Journal of Industrial Microbiology & Biotechnology (1998) 21, 6–10

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Cell membrane effects of some common biocides

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Many antimicrobial compounds exhibit bacterial cell membrane activity as either potassium ion leakage and/or leakage of material that absorbs at 260 nm from the cell. In this experiment a potassium ion selective electrode and spectophotometric observation of 260-nm leakage were used in order to examine cell membrane effects in a selection of common biocides upon both *Escherichia coli* NCIMB 10000 and *Pseudomonas aeruginosa* NCIMB 10548. The observation of potassium ion leakage for pyrithione biocides yielded results which were initially difficult to interpret, but are thought to suggest a species-dependent combination of potassium ion leakage from affected membranes and chelation of those leaked ions in the bathing suspension. Such a result is not, however, supported by the 260-nm material leakage results, which indicate very similar levels of membrane active effects for both species of bacteria.

Keywords: cytoplasmic membrane; biocides; potassium leakage; *Escherichia coli; Pseudomonas aeruginosa; Pseudomonas*-gap

Introduction

The bacterial cytoplasmic membrane is a very delicate organelle and is highly active metabolically. It acts mainly as a selective permeability barrier between the cytosol and the cell's external environment. Any membrane active agent can induce damage by action upon either the membrane potentials, bound enzymes or permeability.

Kuhn and Bielgi [23] suggested that the cationic surface active agents act on the bacterial membrane by dissociating conjugated proteins in a manner analogous to haemolysis. Hotchkiss [17] showed that nitrogen- and phosphorus-containing compounds leaked from staphylococci treated with quaternary ammonium compounds or polypeptide antibiotics. Chlorhexidine causes leakage of intracellular material from *Escherichia coli* and *Staphylococcus aureus* during which a diphasic leakage/concentration pattern is observed. The first part of the curve represents increasing leakage with increasing concentration of the biocide, but at high concentration the protoplasmic contents and/or cytoplasmic membrane become gradually coagulated so that the leakage progressively declines [21]. The interaction between the cytoplasmic membrane and chlorhexidine was found to be through interaction with the acidic lipid components of the membrane [3,5]. This leads to changes in membrane permeability which result in the loss of intracellular potassium [13], 260-nm absorbing materials [20] and phosphates [21,22,27]. This action causes concomitant alterations in the function of certain membrane-associated enzymes [4,22] and transport systems [16,19]. Although not fully elucidated, it was suggested that chlorhexidine interacts with protein moieties of the membrane and perturbs the function of the electron transport chain [19,29].

Centrimide, which is employed extensively in urology and gynaecology as an antiseptic in the form of aqueous and alcoholic solution is also one of the membrane active biocides. In this respect Lambert and Hammond [24] concluded that cetrimide (0.2 mM) causes the release of cell constituents from *E. coli* in the following order: K^+, PO_4^{3+} followed by material absorbing at 260 nm. The release of K^+ ions was completed in 30 min. Using *Staphylococcus aureus*, Denyer and Hugo [10] found that cetrimide (18 μg ml^{-1} or 5.3×10^{-5} M) causes the discharge of pH components of Δp. This concentration is the bacteriostatic concentration and the concentration which caused the maximum leakage of material absorbing at 260 nm.

Fentichlor, which has both antibacterial and antifungal activity, has an application in the treatment of dermatophytic conditions. However, its application as a preservative in cosmetics might be limited by virtue of its photosensitization. Although little work had been done on the mode of action of fentichlor, Hugo and Bloomfield [18] found that it causes leakage of material absorbing at 260 nm in both *E. coli* and *S. aureus*.

Pyrithione is an effective preservative for cosmetics and toiletry products. The membrane effect of pyrithione is due to the disruption of the proton gradient across the cell membrane and thus inhibition of the transport of solutes through the membrane barrier [11,12,14]. Dichlorophen is a preservative for toiletries, textiles and cutting fluids and prevents the growth of bacteria in water cooling systems and humidifying plants. Due to its low toxicity, however, it is used in the treatment of tapeworm in man and domestic animals and for the treatment of athletes foot.

