CHAPTER 15

Chlorhexidine

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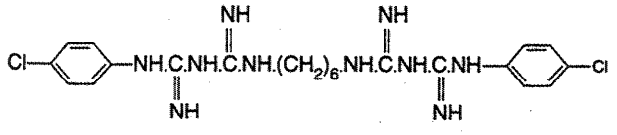
Chlorhexidine was first synthesized in 1950 in the laboratories of ICI Ltd. (England) during antimicrobial research into synthetic antimalarial agents of the proguanil type. It was found to possess a high level of antibacterial activity, low mammalian toxicity, and a strong affinity for binding to skin and mucous membranes. These properties led to the development of chlorhexidine principally as a topical antiseptic for application to such areas as skin, wounds, and mucous membranes and for dental use. In addition, chlorhexidine has been used as a pharmaceutical preservative, particularly in ophthalmic solutions and as a disinfectant for items such as inanimate surfaces and instruments.

CHEMISTRY

Chlorhexidine is 1,6-di(4-chlorophenyl-diguanido) hexane, a cationic bisbiguanide of the following formula:



Study of the related group of bisbiguanides demonstrated that this compound, with a single chlorine substituent in each phenol ring, was the most active (Davies, 1954). Chlorhexidine itself is a strong base, practically insoluble in water (0.008% wt/vol at 20°C), that reacts with acids to form salts of the RX_2 type. The water solubility of the different salts varies widely.

The soluble chlorhexidine digluconate cannot be isolated as a solid and is manufactured as a 20% wt/vol aqueous solution (chlorhexidine gluconate solution BP), higher concentrations being too viscous for convenient use. The diacetate salt has a solubility of 1.9% wt/vol (20°C), whereas the dihydrochloride and other inorganic salts are relatively insoluble (Table 15.1). The low solubility of the inorganic salts may cause problems of precipitation if a water-soluble salt such as digluconate is formulated with, or diluted in, a solution containing inorganic anions such as sulphate or carbonate. Generally, the solubility of chlorhexidine salts in alcohol is higher than that in water; however, chlorhexidine gluconate solution should not be added directly to neat alcohol, because precipitation may occur.

Solutions and powders of chlorhexidine are colorless or almost colorless and usually odorless, although formulations prepared from the diacetate salt occasionally have an odor of acetic acid. Solutions prepared from all salts have an extremely bitter taste that must be masked in formulations intended for oral use.

Chlorhexidine is moderately surface active and forms micelles in solution; the critical micellar concentration of the acetate is 0.01% wt/vol at 25°C (Heard and Ashworth, 1969). Aqueous solutions of chlorhexidine are most stable within the pH range 5 to 8. Above pH 8.0, chlorhexidine base is precipitated, and in more acid conditions there is gradual deterioration of activity because the compound is less stable. Hydrolysis yields p-chloroaniline; the amount is insignificant at room temperature, but it is increased by heating above 100°C, especially at alkaline pH (Goodall et al., 1968).

TABLE 15.1. *Solubility of chlorhexidine base and salts in water at 20°C (% wt/vol)*

Chlorhexidine base	0.008
Diacetate	1.9
Dihydrobromide	0.07
Dihydrochloride	0.06
Dinitrate	0.03
Sulphate	0.01
Carbonate	0.02

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Chemical analysis of chlorhexidine preparations can be performed using a variety of different methods. Samples containing 1 g or more of chlorhexidine may be assayed by the method described in the *British Pharmacopoeia,* that is, by dissolving the evaporated residue in glacial acetic acid (neutralized to crystal violet) and titrating against perchloric acid potentiometrically using a glass electrode. For lower concentrations, a colorimetric method may be used that involves a reaction with alkaline sodium hypobromide (Holbrook, 1958). Chlorhexidine also can be analyzed by gas liquid chromatography (Siefert and Casagrande, 1975) and high-performance liquid chromatography (Huston et al., 1982; Richard et al., 1984; Medlicott et al., 1994).

PHARMACEUTICAL ASPECTS

Compatibility

Chlorhexidine is a cationic molecule and is thus generally compatible with other cationic materials, such as quaternary ammonium compounds (e.g., cetrimide, benzalkonium chloride), although compatibility will depend on the nature and relative concentration of the second cationic species. It is, however, possible for a reaction to occur between chlorhexidine and the counterion of a cationic molecule, resulting in the formulation of a less-soluble chlorhexidine salt, which then may precipitate.

Nonionic substances such as detergents, although not directly incompatible with chlorhexidine salts, may inactivate the antiseptic to varying degrees, according to the chemical type and concentration used. In many cases, a suitable ratio of chlorhexidine to excipient can be chosen to give the required degree of bioavailability and hence activity, and this should be confirmed by suitable microbiologic tests.

Chlorhexidine is incompatible with inorganic anions in all but extremely dilute solutions (Table 15.1). This incompatibility sometimes may be overcome by adding a suitable solubilizing agent in formulations in which this is acceptable. Chlorhexidine is also incompatible with organic anions, such as soaps, sodium lauryl sulphate, sodium carboxymethyl cellulose, alginates, and many pharmaceutical dyes. In certain instances, there will be no visible signs of incompatibility, but the antimicrobial activity may be significantly reduced because of the chlorhexidine being incorporated into micelles.

Effect of pH on Activity

The antimicrobial activity of chlorhexidine is pH dependent; the optimum range of 5.5 to 7.0 corresponds to the pH of the body surfaces and tissues. Within the pH range 5 to 8, however, antibacterial activity will vary with the organism and the type of buffer used. For example, activity against *Staphylococcus aureus* and *Escherichia coli* rises with increased pH, whereas the reverse is true for *Pseudomonas aeruginosa.*

Isotonicity

Dilution of chlorhexidine in physiologic saline to render it isotonic with plasma should be regarded with caution because of the low solubility of chlorhexidine hydrochloride (<1 mg/100 mL). Although solutions may be free of precipitate on preparation, the solutions (normally containing at least 0.02% chlorhexidine) will be supersaturated, and precipitation of the hydrochloride salt is likely to occur on standing.

Sodium acetate may be used to adjust the tonicity of chlorhexidine solutions without the problem of precipitation; however, the pH of the required solution (2.1% wt/vol sodium acetate Ph. Eur) may be as high as 8.0 and should not, therefore, be stored for prolonged periods.

Coloring Solutions

Only a limited number of approved dyes can be used to color chlorhexidine solutions, and even these are anionic in nature and therefore not fully compatible. They usually can be added at low concentrations to tint chlorhexidine solutions for identification purposes but are likely to form a precipitate when used at the higher concentrations necessary to give good skin-staining properties. For example, carmoisine (E122) at a concentration of 0.0005% provides sufficient coloring for identification purposes and will remain stable for long periods. At a concentration of 0.05%, it has good skin-staining properties, but it may precipitate on storage. This solution is usually given a shelf-life of 7 days after preparation.

Packaging

The nature and quality of containers for concentrates and use dilutions are important. Glass, high-density polypropylene, and high-density polyethylene are usually suitable. Low-density polyethylene may be unsuitable because of excessive adsorption, and other packaging materials may interact with the antiseptic. Cork stoppers or cap linings never should be used because water-soluble tannins present may inactivate chlorhexidine (Linton and George, 1966).

Sterilization

Dilute solutions of chlorhexidine (<1.0% wt/vol) may be sterilized by autoclaving at 115°C for 30 minutes or at 121 to 123°C for 15 minutes. Autoclaving of solutions greater than 1.0% can result in the formation of insoluble residues and is therefore unsuitable. If sterile solutions are required at such high concentrations, filtration through a 0.22 micron membrane filter is recommended;

however, the first 10 mL should be discarded because adsorption may occur in the initial stage; fibrous and porcelain filters are unsuitable.

Chlorhexidine hydrochloride powder is stable to dry-heat sterilization at 150°C. The solid salts are stable to sterilizing doses of gamma radiation, but chlorhexidine in solution is decomposed.

Storage

Dilute chlorhexidine solutions may be stored at room temperature, and a shelf-life of at least 1 year can be expected, provided preparation and packaging are adequate. Prolonged exposure to high temperature or light is to be avoided because this can adversely affect stability. All dilute solutions to be stored should be either heat-treated (sterilized or pasteurized) or chemically preserved (4% isopropanol or 7% ethanol) to eliminate the possibility of microbial contamination. For autoclaved solutions, the choice of container material is important, the best results being achieved by using neutral glass or polypropylene. Guidelines on the storage of locally prepared aqueous solutions are as follows:

Untreated solutions: Prepare and use within 24 hours. Do not store.

Chemically preserved solutions: Store unopened for a maximum of 3 months. When opened, use within 7 days.

Sterilized solutions: Store unopened for a maximum of 12 months. When opened use within 24 hours.

Chlorhexidine and Laundering

Chlorhexidine is absorbed onto the fibers of certain fabrics, particularly cotton, and resists removal by washing. If a hypochlorite (chlorine-releasing) bleach is used during the washing procedure, a fast brown stain may develop because of a chemical reaction between the chlorhexidine and the bleach. This problem can be avoided by eliminating the use of a bleach or replacing the chlorine-releasing bleach with one that is peroxide based, such as sodium perborate. Pretreatment of the fabrics with dilute (1%) hydrochloric acid or oxalic acid for 10 to 15 minutes reduces or eliminates staining when a chlorine bleach is subsequently used.

MICROBIOLOGY

The antimicrobial activity of chlorhexidine is directed mainly toward vegetative gram-positive and gram-negative bacteria; it is inactive against bacterial spores except at elevated temperatures, and acid-fast bacilli are inhibited but not killed by aqueous solutions. The infectivity of some lipophilic viruses [e.g., influenzavirus, herpesvirus, human immunodeficiency virus (HIV)] is rapidly inactivated by chlorhexidine, although aqueous solutions are not active against the small protein-coat viruses. Yeasts (including *Candida albicans*) and dermatophytes are usually sensitive, although chlorhexidine's fungicidal action in general is subject to species variation, as are other agents.

Mechanisms of Antibacterial Action

The mechanism of action of chlorhexidine and related biguanides was reviewed by Woodcock (1988). At relatively low concentrations, the action of chlorhexidine is bacteriostatic, and at higher concentrations, it is rapidly bactericidal, with the actual levels varying somewhat from species to species.

The lethal process consists of a series of related cytologic and physiologic changes, some of which are reversible, that culminate in the death of the cell. The sequence is thought to be as follows: (1) rapid attraction toward the bacterial cell; (2) specific and strong adsorption to certain phosphate-containing compounds on the bacterial surface; (3) overcoming the bacterial cell wall exclusion mechanisms; (4) attraction toward the cytoplasmic membrane; (5) leakage of low–molecular weight cytoplasmic components, such as potassium ions, and inhibition of certain membrane-bound enzymes, such as adenosyl triphosphatase; (6) precipitation of the cytoplasm by the formation of complexes with phosphated entities, such as adenosine triphosphate and nucleic acids.

