EXCRETION OF ALKALINE PHOSPHATASE BY BACILLUS SUBTILIS

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Both alkaline phosphatase (Malamy and Horecker, 1961) and ribonuclease (Neu and Heppel, 1964) from E. coli have been recently located in the periplasm (Mitchell, 1961) between the cell membrane and cell wall; these enzymes are liberated by lysozyme-EDTA conversion of cells into spheroplasts. In contrast, two ribonucleases (Nishimura and Nomura, 1959), α-amylase (Nomura et al., 1956), and proteinase (Matsubara et al., 1958) from B. subtilis are exo-enzymes, that is, they are excreted by normal cells. In both organisms, these enzymes must be secreted as such, or as subunits, through the cell membrane. Since to our knowledge no excretion of enzymes through the cell wall has been observed in E. coli, it appeared likely that the difference between peri-enzymes and exo-enzymes is due to a difference in the cell walls of E. coli and B. subtilis rather than a difference in the enzymes themselves. This hypothesis predicts that peri-enzymes in E. coli should be exo-enzymes in B. subtilis and vice versa.

The present paper shows, indeed, that alkaline phosphatase is excreted from intact cells of B. subtilis following derepression by phosphate starvation.

Material and Methods

B. subtilis strain = 60-009, prototroph.

Media: For logarithmic growth, sodium citrate x $2H_2O$, 1 g; $(NH_4)_2SO_4$,

2 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; 0.125 M K_2HPO_4 + KH_2PO_4, pH 6.8; distilled H_2O to 1 liter. For growth in limiting phosphate 1×10^{-4} M phosphate, 0.05 M tris, pH 6.8, and 7.5 g/l KCl, replace the potassium phosphate. Tris = tris(hydroxymethyl)aminomethane, "Trizma Base" from Sigma Chemical Co.

Conditions for growth in limiting phosphate: Bacteria, logarithmically growing with vigorous aeration at 37°C, were centrifuged, when their OD_{600} reached 0.45, and washed twice with an equal volume of 0.05 M tris, pH 6.8. The pellet was resuspended in limiting phosphate medium at OD_{600} = 0.15. During growth, samples were taken for OD_{600} and enzyme determinations.

Alkaline phosphatase assays (Echols et al., 1961) were performed on samples of the growing culture, on centrifugal supernatants of such samples and on lysozyme-DNAase (Worthington) lysates of the pellets that had been washed twice with 0.05 M tris, pH 8.0.

Dehydrogenase assays (Freese and Oosterwyk, 1963) were performed on both the supernatant and twice washed and lysed pellets of samples. Substrates were removed by passage through Sephadex G 25 columns.

Specific activities are given by 1000 times the change of OD_{410} or OD_{340} that would have been produced per minute by a culture of bacteria having an OD_{600} of 1.00 before lysis or centrifugation.

Results and Discussion

Alkaline phosphatase did not appear until at least three hours incubation in the limiting phosphate medium. After this time increasing enzyme activity was observed in samples of either the culture directly or in the centrifugal supernatant, but to only a minor extent in washed cells (Fig. 1). About $2\frac{1}{2}$ hours later the synthesis of alkaline phosphatase stopped, presumably because all the phosphate was used up. From this time on the specific activity of washed cells decreased, apparently because the remaining enzyme was excreted. After five

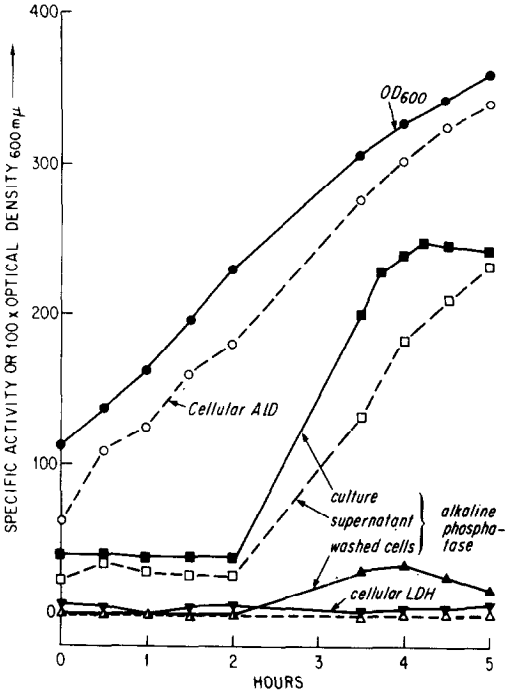

Figure 1. Specific enzyme activities and OD_{600} readings of samples taken from a culture during incubation in limiting phosphate medium. OD_{600} readings are plotted as 1000 times the observed value. No centrifugal supernatant samples contained detectable lactic dehydrogenase or alanine dehydrogenase activity (Δ).

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hours in limiting phosphate, 93% of the total enzyme activity appeared in the supernatant and only 7% remained in the washed cell extract. Throughout this period of alkaline phosphatase excretion, the cells remained intact, as is indicated by the absence of alanine dehydrogenase activity in the supernatant even though this enzyme was also

derepressed by phosphate starvation (Freese and Oosterwyk, 1963). Lactic dehydrogenase served as an additional control since this enzyme was neither derepressed nor found in the supernatant.

This experimental demonstration of the quantitative excretion of alkaline phosphatase from growing cells is in contrast to 15% spontaneous release in E. coli (Torriani, 1960).

Most bacterial exo-enzyme systems are characterized by a very low proportion of activity bound to cells. An exception is induced penicillinase from B. subtilis (Pollock, 1961), 85% of which is bound to the cell membrane during the first three hours; only after this time the enzyme is found in the supernatant.

In conclusion, it appears that the cell wall of E. coli confines peri-plasmic enzymes, whereas the cell wall of B. subtilis allows the exit of similar enzymes which are therefore excreted. Since in general exo-enzymes predominately occur in gram positive organisms (Pollock, 1961), it will be interesting to see whether the corresponding enzymes in gram negative organisms are also located in the periplasm, as they are in E. coli.

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