



Mechanisms of Action of Antibacterial Biocides

S. P. Denyer

Department of Pharmacy, University of Brighton, Lewes Road, Brighton BN2 4GJ, UK

ABSTRACT

Antibacterial biocides are represented by a wide range of chemical agents. This chemical diversity offers a multiplicity of potentially damaging interactions with the bacterial cell. Only rarely, however, are these interactions non-specific in nature; more frequently, the morphology and physiology of the cell, when combined with the physicochemical properties of the biocide, will dictate specific targets or target regions. A knowledge and understanding of these lesions offers a powerful tool in the search for novel chemistries and improved biocidal capabilities. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Chemical biocides fulfil a key role in the preservation of products as diverse as cutting fluids, foods and beverages, cosmetics and pharmaceutical formulations and afford protection against spoilage in a wide range of industrial and environmental applications. Many have entered common usage through a long history of experience. The systematic study of their mechanism of action invites a direction to their future design and development, providing insight into new agents, resistance mechanisms and toxicological problems, and offers guidance on their correct usage.

Mechanism of action studies span many years of investigation (Denyer & Hugo, 1991; Hugo, 1991). This review illustrates the general principles behind these studies and summarizes the major antibacterial lesions attributed to biocide classes.

NATURE OF THE ANTIBACTERIAL EFFECT

Biocides may exert both bacteriostatic and bactericidal effects, although the mechanism of action responsible for each may differ. Bacteriostatic events are generally considered to arise from some metabolic injury which is reversible upon removal or neutralization of the biocide (Fitzgerald *et al.*, 1989) whereas bactericidal action results from irreparable and irreversible damage to a vital cellular structure or function (Fig. 1).

The stages of interaction between biocide and bacterium arise in the following sequence: uptake of biocide by cell; partition/passage of biocide to target(s); concentration of biocide at target(s); damage to target(s). The initiating step is the migration of biocide from the aqueous phase to an association with the cell surface, a process defined as 'uptake' and described by various sorption isotherms (Denyer, 1990). This process is regulated by the physicochemical characteristics of both the cell and biocide, and may subsequently be modified by changes in cellular characteristics brought about by biocide sorption (El-Falaha *et al.*, 1985; Ismaeel *et al.*, 1987; Jones *et al.*, 1991).

An antibacterial effect ultimately arises from the successful interaction of the biocide with, and concentration at, its target or targets (Fig. 2). In progressing towards this target, however, a biocide will encounter intervening structures which account, to varying degrees, for the differing sensitivities of individual bacterial species (Russell, 1991). Thus, Gram-negative cells offer a supplementary barrier, the lipopolysaccharide (LPS) layer, to biocide penetration which Gram-positive cells do not possess. This structure has a significant moderating influence on the penetration of both hydrophilic and hydrophobic molecules, establishing a molecular weight cut-off (*c.* 600 Da) for the passage of the former through water-

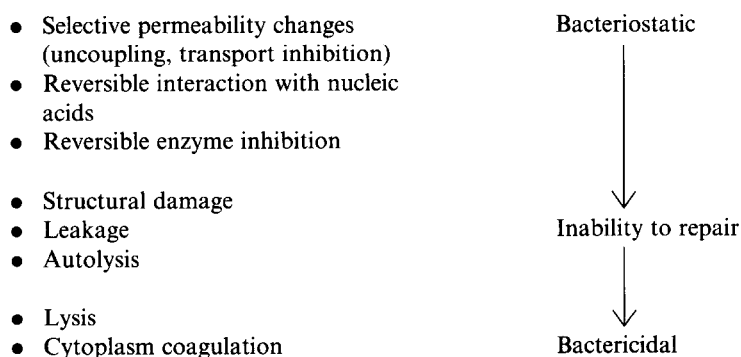


Fig. 1. The antibacterial consequences of biocide-induced damage (modified from Denyer [1990]).