Characteristically, a bacterial cell is negatively charged, the nature of the ionogenic groups varying with bacterial species. It has been shown that, given sufficient chlorhexidine, the surface charge of the bacterial cell is rapidly neutralized and then reversed. The degree of charge reversal is proportional to the chlorhexidine concentration and reaches a stable equilibrium within 5 minutes. The rapid electrostatic attraction of the cationic chlorhexidine molecules and the negatively charged bacterial cell undoubtedly contributes to the rapid rate of kill associated with chlorhexidine, although surface charge reversal is secondary to cell death. Electron microscopy and assay for characteristic outermembrane components, such as 2-keto-3-deoxyoctonate (KDO), demonstrate that sublethal concentrations of chlorhexidine bring about changes in the outer membrane integrity of gram-negative cells. An efflux of divalent cations, especially calcium ions, occurs prior to or during such outer-membrane changes. Chlorhexidine molecules are thought to compete for the negative sites on the peptidoglycan, thereby displacing metallic cations.

In terms of the lethal sequence, the bacterial cytoplasmic membrane appears to be the important site of action. Several changes indicative of damage to the cytoplasmic membrane have been observed in bacterial populations treated with bacteriostatic and bactericidal levels of

chlorhexidine. Leakage of cytoplasmic contents is a classic indication of damage to the cytoplasmic membrane, starting with low molecular weight molecules typified by potassium ions. Electron micrographs of these sublethally treated cells show a shrinkage or plasmolysis of the protoplast. Cells treated with bacteriostatic levels of compound can recover viability despite having lost up to 50% of their K+. This is particularly true if the excess chlorhexidine is removed by a neutralizing agent, as happens in many in vitro testing situations.

As the chlorhexidine concentration is increased, higher molecular weight cell contents, such as nucleotides, appear in the supernatant fluid around the cell. Bacterial cells showing more than a 15% increase in nucleotide leakage have been found to be damaged irreversibly; levels of chlorhexidine producing this effect are therefore bactericidal. The rate of membrane disruption and cell leakage increases with chlorhexidine concentration up to a maximum and then falls back and at concentrations that are rapidly bactericidal (100 to 500 mg/L) release of cell components does not occur. Electron microscopy shows the cytoplasm of these cells to be chemically precipitated, this precipitation having been caused by an interaction between the chlorhexidine and phosphated entities within the cytoplasm, such as adenosine triphosphate and nucleic acids.

Antimicrobial Spectrum

Although numerous publications refer to the bacteriostatic and bactericidal properties of chlorhexidine against particular organisms, the methods used vary, and it is often difficult to compare results. A series of studies were therefore performed to provide a comprehensive spectrum of activity for chlorhexidine using both microbiostatic and microbiocidal methods. The strains of organisms tested include clinical isolates, laboratory strains, and standard culture collection types. Each strain was tested to determine the minimum inhibitory concentration (MIC) of chlorhexidine and its susceptibility to the bactericidal action of 0.05% aqueous chlorhexidine gluconate using a rate-of-kill method.

MIC Method

Twofold dilutions of chlorhexidine gluconate were prepared in Isosensitest agar, the surface of which was inoculated with a suspension of each test organism. After incubation at 37°C for 24 hours, the agar was examined for distinct growth. The MIC was recorded as the lowest chlorhexidine concentration that prevented growth.

Molds and yeasts were tested on Sabouraud's agar incubated at 30°C for 24 to 72 hours. Anaerobes were incubated anaerobically for 2 to 3 days on agar containing 5% lysed blood. Fastidious organisms were incubated in CO_2 for 2 to 3 days (Tables 15.2 and 15.3).

TABLE 15.2. *Bacteriostatic activity of chlorhexidine gluconate*

Test organism	MIC (mg/L)		
	No. of strains	Mean	Range
Gram-positive cocci			
Micrococcus flavus	1	0.5	
Micrococcus lutea	1	0.5	
Staphylococcus aureus	16	1.6	1–4
Staphylococcus epidermidis	41	1.8	0.25–8
Streptococcus faecalis	5	38	32–64
Streptococcus mutans	2	2.5	
Streptococcus pneumoniae	5	11	8–16
Streptococcus pyogenes	9	3	1–8
Streptococcus sanguis	3	9	4–16
Streptococcus viridans	5	25	2–32
Gram-positive bacilli			
Bacillus cereus	1	8	
Bacillus subtilis	2	1	
Clostridium difficile	7	16	8–32
Clostridium welchii	5	14	4–32
Corynebacterium spp	8	1.6	0.5–8
Lactobacillus casei	1	128	
Listeria monocytogenes	1	4	
Propionibacterium acne	2	8	
Gram-negative bacilli			
Acinetobacter anitratus	3	32	16–64
Acinetobacter lwoffi	2	0.5	
Alkaligenes faecalis	1	64	
Bacteroides distastonis	4	16	
Bacteroides fragilis	11	34	8–64
Campylobacter pyloridis	5	17	8–32
Citrobacter freundii	10	18	4–32
Enterobacter cloacae	12	45	16–64
Escherichia coli	14	4	2–32
Gardnerella vaginalis	1	8	
Haemophilus influenza	10	5	2–8
Klebsiella aerogenes	5	25	16–64
Klebsiella oxytoca	2	32	
Klebsiella pneumoniae	5	64	82–128
Proteus mirabilis	5	115	64–>128
Proteus morganii	5	73	16–128
Proteus vulgaris	5	57	32–128
Providencia stuartii	5	102	64–128
Pseudomonas aeruginosa	15	20	16–32
Pseudomonas cepacia	1	16	
Pseudomonas fluorescens	1	4	
Salmonella bredeney	1	16	
Salmonella dublin	1	4	
Salmonella galinarum	1	8	
Salmonella montivideo	1	8	
Salmonella typhimurium	4	13	8–16
Salmonella virchow	1	8	
Serratia marcescens	10	30	16–64

MIC, minimal inhibitory concentration.

Rate-of-Kill Test

The in vitro bactericidal and fungicidal activity of 0.05% chlorhexidine gluconate was determined using a procedure based on British Standard 3286 (1960). One milliliter of a 24-hour broth culture of the test organism was added to 10 mL of aqueous 0.05% wt/vol chlorhexi-

TABLE 15.3. *Fungistatic activity of chlorhexidine*

Organism	No. of strains	Mean MIC (mg/L)
Mold fungi		
Aspergillus flavus	1	64
Aspergillus fumigatus	1	32
Aspergillus niger	1	16
Penicillium notatum	1	16
Rhizopus sp.	1	8
Scopulariopsis spp.	1	8
Yeasts		
Candida albicans	2	9
Candida guillermondii	1	4
Candida parapsilosis	2	4
Candida pseudotropicalis	1	3
Cryptococcus neoformans	1	1
Prototheca zopfii	1	6
Saccharomyces cerevissia	1	1
Torulopsis glabrata	1	6
Dermatophytes		
Epidermophyton floccosum	1	4
Microsporum canis	2	4
Microsporum fulvum	1	6
Microsporum gypseum	1	6
Trichophyton equinum	1	4
Trichophyton interdigitale	2	3
Trichophyton mentagrophytes	1	3
Trichophyton quinkeanum	1	3
Trichophyton rubrum	2	3
Trichophyton tonsurans	1	3

MIC, minimal inhibitory concentration.

dine gluconate solution, which was maintained at ambient temperature (18° to 21°C). One-milliliter aliquots of the mixture were removed after 20 seconds, 1 minute, and 10 minutes and transferred to inactivator broth containing 1.5% soya lecithin and 10% polysorbate 80. A viable count was performed on appropriate further dilutions and, by comparison with an untreated control, a 10-log reduction factor was calculated (Tables 15.4 and 15.5).

Bacterial Susceptibility

The susceptibility of individual bacterial strains to chlorhexidine varies widely; however, few have been found to be capable of surviving concentrations of the antiseptic encountered in use (Pitt et al., 1983; Hammond et al., 1987). It has been suggested that prolonged use of the antiseptic may lead to reduced susceptibility and to the development of resistant bacteria; however, this is not supported by the work of Martin (1969) and Simpson et al. (1989), who found that bacterial strains encountered in areas of prolonged and extensive use of the antiseptic have similar susceptibilities to strains of the same species encountered in areas where there is little or no chlorhexidine.

There is also no good evidence that the plasmid-mediated antibiotic resistance common among gram-negative bacteria is associated with resistance to chlorhexidine.

TABLE 15.4. *Bactericidal activity of 0.05% chlorhexidine gluconate*

Test organism	(No. of strains)	Mean $\log_{10}$ reduction after 1/3 min	1 min	10 min
Gram-positive cocci				
Micrococcus flavus	(1)	0.1	0.4	2.1
Micrococcus lutea	(1)	0.2	0.7	2.9
Staphylococcus aureus	(16)	0.4	0.7	2.5
Staphylococcus epidermidis	(41)	2.2	3.4	>5.1
Streptococcus faecalis	(5)	0.4	0.4	1.1
Streptococcus mutans	(2)	0.8	>4.6	5.8
Streptococcus pneumoniae	(5)	0.8	1.5	>3.5
Streptococcus pyogenes	(9)	1.2	1.8	>3.7
Streptococcus sanguis	(3)	1.1	2.2	>3.9
Streptococcus viridans	(5)	0.4	0.8	2.3
Gram-positive bacilli				
Bacillus cereus	(1)	2.0	2.0	4.7
Bacillus subtilis	(2)	0.5	0.5	0.3
Clostridium difficile	(7)	0.2	0.3	0.3
Clostridium welchii	(5)	2.1	3.1	>4.8
Corynebacterium spp	(8)	1.1	1.4	3.7
Lactobacillus casei	(1)	0.2	0.2	4.1
Listeria monocytogenes	(1)	0.6	2.2	4.8
Propionibacterium acne	(2)	0.7	1.8	3.6
Gram-negative bacilli				
Acinetobacter anitratus	(3)	1.4	2.6	>5.3
Acinetobacter lwoffi	(2)	>4.0	>4.3	>4.8
Alkaligenes faecalis	(1)	1.5	2.7	4.1
Bacteroides distastonis	(4)	0.9	2.7	>4.9
Bacteroides fragilis	(11)	3.0	4.2	5.2
Campylobacter pyloridis	(5)	N.T.	2.8	>4.0
Citrobacter freundii	(10)	3.4	4.9	>6.0
Enterobacter cloacae	(12)	3.5	4.5	>6.3
Escherichia coli	(14)	3.2	5.0	>6.4
Gardnerella vaginalis	(1)	2.3	3.3	>5.8
Haemophilus influenza	(10)	>4.1	>4.1	>4.1
Klebsiella aerogenes	(5)	2.7	3.9	>5.9
Klebsiella oxytoca	(2)	3.2	5.2	>6.4
Klebsiella pneumoniae	(5)	3.0	4.8	>6.2
Proteus mirabilis	(5)	0.8	0.9	2.9
Proteus morganii	(5)	1.0	1.5	4.2
Proteus vulgaris	(5)	0.8	1.0	4.1
Providencia stuartii	(5)	0.6	0.9	1.8
Pseudomonas aeruginosa	(15)	1.7	2.7	4.9
Pseudomonas cepacia	(1)	1.1	1.3	>4.6
Pseudomonas fluorescens	(1)	3.8	5.0	>6.7
Salmonella bredeney	(1)	1.6	3.4	>6.4
Salmonella dublin	(1)	1.5	2.9	3.2
Salmonella galinarum	(1)	2.5	4.0	>6.2
Salmonella montivideo	(1)	2.4	3.8	>6.3
Salmonella typhimurium	(4)	2.0	3.7	>6.0
Salmonella virchow	(1)	1.9	3.9	>6.2
Serratia marcescens	(10)	1.5	3.7	>5.9

