Antiseptics and Disinfectants: Activity, Action, and Resistance

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INTRODUCTION ........................................................................................................................................... 148
DEFINITIONS ................................................................................................................................................ 148
MECHANISMS OF ACTION .......................................................................................................................... 148
Introduction .................................................................................................................................................. 148
Alcohols .......................................................................................................................................................... 151
Aldehydes ...................................................................................................................................................... 151
Anilides .......................................................................................................................................................... 153
Biclanilides .................................................................................................................................................... 153
Chlorhexidine .............................................................................................................................................. 153
Diamidines ................................................................................................................................................... 154
Diamides ...................................................................................................................................................... 155
Halogen-Releasing Agents .......................................................................................................................... 155
Halogen-releasing agents ............................................................................................................................ 155
Iodine and iodophors .................................................................................................................................. 155
Silver Compounds ....................................................................................................................................... 155
Silver nitrate ................................................................................................................................................ 156
Silver sulfadiazine ....................................................................................................................................... 156
Analyses ...................................................................................................................................................... 156
Halophenols ................................................................................................................................................ 157
General Methodology ................................................................................................................................. 148
Haloperoxides ............................................................................................................................................. 157
Haloperoxides ............................................................................................................................................. 157
Hexachlorophene ................................................................................................................................-------- 157
Quaternary Ammonium Compounds ........................................................................................................ 157
Halophenols ................................................................................................................................................ 157
Vapor-Phase Sterilants ................................................................................................................................ 158
MECHANISMS OF RESISTANCE ............................................................................................................... 158
Introduction .................................................................................................................................................. 158
Bacterial Resistance to Antiseptics and Disinfectants .................................................................................. 158
Intrinsic Bacterial Resistance Mechanisms ................................................................................................. 158
Intrinsic resistance of bacterial spores ........................................................................................................ 159
Intrinsic resistance of mycobacteria ............................................................................................................ 160
Intrinsic resistance of other gram-positive bacteria .................................................................................... 161
Intrinsic resistance of gram-negative bacteria .............................................................................................. 161
Physiological (phenotypic) adaption as an intrinsic mechanism ................................................................. 162
Acquired Bacterial Resistance Mechanisms ................................................................................................. 164
Plasmids and bacterial resistance to antiseptics and disinfectants ............................................................. 164
(i) Plasmid-mediated antibiotic resistance in gram-negative bacteria ..................................................... 164
(ii) Plasmid-mediated antibiotic resistance in staphylococci .................................................................... 165
(iii) Plasmid-mediated antibiotic resistance in other gram-positive bacteria .......................................... 166
Mutational resistance to antiseptics and disinfectants ................................................................................. 166
Mechanisms of Fungal Resistance to Antiseptics and Disinfectants ........................................................ 167
Mechanisms of Viral Resistance to Antiseptics and Disinfectants ............................................................ 168
Mechanisms of Protozoal Resistance to Antiseptics and Disinfectants .................................................... 169
Mechanisms of Prion Resistance to Disinfectants ..................................................................................... 169

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INTRODUCTION

Antiseptics and disinfectants are used extensively in hospitals and other health care settings for a variety of topical and hard-surface applications. In particular, they are an essential part of infection control practices and aid in the prevention of nosocomial infections (277, 454). Mounting concerns over the potential for microbial contamination and infection risks in the food and general consumer markets have also led to increased use of antiseptics and disinfectants by the general public. A wide variety of active chemical agents (or “biocides”) are found in these products, many of which have been used for hundreds of years for antisepsis, disinfection, and preservation (39). Despite this, less is known about the mode of action of these active agents than about antibiotics. In general, biocides have a broader spectrum of activity than antibiotics, and, while antibiotics tend to have specific intracellular targets, biocides may have multiple targets. The widespread use of antiseptic and disinfectant products has prompted some speculation on the development of microbial resistance, in particular cross-resistance to antibiotics. This review considers what is known about the mode of action of, and mechanisms of microbial resistance to, antiseptics and disinfectants and attempts, wherever possible, to relate current knowledge to the clinical environment.

A summary of the various types of biocides used in antiseptics and disinfectants, their chemical structures, and their clinical uses is shown in Table 1. It is important to note that many of these biocides may be used singly or in combination in a variety of products which vary considerably in activity against microorganisms. Antimicrobial activity can be influenced by many factors such as formulation effects, presence of an organic load, synergy, temperature, dilution, and test method. These issues are beyond the scope of this review and are discussed elsewhere (123, 425, 444, 446, 451).

DEFINITIONS

“Biocide” is a general term describing a chemical agent, usually broad spectrum, that inactivates microorganisms. Because biocides range in antimicrobial activity, other terms may be more specific, including “-static,” referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and “-cidal,” referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal). For the purpose of this review, antibiotics are defined as naturally occurring or semi-synthetic compounds that are used as antiseptics or disinfectants or both are discussed. Different types of microorganisms are considered, and similarities or differences in the nature of the effect are emphasized. The mechanisms of action are summarized in Table 2.

MECHANISMS OF ACTION

Introduction

Considerable progress has been made in understanding the mechanisms of the antibacterial action of antiseptics and disinfectants (215, 428, 437). By contrast, studies on their modes of action against fungi (426, 436), viruses (298, 307), and protozoa (163) have been rather sparse. Furthermore, little is known about the means whereby these agents inactivate prions (503).

Whatever the type of microbial cell (or entity), it is probable that there is a common sequence of events. This can be envisaged as interaction of the antiseptic or disinfectant with the cell surface followed by penetration into the cell and action at the target site(s). The nature and composition of the surface vary from one cell type (or entity) to another but can also alter as a result of changes in the environment (57, 59). Interaction at the cell surface can produce a significant effect on viability (e.g., with glutaraldehyde (374, 421), but most antimicrobial agents appear to be active intracellularly (428, 451). The outermost layers of microbial cells can thus have a significant effect on their susceptibility (or insusceptibility) to antiseptics and disinfectants; it is disappointing how little is known about the passage of these antimicrobial agents from the extracellular to the intracellular regions. Potentiation of activity of most biocides may be achieved by the use of various additives, as shown in later parts of this review.

In this section, the mechanisms of antimicrobial action of a range of chemical agents that are used as antiseptics or disinfectants or both are discussed. Different types of microorganisms are considered, and similarities or differences in the nature of the effect are emphasized. The mechanisms of action are summarized in Table 2.

General Methodology

A battery of techniques are available for studying the mechanisms of action of antiseptics and disinfectants on microorganisms, especially bacteria (448). These include examination of uptake (215, 428, 459), lysis and leakage of intracellular constituents (122), perturbation of cell homeostasis (266, 445), effects on model membranes (170), inhibition of enzymes, electron transport, and oxidative phosphorylation (162, 272), interaction with macromolecules (448, 523), and effects on macromolecular biosynthetic processes (133), and microscopic examination of biocide-exposed cells (35). Additional and useful information can be obtained by calculating concentration exponents (n values [219, 489]) and relating these to membrane activity (219). Many of these procedures are valuable for detecting and evaluating antiseptics or disinfectants used in combination (146, 147, 202, 210).

Similar techniques have been used to study the activity of antiseptics and disinfectants against fungi, in particular yeasts. Additionally, studies on cell wall porosity (117–119) may provide useful information about intracellular entry of disinfectants and antiseptics (204–208).

Mechanisms of antiprotozoal action have not been widely investigated. One reason for this is the difficulty in culturing some protozoa (e.g., Cryptosporidium) under laboratory conditions. However, the different life stages (trophozoites and cysts) do provide a fascinating example of the problem...
of how changes in cytology and physiology can modify responses to antiseptics and disinfectants. Khunkitti et al. (251–255) have explored this aspect by using indices of viability, leakage, uptake, and electron microscopy as experimental tools.

Some of these procedures can also be modified for studying effects on viruses and phages (e.g., uptake to whole cells and viral or phage components, effects on nucleic acids and proteins, and electron microscopy) (401). Viral targets are

| TABLE 1. Chemical structures and uses of biocides in antiseptics and disinfectants |
|-------------------------------------|---------------------------------|---------------------|
| **Alcohols**                        | **Ethanol**                     | CH₃ —— CH₂OH        |
|                  | **Isopropanol**                | CH₃ —— CH₂OH        |
| **Aldehydes**                       | **Glutaraldehyde**             | OH —— CCH₂CH₂CH₂C —— HO |
|                  | **Formaldehyde**               | H —— C —— HO        |
| **Anilides**                        | **General structure**           | C₆H₅NH.COR          |
|                  | **Trioclocarban**              | Cl —— NHCONH —— Cl   |
| **Biguanides**                      | **Chlorhexidine**              | Cl —— (H₂C₅N₂)₂H₂(C₂H₂)₄N₂(O₅C)₂H₂Cl             |
|                  | **Alexidine, polymeric biguanides** |                         |
| **Bisphenols**                      | **Triclosan**                  | Antisepsis          |
|                  | **Hexachlorophene**            | Antiplaque agents    |
| **Diamidines**                      | **Propamidine**                | Antiseptic          |
|                  | **Dibromopropamidine**         | Preservation         |

Continued on following page
Table 1—Continued

<table>
<thead>
<tr>
<th>Halogen-releasing agents</th>
<th>Chlorine compounds</th>
<th>$\cdot\text{OCl}^-, \text{HOCI}, \text{Cl}_2$</th>
<th>Disinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodine compounds</td>
<td>$\cdot\text{I}_2$</td>
<td>Cleaning</td>
<td></td>
</tr>
<tr>
<td>Halophenols</td>
<td>Chloroxylenol (PCMX)</td>
<td></td>
<td>Antisepsis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Preservation</td>
</tr>
<tr>
<td>Heavy metal derivatives</td>
<td>Silver compounds</td>
<td>Ag</td>
<td>Preservation</td>
</tr>
<tr>
<td>Mercury compounds</td>
<td>Hg</td>
<td>Disinfection</td>
<td></td>
</tr>
<tr>
<td>Peroxygens</td>
<td>Hydrogen peroxide</td>
<td>$\text{H}_2\text{O}_2$</td>
<td>Disinfection</td>
</tr>
<tr>
<td></td>
<td>Ozone</td>
<td>$\text{O}_3$</td>
<td>Sterilization</td>
</tr>
<tr>
<td></td>
<td>Peracetic acid</td>
<td>$\text{CH}_3\text{COOOH}$</td>
<td></td>
</tr>
<tr>
<td>Phenols and cresols</td>
<td>Phenol</td>
<td></td>
<td>Disinfection</td>
</tr>
<tr>
<td></td>
<td>Cresol</td>
<td></td>
<td>Preservation</td>
</tr>
<tr>
<td>General structure</td>
<td>$[\begin{array}{c} \text{N} \ \text{R}^1 \ \text{X} \ \text{R}^2 \ \text{R}^3 \ \text{R}^4 \end{array}]^+$</td>
<td>Disinfection</td>
<td></td>
</tr>
<tr>
<td>Quaternary ammonium compounds</td>
<td>Cetrimide, benzalkonium chloride</td>
<td></td>
<td>Antisepsis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Preservation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cleaning</td>
</tr>
</tbody>
</table>

predominantly the viral envelope (if present), derived from the host cell cytoplasmic or nuclear membrane; the capsid, which is responsible for the shape of virus particles and for the protection of viral nucleic acid; and the viral genome. Release of an intact viral nucleic acid into the environment following capsid destruction is of potential concern since some nucleic acids are infective when liberated from the capsid (317), an aspect that must be considered in viral disinfection. Important considerations in viral inactivation are dealt with by Klein and Deforest (259) and Prince et al.
Alcohols

Although several alcohols have been shown to be effective antimicrobials, ethyl alcohol (ethanol, alcohol), isopropyl alcohol (isopropanol, propan-2-ol) and n-propanol (in particular in Europe) are the most widely used (337). Alcohols exhibit rapid broad-spectrum antimicrobial activity against vegetative bacteria (including mycobacteria), viruses, and fungi but are not sporicidal. They are, however, known to inhibit sporulation and spore germination (545), but this effect is reversible (513). Because of the lack of sporicidal activity, alcohols are not recommended for sterilization but are widely used for both hard-surface disinfection and skin antisepsis. Lower concentrations may also be used as preservatives and to potentiate the activity of other biocides. Many alcohol products include low levels of other biocides (in particular chlorhexidine), which remain on the skin following evaporation of the alcohol, or excipients (including emollients), which decrease the evaporation time of the alcohol and can significantly increase product efficacy (68). In general, isopropyl alcohol is considered slightly more efficacious against bacteria (95) and ethyl alcohol is more potent against viruses (259); however, this is dependent on the concentrations of both the active agent and the test microorganism. For example, isopropyl alcohol has greater lipophilic properties than ethyl alcohol and is less active against hydrophilic viruses (e.g., poliovirus) (259). Generally, the antimicrobial activity of alcohols is significantly lower at concentrations below 50% and is optimal in the 60 to 90% range.

Little is known about the specific mode of action of alcohols, but based on the increased efficacy in the presence of water, it is generally believed that they cause membrane damage and rapid denaturation of proteins, with subsequent interference with metabolism and cell lysis (278, 337). This is supported by specific reports of denaturation of *Escherichia coli* dehydrogenases (499) and an increased lag phase in *Enterobacter aerogenes*, speculated to be due to inhibition of metabolism required for rapid cell division (101).

Aldehydes

Glutaraldehyde. Glutaraldehyde is an important dialdehyde that has found usage as a disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes and surgical equipment and as a fixative in electron microscopy. Glutaraldehyde is used in formaldehyde sterilants, such as formalin, and in ethylene oxide sterilization. It is also used in industrial purposes for the cross-linking of proteins and for the modification of DNA. Glutaraldehyde is a dialdehyde that is used in the manufacture of medical instruments, urological devices, and other disposable medical supplies. It is also used in the food industry for the production of明胶 and in the preservation of fish. Glutaraldehyde is a strong oxidizing agent that can cause tissue damage. It is also a potent mutagen and carcinogen. Glutaraldehyde is a powerful reagent and can cause severe chemical burns if it comes in contact with the skin or eyes. It is also a powerful sensitizer and can cause allergic reactions in some individuals. The use of glutaraldehyde is regulated by the United States Food and Drug Administration (FDA) and the Occupational Safety and Health Administration (OSHA).

