Localization in the Cell and Extraction of Alkaline Phosphatase from Bacillus subtilis

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Study of protoplasts, lysed protoplasts, and cells treated with lysozyme in the absence of osmotic stabilizer suggested that the alkaline phosphatase (EC 3.1.3.1.) of Bacillus subtilis is located in the protoplasmic membrane. Cytochemical evidence in support of this view is presented. The enzyme protein was strongly bound to the membrane structure and could not be solubilized by a number of treatments known to release enzymes from membranes and other lipoprotein structures. Alkaline phosphatase was, however, solubilized by treatment of intact B. subtilis cells or isolated protoplasmic membranes with strong salt solutions at pH 7.2, suggesting that electrostatic forces are responsible for the association between membrane and enzyme protein. Dialysis of alkaline phosphatase solutions against buffer of low ionic strength resulted in precipitation of the enzyme.

The alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1.) of Escherichia coli is released in high yield on converting cells to spheroplasts by treatment with lysozyme and ethylenediaminetetraacetate (10) or lysozyme at pH 9 (13) in the presence of sucrose as osmotic stabilizer. Alkaline phosphatase and a number of other enzymes can also be released by subjecting intact cells to osmotic shock (6, 15, 16). Conversion to spheroplasts liberated about 10% and osmotic shock about 3.5% of the total cell protein. These and other considerations led to the suggestion that, in E. coli, alkaline phosphatase is located in the “periplasmic space” between the cell wall and the cytoplasmic membrane (6). That the enzyme is located near the cell surface was also suggested by histochemical procedures (4).

During an investigation of the properties of Bacillus subtilis alkaline phosphatase (D. Wood, Ph.D. Thesis, Univ. of London, 1968), it became apparent that the enzyme in this species could not readily be solubilized by the procedures mentioned above. The present communication reports histochemical and biochemical evidence suggesting that the enzyme is located in the cytoplasmic membrane, from which it may be removed by extraction of membrane preparations or whole cells with strong salt solutions. These conclusions are in accord with evidence reported while this investigation was in progress (19).

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MATERIALS AND METHODS

Organisms and media. B. subtilis strain A, a prototrophic strain from the culture collection of this department, and strain 60-009, kindly donated by Ernst Freese, were mainly used; B. megaterium KM was used in a few experiments. The alkaline phosphatases of both species are repressible by orthophosphate; cells containing enzyme were produced by incubation in shake flasks at 37°C for 18 hr in a medium (TG/PEP) containing 0.1 M tris(hydroxymethyl)aminomethane (Tris), 0.007 M Na2SO4, 0.02 M KCl, 0.04 M NH4Cl, 4 × 10−4 M MgSO4, 7H2O, 2 × 10−4 M FeSO4, 7H2O, 0.056 M glucose, and 0.5% peptone (Difco), adjusted to pH 7.2 with HCl before sterilization. Growth of B. subtilis was limited by phosphate contained in the peptone, phosphate exhaustion being followed by derepression of alkaline phosphatase synthesis, so that cells displaying high enzyme activity were obtained after 18 hr of incubation. Presumably due to a higher content of phosphate, some batches of peptone proved unsuitable for growth of cells containing alkaline phosphatase. Cells lacking enzyme were produced by growth on phosphate-rich glucose-salts medium (20) modified by the addition of 0.5% peptone (medium 232G/PEP). Plate counts were conducted by serial dilution in sterile physiological saline followed by plating on Lab-Lemco nutrient agar (Oxoid).

