Studies on the Extracellular Alkaline Phosphatase of Micrococcus sodonensis

II. FACTORS AFFECTING SECRETION*

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SUMMARY

The secretion of alkaline phosphatase by Micrococcus sodonensis has been studied by observing the production and accumulation of extracellular enzyme by log phase cells which have been resuspended in fresh growth medium. The accumulation of extracellular alkaline phosphatase activity is the result of a selective permeation process and is totally dependent upon the presence of divalent cation. Although calcium is an actual component of the enzyme and is required for both catalytic activity and enzyme stability, magnesium appears to be the divalent cation required for the over-all process of enzyme secretion. The rate-limiting step in alkaline phosphatase secretion is probably not an enzyme-catalyzed event. A relatively small but significant amount of alkaline phosphatase activity exists in a cell-bound form and can be quantitatively released from cells by treatment with lysozyme. Although lysozyme-sensitive cell wall components are involved in the binding of alkaline phosphatase to the cell, the cell wall is not an obligatory component of the secretion process since lysozyme-prepared, sucrose-stabilized protoplasts are capable of extracellular enzyme production.

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The purification and properties of an extracellular alkaline phosphatase isolated from culture filtrates of Micrococcus sodonensis have been described (1). This calcium-containing enzyme has a molecular weight of 80,000 and accumulates during the logarithmic phase of growth. In an attempt to define the nature of the permeation process responsible for the production of extracellular enzymes by this organism we have examined some of the properties of the secretion of this alkaline phosphatase. In order to facilitate such studies, washed cells were resuspended in a defined medium under conditions which permit substantial rates of extracellular enzyme production over a relatively short period of time. Using this method, we have examined the metal requirement, temperature dependency characteristics, and the sensitivity of the secretion process to various inhibitors of protein synthesis.

EXPERIMENTAL PROCEDURE

Organism and Growth Conditions—Micrococcus sodonensis (ATCC 11880) was grown in the defined medium previously described (2). The medium contained the following in 100 ml of distilled water: biotin, 1 pg; n-lactic acid, 1 g; r-glutamate, 0.5 g; MgSO4·7H2O, 0.2 g; NH4Cl, 50 mg; CaCl2·2H2O, 1 mg; (NH4)2SO4, 4H2O, 80 mg; ZnSO4·7H2O, 1 mg; MnSO4·H2O, 0.8 mg; FeSO4·7H2O, 0.4 mg; H3BO3, 0.4 mg; CuSO4·5H2O, 0.1 mg; CoSO4·7H2O, 0.1 mg; EDTA, 50 mg. Cells were grown in 500 ml of medium in 2-liter Erlenmeyer flasks on a rotary shaker at 30° for 18 hours, harvested by centrifugation at 15,000 × g for 15 min, and washed twice with 0.01 M Tris buffer, pH 8.3. After washing, the cells were resuspended either in 1 volume of Tris buffer (0.3 M, pH 7.2) or in one of the incubation media described below.

Enzyme Secretion by Cell Suspensions—For the study of extracellular enzyme production, twice washed (0.01 M Tris, pH 8.3) cells were usually resuspended in 1/4 volume of the fresh growth medium described above, to which was added CaCl2 (1 mm). In experiments designed to assess the role of divalent cations in secretion, a simplified incubation medium containing glutamic acid (0.5%, w/v), lactic acid (0.08%, w/v), inorganic phosphate-free (3) nutrient broth (0.3%, v/v), ammonium molybdate (0.07 mM), Tris (0.3 M, pH 7.2), and CaCl2 (1.0 mM) was used in place of fresh growth medium. Cell suspensions (3 to 5 ml) were incubated in 25-ml Erlenmeyer flasks on a reciprocating shaker in a water bath maintained at 30°.

EDTA-treated cells were prepared by resuspending twice washed cells in Tris buffer (0.3 M, pH 7.2) containing 1 mM...
The secretion of alkaline phosphatase by washed cells of *M. sodonensis*. Washed logarithmic phase cells were resuspended in fresh growth medium supplemented with CaCl$_2$ (1 mM) and incubated at 30°C. At various intervals during the incubation, extracellular alkaline phosphatase activity was determined as described under “Experimental Procedure.” Cell density was measured by determining the absorbance at 660 nm of a 1:40 dilution of the cell suspension. The pH of the incubation medium was also monitored.

Eq. 2 (center). The effect of EDTA on the secretion of alkaline phosphatase. Washed cells of *M. sodonensis* were treated with EDTA (□) and resuspended in the simplified incubation medium containing glutamic acid, lactic acid, ammonium molybdate, inorganic phosphate-free nutrient broth, and Tris buffer (0.3 M, pH 7.2) at the concentrations stated in the text. Control cells (X) were subjected to the same conditions as the EDTA-treated cells except that EDTA was omitted from the incubation and calcium (1 mM) was present in the final incubation medium. After 225 min of incubation, calcium (1 mM) was added to the EDTA-treated cell suspension. Extracellular alkaline phosphatase activity was determined as described in the text.