**Table 1—Continued**

<table>
<thead>
<tr>
<th>Target</th>
<th>Antiseptic or disinfectant</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell envelope (cell wall, outer membrane)</td>
<td>Glutaraldehyde, EDTA, other permeabilizers</td>
<td>Cross-linking of proteins, Gram-negative bacteria: removal of Mg²⁺, release of some LPS</td>
</tr>
<tr>
<td>Cytoplasmic (inner) membrane</td>
<td>QACs, Chlorhexidine, Diamines, PHMB, alexidine, Phenols</td>
<td>Generalized membrane damage involving phospholipid bilayers, Low concentrations affect membrane integrity, high concentrations cause congealing of cytoplasm, Induction of leakage of amino acids, Phase separation and domain formation of membrane lipids, Leakage; some cause uncoupling</td>
</tr>
<tr>
<td>Cross-linking of macromolecules</td>
<td>Formaldehyde, Glutaraldehyde</td>
<td>Cross-linking of proteins, RNA, and DNA, Cross-linking of proteins in cell envelope and elsewhere in the cell</td>
</tr>
<tr>
<td>DNA intercalation</td>
<td>Acridines</td>
<td>Intercalation of an acridine molecule between two layers of base pairs in DNA</td>
</tr>
<tr>
<td>Interaction with thiol groups</td>
<td>Silver compounds</td>
<td>Membrane-bound enzymes (interaction with thiol groups)</td>
</tr>
<tr>
<td>Effects on DNA</td>
<td>Halogens, Hydrogen peroxide, silver ions</td>
<td>Inhibition of DNA synthesis, DNA strand breakage</td>
</tr>
<tr>
<td>Oxidizing agents</td>
<td>Halogens, Peroxygens</td>
<td>Oxidation of thiol groups to disulfides, sulfoxides, or sulfitoloxides, Hydrogen peroxide: activity due to from formation of free hydroxy radicals (•OH), which oxidize thiol groups in enzymes and proteins, PAA: disruption of thiol groups in proteins and enzymes</td>
</tr>
</tbody>
</table>

**Table 2. Summary of mechanisms of antibacterial action of antiseptics and disinfectants**

<table>
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<th>Target</th>
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<th>Mechanism of action</th>
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</tr>
<tr>
<td>Cross-linking of macromolecules</td>
<td>Formaldehyde, Glutaraldehyde</td>
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</tr>
<tr>
<td>DNA intercalation</td>
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<tr>
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<td>Oxidizing agents</td>
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</tbody>
</table>
Glutaraldehyde has a broad spectrum of activity against bacteria and their spores, fungi, and viruses, and a considerable amount of information is now available about the ways whereby these different organisms are inactivated (Tables 2 and 3). Earlier reviews of its mechanisms of action have been published (179, 182, 374, 482).

The first reports in 1964 and 1965 (182) demonstrated that glutaraldehyde possessed high antimicrobial activity. Subsequently, research was undertaken to evaluate the nature of its action. These bactericidal studies demonstrated (374) a strong association with outer cell layers of organisms such as *E. coli* and *Staphylococcus aureus* (179, 212, 339–341, 343, 344), inhibition of transport in gram-negative bacteria (179), inhibition of dehydrogenase activity (340, 344), and of periplasmic inhibition of transport in gram-negative bacteria (179), inhibition of spheroplast and protoplast lysis in systems, where access of substrate to enzyme is prohibited. Partial or entire removal of the cell wall in hypertonic medium, leading to the production of spheroplasts or protoplasts and the subsequent prevention of lysis by glutaraldehyde when these forms are diluted in a hypotonic environment, suggests an additional effect on the inner membrane, a finding substantiated by the fact that the dialdehyde prevents the selective release of some membrane-bound enzymes of *Micrococcus lysodeikticus* (138).

Glutaraldehyde is more active at alkaline than at acidic pHS. As the external pH is altered from acidic to alkaline, more reactive sites will be formed at the cell surface, leading to a more rapid bactericidal effect. The cross-links thus obtained mean that the cell is then unable to undertake most, if not all, of its essential functions. Glutaraldehyde is also mycobactericidal. Unfortunately, no critical studies have as yet been undertaken to evaluate the nature of this action (419).

The bacterial spore presents several sites at which interaction with glutaraldehyde is possible, although interaction with a particular site does not necessarily mean that this is associated with spore inactivation. *E. coli*, *S. aureus*, and vegetative cells of *Bacillus subtilis* bind more glutaraldehyde than do resting spores of *B. subtilis* (377, 378); uptake of glutaraldehyde is greater during germination and outgrowth than with mature spores but still lower than with vegetative cells. Low concentrations of the dialdehyde (0.1%) inhibit germination, whereas much higher concentrations (2%) are sporicidal. The aldehyde, at both acidic and alkaline pHs, interacts strongly with the outer spore layers (508, 509); this interaction reduces the release of dipicolinic acid (DPA) from heated spores and the lysis induced by mercaptoethanol (or thioglycolate)-peroxide combinations. Low concentrations of both acidic and alkaline glutaraldehyde increase the surface hydrophobicity of spores, again indicating an effect at the outermost regions of the cell. It has been observed by various authors (182, 374, 376, 380) that the greater sporidial activity of glutaraldehyde at alkaline pH is not reflected by differences in uptake; however, uptake per se reflects binding and not necessarily penetration into the spore. It is conceivable that acidic glutaraldehyde interacts with and remains at the cell surface whereas alkaline glutaraldehyde penetrates more deeply into the spore. This contention is at odds with the hypothesis of Bruch (65), who envisaged the acidic form penetrating the coat and reacting with the cortex while the alkaline form attacked the coat, thereby destroying the ability of the spore to function solely as a result of this surface phenomenon. There is, as yet, no evidence to support this theory. Novel glutaraldehyde formulations based on acidic rather than alkaline glutaraldehyde, which benefit from the greater inherent stability of the aldehyde at lower pH, have been produced. The improved sporidical activity claimed for these products may be obtained by agents that potentiate the activity of the dialdehyde (414, 421).

During sporulation, the cell eventually becomes less susceptible to glutaraldehyde (see “Intrinsic resistance of bacterial spores”). By contrast, germinating and outgrowing cells reacquire sensitivity. Germination may be defined as an irreversible process in which there is a change of an activated spore from a dormant to a metabolically active state within a short period. Glutaraldehyde exerts an early effect on the germination process. l-Alanine is considered to act by binding to a specific receptor on the spore coat, and once spores are triggered to germinate, they are committed irreversibly to losing their dormant properties (491). Glutaraldehyde at high concentrations inhibits the uptake of l-[14C]alanine by *B. subtilis* spores, albeit by an unknown mechanism (379, 414). Glutaraldehyde-treated spores retain their refractivity, having the same appearance under the phase-contrast microscope as normal, untreated spores even when the spores are subsequently incubated in germination medium. Glutaraldehyde is normally used as a 2% solution to achieve a sporidical effect (16, 316); low concentrations (<0.1%) prevent phase darkening of spores and also prevent the decrease in optical density associated with a late event in germination. By contrast, higher concentrations (0.1 to 1%) significantly reduce the uptake of l-alanine, possibly as a result of a sealing effect of the aldehyde on the cell surface. Mechanisms involved in the revival of glutaraldehyde-treated spores are discussed below (see “Intrinsic resistance of bacterial spores”).

There are no recent studies of the mechanisms of fungicidal action of glutaraldehyde. Earlier work had suggested that the fungal cell wall was a major target site (179, 182, 352), especially the major wall component, chitin, which is analogous to the peptidoglycan found in bacterial cell walls.

Glutaraldehyde is a potent virucidal agent (143, 260). It reduces the activity of hepatitis B surface antigen (HBsAg) and especially hepatitis B core antigen ([HBCAg] in hepatitis B virus [HBV]) (3) and interacts with lysine residues on the surface of hepatitis A virus (HAV) (362). Low concentrations

<table>
<thead>
<tr>
<th>Target microorganism</th>
<th>Glutaraldehyde action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial spores ..........</td>
<td>Low concentrations inhibit germination; high concentrations are sporidical, probably as a consequence of strong interaction with outer cell layers</td>
</tr>
<tr>
<td>Mycobacteria .............</td>
<td>Action unknown, but probably involves mycobacterial cell wall</td>
</tr>
<tr>
<td>Other nonsporulating bacteria</td>
<td>Strong association with outer layers of gram-positive and gram-negative bacteria; cross-linking of amino groups in protein; inhibition of transport processes into cell</td>
</tr>
<tr>
<td>Fungi ....................</td>
<td>Fungal cell wall appears to be a primary target site, with postulated interaction with chitin</td>
</tr>
<tr>
<td>Viruses ..................</td>
<td>Actual mechanisms unknown, but involve protein-DNA cross-links and capsid changes</td>
</tr>
<tr>
<td>Protozoa ..................</td>
<td>Mechanism of action not known</td>
</tr>
</tbody>
</table>
(<0.1%) of alkaline glutaraldehyde are effective against purified poliovirus, whereas poliovirus RNA is highly resistant to aldehyde concentrations up to 1% at pH 7.2 and is only slowly inactivated at pH 8.3 (21). In other words, the complete poliovirus particle is much more sensitive than poliovirus RNA. In light of this, it has been inferred that glutaraldehyde-induced loss of infectivity is associated with capsid changes (21). Glutaraldehyde at the low concentrations of 0.05 and 0.005% interacts with the capsid proteins of poliovirus and echovirus, respectively; the differences in sensitivity probably reflect major structural variations in the two viruses (75).

Bacteriophages were recently studied to obtain information about mechanisms of virucidal action (298–304, 306, 307). Many glutaraldehyde-treated P. aeruginosa F116 phage particles had empty heads, implying that the phage genome had been ejected. The aldehyde was possibly bound to F116 double-stranded DNA but without affecting the molecule; glutaraldehyde also interacted with phage F116 proteins, which were postulated to be involved in the ejection of the nucleic acid. Concentrations of glutaraldehyde greater than 0.1 to 0.25% significantly affected the transduction of this phage; the transduction process was more sensitive to the aldehyde than was the phage itself. Glutaraldehyde and other aldehydes were tested for their ability to form protein-DNA cross-links in simian virus 40 (SV40); aldehydes (i.e., glyoxal, furfural, pimonaldehyde, acet-aldehyde, and benzylaldehyde) without detectable cross-linking ability had no effect on SV40 DNA synthesis, whereas acrolein, glutaraldehyde, and formaldehyde, which formed such cross-links (144, 271, 297), inhibited DNA synthesis (369).

**Formaldehyde.** Formaldehyde (methanal, CH₂O) is a mono-aldehyde that exists as a freely water-soluble gas. Formaldehyde solution (formalin) is an aqueous solution containing aldehyde that exists as a freely water-soluble gas. Formaldehyde is bactericidal, sporidical, and virucidal, but it works more slowly than glutaraldehyde (374, 482).

Formaldehyde is an extremely reactive chemical (374, 442) that interacts with protein (156, 157), DNA (155), and RNA (155) in vitro. It has long been considered to be sporidical by virtue of its ability to penetrate into the interior of bacterial spores (500). The interaction with protein results from a combination with the primary amide as well as with the amino groups, although phenol groups bind little formaldehyde (155). It has been proposed that formaldehyde acts as a mutagenic agent (291) and as an alkylating agent by reaction with carbonyl, sulfhydryl, and hydroxyl groups (371). Formaldehyde also reacts extensively with nucleic acid (489) (e.g., the DNA of bacteriophage T2) (190). As pointed out above, it forms protein-DNA cross-links in SV40, thereby inhibiting DNA synthesis (369). Low concentrations of formaldehyde are sporostatic and inhibit germination (512). Formaldehyde alters HBsAg and HBeAg of HBV (3).

It is difficult to pinpoint accurately the mechanism(s) responsible for formaldehyde-induced microbial inactivation. Clearly, its interactive, and cross-linking properties must play a considerable role in this activity. Most of the other aldehydes (glutar-aldehyde, glyoxal, succinaldehyde, and o-phthalaldehyde [OPA]) that have sporidical activity are dialdehydes (and of these, glyoxal and succinaldehyde are weakly active). The distance between the two aldehyde groups in glutaraldehyde (and possibly in OPA) may be optimal for interaction of these-CHO groups in nucleic acids and especially in proteins and enzymes (428).

**Formaldehyde-releasing agents.** Several formaldehyde-releasing agents have been used in the treatment of peritonitis (226, 273). They include noxythiolin (oxymethylenethiourea),

**TABLE 4. Mechanisms of antimicrobial action of chlorhexidine**

<table>
<thead>
<tr>
<th>Type of microorganism</th>
<th>Chlorhexidine action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial spores</td>
<td>Not sporicidal but prevents development of spores; inhibits spore outgrowth but not germination</td>
</tr>
<tr>
<td>Mycobacteria</td>
<td>Mycobacteriostatic (mechanism unknown) but not mycobactericidal</td>
</tr>
<tr>
<td>Other nonsporulating</td>
<td>Membrane-active agent, causing protoplast and spheroplast lysis; high concentrations cause precipitation of proteins and nucleic acids</td>
</tr>
<tr>
<td>Yeasts</td>
<td>Membrane-active agent, causing protoplast lysis and intracellular leakage; high concentrations cause intracellular coagulation</td>
</tr>
<tr>
<td>Viruses</td>
<td>Low activity against many viruses; lipid-enveloped viruses more sensitive than nonenveloped viruses; effect possibly on viral envelope, perhaps the lipid moiety</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Recent studies against A. castellanii demonstrate membrane activity (leakage) toward trophozoites, less toward cysts</td>
</tr>
</tbody>
</table>

**Anilides**

The anilides have been investigated primarily for use as antiseptics, but they are rarely used in the clinic. Trielocarban (TCC; 3,4,4′-trichlorocarbanilide) is the most extensively studied in this series and is used mostly in consumer soaps and deodorants. TCC is particularly active against gram-positive bacteria and fungi (30) and lacks appreciable substantivity (persistence) for the skin (37). The anilides are thought to act by adsorbing to and destroying the semipermeable character of the cytoplasmic membrane, leading to cell death (194).

**Biguianides**

**Chlorhexidine.** Chlorhexidine is probably the most widely used biocide in antiseptic products, in particular in handwashing and oral products but also as a disinfectant and preservative. This is due in part to its broad-spectrum efficacy, substantivity for the skin, and low irritation. Of note, irritability has been described and in many cases may be product specific (167, 403). Despite the advantages of chlorhexidine, its activity is pH dependent and is greatly reduced in the presence of organic matter (430). A considerable amount of research has been undertaken on the mechanism of the antimicrobial action of this important bisguianide (389) (Tables 2 and 4), although most of the attention has been devoted to the way in which it
inactivates nonsporulating bacteria (215, 428, 430, 431, 451). Nevertheless, sufficient data are now available to examine its sporostatic and mycobacteriostatic action, its effects on yeasts and protozoa, and its antiviral activity.

Chlorhexidine is a bactericidal agent (120, 215). Its interaction and uptake by bacteria were studied initially by Hugo et al. (222–224), who found that the uptake of chlorhexidine by *E. coli* and *S. aureus* was very rapid and depended on the chlorhexidine concentration and pH. More recently, by using 

\[ ^{[14C]} \text{chlorhexidine gluconate, the uptake by bacteria (145) and yeasts (204) was shown to be extremely rapid, with a maximum effect occurring within 20 s. Damage to the outer cell layers takes place (139) but is insufficient to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, presumably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane or the yeast plasma membrane. In yeasts, chlorhexidine “partitions” into the cell wall, plasma membrane, and cytoplasm of cells (205). Damage to the delicate semipermeable membrane is followed by leakage of intracellular constituents, which can be measured by appropriate techniques. Leakage is not per se responsible for cellular inactivation but is a consequence of cell death (445). High concentrations of chlorhexidine cause coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage (222–224, 290), so that there is a biphasic effect on membrane permeability. An initial high rate of leakage rises as the concentration of chlorhexidine increases, but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol.

Chlorhexidine was claimed by Harold et al. (199) to be an inhibitor of both membrane-bound and soluble ATPase as well as of net K⁺ uptake in *Enterococcus faecalis*. However, only high biocide concentrations inhibit membrane-bound ATPase (83), which suggests that the enzyme is not a primary target for chlorhexidine action. Although chlorhexidine collapses the membrane potential, it is membrane disruption rather than ATPase inactivation that is associated with its lethal effects (24, 272).

