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# Secretion of Alkaline Phosphatase Subunits by Spheroplasts of *Escherichia coli*

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Under conditions that permitted continued protein synthesis, spheroplasts of *Escherichia coli* were unable to form *active* alkaline phosphatase, although they synthesized protein that was antigenically related to alkaline phosphatase subunits. This cross-reacting protein was primarily detected in the medium of the spheroplast culture, and it had properties that closely resembled those of the alkaline phosphatase subunit. These results suggest that formation of the active alkaline phosphatase dimer by intact *E. coli* cells proceeds by a pathway in which inactive subunits released from polyribosomes diffuse through the bacterial cell membrane to a periplasmic space where subsequent dimerization to active enzyme occurs. This pathway provides a possible mechanism for the specific localization of this enzyme to the *E. coli* periplasmic space.

Alkaline phosphatase is one of several hydrolytic enzymes in *Escherichia coli* that appear to be localized in a periplasmic space that lies exterior to the bacterial cell membrane (3). Treatment of *E. coli* by osmotic shock or by lysozyme-ethylenediaminetetraacetic acid (EDTA) leads to a quantitative release of this enzyme (7, 8), and electron micrographs provide evidence for alkaline phosphatase activity near the surface of bacterial cells (6). The purified alkaline phosphatase is an unusually stable zinc metalloprotein composed of two identical subunits (9, 10); thus, synthesis of this enzyme must involve several stages, one of which is the transport of the newly formed protein from the polyribosomes to the periplasmic space. Recent experiments by A. Torriani (*in preparation*) rule out the possibility that active enzyme is formed on polyribosomes and, in addition, show that a small pool of alkaline phosphatase monomers are detectable in the cytoplasm of cells that are synthesizing this enzyme. Transport to the periplasmic space, however, could occur either before or after dimerization of the subunits. With the availability of antibodies that discriminate between the subunit and the active dimer (13), it was possible to determine which form of the enzyme passes through the bacterial cell membrane. To look directly at the material that exits through the membrane, spheroplasts of *E. coli* were prepared with lysozyme under conditions that enabled the treated cells to continue protein synthesis. It was found that spheroplasts can synthesize alkaline phosphatase subunits which were secreted into the cell culture medium. This finding suggests that the pathway for alkaline phosphatase formation in intact cells consists of the transport of the subunit across the bacterial cell membrane and subsequent dimerization to active enzyme in the periplasmic space.

## MATERIALS AND METHODS

*Bacterial strains and growth medium. E. coli* strains CW3747, W3747, and C·10F·27 were obtained from the laboratory of C. Levinthal, Massachusetts Institute of Technology, Cambridge, Mass. The properties of these strains have been described (1). Cells were grown in a medium containing tris(hydroxymethyl) aminomethane (Tris)chloride (*p*H 7.4) and inorganic salts (12), 0.2% glucose, and, per milliliter, 40 μg of inorganic phosphate ($P_i$) as $KH_2PO_4$, 20 μg of L-methionine, and 5 μg thiamine HCl. Spheroplasts were incubated in the same media but contained, in addition, 20% sucrose, 0.1 M $MgSO_4$, and 20 μg each of adenosine, cytidine, guanosine, uridine, and deoxythymidine per ml.

*Antibodies.* Antibodies directed against the active enzyme and against the inactive subunit of alkaline phosphatase were obtained from rabbits. The preparation and properties of these antibodies have been described (13). Antitrinitrophenyl-γ-globulin antibodies were kindly supplied by J. R. Little and were used for controls as a nonspecific rabbit immunoglobulin.

*Chemicals. p*-Nitrophenylphosphate (no. 104), glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP), and nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. Trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J., and uniformly la-

beled $^{14}$C-amino acid mixture was purchased from New England Nuclear Corp., Boston, Mass.