FIG. 1 (left). The secretion of alkaline phosphatase by washed cells of *M. sodonensis*. After 15 min of incubation at 4°C the cell suspension was centrifuged for 15 min at 15,000 × g, washed twice with the same buffer, and resuspended in a volume of Tris buffer (0.3 M, pH 7.2) equal to 1/4 the volume of the original cell culture. The quantity of cell-bound alkaline phosphatase or nuclease activity associated with the washed trichloracetic acid pellet obtained after centrifugation (4 min, 12,000 × g) of the lysozyme-treated cell suspension was then assayed for enzyme activity.

**Protein Synthesis**—Total protein synthesis was determined by measuring the incorporation of L-leucine-$^{14}$C into trichloroacetic acid-insoluble material. An aliquot (0.2 to 1.0 ml) of the incubation medium was added to an equal volume of 20% trichloroacetic acid and held in a boiling water bath for 10 min. The sample was cooled to 4°C and centrifuged at 15,000 × g for 15 min, and the resultant pellet was washed twice with 2 ml of 10% trichloroacetic acid. The washed pellet was dissolved in 2 ml of Hyamine (Packard Instrument Company) and quantitatively transferred to a scintillation vial. Radioactivity was determined by liquid scintillation counting in the Triton scintillator solvent previously described (1). It was established that virtually all of the radioactivity cochromatographed with a leucine standard. Paper chromatography (Whatman No. 1) of a hydrolysate (6 N HCl, 110°C, 22 hours) of the washed trichloroacetic acid pellet obtained from a 2-hour incubation in butanol-acetic acid-water (4:1:5) demonstrated that all radioactivity cochromatographed with a leucine standard.

**Protein Determinations**—Protein was estimated by the ultra violet spectrophotometric method of Waddell (6) as described (1).

**RESULTS**

**Production and Accumulation of Extracellular Alkaline Phosphatase by Washed Cells**—Washed cells of *M. sodonensis* are capable of producing and accumulating extracellular alkaline phosphatase when resuspended in fresh growth medium (Fig. 1). When washed cells from the logarithmic phase of growth (ap...
proximately 18 hours) were resuspended in fresh growth medium at four times the original cell density and incubated at 30°, cell growth was effectively suppressed; the duration of most incubations was usually less than 150 min, during which time the turbidity of the cell suspension (absorbance at 600 nm) had increased by only about 10%. After a lag of approximately 30 min, the rate of accumulation of alkaline phosphatase activity in the extracellular medium was constant for up to 250 min. It is emphasized that use of the term “secretion” refers only to the production and accumulation of enzyme activity in the extracellular medium and is not intended to imply the nature of the mechanism responsible for the release of enzyme from cells. During the course of incubation, the pH of the medium increased from 7.2 to 9.0, which is characteristic of that change observed during the actual growth of the organism in the same medium. Preliminary experiments established that, of the components present in the original growth medium, the omission of only lactate, glutamate, calcium, molybdate, and inorganic phosphate resulted, at most, in a 30% decrease in the rate of extracellular alkaline phosphatase accumulation. Pyruvate or glycerol could substitute for lactate, but sugars such as fructose, glucose, or sucrose at the same concentration could not. The latter observation is consistent with the report (7) that &carbon compounds are not effective carbon sources for the growth of this organism. The omission of α-glycerophosphate from the incubation medium had no effect on secretion and accumulation of extracellular alkaline phosphatase.

