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Review Article

Mechanisms of action of chemical sporicidal and sporistatic agents

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Introduction

Chemical antimicrobial agents are widely used as preservatives in many types of pharmaceuticals (Parker, 1982; Beveridge, 1983), cosmetics (Parker, 1982) and foods (Lueck, 1980; Foegeding and Busta, 1981; Sofos and Busta, 1982) and several other types of industrial products (Bravery, 1982; Hill, 1982a and b; Hugo, 1982; Richardson, 1982; Springle and Briggs, 1982; Weir, 1982). They are also employed as disinfectants or antiseptics (Hugo and Russell, 1982) or, if sufficiently non-toxic, in the systemic treatment of infections. In some instances, chemicals form part of, or contribute to, a sterilization process, e.g. the U.K. process of 'heating with a bactericide' in the sterilization of some injections and some eye preparations (British Pharmacopoeia, 1980). Chemicals may also enhance the effects of γ-radiations on microorganisms.

Comparatively few chemical agents, however, possess sporicidal activity (Waites and Bayliss, 1980; Russell, 1982a; Gould, 1983), although most compounds appear capable of inhibiting either germination or outgrowth of spores in their development into vegetative cells. It is the purpose of this short review to discuss current knowledge of the ways in which chemical sporicidal and sporistatic agents work. Inevitably, this means that some information is necessary on the mechanisms of spore resistance to chemical inhibitors.

Spore structure

The bacterial spore (Fig. 1) may be envisaged as consisting of an outer spore coat (or coats), an intermediate cortex and an inner core or protoplast. Chemically (Table 1), the coat is made up of protein material, whereas the cortex is composed mainly of peptidoglycan some of which occurs as a unique spore lactam. The innermost region, the core, contains dipicolinic acid (DPA), a unique spore constituent possibly associated with heat resistance.

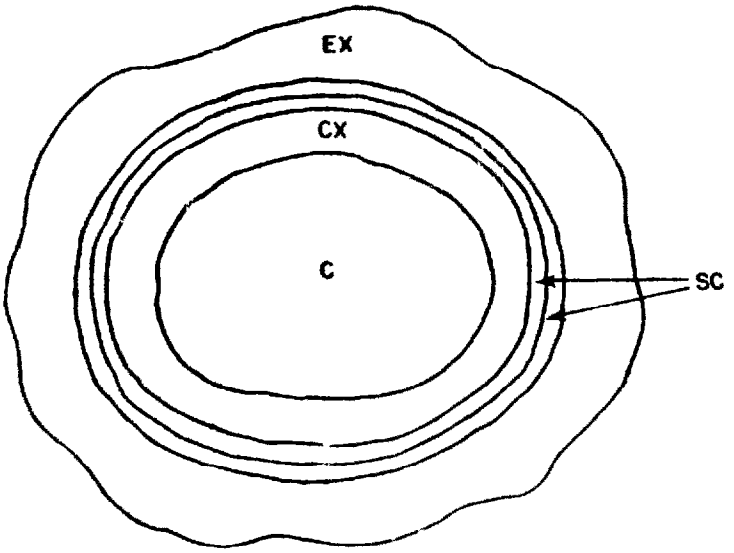

Fig. 1. A 'typical' bacterial spore (not to scale). Ex, exosporium; SC, coats; CX, cortex; C, core.

TABLE 1

CHEMICAL COMPOSITION OF SPORE COMPONENTS

Spore component	Composition	Comment
Outer spore coat	Protein mainly	Alkali-resistant; removed by dithiothreitol (DTT)
Inner spore coat	Protein mainly	Alkali-soluble fraction
Cortex	Mainly peptidoglycan	Presence of internal amide (muramic lactam): see Fig. 3
Core	Protein, DNA, RNA, dipicolinic acid, divalent metals	Unique spore proteins associated with DNA

