

Cytochemistry of Phosphatases in *Myxococcus xanthus*

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An Mg^{2+} -dependent and a K^+ -stimulated adenosine triphosphatase were localized by cytochemistry at or near both surfaces of the cytoplasmic membrane of *Myxococcus xanthus*. An alkaline and an acid phosphatase resided at the external surface of the membrane or in the periplasm. All enzymes could be extracted from partially fixed cells with Mg^{2+} -deficient buffers. Suboptimal external phosphate elicited dissociation of adenosine triphosphatase from the membrane but not that of the unspecific phosphatases. The dissociated enzymes migrated into the cytoplasm where they were associated mainly with cytoplasmic aggregates.

Enzymes which hydrolyze adenosine triphosphate (ATP) are generally reported to reside at the envelopes of bacteria (3-5, 7, 11, 13, 18, 28). Adenosine triphosphatase is associated with the cytoplasmic membrane; it can be solubilized by sonic treatment of cells, multiple washings of protoplasts or isolated membranes with solutions deficient of multivalent cations, or by treatment of cells with lysozyme (1, 2, 12, 17, 19, 21). This enzyme may become soluble in vivo and migrate into the cytoplasm, depending on the stage of development (26) or differences in the general physiological conditions of the cell (20). The observation that the enzyme can be released from the membrane either into the exterior or interior of the cell has led us to investigate whether, in bacteria, adenosine triphosphatase activity and, for comparison, alkaline and acid phosphatases are located at the external or internal layer of the membrane and whether there is evidence for the hypothesis that some phosphatases are located within the membrane.

A variant of *Myxococcus xanthus* was used in which release and migration of the adenosine triphosphatase activity into the cytoplasm, but not that of the unspecific phosphatases, could be induced by suboptimal external phosphate. All three enzymes could be extracted from glutaraldehyde fixed cells with Mg^{2+} -deficient buffers. The results suggest that both an Mg^{2+} -dependent and a K^+ -stimulated, ouabain-sensitive adenosine triphosphatase are located within the membrane, whereas the unspecific phosphatases reside either at the external surface of the membrane or within

the compartment between membrane and cell wall.

MATERIALS AND METHODS

Organism. *M. xanthus* FB was used throughout this investigation. This variant strain, not further characterized by its physiological properties, differs from the original strain (8) in that the phosphate concentration for optimal growth was 5.0×10^{-2} M instead of 1.0×10^{-3} M for the original strain (27).

Determination of enzyme activity. Determinations of adenosine triphosphatase activity and alkaline and acid phosphatase were made alike on (i) whole cells, (ii) broken cells, and (iii) isolated cell fractions. Adenosine triphosphatase activity was determined according to the method of Abrams et al. (3). Since the cells or cell fragments tend to clump, giving inconsistent results when used in smaller quantities, determinations were made in triplicate on 5.0-ml suspensions. All samples were adjusted to approximately 0.5 mg of N per ml of suspension. All determinations were made after incubation of the crude enzyme preparations (whole or broken cells, or cell fragments) in the substrate, consisting of 5.0 mM ATP (Sigma Chemical Co., St. Louis, Mo.), 5.0 mM $MgCl_2$, with or without 1.2 mM KCl, in 0.2 M tris (hydroxymethyl)aminomethane (Tris)-chloride at 37°C for 30 min to 2 hr. The pH of the buffer was adjusted to 7.4 for the K^+ -stimulated and to 9.2 for the Mg^{2+} -dependent adenosine triphosphatase, optimal for each (*unpublished data*). Liberated inorganic phosphate was determined by the method of Fiske and Subbarow (9) and measured at 660 nm against the reagent blank. The readings from the controls (incubation mixture without substrate) were deducted from the readings of the experiments, and the data obtained were calculated from a standard curve. Alkaline and acid phosphatases were determined in 5.0-ml cell or cell fragment suspensions by the method of Torriani (24). The substrate was 0.04 M *p*-nitrophenol phosphate (104, Sigma Chemical Co.)

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in 0.2 M acetate buffer at pH 4.5 or in 0.2 M Tris-Mg buffer at pH 10.0. The amount of liberated *p*-nitrophenol was determined at 420 nm after incubation for 30 min at 37 C. Interval readings were plotted against time, and activity was calculated from the slope of the linear curve obtained. Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as the standard.

