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Cytochemical Localization of Certain Phosphatases in *Escherichia coli*

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Cytochemical studies of *Escherichia coli* at the light and electron microscopic levels have revealed alkaline phosphatase, hexose monophosphatase, and cyclic phosphodiesterase reaction products in the periplasmic space and at the cell surface. In preparations for both light and electron microscopy, reaction product filled polar caplike enlargements of the periplasmic space, such as those described in plasmolyzed cells, indicating significant terminal concentrations of these enzymes; dense substance was often seen within these polar caps in morphological specimens. Staining of the bacterial surface was commonly encountered, but could represent artifactual accumulation of precipitate along the cell wall. Alkaline phosphatase was demonstrated with several substrates (ethanolamine phosphate, glycerophosphate, *p*-nitrophenylphosphate, and glucose-6-phosphate) over a wide *p*H range in a bacterial strain (C-90) known to be constitutive for this enzyme, whereas strains deficient in this enzyme (U-7, repressed K-37), showed no activity with these substrates. Hexose monophosphatase and cyclic phosphodiesterase activities were characterized by reaction-product deposition with specific substrates at acid or neutral, but not at alkaline, *p*H in strains of *E. coli* lacking alkaline phosphatase (U-7 and repressed K-37). Fixation in Formalin or the use of calcium as a capture reagent seemed to interfere with periplasmic staining in cells prepared for electron microscopy. Formalin fixation had little effect on biochemical assays of the phosphatase activity of intact cells in suspension, but partially reduced the activity evident in sonically treated extracts or in suspensions of dispersed cryostat sections. Glutaraldehyde treatment impaired enzyme activity more drastically.

Bacterial cells lack the diverse, membrane-limited organelles which compartmentalize certain enzymes and other constituents within the cells of higher organisms (5, 17, 23, 31). In gram-negative organisms such as *Escherichia coli*, the cytoplasm is limited by a morphologically typical plasma membrane and the cell wall is usually depicted closely surrounding the protoplast, leaving very little periplasmic space between them (11).

Nonetheless, an interesting group of hydrolytic enzymes and binding proteins seems to occupy the periphery of the cell without being tightly bound to the cell wall or the plasma membrane (20, 21). These substances are selectively released by spheroplast production (27) and by osmotic shock (3, 22, 33, 35-37, 51), whereas most cytoplasmic constituents are retained within the cell. Among these apparently peripheral enzymes are phosphatases which, in intact cells, can hydrolyze substrates which do not penetrate the plasma membrane (6). Spheroplasts of *E. coli* secrete precursors of at least one of these phosphatases (alkaline phosphatase) through their plasma membrane into the surrounding medium (40), and alkaline phosphatase is lost into the medium by a mutant strain of *E. coli* with defective cell walls (28). Altogether, the evidence suggests that these peripheral or "surface" enzymes occur external to the plasma membrane and within the periplasmic space (21). This group of enzymes includes an alkaline phosphatase (16) active against many substrates over a broad *p*H range, as well as a hexose monophosphatase (13) and a cyclic phosphodiesterase (2, 33) which display acid *p*H optima; the biochemical properties of these enzymes have been ex-

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tensively investigated in purified extracts by these and other workers.

Several phosphatases (12, 24, 34, 45) and one of the binding proteins (32) have been studied cytochemically at the electron microscope level in gram-negative bacteria. These reports generally agree that enzymatic reaction product appears at the periphery of the cells, but they differ with regard to the precise localization of these enzymes. For example, reaction product indicative of alkaline phosphatase has been convincingly demonstrated in the periplasmic space (12), but other cytochemical studies (24) including our preliminary report of this work (see S. S. Spicer et al., Fed. Proc., p. 539, 1966) localize this enzyme only to the cell surface. Such differences probably reflect variations in the complex cytochemical procedures.

We reaffirmed the cytochemical localization of alkaline phosphatase in the periplasmic space of *E. coli* by tabulating the results from a very large number of separately prepared specimens; hexose monophosphatase and cyclic phosphodiesterase have been similarly localized. Comparable results have also been obtained at the light-microscope level. Various combinations of bacterial strain, substrate, and incubation *p*H have been used to differentiate these enzymes and to confirm the specificity of the methods. In addition, evidence has been obtained that Formalin fixation and the use of calcium as a precipitating cation can interfere with periplasmic staining in specimens prepared for electron microscopy.