Structure and resistance

Some of these structures have been implicated in the resistance of mature spores to various inhibitors (Table 2). These conclusions have been reached mainly from two lines of research; in one, spores with defective coats have been used, whereas in the other (Table 3), a correlation has been made between development of resistance with a particular stage in sporulation. A third and very promising approach has been to use certain diaminopimelic acid$^-$ (Dap$^-$) spores which are unable to synthesize this amino acid, which must be provided in culture media. The extent of cortex development is then a function of external Dap concentration. Experiments with Dap$^-$ mutants of *Bacillus sphaericus* have shown that octanol resistance is linked to cortex development (Imae and Strominger, 1976a and b). It is also conceivable,

TABLE 2

IMPLICATION OF SPORE COMPONENTS IN RESISTANCE TO ANTIBACTERIAL AGENTS

Spore component	Implicated in resistance to	Comment
Coat(s)	Lysozyme	UDS *-spores highly sensitive
	Chlorine	
	Peroxide	Varies with strain
	Ethylene oxide?	No simple relationship
	Glutaraldehyde	UDS-spores highly sensitive
	Iodine	
Cortex	Octanol	25% spore cortex in *B. sphaericus* (Dap$^-$)
	Xylenol	

* UDS = urea + dithiothreitol + sodium dodecyl sulphate.

however, that alterations in the content of cortex could lead to changes elsewhere in the spore (Waites, 1982).

The spore coats appear to play a major role in the resistance of bacterial spores to disinfectants. Sodium hydroxide increases the permeability of bacterial spores to germinants (Wyatt and Waites, 1971, 1974; Labbe et al., 1978). The potentiation of hypochlorite action by sodium hydroxide (Cousins and Allan, 1967) may result from the alkali-induced removal of protein from spore coats (Gould et al., 1970; Kulikovsky et al., 1975). The alkali-soluble fraction of *B. megaterium* spores resides in the inner coat (Nishihara et al., 1981a) and consists predominantly of acidic polypeptides which can be dissociated to their unit components by treatment with sodium dodecyl sulphate (SDS) (Nishihara et al., 1980). Alkali will disrupt or solubilize completely the inner, narrow, electron dense coat of *Cl. perfringens* spores (Labbe et al., 1978), but the cortex of bacterial spores is alkali-resistant.

Exposure of spores to agents that disrupt disulphide bonds, and especially when spores are then treated with alkali, renders the organisms sensitive to lysis by lysozyme or peroxide (Gould et al., 1970).

TABLE 3

RESISTANCE TO ANTIBACTERIAL AGENTS ASSOCIATED WITH SPORULATION STAGE

Agent	Resistance at stage	Biochemical event at stage
Xylene, toluene	III–IV	Forespore engulfment in III, cortex formation in IV
Benzene	IV	
Octanol, butanol	V	Cortex in IV, coat in V
Methanol, ethanol	V	
Chloroform	V–VI	Coat in V. Spore maturation
Phenol	VI	

Based on Milhaud and Balassa (1973) and Balassa et al. (1979).

Another procedure for studying the role of the spore coat is to pretreat spores with SDS plus dithiothreitol (DTT) (Mitani and Kadota, 1976). The components extracted arise almost entirely from the coats, with almost all the alkali-soluble fraction and 40% of the 'resistant fraction' (outer coats) removed from coats of *B. megaterium* (Nishihara et al., 1981a). DTT alone at pH 8.5 substantially removes the outer coat, and partially disrupts the inner coat, of *Cl. perfringens* type A spores and at pH 10 the spores are devoid of coats (Labbe et al., 1978). Hydrogen peroxide will itself remove protein, presumably from the coat, in *Cl. bifermentans* (Wyatt and Waites, 1975); however, removal of coat protein by DTT before exposure of spores to peroxide markedly increases the lethal effect of the disinfectant, whereas *B. cereus* spores are much less affected (Waites and Bayliss, 1979). Thus, the spore coat is likely to offer a protective effect against peroxide to the former, but not to the latter, organism. This is an important finding, since it demonstrates that variations in response do occur with different spore-formers and that one reason for this may be the varying composition of the spore coat.

Two or more of the following have been used for extracting coat components: urea, DTT, mercaptoethanol, thioglycollic acid and SDS. The most potent combination in solubilizing coat protein has been found to be UDS (urea + DTT + SDS), followed by UMS (urea + mercaptoethanol + SDS). UDS-treated spores in particular are highly sensitive to glutaraldehyde, iodine, hydrogen peroxide and chlorine, with urea and SDS believed to facilitate penetration of the disulphide bond-reducing agent (Hutchinson, 1982).

