Interactions of Alkaline Phosphatase and the Cell Wall of *Pseudomonas aeruginosa*

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Spheroplasts prepared by lysozyme treatment of cells of *Pseudomonas aeruginosa*, suspended in 20% sucrose or 0.2 M $MgCl_2$, were examined in detail. Preparation of spheroplasts in the presence of 0.2 M Mg^{2+} released periplasmic alkaline phosphatase, whereas preparation in the presence of 20% sucrose did not, even though untreated cells released phosphatase when suspended in sucrose in the absence of lysozyme. Biochemical characterizations of the sucrose-lysozyme preparations indicated that lysozyme mediated a reassociation of the released phosphatase with the spheroplasts. In addition, the enzyme released from whole cells suspended in 20% sucrose (which represents 20 to 40% of the cell-bound phosphatase) reassociates with the cells in the presence of lysozyme. Electron microscopic examinations of various preparations revealed that phosphatase released in sucrose reassociated with the external cell wall layers in the presence of lysozyme, that sucrose-lysozyme prepared spheroplasts did not dissociate phosphatase which remained in the periplasm of sucrose-washed cells, and that phosphatase was never observed to be associated with the cytoplasmic membrane. A model to account for the binding of *P. aeruginosa* alkaline phosphatase to the internal portion of the tripartite layer of the cell wall rather than to the cytoplasmic membrane or peptidoglycan layer is presented.

It has been found (3, 4) that alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC3.1.3.1.) of *Pseudomonas aeruginosa* is inducible by phosphate starvation and is readily removed from cells after washes in 0.2 M $MgCl_2$, pH 8.4. The enzyme (3) has been localized by electron microscope techniques in the region between the cytoplasmic membrane and the tripartite layer of the cell wall, i.e., the periplasmic space (9). It is well established that periplasmic enzymes from *Escherichia coli* can be removed by either the ethylenediaminetetraacetic acid (EDTA) osmotic shock procedure of Neu and Heppel (10) or by the procedure of Malamy and Horecker (8) involving the formation of spheroplasts in the presence of lysozyme and EDTA. Because EDTA lyses *P. aeruginosa* cells (7), we developed alternative methods for the formation of spheroplasts from *P. aeruginosa* such as the Mg^{2+}-lysozyme method or sucrose-lysozyme method (3). During the formation of spheroplasts by the Mg^{2+}-lysozyme method, the alkaline phosphatase is largely released into the

supernatant fluid, whereas almost no alkaline phosphatase is released during the sucrose-lysozyme spheroplast procedure although suspension of lysozyme-untreated cells in 20% sucrose was previously shown to remove 20 to 40% of the cell-bound alkaline phosphatase.

This communication presents evidence which demonstrates that, in the absence of high concentrations of Mg^{2+}, the lysozyme present in the spheroplast-forming solution mediates a reassociation of alkaline phosphatase with the cell wall of *P. aeruginosa*. In addition, it was possible to establish the specific location of alkaline phosphatase within the periplasmic space of *P. aeruginosa* cells. Electron micrographs of sucrose-lysozyme treated cells and sucrose-lysozyme prepared spheroplasts showed that the alkaline phosphatase is closely associated with the inner region of the tripartite layer and is never observed to be associated with the cytoplasmic membrane.

MATERIALS AND METHODS

Organism and culture conditions. *P. aeruginosa* ATCC 9027 was cultivated at 37 C on a rotary shaker in a glucose-ammonium salt-proteose peptone medium as previously described (4). Under these conditions the culture actively synthesizes alkaline phosphatase be-

tween the 5th and 14th hr. The proteose peptone serves as the only source of organic or inorganic phosphate.

Chemicals. p-Nitrophenylphosphate, tris(hydroxymethyl)aminomethane (Tris) and lysozyme (muramidase) were purchased from the Sigma Chemical Co., St. Louis, Mo. Sodium-β-glycerophosphate was obtained from Fisher Scientific Co., Pittsburgh, Pa.

Release of alkaline phosphatase from cells. Standard quantities of cells obtained from 14-hr cultures grown to 1.2 to 1.4 optical density units (OD, 660 nm; Gilford model 300-N spectrophotometer) were washed with 0.2 M $MgCl_2$ in 0.01 M Tris (pH 8.4) or 20% sucrose in 0.01 M Tris (pH 8.4) as described previously (4). A calibration curve relating OD to dry weight was constructed and it was determined that 1 OD corresponded to 18.5 mg (dry weight). Cell-free extracts were prepared by ultrasonic disruption of cells resuspended in 0.01 M Tris, pH 8.4, as previously described (4).

Enzyme assays. Alkaline phosphatase, reduced nicotinamide adenine dinucleotide (NADH) oxidase, and glucose-6-phosphate (G-6-P) dehydrogenase were assayed as described previously (4). The effect of the various washing components upon alkaline phosphatase activity, as compared to release, were found to be negligible as determined previously (4). One unit of enzyme activity corresponds to the conversion of 1 μmole of substrate to product per minute.

Preparation of spheroplasts. Sucrose-lysozyme spheroplasts were prepared by centrifuging the cells from 20 ml of a 14-hr culture (13,000 $\times$ g) and then resuspending in 20 ml of 20% sucrose (with 0.01 M Tris, pH 8.4); the resulting suspension was 0.5 mg/ml in lysozyme. The cell suspension was incubated at 25 C in a water bath shaker for 30 min; cells were centrifuged and resuspended in 0.01 M $MgCl_2$ and 0.01 M Tris buffer (pH 8.4) for the formation of spheroplasts. Sucrose-0.01 M Mg^{2+}-lysozyme spheroplasts were prepared in the same way except that $MgCl_2$, 0.01 M final concentration, was added to the sucrose. Magnesium-lysozyme spheroplasts were prepared in a similar manner, except that the 20% sucrose was replaced by 0.2 M $MgCl_2$ (pH 8.4) and 0.01 M Tris. During the processes used to convert P. aeruginosa cells to spheroplasts, all supernatant fluids were collected and assayed (as in Table 1) for alkaline phosphatase, NADH oxidase, and G-6-P dehydrogenase.