Isothiazolone biocides such as benzisothiazolone (BIT) are widely used as industrial biocides [1,28]. BIT isothiazolones (Proxel; Zeneca Specialties, Manchester, UK) react mainly with intracellular thiol groups [6–9,15]. Initial reaction between thiol and BIT leads to the formation of a disulphide conjugate. Further reaction of this conjugate with excess thiol leads to the formation of thiol dimers and ring-opened forms of the biocides, which can themselves serve

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Received 27 February 1998; accepted 4 July 1998

as a further source of interactive thiols to give dimerised biocide [6–9,15].

This paper aims to compare and contrast the membrane active nature of these common biocides and the observation of their ability to cause leakage of both K^+ ions and 260-nm absorbing material.

Materials and methods

Organisms and chemicals

Escherichia coli NCIMB 10000 and *Pseudomonas aeruginosa* NCIMB 10548 (PA01) were obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen and were maintained on nutrient agar (Oxoid CM3) slopes at room temperature in a darkened cupboard. Both organisms were incubated at 37°C.

1,2-Benzisothiazolin-3-one (BIT), 2,2′-thiobis(4-chlorophenol) (fentichlor) and 2,2′-methylenebis(4-chlorophenol) (dichlorophen) were the kind gift of Nipa Laboratories Ltd (Pontypridd, UK). Cetrimide was the kind gift of Rhone-Poulenc (Stockport, UK). Zinc pyrithione (ZnPT) was the kind gift of Zeneca Specialties (Manchester, UK). Sodium pyrithione (NaPT) and all other reagents were purchased from Sigma (Poole, UK).

Growth inhibitory activity

The minimal inhibitory concentrations (MIC) of fentichlor, dichlorophen, BIT, pyrithione and cetrimide were determined by the serial dilution (tube dilution) method as described by Bloomfield [2]. Both *E. coli* and *P. aeruginosa* were grown in nutrient broth (Oxoid CM1) for this test.

Preparation of washed cell suspensions

Overnight, liquid cultures of *E. coli* and *P. aeruginosa* were prepared in nutrient broth (50 ml in 100-ml Erlenmeyer flasks; Oxoid CM1). These were incubated in a shaking incubator (200 opm) at 37°C. Cells were harvested, in the late exponential phase of growth, by centrifugation (4000 $\times$ *g*, 10 min) at room temperature, washed twice and resuspended in sterile Tris/HCl buffer (pH 7.2) at an absorbance (OD_{470nm}) of 1.5.

Determination of potassium ion leakage

Fifty millilitres of harvested and washed cells (OD_{470nm} = 1.5) were placed in a clean 100-ml beaker which was magnetically stirred. A volume (5 ml) of ionic strength adjustment buffer (ISAB; 18.37 g of tetraethylammonium chloride in deionised water and made up to 100 ml) was added to the beaker. This ensured that the background ionic strength of all solutions was kept constant. The potassium-ion sensing electrode (Qualiprobe QSE 314, EDT Instruments, Dover, UK) and its reference electrode (Qualiprobe double junction reference electrode E8092, EDT Instruments) were placed into the cell suspension. The potential difference (mV) derived by the electrodes was measured using a Whatman PHA 220 pH/mV meter (Whatman, Maidstone, UK). An aliquot (1 ml) of the biocide, at a predetermined concentration (7.17 mg ml^{-1}, in DMSO) was added to the cell suspension to give a final reaction concentration of 128 μg ml^{-1} in 56 ml. The potassium efflux from the cells in suspension was measured at time intervals over 20 min as a potential difference in mV. These values were converted to concentrations of K^+ ions (M) by reference to a conversion graph which had been constructed earlier using KCl standard solutions. The concentration of K^+ ions released was plotted against time and is given in Figures 1 and 2.