Effect of EDTA on Enzyme Secretion—In order to evaluate the role of metals in the secretion of extracellular enzyme, a study was made of the effect of EDTA on the elaboration and accumulation of enzyme by M. sodonensis. EDTA-treated cells were obtained by incubating washed cells in an EDTA solution for 15 min at 4°. After washing the cells several times with calcium-free buffer, the EDTA-treated cells were resuspended in the simplified incubation medium lacking calcium. As shown in Fig. 2, treatment of cells with EDTA greatly diminished their capacity to release alkaline phosphatase into the extracellular medium. During the first 220 min of incubation the apparent rate of secretion of alkaline phosphatase by EDTA-treated cells was only about 10% that of the control suspension. However, the addition of calcium (at 220 min) led ultimately to a complete restoration of the capacity of the EDTA-treated cells to elaborate alkaline phosphatase. Significant, however, is the fact that after the addition of calcium a substantial lag of some 60 min persisted before control rates of extracellular enzyme production were observed. Despite the fact that no increase in soluble, extracellular alkaline phosphatase was observed during this time, a significant amount of alkaline phosphatase was produced by the cell suspensions during this 60-min period; the increase in enzyme activity was found to be firmly bound to the cells. Cell-bound alkaline phosphatase activity could be rapidly and quantitatively released from cells by treatment with lysozyme. In fact, significant levels of cell-bound enzymes are also observed in growing cultures of the organism. Thus, when log phase cells of M. sodonensis are exhaustively washed free of soluble, extracellular alkaline phosphatase and treated with lysozyme, there is an immediate increase in soluble, nonsedimentable enzyme activity. After a 30-min treatment with lysozyme, no detectable alkaline phosphatase activity can be found in association with the sedimented cell debris, and all enzyme activity can be recovered from the supernatant fluid. Repeated washing of control cells which had not been exposed to lysozyme did not release cell-bound alkaline phosphatase. Lysozyme was therefore employed as a means of evaluating the level of cell-bound enzyme. By simply determining the level of soluble alkaline phosphatase in the presence and absence of lysozyme, the amount of cell-bound enzyme can be determined. As noted in Fig. 3, when EDTA-treated cells were incubated in the absence of calcium, most of the cell-bound (C) alkaline phosphatase was gradually released into the extracellular fluid; after 200 min, the level of cell-bound enzyme had decreased by more than 80%. The cell-bound enzyme which was released upon incubation of EDTA-treated cells could be accounted for in the extracellular fluid (X). Upon addition of calcium to the EDTA-treated cell incubation, there was an immediate increase in the level of cell-bound enzyme despite the fact that 60 min elapsed before an increase in alkaline phosphatase activity was observed in the extracellular medium. It appears, therefore, that upon addition of calcium to EDTA-treated cells which have been depleted of bound enzyme, newly synthesized alkaline phosphatase first saturates and binds firmly to a number of sites on the surface of the cell. Once a substantial number of binding sites have been filled, additional enzyme synthesis results in the appearance of soluble, extracellular alkaline phosphatase. Whether or not these cell-surface binding sites are obligatory intermediates in the process of secretion is not known.

Restoration of Alkaline Phosphatase Secretion in EDTA-treated Cells by Various Metals—The ability of various divalent cations to restore the capacity of EDTA-treated cells to elaborate extracellular alkaline phosphatase was investigated. As noted in Table I, when EDTA-treated cells are resuspended in the simplified medium lacking calcium and incubated at 30°, the rate of appearance of extracellular alkaline phosphatase is reduced by approximately 90%. The low rate of “apparent” release of enzyme from the EDTA-treated cells may reflect only the release of pre-existing cell-bound alkaline phosphatase (Fig. 3), rather than net synthesis and active secretion. The addition of calcium to the incubation medium fully restored the capacity of these cells to secrete alkaline phosphatase. Maximum rates of secretion were achieved with 1 mm calcium. Magnesium and strontium were found consistently to be as effective, or more effective, than calcium in restoring the capacity of EDTA-treated cells to secrete alkaline phosphatase whereas manganese, zinc, and cobalt were completely ineffective. The provision of copper to EDTA-treated cells permitted significant rates (42% of control) of alkaline phosphatase secretion.

It is noteworthy that although magnesium can substitute effectively for calcium in restoring enzyme secretion to EDTA-treated cells, magnesium is actually a potent inhibitor of alkaline phosphatase activity (1). Furthermore, magnesium is completely ineffective in restoring activity to EDTA-inactivated enzyme. On the other hand, treatment of cells with EDTA does not affect their ability to incorporate radioactive leucine into cellular protein. These results are illustrated in Table II. Thus, while EDTA-treated cells secreted alkaline phosphatase at only 12% the rate of control cells, there was no effect on the incorporation of leucine into protein. Incubation of EDTA-treated cells in the presence of either Ca++ or Mg++, however,
secretion were obtained by determining the extracellular level of calcium. Control cells were not treated with EDTA and were incubated in the same medium supplemented with calcium (1 mM). Various divalent cations were added to the incubations as a final concentration of 1 mM. Relative rates of enzyme secretion were obtained by determining the level of extracellular alkaline phosphatase and nuclease between 50 and 120 min of incubation. Results are normalized to the rate of enzyme secretion observed for control cells incubated in the presence of calcium.

Permitted enzyme secretion at control rates but did not significantly affect the incorporation of leucine. It appears, therefore, that EDTA treatment markedly reduces the ability of cells to synthesize and release extracellular alkaline phosphatase without significantly affecting the synthesis of the majority of cellular proteins.

Similar results were obtained when these metals were tested for their ability to restore the capacity of EDTA-treated cells to elaborate and accumulate extracellular nuclease. Manganese appears to be an essential component of the extracellular nuclease of *M. sodonensis*; this metal is required for activity and also affords marked protection of the enzyme against heat activation. Despite the fact that manganese is a component of the extracellular nuclease, this metal was completely ineffective in restoring nuclease secretion to EDTA-treated cells (Table I). The addition of calcium effected complete restoration of the ability of these cells to elaborate extracellular nuclease. Magnesium however, not only restored the ability of EDTA-treated cells to elaborate nuclease, but actually permitted rates of nuclease secretion 2-fold greater than those observed in control cell suspensions which were not treated with EDTA.