Production and fractionation of protoplasts. Cultures were cooled to 0°C, harvested by centrifugation in the cold at 10,000 × g for 10 min, washed by centrifugation in ice-cold 0.1 M Tris buffer (pH 7.2), and resuspended in the same buffer containing 0.5 M sucrose. The suspensions were brought to 37°C, and a solution of lysozyme (EC 3.2.1.17) was added to yield a final lysozyme concentration of 1.2 mg/ml. Proto-
plast formation, which was usually complete in 15 min, was followed by observing the fall in optical density occurring when 0.1-ml samples were pipetted into 1.9 ml of distilled water. Protoplasts were centrifuged at 6,000 × g for 20 min and resuspended in 0.1 M Tris buffer containing 5 μg of ribonuclease per ml and 2.5 μg of deoxyribonuclease per ml. Suspensions were incubated briefly at 37°C, after which they were cooled to 0°C and centrifuged at 40,000 × g for 20 min. The protoplast membranes (pellet) were finally washed in ice-cold buffer. The final washed pellet material appeared, on examination in an electron microscope (see below), to be a pure preparation of protoplasmic “ghosts” similar in size and appearance to those previously described by other workers (24). Membranes were occasionally treated in an ultrasonic disintegrator (Measuring & Scientific Equipment, Ltd., model 60W) for 5 min; the particulate nature of the final material was demonstrated by electron microscopy, the particles being of a range of sizes and of a thickness consistent with their derivation from membranes (8).

**Extraction of alkaline phosphatase.** Suitable portions of washed cells or membranes suspended in 0.01 or 0.1 M Tris buffer (pH 7.2), with or without the addition of 10-4 M magnesium acetate, were brought to the requisite temperature, and salt solutions were added to yield the final concentrations stated. At the end of 10- or 20-min extraction period, the suspensions were rapidly cooled to 0°C and centrifuged at 140,000 × g for 5 min (M.S.E. Super-speed 50; 10 by 10 ml rotor). Supernatants were poured off, and the cell pellets were resuspended in an appropriate volume of the same buffer; both supernatants and resuspended pellets were assayed for enzyme activity.

**Assay of alkaline phosphatase.** Assay of alkaline phosphatase was conducted essentially as described previously (14), except that the assay system contained 6 ml of 1 M diethanolamine-hydrochloride buffer (pH 9.7), and the amount of enzyme preparation added varied according to activity. Enzyme activity varied markedly with buffer concentration (D. Wood, Ph.D. thesis, Univ. of London 1968). Glycine-NaOH buffer (pH 9.7) exerted a strong inhibitory effect, the nature of which is being investigated. Enzyme activity is expressed as micromoles of p-nitrophenyl phosphate hydrolyzed per minute at 37°C.

**Electron microscopy.** Protoplast membrane preparations were deposited on grids coated with Formvar covered with carbon and examined in a Zeiss (EM9) electron microscope after negative staining with 2% potassium phosphotungstate (1). Preparations of membranes treated with ultrasonics were examined after shadowing with gold-palladium.

**Cytochemical localization of alkaline phosphatase.** The method described by a method already described (4). Cells were fixed by the addition of 6.5% glutaraldehyde in the same buffer for 1.5 hr at 4°C. Fixed cells were washed twice in fresh cacodylate buffer and incubated at 37°C for 2 hr in a substrate solution containing (per ml): 0.25 ml of 2% sodium β-glycerophosphate, 0.25 ml of 2% sodium Veronal, 0.05 ml of 2% Ca(NO3)2, 0.05 ml of 2% MgSO4-7H2O, and 0.4 ml of 0.1 M sodium cacodylate buffer (pH 7.4). After incubation, cells were treated with 2% Co(NO3)2 and, after centrifugation, with 2% OsO4 in 0.1 M cacodylate buffer for 2 hr. The osmium-treated cells were dehydrated with water-ethanol-tert-butanol mixtures (7), suspended in two changes of propylene oxide (9), and finally embedded in Araldite (Ciba).

**RESULTS**

Preliminary experiments confirmed that formation of alkaline phosphatase in *B. subtilis* strains A and 60-009 was repressed by orthophosphate. It has been claimed (2) that the enzyme was almost quantitatively excreted into the medium by strain 60-009 and that the release of enzyme was not due to cell lysis. In the present investigation, only 15 to 30% of the total activity was found in the medium when cells of strain A were removed by centrifugation at 10,000 × g for 5 min. With strain 60-009 about 20% of total enzyme activity was found in the 10,000 × g supernatant fluid, though rarely as much as 50% was apparently excreted. More than 90% of the enzyme activity in the medium recovered by low-speed centrifugation of cultures of both strains was sedimented during centrifugation at 140,000 × g for 2 hr, showing that the enzyme in the medium was largely particulate.

