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Cytological aspects of the mode of action of chlorhexidine diacetate

W. B. HUGO AND A. R. LONGWORTH

Electron microscopic examination of ultra-thin sections of *Escherichia coli* cells treated with various concentrations of chlorhexidine diacetate reveal two effects. In low drug concentrations many cells lose electron dense material leaving behind empty shells, in higher concentrations the appearance of the cytoplasm is significantly affected. These results are discussed in the light of biochemical findings under identical conditions.

THE adsorption of chlorhexidine by *Escherichia coli* cells and the resulting leakage of cell constituents and turbidity changes have been described by Hugo & Longworth (1964a). The effect of chlorhexidine on osmotically sensitive forms has also been described (Hugo & Longworth, 1964b). This work suggests that chlorhexidine exerts its bactericidal action by adsorption onto the cell surface and a reaction with the permeability barriers of the cell. Further evidence for the mode of action of chlorhexidine was sought by examining ultra-thin sections of *E. coli* cells, treated with chlorhexidine, by electron microscopy.

Experimental

Materials. The organism used in the work was *E. coli* (formerly NCTC 5934).

Culture media and conditions were as previously described (Hugo & Longworth, 1964a). Chlorhexidine diacetate (Hibitane) was a commercial sample (Imperial Chemical Industries Ltd.).

Araldite resin for embedding specimens was prepared from Araldite 502 CY212. 27 ml, Hardener HY 964. 23 ml, Accelerator DY 064, 2% (Ciba ARL Ltd.) as described by Glauert (1961).

Methods. Cells were harvested, adjusted nephelometrically and suspended at a final concentration of 1·2 mg dry wt cells/ml in 0·013M phosphate buffer pH 7·3 containing various concentrations of chlorhexidine at 20°. After each desired time interval for exposure of the bacteria to chlorhexidine had elapsed, fixation was according to the method of Kellenberger, Ryter & Sechaud (1958).

The samples were dehydrated, embedded in araldite resin and polymerised by the method of Luft (1961).

After sectioning with a diamond knife on a Cambridge Huxley ultramicrotome the sections were collected on copper grids stained for 1 hr in a saturated ethanolic solution of uranyl acetate (Watson, 1958) washed briefly in water and examined in an A.E.I. EM6 electron microscope.

The criteria used for making the choice of cells to record on film were those described by Chapman (1962): (1) Cells recorded are representatives of a definite majority of the cell population. (2) As nearly sagittal or transverse sections as possible are recorded.

From the Department of Pharmacy, University of Nottingham.

Results

Figs 1 and 2 show the appearance of typical *E. coli* cells after 1 and 6 hr suspension in phosphate buffer. Fig. 3 shows cells after 6 hr treatment with 20 μg/ml chlorhexidine. Little, if any, change is observable.

Fig. 4 shows the effect of 6 hr treatment with 90 μg/ml chlorhexidine. Many cells have lost all their electron dense material leaving empty shells believed to be the cell wall. Fig. 5 shows cells after 6 hr treatment with 200 μg/ml chlorhexidine. At this concentration empty cells are not seen but the cytoplasm presents a different appearance from that in the untreated controls.

The appearance of protuberances or adhesions on the cell surface may also be noted. Fig. 6 shows cells treated with 500 μg/ml chlorhexidine

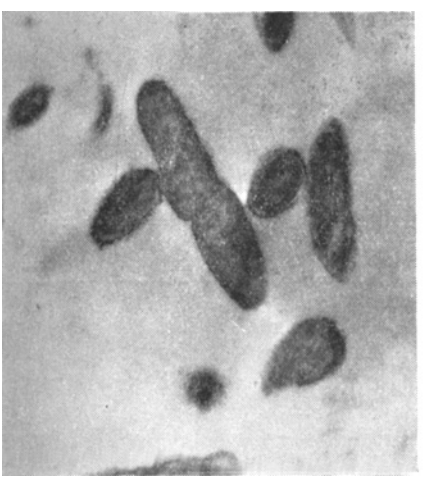

FIG. 1. *E. coli* cells suspended in buffer for 1 hr (× 16,250).

