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Distribution of the Sites of Alkaline Phosphatase(s) Activity in Vegetative Cells of *Bacillus subtilis*

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Sites of alkaline phosphatase activity have been located by an electron microscopic histochemical (Gomori) technique in vegetative cells of a repressible strain SB15 of *Bacillus subtilis*, derepressed and repressed by inorganic phosphate, and in a mutant SB1004 which forms alkaline phosphatase in a medium high in phosphate. The sites of enzyme activity were revealed as discrete, dense, and largely spherical bodies of varying sizes (20 to 150 nm). Cells of both repressible and repression-resistant strains acted on a wide variety of phosphate esters (*p*-nitrophenylphosphate, β-glycerophosphate, adenosine-5'-phosphate, glucose-6-phosphate, glucose-1-phosphate, adenosine triphosphate, and sodium pyrophosphate) to produce inorganic phosphorus under conditions of alkaline phosphatase assay [0.05 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.4) containing 2 mM $MgCl_2$]. The purified alkaline phosphatase also acted on all these esters, although much less effectively on adenosine triphosphate and sodium pyrophosphate than did the cells. Comparison of the relative utilization of the various substrates by repressed and derepressed cells and purified enzyme suggested the presence of multiple enzymes in the cells. Thus, the cytochemical method of trapping the newly generated inorganic phosphorus determines the location of an alkaline phosphatase of broad substrate profile, and in addition locates the sites of other enzymes generating inorganic phosphorus under identical conditions of assay. It is intriguing that all of these enzymes usually exist in a few clusters attached to the peripheral plasma membrane. In addition to this predominant location, there were a few sites of enzyme activity in the cytoplasm unattached to any discernible structure, and also in the cell wall of the repression-resistant and of the derepressed, repressible strains.

Groups of enzymes in eucaryotic cells are frequently compartmentalized into characteristic membrane-bound organelles. Although morphologically identifiable subcellular organelles are rare in procaryotic cells, there might be functionally differentiated areas in the cell which behave like compartments. It has been shown by theoretical treatment that compartmentation is needed for efficient and economic functioning of enzyme systems of even a bacterial cell (15). Significant evidence might be obtained to support this idea of subcellular compartmentalization by studying whether the enzymes inside the bacterial cell are randomly distributed or whether there are localized, isolated sites for individual groups of enzymes. Keeping this general concept in mind, we attempted to localize some enzymes by electron microscopic histochemistry.

We chose alkaline phosphatase because the histochemical procedures for locating phosphatases are well-established and the phosphatases (phosphoric monoester hydrolases EC 3.1.3.1) catalyze the hydrolysis or transphosphorylation of a variety of orthophosphoric monoesters, and consequently participate in diverse physiological functions.

Although various phosphatases have been located in gram-negative organisms (especially *Escherichia coli*) by histochemical methods (5, 9, 13, 14, 20, 21), such studies in gram-positive bacilli are rare (23). We examined the distribution of alkaline phosphatase in gram-positive bacilli for the following reasons. (i) The biochemical properties of alkaline phosphatase have been investigated in the strains under investigation (19).

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(ii) It is already known from the studies on disrupted cells that the enzyme is particle bound (19). (iii) The enzyme can be effectively repressed by inorganic phosphorus (19). (iv) There is a possibility of distribution of this enzyme in different membrane material, i.e., peripheral plasma membrane, mesosomal membrane, or forespore membrane (B. K. Ghosh, J. T. M. Wouters, and J. O. Lampen, Bacteriol. Proc., p. 19, 1970). (v) The alkaline phosphatase of *Bacillus subtilis* can act on a variety of substrates (4).

In this communication, we report that alkaline phosphatase(s) of exponentially growing vegetative cells of *B. subtilis* is located in discrete clusters bound to the peripheral plasma membrane. It is suggested that the clusters of enzyme activity are composed of alkaline phosphatase(s) and other enzyme(s) involved in the biochemical reaction of inorganic orthophosphate (P_i) generation.

MATERIALS AND METHODS

Organism. Two strains of *B. subtilis* were used: the alkaline phosphatase repressible Marburg strain SB15 and its repression-resistant mutant strain SB1004, kindly supplied by A. Tsugita, Osaka University, Japan. These were maintained on the sporulation medium described previously (6).

The growth media used in this investigation are modified from the medium described by Takeda and Tsugita (19). The basal nutritive solution contained peptone (Difco), 10 g; sodium lactate, 10 g; tris(hydroxymethyl)aminomethane (Tris), 6 g; sodium chloride, 3 g; manganese sulfate, 50 mg; and 1 liter of water. The level of inorganic phosphate of this solution was reduced by treatment with magnesia (19). After removal of the precipitate formed by magnesia treatment and autoclaving, the final *p*H was 6.8. This solution was supplemented with 1.2×10^{-4} M magnesium sulfate and 0.9×10^{-4} M calcium chloride which had been autoclaved separately. This low phosphate (LP) medium contained 0.02 to 0.03 mM P_i [determined by the method of Bartlett (1)]. The high phosphate medium (HP) received 10 mM disodium hydrogen phosphate.

