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Activity of biocides against mycobacteria

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1. INTRODUCTION

1.1 General comments

Mycobacteria comprise a fairly diverse group of acid-fast bacteria, the best known members of which are *Mycobacterium tuberculosis* and *Myco. leprae*, the aetiological agents respectively of tuberculosis and leprosy. Other mycobacteria can also cause serious infections (Wolinsky 1979; Wayne 1985; Inderlied *et al.* 1993) and there are many opportunistic species (Collins *et al.* 1984; Grange *et al.* 1990). Opportunistic pathogenic mycobacteria (Collins *et al.* 1984; Collins 1989; Guthertz *et al.* 1989) and potentially pathogenic mycobacteria (Goslee and Wolinsky 1976) may be associated with water supplies.

Early studies revealed that the mycobacteria were more resistant to biocides than other non-sporulating bacteria (Chargaff *et al.* 1931; reviewed by Croshaw 1971). This has been amply substantiated in many subsequent studies, and recent work has demonstrated that some species are particulary resistant.

It is the purpose of this paper to describe the activity of various biocides towards mycobacteria, to assess methods of evaluating this activity, to discuss what is known about their mechanism of antimycobacterial action and about the mechanisms of mycobacterial resistance, and to suggest ways in which mycobactericidal activity can be enhanced,

Some terms need to be defined. A *disinfectant* is used in this paper to denote a substance used in the process of disinfection for the purpose of inactivating micro-organisms, including potentially pathogenic ones but not necessarily bacterial spores, on the surface of inanimate objects. A *biocide* is used generally to denote a substance that kills micro-organisms. A substance that is *mycobactericidal* kills mycobacteria; one that is tuberculocidal inactivates *Myco. tuberculosis* whereas a *mycobacteriostatic* agent inhibits the growth of mycobacteria but does not have a significant lethal effect.

1.2 Transmission of mycobacterial infection

Tuberculosis is on the increase (Blessington and O'Sullivan 1994). Detailed descriptions of the transmission of mycobacterial infection are presented elsewhere in this symposium. Consequently, only a few pertinent comments will be made here insofar as they apply to the inactivation of mycobacteria by biocides.

When deciding upon whether a sterilization or disinfection process is to be undertaken to decontaminate medical devices, it is necessary to consider the nature of these devices and the infection risk associated with decontamination (Favero 1991; Medical Devices Directorate 1993; Russell 1994). Patient care instruments and devices may be classified into: (i) critical

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(high risk), (ii) semi-critical (intermediate risk), (iii) non-critical (low risk). Endoscopes are regarded as being semi-critical items; most are unable to withstand heat and decontamination is often achieved by a high-level chemical disinfectant. Most episodes of endoscopy-acquired infection arise from inadequate cleaning and disinfection, there being a ready transmission of pathogenic bacteria by contaminated endoscopes, with particular problems for immunocompromised patients (Pappas *et al.* 1983; Anon. 1988; Ayliffe 1988; Hanson *et al.* 1988 a, b; Alvardo *et al.* 1991).

Transmission of mycobacterial infection by endoscopy is, however, believed to be rare, despite a marked increase in the use of flexible fibreoptic endoscopes (Axon 1991; Ayliffe *et al.* 1993). Hospital-acquired opportunistic mycobacteria may cause disseminated infection and also lung infections, endocarditis, pericarditis, etc. The source is often associated with haemodialysis and chronic ambulatory peritoneal dialysis (CAPD) (George 1988). There are few documented examples of the hospital transmission of tuberculosis (see Leers 1980; Nelson *et al.* 1983), although there is concern at the spread of this disease in respiratory units (Hanson *et al.* 1992). Bronchoscopy is probably the greatest hazard (George 1988) for the transmission of tuberculosis and other mycobacteria but there are no reports of transmission of *Myco. tuberculosis* by gastrointestinal endoscopy (Carr-Lock and Clayton 1978; Axon and O'Conner 1983; O'Conner and Axon 1983; Ayliffe *et al.* 1986, 1992; Ayliffe 1988).

To ensure that mycobacteria are not transmitted by endoscopy, and especially bronchoscopy, endoscopes must be decontaminated effectively and rapidly (Felmingham *et al.* 1985; Ridgway 1985; Babb 1988; Ferreres 1988; Collignon and Graham 1989; Ayliffe *et al.* 1990, 1993). In addition to *Myco. tuberculosis*, non-tuberculosis mycobacteria such as *Myco. chelonae* and the *Myco. avium intracellulare* (MAI) group must be considered; *Myco. chelonae* is water-associated and MAI are often associated with AIDS patients (Young 1988; Jensen *et al.* 1989; Inderlied *et al.* 1993). The mechanics of endoscope disinfection are described by Babb and Bradley (1991) and Babb (1993).

George (1988) has listed the main requirements for preventing hospital acquired tuberculosis as being (a) an occupational health scheme, (b) constant vigilance by staff, (c) the implementation of appropriate policies, *viz.* isolation of patients, control of infection and disinfection. This paper will examine the effects of biocides on mycobacteria; other workers have previously emphasized the problems associated with the disinfection of endoscopes with particular reference to mycobacteria.

2. SPECTRUM OF ACTIVITY OF BIOCIDES

Different organisms react differently to biocidal agents, and progress continues to be made in understanding the underlying reasons for these responses (Russell and Russell 1995; Russell and Chopra 1996). In this section, the comparative responses of different types of micro-organisms to biocides will be reviewed, together with the antimycobacterial properties of well-known and commonly used chemical agents.