Determination of the leakage of 260-nm-absorbing material

Fifty millilitres of harvested and washed cells (OD_{470nm} = 1.5) were placed in a clean 100-ml beaker and maintained at room temperature whilst stirred. At time t = 0 min, an aliquot (1 ml) of biocide (6.53 mg ml^{-1}, in DMSO) was added to the cell suspension to give a final concentration of 128 μg ml^{-1} in 51 ml. Aliquots (2 $\times$ 1 ml) of the treated cell suspension were removed at regular intervals, placed in Eppendorf tubes and centrifuged at 12 000 $\times$ *g* for 2 min. The supernatant was then decanted from the Eppendorf tubes and pooled into a plastic UV cuvette (1 cm path length). The absorbance of the cuvette was read against a buffer control at 260 nm and plotted against time in Figures 3 and 4.

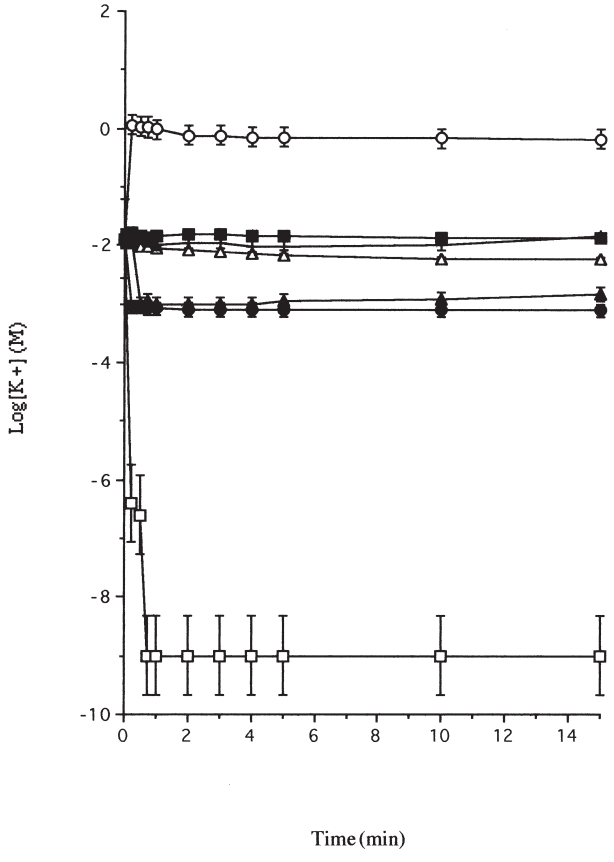

Figure 1 potassium fluxes associated with suspensions of *Escherichia coli* NCIMB 10000 exposed to 128 μg ml^{-1} of (○) cetrimide, (△) sodium pyrithione; (□) dichlorophen, (●) fentichlor, (▲) benzisothiazolone, (■) zinc pyrithione and (+) 18-crown-6 ether. Error bars are calculated and plotted as the standard error of the data set.

