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INTERACTION OF CHLORHEXIDINE WITH YEAST CELLS

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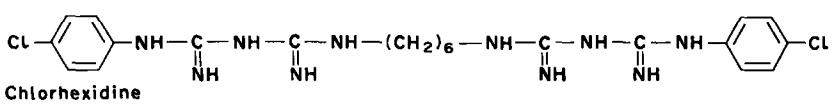
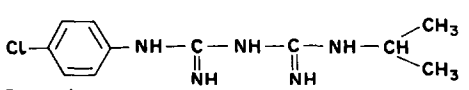
Abstract– The effects on yeast cells of two related compounds, chlorhexidine and paludrine, were compared with those of cetyltrimethylammonium bromide (CTAB). The action of chlorhexidine closely resembles that of CTAB, following an all-or-none pattern. Involvement of membrane lipids is suggested by the similar effect of chlorhexidine and CTAB on liposomes. The possibility, that the disruptive effect of positive surfactants is due to their penetration of the region of the glycerol moieties of membrane lipids is discussed.

MANY surfactants exert their bactericidal action by affecting the cytoplasmic membrane, resulting in leakage of cell constituents and under certain conditions in cell death. Little is known about the details of the process, for instance, whether the surfactant interacts with the protein components or with the lipid components of the membrane. Here the action of chlorhexidine and the closely related compound paludrine on yeast cells is compared with that of cetyltrimethylammoniumbromide (CTAB). These substances affect the cytoplasmic membrane causing leakage of cell constituents although their molecular structure, and even the ratio of charged groups to apolar parts, is very different. In addition the effects of all three compounds on lecithin model membranes were considered.



Chlorhexidine



Paludrine

$C_{16}H_{33}N^{+}(CH_3)_3\ Br^{-}$
Cetyltrimethylammoniumbromide

MATERIALS AND METHODS

Bakers' yeast (Koningsgist, Delft) was washed, aerated for 6 hr, washed again and made up to a 35·5 per cent suspension through which nitrogen was bubbled overnight.

Chlorhexidine diacetate and paludrine chloride were obtained from ICI. Paludrine lactate, which is more soluble than the chloride, was prepared by extracting an alkaline suspension of paludrine chloride with chloroform and by adding lactic acid to the base thus obtained.

Chlorhexidine diacetate (0·01 M), or paludrine lactate (0·01 M), was added to a magnetically stirred suspension of 2·5 g yeast (wet weight) in a volume of 32 ml. The suspension was contained in a double-walled glass vessel, connected to a thermostat (20°).

Conductivity was measured with a Philips PR conductivity meter. The extracellular K^+ concentration was determined with an Eppendorf flamephotometer. Total cellular potassium was estimated by boiling an aliquot of the yeast suspension for 5 min and measuring the potassium in the supernatant.

The extracellular liquid was separated from the yeast cells by filtering a small volume (usually 0·5 ml) diluted with water, through a Millipore filter (HA: 0·45 μm pore size). Chlorhexidine was determined according to the method of Holbrook.[1] Paludrine was determined according to Spacu[2] or Owens.[3]

The percentage of cells killed was determined by selective staining, as described by Maas.[4] Liposomes were prepared according to established procedures.[5,6] Purified egg lecithin in 0·2 M KCl solution (15 mg lipid per ml) was kept at 40° for 30 min, and then shaken on a Vortex mixer for 30 sec. The resulting suspension was dialysed against 0·4 M glucose solution. One ml of liposomes was mixed with 1 ml glucose solution containing surfactant, and the mixture dialysed in a dialysis bag against 5 ml glucose solution. After 4 hr K^+ was estimated in the dialysate.

RESULTS

Chlorhexidine and CTAB rapidly alter the membrane of yeast cells resulting in an instantaneous release of cell constituents (Fig. 1). Chlorhexidine differs from CTAB in that after an initial strong response of the cells to chlorhexidine there is a second phase of gradual leakage. Conductivity changes of the medium parallel K^+ efflux from the cells.