Whole cell assay system for alkaline phosphatase localization studies. P. aeruginosa cells were suspended for 2 min in 0.2 M $MgCl_2$ and 0.01 M Tris buffer (pH 8.4), or 20% sucrose in 0.01 M Tris buffer (pH 8.4) containing 0.5 mg of lysozyme per ml. The suspensions were centrifuged at 13,000 $\times$ g and incubated for 30 min in a modified Gomori (5) mixture of the following composition: sodium-β-glycerophosphate, 0.5%; sodium-barbitol, 0.5%; $Ca(No_3)_2$, 0.02 M; $MgCl_2$, 0.01 M; and Tris buffer, 0.01 M (pH 8.4). Control treatments with cells obtained from inorganic phosphate derepressed and repressed cultures (4), sucrose-lysozyme spheroplasts, magnesium-lysozyme spheroplasts, sucrose-0.01 M $MgCl_2$-lysozyme spheroplasts, and sucrose-lysozyme spheroplasts which had been washed twice with 0.2 M $MgCl_2$ were also incubated with the above mentioned modified Gomori mixture to localize the alkaline phosphatase.

Electron microscopy. The methods for fixation and embedding were those described previously (3). Thin sections were cut with a Sorvall Porter-Blum model MT-2 ultramicrotome, stained with lead citrate (13), and examined with an AEI-EM-(801) electron microscope by using 60-kv electron acceleration voltage.

RESULTS

Release of enzymes during Mg^{2+}lysozyme or sucrose-lysozyme spheroplast formation. The results presented in Table 1 relating to the release of alkaline phosphatase from cells of P. aeruginosa as mediated by 0.2 M $MgCl_2$ or 20% sucrose washing are in agreement with previously reported findings (4). It is further observed that when P. aeruginosa cells are treated with lysozyme in the presence of 0.2 M Mg^{2+}, almost all of the alkaline phosphatase is released to the supernatant fluid as compared to the cell-free extract (Table 1), whereas NADH oxidase and G-6-P dehydrogenase remain entirely within the spheroplasts.

When lysozyme-treated cells of P. aeruginosa are maintained in a medium of high osmotic pressure, i.e., 0.2 M Mg^{2+} which was 0.01 M in Tris (pH 8.4), only some of the cells become spherical. When suspensions of these cells are centrifuged and resuspended in 0.01 M Mg^{2+} and 0.01 M Tris (pH 8.4), 100% of the cells are changed to spheres (Fig. 1 C) as compared to untreated cells (Fig. 1 A). Assay of the supernatant fluid from the 0.01 M Mg^{2+} suspension of spheroplasts showed essentially no further release of alkaline phosphatase and no significant release of the internal enzymes, G-6-P dehydrogenase, or NADH oxidase (Table 1). Spheroplasts prepared by this method are stable overnight, and, furthermore, actinomycin D was unable to block the uptake of ^{14}C-uracil by these preparations (Cheng, Costerton, and Ingram, *unpublished data*). This can be taken as evidence of the intact state of the cytoplasmic membrane of these spheroplasts.

When cells of P. aeruginosa were suspended into 20% sucrose and 0.5 mg of lysozyme per ml, no alkaline phosphatase, NADH oxidase, or G-6-P dehydrogenase was released into the supernatant fluid (Table 1). When the cells obtained by the above treatment were suspended into 0.01 M $MgCl_2$ and 0.01 M Tris (pH 8.4), more than 99% of the cells were converted to spheres (Fig. 1 B). The supernatant fluid obtained after centrifugation of this suspension contained no alkaline phosphatase or NADH oxidase, but there was a considerable amount of G-6-P dehydrogenase (Table 1). This indicated that sucrose-lysozyme spheroplasts were more susceptible to osmotic shock than Mg^{2+}-lysozyme spheroplasts.

TABLE 1. *Enzyme release from cells and spheroplasts of Pseudomonas aeruginosa*

Treatment	Magnesium[a]			Sucrose		
	Alkaline phosphatase	NADH oxidase	G-6-P dehydrogenase	Alkaline phosphatase	NADH oxidase	G-6-P dehydrogenase
0.2 M MgCl₂ or 20% sucrose washes of cells[b]	156.0[c]	0	0	60.0	0	0
Supernatant fluid obtained from cells treated with lysozyme in either 0.2 M MgCl₂ or 20% sucrose ..	154.8	0	0	0	0	0
Supernatant fluid obtained from lysozyme-treated cells after resuspension into 0.01 M MgCl₂ (spheroplast formation)	1.3	0	0	0	0	18.3
0.2 M MgCl₂ wash of spheroplast pellets	0	0	0	165.0	0	0
Cell-free extract	155.8	15.6	144.5	155.8	15.6	144.5

[a] Magnesium and sucrose refer to the wash in which each sample of cells was initially suspended. NADH, reduced nicotinamide adenine dinucleotide; G-6-P, glucose-6-phosphate.