TABLE 15.5. *Fungicidal activity of 0.05% chlorhexidine gluconate*

Test organism	No. of strains	Mean • $\log_{10}$ reduction after 1/3 min	1 min	10 min
Mold fungi				
Aspergillus flavus	1	0.4	0.8	1.7
Aspergillus fumigatus	1	0.7	1.2	2.4
Aspergillus niger	1	0.7	1.2	3.0
Penicillium notatum	1	0.6	2.0	3.5
Rhizopus spp.	1	0.4	0.4	0.5
Scopulariopsis spp.	1	0.6	1.1	2.3
Yeasts				
Candida albicans	2	2.8	>4.1	>4.2
Candida guillermondii	1	3.5	>4.3	>4.3
Candida parapsilosis	2	2.1	3.4	>4.2
Candida pseudotropicalis	1	3.6	>4.4	>4.4
Cryptococcus neoformans	1	4.0	>4.2	>4.2
Prototheca zopfii	1	3.3	>3.6	>3.6
Saccharomyces cerevissia	1	3.7	>3.7	>3.7
Torulopsis glabrata	1	1.3	2.2	>4.4
Dermatophytes				
Epidermophyton floccosum	1	0.7	0.5	>1.8
Microsporum canis	2	0.4	1.0	>2.0
Microsporum fulvum	1	0.2	0.6	>2.4
Microsporum gypseum	1	0.1	0.3	2.0
Trichophyton equinum	1	0.5	1.1	>2.1
Trichophyton interdigitale	2	0.4	0.9	>2.4
Trichophyton mentagrophytes	1	1.3	>2.1	>2.1
Trichophyton quinkeanum	1	0.2	0.9	>2.8
Trichophyton rubrum	2	0.3	0.6	>2.4
Trichophyton tonsurans	1	0.4	0.3	1.6

Michel-Briand (1986), Ahonkai et al. (1984), and Sykes and Matthew (1976) were unable to find any increase in chlorhexidine-resistance amongst antibiotic-resistant strains of *E. coli, P. aeruginosa, Serratia marcescens,* or *Proteus mirabilis*.

Early studies with strains of methicillin-resistant *S. aureus* (MRSA) using bacteriostatic MIC test procedures demonstrated a degree of reduced sensitivity to chlorhexidine compared to methicillin-sensitive strains of this organism (MSSA) (Brumfitt et al., 1985; Mycock, 1985); however, this is considered of little clinical relevance because the highest MIC value for chlorhexidine quoted in these studies is 4 mg/L, and therefore all strains of *S. aureus,* including MRSA, can be regarded as sensitive to user concentrations of chlorhexidine. Both Haley et al. (1985) and Cookson et al. (1989) found the bactericidal activity of a 4% chlorhexidine handwash to be similar for strains of MRSA and MSSA. A number of clinical reports also support the use of chlorhexidine preparations as part of programs for the control of outbreaks with MRSA (Rumbak and Cancio, 1994; Jones and Martin, 1987; Lejeune et al., 1986); however, Kampf et al. (1998) found a chlorhexidine in alcohol handrub to be more effective than a chlorhexidine-based handwash against MRSA and recommend this form of hand disinfection by staff treating MRSA patients.

Depathogenizing Effect

Whereas killing potentially pathogenic bacteria certainly will prevent them from causing infection, certain types of sublethal chemical treatment might also alter or damage bacterial cells in such a way as to reduce their ability to initiate the disease process. Thus, the bacteria could still be viable but less pathogenic. The ability of chlorhexidine to produce such a depathogenizing effect was first investigated by Holloway et al. (1986) using a peritonitis model in mice. Pathogenic strains of *E. coli* and *Klebsiella aerogenes* were treated with sublethal concentrations of chlorhexidine, after which the antiseptic was neutralized and the test suspension injected into susceptible animals. The results of these studies demonstrated that the pathogenicity of bacteria surviving treatment with chlorhexidine was reduced by more than 90%. This was confirmed by Rotter et al. (1988), who could not demonstrate a similar effect with alcohol. The depathogenizing effect of chlorhexidine must be considered secondary to the direct bactericidal activity of the antiseptic; however, it is believed to be an additional, clinically relevant property that is not evident in conventional in vitro studies concerned with viability alone.

Sporicidal Activity

Chlorhexidine will inhibit the growth of the vegetative cells of spore-forming bacteria at relatively low concen-

TABLE 15.6. *Virucidal activity of chlorhexidine gluconate*

Virus	Viral family	Activity	Concentration (%)	Reference
Respiratory syncytial virus	Paramyxovirus	+	0.25	Platt and Bucknall (1985)
Herpes hominis/simplex	Herpesvirus	+	0.02	Bailey and Longson (1972)
Polio virus type 2	Enterovirus	–	0.02	Bailey and Longson (1972)
Adenovirus type 2	Adenovirus	–	0.02	Bailey and Longson (1972)
Equine infectious anaemia virus	Retrovirus	+	2.0	Shen et al. (1977)
Variola virus (smallpox)	Poxvirus	+	2.0	Tanabe and Hotta (1976)
Herpes simplex type 1/type 2	Herpesvirus	+	0.02	Shinkai (1974)
Equine influenza virus	Orthomyxovirus	+	0.001	Eppley (1968)
Hog cholera virus	Togavirus	+	0.001	Eppley (1968)
Bovine viral diarrhoea	Togavirus	+	0.001	Eppley (1968)
Parainfluenza virus	Paramyxovirus	+	0.001	Eppley (1968)
Transmissible gastroenteritis virus	Coronavirus	+	0.001	Eppley (1968)
Rabies virus	Rhabdovirus	+	0.001	Eppley (1968)
Canine distemper virus	Paramyxovirus	+	0.01	Eppley (1968)
Infectious bronchitis virus	Coronavirus	+	0.01	Eppley (1968)
Newcastle virus	Paramyxovirus	+	0.01	Eppley (1968)
Pseudo rabies virus	Herpesvirus	+	0.01	Matishek (1978)
Cytomegalovirus	Herpesvirus	+	0.1	Faix (1986)
Coxsackie virus	Picornavirus	–	0.4	Narang and Codd (1983)
Echo virus	Picornavirus	–	0.4	Narang and Codd (1983)
Human Rota virus	Reovirus	–	1.5	Springthorpe et al. (1986)
Human Immunodeficiency Virus Type I	Retrovirus	+	0.2	Harbison and Hammer (1989)

+, Active in vitro at the concentration stated; –, not active in vitro at the concentration stated.

trations (Table 15.2) and also will inhibit spore germination. It is generally recognized, however, that chlorhexidine has little sporicidal activity except at elevated temperatures. Shaker et al. (1986) investigated the sporicidal activity of an aqueous solution of chlorhexidine gluconate (25 mg/L) against *Bacillus subtilis* spores at various temperatures. At 20°C, 30°C, and 37°C, the antiseptic had little effect on spore viability, even after 120 minutes exposure. At a temperature of 70°C, however, the antiseptic reduced the number of spores by 5 logarithms (i.e., a 99.999% reduction).

Virucidal Activity

Chlorhexidine has good activity against viruses with a lipid component in their coats or with an outer envelope. These include many respiratory viruses, herpes, and cytomegalovirus. In common with many other antiseptics, however, aqueous solutions of chlorhexidine do not have any significant activity against the small protein-coat viruses, which include many of the enteric viruses, poliomyletis, and papilloma (warts) virus.

Human immunodeficiency virus (HIV), the organism responsible for acquired immunodeficiency syndrome (AIDS), is known to be one of the enveloped viruses and can, therefore, be predicted to be sensitive to the action of chlorhexidine. This was confirmed in a series of in vitro studies. A 4% chlorhexidine handwash preparation and 0.5% chlorhexidine in 70% alcohol both were found to be 100% effective against HIV type I after a 15-second contact. Aqueous solutions of chlorhexidine down to a final test concentration of 0.05% were 100% effective within 1 minute (Montefiori et al., 1990). In a more recent series of studies, chlorhexidine at 1 mg/mL (0.1%) was 80% to 100% effective (Harrison and Chantler, 1998). Published data on the activity of chlorhexidine against a wide range of viral agents are summarized in Table 15.6.

CLINICAL APPLICATIONS AND EFFICACY

Skin Disinfection

Chlorhexidine formulated in a detergent base is used extensively for disinfection of the hands of surgeons and nurses and also for whole-body skin disinfection of patients undergoing surgery. Alcohol-based chlorhexidine solutions with emolients also are used by surgeons and nurses for hand disinfection. Alcohol-based chlorhexidine solutions are particularly suitable for final-stage skin preparation of the operation site; the area should be kept wet for at least 2 minutes to achieve the maximal effect. The immediate bactericidal action of chlorhexidine surpasses that of similar preparations containing povidone–iodine, triclosan, hexachlorophane, or parachlorometaxylenol (PCMX). Its valuable persistent (residual) effect, which prevents regrowth of organisms on the skin, is comparable to that of hexachlorophane or triclosan, although chlorhexidine has a broader spectrum of activity, particularly against gram-negative bacteria.

The evidence for efficacy is derived from extensive laboratory testing on volunteers and hospital staff and is generally supported by clinical experience now extending over more than 25 years. Some of this evidence is presented, although differences such as experimental method

inevitably make it difficult to compare data produced by different authors. Furthermore, because the efficacy of any formulation is significantly affected by the excipients present, trials demonstrating activity of one formulation cannot be used as evidence for the efficacy of another.

When assessing the effectiveness of an agent for skin disinfection, several properties of the product must be taken into consideration: immediate bactericidal action against both the resident and transient flora, persistence of action preventing regrowth of skin organisms, and a cumulative effect resulting from regular use. The product also must retain its activity in the presence of blood and have good cosmetic acceptability for the user. The relative importance of these individual properties varies to some degree according to the particular application of the antiseptic.

Surgical Hand Disinfection

The objective of surgical hand disinfection is to render the skin free of bacteria, thus preventing the escape of organisms into the operation wound through the punctures in surgical gloves, which occur frequently during operation (O'Connor, 1984). The procedure must eliminate the transient organisms that are likely to be present on the skin and reduce the resident flora to as low a level as possible. The agent should remain persistent on the skin to maintain the numbers of survivors at this low level throughout the course of the operation.

In the first in-use study involving a 4% chlorhexidine handwash, Smylie et al. (1973) found this antiseptic to be at least as effective as hexachlorophane in reducing the numbers of bacteria on the hands of the surgical team and in maintaining these low numbers for several hours under gloves. A povidone–iodine handwash was less effective initially and allowed the numbers of survivors on the hands to increase dramatically during the course of an operation. The chlorhexidine handwash was more acceptable to users than the other handwashes, undesirable side effects occurring most frequently with the povidone–iodine preparation.

