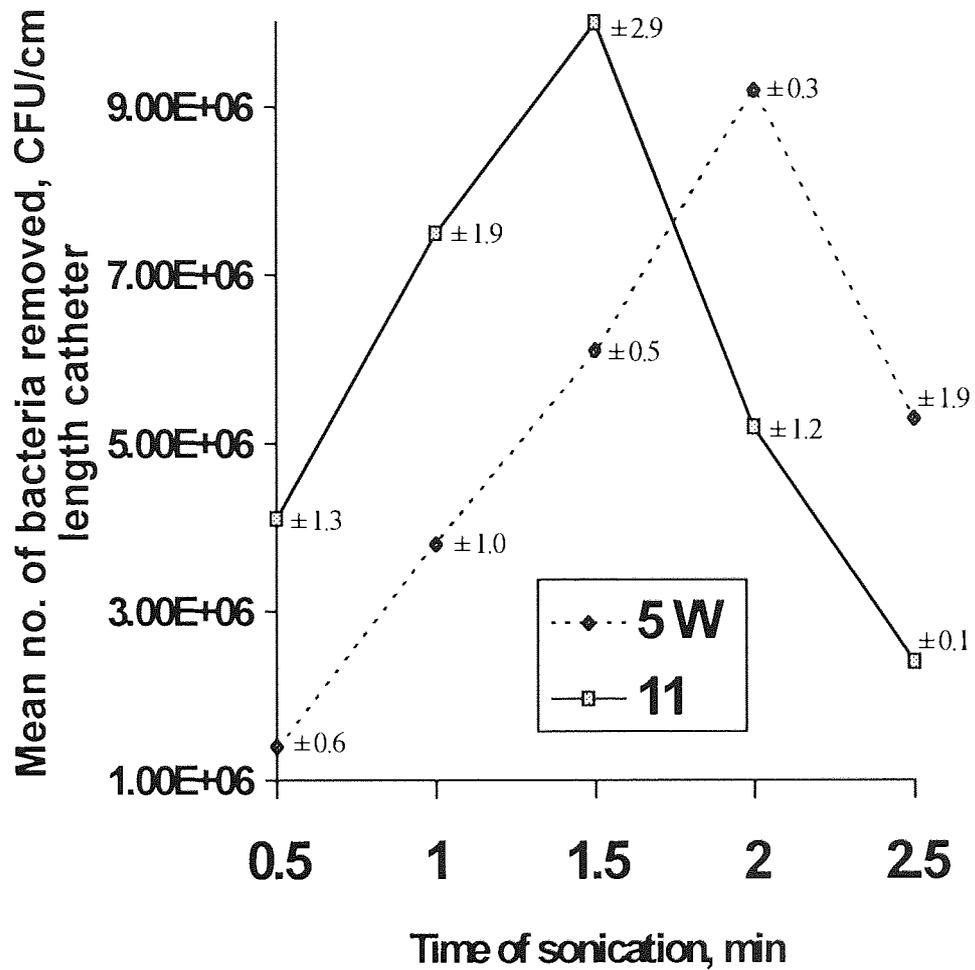
Catheters were colonised for 26 h and were then rinsed by immersion. Examination of the catheter surface by epifluorescence microscopy (section 2.4) showed that micro-colonies and a biofilm began to form. Over the whole length of catheter in one plane of observation, there was a mean of 169 bacteria per HPF (1 *SEM* = 28.9, *n* = 4) and a mean of 24 micro-colonies which consisted of 50 to 100 bacteria per colony.

The catheters that were colonised with *S. epidermidis* NCIMB 12721 for 26 h were rinsed by immersion. Each catheter was then placed in 10 ml MRD and sonicated to remove and disperse the bacteria in the biofilm (section 2.8.4). With the power level of the sonicator set at 5 or 11 W, there was a gradual increase of colony counts over sonication time and reached a maximum (Figure 3-4). After that time, the bacterial count started to decline. The optimal time for 11 W was 1.5 min, and 2.0 min for 5 W. The colony count obtained after optimal time of sonication at either 5 or 11 W did not differ significantly, 9.0 and 9.1×10^6 CFU/ml respectively. Examination by epifluorescence microscopy (section 2.4) of the catheters after sonication for 1.5 min at 5 or 11 W showed that the mean number of bacteria observed was 1.0 per HPF (*SEM* = ,median = 0.2, range: 0.06 to 5.5, *n* = 8) and no micro-colonies. Therefore, this gradual increase in colony count was due to the removal of biofilm from the catheter surface. Furthermore, Gram staining of the bacterial suspension that had been sonicated at 5 or 11 W for 0.5 min showed a mean of 86 % (*SEM* = 0.8%, *n* = 4) of the bacteria were single and a mean of 13.2 % (*SEM* = 0.8 %, *n* = 4) were in pairs with no clumps of four cells observed. Therefore, sonication also broke down clumps to single cells and the percentage of single bacteria was similar to the results in section 3.4.3 (Figure 3-2). Sonication removed bacterial micro-colonies from the catheter surfaces and then dispersed them mostly to single cells. To achieve this, a minimum level of ultrasound energy was required which was related to the power and the time of sonication applied. Hence, the gradual increase of colony count over sonication time. At the same time, sonication reduced the viability of bacteria and the longer the sonication time, the lower the colony count. The optimal time would then be the time that no more micro-colonies were left and most of the clumps of bacteria had been broken up. Any further sonication

would then reduce the colony count. The viability of bacteria that had been sonicated may also be affected by factors such as age and growth conditions of the culture.

Since sonication adversely affected the viability of bacterial cells, it presents a dilemma to the researcher. Physical force was needed to remove bacteria from attached surface and then disperse the clumps of cells in order to enumerate the bacteria in a biofilm. Vortex mixing was not sufficient to remove all micro-colonies and disperse the clumps. The shape of a catheter further complicated the removal of biofilm as the curvature hinders a thorough removal of micro-colonies by scraping. Therefore, sonication at 11 W for 1.5 min was chosen as the compromise procedure to enumerate the number of bacteria in biofilm.

Figure 3-4 Comparison of sonication at 5 & 11 W on the removal of bacteria attached to the surface of catheters which had been colonised for 26 h with *S. epidermidis* NCIMB 12721 (mean \pm 1 SEM, $n = 3$).



Note: Mean no. of CFU/cm length catheter was on arithmetic scale and shown as $NE+06 = N \times 10^6$.

4. Activity of low voltage (DC) electric current: investigation by zone of inhibition test

4.1 Introduction

Most previous reports on the bactericidal activity of electricity have involved current of several hundred mA (Pareilleux & Sicard, 1970; Patermarakis & Fountoukidis, 1990; Shimada & Shimahara, 1982). This is too high an amperage for the prevention of infection associated with CVC as a current in excess of 20 μA passed via a catheter into right side of the heart will cause heart fibrillation (Brown & Smallwood, 1991). Therefore in the present investigation on the application of electricity for the prevention of catheter-associated infection, the amperage has been limited to less than 20 μA that is based on the safety guideline of medical electrical equipment (British Standard BS 5724, 1989).

At 6V, 5 $\mu\text{A}/\text{cm}^2$ of DC applied for 20 h is bactericidal to *S. epidermidis* on the human skin (Bolton *et al.*, 1980). The electricity was applied via a carbon-PVC disc with bacteria in close contact. However, bactericidal activity was observed only when the disc was attached as the anode. Bolton *et al.* (1980) suggested that the activity was due to electrochemical products below the electrode. Similarly, a carbon electrode with 8 μA DC at 0.8 V killed *Sacc. cerevisiae* and bacteria attached to its surface when it was anodic. (Matsunaga *et al.*, 1985). The bactericidal activity was due to the oxidation of intracellular co-enzyme A which led to decrease in all activity and eventually death (Matsunaga & Namba, 1984). This activity was observed only when the bacterial cells were attached to the electrode surface.

However, DC as low as 10 μA also killed bacteria in suspension. When 10 μA DC was applied to 10^4 CFU/ml of *E. coli* or *P. mirabilis* in 10 ml synthetic urine for 24 h, the colony counts were reduced below detectable level (<10 CFU/ml). The time required for this reduction decreased to 4 h when the amperage was increased to 400 μA (Davis *et al.*, 1989). This bactericidal activity was also dependent on the material of the electrode, with carbon the least effective when compared to metals. Moreover, electric current (DC) was applied with

both electrodes in the fluid of the same container, therefore the activity associated with the anode or cathode could not be distinguished.

At 9.0 V, 10 μ A DC repelled *S. epidermidis* from a cathodal surface when they were suspended in saline or blood within 20 min of application of electricity. Furthermore, 10 μ A effects the formation of an inhibition zone to *S. epidermidis* seeded on nutrient agar, and the prevention of migration of bacteria along both the external and internal surface of a catheter (Crocker *et al.*, 1992).

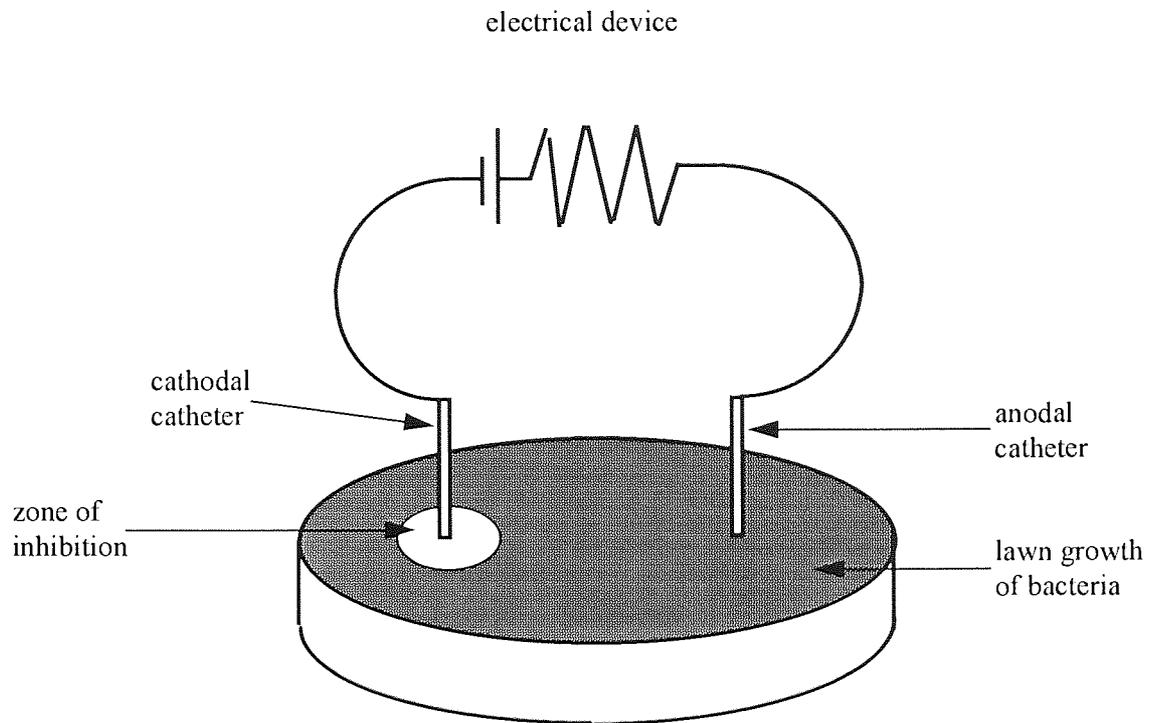
The formation of an inhibition zone on nutrient agar provides a simple and efficient method to investigate the various aspects of the antibacterial activity of low voltage and DC amperage, such as the range of bacteria which are susceptible. Investigation of factors influencing the formation of the inhibition zone and analysis of the inhibition zone could also elucidate the nature of the antibacterial activity of electric current.

4.2 Method

4.2.1 Zone of inhibition test

Five ml of nutrient broth (for staphylococci) or peptone water (for Gram negative bacilli) was inoculated with two colonies of the micro-organisms from an agar plate. The broth was then incubated at 37 °C in air for 2 h. Four μl of the staphylococcal suspension or 3 μl of the suspension of the Gram-negative bacilli was inoculated onto a nutrient agar plate. The bacterial suspension was then spread evenly over the whole plate with a dry swab. Two carbon catheters (2.5 cm lengths) were then placed perpendicularly, 5 cm apart, in each nutrient agar plate. The catheters were then connected via external leads to an electrical device that generated either 2, 10 or 20 μA with one catheter acting as the anode and the other as cathode (Figure 4-1). The plates were then incubated at 37 °C in air for 16 h. After incubation, the diameter of the zone of inhibition around the catheter, if any, was measured with a vernier calliper. The diameter was taken as the total distance between the outer edge of the inhibition zone with the centre of the catheter acting as the mid-point. All organisms were tested on six occasions with each amperage. After removal of catheters and reading the diameters of zone inhibition, at least 20 plates with the staphylococcal strains were re-incubated at 37 °C, in air, for a further 24 h to observe whether organisms would re-grow within the zone after the disconnection of electricity.

Figure 4-1 Application of electric current (DC) to a lawn of bacteria in a zone of inhibition test.



Note: Carbon catheters were placed perpendicularly, 5 cm apart, into a nutrient agar plate that had been seeded with bacteria. The catheters were connected to an electrical device that generated 2, 10 or 20 μA before the plate was incubated at 37 °C for 16 h in air.

4.2.2 Examination of the zone of inhibition

Examination of the zone of inhibition was carried out after the zone of inhibition tests as described in section 4.2.1. At least 20 agar plates that had been seeded with staphylococci and exposed to 10 μA of electric current were examined. After the catheters were removed and the diameter of zone of inhibition had been measured, a block of agar within the zone of inhibition was removed. The seeded surface of the agar was impressed on a sterile glass slide. After fixing with heat, the impression smear was Gram stained and then examined under microscope at $\times 1,000$ magnification. The other agar blocks removed from the zone of inhibition were also impressed on blood agar plates and then placed on fresh blood agar with the side seeded with bacteria up. The blood agar plates were then incubated at 37 °C in air for 16 h to detect bacterial growth.

4.2.3 Effect of 10 μA DC on the ability of agar to support bacterial growth

The effect of 10 μA DC on the subsequent ability of nutrient agar to support bacterial growth was investigated. Ten μA was applied to an uninoculated nutrient agar plate via the carbon catheters as described in section 4.2.1 for 16 h in air at 37 °C. After the electric current had been applied and the catheters removed, 4 μl of a staphylococcal suspension (ca. 10^5 cells) was spread to the whole surface of a nutrient agar plate with a dry swab. The plate was then incubated at 37 °C in air for 16 h and examined for bacterial growth and any zone of inhibition. The test was performed in triplicate with *S. epidermidis* NCIMB 12721, and *S. epidermidis* 811 or 983.

4.2.4 Factors affecting the diameter of zone of inhibition

The effect of inoculum size and current strength on zone size was investigated with *S. epidermidis* 983. Two colonies of the organism were inoculated into 5 ml nutrient broth. After incubation at 37 °C for 4 h, the bacterial suspension was diluted 100 and 10,000 times in saline. Fifty μl of the diluted suspension was added to a nutrient agar plate and then spread with a sterile plastic spreader. After drying in air for 10 min at 37 °C, DC electric current of

either 5, 10, 50 or 100 μA was applied as described in section 4.2.1. The plates were incubated at 37 °C, in air, for 16 h and the diameter of the zone of inhibition, if any, was then measured. The zone of inhibition test at each amperage and inoculum size was repeated six times.

Colony count of the bacterial suspensions (both the 100 and 10,000 times dilution) were determined by the Spiral System™ (section 2.5.1). The number of bacteria was approximately 1×10^3 or 1×10^5 CFU per plate.

4.3 Results

4.3.1 Zone of inhibition test

Carbon impregnated catheters *per se* have previously been shown to have no effect on the growth of bacteria (Crocker, 1993). When 2 or 10 μA DC, via the carbon catheters, was applied to the lawn of bacteria on the nutrient agar for 16 h, no zone of inhibition around the anode was observed with all the organisms tested. When 20 μA was applied, zones of inhibition with diameter ranging 5 to 10 mm were observed with all organisms tested except *K. pneumoniae*, *P. mirabilis* and *C. albicans* (Table 4-1). Conversely, inhibition zones were observed around the cathodes with current as low as 2 μA . However, there was a range of susceptibility to the electric current among the microorganisms tested. Of the five genus of bacteria and a yeast tested, staphylococci were the most sensitive of all, resulting in inhibition zones of diameter of 5 to 8 mm when 2 μA was applied and 11 to 16 mm with 10 μA . The diameter of inhibition zone increased with increased amperage in the range of 2 to 20 μA , but the zone sizes were similar with 10 or 20 μA . Ten μA was the lowest amperage to show any effect for the aerobic Gram-negative bacilli. The zone sizes, in comparison to the staphylococci, were smaller with diameter of zone inhibition up to 8 mm. Frequently, the zone of inhibition was only a narrow rim (< 1 mm from catheter to zone edge) of clear area around the catheter. After the removal of catheters, further incubation of the agar plates did not reduce the zone size, nor was there any bacterial growth within the zone.

When nutrient agar was prepared from nutrient agar no. 2 powder (CM 3) instead of adding 1.5 % (w/v) Agar No. 1 (L11) to nutrient broth (CM 67) and then used in the zone of inhibition test as described in section 4.2.1, no zones of inhibition were observed with any of the organisms tested. Comparison of the formulae of the two media revealed a difference in the ionic content of the agar.

Table 4-1 Zones of inhibition produced around the catheters after the application of electric current and incubation at 37 °C for 16h.

Zone size (mm) at the catheters, mean \pm *S.D.*; *n* = 6

| Micro-organisms | Cathode | | | Anode |
|--------------------------------------|------------------|----------------|----------------|-------------------------|
| | 2 μ A | 10 μ A | 20 μ A | 20 μ A ² |
| <i>S. aureus</i> ATCC 6538 | 0.0 ³ | 11.2 \pm 1.4 | 13.3 \pm 2.1 | 5.5 \pm 0.7 |
| <i>S. aureus</i> NCIMB 6571 | 7.6 \pm 1.9 | 16.0 \pm 1.8 | 18.4 \pm 3.2 | 8.8 \pm 1.4 |
| <i>S. epidermidis</i> NCIMB 12721 | 8.0 \pm 1.9 | 14.2 \pm 2.5 | 16.4 \pm 2.0 | 9.6 \pm 3.4 |
| <i>S. epidermidis</i> 023 | 8.7 \pm 2.9 | 14.6 \pm 0.4 | 13.4 \pm 2.7 | 7.8 \pm 0.9 |
| <i>S. epidermidis</i> 462 | 7.0 \pm 1.9 | 13.1 \pm 2.1 | 11.3 \pm 2.7 | 6.6 \pm 0.6 |
| <i>S. epidermidis</i> 811 | 7.8 ⁴ | 12.3 \pm 1.9 | 13.0 \pm 1.0 | 7.1 \pm 0.2 |
| <i>S. epidermidis</i> 983 | 5.1 \pm 3.9 | 11.1 \pm 2.3 | 17.3 \pm 1.5 | 0.0 |
| <i>S. hominis</i> | 0.0 | 9.6 | 9.9 \pm 3.7 | 6.2 \pm 0.6 |
| <i>E. coli</i> | 0.0 | 7.3 \pm 0.9 | 5.1 \pm 0.5 | 5.1 \pm 0.2 |
| <i>E. coli</i> NCIMB 8879 | 0.0 | 7.6 \pm 1.5 | 8.0 \pm 3.5 | 5.4 \pm 0.8 |
| <i>K. pneumoniae</i> | 0.0 | 5.8 | 0.0 | 0.0 |
| <i>K. pneumoniae</i> ATCC 4352 | 0.0 | 6.7 | 5.1 | 0.0 |
| <i>P. mirabilis</i> | 0.0 | 7.7 \pm 0.9 | 10.5 \pm 1.9 | 0.0 |
| <i>P. aeruginosa</i> | 0.0 | 7.1 | 5.8 | 0.0 |
| <i>C. albicans</i> | 0.0 | 0.0 | 7.9 \pm 0.2 | 0.0 |

1. The zone of inhibition was measured from edge to edge across the centre of the catheter and the external diameter of the catheter was 2.4 mm.
2. No inhibition zone observed around the anode with 2 or 10 μ A.
3. In all cases, zone size of 0.0 mm denoted no inhibition zone observed because the organism grew to the catheter agar interface.
4. Mean zone size given without *S.D.* because occasionally no zone of inhibition was found and so there was less than six measurement, typically *n* = 4

4.3.2 Examination of the zone of inhibition

When impression smears prepared from material within the zone of inhibition were Gram-stained, staphylococci were evident. Bacteria were either single or in pairs and never in clumps of four cells or more. No morphological changes in the stainability, shape or size of the organisms could be observed. The impression culture of the agar block from within the zone of inhibition were sterile after they had been incubated for 16 h.

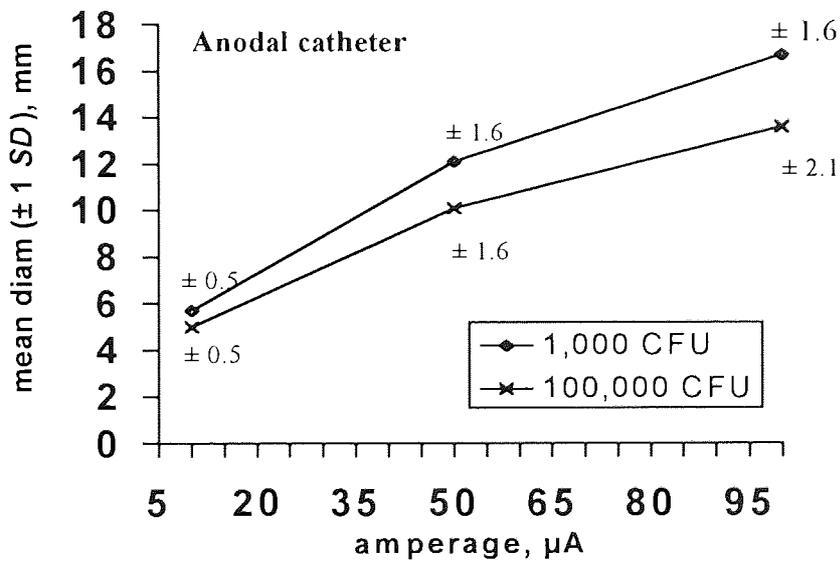
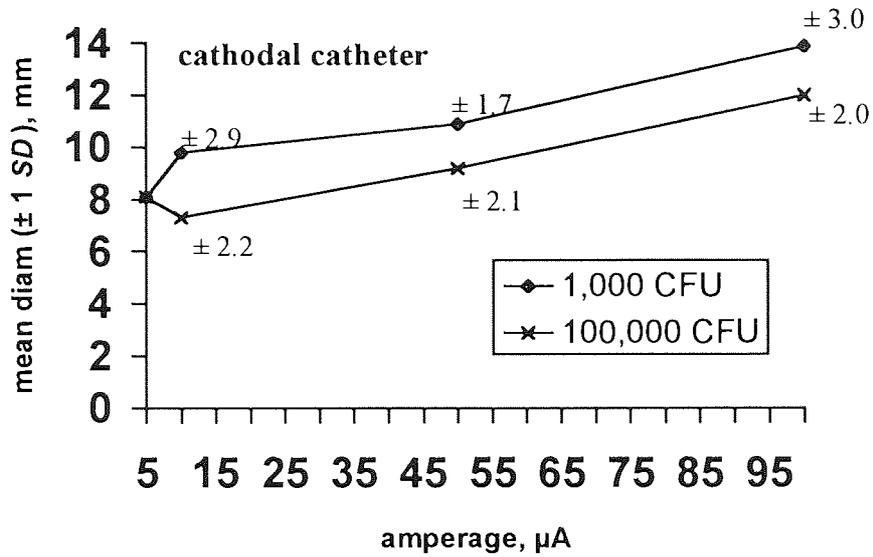
4.3.3 Effect of 10 μ A on the ability of agar to support bacterial growth

The three strains of *S. epidermidis* grew on the agar plates that had been exposed to 10 μ A DC at 37 °C for 16 h. They grew on the entire surface of the agar plates and there were no zones of inhibition around the area where both the cathodal and anodal catheters had been placed.

4.3.4 Factors affecting the zone of inhibition

The zone size of inhibition around the cathodal catheter was related to the amperage applied (5-100 μ A) with either 10^3 or 10^5 CFU of *S. epidermidis* 983 on the plate (Figure 4-2). Although the zones sizes were generally smaller on the plates with 10^5 CFU per plate than the ones with 10^3 CFU per plate, the differences were not significant (Wilcox rank test, *C.L.* = 95 %).

Figure 4-2 Effect of amperage and inoculum size (CFU/plate, test organism: *S. epidermidis* 983) on the diameter of inhibition zones around the catheters after application of electric current for 16 h at 37 °C ($n = 6$).



Note: The zone of inhibition was measured from edge to edge across the centre of the catheter and the external diameter of the catheter was 2.3 mm

4.4 Discussion

This is the first detailed study of the activity of low voltage and amperage (9 V, $\leq 20 \mu\text{A}$) DC using a zone of inhibition test. Antibacterial activities associated with the anode and cathode as a result of application of electric current DC were different. The cathode was more active than the anode when the applied current strength was between 2 to 20 μA . Firstly, the minimum amperage to show any activity at the cathode was 2 μA and at the anode, 20 μA . The cathode was more active than the anode at 20 μA , as demonstrated by the larger zone sizes associated with the cathode. This difference in activity at the electrodes may be due to the opposite polarity at the electrode surfaces. Bacterial cell surfaces on the whole, are electronegative, hence, repulsion may contribute to the formation of the inhibition zone.

Of the 15 species of micro-organisms tested, staphylococci were more susceptible than the aerobic gram-negative bacilli (GNB) with a lower minimum amperage required to form inhibition zones and larger zone sizes. These different susceptibilities may be related to the differences in cell wall and membrane structure of Gram positive and Gram negative bacteria. Although the aerobic GNB also cause catheter-related infections, they represent only <5 % of all infections. Therefore, it is encouraging that low amperage electric current is very effective against staphylococci which is responsible for >95% of CRI. While it is unsafe to apply 20 μA to a CVC to prevent colonisation by bacteria, such level of current may be applied to other medical devices that are further away from the heart such as orthopaedic prosthesis and urinary catheters. Moreover, the activity of electric current is effective against a wider range of bacteria when >20 μA was used.

Crocker *et al.* (1992) applied a continuous electric current to micro-organisms that were suspended in fluid. Bacteria were repelled from the catheter surface to a distance of only 100 μm . They then moved back towards the catheter surface after the disconnection of electric current. In the further study, zone of inhibition was formed around the cathodal catheter when 10 μA DC was applied to a lawn growth of bacteria. The formation of the inhibition zone was thought to be the result of repulsion of organisms away from the catheter to a distance that formed the zone edge (Crocker, 1993). In the present study,

the zone sizes were far greater than the distance resulted from repulsion as observed by Crocker (1993). Moreover, after the disconnection of electric current, reincubation of the plates did not reduce the diameter of inhibition zone. Therefore, the inhibition zone thus formed was stable and suggested that the antibacterial activity was different from repulsion.

Further evidence against repulsion as the main mechanism of the formation of inhibition zone was the examination of the agar surface within the inhibition zone by Gram stained the impression smear of the agar surface. Gram stained smear showed staphylococci were present throughout the inhibition zone. The impression cultures from the inhibition zone were sterile. Therefore, the inhibition zone was due to a bactericidal activity resulting from the application of 10 μA DC, rather than repelling bacteria to a distance to form the inhibition zone edge. Moreover, inhibition zones were also observed around the anodal catheter when 20 μA was applied, which confirmed that the formation of the inhibition zone is unlikely to be due significantly to the repulsion of bacteria.

In a previous study, a zone of bacterial inhibition was observed around the anodal stainless steel needle with an immediate surrounding of concentrated bacterial colonies when 10 μA was applied (Crocker, 1993). However, this may have been due to the release of metallic ions from the anode. A synergistic effect between electric current, DC and silver ions have been documented (Chu *et al.*, 1987; Falcone & Spadaro, 1985). A larger inhibition zone was observed when 0.4 to 4 μA DC was applied to the silver impregnated fabric than the zone around the silver impregnated fabric without electric current. In contrast, the catheters used in the present study were impregnated with carbon only, so this antibacterial activity could not be due to the release of metallic ions but rather to electric current alone.

The formation of an inhibition zone around the cathodal catheter was affected by the constituents, possibly the ions contents, of the media. Agar No. 1 (L11) that was used for all the zone of inhibition tests contained less metal ions, notably divalent ions such as magnesium and calcium ions, than the agar in nutrient agar no. 2 (CM 3). Excluding the ions content of the agars, there were no other differences in the constituents of the media

stated in the manufacturer's label. This suggested that the mechanism of the bactericidal activity of electric current may affect the membrane structure of the organisms.

