International Journal of Pharmaceutics, 55 (1989) 209–215
Elsevier

IJP 01873

Adsorption of alexidine and chlorhexidine to *Escherichia coli* and membrane components

Judi A. Chawner * and Peter Gilbert

Department of Pharmacy, University of Manchester, Manchester (U.K.)

(Received 24 February 1989)
(Modified version received 29 March 1989)
(Accepted 10 April 1989)

Key words: Mechanism of action of bisbiguanides; Chlorhexidine; Alexidine; Bisbiguanide; Antiseptic; Phospholipid interaction; Adsorption

Summary

Activity, uptake and disposition of two related bisbiguanide antiseptics, alexidine and chlorhexidine, were investigated. Rates of onset of membrane damage were faster following alexidine than chlorhexidine treatment. Deep-rough strains of *E. coli* were less sensitive than their smooth counterparts. Whilst the uptake of both agents to whole cells corresponded to 'C-type', alexidine demonstrated additional high affinity uptake ('H-type') at low applied drug concentrations. Distribution studies for the absorbed biocide indicated that the agents must saturate a number of envelope targets before penetration to the cytosol is possible. Alexidine possessed a higher affinity towards these sites than chlorhexidine.

Introduction

Biguanides, notably chlorhexidine (Davies et al., 1954) and vantocil (Davies and Field, 1969), are long established as broad-spectrum biocides and, in the case of chlorhexidine, as preservatives of pharmaceutical and cosmetic products (Hugo and Russell, 1982). The bisbiguanide alexidine (Gjermo et al., 1973) differs from chlorhexidine in that it possesses ethyl hexyl rather than chlorophenol end-groups. All three compounds are thought to interact with components of the cytoplasmic membrane (Chawner and Gilbert, 1989a; Broxton et al., 1983a, 1984; Hugo and Longworth, 1964)) to cause changes in membrane permeability resulting in losses to the environment of intracellular potassium (Elferink and Booij, 1974), 260 nm absorbing materials (Hugo and Longworth, 1964) and phosphates (Rye and Wiseman, 1964; Hugo and Longworth, 1965, 1966). Such actions are thought to cause concomitant alterations in the functions of some membrane-associated enzymes (Hugo and Longworth, 1966; Broxton et al., 1983b; Chawner and Gilbert, 1989a) and transport systems (Hugo and Daltrey, 1974; Harold et al., 1969). It has often been suggested that chlorhexidine interacts directly with protein moieties of the cell membrane, such as ATPase, to perturb the electron transport chain (Harold et al., 1969). Such action can now be discounted as the concentra-

* *Present address*: Departments of Pharmacy and Chemical Engineering, University of Bath, Bath, U.K.
Correspondence: P. Gilbert, Department of Pharmacy, University of Manchester, Oxford Road, Manchester M13 9PL, U.K.

tions required for such effects are considerably above those likely to occur in vivo (Chopra et al., 1987). High concentrations of the drug have, however, been shown to denature and precipitate proteins and such action is thought to be critical at working concentrations (Hugo and Longworth, 1965, 1966). Recently there have been a number of reports of clinical isolates of Gram-negative bacteria which demonstrate a relative insensitivity towards chlorhexidine (El-Moug et al., 1985; Stickler et al., 1983). The concentrations of chlorhexidine to which these strains are insensitive, whilst being far below those recomended for use in clinical applications, suggest that the effects of sublethal exposure to chlorhexidine might relate to specific rather than to non-specific interaction at the cytoplasmic membrane or to alterations in the cell envelopes which affect permeation of chlorhexidine to the cytoplasmic membrane. Additionally, such chlorhexidine tolerance is not demonstrated towards the related bisbiguanides alexidine (Chawner and Gilbert, 1989b) and vantocil (Ismaeel et al., 1987). Alexidine and vantocil have now been demonstrated to have actions at the cytoplasmic membrane distinct from those of chlorhexidine. Unlike chlorhexidine these interact specifically with the acidic-lipid components of the cytoplasmic membrane to cause phase separation and domain-formation of the various phospholipids (Broxton et al., 1984; Ikeda et al., 1984; Chawner and Gilbert, 1989b).