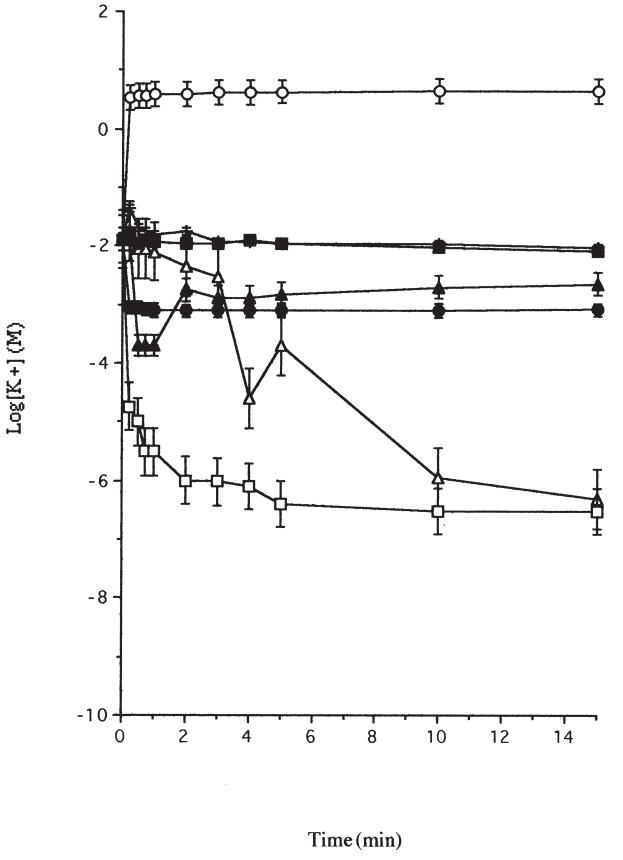

Figure 2 Potassium fluxes associated with suspensions of *Pseudomonas aeruginosa* NCIMB 10548 exposed to 128 μg ml^{-1} of (○) cetrimide, (△) sodium pyrithione, (□) dichlorophen, (●) fentichlor, (▲) benzisothiazolone, (■) zinc pyrithione and (+) 18-crown-6 ether. Error bars are calculated and plotted as the standard error of the data set.

18-Crown-6 ether

18-Crown-6 ether is a compound with K^+ ion chelation properties. This compound was used to repeat both experiments in an attempt to demonstrate the effects of a K^+ ion chelating compound upon the operation of a K^+ ion selective electrode.

Results and discussion

Determination of the minimum inhibitory concentration (MIC) of the various biocides used indicated an observable *Pseudomonas*-gap for five of the six biocides (Table 1). This is expressed as a higher MIC value for the inhibition of *P. aeruginosa* as opposed to that for *E. coli*. Cetrimide exhibited the largest gap where approximately eight times the MIC for *E. coli* was required to inhibit *P. aeruginosa*. This was followed by BIT and fentichlor, both requiring between two and four times the *E. coli* MIC to inhibit *P. aeruginosa*. Dichlorophen's observable *Pseudomonas*-gap was only an increase of about 50% in MIC. NaPT exhibited similar, high MICs against both test microorganisms (100 and 120 μg ml^{-1}), but ZnPT exhibited an obvious *Pseudomonas*-gap. The *Pseudomonas*-gap has been observed elsewhere [25,26] and may be the result of one or more physiological factors expressed by cells of *Pseudomonas* spp. This resistance to both biocides and antibiotics by the pseudo-

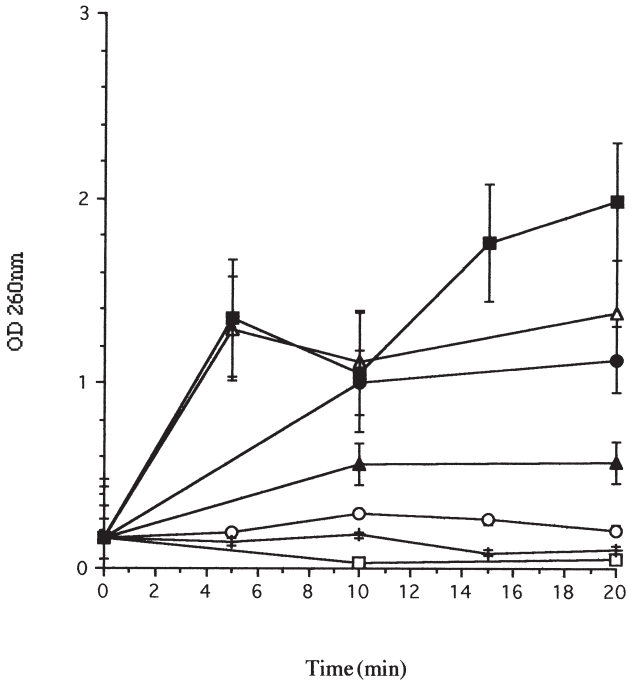

Figure 3 Leakage of 260-nm-absorbing material from cell suspensions of *Escherichia coli* NCIMB 10000 exposed to 128 μg ml^{-1} of (○) cetrimide, (△) sodium pyrithione, (□) dichlorophen, (●) fentichlor, (▲) benzisothiazolone, (■) zinc pyrithione and (+) 18-crown-6 ether. Error bars are plotted as the standard error of the data set.