*Spheroplast preparation.* Portions (40 ml) of an exponential culture of bacteria grown to approximately $3 \times 10^8$ cells/ml were centrifuged, and the pellets were suspended in 0.9 ml of a buffer containing 0.7 ml of 20% sucrose and 0.2 ml of 0.25 M Tris-chloride (*p*H 8.0). Lysozyme (20 μliters of a 2 mg/ml solution) was added, followed by 40 μliters of 0.1 M EDTA (*p*H 8.0). After 10 min at 23 C, 0.1 ml of 30% bovine serum albumin was added and the cells were carefully resuspended with three 10-ml portions of the spheroplast growth medium. The cells were incubated with very slow rotary motion in a water bath (New Brunswick Scientific Co., New Brunswick, N.J.) at 30 C. After 5 min, a uniformly labeled $^{14}$C-amino acid mixture was added to the culture (0.05 μc for each 30 ml of culture). Thirty minutes after the addition of $^{14}$C, the entire culture was centrifuged at 4,000 × *g* and the supernatant fraction was carefully decanted. The spheroplast pellets were resuspended in a small volume of 0.05 M Tris-chloride (*p*H 8.0) and were disrupted by sonic vibration. These 30-fold concentrated extracts were centrifuged at 40,000 × *g* for 30 min, and the supernatant fractions were tested for (i) alkaline phosphatase activity, (ii) protein concentration, (iii) $^{14}$C-labeled protein, (iv) glucose-6-phosphate dehydrogenase activity, and (v) protein that is specifically precipitated by antibodies directed against alkaline phosphatase subunits.

After removal of the spheroplasts, the culture medium was dialyzed for 16 hr at 4 C against 2 liters of buffer containing 0.01 M Tris-chloride (*p*H 7.4) and 0.3 M NaCl, and was subsequently concentrated about 30-fold in a pressure device containing an ultrafilter (UM 1 Diaflo; Amicon Corp., Cambridge, Mass.). This concentrated fluid was clarified by high-speed centrifugation and was then tested for the activities listed above. Because serum albumin had been added to the original medium at such a high concentration, no measurements of protein concentration were made on the concentrated medium.

*Assays.* Alkaline phosphatase activity was measured with *p*-nitrophenylphosphate as substrate (12); 1 unit equals 1 μmole of *p*-nitrophenol formed per min at 37 C ($\epsilon = 16.2 \times 10^3$ at 410 nm). Glucose-6-phosphate dehydrogenase was measured according to Kornberg's procedure (5); 1 unit equals 1 μmole of NADP reduced per min at 37 C. Protein was determined by a micromodification of the Folin-Ciocalteu procedure (4). $^{14}$C-activity in protein was measured by filtering solutions on membrane filters (Millipore Corp., Bedford, Mass.) after the samples in 5% trichloroacetic acid had been heated for 20 min at 80 C. Radioactivity was measured with a low-background gas flow counter.

*Precipitation with antibodies.* Several samples of $^{14}$C-labeled protein solutions, ranging from 0.05 to 0.3 ml, were added to conical centrifuge tubes containing 0.1 ml of a rabbit immunoglobulin preparation consisting of antisubunit antibodies and 0.05 ml of 1 M Tris-chloride (*p*H 7.4). The volume was brought to 1.0 ml with 0.15 M NaCl. For controls, an equal sample of the test solution was added to 0.15 ml of a rabbit immunoglobulin preparation containing antitrinitophenyl-γ-globulin antibodies and 0.05 ml of 1 M Tris-chloride (*p*H 7.4) and brought to 1.0 ml with 0.15 M NaCl. The solutions were mixed and incubated at 4 C for 16 hr. Precipitates were collected by centrifugation at 4 C and were washed three times with cold 0.15 M NaCl. They were finally washed onto a membrane filter (Millipore Corp.), dried, and counted with a low-background gas flow counter. In all calculations, the counts per minute recorded for the precipitate from the control tube was subtracted from that of the precipitate containing the antisubunit antibody. The value for the nonspecific precipitates ranged from 0.5 to 2% of the added radioactivity. Values recorded represent an average obtained over the range of sample volumes for which there was still an excess of antibody present.