2.1 Levels of disinfection

Spaulding *et al.* (1977), Favero (1985, 1991) and Favero and Bond (1991a, b, 1993) have described three levels of disinfection: high, intermediate and low (Table 1). Based on this classification, mycobacteria are more resistant to biocides than other non-sporulating bacteria but less resistant than bacterial spores. Several qualifications are, however, necessary. A biocide with a high level of activity has a lethal action against all types of micro-organisms except high numbers of bacterial spores; one with an intermediate level has a mycobactericidal effect together with inactivation of other vegetative bacteria, fungi and most viruses; one with a low level of activity lacks a mycobactericidal effect but most vegetative bacteria, some fungi and some viruses are inactivated.

This information, and that provided in Table 2, takes no account of the effect of extraneous factors which are considered in Section 2.3.

Clearly, differences exist in the response of different types of micro-organisms to biocides (Medical Devices Directorate 1993) and much current research is being devoted to obtaining a better understanding of the mechanisms involved. Mycobacteria can, on the basis of the information provided here and in Section 2.2, be considered as having a sensitivity towards biocides intermediate between that of bacterial spores on the one hand and cocci on the other.

Table 1 Level of disinfection

Level of disinfection	Activity against
High	Spores*, mycobacteria, non-sporulating bacteria, fungi† and viruses (lipid-enveloped and non-lipid-enveloped)
Intermediate	Mycobacteria, non-sporulating bacteria, fungi† and viruses (lipid-enveloped and non-lipid-enveloped‡)
Low	Non-sporulating bacteria, fungi†, non-lipid viruses‡ and lipid viruses

Based on Favero (1985, 1991) and Favero and Bond (1991a, b, 1993).

* Prolonged periods of time necessary if large numbers of spores present.

† Not necessarily chlamydospores or sexual spores.

‡ Virucidal activity may be limited.

Table 2 Mycobacteriostatic and mycobactericidal activity

Compound(s)	Antimycobacterial activity*	Comment
Alcohols	C	
Aldehydes		
Formaldehyde	C	Chemosterilizer
Glutaraldehyde	C	
Glyoxal	C	
Succinaldehyde	C	
Chlorhexidine	S	High concentrations not mycobactericidal
Halogens		
Chlorine-releasing agents	C	
Iodine-releasing agents	C	
Organomercurials	S	
Peroxygens	S/C	Varies with individual peroxygens
Phenolics	S/C	Varies with individual phenols
Gaseous		
Ethylene oxide	C	Chemosterilizing agents
β-Propiolactone	C	
Quaternary ammonium compounds	S	Even high concentrations normally not mycobactericidal

* S, Mycobacteriostatic; C, mycobactericidal.

2.2 Types of biocides

Several chemically different types of biocides are available. They include aldehydes, alcohols, bisbiguanides, halogen-releasing agents (HRAs), isothazolones, mercurials, organic acids and esters, phenolics and quaternary ammonium compounds (QACs), together with gaseous agents such as ethylene oxide. Some of these demonstrate bactericidal activity towards non-mycobacterial vegetative bacteria, whereas others are also mycobactericidal and sporicidal.

Their anti-mycobacterial properties are summarized in Table 2. It is important, however, to discuss here these properties in rather more detail. Additional information can be found by consulting Russell and Hugo (1987), Gardner and Peel (1991), Rubin (1991), Hugo and Russell (1992) and Russell (1992a, 1994). The information is not always precise, however, with discrepancies sometimes being found as to whether a particular biocidal agent is actually mycobactericidal. Possible reasons for these differences of opinion may reside in the differences in experimental procedure (Section 3) undertaken by various authors.

Croshaw (1971) listed ampholytic surfactants (e.g. the 'Tego' compounds), ethylene oxide gas, iodine, alcohols and especially phenolic compounds, notably cresol-soap formulations, as being mycobactericidal. Notable omissions from this list were formaldehyde and glutaraldehyde. The latter had been found by Bergan and Lystad (1971) to be surprisingly ineffective against tubercle bacilli. Conflicting results had been obtained with formaldehyde, although alcoholic solutions were more potent (Rubbo and Gardner 1965; Rubbo *et al.* 1967). It is now considered (British Standard, 1991) that aldehydes such as formaldehyde, glyoxal, succinaldehyde and glutaraldehyde all have a wide spectrum of activity, including sporicidal. Glutaraldehyde in particular is widely used in the disinfection of flexible fibreoptic endoscopes.

