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Chemical fractionation of phosphorus-32 labelled cells of *Micrococcus lysodeikticus* treated with chlorhexidine

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A method for the chemical fractionation of *Micrococcus lysodeikticus* into "cold trichloroacetic acid soluble", alcohol soluble, ribonucleic acid and residual fractions is described. The results of applying this method to untreated and chlorhexidine-treated ^{32}P labelled cells are discussed. In untreated cells the ^{32}P content of the "cold trichloroacetic acid soluble" fraction, which constitutes the metabolic pool, increases at a rate dependent on temperature increase and there is a corresponding decrease in the ^{32}P content of the ribonucleic acid fraction. At the highest temperature, 40°, the increase of ^{32}P in the pool is followed by a decrease due to leakage from the pool. The release of ^{32}P from chlorhexidine-treated cells maintained at 1°, 20°, 30° and 40° has been measured after 0·5, 2 and 13 hr. There is an initial rapid release which is entirely from the "cold trichloroacetic acid soluble" fraction and a slower secondary release from the ribonucleic acid fraction. This secondary release is almost completely inhibited at 1° and by high (64 and 128 μg/ml) chlorhexidine concentrations.

THE release of radioactive compounds from phosphorus-32 labelled cells of *Micrococcus lysodeikticus* treated with chlorhexidine occurs in two stages. The initial rapid release (1–2 hr) has been attributed to cell membrane damage allowing the leakage of small molecules from the cells, and the slower secondary release to autolytic breakdown in the damaged cells (Rye & Wiseman, 1964). The antibacterial action of chlorhexidine has now been further investigated by studying the effect of temperature on the patterns of release and by a chemical fractionation of untreated and chlorhexidine-treated cells.

Experimental

Materials. The materials, conditions of culture and harvesting were as previously described (Rye & Wiseman, 1964) except that the culture medium used was 1% tryptone agar containing 0·5 μc/ml of sodium phosphate-^{32}P.

Suspending medium. M/15 phosphate buffer pH 7·2 or M/40 tris-hydroxymethylaminomethane (Tris)-HCl buffer pH 7·2.

Reaction mixtures. Bacterial suspensions and equal volumes of buffer or chlorhexidine solutions were maintained at the required temperature for 1 hr before mixing. The final cell concentrations were $1{\cdot}5 \times 10^{10}$/ml.

CHEMICAL FRACTIONATION OF CELLS AND MEASUREMENT OF RADIOACTIVITY

1. *Release of ^{32}P into the suspending medium.* 4 ml samples from each reaction mixture were taken at various time intervals and centrifuged at 5000 rpm for 5 min. The supernatant fluids were removed, recentrifuged and retained.

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2. *Cold trichloroacetic acid soluble fraction.* (*"Cold TCA soluble" fraction*). The walls of the tubes from stage 1 were dried with cellulose tissues to remove traces of liquid and the residual cells suspended in 5 ml of 10% TCA and maintained at 4° for 30 min. The tubes were centrifuged and the supernatant fluids recentrifuged and retained.

3. *Alcohol soluble fraction.* The residues from stage 2 were washed with buffer, centrifuged, suspended in 5 ml of 75% ethanol and maintained at 40° for 30 min. After centrifuging, the residues were resuspended in 4 ml of a mixture of equal volumes of ether and 75% ethanol for 15 min at 40° and recentrifuged. The ethanol and ethanol-ether extracts were then pooled and retained.

4. *Ribonucleic acid (RNA) fraction.* The residues from stage 3 were suspended in 4 ml of M sodium hydroxide for 90 min at 30° and then acidified with 5 ml of M hydrochloric acid. After standing for 15 min at room temperature, the tubes were centrifuged and the supernatant fluids retained.

5. *Residual fraction.* The residues from stage 4 were washed, centrifuged and resuspended in buffer.

The radioactivities of the residual fraction and of 2 ml samples of each of the fractions collected in stages 1–4 were measured and calculated as a percentage of the total radioactivity present. The method used was that described by Rye & Wiseman (1964).