## RESULTS

The addition of lysozyme and EDTA to a culture of *E. coli* suspended in a medium containing a high concentration of sucrose leads to the formation of spheroplasts which are capable of protein synthesis and which are able to support the formation of bacteriophage (2). In the present investigation, freshly prepared spheroplasts incorporated $^{14}$C-amino acids into protein for at least 30 min at an initial rate that was about 20% of that of a similar culture of cells not treated with lysozyme (Fig. 1). In the experiment described in Fig. 1, 95% of the cells were converted into spheroplasts as measured by viable cell count (Table 1).

The incorporation of $^{14}$C-amino acids into protein is indicative of continued protein synthesis by the spheroplasts; however, the level of alkaline phosphatase activity remained essentially constant in *E. coli* strain CW3747, which is constitutive for alkaline phosphatase synthesis (Table 1). In the untreated culture, the alkaline phosphatase activity increased by 46% during the 30-min interval.

The inability of the spheroplasts to make *active* alkaline phosphatase did not result from a general block in protein synthesis. Spheroplasts not only incorporated $^{14}$C-amino acids into material precipitated by hot trichloroacetic acid but also increased their total protein content; e.g., from 44 to 65 μg/ml during the 30-min incubation period in one experiment.

It seemed likely that some form of inactive alkaline phosphatase was synthesized in the spheroplasts, and experiments were performed in order to look for protein antigenically related to alkaline phosphatase. Two preparations of antibodies that recognize forms of *E. coli* alkaline phosphatase are available (13). One of these antibody preparations, obtained from rabbits injected with active enzyme, does not form a

precipitin with the alkaline phosphatase subunit, although it is able to coprecipitate subunits when an enzyme-antibody precipitate is formed. The second antibody preparation precipitates the subunit and does not react under any conditions with either active enzyme or with enzyme that has been inactivated by chelating agents. Only this second preparation (containing antisubunit antibodies) can distinguish subunits from enzyme when active alkaline phosphatase is present; thus, this preparation was used in the present study.

Samples of the extract from the spheroplasts and samples of the dialyzed, concentrated medium were tested for alkaline phosphatase subunits by measuring the amount of $^{14}C$-protein in specific precipitates formed under conditions in which excess antibody was present. Antigenic material was detected both in the culture medium and in the spheroplasts. The amount in the culture fluid accounted for 4 to 5% of the total labeled protein present in the culture fluid (Table 2), and 1 to 2% of the total protein in the spheroplasts formed a precipitate. This latter percentage is about the same as that measured for an isogenic strain of *E. coli* tested under conditions in which the synthesis of alkaline phosphatase was repressed and in which there were very low levels of active enzyme (*see below*).

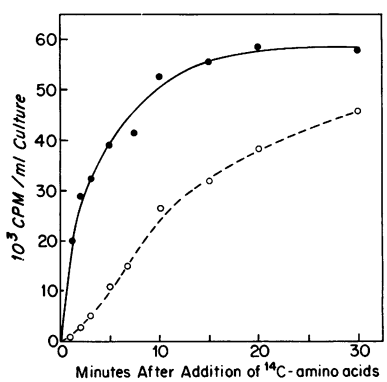


FIG. 1. *Incorporation of $^{14}C$-amino acids into E. coli cells and spheroplasts. An exponentially growing culture of cells was divided into two equal fractions which were harvested by centrifugation. The pelleted cells of one fraction were treated with lysozyme-EDTA and were resuspended in the spheroplast medium (see Materials and Methods). Cells from the other fraction were resuspended in 0.9 ml of the normal growth medium, and after 10 min were diluted with normal growth medium to give the same volume as that of the spheroplasts. Both cultures were agitated slowly at 30 C, and after 5 min 0.05 μc of $^{14}C$ (uniformly labeled) amino acids was added. At the times indicated, 0.1 ml of the culture was removed and added to 1 ml of 5% trichloroacetic acid to measure protein synthesis. Symbols: ●, intact cells; ○, spheroplast culture.*