Inocula were prepared by growing a loopful of spores from the agar slopes in 5 ml of HP medium in a 50-ml Erlenmeyer flask shaken at 200 rev/min on a model 5860 gyrotory shaker (New Brunswick Scientific Co.) at 30 C for 12 hr. The culture was centrifuged, the cells were washed in LP medium, and the entire amount was used to inoculate 50 ml of HP or LP medium. Cells were harvested by centrifugation (after 2 to 6 hr of growth), washed in 0.05 M Tris buffer (*p*H 8.4) containing 2 mM $MgCl_2$ (Tris-Mg), and used for the experiments. All quantities of cells are given as the dry-weight equivalent.

Assay of alkaline phosphatase. The assay was done in 5 ml of Tris-Mg buffer with 0.06 mM *p*-nitrophenyl-phosphate (PNPP) as substrate and containing 0.06 mg of cells. The mixture was incubated at 37 C for 30 min, and the reaction was stopped by the addition of 1 ml of 0.8 M dibasic potassium phosphate. The contents were centrifuged for 10 min at 10,000 × *g* and absorbance of the supernatant fluid was measured at 410 nm in a model 300 Gilford spectrophotometer against a substrate blank. The results are expressed as nanomoles of *p*-nitrophenol produced per milligram of cells.

Inorganic phosphorus, liberated from different substrates, was determined by the method of Bartlett (1). The incubation mixture was identical to that described above, but the reaction was stopped by the addition of 1 ml of 2 N perchloric acid. The net amount of P_i liberated was the difference between the values obtained with and without the addition of cell suspension.

Histochemical procedure. The method of Mölbert (11) was used with minor modifications. The Tris-Mg buffer was supplemented with 30 mM potassium sodium tartrate (Tris-Mg-T) and 3 mM lead nitrate. The latter was added to the buffer just before use in a dropwise manner with continuous stirring to prevent precipitation of lead. Cloudy solutions seriously interfere with the test by forming unspecific deposits on the cell surface.

Electron microscopy. The cells from the histochemical test mixture containing Tris-Mg-T buffer, 0.06 mM PNPP and 3 mM $Pb(NO_3)_2$ were centrifuged at 10,000 × *g* for 10 min, washed twice with Tris-Mg buffer (*p*H 7.0) and prefixed with 3% glutaraldehyde in Tris-Mg buffer (*p*H 7.0) for 15 min at 37 C. These cells were washed once in 0.1 M Veronal-acetate buffer, *p*H 6.2 (8), and further fixed, embedded, and sectioned as described earlier (6), except that the cells before dehydration were washed in 30 and 50% alcohol containing 0.1% sodium chloride. The thin sections were stained with Reynold's lead citrate (16) and examined at 80 kv in a JEM 120 electron microscope (JEOL, Medford, Mass.) with a 30-μm objective aperture.

Purification of alkaline phosphatase. Alkaline phosphatase from the derepressed cells of strain SB15 was isolated by a modification of the procedure of Takeda and Tsugita (19). The purity of this enzyme has not been completely established. This enzyme elutes from a diethylaminoethyl (DEAE)-Sephadex column as a sharp, single peak. The protein present in this peak runs as a single but somewhat broad band in polyacrylamide disc-gel electrophoresis (17). The material from the DEAE-Sephadex column was concentrated and precipitated by dialysis against magnesium-free buffer (19). The precipitate, dissolved in Tris-acetate buffer (*p*H 7.0) containing 0.2 M $MgCl_2$, was the source of the purified enzyme (cf. Tables 1 and 2). The enzyme was purified 15-fold from the crude extract, and a 10% recovery was usually obtained. Detailed work on the purification and subunit constitution of the enzyme is in progress (A. Ghosh, *unpublished data*).

RESULTS

Factors in the histochemical test influencing alkaline phosphatase activity. If components of the histochemical assay medium produce gross inhibition of the alkaline phosphatase, some of the significant enzyme sites in the cell may not be detected. For example, we observed 50 to 80%

TABLE 1. *Liberation of inorganic orthophosphate (P_i) from various phosphate esters by cells and by the purified alkaline phosphatase of Bacillus subtilis*[a]

Source of alkaline phosphatase	P_i liberated[b] (μmoles per mg of cells per min)						
	PNPP	AMP	β-G-P	G-6-P	G-1-P	ATP	PY-P
Whole cells							
Strain SB15 (HP)	4	11	10	9	20	22	69
Strain SB15 (LP)	80	82	54	51	61	70	131
Strain SB1004 (HP)	18	14	15	15	26	29	148
Strain SB100r (LP)	49	32	42	43	77	80	129

[a] Reaction mixture contained 0.06 mg of washed cells in 5 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.4) containing 2 mM $MgCl_2$ and 0.06 mM substrate. The reaction was started with the addition of substrate and continued for 30 min at 37 C. The reaction was stopped by adding 1 ml of 2 N perchloric acid, and the mixture was centrifuged at 10,000 × *g* for 10 min. Inorganic phosphorus in the supernatant fluid was assayed by the method of Bartlett (1).