The role of the spore coats in spore resistance to ethylene oxide is unclear. Pretreatment of *B. subtilis* spores with thioglycollic acid plus urea alters their response to the gas, suggesting that the coat plays a part in resistance (Marletta and Stumbo, 1970). However, resistance to ethylene oxide of *B. cereus* T spores pretreated with alkaline DTT remains unchanged (Dadd and Daley, 1982). Furthermore, *B. subtilis* 4673 (a mutant of strain 4670) with defective coats and outer coat layers thinner and more diffuse, is rather *more* resistant to ethylene oxide than the wild-type strain, whereas strain EV15 which overproduces coat material, thereby possessing an abnormally thick multi-layered coat, has an exceptionally high resistance (Dadd and Daley, 1982). These findings imply on the one hand that increased permeability to ethylene oxide, expected in mutant 4673, does not occur so that the spore coat is not a barrier to entry; and on the other that increased resistance is associated with an excessive coat production.

The increase that occurs in resistance to antibacterial agents during sporulation may be correlated to the stage of spore development (Table 3). In many instances, the antibacterial chemicals studied (xylene, toluene, benzene, etc.) have been organic solvents rather than pharmaceutical preservatives or disinfectants. However, resistance to chloroform and to phenol develops late in the process and before the onset of heat resistance (Milhaud and Halassa, 1973; Balassa et al., 1979). Resistance to methanol and ethanol occurs at the same time as resistance to other alcohols, such as octanol and butanol. Alcohol-resistant sporulation mutants of *B. subtilis* have been described (Bohin and Lubochinsky, 1982) which can sporulate in the presence of alcohols at a frequency of 30–40%.

Germination and outgrowth

Germination is an irreversible process in which an activated spore changes from a dormant to a metabolically active state within a short period of time. The first biochemical step is a biological trigger reaction. During germination there is a loss in resistance to heat and chemicals, alteration of peripheral spore layers, hydration of spore structures, activation of lytic enzymes, degradation of cortex and release of peptidoglycan fragments and calcium dipicolinate.

Outgrowth is defined as the development of a vegetative cell from a germinated spore and takes place in a synchronous and orderly manner. RNA synthesis is closely followed by the onset of protein synthesis, with DNA synthesis occurring some time later.

Inhibitors of germination and outgrowth

Many agents will inhibit the growth of, or kill, vegetative bacterial forms. Most of these also have some effect on bacterial spores, in the context that, at concentrations very similar to bacteriostatic levels, they will inhibit spore germination and/or outgrowth (Russell, 1971, 1982a). Examples include phenols and cresols, parabens (esters of *p*-hydroxybenzoic acid), organic and inorganic mercury compounds and quaternary ammonium compounds. Fig. 2 illustrates the sites at which these and other inhibitors are effective in vegetative cell development. Parker (1969) describes inhibitors of germination as Category 1 agents, whereas Category 2 agents inhibit outgrowth.