After 10 μ A DC was applied to uninoculated nutrient agar for 16 h at 37 °C, no inhibitory effect remained on the agar. Therefore, the application of 10 μ A electric current for 16 h did not alter the ability of the nutrient agar to support bacterial growth or produced toxic substances that are long lasting. Previous results showed that the pH around the electrodes did change and the cathode became alkaline and the anode acidic (Crocker, 1993). In the present study it did not affect the ability of the agar to support bacterial growth.

After the plates were seeded and incubated at 37 °C, a delay of up to 6 h before the application of electric current did not affect the zone size. However, a lapse of 8 h between the incubation of the seeded plates and the application of electric current resulted in no formation of inhibition zone. When the plate seeded with bacteria was incubated, bacteria would start to grow and entered a log phase and also increased in number. Thus a fast growth rate at log phase and with more bacteria on the agar plate as a result of the 8 h incubation may overcome any bactericidal activity of the 10 μ A applied. The diameter of the inhibition zone was smaller with more bacteria (1×10^5 CFU) on the agar than less bacteria (1×10^3 CFU). Hence, the activity of the electric current is affected by the number of bacteria and possibly the growth phase.

While there was an increase in zone sizes when the electric current increased from 2 to 10 μ A, the zone size did not increase significantly from 10 to 20 μ A. A direct linear relation between the zone size and the amperage was more clearly seen when the current was tested between 5 and 100 μ A and the increase was associated with both electrodes (Figure 4-2). At 50 μ A or more, the activity associated with the anode was more prominent than the activity associated with the cathode. This implies that when a higher level of current is used, a more effective antibacterial activity will be obtained with a catheter acting as the anode.

In this study using the zone inhibition test, electric current as low as 2 μ A was bactericidal against staphylococci which are responsible for > 95 % of catheter-related

infections (CRI). At 10 μA , a continuous negative charge via an electro-conducting catheter appeared to be more effective than a positive charge in terms of minimal effective amperage and antibacterial activity. Therefore, in the investigation of the use of electric current in the prevention of CRI there will be a concentration on the use of a cathodal catheter with 10 μA .

5. Activity of low voltage (DC) electric current: effect on bacteria suspended in fluid

5.1 Introduction

The initial stage of colonisation of an intravenous catheter is the adhesion of micro-organisms to the catheter surface. Organisms may reach the catheter surface as a result of inoculation of IV fluid from contaminated hubs (Linares *et al.*, 1985) or they may be impacted onto the catheter surface during insertion (Elliott *et al.*, 1995).

As the organisms come into close proximity of the catheter surface, reversible adhesion can then take place. The Derjaguin-Landau and Verwey Overbeek theory (DLVO) of colloidal particles was proposed to explain this initial stage of colonisation (van Loosdrecht *et al.*, 1990). When a bacterium is in close proximity to the surface (< 10 nm), van der Waals force attracts the bacterium to the surface. But the electrostatic force repels them as a result of the overall negative charges of both the bacterial surface and the substratum. Van der Waals force is dependent on the diameter of the particle and the distance between the surface and the particle. While the electrostatic force is dependent on the surface potential of the substratum and the ionic strength of the aqueous medium. When the summation of these two forces, known as the Gibb's energy, is at a minimum adhesion then takes place. The application of DLVO theory to bacterial adhesion has its limitation since it is based on colloidal particles that are small and with a homogenous surface (Gilbert *et al.*, 1993). Appendages and exopolysaccharide of the bacterium will change the effective diameter of the cell and so consequently alter the van der Waals force. Moreover, surface charge and hydrophobicity of the bacterium vary according to the surface composition which in turn, is greatly influenced by growth conditions (Gilbert *et al.*, 1993).

The initial adsorption of bacteria may also be explained in terms of system free energy (Korber *et al.*, 1995). If the total free energy of a system is reduced by cell contact with a surface, then adsorption of the cell to the substratum will occur. Thus for *S.*

epidermidis, strong surface hydrophobicity correlates well with adhesion. Hence, many researches in the prevention of catheter-related infections have concentrated on the modification of the surface hydrophobicity of the material (Jansen & Peters, 1991) to prevent adhesion of bacteria to catheters.

The other approach to prevent adhesion is to apply electric current continuously to a catheter surface and thus increases its overall negative charge (Elliott *et al.*, 1990). The increased negative charges repelled bacteria from its surface. Crocker (1993) showed that when an electro-conducting catheter was placed in a bacterial suspension, with 10 μA DC applied, organisms moved away from the surface of the negatively charged catheter. The repulsion of bacteria lasted the whole period of examination (50 min). Repulsion of bacteria could lead to a reduction of bacterial adhesion on a negatively charged catheter surface.

As electric current is passed along a catheter into a fluid medium, it may also reduce the viability of bacteria suspended in the medium. Davis *et al.* (1989) demonstrated that when an electric current as low as 40 μA was applied to organisms suspended in synthetic urine, it was bactericidal. In contrast, 200 mA was not bactericidal nor did it affect the growth rate of *E.coli* when the organisms were suspended in nutritious media (Rowley, 1972). These contradicting results may be due to the differences in the test organisms and the constituents of the media. Moreover, staphylococci were not tested in the previous report (Rowley, 1972). In the present study, the antibacterial activity in the zone inhibition test showed that the cathode had better bactericidal activity against staphylococci than *E.coli* (section 4.3) and suggested that 10 μA may have activity against a staphylococcal suspension.

To investigate this phenomenon further, a negatively charged catheter with a continuous current (10 μA) was placed in a bacterial suspension and the number of viable bacteria attached to the catheter determined at timed intervals. Simultaneously, the study investigated whether a cathodal catheter with 10 μA would prevent adhesion of micro-organisms.

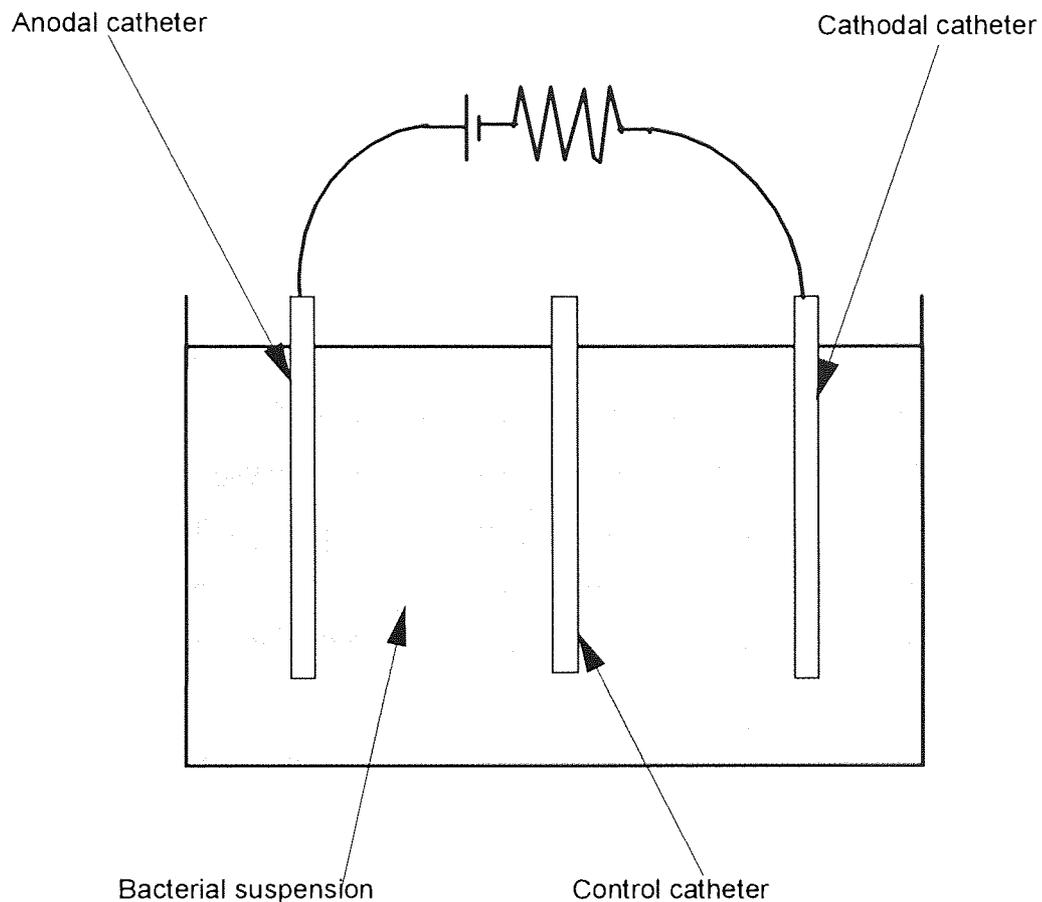
5.2 Method

5.2.1 Prevention of adhesion of bacteria to catheter surface

5.2.1.1 Preparation of bacterial suspension for adhesion and the application of 10 μA to catheters

One colony of *S. epidermidis* NCIMB 12721 was inoculated into 5 ml of nutrient broth. After incubation at 37 °C for 3 to 4 h, the number of bacteria in the suspension was determined by measuring its OD at 570 nm (section 2.5.2). An appropriate volume of the suspension was added to 250 ml of pre-warmed nutrient broth in a 300 ml container to give a final cell density of ca. 1×10^4 CFU/ml. The fresh staphylococcal suspension was then thoroughly mixed on a magnetic stirrer and kept at 37 °C in air. Immediately, two 2.5 cm lengths of sterile catheter with a distance of 4 cm were then immersed vertically in this bacterial suspension. The catheters were connected to an electrical device via external leads with one catheter acting as the cathode and the other as the anode. Another sterile catheter without any electrical connection was also immersed vertically into the broth between the two electrical catheters and served as a control (Figure 5-1). Another identical set of catheters (i.e. one control catheter, one cathodal catheter and the corresponding anodal catheter) was also prepared and immersed in the bacterial suspension.

Figure 5-1 Application of 10 μ A DC to *S. epidermidis* NCIMB 12721 suspended in nutrient broth.



5.2.1.2 Enumeration of bacteria adhered to the catheters after colonisation and then minimal rinsing

After 0.5 and 1.0 h immersion in the bacterial suspension, one set of catheters which consisted of one control catheter, one cathodal catheter and the corresponding anodal catheter, was removed. Each catheter was rinsed by briefly immersed in 30 ml PBS (section 2.7.2). The number of bacteria on the external surface of the catheters was then determined by the roll-plate method (section 2.8.1). Colony counts of the bacterial suspension at the commencement and the end of incubation were also determined

(section 2.5.1) to monitor the growth of the bacteria in the broth. The experiment was carried out in triplicate.

5.2.1.3 Enumeration of bacteria adhered to the catheters after colonisation and then vigorous rinsing

The total number of adherent bacteria remained on a colonised catheter after rinsing was dependent on the rinsing procedure and the total number of rinses. Immersing a colonised catheter in PBS without shaking or inversion removed the least quantity of adherent bacteria (section 3.3), while vigorous rinsing such as repeat inversion for 150 times left with the least number of bacteria on the catheter surface (section 3.3.1). After vigorous rinsing, those bacteria remained attached were probably firmly attached. Therefore, the capacity of 10 μ A DC preventing the firm adhesion of bacteria to the cathodal catheter was further investigated. One control catheter, one cathodal catheter and the corresponding anodal catheters connected to the electrical device generating 10 μ A were immersed in a nutrient broth suspension of *S. epidermidis* NCIMB 12721 as described in section 5.2.1.1. The period of catheters immersed in the bacterial suspension and the simultaneous application of electric current was extended to 4 h so as to allow firm attachment of bacteria to the catheter surface. After 1 and 4 h immersion in the bacterial suspension, one set of catheters was removed and then rinsed by inversion 150 times (section 2.7.1). After rinsing, the number of bacteria on the external surface of the catheters was determined by the roll-plate culture method. The experiment was carried out three times.

5.2.2 Effect of 10 μ A DC on growth of *S. epidermidis* NCIMB 12721 in nutrient broth

Two μ l of an overnight nutrient broth culture of *S. epidermidis* NCIMB 12721 was added to 100 ml of nutrient broth in a container of 300 ml volume so that the final cell density was ca. 1×10^4 CFU/ml. Two such nutrient broth cultures were prepared. Two 2.5 cm lengths of catheters that were connected to an electrical device that generated 10 μ A were placed vertically into the broth of one of the containers (Figure 5-1). Two other catheters that were not connected to any electrical device were immersed vertically into the other broth culture

and served as the control. The cultures were continuously mixed by a magnetic stirrer and incubated at 37 °C in air. Over the initial 8 h of incubation and electric current application, colony counts of the bacteria in the broth were determined by removing 1.0 ml aliquots at timed intervals and then carrying out the Spiral System™ (section 2.5.1). The experiment was repeated twice.

In a separate experiment, the duration of incubation and electric current application was extended to 24 h and the colony count of the bacterial suspension was determined at timed intervals between 14 to 24 h. This experiment was again repeated twice.

5.3 Results

5.3.1 Prevention of adhesion of bacteria to catheter surface

Sterile carbon catheters with 10 μ A DC applied were immersed in the nutrient broth culture of *S. epidermidis* NCIMB 12721 which was incubated at 37 °C. After incubation, the catheters were rinsed by brief immersion in PBS, that removed the fewest numbers of adherent bacteria from the catheter. Brief immersion of the catheter in PBS most likely removed bacteria that were reversibly attached to the catheter, as discussed in Chapter 3 'Evaluation of basic methods' (section 3.3). The number of staphylococci that adhered to both the control and the cathodal catheters increased with time. There was no significant difference in the colony counts of adherent bacteria on the cathodal catheter or the control catheter after 0.5 or 1 h incubation (Wilcoxon signed rank test, 95 % *CL*). After 1 h of incubation of the catheters in the staphylococcal culture, the number of bacteria on the control catheters was too numerous for accurate counting by the roll plate culture method and so the incubation was not extended.

Rinsing the colonised catheters by inversion for 150 times removed more bacteria than immersion as discussed in section 3.3. The population of bacteria which remained on the catheter surface might be the more firmly attached bacteria. After 1 h of incubation, the colony counts on the external surfaces of the catheters were lower when compared to the catheters that had been rinsed by immersion (Table 5-1). Nevertheless, the number of adherent bacteria on the catheters increased over time and there was no significant difference (Wilcoxon signed rank test, 95% *CL*) in the number of bacteria on the external surface of the cathodal catheter as compared to the control catheter after 4 h of incubation and simultaneous application of 10 μ A DC (Table 5-1). Thus, 10 μ A applied to the cathodal catheter did not prevent adhesion of *S. epidermidis* NCIMB 12721 suspended in nutrient broth. After 4 h of incubation, the number of bacteria in the nutrient broth also increased from 2.3×10^6 to 2.1×10^7 CFU/ml so that the number of bacteria adhered was too numerous for an accurate count by the roll plate method.

Table 5-1 Colony count of adherent bacteria on the external surface of catheters after 10 μ A DC applied to the catheters that were immersed in a nutrient broth culture of *S. epidermidis* NCIMB 12721: comparison of control and cathodal catheter.

| Rinsing procedures after colonisation | Period of electric current application <i>h</i> | Mean (± 1 SEM, $n = 3$) colony count, CFU/cm length catheter | |
|---------------------------------------|--|--|-------------------|
| | | Control | Cathodal catheter |
| Immersion | 0.5 | 134 \pm 15 | 274 \pm 97 |
| Immersion | 1.0 | >1,000 ² | 879 \pm 36 |
| Inversion 150 times | 1.0 | 82 \pm 9 | 82 \pm 5 |
| Inversion 150 times | 4.0 | 570 \pm 58 | 628 \pm 85 |

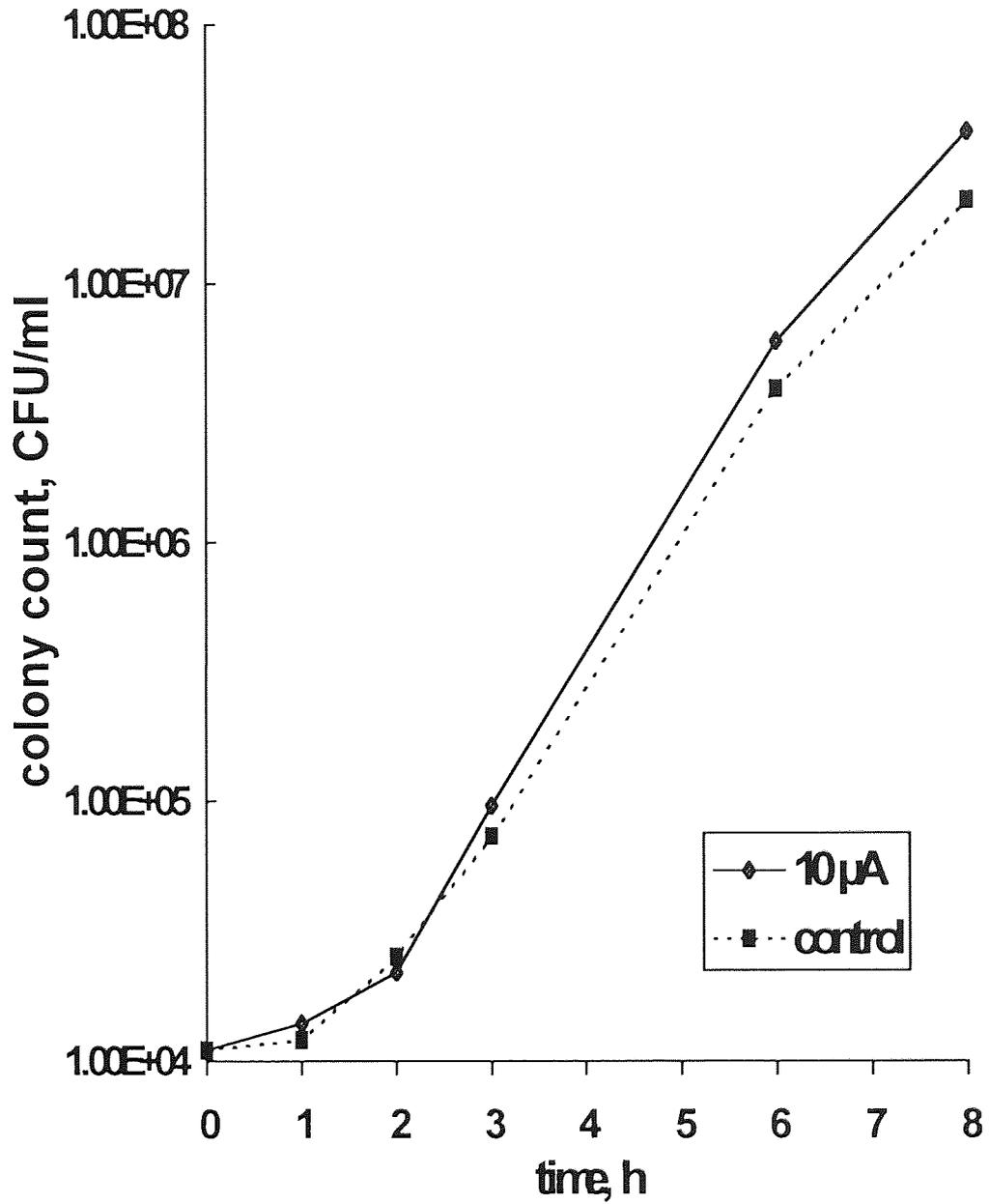
Notes:

1. mean colony count was determined by the roll-plate culture method (section 2.8.1).
2. bacterial growth on the roll-plate culture was confluent and an accurate count was not possible.

5.3.2 Effect of 10 μ A DC to the growth of *S. epidermidis* NCIMB 12721 in nutrient broth

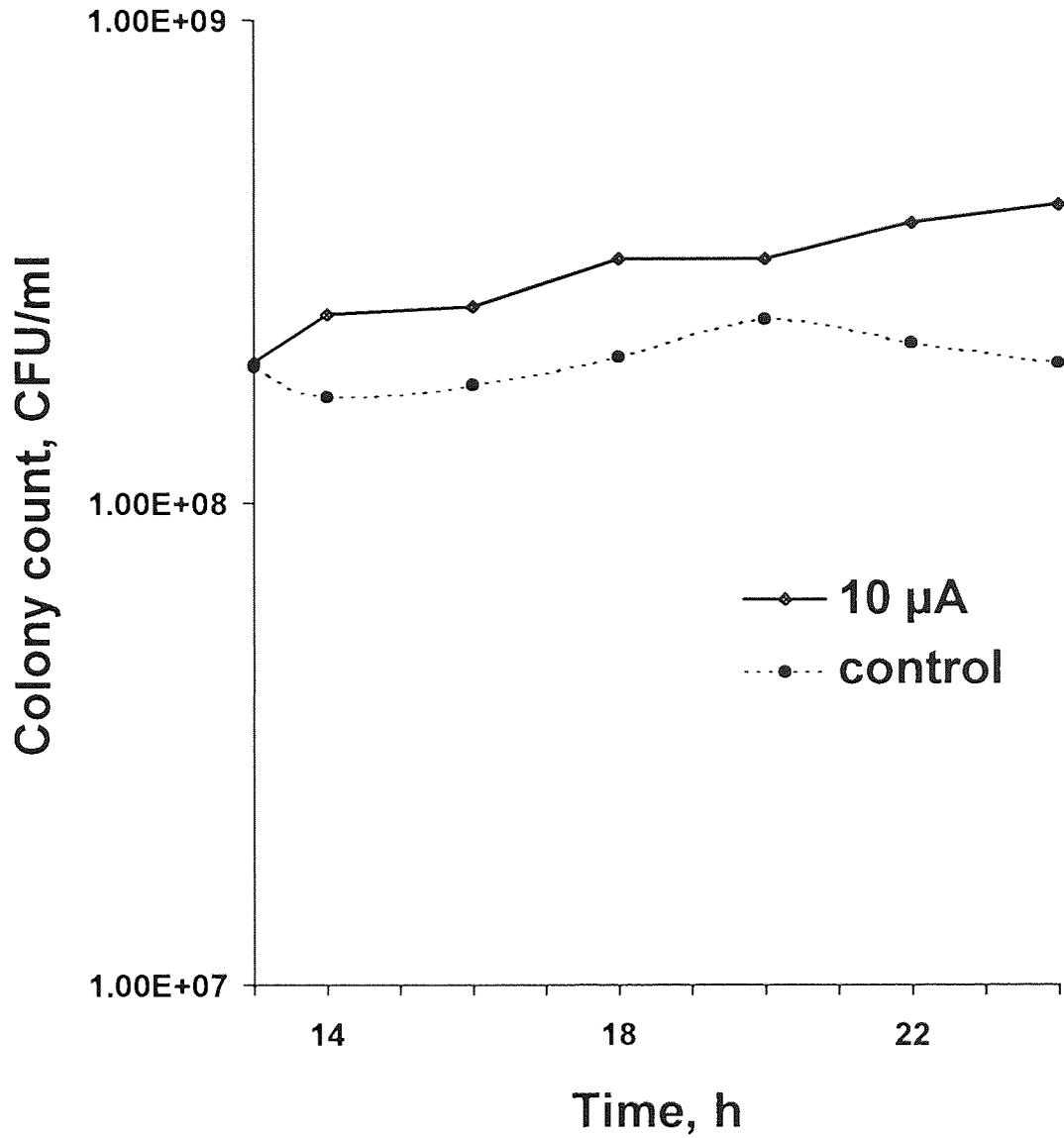
The colony counts of *S. epidermidis* NCIMB 12721 in nutrient broth at 37 °C with or without the application of 10 μ A DC over 24 h were similar. Typical growth curves of the experiments were given in Figure 5-2 and 5-3. Ten μ A did not affect the growth rate for the first 8 h when the staphylococci were grown in nutrient broth. After 14 h, the growth of the staphylococci with or without the application of 10 μ A DC slowed down and the cell density after 24 h were similar and no significant difference was observed (Wilcoxon signed rank test, 95 % CL).

Figure 5-2 Effect of 10 μ A DC on the growth of *S. epidermidis* NCIMB 12721 in nutrient broth for the initial 8 h.



Note: logarithmic scale for the colony count (CFU/ml): $1.00E+N = 1.0 \times 10^N$

Figure 5-3 Effect of 10 μ A DC to the growth of *S. epidermidis* NCIMB 12721 in nutrient broth .



Note: logarithmic scale for the colony count (CFU/ml): $1.00E+N = 1.0 \times 10^N$

5.4 Discussion

When a catheter was placed in the nutrient broth culture of *S. epidermidis* NCIMB 12721, the organisms started to adhere to its surface. This adhesion took place in less than half an hour. After an hour, the adhesion of staphylococci was strong enough to withstand rinsing by repeat inversion 150 times with 10 changes of PBS. Applying 10 μ A DC to the catheters did not prevent adhesion of bacteria to the electrically charged catheters as the colony count of the *S. epidermidis* NCIMB 12721 on the external surface increased with colonisation time. Furthermore, there was no significant differences in the colony counts on the external surface of the cathodal catheter as compared to the control after 4 h incubation. Hence, a negatively charged catheter in nutrient broth with 10 μ A DC applied to it did not appear to prevent nor reduce the extent of adhesion of growing staphylococci to its surface.

This result did not correlate with an earlier findings using a slide model (Crocker, 1993) in which a carbon-impregnated catheter was placed in 0.5 ml nutrient broth of a staphylococcal suspension with a cell density ca. 1×10^6 CFU/ml. The catheter was connected to the negative pole of an electrical device and acted as a cathode. The electric current was completed by connecting the positive pole to a needle immersed in the broth. When 10 μ A DC was applied, bacteria started to move away from the catheter surface and remained at a distance while the current was applied. It was suggested that the movement of the cells away from the catheter surface was due to the repulsion of bacteria as a result of electrostatic forces of the same charge. The movement of bacteria away from the catheter surface inferred that bacteria would not adhere to the catheter, but this was not confirmed in the present study.

The different experimental conditions may explain the differences between the two results. There was only 0.5 ml of bacterial suspension in the slide model as compared to 250 ml in the present experiment. The electric field would be much weaker in the present experiment that used a larger volume of suspension. Moreover, the cathodal catheter and the anode in the slide model were placed in parallel less than 1 cm apart. With such a small volume of suspension and close proximity of electrodes, there may be the additional electrophoretic movements of charged particles such as bacteria or red

blood cells. However, this electrophoretic movement may not prevent adherence of bacteria to the surface in a larger volume test as in the present study. Firstly, the electrodes were farther apart than in the slide model, so that the voltage gradient would be reduced. Secondly, the current flow from one electrode to the other may not be unidirectional as in the slide model.

In the slide model, the bacteria were kept away from the surface of the negatively charged surface only while the current was applied. In the present study, electric current had to be disconnected before the number of staphylococci adhered to the catheter could be counted. Breaking of electric circuit occurred when the catheters were removed from the broth. Once the catheter was out of the fluid, the discontinuity of electric circuit resulted in the loss of its negative charges. Moreover, when the catheter was removed from the broth, a thin film of fluid would adhere to the catheter due to surface tension and this may bring the organisms near to the surface. Consequently, those bacteria that were repelled from the surface in the bacterial suspension might be able to adhere to the surface again. Although the overall charge of the bacterial surface is negative, there are regions that are positively charged, and these positive charges may also bring the bacteria to a negatively charged surface. It is also possible that a negatively charged catheter surface may prevent firm attachment of bacteria but the present study was not sensitive enough to detect the differences.

Ten μA DC electric current did not affect the growth of *S. epidermidis* NCIMB 12721 in nutrient broth including the growth rate, and the duration of the growth phases of cell cycle, since the growth curves of the staphylococci in nutrient broth with or without the application of electric current were similar. The final cell density in the broth was also similar with or without 10 μA DC. This is similar to a previous study of the effect of electric current on *E. coli* (Rowley, 1972). It is probable that the large volume, the nutritious constituents of the broth and the low amperage of electric current resulted in no bactericidal activity by electric current.

6. Activity of low voltage (DC) electric current: effect on bacteria attached on catheter surface

6.1 Introduction

The insertion wound is a major source of bacteria that may subsequently colonise an intravascular catheter (Egebo *et al.*, 1996). Reduction of this bacterial population is one of the strategies in the prevention of catheter-related infections (Elliott *et al.*, 1995). However, procedures such as thorough skin disinfection do not completely eliminate the bacterial population. Some of these micro-organisms may be impacted onto the catheter surface during insertion (Elliott *et al.*, 1995). Following placement of a catheter, a positive electric potential gradient across the tissue may also attract organisms on the skin surface to the catheter-tissue interface (Dealler *et al.*, 1988). Another major source of bacteria is from the operators' hands. Manipulation of hubs, connectors and other components of the catheter in-use can lead to their contamination (Linares *et al.*, 1985). For example, more than 20 % of Swan-Ganz catheters were found to be colonised with bacteria by the fifth day of insertion and these potentially can lead to catheter-related infections (Raad & Bodey, 1992).