Alkaline glutaraldehyde, 2%, is now widely regarded as being an effective mycobactericidal agent against *Myco. tuberculosis*, *Myco. smegmatis*, *Myco. fortuitum* and *Myco. terrae* (Collins and Montalbine 1976; Miner *et al.* 1977; Axon and O'Conner 1983; O'Conner and Axon 1983; Collins 1986a, b, 1987; van Klingeren and Pullen 1987; Axon 1991; Babb and Bradley 1991; Broadley *et al.* 1991; British Standard, 1991; Bruch 1991; Rubin 1991; Rutala *et al.* 1991; Uttley and Pozniak 1993; Ayliffe *et al.* 1993; Uttley and Simpson 1994). Ayliffe *et al.* (1992), however, have stated that only small numbers of *Myco. tuberculosis* are inactivated after 10 min, and Carson *et al.* (1978) have noted that there was a variation in resistance of strains to glutaraldehyde and formaldehyde and found strains of *Myco. chelonae* (*Myco. chelonei*) and *Myco. fortuitum* grown in commercial distilled water (CDW) to be very resistant to chlorine; some of the CDW-grown strains of *Myco. chelonae* gave only a 2·5 $\log_{10}$ reduction in viability after 60 min exposure to 2% alkaline glutaraldehyde. Van Klingeren and Pullen (1993) have isolated *Myco. chelonae* subsp. *abscessus* from endoscope washers

which were not killed by a 60 min exposure to alkaline glutaraldehyde, whereas a reference strain showed an inactivation of 5 $\log_{10}$ after 10 min. They hypothesized that resistance to glutaraldehyde might be developing. It is of interest that the resistant *Myco. chelonae* was also resistant to peracetic acid (cf. Lynam *et al.* 1995) but not to a phenolic or to the chlorine-releasing agent (CRA), sodium dichloroisocyanurate (NaDCC). Other workers have also noticed an above-average resistance of *Myco. chelonae* to glutaraldehyde (Nye *et al.* 1990; Uttley and Simpson 1994). This organism has a particular propensity to adhere to smooth surfaces (Uttley and Simpson 1994) which might well be a contributory factor to its resistance. A particular problem with many alkaline glutaraldehyde formulations is their comparatively poor stability (Robinson *et al.* 1988; Table 3).

MAI are the most frequent type of mycobacteria isolated from AIDS patients (Hanson 1988). These organisms have been found to be much more resistant to alkaline glutaraldehyde than *Myco. tuberculosis* (Collins 1986b; Hanson 1988; Broadley *et al.* 1991; Coates and Hutchinson 1994; Holton *et al.* 1994). Relyveld (1977) found that glutaraldehyde was less effective than hypochlorite against mycobacteria.

Favero (1985, 1991) and Favero and Bond (1991a, b, 1993) cited 2% alkaline glutaraldehyde, 8% formaldehyde in 70% alcohol, 6–10% stabilized hydrogen, gaseous ethylene oxide, alcohol (70–90%), 0·5% iodine in alcohol, 1% aqueous iodine, chlorine compounds and phenolics as being tuberculocidal. Alcohol has been known for many years as being in this category (Smith 1974) and probably enhances the activity of other agents. Phenolics are also highly effective as mycobactericidal agents (Hegna 1977; Richards and Thoen 1979). Chlorhexidine and QACs are low-level disinfectants and inhibit the growth of some mycobacteria but are not lethal (Hirsch 1954; Patterson *et al.* 1956; Fodor and Szabo 1980; Broadley *et al.* 1991). However, according to Ascenzi *et al.* (1986, 1987) a QAC had a similar tuberculocidal activity to 2% glutaraldehyde and Holton *et al.* (1994) have recently claimed that a high concentration QAC product was also mycobactericidal. Other workers (Best *et al.* 1990) have demonstrated the lack of mycobactericidal activity of QACs and in the author's experience this conclusion is correct. A new product containing a mixture of alkyl polyguanides and alkyl quaternaries is claimed to be mycobactericidal under in-use conditions (Nicholson *et al.* 1995).

Possible mycobacterial activity of peroxygens was not considered by Baldry and Fraser (1988). Current opinion (Broadley *et al.* 1993; Cutler and Wilson 1993; Holton *et al.* 1994; Taylor *et al.* 1994) suggests an activity well below that of glutaraldehyde against mycobacteria. Nevertheless, peracetic acid is sporicidal (Baldry and Fraser 1988; Bradley *et al.* 1995), which implies that it is also mycobactericidal, and has been claimed to be effective against glutaraldehyde-resistant mycobacteria (Lynam *et al.* 1995).

Alcohols are usually considered to be mycobactericidal when used at appropriate concentrations (Favero 1985, 1991; Favero and Bond 1991a, b), although some earlier studies (Frobisher and Sommermeyer 1953) suggested a reduced effect on organisms in sputum. Ethylene oxide has been employed (Newman *et al.* 1955) to decontaminate materials handled by tuberculosis patients.

2.3 Factors influencing antimycobacterial activity

The antimicrobial activity of biocides is influenced by many factors. These include concentration and temperature at which the biocide is used, its pH, period of contact and whether any interfering substances are present. Such interfering agents include organic soiling matter (e.g. blood,

Table 3 Stability and activity of glutaraldehyde solutions

Parameter	Result
Stability of solutions	
Storage length	Alkaline solutions lose potency on storage
pH	Stable at acid pH, rapid decay at alkaline
Temperature	Stability of alkaline solutions decreases as temperature rises
Activity of solutions	
Storage period	Usually *ca* 2 weeks, 'long-life' *ca* 4 weeks
pH	Optimum pH 8–8·5
Temperature	Increased activity
Organic soil	Activity reduced
Type of organism	Mycobacteria generally intermediate in susceptibility between other non-sporulating bacteria (most sensitive) and spores (most resistant)

Based on Russell (1994).

serum, pus, urine, milkstone), non-ionic or anionic surface-active agents and containers and closures.