TABLE 1. *Alkaline phosphatase activity in cells and spheroplasts of E. coli CW3747*[a]

| Culture | Viable cells per ml | | Alkaline phosphatase activity (units per ml) | |
|---|---|---|---|---|
| | Initial | Final | Initial | Final |
| Intact cells.... | $3.9 \times 10^8$ | $7.0 \times 10^8$ | 0.29 | 0.42 |
| Spheroplasts.. | $0.20 \times 10^8$ | $0.37 \times 10^8$ | 0.28 | 0.28 |

[a] Fractions (1 ml) of the cultures described in Fig. 1 were examined 1 min before and 30 min after the addition of $^{14}C$-amino acids. A 0.1-ml amount of the fraction was diluted and samples were plated on nutrient agar for measurement of the viable bacteria. The remaining 0.9 ml was disrupted by sonic treatment and assayed for alkaline phosphatase (*see* Materials and Methods). Spheroplasts were prepared from the balance of the culture; 92% of the total alkaline phosphatase activity was released into the culture medium, and less than 10% of the total glucose-6-phosphate dehydrogenase was detected in the 25-fold concentrated culture medium (*see* Table 3).

These experiments were repeated a number of times, and the results, together with an estimate of the fraction of spheroplasts lysed during the incubation period and the per cent of bacterial cells not converted to spheroplasts, are summarized in Table 3. The fraction of lysed cells was measured by the relative amount of glucose-6-phosphate dehydrogenase that appeared in the culture medium. This enzyme is localized in the bacterial cell cytoplasm and is not released in the preparation of spheroplasts (3). In all but one of the experiments recorded in Table 3, there was less than 20% lysis of the culture. The fraction of cells still intact after the spheroplast procedures was estimated by the amount of alkaline phosphatase that remained associated with the spheroplasts after the medium was removed by centrifugation, as this enzyme is quantitatively released by spheroplasts. By this criterion, less than 10% of the culture consisted of intact cells. In two experiments, colony counts of the spheroplast preparation showed that less than 5% of the cells could form colonies on agar plates.

Three sets of control experiments were performed, and the results of these experiments are included in Table 3. In one set of experiments, the isogenic strain W3747, in which alkaline phosphatase synthesis is repressed during the

exponential phase of growth, was examined. One to 2% of the protein in the spheroplasts of this strain showed cross-reaction with the antisubunit antibodies and about the same percentage of protein could be detected in the medium. This precipitating protein is attributed to the presence, in the immunoglobulin preparation, of antibodies to proteins other than alkaline phosphatase.

TABLE 2. *Amount of protein with the antigenic specificity of alkaline phosphatase subunits in E. coli spheroplasts*[a]

| Sample | $^{14}$C-protein added | $^{14}$C-protein precipitated by nonspecific γ-globulin | $^{14}$C-protein precipitated by γ-globulin containing antisubunit antibodies | Total protein specifically precipitated |
|---|---|---|---|---|
| | *counts/min* | *counts/min* | *counts/min* | % |
| Spheroplasts | 14,000 | 158 | 298 | 1.0 |
| | 20,000 | 208 | 456 | 1.2 |
| | 35,000 | 380 | 976 | 1.7 |
| | 70,000 | 442 | 959 | 0.8 |
| Medium | 6,000 | 65 | 320 | 4.2 |
| | 8,000 | 75 | 384 | 3.9 |
| | 12,000 | 80 | 663 | 4.9 |
| | 12,000 | 110 | 754 | 5.4 |
| | 16,000 | 127 | 1005 | 5.5 |

[a] Spheroplasts were prepared and incubated according to procedures described in Materials and Methods. An 80-ml amount of culture was used in the analysis; the medium was concentrated to 4.5 ml, and the volume of the extract from the spheroplasts was 2.2 ml. The specific radioactivity of the protein (based on the amount of protein formed *after* the spheroplasts had been prepared) was 475 counts per min per μg. In this experiment, 95% of the cells were converted to spheroplasts according to viable count, and less than 10% of these spheroplasts were lysed (see Table 3).