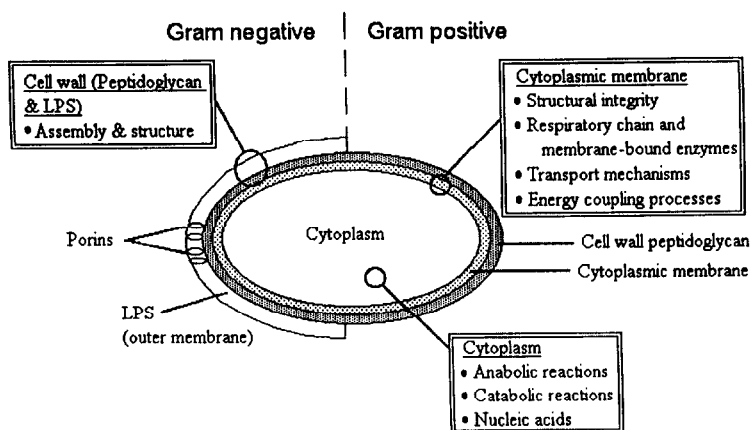


Fig. 2. Potential targets for biocides.

filled pores (porins) and requiring optimal lipophilic properties for the progress of hydrophobic biocides (Gilbert & Wright, 1987; Russell, 1991). This barrier effect can be relieved by the introduction of agents such as ethylenediaminetetraacetic acid (EDTA), which increases the permeability of the Gram-negative outer membrane (Russell, 1991). Irrespective of Gram stain, all intervening structures offer opportunities for non-specific binding of biocide thereby depleting the chemical challenge. The potential for phenotypic variation in these barriers, as well as the target site(s), has been proposed as the basis of variation in biocide sensitivity due to inoculum history (Al-Hiti & Gilbert, 1980; Wright & Gilbert, 1987; Brown *et al.*, 1990; Stewart & Olson, 1992).

Models of biocide penetration have been described (e.g. Gilbert & Wright, 1987) which seek to build a relationship between the physico-chemical characteristics of a biocide and bacterial sensitivity. Undoubtedly, factors influencing biocide chemistry and/or microbial physicochemistry, such as pH, will significantly affect the outcome of the microbe-biocide interaction; for this reason, weak acids are most active at pHs below their pKa and cationic surfactants at pHs which ensure the surface negative charge of the bacterium (Russell, 1992; Richards *et al.*, 1995).

The nature and extent of any antibacterial effect will be determined naturally by the progress of the biocide through the stages of interaction and the final damaging event(s). None of these stages are instantaneous although the time taken for their completion may vary between different classes of biocide, even where they share the same eventual target. Thus, theoretically, all antibacterial events may be reversed if redressed quickly enough. In practice, however, some damaging events are compounded to