Such differences in the action of these related biguanides with respect to resistance and molecular action prompted the current study which compared uptake, disposition, binding affinities and activity of the bisbiguanides, alexidine and chlorhexidine towards wild-type and various envelope-deficient strains of *Escherichia coli*.

Materials and Methods

Organisms and chemicals

Escherichia coli ATCC 8739 was obtained from the American Type Culture Collection (Maryland, U.S.A.). *E. coli* K12, strains D212 and D21F2 were obtained from the laboratory of Dr. H. Boman (University of Umea, Sweden) and represent extremes of a series of lipopolysaccharide-defective mutants. Stock cultures were maintained on nutrient agar (Oxoid CM3) slopes at room temperature in the dark, after overnight incubation at 35 °C.

Chlorhexidine diacetate and alexidine were kindly given by ICI plc Organics Division. Cardiolipin, protease (Type XVI, *Staphylococcus aureus*) and lipopolysaccharide (*E. coli* 0127: B8) were obtained from Sigma Chemicals (Poole, U.K.). Uniformly ^{14}C-labelled chlorhexidine (30 mCi/mmol) was obtained from Amersham International (Bucks, U.K.). 'Optiphase' MP liquid scintillant was obtained from LKB (Fisons, Loughborough, U.K.). All other chemicals were of the purest available grade and obtained from BDH Ltd (London, U.K.).

Preparation of cell suspensions

Chemically defined liquid media based on those described by Gilbert and Brown (1978) were employed for the growth of *E. coli* ATCC 8739. This was designed so that the growth of the cultures ceased at an optical density (E_{470} nm) of 1.0 due to depletion of glycerol. All other nutrients were present to a known excess. For the growth of the D21 and D21F2 strains of *E. coli* K12 a medium based on that of Vogel and Bonner (1956) and Lugtenberg et al. (1976) was employed. This supplied thiamine, yeast extract and tryptone in addition to the salt requirements and permitted growth to an optical density (E_{470} nm) of 4.0 at 16 h. Liquid cultures were grown overnight at 35 °C, in 250 ml Erlenmeyer flasks containing 100 ml of medium, in an orbital incubator (250 osc/min). Cultures were harvested by centrifugation (35 °C, 15,000 *g*, 15 min), washed 3 times and finally resuspended to an appropriate optical density in sterile saline (0.9% w/v).

Assay of bisbiguanides

Concentrations of alexidine were assessed colorimetrically at 540 nm following reaction of sample (2 ml) with sodium acetate (0.1 ml, 10% w/v), Eosin Y (0.25 ml, 0.024% w/v) and water (0.15 ml) for 15 min at room temperature. The assay utilises the property of polycationic materials to

affect the pK of certain indicators (Baumgartner et al., 1974).

Chlorhexidine concentrations were determined by transfer of samples (0.2 ml) to scintillation vials containing Optiphase MP liquid scintillant (4 ml) and assessment of the level of ^{14}C radiolabel. A 1218 Rack beta counter (LKB Wallac) with appropriate quench correction was used.

Assessment of cytoplasmic membrane damage

The extent of damage to cell membranes was assessed by measuring the degree and rate of potassium-ion loss from the cells to suspension supernatants. Washed suspensions of the *E. coli* ATCC 8739 and D21 and D21F2 (2×10^9 cells/ml) in saline (0.9% w/v) were maintained, with stirring, at 35 °C and monitored by potassium-specific ion electrode (Model 93-91, Orion Research Inc., Cambridge, U.S.A.) for the appearance of potassium within extracellular fluids (Lambert and Hammond, 1973). Untreated cell suspensions were sonicated to give complete lysis and the potassium ion concentrations within cell lysates determined. These values indicated the total possible losses to supernatant of which the results were expressed as percentages.