Fractionation of cells. Merthiolate-killed cells, washed twice with 0.02 M Tris-Mg, were suspended to one-fourth of the original volume in the buffer. The cells were broken by freezing and thawing six times (-10 to 37 C). Deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.) was added to a final concentration of 100 μ g/g (wet weight) of broken cells and was maintained at 4 C for 5 min. The broken cells were then washed twice with 0.02 M Tris-Mg by centrifugation at 105,000 \times *g* at 0 C for 4 hr to insure complete sedimentation of all cell fragments. Large cell envelope fragments were obtained by centrifugation of broken cells at 8,500 \times *g* for 30 min at 0 C; smaller fragments, by centrifugation of the 8,500 \times *g* supernatant fluid at 20,000 \times *g* for 30 min at 0 C. The sediments were pooled, washed twice in the buffer by centrifugation at 10,000 \times *g* for 30 min at 0 C, suspended in the original volume, and tested for phosphatase activity. The 20,000 \times *g* supernatant fraction, containing the cytoplasmic fraction, was tested for enzyme activity without further treatment.

Electron microscopy and cytochemistry. Cultures in their respective exponential phase were harvested by centrifugation. The cells or isolated wall membrane fragments were fixed for 30 min in Tris-Mg buffered 1% glutaraldehyde (50% w/w, biological grade, Fisher Scientific Co., Pittsburgh, Pa.) at pH 7.2 in the cold. The Ryter and Kellenberger technique (23) was followed for fixation with OsO₄. The specimens were stained with 0.5% uranyl acetate in the Ryter and Kellenberger buffer, dehydrated in a series of graded alcohol, and embedded in Epon 812. Sections were cut on a Porter-Blum model MT 2 microtome with DuPont diamond knives, mounted on carbon films, and observed in a JEM-T6S or in an RCA EMU-3G electron microscope. For localization of adenosine triphosphatase activity, cells or wall membrane fractions were washed five times with cold Tris-Mg after fixation with glutaraldehyde. For localization of the K⁺-stimulated adenosine triphosphatase, the substrate mixture contained 5 mM ATP, 0.2 M Tris-maleate, 1.2 mM KCl, and 0.5 mM PbNO₃, added in that order with stirring. The pH was adjusted to 7.2 with KOH. In preliminary experiments, various concentrations of ethylenediaminetetraacetic acid, up to 2.0 mM, known to prevent nonenzymatic hydrolysis of ATP by PbNO₃ (16), were added to the mixture but were unnecessary at a pH lower than 7.5 as long as the PbNO₃ concentration was maintained at 0.5 mM. Incubation was for 30 min to 2 hr at 37 C. The control mixtures contained in addition 10 mM NaCN, or the substrate (ATP) was omitted.

The Mg²⁺-dependent adenosine triphosphatase was localized at pH 8.5 with a slightly modified calcium method of Padykula and Herman (22).

The substrate mixture was composed of 5 mM ATP, 0.2 M Tris-chloride, 10 mM sodium barbital, 5 mM MgCl₂, and 0.4 mM Ca(NO₃)₂. After incubation for 20 min at 37 C, the specimens were treated with 2% (w/v) aqueous Co(NO₃)₂ for 3 to 5 min at room temperature, followed by a 1-min treatment with freshly prepared 1% yellow ammonium sulfide. The specimens then were washed once or twice in Tris-Mg, fixed in OsO₄, and processed for electron microscopy.

The procedure of Done et al. (6) using calcium as capturing ion was followed for localization of alkaline phosphatase. The only modification was the use of a mixture (1:3) of α - and β -sodium glycerophosphate as substrate (controls without substrate). Acid phosphatase was localized by incubating cells in a mixture of (1:3) α - and β -sodium glycerophosphate as substrate at 37 C for 30 min. The substrate mixture contained 0.2 M Tris-maleate, 5 mM MgCl₂, and 2.5 mM PbNO₃, adjusted with HCl to pH 5.2 (controls without substrate). Posttreatment and processing of the specimens for electron microscopy were as described above.

Incubation mixtures used for localizing phosphatase activity were freshly prepared and filtered before use. Specimens were dispersed in the incubation mixture. The chemicals used in all experiments were of analytical grade.

RESULTS

Cytochemistry of whole cells. Cells, used for the cytochemical experiments, were taken from cultures of their respective midexponential phase, regardless of their growth pattern under suboptimal conditions. In cells grown at the optimal phosphate concentration of 5.0×10^{-2} M, lead phosphate precipitate, indicative of adenosine triphosphatase activity, was found in the periplasm, i.e., the compartment between cell wall and membrane (Fig. 1). The precipitate was discontinuous without any regular pattern of distribution. The same location and scattered distribution of adenosine triphosphatase activity in serial sections were observed when the calcium phosphate method was used (Fig. 2). No increased amounts of precipitates were noticed at any particular portions of the cell, e.g., division plane or cell poles. Activity was exclusively in the periplasm when the cells were tested for alkaline or acid phosphatase.