The effects of chlorhexidine on yeast cells are probably similar to those previously described for bacteria (204–207). Chlorhexidine has a biphasic effect on protoplast lysis, with reduced lysis at higher biocide concentrations. Furthermore, in whole cells, the yeast cell wall may have some effect in limiting the uptake of the biocide (208). The findings presented here and elsewhere (47, 136, 137, 527) demonstrate an effect on the fungal plasma membrane but with significant actions elsewhere in the cell (47). Increasing concentrations of chlorhexidine (up to 25 μg/ml) induce progressive lysis of *Saccharomyces cerevisiae* protoplasts, but higher biocide concentrations result in reduced lysis (205).

Work to date suggests that chlorhexidine has a similar effect on the trophozoites of *Acanthameoba castellanii*, with the cysts being less sensitive (251–255). Furr (163) reviewed the effects of chlorhexidine and other biocides on *Acanthameoba* and showed that membrane damage in these protozoa is a significant factor in their inactivation.

Mycobacteria are generally highly resistant to chlorhexidine (419). Little is known about the uptake of chlorhexidine (and other antiseptics and disinfectants) by mycobacteria and on the biochemical changes that occur in the treated cells. Since the MICs for some mycobacteria are on the order of those for chlorhexidine-sensitive, gram-positive cocci (48), the inhibitory effects of chlorhexidine on mycobacteria may not be dissimilar to those on susceptible bacteria. *Mycobacterium avium-intracellularare* is considerably more resistant than other mycobacteria (48).

Chlorhexidine is not sporicidal (discussed in “Mechanisms of resistance”). Even high concentrations of the bisbiguanide do not affect the viability of *Bacillus* spores at ambient temperatures (473, 474), although a marked sporicidal effect is achieved at elevated temperatures (475). Presumably, sufficient changes occur in the spore structure to permit an increased uptake of the biocide, although this has yet to be shown experimentally. Little is known about the uptake of chlorhexidine by bacterial spores, although coatless forms take up more of the compound than do “normal” spores (474).

Chlorhexidine has little effect on the germination of bacterial spores (414, 422, 432, 447) but inhibits outgrowth (447). The reason for its lack of effect on the former process but its significant activity against the latter is unclear. It could, however, be reflected in the relative uptake of chlorhexidine, since germinating cells take up much less of the bisbiguanide than do outgrowing forms (474). Binding sites could thus be reduced in number or masked in germinating cells.

The antiviral activity of chlorhexidine is variable. Studies with different types of bacteriophages have shown that chlorhexidine has no effect on MS2 or K coliphages (300). High concentrations also failed to inactivate *Pseudomonas aeruginosa* phage F116 and had no effect on phage DNA within the capsid or on phage proteins (301); the transduction process was more sensitive to chlorhexidine and other biocides than was the phage itself. This substantiated an earlier finding (306) that chlorhexidine bound poorly to F116 particles. Chlorhexidine is not always considered a particularly effective antiviral agent, and its activity is restricted to the lipid-enveloped viruses (361). Chlorhexidine does not inactivate nonenveloped viruses such as rotavirus (485), HAV (315), or poliovirus (34). Its activity was found by Ranganathan (389) to be restricted to the nucleic acid core or the outer coat, although it is likely that the latter would be a more important target site.

**Alexidine.** Alexidine differs chemically from chlorhexidine in possessing ethylhexyl end groups. Alexidine is more rapidly bactericidal and produces a significantly faster alteration in bactericidal permeability (79, 80). Studies with mixed-lipid and pure phospholipid vesicles demonstrate that, unlike chlorhexidine, alexidine produces lipid phase separation and domain formation (Table 2). It has been proposed (80) that the nature of the ethylhexyl end group in alexidine, as opposed to the chlorophenol one in chlorhexidine, might influence the ability of a biguane to produce lipid domains in the cytoplasmic membrane.

**Polymeric biguanides.** Vantocil is a heterodisperse mixture of polyhexamethylene biguanides (PHMB) with a molecular weight of approximately 3,000. Polymeric biguanides have found use as general disinfecting agents in the food industry and, very successfully, for the disinfection of swimming pools. Vantocil is active against gram-positive and gram-negative bacteria, although *P. aeruginosa* and *Proteus vulgaris* are less sensitive. Vantocil is not sporicidal. PHMB is a membrane-active agent that also impairs the integrity of the outer membrane of gram-negative bacteria, although the membrane may also act as a permeability barrier (64, 172). Activity of PHMB increases on a weight basis with increasing levels of polymerization, which has been linked to enhanced inner membrane perturbation (173, 174).

Unlike chlorhexidine but similar to alexidine (Table 2), PHMB causes domain formation of the acidic phospholipids of the cytoplasmic membrane (61–64, 172, 173, 227). Permeability changes ensue, and there is believed to be an altered function of some membrane-associated enzymes. The proposed sequence of events during its interaction with the cell envelope of *E. coli* is as follows: (i) there is rapid attraction of...
PHMB toward the negatively charged bacterial cell surface, with strong and specific adsorption to phosphate-containing compounds; (ii) the integrity of the outer membrane is impaired, and PHMB is attracted to the inner membrane; (iii) binding of PHMB to phospholipids occurs, with an increase in inner membrane permeability (K’ loss) accompanied by bacteriostasis; and (iv) complete loss of membrane function follows, with precipitation of intracellular constituents and a bactericidal effect.

**Diamidines**

The diamidines are characterized chemically as described in Table 1. The isethionate salts of two compounds, propamidine (4,4-diaminodiphenoxypyracne) and dibromopropamidine (2,2-dibromo-4,4-diaminodiphenoxypyracne), have been used as antibacterial agents. Their antibacterial properties and uses were reviewed by Hugo (213) and Hugo and Russell (226). Clinically, diamidines are used for the topical treatment of wounds.

The exact mechanism of action of diamidines is unknown, but they have been shown to inhibit oxygen uptake and induce leakage of amino acids (Table 2), as would be expected if they are considered as cationic surface-active agents. Damage to the cell surface of *P. aeruginosa* and *Enterobacter cloacae* has been described (400).

**Halogen-Releasing Agents**

Chlorine- and iodine-based compounds are the most significant microbialidal halogens used in the clinic and have been traditionally used for both antiseptic and disinfectant purposes.

**Chlorine-releasing agents.** Excellent reviews that deal with the chemical, physical, and microbiological properties of chlorine-releasing agents (CRAs) are available (42, 130). The most important types of CRAs are sodium hypochlorite, chlorine dioxide, and the N-chloro compounds such as sodium dichloroisocyanurate (NaDCC), with chlorine-T being used to some extent. Sodium hypochlorite solutions are widely used for hard-surface disinfection (household bleach) and can be used for disinfecting spillages of blood containing human immunodeficiency virus or HBV. NaDCC can also be used for this purpose and has the advantages of providing a higher concentration of available chlorine and being less susceptible to inactivation by organic matter. In water, sodium hypochlorite ionizes to produce Na⁺ and the hypochlorite ion, OCI⁻, which establishes an equilibrium with hypochlorous acid, HOCl (42). Between pH 4 and 7, chlorine exists predominantly as HClO, the active moiety, whereas above pH9, OCI⁻ predominates. Although CRAs have been predominantly used as hard-surface disinfectants, novel acidified sodium chloride (a two-component system of sodium chloride and mandelic acid) has been described as an effective antiseptic (248).

Surprisingly, despite being widely studied, the actual mechanism of action of CRAs is not fully known (Table 2). CRAs are highly active oxidizing agents and thereby destroy the cellular activity of proteins (42); potentiation of oxidation may occur at low pH, where the activity of CRAs is maximal, although increased penetration of outer cell layers may be achieved with CRAs in the unionized state. Hypochlorous acid has long been considered the active moiety responsible for bacterial inactivation by CRAs, the OCI⁻ ion having a minute effect compared to undissolved HOCl (130). This correlates with the observation that CRA activity is greatest when the percentage of undissolved HOCl is highest. This concept applies to hypochlorites, NaDCC, and chloramine-T.

Deleterious effects of CRAs on bacterial DNA that involve the formation of chlorinated derivatives of nucleotide bases have been described (115, 128, 477). Hypochlorous acid has also been found to disrupt oxidative phosphorylation (26) and other membrane-associated activity (70). In a particularly interesting paper, McKenna and Davies (321) described the inhibition of bacterial growth by hypochlorous acid. At 50 μM (2.6 ppm), HOCl completely inhibited the growth of *E. coli* within 5 min, and DNA synthesis was inhibited by 96% but protein synthesis was inhibited by only 10 to 30%. Because concentrations below 5 mM (260 ppm) did not induce bacterial membrane disruption or extensive protein degradation, it was inferred that DNA synthesis was the sensitive target. In contrast, chlorine dioxide inhibited bacterial protein synthesis (33).

CRAs at higher concentrations are sporidical (44, 421, 431); this depends on the pH and concentration of available chlorine (408, 412). During treatment, the spores lose refractivity, the spore coat separates from the cortex, and lysis occurs (268). In addition, a number of studies have concluded that CRA-treated spores exhibit increased permeability of the spore coat (131, 268, 412).

CRAs also possess virucidal activity (34, 46, 116, 315, 394, 407, 467, 485, 486). Olivieri et al. (359) showed that chlorine inactivated naked f2 RNA at the same rate as RNA in intact phage, whereas f2 capsid proteins could still adsorb to the host. Taylor and Butler (504) found that the RNA of poliovirus type 1 was degraded into fragments by chlorine but that poliovirus inactivation preceded any severe morphological changes. By contrast, Floyd et al. (149) and O’Brien and Newman (357) demonstrated that the capsid of poliovirus type 1 was broken down. Clearly, further studies are needed to explain the antiviral action of CRAs.

**Iodine and iodophors.** Although less reactive than chlorine, iodine is rapidly bactericidal, fungicidal, tuberculocidal, virucidal, and sporidical (184). Although aqueous or alcoholic (tincture) solutions of iodine have been used for 150 years as antiseptics, they are associated with irritation and excessive staining. In addition, aqueous solutions are generally unstable; in solution, at least seven iodine species are present in a complex equilibrium, with molecular iodine (I₂) being primarily responsible for antimicrobial efficacy (184). These problems were overcome by the development of iodophors (“iodine carriers” or “iodine-releasing agents”); the most widely used are povidone-iodine and poloxamer-iodine in both antiseptics and disinfectants. Iodophors are complexes of iodine and a solubilizing agent or carrier, which acts as a reservoir of the active “free” iodine (184). Although germcidal activity is maintained, iodophors are considered less active against certain fungi and spores than are tinctures (454).

Similar to chlorine, the antimicrobial action of iodine is rapid, even at low concentrations, but the exact mode of action is unknown. Iodine rapidly penetrates into microorganisms (76) and attacks key groups of proteins (in particular the free-sulfur amino acids cysteine and methionine [184, 267]), nucleotides, and fatty acids (15, 184), which culminates in cell death (184). Less is known about the antiviral action of iodine, but nonlipid viruses and parvoviruses are less sensitive than lipid enveloped viruses (384). Similarly to bacteria, it is likely that iodine attacks the surface proteins of enveloped viruses, but they may also destabilize membrane fatty acids by reacting with unsaturated carbon bonds (486).

**Silver Compounds**

In one form or another, silver and its compounds have long been used as antimicrobial agents (55, 443). The most important silver compound currently in use is silver sulfadiazine...
(AgSD), although silver metal, silver acetate, silver nitrate, and silver protein, all of which have antimicrobial properties, are listed in Martindale, The Extra Pharmacopoeia (312). In recent years, silver compounds have been used to prevent the infection of burns and some eye infections and to destroy warts.

Silver nitrate. The mechanism of the antimicrobial action of silver ions is closely related to their interaction with thiol (sulfhydryl, −SH) groups (32, 49, 161, 164), although other target sites remain a possibility (397, 509). Liu et al (287) demonstrated that amino acids such as cysteine and other compounds such as sodium thioglycolate containing thiol groups neutralized the activity of silver nitrate against P. aeruginosa. By contrast, amino acids containing disulfide (SS) bonds, non-sulfur-containing amino acids, and sulfur-containing compounds such as cystathionine, cysteic acid, L-methionine, taurine, sodium bisulfite, and sodium thiosulfate were all unable to neutralize Ag⁺ activity. These and other findings imply that interaction of Ag⁺ with thiol groups in enzymes and proteins plays an essential role in bacterial inactivation, although other cellular components may be involved. Hydrogen bonding, the effects of hydrogen bond-breaking agents, and the specificity of Ag⁺ for thiol groups were discussed in greater detail by Russell and Hugo (443) (Table 2). Virucidal properties might also be explained by binding to −SH groups (510).

Lukens (292) proposed that silver salts and other heavy metals such as copper act by binding to key functional groups of fungal enzymes. Ag⁺ causes the release of K⁺ ions from microorganisms; the microbial plasma or cytoplasmic membrane, with which is associated many important enzymes, is an important target site for Ag⁺ activity (161, 329, 392, 470).

In addition to its effects on enzymes, Ag⁺ produces other changes in microorganisms. Silver nitrate causes marked inhibition of growth of Cryptococcus neoformans and is deposited in the vacuole and cell wall as granules (60). Ag⁺ inhibits cell division and damages the cell envelope and contents of P. aeruginosa (398). Bacterial cells increase in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibit structural abnormalities, although without any blebs (protuberances) (398). Finally, the Ag⁺ ion interacts with nucleic acids (543); it interacts preferentially with the bases in DNA rather than with the phosphate groups, although the significance of this in terms of its lethal action is unclear (231, 387, 510, 547).

Silver sulfadiazine. AgSD is essentially a combination of two antibacterial agents, Ag⁺ and sulfadiazine (SD). The question whether the antibacterial effect of AgSD arises predominantly from only one of the compounds or via a synergistic interaction has been posed repeatedly. AgSD has a broad spectrum of activity and, unlike silver nitrate, produces surface and membrane-blebs in susceptible (but not resistant) bacteria (96). AgSD binds to cell components, including DNA (332, 404). Based on a chemical analysis, Fox (153) proposed a polymeric structure of AgSD composed of six silver atoms bonding to six SD molecules by linkage of the silver atoms to the nitrogens of the SD pyrimidine ring. Bacterial inhibition would then presumably be achieved when silver binds to sufficient base pairs in the DNA helix, thereby inhibiting transcription. Similarly, its antiphage properties have been ascribed to the fact that AgSD binds to phage DNA (154, 388). Clearly, the precise mechanism of action of AgSD has yet to be solved.

Peroxogens

Hydrogen peroxide. Hydrogen peroxide (H₂O₂) is a widely used biocide for disinfection, sterilization, and antisepsis. It is a clear, colorless liquid that is commercially available in a variety of concentrations ranging from 3 to 90%. H₂O₂ is considered environmentally friendly, because it can rapidly degrade into the innocuous products water and oxygen. Although pure solutions are generally stable, most contain stabilizers to prevent decomposition. H₂O₂ demonstrates broad-spectrum efficacy against viruses, bacteria, yeasts, and bacterial spores (38). In general, greater activity is seen against gram-positive than gram-negative bacteria; however, the presence of catalase or other peroxidases in these organisms can increase tolerance in the presence of lower concentrations. Higher concentrations of H₂O₂ (10 to 30%) and longer contact times are required for sporicidal activity (416), although this activity is significantly increased in the gaseous phase. H₂O₂ acts as an oxidant by producing hydroxyl free radicals (‘•OH) which attack essential cell components, including lipids, proteins, and DNA. It has been proposed that exposed sulphydryl groups and double bonds are particularly targeted (38).