The effects of concentration of the biocide, period of contact and temperature of usage have been quantified mathematically (Russell 1992b). However, these aspects have been considered with organisms such as *Escherichia coli* and *Staphylococcus aureus* and there is no evidence to suggest that these factors would similarly affect the antimycobacterial activity of biocides. Mycobacteria may be killed more slowly than other non-sporulating bacteria.

Organic soiling matter is known to reduce the activity of many biocides, as demonstrated in Tables 3 and 4. This reduction in activity is probably due to an interaction between a biocide and organic soil, and this must clearly be taken into account in the design of mycobactericidal test methods (Section 3.2).

The activity of many biocides is also dependent upon the environmental pH (Tables 3 and 4). This is particularly true of mycobacteriostatic agents such as chlorhexidine and QACs and, more importantly, of mycobactericidal agents such as glutaraldehyde, CRAs and iodine-releasing agents (Russell 1992b). Glutaraldehyde (Table 3) is most stable at acid pH, the form in which it is purchased, but most active against mycobacteria and other organisms at alkaline pH, around 8–8·5 (Stonehill *et al.* 1963; Borick *et al.* 1964; Borick 1968; Gorman *et al.* 1980; Scott and Gorman 1991; Russell 1994). In practice, it is usual to 'activate' (alkalinate) an acid solution immediately before use. Such activated solutions lose activity in storage and should not be used after 1–2 weeks. Various reports have appeared in order to assess the biocidal (including mycobactericidal) activity of reused alkaline glutaraldehyde solutions in endoscopy units (Bageant *et al.* 1981; Isenberg *et al.* 1988; Mbithi *et al.* 1993). Two per cent alkaline glutaraldehyde solutions became ineffective against mycobacteria in much less than 14 d in reuse baths (Mbithi *et al.* 1993). Alkaline glutaraldehyde formulations have been developed with a shelf-life of up to 28–30 d.

Table 4 Factors influencing antimycobacterial activity of biocides

Factor	Influence on antimycobacterial activity
Concentration	Activity rises as concentration increases; mathematical relationship unknown (unlike several other bacteria)
Period of contact	Long periods may be necessary to achieve mycobacterial effect
Temperature of treatment	Activity generally increases with rise in temperature
pH	Depends on individual agents, e.g. CRAs, phenols: more active at acid pH; chlorhexidine, QACs, glutaraldehyde: more active at alkaline pH
Presence of organic matter	Several agents adversely affected, notably chlorhexidine and QACs; glutaraldehyde less affected
Other factors	Interfering substances, e.g. rubber, containers and closures, presence of non-ionic surfactants may reduce activity of some antimycobacterial agents

Based on Russell (1992b).

3. EVALUATION OF ANTIMYCOBACTERIAL ACTIVITY

There is no universal test method for evaluating the activity of biocidal agents towards mycobacteria although various official bodies have published their own procedures. In his excellent account of the evaluation of antibacterial and antifungal activity of disinfectants, Reybrouck (1992) has described suspension, capacity and carrier tests as well as tests under practical conditions and has stated that several of these have been adapted for testing mycobactericidal activity. One problem associated with mycobacteria is the fact that most pathogenic types grow very slowly so that long periods of incubation may be necessary for completion of tests. Rapidly growing strains of mycobacteria are thus on occasion employed as 'indicators' (models) of *Myco. tuberculosis* (Section 3.3).

3.1 Mycobacteriostatic activity

Mycobacteriostatic tests are equivalent to those that determine the minimum inhibitory concentrations (MICs) of antibacterial and antifungal agents, although (as stated above) much longer periods of incubation are necessary with mycobacteria. MIC values are not necessarily significant but they do provide preliminary information about those concentrations that could be tested in mycobactericidal procedures. Furthermore, it is interesting to note that MICs *vs* mycobacteria tend generally to be of the same order as those against other bacteria (Table 5), although *Myco. avium* may show high resistance.

3.2 Mycobactericidal activity

As with biocidal agents generally, a whole gamut of tests has been devised to evaluate mycobactericidal activity. These range from suspension tests to carrier tests with more recent ventures which used different turbidometric assessments. It

Table 5 Minimum inhibitory concentrations (MICs) of antibacterial agents towards mycobacteria and other bacteria

	MIC (μg ml^{-1}) *vs*			
	Mycobacteria	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*
Cetylpyridinium chloride	5–10‡	0·5	100	>100
Chlorhexidine diacetate	0·5–1*	0·5–1	1	5–60
Cetrimide	5–10	4	32	64–128
Chlorocresol	100–250	625	1250	
Hydroxybenzoates				
methyl	2500	800	800	1000
ethyl	500	500	560	700
propyl	320	150	350	350
butyl	225	120	160	150

Based on Broadley *et al.* (1991), Hugo and Russell (1992) and Russell (1992b).
* *Mycobacterium avium* is more resistant.

is neither possible nor relevant to present here a detailed account of all of the methods employed by various authors. Instead, Table 6 summarizes the types of methods and their salient features and lists official and other sources that can be consulted for further information.