**Biochemical evidence of enzyme localization.** Conversion of *B. subtilis* A and *B. megaterium* KM to protoplasts did not result in liberation of alkaline phosphatase into the suspension medium. Lysis of protoplasts followed by differential centrifugation revealed that, in both species, 90% of the total enzyme activity of the cells resided in the protoplasmic membrane fraction (Table 1). This conclusion was substantiated by treatment of strain A with lysozyme in the absence of sucrose, separation of membrane fragments by centrifugation at 40,000 × g (20 min), and assay of both membrane and soluble fractions for enzyme activity. About 95% of the total alkaline phosphatase activity was found in the membrane fraction. Neuf and Heppel (16) showed that alkaline phosphatase was almost quantitatively released from *E. coli* by osmotic shock. When washed cells of *B. subtilis* A were subjected to this treatment there was no detectable release of enzyme activity.

**Cytochemical evidence of enzyme localization.** Strain A was grown in low-phosphate (TG/PEP) medium and phosphate-rich (232G/PEP) medium; cells grown in the latter medium were devoid of alkaline phosphatase activity. Cells grown in each medium were fixed, incubated in the presence and absence of β-glycerophosphate,
and further treated as already described. Only micrographs from cells grown in TG/PEP and subsequently incubated with β-glycerophosphate showed deposition of electron-opaque material (Fig. 1). Sections from cells grown in 232G/PEP, whether incubated with substrate or not, did not contain electron-opaque deposits. The electron-opaque material detected after cobalt staining of TG/PEP-grown cells which had been incubated with substrate was apparently located in the region of the protoplasmic membrane, thus confirming the results of the fractionation experiments reported above.

Solubilization of alkaline phosphatase. Various procedures were used in attempts to remove alkaline phosphatase from its association with the protoplasmic membrane to produce a soluble preparation amenable to further purification. Ultrasonic treatment of whole cells or membrane preparations of B. subtilis A liberated, respectively, about 90 and 70% of the total enzyme activity into the 30,000 × g supernatant, but the activity so released was sedimented quantitatively during high-speed centrifugation (130,000 × g for 2 hr), indicating that the alkaline phosphatase was still particle-bound. Similarly, attempts to solubilize the enzyme by passage of whole cells through a French pressure cell (5) failed to yield a soluble preparation.

Yamaguchi and his co-workers (26) removed cytochrome c from membranes of B. megaterium by bringing a suspension of membranes in distilled water to pH 8 with dilute NaOH. The same protein was solubilized by the action of lipase on B. megaterium and B. subtilis membranes (21). Neither of these methods, when applied to membrane preparations from B. subtilis A under conditions recommended by the authors, yielded soluble preparations of alkaline phosphatase. A number of other methods, previously found to remove enzymes from mitochondrial membranes or other lipoprotein complexes, were similarly unsuccessful. These included repeated freezing and thawing or treatment with n-butanol of both whole cells and isolated membranes, treatment of membranes with cold acetone or of whole cells with sodium deoxycholate, all under the conditions recommended by Morton (12). By using these methods, various amounts of enzyme were liberated into the 30,000 × g supernatant, but they always sedimented on centrifugation at 130,000 × g for 2 hr. Similar results were obtained on treating membrane suspensions with sodium dodecyl sulfate, sodium dodecyl sulfate plus n-butanol, Tween 80, phospholipase D, or lipase plus deoxycholate (for further details, see D. Wood, Ph.D. Thesis, Univ. of London, 1968).