Once adhered to the catheter surface, bacteria may derive nutrients from catheter material to support their growth (Kristinsson, 1989). One approach to inhibit growth is to load catheters with antimicrobial agents such as benzalkonium chloride which reduces bacterial colonisation of catheters (Tebbs & Elliott, 1993; Mermal *et al.*, 1993). Heparin bonded to catheters by benzalkonium chloride also reduced the infection rate of catheter-related infections (Mermal *et al.*, 1993). However, the limited quantity of incorporated antimicrobial agent leached out from the material and antimicrobial activity was lost.

In contrast, bacteria attached to an electro-conducting catheter would be exposed continuously to the antibacterial effect of electric current whilst the electric current was applied. The antibacterial effect of electric current was bactericidal to organisms in the vicinity of the cathodal catheters as demonstrated in the zone inhibition test (section

4.2.2). This suggests that an electric current when applied continuously to a cathodal catheter would be bactericidal to adhering organisms. Bacteria in 100 ml nutrient broth into which electric current carrying catheters were not however affected (section 5.3.2). It appears that the bactericidal activity associated with the cathodal catheter may only affect micro-organisms that are near to or attached to the catheter surface and not in the surrounding medium.

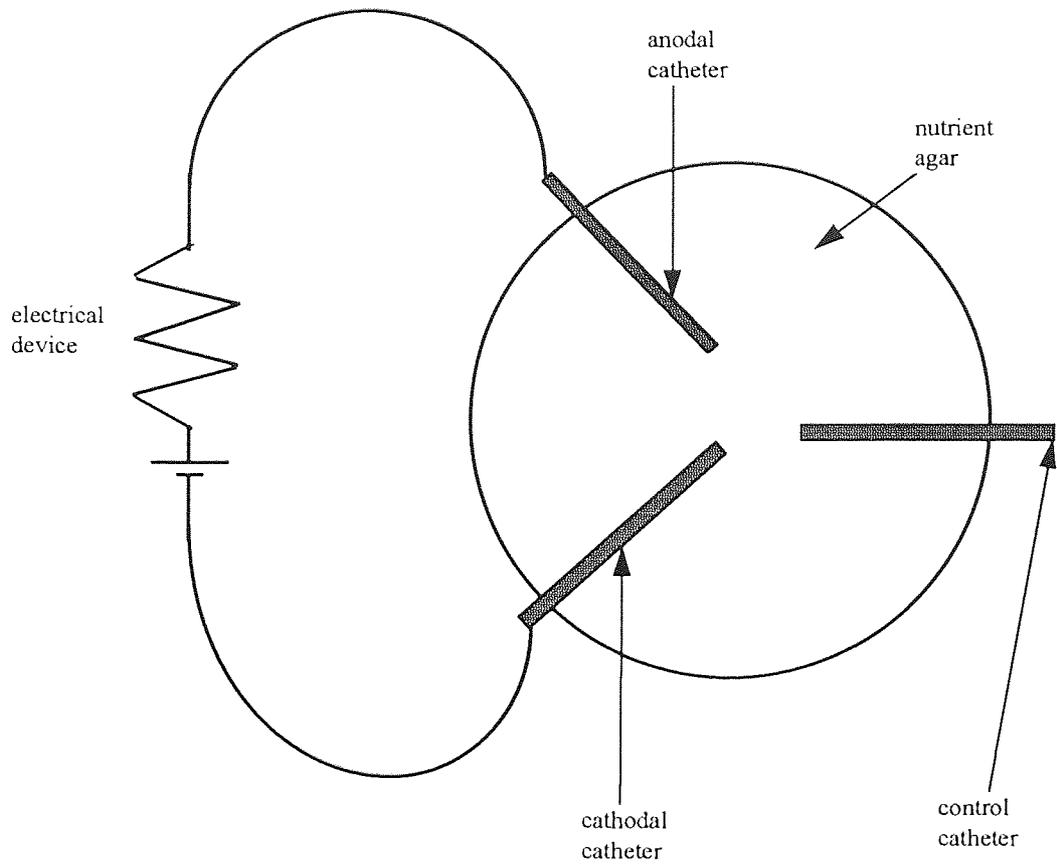
Therefore, the effect of 10 μ A applied to an electro-conducting catheter on the growth and viability of attached micro-organisms was investigated.

6.2 Method

6.2.1 Effect of 10 μ A on bacterial growth

Catheters of 2.5 cm length were colonised with bacteria by incubating them in a bacterial suspension of 1×10^5 CFU/ml in nutrient broth for 2.5 h (section 2.6.1). After colonisation, loosely attached bacteria were removed by centrifugation three times (section 2.7.2). Three colonised catheters were placed through apertures in the side wall of a sterile Petri dish (Figure 6-1). Twenty ml of molten nutrient agar at 44 °C containing triphenyl tetrazolium (0.01 % w/v) was slowly poured over the catheters. After the agar had set, the catheters were connected to the electrical device via the external leads and clips. One catheter acted as the cathode, one as the anode and the third with no electrical connection as the control. The plate was then incubated at 37 °C, in air, for 48 h. The external surfaces of the catheters were examined daily for bacterial growth with a stereomicroscope (model SV8, Zeiss, Germany). Each of the following bacteria was tested three times: two strains of *S. aureus* (NCIMB 6571 and ATCC 6538), 5 strains of *S. epidermidis* (NCIMB 12721, clinical isolates 023, 462, 811 & 983) and *E. coli* NCIMB 8879.

Figure 6-1 Application of 10 μ A DC to catheters colonised with bacteria and then embedded in nutrient agar.



6.2.2 Viability of *S. epidermidis* NCIMB 12721 in medium A

To investigate the effect of an electric current on the number of viable bacteria attached to a catheter surface, catheters colonised with bacteria were suspended in medium A and then 10 μ A DC was applied. Medium A is the fluid equivalent of Stuart's medium that maintains viability of bacteria up to 48 h at 25 °C including fastidious bacteria such as *Neisseria gonorrhoea* and *Haemophilus influenzae* (Oxoid Manual, 1990).

The viability of *S. epidermidis* NCIMB 12721 in medium A was also studied. One colony of *S. epidermidis* NCIMB 12721 was inoculated into 5 ml of nutrient broth and then incubated at 37 °C for 2 to 3 h. The bacterial suspension was then centrifuged at 660 g for 5 min and the supernatant discarded. The deposit of bacteria was then resuspended in 20 ml medium A to give a final cell density of 1×10^4 to 1×10^5 CFU/ml. The bacteria suspension was then incubated at 37 °C in air, in the same condition as the experiment described in section 6.2.3. At timed intervals, the suspension was mixed by gentle inversion, an aliquot removed and the colony count determined.

6.2.3 Effect of 10 μ A on the number of viable bacteria on the catheter surfaces

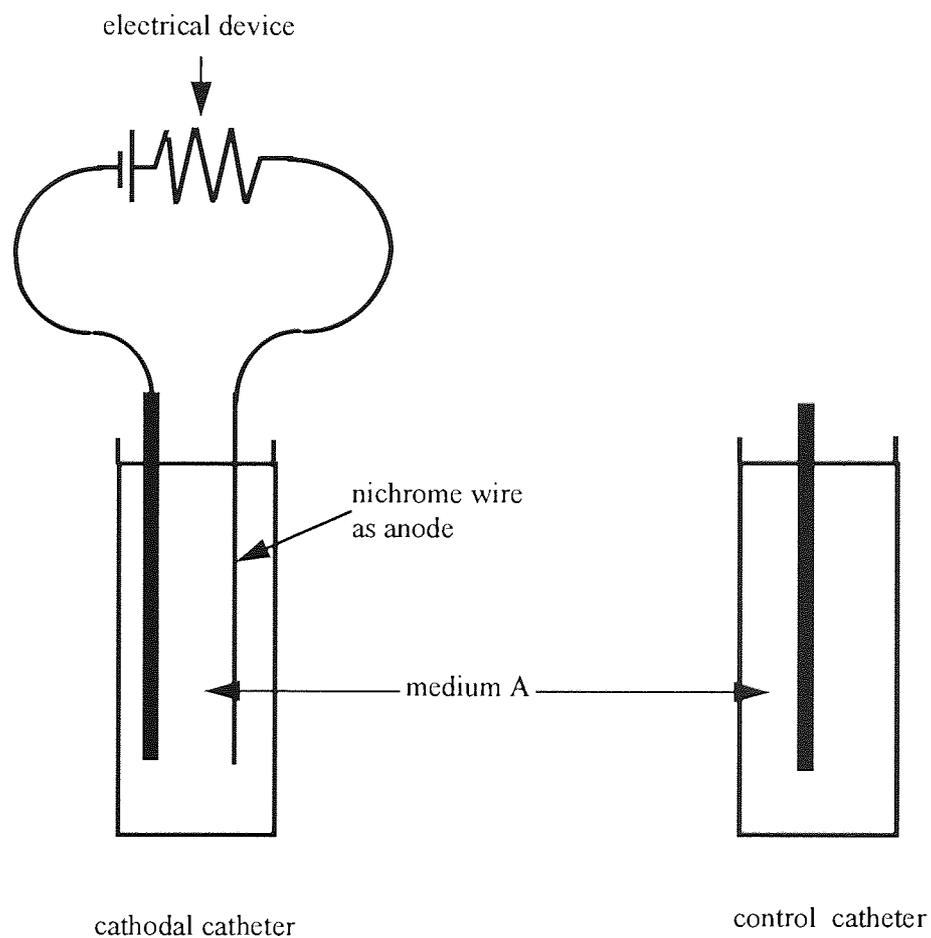
Catheters (2.5 cm lengths) were colonised with bacteria for 2.5 h (section 2.6.1) and then loosely attached bacteria were removed by centrifugation three times (section 2.7.2). One catheter was then connected to the negative pole of an electrical device and completely immersed vertically in 15 ml medium A. A sterile nichrome wire was connected to the positive pole and immersed in the fluid to complete the circuit (Figure 6-2). Another catheter that was colonised with bacteria was similarly placed in another 15 ml medium A without electric current being applied, and served as control. After incubation at 37 °C for 16 h in air, the catheters were removed and the number of bacteria attached to their surfaces determined by the roll plate culture (section 2.8.1).

After each catheter was rolled on the blood agar to remove the organisms on the external surface, it was then cut longitudinally into two halves to expose the internal surface. Each

half of the catheter was then gently pressed onto another blood agar plate so that organisms on the internal surface would be impressed onto the agar surface. Ten imprints of each half of the catheter were made.

All the plates were then incubated for 16 h at 37 °C in air and the number of bacterial colonies determined. *S. epidermidis* NCIMB 12721, *S. epidermidis* 023 and 983 were each tested individually on six occasions.

Figure 6-2 Application of 10 μ A DC to catheters pre-colonised with *S. epidermidis* and then suspended in medium A.



6.2.4 Susceptibility of bacteria to 10 μ A DC after various catheter colonisation times

Catheters (2.5 cm lengths) were colonised with *S. epidermidis* NCIMB 12721 for 2.5 or 16 h (section 2.6.1). After colonisation, any loosely attached bacteria to the catheter were removed by centrifugation (section 2.7.2). One catheter was connected to the negative pole of an electrical device thereby acting as the cathode. It was then completely immersed vertically in 15 ml of medium A. The circuit was completed by connecting a sterile nichrome wire to the positive pole and immersed in the medium A (Figure 6-2). Another catheter that had been colonised with bacteria was similarly immersed in the medium A, but without the electric current being applied. This served as the control. Five identical sets of catheters were prepared simultaneously. After 2, 4, 6, 8, or 24 h incubation at 37 °C in air, the catheters were removed and the number of viable bacteria on the external surfaces were determined by the roll-plate culture (section 2.8.1) for. This experiment was carried out in duplicate. Statistical analysis was performed by means of the unpaired *t*-test (two-tailed) applied to log₁₀ CFU values.

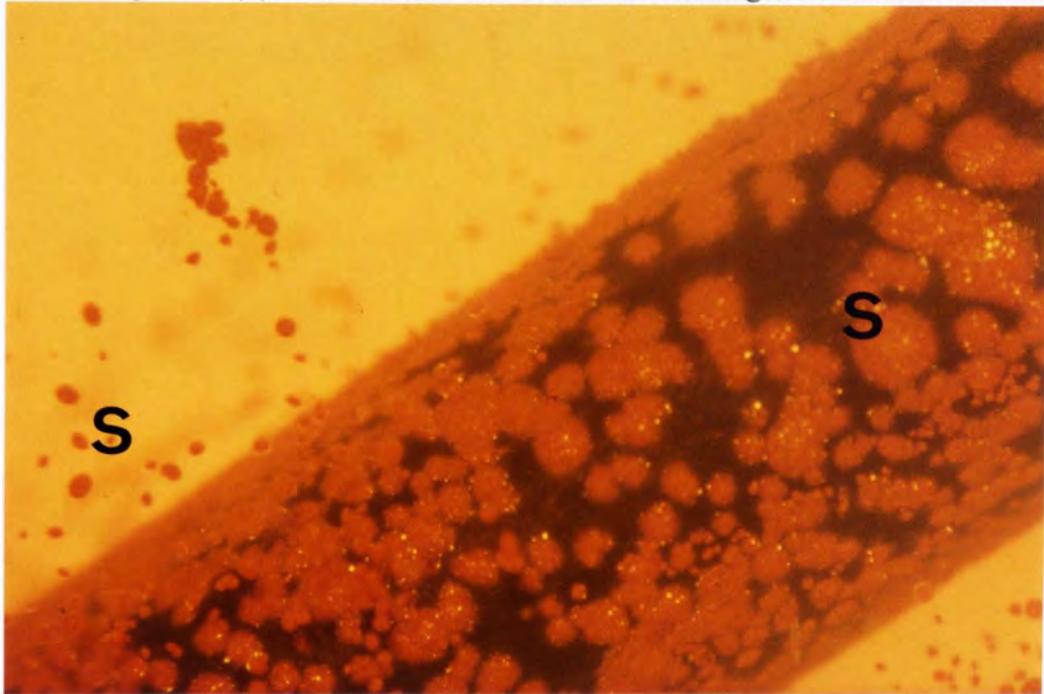
6.3 Results

6.3.1 Effect of 10 μ A DC on the growth of bacteria attached to catheters

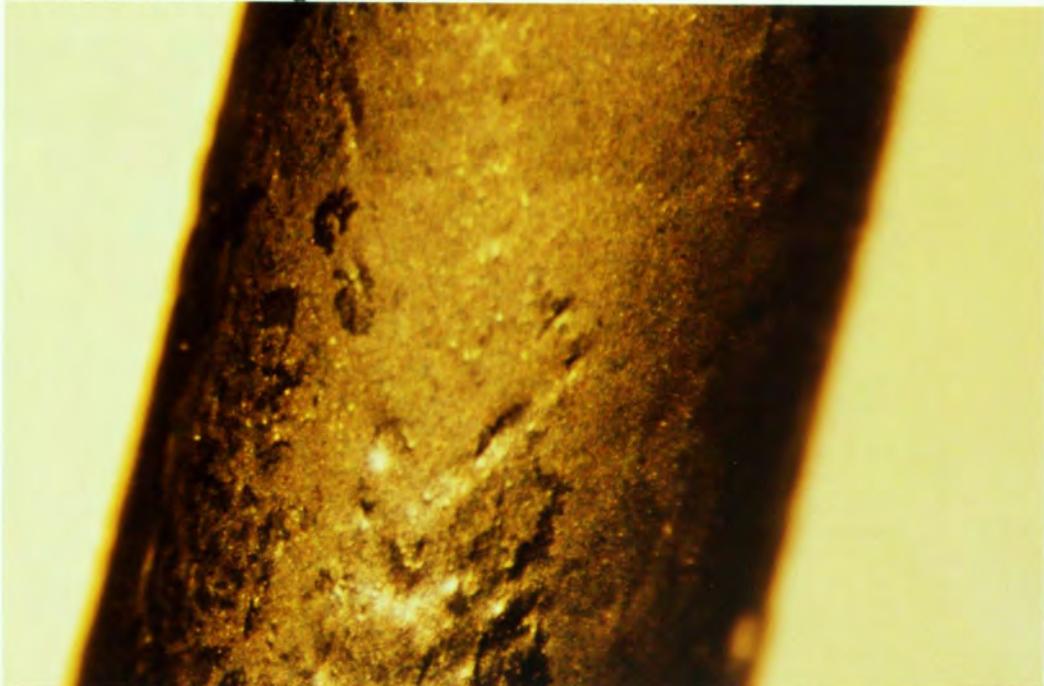
Observation of bacterial growth on the catheter surface by stereo-microscopy was aided by the incorporation of triphenyl tetrazolium in the agar. As bacteria grew, the salt was reduced to a red compound and so any bacterial colonies appeared red. After incubation for 16 h in nutrient agar, bacteria grew as discrete colonies on the surfaces of all the anodal and control catheters and also in the agar surrounding the catheters. Growth of the two strains of *S. aureus* (NCIMB 6571 and ATCC 6538), 5 strains of *S. epidermidis* (NCIMB 12721, clinical isolates 023, 462, 811 & 983) and *E. coli* NCIMB 8879 were inhibited on the surfaces of the cathodal catheters but not on the anodal or the control catheters. The inhibition of growth of *S. aureus* NCIMB 6571 on the cathodal catheter but not on the control catheter is a typical result and is shown in Figure 6-3. The entire length of the catheter surface was covered with bacterial colonies. When nutrient agar was poured over the colonised catheters, organisms detached from the surface were trapped in the agar surrounding the catheters. They grew as lancet shape colonies in the agar near the catheters. The size of the colonies was up to 2 mm in diameter. In contrast, bacterial growth was not detected on the entire surface nor near the vicinity of the cathodal catheters attached to the electrical device for 48 h. Identical results were observed for all the bacterial strains tested. Therefore, the cathodal catheter with a continuous 10 μ A inhibited bacterial growth on its surface and in the agar surrounding the catheter.

Figure 6-3 Inhibition of growth of *S. aureus* NCIMB 6571 on cathodal catheters after 48 h exposure to 10 μ A DC.

A) Bacterial growth (S) on the surface and in the surrounding of the control catheter.



B) Inhibition of bacterial growth on cathodal catheter.

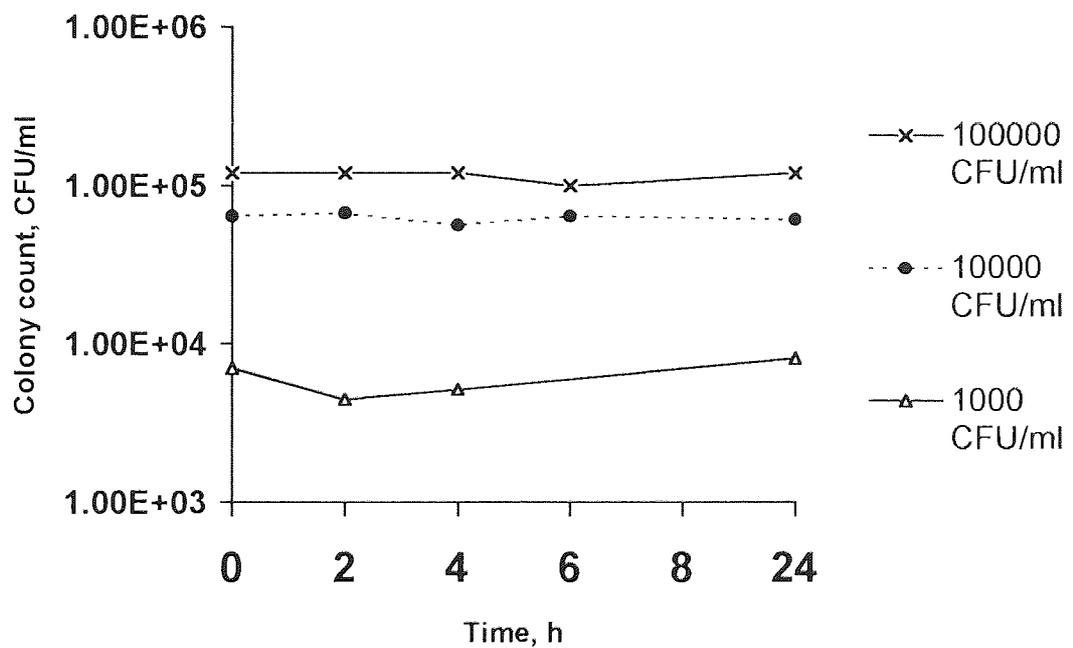


Note: Ten μ A DC was applied to colonised catheters after they were embedded in nutrient agar. External diam. of catheters was 2.4 mm. Photographs taken with catheters *in situ* after 48 h incubation.

6.3.2 Viability of *S. epidermidis* NCIMB 12721 in medium A

The colony counts of *S. epidermidis* NCIMB 12721 in medium A over the 24 h period did not change significantly (Figure 6-4). Medium A was therefore suitable to test the effect of 10 μ A DC on the viability of adhered staphylococci on catheter surfaces.

Figure 6-4 Viability of *S. epidermidis* NCIMB 12721 in medium A at 37 °C.



Note:

1. Colony count, CFU/ml was on a logarithmic scale, $1.00E+N = 1.0 \times 10^N$
2. Viability of *S. epidermidis* NCIMB 12721 was tested at three different inoculum sizes: ca. 1×10^3 , 1×10^4 and 1×10^5 CFU/ml ($n = 2$ for each concentration).

6.3.3 Effect of the electric current on the number of the viable bacteria attached to catheter surfaces

The number of bacteria attached to the external surface of the cathodal catheters after 2.5 h colonisation in nutrient broth, as determined by the roll plate method, was significantly reduced as compared to the control catheters after application of the 10 μA DC for 16 h ($p < 0.01$) (Table 6-1). By comparison, the rolled imprints made from the control catheters showed a semi-confluent growth of staphylococci which was not possible to accurately count. An estimate of approximately $>1,000$ CFU per cm of catheter was recorded. The number of CFU on the internal surface on the control catheter was less than the number of CFU on the external surface. Nevertheless, the number of bacteria attached to the internal surface of the cathodal catheters were also significantly reduced when compared with the control. Similar results were obtained for the three strains of staphylococcal tested.

Table 6-1 Number of bacteria on the catheter surfaces after application of 10 μA for 16 h, as determined by the roll plate method.

| Organisms | Mean number (range) of CFU/cm length of catheter | | | |
|--------------------------------------|--|----------------|------------------|----------------|
| | External surface | | Internal Surface | |
| | Control | Cathodal | Control | Cathodal |
| <i>S. epidermidis</i> NCIMB 12721 | >1000 | 24* (0-77) | 337 (180-600) | 60* (0-200) |
| <i>S. epidermidis</i> 983 | >1000 | 95* (0-440) | 280 (23-600) | 7* (0-35) |
| <i>S. epidermidis</i> 023 | >1000 | 10* (0-45) | 506 (150-700) | 23* (0-88) |

* $p < 0.01$ compared to control, $n = 6$

6.3.4 Susceptibility of bacteria after various catheter colonisation times

The number of viable bacteria present on the surfaces of both the control and cathodal catheters which were colonised for 2.5 and 16 h are shown in Table 6-2. When 10 μ A DC was applied to bacteria that had colonised the catheters for 2.5 h, a significant reduction on bacterial count was detected as early as 6 h ($p < 0.05$). The bacteria that had colonised the catheters for 16 h, however, appeared to be more resistant to the killing effect of the electric current. After 8 h of electrical application, confluent growth of bacteria was still obtained from the cathodal catheter, although bacterial growth was clearly reduced. However, after 24 h of electrical application, the viable count related to the cathodal catheter was significantly reduced when compared with that of the control catheter ($p < 0.05$).

Table 6-2 Number of *S. epidermidis* NCIMB 12721 attached to a catheter surface after either 2.5 or 16 h colonisation, followed by exposure to 10 μ A DC for various periods of time.

| Duration of electrical application, h | Mean number of CFU/cm catheter (range), $n=4$ 2.5 h colonisation | | Mean number of CFU/cm catheter (range), $n=4$ 16 h colonisation | |
|---------------------------------------|---|-----------------|--|--------------|
| | Control | Cathodal | Control | Cathodal |
| 2 | 75 (60-82) | 72 (37-100) | >1000 | >1000 |
| 4 | 226 (40-514) | 56 (4-110) | >1000 | >1000 |
| 6 | 341 (100-690) | 42 (0-130)* | >1000 | >1000 |
| 8 | 138 (46-220) | 36 (11-60)* | >1000 | >1000 |
| 24 | ND [#] | ND [#] | >1000 | 76 (25-282)* |

* $p < 0.05$ compared to the control catheter

ND= not done

6.4 Discussion

In this study, the investigation was concentrated on the activity of 10 μA against bacteria that had already adhered to the catheter surface. A cathodal catheter with 10 μA DC applied did not prevent adhesion nor did it affect the growth of staphylococci suspended in nutrient broth, but it inhibited growth of adherent bacteria embedded in nutrient agar.

To study the effect of electric current on bacteria adhered to an electro-conducting catheter, carbon catheters were incubated in a bacterial suspension in nutrient broth. After 2.5 h incubation, sufficient number of staphylococci remained adhered to the catheter surface following rinsing. The staphylococci adhered to the surface of catheters were either in pairs or clumps of four cells, so they were in the early stage of the growth cycle. Each rinsing procedure removed a certain proportion of the bacteria originally attached. Similarly, when molten nutrient agar was poured over the colonised catheters, a proportion of organisms detached from the catheter surface. These detached organisms were however trapped in the agar and then grew as discrete colonies after incubation. The number of bacteria detached from the catheters depended on the procedure that rinsed the colonised catheters. Rinsing by centrifugation was found to be most suitable as it resulted in the fewest bacteria detached so the least colonies formed. With other rinsing procedures, many more bacteria detached from the surface. They then grew as colonies on or near the catheters and so obscured the observation of bacterial growth actually on the catheter surface.

Adherent bacteria were inhibited from growth on the surfaces of cathodal catheters with a continuously applied 10 μA DC. This inhibitory activity was throughout the whole length of the catheter. All the bacterial strains tested including *E. coli* which was one of the least susceptible bacteria in the zone inhibition test were inhibited from growth. Moreover, the bacteria trapped in the agar in the surrounding of the cathodal catheter were also inhibited whilst those in the surrounding of the anodal or control catheter grew as discrete colonies. This agreed with the results from the zone inhibition test that the cathodal catheter with 10 μA DC inhibited growth of bacteria near or on the surface of the catheter.

To investigate whether the inhibition of bacterial growth on the cathodal catheter surface was bactericidal or bacteriostatic, the colonised catheter was immersed in culture broth. Electric current was then applied to the catheters for 16 h and a colony count of the bacteria attached to the surface of the catheter was performed. Nutrient broth was not used because 10 μA did not inhibit bacterial growth when they were suspended in it. Hence, any bacteria detached from the catheter surface would multiply in the broth. The resultant high cell density in the broth in which the cathodal catheters were suspended would conceal any inhibitory effect that it may have. Medium A was therefore chosen because the colony count of *S. epidermidis* NCIMB 12721 did not change significantly over 24 h at 37 °C (Figure 6-4). After application of electric current to colonised catheters, colony counts of the catheter surfaces were carried out by the roll-plate culture. The method was efficient in the removal of bacteria from the catheter surface and it used the least physical forces in comparison with sonication and vortex mixing (section 3.4.1). Moreover, sonication is synergistic with other antimicrobial agents (Ahmed & Russell, 1985) and it may amplify the bactericidal activity of electric current. At this stage, the activity and the mechanisms of the electric current were still under investigation and so a more conservative method of removal of organisms was adopted.

When colonised catheters were suspended in medium A, the colony count on the external and the internal surfaces of the cathodal catheters were greatly reduced (Table 6-1). This demonstrated that the cathodal catheter with a continuous 10 μA DC was bactericidal to bacteria attached to its surface. The lower bacterial density on the internal surface may be due to restricted access of organisms to the internal surface during colonisation or the impression imprints of the catheter not removing as many as organisms as the roll plate culture method. Nevertheless, bactericidal activity was also demonstrated on the internal surface of the cathodal catheter.

This bactericidal activity was apparent after 6 h of application of electric current. When the colonisation time was extended to 16 h, 24 h of electric current application was required to achieve bactericidal activity. This extra period of application of electric current may have been required because of the inoculum effect as a result of the increased number of organisms. Moreover, after 16 h of incubation in the staphylococcal

culture, micro-colonies would have formed on the catheter surface. This was inferred from section 6.3.1 in which colonised catheters embedded in nutrient agar after incubation for the same period were covered with colonies. Hence, the bacteria inside the colonies may be protected from any deleterious effect of electric current by the slime and exopolymers surrounding the cells.