[b] Abbreviations: PNPP, *p*-nitrophenylphosphate; AMP, adenosine-5'-monophosphate; β-G-P, beta-glycerophosphate; G-6-P, glucose-6-phosphate; G-1-P, glucose-1-phosphate; ATP, adenosine triphosphate; PY-P, sodium pyrophosphate; HP, cells grown in high phosphate medium; LP, cells grown in low phosphate medium. For purified alkaline phosphatase, P_i-liberated values (micromoles per milligram of protein per minute) were: PNPP, 10,967; AMP, 11,183; β-G-P, 8,581; G-6-P, 7,516; G-1-P, 6,451; ATP, 3,226; PY-P, 1,074.

TABLE 2. *Liberation of inorganic orthophosphate (P_i) from various phosphate esters by cells and by the purified alkaline phosphatase of Bacillus subtilis*

Source of alkaline phosphatase	Ratio of the cleavage of test substrates to PNPP[a]						
	PNPP	AMP	β-G-P	G-6-P	G-1-P	ATP	PY-P
Purified enzyme	1.0	1.0	0.8	0.7	0.6	0.3	0.1
Whole cells							
Strain SB15 (HP)	1.0	2.5	2.2	2.0	4.7	5.0	16.0
Strain SB15 (LP)	1.0	1.0	0.7	0.6	0.7	0.9	1.6
Strain SB1004 (LP)	1.0	0.7	0.9	0.9	1.6	1.6	2.7
Strain SB1004 (HP)	1.0	0.8	0.8	0.8	1.4	1.6	8.1

[a] For abbreviations and description of reaction, see footnotes to Table 1.

inhibition of alkaline phosphatase activity on prolonged exposure to buffer containing lead salts. By using a low lead concentration (3 mM) and minimizing the time of exposure (present only during assay), this inhibition could be reduced to 20 to 30% (B. K. Ghosh, *unpublished data*).

Published literature (10) on the cytochemical demonstration of enzymes indicates that glutaraldehyde prefixation does not significantly inhibit the enzymes. Contrary to this general finding, the alkaline phosphatase activity of *B. subtilis* was reduced 60 to 90% by short treatment with 0.1 to 3% glutaraldehyde even at 4 C. Comparable inhibitions of *E. coli* 5'-nucleotide phosphatase (14) have been reported. For this reason, we fixed the cells with glutaraldehyde only after the histochemical reaction had been carried out.

Comparison of alkaline phosphatase activity of repressed and derepressed cells in histochemical and biochemical assay conditions. Incubation for 30 min with substrate (PNPP) was used instead of the usual 90 min or more (9, 22) because longer incubation caused autolytic disorganization of the cell structure. It is evident from Fig. 1 that the activity of derepressed cells is linear with time in both biochemical and histochemical reaction mixtures, but the low activity present in repressed cells shows only an initial linearity and falls after 15 to 20 min. The cause of this drop of activity cannot be explained at present. Repressed cells show 40% and derepressed cells 60% activity in the histochemical assay medium compared to that in the biochemical reaction mixture (Fig. 1). The activity of strain SB15 cells grown in HP medium is only about 6% of that found in cells grown in LP medium. In contrast, cells of the repression-resistant mutant SB1004 grown in HP medium showed about 40% of the enzyme activity present in the cells grown in LP medium (Tables 1 and 2).

Variation of PNPP concentration influenced the enzyme reaction in a complex manner. Concentrations higher than 0.15 mM could not be used in the assay because under these conditions the repressed cells exhibited a much greater ac-

tivity relative to that shown by derepressed cells, possibly due to nonspecific breakdown of the substrate.

Utilization of different substrates. Detection of alkaline phosphatase by histochemical test depends on the generation of orthophosphate which may result from the action of different enzymes on a variety of substrates under varying conditions. Consequently, it is helpful to know the extent of hydrolysis of various phosphate esters to liberate P_i under the conditions of histochemical assay of alkaline phosphatase. It is obvious from the results shown in Tables 1 and 2 that both repressed and derepressed cells can liberate P_i from a large variety of phosphate esters. The broad substrate profile of the cells might be due either to a single enzyme of low specificity or to different enzymes acting under the assay conditions. The purified alkaline phosphatase from derepressed cells of strain SB15 (Tables 1 and 2) clearly has a wide substrate spectrum, but, unlike the whole cells, the pure enzyme has low activity on adenosine triphosphate (ATP) and almost none on pyrophosphate.

