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Effect of chlorhexidine diacetate on "protoplasts" and spheroplasts of *Escherichia coli*, protoplasts of *Bacillus megaterium* and the Gram staining reaction of *Staphylococcus aureus*

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Chlorhexidine prevents the transformation of *Bacillus megaterium* cells to protoplasts by lysozyme, of *E. coli* cells to spheroplasts by penicillin and causes lysis of "protoplasts" and spheroplasts of *E. coli* stabilised in hypertonic sucrose solution. Transformation of *Staphylococcus aureus* cells to the Gram-negative condition occurred in contact with 10 to 800 μg/ml of chlorhexidine and 10, 50 and 100 μg/ml of cetyltrimethylammonium bromide but higher concentrations of the latter prevented this change. Results indicate that chlorhexidine damages the permeability barrier of bacterial cells.

THE adsorption of chlorhexidine by suspensions of *Escherichia coli* and *Staphylococcus aureus* and the leakage and turbidity changes caused by the drug have been described previously and chlorhexidine was thought to owe its bactericidal activity to a physical disorganisation of the permeability barriers of the cell (Hugo & Longworth, 1964).

To examine this hypothesis experiments were made on bacterial forms deprived of some or all of their cell walls.

Definitions. The following terms are used (McQuillen, 1960a): The term protoplast is reserved for the structure derived from a vegetative cell by removal of the cell wall. Where the cell wall is modified to render the organism spherical but it is not known whether the cell wall is entirely absent the term "protoplast" is used. The globular forms produced by growth in penicillin are termed spheroplasts. The feature common to the three forms is a deficiency of a component of the cell wall responsible for structural rigidity so these forms show greater susceptibility to lysis but can be stabilised by suspension in a solution of a non-permeable solute at sufficient concentration to balance the pressure within the depleted cell, for example with sucrose.

Experimental and results

Organisms used in the work were *Escherichia coli* (formerly NCTC 5934), *Bacillus megaterium* NCTC 6005 and *Staphylococcus aureus* (Oxford strain).

Culture media were prepared from Oxoid materials. Nutrient broth consisted of Lab Lemco 1 g, peptone 1 g, sodium chloride 0·5 g, distilled water to 100 ml. When a solid medium was required 1·8% of agar No. 3 was included. Liquid conversion medium for spheroplast preparation was the nutrient broth plus 0·25% w/v $MgSO_4.7H_2O$ and 0·33M sucrose. In all cases the pH after adjustment and sterilisation was 7·3.

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Chlorhexidine diacetate (I.C.I.) and cetyltrimethylammonium bromide, CTAB (B.D.H.), were obtained commercially. Egg white lysozyme (L. Light & Co. Ltd.) was used throughout.

The bacterial suspensions were prepared by washing 18 hr cultures grown at 37° for *E. coli* and *Staph. aureus* and 30° for *B. megaterium* from the surface of agar, centrifuging at 1,000 *g* for 2 min to remove agar and large clumps, and washing twice with water before resuspension and nephelometric standardisation.

The extinction was determined at a wavelength of 500 mμ in a Unicam SP500 spectrophotometer at 20° $\pm$ 1° using 1 cm silica cells against a reference cell containing water. For one experiment an E.E.L. nephelometer was used.

Microscopic examinations were made with a Baker interference microscope and for total counts a Helber counting chamber, 0·02 mm deep, was used. Experiments were in duplicate and representative results are shown.

PREPARATION OF "PROTOPLASTS" OF *E. coli.*

Repaske (1956) discovered that *E. coli* is sensitive to lysozyme in 2-amino-2-hydroxymethylpropane-1,3-diol(tris) buffer in the presence of ethylenediaminetetra-acetic acid (EDTA) and later Repaske (1958) pointed out that the relative amounts and the order of addition of the compounds are also critical. Mahler & Fraser (1956) showed that with *E. coli*, lysis could be prevented if sucrose 0·5M was present when spherical "protoplasts" were produced; lysis occurred upon dilution of the stabilising solution.