Adsorption and disposition of biocides within suspensions

Washed cell suspensions (20 ml, in phosphate buffer pH 7.0, 0.1 M, 6×10^5 cells/ml) were equilibrated at 35 °C in a shaking incubator. Various concentrations of [U-^{14}C]chlorhexidine or alexidine were added. Preliminary experiments had established that adsorption of the biocides was complete within 5 min contact with cells. After 15 min contact, therefore, the suspensions were separated by centrifugation (30,000 *g*, 1 min), supernatants collected and the pellets resuspended in buffer (1 ml). For chlorhexidine-treated cell suspensions, then the suspension, supernatants and resuspended pellets were assayed by the level of radioactivity. Supernatants of alexidine-treated suspensions were assayed colorimetrically for the equilibrium concentrations of biocide.

Disposition of the biocides within treated cells was examined, following exposure to radiolabelled chlorhexidine, by fractionation. Experiments were performed, as above, except that only 3 concentrations of chlorhexidine were employed (10, 50 and 100 μg/ml). Cell pellets generated were resuspended in the supernatants of duplicate suspensions treated with unlabelled chlorhexidine. This process was intended to minimise desorption and redistribution of the biocide. Cell pellets were sonicated on ice and unbroken cells were removed by centrifugation (5000 *g*, 15 min). The cell envelopes were collected by ultracentrifugation (80,000 *g*, 1 h, 0 °C) and these supernatants and pellets assayed with respect to the ^{14}C radiolabel. Since no radiolabel was available for alexidine, the experiments were repeated at a fixed chlorhexidine concentration (50 μg/ml) for cells pretreated for 30 min with various concentrations of alexidine. Fractions were analysed as before and competition for binding between the two compounds assessed.

Determination of binding affinities

Binding of the bisbiguanides to diphosphatidyl glycerol (DPG), lipopolysaccharide (LPS) and protein was estimated by the use of dialysis chambers with donor and recipient chambers of 30 ml and 5 ml, respectively, separated by a cellulose Visking membrane. Preliminary experiments showed that the biocides could equilibrate across the membranes within 24 h but that the absorbents could not. Colloidal solutions of each material were prepared in sterile distilled water (DPG, 0.3 mg/ml; LPS, 0.4 mg/ml; protease, 50 μg/ml) and placed in the recipient chamber of the dialysis apparatus. Solutions of biocide were applied to the donor chamber. Both chambers were sealed and the apparatus was left to equilibrate for 24 h at room temperature. At this time concentrations of biocide in the donor solutions were analysed. After correction for dilution by the recipient solution, the equilibrium concentrations of biocide and amounts bound could be calculated.

Results

Sensitivity determinations

Sensitivity of a number of LPS-deficient strains of *Escherichia coli* towards the two biguanides was

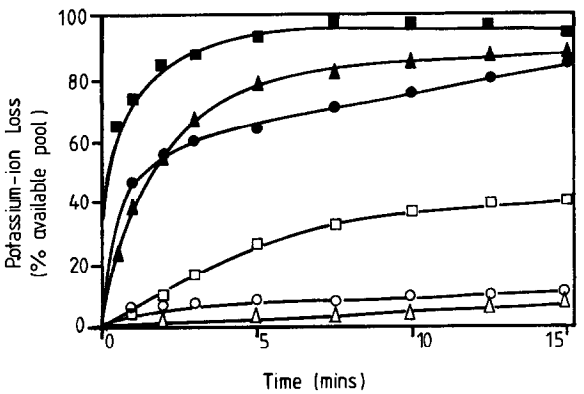

Fig. 1. Comparison of the rates of potassium ion loss following treatment of cell suspensions of *Escherichia coli* (□, ■) D21; (△, ▲) D21F2; and (○, ●) ATCC 8739 with alexidine (10 μg/ml, solid symbols) or chlorhexidine (10 μg/ml, open symbols).