A second control experiment was performed with strain CW3747 that had been subjected to the sucrose-lysozyme-EDTA treatment *after* 30 min of incubation with $^{14}$C-amino acid. In these cells, 2.6% of the protein in spheroplasts was precipitated by the antisubunit antibodies. Only 1% of the total $^{14}$C-protein present in the culture appeared in the culture fluid, and no significant part of this was specifically precipitated. Most of the precipitated protein was probably not related to alkaline phosphatase, but a small fraction of it could have resulted from the pool of subunits that has been detected in the cytoplasm of cells that synthesize alkaline phosphatase (A. Torriani, *in preparation*).

The third control experiment was carried out with one of the alkaline phosphatase-negative mutants (C.10 F.27) known to form an abnormal subunit that does not dimerize except under special conditions (12). Results of this "positive" control showed that 5.4% of the protein in the medium was precipitated as compared to 1.5% in the spheroplasts.

These control experiments indicated that a major part of the antigenic $^{14}$C-protein in the medium is related to alkaline phosphatase. To characterize this antigenic material further, the concentrated medium was centrifuged through a

TABLE 3. *Distribution of alkaline phosphatase subunits and enzyme between spheroplasts (S) and medium (M)*[a]

| Bacterial strain | Protein that precipitates with antisubunit antibody[b] | | Alkaline phosphatase activity (total units) | | Glucose-6-phosphate dehydrogenase activity (total units[c]) | | $^{14}$C-labeled protein (per cent total) | |
|---|---|---|---|---|---|---|---|---|
| | S | M | S | M | S | M | S | M |
| CW 3747 (80)[d] | 1.3 | 5.0 | 2.6 | 31.0 | .14 | <.01 | 75 | 25 |
| CW 3747 (120) | 2.0 | 7.4 | 5.6 | 31.4 | .17 | .04 | 81 | 19 |
| CW 3747 (120) | 1.2 | 4.6 | 3.3 | 38.6 | | | 75 | 25 |
| CW 3747 (120) | 0.8 | 6.0 | 1.9 | 33.4 | .32 | .03 | 79 | 21 |
| CW 3747 (120) | 1.0 | 4.3 | 1.7 | 42.0 | .24 | .05 | 76 | 24 |
| W 3747 (30) | 1.6 | <0.1 | <0.005 | 0.01 | .08 | .01 | 85 | 15 |
| W 3747 (120) | 2.0 | 2.0 | <0.005 | 0.006 | .11 | .15 | 83 | 17 |
| W 3747 (60) | 1.0 | 1.0 | <0.005 | 0.01 | .08 | .01 | 80 | 20 |
| CW 3747 (30)[e] | 2.6 | <0.1 | 8.25 | 0.06 | .06 | <.01 | 99 | 1 |
| C 10 F27 (60) | 1.5 | 5.4 | 0.06 | 1.2 | .06 | .02 | 71 | 29 |

[a] Refer to Materials and Methods for experimental details.

[b] Per cent $^{14}$C added found in precipitate. Corrected for nonspecific precipitation.

[c] Values between experiments have been normalized to a standard control carried out with glucose-6-phosphate-dehydrogenase noted in Materials and Methods.

[d] The numbers in parentheses refer to the volume of the culture that was labeled with $^{14}$C-amino acids.

[e] This culture was not treated with lysozyme.

linear sucrose gradient. Figure 2 shows that the peak of antigenic material sediments slower than enzymatically active alkaline phosphatase, at a rate close to that previously measured for the alkaline phosphatase subunit [$S_{20,w}$ = 3.5 (11)]. The antigenic protein in the medium was also digested by trypsin (Table 4), which is consistent with the known lability of alkaline phosphatase subunits to proteolysis (11).

An attempt was made to convert the inactive alkaline phosphatase-like $^{14}C$-protein in the concentrated medium into the active enzyme which is stable to proteolysis. The medium was divided into two fractions; one of these fractions was dialyzed for 16 hr at 24 C against a solution of $10^{-4}$ M zinc sulfate and 0.01 M Tris (pH 7.4), conditions which enable subunits to associate to active protein. The other fraction was kept at 0 C in the presence of 0.3 M NaCl, conditions which greatly inhibit reactivation of subunits. Both fractions were then treated with 0.2 mg of Pronase per ml at 37 C for 60 min and dialyzed against 0.01 M Tris (*p*H 7.4) -0.001 M $MgSO_4$. The two samples were chromatographed separately on diethylaminoethyl (DEAE) cellulose, and the $^{14}C$ activity was measured over the peak that contained the nonlabeled active enzyme present before the spheroplasts were made. No significant differences in the distribution of radioactivity were found between the two samples. Possibly, the secreted subunits were irreversibly denatured as a result of the dialysis and the subsequent concentration steps.