Results

EFFECT OF TEMPERATURE ON THE RELEASE OF ^{32}P FROM UNTREATED AND CHLORHEXIDINE-TREATED CELLS

Table 1 shows the percentage of ^{32}P released from untreated and chlorhexidine-treated cells maintained at 1°, 20°, 30° and 40° after 0·5, 2 and

TABLE 1. RELEASE OF ^{32}P FROM *Micrococcus lysodeikticus* TREATED WITH CHLORHEXIDINE AT VARIOUS TEMPERATURES, EXPRESSED AS PERCENTAGE OF TOTAL CELLULAR ACTIVITY. CELL CONCENTRATION $1{\cdot}5 \times 10^{10}$/ml. SUSPENDING MEDIUM M/15 PHOSPHATE BUFFER (pH 7·2).

	1° Chlorhexidine concentration µg/ml					20° Chlorhexidine concentration µg/ml				
Time	0	8	16	32	128	0	8	16	32	128
30 min	0·7	1·2	3·3	7·8	10·8	0·4	0·7	3·1	10·5	14·8
2 hr	1·0	1·8	4·7	8·1	12·7	0·6	1·2	8·5	15·7	15·7
13 hr	1·3	2·0	5·8	11·0	12·6	2·3	3·1	12·3	32·0	17·3
	30° Chlorhexidine concentration µg/ml					40° Chlorhexidine concentration µg/ml				
Time	0	8	16	32	128	0	8	16	32	128
30 min	0·5	1·0	4·9	13·7	15·5	1·7	3·3	10·6	31·2	30·0
2 hr	1·2	1·9	8·5	22·5	17·4	3·4	4·7	15·8	35·0	30·0
13 hr	6·1	7·8	20·2	34·8	16·8	20·4	23·4	46·5	56·0	36·0

13 hr. There is a gradual release of ^{32}P from untreated cells at all temperatures. This release is small except after 13 hr at 30° and 40°. Treatment of cells with chlorhexidine increases the amount of ^{32}P released. At 1°, 20° and 30°, the extent of release after 30 min increases with increasing concentrations of chlorhexidine and at 20° and 30° the subsequent secondary release is similar to that previously described (Rye & Wiseman, 1964). At 1° the secondary release is almost completely inhibited. At 40° both the initial release and the secondary release are greater than at the other temperatures.

EFFECT OF TEMPERATURE ON THE ^{32}P RELEASE AND ^{32}P CONTENT OF THE "COLD TCA SOLUBLE" FRACTION IN UNTREATED CELLS

Fig. 1A shows (*a*) the amount of ^{32}P released into the suspended medium from untreated-cells maintained at 20° and (*b*) that remaining in the "cold TCA soluble" fraction which constitutes the metabolic pool of these cells.

