J. Pharm. Pharmac., 1966, **18**, *Suppl.*, 114S–118S Received May 2, 1966

Effect of chlorhexidine upon ^{32}P release and cell viability in *Escherichia coli*

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The amount of the cold trichloroacetic acid soluble fraction (metabolic pool), expressed as a percentage of its ^{32}P content, released from labelled cells of *Escherichia coli* treated with chlorhexidine has been compared with the percentage of cells killed by the chlorhexidine. Treatment was with amounts varying from 0–64 μg/ml for 5 min at 20°. Approximately 100% of the cells were killed and 100% of the metabolic pool released by treatment with 64 μg/ml of chlorhexidine. At 4, 8 and 16 μg/ml the percentage of cells killed by chlorhexidine is significantly lower than the percentage of the metabolic pool released.

CHLORHEXIDINE causes the release of ^{32}P from labelled cells of *Micrococcus lysodeikticus* (Rye & Wiseman, 1964) and of pentose and material absorbing at 260 mμ from *M. lysodeikticus* (Wiseman, 1964), *Staphylococcus aureus* and *Escherichia coli* (Hugo & Longworth, 1964). The pattern of release is similar in all instances. As the concentration of chlorhexidine is increased, the extent of release increases to a maximum then decreases at higher concentrations.

Using chlorhexidine at concentrations up to that causing the maximum release of ^{32}P from *M. lysodeikticus*, Rye & Wiseman (1965) showed that the initial release came from the metabolic pool which is that fraction soluble in cold trichloroacetic acid. They suggested that if the ^{32}P content of this fraction was not completely released only a proportion of the cells were leaking.

Hugo & Longworth (1964) determined the mean single survivor times of *Staph. aureus* and *E. coli* after treatment with chlorhexidine concentrations very much greater than those producing maximum release. These authors concluded that over the range of concentrations examined there was no relationship between the amount of cellular constituents released and the number of organisms killed, although they recognised that such a relationship might be present at lower concentrations of chlorhexidine.

This paper reports an investigation of the relation between the extent of ^{32}P release from labelled cells of *E. coli* and the number of organisms killed after 5 min treatment at 20° with concentrations of chlorhexidine up to that causing maximum release.

Experimental

MATERIALS

Chlorhexidine diacetate. Imperial Chemical (Pharmaceuticals) Ltd.
Escherichia coli (NCTC 86).

Growth medium. Ammonium chloride 5×10^{-2}M, magnesium chloride 5×10^{-4}M, sodium sulphate 5×10^{-4}M, potassium dihydrogen phosphate 10^{-3}M, trishydroxymethylaminomethane 10^{-1}M, and glucose 1 mg/ml. The pH was adjusted to 7·7 using M hydrochloric acid.

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Glucose-free medium was growth medium from which glucose had been omitted. *Low-phosphate medium* was growth medium without added phosphate and contained approximately 4×10^{-5}M phosphate as impurity.

Egg yolk solution. The separated yolks from two eggs were mixed with 200 ml of Oxoid tryptone soya broth and 6 g of kaolin. The resulting suspension was clarified by centrifuging at 6,000 rpm for 10 min.

METHODS

Absorbance measurements were made with a 1 cm path length at 650 mμ using a Unicam SP500 spectrophotometer.

Preparation of ^{32}P-labelled bacterial cells. E. coli was grown at 37° with aeration by shaking at 120 throws/min. 100 ml of cells in the log phase (absorbance approx. 0·4) were harvested by filtration through an 8 cm diameter Oxoid membrane filter, washed with and then suspended in low-phosphate medium at 37°. 0·5 μc of ^{32}P orthophosphate (specific activity 5 c/mg phosphorus) was added followed 30 sec later by 5 ml of 10^{-1}M potassium dihydrogen phosphate. After a further 1 min the labelled suspension was filtered, washed with and then suspended in glucose-free medium at 20°. The absorbance was adjusted to 0·600 with more glucose-free medium.

Reaction mixtures. 5 ml samples of the labelled cell suspensions were added to tubes each containing 5 ml of suitable dilutions of chlorhexidine in glucose-free medium at 20°. Five min after adding the cells, samples were removed for radioactivity measurements and for viable counting. Two min intervals were allowed between inoculations to permit all measurements to be made after exactly 5 min reaction time.

Radioactivity measurements. 2 ml samples in duplicate of the reaction mixtures and of untreated cell suspensions were filtered through 30 mm Oxoid membrane filters and washed with 4 ml of phosphate buffer pH 7·0. The washing of samples was completed within 15 sec. The membranes were attached to flat aluminium planchets with "Durofix" adhesive, dried and the ^{32}P content determined by counting for 50 min or until 50,000 counts were recorded, using a Beckman "Lowbeta" automatic planchet counter. The radiochemical statistical error was less than 1% ($P = 0{\cdot}95$).