### Table 1. Localization of alkaline phosphatase activity in B. subtilis A and B. megaterium KM protoplasts and protoplast lysates

<table>
<thead>
<tr>
<th>Fraction</th>
<th>B. subtilis A</th>
<th>B. megaterium KM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total EU</td>
<td>Initial activity</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>5.600</td>
<td>100</td>
</tr>
<tr>
<td>Protoplasts*</td>
<td>5.500</td>
<td>98</td>
</tr>
<tr>
<td>Suspension fluid*</td>
<td>0.083</td>
<td>1.3</td>
</tr>
<tr>
<td>Membrane suspension</td>
<td>5.000</td>
<td>90</td>
</tr>
<tr>
<td>Protoplast lysate*</td>
<td>0.049</td>
<td>8.9</td>
</tr>
<tr>
<td>Washed membranes*</td>
<td>5.050</td>
<td>91</td>
</tr>
<tr>
<td>Wash fluid*</td>
<td>0.033</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Enzyme units.
* Cells suspended in 0.1 M Tris buffer (pH 7.2) containing 0.5 M sucrose, preparatory to lysozyme treatment.
* Pellet (6,000 × g) after lysozyme treatment.
* Supernatant (6,000 × g) containing lysozyme-digested wall components.
* Pellet (40,000 × g) after lysis of protoplasts in 0.1 M Tris buffer (pH 7.2).
* Supernatant (40,000 × g) after lysis of protoplasts.
* Pellet (40,000 × g) washed in 0.1 M Tris (pH 7.2) by centrifugation at 40,000 × g.
* Final 40,000 × g supernatant.

During the course of this investigation, Takeda and Tsugita (19) reported the extraction in soluble form, by 1.0 M magnesium acetate, of alkaline phosphatase from the debris after lysozyme lysis of the Marburg strain of B. subtilis. This observation was substantiated by using the isolated membrane fraction from B. subtilis A. Suspensions of purified membranes in 0.1 M Tris buffer (pH 7.2) were stirred with 0.5 M MgSO₄·7H₂O or 1.0 M NaCl for 20 min at room temperature, followed by centrifugation at 140,000 × g for 2 hr. A large part of the total enzyme activity of the membrane preparation was present in the 140,000 × g supernatant (Table 2). In a number of experiments, between 50 and 75% of the total activity was solubilized by extraction of membranes with 0.5 M Mg²⁺. The extraction of alkaline phosphatase from membranes by solutions of high ionic strength was not confined to magnesium salts. About 70% of the total enzyme activity was liberated in soluble form by treatment with 1.0 M NaCl (Table 2). On dialysis of both 140,000 × g supernatants against solutions of low ionic strength (0.01 or 0.1 M Tris buffer, pH 7.2) at 4 C, more than 90% of the enzyme...
activity was precipitated and sedimented on centrifugation at 20,000 × g for 20 min. This precipitation was not observed when a high ionic strength was maintained during dialysis.

Extraction from intact cells. Salt solutions of high ionic strength also extracted alkaline phosphatase from intact cells. Cells of *B. subtilis* 60-009 were washed in ice-cold 0.01 M Tris (pH 7.2) and resuspended in the same buffer. The suspension was divided into two portions, one of which was treated with lysozyme (0.1 mg/ml) at 37 °C in the absence of osmotic stabilizer. When lysis was complete, the suspension was subdivided, and suitable additions were made to yield samples containing 0.5 M magnesium acetate, 1.0 or 5.0 M NaCl. The other portion of the original suspension was subdivided and treated similarly, except that the cells were not exposed to lysozyme before addition of magnesium acetate or NaCl. All preparations were incubated at 37 °C for 20 min; after incubation, total alkaline phosphatase activity was assayed in each of the suspensions, in the supernatant fluids after centrifugation at 140,000 × g, and in the resuspended pellets. Enzyme was liberated from intact cells by both salts at the concentrations tested, but was solubilized more effectively from lysozyme-treated cells than from intact cells (Table 3).

Other salts were also active in solubilizing the enzyme protein from intact cells of *B. subtilis* 60-009. Although the amount of enzyme released by a given salt concentration varied between experiments, the results were qualitatively
Table 2. Distribution of alkaline phosphatase after extraction of B. subtilis A protoplast membranes with magnesium sulfate or sodium chloride solutions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>+ 0.5 M MgSO₄·7H₂O</th>
<th>+ 1 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total EUa Initial activity</td>
<td>Total EU Initial activity</td>
</tr>
<tr>
<td>Membrane suspension</td>
<td>46.7 100</td>
<td>20.0 100</td>
</tr>
<tr>
<td>Supernatant (140,000 x g)</td>
<td>26.7 57</td>
<td>13.8 69</td>
</tr>
<tr>
<td>Pellet (140,000 x g)</td>
<td>17.4 37</td>
<td>5.7 28</td>
</tr>
</tbody>
</table>