In these studies of low voltage and low amperage electric current to prevent CVC associated infections, 10 μA DC via a cathodal catheter was shown to be bactericidal to organisms attached on their surfaces and also in the vicinity of the catheter. The size of the inhibition zone may give an indication of how close organisms have to be to the catheter surface in order to be affected. Ten μA was active against a wide range of bacteria, in particular staphylococci which commonly cause catheter-related infections. The duration required for killing was dependant on the number of organisms attached to the catheter. Previous studies showed that the cathodal catheter also repels bacteria from its surface and prevents migration (Crocker *et al.*, 1992). Therefore, a cathodal catheter with 10 μA DC has a high potential for use in the prevention of catheter-related infections.

Ten μA was the lowest possible amperage of electric current that had consistent bactericidal activity and is also within the safety limit for an electro-conducting device to be placed near the heart (BS 5724, 1989). Electric current of amperage more than 10 μA was more active around the electrodes in the zone of inhibition test and so may be useful in other body sites to prevent and treat infections. Davis *et al.* (1989) used 50 or more μA to eradicate bacteria in synthetic urine and suggested that this would be useful for the prevention of urinary tract infections caused by urinary catheters. One hundred CFU of *E. coli* and *P. mirabilis* in 10 ml of synthetic urine were reduced to an undetectable level after 4 h application of 50 μA . This bactericidal activity was dependent on the material of the electrodes with carbon the least active when compared to metallic electrodes. The bactericidal activity of electric current in synthetic urine was considered to be partly due to metallic ions. Indeed, silver impregnated fabric has been shown to be synergistic with electric current (Chu *et al.*, 1987; Falcone & Spadaro, 1986). This high bactericidal activity of electric current in synthetic urine is in complete

contrast to the lack of activity observed in the present study of electric current against staphylococci suspended in nutrient broth (section 5.3.2). These differences may be due to the higher amperage (50 to 400 μA), the smaller bacterial population (100 CFU), and volume of bacterial suspension (10 ml) in a broth with no protein (synthetic urine) and the nature of electrodes (metal) which Davis *et al.* (1992) used as compared to the present investigations (10 μA , 10^6 CFU, 100 ml, nutrient broth and carbon electrodes respectively) (section 5.3.2).

Another study akin to the present investigation was the application of electric current (DC) to bacteria on intact skin (Bolton *et al.*, 1980). After inoculation of the subject's skin with *S. epidermidis*, the area was covered with a piece of carbon impregnated polyolefin or polyvinyl chloride (PVC) plastic film (10 cm^2) with a piece of rayon fabric separating the skin and the film. The fabric was soaked with 0.4 ml of saline to provide a moist interface between the electrode and the skin. When 5 or more $\mu\text{A}/\text{cm}^2$ (up to a total of 50 μA) DC was applied to the plastic film for 20 h, the number of *S. epidermidis* on the skin was reduced from ca. 1×10^4 CFU to <10 CFU. This bactericidal effect was observed only when the carbon plastic film acted as the anode and no noticeable effect was obtained when it acted as the cathode. Hence, the bactericidal effect associated with the electrode is in complete contrast to the present study. Bactericidal activity was associated with the cathode as low as 2 μA in the zone of inhibition test and the zone sizes increased with the amperage applied. At 50 μA or more, both anode and cathode showed bactericidal activity as demonstrated by the presence of inhibition zones around both electrodes. This difference in the activity associated with electrodes may indicate different mechanisms of the bactericidal effect of DC electric current. Bolton *et al.* (1980) observed that the bactericidal activity associated with the anode diminished when the skin was separated from the rayon fabric with a piece of dialysis membrane. They inferred that the bactericidal activity was electrochemical in nature.

Khoury *et al.* (1992) also investigated the use of electric current in the prevention of infections associated with medical devices. They used the electrical Robbins device to study the effect of electric current to bacteria in a biofilm. The electrical Robbins device consisted of a series of removable stainless steel studs embedded in a rectangular box made of Perspex. A length of platinum wire was placed longitudinally at the bottom of

the box. An AC electric current was applied to the stud and the circuit completed via the platinum wire. Bacterial culture or fresh medium flowed from one end of the box, passing the surfaces of the studs, to the other end of the box as waste. Fifteen to 400 $\mu\text{A}/\text{cm}^2$ of electric current when applied alone did not reduce the number of cells in the biofilm that had already formed on the surface of the stainless steel studs, but it inhibited further growth. However, when antibiotics at a concentration that did not sterilise a biofilm combined with electric current were applied, the cells in the biofilm were sterilised after 8 to 10 h (Khoury *et al.*, 1992). This is not surprising as cells in biofilms are recalcitrant to treatment possibly due to penetration barriers (Costerton *et al.*, 1987) and slow growth rates (Brown & Gilbert, 1993). Moreover, the flow rate of the fresh medium at 60 ml/h would have been slow enough for the cells detached from the studs to grow to a high density. Electric current would not have any deleterious effect to these organisms suspended in the broth and hence they may be able to re-colonise the surface. In the present study, electric current was applied to the catheters after 2.5 h colonisation and was effective in killing them. Therefore, it appears that for an electric current to be effective in preventing bacterial colonisation of catheters, it should be applied immediately after the adhesion of organisms. The bactericidal activity of electric current to newly adhered bacteria and its synergistic effect with antimicrobial agents would make the use of electric current a very promising approach to the prevention and treatment of medical device related infections.

7. Mechanisms of bactericidal activity of electric current by the zone of inhibition test

7.1 Introduction

The present study demonstrated that 10 μA DC was bactericidal around the cathode and that there were differences in the bactericidal activity between the anode and the cathode. In the zone of inhibition tests, the bactericidal activity associated with the cathode was dependent on the amperage and staphylococci were more susceptible than aerobic Gram negative bacilli. Repulsion of bacteria from a negatively charged surface was first thought to be responsible for the formation of the inhibition zone (Crocker, 1993). The presence of non-viable staphylococci inside the inhibition zone (section 4.3.2) inferred that other mechanisms besides repulsion were taking place causing the bactericidal activity.

There are only a limited number of reports on bactericidal activity of low amperage ($<20 \mu\text{A}$) electric current and its mechanisms. The majority of the reports used electric current AC of 400 mA or more, and with *E. coli* as the test organism (Stoner *et al.*, 1982; Shimada & Shimahara, 1982 & 1985). The mechanisms reported in these investigations where 600 mA AC were used (Shimada & Shimahara, 1982 & 1985) may not be strictly applicable to the present findings using 20 μA . In the present study, a difference in amperage affecting the antimicrobial activity of electric current was clearly demonstrated in the zone of inhibition tests. There was no inhibition zone formed around the anode when 10 μA was applied. However, inhibition zones comparable in sizes to those around the cathodes were observed when 100 μA was applied (section 4.3.4). Therefore, even a 10 times difference in amperage resulted in a difference in antimicrobial activity which may reflect changes in mechanisms as well. Nevertheless, those reports on the mechanisms of bactericidal activity using hundreds of mA of electric current AC may give indications of the possible mechanisms when a much lower quantity of electric current was used.

Previous reports of the mechanisms of the bactericidal activity of electric current have demonstrated toxic substances produced by electrolysis as the main vehicles of bactericidal

activity. Stoner *et al.* (1982) detected free chlorine and bromine by the O-tolidine method after the application of AC electric current (0.1 to 1.0 A in total). The electricity was discharged in a suspension of *E. coli* in sodium chloride or sodium bromide solutions (0.05 % - 0.85 % aq.) via graphite electrodes. Without the halogen compounds in the solution, there was no bactericidal activity. It was suggested that the active agents at pH 7.0 were hypochlorous acid, HClO and hypobromous acid, HBrO rather than the free molecules. This hypothesis was based on the observation that deoxygenation of the medium significantly reduced the bactericidal activity and the optimal bactericidal activity at pH 7.0 coincided with the acid dissociation constant of HClO and HBrO.

Hydrogen peroxide which is another oxidising agent, was detected in solutions containing no chloride ions after the application of 100 to 300 mA/cm² AC (Shimada & Shimahara, 1982). The amount of H₂O₂ produced in the phosphate buffer solutions was proportional to the amperage and the duration of exposure to the electric current. The survival rate of *E. coli* increased 2000 times when the test was carried out under anaerobic conditions as compared to under aerobic conditions. The antimicrobial activities were further investigated by exposing *E. coli* to 600 mA/cm² AC under anaerobic conditions (Shimada & Shimahara, 1985a). After 5 h exposure, the survival rate of *E. coli* was 40 %. These viable and non-viable cells showed an increase in negative surface charges, a reduction in respiratory rate and possibly a modification of membrane permeability. Further experiments under anaerobic conditions demonstrated the leakage of DNA-related materials from the cells. Moreover, aggregation of intracellular DNA-related materials was enhanced as observed by thin section electron microscopy (Shimada & Shimahara, 1985b). It was concluded that 600 mA AC altered the structural, morphological and physiological properties of the bacteria and the production of H₂O₂ from electrolysis led to high bactericidal efficiency. The electrodes used in these experiments were carbon. Therefore, the antibacterial activity observed would be most likely due to electricity alone.

When metallic electrodes were used, electrolysis products of metallic origin may contribute to the bactericidal activity of electricity. Metals such as silver are known to be bactericidal and the application of electric current to silver compounds enhanced their bactericidal activity (Berger *et al.*, 1976; Chu *et al.*, 1987). Even a noble metal that is considered inert could be

electrolysed and produced antibacterial substances. Rosenberg *et al.* (1965) found that platinum electrodes were electrolysed to form complexes, $[\text{PtCl}_6]^-$, with chloride and ammonium ions. The double negatively charged species of the complexes were bactericidal and the neutral species inhibited cell division (Rosenberg *et al.*, 1967). The latter discovery led to the development of cisplatin and its extensive use as an anticancer drug (Rosenberg 1980).

Davis *et al.* (1991) investigated the bactericidal activity of electric current in synthetic urine and similarly reported that the nature of the electrodes affected the efficiency of the bactericidal activity. The carbon electrodes were the least effective and the gold electrodes the most effective in terms of minimal duration of electricity application. Further experiments also confirmed the previous findings (Pareilleux & Sicard, 1970) that there was residual toxicity in the media after electric current application and that chloride ions in the media were essential for the bactericidal activity (Davis *et al.*, 1992). Chlorine and chlorine based compounds were then identified as the main bactericidal agents (Davis *et al.*, 1994).

However, electrolysis products were not the only vehicles of bactericidal activity of electric current reported. Matsunaga *et al.* (1984) investigated the respiratory function of the cell under the effect of electric current, DC. The respiratory rate of the micro-organisms attached to an anodic electrode surface was reduced to ca. 25 % as compared to cells without electric current. After 10 min of application of +0.74 V (maximum current 10 μA), the intracellular enzyme CoA became dimerised and led to the reduction of the respiratory rate (Matsunaga *et al.*, 1985). It was suggested that the dimerisation of enzyme CoA was due to direct electron transfer between the electrode and the cell, that led to the inhibition of the respiratory function of the cell and eventually cell death. The electric potential at the anode may also affect the bacterial membrane structure since the application of +1.4 V for 1.0 min led to the lysis of sheep erythrocytes (Shinohara *et al.*, 1989).

Most reports used simple salt solutions such as 0.05 % NaCl or phosphate buffers (Stoner *et al.*, 1982; Shimada & Shimahara, 1982 & 1985). The effect of electric current in complex medium such as nutrient agar has not been investigated. Using the zone of inhibition test

developed in the present study (section 4.2), the mechanisms of the bactericidal activity of electric current were studied.

7.2 Materials and methods

7.2.1 Effect of anaerobic conditions, addition of catalase and MgSO_4 to the medium on the zone sizes

The effects of anaerobic conditions, addition of catalase and MgSO_4 to the medium on the size of the inhibition zones were investigated with four staphylococcal strains: *S. aureus* NCIMB 6571, *S. epidermidis* NCIMB 12721, *S. epidermidis* 983 and 811. Three colonies obtained from cultures on nutrient agar slopes were inoculated into 5 ml of nutrient broth. The resulting bacterial suspension was then incubated at 37 °C for 2 h. Four μl of the staphylococcal suspension (ca. 10^5 cells) was inoculated onto the surface of a nutrient agar plate. Four nutrient agar plates were prepared for each bacterium studied. Two carbon catheters, (2.5 cm long), were placed perpendicularly in one nutrient agar plate. The carbon catheters were then connected to an electrical device which generated 10 μA DC via external leads with one catheter acting as a cathode and the other as anode (Figure 4-1). The agar plate was then incubated at 37 °C, in air for 16 h. The second agar plate was flooded with 0.5 ml of a freshly prepared catalase solution (EC 1.11.1.6 of bovine liver origin, Sigma Chemical Co. Ltd., Poole, UK) which had 1500 units of activity (one unit decomposes 1.0 μmole of H_2O_2 per min at pH 7.0 at 25 °C at 10.3 mM H_2O_2). This agar plate was then left at room temperature for up to 5 min to allow the catalase to be absorbed by the agar. Two catheters were then placed perpendicularly in the agar, and electric current of 10 μA was applied as described above. This plate was then incubated at 37 °C, in air, for 16 h. The third agar nutrient agar plate was prepared by adding 10 μl of sterile 1.0 M MgSO_4 into 20 ml nutrient agar that had been melted and then cooled to 44 °C. After thorough mixing, it was then poured into a Petri dish and left to set. Two catheters were then placed perpendicularly in the agar, and electric current of 10 μA was applied as described above. The final agar plate of the set was placed in an anaerobic cabinet (Model Anaerobic 22290, Microflow Anaerobic System, Andover, UK) with an atmosphere of 10 % CO_2 , 10 % H_2 and 80 % N_2 at 37 °C. After 10 min, two catheters were placed in the nutrient agar plate and connected to one electrical device as described above. The plate was then incubated under these conditions for 16 h.

Following incubation, catheters were removed from the plates and the diameters of any zones of inhibition around the catheter insertion sites into the agar were measured with a vernier calliper. The diameter was taken as the total distance between the outer edges of the inhibition zone with the centre of the catheter acting as the mid-point. The staphylococcal strains were tested on six separate occasions. Oxygen concentration in the anaerobic cabinet was measured with an oxygen meter (OX 20, Walden Precision Apparatus LTD, Cambridge, UK).

7.2.2 Effect of various concentrations of catalase on the inhibition zone

To quantify the effect of catalase on the inhibition zone, various amounts of the enzyme were dissolved in PBS and added to 20 ml molten nutrient agar that had been cooled to 44 °C (final concentration of catalase ranged from 50 to 2500 units/ml agar). After thorough mixing, the plates were allowed to set. Four µl of staphylococcal suspension prepared as above was then spread onto each plate. Two carbon catheters were perpendicularly placed in the agar and connected to the electrical device which generated 10 µA DC. All plates were incubated in air, at 37 °C for 16 h. After the catheters were removed, the diameter of the inhibition zone was measured. *S. aureus* NCIMB 6571 and *S. epidermidis* NCIMB 12721 were tested six times at each concentration of catalase.

7.2.3 Effect of microaerophilic and anaerobic atmospheres on the inhibition zone

Four µl of staphylococcal suspension were spread onto each of five nutrient agar plates. Two catheters were then placed in each nutrient agar plate as described above. An electrical device which generated 10 µA was connected to the catheters of one of the five plates and was then incubated in air, at 37 °C. Another plate with the two catheters connected to an electrical device which generated 10 µA was placed in an anaerobic container. A palladium catalyst pack and a Gas Generating Kit, Campylobacter System (Oxoid BR 60) which was prepared according to the manufacturer's instructions were immediately placed in the container which was then sealed and incubated at 37 °C. Microaerophilic condition which has approximately 5 % oxygen was achieved within 30 min. The remaining three nutrient agar plates with the catheters were placed in an anaerobic cabinet. An electrical device which generated either 10,

75, or 100 μA DC was then connected to the catheters of each plate via external leads. All 5 nutrient agar plates were incubated for 16 h and then the diameter of the inhibition zone measured as described above. *S. aureus* NCIMB 6571 and *S. epidermidis* NCIMB 12721 were each tested six times.

7.2.4 Detection of hydrogen peroxide in the agar within the inhibition zone

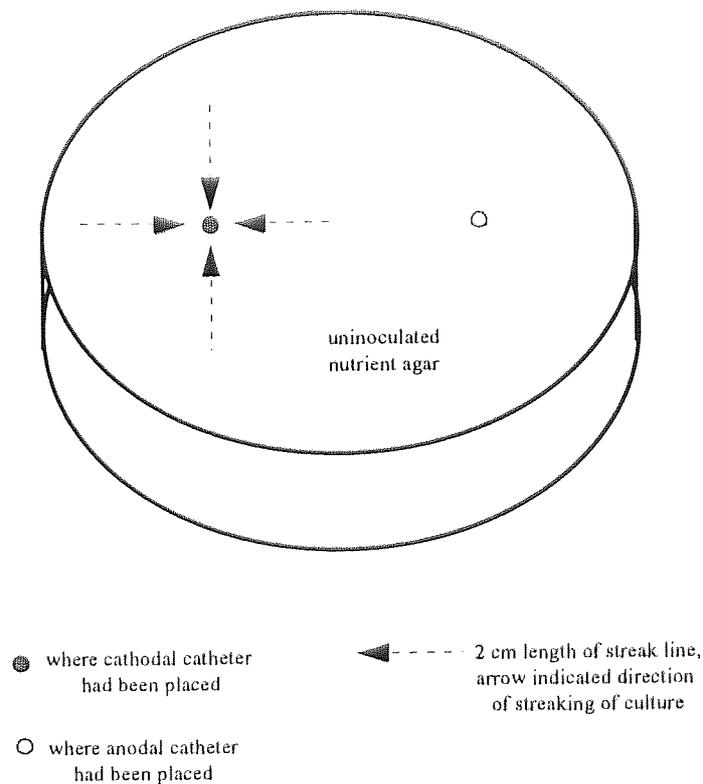
After measuring the diameter of the inhibition zone, a Merckoquant[®] strip (E. Merck, Darmstadt, Germany) to detect H_2O_2 was placed on the nutrient agar within the inhibition zone. The strip contained a peroxide which transfers oxygen from a peroxide or a hydroperoxide group to an organic redox indicator. The indicator is then converted to a blue-coloured oxidation product which can be visually detected. The strip measures between 0.5 to 25.0 mg/l hydrogen peroxide semi-quantitatively. The presence of hydrogen peroxide less than 0.5 mg/l was indicated by the presence of blue colour on the reaction area.

7.2.5 Demonstration of H_2O_2 in uninoculated nutrient agar that had been exposed to 10 μA DC

In the earlier experiments of the present study (section 4.3.3), uninoculated nutrient agar plates that had been exposed to 10 μA for 16 h were not inhibitory to subsequent bacterial growth. This is in contrast with the results in this section that H_2O_2 was the principal bactericidal agent associated with the cathode. To confirm the presence of H_2O_2 in uninoculated nutrient agar that had been exposed to 10 μA DC for 16 h, the experiment in section 4.2.3 was repeated. Two carbon catheters were placed perpendicularly at a distance of 5 cm into a nutrient agar plate that was not inoculated with bacteria. The catheters were then connected to an electrical device that generated 10 μA . Five agar plates were prepared and they were then incubated at 37 °C for 16 h in air. After incubation, the catheters were removed from the agar plate and immediately a Merckoquant[®] strip was impressed onto the surfaces of two agar plates surrounding the cathode and the anode. To determine whether the agar surrounding the cathodal catheter had been placed could support subsequent bacterial growth, a staphylococcal broth culture was inoculated onto the surface of the three remaining agar plates within 5

min after the removal of plates from the incubator. Instead of spreading the bacterial suspension by a dry swab as described in section 4.2.3, a loopful (2 μ l) of a 1 in 100 dilution of an overnight broth culture (ca. 10^5 cells) of *S. aureus* NCIMB 6571 or *S. epidermidis* NCIMB 12721 was streaked across the agar surface where the cathodal catheter had been placed (Figure 7-1). After inoculation, the plates were then incubated at 37 °C in air. The plates were examined for any inhibition zone after 8 and 24 h incubation.

Figure 7-1 Demonstration of H_2O_2 in uninoculated nutrient agar that had been exposed to 10 μ A DC for 16 h.

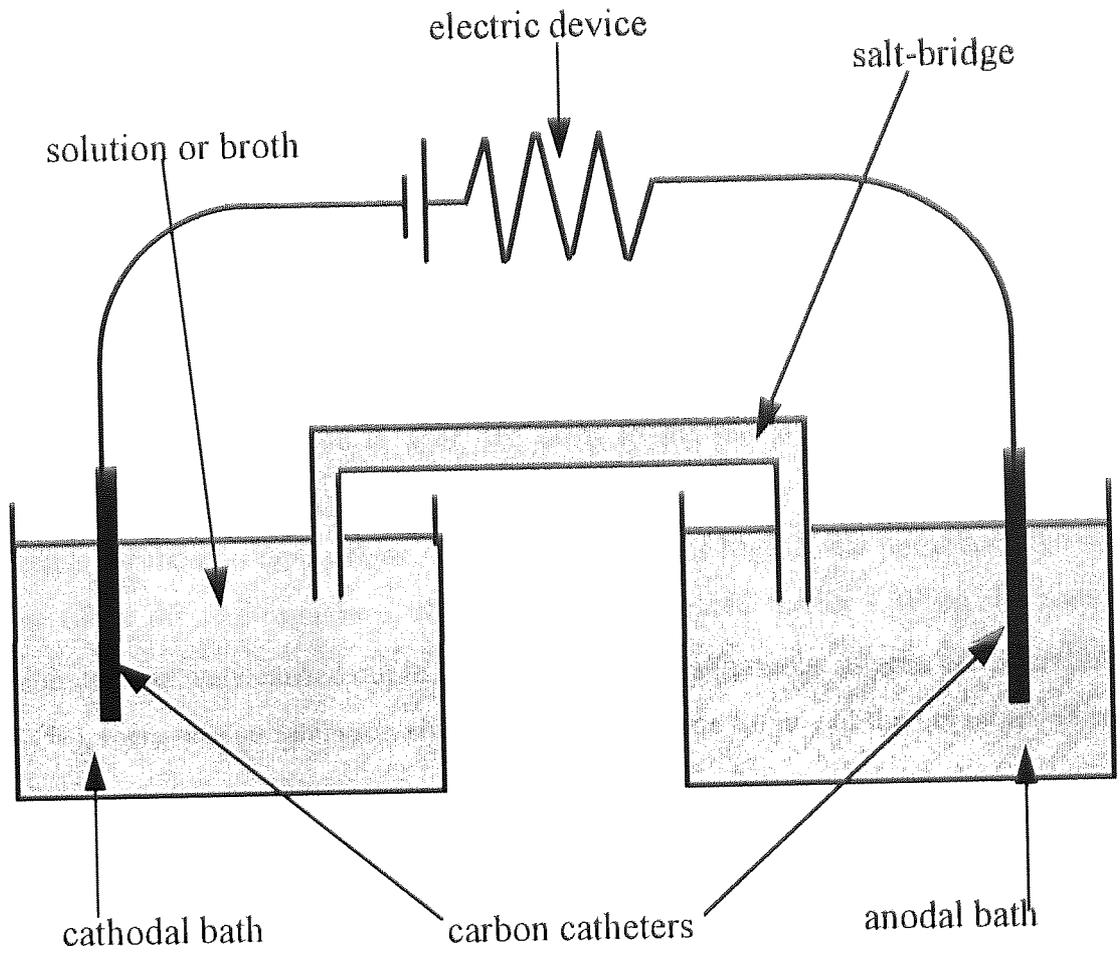


Note: After the staphylococcal culture had been streaked on the surface, the plate was incubated for 24 h and examined for an inhibition zone.

7.2.6 Detection of H₂O₂ and chlorine by the salt-bridge apparatus

The salt-bridge is a standard apparatus for investigating electrolysis (Figure 7-2). It consisted of two glass universal containers of 30 ml volume. Fifteen ml PBS, nutrient broth or medium B was placed in each container, and they were connected via the salt-bridge which consisted of a polyurethane tube (internal diameter 1.5 mm, and 150 mm length) filled with the same solution as in the containers. One carbon catheter (2.5 cm long) which acted as an electrode was then placed in the broth of each container. The catheters were then connected to an electrical device which completed the circuit. The ions in the solutions acted as conductors of electric current. Either 10 or 100 μ A were applied for 14 h and hydrogen peroxide was detected by a Merckoquant[®] strip. Free chlorine was detected by the *N,N*-diethyl-*p*-phenylene diamine colorimetric method using a Lovibond[®] chlorine kit (Tintometer Ltd, Salisbury, Wilts., UK) with a minimum detection limit of 0.1 mg/l free chlorine.

Figure 7-2 Salt-bridge apparatus.



7.3 Results

7.3.1 Mechanisms of bactericidal activity of electricity as investigated by the zone inhibition test

In the majority of experiments, there was no zone of inhibition around the anode with all the staphylococcal strains tested. On a few occasions, a rim (<1 mm width) of clear area around the catheter-agar interface was observed. The diameter of the inhibition zones produced around the cathode in air, in air with catalase, in air with 0.5 mM MgSO₄ and in anaerobic condition without catalase is shown in Table 7-1. The diameter of the inhibition zones in air for all staphylococcal strains was similar (mean 10.2 - 11.5 mm). Addition of catalase to the agar reduced the zone size for the three strains of *S. epidermidis* and there was no inhibition zone with *S. aureus* NCIMB 6571. Addition of MgSO₄ (final concentration in agar, 0.5 mM) to the media reduced the sizes of the inhibition zone with *S. epidermidis* 983 and 811 only, but there were no significant changes for the two type strains. Since the addition of MgSO₄ did not reduce the zone sizes of all the test organisms, this was not investigated further. When the zone of inhibition tests were carried out in anaerobic conditions (gaseous oxygen concentration < 0.1 %), there was no inhibition zone around the cathode for any of the organisms tested.

Table 7-1 Zones of inhibition produced around the cathode under aerobic and anaerobic conditions with or without catalase and with or without MgSO₄ (final concentration in agar, 0.5 mM).

Zone diam.¹ (mm): mean \pm S.D., $n = 6$

| Organisms | Aerobic | Aerobic, with catalase ⁵ | Aerobic, with 0.5 mM MgSO ₄ | Anaerobic, without catalase ⁵ |
|--------------------------------------|----------------|---|--|--|
| <i>S. aureus</i> NCIMB 6571 | 11.5 \pm 1.2 | 0.0 ² | 12.4 \pm 4.0 | 0.0 |
| <i>S. epidermidis</i> NCIMB 12721 | 10.2 \pm 1.0 | 5.4 \pm 2.1 ³ | 10.7 \pm 2.3 | 0.0 |
| <i>S. epidermidis</i> 983 | 11.3 \pm 1.0 | 7.2 \pm 1.1 ³ | 8.8 \pm 2.5 ³ | 0.0 |
| <i>S. epidermidis</i> 811 | 11.1 \pm 3.2 | < 4.0 ⁴ | 7.7 \pm 2.8 ³ | 0.0 |

Notes:

1. The zone of inhibition was measured from edge to edge across the centre of the catheter and the external diameter of the catheter was 2.3 mm.
2. No zone of inhibition observed, as the bacteria grew up to catheter-agar interface.
3. Significantly different from the zone sizes under aerobic conditions, $p < 0.01$, Wilcoxon Signed Rank Test.
4. No zone of inhibition observed ($n = 3$) and mean = 4.0 mm ($n = 3$)
5. The catalase (1500 units in total) was spread over the nutrient agar plate.