The existence of multiple enzymes is indicated by the data presented in Table 1. (i) If the decrease of cleavage of any substrate is accepted as an index of repression of the enzyme acting on that substrate, there are significant differences in the repression for ATP, pyrophosphate or glucose-1-phosphate (48 to 70%), and for PNPP (95%). This strongly suggests the existence of enzymes less sensitive to repression by P_i than is the phosphatase acting on PNPP. (ii) The ratios (Table 1) of activity on the test substrates to that on PNPP for the pure enzyme and for whole cells varied significantly. For ATP and pyrophosphate, these ratios were 0.9 and 1.6 in derepressed cells against 0.3 and 0.1 with pure enzyme. These variations were not due to permeability restrictions, since the values obtained with lysed cells were comparable (data not presented in detail). It is probable, therefore, that the altered ratios obtained from whole cells are due to the presence of an additional enzyme(s) acting preferentially on those substrates.

In repressed cells of SB15, the ratio of activity on other phosphate esters to that on PNPP is higher than 2 (5 for ATP and 16 for pyrophosphate). These high values again indicate the presence of a relatively strong P_i-repressible enzyme acting preferentially on PNPP. It should also be noted that the ratios for the repression-resistant strain SB1004, grown in the presence or absence of P_i, are comparable to the values for derepressed cells of SB15 (the only appreciable difference is with pyrophosphate). Thus, repressed cells of SB15 probably have a different mixture of enzymes involved in their (slow) formation of orthophosphate than do the derepressed or repression-resistant cells. The obvious relevance to interpretation of the present results is that the repressed cells could not be expected to be totally free of enzyme sites in the histochemical test and, further, that the sites of enzyme activity are almost certainly due to multiple enzymes which act on diverse endogenous phosphate esters.

Semi-quantitative estimates of the distribution of alkaline phosphatase activity in the vegetative cells of B. subtilis. The sites of enzyme activity can be detected in thin sections as highly electron-dense deposits associated with different subcellular structures. The thin sections were prepared from cells incubated either in the presence or absence of exogenous substrates (e.g., PNPP). It is likely that the cells grown in these rich media have a substantial amount of substrate which could be utilized by the enzyme. Attempts to deplete endogenous substrates by incubating the cells in buffered saline caused gross autolytic damage.

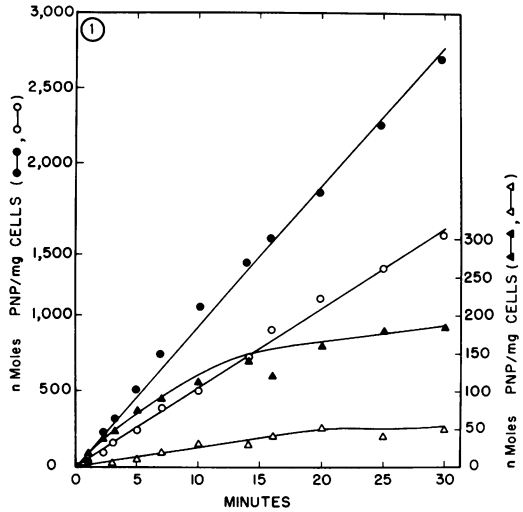

FIG. 1. *Rate of utilization of p-nitrophenylphosphate by repressed and derepressed cells of B. subtilis in the biochemical and histochemical reaction mixtures. The assay mixture contained 0.06 mg of washed cells in 5 ml of 0.05 M Tris buffer (pH 8.4), containing 2 mM $MgCl_2$ and 0.06 mM p-nitrophenylphosphate; 30 mM potassium sodium tartrate and 3 mM lead nitrate were also added for the histochemical test. The reaction was started by the addition of the substrate and stopped at the required time intervals with the addition of 1 ml of 0.8 M dibasic potassium phosphate. This mixture was centrifuged at 10,000 × g for 10 min, and the absorbance of the supernatant fluid was measured at 410 nm. Symbols: △, repressed cells in histochemical reaction mixture; ▲, repressed cells in biochemical assay medium; ○, derepressed cells in histochemical reaction mixture; ●, derepressed cells in biochemical assay medium.*

Alkaline phosphatase activity was found in the following locations: (i) in the cytoplasmic material but bound to peripheral plasma membrane; (ii) in the peripheral plasma membranes; (iii) in the cytoplasmic material but bound to mesosomal membranes; (iv) in the mesosomal membrane; (v) in the intramesosomal space; (vi) apparently free in the cytoplasm; (vii) in the periplasm, and (viii) in the cell wall. For an enzyme activity associated to such a variety of subcellular structures, conclusive information on the significance of an individual location can only be obtained if the frequency of association of the activity with individual structures is determined. The number of sites found in the different locations were counted in 200 longitudinal thin sections (100 nm thick) of vegetative cells without any forespore. Though theoretically it is possible to have two to three longitudinal sections of 100 nm thickness per bacterium, it is rare to obtain two longitudinal sections from the same bacterium due to randomness of the collection of sections. Thus, information from one longitudinal section is accepted as representative of one bacterium. Mesosomal structures appear with irregular frequency (i.e., sections may or may not have such structures); consequently, 100 individual mesosomes were counted and the percentage of such structures containing deposits was determined (see above).