* Enzyme units.
+ Cells converted to protoplasts, lysed by suspension in 0.1 M Tris (pH 7.2); membranes washed and resuspended in the same buffer containing 0.5 M MgSO₄·7H₂O or 1 M NaCl for 20 min at room temperature. Centrifuged at 140,000 x g for 2 hr.
* Resuspended in 0.1 M Tris buffer (pH 7.2).

reproducible. High concentrations of sucrose, glycerol, or glycine failed to liberate significant amounts of enzyme (Table 4). The presence of 10⁻³ M magnesium acetate in the suspension buffer was without effect on the liberation of enzyme from intact cells by sodium salts. In several experiments of the type described in Table 4, the fall in viability did not exceed 10% even in the highest salt concentrations. The release of enzyme from intact cells by salt solutions was not markedly temperature-dependent between 0 and 37 C. A study of the time course of enzyme extraction from intact cells was difficult with the method used here, owing to the time occupied by centrifugation. However, the process was relatively rapid and was probably complete within 5 min. The degree of solubilization of alkaline phosphatase depended on salt concentration (Table 4). This effect was studied in detail over a wide range of NaCl and MgCl₂ concentrations (Fig. 2). Significant amounts of enzyme were never liberated at NaCl concentrations less than 1.0 M. On a molar basis, MgCl₂ was considerably more effective than NaCl in liberating enzyme from intact cells. Nitrates were particularly effective in solubilizing the enzyme (Table 4).

Extraction of alkaline phosphatase from intact cells by the action of 5 M NaCl has also been utilized in large-scale extraction and purification of the enzyme. For instance, the cells from a 5-liter culture of strain 60-009 were harvested, washed in 0.01 M Tris buffer (pH 7.2), and resuspended in 30 ml of the same buffer containing 5 M NaCl. After incubation at 37 C for 20 min, the suspension was centrifuged at 40,000 x g for 20 min, and the supernatant was further centrifuged at 140,000 x g for 2 hr. In two separate experiments, 80 and 86% of the total cell-bound enzyme was solubilized by this treatment.

### DISCUSSION

Alkaline phosphatase may be liberated from *E. coli* cells either by conversion to spheroplasts (10, 13) or by osmotic shock (16). Neither of these procedures freed appreciable amounts of the enzyme from *B. subtilis*, suggesting that the association of the enzyme protein with other cell components is of a different nature in the two organisms.

The alkaline phosphatases of *B. subtilis* A and *B. megaterium* KM were associated with the protoplast membrane. From the results of cell fractionation presented here, it is not possible to distinguish between a possible mesosomal or cytoplasmic membrane location of alkaline phosphatase. Cytochemical techniques for investigating the location of alkaline phosphatase appear to confirm the membrane location of the enzyme in *B. subtilis* A. In thin sections from derepressed cells incubated with β-glycerophosphate, cobalt phosphate, the electron-opaque material representing the site of alkaline phosphatase, appeared to be localized in a discontinuous fashion, apparently in random areas of the membrane. This observation is of some interest since alkaline...
TABLE 4. Extraction of alkaline phosphatase from intact cells of B. subtilis 60-009 by salt solutions

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conca</th>
<th>Per cent of total enzyme in extract(^a)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Expt 1(^b)</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>3.6</td>
</tr>
<tr>
<td>NaCl</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>2.0</td>
<td>63.6</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>1.0</td>
<td>33.4</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0</td>
<td>58.0</td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>2.0</td>
<td>95.2</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1.0</td>
<td>75.4</td>
</tr>
<tr>
<td>MgSO(_4)</td>
<td>1.0</td>
<td>80.9</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>1.0</td>
<td>54.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.0</td>
<td>31.5</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Cells were suspended in 0.1 M Tris buffer (pH 7.2) containing salts at the concentrations indicated. Incubation was at 37 C for 10 min, followed by centrifugation at 140,000 \(\times\) g for 5 min.

\(^b\) With 0.64 mg (dry weight) of cells/ml.