^{32}P content of the cold trichloroacetic acid soluble fraction (metabolic pool). Samples (2 ml) of bacterial suspensions were mixed with equal volumes of 10% trichloroacetic acid and maintained at 4° for 30 min. The radioactivity remaining in the cells after this treatment was measured using the method described above and the ^{32}P content of the metabolic pool determined by subtraction from the total cellular radioactivity.

Determination of the percentage of organisms killed. Samples (0·2 ml) of the reaction mixtures were transferred to 10 ml of egg yolk solution or to 10 ml of glucose-free medium. Suitable dilutions in tryptone-soya broth or glucose-free medium respectively were then counted by the pour plate method using Oxoid tryptone-soya agar and 24 hr incubation at 37°. The number of organisms killed was calculated and expressed as a percentage of the number of viable organisms in untreated suspensions.

Results and discussion

When *E. coli* is labelled by cultivation in a synthetic medium containing ^{32}P labelled phosphate, approximately 20% of the radioactivity is present in the metabolic pool (Roberts, Abelson, Cowie, Bolton & Britten, 1957). The method of labelling described in this paper produces cells in which 75–85% of the radioactivity is in the metabolic pool and thus enables changes in this pool to be more accurately measured. Fig. 1 shows the changes in the total cellular radioactivity and of that fraction insoluble in cold trichloroacetic acid over a period of 3 hr at 20° in untreated cells suspended in glucose-free medium. The decrease in the ^{32}P content of the metabolic pool is due either to leakage from the cells or to an exchange with non-labelled phosphate in the suspending medium. This rate of decrease (0·23%/min) is approximately linear over 3 hr.

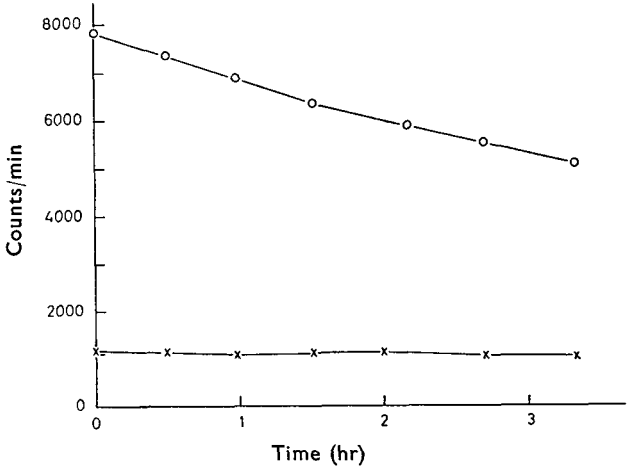

FIG. 1. The changes with time of the ^{32}P content of labelled cells of *Escherichia coli* suspended in glucose-free medium pH 7·7 at 20°. Cell concentration 3·2 × 10^8/ml. O——O, total cellular radioactivity. X——X, radioactivity remaining after treatment with 5% trichloroacetic acid for 30 min at 4°.

Fig. 2 shows the effect on the total cellular radioactivity when *E. coli* cells are treated with various concentrations of chlorhexidine. The amount of radioactive material released from the cells increases with chlorhexidine concentrations up to 64 μg/ml when it becomes constant and is approximately equal to the ^{32}P content of the metabolic pool.

Viable counts and ^{32}P release. Experiments to assess the efficiency of several agents for the inactivation of chlorhexidine showed that the highest recovery was obtained by using solutions of fresh egg yolk in tryptone-soya broth as the primary inactivator and tryptone-soya broth for the remaining dilutions.

Fig. 3 shows the average of six experimental determinations of the percentage of organisms killed after 5 min treatment with chlorhexidine, at concentrations up to 64 μg/ml, when both egg yolk and dilution were

used to inactivate the chlorhexidine. Fig. 3 also shows the extent of ^{32}P release expressed as a percentage of the metabolic pool after 5 min treatment with chlorhexidine. These results are the average of seven duplicate experiments. Table 1 shows the average of five experimental determinations of the apparent percentage of organisms killed after 5 min treatment with chlorhexidine when dilution only was used to inactivate the chlorhexidine.

TABLE 1. EFFECT OF TREATMENT WITH CHLORHEXIDINE FOR 5 MIN AT 20° UPON THE APPARENT VIABILITY OF *Escherichia coli*

Chlorhexidine concentration μg/ml	2	4	8	16	32	64
Apparent percentage of cells killed with estimated standard deviation	13 ± 6·5	32 ± 6·5	39 ± 12	80 ± 20	>99	>99

Suspending medium glucose-free medium pH 7·7. Cell concentration 3·2 × 10^8/ml. Chlorhexidine inactivated by dilution alone.