In addition to the ineffectiveness of manganese and zinc in restoring the ability of EDTA-treated cells to elaborate extracellular nuclease and alkaline phosphatase, these metals are also potent inhibitors of enzyme secretion when added to calcium-containing cell suspensions. When manganese or zinc (1 mM) permitted enzyme secretion at control rates but did not significantly affect the incorporation of leucine. It appears, therefore, that EDTA treatment markedly reduces the ability of cells to synthesize and release extracellular alkaline phosphatase without significantly affecting the synthesis of the majority of cellular proteins.

### Table I

<table>
<thead>
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<th>Metal addition</th>
<th>Relative rate of secretion</th>
<th>Alkaline phosphatase</th>
<th>Nuclease</th>
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<td>EDTA-treated cells</td>
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<tr>
<td>None</td>
<td>0.12</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Ca++</td>
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<td>Mg++</td>
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<td>Zn++</td>
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<tr>
<td>Cu++</td>
<td>1.00</td>
<td>1.00</td>
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</tr>
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</table>

For a Cell suspensions were prepared and cells were treated with EDTA as described in "Experimental Procedure."

Table II

### Table II

<table>
<thead>
<tr>
<th>Cells*</th>
<th>Metal added (1 mM)</th>
<th>Relative rates of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
<td>Incorporation</td>
</tr>
<tr>
<td>Control</td>
<td>Ca++</td>
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<tr>
<td>EDTA-treated</td>
<td>None</td>
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</tr>
<tr>
<td>EDTA-treated</td>
<td>Ca++</td>
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<tr>
<td>EDTA-treated</td>
<td>Mg++</td>
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* Cell suspensions were prepared and cells were treated with EDTA as described in “Experimental Procedure.”

was added to 1 mM calcium-containing cell suspensions which were actively elaborating extracellular nuclease and alkaline phosphatase, prompt and complete inhibition of further enzyme secretion resulted.

From these results it is clear that the presence of divalent cation is required for the elaboration of at least two extracellular enzymes by *M. sodonensis*. Furthermore, in the case of the two enzymes examined, it appears that magnesium ion is most effective in stimulating the actual release and accumulation of both the extracellular enzymes while, in each instance, the particular enzyme specifically requires a cation other than magnesium as a structural component (phosphatase, calcium; and nuclease, manganese).

### Comparison of Effect of EGTA and EDTA on Secretion of Alkaline Phosphatase—The results presented above suggest that calcium or magnesium can satisfy the divalent cation requirement for the elaboration of at least two extracellular enzymes (nuclease and alkaline phosphatase) by *M. sodonensis*. In an attempt to evaluate further the divalent cation specificity of the secretion process, a comparison was made of the effect of EGTA and EDTA on the secretion of extracellular alkaline phosphatase. The binding constants for the EDTA-Ca++ and EDTA-Mg++ complexes are approximately equivalent whereas the binding constant for the EDTA-Mg++ complex is several orders of magnitude less than that for the EDTA-Ca++ complex (8). Therefore, if the release of extracellular enzyme from the cell surface is magnesium-dependent, enzyme secretion should be much more sensitive to inhibition by EDTA than by EGTA. On the other hand, if the divalent cation requirement for secretion is calcium-specific, then comparable inhibition of the accumulation of extracellular enzyme should be achieved with EGTA and EDTA. It is apparent from Fig. 4 that the presence of 2 mM EDTA in the incubation medium resulted in 80% inhibition of alkaline phosphatase secretion, whereas EGTA at the same concentration was without effect. This observation supports the contention that the release of alkaline phosphatase from cells of *M. sodonensis* is dependent upon magnesium rather than calcium.}

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*S. T. Brownlee and E. C. Heath, unpublished observations."
than calcium. Enzyme secretion was significantly stimulated by lower concentrations (0.35 mM) of both of these chelating agents; the presence of 0.35 mM EDTA and EGTA resulted in 5.0- and 2.5-fold stimulation, respectively, in the rate of accumulation of extracellular alkaline phosphatase. The calcium content of the incubation medium was 0.1 mM. An explanation for the enhanced rate of secretion in the presence of relatively low concentrations of these chelating agents is not readily apparent. Perhaps at relatively low concentrations, EDTA and EGTA chelate and consequently reduce the effective concentration of certain divalent cations which are inhibitory to the production of extracellular alkaline phosphatase.