The structure of the lead phosphate deposits was of some interest. Although apparently amorphous in longitudinal or cross sections through cells, in oblique sections the deposits were often cut through their sites at the membrane, revealing structural details (Fig. 3). A centrally located dense "nucleus," about 7 nm in diameter, is surrounded by a less dense halo, which is limited by a dense outer circle. The outer circle seems to

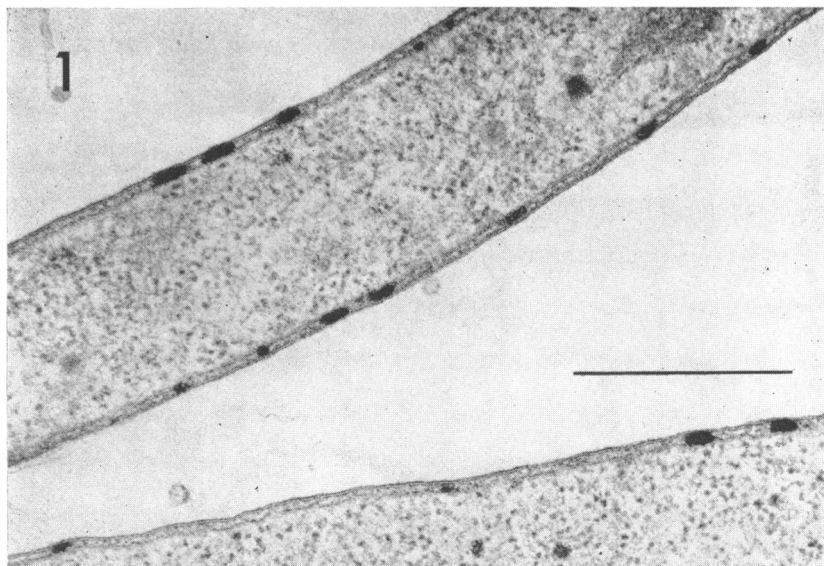


FIG. 1. Site of K^+ -stimulated adenosine triphosphatase in cells grown in 5.0×10^{-2} M phosphate. Lead phosphate precipitate is located in the compartment between cell wall and membrane. Scale marker $0.5 \mu\text{m} \times 57,500$

be connected with the dense "nucleus" by radial spokes.

No difference in the site of the two adenosine triphosphatases were found. Both the Mg^{2+} -dependent and the K^+ -stimulated adenosine triphosphatases were apparently located at the membrane. All following experiments were thus carried out with lead as capturing ion only.

Cytochemistry of isolated cell envelopes. Cells were lysed by freezing and thawing six times, and the wall membrane complex was, after separation from the cytoplasm, incubated in the enzyme substrate (ATP or sodium glycerophosphate). Stabilization of the enzymes at the membrane was achieved with 0.02 M Mg^{2+} in Tris, in which the cells were washed and lysed. The lead phosphate precipitate was mainly bound to the membrane but not to the wall (Fig. 4). This was particularly apparent at places where the gap between wall and membrane was artificially widened during the procedure for isolation of the cell envelopes. Activity was observed on both surfaces of the membrane despite some distortion of the wall membrane fragments as a result of the preparative procedures. The number of precipitates per unit length of the membrane was markedly increased compared with that of precipitates in undisrupted cells. Under the same preparational conditions, both alkaline and acid phosphatase remained in the periplasm (Fig. 5); depicted is an envelope fragment in which calcium phosphate (alkaline phosphatase) is partially adhering to the wall and to the membrane.

In vivo migration of enzymes. Suboptimal phosphate concentration in the growth medium (5.0×10^{-4} M) elicited release of adenosine triphosphatase activity from the membrane (Fig. 6). No activity was excreted into the growth medium or released into the washing fluid during preparation of the specimen for electron microscopy. Most of the released enzymes were apparently concentrated at cytoplasmic aggregates which were concomitantly formed with the migration of the enzymes during growth of cells in suboptimal phosphate concentrations. Under these conditions, alkaline and acid phosphatases were not dissociated from the membrane (Fig. 7). This observation was confirmed by determining phosphatase activity of separated cell envelopes and cytoplasm of cells grown in various phosphate concentrations. The dissociation of adenosine triphosphatase from the membrane was directly proportional to decreasing amounts of external phosphate, whereas the activity of the unspecific phosphatases remained primarily membrane bound (Fig. 8).