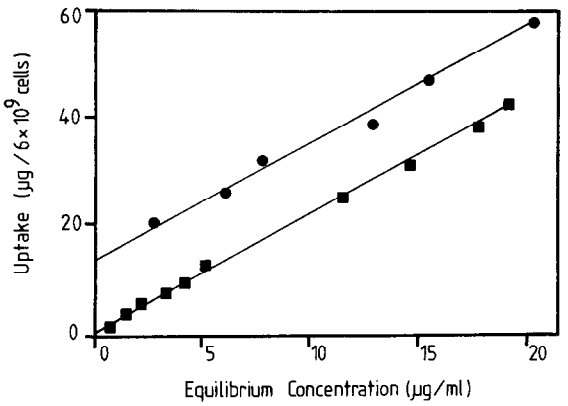

Fig. 2. Absorption/adsorption of the bisguanides alexidine (●) and chlorhexidine (■) by thick washed suspensions (6×10^9 cells/ml) of *Escherichia coli*.

assessed as loss of intracellular potassium. Previous studies had indicated that such loss is rapid and a good indicator of the extent of biocide-induced killing (Broxton et al., 1984). Loss of potassium following treatment with alexidine was rapid, was complete within 5 min and followed first-order kinetics. The extent of potassium ion loss was greater for D21 wild type cells than for the deep rough D21F2 variant or for *E. coli* B. Chlorhexidine-induced losses of potassium ions was considerably slower than that for alexidine and occurred at higher concentrations. Once again the extent of damage was greater for the D21 strain than for the other strains. These results are illustrated in Fig. 1, as time courses, following treatment of the 3 strains with a fixed concentration of biocide (10 μg/ml). This concentration corresponded to that giving a 90% kill of the E. coli B strain within 1 min (Chawner and Gilbert, 1989a).

Biocide uptake by cell suspensions

Uptake of the biocides by thick washed cell suspensions of *E. coli* ATCC 8739 was determined and the results expressed as adsorption isotherms (Fig. 2). The absorption of both agents closely followed C-type adsorption patterns, according to the classification of Giles et al. (1974). C-type adsorption corresponds to a constant partitioning of solute into the cells. Alexidine showed, in addition, a high-affinity uptake at low equilibrium concentrations (H-type adsorption). This is suggestive of high-affinity binding targets for the agent which must be saturated prior to partitioning of the agent throughout the cell. An implication of the data is that such high affinity sites are not shared by chlorhexidine. The slopes of the C phases for the two agents were, however, similar.

For the chlorhexidine studies it was possible, through the use of radiolabel, to study the distribution throughout the cell, of the agent following its adsorption. Cells were fractionated into envelopes and cytosol and the relative amounts of chlorhexidine associated with each fraction determined for various applied drug concentrations (Table 1). The fraction of the adsorbed chlorhexidine associated with the cytosol increased

TABLE 1

Binding and distribution of chlorhexidine within Escherichia coli cell suspensions

Chlorhexidine uptake	Applied chlorhexidine concentration		
	10 μg/ml	50 μg/ml	100 μg/ml
Equilibrium concentration	2.5	18.2	28.6
Uptake (μg) per 6×10^9 cells	7.8	32.4	70.9
Percentage of total uptake in cytosol	16.6	22.6	49.4
Percentage of total uptake in envelope	83.4	77.4	50.6

TABLE 2

Binding and distribution of chlorhexidine within Escherichia coli cell suspensions in the presence of alexidine at various concentrations

Chlorhexidine	Applied alexidine concentration				
	0 μg/ml	20 μg/ml	50 μg/ml	100 μg/ml	150 μg/ml
Whole cell uptake (% of applied concn.)	64.5	63.2	62.4	67.1	64.4
Uptake into cytosol (% total uptake)	16.7	14.8	22.7	37.1	55.5
Uptake into envelope (% total uptake)	83.3	85.2	77.3	62.9	44.5

Chlorhexidine was applied at a concentration of 25 μg/ml.

with increasing applied concentrations from 4.2% for concentrations of 10 μg/ml to 13.2% for 100 μg/ml. This indicated that whilst the major adsorptive target was the cell envelope, this became saturated at higher chlorhexidine concentrations or damage to the envelope permitted greater penetration to the cytosol.

Binding and disposition of chlorhexidine (50 μg/ml) within cells was reinvestigated for cell suspensions pretreated with varying concentrations of alexidine (Table 2). The total amounts of chlorhexidine bound to the cells were unaffected even by the highest alexidine concentrations employed (100 μg/ml). This might be expected if both agents followed C-type adsorption. Amounts of chlorhexidine associated with the cell envelope fraction, however, decreased with increasing alexidine concentration. This, together with the observation of high-affinity uptake for alexidine, is compatible with the notion that high-affinity binding targets in the envelope for alexidine represent low-affinity binding targets for chlorhexidine.