Precise localization of the sites of enzyme activity depends on several factors. (i) Two serial sections were compared to ascertain the association of the deposit to a structure. (ii) When the distance of the center of a deposit from the membrane is less than its greatest diameter, it is considered to be membrane bound. (iii) Frequently, visualization of deposits is difficult due to apparently similar densities of the deposits and other cellular structures (e.g., cell wall or membrane) on the initial print of the micrograph. But reprinting the micrograph with a substantially reduced exposure time facilitated the detection of the highly dense deposits.

The distribution studies (Table 3) unequivocally show that in early exponential phase the phosphatases of both repressed and derepressed organisms are usually bound to the peripheral plasma membrane. There are also a considerable number of sites in the cytoplasm apparently free of any membrane. Although other locations are statistically insignificant, the loci in the cell wall and mesosomes might have physiological importance. Alkaline phosphatase activity in the cell wall was seen only in the derepressed cells (60% of the thin sections showed this). It is also remarkable that 60 to 70% of the mesosomes contained loci of enzyme activity, usually on the cytoplasmic surface of the mesosomal membrane (Fig. 10).

Morphological characteristic of the sites of enzyme activity. The accompanying electron micrographs (Fig. 2 to 13) illustrate typical sites of activity, as obtained from the survey of 200 to 500 thin sections of each strain. They are collected from derepressed, repressed, and repression-resistant cells, but do not signify any quantitative relationships with the different cell types.

Loci of enzyme activity in the cytoplasmic material attached to the peripheral membrane of repressed (Fig. 2 and 7) and derepressed (Fig. 4 to 6) cells could be detected in the presence of PNPP. The deposits, which form in the absence of exogenous substrate, are loose aggregates of small units; their electron densities are also lower than those formed in the presence of exogenous substrate (B. K. Ghosh and A. Ghosh, *unpublished data*). The sites of enzyme activity are discrete, approximately spherical with variable diameter, and randomly distributed (Fig. 6 and 7). The deposits in derepressed cells (Fig. 4 to 6) have a larger diameter (20 to 150 nm) than those in repressed cells 20 to 40 nm, (Fig. 2 and 7), and frequently have irregular profiles resulting from the aggregation of smaller deposits (Fig. 6 and 8, arrows). Further, many such deposits bind to the membrane at multiple points (Fig. 8).

The average number of deposits is two in repressed cells and four in derepressed cells; in both cases, there is a broad spread of distribution from 1 to 15 deposits per cell (B. K. Ghosh and A. Ghosh, Bacteriol. Proc., p. 31, 1971). A typical low-magnification view of a preparation of derepressed cells is given in Fig. 11. The sections similar to those shown by arrows have been used for counting. In Fig. 11, the number of deposits varies between one and five and most of them are associated to peripheral plasma membrane. We have also presented thin sections representing the lower (Fig. 2, 4, and 5) and higher (Fig. 6 and 7) frequencies. In both cases, most of the loci of enzyme activity are attached to the peripheral plasma membrane.

Figure 3 represents a control section from cells that were not treated with histochemical reaction mixture. It illustrates the intactness and similarity of the ultrastructure of the cells both with and without the histochemical procedure. A typical unstained section from preparations treated by the histochemical procedure but fixed only in 3% glutaraldehyde (no OsO_4 postfixation) shows the accumulation of electron-dense products due only to the enzyme activity (Fig. 5). The deposits are attached to the inner edge of the halo representing the peripheral plasma membrane.

Besides the predominant plasma membrane-

TABLE 3. *Percentage distribution of the sites of alkaline phosphatase activity in association with various subcellular structures of early log-phase Bacillus subtilis*

Strain	Distribution[a]						
	Pm.B	Cy	Me-M	Me-V	Pm	Per	CW
SB15 (LP)	65	19	4	2	0	0	10
SB15 (HP)	76	18	4	1	0	0	0
SB1004 (LP)	61	22	2	2	1	1	11

[a] Results are expressed as percentages of total enzyme sites associated with different subcellular structures in 200 longitudinal thin sections examined. Abbreviations: Pm.B, bound to peripheral membrane; Cy, free in cytoplasm; Me-M, bound to mesosome membrane; Me-V, inside the vesicle of mesosome; Pm, in plasma membrane; Per, in periplasmic space; CW, in cell wall; LP, cells grown in low phosphate medium; HP, cells grown in high phosphate medium.

bound sites, enzyme activity is found in association to other specific structures. The following describes some of their morphological features.

In a few cells, electron-dense material can be seen in the peripheral plasma membrane without appreciable accumulation of dense material into the cytoplasm. Some portions of the membrane were thickened (Fig. 13, boxed area) and isolated, small sites (10 nm) similar to those scattered in the cytoplasm (Fig. 13, arrows) could be seen in the peripheral plasma membrane.

Phosphatase activity of mesosomes is usually attached to the cytoplasmic surface of mesosomal membrane leaving the intramesosomal space free of deposits (Fig. 10, arrows). Occasionally, in derepressed cells, the sites of enzyme activity were also found in the mesosomal membrane and in the lumen of the mesosome (Fig. 9, arrow).