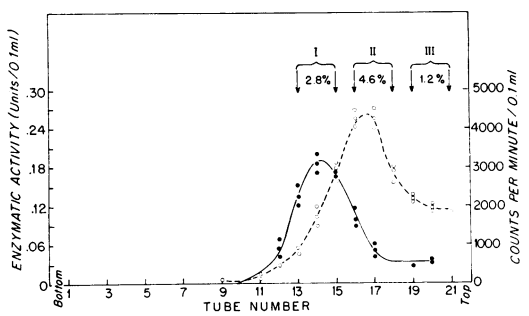


FIG. 2. *Zonal centrifugation in a sucrose gradient of the 50-fold concentrated media from a culture of spheroplasts labeled with $^{14}C$-amino acids for 30 min. Fractions (0.3 ml) of the supernatant fluid were layered onto a 15 to 30% sucrose gradient containing 0.2 M NaCl and 0.05 M Tris-chloride (pH 7.4). After 16 hr of centrifugation at 39,000 rev/min in the SW 39 rotor of a spinco model L centrifuge (temperature = 14 F), the three tubes were punctured and 20 equal fractions were collected. The fractions (indicated by the brackets) from the three gradients were pooled, dialyzed against 0.2 M NaCl-0.05 M Tris-chloride (pH 7.4), and tested against the antisubunit antibodies; the numbers indicate the percentages of $^{14}C$-protein specifically precipitated by antisubunit antibodies (see Materials and Methods). Symbols: ●, alkaline phosphatase activity; ○, counts per min in protein.*

To test the stability of subunits and to measure their recovery in the experimental procedure, a reconstruction experiment was carried out with $^{14}C$-labeled subunits prepared from purified enzyme. Only 35% of the $^{14}C$-protein subunits initially present as antigenic material were recovered after dialyzing and concentrating the solution (Table 5). In a similar experiment, except that active enzyme was not added to the subunits, only 20% of the added subunits could be reassociated to form active enzyme after

TABLE 4. *Effect of trypsin on the precipitin reaction between antisubunit antibodies and protein in the spheroplast medium*[a]

| Treatment | Protein added (counts/min) | Protein precipitated[b] | |
|---|---|---|---|
| | | Counts/min | Per cent |
| Without trypsin.. | 20,000 | 1,465 | 7.3 |
| Without trypsin.. | 30,000 | 2,408 | 8.0 |
| Trypsin added.... | 20,000 | 231 | 1.1 |
| Trypsin added.... | 30,000 | 293 | 1.1 |

[a] Portions of a 27-fold concentrated medium from a spheroplast culture of CW3747 were preincubated with 50 μg of trypsin in 0.05 M Tris (*p*H 7.4)-0.15 M NaCl at 24 C for 30 min; the precipitin reaction was carried out as described in Materials and Methods.

[b] Nonspecific precipitation has been subtracted.

TABLE 5. *Reconstruction experiment*[a]

| Phase of expt | Enzyme | $^{14}C$-protein | Antigenic protein |
|---|---|---|---|
| | *units* | *counts/min* | *μg* |
| Initial..... | 8.0 | 4,500 | 41 |
| After dialysis and concentration..... | 6.5 | 3,150 | 14.5 |
| Recovery (%)..... | 81 | 70 | 35 |