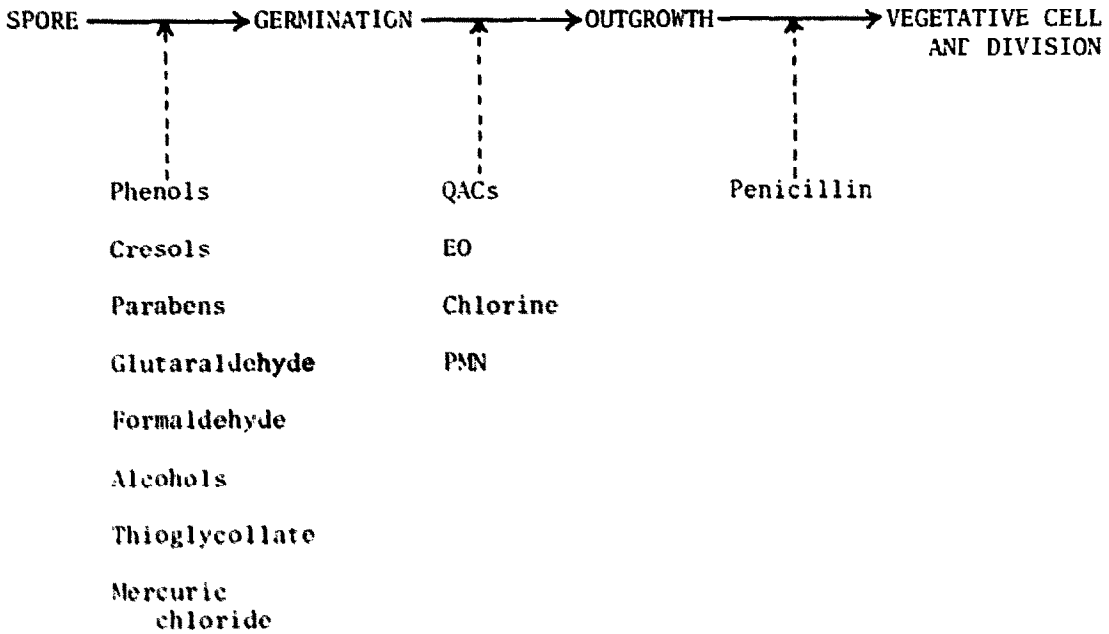

Fig. 2. Effect of some antimicrobial agents on germination and outgrowth. EO, ethylene oxide; QACs, quaternary ammonium compounds; PMN, phenylmercuric nitrate.

The most rapid method of detecting germination and outgrowth is by measuring changes in optical density of suspensions (Gould, 1971). Inhibitors can then be tested at different stages, e.g. by adding to the system at zero time, during germination, at the end of germination (immediately prior to outgrowth) or during outgrowth.

The effects of inhibitors of spore germination may be reversible. This is clearly apparent from studies on phenols (Parker and Bradley, 1968; Lewis and Jurd, 1972; Jones and Russell, 1981), formaldehyde (Trujillo and David, 1972), alcohols (Trujillo and Laible, 1970) and parabens (Watanabe and Takesue, 1976). In practice, it is necessary to achieve removal of the inhibitor, e.g. by the addition of an appropriate neutralizing agent or by use of membrane filtration. These findings suggest a fairly loose binding of these agents to a site (or sites) on the spore surface since mere washing is sufficient to dislodge the inhibitor.

Mercuric chloride is a powerful inhibitor of the germination of spores of *Cl. botulinum* Type A (Ando, 1973) and of *Bacillus* spp. (Gould and Sale, 1970; Vinter, 1970) and appears to inhibit some reactions in germination before the loss in heat resistance but not the subsequent release of peptidoglycan (Hsieh and Vary, 1975). In contrast, phenylmercuric nitrate has been shown (Parker, 1969; Jones and Russell, 1981) to have little effect on germination of *B. subtilis* spores, but a pronounced inhibitory action on their outgrowth. It is difficult at present to propose that these apparently contradictory findings represent a fundamental difference in action of inorganic and organic mercury compounds.

Quaternary ammonium compounds are sporistatic and not sporicidal. Cetrimide appears to bind strongly to spores, since its removal cannot be achieved by simple washing procedures but only by the use of lubrol plus lecithin (Chiori et al., 1965). Nevertheless, it does not inhibit germination although outgrowth is affected (Lund, 1962). More recently, it has been shown (Jones and Russell, 1981) that another quaternary ammonium compound, benzalkonium chloride, also inhibits outgrowth, but not germination, and that spores removed by membrane filtration are still prevented from undergoing outgrowth when transferred to an appropriate medium.

Despite its lack of sporicidal activity, ethanol inhibits spore germination in *Bacillus* and *Clostridium* spp. (Koransky et al., 1978; Yasuda-Yasaki et al., 1978). Its selective action against vegetative cells as opposed to spores has been used to facilitate isolation of the latter (Johnston et al., 1964). Other alcohols (including the extensively studied methanol and octanol) will also inhibit germination, the extent depending on alcohol concentration.