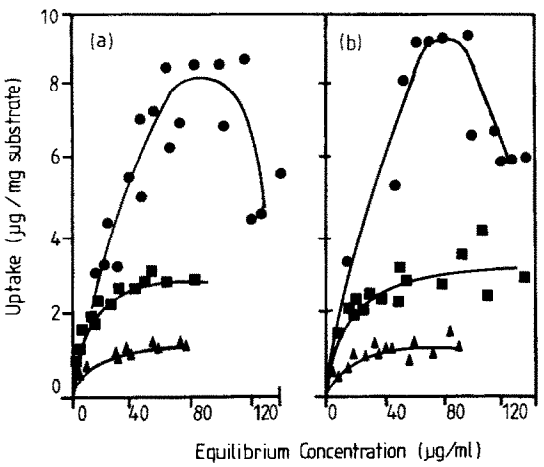

Fig. 3. Uptake isotherms for the absorption/adsorption of alexidine (a) and chlorhexidine (b) onto protease (●), lipopolysaccharide (▲) and diphosphatidyl glycerol (■).

Binding to isolated envelope components

Adsorption/absorption isotherms for alexidine and chlorhexidine and protein (protease), lipopolysaccharide and diphosphatidylglycerol are illustrated in Fig. 3. In all instances binding corresponded to L-type (Langmuirian) and plateaued at elevated biocide concentrations. These plateau values (biocide/mg) give an indication of the antiseptic binding capacity for each cellular component. Binding was considerably greater to protease than by either LPS or phospholipid. The extent of binding to phospholipid was approximately 3 times that of LPS. There was no indication of high-affinity binding of alexidine to any of the substrates tested and the results for the two biguanides were similar.

Discussion

Onset of potassium ion loss following treatment of the various cell types was rapid and for alexidine-treated cell suspensions it was complete within several minutes. The rates of potassium ion loss following treatment of the cell suspensions were slower for chlorhexidine treatment and persisted for up to 30–45 min. Surprisingly the D21 wild type strain of *E. coli* K12, which contained a

complete LPS-polysaccharide region and capsule, was the most sensitive of the strains towards these biocides. This suggested that the presence of the polysaccharide region in some way facilitated access of the biocides to the cytoplasmic membrane. Deep-rough strains were the most resistant of the cell types.

Uptake of both bisbiguanides, by cell suspensions, corresponded to 'C-type' adsorption (Fig. 2). Such adsorptive processes are commonly observed with biocides and indicate a partitioning of the agent into the cells (Giles et al., 1974). In this instance the slopes of the isotherms were similar for the two agents which suggested equivalent binding affinities between both biocides and the bacteria. Previous reports of chlorhexidine adsorption by whole cell suspensions (Hugo and Longworth, 1964; Hugo and Daltrey, 1974; Nadir and Gilbert, 1979; Daham and Wiseman, 1987) have noted 'S-type' and 'L-type' adsorption in addition to 'C-type'. The presence of metal cations has been reported to dramatically affect the pattern and extent of chlorhexidine uptake (Nadir and Gilbert, 1979). Thus Daham and Wiseman (1987) observed 'S-type' uptake in the presence of magnesium but 'C-type' in the presence of EDTA with no added magnesium. We believe that such patterns reflect competition between the bisbiguanide and cations for available binding sites on the cell surface. Increased sensitivity of the smooth strains (Fig. 1) would suggest that, in addition, the presence of complete polysaccharide chains on the LPS reduce these competitive effects.

Alexidine adsorption showed an initial high-affinity uptake by the cells and suggested alexidine to possess high-affinity binding sites on the cells. The presence of such high-affinity binding sites might explain the more rapid onset of membrane damage caused by this agent.