Extraction of enzymes. Cells grown at optimal phosphate concentration were processed for cytochemistry in the usual manner, but Mg^{2+} was omitted from the buffer used for washing the cells after fixation in glutaraldehyde. When washed three times with Mg^{2+} -deficient buffer, adenosine triphosphatase activity was present partially in the periplasm and partially adhering to the surface layer of the cell wall (Fig. 9). Five washings removed all of the activity from the

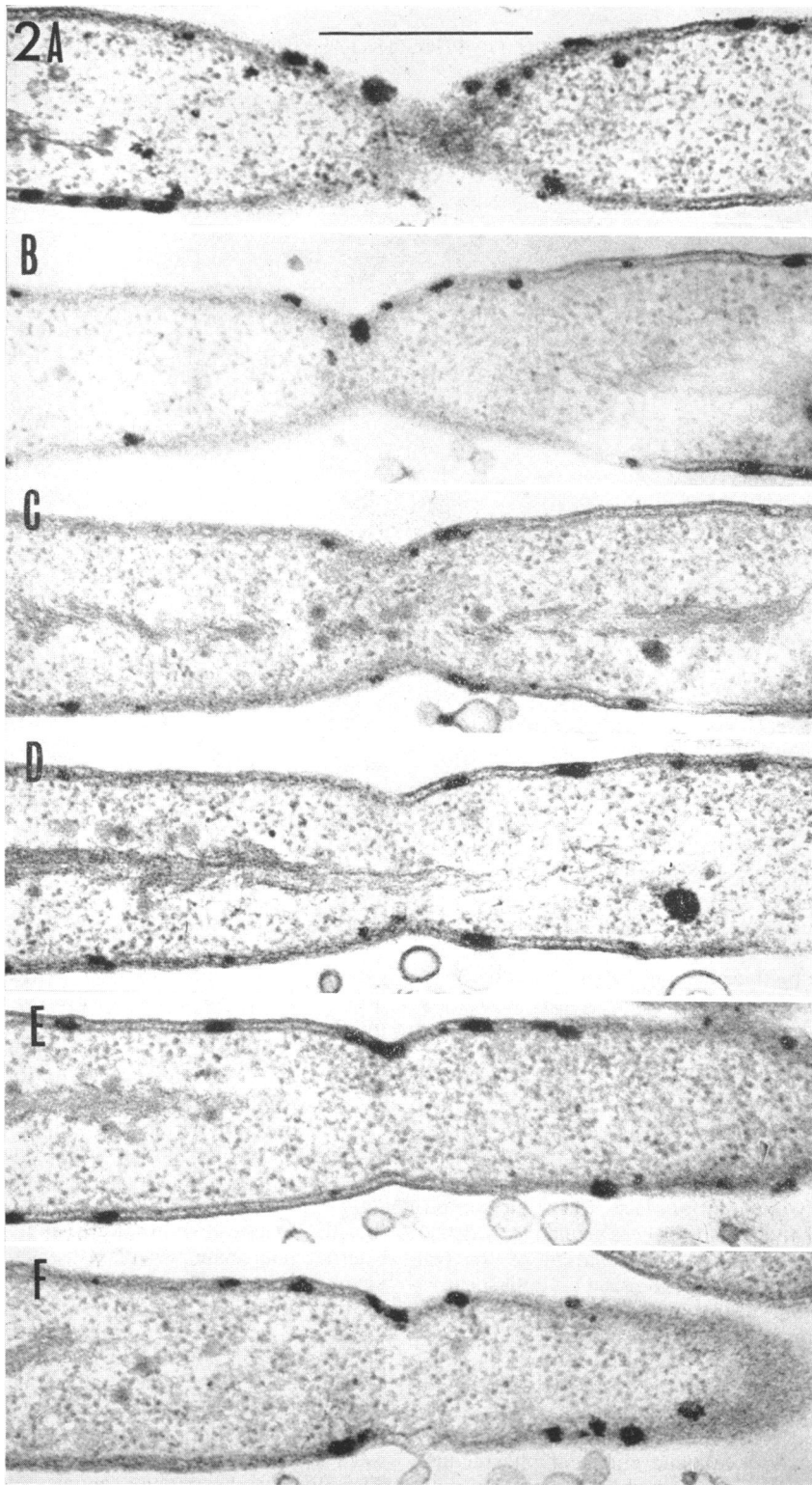


FIG. 2. Site of Mg^{2+} -dependent adenosine triphosphatase activity in a series of sections through a dividing cell. Calcium phosphate precipitate in the compartment between wall and membrane. Number of precipitates not enhanced at division plane. Scale marker $0.5 \mu m \times 57,500$.