Peroxacetic acid. Peracetic acid (PAA) (CH₃COOOH) is considered a more potent biocide than hydrogen peroxide, being sporicidal, bactericidal, virucidal, and fungicidal at low concentrations (<0.3%) (38). PAA also decomposes to safe by-products (acetic acid and oxygen) but has the added advantages of being free from decomposition by peroxidases, unlike H₂O₂, and remaining active in the presence of organic loads (283, 308). Its main application is as a low-temperature liquid sterilant for medical devices, flexible scopes, and hemodialyzers, but it is also used as an environmental surface sterilant (100, 308).

Similar to H₂O₂, PAA probably denatures proteins and enzymes and increases cell wall permeability by disrupting sulphydryl (–SH) and sulfur (–S–) bonds (22, 38).

Phenols

Phenolic-type antimicrobial agents have long been used for their antiseptic, disinfectant, or preservative properties, depending on the compound. It has been known for many years (215) that, although they have often been referred to as “general protoplasmic poisons,” they have membrane-active properties which also contribute to their overall activity (120) (Table 2).

Phenol induces progressive leakage of intracellular constituents, as the release of K⁺, the first index of membrane damage (273), and of radioactivity from ¹⁴C-labeled E. coli (242, 265). Pulvertaft and Lumb (386) demonstrated that low concentrations of phenols (0.032%, 320 μg/ml) and other (non-phenolic) agents lysed rapidly growing cultures of E. coli, staphylococci, and streptococci and concluded that autolytic enzymes were not involved. Srivastava and Thompson (487, 488) proposed that phenol acts only at the point of separation of pairs of daughter cells, with young bacterial cells being more sensitive than older cells to phenol.

Hugo and Bloomfield (216, 217) showed with the chlorinated bis-phenol fenticonal that there was a close relationship between bactericidal activity and leakage of 260-nm-absorbing material (leakage being induced only by bactericidal concentrations). Fentichlor affected the metabolic activities of S. aureus and E. coli (217) and produced a selective increase in permeability to protons with a consequent dissipation of the proton motive force (PMF) and an uncoupling of oxidative phosphorylation (41). Chlororesol has a similar action (124). Coagulation of cytoplasmic constituents at higher phenol concentrations, which causes irreversible cellular damage, has been described by Hugo (215).

The phenolics possess antifungal and antiviral properties. Their antifungal action probably involves damage to the plas-
ma membrane (436), resulting in leakage of intracellular constituents. Phenol does not affect the transduction of S. aureus PAO by bacteriophage F116 (301), has no effect on plasmid DNA within the capsid, and has little effect on several of the phage band proteins unless treatments of 20 min or longer are used (303, 304).

**Bis-Phenols**

The bis-phenols are hydroxy-halogenated derivatives of two phenolic groups connected by various bridges (191, 446). In general, they exhibit broad-spectrum efficacy but have little activity against P. aeruginosa and molds and are sporostatic toward bacterial spores. Triclosan and hexachlorophane are the most widely used biocides in this group, especially in antisep tic soaps and hand rinses. Both compounds have been shown to have cumulative and persistent effects on the skin (313).

**Triclosan.** Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether; Irgasan DP 300) exhibits particular activity against gram-positive bacteria (469, 521). Its efficacy against gram-negative bacteria and yeasts can be significantly enhanced by formulation effects. For example, triclosan in combination with EDTA caused increased permeability of the outer membrane (282). Reports have also suggested that in addition to its antibacterial properties, triclosan may have anti-inflammatory activity (25, 522). The specific mode of action of triclosan is unknown, but it has been suggested that the primary effects are on the cytoplasmic membrane. In studies with E. coli, triclosan at subinhibitory concentrations inhibited the uptake of essential nutrients, while higher, bactericidal concentrations resulted in the rapid release of cellular components and cell death (393). Studies with a divalent-ion-dependent E. coli triclosan mutant for which the triclosan MIC was 10-fold greater than that for a wild-type strain showed no significant differences in total envelope protein profiles but did show significant differences in envelope fatty acids (370). Specifically, a prominent 14:1 fatty acid was absent in the resistant strain, and there were minor differences in other fatty acid species. It was proposed that divalent ions and fatty acids may adsorb and limit the permeability of triclosan to its site of action (370). Minor changes in fatty acid profiles were recently found in both E. coli and S. aureus strains for which the triclosan MICs were elevated; however, the MBCs were not affected, suggesting, as for other phenols, that the cumulative effects on multiple targets contribute to the bactericidal activity (318, 319).

**Hexachlorophane.** Hexachlorophane (hexachlorophene; 2,2′-dihydroxy-3,5,6,3′,5′,6′-hexachlorodiphenylmethane) is another bis-phenol whose mode of action has been extensively studied. The primary action of hexachlorophane, based on studies with Bacillus megatherium, is to inhibit the membrane-bound part of the electron transport chain, and the other effects noted above are secondary ones that occur only at high concentrations (92, 158, 241, 481). It induces leakage, causes protoplast lysis, and inhibits respiration. The threshold concentration for the bactericidal activity of hexachlorophane is 10 μg/ml (dry weight), but peak leakage occurs at concentrations higher than 50 μg/ml and cytological changes occur above 30 μg/ml. Furthermore, hexachlorophane is bactericidal at 0°C despite causing little leakage at this temperature. Despite the broad-spectrum efficacy of hexachlorophane, concerns about toxicity (256), in particular in neonates, have meant that its use in antisep tic products has been limited.

**Halophenols**

Chloroxylenol (4-chloro-3,5-dimethylphenol; p-chloro-m-xylene) is the key halophenol used in antiseptic or disinfectant formulations (66). Chloroxylenol is bactericidal, but P. aeruginosa and many molds are highly resistant (66, 432). Surprisingly, its mechanism of action has been little studied despite its widespread use over many years. Because of its phenolic nature, it would be expected to have an effect on microbial membranes.

**Quaternary Ammonium Compounds**

Surface-active agents (surfactants) have two regions in their molecular structures, one a hydrocarbon, water-repellent (hydrophobic) group and the other a water-attracting (hydrophilic or polar) group. Depending on the basis of the charge or absence of ionization of the hydrophilic group, surfactants are classified into cationic, anionic, nonionic, and ampholytic (amphoteric) compounds. Of these, the cationic agents, as exemplified by quaternary ammonium compounds (QACs), are the most useful antiseptics and disinfectants (160). They are sometimes known as cationic detergents. QACs have been used for a variety of clinical purposes (e.g., preoperative disinfection of unbroken skin, application to mucous membranes, and disinfection of noncritical surfaces). In addition to having antimicrobial properties, QACs are also excellent for hard-surface cleaning and deodorization.

It has been known for many years that QACs are membrane-active agents (221) (Table 2) (i.e., with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane in yeasts) (215). Salton (460) proposed the following sequence of events with microorganisms exposed to cationic agents: (i) adsorption and penetration of the agent into the cell wall; (ii) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (iii) leakage of intracellular low-molecular-weight material; (iv) degradation of proteins and nucleic acids; and (v) wall lysis caused by autolytic enzymes. There is thus a loss of structural organization and integrity of the cytoplasmic membrane in bacteria, together with other damaging effects to the bacterial cell (120).

Useful information about the selectivity of membrane action can be obtained by studying the effects of biocides on proplasts and spheroplasts suspended in various solutes. QACs cause lysis of spheroplasts and protoplasts suspended in sucrose (107, 215, 243, 428). The cationic agents react with phospholipid components in the cytoplasmic membrane (69), thereby producing membrane distortion and protoplast lysis under osmotic stress. Isolated membranes do not undergo disaggregation on exposure to QACs, because the membrane distortion is not sufficiently drastic. The non-QAC agents TCC and trichlorosalicylanide have specific effects: TCC induces protoplast lysis in ammonium chloride by increasing CI− permeability, whereas trichlorosalicylanide induces lysis in ammonium nitrate by increasing NO3− permeability (428). In contrast, QACs (and chlorhexidine) induce lysis of protoplasts or spheroplasts suspended in various solutes because they effect generalized, rather than specific, membrane damage.

The bacterial cytoplasmic membrane provides the mechanism whereby metabolism is linked to solute transport, flagellar movement, and the generation of ATP. Protons are extruded to the exterior of the bacterial cell during metabolism. The combined potential (concentration or osmotic effect of the proton and its electropositivity) is the PMF, which drives these ancillary activities (428). The QAC cetrimide was found (121) to have an effect on the PMF in S. aureus. At its bacteriostatic concentration, cetrimide caused the discharge of the pH component of the PMF and also produced the maximum amount of 260-nm-absorbing material.
QACs are also believed to damage the outer membrane of gram-negative bacteria, thereby promoting their own uptake. This aspect of QACs is considered below (see “Intrinsic resistance of gram-negative bacteria”).

The QAC cetylpyridinium chloride (CPC) induces the leakage of K+ and pentose material from the yeast S. cerevisiae and induces protoplast lysis as well as interacting with crude cell sap (205). Unlike chlorhexidine, however, no biphasic effect on protoplast lysis was observed. The initial toxic effect of QACs on yeast cells is a disorganization of the plasma membranes, with organized lipid structures in the membranes (and in lipid bilayers) being disrupted.

QACs are sporostatic; they inhibit the outgrowth of spores (the development of a vegetative cell from a germinated spore) but not the actual germination processes (development from dormancy to a metabolically active state), albeit by an unknown mechanism (414). Likewise, the QACs are not mycobactericidal but have a mycobacteriostatic action, although the actual effects on mycobacteria have been little studied (419).

The QACs have an effect on lipid, enveloped (including human immunodeficiency virus and HBV) but not nonenveloped viruses (394, 485, 486). QAC-based products induced disintegration and morphological changes of human HBV, resulting in loss of infectivity (382). In studies with different phages (298–301, 303–305, 307), CPC significantly inhibited transduction by bacteriophage F116 and inactivated the phage particles. Furthermore, CPC altered the protein bands of F116 but did not affect the phage DNA within the capsid.

**Vapor-Phase Sterilants**

Many heat-sensitive medical devices and surgical supplies can be effectively sterilized by liquid sterilants (in particular glutaraldehyde, PAA, and hydrogen peroxide) or by vapor-phase sterilization systems (Table 1). The most widely used active agents in these “cold” systems are ethylene oxide, formaldehyde, and more recently developed, hydrogen peroxide and PAA. Ethylene oxide and formaldehyde are both broad-spectrum alkylating agents. However, their activity is dependent on active concentration, temperature, duration of exposure, and relative humidity (87). As alkylating agents, they attack proteins, nucleic acids, and other organic compounds; both are particularly reactive with sulfhydril and other enzyme-reactive groups. Ethylene oxide gas has the disadvantage of being mutagenic and explosive but is not generally harsh on sensitive equipment, and toxic residuals from the sterilization procedure can be routinely eliminated by correct aeration. Formaldehyde gas is similar and has the added advantage of being nonexplosive but is not widely used in health care. Vapor-phase hydrogen peroxide and PAA are considered more active (as oxidants) at lower concentrations than in the liquid form (334). Both active agents are used in combination with gas plasma in low-temperature sterilization systems (314). Their main advantages over other vapor-phase systems include low toxicity, rapid action, and activity at lower temperature; the disadvantages include limited penetrability and applications.

**MECHANISMS OF RESISTANCE**

**Introduction**

As stated above, different types of microorganisms vary in their response to antiseptics and disinfectants. This is hardly surprising in view of their different cellular structure, composition, and physiology. Traditionally, microbial susceptibility to antiseptics and disinfectants has been classified based on these differences; with recent work, this classification can be further extended (Fig. 1). Because different types of organisms react differently, it is convenient to consider bacteria, fungi, viruses, protozoa, and prions separately.

**Bacterial Resistance to Antiseptics and Disinfectants**

In recent years, considerable progress has been made in understanding more fully the responses of different types of bacteria (mycobacteria, nonsporulating bacteria, and bacterial spores) to antibacterial agents (43, 84, 414, 415, 419, 422, 496). As a result, resistance can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids (self-replicating, extrachromosomal DNA) or transposons (chromosomal or plasmid integrating, transmissible DNA cassettes). Intrinsic resistance is demonstrated by gram-negative bacteria, bacterial spores, mycobacteria, and, under certain conditions, staphylococci (Table 5). Acquired, plasmid-mediated resistance is most widely associated with mercury compounds and other metallic salts. In recent years, acquired resistance to certain other types of biocides has been observed, notably in staphylococci.

**Intrinsic Bacterial Resistance Mechanisms**

For an antiseptic or disinfectant molecule to reach its target site, the outer layers of a cell must be crossed. The nature and composition of these layers depend on the organism type and may act as a permeability barrier, in which there may be a reduced uptake (422, 428). Alternatively but less commonly, constitutively synthesized enzymes may bring about degradation of a compound (43, 214, 358). Intrinsic (innate) resistance...
is thus a natural, chromosomally controlled property of a bacterial cell that enables it to circumvent the action of an antiseptic or disinfectant. Gram-negative bacteria tend to be more resistant than gram-positive organisms, such as staphylococci septic or disinfectant. Gram-negative bacteria tend to be more resistant to antiseptics and disinfectants (43, 46, 150, 414, 418, 420, 422, 423, 457). Although Bacillus species are generally not pathogenic, their spores are widely used as indicators of efficient sterilization. Clostridium species are significant pathogens; for example, C. difficile is the most common cause of hospital-acquired diarrhea (478). Many biocides are bactericidal or bacteriostatic at low concentrations for nonsporulating bacteria, including the vegetative cells of Bacillus and Clostridium species, but high concentrations may be necessary to achieve a sporidical effect (e.g., for glutaraldehyde and CRAs). By contrast, even high concentrations of alcohol, phenolics, QACs, and chlorhexidine lack a sporidical effect, although this may be achieved when these compounds are used at elevated temperatures (475).

A typical spore has a complex structure (29, 151). In brief, the germ cell (protoplast or core) and germ cell wall are surrounded by the cortex, outside which are the inner and outer spore coats. A thin exosporium may be present in the spores of some species but may surround just one spore coat. RNA, DNA, and DPA, as well as most of the calcium, potassium, and phosphorus, are present in the spore protoplast. Also present are large amounts of low-molecular-weight basic proteins (small acid-soluble spore proteins [SASPs]), which are rapidly degraded during germination. The cortex consists largely of peptidoglycan, including a spore-specific muramic lactam. The spore coats comprise a major portion of the spore. These structures consist largely of protein, with an alkali-soluble fraction made up of acidic polypeptides being found in the inner coat and an alkali-resistant fraction associated with the presence of disulfide-rich bonds being found in the outer coat. These aspects, especially the roles of the coat(s) and cortex, are all relevant to the mechanism(s) of resistance presented by bacterial spores to antiseptics and disinfectants.

Several techniques are available for studying mechanisms of spor resistance (428). They include removing the spore coat and cortex by using a “step-down” technique to achieve a highly synchronous sporulation (so that cellular changes can be accurately monitored), employing spore mutants that do not sporulate beyond genetically determined stages in sporulation, adding an antiseptic or disinfectant at the commencement of sporulation and determining how far the process can proceed, and examining the role of SASPs. Such procedures have helped provide a considerable amount of useful information. Sporulation itself is a process in which a vegetative cell develops into a spore and involves seven stages (designated 0 to VII). During this process, the vegetative cell (stage 0) undergoes a series of morphological changes that culminate in the release of a mature spore (stage VII). Stages IV (cortex development) to VII are the most important in the development of resistance to biocides.

