EVALUATION OF CYTOCHEMICAL METHODS FOR STUDYING ALKALINE PHOSPHATASE LOCALIZATION IN Bacillus subtilis

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The possibility of using "lead" and calcium" methods for determining the localization of alkaline phosphatase in bacterial cells was studied. The unsuitability of a cytochemical medium containing lead nitrate for determining the true localization of alkaline phosphatase in microorganisms was demonstrated.

KEY WORDS: *Bacillus subtilis; alkaline phosphatase.*

There have been few cytochemical investigations of bacterial phosphatases with the aid of the electron microscope. The use of calcium as phosphate acceptor enables phosphatase to be determined in unfixed bacterial cells, without loss of enzyme activity. However, the use of the "calcium" method in conjunction with electron microscopy is restricted by limitations imposed by the staggered nature of the reaction. This disadvantage has been overcome by a modification of the method [3].

The "lead" method, based on a cytochemical reaction in the presence of lead nitrate, was first used for electron microscopy in 1966 [15].

Both methods have been used to determine the site and mechanism of secretion of alkaline phosphatase by bacterial cells [2, 3, 7-9, 12, 13]. However, in some of these publications the suggestion was made that the "lead" method is unsuitable for the demonstration of alkaline phosphatase [2, 4, 13, 16, 17].

The object of this investigation was to compare the usefulness of the "lead" and "calcium" methods for determining the localization of alkaline phosphatase in bacterial cells.

EXPERIMENTAL METHOD

Bacillus subtilis strain No. 83, possessing alkaline phosphatase activity when grown on medium restricted in inorganic phosphorus, was used. The nutrient medium for repression and derepression of alkaline phosphatase synthesis was a salt medium [6] with certain modifications. The "control" medium contained the same components but ten times more inorganic phosphorus. The medium was poured into flasks and sterilized, after which sterile 40% glucose was added to each flask to a final concentration of 0.1%.

Bacillus subtilis cells from a culture grown on agar for 18 h at 37°C was seeded into the flasks with the control and experimental media. Growth continued for 18 h at 37°C. After growth the cells were washed once with distilled water.

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Alkaline phosphatase activity was determined by two methods. 1) The cells were incubated in medium containing paranitrophenyl phosphate [6]. Activity was expressed in micrograms paranitrophenol per 100 million bacteria in 30 min. 2) The cells were incubated in medium containing β-glycerophosphate [5] and this was followed by determination of inorganic phosphate [1]. Protein was determined by Lowry's method [10]. Activity of the enzyme was expressed in micrograms inorganic phosphate per milligram protein.

The cytochemical determination of the localization of alkaline phosphatase activity in conjunction with electron microscopy was carried out by "calcium" [3] and "lead" [7] methods. After incubation, samples were taken for electron-microscopic examination of whole cells and for fixation by Ryter's method [14]. The fixed cells were embedded in a mixture of Epon and Araldite [11]. Ultrathin sections were obtained on the OmY-2 ultramicrotome and examined in the JEM 100B electron microscope with a voltage of 80 kV and instrumental magnification of 15,000-50,000 times.

EXPERIMENTAL RESULTS

The dynamics of growth of a culture of B. subtilis strain No. 83 under conditions of repression and derepression of alkaline phosphatase synthesis is shown in Fig. 1a. Clearly the conditions of cultivation affected the dynamics of growth. Cells from an 18-h culture were therefore used.

The dynamics of alkaline phosphatase activity under different conditions of cultivation is shown in Fig. 1b. When grown in a medium deficient in phosphorus, B. subtilis strain No. 83 synthesizes alkaline phosphatase.

The electron-microscopic investigations showed that when the localization of the enzyme was studied by the "calcium" method in cells grown with derepression of alkaline phosphatase synthesis (Fig. 2a, b), a clear and specific reaction revealing the site of enzyme activity was found. The reaction product lay on the surface of the inner and outer membranous structures of the cell, in agreement with observations by other workers [4, 5, 13].

In control specimens (Fig. 2c, d), in which the bacteria were grown with repression of alkaline phosphatase synthesis and stained by the "calcium" method, no calcium phosphate was deposited, in agreement with the results of biochemical analysis (Fig. 1b).

Cells of B. subtilis grown with repression of alkaline phosphatase synthesis and stained by this "lead" method are shown in Fig. 2e, f. Clearly lead phosphate was deposited in the control preparations, showing that the "lead" method is unsuitable for the demonstration of enzyme activity under these conditions.

Nonspecific staining of cell structures by the use of the "lead" method of detecting ATPase activity has also been demonstrated previously [2]. Other workers [16] found that lead is adsorbed by the metachromatic granules of bacteria in the absence of substrate for phosphatase activity. In these workers' opinion, lead stains the "polar" bodies or preformed phosphates rather than phosphatases.