One aspect that must be mentioned, however, which is applicable to all methods is the necessity of ensuring adequate neutralization or removal of the antibacterial agent at the time of sampling. This simple, but fundamental, point is not always considered adequately, with a consequent overestimation of mycobactericidal activity. Neutralization procedures are essentially of three types (Russell 1981): chemical neutralization, quenching by dilution to a subinhibitory concentration or the use of membrane filtration. Compounds with high dilution coefficients (η-values) such as phenolics rapidly lose activity on dilution whereas those with low (e.g. mercurials) or intermediate (e.g. QACs, chlorhexidine) η-values are best quenched chemically. Glycine (2% w/v) is an adequate neutralizer for glutaraldehyde in mycobactericidal testing procedures (Broadley *et al.* 1991) although some authors have used 1% w/v sodium bisulphite (Collins and Montalbine 1976; Collins 1986a, b; Hardie 1986) and others, for reasons not stated, used Letheen (a mixture of lecithin and polysorbate: Isenberg *et al.* 1988). Sodium bisulphite is

Table 6 Methods for evaluating mycobactericidal efficacy*

Test method	Principle of procedure	Reference(s)
AFNOR	Suspension test (dilution-neutralization and membrane filtration) *vs Mycobacterium smegmatis*	AFNOR 1988
DGHM	Qualitative suspension test *vs Myco. tuberculosis*	DGHM 1981
MAFF	Suspension test (60 min, 4°C) *vs Myco. fortuitum*	British Standard 6734, 1986
AOAC	Carrier test (a) Presumptive *vs Myco. smegmatis* (b) Confirmatory *vs Myco. bovis* BCG†	AOAC 1984
Other		
(1) Carrier	Combined test using *Myco. bovis* BCG, *Staphylococcus aureus*, *Bacillus stearothermophilus* spores, *Trichophyton mentagrophytes* and poliovirus type 1 (Sabin)	Best *et al.* 1994
(2) Endoscopes	Activity of biocide *vs* organisms dried on to endoscope Endoscopes heavily contaminated with *Myco. tuberculosis*	Cutler and Wilson 1993 Hanson *et al.* 1992
(3) Bactec	Measurment of release of $^{14}CO_2$ from radiolabelled amino acid	Broadley *et al.* 1993
(4) [^{35}S]-methionine	Incorporation of [^{35}S]-methionine into protein	Holton *et al.* 1994
(5) Membrane filtration	Counting of colonies on the surface of membrane filters	Ascenzi *et al.* 1987; Collins 1987

* For a detailed resumé of 'Official' methods, see Cremieux and Florette (1991).
† Preliminary check of resistance of organism to phenol necessary.

itself an antibacterial agent, although not necessarily mycobactericidal, but there is some evidence that it might actually enhance the lethal activity of glutaraldehyde (Collins 1986a). Some workers use dilution to quench the action of glutaraldehyde (Davis *et al.* 1984; Holton *et al.* 1994) and of QACs (Garcia de Cabo *et al.* 1978; Holton *et al.* 1994) and certainly there have been instances of false claims being made about the postulated mycobactericidal activity of QACs because of a failure to prevent bacteriostasis in subculture media (Leers *et al.* 1974; Garcia de Cabo *et al.* 1978).

Many mycobactericidal suspension test procedures are based on the enumeration of survivors by colony counting. Alternative methods are now being sought, in particular the use of biochemical techniques such as the release of ^{14}C-CO_2 from a radiolabelled amino acid in a growth medium, e.g. the 'BACTEC' apparatus. As yet, however, this gives only a qualitative response but it is not inconceivable that a more quantitative assessment could be obtained by linking growth with viable numbers (Broadley *et al.* 1995). Another non-counting procedure that merits consideration is the incorporation of ^{35}S-methionine into protein (Holton *et al.* 1994): in this method, bacteria are grown on a medium containing the labelled amino acid before exposure to biocide. After exposure, incorporation of ^{35}S-methionine into protein is measured. It is unlikely, however, that this procedure will be used routinely.

A membrane filtration method, in which washing *in situ* removes residual biocide and in which colony counting is facilitated on the membrane itself, has been described by Ascenzi *et al.* (1986) and by Collins (1987). Carrier methods are considered by Leers *et al.* (1974), Davis *et al.* (1984), Collins (1986a), Best *et al.* (1988, 1990, 1994) and Mbithi *et al.* (1993).

'Official' methods of evaluating mycobactericidal, or other, activity of test compounds are well documented by Cremieux and Fleurette (1991; Table 6). These include the suspension tests listed by AFNOR (1988), DGHM (1981) and British Standard 6734 (1986) and the carrier method recommended by the AOAC (1984). It is noticeable (see Section 3.3) that some of these procedures use *Myco. smegmatis* as the test organism. The AOAC (1984) method has been criticized by various workers (Hardie 1986; Ascenzi 1991).

Standardization of mycobactericidal test methods is undoubtedly needed (Ayliffe 1989; Ascenzi 1991). Cutler and Wilson (1993) have transposed this point to the standardization of disinfectant testing of contaminated endoscopes and have posed the following questions: what strain, inoculum size, type of organic matter, contact time, sampling technique, washing technique, recovery medium and period of incubation should be employed? The subject of a suitable recovery medium has actually received little attention, although Ascenzi *et al.* (1987) have demonstrated the superiority of Middlebrook 7H10 and 7H11 agars over Lowenstein-Jensen medium. These are all important points that need to be addressed.

For additional information on test methods of evaluating mycobactericidal activity, the interested reader is referred to Sonntag (1978), Schleiser (1979), Borneff (1981), Parkinson (1981), Lind *et al.* (1986), Quinn (1987), Eigner (1988), Ayliffe (1989), Cole *et al.* (1990), Babb *et al.* (1992) and Reybrouck (1992).