Resistance to antiseptics and disinfectants develops during sporulation and may be an early, intermediate, or (very) late event (103, 375, 378, 429, 474). Useful markers for monitoring the development of resistance are toluene (resistance to which is an early event), heat (intermediate), and lysozyme (late) (236, 237). Studies with a wild-type B. subtilis strain, 168, and its Spo2− mutants have helped determine the stages at which resistance develops (262, 375, 474). From these studies (Fig. 2), the order of development of resistance was toluene (resistance to which is an early event), heat (intermediate), and lysozyme (late) (236, 237). Studies with a wild-type B. subtilis strain, 168, and its Spo2− mutants have helped determine the stages at which resistance develops (262, 375, 474). From these studies (Fig. 2), the order of development of resistance was toluene (marker), formaldehyde, sodium lauryl sulfate, phenol, and phenylmercuric nitrate; m cresol, chlororesol, chlorhexidine gluconate, cetylpyridinium chloride, and mercuric chloride; and moist heat (marker), sodium dichloroisocyanurate, sodium hypochlorite, lysozyme (marker), and glutaraldehyde. The association of the onset of resistance to a particular antiseptic or disinfectant with a particular stage(s) in spore development is thereby demonstrated.

Spore coat-less forms, produced by treatment of spores un-
der alkaline conditions with urea plus dithiothreitol plus sodium lauryl sulfate (UDS), have also been of value in estimating the role of the coats in limiting the access of antiseptics and disinfectants to their target sites. However, Bloomfield and Arthur (44, 45) and Bloomfield (43) showed that this treatment also removes a certain amount of cortex and that the amount of cortex remaining can be further reduced by the subsequent use of lysozyme. These findings demonstrate that the spore coats have an undoubted role in conferring resistance but that the cortex also is an important barrier since (UDS plus lysozyme)-treated spores are much more sensitive to chlorine- and iodine-releasing agents than are UDS-exposed spores.

The initial development and maturity of the cortex are implicated in the development of resistance to phenolics. Likewise, it is now clear that cortex development is at least partially responsible for resistance to chlorhexidine and QACs; this resistance is enhanced in developing spores by the initiation of spore coat synthesis (262). The effect of various concentrations of chlorhexidine, sublethal to vegetative bacteria, on the development of spores of Bacillus subtilis 168 MB2 were investigated by Knott and Russell (261). They found that chlorhexidine affected spore development; as concentrations of the biguanide remaining in cortex increased, spore index values (the percentage of cells forming spores) decreased and sensitivity to both heat and toluene increased. By contrast, the control (untreated) culture was highly resistant to both of these agents and had a high spore index value, indicative of high levels of mature spores. The slightly increased resistance to toluene compared to resistance to heat was not surprising, since cells must reach stages V to VI (synthesis of spore coats and maturation) to attain heat resistance but only stage III (forespore engulfment) to attain toluene resistance (Fig. 2); in other words, if sporulation is inhibited by chlorhexidine, more cells are likely to reach stage III than the later stages. While less definitive than the earlier approaches, these procedures provide further evidence of the involvement of the cortex and coats in chlorhexidine resistance.

Development of resistance during sporulation to formaldehyde was an early event but depended to some extent on the concentration (1 to 5% [vol/vol]) of formaldehyde used. This appears to be at odds with the extremely late development of resistance to the dialdehyde, glutaraldehyde. Since glutaraldehyde and the monoa diehyde, formaldehyde, contain an aldehyde group(s) and are alkylating agents, it would be plausible to assume that they would have a similar mode of sporidal action, even though the dialdehyde is a more powerful alkylating agent. If this were true, it could also be assumed that spores would exhibit the same resistance mechanisms for these disinfectants. In aqueous solution, formaldehyde forms a glycol in equilibrium (512, 524); thus, formaldehyde could well be acting poorly as an alcohol-type disinfectant rather than as an aldehyde (327). Alkaline glutaraldehyde does not readily form glycols in aqueous solution (178). Resistance to formaldehyde may be linked to cortex maturation, and resistance to glutaraldehyde may be linked to coat formation (262).

Setlow and his coworkers (472) demonstrated that α/β-type SASPs coat the DNA in wild-type spores of B. subtilis, thereby protecting it from attack by enzymes and antimicrobial agents. Spores (α/β- lacking these α/β-type SASPs are significantly more sensitive to hydrogen peroxide (471) and hypochlorite (456). Thus, SASPs contribute to spore resistance to peroxide and hypochlorite but may not be the only factors involved, since the coats and cortex also play a role (428).

Two other aspects of spores should be considered: the revival of injured spores and the effects of antiseptics and disinfectants on germinating and outgrowing spores. Although neither aspect is truly a resistance mechanism, each can provide useful information about the site and mechanism of action of sporidical agents and about the associated spore resistance mechanisms and might be of clinical importance.

The revival of disinfectant-treated spores has not been extensively studied. Spicher and Peters (483, 484) demonstrated that formaldehyde-exposed spores of B. subtilis could be revived after a subsequent heat shock process. A more recent finding with B. stearothermophilus casts further doubt on the efficacy of low-temperature steam with formaldehyde as a sterilizing procedure (541). The revival of spores exposed to glutaraldehyde, formaldehyde, chlorine, and iodine was examined by Russell and his colleagues (103, 376, 377, 424, 532–537). A small proportion of glutaraldehyde-treated spores of various Bacillus species were revived when the spores were treated with alkali after neutralization of glutaraldehyde with glycine (103, 379, 380). Experiments designed to distinguish between germination and outgrowth in the revival process have demonstrated that sodium hydroxide-induced revival increases the potential for germination. Based on other findings, the germination process is also implicated in the revival of spores exposed to other disinfectants.

Intrinsic resistance of mycobacteria. Mycobacteria are well known to possess a resistance to antiseptics and disinfectants that is roughly intermediate between those of other nonsporulating bacteria and bacterial spores (Fig. 1) (177, 345, 419). There is no evidence that enzymatic degradation of harmful molecules takes place. The most likely mechanism for the high resistance of mycobacteria is associated with their complex cell walls that provide an effective barrier to the entry of these agents. To date, plasmid- or transposon-mediated resistance to biocides has not been demonstrated in mycobacteria.

The mycobacterial cell wall is a highly hydrophobic structure with a mycolarabinogalactan-peptidoglycan skeleton (27, 105, 106, 322, 389, 390, 461, 530). The peptidoglycan is covalently linked to the polysaccharide copolymer (arabinogalactan) made up of arabinose and galactose esterified to mycolic acids. Also present are complex lipids, lipopolysaccharides (LPSs), and proteins, including porin channels through which hydrophilic molecules can diffuse into the cell (232, 556). Similar cell wall structures exist in all the mycobacterial species examined to date (228). The cell wall composition of a particular species may be influenced by its environmental niche (27). Pathogenic bacteria such as Mycobacterium tuberculosis exist in a relatively nutrient-rich environment, whereas saprophytic mycobacteria living in soil or water are exposed to natural antibiotics and tend to be more intrinsically resistant to these drugs.
Antiseptics or disinfectants that exhibit mycobacterial activity are phenol, PAA, hydrogen peroxide, alcohol, and glutaraldehyde (16, 17, 99, 419, 425, 455). By contrast, other well-known bactericidal agents, such as chlorhexidine and QACs, are mycobacteriostatic even when used at high concentrations (51, 52, 419, 425, 455). However, the activity of these can be substantially increased by formulation effects. Thus, a number of QAC-based products claim to have mycobacterial activity. For example, a newer formulation (Sactimed-I-Sinald) containing a mixture of alkyl polyguanides and alkyl QACs is claimed to be mycobactericidal (211, 353). However, there is some doubt whether the antibacterial agents had been properly quenched or neutralized to prevent carryover of inhibitory concentrations into recovery media.

Many years ago, it was proposed (T. H. Shen, cited in reference 99) that the resistance of mycobacteria to QACs was related to the lipid content of the cell wall. In support of this contention, Mycobacterium phlei, which has a low total cell lipid content, was more sensitive than M. tuberculosis, which has a higher lipid content. It was also noted that the resistance of various species of mycobacteria was related to the content of waxy material in the wall. It is now known that because of the highly hydrophobic nature of the cell wall, hydrophobic biocides are generally unable to penetrate the mycobacterial cell wall in sufficiently high concentrations to produce a lethal effect. However, low concentrations of antiseptics and disinfectants such as chlorhexidine must presumably traverse this permeability barrier, because the MICs are of the same order as those concentrations inhibiting the growth of nonmycobacterial strains such as S. aureus, although M. avium-intracellulare may be particularly resistant (51, 52). The component(s) of the mycobacterial cell wall responsible for the high biocide resistance are currently unknown, although some information is available. Inhibitors of cell wall synthesis increase the susceptibility of M. avium to drugs (391); inhibition of mycocide C, arabinogalactan, and mycic acid biosynthesis enhances drug susceptibility. Treatment of this organism with m-fluoro-dl-phenylalanine (m-FL-phen), which inhibits mycicide C synthesis, produces significant alterations in the outer cell wall layers (106). Ethambutol, an inhibitor of arabinogalactan (391; 501) and phospholipid (461, 462) synthesis, also disorganizes these layers. In addition, ethambutol induces the formation of ghosts without the dissolution of peptidoglycan (391). Methyl-4-(2-octadecyloctylcyclopropen-1-yl) butanoate (MOCB) is a structural analogue of a key precursor in mycic acid synthesis. Thus, the effects of MOCB on mycic acid synthesis and m-FL-phen and ethambutol on outer wall biosynthetic processes leading to changes in cell wall architecture appear to be responsible for increasing the intracellular concentration of chemotherapeutic drugs. These findings support the concept of the cell wall acting as a permeability barrier to these drugs (425). Fewer studies have been made of the mechanisms involved in the resistance of mycobacteria to antiseptics and disinfectants. However, the activity of chlorhexidine and of a QAC, cetlypyridinum chloride, against M. avium and M. tuberculosis can be potentiated in the presence of ethambutol (52). From these data, it may be inferred that arabinogalactan is one cell wall component that acts as a permeability barrier to chlorhexidine and QACs. It is not possible, at present, to comment on other components, since these have yet to be investigated. It would be useful to have information about the uptake into the cells of these antiseptic agents in the presence and absence of different cell wall synthesis inhibitors.

One species of mycobacteria currently causing concern is M. chelonae, since these organisms are sometimes isolated from endoscope washes and dialysis water. One such strain was not killed even after a 60-min exposure to alkaline glutaraldehyde; in contrast, a reference strain showed a 5-log-unit reduction after a contact time of 10 min (519). This glutaraldehyde-resistant M. chelonae strain demonstrated an increased tolerance to PAA but not to NaDCC or to a phenolic. Other workers have also observed an above-average resistance of M. chelonae to glutaraldehyde and formaldehyde (72) but not to PAA (187, 294). The reasons for this high glutaraldehyde resistance are unknown. However, M. chelonae is known to adhere strongly to smooth surfaces, which may render cells within a biofilm less susceptible to disinfectants. There is no evidence to date that uptake of glutaraldehyde by M. chelonae is reduced.

Intrinsic resistance of other gram-positive bacteria. The cell wall of staphylococci is composed essentially of peptidoglycan and teichoic acid. Neither of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high-molecular-weight substances can readily traverse the cell wall of staphylococci and vegetative Bacillus spp., this may explain the sensitivity of these organisms to many antibacterial agents including QACs and chlorhexidine (411, 417, 422, 428, 451). However, the plasticity of the bacterial cell envelope is a well-known phenomenon (381). Growth rate and any growth-limiting nutrient will affect the physiological state of the cells. Under such circumstances, the thickness and degree of cross-linking of peptidoglycan are likely to be modified and hence the cellular sensitivity to antiseptics and disinfectants will be altered. For example, Gilbert and Brown (171) demonstrated that the sensitivity of Bacillus megaterium cells to chlorhexidine and 2-phenoxethanol is altered when changes in growth rate and nutrient limitation are made with chemostat-grown cells. However, lysozyme-induced protoplasts of these cells remained sensitive to, and were lysed by, these membrane-active agents. Therefore, the cell wall in whole cells is responsible for their modified response.

In nature, S. aureus may exist as mucoid strains, with the cells surrounded by a slime layer. Nonmucoid strains are killed more rapidly than mucoid strains by chloroxylenol, cetrimide, and chlorhexidine, but there is little difference in killing by phenols or chlorinated phenols (263); removal of slime by washing rendered the cells sensitive. Therefore, the slime plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules.

There is no evidence to date that vancomycin-resistant enterococci or enterococci with high-level resistance to aminoglycoside antibiotics are more resistant to disinfectants than are antibiotic-sensitive enterococcal strains (9, 11, 48, 319). However, enterococci are generally less sensitive to biocides than are staphylococci, and differences in inhibitory and bacterial concentrations have also been found among enterococcal species (257).

Intrinsic resistance of gram-negative bacteria. Gram-negative bacteria are generally more resistant to antiseptics and disinfectants than are nepromulating, nonmycobacterial gram-positive bacteria (Fig. 2) (428, 440, 441). Examples of MICs against gram-positive and -negative organisms are provided in Table 6. Based on these data, there is a marked difference in the sensitivity of S. aureus and E. coli to QACs (benzalkonium, benzethonium, and cetrimide), hexachlorophene, diamidines, and triclosan but little difference in chlorhexidine susceptibility. P. aeruginosa is considerably more resistant to most of these agents, including chlorhexidine, and (not shown) Proteus spp. possess an above-average resistance to cationic agents such as chlorhexidine and QACs (311, 440).

The outer membrane of gram-negative bacteria acts as a barrier that limits the entry of many chemically unrelated types
of antibacterial agents (18, 169, 196, 197, 355, 366, 440, 516, 517). This conclusion is based on the relative sensitivities of staphylococci and gram-negative bacteria and also on studies with outer membrane mutants of *E. coli*, *S. typhimurium*, and *P. aeruginosa* (134, 135, 433–435, 438). Smooth, wild-type bacteria have a hydrophobic cell surface; by contrast, because of the phospholipid patches on the cell surface, deep rough (heptose-less) mutants are hydrophobic. These mutants tend to be hypersensitive to hydrophobic antibiotics and disinfectants. Low-molecular-weight (MW < ca. 600) hydrophilic molecules readily pass via the porins into gram-negative cells, but hydrophobic molecules diffuse across the outer membrane bilayer (Table 7). In wild-type gram-negative bacteria, intact LPS molecules prevent ready access of hydrophobic molecules to phospholipid and thence to the cell interior. In deep rough strains, which lack the O-specific side chain and most of the core polysaccharide, the phospholipid patches at the cell surface have their head groups oriented toward the exterior.

In addition to these hydrophilic and hydrophobic entry pathways, a third pathway has been proposed for cationic agents such as QACs, biguanidies, and diamidines. It is claimed that these damage the outer membrane, thereby promoting their own uptake (197). Polycations disorganize the outer membrane of *E. coli* (520). It must be added, however, that the QACs and diamidines are considerably less active against wild-type strains than against deep rough strains whereas chlorhexidine has the same order of activity (MIC increase about 2 to 3 fold) against both types of *E. coli* strains (434, 435, 439). However, *S. typhimurium* mutants are more sensitive to chlorhexidine than are wild-type strains (433).