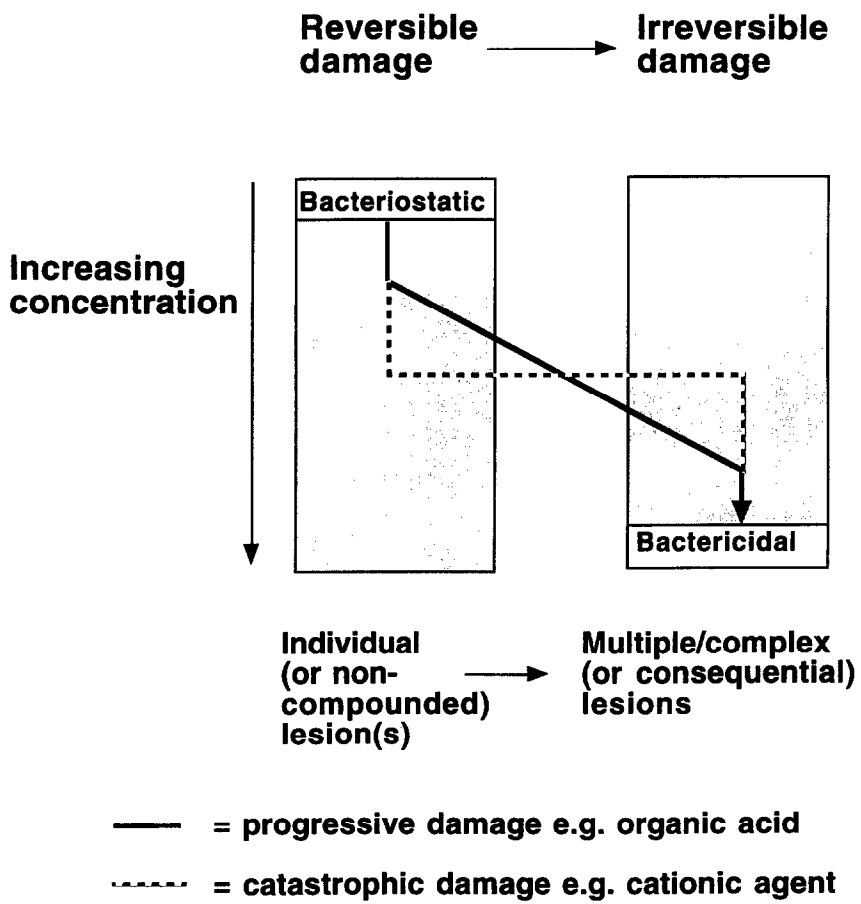


Fig. 3. Relationship between bacteriostatic and bactericidal effects.

such magnitude or are of such rapidity of onset that they initiate consequential effects which cannot be reversed. Such damage may be enhanced by increasing the applied concentration of biocide (Fig. 3). Inevitably, most biocide-induced damage, if sustained for long enough and of sufficient severity, will lead to accelerated cell death.

MECHANISM OF ACTION STUDIES

Methodological approach

A variety of methods have been established by which to follow and assess damage to bacteria and these have been reviewed comprehensively (Russell *et al.*, 1973; Denyer & Hugo, 1991). Mechanism of action studies

seek to undertake the quantitative assessment and comparison of biocide activity at both the whole cell and subcellular/biochemical level. This approach, while classical in its application, must be used with careful attention to detail, in particular to eliminate, or account for, experimentally-induced effects.

In practice, biocides are employed frequently in environments capable of sustaining microbial metabolism and often growth. In the laboratory environment, this is modelled in nutrient media and measures of whole cell sensitivity, such as the minimum growth inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) are determined (Bloomfield, 1991). These values give important reference points when examining dose-related effects and a causal relationship between antibacterial action and target damage is often presumed where similar concentration dependencies apply. In mechanism of action studies, however, the investigator will frequently seek to eliminate repair and recovery processes since these will often serve to obscure determination of the metabolic/structural injury. Thus, many studies are pursued in non-nutrient buffered media with the consequent risk of underestimating or undervaluing the importance of recovery processes and perhaps underappreciating the complexity of the mechanism of action.

A further complication arising in the study of mechanisms of action is population variability. In an asynchronous batch culture, cells develop at different rates meeting different nutritional gradients throughout their growth cycle leading to variations in biocide sensitivity. This, taken together with the collision theory of biocide–bacterium interaction, will mean that in any population there may be wide variation in the extent of individual cell damage at any particular stage of biocide treatment. An inevitable consequence of this is that any attempt to reverse the process of biocide damage in a population, through biocide neutralization or some other process, will lead to the rescue of relatively ‘uninjured’ bacteria, but the loss of other cells. The success of the rescue will inevitably depend upon the efficiency of the recovery process.

In the interpretation of mechanism of action studies, therefore, it is important to recognize such factors as the influence of nutrient on recovery, population variability, inoculum history (Russell, 1992), and the possibility of stressed cells arising prior to experimentation (Gilbert & Brown, 1991; Wyber *et al.*, 1994).

Examples of biocide-induced damage

Notwithstanding the important methodological considerations described above, it is possible to identify key lesions responsible for the antibacterial

activity of many biocides. For convenience, the target regions are often classified as the cell wall, cytoplasmic membrane, and cytoplasm (Fig. 2) although these do not represent mutually exclusive areas for biocide action. The consequences of damage to these regions are given in Table 1 and this leads to the classification of biocides by target.