The parabens inhibit germination (Lewis and Jurd, 1972; Watanabe and Takesue, 1976; Robach and Pierson, 1978) as do similar substances (Prasad, 1974). Higher concentrations are needed to inhibit outgrowth.

Chlorine at high concentrations will prevent germination, whereas moderate concentrations markedly retard outgrowth and low concentrations have only slight effects on either (Wyatt and Waites, 1975).

Sublethal concentrations of ethylene oxide inhibit outgrowth, but not germination, of spores (Reich, 1980). Dadd and Daley (1982) have found that the resistance of spores to ethylene oxide is not lost rapidly following germination, so that

hydration of the spore core and alteration of spore coat layers do not appear to be linked to sensitivity to this gaseous disinfectant.

A particularly interesting compound, although not in the pharmaceutical context, is the curing agent, sodium nitrite. High concentrations are needed to inhibit growth of bacterial vegetative and spore forms (see Russell, 1982a for a comprehensive discussion). Ando (1980) has shown that low concentrations of nitrite do not inhibit germination, and that high concentrations actually stimulate germination, of *Cl. perfringens* spores. This effect is both pH- and temperature-dependent, with maximum germination at pH 4 and least at pH 7; the least amount occurred at 0°C and the most at 60°C. At pH 4, sodium nitrite exists as undissociated nitrous acid, HNO_2. Spores pretreated with DTT show an increasing rate of nitrite-induced germination at pH 4, which again increases as the temperature rises (Ando, 1980). Nitrous acid affects the cortex, and induces lysis of cortical fragments (see later).

Although sodium thioglycollate is widely used as a constituent of anaerobic culture media, for example, in sterility testing, and as a means of neutralizing the antibacterial activity of mercury preservatives (Russell et al., 1979) it has been shown to inhibit the germination of spores of various *Clostridium* spp. (Treadwell et al., 1958; Mossel and Beerens, 1968; Hibbert and Spencer, 1968; Bester and Claassens, 1970). Thioglycollate medium is inhibitory to spores damaged by ethylene oxide (Davis et al., 1979). Due attention should be paid to these findings in designing appropriate media for estimating the recovery of spores injured by chemical agents.

Examples of other inhibitors (Russell, 1982a) are ethylenediamine tetraacetic acid (effect on germination and outgrowth) and acetic (ethanoic) acid which, by virtue of producing an acid pH, is an important factor in limiting the growth of *Cl. botulinum* spores in food: spore outgrowth is the inhibited process.

Mechanisms of sporicidal action

Sykes (1970) discussed the permeability of bacterial spores to various disinfectants, and proposed that sporicidal agents could enter the spore, whereas others were held at the spore surface. This rather arbitrary division does not, of course, explain the manner in which sporicides actually achieve their effect.

Glutaraldehyde ($CH_2CHO \cdot CH_2 \cdot CH_2CHO$) is an important antimicrobial agent, with an enhanced effect at alkaline pH (Russell and Hopwood, 1976; Gorman et al., 1980; Russell, 1982b, 1983a and b) which is believed to result from changes in the surface of the cell. Glutaraldehyde interacts with the α-amino groups of amino acids, the rate of reaction being pH-dependent and increasing considerably over the pH range 4–9. Low concentrations of glutaraldehyde inhibit spore germination, and much higher aldehyde levels are necessary to achieve death of spores (Thomas and Russell, 1974, 1975). Interaction of the dialdehyde at acid and alkaline pH appears to occur to a considerable extent with the outer spore layers. On the basis of measurements of electrophoretic mobilities, Munton and Russell (1970) proposed tentatively that acid glutaraldehyde interacts with, and remains at, the spore surface, whereas alkaline glutaraldehyde penetrates into the spore interior. The surface of

bacterial spores is negatively charged (Nishihara et al., 1981b). There appears to be a possible role for the alkalinating activator, sodium bicarbonate, in facilitating penetration and interaction of glutaraldehyde with inner spore constituents. Support for this hypothesis comes from the recent study of Hutchinson (1982) whose findings demonstrate that in the acid state, glutaraldehyde is confined to the spore coats, whereas in the alkaline state it can penetrate beyond the coats. She further found that coatless spores were less sensitive to lysozyme after their pretreatment with alkaline but not acid glutaraldehyde, and postulated that the inhibition of germination and outgrowth observed with sub-sporicidal concentrations of alkaline glutaraldehyde resulted from a prevention of cortical breakdown.

