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Release of phosphorus-32-containing compounds from *Micrococcus lysodeikticus* treated with chlorhexidine

R. M. RYE AND DAVID WISEMAN

Chlorhexidine has been shown to cause the release of cellular constituents from phosphorus-32 labelled cells of *Micrococcus lysodeikticus* suspended in distilled water or in phosphate buffer. An initial rapid release is followed by a slower secondary release. This secondary release is inhibited by high concentrations of chlorhexidine. The release depends on the chlorhexidine to cell ratio and not on the absolute bactericide concentration.

CHLORHEXIDINE, 1,6-di-(4-chlorophenyldiguanido)hexane, an antibacterial agent, is the most active of a large number of related biguanides which were synthesised (Davies, Francis, Martin, Rose & Swain, 1954; Rose & Swain, 1956). It is bactericidal against a wide range of both Gram-positive and Gram-negative organisms although the Gram-positive organisms are the more sensitive (Calman & Murray, 1956; Lawrence, 1960). Its activity is highly dependent upon its chemical structure, small changes in which cause a marked decrease in activity (Davies & others, 1954).

Experimental

MATERIALS

Chlorhexidine diacetate, Imperial Chemical (Pharmaceuticals) Ltd., Wilmslow, Cheshire.

Sodium phosphate-^{32}P, the Radiochemical Centre, Amersham, Buckinghamshire.

Micrococcus lysodeikticus, (NCTC 2665).

Suspending medium, distilled water or M/15 phosphate buffer pH 7·2.

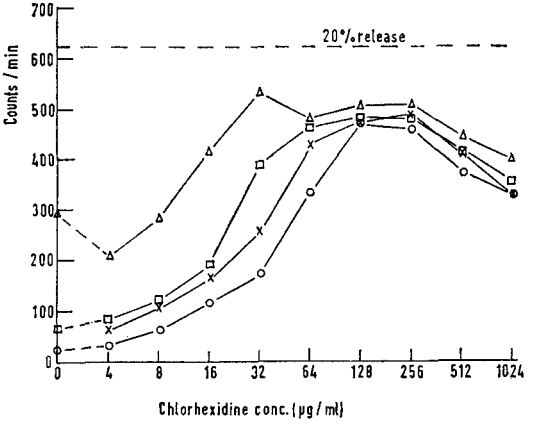

FIG. 1. Release of phosphorus-32 from *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium distilled water. Cell concentration $1{\cdot}7 \times 10^{10}$/ml. Contact times: ○ ½ hr, × 2 hr, □ 6 hr, △ 21 hr.

From the Department of Pharmacy, Bradford Institute of Technology

METHODS

Conditions of culture and harvesting. The organisms were grown for 48 hr at 30° on Oxoid tryptone soya agar containing 0·125 μc/ml of sodium phosphate-^{32}P. The cells were harvested, washed three times with the suspending medium and the total count of the final suspension determined by opacity measurements.

Treatment of cells and measurement of released radioactivity. Reaction mixtures were prepared from samples of the bacterial suspensions and equal volumes of suitable dilutions of chlorhexidine dissolved in suspending medium. The mixtures were maintained at room temperature (18–20°).

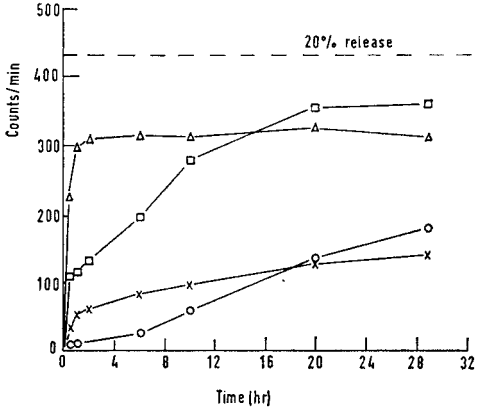

FIG. 2. Rate of release of phosphorus-32 from *Micrococcus lysodeikticus* suspended in distilled water alone and in the presence of chlorhexidine. Cell concentration 1·8 × 10^{10}/ml. ○ Distilled water alone: × 8, □ 32, △ 128 μg chlorhexidine/ml.