**Effect of Inhibitors of Protein Synthesis on Alkaline Phosphatase Secretion**—A comparison was made of the effect of actinomycin D, an inhibitor of protein synthesis at the transcriptional stage; and several inhibitors which act at the translational stage of protein synthesis (sparsomycin, chloramphenicol, puromycin), on the ability of cells to secrete alkaline phosphatase. In order to provide a basis for comparison, the effects of these inhibitors on the incorporation of leucine-4,5-3H into total cell protein was determined. Various concentrations of each inhibitor were added to incubation mixtures containing leucine-4,5-3H; at intervals, aliquots of the suspension were removed, and the amount of extracellular alkaline phosphatase activity produced and the amount of leucine-4,5-3H incorporated into protein were determined. The results of such an experiment appear in Fig. 5, in which actinomycin D was employed as the inhibitor. After a lag of approximately 30 min, constant rates of alkaline phosphatase production and leucine-4,5-3H incorporation were observed in both the control and inhibitor-containing incubations. Rates of enzyme secretion and protein synthesis were evaluated during the linear period between 30 and 90 min; from experiments of this type, the concentration of inhibitor required for 50% inhibition of alkaline phosphatase secretion and leucine 4,5-3H incorporation was obtained. It is apparent from Table III that, of the compounds tested, only actinomycin D affected a comparable degree of inhibition of both protein synthesis and alkaline phosphatase secretion at any given concentration. With respect to the other inhibitors of protein synthesis, however, alkaline phosphatase secretion was much more sensitive to inhibition than was total cellular protein synthesis; 6 to 17 times as much inhibitor was required in order to achieve 50% inhibition of leucine-4,5-3H incorporation as was required for comparable inhibition of alkaline phosphatase secretion. Essentially the same results were obtained when the extracellular nuclease was studied; that is, nuclease secretion was much more sensitive

**TABLE III**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Leucine-4,5-3H Incorporation</th>
<th>Alkaline Phosphatase Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycin D</td>
<td>0.05</td>
<td>0.00</td>
</tr>
<tr>
<td>Puromycin</td>
<td>48</td>
<td>4.3</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>43</td>
<td>2.5</td>
</tr>
<tr>
<td>Sparsomycin</td>
<td>14</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Fig. 4. The effect of EGTA and EDTA on the secretion of alkaline phosphatase. Washed cells of *M. sodonensis* were re-suspended in fresh growth medium containing 0.1 mm calcium. Varying amounts (0 to 2 mmoles) of EGTA (○) and EDTA (●) were added to the cell suspensions at the start of the incubation. Extracellular alkaline phosphatase was determined, and rates of secretion are expressed relative to that observed in the absence of chelating agent.

Fig. 5. Effect of actinomycin D on the secretion of alkaline phosphatase and on the incorporation of leucine 4,5-3H into protein. Washed cell suspensions (5 ml) of *M. sodonensis* were re-suspended in fresh growth medium supplemented with calcium (1 mm) and incubated in the presence of leucine 4,5-3H (5 µCi, 29.1 µCi per µmole) and actinomycin D (1 and 5 µg per ml). At various intervals during the incubation the level of extracellular alkaline phosphatase (A) and the incorporation of radioactivity into protein (1-ml aliquot) (B) were determined.
than protein synthesis to the three inhibitors of protein synthesis studied (chloramphenicol, sparsomycin, and puromycin).

Effect of Temperature on Secretion of Alkaline Phosphatase—The fact that linear rates of alkaline phosphatase production are observed for a considerable period of time permits a study of the effect of temperature on enzyme secretion. The secretion of alkaline phosphatase was studied over a range of temperatures from 8 to 33° and the results are presented in Fig. 6. No accumulation of extracellular enzyme was observed at 8°; however, at higher temperatures, after a lag of 30 to 50 min, the release of alkaline phosphatase with time was linear. From these results it is clear that temperature has a pronounced effect on the rate of alkaline phosphatase secretion; by increasing the temperature of incubation approximately 8° (from 25.2 to 33.3°), the rate of alkaline phosphatase secretion was increased 12- to 15-fold. An Arrhenius plot of the rates of secretion calculated from data obtained during the linear period (50 to 140 min) of secretion is presented in Fig. 7. From the slope of this plot, the activation energy (ΔH++) for the over-all secretion process was calculated to be 54.7 kcal per mole. In the same experiments, the temperature dependency of protein synthesis was also evaluated by determining rates of incorporation of leucine-4,5-14C into total cellular protein; these results are presented in Fig. 8, in which the logarithm of the relative rate of leucine-4,5-14C incorporation into cellular protein is plotted as a function of the reciprocal of the absolute temperature (8-34°). From these data an activation energy of 19.9 kcal per mole was obtained.