Formaldehyde is claimed (Sykes, 1970) to penetrate into bacterial spores, but evidence in support of this contention is lacking. Formaldehyde is a highly reactive chemical, interacting with protein, DNA and RNA in vitro. It is unclear, however, what reaction on or in the spore is responsible for its sporicidal efficacy. After exposure to formaldehyde at 20°C, *B. subtilis* spores are incapable of reproduction. If, however, the formaldehyde is removed and the spores heated before being pour-plated then the numbers of colony-forming units increases dramatically, the maximum effect resulting from a heat activation at 70°C (Spicher and Peters, 1981). These findings imply that formaldehyde, at least under the conditions tested, is not sporicidal but merely damages the spores to such an extent that subsequent germination and/or outgrowth are impaired. These results show the importance of providing adequate facilities for the recovery and revival of damaged cells.

The effect of undissociated nitrous acid (sodium nitrite at pH 4) on germination was discussed earlier. Nitrous acid is believed to interact with spore coats, probably penetrating as an undissociated molecule to what is believed to be its site of action, the cortex (Ando, 1980). Here, it breaks the peptidoglycan chain at the spore-unique muramic lactam residues (Fig. 3) giving free hexosamine in nitrite digests of cortical fragments (Warth, 1978). It is well known that lysozyme hydrolyses glycosidic β1,4-linkages between N-acetylmuramic acid and N-acetylglucosamine; it does not,

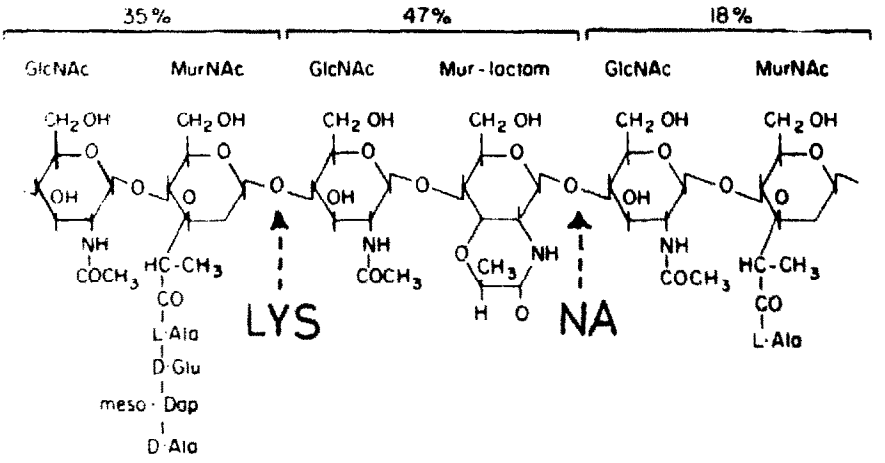

Fig. 3. Spore peptidoglycan and the actions of lysozyme (LYS) and nitrous acid (NA).

however, hydrolyze glycosidic bonds adjacent to a muramic lactam (Fig. 3) and thus its action is different from that of nitrite (Ando, 1980).

Hypochlorites solubilize the cell walls of Gram-positive bacteria, *B. megaterium* spores and 'spore integuments' (Rode and Williams, 1966). During this treatment, the spores lose refractility and darken, there is a significant separation of spore coat from cortex and eventually lysis occurs (Kulikovsky et al., 1975). When exposed to hypochlorites, bacillus or clostridial spores leak DPA which suggests an increase in spore permeability, although not all the spore calcium, RNA and DNA are released by this treatment. Chlorine will also remove spore coat protein, thereby allowing lysozyme to degrade underlying peptidoglycan and initiate germination. An interesting finding is the increase in sensitivity to heat in spores that have been pretreated with sublethal concentrations of chlorine (Dye and Mead, 1972). Since spores with and without coats are equally sensitive to heat, and because the cortex appears to regulate the water content of the spore core (Gould, 1978) then the above findings imply that the main action of chlorine is on the spore cortex (Waites et al., 1976). Further evidence for this is provided by the release of hexosamine from the cortex of intact spores and from cortical fragments (Hutchinson, 1982).