The response of the yeast cells to CTAB follows an all-or-none pattern: the percentage of dead cells approximately equals the percentage K^+ loss. Chlorhexidine (Fig. 2) causes some leakage of K^+ ions before cell death but the evidence still suggests an all-or-none effect, combined with a slow gradual release of K^+. The action of paludrine (Fig. 3) closely resembles that of a positive detergent with a shorter chain (e.g. dodecylpyridinium chloride).[7] While a long-chain positive surfactant such as CTAB is immediately and completely adsorbed to the cell, the amount of a short-chain positive surfactant that is bound gradually increases with time. In the latter case potassium is gradually lost also; Maas[7] considers the action of short-chain cationics as an all-or-none effect.

Paludrine adsorbs to yeast cells to a lesser extent than chlorhexidine which in the concentration range considered is completely adsorbed. Since the curves for K^+ leakage and cell viability diverge the process may not be an all-or-none effect, though the levelling off with time of K^+ leakage supports this view.

The K^+ loss remained unchanged when the amount of chlorhexidine or paludrine added was increased to five times the maximum concentration shown in Figs. 2 and 3.

Riemersma[8] observed that at low concentrations CTAB and other cationic surfactants are bound without any change in membrane permeability. Lysis of yeast cells, i.e. the loss of membrane impermeability, starts at a critical concentration of bound

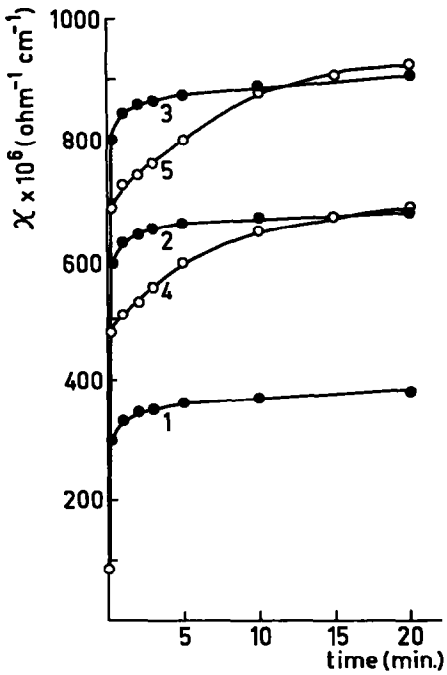


FIG. 1. Specific conductance of a yeast suspension as a function of time after treatment with CTAB or chlorhexidine. In a total volume of 32 ml 2·5 g yeast was treated with 0·01 M surfactant. (●) CTAB; (○) chlorhexidine 1, 2 and 3: 1, 2 and 3 ml CTAB respectively; 4 and 5: 1 and 2 ml chlorhexidine respectively.

surfactant; this quantity is strongly temperature-dependent. At 20 no lytic threshold for chlorhexidine could be demonstrated. Paludrine (Fig. 3) is practically nonlytic at low concentrations.

It has been known for some time that several cations can inhibit lysis of yeast cells by positive surfactants or dyes.[9, 10] The order of effectiveness of metal ions was found

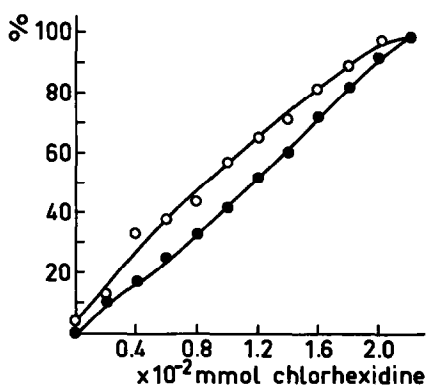


FIG. 2. Loss of viability and leakage of K^+ from the cells as a function of the amount of chlorhexidine added (in this range completely absorbed by the cells). Samples for viability determination and K^+ estimation were taken one hour after mixing the chlorhexidine solution and yeast suspension. (○) K^+ leakage, expressed as a percentage of maximal leakage; (●) percentage dead cells.