[b] Twenty milliliters of 14-hr cells [480 mg (dry weight) of a culture grown to 1.3 optical density units] were centrifuged and resuspended in an equal volume of wash solution as a control to determine the release of periplasmic, cytoplasmic membrane, and cytoplasmic marker enzymes. A similar volume of cells was resuspended in each of the two wash solutions to which lysozyme (0.5 mg/ml) was added, and the suspension was incubated at 25 C for 30 min in a water bath shaker. The suspension was centrifuged, the supernatant fluids were assayed for each enzyme, and the cell pellets were resuspended in 0.01 M MgCl₂ and 0.01 M tris(hydroxymethyl)aminomethane, pH 8.4, for the formation of spheroplasts. The suspension was centrifuged and the supernatants were assayed for each enzyme. Spheroplast pellets were washed with 0.2 M MgCl₂, a treatment which was previously shown (4) to effectively remove all remaining cell-bound alkaline phosphatase, and the supernatant fluids obtained after this treatment were assayed for each enzyme.
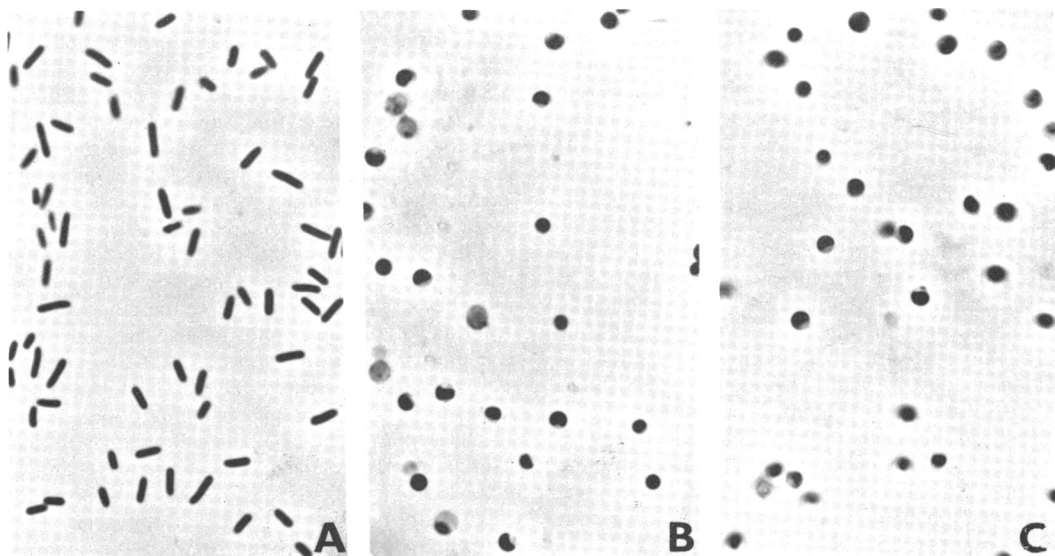
[c] Units of enzyme per 25 g (dry weight).

FIG. 1. *Phase contrast micrographs of (A) whole cells; (B) spheroplasts prepared in sucrose; (C) spheroplasts prepared in 0.2 M Mg²⁺. ×2,000.*

It was also noted that when 20% sucrose-lysozyme or 0.2 M Mg²⁺-lysozyme cells were suspended in distilled water, the Mg²⁺-lysozyme cells changed to spheres and were stable for more than 1 hr, but sucrose-lysozyme cells were lysed within 10 min. This result demonstrates the importance of Mg²⁺ in the maintenance of spheroplast integrity. The uptake of ¹⁴C-uracil by sucrose-lysozyme spheroplasts was not blocked by actinomycin D (Cheng, Costerton, and Ingram, *unpublished data*) and the amount of G-6-P dehydrogenase released did not increase with time. It is obvious, therefore, that the G-6-P dehydrogenase released into the medium initially

is due to the bursting of some cells during osmotic shock. The phase microscopic count of these spheroplasts also confirmed this conclusion. The sucrose-lysozyme spheroplasts which are not lysed by osmotic shock are stable in low concentrations of $MgCl_2$, i.e., 0.01 M, for more than 1 hr.

From these results, it is apparent that, even though some sucrose-lysozyme spheroplasts burst, the alkaline phosphatase is still associated with the cells. Since we have shown that suspension of whole cells in 20% sucrose releases approximately one-third of the cell-bound alkaline phosphatase if lysozyme is not present (Table 1), it is clear that lysozyme either prevents the release of alkaline phosphatase or mediates its reassociation with the cell.

Reassociation of alkaline phosphatase with cells of P. aeruginosa. To delineate between the possibilities (i) that lysozyme prevents the dissociation of cell-bound alkaline phosphatase from 20% sucrose-treated cells or (ii) that lysozyme mediates a reassociation of released alkaline phosphatase with cells, a method was required whereby the reassociation of dissociated alkaline phosphatase could be observed in the presence of cells and lysozyme. Based upon our evidence, a reasonable approach was to attempt to recombine the phosphatase released by the 20% sucrose washing procedure (reference 3, and experiment 2, Table 2) with washed cells in the presence and absence of 0.5 mg of lysozyme per ml.

The results in Table 2 show that the alkaline phosphatase released by washing cells in 20% sucrose is converted almost instantly, upon the addition of lysozyme, to a state which sediments after a 10-min centrifugation at 13,000 × g (experiment 6, Table 2). Since the reassociation is almost instantaneous, there is little likelihood of damage to the cells due to the lysozyme, and the only logical site for alkaline phosphatase reassociation is the cell wall. The reaction also shows an absolute requirement for washed cells; without washed cells the addition of lysozyme to a 20% sucrose supernatant solution containing alkaline phosphatase did not affect its removal from solution upon centrifugation for 10 min at 13,000 × g (experiment 4, Table 2).

Once the reassociation of alkaline phosphatase with the sucrose-washed cells occurred, the complex was stable to suspension in 0.01 M Mg^{2+} solution, but a 0.2 M Mg^{2+} wash completely removed the bound enzyme (experiment 8, Table 2). The enzyme removed by this 0.2 M Mg^{2+} wash includes the component which was not released upon initial exposure to 20% sucrose and the component which was reassociated upon the addition of lysozyme (Table 2). Table 2 also

shows that added lysozyme cannot mediate the reassociation of the enzyme with cells washed and suspended in the presence of 0.2 M $MgCl_2$ (experiments 5 and 7, Table 2). This clearly indicates that the lysozyme-mediated reassociation of alkaline phosphatase with cells of P. aeruginosa requires low ionic concentration.