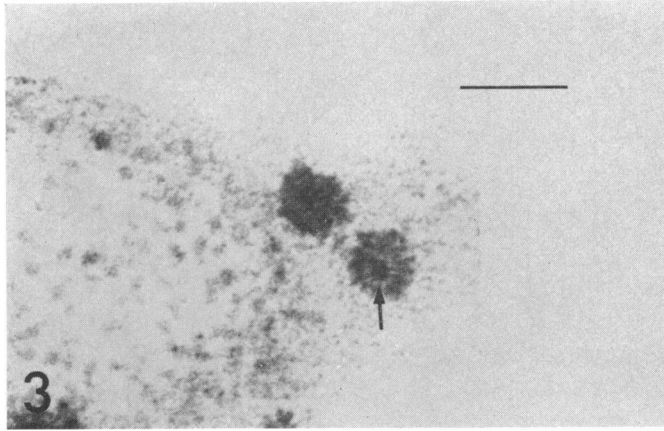


FIG. 3. Oblique section through a portion of a cell with adenosine triphosphatase activity. Precipitated lead phosphate apparently cut at site of its deposition at the surface of the membrane (arrow). Scale marker $0.1 \mu\text{m} \times 138,000$.

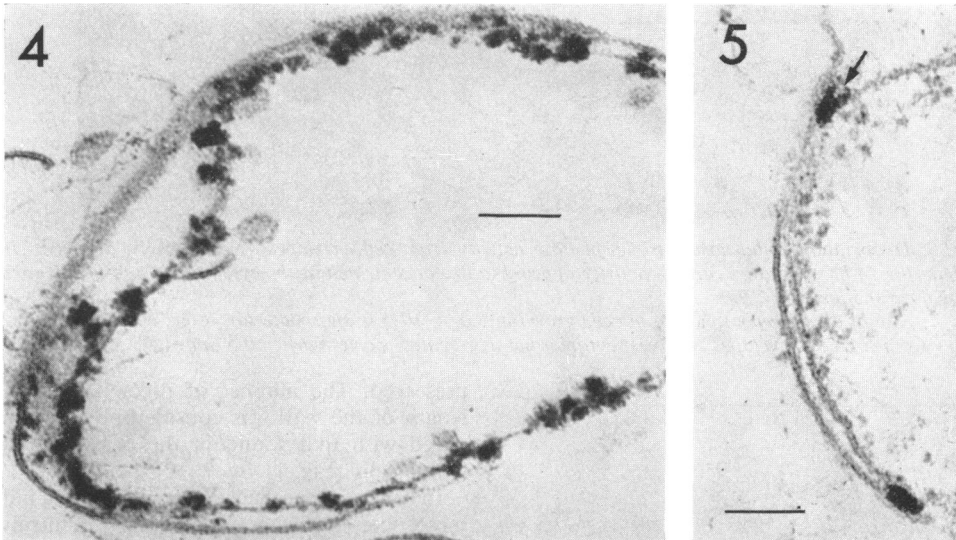


FIG. 4. Isolated wall membrane fraction with adenosine triphosphatase activity. Enzyme activity at both surfaces of the membrane. No precipitate adhering to the wall. Scale marker $0.1 \mu\text{m} \times 103,500$.

FIG. 5. Isolated wall membrane fraction with alkaline phosphatase activity. At separation of the wall from the membrane, calcium phosphate precipitate appears to be split, partially adhering to the wall and to the membrane (arrow). Scale marker $0.1 \mu\text{m} \times 105,000$.

membrane. Precipitates exclusively adhered then to the external layer of the wall (Fig. 10). Essentially the same observation was made for alkaline and acid phosphatase. The dislodged enzymes apparently were trapped within or at the cell wall, and the enzymatically liberated phosphate was precipitated at the surface, since little activity was detectable in the washing fluids. After eight washings, the enzymes were quantitatively solu-

bilized. No lead or calcium phosphate was deposited within the cells or at the cell surfaces. There seemed to be a loss of structural integrity of the membrane, and the normally well-defined triple-layered structure appeared to have collapsed. In some areas, the width of the membrane was reduced to about one-half. The inner layer stained much less than the outer one, and the three outer layers of the cell wall were fairly well