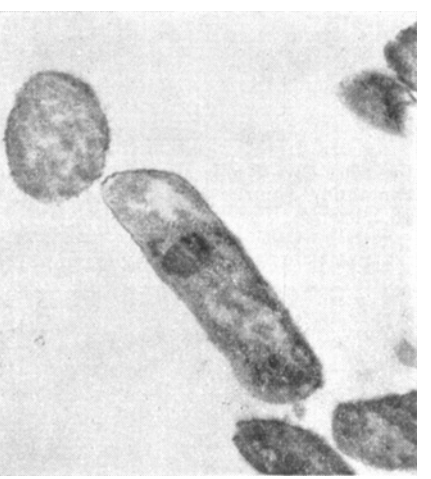

FIG. 2. *E. coli* cells suspended in buffer for 6 hr (× 20,000).

for 6 hr. Again, empty cells are not seen and the cytoplasm appears very granular. The protuberances or adhesions on the cell surface are larger and more numerous.

Further series of photographs taken after 1 and 3 hr contact with the same concentrations of drug as used above produced similar results and it would appear that of the two parameters, time and drug concentration, the second is more important with respect to chlorhexidine induced cytological damage.

Whilst it is accepted that in any population of cells the reaction to a stress will be variable it appears that three distinct effects of the drug depending on concentration may be differentiated. (1) Low concentrations, around 20 μg/ml, cause little or no observable cytological damage. (2) At concentrations around 90 μg/ml the drug causes cytological damage and loss of cell constituents. (3) At high concentrations, 200 and 500

μg/ml, cells do not appear to lose their cytoplasmic constituents. The cytoplasm, however, appears markedly different from that in the controls

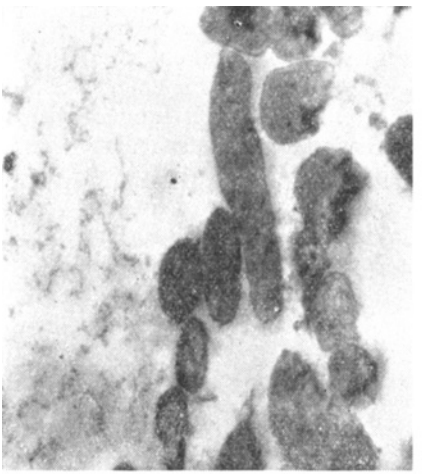

FIG. 3. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 20 μg/ml (× 16,250).

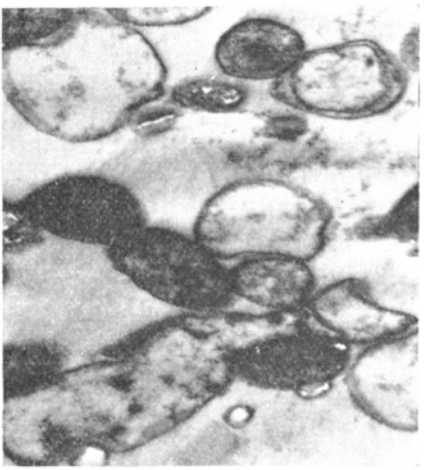

FIG. 4. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 90 μg/ml (× 20,000).

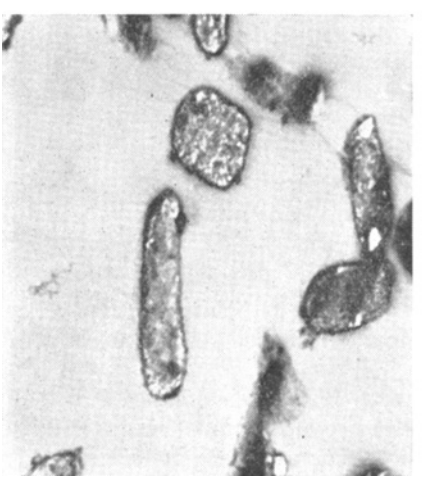

FIG. 5. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 200 μg/ml (× 16,250).