Ultrastructural localization of alkaline phosphatase. Previously (3) it was shown that alkaline phosphatase of untreated cells is completely localized between the cytoplasmic membrane and the tripartite layer of the cell wall, and the same distribution is seen in the untreated cells used in these experiments (Fig. 2). Similar studies performed with inorganic phosphate-repressed cells, or with cells which were devoid of alkaline phosphatase activity after washes with 0.2 M $MgCl_2$ showed a complete absence of lead phosphate deposits within the periplasmic space, indicating that such deposits are indeed the result of alkaline phosphatase activity (3). Since enzyme analysis showed that washing in 20% sucrose released approximately 30% of the alkaline phosphatase present in the cell (Table 2), whereas all of the enzyme remains bound to the cells after suspension in 20% sucrose containing 0.5 mg of lysozyme per ml, it became desirable to localize the alkaline phosphatase in these treated cells. Cells treated by the modified Gomori reaction and initially suspended in sucrose and lysozyme for 2 min showed that some of the enzyme is still located between the cytoplasmic membrane and the tripartite layer of the cell wall, i.e., the periplasmic space (reference 9 and Fig. 3, arrows), whereas a substantial proportion is also associated with the outer surface of the cells.

When sucrose-lysozyme treated cells are resuspended into 0.01 M Mg^{2+} and Tris, 0.01 M (pH 8.4), they assume a spherical shape, and the distribution of alkaline phosphatase in their cell envelopes remains the same as that observed in sucrose-lysozyme treated cells (Fig. 4). An inner dense deposit of lead phosphate is observed inside the tripartite layer of the cell wall where this layer is clearly resolved (Fig. 4, arrows), and a more loosely packed mass of crystals is associated with the outer surface of the cells. The amorphous aggregates seen between the spheroplasts are probably cellular debris, and, although the most striking reassociation of the alkaline phosphatase is to the surface of the cell wall, there is probably some association of alkaline phosphatase with debris.

When the cytoplasmic membrane is separated from the cell wall (Fig. 4, "S"), both the inner and outer deposits of lead phosphate are associated with the cell wall. In sucrose-plasmolyzed cells (Fig. 5) and in fragments of the cell enve-

lope (Fig. 5, inset), both layers of electron-dense material are associated with the cell wall and no alkaline phosphatase activity is associated with the cytoplasmic membrane.

When spheroplasts are formed by incubating cells in 20% sucrose, 0.5 mg of lysozyme per ml, and 0.01 M Mg^{2+} with subsequent resuspension into 0.01 M $MgCl_2$, an enzyme localization pattern identical with that of sucrose spheroplasts is obtained (Fig. 6). The inclusion of 0.01 M Mg^{2+} in the sucrose-lysozyme treatment solutions appears to enhance the separation of the cytoplasmic membrane from the cell wall in a large number of cells and, as in the case of the sucrose-lysozyme spheroplasts, the enzyme activity is exclusively associated with the cell wall. The deposition of electron-dense material in the cell envelope of this preparation shows that low Mg^{2+} concentration neither increases the release of alkaline phosphatase nor interferes with the lysozyme-mediated reassociation to the outer surface of the cell. Examinations of electron micrographs of magnesium-lysozyme spheroplasts showed no evidence of electron-dense deposits indicating the complete absence of alkaline phosphatase, a result consistent with the biochemical data.

Enzyme assays showed that, when either sucrose-lysozyme spheroplasts or sucrose-0.01 M Mg^{2+}-lysozyme spheroplasts are resuspended into 0.2 M Mg^{2+}, the alkaline phosphatase was completely released (Table 1), and magnesium washed spheroplasts after treatment by Gomori reaction show a complete absence of lead phosphate deposits in the cell envelope (Fig. 7). Taken with our previous finding (3) that the Gomori reaction occurs only in cells induced to synthesize alkaline phosphatase, the absence of reaction in cells treated to remove the enzyme establishes that the original deposition of the electron-dense material described here is due to the production of inorganic phosphate from β-glycerophosphate by this enzyme.

DISCUSSION

In a previous study (3), we reported the production of spheroplasts of *P. aeruginosa* after washes of the cells in either 0.2 M $MgCl_2$ or 20% sucrose, 0.5 mg of lysozyme per ml, and resuspension of the cells into 0.01 M $MgCl_2$. The present investigations were undertaken in an effort to describe more fully some of the characteristics of spheroplasts produced by these two methods. From the results presented here, apparently alkaline phosphatase, a periplasmic enzyme of *P. aeruginosa*, is released by 0.2 M $MgCl_2$ prior to the production of spheroplasts. Spheroplasts prepared in this manner do not release

TABLE 2. *Conditions for release and reassociation of alkaline phosphatase with cells of Pseudomonas aeruginosa*

Expt no.	Treatment	Alkaline phosphatase in supernatant fluid	Alkaline phosphatase remaining in cells
1	0.2 M Mg^{2+} wash of cells[a]	150.0[b]	1.3
2	20% Sucrose wash of cells	48.7	101.8
3	Supernatant fluid from experiment 1 + lysozyme (0.5 mg/ml)	150.0	–
4	Supernatant fluid from experiment 2 + lysozyme (0.5 mg/ml)	48.7	–
5	Supernatant fluid from experiment 3 + 0.2 M Mg^{2+}-washed cells	150.0	1.3
6	Supernatant fluid from experiment 4 + 20% sucrose-washed cells	0	147.4
7	0.2 M Mg^{2+} wash of cells from experiment 5	1.3	0
8	0.2 M Mg^{2+} wash of cells from experiment 6	147.0	1.3
9	Cell-free extract	145.0	–

[a] Twenty milliliters of a 14-hr culture [480 mg (dry weight) of a culture grown to 1.3 optical density units] was centrifuged and resuspended in 20 ml of the washing solutions as indicated for 10 min. Suspensions from each experiment were centrifuged and the supernatant fluids were assayed for alkaline phosphatase activity. The cell pellets obtained were resuspended into 0.01 M Tris, pH 8.4, and subjected to sonic disintegration to determine the alkaline phosphatase remaining in the cells. Experiments 1 and 2 were performed in duplicate to obtain the appropriately treated cells used in experiments 4 and 6. Similarly, experiments 5 and 6 were performed in duplicate to obtain the cell pellets used in experiments 7 and 8. A dash (–) indicates that the test was not performed.