In a large volunteer study using the official U.S. Food and Drug Administration (FDA) guidelines for assessing surgical hand scrubs, Peterson et al. (1978) found that a chlorhexidine handwash produced significantly greater reductions in numbers of resident bacteria than either hexachlorophane or povidone–iodine preparations and maintained these low numbers for up to 6 hours under gloves. Povidone–iodine was not persistent, allowing a significant increase in bacterial numbers with time. This particular chlorhexidine handwash (Hibiclens) was also the first surgical hand scrub to be approved as safe and effective by the Topical Antimicrobials Committee of the FDA.

Aly and Maibach (1983) examined the effectiveness of sponge brushes impregnated with either chlorhexidine or povidone–iodine handwashes in a volunteer surgical hand-disinfection study. The immediate and persistent effects of the agents and also the effect of blood was assessed. Chlorhexidine proved to be significantly more effective than povidone–iodine in both the presence and absence of blood, thus confirming an earlier study by Lowbury and Lilly (1974), who found that blood significantly reduced the effectiveness of povidone-iodine, but not of chlorhexidine.

Pereira et al. (1997) evaluated several procedures for surgical hand disinfection with detergent hand scrubs and alcohol-based hand rubs. The most effective treatments were a 5-minute scrub with 4% chlorhexidine detergent solution and combination treatments consisting of an initial 2- minute scrub with 4% chlorhexidine detergent solution, followed by a 30-second hand rub with chlorhexidine in alcohol solution. The authors considered the alcohol-based handrubs to be as effective as the detergent-based handscrub solution and no more damaging to the skin.

In an evaluation of duration of surgical scrub techniques, Okubo et al. (1994) found that a 3-minute "scrub" with chlorhexidine detergent solution without brushes was as effective as 3- or 6-minute scrubs with brushes, and there was much less risk of injury to the skin.

Hygienic Hand Disinfection

The major source of infectious organisms within the hospital is the infected or heavily colonized patient, and the principal route of transmission is via the hands of hospital personnel. Handwashing or disinfection is therefore considered to be the single most important measure to prevent nosocomial infection. The primary objective of hygienic hand disinfection is to eliminate the transient organisms that have been acquired on the hands, thus preventing their transfer between patients. The agent must act rapidly because busy hospital staff wash for only a short time. Persistence of antibacterial action on the skin is considered desirable to help prevent colonization with hospital pathogens and reduce the level of contaminants acquired between handwashes. User acceptability of a formulation is of particular importance in the ward situation, where frequent handwashing is often necessary. Factors such as soreness and dryness of the hands can have more influence on the use of a handwash product than its antimicrobial efficacy (Larson et al., 1986).

Rapidity of action of chlorhexidine against transient contaminants artificially applied to the skin was demonstrated by La Rocca et al. (1985). In a study designed to mimic a ward handwash, the hands of subjects were repeatedly contaminated with *S. marcescens* and washed with chlorhexidine skin cleanser for 15 seconds. A 99.9% reduction of the contaminants was demonstrated after a single 15-second wash with chlorhexidine. Progressively greater reductions were obtained following repeated

washing, owing to the cumulative activity of residual chlorhexidine.

Larson et al. (1986) compared the microbiologic and physiologic changes to skin brought about by frequent handwashing (15-second wash, 24 times daily). A chlorhexidine handwash significantly reduced the bacterial hand flora compared with all other agents tested and produced no more skin trauma than nonmedicated soap.

Maki et al. (1979) compared the effectiveness of chlorhexidine skin cleanser with other handwash preparations in a controlled trial with nurses in a neurosurgical unit. Following a single 15-second wash, chlorhexidine significantly reduced both the total skin flora and gram-negative organisms acquired naturally during patient contact. No significant reductions in bacterial counts were found following washing with unmedicated soap or an iodophor-containing preparation. The effect of regular use of the agents also was investigated. Each agent was used exclusively on the unit for 4 weeks, during which period repeated hand samples were taken. Regular use of chlorhexidine produced the lowest total bacterial counts and lowest mean numbers of gram-negative bacilli and *S. aureus*. This finding confirmed the marked effectiveness of chlorhexidine skin cleanser in a single brief handwashing and demonstrates its persistent effect between repeated handwashes.

In a later study, Maki and Hecht (1982) investigated the effect of different handwash preparations on the incilence of nosocomial infection in a large intensive care unit. Lower rates of infection were obtained with both chlorhexidine and povidone–iodine handwash preparations than with unmedicated soap; however, chlorhexidine was better tolerated and was considered more acceptable for routine use. Stanley et al. (1989) also demonstrated that the rate of infection in an intensive care unit was significantly lower when hands were washed with a 4% chlorhexidine preparation than when liquid soap and an alcohol rinse were used.

Preoperative Whole-Body Disinfection

A significant proportion of postoperative wound infections are caused by microorganisms from the patient's own skin, which may be derived from sites remote from that of the operation. Reducing the level of the skin microflora over the whole body is, therefore, thought to be of benefit in reducing the incidence of infection from these sources.

Brandberg and Anderson (1980) demonstrated a significant reduction in total skin flora following a shower bath with a 4% chlorhexidine skin cleanser and found that this reduction was maintained for up to 1 week, emphasizing the persistent properties of the antiseptic. These results were confirmed by Kaiser et al. (1988), who also demonstrated a beneficial cumulative effect with repeated application of chlorhexidine.

Following earlier microbiologic studies, Brandberg et al. (1980) performed a clinical investigation to determine the effect of preoperative showering with chlorhexidine skin cleanser on infection rates in patients undergoing vascular surgery. On admission, patients bathed daily with the antiseptic (three to eight baths, depending on the length of preoperative stay). Immediately prior to surgery, the operation site was washed with this same solution and finally prepared using 0.5% chlorhexidine in 70% alcohol. A control group of patients did not perform daily bathing but received the same operation-site preparation. The infection rate was reduced from 17.5% in the control group to 8% in the patients who showered preoperatively with chlorhexidine.

In a closely monitored clinical study involving more than 2,000 surgical patients, Hayek et al. (1987) demonstrated a significant reduction in the infection rate in patients who bathed with chlorhexidine skin cleanser on two occasions in the 24 hours prior to surgery. The overall infection rate was 9% for patients who bathed with chlorhexidine compared with 12.5% for those using unmedicated bar soap.

In addition to reducing patient morbidity, this infection control measure was considered to confer significant health economic benefits to the hospital in terms of improved bed use and cost effectiveness. Hayek and Emerson (1988) reported that the reduction in infection rate achieved by preoperative bathing with chlorhexidine would save 22 bed days per 100 patients, allowing almost 3% more patients to be admitted for elective surgery.

Other workers investigated the effect of both preoperative and postoperative bathing with chlorhexidine. Randall and co-workers reported the results of two prospective studies in men undergoing vasectomy. In the first of these (Randall et al., 1983), preoperative bathing with chlorhexidine skin cleanser was microbiologically effective, but it did not reduce the postoperative infection rate, which was in excess of 30%. In the second study (Randall et al., 1985), preoperative bathing with chlorhexidine was retained, and the effect of additional postoperative bathing on the days following operation was examined. This procedure, which involved both preoperative and postoperative bathing, reduced the infection rate from 37.8% to 6.7%. Wound infection in this group of patients was considered largely a secondary phenomenon occurring after the patient left the hospital.

Some trials failed to demonstrate a clinical benefit, although these either have not followed the recommended skin disinfection method or have included too few patients to show statistical significance. In a review of all studies on the use of chlorhexidine for whole-body disinfection, Brandberg (1989) concluded that the procedure is a valuable adjunct to existing antiseptic and aseptic measures that will contribute toward a reduction in infections caused by organisms derived from the patient's own skin.

Urology

Numerous reports have described the effectiveness of chlorhexidine preparations in preventing urinary tract infections. A solution of 0.05% chlorhexidine in either glycerine (Miller et al., 1960) or ethylene glycol (Gillespie, 1962) was used successfully as a combined urethral antiseptic and lubricant. Intermittent bladder irrigation with 0.02% aqueous solution of chlorhexidine was effective in reducing the incidence of bacteria in catheterized patients (Ball, 1987; Kirk, 1979; Bruun and Digranes, 1978). Solutions used in the urinary tract should be prepared from chlorhexidine gluconate solution BP, which contains no additives. The inadvertent use of solutions containing surfactants causes hematuria in some patients.

Obstetrics and Gynecology

In one of the earliest reports involving chlorhexidine, Calman and Murray (1956) reported on the suitability of several antiseptics for use in obstetrics. They considered that the most exacting test, and the one most relevant to practical obstetrics, was a 2 1/2-minute bactericidal test in the presence of fresh blood. By this standard, only chlorhexidine achieved a complete kill of all organisms at its recommended user dilution of 1 in 2,000 (0.05%). On the basis of this work and on the experience of 2 1/2 years of clinical use, chlorhexidine was considered the most satisfactory antiseptic available for use in midwifery.

Byatt and Henderson (1973) compared the ability of different antiseptics to disinfect the perineum. The most effective aqueous preparation was a 4% chlorhexidine detergent preparation both undiluted and at a dilution of 1:80, which brought about reductions in recoverable bacteria of 98% and 82%, respectively. An equivalent concentration of chlorhexidine without added cleansing agents was less effective, suggesting that the mucous secretions and fatty acid exudates present in this situation prevent penetration of antiseptics to the bacteria.

Christensen et al. (1985) investigated the effectiveness of vaginal washing with a 0.2% chlorhexidine solution for the prevention of neonatal colonization with group B streptococci. A significant reduction in colonization with this organism was noted in the infants of treated mothers, and the investigators concluded that this procedure may prevent serious infection in the early neonatal period. Taha et al. (1997) reported that neonatal admissions and mortality due to sepsis were reduced when a sterile aqueous solution of chlorhexidine 0.25% was used to cleanse and disinfect the birth canal.

Wounds and Burns

Platt and Bucknell (1984), using a wound model in guinea pigs, demonstrated that irrigation with 0.05% chlorhexidine was highly effective in preventing wound sepsis. This finding was confirmed in the clinical situation by Colombo et al. (1987), who demonstrated a significant reduction in postoperative infection in patients whose wounds were sprayed with 0.05% chlorhexidine during suturing. Groups of workers in France also have found irrigation with chlorhexidine to be effective for controlling existing wound infection (Carrier-Clerambault, 1978; Gerard et al., 1979).

Chlorhexidine preparations have been used extensively in the management of burns for cleansing and antisepsis. In addition, several groups of workers have used the antiseptic for balneotherapy of major burns. The patients were immersed in baths containing aqueous solutions of chlorhexidine gluconate at concentrations ranging from 0.01% to 0.05%. This treatment was considered a valuable adjunct to the existing infection control measures (Collier et al., 1978; Jouglard et al., 1979).

Oral Disease

The role of chlorhexidine as an effective agent for the prevention and treatment of oral disease has been widely recognized for a number of years. With the exception of fluoride, no chemical agent for use in the mouth has been so extensively studied. The effectiveness of chlorhexidine stems from its ability to adsorb to negatively charged surfaces in the mouth (e.g., tooth, mucosa, pellicle, restorative materials), maintaining prolonged antimicrobial activity for many hours (Gjermo et al., 1974). Retention studies with radiolabeled chlorhexidine show that approximately one-third of the dose is retained after a 60-second mouth rinse with 10 mL of a 0.2% solution (Bonesvoll, 1977). Clinical studies demonstrated that this treatment, applied twice daily, produces optimum uptake and retention in the mouth (Cancro et al., 1974; Agerbaek et al., 1975).