3.3 Indicator organisms

Several species of mycobacteria are highly pathogenic and are also slow-growing. Attempts have therefore been made to replace organisms such as *Myco. tuberculosis* with other, less pathogenic, faster-growing strains. A major issue also, is that such an indicator organism should respond to biocides in a manner that describes the mycobacteria in general and probably the most resistant member in particular. *Mycobacterium tuberculosis* is still regarded as the most important member of the genus but is not necessarily the most resistant to biocides. Extrapolating the findings from an indicator organism to the clinical situation can be fraught with danger. Many of the 'Official' test procedures described in Section 3.2 use *Myco. smegmatis* as the test organism. There is ample evidence, however, which shows that *Myco. smegmatis* might not be a suitable indicator organism because it is much more sensitive to biocides than *Myco. tuberculosis* (van Klingeren and Pullen 1987). On the other hand, *Myco. terrae* and *Myco. tuberculosis* had a similar response to biocides in a quantitative suspension test involving the calculation of ME (microbicidal effect) values (van Klingeren and Pullen 1987) and *Myco. terrae* might thus be a suitable indicator organism when assessing biocides for possible tuberculocidal activity.

However, *Myco. tuberculosis* is not necessarily the most resistant mycobacterial species to biocides, problems frequently being encountered with MAI strains (Collins 1986b; Hanson 1988; Woodcock *et al.* 1989) and *Myco. chelonae* (Carson *et al.* 1978; George 1988; Nye *et al.* 1990; van Klingeren and Pullen 1993; Taylor *et al.* 1994; Uttley and Simpson 1994). Thus, studies with *Myco. terrae* alone will not suffice when information about these other mycobacteria is needed.

3.4 Practical situation: endoscopes

Most of the methods discussed above are *in vitro* ones. Tests on actual endoscopes experimentally contaminated with mycobacteria (Hanson *et al.* 1992; Cutler and Wilson 1993) are likely to provide more meaningful data about disinfection processes, including biocide concentration and periods of contact.

4. MECHANISMS OF MYCOBACTERIAL INACTIVATION BY BIOCIDES

Mechanisms of inactivation of most non-sporulating bacteria by biocides have been examined comprehensively (Hugo 1992). Progress with bacterial spores is also proceeding satisfactorily, if slowly. Very few concerted attempts have, however, been made to explain the mechanisms of action of the comparatively few agents that inactivate mycobacteria (Russell 1992a).

To discuss this aspect adequately, it is necessary to consider the mycobacterial cell (and in particular the structure and composition of the cell wall), the uptake of biocides into the cell and the potential target site(s) at which interaction produces stasis or a lethal effect.

4.1 The mycobacterial cell

The mycobacterial cell contains a comparatively thick cell wall which consists of several components (Draper 1984). The 'covalent cell wall skeleton' (Fig. 1a, b) is made up of two covalently linked polymers, *viz.* peptidoglycan and a mycolate of arabinogalactan (David 1981). The peptidoglycan contains *N*-glycolmuramic acid instead of the more widely found *N*-acetylmuramic acid, whereas the arabinogalactan mycolate contains D-arabinose and D-galactose (ratio *ca* 5 : 2), with about 10% of the arabinose residues esterified by a molecule of mycolic acid (Fig. 1a). Cell wall lipids occur as free lipids, wax D and cord factors (Petit and Lederer 1978; Inderlied *et al.* 1993).

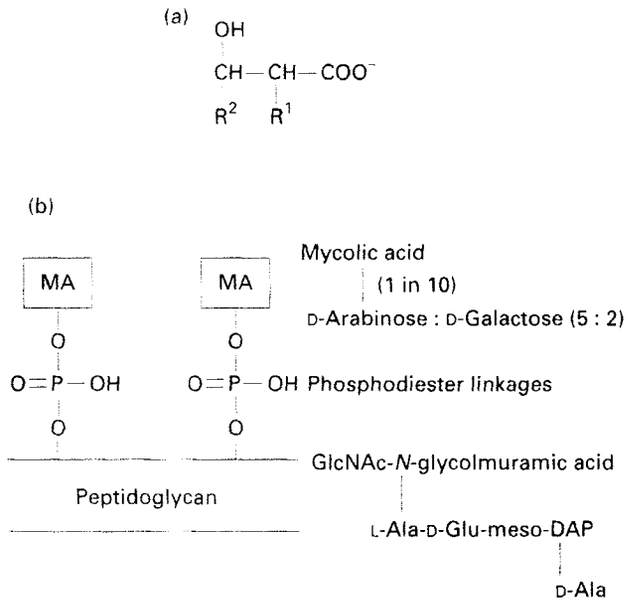

Fig. 1 (a) General structure of mycolic acids: R^1 and R^2 are alkyl groups that may be saturated or unsaturated. (b) Diagrammatic representation of the mycobacterial cell wall. MA, Mycolate of arabinogalactan

The mycolic acids make up about 50% by weight of this lipopolysaccharide core of mycoylarabinogalactanpeptidoglycan. The mycobacterial cell wall is highly hydrophobic in nature (Nikaido *et al.* 1993) and the mycolic acids have an important role to play in reducing permeability to hydrophilic molecules. Porins are also present, thereby enabling hydrophilic molecules to cross the wall, and these appear to be similar (Jarlier and Nikaido 1990) to those found in *Pseudomonas aeruginosa* cell envelopes so that only low molecular weight hydrophilic substances can enter the cell by this route.