Gram-negative bacteria that show a high level of resistance to many antiseptics and disinfectants include *P. aeruginosa*, *Burkholderia cepacia*, *Proteus* spp., and *Providencia stuartii* (428, 440). The outer membrane of *P. aeruginosa* is responsible for its high resistance; in comparison with other organisms, there are differences in LPS composition and in the cation content of the outer membrane (54). The high Mg$^{2+}$ content aids in producing strong LPS-LPS links; furthermore, because of their small size, the porins may not permit general diffusion through them. *B. cepacia* is often considerably more resistant in the hospital environment than in artificial culture media (360); the high content of phosphate-linked arabinose in its LPS decreases the affinity of the outer membrane for polymyxin antibiotics and other cationic and polycationic molecules (97, 516). *Pseudomonas stutzeri*, by contrast, is highly sensitive to many antibiotics and disinfectants (449), which implies that such agents have little difficulty in crossing the outer layers of the cells of this organism.

Members of the genus *Proteus* are invariably insensitive to chlorhexidine (311). Some strains that are highly resistant to chlorhexidine, QACs, EDTA, and diamidines have been isolated from clinical sources. The presence of a less acidic type of outer membrane LPS could be a contributing factor to this intrinsic resistance (97, 516).

A particularly troublesome member of the genus *Providencia* is *P. stuartii*. Like *Proteus* spp., *P. stuartii* strains have been isolated from urinary tract infections in paraplegic patients and are resistant to different types of antiseptics and disinfectants including chlorhexidine and QACs (492, 496). Strains of *P. stuartii* that showed low-, intermediate-, and high-level resistance to chlorhexidine formed the basis of a series of studies of the resistance mechanism(s) (86, 422, 428). Gross differences in the composition of the outer layers of these strains were not detected, and it was concluded that (i) subtle changes in the structural arrangement of the cell envelopes of these strains was associated with this resistance and (ii) the inner membrane was not implicated (230).

Few authors have considered peptidoglycan in gram-negative bacteria as being a potential barrier to the entry of inhibitory substances. The peptidoglycan content of these organisms is much lower than in staphylococci, which are inherently more sensitive to many antiseptics and disinfectants. Nevertheless, there have been instances (discussed in reference 422) where gram-negative organisms grown in subinhibitory concentrations of a penicillin have deficient permeability barriers. Furthermore, it has been known for many years (215, 409, 411) that penicillin-induced spheroplasts and lysozyme-EDTA-Tris “protoplasts” of gram-negative bacteria are rapidly lysed by membrane-active agents such as chlorhexidine. It is conceivable that the stretched nature of both the outer and inner membranes in β-lactam-treated organisms could contribute to this increased susceptibility.

The possibility exists that the cytoplasmic (inner) membrane provides one mechanism of intrinsic resistance. This membrane is composed of lipoprotein and would be expected to prevent passive diffusion of hydrophilic molecules. It is also known that changes in membrane composition affect sensitivity to ethanol (159). Lannigan and Bryan (275) proposed that decreased susceptibility of *Serratia marcescens* to chlorhexidine was linked to the inner membrane, but Ismaeel et al. (230) could find no such role with chlorhexidine-resistant *P. stuartii*. At present, there is little evidence to implicate the inner membrane in biocide resistance. In addition, chlorhexidine degradation was reported for *S. marcescens*, *P. aeruginosa*, and *Achromobacter/Alcaligenes xylosoxidans* (358).

**Physiological (phenotypic) adaption as an intrinsic mechanism.** The association of microorganisms with solid surfaces leads to the generation of a biofilm, defined as a consortium of organisms organized within an extensive exopolysaccharide exopolymer (93, 94). Biofilms can consist of monocultures, of several diverse species, or of mixed phenotypes of a given species (57, 73, 381). Some excellent publications that deal with the nature, formation, and content of biofilms are available (125, 178, 276, 538). Biofilms are important for several reasons,

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### TABLE 7. Possible transport of some antiseptics and disinfectants into gram-negative bacteria*  

<table>
<thead>
<tr>
<th>Antiseptic/disinfector</th>
<th>Passage across OM*</th>
<th>Passage across IM*</th>
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<tbody>
<tr>
<td>Chlorhexidine</td>
<td>Self-promoted uptake(?)</td>
<td>IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs</td>
</tr>
<tr>
<td>QACs</td>
<td>Self-promoted uptake(?)</td>
<td>IM is a major target site; damage to IM enables biocide to enter cytosol, where further interaction occurs</td>
</tr>
<tr>
<td>Phenolics</td>
<td>Hydrophobic pathway (activity increases as hydrophobicity of phenolic increases)</td>
<td>IM is a major target site, but high phenolic concentrations coagulate cytoplasmic constituents</td>
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</table>

* Data from references 197, 433 to 435, 438, and 439.

a OM, outer membrane; IM, inner membrane.
notably biocorrosion, reduced water quality, and foci for contamination of hygienic products (10, 12–14). Colonization also occurs on implanted biomaterials and medical devices, resulting in increased infection rates and possible recurrence of infection (125).

Bacteria in different parts of a biofilm experience different nutrient environments, and their physiological properties are affected (57). Within the depths of a biofilm, for example, nutrient limitation is likely to reduce growth rates, which can affect susceptibility to antimicrobial agents (98, 142, 171, 172). Thus, the phenotypes of sessile organisms within biofilms differ considerably from the planktonic cells found in laboratory cultures (73). Slow-growing bacteria are particularly insusceptible, a point reiterated recently in another context (126).

Several reasons can account for the reduced sensitivity of bacteria within a biofilm (Table 8). There may be (i) reduced access of a disinfectant (or antibiotic) to the cells within the biofilm, (ii) chemical interaction between the disinfectant and the biofilm itself, (iii) modulation of the microenvironment, (iv) production of degradative enzymes (and neutralizing chemicals), or (v) genetic exchange between cells in a biofilm. However, bacteria removed from a biofilm and recultured in culture media are generally no more resistant than the “ordinary” planktonic cells of that species (57).

Several instances are known of the contamination of antimicrobial solutions by bacteria. For example, Marrie and Costerton (310) described the prolonged survival of S. marcescens in 2% chlorhexidine solutions, which was attributed to the embedding of these organisms in a thick matrix that adhered to the walls of a storage container. Similar conclusions were reached by Hugo et al. (225) concerning the survival of B. cepacia in chlorhexidine and by Anderson et al. (10, 12–14) concerning the contamination of iodophor antisepsics with Pseudomonas. In the studies by Anderson et al., Pseudomonas biofilms were found on the interior surfaces of polyvinyl chloride pipes used during the manufacture of providone-iodine antisepsics. It is to be wondered whether a similar reason could be put forward for the contamination by S. marcescens of a benzalkonium chloride solution implicated in meningitis (468). Recently, a novel strategy was described (540) for controlling biofilms through generation of hydrogen peroxide at the biofilm-surface interface rather than simply applying a disinfectant extrinsically. In this procedure, the colonized surface incorporated a catalyst that generated the active compound from a treatment agent.

Gram-negative pathogens can grow as biofilms in the catheterized bladder and are able to survive concentrations of chlorhexidine that are effective against organisms in noncatheterized individuals (493, 494). Interestingly, the permeability agent EDTA has only a temporary potentiating effect in the catheterized bladder, with bacterial growth subsequently recurring (495). B. cepacia freshly isolated from the hospital environment is often considerably more resistant to chlorhexidine than when grown in artificial culture media, and a glycolcalyx may be associated with intrinsic resistance to the bisbiguanide (360). Legionella pneumophila is often found in hospital water distribution systems and cooling towers. Chlorination in combination with continuous heating (60°C) of incoming water is usually the most important disinfection measure; however, because of biofilm production, contaminating organisms may be less susceptible to this treatment (140). Increased resistance to chlorine has been reported for Vibrio cholerae, which expresses an amorphous exopolysaccharide causing cell aggregation (“rugose” morphology [336]) without any loss in pathogenicity.

One can reach certain conclusions about biofilms. The interaction of bacteria with surfaces is usually reversible and eventually irreversible. Irreversible adhesion is initiated by the binding of bacteria to the surface through exopolysaccharide glycolcalyx polymers. Sister cells then arise by cell division and are bound within the glycolcalyx matrix. The development of adherent microcolonies is thereby initiated, so that eventually a continuous biofilm is produced on the colonized surface. Bacteria within these biofilms reside in specific microenvironments that differ from those of cells grown under normal laboratory conditions and thus show variations in their response to antisepsics and disinfectants.

Recent nosocomial outbreaks due to M. chelonae (discussed under “Intrinsic resistance of mycobacteria”), M. tuberculosis (4, 323) and HCV (53) underscore the importance of pseudobiofilm formation in flexible fiberoptic scope contamination. These outbreaks were associated with inadequate cleaning of scopes, which compromised subsequent sterilization with glutaraldehyde. While these organisms do not form a true biofilm, the cross-linking action of glutaraldehyde can cause a buildup of insoluble residues and associated microorganisms on scopes and in automated reprocssors.

Biofilms provide the most important example of how physiological (phenotypic) adaptation can play a role in conferring intrinsic resistance (57). Other examples are also known. For example, fattened cells of S. aureus produced by repeated subculturing in glycerol-containing media are more resistant to alkyl phenols and benzylpenicillin than are wild-type strains (220). Subculture of these cells in routine culture media resulted in reversion to sensitivity (218). Planktonic cultures grown under conditions of nutrient limitation or reduced growth rates have cells with altered sensitivity to disinfectants, probably as a consequence of modifications in their outer membranes (56, 59, 98). In addition, many aerobic microorganisms have developed intrinsic defense systems that confer tolerance to peroxide stress (in particular H₂O₂) in vivo. The so-called oxidative-stress or SOS response has been well studied in E. coli and Salmonella and includes the production of neutralizing enzymes to prevent cellular damage (including peroxidases, catalases, glutathione reductase) and to repair DNA lesions (e.g., exonuclease III) (112, 114, 497). In both organisms, increased tolerance can be obtained by pretreatment with a subinhibitory dose of hydrogen peroxide (113, 539). Pretreatment induces a series of proteins, many of which are under the positive control of a sensor/regulator protein (OxyR), including catalase and glutathione reductase (497).

<table>
<thead>
<tr>
<th>TABLE 8. Biofilms and microbial response to antimicrobial agents</th>
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<tr>
<td>Mechanism of resistance associated with biofilms</td>
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<tr>
<td>Exclusion or reduced access of antiseptic or disinfectant to underlying cell</td>
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<tr>
<td>Modulation of microenvironment</td>
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<tr>
<td>Increased production of degradative enzymes by attached cells</td>
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<tr>
<td>Plasmid transfer between cells in biofilm</td>
</tr>
</tbody>
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and further nonessential proteins that accumulate to protect the cell (338). Cross-resistance to heat, ethanol, and hypochlo-
rous acid has also been reported (81, 128, 335). The oxidative
stress response in gram-positive bacteria is less well studied,
but Bacillus tolerance to H2O2 has been described to vary dur-
ing the growth phase (127) and in mutant strains (67, 200).
Similar inducible defense mechanisms were described for
Campylobacter jejuni (185), Deinococcus (528), and Haemophi-
lus influenzae (36). However, the level of increased tolerance to
H2O2 during the oxidative stress response may not afford sig-
ificant protection to concentrations used in antiseptics and
disinfectants (generally >3%). For example, B. subtilis mutants
have been described to be more resistant at ~0.5% H2O2 than
are wild-type strains at ~0.34% H2O2 (200).

Acquired Bacterial Resistance Mechanisms

As with antibiotics and other chemotherapeutic drugs, ac-
quired resistance to antiseptics and disinfectants can arise by
either mutation or the acquisition of genetic material in the
form of plasmids or transposons. It is important to note that
“resistance” as a term can often be used loosely and in many
cases must be interpreted with some prudence. This is partic-

TABLE 9. Possible mechanisms of plasmid-encoded resistance to antiseptics and disinfectants

<table>
<thead>
<tr>
<th>Chemical agent</th>
<th>Examples</th>
<th>Mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiseptics or disinfectants</td>
<td>Chlorhexidine salts</td>
<td>(i) Inactivation: not yet found to be plasmid mediated; chromosomally mediated inactivation; (ii) Efflux: some S. aureus, some S. epidermidis; (iii) Decreased uptake?</td>
</tr>
<tr>
<td></td>
<td>QACs</td>
<td>(i) Efflux: some S. aureus, some S. epidermidis; (ii) Decreased uptake?</td>
</tr>
</tbody>
</table>
|                        | Silver compounds              | (i) Inactivation by formaldehyde dehydrogenase; (ii) Cell surface alterations (outer mem-
|                        | Formaldehyde                  | brane proteins)                                                             |
|                        | Acridinesa                    | Efflux: some S. aureus, some S. epidermidis                                 |
|                        | Diamidines                    | Efflux: some S. aureus, some S. epidermidis                                 |
|                        | Crystal violet*               | Efflux: some S. aureus, some S. epidermidis                                 |
| Other biocides         | Mercurialsb                   | Inactivation (reductases, lyses)                                            |
|                        | Ethidium bromide              | Efflux: some S. aureus, some S. epidermidis                                 |

a Now rarely used for antiseptic or disinfectant purposes.
b Organomercurials are still used as preservatives.

Plasmids and bacterial resistance to antiseptics and disin-
fecteds. Chopra (82, 83) examined the role of plasmids in en-
coding resistance (or increased tolerance) to antiseptics and
disinfectants; this topic was considered further by Russell (413). It
was concluded that apart from certain specific examples such as
silver, other metals, and organomercurials, plasmids were
not normally responsible for the elevated levels of antiseptic or
disinfectant resistance associated with certain species or strains.
Since then, however, there have been numerous reports linking
the presence of plasmids in bacteria with increased tolerance
to chlorhexidine, QACs, and triclosan, as well as to diamidines,
acridines and ethidium bromide, and the topic was reconsider-
ed (83, 423, 427) (Table 9).

Plasmid-encoded resistance to antiseptics and disinfectants
had at one time been most extensively investigated with mer-
curials (both inorganic and organic), silver compounds, and
other cations and anions. Mercurials are no longer used as
disinfectants, but phenylmercuric salts and thiomersal are still
used as preservatives in some types of pharmaceutical products
(226). Resistance to mercury is plasmid borne, inducible, and
may be transferred by conjugation or transduction. Inorganic
mercury (Hg2+) and organomercury resistance is a common
property of clinical isolates of S. aureus containing penicillinase
plasmids (110). Plasmids conferring resistance to mercurials
are either narrow spectrum, specifying resistance to Hg2+ and
to some organomercurials, or broad-spectrum, with resistance
to the above compounds and to other organomercurials (331).
Silver salts are still used as topical antimicrobial agents
(50, 443). Plasmid-encoded resistance to silver has been found in
Pseudomonas stutzeri (192), members of the Enterobacteri-
aceae (479, 480, 511), and Citrobacter spp. (511). The mecha-

(i) Plasmid-mediated antiseptic and disinfectant resistance
in gram-negative bacteria. Occasional reports have examined
the possible role of plasmids in the resistance of gram-negative
bacteria to antiseptics and disinfectants. Sutton and Jacoby
(498) observed that plasmid RP1 did not significantly alter the
resistance of P. aeruginosa to QACs, chlorhexidine, iodine, or
chlorinated phenols, although increased resistance to hexa-
chlorophene was observed. This compound has a much greater
effect on gram-positive than gram-negative bacteria, so that it
is difficult to assess the significance of this finding. Transfor-
mation of this plasmid (which encodes resistance to carbeni-
cillin, tetracycline, and neomycin and kanamycin) into E. coli
or P. aeruginosa did not increase the sensitivity of these orga-

Strains of Providencia stuartii may be highly tolerant to Hg2+,
cationic disinfectants (such as chlorhexidine and QACs), and
antibiotics (496). No evidence has been presented to show that
there is a plasmid-linked association between antibiotic resis-
tance and disinfectant resistance in these organisms, pseudo-

onads, or Proteus spp. (492). High levels of disinfectant resis-
tance have been reported in other hospital isolates (195),
although no clear-cut role for plasmid-specified resistance has
emerged (102, 250, 348, 373, 518). High levels of tolerance to
chlorhexidine and QACs (311) may be intrinsic or may have
resulted from mutation. It has been proposed (492, 496) that
the extensive usage of these cationic agents could be respon-
sible for the selection of antiseptic-disinfectant-, and antibiot-
ic-resistant strains; however, there is little evidence to support
this conclusion. All of these studies demonstrated that it was dif-
ficult to transfer chlorhexidine or QAC resistance under nor-
Methicillin-resistant staphylococci. Methicillin-resistant S. aureus (MRSA) strains are a major cause of sepsis in hospitals throughout the world, although not all strains have increased virulence. Many can be referred to as “epidemic” MRSA because of the ease with which they can spread (91, 295, 317). Patients at particularly high risk are those who are debilitated or immunocompromised or who have open sores.