[a] A preparation of purified $^{14}C$-alkaline phosphatase (73 counts per min per μg of protein) was acidified, and the labeled subunits were diluted to a volume of 60 ml, containing 0.3 M NaCl, 0.01 M Tris-chloride (*p*H 7.4), and 20% sucrose. Bovine serum albumin was added to 1 mg/ml, and 6.2 units of active alkaline phosphatase was also added. This solution was dialyzed and concentrated to 3.5 ml, and the $^{14}C$-protein precipitable by antisubunit antibodies was measured. In the original acidified enzyme preparation, prior to dilution, 70% of the $^{14}C$-protein was specifically precipitable.

dialysis and concentration. There was a loss of antigenic material as well as a considerable amount of irreversible denaturation to the subunit in the process of concentrating the culture medium. These results were not unexpected; earlier studies of the alkaline phosphatase subunit have shown it to be unstable (11).

The data from these reconstruction experiments made it possible to estimate the amount of inactive alkaline phosphatase material secreted by spheroplasts. In an experiment cited previously (*see* Table 3), the increase in total protein during the $^{14}C$-labeling period was 1,680 $\mu$g. The specific radioactivity of this protein was 475 counts per min per $\mu$g, and 0.4 ml of the concentrated medium gave a precipitate with antibody that contained 878 counts/min. If 80% of this was alkaline phosphatase, the total amount of antigenic protein present in the concentrated medium (4.5 ml) would be $(0.80) \times (878) \times (4.5)/(475) \times (0.4) = 16.5$ $\mu$g. After correcting for recovery (0.35), we would obtain a value of 47 $\mu$g as protein related to alkaline phosphatase. This amounts to 2.8% of the total protein formed by the spheroplasts. In a control experiment with intact cells, 3% of the protein formed was active alkaline phosphatase. Thus, the spheroplasts produce the same relative amount of alkaline phosphatase protein as do intact cells, but the protein appears as inactive subunits in the culture medium.

## DISCUSSION

The synthesis of alkaline phosphatase by *E. coli* is repressed when the cells are grown in a medium supplied with inorganic phosphate (15). About one molecule of active enzyme per cell can be detected under these conditions, and there is no evidence for a substantial precursor pool of inactive protein in repressed cells. When synthesis of the enzyme is stimulated by withdrawal of $P_i$, the active form of the protein is detected as a dimer of identical subunits localized exterior to the bacterial cell cytoplasm but within the cell wall. Recent evidence has indicated that there are subunits of alkaline phosphatase present in the cytoplasm of cells making active enzyme (Torriani, *in preparation*). The question examined here is whether subunits or dimer pass through the cell membrane.

Previous studies with a mutationally altered form of the *E. coli* alkaline phosphatase have suggested that these altered subunits, which were released into the culture medium by spheroplast formation, are capable of traversing the cell membrane (12). Similar results were obtained when alkaline phosphatase was synthesized in the presence of the histidine analogue, triazolealanine (14). In this case, the substitution of the analogue for histidine produced a subunit of alkaline phosphatase incapable of associating to a dimeric form, and this subunit was released into the medium by lysozyme treatment of the cells. (S. Schlesinger, *unpublished data*). The results of the present study are consistent with these earlier observations, since they show that normal subunits formed by spheroplasts are detectable in the culture medium.

Although the protein in the medium that cross-reacts with antisubunit antibodies has properties identical to those of the subunit, the results do not eliminate the possibility than an inactive dimer which is altered in shape and which is recognizable by these antibodies is formed in the cytoplasm. This form of inactive dimer would be quite distinct from any form of the wild-type alkaline phosphatase detected thus far. Studies of the inactive zincless dimer, obtained by treating enzyme with chelators, have shown that this protein does not cross-react with antisubunit antibodies nor does it differ in sedimentation coefficient from the dimer.

Transport of this enzyme to the periplasmic space as an inactive subunit would provide one means of protecting the bacterial cell from the presence of an active alkaline phosphatase in its cytoplasm. It would also enable the transport system to handle a much smaller protein molecule than the active form of this enzyme. This transport process may require specific carriers, but it may depend only on the primary amino acid sequence of the polypeptide chain. Experiments to test these alternatives are in progress; these experiments should help in elucidating the form of alkaline phosphatase required for transport from the cytoplasm to the periplasmic space.

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