5 ml samples were taken from the mixtures after various time intervals, centrifuged at 4,500 rpm for 10 min and the radioactivity of the supernatant liquids measured by counting 3 ml samples using a Mullard MX 124/01 Liquid sample tube. In the experiments with "dilute" bacterial suspensions, 10 ml samples were taken and 7 ml of the supernatant liquid was counted. The total radioactivity of the bacteria was determined by counting 1 ml samples of the original suspensions. Each sample was counted for 15 min which in most cases reduced the random error (P = 68·3%) to below 2% of the observed count.

Results

Release from cells suspended in distilled water. Cells suspended in distilled water alone gradually release compounds containing ^{32}P.

The addition of chlorhexidine causes an initial rapid release during the first hr. The amount released during this period increases with increasing concentrations of chlorhexidine up to a maximum at 128 μg/ml. Concentrations in excess of 256 μg/ml cause less than this maximum release (Fig. 1).

In low concentrations of chlorhexidine, the initial release is followed by a slower secondary release extending over at least 30 hr. A secondary release was not observed in concentrations of chlorhexidine above 128 μg/ml (Fig. 2).

Release from cells suspended in M/15 *phosphate buffer.* Cells suspended in phosphate buffer alone show virtually no release over 30 hr. In the presence of chlorhexidine, the initial release is similar to that obtained with cells treated in distilled water. The subsequent secondary release however, differs from that occurring in water. This release is small with concentrations of chlorhexidine up to 8 μg/ml but with concentrations between 16 and 32 μg/ml, the secondary release is much greater and a peak in the release curve is observed (Figs 3 and 4).

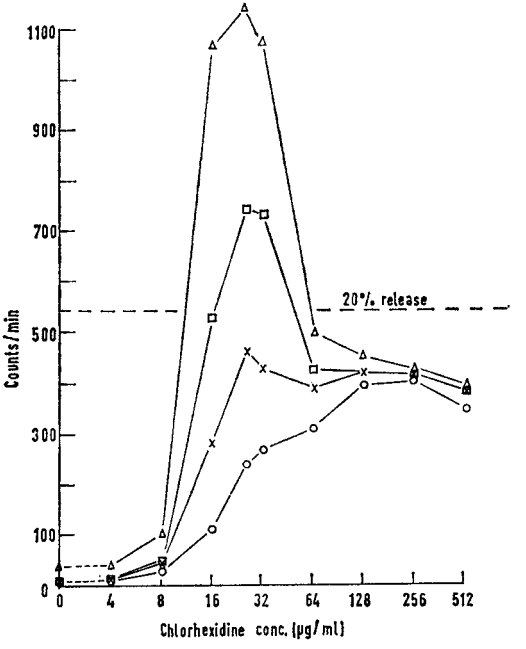

FIG. 3. Release of phosphorus-32 from *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium M/15 phosphate buffer (pH 7·2). Cell concentration $1{\cdot}5 \times 10^{10}$/ml. Contact times: ○ ½ hr, × 2 hr, □ 6 hr, △ 21 hr.

Release from "dilute" cell suspensions. The experiments in phosphate buffer were repeated using a much lower cell concentration. The results are given in Fig. 5. A similar pattern of release was observed but occurred at proportionately lower chlorhexidine concentrations.

Discussion

Several antibacterial agents have been shown to initiate or accelerate the release of cellular constituents. These compounds fall into three main groups:

(i) *Surface-active agents.* Hotchkiss (1946), Gale & Taylor (1947), Salton (1950, 1951), Newton (1953), Stedman, Kravitz & King (1957).

(ii) *Phenolic compounds.* Hotchkiss (1946), Gale & Taylor (1947), Beckett, Patki & Robinson (1959), Joswick (1962), Judis (1962, 1963).

(iii) *Polypeptide antibiotics.* Hotchkiss (1946), Gale & Taylor (1947), Newton (1953), Few & Schulman (1953).

Chlorhexidine does not readily fit into any of the above groups. It bears closest resemblance to the quaternary ammonium compounds in its chemical structure, the concentrations required for activity and in its antibacterial spectrum. Unlike the quaternary compounds however, chlorhexidine has little effect on the surface tension of water. A concentration of 256 μg/ml chlorhexidine diacetate in distilled water gives readings between 68–70 dynes/cm using a Du Nouy Tensiometer.