About 20% of deposits were randomly distributed in the ctyoplasm apparently unbound to any structure (Table 3). These sites can either be small (20 to 40 nm) and isolated from each other or a large cluster (Fig. 13, C) formed by the aggregation of smaller units.

Derepressed cells (especially LP cells of strain SB1004) demonstrated phosphatase activity in the wall (Fig. 12, arrows). The electron-dense deposits generally accumulate throughout the thickness of the wall and in the adjacent area of plasma membrane; but the cytoplasmic sites associated to peripheral plasma membrane were not found near these wall loci.

Loci of enzyme activity were rarely found in the periplasmic space of the vegetative cells. In the under-exposed prints, the region of the highest density of these sites could be seen in the periplasmic space, and, unlike usual membrane-associated loci, these deposits do not extend into the cytoplasm. In a subsequent publication, it will be shown that this space becomes a significant location of the enzyme activity in the early sporulating cells (B. K. Ghosh, *unpublished results*).

DISCUSSION

Danielli (3), Casselman (2), Moses and Rosenthal (12) have discussed different parameters of the cytochemical demonstration of phosphatases by phosphate precipitation as originally developed by Gomori (7) and Takamatsu (18). The basic method was adapted to electron microscopy by Mölbert et al. (11). From theoretical considerations, it has been concluded that the phosphate deposits form surrounding the location of enzyme inside the cell. Furthermore, according to Moses and Rosenthal (12), the mechanism of lead salt deposition does not appear to be an adsorption of lead phosphate to the tissue. It can be concluded that the technique followed in our study defines the sites of generation of orthophosphate and indirectly locates the enzyme(s) participating in the biochemical reaction forming this product. This statement can be applied to an individual system only if the conditions used for the histochemical test have been correlated with the biochemical assay. Another important point is that the histochemical procedure chiefly provides information on the location of the enzyme(s) and will detect only gross quantitative differences in the amount of enzyme present in the repressed and derepressed cells. Recently, Wetzel et al. (22) have demonstrated an excellent correlation of biochemical data and cytochemical localization of phosphatases in a study on a variety of strains of *E. coli*.

The purified alkaline phosphatase from strain SB15 has a broad substrate profile, although it is relatively inactive on ATP and pyrophosphate. The existence of multiple enzymes forming orthophosphate from a variety of substrates under the assay conditions is suggested from a comparison of the substrate profile of repressed and derepressed cells and of the purified enzyme. All of these enzymes are repressible by P_i, although the relative degree of repression varies widely. It is intriguing that these enzymes, singly or collectively, exist in clusters usually bound to the pe-