\(^c\) With 1.44 mg (dry weight) of cells/ml.

Phosphatase is synthesized only after phosphate exhaustion and is presumably incorporated into a preexisting membrane structure. Alternatively, since there is limited cell division during phosphate starvation, enzyme molecules may be incorporated only into newly synthesized areas of membrane, though there appeared to be no concentration of enzyme at the cell poles or in positions where lateral wall formation might occur. However, little is known of the detailed structure of cell membranes (18), and the discontinuous distribution of alkaline phosphatase may merely mean that a membrane-associated protein is normally confined to certain membrane regions. A similar discontinuous distribution of adenosine triphosphatase in *M. xanthus* was described by Voelz and Ortigoza (23), though the enzyme in *B. cereus* appeared to be distributed over the whole membrane (22).

It is probable that the alkaline phosphatase molecules of *B. subtilis* are oriented in the protoplasmic membrane in such a way that the enzymic substrate can reach the active site without actually passing into the cytoplasm since, with a few exceptions, monophosphate esters cannot be transported across the permeability barrier. In the investigation of *M. xanthus* mentioned earlier (24), it was conjectured that both Mg\(^{2+}\)-dependent and K\(^+\)-stimulated adenosine triphosphatases were located at both inner and outer membrane surfaces, but that acid and alkaline phosphatases resided at the external surface only, or alternatively in the periplasm.

In studies on protein liberation from intact cells the possibility of cell lysis must be considered. Lysis is unlikely to account for the solubilization of alkaline phosphatase by salt solutions in the experiments reported here since treatment with strong NaCl or MgCl\(_2\) solutions, leading to the release of 90 to 95% of the total enzyme activity of the cells, resulted in only a slight fall in viability.

 Liberation of enzymes as a result of washing intact cells with solutions of high ionic strength has been observed by other workers. For example, acid phosphatase was liberated from stationary-phase *Saccharomyces mellis* by high concentrations of KCl (25). It was suggested that the enzyme was bound to some cell component by electrostatic forces and possibly by disulfide bridges. Noncovalent binding of enzyme proteins to some cell structure was also suggested.

![Fig. 2. Effect of NaCl and MgCl\(_2\) concentration on extraction of alkaline phosphatase from intact cells of *B. subtilis* 60-009. Cells were suspended in 0.1 M Tris buffer (pH 7.2) containing NaCl or MgCl\(_2\) concentrations indicated. Incubation was at 37 C for 10 min. Each tube contained 0.069 enzyme units (14) in a total volume of 5 ml.](http://jb.asm.org/)
by Coles and Gross (3) to explain the liberation of cell-bound penicillinase of \textit{Staphylococcus aureus} by treatment of intact cells with salt solutions. The loosely bound acid phosphatase of this organism could also be released by treatment with strong KCl solutions (11). An important difference between these latter observations and those reported here is the fact that the yeast and staphylococcal enzymes are believed to be quite loosely bound, possibly located in the cell wall; on the other hand, the alkaline phosphatase of \textit{B. subtilis} has been shown to be located in the protoplasmic membrane, possibly an integral part of the membrane and certainly sufficiently strongly bound to resist repeated washing and various treatments known to remove proteins from membrane structures. The extent of release and nature of any other proteins released from \textit{B. subtilis} membranes by solutions of high ionic strength are being investigated.

The excretion of more than 90\% of the total alkaline phosphatase during growth of cultures of \textit{B. subtilis} 60-009, previously reported (2), was not observed in the present investigation. Normally less than 25\% of the total activity was present in the growth medium; further, this activity sedimented at 140,000 \times g. As also noted by others (19), high salt concentrations were necessary to maintain alkaline phosphatase in solution; the enzyme protein was precipitated on lowering the ionic strength of the solute. Without further investigation it is impossible to decide whether the phosphatase in the medium was bound to particles derived from membranes after lysis or was excreted into a medium, the ionic strength of which was too low to maintain the enzyme protein in solution. Alternatively, the enzyme may be excreted as a particulate entity, possibly as a complex with other excreted proteins.

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\textbf{LITERATURE CITED}