Preliminary experiments showed optimal conditions to be when 1 ml of an aqueous suspension of *E. coli* containing 50 $\times$ 10^8 cells/ml was added to 9 ml of the lytic system such that the final suspension contained 0·033M tris buffer pH 7·3 10 μg/ml lysozyme and 200 μg/ml EDTA, Lysis at 20° as estimated by spectrophotometric analysis, was complete in 30 min. Using the conditions outlined above and including 0·5M sucrose in the medium, small spherical bodies were evident on microscopical examination and the conversion appeared to be complete within 30 min of inoculation.

EFFECT OF CHLORHEXIDINE ON "PROTOPLASTS" OF *E. coli*

2 ml of a suspension of *E. coli* containing 50 $\times$ 10^9 cells/ml was added to 198 ml of the Tris/EDTA/lysozyme/sucrose system previously described. After 30 min contact at 20°, 9 ml quantities were removed and added to 1 ml quantities of chlorhexidine in 0·5M sucrose and changes in extinction over a period of time estimated. A control experiment with "protoplasts" added to 0·5M sucrose solution was also made. After 1 hr contact with chlorhexidine, samples were removed and examined by interference microscopy. The extinction determinations are presented in Fig. 1.

Low concentrations of chlorhexidine 5–20 μg/ml caused a rapid decrease in extinction (indicative of lysis) and over this range of

concentrations microscopic examination showed that no "protoplasts" were present but much lysed matter was evident. Higher concentrations of chlorhexidine 40–100 μg/ml produced effects ranging from a slight reduction to an increase in extinction as compared to "protoplasts" suspended in buffer. Microscopic examination showed that whilst no true spherical forms were present there was little evidence of lysis.

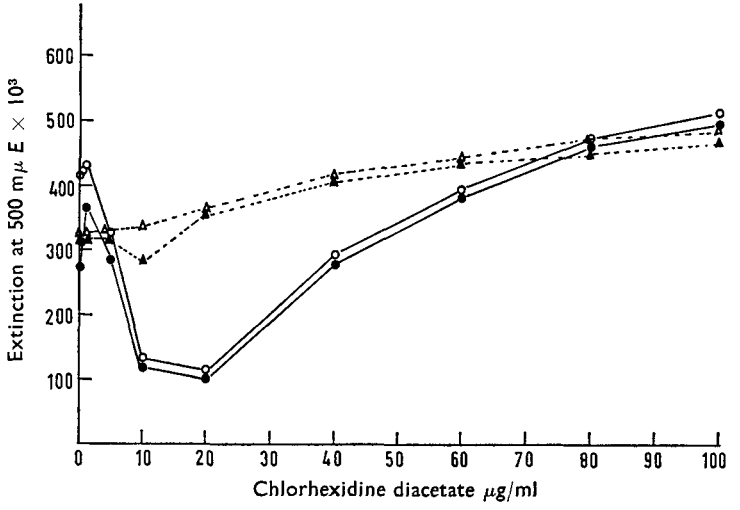

FIG. 1. Effect of concentration of chlorhexidine diacetate on the extinction ($E \times 10^3$ at 500 mμ) of "protoplasts" of *E. coli* in 0·5M sucrose. O—O Extinction after 5 min contact, ●—● extinction after 1 hr contact; and on the extinction of *E. coli* cells suspended in tris buffer pH 7·3. △ - - - △ Extinction after 5 min contact, ▲ - - - ▲ extinction after 3 hr contact.

Fig. 1 also shows the extinction of an *E. coli* cell suspension 50×10^7 cells/ml in 0·033M tris buffer read against a reference cell containing water after 5 min and 180 min contact at 20°. The slight decrease in extinction which occurred after 180 min over the concentration range 5–20 μg/ml is indicative of loss of cytoplasmic constituents from the cells. The increase in extinction of "protoplast" and cell suspensions which occurred in high drug concentrations is indicative of increased light scattering properties of the forms possibly caused by adsorbed chlorhexidine molecules (Hugo & Longworth, 1964).