The lysis of some bacterial spores by hydrogen peroxide (Bayliss and Waites, 1976, 1979) increases in the presence of certain divalent cations such as Cu^{2+} and Co^{2+} (Gould and Hitchins, 1963; Waites, 1982). In the presence of Cu^{2+} ions, the lethal effect of peroxide against *Cl. bifermentans* spores is increased. Although these spores and *B. subtilis* var. *niger* spores take up Cu^{2+} at approximately the same rate, only the protoplasts of the former bind the cation, so that the effect of peroxide against the latter organisms is not increased significantly in the presence of Cu^{2+} (see Waites, 1982; Russell, 1982a). For sporicidal action to occur, activation of hydrogen peroxide to hydroxyl radicals is necessary (King and Gould, 1969).

The alkylating agents ethylene oxide and propylene oxide are believed to inactivate bacteria and their spores by combining with the amino, carboxyl, sulphydryl and hydroxyl groups of proteins (Hoffman, 1971) and/or interaction with nucleic acids, notably with N-7 of guanine moieties in DNA to produce 7-(2'-hydroxyethyl) guanine (see Russell, 1982a). Alkylation of spore DNA with propylene oxide results in single strand breaks (Tawaratani et al., 1979).

Enhancement of sporicidal activity

Improved knowledge of mechanisms of sporicidal action and of spore resistance should ideally lead to the design of more effective sporicides. This goal has yet to be achieved. Sykes (1970) showed that neither alcohol nor acid was sporicidal, whereas acid alcohol rapidly killed spores. Potentiation of action also occurs with a buffered methanol/sodium hypochlorite mixture, which kills spores more rapidly than buffered hypochlorite alone (Coates and Death, 1978). This is claimed (Coates and Death, 1978; Death and Coates, 1979) to result from an effect of the alcohol on spore coats, although there is little direct evidence to support this contention.

The best known example of potentiation of chemical activity is by heat (Briggs and Yazdany, 1974) but other instances are also well documented, e.g. chemical plus irradiation, peroxide plus ultraviolet radiation (Russell, 1982a; Waites and Bayliss, 1983).

TABLE 4

SUMMARY OF SITES OF ACTION OF SPORICIDAL AGENTS

Sporicidal agent	Probable site or mechanism of action
Hydrogen peroxide	Protoplast?
Chlorine	Cortex
Nitrous acid	Cortex (at muramic lactam residues)
Lysozyme *	Cortex (β1,4-linkages in peptidoglycan)
Alkali	Coats (inner only)
Glutaraldehyde (alkaline)	Intracellular penetration: cortex?
Ethylene oxide	Alkylation of protein and DNA

* Against coat-less spores

Conclusions

This short paper has reviewed the relationship between spore structure and resistance and has considered the mechanisms of action of various sporicidal and sporistatic agents (Table 4).

An impressive array of evidence implicates the spore coat in resistance to most chemical agents, and further research could with benefit be directed towards means of improving the sporicidal activity of disinfectants.

Knowledge of the mechanism of action of sporicides remains somewhat disappointing, although some progress has been made in elucidating the ways in which they achieve their effect. A better understanding of their mechanisms of action has undoubtedly been retarded by the complex nature of the spore structure and often by the need to consider the role presented by the coat(s) in limiting entry to the underlying areas. Further studies with mutants, defective in coat formation or in spore development, could yield promising information.

Various agents, whether sporicidal or merely sporistatic, will inhibit the germination and/or outgrowth of bacterial spores. There is no relationship between sporicidal action and the stage at which that sporicide acts on germination or outgrowth, e.g. of the sporicidal agents glutaraldehyde, formaldehyde, ethylene oxide and chlorine, the first two inhibit germination and the last two act predominantly on outgrowth. It is likely that further studies on the actual mechanism of inhibition by preservatives and disinfectants would be revealing.

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