By far the most frequently-cited target region is the bacterial cytoplasmic membrane. This is not surprising given its fundamental metabolic and structural role within the cell, its large surface area for interaction, and its (relative) proximity to the external aqueous environment. A wide range of biocides of different chemical classes will damage the membrane, albeit by different mechanisms. It will also be noted from Table 1 that several agents have plurality of action, often reflecting a more generalized reactivity. This leads to an alternative classification of biocides by reference to their physicochemical mechanism of interaction with their target (Table 2). This affords some explanation for the target specificity of some agents and the apparent promiscuity of others.

Mechanisms of interaction

Biocides which interact strongly by chemical or electrostatic bonding with their target(s) are generally difficult to neutralize by dilution and will require some form of surrogate compound with which to interact; this is the basis of action for the specific inactivating agents listed in Table 3. Conversely, those agents mediating their effects through weakly physical interactions with cellular lipid are inactivated readily by dilution. The behaviour of a biocide on dilution is described by its concentration exponent; this parameter may be a useful indicator of the mechanism of interaction between biocide and target (Hugo & Denyer, 1987).

Chemically-reactive agents may display some target specificity (e.g. membrane thiol groups; Morris *et al.*, 1984), but frequently are of sufficient reactivity to interact with several different cellular components obscuring the primary lesion (if indeed there is one). Most likely in these situations, it is target accessibility which determines the sequence of inhibiting events and not necessarily the crucial nature of any particular cellular component. Under these circumstances, the characteristics of the biocidal agent which determine its passage *in active form* to susceptible areas is key; in some instances, the active agent may be released from a donor molecule (Rossmore & Sondossi, 1988). Excessive reactivity can lead to competing non-specific interactions between the biocide and bacterium or the surrounding medium which would serve to decrease overall antibacterial activity (Paulus & Kühle, 1986). A special case pertains for agents which cause the oxidation of thiol groups to disul-

TABLE 1
Consequences of Biocide-induced Damage

Target region	Damaging event	Consequence	Example biocides	Selected references
Cell wall	Structural/functional changes; release of wall components; initiation of autolysis	Abnormal morphology and construction; non-specific increase in cell permeability; lysis	Phenol Sodium hypochlorite	Pulvertaft & Lumb (1948)
			Formaldehyde	Pulvertaft & Lumb (1948) Douglas <i>et al.</i> (1990) Douglas <i>et al.</i> (1990)
			Formaldehyde-releasing agents	Pulvertaft & Lumb (1948) Schaechter & Santomassino (1962)
			Mercurials	Salton (1957)
			Cetytrimethylammonium bromide (CTAB)	Ingram & Buttke (1984)
			Aliphatic alcohols	Halegoua & Inouye (1979)
			2-Phenylethanol	Leive (1974)
			EDTA	Bolle & Kellenberger (1958) El-Falaha <i>et al.</i> (1989)
			Sodium dodecyl sulphate (SDS)	Richards <i>et al.</i> (1995)
			4-Aminobenzoic acid	

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TABLE 1—*contd.*

Cytoplasmic membrane	1. Loss of structural organisation and integrity	Progressive leakage of intracellular material (e.g. potassium ions, inorganic phosphate, amino acids, pentoses, nucleotides, protein); initiation of autolysis	Quaternary ammonium compounds (QACs)	
			Phenol	Hotchkiss (1944) Salton (1950, 1951) Judis (1962) Kroll & Anagnostopoulos (1981)
			4-Ethylphenol	Hugo & Bowen (1973)
			Tetrachlorosalicylanilide (TCS)	Woodroffe & Wilkinson (1966)
			Fentichlor	Hugo & Bloomfield (1971)
			SDS	Gilby & Few (1960)
			Ethanol	Salton (1963)
			Chlorhexidine	Hugo & Longworth (1964)
			Polymeric biguanides and alexidine	Broxton <i>et al.</i> (1983) Chawner & Gilbert (1989b)
			2-Phenylethanol	Silver & Wendt (1967) Denyer <i>et al.</i> (1986)
			<i>N</i> -dodecylmethanolamine	Lambert & Smith (1976a, b)
			Triclosan	Regos & Hitz (1974)
			Polyethoxyalkylphenols (Tritons)	Lamikanra & Allwood (1977)