EFFECT OF PRETREATMENT OF *B. megaterium* CELLS WITH CHLORHEXIDINE ON SUBSEQUENT TRANSFORMATION TO PROTOPLASTS

B. megaterium cells were suspended in water to contain 3·88 mg dry wt cells/ml and 5 ml of this suspension was added to 5 ml volumes of chlorhexidine solutions. After 30 min contact at 20° the cells were harvested by centrifugation washed in 10 ml of water and again harvested and resuspended in 10 ml water. 1 ml of this suspension was then added to 9 ml of a solution such that the final suspension contained 0·194 mg dry wt cells/ml, 20 μg/ml lysozyme 0·5M sucrose in 0·013M phosphate

buffer pH 7·3 at 20°, conditions previously determined as optimal for protoplast production. The control experiments consisted of cells treated with water before addition to lysozyme and cells examined after chlorhexidine treatment, omitting lysozyme treatment. After various time intervals in the lysozyme medium samples were examined by interference microscopy. The results are presented in Table 1.

TABLE 1. MICROSCOPIC APPEARANCE OF *B. megaterium* CELLS AFTER LYSOZYME TREATMENT (20 μg/ml) FOLLOWING 30 min CONTACT WITH CHLORHEXIDINE DIACETATE SOLUTIONS

Conc. of chlorhexidine diacetate pretreatment μg/ml	Period of contact with lysozyme		
	1 hr	3 hr	18 hr
0	conversion to spherical forms complete	spherical forms survive but some lysis evident	few spherical forms, much lysed matter
5	95% conversion to spherical forms	,, ,,	,, ,,
10	some spherical forms and lysed cells	much lysed matter	much lysed matter
20	rods and lysed cells	lysed matter and a few rods	lysed matter
50			
100			
200	rod forms only, no lysis	rod forms only, no lysis	rod forms only, no lysis
400			
500			

Cells treated with chlorhexidine but not exposed to lysozyme remained unchanged as rods. Lysozyme treatment of chlorhexidine-treated cells showed that in low concentrations the drug destroyed the permeability barrier which is responsible for maintaining lysozyme-produced protoplasts in hypertonic media. High concentrations of the drug rendered the cells insensitive to lysozyme action and inhibited lysis.

TABLE 2. EFFECT OF CHLORHEXIDINE DIACETATE AND CTAB ON THE GRAM STAINING REACTION OF *Staph. aureus*

Concn. of drug μg/ml	% of cells remaining Gram-positive after contact with drug, hr.							
	Chlorhexidine				CTAB			
	1	3	6	24	1	3	6	24
0	100	100	90	50	100	100	98	50
10	100	100	80	20	100	75	50	30
20	100	90	70	30				
30	100	90	60	10				
40	100	90	50	20				
50	100	90	50	5	100	95	50	40
100	100	90	50	5	100	95	70	50
200	100	90	50	5				
300	100	90	50	2				
500	100	90	50	2	100	100	100	98
800	100	90	40	2	100	100	100	98

PREPARATION OF SPHEROPLASTS OF *E. coli*

The method used was that of Hugo & Russell (1960): 10 ml of an 18 hr culture of *E. coli* in nutrient broth was added to 190 ml of the conversion medium containing 100 units/ml benzylpenicillin (sodium salt) and the

turbidity of the culture and the number of spheroplasts were determined hourly after incubation at 37°. The experiment was repeated but the cells were treated with 100 μg/ml chlorhexidine for 10 min before addition to the conversion medium. The results are presented in Fig. 2; no spheroplasts were produced when the cells were pretreated with chlorhexidine.