The use of lead salts also leads to inhibition of enzyme activity and to nonenzymic hydrolysis of phosphoric esters [13].

Similar results have also been obtained for tissues during light microscopy [17]. However, despite the disadvantages of the "lead" method, some workers [7] use it to determine the localization of alkaline phosphatase without the necessary controls and with failure to take into account the possible appearance of artefacts through the nonspecific deposition of lead.

The results described above show that the true picture of enzyme localization was observed by the use of the "calcium" staining method, in good agreement with results described by many other workers [4, 5, 13, 16], but in disagreement with the observations of Ghosh et al. [7].

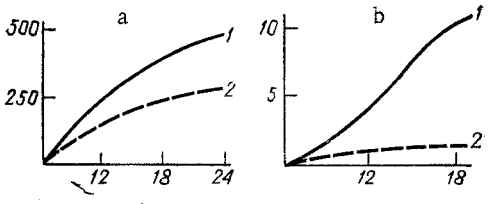

Fig. 1. Dynamics of growth of cells and alkaline phosphatase activity: a) dynamics of growth of B. subtilis strain No. 83 cells at 37°C: 1) cultivation with repression of alkaline phosphatase synthesis; 2) cultivation with derepression of alkaline phosphatase synthesis; b) dynamics of alkaline phosphatase activity at 37°C: 1) enzyme activity during incubation of cells with derepression of alkaline phosphatase synthesis; 2) enzyme activity during incubation of cells with repression of alkaline phosphatase synthesis. Abscissa, time (in h); ordinate: a) number of cells (in millions/ml), b) enzyme activity (in μg paranitrophenol/100 million cells).

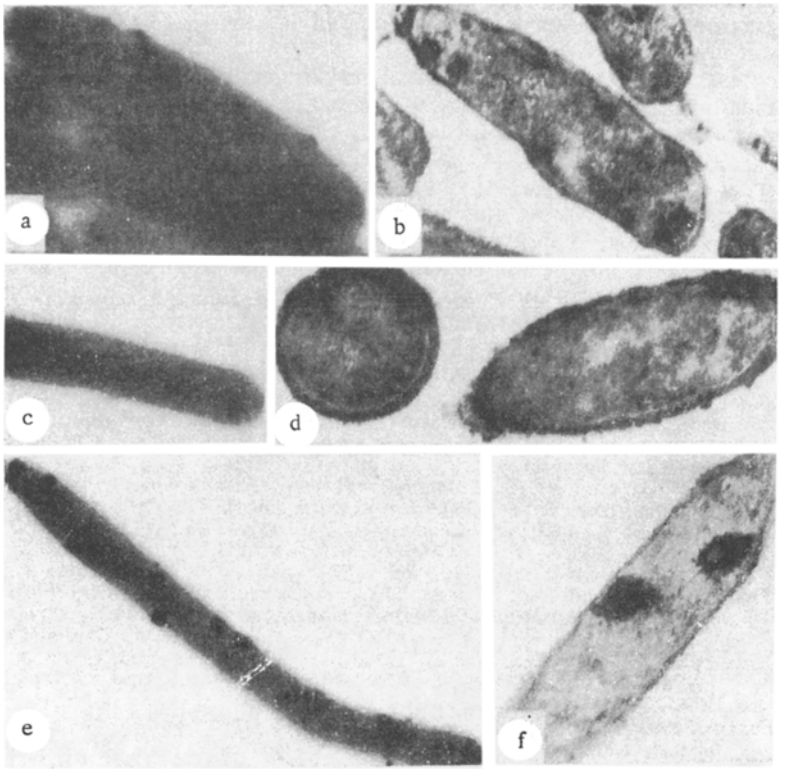

Fig. 2. Electron micrographs of stained cells of B. subtilis strain No. 83: b) cells grown with derepression of alkaline phosphatase synthesis and stained by "calcium" method (a: 20,000×, b: 50,000×); c, d) cells grown with repression of alkaline phosphatase synthesis, stained by "calcium" method (c: 15,000×, d: 50,000×); e, f) cells grown with repression of alkaline phosphatase synthesis, stained by "lead" method (e: 17,000×, f: 50,000×).

The system of induced synthesis of alkaline phosphatase thus provides a convenient model for the selection and evaluation of a cytochemical method of determining the activity of the enzyme in bacteria; however, the combination of biochemical and cytochemical methods of determination of alkaline phosphatase activity in the cells of B. subtilis strain No. 83 clearly demonstrates the unsuitability of the "lead" method, so that the "calcium" method must be recommended for electron-microscopic investigations of enzyme localization.

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