Fractionation of cells following treatment with chlorhexidine demonstrated that the agent must saturate the cell envelope prior to entry into the cytosol (Table 1). Competition studies for the binding of alexidine and chlorhexidine indicated that alexidine would preferentially occupy such envelope sites (Table 2). Such data suggest that the high-affinity binding sites for alexidine might also bind chlorhexidine, but less strongly, and that these sites are within the cell envelope.

Binding of the bisbiguanides to isolated cellular components such as protein, LPS and DPG occurred to similar extents for the two biocides and displayed similar relative binding capacities between the 3 macromolecules. In all instances binding followed a Langmuirian pattern and plateaued at elevated levels. These plateau levels (biocide/mg) gave an indication of the relative binding capacities of each component and were considerably higher for protein than for either DPG or LPS. The extent of binding to the LPS was 3 times less than that to the phospholipid. This is surprising since the presence of complete polysaccharide regions in the D21 wild-type strain appeared to facilitate uptake and action of the biocides. High binding capacity of the protein might well reflect a high number of potential binding sites on these molecules rather than high binding affinity, but nevertheless the potential of proteins rather than phospholipids as neutralisers of bisbiguanide action is clear. Relative binding affinities to major cellular and envelope components fails to explain the selectivity, between alexidine and chlorhexidine, of chlorhexidine-tolerant isolates. Such selectivity probably resides in the presence on the envelope of high-affinity targets for alexidine but not chlorhexidine and also with the different molecular actions of the agents at the cytoplasmic membrane (Chawner and Gilbert, 1989a and b).

Acknowledgements

We would like to acknowledge the award of an SERC studentship to J.A.C.

References

Baumgartner, E., Fernandez-Princi, R. and Turyn, D., Change of apparent acidity of indicators in polyelectrolyte solutions. *J. Chem. Soc. Faraday Trans.*, I (1974) 1518–1526.

Broxton, P., Woodcock, P.M. and Gilbert, P., A study of the antibacterial activity of some polyhexamethylene biguanides towards *Escherichia coli* ATCC 8739. *J. Appl. Bacteriol.* 54 (1983a) 345–353.

Broxton, P., Woodcock, P.M. and Gilbert, P., Action of some polyhexamethylene biguanides upon respiration of *Escherichia coli* ATCC 8739. *J. Pharm. Pharmacol.*, Suppl. 35 (1983b) 67P.

Broxton, P., Woodcock, P.M. and Gilbert, P., Interaction of some polyhexamethylene biguanides and membrane phospholipids in *Escherichia coli*. *J. Appl. Bacteriol.*, 57 (1984) 115–124.

Chawner, J.A. and Gilbert, P., A comparative study of the bactericidal and growth inhibitory activities of the bisbiguanides alexidine and chlorhexidine. *J. Appl. Bacteriol.*, 66 (1989a) 243–252.

Chawner, J.A. and Gilbert, P., Interaction of the bisbiguanides alexidine and chlorhexidine with phospholipid vesicles: evidence for separate modes of action. *J. Appl. Bacteriol.*, 66 (1989b) 253–258.

Chopra, I., Johnson, S.C. and Bennett, P.M., Inhibition of *Providencia stuartii* cell-envelope-enzymes by chlorhexidine. *J. Antimicrob. Chemother.*, 19 (1987) 743–751.

Danam, S.A.M. and Wiseman, D., The uptake of chlorhexidine by *Escherichia coli* and its effects upon viability in the absence and presence of EDTA. *J. Pharm. Pharmacol.*, Suppl., 39 (1987) 16P.

Davies, A. and Field, B.S., Action of biguanides, phenols and detergents upon *Esch. coli* and its sphaeroplasts. *J. Appl. Bacteriol.* 32 (1969) 233–243.

Davies, G.E., Francis, J., Martin, A.R., Rose, F.L. and Swain, G., 1 : 6-Di-4′-chlorophenyldiguanidohexane (Hibitane): laboratory investigation of a new antibacterial agent of high potency. *Br. J. Pharmacol.*, 9 (1954) 192–196.

Elferink, J.G.R. and Booij, H.L., Interaction of chlorhexidine with yeast cells. *Biochem. Pharmacol.*, 23 (1974) 1413–1419.