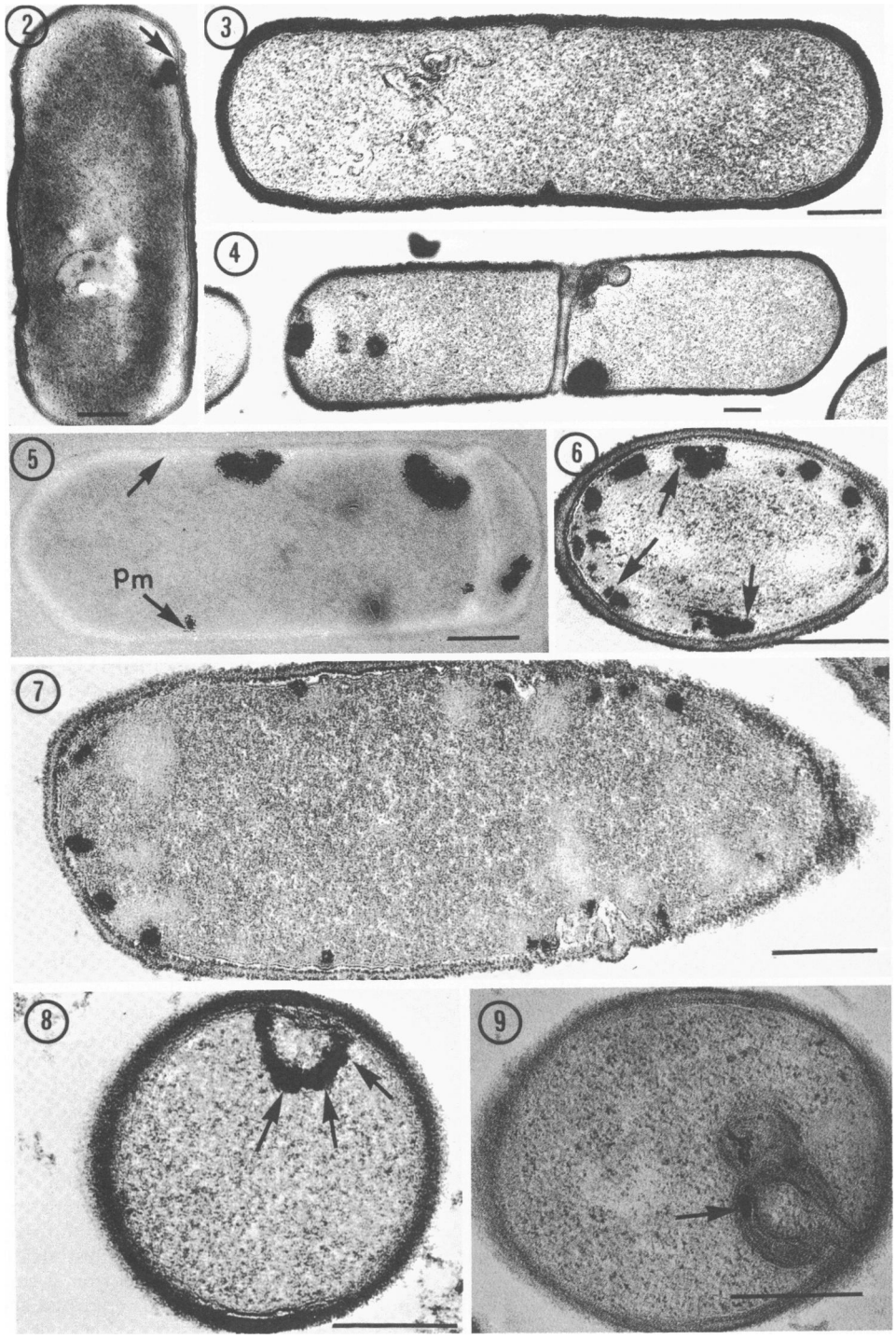

FIG. 2–9

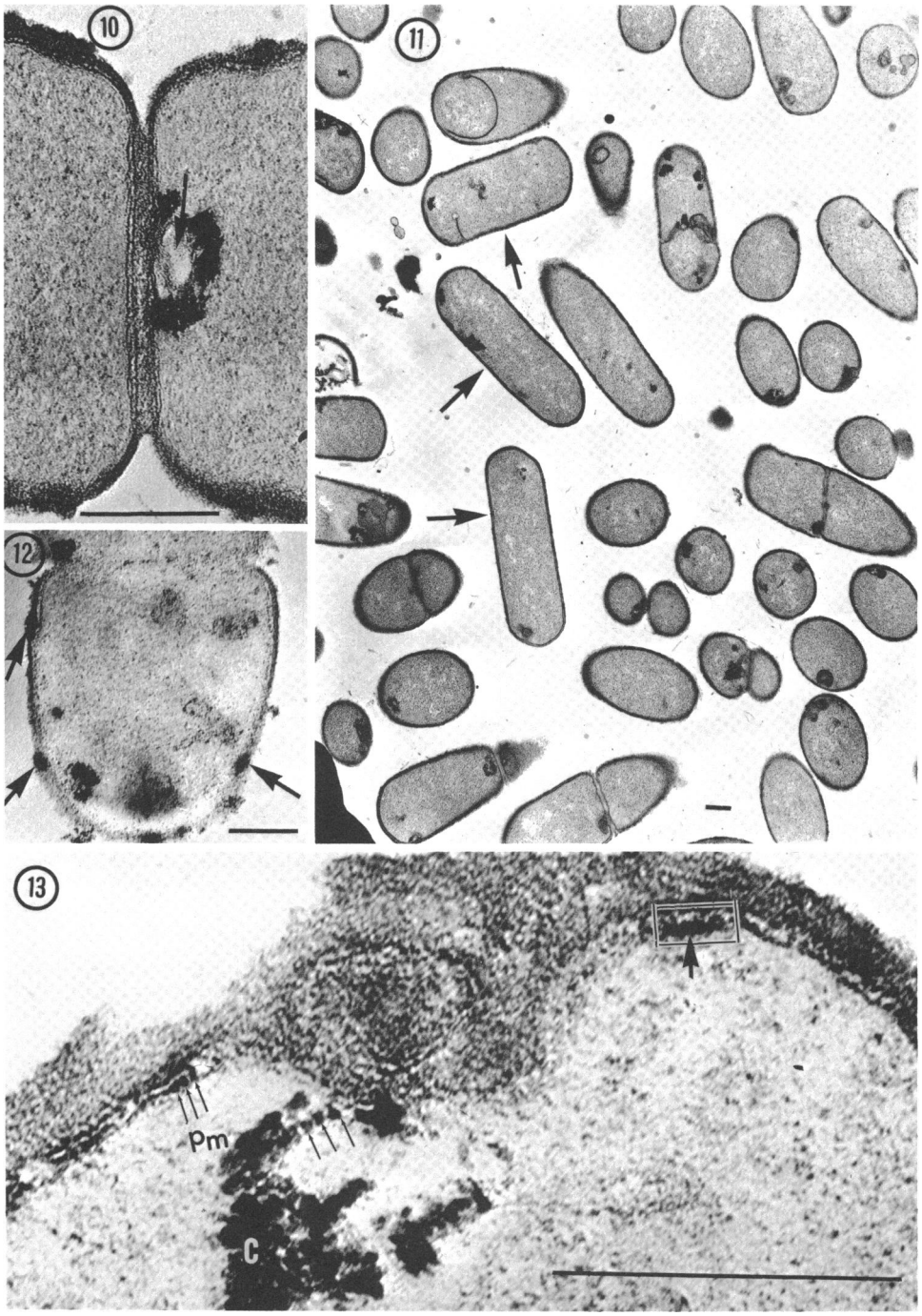

FIG. 10–13

ripheral plasma membrane of log-phase (vegetative) cells. Though these sites of enzyme activity are bound to the plasma membrane, they are rarely a part of the membrane structure. Preliminary experiments suggest that they are composed of groups of polyribosomes. The portions of the membrane associated with the clusters may either be specific binding sites or be the point of synthesis of the enzyme(s). The simplest explanation is that these clusters are organized synthetic units, but, in fact, the available data do not enable a choice to be made between these and perhaps other explanations. Nevertheless, it should be noted that evidence for electrostatic binding of alkaline phosphatase to the plasma membrane of *B. subtilis* has been obtained (23). The membrane-bound alkaline phosphatase from the protoplast membrane of cells of strain SB1004 grown in LP medium could be extracted with concentrated salt solutions (J. T. M. Wouters, *unpublished data*), suggesting electrostatic binding of the enzyme to the membrane.

Fortuitous binding of the enzyme-containing clusters to the membrane can be discounted since the number of sites per individual cell did not vary significantly; furthermore, an appreciable shift in the distribution of sites was seen at later periods of growth.

Since loci of enzyme activity are present in both repressed and derepressed cells, the enzyme-forming sites must function under both conditions, but probably differ widely in the amounts of enzyme(s) produced. The larger size of individual sites in derepressed cells may account for the higher amounts of enzyme formed. A detailed statistical analysis of the apparent volume of the clusters in repressed, derepressed, and repression-resistant organisms is in progress (B. K. Ghosh and A. Ghosh, Bacteriol. Proc., p. 31, 1971).

The results presented in this paper show the need for (at least) semiquantitative studies to evaluate histochemical data. Although the phosphatase(s) can be located in diverse cellular structures, about two-thirds of the sites were attached to the peripheral plasma membrane (Table 3). Despite the fact that they occur much less frequently, the phosphatase clusters bound to the cytoplasmic surface of the mesosomal membrane warrant further investigation. Finally, the obvious mosaic distribution of the alkaline phosphatase in the cell suggests the possibility of functional compartmentation of an individual type of enzyme.