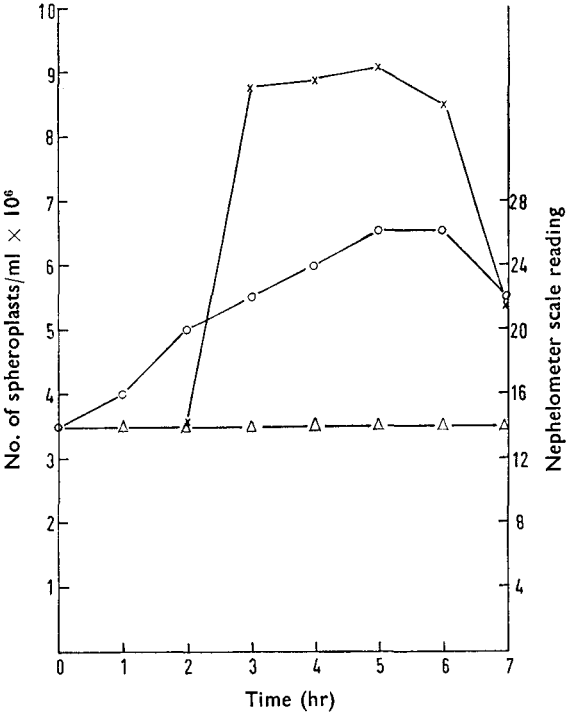

FIG. 2. Changes in turbidity (O—O) and number of spheroplasts (X—X) when culture of *E. coli* is treated with 100 units/ml benzylpenicillin in hypertonic medium. Also, changes in turbidity (△—△) of *E. coli* culture, pretreated with chlorhexidine diacetate 100 μg/ml for 10 min, in hypertonic medium containing 100 units/ml benzylpenicillin.

EFFECT OF CHLORHEXIDINE ON PENICILLIN-INDUCED SPHEROPLASTS OF *E. coli*

Spheroplasts were produced as described above and 4 hr after inoculation 9 ml samples were removed and added to 1 ml volumes of chlorhexidine in 0·33M sucrose at 20°, and the changes in extinction at 500 mμ followed. The results, presented in Fig. 3, show that chlorhexidine causes lysis of spheroplasts.

EFFECT OF CHLORHEXIDINE AND CTAB ON THE GRAM STAINING REACTION OF *Staph. aureus*

Dawson, Lominski & Stern (1953), found that low concentrations of CTAB caused loss of ability of *Staph. aureus* cells to retain the crystal violet–iodine complex of the Gram staining reaction whilst high

concentrations of the drug inhibited the conversion of cultures suspended in water to the gram-negative state.

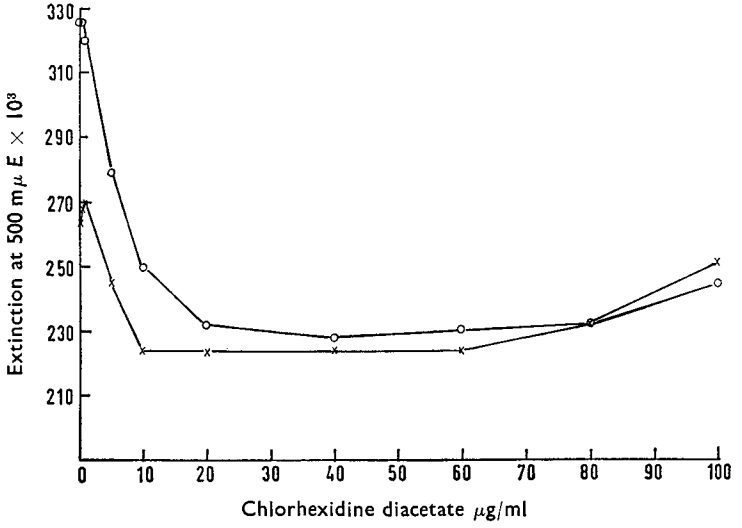

FIG. 3. Effect of chlorhexidine diacetate on penicillin-induced spheroplasts of *E. coli*. O—O Extinction ($E \times 10^3$) after 5 min contact. X—X Extinction ($E \times 10^3$) after 1 hr contact.