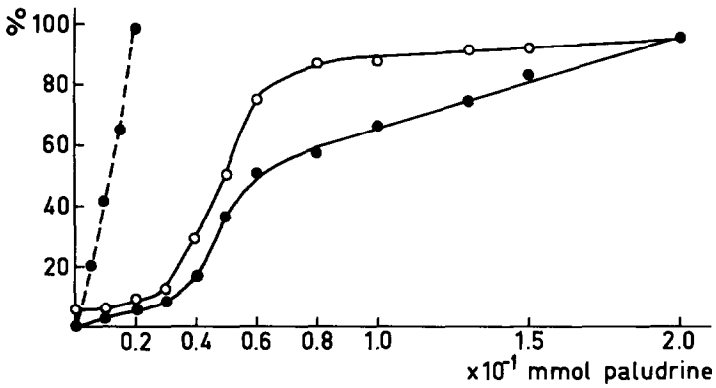


FIG. 3. Action of paludrine, represented in the same way as for chlorhexidine in Fig. 2. In comparison the action of chlorhexidine (percentage dead cells, dotted line) is represented on the same scale. (○) K^+ leakage; (●) percentage dead cells.

to be: Th^{4+}, UO_2^{2+}, La^{3+}, $Ce^{3+} > Al^{3+} \gg Ni^{2+}$, Co^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}. The protective action of Th^{4+} against chlorhexidine induced lysis is represented in Fig. 4. Below a certain threshold concentration of surfactant there is no efflux of cell constituents. This threshold concentration is increased in the presence of increasing concentrations of metal ions.

Chlorhexidine was more effective in inducing K^+ leakage from liposomes than CTAB as is shown in Fig. 5. The surfactant-lipid ratio which caused leakage in liposomes was approximately the same as in living cells. (Extraction of lipids from 2·5 g wet yeast yields about 25 mg lipid.) Paludrine also caused leakage from liposomes but, as might be expected, in higher concentrations (Fig. 6).

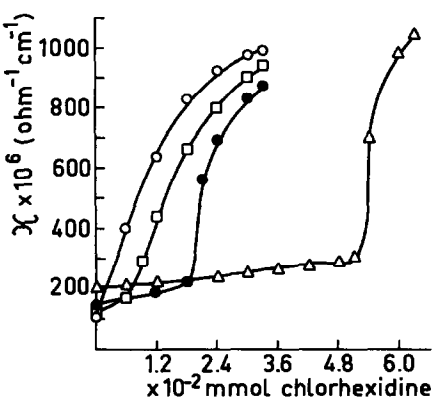


FIG. 4. Specific conductance in yeast suspension (2·5 g yeast in 32 ml) during stepwise addition of 0·01 M chlorhexidine acetate in the presence of thorium ions. (○) without Th^{4+}; (□) 1 μmole Th^{4+}; (●) 2 μmole Th^{4+}; (△) 4 μmole Th^{4+}, added per g of yeast before chlorhexidine addition.

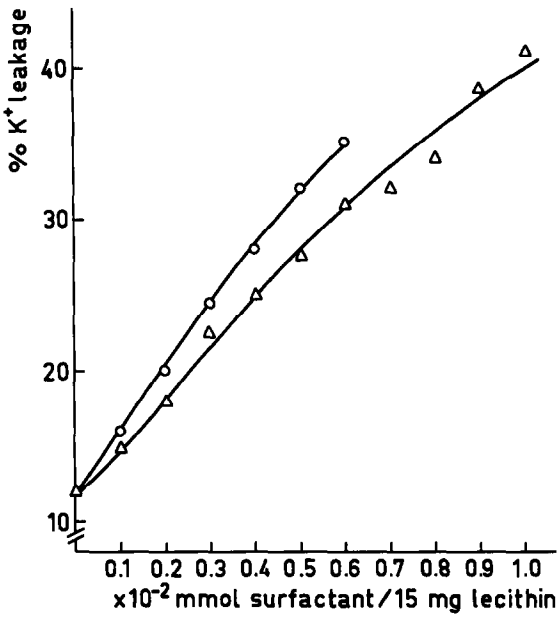


FIG. 5. Leakage of K^+ ions from liposomes by the action of CTAB and chlorhexidine. Maximal K^+ leakage was estimated after addition of Triton X-100, which leads to lysis of liposomes. (△) CTAB; (○) chlorhexidine. Higher concentrations of chlorhexidine gave irreproducible values, probably as a consequence of precipitation of chlorhexidine chloride.