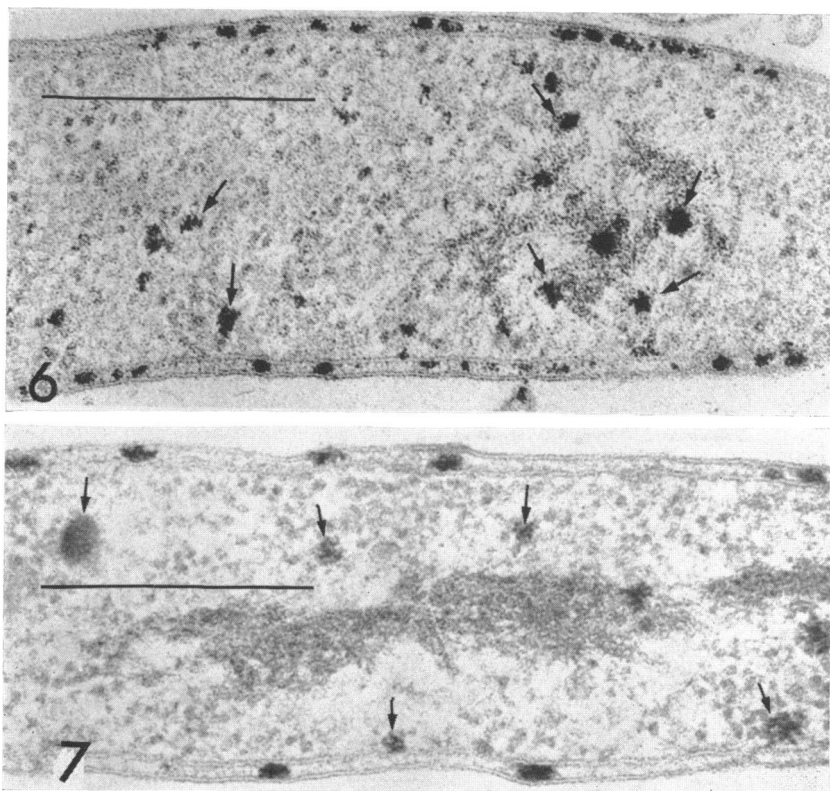


FIG. 6. Dissociation of adenosine triphosphatase activity from cell envelopes of cells grown at 5.0×10^{-4} M phosphate for 24 hr. Migrated enzyme activity appears to be concentrated at the cytoplasmic aggregates (arrows). Scale marker $0.5 \mu\text{m} \times 72,300$.

FIG. 7. Site of alkaline phosphatase in cells grown in 5.0×10^{-4} M phosphate for 48 hr. No dissociation of the enzymes from the cell envelopes. Arrows, cytoplasmic aggregates. Scale marker $0.5 \mu\text{m} \times 72,300$.

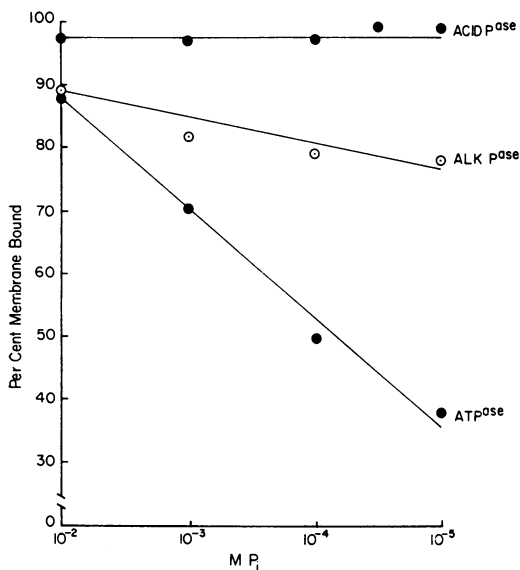


FIG. 8. Dissociation of phosphatase activity from cell envelopes of cells grown in various phosphate concentrations.

preserved. The number of precipitates per unit length of the wall was considerably higher compared with that found in the periplasm of the whole cells (Fig. 1).

In cells of a parallel culture, containing induced mesosomes (25), activity remained firmly attached to the involuted membrane (Fig. 11, 12). No precipitate was located in the periplasm, and very little adhered to the cell surface. The involuted portion of the membrane seemed better preserved than the membrane itself. Precipitates were between the outer leaflets of juxtaposed segments of the involuted membrane (Fig. 11) or at the inner surface of a single membrane in the mesosome (Fig. 12).