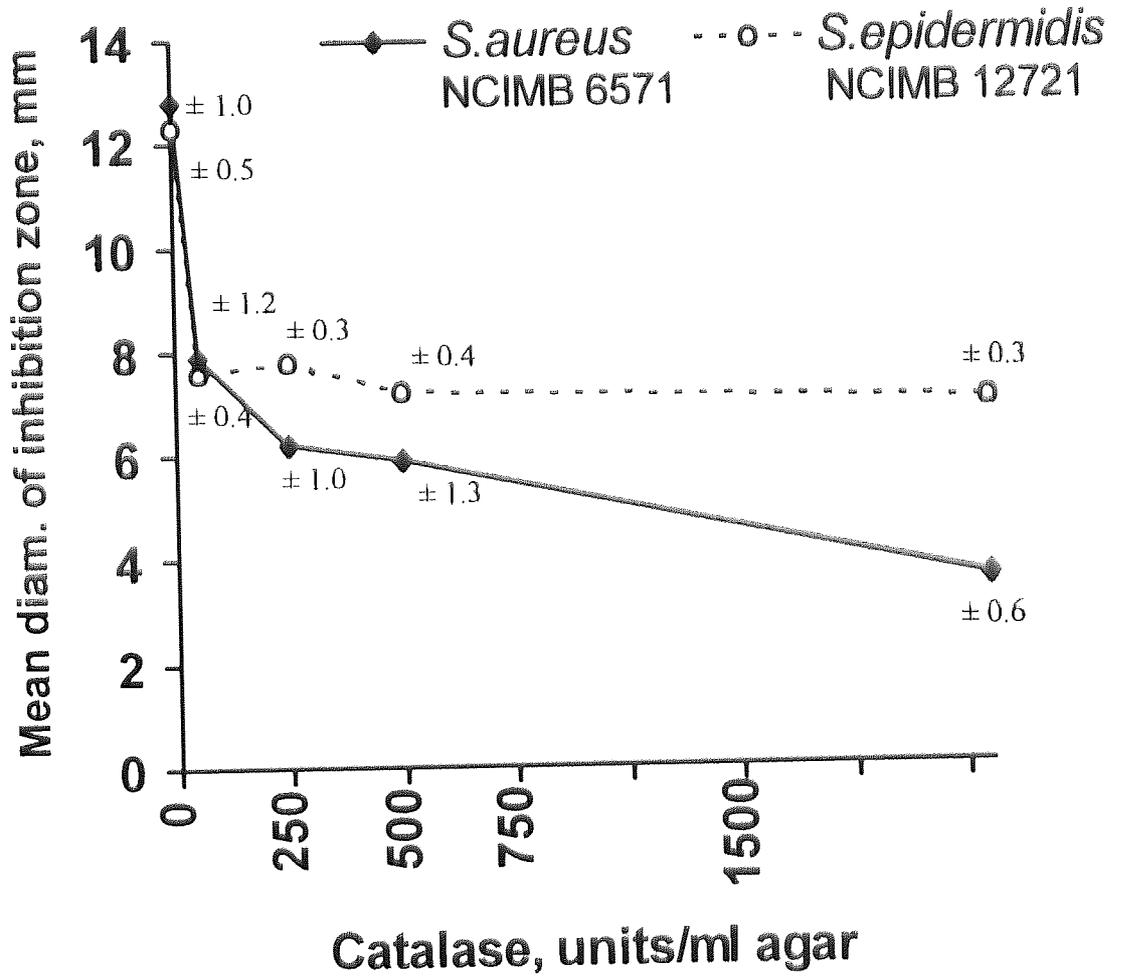
7.3.2 Effect of various concentrations of catalase on the diameter of the inhibition zone

Addition of catalase as low as 50 units/ml agar significantly reduced the zone size ($p < 0.01$, Wilcoxon Signed Rank Test) as compared to the control (Figure 7-3). When *S. epidermidis* NCIMB 12721 was tested, increasing the amount of catalase from 50 to 2,500 units/ml agar did not reduce the diameter of the inhibition zone any further nor eliminated it completely. With *S. aureus* NCIMB 6571, increasing the amount of catalase reduced the inhibition zone gradually, but still did not eliminate the inhibition zone completely even at the highest concentration (2,500 units/ml agar) of the enzyme.

7.3.3 Effect of microaerophilic and anaerobic atmospheres on the inhibition zone

The diameter of the inhibition zone around the cathode was reduced as oxygen concentration decreased under microaerophilic conditions (Table 7-2). When the zone of inhibition tests were carried out in anaerobic conditions, no inhibition zone was formed around the cathode and the organisms grew up to the catheter-agar interface. Increasing the electric current to 100 μA did not alter this effect. However, the absence of oxygen had no influence on the activity of the anode. In addition, as the amperage increased under anaerobic conditions, the zone size similarly increased.

Figure 7-3 Effect of various concentrations of catalase added to media on the inhibition zone size around the cathode (mean diameter ± 1 SEM, $n = 6$).



Note: The zone of inhibition was measured from edge to edge across the centre of the catheter and the external diameter of the catheter was 2.3 mm.

Table 7-2 Zones of inhibition produced under different atmospheric conditions.

Zone diameter¹
(mean \pm 1 S.D., mm; $n = 6$)

| Atmospheric conditions ² | Current, μ A | <i>S. aureus</i> , NCIMB 6571 | | <i>S. epidermidis</i> , NCIMB 12721 | |
|-------------------------------------|------------------|----------------------------------|----------------|--|----------------|
| | | Cathode | Anode | Cathode | Anode |
| Aerobic | 10 | 12.3 \pm 1.0 | 4.3 \pm 0.5 | 8.0 \pm 0.9 | 2.6 \pm 1.0 |
| Micro-aerophilic | 10 | 6.8 \pm 0.8 ³ | 3.2 \pm 1.3 | 6.0 \pm 1.4 ³ | 0.0 |
| Anaerobic | 10 | 0.0 ⁴ | 3.8 \pm 2.2 | 0.0 | 3.8 \pm 1.0 |
| Anaerobic | 75 | 0.0 | 12.8 \pm 3.5 | 0.0 | 13.6 \pm 1.0 |
| Anaerobic | 100 | 0.0 | 16.2 \pm 1.2 | 0.0 | 15.7 \pm 1.4 |

Note:

- The zone of inhibition was measured from edge to edge through the centre of the catheter and the external diameter of the catheter was 2.3 mm
- Oxygen concentrations under different conditions were: aerobic = 20.9 %, microaerophilic = ca.5 %, anaerobic = 0.1 %
- Significantly different from the zone sizes under aerobic conditions, $p < 0.01$, Wilcoxon Signed Rank test
- No zone of inhibition observed, as the bacteria grew up to the catheter-agar interface

7.3.4 Detection of hydrogen peroxide in agar within the inhibition zone

Hydrogen peroxide was detected in the agar within the inhibition zone around the cathode only. The concentration was up to 20 mg/l (0.59 mmol/l) at the catheter-agar interface and gradually diminished over a 2-3 mm distance from the catheter surface. Within the zone of inhibition in the agar with added catalase, hydrogen peroxide was not detected.

7.3.5 Detection of H₂O₂ in uninoculated nutrient agar that had been exposed to 10 μ A DC for 16 h

With the Merckoquant[®] strip, hydrogen peroxide was detected on the surface of the agar surrounding where the cathodal catheter had been placed. The concentration at the agar-catheter interface was up to 10 mg/l and gradually diminished as observed above (section 7.3.4). The other agar plates were inoculated with a loopful of the staphylococcal culture and then incubated. On the plates inoculated with *S. aureus* NCIMB 6571, inhibition zone was evident after incubation for 8 h. The plates were then incubated for a further 16 h. The inhibition zone sizes reduced and between 5 to 10 colonies were found within the inhibition zone in about half of the plates. In contrast, there were no inhibition zones on all the agar plates inoculated with *S. epidermidis* NCIMB 12721 after 24 h incubation, though within the 2 to 3 mm distance from the cathodal catheter the colony sizes were obviously reduced.

Table 7-3 Effect of a 16 h-discharge of 10 μ A DC in nutrient agar to its subsequent ability to support bacterial growth.

| Incubation period of inoculated plate at 37 °C , h | Mean diam. of inhibition zone ¹ around the cathodal catheter site, mm (± 1 SEM, $n = 8$) | |
|--|---|--------------------------------------|
| | <i>S. aureus</i> NCIMB 6571 | <i>S. epidermidis</i> NCIMB 12721 |
| 8 | 24.8 \pm 2.4 | not observable ² |
| 24 | 21.6 \pm 1.1 ³ | no zone ⁴ |

- Note:
1. The zone of inhibition was measured from edge to edge through the centre of the catheter and the external diameter of the catheter was 2.3 mm.
 2. After 8 h of incubation, no bacterial growth was observable.
 3. $n = 5$, because no zone of inhibition observed occasionally ($n = 3$)
 4. Organisms grew up to the edge of catheter-agar interface.

7.3.6 Detection of H₂O₂ and chlorine by the salt-bridge apparatus

After the application of 10 μ A DC, hydrogen peroxide was detected only in the broth associated with the cathode. It was detected semi-quantitatively by the Merckoquant[®] strip after 4 h of application of electric current and increased to 5-10 mg/l (0.15 - 0.30 mmol/l) after 16 h. However, chlorine was not detected in the broth associated with the anode throughout the 16 h. When 100 μ A was applied for 16 h, between 10 to 20 mg/l hydrogen peroxide was detected in the broth associated with the cathode, and 0.1 mg/l chlorine in the broth associated with the anode.

7.4 Discussion

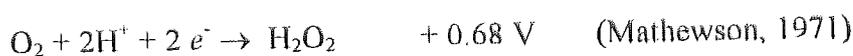
Toxic substances such as H_2O_2 and chlorine produced as a result of electrolysis have been demonstrated when hundreds of mA of AC electric current was applied to solutions (Shimada and Shimahara, 1982; Stoner *et al.*, 1982). Electrolysis may also have taken place when low amperage ($10 \mu\text{A}$) DC was applied. At the cathode, oxygen may be electrolysed to form H_2O_2 (Mathewson, 1971). Formation of an inhibition zone around a current carrying catheter placed in a lawn of bacteria suggested the effect of a concentration gradient and inferred the presence of a diffusible substance. Therefore, experiments in the present study were designed to detect H_2O_2 around the cathode in the zone of inhibition test. This was examined firstly, by spreading catalase which is a specific enzyme decomposing H_2O_2 on the agar to the diameter of inhibition zone.

The presence of catalase on the agar surface reduced the diameter of the inhibition zones around the cathodal catheter, but the four staphylococcal strains showed various degrees of reduction in zone diameter. Catalase is one of the most efficient enzymes with a turnover number of 4×10^7 (mole substrate)/(mole enzyme)/sec, which rapidly converts hydrogen peroxide to water and oxygen (Brill, 1966). With 1,500 units of catalase spread on the agar surface, no zone of inhibition was recorded with *S. aureus*. This was probably due to the higher intracellular content of catalase which some strains of *S. aureus* contain (Amin & Olson, 1968). This intrinsic catalase in combination with the added enzyme may have been sufficient to nullify completely the effect of any hydrogen peroxide produced. In contrast, a zone of inhibition was always formed with the three *S. epidermidis* strains, albeit much reduced zone sizes.

The high efficiency of catalase was further demonstrated by the effect of adding various amounts of catalase in the agar to the diameter of the inhibition zone. Concentrations as low as 50 units of catalase per ml agar reduced the zone size significantly, and increasing the catalase from 50 to 2,500 units/ml agar did not drastically reduce the zone sizes. The production of hydrogen peroxide must be very rapid and its concentration near the catheter-agar interface be so high that low concentration of catalase could not decompose it fast enough to completely eliminate an inhibition zone. The presence of

hydrogen peroxide in the inhibition zone was confirmed with the Merckoquant® strip which detect peroxide and hydroperoxide groups. The highest concentration (up to 0.6 mmol/l) of H₂O₂ produced was at the catheter-agar interface and diminished outwardly over a distance of 2-3 mm. Therefore, it appeared that the zone size around the cathode in the presence of media catalase was determined by the rates of diffusion and of catalytic decomposition of hydrogen peroxide.

The production of hydrogen peroxide at the cathode required oxygen. This is consistent with the half-cell reaction describing the electrolysis of hydrogen ions in the presence of oxygen:



The amount of hydrogen peroxide produced is in a stoichiometric relation with the concentration of oxygen. If hydrogen peroxide is the main agent for inhibition, the zone size should reduce in the absence of oxygen. Under microaerophilic conditions less hydrogen peroxide is produced as predicted by the half-cell equation and so the zone size decreased. This was confirmed in experiments carried out under anaerobic conditions, where no zones of inhibition were observed even when the amperage was increased to 100 µA (Table 7-2). Previous studies demonstrated the production of hydrogen peroxide when relatively high (400 mA) AC was applied to phosphate buffers without chloride ions (Shimada & Shimahara, 1982). However, the production of hydrogen peroxide was not related to either the cathode or anode because AC was used. In the present study, using the zone of inhibition test, hydrogen peroxide was shown for the first time to be associated with the cathode when lower levels of DC (10 µA) was applied.

In the demonstration of the antibacterial effect of 10 µA DC by the zone of inhibition test, the use of nutrient agar (CM3) that had a high divalent ions content completely neutralised the bactericidal activity around the cathode (section 4.3.1). It was hypothesised that one of the effects of electric current on bacteria was the disruption of the bacterial membrane. Since magnesium ions are essential to maintain the structure and function of bacterial membranes, the high concentrations of magnesium ions in the agar would protect the staphylococci from the disruption of the membrane by electric

current. Therefore, an inhibition zone test was carried out in nutrient agar with added magnesium ions to test this hypothesis. In comparison to the zone sizes in the nutrient agars with low magnesium ions content, the zone sizes around the cathodes were reduced when 0.5 mM MgSO_4 (final concentration) was added to nutrient agar. However, the reduction in the inhibition zone sizes was observed in the clinical strains only but not the type cultures. Thus, the protection by magnesium ions were not conclusive in the zone of inhibition test. Further experiments (Chapter 8) were carried out to investigate the effect of magnesium ions on the bactericidal activity of electric current with a special reference to H_2O_2 .

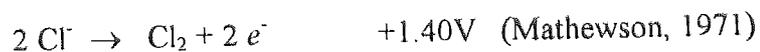
In the investigation of the bactericidal activity of 10 μA DC by the zone of inhibition test, nutrient agar that had been exposed to electric current did not inhibit subsequent bacterial growth (section 4.3.3). Hence, it was concluded that toxic substances were either not produced, or if they were produced, their quantity would be minute or the toxic substances were not long lasting enough to affect the nutrient agar to support subsequent bacterial growth. However, the present investigation (section 7.3.2) demonstrated that H_2O_2 was produced around the cathode. The amount of H_2O_2 produced after 16 h of discharge of 10 μA DC was sufficient to inhibit the growth of *S. aureus* NCIMB 6571 that was inoculated after the application of electric current. In contrast, there were no zones of inhibition formed with *S. epidermidis* NCIMB 12721 which confirmed the findings of the earlier experiment (section 4.3.3). The staphylococcal strains tested then were all *S. epidermidis* and none of the three strains had inhibition zones formed on the agar that had been exposed to electric current (section 4.3.3). Hence, the susceptibility of *S. epidermidis* to electricity was not due to H_2O_2 exclusively, but the concurrent application of electric current may be an important factor. The direct antibacterial effects of electric current on *S. epidermidis* was further investigated in later experiments (Chapter 9).

The use of only one species of staphylococci in the earlier experiment (section 4.3.3) led to a wrong conclusion. Another factor of not detecting the production of H_2O_2 in the earlier experiment (section 4.3.3) was the use of dry swabs to spread the bacterial suspension. Although the concentration of H_2O_2 detected by the Merckoquant® was as

high as 10 mg/l, it was concentrated at the agar-catheter interface and the total quantity produced and remained in the agar was small. So when the staphylococcal culture was spread by the dry swab (section 4.3.3), the H_2O_2 thus produced would have been spread over a large area of the agar surface and the resultant concentration was not high enough to inhibit bacterial growth. When the bacterial suspension was inoculated with a wire-loop without removing the pre-formed H_2O_2 , after 8 h incubation of the plate inhibition zones were observed around the site where the cathodal catheter was. However, the diameter of the inhibition zones reduced after further incubation of the agar plate. This is probably because the H_2O_2 formed was a limited quantity and also could be decomposed naturally or by the growing staphylococci. This further illustrates the advantage of a continuously applied electric current over an antimicrobial agent-loaded catheter, since it would ensure a continuous production of bactericidal substances such as H_2O_2 while electric current is applied.

The formation of inhibition zones around the anodal catheters was observed when 20 μA or more was applied (Table 4-1 & Figure 4-2). The zone size was not affected by the absence of oxygen (Table 7-2) and hydrogen peroxide was not detected inside the inhibition zone. It is likely that chlorine, or chlorine related compounds, were responsible for the formation of inhibition zones around the anode. Firstly, H_2O_2 was produced by electrolysis at the cathode and it would be logical that electrolysis also took place at the anode, which included electrolysis of chloride ions to chlorine molecules. Chlorine and chlorine-related compounds have been previously shown to be the bactericidal agents when 400 μA DC was applied in synthetic urine, but its production was not related either to anode or cathode (Davis *et al.*, 1994). As there was no strip method to detect chlorine in agar, the role of chlorine when low current was applied was investigated in a salt-bridge apparatus in this study. By linking the broth of the two containers, each with an electrode, via a salt-bridge, their contents remained separate but a complete electrical circuit was achieved. Any antibacterial substances produced in either container would therefore be independent of the corresponding electrode. When 10 μA was applied, no chlorine was detected in either containers. However, 0.1 mg/l chlorine was produced in the broth containing the anode when the electric current was increased to 100 μA DC. It is therefore likely that the inhibition zone observed around

the anode at $\geq 75 \mu\text{A}$ is partly due to chlorine. At the anode, chloride ions lose their electrons to produce chlorine molecules:



In a multi-ion situation such as a bacterial culture broth, all ions including chloride and hydronium ions may be electrolysed. However, the electrolysis reaction which will take place depends on factors such as the half-cell potential, the concentrations of the ions, and the physical state of the electrolysis products (Mathewson, 1971). The lower the half-cell potential, the easier for the reaction to take place. The potential for the formation of hydrogen peroxide (+ 0.68 V) is lower than the formation of oxygen from water at the anode (+ 1.23 V) or chlorine from chloride ions (+1.40 V) (Mathewson, 1984). Furthermore, oxygen is available in large quantity in aerobic conditions, and so facilitates the electrolysis of oxygen to hydrogen peroxide. As the half-cell potential for the production of chlorine from chloride by electric current is higher than that of hydrogen peroxide, chlorine was probably present only when an electric current of $\geq 75 \mu\text{A}$ was applied as observed in the experiment. The amount of electrolysis product is also proportional to the amount of electric charge available as is evident by the increase in zone size around the anode as the current increased.

This is the first report to demonstrate that electric current as low as $10 \mu\text{A}$ when applied to a multi-ion solution, electrolysed ions in the solution and consequently produced the antibacterial substances hydrogen peroxide and chlorine, at the cathode and anode respectively. Furthermore, generation of hydrogen peroxide (+ 0.68V) appeared to precede the production of chlorine (+ 1.4V) as current increased from 10 to $100 \mu\text{A}$. This may be due to the relatively instability and minute quantity of free chlorine produced which did not result in a zone of inhibition at $10 \mu\text{A}$. As the current increased to $50 \mu\text{A}$, both reactions took place concurrently. It is possible that other bactericidal substances such as HO_2 , ozone, OCl^- are also produced. Their presence is difficult to demonstrate since they are unstable and no practical specific methods are available.

The differentiation of H_2O_2 produced at the cathode and chlorine produced at the anode also explained the anomalous results that bactericidal activity of electric current was associated with the anode only but not with the cathode when it was tested on human

skin (Bolton *et al.*, 1980). Pieces of carbon impregnated plastic circle (10 cm²) were used as electrodes and staphylococci were inoculated between the circle and the skin. Up to 100 $\mu\text{A}/\text{cm}^2$ DC was then applied to the carbon-impregnated plastic circle for 24 h. The occlusive plastic circle would have prevented the penetration of oxygen. The skin bacterial flora between the skin surface and the plastic circle would also further reduce the oxygen concentration. Without oxygen, H_2O_2 would not be produced under the cathodal plastic circle. Conversely, with more than 100 μA DC applied to the anode, there would have sufficient quantity of chloride in the saline and sweat from the skin for electrolysis to take place to produce chlorine.

Hydrogen peroxide has not been considered as the main bactericidal agent by other workers. Stoner *et al.* (1982) reported the reduction of bactericidal activity of 0.1 to 1.0 A AC electric current when oxygen was removed from the bacterial suspension. This is possibly due to the absence of oxygen which led to H_2O_2 not being produced and so the bactericidal activity would drastically decrease. Davis *et al.* (1994) calculated the efficiency for the production of chlorine and chlorine based molecules by iontophoresis and stated that only a small percentage of the electrical energy in the system was used in the production of chlorine and related substances. It was suggested that the remaining electrical energy may have gone into heat or production of nonoxidant substances. Furthermore, the same quantity of chlorine and chlorine-based substances added to the bacterial suspension was not as effective as the chlorine and related compounds produced by iontophoresis. The production of H_2O_2 at the cathode could account for some of this apparent missing electric energy and the increased bactericidal activity of chlorine and chlorine based compounds in iontophoresis than the compounds alone.

Production of antibacterial substances as a result of electrolysis over a range of electric current (10 μA to 1.0 A), both DC and AC, has been conclusively demonstrated in the present study and previous studies (Stoner *et al.*, 1982; Shimada & Shimahara, 1982). Electrolysis takes place at the surface of the electrodes (Mathewson, 1971) which may mean the bacteria attached to a current carrying catheter would be exposed to high concentrations of these oxidative compounds. Yet their concentrations have not been so high that they were toxic to the surrounding cells, since bacteria in a 100 ml nutrient broth were not inhibited

(section 5.3.2). Therefore, an electric current carrying catheter has many potentials in the prevention of catheter related infections.

8. Magnesium ions and bactericidal activity of electric current

8.1 Introduction

In the study of the activity of electric current by the zone of inhibition test (section 4.2.1), replacing nutrient broth no. 2 (CM 67) and agar no. 1 (L11) with nutrient agar (CM3) reduced the susceptibility of staphylococci to the bactericidal activity associated with the cathodal catheter (section 4.3.1). The reduction of the inhibition zones was dramatic, as no zones of inhibition were obtained when nutrient agar (CM3) was used. The two media contained identical but different quantities of proteinous constituents (Bridson, 1990a). Agar No. 1 (L11) is specified as low in Mg^{2+} and Ca^{2+} ion contents and suitable for antimicrobial susceptibility testing in agar (Bridson, 1990b) whereas the cations contents of the agar in nutrient agar (CM3) has not been specified but is likely to be high. The probable high concentrations of Mg^{2+} and Ca^{2+} ions in the nutrient agar (CM3) were hypothesised as the cause of the elimination of the inhibition zone around the cathodal catheter (section 4.3.2).

The susceptibility of bacteria to antimicrobial agents in the agar diffusion test influenced by divalent cations in the media have long been recognised (Gilbert *et al.*, 1971). *P. aeruginosa* for example was shown to be more susceptible to the aminoglycosides when grown in a medium deficient in calcium and magnesium ions (Shand *et al.*, 1988). Mechanisms of the altered susceptibility to antimicrobial agents may be due to the chelation of the cations by the agar and so reduce the availability of the ions. The metallic cations might also affect the activity of antimicrobial agents indirectly by altering some constituents in the medium which in turn affects the drug or the micro-organisms. Finally, it may be the interaction between cations and the bacterium (Barry, 1986).

Divalent cations are essential for bacterial growth and magnesium ions are an absolute requirement (Jasper & Silver, 1977). In general, the intracellular content of magnesium in bacterial cells is 10 - 20 mM and the concentration increases to as high as 100 mM in exponentially growing *E. coli* (Hughes & Poole, 1989a). Many enzymes are activated by magnesium, including phosphohydrolases and phosphotransferases (Jasper & Silver, 1977). In addition to activation of enzymes, Mg^{2+} ions control the structure of nucleic

acid by neutralising the negative charges of the phosphate group of nucleic acids. The general folding and conformation of tRNA are maintained by Mg^{2+} ions, which in turn affect the binding of tRNA to amino acids. In protein synthesis, the dissociation and association equilibrium of ribosome is stabilised by Mg^{2+} and potassium ions (Hughes & Poole, 1989b). Mg^{2+} ions also stabilise the cell wall structure of *Halobacterium spp.* as a result of surface binding. In *E. coli*, Mg^{2+} ions bind firmly to lipopolysaccharide on the outer monolayer and the underlying peptidoglycan (Beveridge, 1989). Furthermore, the structural integrity of mitochondria depends upon Mg^{2+} (Hughes & Poole, 1989b).

The essential role of magnesium ions in bacterial membrane integrity is also reflected in the alteration of susceptibility of bacteria to antimicrobial agents. When *P. aeruginosa* was grown in Mg^{2+} -depleted culture medium, the components in the cell envelopes changed and subsequently altered the membrane permeability. Cells that are Mg^{2+} deficient are more resistant to polymyxin and EDTA (Kenward *et al.*, 1979; Shand *et al.*, 1988). In contrast, excess Mg^{2+} reduced the enhancing effect of bacitracin to toxicity of divalent cations in *Bacillus licheniformis* (Haavik, 1976). The significance of Mg^{2+} in the cell wall of gram-positive bacteria is also clearly shown in the study of sublethal heat injury of *S. aureus*. Up to 40 % of Mg^{2+} along with ester bound D-alanine of teichoic acid were lost. Repair of injured cells began with rapid and strong binding of Mg^{2+} ions resulting in the intracellular Mg^{2+} concentration reaching normal levels within 1 h during recovery (Hurst *et al.*, 1975).

In this present study the effect of magnesium ions on the susceptibility of staphylococci to 10 μ A DC was investigated. The viability of *S. epidermidis* NCIMB 12721 when exposed to H_2O_2 alone in the presence of Mg^{2+} ions was also studied. To investigate the mechanisms by which Mg^{2+} protects bacteria from H_2O_2 , the catalytic decomposition of H_2O_2 by Mg^{2+} was also examined.

8.2 Methods

8.2.1 Effect of Mg^{2+} ions on the susceptibility of a staphylococcal suspension to 10 μA DC

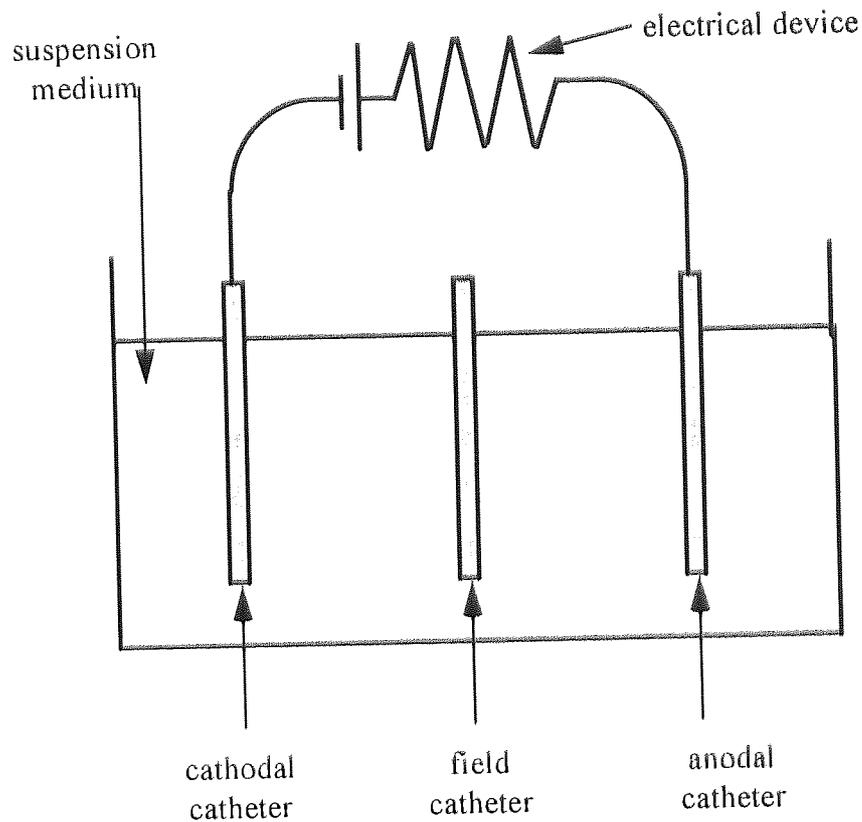
One colony of either *S. aureus* NCIMB 6571 or *S. epidermidis* NCIMB 12721 was inoculated into 20 ml of nutrient broth and incubated overnight at 37 °C in air. After incubation, the bacterial suspension was centrifuged at 660 g for 5 min. The supernatant was then removed and the cell deposit resuspended in 30 ml PBS. The cell suspension was again centrifuged and resuspended as described above to remove any Mg^{2+} . The staphylococci were then resuspended in 100 ml of medium A to a final cell density of ca. 1×10^7 CFU/ml. Three aliquots of 100 ml of staphylococcal suspension were prepared. One of the suspensions was the control which was not exposed to electric current. Two sterile carbon catheters were immersed vertically into each of the remaining two bacterial suspensions and each pair of catheters was then connected to an electrical device that generated 10 μA DC (Figure 5-1). Sterile $MgSO_4$ solution was added to one of the staphylococcal suspension (final concentration of Mg^{2+} in medium A: 0.125 mM) with 10 μA applied. The bacterial suspensions were then incubated at 37 °C in air for 24 h with continuous stirring on a magnetic stirrer (speed of stirring: 100 rpm). Colony counts of each suspension were carried out at timed intervals. The experiment was repeated once for *S. epidermidis* NCIMB 12721 and four times for *S. aureus* NCIMB 6571.