Properties of Cell-bound Alkaline Phosphatase—Results presented in an earlier section demonstrated the existence of significant levels of cell-bound alkaline phosphatase which could be released in a soluble, non-sedimentable form by treatment of the cells with lysozyme. The level of cell-bound enzyme, estimated in cells from an 18-hour culture of _M. sodonensis_ which extracellular alkaline phosphatase activity had reached a maximum, was found to be about 6% of the total activity present in the culture. As noted earlier, lysozyme treatment solubilized all of the cell-bound enzyme, and no detectable activity was found in association with sedimented cell debris. Cell-bound alkaline phosphatase which has been rendered soluble by lysozyme treatment is indistinguishable from the enzyme which is isolated from the extracellular medium in that the following properties of the two enzymes are identical: heat stability, pH optimum, sedimentation in sucrose density gradients, and chromatographic behavior on molecular sieve columns (Sephadex G-200). However, an interesting property of the cell-bound enzyme is the fact that it is significantly more labile to heat inactivation when bound to cells than when free in solution after release and solubilization by lysozyme. Fifty per cent inactivation of the soluble enzyme at all stages of purification is obtained by heating for 2 min at 80°; comparable inactivation of the cell-bound enzyme is obtained by heating for 2 min at 54°.

The following observations are consistent with the concept that the cell-bound alkaline phosphatase activity exists external to the membrane surface of the bacterial cell. First, thoroughly washed cells of _M. sodonensis_ are capable of catalyzing the hydrolysis of NP and releasing p-nitrophenol into the extracellular medium (Fig. 9). Of interest is the observation that equivalent amounts of p-nitrophenol and phosphate were not found in the extracellular medium; in fact, only p-nitrophenol could be detected in the supernatant fluids. This observation suggests that the actual hydrolysis of organic phosphate by

![Fig. 6. The effect of temperature on the accumulation of extracellular alkaline phosphatase. Washed cells of _M. sodonensis_ were resuspended in fresh growth medium supplemented with calcium (1 mM) and incubated with shaking at 8.3° (○), 25.2° (●), 30.3° (△), and 33.3° (×). At various intervals, the level of extracellular alkaline phosphatase was determined.](http://www.jbc.org/content/249/4/1571/F6)

![Fig. 7. Arrhenius plot of the temperature dependence of alkaline phosphatase secretion. Rates of alkaline phosphatase secretion were determined at various temperatures between 8 and 34° in three separate experiments, (●, ○, and △) according to the method described in the legend to Fig. 6. Rates of secretion were calculated in the linear period between 50 and 140 min and normalized to the rate observed at 33.3°. The logarithm of the relative rate of secretion was plotted as a function of the reciprocal of the absolute temperature. An activation energy (ΔH++) of 54.7 kcal was calculated from the following expression:](http://www.jbc.org/content/249/4/1571/F7)
alkaline phosphatase may be intimately coupled to the uptake and transport of the inorganic phosphate. An additional observation supporting the idea that cell-bound alkaline phosphatase exists external to the cell membrane is that conditions which do not cause destruction of the cell membrane can effect the release of cell-bound alkaline phosphatase. Treatment of cells with 0.1% sodium dodecyl sulfate or lithium chloride (5 mM) releases essentially all of the cell-bound enzyme in a soluble, non-sedimentable form. Cells which have been stripped of bound enzyme with sodium dodecyl sulfate are unaffected in their ability to secrete alkaline phosphatase when resuspended in fresh growth medium.

**Secretion of Alkaline Phosphatase by Protoplasts**—In order to assess the role of cell wall constituents in alkaline phosphatase secretion, the ability of lysozyme-prepared protoplasts to secrete alkaline phosphatase was studied. Phase contrast microscopy indicated that protoplasts of *M. sodonensis* could be prepared and maintained if lysozyme treatment was carried out in the presence of 0.6 M sucrose. Protoplasts prepared in this manner were capable of synthesizing and accumulating extracellular alkaline phosphatase (Fig. 10) at a rate comparable to that of control cells. After 100 min, the rate of accumulation of extracellular enzyme by the protoplast preparation decreased, presumably owing to the lability of protoplasts to prolonged incubation at 20°C. Conversely, any significant dilution of the sucrose solution in which the stabilized protoplasts were suspended resulted in rupture of the protoplasts and complete destruction of their ability to secrete alkaline phosphatase. These observations suggest that, while the lysozyme-sensitive cell wall components may be significant in binding the cell-bound enzyme, their integrity is not essential to production of the enzyme.

**DISCUSSION**

Alkaline phosphatase isolated from culture filtrates of organic phosphate-grown cells of *Micrococcus sodonensis* appears to arise by selective secretion of the enzyme into the extracellular medium rather than by autolysis of cells. The enzyme is elaborated into the extracellular medium soon after the culture enters the stationary phase of growth, production of extracellular alkaline phosphatase ceases. Furthermore, comparison of the disc gel electrophoresis patterns obtained with soluble intracellular and extracellular proteins revealed marked quantitative and qualitative differences with respect to the mobility and intensity of protein bands (1). In addition, efforts to detect intracellular alkaline phosphatase activity have been unsuccessful. These observations satisfy the criteria for extracellularity as defined by Pollock (10) and suggest that selective permeation is responsible for the production of extracellular proteins by *M. sodonensis*.