DISCUSSION

Cytochemical evidence is presented that, under optimal growth conditions, both a K^+ -stimulated and an Mg^{2+} -dependent adenosine triphosphatase were associated with the cytoplasmic membrane. Lead or calcium precipitate, indicative of enzyme activity, was deposited externally to the membrane of undisrupted cells. Deposits were

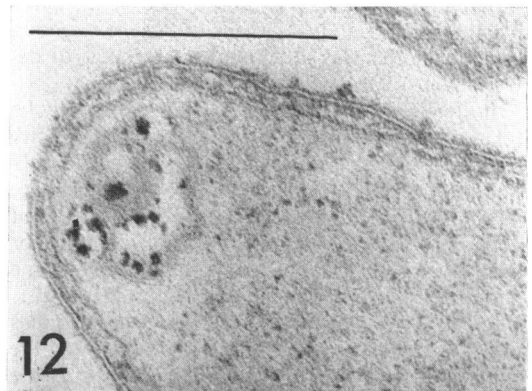
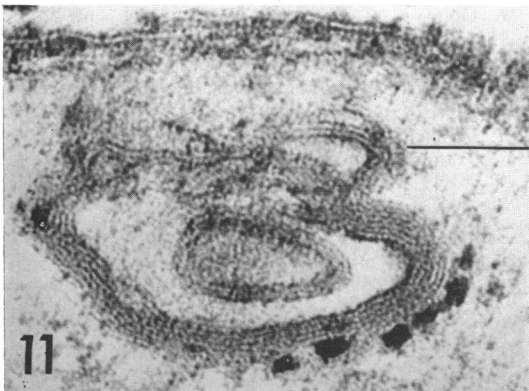
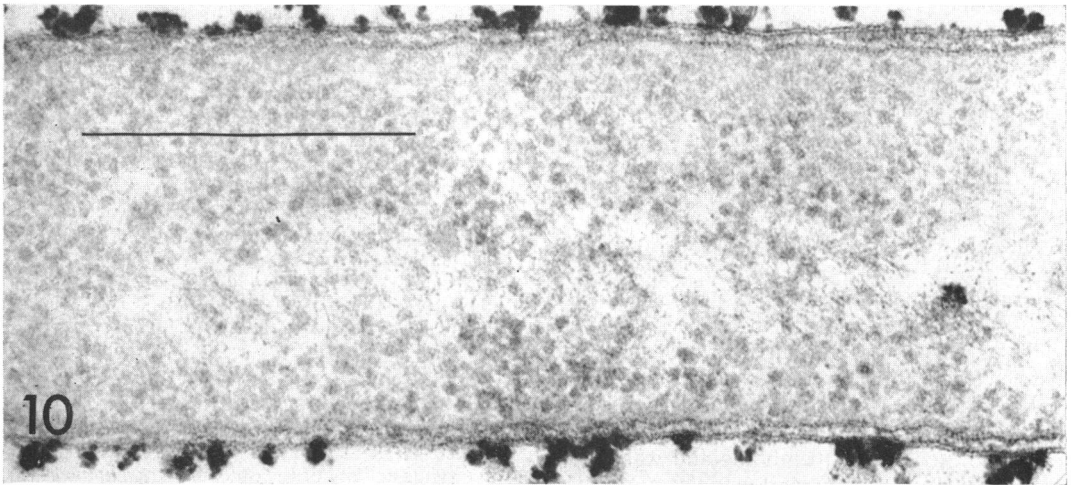
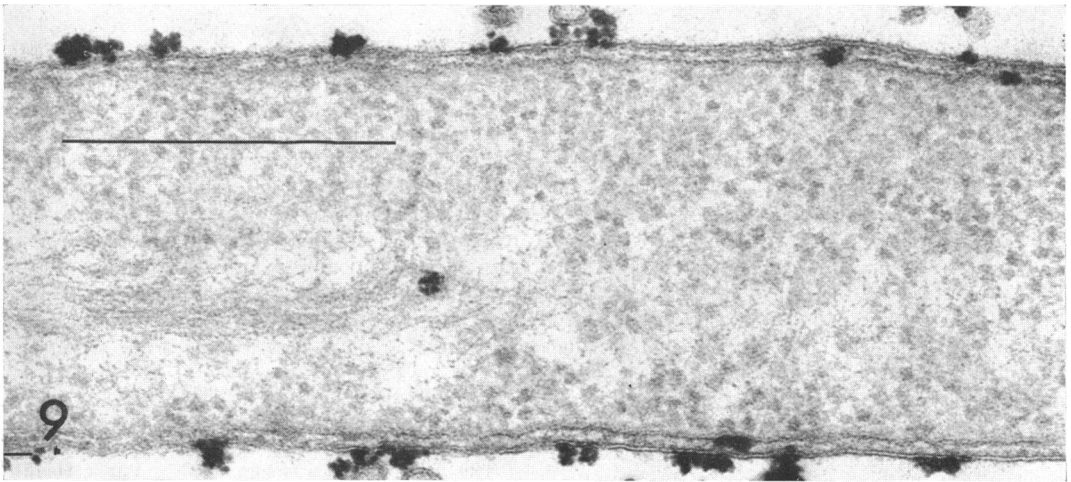