8.2.2 Effect of magnesium and calcium ions in the suspension medium on the susceptibility of adherent bacteria to 10 μA DC

Twelve catheters, each of 2.5 cm length, were placed in 100 ml of nutrient broth containing 1×10^6 CFU/ml of *S. epidermidis* NCIMB 12721. Colonisation of the catheters was carried out by incubating the broth at 37 °C, in air with stirring for 2.5 h (section 2.6.2). The catheters were then rinsed in PBS by centrifugation three times to remove loosely attached organisms (section 2.7.2). To determine the number of bacteria adhered to the catheter, three catheters were cultured by the roll plate method (section 2.8.1). The remaining nine catheters were divided into three sets. One set of catheters

was immersed in 100 ml medium B without magnesium or calcium ions. They were not connected to any electricity source and served as the control. The second set of catheters were immersed vertically in medium B without magnesium or calcium ions. Two of the catheters were connected to an electrical device that generated 10 μ A DC with one catheter acted as the cathode and the other as the anode. The third catheter was not connected to the electricity source but immersed in the broth. This was the field catheter that was effected by the potential and field effect but not directly by the electric current (Figure 8-1). The third set of catheters were immersed in medium B with calcium chloride or magnesium sulphate added (final concentrations of both in broth: 0.2 mM). Two catheters were connected to the electrical device as described above and the third catheter was the field catheter. All the broth containing the catheters were then incubated at 37 °C, in air, with stirring on a magnetic stirrer (speed of stirring: 100 rpm). After 16 h, all the catheters were then removed and cultured by the roll plate method (section 2.8.1) to determine the number of bacteria on the catheter surfaces. The experiment was repeated three times.

Figure 8-1 Application of 10 μA DC to pre-colonised catheters suspended in broth.



8.2.3 Effect of Mg^{2+} ions on the susceptibility of *S. epidermidis* NCIMB 12721 to various concentrations of H_2O_2

One colony of *S. epidermidis* NCIMB 12721 was inoculated into 5 ml of nutrient broth and incubated at 37 °C for 2 h. After incubation, the number of bacteria was estimated by turbidity (section 2.5.2). The bacterial suspension was then filtered through a sterile cellulose acetate membrane of 0.2 μm pore size (Acrodisc®, Gelman Sciences, Ann Arbor, USA). The staphylococci on the membrane was resuspended in 5 ml of medium B without Mg^{2+} ions and then filtered again with a new membrane filter. This was repeated to remove Mg^{2+} loosely attached to the bacteria. After the final filtration, the bacteria were resuspended in medium B without Mg^{2+} to give a final cell density of ca. 2.5×10^7 CFU/ml.

A 1 in 30 aqueous dilution of a 3 % solution of H_2O_2 (Thornton & Ross, Huddersfield, England) was prepared immediately before use to give a 1.0 g/l solution. From this 1.0 g/l solution, various concentrations of H_2O_2 in medium B without Mg^{2+} were prepared according to the table below:

| H_2O_2 , final conc., mg/l | 0 | 1 | 10 | 20 | 40 | 80 |
|--|-----|------|------|-----|-----|-----|
| 1000 mg/l H_2O_2 , ml | 0.0 | 0.05 | 0.5 | 1.0 | 2.0 | 4.0 |
| medium B without Mg^{2+} , ml | 50 | 50 | 49.5 | 49 | 48 | 46 |

Two series of H_2O_2 solutions were prepared and 0.125 ml of 0.2 M MgSO_4 was added to each container of the second series of H_2O_2 solution (final concentration of Mg^{2+} : 0.5 mM). Two hundred μl of the rinsed suspension of *S. epidermidis* NCIMB 12721 (ca. 2.5×10^7 CFU/ml) was then added to each container of the series of H_2O_2 solutions in timed intervals. The bacterial suspensions were incubated at 37 °C in air for 20 h. At timed intervals, colony counts of each staphylococcal suspension were carried out by removing 0.5 ml of the well mixed suspension to 4.5 ml of MRD containing ca. 15000 units of catalase (EC 1.11.1.6, Sigma Chemical Co. Ltd. Poole, UK). After thorough mixing, colony counts of the diluted bacterial suspension were determined by the Spiral System™ immediately. The experiment was repeated once.

8.2.4 Effect of various concentrations of Mg^{2+} on the susceptibility of *S. epidermidis* NCIMB 12721 to 20 mg/l H_2O_2

One colony of *S. epidermidis* NCIMB 12721 was inoculated into 5 ml of nutrient broth and incubated at 37 °C for 2.5 h. After the incubation, the number of bacteria was estimated by turbidity (section 2.5.2). The bacterial suspension was then filtered through a sterile cellulose acetate membrane (pore size: 0.2 μm , Acrodisc®, Gelman Sciences). The staphylococci on the membrane were resuspended in 5 ml of medium B without Mg^{2+} and then filtered again with a new membrane filter. This was repeated once more to remove any Mg^{2+} derived from the nutrient broth. After the final filtration, the

bacteria were then resuspended in medium B without Mg^{2+} to give a cell density of ca. 2.5×10^7 CFU/ml.

Sterile 0.01 and 1.0 M MgSO_4 solutions were prepared and then aliquots were added to 48 ml of medium B to give a final concentrations of 50, 500 and 5000 μM . Five hundred μl of the staphylococcal suspension was added to each of the 49 ml medium B containing various concentrations of MgSO_4 and also to 49 ml of medium B without the addition of MgSO_4 .

A 1 in 30 aqueous dilution of a 3 % solution of H_2O_2 (Thornton & Ross, Huddersfield, England) was prepared immediately before use to give a 1.0 g/l solution. One ml of the H_2O_2 was added to each of the 49 ml bacterial suspensions prepared above to give a final concentration of 20 mg/l H_2O_2 . The broth was then thoroughly mixed and incubated at 37 °C in air for 6 h. At timed intervals, 0.5 ml of the bacterial suspension was removed and added to 4.5 ml of MRD which contained ca. 15,000 units of catalase (section 8.2.6). After thorough mixing, colony counts of the diluted bacterial suspension was carried out using the Spiral System™ (section 2.5.1). The experiment was repeated once.

8.2.5 Decomposition of H_2O_2 in the presence of Mg^{2+}

From a freshly prepared 150 mg/l H_2O_2 solution, 3 tubes of 10 ml diluted H_2O_2 (final concentration: 15 mg/l) were prepared. Various aliquots of an one molar solution of MgSO_4 were then added to two of the three tubes to give a final concentration of MgSO_4 of either 1 or 100 mM. The tubes were placed at 37 °C and the H_2O_2 concentrations was measured with the Merkoquant® strips at timed intervals for 8 h. The experiment was repeated twice.

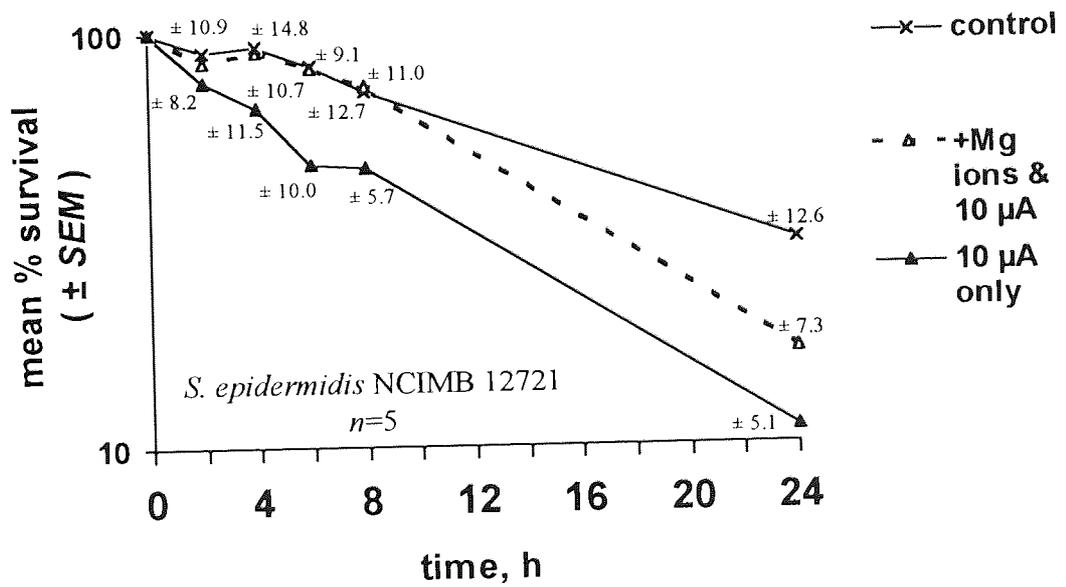
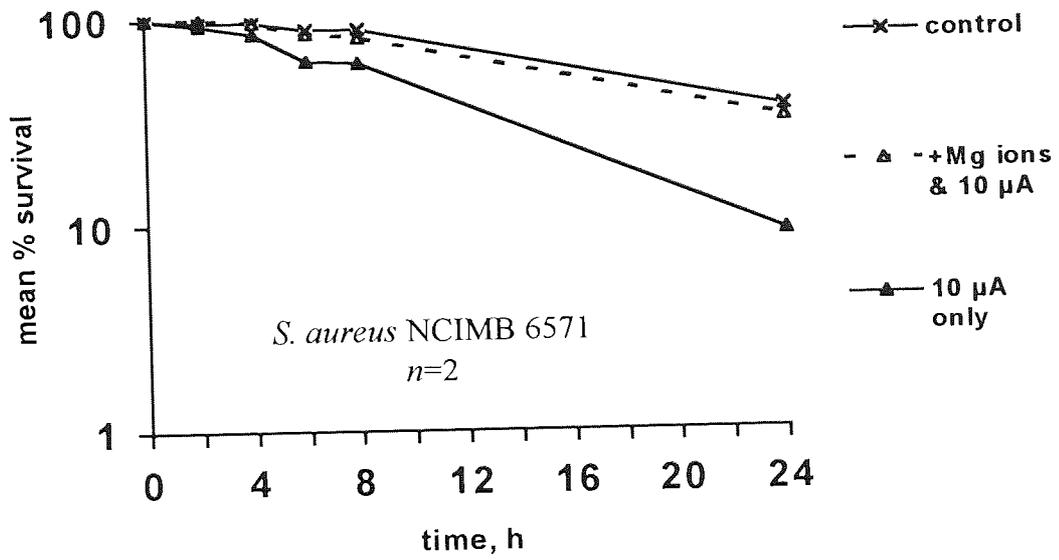
8.3 Results

8.3.1 Effect of Mg^{2+} in medium A on the susceptibility of staphylococci to 10 μA DC

Staphylococci were suspended in medium A which contained no magnesium ions and it maintained the viability of bacteria. During incubation in air and with continuous stirring, the colony counts of the control suspension of *S. aureus* NCIMB 6571 that had no electric current application remained constant for the first 8 h and then gradually decreased. In comparison, under the same conditions, the colony count of *S. epidermidis* NCIMB 12721 showed a more gradual decline from the commencement of the experiment (Figure 8-2).

When the staphylococcal suspension was exposed to 10 μA DC, the colony counts decreased more rapidly than the control and was significantly lower ($p < 0.01$) than the control after 4 h of electricity application. After 24 h application of electricity, the colony count was reduced to 10 % of the control. In comparison, addition of $MgSO_4$ (final concentration: 0.125 mM) to the suspension reduced the susceptibility of staphylococci to electric current. The survival curve of the staphylococci suspended in medium A with Mg^{2+} was similar to the control (Figure 8-2).

Figure 8-2 Effect of $MgSO_4$ (final concentration: 0.125 mM) on the susceptibility of staphylococci suspended in medium A to 10 μA DC.



8.3.2 Effect of magnesium and calcium ions in the suspension medium on the susceptibility of adherent bacteria to 10 μ A DC

Carbon catheters were colonised with *S. epidermidis* NCIMB 12721 and then rinsed by centrifugation (section 2.7.2). The number of bacteria on the surfaces of the catheters was determined by the roll plate method (section 2.8.1). After incubation, the bacterial growth on the roll-plate imprint was semi-confluent, so the colony count was recorded as >1000 CFU/cm length catheter.

One set of catheters was immersed in medium B without Mg^{2+} nor Ca^{2+} ions and served as the control. No electric current was applied to the control catheters whilst incubated at 37 °C. The colony counts of the *S. epidermidis* on the external surface of the catheters after incubation were again >1000 CFU/cm length catheter. Hence, there was no apparent decrease in the colony counts of bacteria on the colonised catheters after 16 h at 37 °C in the absence of Mg^{2+} and Ca^{2+} ions .

Three catheters were immersed in medium B with or without Mg^{2+} or Ca^{2+} ions added. Two of them were connected to an electrical device that generated 10 μ A and the third catheter that was not connected to the electrical device acted as the field catheter. Ten μ A DC applied to the catheters for 16 h at 37 °C reduced the colony counts of the cathodal catheters in the absence of Mg^{2+} and Ca^{2+} ions (Table 8-1). The colony counts on the surface of the anodal catheter with or without Mg^{2+} ions were reduced to an even lower count than the cathodal catheter. With the addition of $MgSO_4$ (final concentration: 0.2 mM), but in the absence of Ca^{2+} , to the medium, the number of bacteria on the surface of the cathodal catheters did not decrease. With the addition of $CaCl_2$ only (final concentration: 0.2 mM), the number of bacteria on the surfaces of all the catheters were reduced to a similar degree.

Table 8-1 Effect of Mg^{2+} and Ca^{2+} ions in the suspension medium on the susceptibility of adherent bacteria that had been exposed to 10 μ A DC for 16 h.

| | Mean no. (± 1 SEM, $n = 3$) of <i>S. epidermidis</i> NCIMB 12721 on catheters immersed in medium B with different ion content ¹ , CFU/cm length of catheter | | |
|--------------------|--|--|--|
| Catheters | without Mg^{2+} nor Ca^{2+} ions | $MgSO_4$ added final conc., 0.2 mM | $CaCl_2$ added final conc., 0.2 mM |
| Cathodal | 256.7 ± 19.1 | >1000 ³ | 478.7 ± 216.3 |
| Anodal | 47.7 ± 10.1 | 78.3 ± 49.7 | 433.0 ± 190.5 |
| Field ² | >1000 | >1000 | 256.0 ± 144.6 |

Notes: 1. Medium B consisted of glycerol, 10 mM; $(NH_4)_2SO_4$, 6 mM; $MgSO_4$, 0.5 mM; KCl, 13.5 mM; KH_2PO_4 , 28 mM; Na_2HPO_4 , 72 mM; thiamine, 1 mg/l; biotin, 0.5 mg/l; peptone, 0.5 g/l.

2. Field catheter was placed in the same broth as the two electrodes, but not connected to the electrical device.

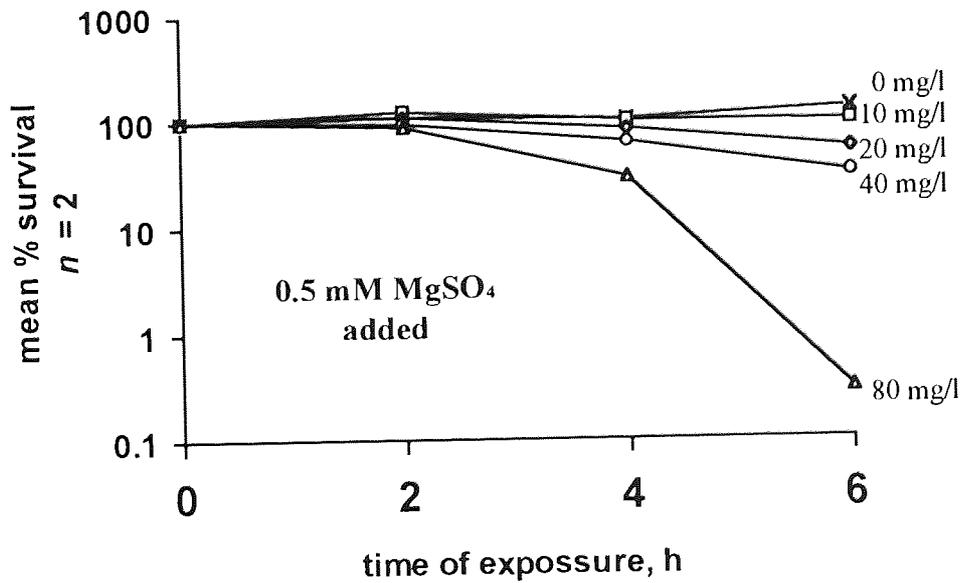
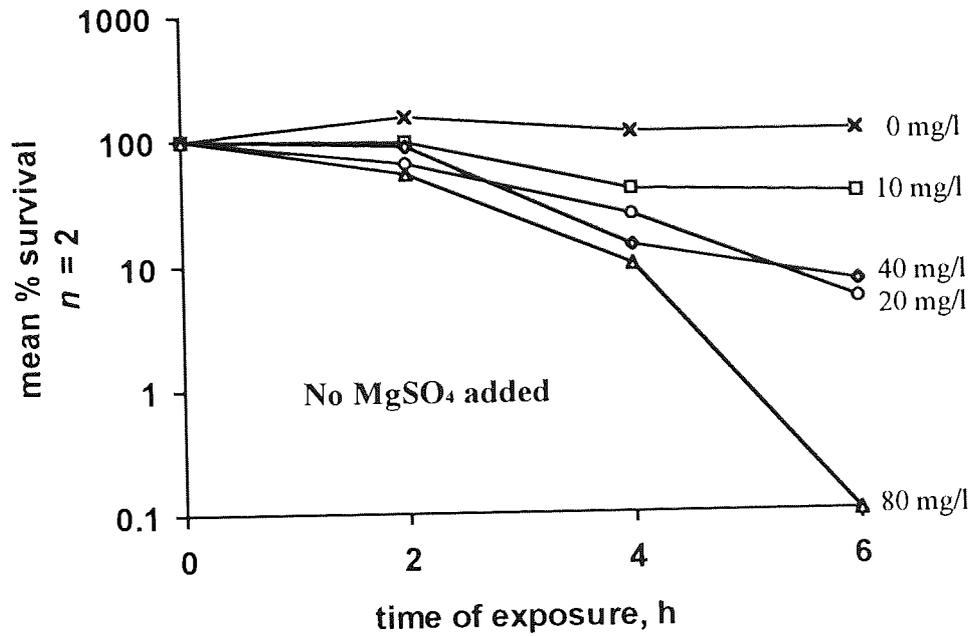
3. >1000 CFU/ cm length of catheter and no SEM was given because semi-confluent growth on the roll-plate imprints was observed in all three experiments.

8.3.3 Effect of 0.5 mM MgSO₄ on the susceptibility of *S. epidermidis* NCIMB 12721 to various concentrations of H₂O₂

S. epidermidis NCIMB 12721 was grown in nutrient broth and then the cells were rinsed to remove Mg²⁺ derived from the broth. The staphylococci (total number of cells, ca. 5.0×10^5) were then exposed to various concentrations of H₂O₂ with or without Mg²⁺ in the suspension medium, medium B. Colony counts of *S. epidermidis* NCIMB 12721 were determined at timed intervals and the percentage survival calculated.

The survival curves of *S. epidermidis* NCIMB 12721 after the initial 6 h exposure to various concentrations of H₂O₂ with or without Mg²⁺ are shown in Figure 8-3. The colony counts of the bacterial suspension in the medium B with or without Mg²⁺ did not change significantly during the initial 6 h of incubation, but reduced to ca. 50 % of the original inoculum after 20 h incubation. In comparison, there was no bactericidal activity observed when 1 mg/l H₂O₂ was added to the staphylococcal suspension even after 20 h incubation. The colony counts of the suspension exposed to 1 mg/l H₂O₂ were similar to the control (no H₂O₂). The lowest susceptible concentration of H₂O₂ for *S. epidermidis* NCIMB 12721 without Mg²⁺ was 10 mg/l. As the concentration of H₂O₂ increased, the killing time decreased. In the presence of 0.5 mM Mg²⁺, the survival rate of the staphylococci increased for the same exposure period. However, this increase in survival rate diminished when the concentration of H₂O₂ was 80 mg/l. After 20 h exposure, no staphylococci were recovered (detection limit: 100 CFU/ml) when the H₂O₂ concentration was ≥ 20 mg/l.

Figure 8-3 Effect of MgSO_4 (final concentration: 0.5 mM) in medium B on the susceptibility of *S. epidermidis* NCIMB 12721 to various concentrations (0 - 80 mg/l) of H_2O_2 .



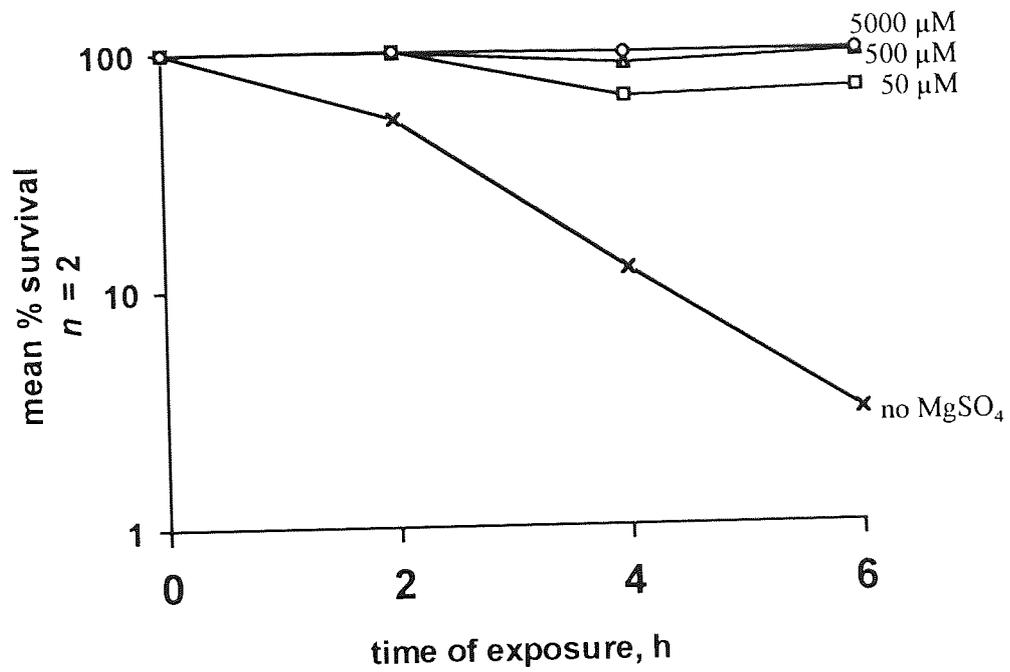
8.3.4 Effect of various concentrations of MgSO_4 on the susceptibility of *S. epidermidis* NCIMB 12721 to 20 mg/l H_2O_2

S. epidermidis NCIMB 12721 was grown in nutrient broth for 2.5 h and then washed with Mg^{2+} free medium B. The cells were resuspended in medium B with various concentrations of MgSO_4 . Hydrogen peroxide (final concentrations: 20 mg/l) was then added to the bacterial suspension and incubated at 37 °C. At timed intervals, colony counts of the bacterial suspension were carried out and the percentage survival of *S. epidermidis* NCIMB 12721 to 20 mg/l H_2O_2 is shown in Figure 8-4. Without MgSO_4 added, the colony count rapidly declined to 3 % of the original inoculum after 6 h exposure. In comparison, the colony count decreased very gradually with the addition of MgSO_4 in medium B. There was at least 1 log cycle difference in the colony counts between the suspensions that had no MgSO_4 and 50 μM MgSO_4 . With the addition of 5 mM MgSO_4 in the medium, the colony counts remained virtually the same as the original inoculum.

8.3.5 Decomposition of H_2O_2 in the presence of Mg^{2+}

The decomposition of H_2O_2 at 37 °C in the presence of Mg^{2+} was investigated by mixing H_2O_2 solution with or without MgSO_4 (final concentrations: 1 or 100 mM) and then incubating at 37 °C. The concentration of H_2O_2 measured by the Merckoquant® strip was 10 mg/l in all cases and there were no significant changes in any of their concentrations up to 8 h.

Figure 8-4 Effect of various concentrations (50 to 5000 μM) of MgSO_4 on the susceptibility of *S. epidermidis* NCIMB 12721 in medium B to 20 mg/l H_2O_2 .



8.4 Discussion

In the study on the activity of electric current by the zone of inhibition test, the use of different agars were shown to influence the size of the inhibition zones around the cathode (section 4.2.1). Variation in divalent cations content in different batches of the same agar from the same manufacturer has been reported (Barry, 1986). Comparison of the formulae of the nutrient agars used in the earlier experiments (section 4.3.1) suggested that there may be differences in the concentrations of divalent cation in the agar. Since the susceptibility of bacteria to antimicrobial agents in agar diffusion test are influenced by the cation contents of the medium (Gilbert *et al.*, 1971), it was postulated that divalent cations such as Mg^{2+} may similarly affect the bactericidal activity of electric current in the zone inhibition test.

Results of the earlier experiments in the present study also inferred that there may be an indirect effect of Mg^{2+} on the antibacterial activity of electricity. Ten μA DC was bactericidal to adherent bacteria on the electrode (section 6.3.3) but did not inhibit growth of bacteria in fluid suspension (section 5.3.2). Bacteria adherent to the electrode would be directly exposed to certain properties of electricity including the potential gradient. In comparison, bacteria in a fluid suspension would not be subjected to these factors. The primary target site of the direct effect of electricity is likely to be the bacterial membrane. Previous report has shown that electric current can disrupt the membrane of RBC which subsequently lysed (Shinohara *et al.*, 1989). Other direct effects of electricity included the dimerisation of enzyme CoA in bacteria adhered to an anodic electrode (Matsunaga *et al.*, 1992b). Similar antibacterial activities that affect bacterial membranes may also occur at the cathode. Magnesium ions are essential for the integrity of the bacterial cell envelope and the function of enzymes (Hughes & Poole, 1989b). The effect of Mg^{2+} on the antibacterial activity of electric current was therefore, investigated in the present study.

The effect of Mg^{2+} on the susceptibility of a suspension of staphylococci to electricity was investigated. Bacteria were suspended in medium A which was a non-nutritious broth and did not contain Mg^{2+} . Addition of Mg^{2+} to medium A thus provided a convenient means to compare the survival rates of staphylococci to electric current with

or without Mg^{2+} ions. *S. aureus* NCIMB 6571 and *S. epidermidis* NCIMB 12721 were susceptible to 10 μA DC discharged into the 100 ml medium A (Figure 8-2). This was in contrast to the application of 10 μA DC to 100 ml of nutrient broth culture of staphylococci in the earlier experiment of the present study in which the growth rate and the final cell density of the bacteria in Nutrient Broth no. 2 (CM 67) were not affected by 10 μA DC (section 5.3.2, Figure 5-2 & 5-3). This may be due to the rapid bacterial growth in Nutrient Broth no. 2 which exceeded any deleterious effect that 10 μA DC might have. In contrast, medium A maintained viability of bacteria rather than inducing growth (Bridson, 1990). In the presence of Mg^{2+} , the susceptibility of the two type cultures: *S. epidermidis* NCIMB 12721 and *S. aureus* NCIMB 6571 to 10 μA DC discharged into 100 ml medium A was reduced (Figure 8-2). The differences in the colony counts between the control and the broth with the addition of Mg^{2+} were less than 1 log cycle. The phenomenon was reproducible.

The protective effect of Mg^{2+} was confirmed in the investigation into the bactericidal activity of electric current to adherent bacteria on the cathodal catheter. Magnesium ions reduced the susceptibility of adherent *S. epidermidis* NCIMB 12721 on the cathodal catheter to the bactericidal activity of 10 μA DC. The colony counts of the number of bacteria on the surface of the cathodal catheter were similar to the control catheter (Table 8-1). Hence, this protective effect of Mg^{2+} appeared to be more prominent for the adherent bacteria than for the bacteria suspended in medium A. This suggested that the bactericidal activity of 10 μA DC may be due to a direct effect of electricity.

Calcium also protected adherent bacteria from the bactericidal activity of electric current but to a lesser degree than Mg^{2+} ions since the colony counts of the cathodal catheters were still reduced to half of the control. Addition of 0.2 mM $CaCl_2$ to the test medium did not restore the colony count of bacteria on the cathodal catheter to a level comparable to the control. This limited protection from Ca^{2+} ions became apparent only in the absence of Mg^{2+} . This was consistent with the observation that bacteria suspended in medium A were susceptible to 10 μA DC discharged into the test menstrum since this medium contained 0.9 mM $CaCl_2$, and the addition of Mg^{2+} reduced their susceptibility to electric current. In comparison to Mg^{2+} , addition of calcium ions to culture media did

not protect the bacteria exposed to electricity to the same extent. This is possibly due to calcium requirement of bacteria is small since the intracellular content of Ca^{2+} is very low ($< 100 \text{ nM}$). This tiny quantity of calcium ions is easily met without the addition of calcium salts to culture media because the constituents of culture media are usually contaminated with sufficient calcium ions (Hughes & Poole, 1989). The role of calcium in bacterial physiology is not as well understood as Mg^{2+} ions, but it is an important growth regulator (Hughes & Poole, 1989).