[b] Units of enzyme per 25 g (dry weight) of cell culture.

cytoplasmic membrane or cytoplasmic marker enzymes such as NADH oxidase or G-6-P dehydrogenase, a result consistent with that found after whole cell washing with 0.2 M $MgCl_2$ (4). In addition, phase microscopic examinations and internal enzyme release suggest that cells treated by the above procedures and diluted into 0.01 M $MgCl_2$ yield spheroplasts which are stable for at least 24 hr.

Spheroplasts of *P. aeruginosa* prepared by suspending cells in 20% sucrose and 0.5 mg of lysozyme per ml appear to be similar to those prepared after suspension in 0.2 M $MgCl_2$. Neither internal nor membrane marker enzymes are released during the spheroplast formation step.
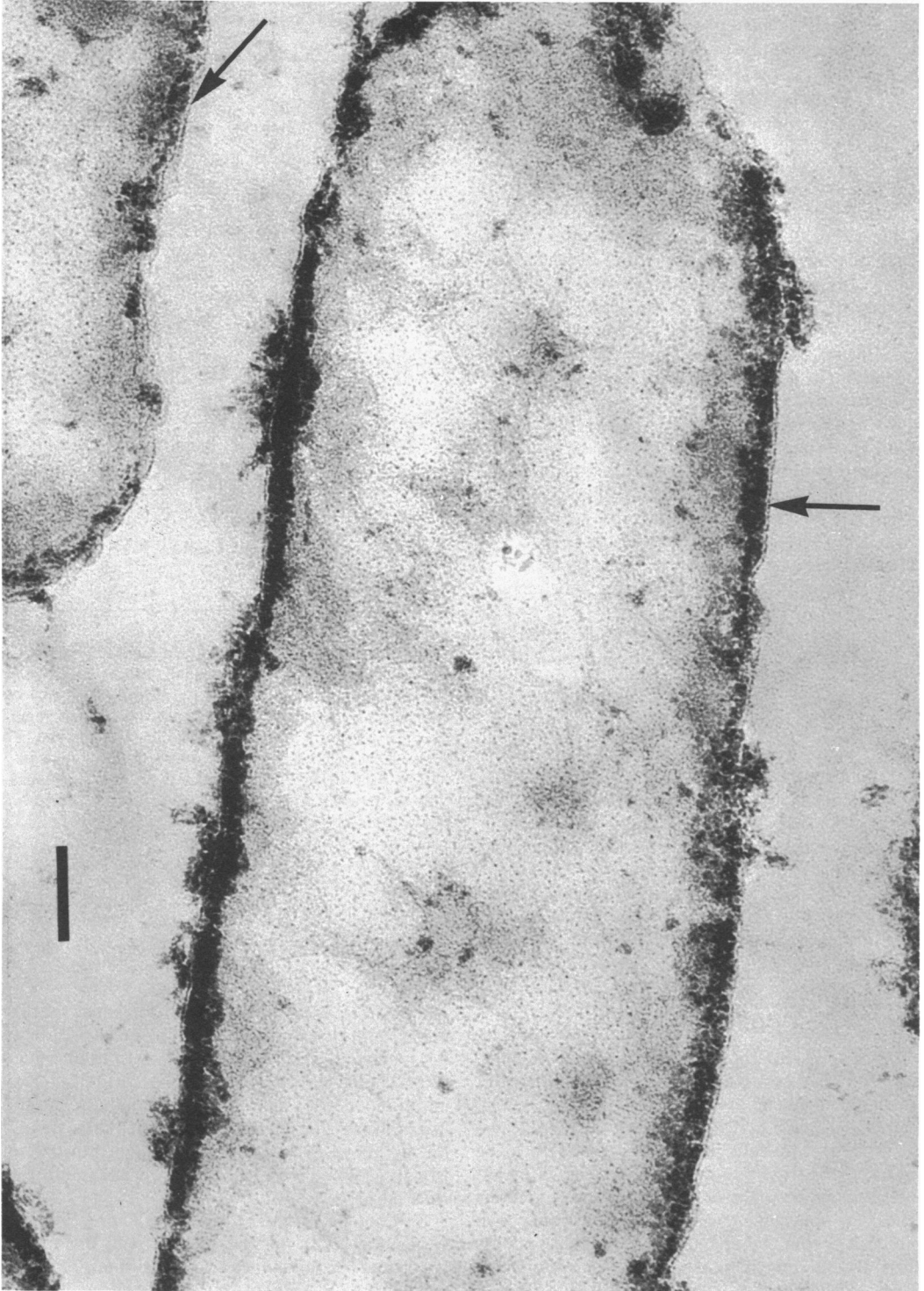
Fig. 2. *Electron micrograph of a thin section of cells grown at pH 6.8 and subjected to the Gomori reaction for the localization of alkaline phosphatase. Note the heavy deposition of lead phosphate inside the tripartite layer of the cell wall (arrows). ×139,000. Bar represents 0.1 μm.*