El-Moug, T., Furr, J.R. and Russell, A.D., Effect of chlorhexidine on a chlorhexidine sensitive and a chlorhexidine resistant strain of *Providencia stuartii*. *Lett. Appl. Microbiol.*, 1 (1985) 37–40.

Gilbert, P. and Brown, M.R.W., Effect of R-plasmid RP1 and nutrient depletion on the gross cellular composition of *Escherichia coli* and its resistance to some uncoupling phenols. *J. Bacteriol.*, 133 (1978) 1062–1065.

Giles, C.H., Smith, D. and Huitson, A., A general treatment and classification of the solute adsorption isotherm. *J. Colloid Interface Sci.*, 47 (1974) 755–765.

Gjermo, P., Rolla, G. and Arskaug, L., The effect on dental plaque formation and some in vitro properties of 12 bis-biguanides. *J. Periodont. Res.*, 8 (1973) 81–88.

Harold, F.M., Baarda, J.R. and Abrams, A., Dio 9 and chlorhexidine: inhibitors of membrane bound ATPase and of cation transport in *Streptococcus faecalis*. *Biochim. Biophys. Acta*, 183 (1969) 129–136.

Hugo, W.B. and Daltrey, D.C., Studies on the mode of action of the antibacterial agent chlorhexidine on *Clostridium perfringens*. 2. Effect of chlorhexidine on metabolism and on the cell membrane. *Microbios*, 11 (1974) 131–146.

Hugo, V.B. and Longworth, A.R., Some aspects of the mode of action of chlorhexidine. *J. Pharm. Pharmacol.*, 16 (1964) 655–662.

Hugo, W.B. and Longworth, A.R., Cytological aspects of the mode of action of chlorhexidine diacetate. *J. Pharm. Pharmacol.*, 17 (1965) 28–32.

Hugo, W.B. and Longworth, A.R., The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic membrane constituents, dehydrogenase activity and cell walls of *E. coli* and *S. aureus*. *J. Pharm. Pharmacol.*, 18 (1966) 569–578.

Hugo, W.B. and Russell, A.D., Types of antimicrobial agents. In (Eds.) Russell, A.D., Hugo, W.B., Ayliffe, G.A.J., *Principles and Practice of Disinfection, Preservation and Sterilisation*, Blackwell, Oxford, 1982, pp. 8–106.

Ikeda, T., Tazuke, S., Bamford, C.H. and Ledwidth, A., Interaction of a polymeric biguanide biocide with phospholipid membranes. *Biochim. Biophys. Acta*, 769 (1984) 57–66.

Ismaeel, N., Furr, J.R. and Russell, A.D., Inhibitory and lethal effects of chlorhexidine and a polymeric biguanide on some strains of *Providencia stuartii*, *Lett. Appl. Microbiol.*, 5 (1987) 23–26.

Lambert, P.A. and Hammond, S.M., Potassium fluxes, first indications of membrane damage in microorganisms, *Biochim. Biophys. Acta*, 54 (1973) 796–799.

Lugtenberg, B., Peters, R., Bernheimer, H. and Berendsen, W., Influence of cultural conditions and mutations on the composition of the outer membrane of *E. coli*. *Mol. Gen. Genet.*, 147 (1976) 251–258.

Nadir, M.T. and Gilbert, P., Influence of potassium chloride upon the binding of chlorhexidine diacetate and (ethoxy)5-octyl pheol (Triton X45) towards *Bacillus megaterium*. *Microbios.*, 26 (1979) 51–63.

Rye, R.M. and Wiseman, D., Release of phosphorus-32-containing compounds from *Micrococcus lysodeikticus* treated with chlorhexidine. *J. Pharm. Pharmacol.*, 20 (1964) 14s–17s.

Stickler, D.J., Thomas, B., Clayton, C.L. and Chawla, J.C., Studies on the genetic basis of chlorhexidine resistance, *Br. J. Clin. Practice*, Symposium, 25 (1983) 23–28.

Vogel, H.J. and Bonner, D.M., Acetylornithinase of *E. coli*: Partial purification and some properties. *J. Biol. Chem.*, 218 (1956) 97–106.