Plaque formation is a continual process, and an agent showing persistent activity offers an advantage over agents with only a limited duration of effect. Chlorhexidine remains the agent of choice for supragingival plaque control (Addy, 1986; Kornman, 1986). Treatment with chlorhexidine mouthwash or gel reduces the duration of and discomfort from minor aphthous ulceration and increases the total number of ulcer-free days (Hunter and Addy, 1987; Addy et al., 1976).

Preoperative and postoperative use of chlorhexidine mouthwash reduces the oral microflora and the incidence of postextraction bacteremia (Martin and Nind, 1987; Field et al., 1988). Postoperative gingival healing is enhanced by the use of chlorhexidine applied as either a mouthwash or a gel. The antiseptic is thought to achieve this by reducing the level of microbial contamination of the wound (Langebaek and Bay, 1976; Bakaeen and Strahan, 1980).

Oral hygiene can prove difficult to maintain in certain compromised groups. Persons who have mental or physi-

cal disabilities may be unable either to understand or perform adequate control measures. Immunocompromised patients are particularly susceptible to oral infections, especially with *Candida* species, and the same is true of denture wearers. Chlorhexidine mouthwash, gel, and spray have been effective in helping maintain the oral hygiene of mentally and physically handicapped groups (Francis et al., 1987; Usher, 1975); the mouthwash and gel have been used successfully in immunocompromised patients to control candidiasis and mucositis (Ferretti et al., 1987; Jacobsen et al., 1979).

Soaking dentures in chlorhexidine is effective in reducing colonization with *Candida* species (Olsen, 1975; Budtz-Jorgensen, 1977). It is suggested that the antiseptic exerts both antifungal and antiadhesive effects, which may last for several days (McCourtie et al., 1986).

Persons who have an increased caries risk can be identified from the high salivary levels of *Streptococcus mutans*. Use of chlorhexidine gel (Zickert et al., 1982) or chlorhexidine fluoride mouthwash (Luoma et al., 1978), along with other preventive measures such as fluoride treatment and diet control, has been shown to reduce significantly the incidence of caries in these patients. Radiation caries also can be controlled by a chlorhexidine/fluoride mouthwash (Katz, 1982).

The most common side effect associated with oral use of chlorhexidine is extrinsic tooth staining, which seems to be an unavoidable consequence of having clinically effective chlorhexidine available at the tooth surface. The highly reactive molecule combines with dietary chromogens, which then are precipitated onto the tooth surface (Addy et al., 1985); it is this chlorhexidine/chromogen complex that forms the characteristic stain. Maximum staining occurs in vitro at 0.1% chlorhexidine (Prayitno and Addy, 1979); however, reducing the concentration to limit staining requires a corresponding increase in volume to maintain clinical efficacy. Thus, clinically equivalent doses of chlorhexidine have equivalent levels of stain.

The stain is extremely variable from individual to individual and may be minimized by prior toothbrushing with a dentifrice (Kornman, 1986). Although tooth staining limits the long-term oral use of chlorhexidine, for some patient groups, the benefits outweigh the disadvantages. Indeed, for the physically and mentally handicapped, chlorhexidine may be the only option for effective oral hygiene (Storhaug, 1977).

SAFETY

Chlorhexidine has been in use throughout the world for more than 30 years in a wide range of clinical situations; during this period, reports of adverse reactions have been relatively few. Extensive studies involving experimental animals and human volunteers as well as observations of hospital staff and patients have been carried out to determine the nature and probability of untoward reactions that may be associated with the varied applications of chlorhexidine to living tissues. These range from disinfection of intact adult skin to delicate mucous membranes (e.g., bladder and eye) and traumatic or surgical wounds. Systemic (oral, intramuscular, intravenous) and topical routes of administration have been investigated for short- and long-term adverse effects.

Acute effects of accidental ingestion or injection have been rarely reported and are associated only with high doses of chlorhexidine. Long-term effects of animal feeding and topical (human) use showed that absorption from the alimentary tract or through the skin is negligible or absent. There is no evidence of carcinogenicity. The incidence of skin irritation and hypersensitivity is low when chlorhexidine is applied at its recommended concentrations. There is no deleterious effect on healing of wounds or grafting of burns. As with most disinfectants, a high probability of total deafness rules out the use of chlorhexidine during surgery on the inner or middle ear. Chlorhexidine is toxic to nerve tissue, and therefore contact with the brain and meninges must be avoided. Strong solutions of chlorhexidine and preparations that are formulated with other excipients, such as alcohols and surfactants, also should be kept away from the eyes.

Acute Toxicity

Systemic administration of formulations of chlorhexidine by oral, intravenous, and subcutaneous routes has been performed in rats and mice. Oral median lethal dose (LD50) values are high because chlorhexidine is poorly absorbed from the gut and is excreted mainly unchanged in the feces. Administration by the intravenous route results in greater toxicity than by either the oral or subcutaneous routes because of a stromalytic effect on red blood cells resulting from its surfactant activity.

Subacute and Chronic Toxicity

In longer-term studies (up to 3 months), there is a dose-related decrease in body weight and a decrease in consumption of drinking water that results from the unpalatable taste of chlorhexidine. Rats receiving 50, 100, or 200 mg/kg of chlorhexidine daily in their drinking water for 90 days produced only evidence of reactive histiocytosis of the mesenteric lymph nodes; however, this is known to occur in aging rats. The change was not neoplastic, and phagocytic function was normal. In longer-term tests of up to 2 years, the effect on the mesenteric lymph nodes was nonprogressive.

The effect of chlorhexidine gluconate at doses of 5 and 50 mg/kg daily on the reproductive performance of male and female rats also was studied. Mating performance, pregnancy rate, duration of gestation, litter parameters,

and the condition of the dams at terminal necropsy were unaffected by the treatment.

Oncogenicity

Carcinogenicity studies have been performed in both rats and mice given oral chlorhexidine plus artificially increased levels of its degradation product p-chloroaniline. No evidence of carcinogenicity was found in rats after 2 years of up to 40 mg/kg of chlorhexidine plus 0.6 mg/kg/day p-chloroaniline daily.

Several reports of mutagenicity tests with chlorhexidine have ben done based on modifications of the microbiologic Ames test procedure. This type of microbiologic test is considered to be fundamentally inappropriate for use with known antimicrobial agents because it is difficult, if not impossible, to separate the legitimate lethal action of the drug on the bacterial cell from any toxicologic activity that the drug may possess. Although some reports claim to have demonstrated positive mutagenicity with chlorhexidine by this means (Ackermann-Schmidt, 1982), the significance of the findings is considered extremely doubtful, particularly in view of the complete lack of carcinogenicity demonstrated in more meaningful animal studies. From the evidence available, it is concluded that chlorhexidine is not a carcinogen.

Dermal Absorption

All the available information on chlorhexidine suggests that the antiseptic is absorbed through skin to an extremely small degree, if at all.

Case (1980) reported on a series of studies to detect percutaneous absorption of chlorhexidine following application to the skin of volunteers. Radiolabeled chlorhexidine formulated as a 4% handwash or 5% aqueous solution was applied to forearm skin and left in contact for 3 hours. Levels ranging from 96% to 98% of the radioactivity subsequently were recovered from the skin. No radioactivity was detected in blood or urine. The equivalent of 0.007% of the administered dose was detected in only one of the fecal samples. In a further study, subjects followed an exaggerated surgical scrub schedule using a 4% chlorhexidine handwash five times daily for three 5-day weeks. Each treatment lasted 6 minutes and included 3 minutes of brush scrubbing. Blood samples were taken throughout the test period and analyzed for chlorhexidine using a gas liquid chromatography method with a sensitivity of 0.01 to 0.05 mg/L. No detectable blood levels were found in any of the volunteers at any time. Blood samples also were taken from hospital staff members who had been regular users of 4% chlorhexidine for surgical hand disinfection for at least 6 months. No detectable levels of chlorhexidine were found in any of the subjects.

Husak et al. (1981), who used 4% chlorhexidine handwash for bathing infants, were unable to detect skin absorption of chlorhexidine on each of the 3 days following bathing. Cowen et al. (1979), who also bathed infants with this preparation, did find significant levels of chlorhexidine in the blood when samples were taken by heel prick, but this was thought to be due to contamination from the treated skin. Samples of venous blood taken in an attempt to avoid this contamination showed extremely low levels of chlorhexidine in some infants and none in others.

Gongwer et al. (1980) bathed neonatal rhesus monkeys for 90 days with an experimental handwash formulation containing 8% chlorhexidine gluconate. No adverse effects on the brain or other tissues were found. Minimal amounts of chlorhexidine were detected in a few tissues and in the feces, and it was suggested that the antiseptic had entered the body by oral ingestion following grooming.

From the results of all the studies listed, it is clear that percutaneous absorption of chlorhexidine, if it occurs at all, is minimal. There is no evidence that any chlorhexidine that may be absorbed will be toxic.

Skin Irritation and Sensitization

Primary dermal irritancy studies with aqueous chlorhexidine gluconate solutions (20%, 0.5%) or a 4% chlorhexidine handwash were performed in rabbits using the Draize test method. The results showed that none of the formulations was a primary skin irritant. A repeated-application dermal toxicity study was performed in rabbits with aqueous chlorhexidine gluconate (20%) applied once daily, 5 days a week for 3 weeks. The concentrated solution caused damage to both intact and abraded skin, which healed within 2 weeks of stopping treatment. Tests in human volunteers to detect allergic contact sensitization and photosensitization following repeated application of chlorhexidine formulations proved negative.

Local skin irritation reactions occasionally are reported with chlorhexidine use. Long-term experience demonstrated an extremely low incidence of sensitization reactions. There have been isolated reports of generalized allergic reactions, and in the most severe cases shock has occurred (fall in blood pressure, nausea, dizziness, dyspnea). Anaphylactoid and other hypersensitivity reactions have been reported with topically applied chlorhexidine solutions (Kimura et al., 1994; Okano et al., 1989), and with the use of chlorhexidine-impregnated catheters (Nightingale, 1998; Terazawa et al., 1998). Considered against the extensive use of the antiseptic over more than 30 years, however, the incidence of such side effects must be considered extremely low.

Wound Healing

Saatman et al. (1986) assessed the effects of 0.05% and 4% chlorhexidine gluconate solutions on the healing of surgically induced wounds in guinea pigs. The wounds

were irrigated after surgery and daily thereafter until necropsy. Daily progress of the wound appeared normal in all treatment groups, with no gross evidence of treatment effects. On histopathologic evaluation, the treated animals exhibited a slight delay in healing compared with saline-treated animals on days 3, 6, and 9; however, by days 14 and 21, no differences were detectable.

Hirst (1973) investigated the effect of chlorhexidine on gingival wound healing in dogs. Biopsy sites were treated with 20% chlorhexidine or saline for 42 days, after which the sites were graded for inflammatory response. Sites exposed to chlorhexidine had less evidence of inflammation than the control sites, and healing was judged as complete.