It has been known for several years that some antibiotics and disinfectants are, on the basis of MICs, somewhat less inhibitory to S. aureus strains that contain a plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamicin (Table 10). These biocidal agents include chlorhexidine, diamidines, and QACs, together with ethidium bromide and acridines (8, 238, 289, 423, 427, 463). According to Mycock (346), MRSA strains showed a remarkable increase in tolerance (at least 5,000-fold) to povidone-iodine. However, there was no decrease in susceptibility of antibiotic-resistant strains to phenolics (phenol, cresol, and chlorocresol) or to the preservatives known as parabens (8).

Tennent et al. (505) proposed that increased resistances to cetyltrimethylammonium bromide (CTAB) and to propamidine isethionate were linked and that these cationic agents may be acting as a selective pressure for the retention of plasmids encoding resistance to them. The potential clinical significance of this finding remains to be determined.

Staphylococci are the only bacteria in which the genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been described (466). In S. aureus, these mechanisms are encoded by at least three separate multidrug resistance determinants (Tables 10 and 11). Increased antibiotic MICs have been reported to be widespread among MRSA strains and to be specified by two gene families (qacAB and qacCD) of determinants (188, 280, 281, 289, 289, 363–365, 367, 506). The qacAB family of genes (Table 11) encodes proton-dependent export proteins that develop significant homology to other energy-dependent transporters such as the tetracycline transporters found in various strains of tetracycline-resistant bacteria (405). The qacA gene is present predominantly on the pSK1 family of multiresistance plasmids but is also likely to be present on the chromosome of clinical S. aureus strains as an integrated family plasmid or part thereof. The qacB gene is detected on large heavy-metal resistance plasmids. The qacC and qacD genes encode identical phenotypes and show restriction site homology; the qacC gene may have evolved from qacD (288).

Interesting studies by Reverdy et al. (395, 396), Dussau et al. (129) and Behr et al. (31) demonstrated a relationship between increased S. aureus MICs to the β-lactam oxacillin and four antiseptics (chlorhexidine, benzalkonium chloride, hexamine, and acriflavine). A gene encoding multidrug resistance was not found in susceptible strains but was present in 70% of S. aureus strains for which the MICs of all four of these antiseptics were increased and in 45% of the remaining strains resistant to at least one of these antiseptics (31). Genes encoding increased QAC tolerance may be widespread in food-associated staphylococcal species (203). Some 40% of isolates of coagulase-negative staphylococci (CNS) contain both qacA and qacC genes, with a possible selective advantage in possessing both as opposed to qacA only (281). Furthermore, there is growing evidence that S. aureus and CNS have a common pool of resistance determinants.

Triclosan is used in surgical scrubs, soaps, and deodorants. It is active against staphylococci and less active against most gram-negative organisms, especially P. aeruginosa, probably by virtue of a permeability barrier (428). Low-level transferable resistance to triclosan was reported in MRSA strains (88, 90); however, no further work on these organisms has been described. According to Sasatsu et al. (465), the MICs of triclosan against sensitive and resistant S. aureus strains were 100 and

### TABLE 10. qac genes and susceptibility of S. aureus strains to some antiseptics and disinfectants

<table>
<thead>
<tr>
<th>qac gene</th>
<th>MIC ratios of:</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>qacA</td>
<td>&gt;16 2.5 &gt;16 &gt;16 4 &gt;3 &gt;4 2</td>
<td>sensitive</td>
</tr>
<tr>
<td>qacB</td>
<td>8 1 &gt;4 2 &gt;3 &gt;2 2</td>
<td>sensitive</td>
</tr>
<tr>
<td>qacC</td>
<td>1 1 ca.1 1 6 &gt;3 &gt;4 1</td>
<td>sensitive</td>
</tr>
<tr>
<td>qacD</td>
<td>1 1 ca.1 1 6 &gt;3 &gt;4 1</td>
<td>sensitive</td>
</tr>
</tbody>
</table>

a qac genes are otherwise known as nucleic acid binding (NAB) compound resistance genes (88).

b Calculated from the data in reference 289. Ratios are MICs for strains of S. aureus carrying various qac genes divided by the MIC for a strain carrying no gene (the actual MIC for the test strain is shown in the bottom row).

### TABLE 11. qac genes and resistance to quaternary ammonium compounds and other antiseptics and disinfectants

<table>
<thead>
<tr>
<th>Multidrug resistance determinant</th>
<th>Gene location</th>
<th>Resistance encoded to</th>
</tr>
</thead>
<tbody>
<tr>
<td>qacA</td>
<td>pSK1 family of multiresistant plasmids, also β-lactamase and heavy-metal resistance families</td>
<td>QACs, chlorhexidine salts, diamidines, acridines, ethidium bromide</td>
</tr>
<tr>
<td>qacB</td>
<td>β-Lactamase and heavy-metal resistance plasmids</td>
<td>QACs, acridines, ethidium bromide</td>
</tr>
<tr>
<td>qacC</td>
<td>Small plasmids (&lt;3 kb) or large conjugative plasmids</td>
<td>Some QACs, ethidium bromide</td>
</tr>
<tr>
<td>qacD</td>
<td>Large (50-kb) conjugative, multiresistance plasmids</td>
<td>Some QACs, ethidium bromide</td>
</tr>
</tbody>
</table>

a The qacK gene has also been described, but it is likely to be less significant than qacAB in terms of antiseptic or disinfectant tolerance.

b These genes have identical target sites and show restriction site homology.
There was an increase in the copy number of the ebr (qacCD) gene whose normal function was to remove toxic substances from normal cells of staphylococci and enterococci. Based on DNA homology, it was proposed that qacA and related genes carrying resistance determinants evolved from preexisting genes responsible for normal cellular transport systems (405) and that the antibiotic resistance genes evolved before the introduction and use of topical antimicrobial products and other antibiotics and disinfectants (288, 289, 365, 367, 368, 405).

Baquero et al. (23) have pointed out that for antibiotics, the presence of a specific resistance mechanism frequently contributes to the long-term selection of resistant variants under in vivo conditions. Whether low-level resistance to cationic antibiotics, e.g., chlorhexidine, QACs, can likewise provide a selective advantage on staphylococci carrying qac genes remains to be elucidated. The evidence is currently contentious and inconclusive.

(iii) Plasmid-mediated antibiotic and disinfectant resistance in other gram-positive bacteria. Antibiotic-resistant corynebacteria may be implicated in human infections, especially in the immunocompromised. ‘Group JK’ coryneforms (Corynebacterium jeikeium) were found to be more tolerant than other coryneforms to the cationic disinfectants ethidium bromide and hexachlorophene, but studies with plasmid-containing and plasmid-cured derivatives produced no evidence of plasmid-associated resistance (285). Enterococcus faecium strains showing high level resistance to vancomycin, gentamicin, or both are not more resistant to chlorhexidine or other nonantibiotic agents (9, 11, 20, 319). Furthermore, despite the extensive dental use of chlorhexidine, strains of Streptococcus mutants remain sensitive to it (235). To date, therefore, there is little or no evidence of plasmid-associated resistance of nonstaphylococcal gram-positive bacteria to antibiotics and disinfectants.

Mutational resistance to antibiotics and disinfectants. Chromosomal mutation to antibiotics has been recognized for decades. By contrast, fewer studies have been performed to determine whether mutation confers resistance to antibiotics and disinfectants. It was, however, demonstrated over 40 years ago (77, 78) that S. marcescens, normally inhibited by QACs at <100 μg/ml, could adapt to grow in 100,000 μg of a QAC per ml. The resistant and sensitive cells had different surface characteristics (electrophoretic mobilities), but resistance could be lost when the cells were grown on QAC-free media. One problem associated with QACs and chlorhexidine is the turbidity produced in liquid culture media above a certain concentration (interaction with agar also occurs), which could undoubtedly interfere with the determination of growth. This observation is reinforced by the findings presented by Nicoletti et al. (354).

Prince et al. (383) reported that resistance to chlorhexidine could be induced in some organisms but not in others. For example, P. mirabilis and S. marcescens displayed 128- and 258-fold increases, respectively, in resistance to chlorhexidine, whereas it was not possible to develop resistance to chlorhexidine in Salmonella enteritidis. The resistant strains did not show altered biochemical properties of changed virulence for mice, and some strains were resistant to the QAC benzalkonium chloride. Moreover, resistance to chlorhexidine was stable in S. marcescens but not in P. mirabilis. Despite extensive experimentation with a variety of procedures, Fitzgerald et al. (148) were unable to develop stable chlorhexidine resistance in E. coli or S. aureus. Similar observations were made by Cookson et al. (89), who worked with MRSA and other strains of S. aureus, and by McDonnell et al. (319), who worked with MRSA and enterococci. Recently, stable chlorhexidine resistance was developed in P. stutzeri (502); these strains showed...
various levels of increased tolerance to QACs, triclosan, and some antibiotics, probably as a result of a nonspecific alteration of the cell envelope (452). The adaptation and growth of *S. marcescens* in contact lens disinfectants containing chlorhexidine, with cross-resistance to a QAC, have been described previously (166).

Chloroxylenol-resistant strains of *P. aeruginosa* were isolated by repeated exposure in media containing gradually increasing concentrations of the phenolic, but the resistance was unstable (432). The adaptation of *P. aeruginosa* to QACs is a well-known phenomenon (1, 2, 240). Resistance to amphoteric surfactants has also been observed, and, interestingly, cross-resistance to chlorhexidine has been noted (240). This implies that the mechanism of such resistance is nonspecific and that it involves cellular changes that modify the response of organisms to unrelated biocidal agents. Outer membrane modification is an obvious factor and has indeed been found with QAC-resistant and amphoteric compound-resistant *P. aeruginosa* (240) and with chlorhexidine-resistant *S. marcescens* (166). Such changes involve fatty acid profiles and, perhaps more importantly, outer membrane proteins. It is also pertinent to note here the recent findings of Langsud and Sundheim (274). In this study, resistance of *P. fluorescens* to QACs was reduced when EDTA was present with the QAC (although the lethal effect was mitigated after the cells were grown in medium containing QAC and EDTA); similar results have been found with laboratory-generated *E. coli* mutants for which the MICs of triclosan were increased (318). EDTA has long been known (175, 410) to produce changes in the outer membrane of gram-negative bacteria, especially pseudomonads. Thus, it appears that, again, the development of resistance is associated with changes in the cell envelope, thereby limiting uptake of antiseptics and disinfectants.

Hospital (as for other environmental) isolates of gram-negative bacteria are invariably less sensitive to disinfectants than are laboratory strains (196, 209, 279, 286, 492). Since plasmid-mediated transfer has apparently been ruled out (see above), selection and mutation could play an important role in the presence of these isolates. Subinhibitory antibiotic concentrations may cause subtle changes in the bacterial outer structure, thereby stimulating cell-to-cell contact (109); it remains to be tested if residual concentrations of antiseptics or disinfectants in clinical situations could produce the same effect.

Another insusceptibility mechanism has been put forward, in this instance to explain acridine resistance. It has been proposed (270, 351) that proflavine-sensitive and -resistant cells are equally permeable to the acridine but that resistant cells possessed the ability to expel the bound dye. This is an important point and one that has been reinforced by more recent studies that demonstrate the significance of efflux in resistance of bacteria to antibiotics (284, 330, 355). Furthermore, multidrug resistance (MDR) is a serious problem in enteric and other gram-negative bacteria. MDR is a term used to describe resistance mechanisms used by genes that form part of the normal cell genome (168). These genes are activated by induction or mutation caused by some types of stress, and because they are distributed ubiquitously, genetic transfer is not needed. Although such systems are most important in the context of antibiotic resistance, George (168) provides several examples of MDR systems in which an operon or gene is associated with changes in antiseptic or disinfectant susceptibility; e.g., (i) mutations at an *acr* locus in the Acr system render *E. coli* more sensitive to hydrophobic antibiotics, dyes, and detergents; (ii) the *robA* gene is responsible for overexpression in *E. coli* of the RobA protein that confers multiple antibiotic and heavy-metal resistance (interestingly, Ag⁺ may be effluxed [350]); and (iii) the MarA protein controls a set of genes (*mar* and *sorRS* regulons) that confer resistance not only to several antibiotics but also to superoxide-generating agents. Moken et al. (333) have found that low concentrations of pine oil (used as a disinfectant) could select for *E. coli* mutants that overexpressed MarA and demonstrated low levels of cross-resistance to antibiotics. Deletion of the *mar* or *acrAB* locus (the latter encodes a PMF-dependant efflux pump) increased the susceptibility of *E. coli* to pine oil; deletion of *acrAB*, but not of *mar*, increased the susceptibility of *E. coli* to chloroxylenol and to a QAC. In addition, the *E. coli* MdfA (multidrug transporter) protein was recently identified and confers greater tolerance to both antibiotics and a QAC (benzalkonium) (132). The significance of these and other MDR systems in bacterial susceptibility to antiseptics and disinfectants, in particular the issue of cross-resistance with antibiotics, must be studied further. At present, it is difficult to translate these laboratory findings to actual clinical use, and some studies have demonstrated that antibiotic-resistant bacteria are not significantly more resistant to the lethal (or bactericidal) effects of antiseptic and disinfectants than are antibiotic-sensitive strains (11, 88, 89, 319).

### Mechanisms of Fungal Resistance to Antiseptics and Disinfectants

In comparison with bacteria, very little is known about the ways in which fungi can circumvent the action of antiseptics and disinfectants (104, 111, 296). There are two general mechanisms of resistance (Table 12): (i) intrinsic resistance, a natural property or development of an organism (201); and (ii) acquired resistance. In one form of intrinsic resistance, the cell wall presents a barrier to reduce or exclude the entry of an antimicrobial agent. The evidence to date is somewhat patchy, but the available information links cell wall glucan, wall thickness, and relatively porosity to the susceptibility of *Saccharomyces cerevisiae* to chlorhexidine (Table 13) (204–208). Protoplasts of this organism prepared by glucuronidase in the presence of β-mercaptoethanol are lysed by chlorhexidine concentrations well below those effective against “normal” (whole) cells. Furthermore, culture age influences the response of *S. cerevisiae* to chlorhexidine; the cells walls are much less sensitive at stationary phase than at logarithmic growth phase (208), taking up much less [14C]chlorhexidine gluconate (206). Gale (165) demonstrated a phenotypic increase in the resistance of *Candida albicans* to the polyenic antibiotic amphotericin B as the organisms entered the stationary growth phase, which was attributed to cell wall changes involving tighter cross-linking (74). Additionally, any factor increasing glucanase activity increased amphotericin sensitivity.