2. Selective increase in permeability to protons and other ions	Dissipation of proton motive force; uncoupling of oxidative phosphorylation; inhibition of active transport; loss of metabolic pools	Lipophilic weak acids (e.g. acetic, propionic, sorbic, benzoic)	Freese <i>et al.</i> (1973)
			Sheu <i>et al.</i> (1975)
		Parabens	Eklund (1980, 1985)
		Alkylphenols	Hugo & Bowen (1973) Denyer <i>et al.</i> (1980)
		Chlorocresol	Denyer <i>et al.</i> (1986)
		2-Phenoxyethanol	Gilbert <i>et al.</i> (1977b)
		Fentichlor	Bloomfield (1974)
		TCS	Hamilton (1968)
		1-Dodecylpiperidine <i>N</i> -oxide	Mlynarcik <i>et al.</i> (1981)
		3. Inhibition of membrane-bound enzymes	Inhibition of respiration and energy transfer; inhibition of ATP synthesis; inhibition of substrate oxidation; inhibition of transport processes
2-Phenoxyethanol	Gilbert <i>et al.</i> (1977c)		
Azide	Heinen (1971)		
Bronopol	Stretton & Manson (1973) Shepherd <i>et al.</i> (1988)		
CTAB	Rosenthal & Buchanan (1974)		

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TABLE 1—contd.

Cytoplasm	1. Inhibition of cytoplasmic enzymes; interaction with functional biomolecules (e.g. DNA, RNA)	Inhibition of catabolic and anabolic processes	Isothiazolones (e.g. 5-chloro-2-methyl-4-isothiazolin-3-one; 1,2-benzisothiazolin-3-one)	Fuller <i>et al.</i> (1985) Chapman & Diehl (1995)
			Iodoacetate	Morris <i>et al.</i> (1984)
			Organomercurials	Stretton & Manson (1973)
			Heavy metal salts (e.g. silver, copper, mercury)	Reviewed in Harold (1970)
			Inhibition of catabolic and anabolic processes	Reviewed in Hugo (1992)
			Formaldehyde	
			Formaldehyde releasing agents	
			Acridine dyes	
			Oxidizing agents (e.g. hydrogen peroxide, peracetic acid)	
			Parabens	Ness & Eklund (1983)
	2. Coagulation and precipitation of cytoplasmic constituents (usually at high biocide concentrations)	Denaturation of enzymes; destruction of biomolecules	Chloroacetamide	Serry <i>et al.</i> (1986)
			Chlorhexidine and other biguanides	Hugo & Longworth (1964) Davies & Field (1969)
			QACs	Kopecká-Leitmanová <i>et al.</i> (1989)
			Some phenolics Some heavy metals	Reviewed in Hugo (1992)

TABLE 2
Mechanisms of Interaction Between Selected Biocides and Their Prospective Targets

<i>Mechanisms of interaction</i>	<i>Example biocides</i>	<i>Typical targets</i>
<i>Chemical reactions:</i>		
Oxidation of (predominantly) thiol groups	Isothiazolones, organomercurials, heavy metal salts, hypochlorites, organochlorine derivatives, bronopol, metallo-compounds (e.g. oxines)	Thiol-containing cytoplasmic and membrane-bound enzymes e.g. dehydrogenases
General alkylation reactions	Glutaraldehyde, formaldehyde (and by implication formaldehyde-releasing agents)	Biomolecules (e.g. protein, RNA, DNA) containing amino, imino, amide, carboxyl and thiol groups. Intermolecular cross-linking may occur
Halogenation	Hypochlorites, chlorine-releasing agents	Amino groups in proteins
Free-radical oxidation (e.g. hydroxyl radicals)	Hydrogen peroxide, peracetic acid	Enzyme and protein thiol groups
Metal ion chelation	EDTA	Divalent cation-mediated outer membrane integrity; principal target region Gram-negative cell wall
Intercalation	Aminoacridines	Intercalation between DNA base pairs
<i>Physical interactions:</i>		
Penetration/partition into phospholipid bilayer; possible displacement of phospholipid molecules	Phenols, weak acids, parabens, TCS	Transmembrane pH gradient; membrane integrity
Solvation of phospholipids	Aliphatic alcohols	Membrane integrity
Electrostatic (ionic) interaction with phospholipids	QACs, biguanides	Cytoplasmic membrane integrity; membrane-bound enzyme environment and function
Membrane-protein solubilization	Anionic surfactants	Cytoplasmic membrane integrity; membrane-bound enzyme environment and function