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FIG. 2–9. *Repressed cells of strain SB15; such low number of deposits per section represents majority of population; an underexposed print of this micrograph showed the association of deposit to the inner surface of peripheral plasma membrane but not to the wall (arrow). ×35,000. The marker in this and subsequent pictures indicates 0.2 μm unless otherwise shown on the micrograph.* FIG. 3. *Control repressed (SB15) cell; not incubated in the histochemical reaction mixture. × 53,000.* FIG. 4. *Derepressed cell (SB15) with low number of deposits representative of the majority of the population; note the deposits are larger than those of repressed cells (Fig. 2). ×28,000.* FIG. 5. *This unstained section (SB15) was obtained from cells incubated in the histochemical reaction mixture and fixed in 3% glutaraldehyde but without OsO_4 postfixation. The halo (arrow) includes peripheral plasma membrane, periplasm, and part of the cell wall. Note that the deposits are associated to the inner edge of the "halo", i.e., the peripheral plasma membrane (pm). ×50,000.* FIG. 6 and 7. *Derepressed and repressed cells, respectively, of strain SB15 deposits representative of a minority of the population. Note that the discrete sites are clearly associated with the peripheral plasma membrane. Arrows indicate deposits formed by aggregation of smaller units. ×80,000.* FIG. 8. *Repression-resistant strain SB1004 grown in low phosphate medium; a large, aggregated deposit with multiple points of attachment to the membrane; ×92,000.* FIG. 9. *Deposit in the intramesosomal space (arrow) of a derepressed SB15 cell. ×92,000.*

FIG. 10. *Site of activity attached to the mesosomal membrane of a repressed cell of strain SB15; the intramesosomal space is free of deposits (arrow). This is the typical distribution of activity associated with mesosomes. ×98,000.* FIG. 11. *A section through a group of derepressed cells of strain SB15; the longitudinal sections (arrows) are used for quantitation. ×18,000.* FIG. 12. *Cells of strain SB1004 grown in low phosphate medium; note electron dense deposits in the wall (arrows). ×48,000.* FIG. 13. *High-magnification micrograph of a derepressed cell of strain SB15 showing alkaline phosphatase activity in the plasma membrane. Membrane area having activity (boxed area, arrow) is thickened. Circular deposits of 10 nm diameter are seen in the cytoplasm (arrows); similar small deposits are also present in the plasma membrane (pm, arrows). These highly dense portions of membrane could more easily be recognized in underexposed prints. ×240,000.*

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