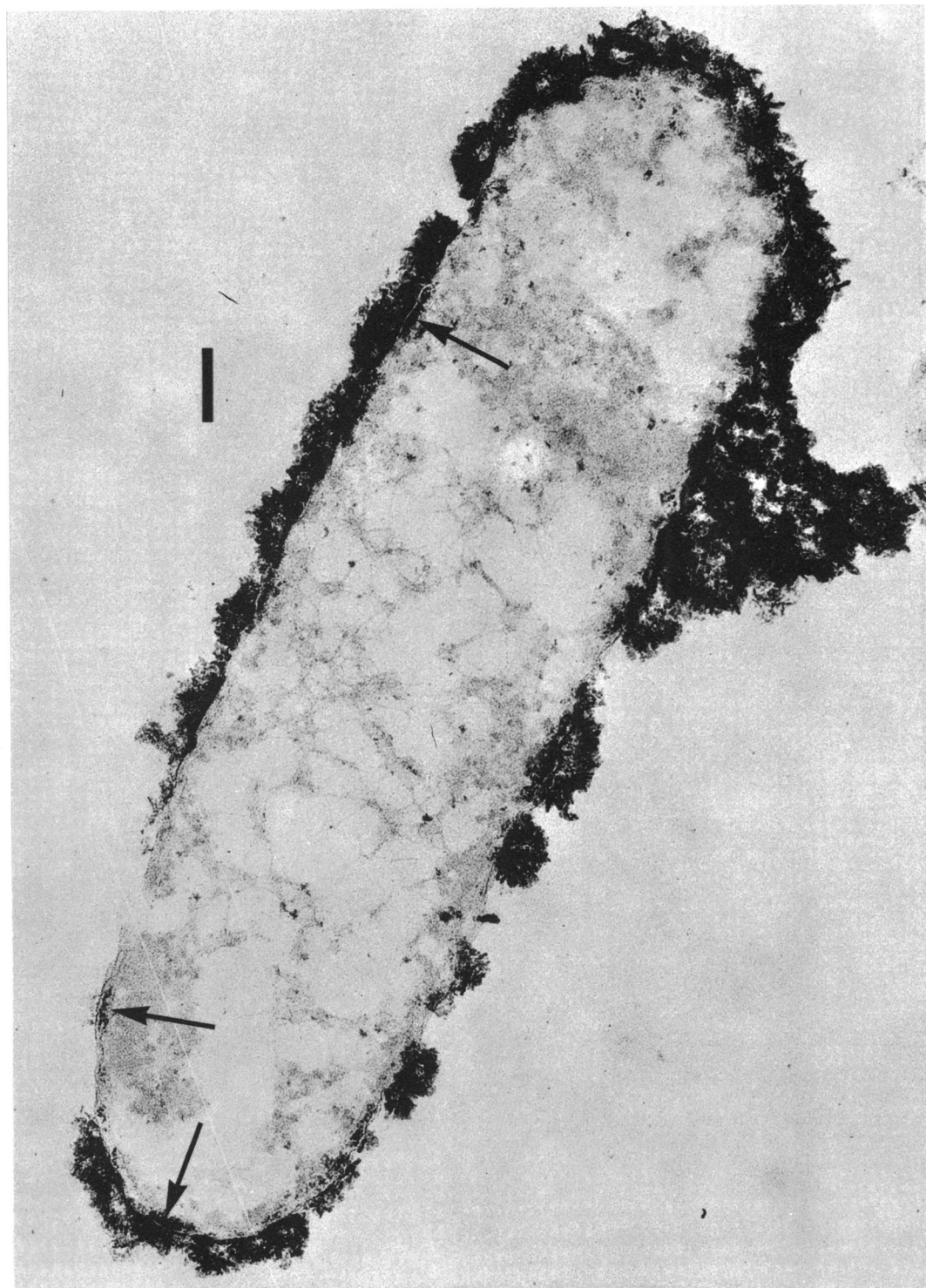
FIG. 3. *Electron micrograph of a sucrose-lysozyme treated cell which was subjected to the Gomori reaction. Note the dense deposits of lead phosphate lying inside the tripartite layer of the cell wall (arrows) and large, loosely organized deposits at the cell surface.* ×112,000. *Bar represents 0.1 μm.*
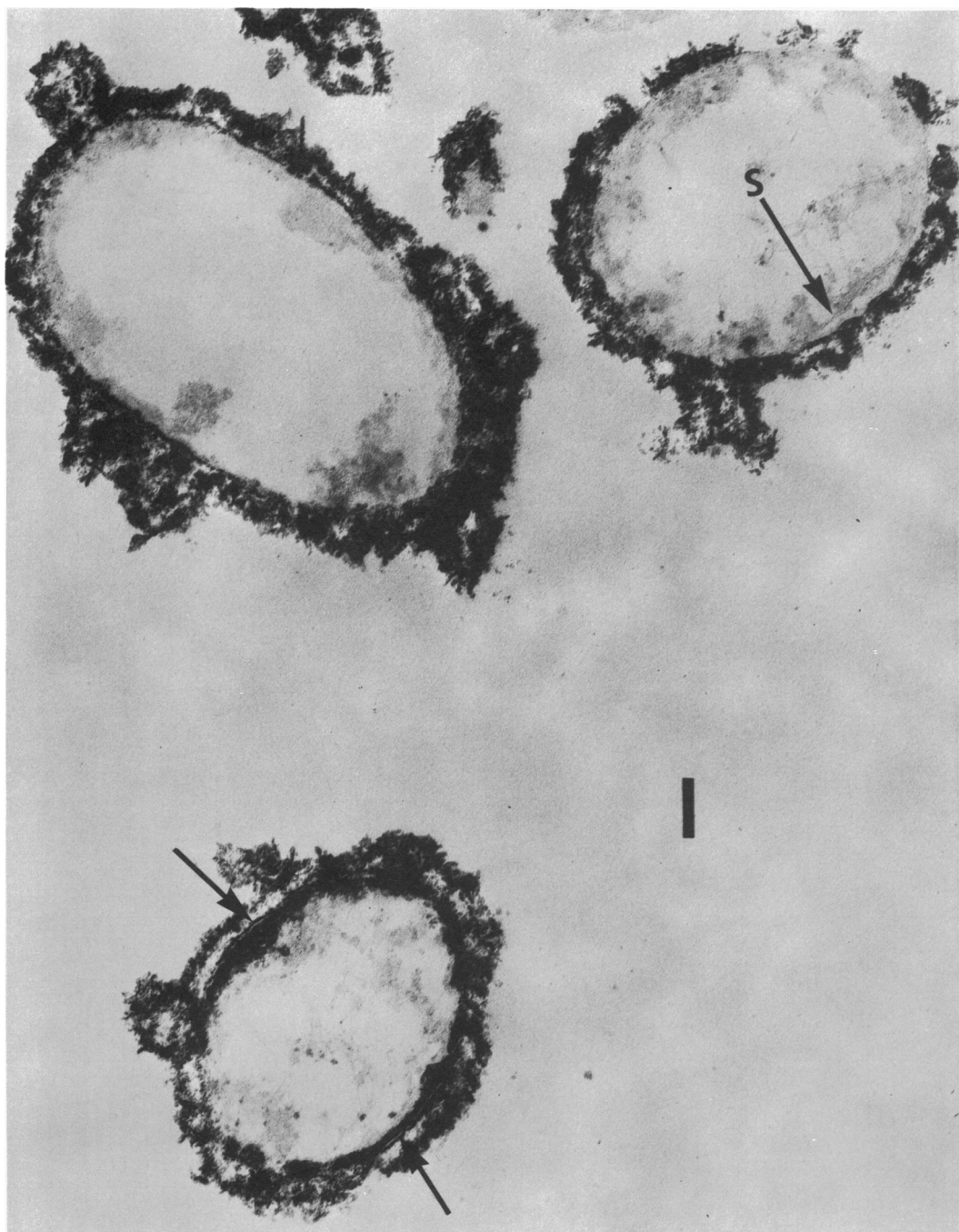
FIG. 4. *Electron micrograph of sucrose-lysozyme spheroplasts which were subjected to the Gomori reaction. Note the inner dense layer of lead phosphate which clearly lies inside the tripartite layer of the cell wall (arrows) and the loosely organized deposits on the surface of the cells and on the aggregates of cellular debris. In the area (S) where the cytoplasmic membrane is separated from the cell wall, the enzyme activity is clearly associated with the wall alone. ×84,000. Bar represents 0.1 μm.*