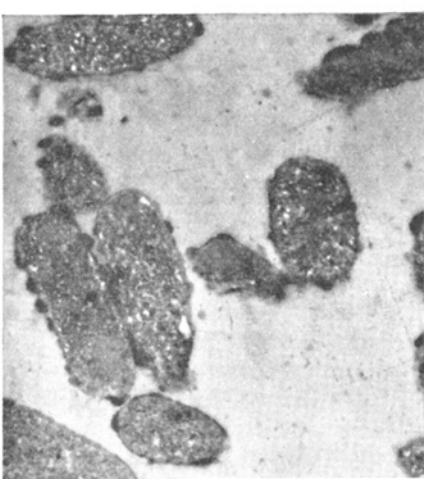

FIG. 6. *E. coli* cells after 6 hr in buffer containing chlorhexidine, 500 μg/ml (× 20,000).

in presenting a granular appearance. The appearance of swellings on the cell surface at these concentrations is not fully understood but could be due to cellular extrusion or to the accumulation of drug aggregates on the cell surface.

Discussion

Hugo & Longworth (1964a) showed that treatment of *E. coli* cells with chlorhexidine causes a leakage of cell constituents into the suspending medium, indicating damage to the permeability barriers of the cell and that certain concentrations corresponding to a particular drug adsorption level cause maximal leakage and changes in turbidity. Higher concentrations of drug seemed to cause a low initial level of leakage which was not followed by a secondary leakage even though the drug, as would be expected, was more rapidly bactericidal, as estimated by extinction data, at high concentrations.

The results of the present communication show that, at the concentration of chlorhexidine which causes maximal leakage, cytological damage in the form of cell rupture leaving empty ghosts of cells is evident, whilst high concentrations seem to cause a change in the appearance of the cytoplasm without causing loss of cytoplasmic constituents. Newton (1953), using polymyxin E, which shows a diphasic leakage effect similar to chlorhexidine, also noted that shadowed preparations of *Pseudomonas aeruginosa* lose their "electron-dense" material when treated with a concentration of polymixin which causes maximal leakage but retain all electron dense-material when treated with high concentrations, whilst showing marked surface damage also in the form of surface swelling.

Chapman (1963), using colomycin which may be of an identical structure to polymyxin E (Wilkinson, 1963; Hugo & Stretton, 1963), showed that at a constant dose level of 1000 μg/ml, which is far in excess of the concentration causing maximum leakage (Hugo and Stretton, unpublished observations), the cytological effects can be divided into three types which, he suggests, result from an altered intracellular ionic milieu in turn due to a primary effect of the antibiotic agent upon the plasma membrane, the site of selective permeability.

If it is accepted that the cytoplasmic membrane in bacterial cells is responsible both for osmotic regulation and enzymic co-ordination of the cell then it is unnecessary to invoke two separate modes of action, one for low and the other for high concentrations of the drug. That a reaction occurs with the permeability barriers of the cell is evident both from biochemical and cytological studies, and the manifestations of cell death so caused, in terms of release of cell constituents, depends upon the level of drug adsorption. The gross cytoplasmic disorganisation occurring in high concentrations of the drug could be caused by disruption of the enzymic co-ordinating function of the cytoplasmic membrane which could in turn be caused by a disruption of the structure of the membrane, although, in contrast to low concentrations, this damage is not revealed by the loss of cytoplasmic constituents.

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References

Chapman, G. B. (1962). *J. Bact.*, **84**, 169–185.
Chapman, G. B. (1963). *Ibid.*, **86**, 536–543.
Glauert, A. M. (1961). *Techniques for Electron Microscope*, p. 179, Editor, Kay, D., Oxford: Blackwell.
Hugo, W. B. & Longworth, A. R. (1964a). *J. Pharm. Pharmacol.*, **16**, 655–662.
Hugo, W. B. & Longworth, A. R. (1964b). *Ibid.*, **16**, 751–758.
Hugo, W. B. & Stretton, R. J. (1963). *Ibid.*, **15**, 489–490.
Kellenberger, E., Ryter, A. & Sechaud, J. (1958). *J. Biophys. Biochem. Cytol.*, **4**, 671–678.
Luft, J. H. (1961). *Ibid.*, **9**, 409–414.
Newton, B. A. (1953). *J. gen. Microbiol.*, **9**, 54–64.
Watson, M. L. (1958). *J. Biophys. Biochem. Cytol.*, **4**, 475–478.
Wilkinson, S. (1963). *Lancet*, **1**, 922–923.