phides (e.g. isothiazolones). This reaction may be reversed by intracellular sulphhydryl compounds or through the process of active oxidative metabolism (Fuller *et al.*, 1985; Collier *et al.*, 1990a,b; Chapman & Diehl, 1995). Progressive oxidation to sulfoxides and disulphoxides by more powerful oxidizing agents is not reversible.

Membrane-disruptive agents elicit their effects through diverse interactions with this organelle (Table 2) involving both the hydrophobic and polar regions of the phospholipid bilayer and membrane-bound proteins (Denyer, 1990). The precise nature of these interactions is unclear, but key characteristics such as lipophilicity and delocalization of charge (Finkelstein, 1970; Kroll & Patchett, 1991; Buckton *et al.*, 1991) imply partitioning into the hydrophobic region for phenolics, weak acids and their esters, while the high affinity of cationic agents for the membrane suggests a strong interaction with the negatively-charged polar head group of phospholipids, an affinity moderated by alkyl chain length in the QACs (Brown & Tomlinson, 1979; Gilbert & Al-Taae, 1985). Changes in phospholipid packing and phase separation arise from these electrostatic interactions (Broxton *et al.*, 1984; Chawner & Gilbert, 1989a); cellular damage may possibly be aided by polymeric heterogeneity in the biocide formulation (Gilbert *et al.*, 1990). For the QACs, a successive interplay of polar and hydrophobic interactions with the membrane may determine the progress of antibacterial events (Kopecká-Leitmanová *et al.*, 1989). In some studies, monolayer uptake is co-incident with concentrations first eliciting significant inhibitory activity (Salt & Wiseman, 1968, 1991); uptake and subsequent binding of cationic agents may be encouraged by the formation of biocide aggregates (Kanazawa *et al.*, 1995).

Irrespective of the precise mechanism of action, the efficacy of any biocide is as dependant upon the physicochemical characteristics of that agent as it is on the significance of the targets to which it is disposed.

Enhancement of action

Accessibility of target to biocide may be improved by the coincident use of cell permeabilizing agents (Hart, 1984; Vaara, 1992; Bloomfield & Arthur, 1994) or by modification to the chemical structure of biocides permitting pro-drug delivery or portage transport (Denyer *et al.*, 1991). Further, judicious combination of biocides having biochemically or physicochemically complementary mechanisms of action may lead to synergistic activity (Denyer *et al.*, 1985b, 1986; Denyer & King, 1988; Lehmann, 1988; Pons *et al.*, 1992). Thus, a knowledge of mechanisms of action may more readily allow biocides to be combined to best advantage.

TABLE 3
Inactivating/Neutralizing Processes for Selected Biocides

<i>Inactivating/neutralizing process</i>	<i>Biocide</i>
Dilution ($\pm$ Tween 80)	Phenols Cresols Parabens Alcohols
<i>Specific inactivators:</i> Cysteine/thioglycollate	Mercurials Bronopol Isothiazolones
Lecithin (+ Tween 80/Lubrol W)	QACs Biguanides
Sodium thiosulphate	Halogens
Glycine	Glutaraldehyde Formaldehyde

CONCLUSION

Biocides exhibit a multiplicity of antibacterial mechanisms. Specific lesions may be identifiable, while consequential and supplementary damage may arise through continued intracellular interactions (Chapman & Diehl, 1995). A knowledge of mechanisms of action, combined with an understanding of quantitative structure–activity relationships, provides an important platform from which novel biocides may emerge offering enhanced activity and environmental acceptability (Lindstedt *et al.*, 1990; Cupková *et al.*, 1993; Ahlström *et al.*, 1995).

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