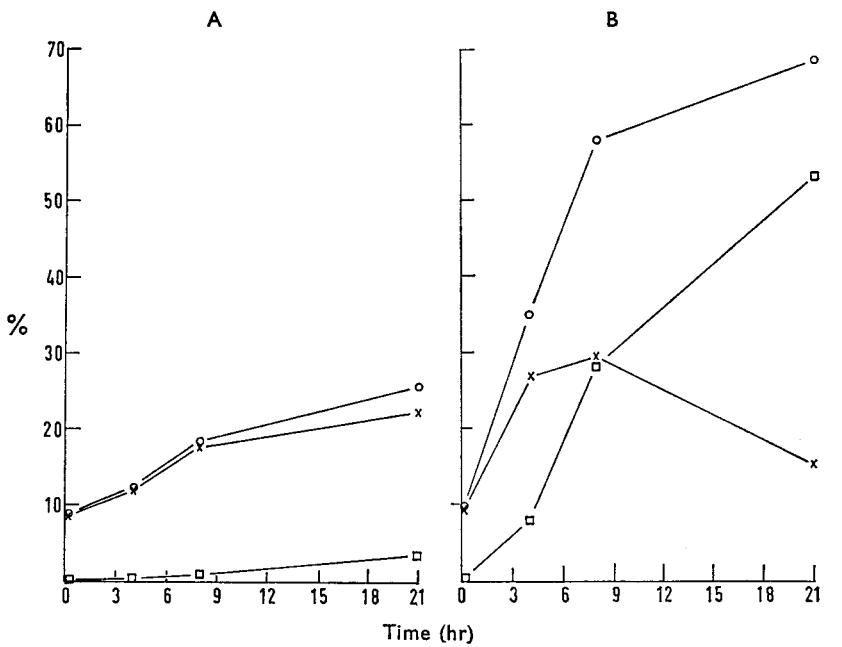

FIG. 1. Changes (%) in the amount of ^{32}P present in the cold TCA soluble fraction and released from *Micrococcus lysodeikticus* on storage in phosphate buffer (pH 7·2) at 20° (A) and 40° (B). □ Release (*a*), × Cold TCA soluble (*b*), ○ Sum of (*a*) and (*b*).

Both (*a*) and (*b*) gradually increase over 21 hr. Fig. 1B shows the results of similar measurements at 40°. Considerable release of ^{32}P occurs and the ^{32}P content of the metabolic pool at first increases but then decreases due to leakage from the cells.

EFFECT OF CHLORHEXIDINE ON THE DISTRIBUTION OF ^{32}P IN THE CELL FRACTIONS

Fig. 2A shows (*a*) the amount of ^{32}P released from cells treated with chlorhexidine for 15 min at 20° and (*b*) that remaining in the "cold TCA soluble" fraction of these cells. As the concentration of chlorhexidine

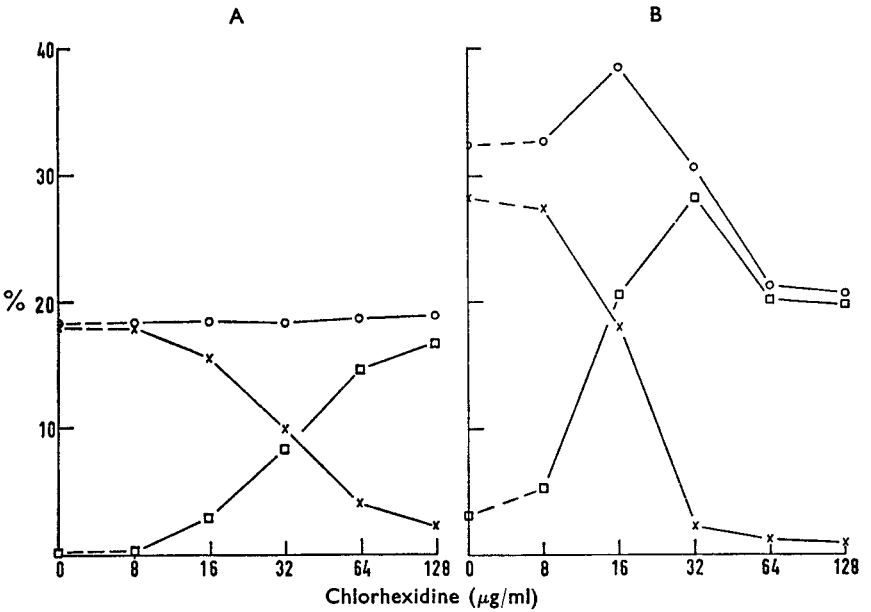

FIG. 2. Distribution of ^{32}P between the released material and the cold TCA soluble fraction of *Micrococcus lysodeikticus* treated with chlorhexidine in Tris buffer (pH 7·2) at 20° for 15 min (A) and for 22 hr (B). □ Release (*a*), × Cold TCA soluble (*b*), ○ Sum of (*a*) and (*b*).

is increased, (*a*) increases and there is a corresponding decrease in (*b*), the sum of (*a*) and (*b*) remaining constant. After 22 hr at 20° an increase occurs in the sum of (*a*) and (*b*) at low chlorhexidine concentrations but not at 64 and 128 μg/ml (Fig. 2B). The rate of increase of the sum of (*a*) and (*b*) at 30° is greater than at 20°. The results at 30° between 15 min and 22 hr are shown in Fig. 3.