Although the majority of the alkaline phosphatase exists in the culture filtrate, a significant amount of the active enzyme is firmly bound to the cell. At the time cultures enter the stationary phase, about 6% of the alkaline phosphatase activity in the culture exists in a cell-bound form and cannot be solubilized or released by repeated washing. Several observations suggest that the cell-bound enzyme exists external to the primary cell membrane and that binding is dependent upon constituents of the cell surface. First, cells which have been extensively washed free of extracellular enzyme are capable of catalyzing the phosphorolysis of organic phosphates (e.g. *p*-nitrophenyl phosphate), although it is of interest to note that only the non-phosphate portion (i.e. *p*-nitrophenol) of the original substrate can be recovered in the extracellular medium (Fig. 9); whether or not the cell-bound enzyme plays an active role in phosphate...
transport is not known. It may be that the cell-bound enzyme simply releases both products of the phosphorolysis reaction into the medium and that a very high affinity permease concentrates phosphate from the medium. Further evidence to support the contention that the cell-bound enzyme is situated external to the cell membrane is the observation that treatment with detergent (e.g., sodium dodecyl sulfate) or salt (5 M LiCl) rapidly and quantitatively displaces the cell-bound enzyme without altering the viability of the cells or their ability to secrete enzyme. Furthermore, lysozyme-prepared, sucrose-stabilized protoplasts are capable of secretion of alkaline phosphatase at rates comparable to those of intact cells. The same conditions of protoplast formation (0.6 M sucrose, lysozyme) cause the release and solubilization of all of the cell-bound alkaline phosphatase and no detectable activity is found in association with sedimentable membranes. The latter observation suggests that lysozyme-sensitive cell wall constituents are involved in binding the enzyme and that the cell wall itself plays no obligatory role in the secretion of alkaline phosphatase. However, the possibility remains that the external surface of the cell membrane, as well as lysozyme-sensitive cell wall constituents, may be involved in the binding of enzyme to the cell in such a way that alkaline phosphatase is localized between the membrane and the cell wall.

The cell-bound enzyme, when studied after release from cells by lysozyme treatment, is indistinguishable from that isolated from culture filtrates in terms of molecular weight, pH optimum, and heat stability. Of interest, but of unknown significance, is the fact that cell-bound alkaline phosphatase is significantly more labile to heat inactivation than when free in solution. The results presented indicate that divalent cations probably play a dual role in the phenomenon of secretion. In the case of alkaline phosphatase, it has been established that calcium is an integral component of the protein and that catalytic activity and enzyme stability are calcium-dependent (1). On the other hand, magnesium was shown to be a potent inhibitor of alkaline phosphatase activity and, in addition, could not substitute for calcium in restoring activity to the EDTA-inactivated enzyme. However, the results obtained in the present study indicate that magnesium is actually required for the production and accumulation of alkaline phosphatase in the extracellular medium. This conclusion is supported by the observation that magnesium and calcium are equally effective in restoring the capacity for alkaline phosphatase secretion to EDTA-treated cells and by the marked difference in the response of secreting cells to inhibition by EDTA and EGTA. Similar studies involving the extracellular nuclease, which is secreted simultaneously with alkaline phosphatase, reinforce the belief that magnesium plays a primary role in enzyme secretion. It appears, therefore, that the synthesis and secretion of at least two extracellular enzymes by M. sodonensis are dependent upon magnesium, a metal distinct from that which is actually incorporated into each of these extracellular enzymes. These observations are consistent with the concept that membrane-dependent processes essential to the synthesis, permeation, or release of extracellular enzymes from cells of M. sodonensis are magnesium-dependent and that various divalent cations are incorporated into the specific enzymes at some point after they are presented to the external surface of the cell membrane.