In earlier work of the present study (Chapter 7) the zone of inhibition tests were carried out with the addition of MgSO_4 to nutrient agar during the investigation on the mechanism of the electric current. The zone sizes, when compared to those obtained in nutrient agar without the addition of MgSO_4 , were significantly reduced with the two clinical strains of *S. epidermidis* tested but not with the type cultures: *S. epidermidis* NCIMB 12721 and *S. aureus* NCIMB 6571 (section 7.3.1). Magnesium ions therefore appeared to reduce the susceptibility of *S. epidermidis* to the bactericidal activity of electric current associated with the cathode, though the protective effect was not uniformly observed among the staphylococci.

Since H_2O_2 was shown to be the bactericidal agent associated with the cathode (section 7.3.1), it implied that the protection of bacteria by Mg^{2+} ions against the activity of electric current was from the action of H_2O_2 . This was investigated by exposing *S. epidermidis* NCIMB 12721 to H_2O_2 alone with and without the addition of Mg^{2+} ions in the test menstrum. The colony counts of the bacterial suspension with 0.5 mM MgSO_4 were higher than the bacterial suspension without Mg^{2+} after the same duration of exposure to 10 to $40 \text{ mg/l H}_2\text{O}_2$ (Figure 8-3). After 6 h exposure, the decrease in colony counts was the least when the cells were exposed to 20 mg/l of H_2O_2 , as 97% of the cells in 0.5 mM Mg^{2+} ions remained viable after a 6 h exposure, as compared to a 35% survival in the control. As the concentration of H_2O_2 increased, the protective effect of Mg^{2+} diminished. When *S. epidermidis* NCIMB 12721 was exposed to 80 mg/l of H_2O_2 for 6 h , the $\%$ survival of bacteria in the presence of Mg^{2+} was similar to the suspension without magnesium ions. This protective effect of Mg^{2+} was therefore only observed below $80 \text{ mg/l H}_2\text{O}_2$. This is similar to the zone of inhibition test in which the

concentration of H_2O_2 was the highest at the agar-catheter interface and gradually diminished centripedally. With a finite amount of Mg^{2+} in the agar, inhibition zones around the cathodal catheter were still formed as long as the concentration of H_2O_2 was higher than the critical concentration. Hence, adherent bacteria on a cathodal catheter would still be susceptible to the bactericidal activity of 10 μA DC since the surface of the catheter would be the site of electrolysis with the resultant formation of H_2O_2 .

The protective effect by Mg^{2+} was not dependent on the concentrations of this ion. When *S. epidermidis* NCIMB 12721 was exposed to 20 mg/l of H_2O_2 , addition of 50 μM MgSO_4 increased the viability of the bacteria to nearly 90 % of the control (Figure 8-4). A 100 fold increase of MgSO_4 did not significantly increase the percentage survival of the bacteria. This was probably due to the ability of the gram-positive bacterial cell wall to bind cations strongly as sublethally affected staphylococci are able to restore the intracellular Mg^{2+} ions level within an hour during recovery (Hurst *et al.*, 1975).

The protective effect of Mg^{2+} against the action of H_2O_2 on micro-organisms has not been previously reported. The mechanism of Mg^{2+} ions protecting bacteria from the action of H_2O_2 need further investigations. Preliminary experiments in this study demonstrated that the protective effect was unlikely due to a catalytic decomposition of H_2O_2 by Mg^{2+} since mixing H_2O_2 with MgSO_4 did not reduce the concentration of H_2O_2 for 20 h even at 37 °C (section 8.3.5). Hydrogen peroxide is a strong oxidising agent and acts as a generator of free hydroxyl radicals (OH^\cdot) which may result in damage of surface membranes (Baldry & Fraser, 1988). Hydrogen peroxide has multiple activities on bacterial cells including the oxidation of the thiol groups in lipids and proteins in both the cytoplasm and the membrane. Intracellularly, H_2O_2 causes dissociation of ribosomes (Nakamura & Tamaoki, 1968) and may also cause DNA breakage (Hugo & Russell, 1992). In the present study, the staphylococci were grown in nutrient broth that had sufficient Mg^{2+} ions, so it is unlikely that their cell wall or membrane were modified. These Mg^{2+} -sufficient cells were then exposed to electric current or H_2O_2 . Heat at sublethal level causes loss of large quantity of Mg^{2+} and was suggested as the first critical injury point (Hurst *et al.*, 1975). Hydrogen peroxide may oxidise the cell wall

components and cause similar leakage of Mg^{2+} ions from the cell wall. The extracellular Mg^{2+} in the test menstium may then compensate this loss as a result of rapid binding of the ions (Hurst *et al.*, 1975). The oxidation of thiol groups in the envelope by H_2O_2 inactivates enzymes (Hugo & Russell, 1992) and the Mg^{2+} in the suspension medium may also restore their activities. Similarly, the extracellular Mg^{2+} ions may penetrate the cells and reverse the dissociation of ribosomes. Moreover, Mg^{2+} can block the action of H_2O_2 that causes the dissociation of ribosome (Nakamura & Tamaoki, 1968).

In this study (section 8.3.2), bactericidal activity was also demonstrated on surface of the anodal catheter when they were suspended in medium B (Table 8-1), which was not observed in the zone of inhibition test (section 4.3.1) nor when the colonised catheters were embedded in nutrient agar (section 6.3.1). Furthermore, colony counts on the surface of the anodal catheter were significantly lower than the counts on the cathodal surface. In contrast to the bactericidal activity associated with the cathode, the activity associated with the anode was not affected by the addition of Mg^{2+} ions. The bactericidal activity associated with the anodal catheter was demonstrated to be partly due to the production of free chlorine when 100 μA DC was applied (section 7.3.6). Chlorine would also be produced when 10 μA DC was applied, though the quantity of chlorine would be much less than with 100 μA . The potency of chlorine is reduced in the presence of proteinous materials. Thus, the minute quantity of chlorine produced at the anode when 10 μA was applied would be neutralised rapidly in the zone of inhibition test or when the anodal catheter was embedded in nutrient agar. Consequently no antibacterial activity was observed in the earlier experiments (section 4.3.1 & 6.3.1). However, the restricted quantity of chlorine on the electrode surface was bactericidal to the adherent bacteria when the catheter was suspended in a simple salts medium, medium B. The susceptibility of staphylococci to chlorine was not affected by Mg^{2+} ions which suggested that the target sites of the lethal action of H_2O_2 and chlorine were different.

Both chlorine and H_2O_2 are highly reactive compounds and cause multiple injuries to the bacterial cell that are common to both (Hugo & Russell, 1992). Target sites unique to chlorine include lysis of the cell wall in low concentration and oxidation of amino groups of compounds in the cytoplasm. This difference in the target sites strongly suggested

that the protection of bacteria by Mg^{2+} to H_2O_2 is due to its protection of ribosomes. Further work would be needed to verify this hypothesis.

9. Direct effects of 10 μ A DC on bacteria attached to an electrode

9.1 Introduction

The antibacterial activities of electricity include indirect effects such as electrolysis, and other direct effects such as repulsion of bacteria from the cathode (Elliott *et al.*, 1990). Shimada and Shimahara (1985a & b) reported leakage of intracellular DNA materials, inhibition of respiratory functions, increase in surface negative charges and aggregation of nuclear materials when *E. coli* were exposed to 600 mA/cm² AC. By exposing the cells under anaerobic conditions and in phosphate buffers that contained no chloride ions, production of H₂O₂ and chlorine as a result of electrolysis was excluded. In their experiments, even without the production of H₂O₂ or chlorine, there was 50 % mortality of the bacteria. However, Khoury *et al.* (1992) and Blenkinsopp *et al.* (1992) reported electric current did not reduce the number of bacteria in an established biofilm but it acted synergistically with antibiotics to sterilise the biofilm. After the application of up to 50 mA electric current for 48 h, they examined by scanning electron microscopy the surface of the electrodes where the biofilm had formed. Since the killing of the biofilm and its denudation from the surface occurred on the entire area of the electrode and the conductive metal in the electric field, they proposed that the enhanced efficacy of antibiotics by electric current was not due to the production of highly reactive radicals or compounds but to electrophoretic mobility of antimicrobial agents into the biofilm under electric field (Costerton *et al.*, 1994).

Among the reports cited, Matsunaga *et al.* (1985) applied the lowest amperage (< 20 μ A) to bacteria. With low amperage and at + 0.74 V, the direct electron transfer between the electrode and the bacteria caused the dimerisation of the enzyme CoA of the organism and led to respiratory arrest and eventual cell death. However, this bactericidal activity was demonstrated only when the electrode surface was anodic to the reference electrode. In contrast, 10 μ A DC applied to a cathodal catheter repelled bacteria from its surface (Elliott *et al.*, 1990).

In the present study, 10 μA DC was bactericidal to adherent bacteria on both the cathodal and anodal surfaces due to the indirect effect of electrolysis. To investigate whether the direct effect of 10 μA DC was bactericidal, further experiments were designed to eliminate as far as possible the production of H_2O_2 and chlorine so as to observe any direct bactericidal effects of 10 μA DC.

9.2 *Methods*

9.2.1 **Influence of anaerobic conditions on the antibacterial activity of electric current on organisms attached to a catheter surface**

A salt-bridge apparatus (Figure 7-2) placed in an anaerobic cabinet (section 7.2.1) was used to study the antibacterial activity of electric current on organisms attached to a catheter surface. Under anaerobic conditions, H_2O_2 was not be produced at the cathode because of lack of oxygen (section 7.3.3). The containers and the linking bridge were filled with medium B with or without magnesium ions. Two colonised catheters, one of which acted as the cathode, were placed vertically in a glass container of 300 ml volume, containing 100 ml broth. The other catheter which was not connected to any electrical source, acted as the field catheter. All the broth had been previously stored in the anaerobic cabinet for at least 24 h. A sterile, non-colonised catheter which acted as the anode was placed in another container, which also contained 100 ml broth. The broth of the two containers were connected with a salt-bridge as described above. An electrical device was connected to one of the two catheters in both containers to generate a constant current of 10 μA . After 14 h all catheters were removed and examined by the roll-plate method (section 2.8.1). The oxygen concentration of the atmosphere in the anaerobic cabinet and the dissolved oxygen concentration was measured by the oxygen meter (section 7.2.1).

9.2.2 **Influence of chloride ions in the test medium on the antibacterial activity of electric current on organisms attached to a catheter surface under aerobic conditions**

The salt-bridge apparatus was prepared as described above, but used under aerobic conditions. One hundred ml medium B with or without potassium chloride (final concentration of KCl, 1.35 mM) was added to each container and the linking salt-bridge was filled with the same broth. Two colonised catheters were placed in the first container, with one catheter acting as the anode and the other the field catheter. One sterile non-colonised catheter was placed in the second container as the cathode. An

electrical device which generated 100 μ A DC was applied to the catheters via external leads and the apparatus was incubated at 37 °C, in air, for 14 h. All the catheters were then removed and the number of bacteria on the external surface determined by the roll plate method. Chloride concentration in the sterile biofilm broth without potassium chloride was measured by ion chromatography using Dionex Chromatograph (Sunnyville, California, USA) with the detection limit of 0.01 mg/l.

9.3 Results

9.3.1 Influence of anaerobic conditions on the antibacterial activity of electric current on organisms attached to a catheter surface

Ten μA DC was bactericidal to organisms attached to the surface of a electro-conducting catheter when the test was carried out in aerobic conditions. If the activity of electric current applied to the organisms attached on the catheter surface was examined under anaerobic conditions, any bactericidal activity present at the cathode would indicate mechanisms other than electrolysis are probably involved. When the activity of electric current (10 μA DC) on organisms attached to catheter surface was carried out in anaerobic conditions, the mean colony count on the cathodal catheter surface was reduced with or without the addition of Mg^{2+} (Table 9-1). The colony counts were significantly lower ($n = 4$, $p < 0.01$) than the control catheter and the field catheter since both viable counts were $>1,000$ CFU/cm length catheter. The dissolved oxygen concentration in the broth was 0.1 mg/l and H_2O_2 was not detected by the Merckoquant[®] strip.

Table 9-1 Bactericidal activity associated with the cathodal catheter under anaerobic conditions after 16 h of application of 10 μA DC

| Mg ²⁺ ions content of medium B | number of bacteria on the catheter surface, CFU/cm length catheter (mean \pm 1 SEM, $n = 4$) | |
|---|---|---------|
| | Cathodal | Control |
| without MgSO ₄ | 241 \pm 66* | > 1000 |
| with 0.2 mM MgSO ₄ | 629 \pm 130* | > 1000 |

* significantly lower than the control, $p < 0.01$,

9.3.2 Influence of chloride ions in the test medium on the antibacterial activity of electric current on organisms attached to a catheter surface

The removal of oxygen from the test medium did not eliminate completely the bactericidal activity of the cathode. The effect of omitting chloride ion from the medium B on the antibacterial activity of the anode was also examined. At 10 μA , the antibacterial activity at the anode was not doubtful in the zone inhibition test (section 4.3.1) and when the anodal catheter was embedded in nutrient agar (section 6.3.1), therefore 100 μA was applied. With or without the addition of KCl to medium B, the colony count at the anode surface was also significantly reduced as compared to the control when 100 μA DC was applied (Table 9-2). Without the addition of KCl, colony counts of both the control catheters and the field catheters were significantly lower than when the catheters were immersed in the broth with added chloride. The chloride concentration in the broth without potassium chloride contained 1.25 mg/l chloride ions.

Table 9-2 Colony counts of *S.epidermidis* NCIMB 12721 on the catheter surfaces in test media with/without chloride ions added, after 14 h of application of 100 μ A DC under aerobic conditions (CFU/cm length of catheter)

| | 1.35 mM KCl added | | | | no KCl added | |
|--------------|----------------------|--------------------|-------|------------------|------------------|-------|
| | control ¹ | field ² | anode | control | field | anode |
| <i>N</i> = 6 | | | | | | |
| Mean | 975 | 645 | 7 | 794 ³ | 469 ⁴ | 36 |
| Median | 1000 | 704 | 4 | 818 | 409 | 4 |
| Range | 800->1000 | 88-100 | 0-19 | 489->1000 | 74-1000 | 0-207 |

- Note:**
1. Control catheter was placed in their respective broth in the same condition as tests, but without electric current,
 2. Field catheter was placed in the anodal bath, but not connected to the electrical device
 3. Significantly lower than the control with 1.35 mM KCl added, $p < 0.01$, Wilcoxon Signed Rank test
 4. Significantly lower than the viable count of the field catheter with 1.35 mM KCl, $p < 0.01$, Wilcoxon Signed Rank test

9.4 Discussion

The present study firmly established that even at low amperage, 10 μA DC, electrolysis occurred and produced highly reactive compounds that are bactericidal (Chapter 7). However, electricity *per se* may have antibacterial activity. Bacteria attached to the surface of an electrode are exposed to the effects of electric current including electric field and potential gradient. In comparison, bacteria suspended in the associated broth would not be exposed to the same degree. To investigate this intrinsic activity of electricity, electric current was applied to bacteria attached to a catheter surface and also in broth after the removal of chloride ions and oxygen. When these ions and molecules were excluded, the colony counts of bacteria on the catheter surfaces were also significantly reduced as compared to the catheters tested in the presence of oxygen and chloride. This bactericidal activity associated with the cathode cannot be attributed to hydrogen peroxide despite the presence of residual oxygen in the test medium. The dissolved oxygen concentration in the test medium was 0.1 mg/l (6 $\mu\text{mol/l}$) and if all the oxygen were to be converted to hydrogen peroxide, there would be only 3 $\mu\text{mol/l}$ produced. This concentration of hydrogen peroxide is below the MIC of *S. aureus* (0.15 mmol/l) (Baldry, 1983). The sensitivity of *S. epidermidis* NCIMB 12721 to hydrogen peroxide is similar to *S. aureus* NCIMB 6571 in the zone of inhibition test and so the bactericidal effect cannot be due to hydrogen peroxide. The concentration of residual chlorine in the medium B was 1.25 mg/l, and if all these chloride ions were to be electrolysed to chlorine there would be 0.6 mg/l chlorine molecule produced. This concentration of chlorine would only be bactericidal if it was all concentrated on the catheter surface. This is unlikely as the broth in the experiment was continuously mixed. Therefore, it appears that electricity *per se* at the anode also has antibacterial activity. This bactericidal activity was effective only on the electrode surface as the bacteria attached to the field catheter were still viable even when 100 μA DC was applied.

The bactericidal activity associated with the cathode under anaerobic conditions was also affected by the presence of Mg^{2+} . Addition of Mg^{2+} reduced the susceptibility of the staphylococci (Table 9-1). Thus it is possible that the mechanism of this activity may include the disruption of the integrity of the bacterial membrane or electrolysis of molecules on the cell surface.

The present study demonstrated that 10 to 100 μA DC applied via the carbon catheters was bactericidal to staphylococci under anaerobic conditions and exclusion of chloride to eliminate the production of these reactive radicals and compounds. Such test conditions would simulate the environment within the biofilm. Within the biofilm, oxygen content is low and ions concentrations may be low due to diffusion barrier. Electric current associated with the cathode under conditions in which H_2O_2 was not produced would therefore eradicate bacteria that are attached to the electrode surface but underneath the biofilms.

Moreover, earlier experiments in the present study (section 6.3) demonstrated that when electric current was applied as soon as the bacteria attached to the surface, it was bactericidal. Therefore, 10 μA DC applied via the carbon catheters may prevent the formation of biofilm. However, results from Khoury *et al.* (1992) and Blenkinsopp *et al.* (1992) showed that electricity (DC) alone did not prevent formation of biofilm though it reduced the number of bacteria in the biofilm for 24 h. Therefore, further investigations into the activity of electric current in the prevention of formation of biofilm is necessary.

10. Low amperage electric current and biofilm formation

10.1 Introduction

Medical devices including CVC associated infections are often recalcitrant to treatment by antibiotics and necessitate the removal of the device. The failure of treatment despite seemingly adequate level of antibiotics is largely due to the biofilm mode of bacterial growth on devices (Christensen *et al.*, 1994). Bacteria in the biofilm may become embedded in a matrix of exopolysaccharide (Costerton & Lappin-Scott, 1995). This slime layer or glycocalyx is a permeability barrier which prevents antibiotics reaching the bacteria (Costerton *et al.*, 1987). It also interferes with the immune system preventing both humoral and cellular activity against bacteria (Christensen *et al.*, 1994). Moreover, the recalcitrance of the bacteria in the biofilm may be due to its low growth rate (Gilbert & Brown, 1995). It is important to test any antibacterial therapeutic agents against this biofilm mode of bacterial growth (Anwar *et al.*, 1990) in addition to conventional MIC and MBC determination.

Biofilms are functional consortia of microbial cells organised within extracellular polymer matrices and associated with surfaces. Their physiology, metabolism and organisation are greatly dependent on the nature of these surfaces, and also the prevailing physicochemical environment. There are various in-vitro models which have been used to produce biofilms and to study the activity of antibacterial agents (Gilbert & Allison, 1993). Individual models reproduce particular physiological constraints upon biofilm growth and development, take into consideration physical forces such as shearing force in catheter, nutrient availability, growth rate, single or multi species of micro-organisms in the matrices, and surfaces interface (solid/liquid and solid/air). Each of these factors will affect the nature of the developed biofilms, and also their susceptibility to treatment with antibiotics or biocides (Brown *et al.*, 1988). Several different models would be necessary to mimic all the biofilm functional activities in their in vivo situation (Gilbert & Allison, 1993). One of these models is the modified Robbins device which provides samples at various stages of the biofilm development and control of flow of organisms over a surface (Lappin-Scott *et al.*, 1993).

The effect of electricity on microbial biofilm was investigated using the electrical modified Robbins device (Blenkinsopp *et al.*, 1992; Khoury *et al.*, 1992). It consisted of a perspex rectangular block with a central flow channel in which removable stainless steel studs were embedded and exposed to the flowing liquid. Biofilms were produced on the surfaces of the studs in the device by passing fluid from batch cultures of bacteria. A platinum wire was set in the bottom of the flow channel and directly below the row of studs. Electric current DC (15 to 400 $\mu\text{A}/\text{cm}^2$) was then applied to the stainless steel studs and directly to the biofilm on the surfaces of the studs. However, the polarities of the steel studs and the platinum wire were changed every 64 s to prevent accretion of metallic ions. Electricity alone did not reduce the number of cells in the biofilm that had developed for 24 h but it inhibited further growth (Khoury *et al.*, 1992). However, when antibiotics at a concentration that did not sterilise a biofilm combined with electric current were applied, the bacteria in the biofilm were killed after 8 to 10 h exposure. It was demonstrated further that the synergism between electricity and antimicrobial agents also took place on the surfaces of stainless steel studs that were placed between the electrodes but not connected to the electricity (Costerton *et al.*, 1994). The mechanisms of the synergism of electricity and antimicrobial agents was investigated by examining the surfaces where the biofilm had formed and later subjected to electric current and antibiotics with scanning electron microscopy. Biofilms were removed only from the surfaces which had been exposed to both electricity and antibiotic. Bacteria that remained attached to the electrode surface were disrupted. In comparison, biofilms on the surfaces of the electrodes that had been exposed to electricity or antibiotic alone remained intact. The number of viable cells on the surfaces of the electrode or the insert between the electrodes were not significantly different. The number of viable cells on the upstream electrode was also not significantly different from the electrode in the downstream. It was therefore, concluded that any toxic products produced from electrolysis was not significant in the synergism observed. It was suggested that the enhanced efficacy of antibiotics against biofilms was due to electrophoretic mobility of antibiotic penetrating the biofilm matrix under the influence of electric field (Costerton *et al.*, 1994). However, electrolysis products were not determined chemically.

The synergism between electric current and antibiotics was also investigated by positioning the biofilm away from the electrode surface and so the biofilm was indirectly exposed to electric current (Jass & Lappin-Scott, 1996). A *P. aeruginosa* biofilm was grown onto one side of a dialysis membrane and then positioned between the stainless steel electrodes in a chamber. The surfaces of the electrodes and the membrane were parallel to each other with a distance of 8 mm between the membrane and each electrode surface. Electric current DC (total current: 45 to 100 mA) was then applied to the stainless steel electrodes, thus the flow of electric field became perpendicular to the membrane (Jass, 1995). Electric current or antibiotics alone did not have any effect on the established biofilm. Activity of tobramycin (10 µg/ml) was significantly enhanced by application of 45 mA DC (total current) for 24 to 48 h but the reduction in the number of viable cells in the biofilm (decrease by 1 log order in colony count) was not as pronounced as the results obtained in the electrical Robbins device (decrease of 4 to 5 log order of colony count to sterility of biofilm) (Blenkinsopp *et al.*, 1992). Moreover, electric current could only enhance the activity of those antibiotics that were effective against planktonic (free floating) cells (Jass & Lappin-Scott, 1996).

The synergistic effect between electricity and antibiotics was investigated further with the flow of electric current parallel to and not directly to the biofilm (Wellman *et al.*, 1996). A mixed species (*P. aeruginosa* and *K. pneumoniae*) biofilm on a polycarbonate tile was placed in a chamber with two platinum wires at each end of the tile. Application of total current of 1 mA DC for 24 h enhanced the activity of tobramycin (5 mg/l) and reduced the number of viable bacteria in the biofilm by 6 to 8 log order.

Using a design similar to the electrical Robbins device, Whitham (1995) reported that no enhancement of efficacy of biocides by electric current was observed. A pair of platinum wires as electrodes were placed on both sides of the steel studs so that the steel stud did not act as the electrode and that the electric field flowed in parallel to the biofilm formed on the stud surface. The distance of the electrodes was also increased from 2 mm as in the original electrical Robbins device (Blenkinsopp *et al.*, 1992, Khoury *et al.*, 1992) to 15 mm. There was no enhanced efficacy of biocides by electric current when electric current (total current: 20 to 50 mA) and biocides were applied simultaneously to biofilms

of 1 to 30 day old. Unfortunately, details of the experiment such as the culture media, the volume of the broth in flow chamber and the duration of electric current application were not given (Whitham, 1995) and so comparison with the other reports is difficult.

There are many similarities among the reports cited above. The total electric current applied to the biofilm ranged from tens to several hundreds of mA which would be too high an amperage to be used in human body. The electrodes used were metals, either single element (such as platinum) or composite material (such as stainless steel). In order to avoid accretion and corrosion of the electrodes, the polarity was alternated. The distance between the electrodes were also small, ranging from 2 mm (electrical Robbins device) to 15 mm (Whitham, 1995). Electric current was applied after a biofilm (1 to 30 days old) had formed and so it was much more difficult to eradicate an established biofilm. The test bacterial cultures were mainly *P. aeruginosa*, other micro-organisms tested included sulphite-reducing bacteria, *S. epidermidis*, *E. coli*, *K. pneumoniae* and *C. albicans* (Khoury *et al.*, 1992; Whitham, 1995; Wellman *et al.*, 1996). The volume of fluid in the flow chamber were likely to be small, ranging from 5 ml in the electrical Robbins device (Khoury *et al.*, 1992) to 8.6 ml in the electrical colonisation cell (Jass, 1995). A small volume of fluid in the flow chamber and a slow flow rate of culture medium at 60 ml/h would easily accumulate electrolysis products from the electrodes.

The reports cited above (Blenkinsopp *et al.*, 1992; Khoury *et al.*, 1992; Jass & Lappin-Scott, 1996) stated that electricity alone had only a minimal effect on the biofilm. However, in the present study, 10 μ A DC was bactericidal to adherent bacteria (Chapter 6) to the cathodal catheter surface. The bactericidal activity was due to electrolysis which took place at the electrode surface (Mathewson, 1971). Therefore, adherent bacteria would be exposed to the bactericidal substances such as H₂O₂ and free chlorine as a result of electrolysis. The bactericidal activity to adherent bacteria was not entirely due to electrolysis since both the cathodal and anodal surface reduced the number of bacteria attached to their surface even after the production of H₂O₂ and chlorine were excluded (Chapter 9). Therefore, 10 μ A DC may inhibit the formation of a biofilm on the catheter surface. The effect of 10 μ A DC on the development of a biofilm on the catheter surfaces was investigated in a batch culture.

10.2 Methods

The effect of 10 μA DC on the formation of a biofilm on carbon-impregnated catheter was studied in a batch culture. Sterile carbon catheters (2.5 cm lengths) were placed in 100 ml of nutrient broth containing ca. 1×10^4 CFU/ml of *S. epidermidis* NCIMB 12721. The bacterial suspension with the catheters were incubated at 37 °C, in air with continuous stirring, on a magnetic stirrer (100 rpm) for 6 h. The catheters were then removed and each was immersed in 30 ml of PBS to remove loosely attach organisms (section 2.7.2).

Three of these colonised catheters were removed and the number of attached bacteria was determined by the Spiral System™ after the catheter was sonicated (section 2.8.4). The remaining colonised catheters were divided into four groups of three catheters and all were immersed in 1 litre of medium B in a 2.5 litre flask. Two of the catheters from each group were connected to an electrical device that generated 10 μA DC with one catheter acting as the anode and the other, the cathode. The third catheter in each group was not connected to an electrical device and served as the control catheter. The electrical catheters were placed at least 15 mm apart. There were therefore, four groups of catheters in the flask with each pair of catheters connected to an electrical device. Four sterile but not colonised carbon catheters ('sterile' catheters) were also immersed in the medium in the flask. The broth was maintained at 37 °C with continuous stirring on a magnetic stirrer (speed of stirring: 100 rpm).

The number of viable organisms in the broth in which the catheters were suspended were determined by the Spiral System™ at timed intervals. The batch culture was kept for four days. After each day, one group of the catheters that included the electrical catheters, one control catheter and one 'sterile' catheter was removed, rinsed by immersion in 30 ml PBS (section 2.7.2), and then placed in 10 ml MRD in which they were sonicated for 1.5 min at 11 W (section 2.8.4). Colony forming counts of the resulting bacterial suspension was determined by the Spiral System™. Daily after the removal of the catheters, the concentrations of H_2O_2 and free chlorine were checked with their appropriate reagents (section 7.2.6).

To determine whether or not the sonication procedure was sufficient to remove the biofilm and disperse the bacteria, the catheters that had a 3 or 4 days old biofilm were stained and examined by the acridine orange method (section 2.4) after sonication. Ten μl of the resultant bacterial suspension in the sonicate was also spread on a glass slide. After air drying, the slide was gram-stained and then examined under the microscope to assess the clumping of the cells (section 2.8.3).