FIG. 9. Adenosine triphosphatase activity at pH 7.2 in cells grown in optimal phosphate concentrations, washed three times in Tris-chloride (no Mg^{2+}) after fixation in glutaraldehyde. Scale marker $0.5 \mu m \times 88,000$.

FIG. 10. Same as Fig. 9. Cells washed five times in Tris-chloride. Scale marker $0.5 \mu m \times 88,000$.

FIG. 11. Adenosine triphosphatase activity at pH 7.2. Precipitate at induced mesosomes of cells grown in optimal phosphate concentration, washed five times in Tris-chloride after fixation in glutaraldehyde. Scale marker $0.1 \mu m \times 160,000$.

FIG. 12. Same as Fig. 11. Activity at the site of the invaginated membrane, none in the periplasm. Scale marker $0.5 \mu m \times 80,000$.

at both membrane surfaces when the enzymes were localized in isolated cell envelopes. The alkaline and the acid phosphatases could be localized only externally to the membrane in either case. Precipitate even adhered partially to the wall at places where the gap between wall and membrane was artificially widened. Solely from these observations it could be conjectured that both adenosine triphosphatases were located at both membrane surfaces, whereas the alkaline and the acid phosphatases resided at the external membrane surface only or in the periplasm.

Until recently, it was believed that the adenosine triphosphatase in eucaryotic cells was located externally at surface membranes (10). Marchesi and Palade (16) recently demonstrated both an Mg^{2+} -adenosine triphosphatase and an Na^+ - K^+ -adenosine triphosphatase at the inner surface of erythrocyte ghost membranes. Adenosine triphosphatase activity was localized internally to the membrane of the wall-less *Mycoplasma gallisepticum*, whereas the acid phosphatase was not membrane associated (18). The alkaline phosphatase of *Escherichia coli* was found by Done et al. (6) to reside in the periplasm, whereas Spicer et al. (Federation Proc. 25:539, 1966) and Kushnarev and Smirnova (14) localized the enzyme in the cell wall. In the same species, enzymes which hydrolyze glucose-6-phosphate and uridine cyclic phosphate were located in the compartment between wall and membrane. We suggested (26) that adenosine triphosphatase in *E. coli* is located at the membrane and wall, in *B. cereus* at the membrane and cytoplasm, whereas in *M. xanthus* all activity was found in the cytoplasm. The variability in sites of enzymes among various species was attributed to the stage of development of the cells.

It appears then that the site of enzymes may be related not only to species or even strain specificity but also to the physiological state of the cell. Suboptimal concentrations of external phosphate in *M. xanthus*, e.g., resulted in a dissociation of adenosine triphosphatase, but not alkaline or acid phosphatase, from its site at the membrane and migration into the cytoplasm. It is not understood why, under such conditions, the enzymes were not secreted but were released into the cytoplasm. It is also not known whether only the enzyme molecules internal to the membrane migrated into the cytoplasm or whether those located externally permeated through the core lipids of the membrane after extended growth of cells in suboptimal phosphate. After 24 hr, the latter were still partially present at the external surface of the membrane but not after 48 hr of growth. The possibility cannot be excluded, therefore, that the adenosine triphosphatases are lo-

cated within the membrane rather than at its surfaces, whereas the alkaline and acid phosphatases may reside at the external surface of the membrane. Lead or calcium phosphate was then deposited at a distance from the enzyme. Unfortunately, the scarcity of information on the mode of deposition of enzymatically liberated phosphate and on permeation of enzyme molecules through partially fixed membranes limits further discussion on the relation of enzyme sites to membrane structure.

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