The results obtained with various inhibitors of protein synthesis are pertinent when considering the site of synthesis of alkaline phosphatase. Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, was equally effective in inhibiting both the synthesis of cellular proteins (leucine-4,5-3H incorporation) and the secretion of alkaline phosphatase. Assuming that actinomycin D-inhibited secretion reflects an inhibition of the actual synthesis of alkaline phosphatase, this observation is consistent with the concept that a single site of transcription functions to produce messenger RNA which directs the synthesis of both extracellular and intracellular proteins. Studies with inhibitors which affect later stages of protein synthesis (e.g., puromycin), on the other hand, are consistent with the concept that two sites of protein synthesis may exist within the bacterial cell: the incorporation of leucine-4,5-3H into cellular protein was considerably more resistant to inhibition than was the secretion of alkaline phosphatase. In view of the improbability that the chemical characteristics of the synthesis of intracellular and extracellular proteins are inherently different, the concept that multiple sites of protein synthesis exist within the bacterial cell is more attractive. Furthermore, the fact that alkaline phosphatase secretion is considerably more sensitive than cellular protein synthesis to inhibition by inhibitors of protein synthesis suggests that the site of synthesis of extracellular proteins is more peripherally oriented, or more accessible to the extracellular medium, than is the site of intracellular protein synthesis; thus, as inhibitors permeate the cell, the former sites are preferentially titrated by the inhibitor, thereby resulting in a greater sensitivity to the inhibitor of protein synthesis. Such a hypothesis involving a single site of transcription and multiple sites of translation leads inescapably to a consideration of specificity; that is, by what mechanism do the various families of messenger RNA coding for intracellular and extracellular proteins recognize their distinct and respective sites of protein synthesis? Two recent reports support the concept that multiple sites of protein synthesis exist within a single cell (11, 12). The existence of a specific membrane site of protein synthesis for extracellular proteins could account for the selectivity of the secretion phenomenon if the process of peptide bond formation resulted in the externalization of the growing polypeptide such that the completed protein was released into the extracellular medium. Alternatively, if the actual synthesis of a particular extracellular protein at the membrane surface does not lead to externalization of the protein, then the selective nature of the secretion phenomenon must be explained in some other way. The specificity of the permeation process could be determined by a component of the membrane-permeability barrier itself. This membrane component could be either a protein or a lipid which binds specifically to the protein that is to be secreted. On the other hand, the specificity for secretion may be conferred by a particular macromolecule which binds to the secreted protein to form an intracellular complex which is then able to pass the permeability barrier into the extracellular medium. This hypothesis predicts that the cosecreted macromolecule should ultimately be found in the extracellular medium. It is interesting to note that M. sodonensis elaborates soluble, extracellular polysaccharide which is associated with the extracellular alkaline phosphatase through a number of steps in the purification of the latter.

Studies on the effect of temperature on alkaline phosphatase secretion suggest that an enzyme-catalyzed reaction involving covalent bond exchange is probably not rate limiting to the process of enzyme secretion. Although the activation energy
for most enzymatic processes is about 12 kcal per mole (13) and certainly less than 20 kcal per mole, the over-all secretion of alkaline phosphatase is characterized by an activation energy of 55 kcal per mole. Consider in the simplest case that selective protein secretion is the result of three distinct events: (a) transport into the cell of amino acids and other low molecular weight compounds required for protein synthesis; (b) protein synthesis from free amino acids; and (c) permeation or release of the enzyme from the cell. Studies on the effect of temperature on total cellular protein synthesis in secreting cells of \textit{M. sodonensis} yielded an activation energy of 19.9 kcal per mole, a value similar to the value of 15.0 kcal per mole calculated from the data in \textit{vivo} of Friedman (14). Furthermore, membrane transport processes for inorganic ions (15), amino acid (16), and sugars (17) in the temperature range 15–30°C are characterized by activation energies which are also less than 20 kcal per mole. The fact that the over-all secretion of alkaline phosphatase is characterized by an activation energy of 55 kcal per mole suggests that permeation, the final phase of enzyme secretion, is rate-limiting and that it probably involves a process which is not enzyme-dependent. Two possible events which could be rate-limiting to the process of secretion and which would be compatible with the experimental temperature dependency data are: (a) conformational changes in protein structure and (b) a permeation process involving the passage of a relatively hydrophilic molecule such as a protein through a hydrophobic barrier such as the bacterial membrane. According to the first suggestion and assuming that alkaline phosphatase is synthesized within the cell, it is conceivable that a conformational change in the structure of the enzyme (or membrane components) is a prerequisite to the actual permeation event and that the rate of the protein into the extracellular fluid is limited by this conformational change. Conformational transitions in protein structure are often characterized by activation energies in excess of 30 kcal per mole (18). Recently, Sargent and Lampen (19) proposed that an essential step in protein secretion is one involving conformational changes in the protein itself. The second suggestion as to the nature of the rate-limiting step in enzyme secretion is also consistent with the thermodynamic data which characterize hydrophobic-hydrophilic mixing processes. Price (20), for example, established that the transport of a single hydrophilic water molecule across synthetic lipid bilayers requires an activation energy of 13 kcal per mole.

Recently, several mutants of \textit{M. sodonensis} were selected solely on the basis of their inability to produce extracellular nuclease on solid media. When these mutants were examined for their ability to elaborate various extracellular enzymes in liquid media, it was found that they were defective not only in their ability to secrete nuclease but also in alkaline phosphatase and protease activity as well. Studies are in progress to determine the nature of the defect in these mutants and should provide a more clear understanding of the details of protein secretion.

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