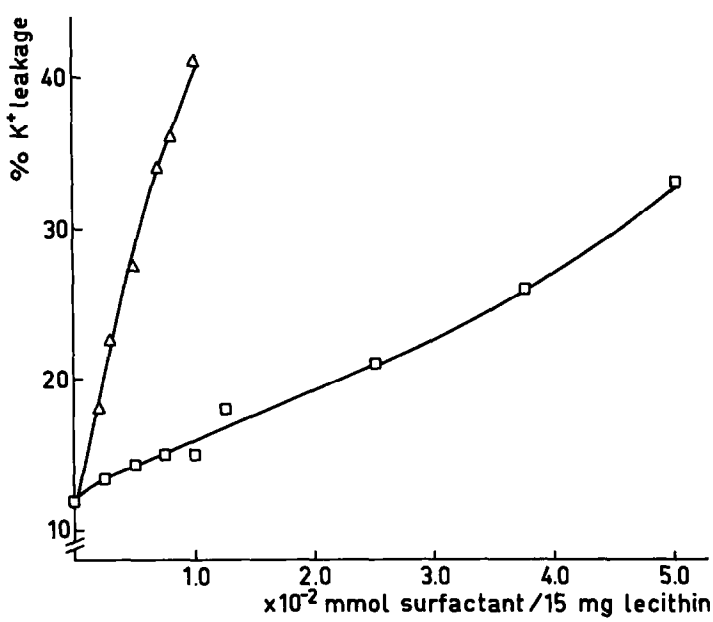


FIG. 6. Leakage of K^+ ions from liposomes by the action of paludrine. For comparison the action of CTAB is given on the same scale. (□) paludrine; (△) CTAB.

DISCUSSION

The instantaneous effect of chlorhexidine (Fig. 1) supports Hugo's[11] idea, that its primary action is a disorganization of the structure of the plasma membrane. Chlorhexidine may cause intracellular damage as well, but at higher concentrations than required for membrane disruption.[12,13]

The actions of chlorhexidine and CTAB are similar, e.g. in both cases the leakage of potassium and the decrease of viability parallel each other. However, since they differ in structure it is unlikely that both compounds effect the breakdown of the permeability barrier by interacting with a specific membrane protein.

Most investigators have supposed that cationic surfactants like CTAB disturb membrane integrity by interacting with the lipid components.[2,8,9,14,15] This hypothesis is supported by the action of CTAB on black lipid membranes.[18,19] Liposomes, composed of concentric bimolecular phospholipid layers possess permeability properties which are similar to those of biomembranes.[5,6,20] Our experiment with liposomes shows that CTAB is able to disrupt an organized lipid structure in such a way that it loses its impermeability to ions. The results support the view that the membrane lipids interact with CTAB. The action of chlorhexidine parallels that of CTAB; it disrupts lipid-bilayers at even lower concentrations. This suggests that the membrane damaging effect of chlorhexidine is in the main a consequence of its interaction with membrane lipids.

In view of the molecular differences between CTAB and chlorhexidine it is still not certain how the interaction with lipids takes place. In considering the lipid action of surfactants it is often tacitly assumed that the long chain disturbs the organization of the apolar part of the membrane. However, Maas[7] investigating alkylpyridinium chlorides with different chain lengths, showed that the lytic effect was independent of the chain length. Experiments with oleate micelles also support the view that disorganization of the apolar part of the membrane is not the main cause of lysis.[21]

However, the mere binding of a compound, causing a shift in the balance of the charged groups on the outside of the bilayer is not the primary cause of breakdown of the permeability barrier, as can be seen with the strongly bound ions such as Th^{4+} and UO_2^{2+}.

Recent studies suggest that in bilayers and biomembranes the phospholipid packing of the molecules is strongest at the glycerol parts of the phospholipids.[22,23] The course of the lytic process can be postulated as follows. In the yeast cell, polyphosphates[24,25] and anionic lipids contribute to the charge of the outward face of the cytoplasmic membrane, hence positively charged compounds will be concentrated here. Provided the adsorbed ion has a shorter or longer hydrophobic part, a lytic action follows by penetration of the most vulnerable site of the permeability barrier i.e. the region of the glycerol moieties. The presence of a positive charge (to promote the binding) and an apolar part sufficient to give an interaction with the glycerol moieties, are essential for this process.

It may be said that by chance CTAB and chlorhexidine have about the same lytic activity, the first having one charge and a long apolar chain, while the second has two charges and a less pronounced apolar part. Shortening the chain (dodecylpyridinium chloride)[7] or decreasing the molecular weight (paludrine) reduces the binding and decreases the lytic effect.

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