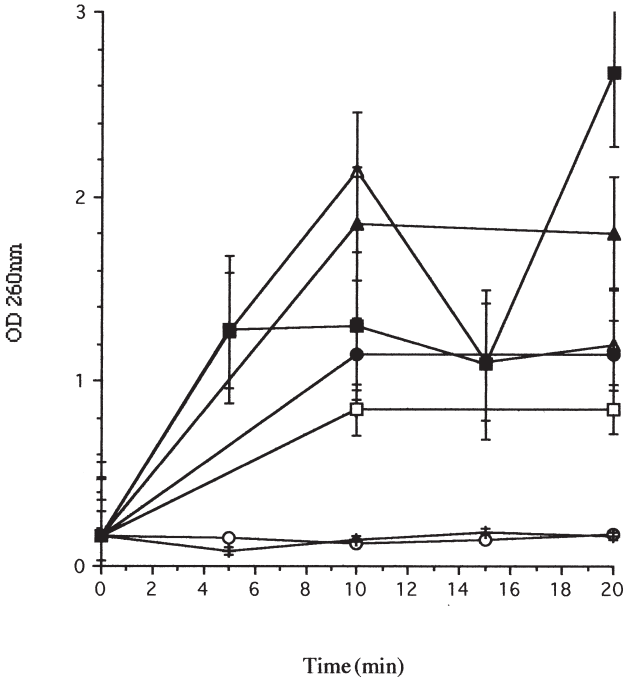

Figure 4 Leakage of 260-nm-absorbing material from cell suspensions of *Pseudomonas aeruginosa* NCIMB 10548 exposed to 128 μg ml^{-1} of (○) cetrimide, (△) sodium pyrithione, (□) dichlorophen, (●) fentichlor, (▲) benzisothiazolone, (■) zinc pyrithione and (+) 18-crown-6 ether. Error bars are plotted as the standard error of the data set.

Table 1 Minimal inhibitory concentrations of various biocides against *Escherichia coli* NCIMB 10000 and *Pseudomonas aeruginosa* NCIMB 10548

Organism	Minimal inhibitory concentration (μg ml^{-1})					
	NaPT	ZnPT	Fen	Dic	BIT	Cet
Escherichia coli NCIMB 10000	120	4.5	30	25	20	16
Pseudomonas aeruginosa NCIMB 10548	100	13	80	35	80	128

NaPT = sodium pyrithione; ZnPT = zinc pyrithione; Fen = fentichlor; Dic = dichlorophen; BIT = benzisothiazolone; Cet = cetrimide.

monads is thought to be intrinsic and related to the nature of the cell envelope, in particular the structure and composition of the Gram-negative outer membrane. The cation content of the *Pseudomonas* spp outer membrane is significantly higher than that of other Gram-negative organisms. In particular, Mg^{2+} is thought to help maintain the integrity of the outer membrane by ensuring strong lipopolysaccharide–lipopolysaccharide linkage and subsequent resistance to membrane active antimicrobials such as the quaternary ammonium compounds [26].

Figures 1 and 2 represent the effects of the various biocides upon the membranes of *E. coli* (Figure 1) and *P. aeruginosa* (Figure 2) as indicated by potassium (K^+) leakage. These figures indicate that cetrimide has observable and marked leakage effects upon both organisms and that the onset of this effect is rapid. The initial K^+ ion concentration of the bathing solution for both microorganisms in all experiments was 1.1×10^{-4} M. The maximum rate of cetrimide-induced leakage was achieved within 1 min for *E. coli* (1.15×10^{-2} M) and then the levels of K^+ ions fell slowly over the rest of the period of observation, finally reaching a concentration of 6.7×10^{-3} M at 15 min. However, for *P. aeruginosa* the onset of leakage appeared to be as rapid as that for *E. coli*, 3.8×10^{-2} M at 1 min, but continued to exhibit leakage over the rest of the period of observation, eventually reaching a level of 4.75×10^{-2} M at 15 min. This result indicates that whilst the MIC for cetrimide against *P. aeruginosa* is considerably higher than that for cetrimide against *E. coli*, the leakage it causes at the same concentration, is similar for both organisms.

Of the other biocides tested, only BIT gave observable leakage of K^+ ions and this was at a much lower rate than that for cetrimide at the same concentration, 128 μg ml^{-1}. The onset of leakage with BIT only became apparent after an initial reduction in K^+ concentration followed by a small, gradual increase to a level of only 1.4×10^{-4} M with *E. coli* and 2.3×10^{-4} M with *P. aeruginosa*.