The alcohol-soluble fraction was found in all our experiments to contain between 2–3·5% of the total ^{32}P content of the cells.

The ^{32}P content of the RNA fraction decreases to varying extents over a period of 22 hr in both untreated and chlorhexidine-treated cells. Results obtained after 22 hr at 30° are shown in Fig. 4. At 64 and 128 μg/ml of chlorhexidine, little change in the ^{32}P content of the RNA fraction occurs, but at lower concentrations (16 and 32 μg/ml) the decrease in the ^{32}P content of this fraction is significantly greater than that occurring in untreated cells. Fig. 4 also shows that a decrease in the ^{32}P content of the RNA fraction is accompanied by a corresponding increase in the total of ^{32}P released and ^{32}P present in the "cold TCA soluble" fraction, and that the residual fraction remains unchanged.

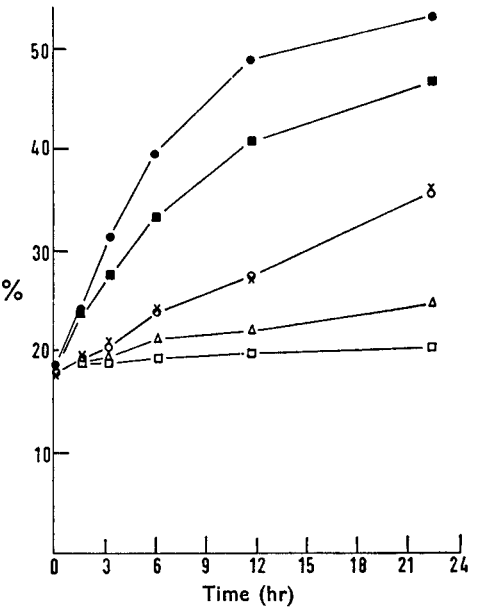

FIG. 3. Effect of chlorhexidine on the rate of change of the sum of the amount (%) of ^{32}P released and that present in the cold TCA soluble fraction of *Micrococcus lysodeikticus* suspended in Tris buffer (pH 7·2) at 30°. Chlorhexidine concentrations, ○ 0, × 8, ● 16, ■ 32, △ 64, □ 128 μg/ml.

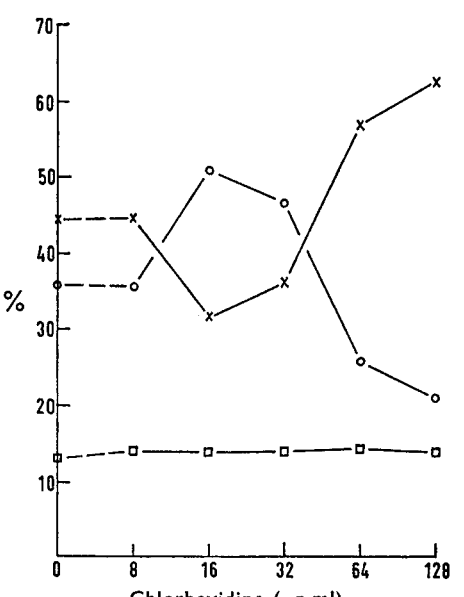

FIG. 4. The distribution of ^{32}P in *Micrococcus lysodeikticus* suspended in Tris buffer (pH 7·2) at 30° after 22 hr treatment with chlorhexidine. × RNA fraction, □ Residual fraction, ○ Sum of ^{32}P release and that present in the cold TCA soluble fraction.

Discussion

FRACTIONATION TECHNIQUES

The distribution of phosphorus among various broad classes of compounds present in bacteria can be determined after chemical fractionation of the cells. The fractionation methods reported in the literature, although similar in principle, have varied in detail (Hutchison & Munro, 1961). We selected the concentrations of reagents and conditions of reaction used in each stage after studying the effect of varying these factors upon the rate and extent of extraction from *M. lysodeikticus*.