10.3 Results

The mean number of bacteria attached to the carbon catheters was 1.4×10^5 CFU/cm length of catheter after they were incubated for 6 h in the nutrient broth culture of *S. epidermidis* NCIMB 12721. The effect of 10 μ A DC on the biofilm growth on the carbon catheter was investigated by comparing the number of bacteria on the electrical catheters against the control catheters over four days.

The efficiency of the sonication procedure to remove the biofilms was assessed by examining the catheter surface after sonication. The dispersal of bacteria in the biofilm of the resultant bacterial suspension was also examined. The number of bacteria remained on the catheter surface and the dispersal of bacteria by sonication of the 3 and 4 days old biofilm are shown in Table 10-1.

Table 10-1 Efficiency of sonication on the removal of biofilm from carbon catheters and the dispersion of bacteria in the sonicate.

| Age of biofilm (days) | Percentages of bacterial units in sonicate (mean \pm SEM, n = 8) | | | | No. of bacteria on the catheter surface (mean \pm SEM, n = 4) |
|-----------------------|---|----------------|---------------|---------------|--|
| | single | pairs | quadruples | clumps | |
| 3 | 39.8 \pm 6.8 | 53.0 \pm 4.5 | 6.8 \pm 2.8 | 0.5 \pm 0.4 | 3.1 \pm 0.7 |
| 4 | 21.8 \pm 1.7 | 75.0 \pm 0.8 | 3.0 \pm 1.1 | 0.3 \pm 0.3 | 13.9 \pm 3.8 |

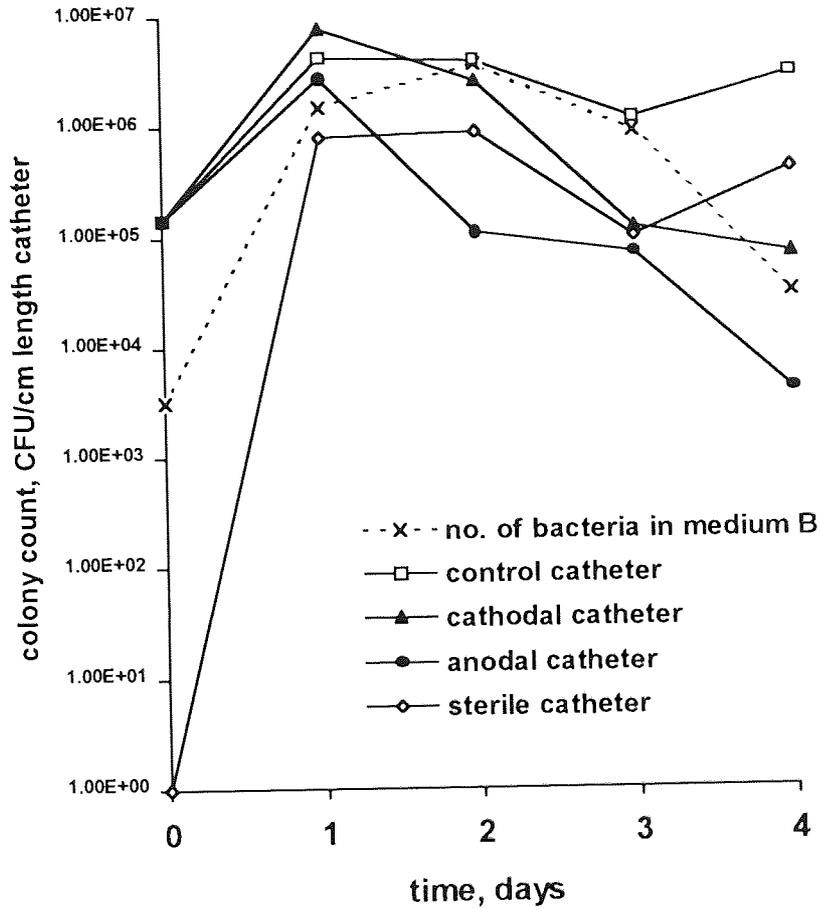
After sonication, the remaining bacteria attached to the catheter surface were mainly single organisms. Occasional pairs were also present, however no micro-colonies nor biofilms were seen attached to the catheter surface. In the bacterial suspension after sonication, the bacteria were mainly in pairs (53 and 75 % of the 3 and 4 days old biofilm respectively). This compared to > 70 % which were single cells when a 16 h biofilm was sonicated for the same period (section 3.4.3, Figure 3-3). The efficiency of the

sonication procedure in the removal of the biofilm and the dispersal of bacteria in the biofilm decreased as the age of the biofilm increased. However, the decrease in efficiency of the sonication procedure was similar for all the catheters that had biofilms of the same age. The colony counts of catheters that had biofilm of the similar age appeared therefore to be similarly affected by sonication.

The colony counts of the number of bacteria on the electrical, control and the initially sterile catheters over 4 days is shown in Figure 10-1. Staphylococci which adhered to all the catheters multiplied and the total cell densities on the catheter surface were similar after 24 h. Bacteria detached from the colonised catheters and grew in the culture medium as planktonic cells. These cells in the suspension also adhered to the initially sterile catheters ('sterile' catheter) and then grew on the sterile catheter. The total number of bacteria on the 'sterile' catheters after 1 day was similar to the control catheter. After 24 h incubation, the colony counts of all the catheters decreased as time increased. The number of bacteria on the anodal catheter was consistently lower than the control catheter by ca. 1 log order from day 2 onwards. The number of bacteria on the cathodal catheter also reduced over the same period but at a slower rate.

Free chlorine and H_2O_2 were not detected in the broth over the 4 days incubation (detection limit of free chlorine was 0.1 mg/l and H_2O_2 was 0.1 mg/l).

Figure 10-1 Effect of 10 μA DC on biofilm formation by *S. epidermidis* NCIMB 12721 on carbon catheters ($n = 2$).



Notes: 1. Colony count was in logarithmic scale, and $1.00\text{E}+N = 1.00 \times 10^N$

2. Number of bacteria in medium B was CFU/ml

10.4 Discussion

Staphylococci adhered to the catheters during the 6 h incubation in the nutrient broth culture. During this period the cells would have been in the early log phase of growth. At this stage, micro-colonies would not have been formed on the catheters. The staphylococci on the catheters therefore were still in the very early stage of biofilm formation when the catheters were transferred to medium B and exposed to 10 μ A DC.

When the colonised catheters were immersed in medium B, the shearing force of the stirring assisted the detachment of staphylococci from the surfaces. This was demonstrated by the increase in number of organisms in the broth. The detached organisms then grew in the broth and also adhered to the surfaces of all the catheters including the initially sterile catheters ('sterile' catheters). During the first 24 h, growth of staphylococci on all the colonised catheters and the planktonic cells were in the fast growing log phase. The number of bacteria in the biofilm on all the catheters including the 'sterile' catheters after 24 h incubation were similar (8.0×10^5 to 7.8×10^6 CFU/cm length catheter). However, the increase in number of bacteria on the surface of the 'sterile' catheters included the deposition of planktonic cells and then the growth of the adhered cells. There were no bacteria on the surface of the 'sterile' catheters at the commencement of the experiment. Within 24 h, the colony count of the 'sterile' catheters increased to 8.0×10^5 CFU/cm length of catheter, while the colony count of the control catheters increased by just 2 log order. Therefore, the deposition of bacteria on the surface of a 'sterile' catheter must be substantial in the beginning, then continuous throughout the whole incubation period.

For the first 24 h, the increase in colony counts on the electrical catheters and the control catheters were similar. The increase most likely represented both the growth of already adherent bacteria and the deposition of further planktonic cells. Earlier experiments in this study showed that 10 μ A DC did not affect the growth of bacteria in the culture medium nor did it prevent adhesion (section 5.3) but inhibited the growth of adherent bacteria (section 6.3.1). Therefore, the number of cells adherent to the catheters should be similar in all cases. Thus, in this phase of rapid growth of the planktonic and adherent

cells, 10 μ A DC did not prevent the further growth of a biofilm by *S. epidermidis* NCIMB 12721 on the electrical catheters.

After the first day, nutrients in the broth would start to run out because no fresh medium was added and so bacterial growth rate would slow down. The colony count of the planktonic cells increased by less than 1 log order during the second 24 h and then started to decrease. The colony counts of the control catheters and the 'sterile' catheters remained the same during the second and third day of incubation. The decline in the number of cells on the control and the 'sterile' catheters after 48 h incubation were gradual and the final cell density was 10^5 to 10^6 CFU/cm length of catheter. However, the cell density of the two electrical catheters started to decrease after 24 h incubation. After 3 days of incubation, the colony counts of both the anodal and cathodal catheters were 1 log order lower than the control catheter. Thus, 10 μ A DC appeared to reduce the number of viable cells in a slow growing biofilm. After 4 days, the biofilms on the electrical catheters were not sterilised nor greatly reduced (less than 4 log order reduction in cell count). This degree of reduction of the viable count of cells in a 24 h old biofilm was similar to the findings of Blenkinsopp *et al.* (1992).

Throughout the experiment, H_2O_2 and chlorine were not detected in the broth. It is likely despite their continuous production, the volume of the broth (1 litre) would dilute both H_2O_2 and chlorine to below detectable concentrations. The continuous stirring would also constantly remove any H_2O_2 , chlorine or reactive radicals that formed on the electrode surface. Furthermore, the bacteria in the broth would also decompose any H_2O_2 thus formed. Therefore, the exposure of the biofilm to the bactericidal electrolysis products must be relatively brief in the present study. Nevertheless, they reduced the number of viable cells of the biofilm by 1 log order.

In contrast, in the electrical Robbins device (Costerton *et al.*, 1994) and the electrical colonisation cells (Jass, 1995), the volume of the broth used in the test chamber was small (< 10 ml) and the flow rate of fluid was slow (60 ml/h). Hydrogen peroxide would have accumulated in these test systems. Although H_2O_2 was not measured in the

electrical colonisation cell, the accumulation of H_2O_2 was indirectly demonstrated by Jass (1995) as an increase in oxygen tension in the broth since H_2O_2 decomposed to oxygen and water. The accumulation of H_2O_2 may be the cause of reduction in the number of bacteria in the young biofilm after 24 h exposure to electric current in the electrical Robbin's device (Blenkinsopp *et al.*, 1992) despite pseudomonads being ten times more resistant to H_2O_2 than staphylococci (Baldry, 1983). Moreover, the bacteria in these studies of biofilm and electric current were cultured in Mg^{2+} -depleted ($40 \mu\text{M MgSO}_4$) media (Blenkinsopp *et al.*, 1992; Khoury *et al.*, 1992; Jass, 1995; Wellman *et al.*, 1996). The magnesium deficient growing conditions would alter the cell envelope resulting in changes in their susceptibility to antibiotics (Kenward *et al.*, 1979; Shand *et al.*, 1988). In the present study, staphylococci maintained in low Mg^{2+} condition were more susceptible to H_2O_2 (section 8.3.3) and so pseudomonads may be similarly affected.

The different design of the models in the study of the effect of electricity on biofilm development may also affect the results. Biofilms were directly exposed to the electrolysis products including H_2O_2 and metallic ions in the electrical Robbins device since the biofilm was on the electrode surface (Khoury *et al.*, 1992). In contrast, the biofilm was 8 mm away from the electrodes in the electrical colonisation cell (Jass, 1995). Any electrolysis products formed would need to pass the dense bacterial suspension in the chamber and so the concentrations of any of these oxidative compounds would be lower in the suspension than at the electrode surface. This is evident by the reduction in the enhancing effect of electric current to the antibiotics in the electrical colonisation cell. The reduction of the total viable cells in the biofilm in the electrical colonisation cell was only 1 log order (Jass & Lappin-Scott, 1996) when compared to a sterilisation of biofilm in the electrical Robbins device (Khoury *et al.*, 1992; Blenkinsopp *et al.*, 1992). Whitham (1995) similarly placed the electrodes 15 mm away from the biofilm and reported no enhancement of activities of biocides by electric current.

In the present study, 0.5 mM MgSO_4 was added to medium B as originally described (Duguid *et al.*, 1992) and this would affect the bactericidal activity associated with the

cathode. In the previous chapter (Chapter 8), Mg^{2+} ions were shown to affect the activity of H_2O_2 in the concentration range of 10 to 40 mg/l, therefore the efficacy of 10 μA DC against the staphylococci in the biofilm would be diminished in the present experiment despite the bacteria adhered to the cathode surface.

However, the colony count of the bacteria on the anodal catheter was not only reduced when compared to the control catheter but also significantly lower. This is in contrast to the earlier experiments (section 4.3.1) of the present study in that 10 μA DC applied via the anodal catheter did not consistently produce an inhibition zone. This is possibly due to the amount of chlorine produced being so minute that there was insufficient quantity to effect an inhibition zone. Therefore, electric current carrying catheters appeared to reduce the number of viable cells in a 24 h old biofilm, though only by 1 log order.

Study of biofilms in a batch culture does not control factors such as growth rate (Gilbert & Allison, 1993) and the culture is constantly changing. As in this study, both planktonic and adherent bacteria went through different growth rates at the different stages of the growth cycle. The thickness of the biofilm, the interchange of planktonic and adherent cells and the accumulation of toxic metabolites and in this study, antimicrobial agents (H_2O_2 , chlorine) were factors affecting the results. The present batch culture study is also different from the in vivo situation. The electrical catheters that had been colonised with bacteria were placed in a broth. Bacteria detached from the catheters began to grow in the broth and so the electrical catheters were constantly immersed in a bacterial suspension with a high cell density. Bacteria adhered to a CVC would not be immersed in a bacterial suspension of such high cell density. Therefore, the batch culture experiment of the present study may underestimate the potential of the activity of electric current. Recently, bacteria trapped in agar gel was investigated as a model for biofilm study (Jouenne *et al.*, 1994). This method may eliminate the problem associated with batch culture.

11. General Discussion

In the present study, the zone of inhibition test was set up to screen a wide range of organisms because it is a relatively straight forward procedure. Previous reports on the antibacterial activities of electric current had been tested mainly on gram-negative aerobic bacilli such as *E. coli* (Shimada & Shimahara, 1982, 1985; Pareilleux & Sicard, 1970) and *P. aeruginosa* (Blenkinsopp *et al.*, 1992). In addition to the aerobic gram-negative bacilli, 8 species of staphylococci including 5 species of *S. epidermidis* were tested in the present study. The coagulase negative staphylococci are the most common organisms associated with medical device related infections (Elliott, 1993). Using the zone of inhibition test, 10 μ A applied via the cathodal catheter was bactericidal against a wide range of organisms, particularly the staphylococci.

In the zone of inhibition test, the anode and the cathode were placed apart and therefore their separate activities could be observed simultaneously. The separation of the electrodes and the concomitant use of low current (10 μ A DC) demonstrated a difference in the bactericidal activities associated with the electrodes. This would not be demonstrable in a test system that the electrodes were placed in the same broth or when AC was used.

Bactericidal activities associated with the cathode was effective only against adherent bacteria that attached to the cathodal surface. The bactericidal agent associated with the cathode was H_2O_2 and the anode, Cl_2 . The production of both bactericidal agents were a result of electrolysis of oxygen and chloride ions respectively. If electrolysis process occurs at the electrodes at 10 μ A as shown in the present study, it is likely to take place in higher amperage. This was not considered in the investigations of the mechanisms of the enhancement effect of antibiotics by tens of mA electric current (Costerton *et al.*, 1995) nor the bactericidal activity of an anodic surface (Matsunaga *et al.*, 1985).

The composition of the electrodes also needs to be taken into account when considering the antimicrobial activity of electric current. Metal electrodes, for example, may be

electrolysed to ionic forms with relatively high concentration accumulating on the electrode surface. Metals that are essential for microbial growth can be toxic when they are at high concentrations (Hughes & Poole, 1989). Even inert metals such as platinum can be electrolysed to form compounds that are bactericidal or inhibitory to bacterial cell division (Rosenberg *et al.*, 1965). The improved bactericidal activity of metal electrodes with 400 μA DC in synthetic urine as compared to carbon electrodes suggests even gold can be electrolysed to produce toxic compounds (Davis *et al.*, 1991). The present study benefited from the advanced technology of fine carbon particles incorporated into polymers to conduct electricity. Carbon is inert but is a good conductor of electricity. Without the interference from metal, the phenomena observed in the present study, unlike previous reports, could be attributed to electricity alone.

Electrolysis takes place on the surface of the electrodes (Mathewson, 1971). When the end-products are gaseous such as H_2O_2 and Cl_2 , the electrolysis process is facilitated by the surface rugosities, which act as nucleation points for gas formation. Organisms attached to a catheter surface may also act as nucleation points for the electrolysis process and therefore be inadvertently exposed to the oxidative compounds. The first law of electrolysis states that the mass of any given substance which is liberated or dissolved as a result of electrolysis is proportional to the quantity of electric charge that has flowed (Mathewson, 1971). This was observed in the increase in zone sizes around the cathodal catheter when the current was increased (section 4.3.4).

Since toxic substances such as H_2O_2 and Cl_2 resulting from electrolysis are the principal bactericidal agents in liquid culture, the volume of the test menstrum into which the electric current was discharged also influence efficacy. The concentrations of H_2O_2 and Cl_2 throughout the medium would depend on diffusion or stirring of the solutions. This in turn would influence the duration of exposure of the adherent organisms to the toxic substances.

In the present study, the activity of the cathodal catheter was studied by three different *in vitro* models that simulate an intravenous catheter in use. Firstly, the catheters that had been colonised with bacteria were embedded in nutrient agar and then exposed to 10 μA

DC. Bacterial growth on the external surface of the cathodal catheter was inhibited for 2 days (section 6.3.1). The length of catheter embedded in the nutrient agar is analogous to the section of a catheter in the subcutaneous tissue which would be surrounded by tissue and constantly bathed in body fluid. Extraluminal colonisation of catheter is one of the major routes of infection (Mermal *et al.*, 1991; Raad & Bodey, 1992). Bacteria of the skin flora may enter the catheter-tissue interface possibly aided by electro-potential gradient (Dealler *et al.*, 1988) or capillary action (Cooper *et al.*, 1991). Ten μA DC prevented the migration of organisms from the surface along the catheter (Crocker *et al.*, 1992). In the present study, 10 μA DC also inhibited growth of already attached bacteria on the catheter (section 6.3.2). The application of electric current may therefore also prevent the bacterial colonisation of the catheter via the extraluminal route.

Bacteria can also gain access to and then colonise the internal lumen of an intravenous catheter via a contaminated hub (Linares *et al.*, 1985). The internal lumen of a catheter is constantly filled with fluid and bolus flushed only periodically. Therefore, any organisms on the internal surface of a catheter are immersed in a small quantity of stationary fluid for most of the time. Adherent bacteria on the internal lumen would be similar to those that attached to the cathodal catheter and immersed in 15 ml of fluid medium without stirring (section 6.3.2). The adherent bacteria on both the internal and external surfaces of the cathodal catheter were killed after 6 h exposure to 10 μA DC. Thus, a cathodal catheter may prevent the intraluminal colonisation of the catheter, which is another major route of infection (Raad & Bodey, 1992).

The experiment in which the catheters were immersed in 1 litre of broth with constant stirring as in the batch culture of the biofilm study (section 10.3), were similar to the situation on the external surface of a catheter in a vein. The large volume and constant flow of blood would constantly remove any traces of electrolysis products formed on the catheter surface and so contact time between the adherent bacteria and the toxic substances would be limited. Ten μA DC did not prevent the further growth of bacteria attached to the catheter when they were in the log phase of growth (section 10.3) but reduced the number of the organisms in the biofilm when the growth decreased. It therefore appeared that 10 μA DC may inhibit the

further growth of bacteria with a slow growth rate which is thought to be one of the major physiological change conferring the resistance to treatment.

Bactericidal activity associated with the anode in the zone of inhibition test was only demonstrable when 20 or more μA was applied (section 4.3.1, Table 4-1). However, when 10 μA was applied to the anodal catheter that had been colonised with *S. epidermidis* NCIMB 12721, the number of bacteria in a 24 h biofilm was reduced (section 10.3) by more than 2 log order when compared to the cathode (section 10.3). The bactericidal activity associated with the anode was due to the production of free chlorine and possibly other chlorine based compounds when 400 μA DC was applied (Davis *et al.*, 1994). This was also demonstrated in the present study by the salt-bridge apparatus when 100 μA was applied (section 7.3.6). Therefore, it is likely that chlorine and related compounds were also produced when 10 μA was applied to the catheters since the amount of electrolysis products produced is proportional to the current applied. The amount of chlorine produced when 10 μA DC was applied for 16 h may have been so small that it was insufficient to diffuse to produce an inhibition zone but remained effective in killing the adherent bacteria

The present study clearly established that electrolysis products such as H_2O_2 and Cl_2 were responsible for the bactericidal activities of electricity. Electric current *per se* also showed antibacterial activity (section 9.3). Adherent bacteria were exposed to both the electrolysis products such as H_2O_2 and Cl_2 , and the direct effect of electricity. However, it would be difficult to delineate the proportion of contribution of each element.

The susceptibility of staphylococci to H_2O_2 was dependent on Mg^{2+} ions concentration (section 8.3.2). *S. epidermidis* NCIMB 12721 was grown in Mg^{2+} -sufficient medium and then exposed to H_2O_2 . The susceptibility of the staphylococci to H_2O_2 reduced in the presence of Mg^{2+} ions. However, this was demonstrable only when staphylococci were exposed to low (<80 mg/l) concentration of H_2O_2 . This is consistent with previous reports that electricity was very effective in non-nutritious test media (Pareilleux & Sicard, 1970; Shimada & Shimahara, 1982, 1985) since in all those reports bacteria were first grown in

nutritious medium and then suspended in either simple salt solutions or water. The organisms were therefore exposed to the effect of electricity in test menstra containing no Mg^{2+} ions.

New developments in the prevention of CVC related infections

In the prevention of CVC related infections, the main thrust is still the incorporation of antimicrobial agents into the catheter such as minocycline and rifampicin (Raad *et al.*, 1996). However, the rationale of incorporating antibiotics in catheters as a preventive measure has been questioned (Goldman *et al.*, 1993) as the wide spread prophylactic use of antibiotics may increase the emergence of resistant organisms. Therefore, catheters impregnated with antimicrobial agents other than antibiotics has been received with enthusiasm. However, no detailed clinical trials of any of these catheters have been published to date. Though the silver sulphadiazine and chlorhexidine impregnated polyurethane catheter had been demonstrated to reduce colonisation and biofilm formation in animal models (Bach *et al.*, 1994; Greenfield *et al.*, 1995), the results of full clinical trial are awaited.

Another development using electricity was the silver iontophoretic catheter. A pair of silver wires were helically coiled round a catheter. The silver wires were connected to a battery that generated electric current. In an animal model, the catheter was protected from bacterial colonisation up to 30 days and was superior to the silver sulphadiazine and chlorhexidine impregnated polyurethane catheter (Raad *et al.*, 1996b). They suggested the bactericidal activity might be due to both the silver ions and to the bactericidal activity from electric current.

Further work

Susceptibility of streptococci and enterococci to electricity

Fourteen species of bacteria and a *C. albicans* were tested in the zone of inhibition test but the streptococci and the enterococci were not tested. Although the streptococci and the enterococci are not the most commonly isolated organisms from catheters, together they are responsible for ca. 10 % of catheter related infections (Table 1-3). The recent emergence of enterococci that are resistant to glycopeptides (Johnson *et al.*, 1990) and to multiple antibiotics would increase the urgency to search for alternative approaches to the prevention of infections caused by these organisms. Since they do not have catalase which was considered as one of the resistance factors to H₂O₂ (Amin & Olson, 1968), electric current would be a promising approach. Therefore, susceptibility testing of streptococci and enterococci to electric current would be of interest.

Electrolysis using low voltage and low amperage electricity

The bactericidal activities of the electrical catheters were largely a result of electrolysis of ions surrounding the electrodes. Electrolysis has been well established as a process for production of chemicals in industry but its application to clinical situation has not been studied. Therefore, it would be a prerequisite to identify and quantify the factors that affect the electrolysis process on the electro-conducting catheters. This would include optimising the voltage, determining the relationship between the total current, the current density and the surface area of the device which needs protection, the minimum duration of electrolysis to be effective and the use of pulses of electric current instead of continuous electricity.

Bactericidal activity associated with the anode and due to chlorine

In the zone of inhibition test, 20 or more μA DC was required to produce inhibition around the anodal catheter (Table 4-1). Therefore, the activity associated with the anode was not investigated fully since this amperage exceeded the recommended safety amperage for any apparatus carrying electricity placed near the heart, which is less than 15 μA DC (BS 1989). However, 10 μA DC applied via the anodal catheter was effective in significantly reducing the number of bacteria in a 24 h biofilm. The reduction in the number of bacteria was partly due to the production of chlorine, though its

quantity was small. The factors affecting the efficacy of these oxidative compounds when they are used in very low concentrations are different when used as sterilising agents in inanimate objects. Therefore, the bactericidal activity associated with the anode and the small quantity of chlorine would need to be investigated further. The amount of chlorine produced by 10 μA was too low to be detected by the DPD method. A more sensitive method is required to measure this small quantity of chlorine. From these results, other antimicrobial agents that would act synergistically with chlorine at this concentration could be investigated.

Electricity and biofilm

Catheters inserted into a central vein would pass via the skin and the subcutaneous tissue before it enters the vascular cannulated vessel. The venous section of the catheter would be subjected to the continuous flow of blood. Immersing the catheters in a 1 litre broth with constant stirring in the batch culture (section 10.2) was a rudimentary method to mimic the in-vivo situation. Ten μA DC did not prevent the further development of nor did it sterilise a new biofilm in the batch culture system (section 10.3). This may be due to the minute quantities of the antimicrobial agents produced and the further dilution of the agents in a 1 litre test medium. The high growth rate of the organisms in the broth also rapidly outgrew the bactericidal activity of the minute quantities of the H_2O_2 and Cl_2 produced. A more suitable model would be the electrical Robbins device in which the surface colonised by bacteria would be exposed to the continuous flow of fluid. However, the growth rate cannot be accurately controlled in the electrical Robbin's device.

In the batch culture of the present study (section 10.3), when the organisms entered the stationery phase of growth, the number of bacteria in the 24 h biofilm on the electrical catheter decreased. Therefore, it appeared that the failure of 10 μA DC to inhibit biofilm growth in the initial 24 h was also due to the fast growth rate of the organisms. The efficacy of 10 μA DC to eradicate biofilm needs to be re-assessed in a growth-rate controlled situation.

Catheters embedded in nutrient agar (section 6.3.1) was analogous to the subcutaneous section of the catheter. In the present study, adherent bacteria on the catheter when embedded in nutrient agar and then exposed to 10 μA DC were inhibited from growth (section 6.3.1). However, this inhibition of adherent bacteria from further growth for 2 days by 10 μA DC would need further investigations such as the viability of the cells in the biofilm embedded in the agar. These results would also be applicable to the prevention of colonisation of indwelling prosthetic devices by bacteria since these devices are also surrounded by tissues.

Synergism with other antimicrobial agents

Fifteen to 400 mA electric current enhanced the activities of antibiotics against bacteria in the biofilm (Blenkinsopp *et al.*, 1992; Khoury *et al.*, 1992; Wellman *et al.*, 1996), similar synergism may be observed with lower amperage such as 10 μA DC.

Electric current (DC) carrying catheters may also be applied to electro-conducting catheters that are also incorporated with antimicrobial agents to enhance their bactericidal action. The bactericidal effect associated with the anode and the cathode were due to different reactive compounds, both of which may act synergistically with different antimicrobial agents. Combination of electric current and certain antimicrobial agents could be specifically selected to eradicate biofilms on catheters. Moreover, the charge of the catheter may be optimised so that it will augment any antibiotics that the patient is receiving. The different charges of the electrical catheters may also be utilised to extend the antimicrobial half-life of a catheter loaded with antimicrobial agents. The cathodal catheter may retain anionic compounds and the anodal catheter, cationic antimicrobial agents better and thus retard their leaching into the surrounding.

Conclusion

Ten μA DC applied to carbon catheters did not prevent adhesion of bacteria nor did it inhibit growth when the bacteria were suspended in broth. However, it was bactericidal to adherent bacteria that were still in the early stage of biofilm formation. The electrical catheters were effective in preventing both the intraluminal and extraluminal routes of infection. The bactericidal activity associated with the cathode was H_2O_2 and with the

anode, chlorine. Ten μA DC reduced the number of bacteria in a 24 h biofilm attached to an electrical catheter. The bactericidal activities associated with the cathode were further characterised and would be valuable to maximise the efficacy of electrical catheters in the prevention of catheter associated infections. The results of the present study would also be applicable to other medical device-related infections.

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