Interior to the mycobacterial cell wall is the cytoplasmic (plasma) membrane which surrounds the cytoplasm. The membrane is lipoprotein in nature (Inderlied *et al.* 1993). The cytosol contains DNA, RNA, protein and enzymes.

4.2 Uptake of biocides

The uptake of antibiotics into Gram-positive and Gram-negative bacteria has been well documented (Russell and Chopra 1996) and a reasonable amount of information is also available about biocide uptake. In contrast, mechanisms of the uptake of biocides into mycobacteria are poorly understood, although progress is being made, mainly due to an improved knowledge of the mycobacterial cell wall (Section 4.1) and also to studies undertaken with hydrophilic substances (Jarlier and Nikaido 1990).

The first stage in the uptake of a biocide by a mycobacterial cell probably involves an adsorption process to the cell wall. This has been amply demonstrated with various biocides and other non-sporulating bacteria (Hugo 1992) but has yet to be shown with mycobacteria. Passage across the cell wall could then take place with interaction with sensitive target site(s) at the cytoplasmic membrane or deeper within the cell.

It would be interesting to examine the uptake of biocides by various species of mycobacteria ranging from the more sensitive type (*Myco. smegmatis*) to the more resistant ones (MAI and *Myco. chelonae*). Adsorption *per se* does not necessarily correlate with sensitivity or resistance but does, nevertheless, provide information about the primary interaction of a biocide with a cell. It would also be interesting to try to relate biocide uptake with the hydrophobic nature of the cell surface of different species, although Hardham and James (1981) could find no difference in the cell surface properties of different species of mycobacteria.

4.3 Potential target sites

Whilst the mycobacterial cell wall is a prime target site for chemotherapeutic drugs that can inhibit the biosynthesis of a specific cell wall polymer (Draper 1984), it is unlikely that

Table 7 Possible cell wall components involved in mycobacterial resistance to biocides

Cell wall component	Inhibitor of synthesis*	Relevance to resistance to	
		Antibiotics	Biocides
Mycoside C	*m*-Fl-phe	Yes	Unknown†
Arabinogalactan	Ethambutol	Yes	Yes
Mycolic acid	MOCB	Yes	Unknown†

* *m*-Fl-phe, *m*-Fluoro-D-phenylalanine; MOCB, methyl 4-(2-octadecylcyclopropen-1-yl) butanoate.
† Not yet tested.

the wall is a major target site for many biocidal agents. An exception might be glutaraldehyde, as it binds strongly to the surface of bacterial cells (Eager *et al.* 1986). Unfortunately, no detailed studies of the effects of the dialdehyde on mycobacterial cell walls have been made.

The underlying cytoplasmic membrane is a potential target site for the so-called 'membrane-active' agents such as chlorhexidine and QACs. In non-sporulating bacteria these chemicals damage the membrane, with higher concentrations causing intracellular precipitation of proteins and nucleic acids (Russell and Chopra 1996). Low concentrations of chlorhexidine and the QAC cetylpyridinium chloride (CPC) are tuberculostatic at concentrations of the same order as those that inhibit other bacteria (Broadley *et al.* 1991; Table 5), but are not mycobactericidal. These findings imply that chlorhexidine and CPC must cause membrane damage and that either this is insufficient to render the cells non-viable or too small a concentration is available to produce cytoplasmic damage or both. MAI is more resistant to chlorhexidine and CPC but the reason for this is probably reduced uptake (Section 5).

Within the cytosol are several potential target sites: proteins and enzymes, DNA and RNA. Although information is readily available about the effects of biocides on non-sporulating bacteria in general (Russell and Russell 1995; Russell and Chopra 1996), data on interaction of biocides with such targets in mycobacteria are sparse.

5. MECHANISMS OF MYCOBACTERIAL RESISTANCE TO BIOCIDES

5.1 Resistance mechanisms: general aspects

Bacterial resistance to biocides is generally of two types: intrinsic (innate), a natural property of an organism, or acquired (either by mutation or by the acquisition of plasmids or transposons) (Russell and Russell 1995; Russell and Chopra 1996). Intrinsic resistance occurs either by enzymatic inactivation or more usually by diminished cellular permeability; the latter is exemplified by the reduced sensitivity of Gram-negative bacteria, and especially of organisms such as *Pseudomonas aeruginosa* and *Proteus* and *Providencia* spp. in comparison with staphylococci, and the markedly enhanced resistance to biocides of bacterial spores. Mycobacteria also fall into this category and there are no published data about plasmid- or transposon-mediated resistance to biocides.

5.2 Intrinsic resistance: the mycobacterial cell wall as a barrier

As pointed out in Section 4, the mycobacterial cell wall is highly hydrophobic with a mycoylarabinogalactanpeptidoglycan skeleton (Fig. 1). Hydrophilic antibacterial agents are thus unable to penetrate the cell wall in sufficiently high concentrations to achieve a mycobactericidal effect. The wall thus acts as a permeability barrier to such compounds. Low concentrations must traverse the wall because MICs against mycobacteria are generally of the same sort of order as those found with other types of bacteria (Table 5), although *Myco. avium* may be particularly resistant (Broadley *et al.* 1991).