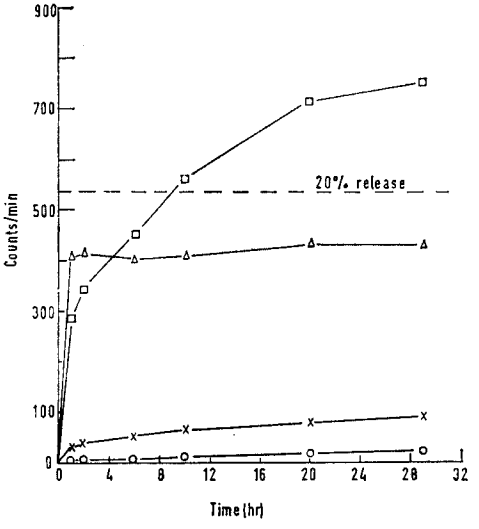

FIG. 4. Rate of release of phosphorus-32 from *Micrococcus lysodeikticus* suspended in M/15 phosphate buffer (pH 7·2) alone and in the presence of chlorhexidine. Cell concentration $1{\cdot}8 \times 10^{10}$/ml. ○ Buffer alone: × 8, □ 32, △ 128 μg chlorhexidine/ml.

The release of cellular constituents from bacteria is regarded by many workers as occurring in two stages.

Hotchkiss (1946) states that adsorption of the surface-active agent onto the bacterial surface results in irreversible damage to the cellular membrane so that the total content of soluble nitrogen and phosphorus compounds is released from the cell. The membrane injury is the signal for the beginning of a series of enzymatic processes which may lead to the virtual dissolution of the cell. The exposure of staphylococci to various bactericides for 20 min at 25° is sufficient to allow autolysis.

Salton (1951), studying the release of 260 mμ absorbing materials from *Staphylococcus aureus* treated with cetyltrimethylammonium bromide

found the primary release to be completed in 3–4 hr. The autolytic breakdown follows this and does not appear to make a significant contribution during the initial leakage.

Newton (1953), studying the release of cellular constituents from *Pseudomonas aeruginosa* treated with cetyltrimethylammonium bromide obtained results similar to those of both Salton and Hotchkiss, and showed that the release of 260 mμ absorbing material, pentose and total phosphate all occurred at the same rate.

Stedman, Kravitz & King (1957) also observed a two stage release of carbon-14 labelled compounds from *Serratia marcescens* treated with a quaternary ammonium compound.

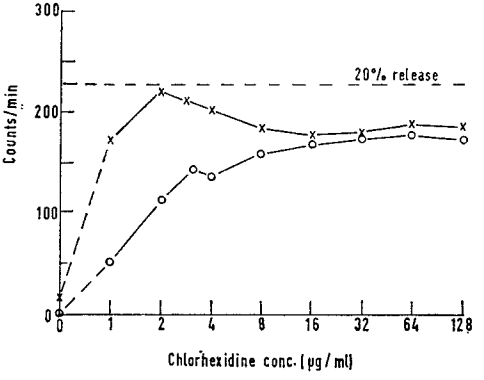

FIG. 5. Release of phosphorus-32 from "dilute" suspensions of *Micrococcus lysodeikticus* treated with chlorhexidine. Suspending medium M/15 phosphate buffer (pH 7·2). Cell concentration 9×10^8/ml. Contact times ○ 3 hr, × 20 hr.

Our results using chlorhexidine show that the release of phosphorus-containing compounds occurs in two stages. The rate and extent of release depends on the ratio of chlorhexidine to bacterial cells and not upon the absolute concentration of chlorhexidine. The initial release is rapid, being completed in 1–2 hr, and is compatible with the hypothesis that cell membrane damage has occurred. We found the maximum initial release produced by chlorhexidine to be between 12% and 22% of the total activity.

The secondary release, which is observed to occur only at low ratios of chlorhexidine to bacteria, takes place over at least 30 hr. This secondary release, which may be due to autolysis in the damaged cells, contributes little to the amount released during the first 2 hr.

Our observations that the autolysis is inhibited by high concentrations of chlorhexidine resemble the findings of Newton (1953) with cetyltrimethylammonium bromide and Joswick (1962) with hexachlorophane.

The increased rate of autolysis in phosphate buffer may result from the involvement of phosphate-requiring enzymatic processes, or it may be due simply to the effect of pH.

The apparent decreased release at concentrations of chlorhexidine in excess of 256 μg/ml may be attributable to a precipitation of labelled compounds by the chlorhexidine and to their removal during centrifuging.

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