At concentrations of chlorhexidine between 2 and 32 μg/ml the percentage of the metabolic pool released is significantly greater than the percentage of cells killed. A possible explanation is that when chlorhexidine is adsorbed by bacteria it produces cellular damage resulting initially in leakage of the metabolic pool and finally in death. The chlorhexidine-cell ratio probably governs both the rate at which this

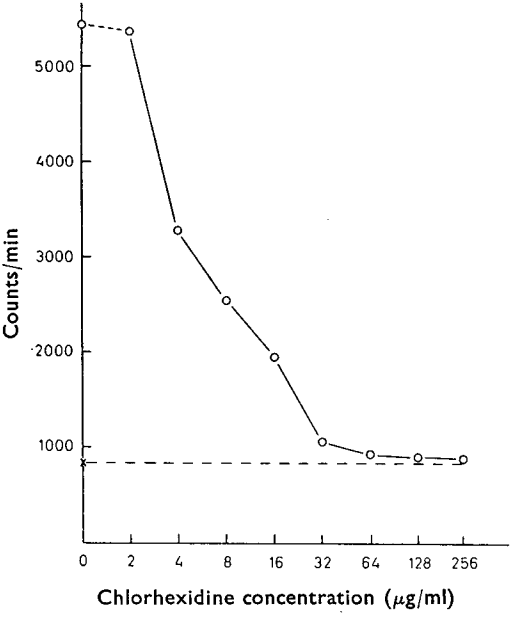

FIG. 2. The effect of treatment with chlorhexidine for 5 min at 20° on the ^{32}P content of labelled cells of *Escherichia coli* suspended in glucose-free medium pH 7·7. Cell concentration 3·2 × 10^8/ml. ○——○, total cellular radioactivity. X——X, radioactivity remaining after treatment with 5% trichloroacetic acid for 30 min at 4°.

process occurs and the proportion of cells damaged. Thus at low chlorhexidine concentrations (4, 8 and 16 μg/ml), if the adsorbed bactericide molecules are removed or inactivated by egg yolk before death has occurred, then some of the cells which have lost all or part of their metabolic pool are able to recover. This interpretation recalls that of Judis (1962) who studied the action of phenolic disinfectants on *E. coli* and suggested that the leakage of cellular constituents probably precedes the death of the organisms and that a certain amount of damage to the cell membrane can be tolerated and repaired.

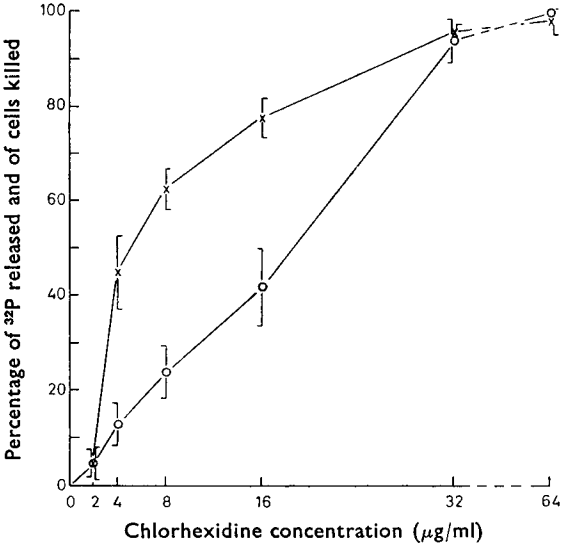

FIG. 3. Effect of chlorhexidine after 5 min at 20° on the release of ^{32}P from, and the cell viability of *Escherichia coli* suspended in glucose-free medium pH 7·7. Cell concentration 3·2 × 10^8/ml. X——X, ^{32}P release as a percentage of the metabolic pool. ○——○, percentage of cells killed. The results are the mean of six experiments and estimated standard deviations are indicated.

Within 5 min at higher concentrations of chlorhexidine (32 and 64 μg/ml) most of the cells appear to be damaged beyond recovery; little increase in the percentage of survivors recovered is observed on inactivating the chlorhexidine with egg yolk.

Acknowledgement. The authors are grateful to Mrs. S. Kaye for valuable technical assistance.

References

Hugo, W. B. & Longworth, A. R. (1964). *J. Pharm. Pharmac.*, **16**, 655–662.
Judis, J. (1962). *J. pharm. Sci.*, **51**, 261–265.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1957). *Studies of Biosynthesis in* Escherichia coli. Washington: Kirby Lithographic Co.
Rye, R. M. & Wiseman, D. (1964). *J. Pharm. Pharmac.*, **16**, 516–521.
Rye, R. M. & Wiseman, D. (1965). *Ibid.*, **17**, 295–301.
Wiseman, D. (1964). *Ibid.*, **16**, *Suppl.* 56*T*–57*T*.