Fentichlor gave no observable rate of K^+ ion leakage for either microorganism. The closely related compound dichlorophen, however, gave evidence of K^+ ion uptake or chelation. In the case of *E. coli* the concentration of K^+ ions in the bathing solution fell from 1.1×10^{-4} M to 5.0×10^{-9} M and for *P. aeruginosa* the fall was from 1.1×10^{-4} M to 3.0×10^{-8} M. This observation suggests that dichlorophen acts as an ion chelating agent in the presence of bacterial cells. This is an unusual result as dichlorophen is often cited as a membrane-active agent [25].

NaPT exhibited effects similar to those of dichlorophen in that it apparently chelated K^+ ions from the bathing solutions of both *E. coli* and *P. aeruginosa*. In the case of *E. coli* this represented a loss of ions from an initial concentration of 1.1×10^{-4} M to 5.22×10^{-5} M at 15 min. Such a reduction is equivalent to a halving of available bathing K^+ ions. However, unlike dichlorophen, the pyrithiones appeared to exhibit species-specific chelation properties. In the case of *P. aeruginosa*, the concentration of K^+ ions was reduced from 1.1×10^{-4} M to 3.2×10^{-8} M. Such a result indicates that *P. aeruginosa* cells are more susceptible to loss of K^+ ions from their external environment by chelation. Such an implication is, however, unlikely to be the case in reality. It is more likely that this result was the simultaneous observation of two separate, but related events involving K^+ ions.

The first of these events would be the straightforward chelation of K^+ ions from the bathing medium by the NaPT. This event should not be species-specific and should have exhibited a result similar to that for dichlorophen. The second event, however, is the simultaneous membrane activity of the biocide upon the bacterial cell. This would result in the leakage of K^+ ions from the cell and their subsequent chelation by excess pyrithione. This would result in an apparent differential K^+ ion loss between the two species of microorganism dependent upon the differing sensitivity of the two species towards the biocide. In effect, *P. aeruginosa* would exhibit a much greater level of K^+ ion loss from the bathing solution if it were less sensitive to the membrane active effects of the biocide, whereas *E. coli* would exhibit a lower level of K^+ ion loss due to its corresponding greater K^+ ion leakage. This suggestion is not supported by the MIC data. These data show that the MIC values for *E. coli* and *P. aeruginosa* are very similar at 120 μg ml^{-1} and 100 μg ml^{-1} respectively and that, if anything, the *P. aeruginosa* cells should be more susceptible to the effects of this compound. The results for ZnPT against both microorganisms, however, exhibit no obvious signs of either K^+ ion leakage or chelation. This is not surprising as K^+ will not displace the Zn atom from ZnPT. Such a result indicates that either this compound has no membrane effect upon either microorganism or that its induced potassium leakage is exactly balanced by its K^+ ion chelation. In the light of the 260-nm absorbing material leakage results this latter theory is the more probable, as these results indicate that both NaPT and ZnPT exhibit marked membrane activity against both *E. coli* and *P. aeruginosa*. Indeed most of the biocides tested gave higher levels of leakage than the positive control compound, cetrimide. The 18-crown-6-ether exhibited no apparent K^+ ion or 260-nm-absorbing material leakage in either experiment.

Conclusions

The results of these studies indicate that the pyrithione biocides are able to disrupt membrane function of Gram-negative bacteria. In addition to and simultaneously with this disruption, these compounds can also chelate K^+ ions from the bathing medium. A combination of these two

events may explain some of the inhibitory effects of these compounds upon bacterial cells. Of the other biocides, only BIT exhibited any obvious K^+ ion leakage effects. However, NaPT, ZnPT, dichlorophen and fentichlor all exhibited leakage of 260-nm-absorbing material at a higher level than that observed with cetrimide.

Acknowledgements

We thank Miss Nancy Hakooz, Mr A AL-Mowaswess, Mr S AL-Najjar, Mr F Abu Hiyyeh, Mr G Molloy, Ms D Franklin, Mr D Flynn and Mr M Dorward for their help in both technical and academic areas of this research.

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