It has been reported that chlorhexidine is cytotoxic to exposed fibroblasts when assessed in vitro using tissue culture methods; however, Sanchez et al. (1988) demonstrated that this cytotoxicity observed in vitro does not occur in vivo. Using an infected-wound model, they found that chlorhexidine actually accelerated the rate of healing compared with saline-treated controls.

Ototoxicity

Sensorineural deafness was found to occur in patients who had undergone vascular myringoplasty operations; the common factor was the use of 0.05% chlorhexidine in 70% alcohol for perioperative disinfection of the middle ear. It was thought that the solution had penetrated the membrane of the round window, entering the inner ear and causing damage to the cochlea, which resulted in permanent hearing loss (Bicknell, 1971). This has since been extensively investigated using animal models. Severe vestibular and cochlear damage has been found to occur, the extent of which is related to concentration and duration of exposure (Morizono et al., 1973; Igarashi and Suzuki, 1985). Other antiseptics have been similarly ototoxic when administered in this way. Toxicity is significantly enhanced in the presence of high levels of alcohol or detergent (Morizono et al., 1974).

Neurotoxicity

The neurotoxicity of chlorhexidine was investigated by Henschen and Olson (1984) using a rat model. A dose-dependent degeneration of the adrenergic nerves occured following the application of chlorhexidine, and these researchers suggested that neurotoxic effects on thin unmyelinated fibers should be looked for in the central nervous system. In an earlier study, Hurst (1955) had found that chlorhexidine, along with most other antiseptics, was toxic when injected into the cerebrospinal fluid of monkeys.

Ocular Toxicity

Although dilute solutions of pure chlorhexidine have been used for eye irrigation (0.05%) and as a preservative for such preparations as eye drops (0.01%), higher concentrations and preparations containing other excipients may cause eye damage.

Following direct topical application of up to 2% chlorhexidine to the eye, no changes to the cornea were detected by either direct observation or light microscopy; however, superficial epithelial changes were noted by electron microscopy following application of 0.1% and 0.5% chlorhexidine (Browne et al., 1975; Dormans and van Logten, 1982).

Multiple concentrations of chlorhexidine ranging from 0.1% to 4% were evaluated for their toxic effects on regrowth of corneal epithelium. Concentrations greater than 2% were clearly toxic to both the corneal epithelium and conjunctiva; a concentration of 1% produced no significant delay in epithelial healing but did cause mild conjunctivitis. Concentrations less than 1% were not statistically different from the control group, either in reepithelialization or visible toxic effects (Hamill et al., 1984).

Isolated reports have been received of injury resulting from accidental introduction into the eye of an antiseptic handwash containing 4% chlorhexidine in a detergent base. Accidental splashes entering the eye during normal handwashing cause irritation, which has resolved completely following prompt and thorough washing the affected eye with water. Irreversible corneal damage occurred following more prolonged ocular exposure, however, as a result of misuse of the antiseptic handwash on anesthetized patients for skin preparation of the periorbital area and eyelids. The product had pooled on the eye surface and remained there for up to 1 hour (Hamed et al., 1987; Phinney et al., 1988). From the information available, it would appear that the excipients present in the handwash formulation, or possibly a combined effect of the excipients and chlorhexidine, are a more likely cause of permanent ocular damage than the chlorhexidine alone.

ACRONYMS AND ABBREVIATIONS

MIC minimum inhibitory concentration
MRSA methicillin-resistant *Staphylococcus aureus*
MSSA *meticillin-sensitive Staphylococcus aureus*

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3

Neutralizer Evaluations as Control Experiments for Antimicrobial Efficacy Tests

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I. INTRODUCTION

The study of biocidal efficacy requires a distinction between the potential forms of antimicrobial effects. An antimicrobial agent can be either biocidal or biostatic. A disinfectant or preservative is said to be *biocidal* when exposure of the index microorganism to the biocide results in cell death. A *biostatic* agent prevents the growth of the organism under conditions that would normally allow growth. A biocidal test measures the efficacy of an antimicrobial agent by an apparent decrease in the number of viable microorganisms. The primary goal of a biocidal efficacy assay is the accurate determination of cell survival with time. This requires effective neutralization of the biocidal agent at the specific sampling time points [1–4].

A carefully designed biocidal test measures microbial kill, not biostasis.

This is done by measuring the numbers of organisms able to grow after exposure to the antimicrobial agent. Carryover of residual disinfectant from the test could inhibit growth in the recovery medium, leading to an overestimation of kill. It is necessary to demonstrate the adequacy of neutralization to establish the reliability of the biocidal data [1,4].

Three methods have recently been published detailing neutralizer evaluation protocols. These methods have different goals, and strengths. Dey and Engley describe a procedure, with *Staphylococcus aureus* as the index organism, that measures survival with time. The challenge organism is inoculated directly into the disinfecting solution, then sampled with time [5]. The efficacy of the neutralizer was measured by increased recovery of the challenge organism among different treatments. This protocol is useful in identifying neutralizers. However, it lacks the ability to distinguish between improved neutralization of the disinfectant and improved recovery of the index organism.

Terleckyj and Axler describe a second method that uses *Candida albicans* as the index organism [6]. This protocol was designed as a control procedure to demonstrate neutralization for a fungicidal experiment. The initial step of this method was a neutralization period. The biocide was exposed to the neutralizer before addition of the challenge microorganism. The challenge organism was then added at a concentration of approximately 10^6 colony-forming units (CFU)/ml to the suspension after this initial incubation. Survival of the challenge organism was assayed after an additional 15-min incubation. The basic design was first described in 1972 by Bergan and Lystad [7]. An assumption of these methods is that all index organisms will behave identically, and so only one organism needs to be tested. This is not a valid assumption, as different organisms will differ in their sensitivity to biocides, and it is this sensitivity that is of concern in a neutralizer evaluation study. In addition, the method of Terleckyj and Axler involves a centrifugation step after exposure to the biocide. The cells are resuspended before plating, diluting the biocidal agent. Further dilution occurs because of the high number of cells inoculated in the solution. This high concentration (10^6 CFU/ml) requires dilution for the determination of viable colony counts. These dilutions compromise the stringency of the procedure.

The final method was described by myself and colleagues [8]. This method is similar in overall design to that described by Bergan and Lystad. However, it employs a smaller inoculum size, statistical analysis, and evaluation of the neutralizer with all index organisms. This procedure lends itself to specific modifications of the general procedure that are included to mimic the different biocidal assays. Details of these methods are described in Section V.

One specific function of a neutralizer evaluation is to serve as a control experiment to the biocidal evaluation. Therefore, replication of all critical parameters of the biocidal experiment is critical to the neutralizer evaluation. The importance of this point cannot be overstated.

This review will examine the neutralizer evaluation solely as a control experiment of the biocidal study. However, it must be noted, in passing, that many of the same concerns apply to sterility testing of biocides, disinfectants, and preserved solutions [9].

II. METHODS OF NEUTRALIZING BIOCIDES

A. Neutralization by Chemical Inhibition

Many biocides can be chemically inactivated. Table 1 provides a listing of known neutralizers and the biocides affected. Several neutralizing and dilution broth media have been formulated to take advantage of these neutralizers. Among the more popular of these broths are Dey-Engley (D/E), Letheen, and thioglycolate. A full listing of the formulations for currently used neutralizing broths is provided in Table 2. It is important to remember that the efficacy of the compounds listed in Table 1 and of the broths listed in Table 2, were established at specific concentrations of neutralizer and biocide. Additionally, particular challenge organisms were employed to demonstrate efficacy. The efficacy of any neutralizer must be demonstrated for the specific test system in use.

The D/E broth was formulated to neutralize a wide range of biocidal agents (compare ingredients as listed in Table 2 with Table 1) [3,5]. The neutralizer efficacy of the formulation was originally demonstrated with *Staphylococcus aureus* [5], then later with a variety of microorganisms [6,8]. Letheen broth is effective in recovering bacteria exposed to quaternary ammonium compounds and biguanides [1,43]. The thioglycolate medium is effective against mercurials [1]. Other recommended neutralization (or dilution) broths include TAT [44], and AOAC Disinfectant Neutralization Solution [45].

One concern with chemical inactivation of biocides is that the neutralizer itself may be harmful to some of the bacteria of interest [1,2,46]. Table 3 lists bacteria sensitive to specific neutralizers.

Despite the concerns over neutralizer toxicity, the convenience and quick action of chemical neutralizers encourage their use. Chemical inactivation of biocides is the preferred method for most applications. Performing the proper neutralizer evaluation before the biocidal assay is critical to any demonstration of disinfection efficacy.

B. Neutralization by Dilution

The concentration of a disinfectant exerts a large effect on its potency. The relation between concentration and antimicrobial effect differs among biocidal agents, but is relatively constant for a particular biocide. This relation is exponential in nature, with the general formula:

$$C^n t = k$$

TABLE 1 Biocide Neutralizers

Biocide	Neutralizers/Inactivators	Ref.
Alcohols		
Isopropanol, phenoxyethanol	Polysorbate 80	10
	Dilution	11
Aldehydes		
2-Bromo-2-nitropropane-1,3-diol (bronopol)	Serum, cysteine, thiosulfate, thioglycolate, metabisulfite	12
Formaldehyde	Sodium sulfite, ammonia	1
	Histamine	13
Glutaraldehyde	Dilution	14
	Sodium bisulfite	15
	Sodium sulfite, glycine	2
	Cystine or cyteine	16
	Glycine	17
Chloralkytriazaazoniadamantane (Dowicil 200)	Dilution	18
Dimethylol dimethyl hydatoin (Glydant)	Dilution	19
Biguanides and bis-biguanides		
Chlorhexidine	Lecithin/polysorbate 80, 0.5% polysorbate 80	20 21,22
Polyhexamethylene biguanide HCl (Cosmocil CQ)	Polysorbate 80/lecithin	4
Phenolics		
Phenylphenol, chloroxylenol, cresols, chlorocresols, phenol	Nonionic surfactants	1
	Polysorbate 80	7
	Dilution	23
Quaternary ammonium compounds		
Cetrimide, benzalkonium and benzethonium chloride	Lecithin/polysorbate	24
	Suramin sodium	25
	Organic material	26
	0.5% polysorbate 80	27
	Cyclodextrins	28
Mercurials	Sulfhydryl compounds	1
	Thioglycolic acid	26
	Thiosulfate, bisulfite	29
	Ammonium sulfite	30
Organic acids		
Benzoic, propionic sorbic	Nonionic surfactants	10
	Dilution	1
	pH 7 or above	24
Halogens		
Hypochlorite	Thiosulfate	31
	Dilution	32
Iodine	Thiosulfate	33
	Polysorbate 80	32
	Skim milk	34
EDTA	Mg^{+2} or Ca^{+2} ions	35
Imadazolidinyl urea	Dilution	19
Diazolidinyl urea (Germall 115 or II)		
Methyl-, and methylcholoroisothiazolinone (Kathon)	Amines, sulfites, mercaptans, sodium bisulfite	36
	Heparin	37
Parabens		
methyl-, ethyl-, propyl-, butyl-parahydroxybenzoic	Lecithin, filtration, dilution	1
	Polysorbate surfactants	38
	1% polysorbate 80 or 20	21,39–41
	Dilution	42

TABLE 2 Composition of Available Neutralizer Broths

Ingredient	D/E Broth	Letheen	TAT + Tween 20	NIH thioglycolate	AOAC disinfectant neutralizer solution
Cystine				0.5 g	
Polysorbate 80	5.0 g	5.0 g			28.0 ml
Polysorbate 20			40.0 ml		
Lecithin	7.0 g	0.7 g	5.0 g		4.0 g
Sodium thioglycolate	1.0 g			0.5 g	
Sodium thiosulfate	6.0 g				
Sodium bisulfite	2.5 g				
Tryptone	5.0 g		20.0 g		
Yeast extract	2.5 g			5.0 g	
Dextrose	10.0 g			5.5 g	
Peptamin		10.0 g			
Beef extract		5.0 g			
Sodium chloride		5.0 g		2.5 g	
Soytone					
Casitone				15.0 g	
KH_2PO_4					42.5 mg

TABLE 3 Potential Toxicity of Neutralizers

Inactivating agent	Disinfectant	Potential toxicity	Ref.
Sodium bisulphite	Glutaraldehyde	Nonsporing bacteria	14
Sodium thioglycolate	Mercurials	Bacteria and spores	47,48
Sodium thiosulfate	Iodine and chlorine	Staphylococci	49
Lecithin + Lubrol W	QACs	Bacteria	50
Glycine	Glutaraldehyde	Growing cells	51
Lubrol W	QACs	*Pseudomonas* spp.	4

where

C is the concentration
t is the time required to kill a standard inoculum
k is a constant
η is gradient of the plot of log t against log C

The letter η is described as the *concentration exponent*. Excellent reviews on this subject have been prepared by Cowles [52], Tilley [53], and Hugo [54]. These articles are recommended to the interested reader for more discussion.