Treatment of cells with cold TCA extracts the metabolic pool (Britten & McClure, 1962) and in several species of bacteria the phosphorus-containing compounds in this pool have been shown to consist of inorganic phosphate and nucleoside phosphates (Roberts, Abelson, Cowie, Bolton & Britten, 1957; Franzen & Binkley, 1961; Smith & Maaloe, 1964). We found that when using 10% TCA at 4° the percentage of ^{32}P extracted from labelled cells of *M. lysodeikticus* remained constant between 5 and 60 min. The radioactivity extracted by a second treatment with 10% TCA was approximately 6% of that removed by the first treatment; this can be attributed to the intercellular liquid remaining from the first extraction.

The alcohol-soluble fraction was found to contain only 2–3·5% of the total ^{32}P and no conclusions could be drawn from the variations observed under different conditions of treatment.

RNA is extracted from cells by sodium hydroxide solution, and after investigating the rate of this extraction we selected 90 min treatment at 30° with M sodium hydroxide followed by acidification for the separation of RNA from deoxyribonucleic acid and protein which thus comprise the residual fraction.

INITIAL RELEASE OF ^{32}P FROM CHLORHEXIDINE-TREATED CELLS

The ^{32}P released from cells treated with chlorhexidine for 15 min at 20° comes entirely from the metabolic pool and the proportion of this pool that is released depends on the chlorhexidine concentration (Fig. 2A). Thus under conditions where breakdown of other bacterial constituents does not take place, the maximum release of ^{32}P which can occur is governed by the content of this pool. Leakage of the pool from only a proportion of the cells may account for the incomplete release of ^{32}P from the "cold TCA soluble" fraction at low chlorhexidine concentrations.

RNA BREAKDOWN IN WASHED CELLS

During endogenous metabolism in starved bacteria, RNA can be utilised as a substrate, being degraded into smaller molecules which are subsequently oxidised (Dawes & Ribbons, 1964). This process results in the release of degradation products from the cells (Strange, Wade & Ness, 1963) and in an accumulation of material in the metabolic pool (Holden, 1958; Gronlund & Campbell, 1963). Figs 1A and B suggest that breakdown of RNA occurs in washed cells of *M. lysodeikticus* suspended in buffer. The rate of this breakdown is temperature dependent, being

negligible at 1° and occurring at between 6–10% per hr at 40° during the first 8 hr. Rapid breakdown of RNA during the preincubation of cells at 40° accounts for the high initial release (Table 1) on subsequent treatment with chlorhexidine at this temperature. In untreated cells at 20°, RNA degradation products accumulate in the "cold TCA soluble" fraction with little ^{32}P-containing material being released (Fig. 1A). At 40° an extensive release of ^{32}P into the suspending medium occurs, probably due to cell membrane damage. The ^{32}P content of the "cold TCA soluble" fraction thus depends both on the rate of RNA breakdown and on the rate of release of ^{32}P from the metabolic pool (Fig. 1B).

SECONDARY RELEASE OF ^{32}P FROM CHLORHEXIDINE-TREATED CELLS

The secondary release of ^{32}P from chlorhexidine-treated cells results from the breakdown of RNA and subsequent leakage of degradation products into the medium. The rate of this breakdown in chlorhexidine-treated cells differs from that in untreated cells (Fig. 3). At concentrations of 16 and 32 μg/ml chlorhexidine accelerates this breakdown and the consequent leakage, but at 64 and 128 μg/ml the breakdown is inhibited and virtually no secondary release occurs. *M. lysodeikticus* has been shown to contain the enzyme polynucleotide phosphorylase which can degrade RNA to nucleoside diphosphates, the activity of this enzyme depends on the Mg^{++} concentrations (Olmsted & Lowe, 1959). The differences in the rates of breakdown of RNA in chlorhexidine-treated cells compared with the rate in untreated cells may be due to a reduction in the availability of Mg^{++} in the treated cells. The different patterns of release of ^{32}P from labelled cells thus result from the dependence of RNA breakdown upon temperature, time and chlorhexidine concentration.

Our results also show that in experiments designed to study the release of material from cells treated with bactericides, the conditions before and during treatment should be strictly controlled and the extent of the release related to the content of the metabolic pool and to the rate of breakdown of cellular constituents in untreated cells.

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