The component/components of the mycobacterial cell wall responsible for high biocidal resistance is/are unknown. There are, however, certain clues (Table 7) which, if developed properly, could lead to a better understanding of this mechanism of resistance. Inhibitors of cell wall synthesis have been shown to increase the susceptibility of *Myco. avium* to drugs (Rastogi *et al.* 1990). These inhibitors can be considered on the basis of which wall component they inhibit; for example, *m*-fluoro-DL-phenylalanine (*m*-Fl-phe) is an inhibitor of mycoside C biosynthesis in this organism (David *et al.* 1988). Ethambutol (EB) is an inhibitor of phospholipid (Sareen and Khuller 1988, 1990) and arabinogalactan (Takayama and Kilburn 1988; Rastogi *et al.* 1990) biosynthesis, with the latter believed to be its major effect. Methyl 4-(2-octadecylcyclopropen-1-yl) butanoate (MOCB) is a structural analogue of a key precursor in mycolic acid biosynthesis (Wheeler *et al.* 1993). Treatment of *Myco. avium* with *m*-Fl-phe produces significant alterations in the outer cell wall layers (David *et al.* 1988). EB also disorganizes these layers

and, in addition, can induce the formation of bacterial ghosts without the dissolution of the peptidoglycan (Rastogi *et al.* 1990). Thus, effects on cell wall architecture induced by *m*-Fl-phe and EB are responsible for increasing the intracellular penetration of chemotherapeutic drugs and support the concept of the cell wall acting as a barrier (Chargaff *et al.* 1931; David 1981; David *et al.* 1987a, b; Rastogi *et al.* 1981, 1986; McNeil and Brennan 1991; Russell 1992a; Stickler and King 1992). Croshaw (1971) quotes the work of T. H. Shen who, in 1934, correlated resistance with the content of material in the wall.

Ethambutol has been tested in combination with two biocides, chlorhexidine diacetate (CHA) and cetylpyridinium chloride (CPC). The activities of both CHA and CPC against *Myco. avium* increased significantly in the presence of EB (Russell *et al.* 1994; Broadley *et al.* 1995). These findings provide tentative evidence that the arabinogalactan component of the mycobacterial cell wall skeleton is involved in the exclusion mechanism but do not, of course, rule out the possibility that other wall components are also contributory factors. What is clearly needed is a series of experimental approaches that utilize specific cell wall synthesis inhibitors so that the relative importance of the wall components in conferring resistance to biocides can be determined. In the end, of course, a mere 'opening-up' of the cell wall architecture, however achieved, may be all that is needed for CHA, CPC and other biocidal agents to penetrate through the wall.

Spheroplasts and L-forms of bacteria can be produced under specific conditions (Willett and Thacore 1966, 1967). These modified cell wall forms might be useful in studying intrinsic resistance of mycobacteria to biocides although they do not, as yet, appear to have been used for this purpose.

A further problem in the inactivation of mycobacteria by biocides may be presented by the incorporation of these organisms into biofilms (Uttley and Simpson 1994). The specific case of *Myco. chelonae* has been alluded to frequently, particularly the high resistance presented by some strains to glutaraldehyde. The mechanism of this resistance is unknown, but could be associated with biofilm production, cell wall barrier or reduced aldehyde uptake.

6. POSSIBLE METHODS OF POTENTIATING MYCOBACTERICIDAL ACTIVITY

Previous sections (4 and 5) have considered the mechanism of antimycobacterial action of, and mycobacterial resistance to, antibacterial agents. In each instance, the sparse information available was emphasized. Nevertheless, the data do suggest some ways whereby mycobactericidal activity could be increased.

Very few new biocidal agents are likely to be produced in the foreseeable future. However, one such compound is orthophthalaldehyde, which is claimed to be a potent microbicidal agent (Alfa and Sitter 1994) although its activity against mycobacteria has yet to be assessed.

One likely possibility is the use of a combination of two biocides to produce an enhanced response, another is to combine a potent agent with other compounds, as recently done by Gordon *et al.* (1994) who increased the mycobactericidal activity of glutaraldehyde with α,β-unsaturated and aromatic aldehydes. An improved knowledge of those components of the mycobacterial cell responsible for causing intrinsic resistance could also be important, but the use of specific cell wall synthesis inhibitors to improve intracellular penetration is likely to be of more significance in the field of chemotherapy than with biocidal compounds (Hoffner *et al.* 1989; Russell and Chopra 1996)

7. CONCLUSIONS

Comparatively few disinfectant-type agents are actively mycobactericidal. Even with those that are mycobactericidal, problems can arise practically because of (a) limited use, e.g. ethylene oxide, or (b) high toxicity to personnel, e.g. glutaraldehyde. The dialdehyde has been employed extensively in hospital endoscopy units but concerted attempts are now being made to replace it by other equally active, less toxic agents. Peracetic acid-based formulations might have a role to play here.

The mechanisms of mycobactericidal action of antibacterial agents are poorly understood and there is scope for much further study in this area. Some progress has been reported in the understanding of the nature of the resistance presented to biocides by the mycobacterial cell wall but this has yet to be translated into improvements in the design of new mycobactericidal agents or in the development of combinations of existing biocides. With the isolation of mycobacterial strains that occasionally show enhanced resistance to glutaraldehyde, and possibly to other antibacterial agents also, it is clear that further research is needed urgently.

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