Concentration exponents are determined experimentally. Two concentrations of biocide are tested in the same formulation, C_1 and C_2. Identical inocula are added to each, and the minimum "kill time" determine for each (t_1 and t_2): η is then determined by the equation

$$\eta = \frac{\log(t_2) - \log(t_1)}{\log(C_1) - \log(C_2)}$$

The concentration exponents for several biocides are provided in Table 4. Note that biocides with high η values are rapidly neutralized by dilution, whereas those with low η values are less dramatically effected.

It is important to demonstrate that dilution is sufficient for neutralization in the test system even with biocides of high η values. A high η value is no assurance of adequate neutralization without experimental data.

C. Neutralization by Filtration

Dilution of the biocide by filtration is another means to neutralize a disinfecting solution. This procedure relies on filtration to separate the microorganisms in

TABLE 4 Concentration Exponent of Common Biocides

Compound	η Value	Increased time factor when concentration reduced to 1/2	1/3
Phenolics	6	2^6	3^6
Alcohol	10	2^{10}	3^{10}
Parabens	2.5	$2^{2.5}$	$3^{2.5}$
Chlorhexidine	2	2^2	3^2
Mercury compounds	1	2	3
QACs	1	2	3
Formaldehyde	1	2	3

Source: Ref. 54.

suspension from the disinfecting solution. The filter is then removed, and placed on the surface of an agar plate for incubation. Nutrients leach up through the membrane, and discrete colonies arise on the surface of the filter, allowing quantification of survivors.

Filtration alone may not remove sufficient quantities of the biocidal agent to allow growth of surviving microorganisms. Growth inhibition may occur owing to adherence of residual preservative to the filter membrane [55–60]. Filtration through a low-binding filter material, such as polyvinylidene difluoride, helps lessen this adherence [9]. Additionally, the preservative may be diluted or flushed from the filter by rinsing with a benign fluid, such as 0.1% peptone [61]. Chemical neutralizers included in a rinse of the filter can be useful in assuring complete neutralization. Filtration alone cannot be assumed to be an effective means of neutralization. Effective recovery of survivors by this procedure requires demonstration of neutralization efficacy in the test system.

III. FACTORS AFFECTING NEUTRALIZATION OF ANTIMICROBIALS

There are four criteria to be met in designing a study of potential neutralizers [2]:

1. The neutralizer must effectively inhibit the action of the biocidal solution.
2. The neutralizer must not itself be unduly toxic to the challenge organisms.
3. The neutralizer and active agent must not combine to form a toxic compound.
4. These first three criteria must be demonstrated under conditions that mimic the actual conditions of the assay for disinfecting efficacy.

These criteria can be met by consideration of three factors. The first is *neutralizer efficacy*, or the ability of the neutralizer to inhibit the action of the biocidal agent against a specific microorganism. The second is *neutralizer toxicity*, or the inherent toxicity of the neutralizer for the organisms under study. These two criteria can be analyzed by the comparison of three populations [2,8, 9,62,63] (Fig. 1). The final factor addresses the adequate recovery of organisms sublethally injured by exposure to the biocidal agent.

A. Neutralizer Efficacy

The inherent efficacy of neutralizers used in biocidal experiments will vary with both the organism under study and the biocide. In addition, the sensitivity of the organism to that biocide and the concentration of the biocide to be neutralized can have an enormous effect on the efficacy of the neutralizing treatment. The efficacy of neutralization can be demonstrated by comparing recovery of organisms from

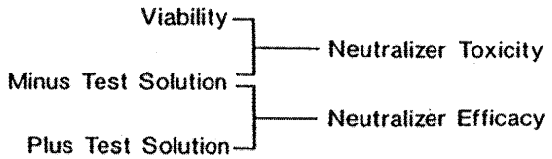

FIGURE 1 Population comparisons in neutralizer evaluation studies designed to control assays of antimicrobial efficacy. Neutralizer toxicity and neutralizer efficacy are defined as the similarity in the recovery between the two population pairs described.

two treatment populations. The first treatment consists of the neutralizer with the appropriate volume of biocide. This is the "plus-disinfectant" group. The second treatment consists of the neutralizer diluted with an equivalent volume of phosphate-buffered saline ("minus-disinfectant" group). Both populations are then inoculated with low numbers of microorganisms and assayed for survivors.

It is important to perform this experiment several times to allow statistical treatment of the recovery data. It is also important to compare the recovery of microorganisms from the neutralizer in the presence and the absence of the biocide. This comparison avoids confusing toxic effects of the neutralizer with inadequate inhibition of the biocidal agent (see following section).

B. Neutralizer Toxicity

Several chemical inhibitors of antimicrobials are themselves toxic (see Table 3). Care must be taken to avoid enhancing the apparent kill by an artifact of the recovery system. This factor can be estimated as a comparison of recovery between two populations, as was the factor of neutralizer efficacy. The two treatment groups are somewhat different. The first treatment consists of the minus-disinfectant group from the foregoing. The second treatment is the viability control and consists of an equivalent volume of phosphate-buffered saline (PBS) or other benign diluent. Both populations are then inoculated with low numbers of microorganisms and incubated for survivors. As in the previous section, it is important to perform this experiment several times to allow statistical treatment of the recovery data.

If the neutralizer is both effective and nontoxic, then all treatment groups will behave in identical manners. However, this is rarely so, and usually, some measure of neutralizer toxicity must be accepted to allow adequate neutralization.

C. Recovery of Injured Organisms

The recovery of injured organisms is not addressed by neutralizer evaluation studies. This limitation of a neutralizer evaluation study must be addressed at some point in preparation for the biocidal experiment. Although a sensitive assay

for the neutralizing efficacy of a medium, a neutralizer evaluation study uses healthy cells that have not been exposed to disinfectants. This is not the situation in the actual experiment, where many viable cells will be damaged to varying degrees by exposure to the biocidal agent. The recovery of damaged microorganisms is extremely difficult to quantify [64,65]. One method used to estimate the number of crippled organisms in a biocidal study compares recovery in a rich nutrient medium with recovery in a stressful medium [66,67]. Trypticase soy agar medium is an example of a nutrient medium suitable for a variety of bacteria. Examples of stressful media might be medium supplemented with 5.5% KCl, or a medium with a low pH value. The fraction of the population unable to grow on the stressful medium is defined as the fraction of crippled organisms.

One way to demonstrate recovery of crippled organisms in a biocidal assay would be to compare the "kill curves" generated by plating on several different nutrient media. The optimal conditions for growth are those that give the least apparent kill [69]. Obviously, the neutralizer evaluation described earlier is a necessary prerequisite to this experiment.

IV. FACTORS AFFECTING NEUTRALIZER EVALUATIONS

The purpose of a neutralizer evaluation is to serve as a control experiment for the biocidal efficacy assay. Therefore, it is important to test the neutralizer under the conditions of the biocidal test. In general, any factor that affects the apparent disinfecting efficacy of a treatment must be a concern for the neutralizer evaluation. These factors include the nature of the challenge organism, pretreatment factors, treatment factors, and method of recovery [70,71].

A. Nature of the Challenge Organism

The nature of the challenge organism exerts a strong effect on the response to antimicrobial challenge. The *United States Pharmacopeia* (*USP*) [72] and American Society for Testing and Materials (ASTM) [73] preservative challenge tests use five different microorganisms. Represented among these index organisms for each test are gram-positive bacteria, gram-negative bacteria, yeast, and molds. These particular species serve as index organisms for the major prokaryotic and eukaryotic groups. Similarly, the manual of the Association of Official Analytic Chemists (AOAC) uses different tests for specific biocidal claims [45]. In other words, antistaphylococcal activity is a specific claim and is not taken as proof of tuberculocidal activity. These differences must be taken into account in the design of neutralizer evaluation studies.

Differences among the index organisms may well influence the efficacy and toxicity of the neutralizer, as discussed earlier. All microorganisms to be used as index organisms in the biocidal assay must be tested in the neutralizer evaluation. There are two reasons for this concern. First, many commonly used neutralizers

are toxic to certain species (see Table 3). Second, the differing efficacy of the biocide among challenge organisms will require differing levels of neutralization. These differences among organisms can be clearly seen in differing responses to neutralization.

B. Pretreatment Factors

The growth and preparation of the challenge organism determines the physiological state of the cell. This state has a direct influence on the results of any assay of disinfecting efficacy. The conditions of organism preparation and storage must be standardized for the neutralizer evaluation and reflect the conditions of the antimicrobial assay.

Biocidal tests do not use individual cells. Rather, populations of cells are harvested for study. The data generated from these studies is less variable if the cell populations are homogeneous. Liquid cultures or confluent growths on solid medium are best suited for the reproducible preparation cultures [74].

C. Treatment Factors

All factors of the biocidal test must be duplicated in the neutralizer evaluations. The constituents of the suspension can have a dramatic effect on the efficacy of the test solution; organic load decreases the efficacy of oxidative antimicrobials [75]. Additionally, the manner and storage of organic load can affect the efficacy of antimicrobials. Even factors such as the water used to make up the solutions need to be standardized.

The concentration of the biocidal agent exerts a logarithmic effect on the efficacy of the formulation. This effect, known as the concentration exponent, was discussed earlier. A biocidal evaluation may involve plating 1 ml or 10^{-1}–10^{-5} dilutions onto an agar recovery medium. The neutralizer evaluation should test this recovery under the most stringent conditions to be seen. This is reflected by the 10^{-1} dilution, requiring growth in the highest concentration of biocide.