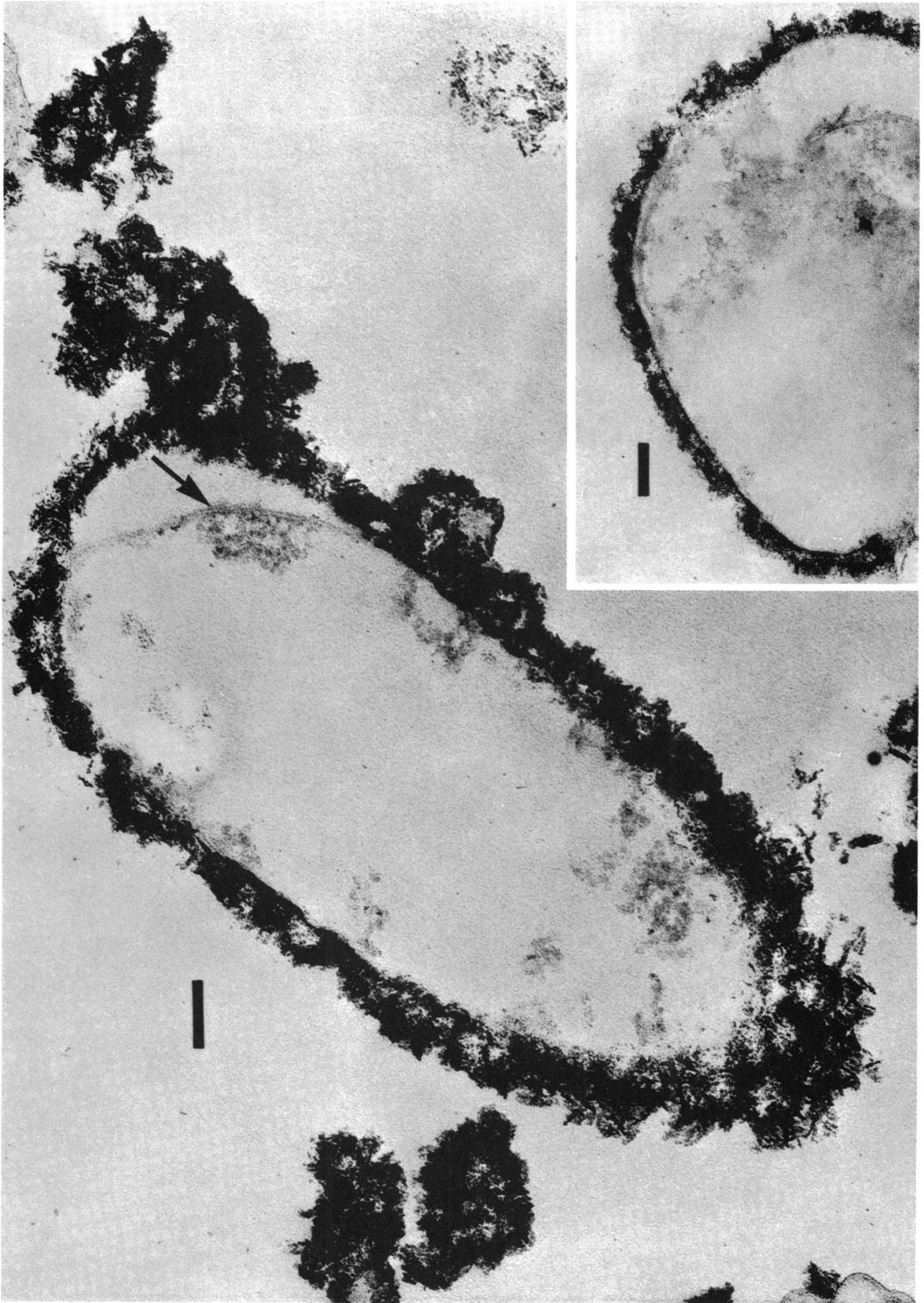
FIG. 5. *Electron micrograph of a sucrose-lysozyme treated cell which was subjected to the Gomori reaction. The cytoplasmic membrane is clearly resolved (arrow) in this plasmolyzed cell and the enzyme activity is associated only with the cell wall. Inset shows that both the inner dense deposit of lead phosphate and the outer, loosely organized deposit have remained associated with a fragment of the cell envelope even though the cytoplasmic membrane has been essentially lost.* ×102,600. *Bar represents 0.1 μm.*
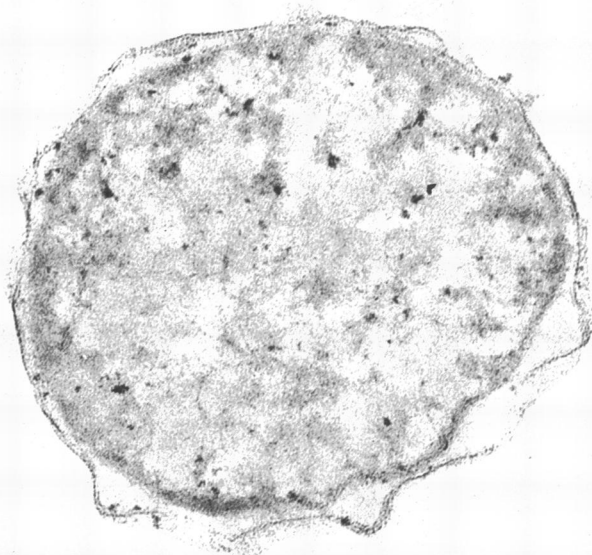
FIG. 6. *Electron micrograph of sucrose-0.01 M Mg²⁺ lysozyme spheroplasts which were subjected to the Gomori reaction. Note that both inner dense and outer, loosely organized deposits of lead phosphate are formed and that both of these deposits are clearly associated with the cell wall when this structure is separated from the cytoplasmic membrane.* ×75,000. *Bar represents 0.1 μm.*