Cell suspensions containing *Staph. aureus* (1·2 mg dry wt cells/ml) were treated with various concentrations of chlorhexidine or CTAB at 20°, samples were Gram stained by the method of Preston (1962) and an estimate made of the percentage of Gram-positive cells. Table 2 shows that both compounds caused transformation to Gram-negative forms in low concentrations but the change was inhibited in high concentrations of CTAB.

Discussion

It is generally accepted that, underlying the rigid wall of a bacterial cell, there exists an osmotic barrier which is semipermeable and which bounds the contracting cytoplasm during plasmolysis (McQuillen, 1960b; Hughes, 1962). The membrane is also thought to be responsible for the control of diffusion into and out of the cell and is believed to be the site of action of many enzyme systems (Hughes, 1962).

Mitchell (1962) points out that to produce the spatial and metabolic organisation of the organism the enzymes must be supported upon or within a specifically locating framework and that the framework or backbone of the whole spatial metabolic organisation is in bacterial cells, the cell wall and cytoplasmic membrane. Hence any compound or circumstance which causes damage to this framework will upset the ordered reactions of the cell.

Our results show that chlorhexidine in low concentrations caused lysis

of osmotically sensitive forms suspended in hypertonic media and that higher concentrations affected the cell and rendered it insensitive to osmotic shock. Further it may be noted that treatment of cells with chlorhexidine destroyed their ability to undergo transformation to spherical forms on removal of, or damage to, the cell wall. Low concentrations of drug appeared to damage the cytoplasmic membrane of *B. megaterium* so that subsequent lysozyme treatment produced lysis and high concentrations fixed the cells in the rod shaped form. Possible mechanisms for this latter effect are: prevention of the action of lysozyme; denaturation or destruction of the cytoplasmic membrane; or physical sealing in of the cell surface caused by a build up of multilayers of drug around the cell.

If one assumes that only living bacteria possessing an intact cytoplasmic membrane and cytoplasm are able to undergo spherical transformation, then lack of this ability is indicative of death. The inability of chlorhexidine-treated cells to undergo spherical transformation in the presence of penicillin may be explained by the fact that spheroplasts are believed to be formed as a result of unbalanced cell wall synthesis, that is, where synthesis of the backbone mucopeptide of the cell wall is prevented by penicillin. If chlorhexidine reacts with the cells so as to destroy their viability, then no such transformation could occur.

Dawson, Lominski & Stern (1953) suggest that the inhibition of leakage of cell constituents caused by high concentrations of CTAB is a result of protein denaturation and inactivation of autolytic enzymes. It is accepted that damaged cells of *Staph. aureus* do not retain the crystal violet-iodine complex of the Gram stain and that damage to the cytoplasmic membrane and cell wall of this organism will cause a loss of Gram-positive staining (Salton, 1961). Dawson & others (1953) explain the retention of the crystal violet-iodine complex by cells treated with high concentrations of CTAB in terms of protein denaturation and cytoplasmic coagulation. However, no such effect is noted with chlorhexidine and high concentrations of this drug, which prevent lysis of osmotically sensitive forms and tend to inhibit leakage of cell constituents (Hugo & Longworth, 1964) are more effective in transforming cells to the Gram-negative state than are low concentrations which cause both lysis and leakage.

Autolytic enzymes cannot be involved in the observed lysis of "protoplasts" and spheroplasts, since where lysis occurred it was practically complete within 1 hr. It may be that inhibition of lysis and leakage by chlorhexidine is caused by a build up of multilayers of the drug on the cell surface; these layers are washed off by the iodine-acetone used in the decolourisation stage of the Gram staining procedure so that the reagent can then penetrate to decolourise the cell.

It would appear, therefore, that chlorhexidine exerts its bactericidal action by combination with the cell surface and disruption of the permeability barriers of the cell and that the subsequent manifestation of this damage in terms of the release of cell constituents plays only a minor role in the mode of action. The primary action of the drug appears to be

a disruption and disorganisation of the structure and function of the cytoplasmic membrane.

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