D. Posttreatment Factors

Two posttreatment factors influence neutralizer evaluations. The first is the recovery medium used to support the growth of survivors. This concern was discussed in the foregoing. The second consideration is the incubation conditions. Optimal conditions for growth must be employed to ensure complete growth and reproducible results.

V. NEUTRALIZER EVALUATION DESIGNS

Three methods to estimate the survivors of exposure to a disinfectant will be discussed. These are differentiated by recovery media; recovery on agar plates,

recovery in liquid medium, and the use of membrane filtration as a means to concentrate survivors for enumeration on agar plates.

A. Recovery on Solid Medium

Suspension tests of antimicrobial efficacy usually quantify surviving microorganisms on solid (agar) medium. Challenge organisms are physically suspended in the test solution, and aliquots are removed with time to determine the number of surviving microorganisms by their recovery on agar plates.

The general procedure for these tests involves preparing a suspension of challenge organism in the test solution, usually at a concentration of approximately 10^6 CFU/ml. The samples are removed with time and diluted tenfold in a suitable neutralizing and dilution broth, typically Dey-Engley broth. This step should accomplish the neutralization of the biocide. The broth dilution is continued in series to allow appropriate dilution of the sample for plating. Several dilutions are plated in a recovery agar to quantify the number of viable organisms in the original sample.

Figure 2 describes one method for the evaluation of neutralizers in this system. The plus-disinfectant population for each test organism is constructed by adding 1 ml of biocide to 9 ml of neutralizing medium. These mixtures are incubated on the benchtop for approximately 5 min, then inoculated with challenge organism. The inoculated suspensions are incubated for approximately 10

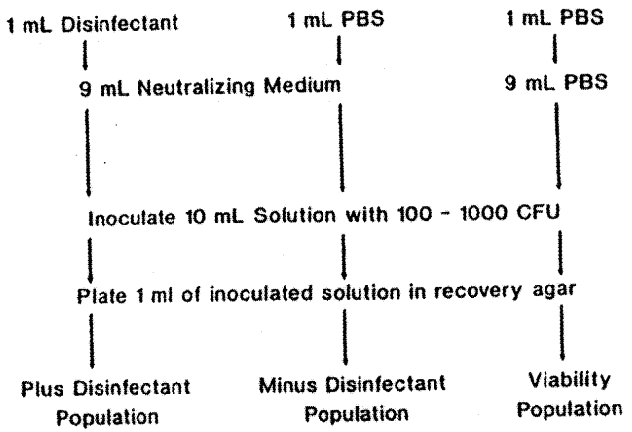

FIGURE 2 Diagram of a design for a neutralizer evaluation study to control a quantitative assay of antimicrobial efficacy. The important considerations in this design are the ratio of disinfectant/neutralizer-broth, recovery medium, and index organisms. This design can be used to validate a protocol for a test of preservative efficacy.

min, and plated in replicate. The minus-disinfectant population is treated in a similar fashion, substituting PBS for the disinfectant. Several different recovery media may be employed to ensure maximal recovery.

This technique has the advantage of allowing the separation of various critical operations in recovery. These operations include biocide neutralization, dilution of survivors to countable levels, and plating survivors for recovery. This separation is important, as optimal recovery of survivors may require neutralization in one medium, and plating for recovery on a second. Comparisons among the recoveries in each population can be statistically analyzed as described in Section V.D.

This procedure uses low numbers (30–100 CFU) of index organisms. The use of a low inoculum provides two advantages. First, the use of low numbers increases the effect of low levels of biocides, as a small reduction in measured CFU takes on increased significance. Second, these low numbers allow direct plating of solutions, avoiding dilution of the disinfectant during plating.

B. Recovery In Liquid Medium

Two common disinfection efficacy tests recover surviving organisms in liquid medium. The multi-item microbial challenge test is a carrier assay of contact lens disinfection regimens [76]. The use-dilution test is a carrier assay of surface disinfectants [45,77]. Both tests involve inoculating a carrier, then assaying the carrier for viable microorganisms after disinfection in liquid medium. The assumption made in these tests is that the recovery medium permits growth of all surviving microorganisms at the end of the disinfection period. The final determination of efficacy is based on the absence of growth in liquid culture. Therefore, this broth must serve both to neutralize the disinfectant and to support the growth of all index organisms. The quality and properties of the recovery medium are of critical importance to the accuracy of the test.

A method to examine neutralizer toxicity and efficacy for this type of test is shown in Figure 3. A small inoculum (10–100 CFU) in each of the three different media configurations (populations) is incubated under the proper conditions. Comparisons of growth between the different media configurations provide an appropriate measure of both neutralizer efficacy and neutralizer toxicity.

The viability population consists of an inoculum in a rich broth medium, establishing the viability and growth characteristics of the particular challenge organism. The minus-disinfectant population consists of organisms in the neutralizing–recovery broth diluted with phosphate buffered saline at the same dilution ratio as in the plus-disinfectant population. This must also be no less than the concentration of biocide seen in the final use-dilution or multi-item microbial challenge test. Comparison between growth in the rich broth and growth in the neutralizing–recovery broth medium without the disinfectant allows the deter-

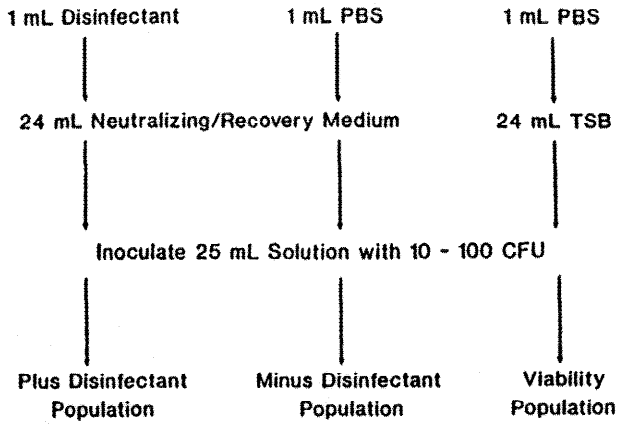

FIGURE 3 Diagram of a design for a neutralizer evaluation study to control a qualitative assay of antimicrobial efficacy. The important considerations in this design are the broth nature of the recovery medium, requiring a broth suitable for both neutralization and recovery, the ratio of disinfectant/neutralizer broth, and the index organisms.

mination of the neutralizer toxicity. The comparison of growth with, and without, the disinfecting solution determines neutralizer efficacy.

C. Recovery by Membrane Filtration

A neutralizer evaluation for a membrane filtration protocol requires the same comparisons and involves concerns similar to those previously described. Considerations in the neutralization evaluation include the test system, the membrane type, the filtration apparatus, the medium, the diluting (neutralization) fluid, and the solution being tested. Additionally, an estimate of the loss of cells through the mechanics of filtration should be determined [9,78–80].

One protocol of neutralizer evaluation, as patterned on the *USP* sterility test, is described in Figure 4 [9]. This procedure requires filtration of the determined amount of biocidal solution, followed by two, 100-ml volumes of diluting–neutralizing fluid. A third 100-ml volume, inoculated with 10–100 CFU of the index organism is then passed through the filter. Finally, the inoculated filter is placed on the surface of a freshly poured agar plate and incubated for growth.

Neutralizer toxicity is evaluated by comparison between the recovery of the membrane without the test solution and those filtered with *USP* diluting fluid A (DFA–0.1% meat peptone) [61]. Neutralizer efficacy is estimated by comparison between the recovery on the membrane both with and without the test solution. Filtration may lead to reduced recovery of organisms through death or adherence

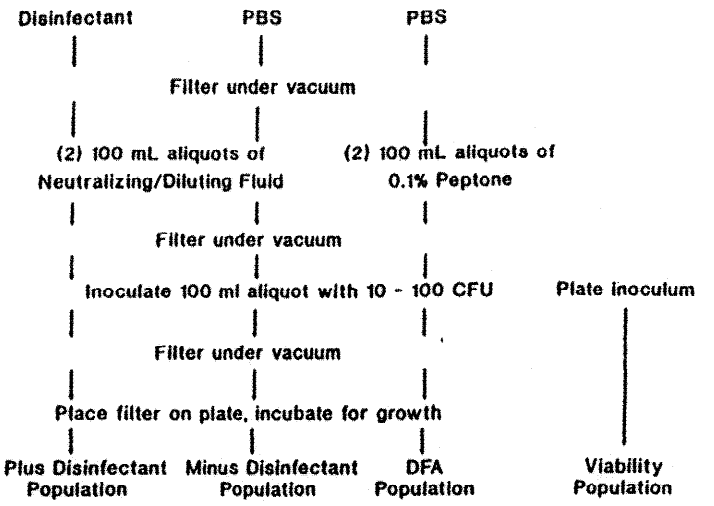

FIGURE 4 Diagram of a design for a neutralizer evaluation study to control an assay involving membrane filtration of an antimicrobial. The important considerations in this design are the amount of antimicrobial to be filtered, the amount of neutralizing/diluting fluid used to wash the membrane, the nature of the membrane filter material, the recovery medium, and the index organisms. This design can be used to validate a protocol for sterility testing, or an assay of antimicrobial efficacy.

to the filtration vessel walls. This technique-specific loss can be estimated by comparing recovery in the DFA population with the viable count. Membrane filtration allows for two methods to enhance neutralizer efficacy. The first is to incorporate a specific neutralizer in the diluting fluid to overcome the effect of the antimicrobial agent. The second is to test several different types of filters to find a filter type that is effective in the specific system. Several filter types are now available with different surface properties. These different properties will affect the relative binding of the active agent to the membrane.

D. Statistical Analysis

Assays such as the use-dilution test and the multi-item microbial challenge test require recovery in liquid media. These data are nominal (growth or no growth) and so can be analyzed by a χ^2 test [81], the sign test [82], or McNemar's test [83; reviewed in Ref. 84].

A second type of data is provided by tests measuring recovery as colony-forming units on agar plates. These natural data (CFU recovered) follow a Poisson distribution. They can be transformed to approximate a normal distribution either

by taking the $\log_{10}$ value of each datum, or by the modified square root transformation of Anscombe [85]. These transformed data can be analyzed in several ways. Student's *t*-test can be used for simple pair-wise comparisons in this analysis. However, if several different neutralizers and recovery media are being evaluated, it would be more appropriate to analyze the data initially by analysis of variance (ANOVA). If significant differences were indicated by this analysis, further information on specific differences could be determined by Dunnett's test using the minus-disinfectant population as the control [81].

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Antiseptics and Their Role in Infection Control

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I. INTRODUCTION

The positive influence of antiseptics in the control of nosocomial infections has long been debated. Studies that definitely show antiseptics do aid in the control of hospital-acquired infections are almost nonexistent. Because infection rates are often low to begin with, such studies often require large numbers of patients and, as such, are (a) difficult to control, (b) must be carried out over a long time period, and (c) are expensive. In addition to these factors, since antisepsis is a routine procedure, it may not be ethical to withhold these procedures from patients as a control group. There are several in vitro and clinical trials comparing the various products that show the positive influence of antiseptics and, by deduction, the information indicates that the proper use of antiseptics in a variety of situations can be helpful in controlling cross-infection of patients by health care personnel. The first demonstration of the link between handwashing and reductions in postoperative infections was made by Semmelweis [1]. It is still believed that most