FIG. 7. *Electron micrograph of a sucrose-lysozyme spheroplast which was washed in 0.2 M Mg²⁺ and subjected to the Gomori reaction. Note the absence of enzyme activity and the distortion of the cell wall.* ×111,500. *Bar represents 0.1 μm.*

Based upon the small, significant release of G-6-P dehydrogenase from the sucrose-lysozyme preparations, however, it appears that these spheroplasts are more fragile than those obtained by suspension in 0.2 M MgCl$_2$.

From the results obtained in this study, apparently lysozyme mediates a reassociation of alkaline phosphatase with the cell wall of *P. aeruginosa*. The electron micrographs suggest that the reassociated alkaline phosphatase is confined to the outer cell wall layers, whereas enzyme which is not disssociated by 20% sucrose remains in the periplasmic space. Since the reassociated alkaline phosphatase appears as nonuniform, loosely bound fragments attached to the outer cell wall layers and since periplasm-localized enzyme within the spheroplasts appears uniform, it is concluded that neither lysozyme nor spheroplast formation releases enzyme from the periplasmic space.

The involvement of lysozyme as a direct or mediating binding agent has been described in other systems. Lysozyme is known to bind ribonucleic acid (12), deoxyribonucleic acid (2), the cell surface of *E. coli* (15), membranes and ribosomes of *E. coli* (11), and liposomes (14). It has also been suggested that this enzyme binds to the cell surface of *P. aeruginosa* (6). From these discussions, it appears that the reassociation of released alkaline phosphatase with 20% sucrose-washed cells of *P. aeruginosa* is mediated by lysozyme and that the binding may be to the lipopolysaccharide or lipoprotein components of the cell wall. This latter conclusion is strengthened by the fact that even cell debris reassociates with the phosphatase in the presence of lysozyme. The possibility of cross reactivity between *E. coli* alkaline phosphatase and *P. aeruginosa* cell wall, as mediated by lysozyme, is currently under investigation.

Although previous studies with *P. aeruginosa* (3) and *E. coli* (10) have shown that alkaline phosphatase is a periplasmic enzyme that is located between the cytoplasmic membrane and the outer tripartite layer, it is not clear at present whether the enzyme is bound to the cytoplasmic membrane, or the peptidoglycan layer, and projects outwardly to the periplasm or whether it is bound to the tripartite layer and inwardly projects into the periplasm. The fact that sucrose-lysozyme spheroplasts of *P. aeruginosa* still contain uniformly located periplasmic alkaline phosphatase as already mentioned suggests that, in this organism, at least, the peptidoglycan is not responsible for phosphatase binding. Even more important is the fact that in cells treated for a short time with sucrose and lysozyme (Fig.
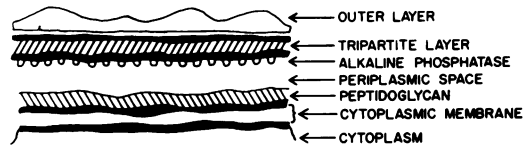

FIG. 8. *Diagrammatic representation of alkaline phosphatase location within the periplasmic space of P. aeruginosa. The peptidoglycan layer is illustrated next to the cytoplasmic membrane for convenience only, since its true location has not been established in this organism.*

5), sucrose-lysozyme spheroplasts (Fig. 4 "S"), sucrose-0.01 M Mg^{2+}-lysozyme spheroplasts which show enhanced separations of the cytoplasmic membrane from the tripartite layer (Fig. 6), or sucrose-plasmolyzed cells (Fig. 5, inset), unreleased phosphatase is not associated with the cytoplasmic membrane but rather with the internal region of the tripartite layer. Although not entirely ruled out by the discussions presented here, the remote possibility exists that lysozyme itself reassociates periplasmic alkaline phosphatase with the internal region of the tripartite layer of the cell wall. However, based upon considerations such as the uniform distribution of undissociated periplasmic alkaline phosphatase in sucrose-lysozyme spheroplasts, the complete absence of alkaline phosphatase from the cytoplasmic membrane, and the translocation of alkaline phosphatase from its periplasmic location to an external cell wall location (3) after glutaraldehyde fixation of untreated cells, it is concluded that the possibility of a lysozyme-mediated binding and translocation of alkaline phosphatase to the inner tripartite layer is unlikely. It is proposed, therefore, that in untreated cells, periplasmic alkaline phosphatase is not bound by the cytoplasmic membrane or the peptidoglycan layer but rather by the internal region of the tripartite layer. The proposed model for the specific location of alkaline phosphatase within the periplasmic space of *P. aeruginosa* cells is illustrated in Fig. 8.

Studies on the forces responsible for the binding to this layer as well as the actual component of the layer involved in binding phosphatase are currently under investigation.

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