The porosity of the yeast cell wall is affected by its chemical

<table>
<thead>
<tr>
<th>Type of resistance</th>
<th>Possible mechanism</th>
<th>Example(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrinsic</strong></td>
<td>Exclusion</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td></td>
<td>Enzymatic inactivation</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td></td>
<td>Phenotypic modulation</td>
<td>Ethanol</td>
</tr>
<tr>
<td></td>
<td>Efflux</td>
<td>Not demonstrated to date*</td>
</tr>
<tr>
<td><strong>Acquired</strong></td>
<td>Mutation</td>
<td>Some preservatives</td>
</tr>
<tr>
<td></td>
<td>Inducible efflux</td>
<td>Some preservatives*</td>
</tr>
<tr>
<td></td>
<td>Plasmid-mediated responses</td>
<td>Not demonstrated to date</td>
</tr>
</tbody>
</table>

* Efflux is now known to be one mechanism of fungal resistance to antibiotics (531).
composition, with the wall acting as a barrier or modulator to the entry and exit of various agents. DeNobel et al. (117–119) used the uptake of fluorescein isothiocyanate (FITC) dextrans and the periplasmic enzyme invertase as indicators of yeast cell wall porosity. Intact S. cerevisiae cells were able to endocytose FITC dextrans of 70 but not of 150. A new assay for determining the relative cell wall porosity in yeast based upon polycation-induced leakage of UV-absorbing compounds was subsequently developed. Hiom et al. (206, 208) found that the relative porosity of cells decreases with increasing culture age and that there was a reduced uptake of radiolabeled chlorhexidine gluconate. As the age of an S. cerevisiae culture increases, there is a significant increase in the cell wall thickness, with values of 0.19, 0.25, and 0.31 μm recorded for cells from 1-, 2-, and 6-day old cultures, respectively (206).

These findings (Table 13) can provide a tentative picture of the cellular factors that modify the response of S. cerevisiae to chlorhexidine. Mannan mutants of S. cerevisiae show a similar degree of sensitivity to chlorhexidine as the parent strain (204). The glucan layer is shielded from &-glucuronidase by mannoproteins, but this effect is overcome by &-mercaptoethanol (119). The mannoprotein consists of two fractions, sodium dodecyl sulfate-soluble mannoproteins and sodium dodecyl sulfate-insoluble, glucanase-soluble ones: the latter limit cell wall porosity (119). Thus, glucan (and possibly mannoproteins) plays a key role in determining the uptake and hence the activity of chlorhexidine in S. cerevisiae. C. albicans is less sensitive and takes up less [14C]chlorhexidine overall (206), but only recently has been reported to have a reduced uptake of radiolabeled chlorhexidine.

Yeasts grown under different conditions have variable levels of sensitivity to ethanol (176, 402). Cells with linoleic acid-enriched plasma membranes are more resistant to ethanol than are cells with oleic acid-enriched ones, from which it has been inferred that a more fluid membrane enhances ethanol resistance (6).

There is no evidence to date of antiseptic efflux (although benzoic acid in energized cells is believed to be eliminated by flowing down the electrochemical gradient [529]) and no evidence of acquired resistance by mutation (except to some preservatives [436]) or by plasmid-mediated mechanisms (426, 436). It is disappointing that so few rigorous studies have been performed with yeasts and molds and antiseptics and disinfectants (see also Miller’s [328] treatise on mechanisms for reaching the site of action). Molds are generally more resistant than yeasts (Table 14) and considerably more resistant than nonsporulating bacteria (Table 15). Mold spores, although more resistant than nonsporulating bacteria, are less resistant than bacterial spores to antiseptics and disinfectants (436). It is tempting to speculate that the cell wall composition in molds confers a high level of intrinsic resistance on these organisms.

### Mechanisms of Viral Resistance to Antiseptics and Disinfectants

Early studies on the effects of disinfectants on viruses were reviewed by Grossgebauer (189). Potential viral targets are the viral envelope, which contains lipids and is a typical unit membrane; the capsid, which is principally protein in nature; and the genome. An important hypothesis was put forward in 1963 (258) and modified in 1983 (259) in which it was proposed that viral susceptibility to disinfectants could be based on whether viruses were “lipophilic” in nature, because they possessed a lipid envelope (e.g., herpes simplex virus [259]) or “hydrophilic” because they did not (e.g., poliovirus [514]). Lipid-enveloped viruses were sensitive to lipophilic-type disinfectants, such as 2-phenylphenol, cationic surfactants (QACs), chlorhexidine, and isopropanol, as well as to ether and chloroform. Klein and Deforest (259) further classified viruses into three groups (Table 16), A (lipid containing), B (nonlipid picornaviruses), and C (other nonlipid viruses larger than those in group B) and disinfectants into two groups, broad-spectrum ones that inactivated all viruses and lipophilic ones that failed to inactivate picornaviruses and paroviruses.

Capsid proteins are predominantly protein in nature, and biocides such as glutaraldehyde, hypochlorite, ethylene oxide, and hydrogen peroxide, which react strongly with amino or sulphydryl groups might possess virucidal activity. It must, however, be added that destruction of the viral capsid may result in the release of a potentially infectious nucleic acid and that viral inactivation would only be complete if the viral nucleic acid is also destroyed.

Unfortunately, the penetration of antiseptics and disinfectants into different types of viruses and their interaction with viral components have been little studied, although some information has been provided by investigations with bacteriophages (307). Bacteriophages are being considered as “indicator species” for assessing the virucidal activity of disinfectants (258). Klein and Deforest (259) further classified viruses into three groups in 1963 (258) and modified in 1983 (259) in which it was proposed that viral susceptibility to disinfectants could be based on whether viruses were “lipophilic” in nature, because they possessed a lipid envelope (e.g., herpes simplex virus [259]) or “hydrophilic” because they did not (e.g., poliovirus [514]). Lipid-enveloped viruses were sensitive to lipophilic-type disinfectants, such as 2-phenylphenol, cationic surfactants (QACs), chlorhexidine, and isopropanol, as well as to ether and chloroform. Klein and Deforest (259) further classified viruses into three groups (Table 16), A (lipid containing), B (nonlipid picornaviruses), and C (other nonlipid viruses larger than those in group B) and disinfectants into two groups, broad-spectrum ones that inactivated all viruses and lipophilic ones that failed to inactivate picornaviruses and paroviruses.

### Table 13. Parameters affecting the response of S. cerevisiae to chlorhexidine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Role in susceptibility of cells to chlorhexidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall composition</td>
<td>Increased activity possibly responsible for decreased activity (?).</td>
</tr>
<tr>
<td>Glucan</td>
<td>Glucan (possibly mannoproteins) plays a key role in determining the uptake and hence the activity of chlorhexidine in S. cerevisiae.</td>
</tr>
<tr>
<td>Cell wall thickness</td>
<td>Decreased activity possibly responsible for decreased activity (?).</td>
</tr>
<tr>
<td>Relative porosity</td>
<td>Decreased activity possibly responsible for decreased activity (?).</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>Changes altering CHG susceptibility (?). Not investigated to date.</td>
</tr>
</tbody>
</table>

* Data from references 204 to 208 and 436.

### Table 14. Lethal concentrations of antiseptics and disinfectants toward some yeasts and molds

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Yeast (Candida albicans)</th>
<th>Molds (Penicillium chrysogenum, Aspergillus niger)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzalkonium chloride</td>
<td>10</td>
<td>100–200</td>
</tr>
<tr>
<td>Cetrimide/CTAB</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Chlorhexidine</td>
<td>20–40</td>
<td>400</td>
</tr>
</tbody>
</table>

* Derived in part from data in reference 525.

b CTAB, cetyltrimethylammonium bromide.
disinfection system could be an efficient means of viral inactivation while overcoming the possibility of multiplicity reactivation (first put forward by Luria [293]) to explain an initial reduction and then an increase in the titer of disinfectant-treated bacteriophage. Multiplicity reactivation as a mechanism of resistance was supported by the observation of Young and Sharp (546) that clumping of poliovirus following partial inactivation by hypochlorite significantly increased the phage titer. It is envisaged as consisting of random damage to the capsid protein or nucleic acid of clumped, noninfectious virions from which complementary reconstruction of an infectious particle occurs by hybridization with the gene pool of the inactivated virions (298).

Another resistance mechanism also involves viral aggregation, e.g., the persistence of infectivity of formaldehyde-treated poliovirus (458) and the resistance of Norwalk virus to chlorination (249). A typical biphasic survival curve of enterovirus and rotavirus exposed to peracetic acid is also indicative of the presence of viral aggregates (198).

Finally, there remains the possibility of viral adaptation to new environmental conditions. In this context, Bates et al. (28) described the development of poliovirus having increased resistance to chlorine inactivation. Clearly, much remains to be learned about the mechanism of viral inactivation by and viral resistance to disinfectants.

Mechanisms of Protozoal Resistance to Antiseptics and Disinfectants

Intestinal protozoa, such as Cryptosporidium parvum, Entamoeba histolytica, and Giardia intestinalis, are all potentially pathogenic to humans and have a resistant, transmissible cyst (or oocyst for Cryptosporidium) (233, 234). Of the disinfectants available currently, ozone is the most effective protozoan cysticide, followed by chlorine dioxide, iodine, and free chlorine, all of which are more effective than the chloramines (234, 264). Cyst forms are invariably the most resistant to chemical disinfectants (Fig. 1). The reasons for this are unknown, but it would be reasonable to assume that cysts, similar to spores, take up fewer disinfectant molecules from solution than do vegetative forms.

Some recent studies have compared the responses of cysts and trophozoites of Acanthamoeba castellanii to disinfectants used in contact lens solutions and monitored the development of resistance during encystation and the loss of resistance during excystation (251–255). The lethal effects of chlorhexidine and of a polymeric biguanide were time and concentration dependent, and mature cysts were more resistant than precystment trophozoites or precystment cysts. The cyst “wall” appeared to act as a barrier to the uptake of these agents, thereby presenting a classical type of intrinsic resistance mechanism (163). Acanthamoebae are capable of forming biofilms on surfaces such as contact lenses (186). Although protozoal biofilms have yet to be studied extensively in terms of their response to disinfectants, it is apparent that they could play a significant role in modulating the effects of chemical agents.

Mechanisms of Prion Resistance to Disinfectants

The transmissible degenerative encephalopathies (TDEs) form a group of fatal neurological diseases of humans and other animals. TDEs are caused by prions, abnormal proteinaceous agents that appear to contain no agent-specific nucleic acid (385). An abnormal protease-resistant form (PrP<sup><em>res</em></sup>) of a normal host protein is implicated in the pathological process. Prions are considered highly resistant to physical and chemical agents (Fig. 1), although the fact that crude preparations are often studied means that extraneous materials could, at least to some extent, mask the true efficacy of these agents (503). According to Taylor (503), there is currently no known decontamination procedure that will guarantee the complete absence of infectivity in TDE-infected tissues processed by histopathological procedures. Prions survive acid treatment, but a synergistic effect with autoclaving plus sodium hydroxide treatment is observed. Formaldehyde, unbuffered glutaraldehyde (acidic pH), and ethylene oxide have little effect on infectivity, although chlorine-releasing agents (especially hypochloriters), sodium hydroxide, some phenols, and guanidine thiocyanate are more effective (141, 309, 503).

With the information presently available, it is difficult to explain the extremely high resistance of prions, save to comment that the protease-resistant protein is abnormally stable to degradative processes.

CONCLUSIONS

It is clear that microorganisms can adapt to a variety of environmental physical and chemical conditions, and it is therefore not surprising that resistance to extensively used antiseptics and disinfectants has been reported. Of the mechanisms that have been studied, the most significant are clearly intrinsic, i.e., the ability to sporulate, adaptation of pseudomonads, and the protective effects of biofilms. In these cases, “resistance” may be incorrectly used and “tolerance,” defined as developmental or protective effects that permit microorganisms to survive in the presence of an active agent, may be more correct. Many of these reports of resistance have often paralleled issues including inadequate cleaning, incorrect product use, or ineffective infection control practices, which cannot be underestimated. Some acquired mechanisms (in particular with heavy-metal resistance) have also been shown to be clinically significant, but in most cases the results have been spec-
TABLE 16. Viral classification and response to some disinfectants

<table>
<thead>
<tr>
<th>Viral group</th>
<th>Lipid envelope</th>
<th>Examples of viruses</th>
<th>Effects of disinfectants</th>
<th>Lipophilic spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>HSV, HIV, Newcastle disease virus, rubies virus, influenza virus</td>
<td>S S</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>−</td>
<td>Non-lipid picornaviruses (poliovirus), Coxsackie virus, echovirus</td>
<td>R S</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>−</td>
<td>Other larger nonlipid viruses (adenovirus, reovirus)</td>
<td>R S</td>
<td></td>
</tr>
</tbody>
</table>

* Data from reference 259; see also reference 444. For information on the inactivation of poliovirus, see reference 514.

† Present (+) or absent (−).

‡ Lipophilic disinfectants include QACs and chlorhexidine. S, sensitive; R, resistant.

ulative. Increased MICs have been confirmed, in particular for staphylococci. However, few reports have further investigated increased bactericidal concentrations or actual use dilutions of products, which in many cases contain significantly higher concentrations of biocides, or formulation attributes, which can increase product efficacy; in a number of cases, changes in the MICs have actually been shown not to be significant (9, 88, 89, 319, 428). Efflux mechanisms are known to be important in antibiotic resistance, but it is questionable if the observed increases in MICs of biocides could have clinical implications for hard-surface or topical disinfection (423, 428). It has been speculated that low-level resistance may aid in the survival of microorganisms at residual levels of antiseptics and disinfectants; any possible clinical significance of this remains to be tested. With growing concerns about the development of biocide resistance and cross-resistance with antibiotics, it is clear that clinical isolates should be under continual surveillance and possible mechanisms should be investigated.

It is also clear that antiseptic and disinfectant products can vary significantly, despite containing similar levels of biocides, which underlies the need for close inspection of efficacy claims and adequate test methodology (183, 423, 428). In addition, a particular antiseptic or disinfectant product may be better selected (as part of infection control practices) based on particular circumstances or nosocomial outbreaks; for example, certain active agents are clearly more efficacious against gram-positive than gram-negative bacteria, and C. difficile (despite the intrinsic resistance of spores) may be effectively controlled physically by adequate cleaning with QAC-based products.

In conclusion, a great deal remains to be learned about the mode of action of antiseptics and disinfectants. Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Studies of the mechanisms of action of and microbial resistance to antiseptics and disinfectants are thus not merely of academic significance. They are associated with the more efficient use of these agents clinically and with the potential design of newer, more effective compounds and products.

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Antiseptics and Disinfectants: Activity, Action, and Resistance
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Volume 12, no. 1, p. 147–179, 1999. Page 168, Table 14, spanner: “Lethal concn (µg/µl)” should read “Lethal concn (µg/ml).”