MATERIALS AND METHODS

Organisms and growth conditions. Three strains of *E. coli* were employed as follows: C-90 constitutive for alkaline phosphatase; U-7 lacking alkaline phosphatase through genetic deletion (both kindly provided by A. Garen); and K-37, a derivative of strain K-10 obtained from N. D. Zinder, in which alkaline phosphatase could be repressed by growth in a phosphate-enriched medium (42). The cells were grown according to published methods (6). Preliminary comparisons of cells in logarithmic and stationary growth phases revealed similar cytochemical staining, so more convenient stationary phase cultures were generally used. The cells were collected by centrifugation and washed at 3 C three times in 0.01 M (*p*H 7.3) tris(hydroxymethyl)aminomethane (Tris)-maleate buffer containing 0.15 M NaCl.

Light microscopy. Freshly harvested cells (strains C-90, K-37 induced, K-37 repressed, and U-7) were washed twice in buffer [0.01 M Tris-hydrochloride (*p*H 7.3)-0.03 M sodium chloride], and were subsequently handled according to several different protocols, all of which led to a similar localization of alkaline phosphatase reaction product. Suspensions (1 g, wet weight, in 80 ml of buffer) of cells which had been unfixed or fixed (10% Formalin and 2% $CaCl_2$ for 15 min at 4 C) and washed twice in buffer were incubated in cytochemical reaction mixtures by addition of 0.2 ml of bacterial suspension to 2 ml of reaction mixture. Alternatively, drops of unfixed or fixed bacterial suspension were air-dried on clean glass slides for cytochemical treatment. Some unfixed bacteria were fixed in Formalin or acetone after drying on slides. Incubations for alkaline phosphatase localization were carried out at 37 C for intervals of 1 to 30 min by one of the following published procedures: (i) a calcium-cobalt method (18), (ii) a lead method (50), or (iii) a Naphthol-AS method (7). Cells reacted in suspension were washed twice in buffer and were either dried on glass slides or examined as wet preparations.

Penicillin spheroplasts of C-90 log-phase cells, prepared by the method of Lederberg (25), were fixed in Formalin containing 20% sucrose and reacted cytochemically as above.

Fine structure and cytochemistry. To obtain a cohesive mass of bacteria for subsequent cytochemical processing, 5% bovine fibrinogen (Armour Pharmaceutical Co., Chicago, Ill.) was added to the last wash solution and, after centrifugation and removal of the supernatant fluid, drops of 1% bovine thrombin (Mann Research Laboratories, New York, N.Y.) were gently layered on the pellet. The pellet was allowed to clot for 5 min at room temperature and was then fixed in situ for 15 min at 4 C; it was withdrawn from the centrifuge tube intact and cut into vertical slices 2 mm wide to include the entire density range of particles in the pellet.

For morphological examination, the tissue was fixed by one of the following procedures: (i) at 4 C for 90 min in 6.25% glutaraldehyde in 0.1 M sodium cacodylate buffer [*p*H 7.4 (39)] followed by 45 min in phosphate-buffered osmium (29); (ii) at 4 C for 90 min in phosphate-buffered osmium alone (29); or (iii) by the method of Kellenberger and Ryter (23).

For cytochemical examination, tissue was fixed at 4 C for 90 min in sodium cacodylate-buffered 6.25% glutaraldehyde (39), in cacodylate-buffered 12.5% β-hydroxyadipaldehyde (39), or in a solution of 10% Formalin with 2% calcium acetate. Shorter fixation times (5, 30, and 60 min) were without effect on reaction-product localization. The slices of clotted, fixed bacterial pellets were rinsed three times in cold 7.5% sucrose and were frozen with dry ice and mounted so that each cryostat section would display the full depth of the centrifuged pellet. Cryostat sections were cut approximately 40 μm thick and transported between layers of lens paper tied over one end of a 10-cm length of 10-mm glass tubing until final embedding. After three rinses in cold 11.5% sucrose, the sections were immersed in freshly prepared enzyme incubation media maintained at 37 C. Most sections were incubated for 90 min with occasional agitation, although some duplicates were incubated for only 15 or 30 min.

Incubation media employing calcium as a capture reagent were formulated by the method of Gomori (18)

with one of the following substrates at the indicated *final* concentrations: ethanolamine phosphate (0.01 M), β-glycerophosphate (0.025 M), *p*-nitrophenylphosphate (0.025 M), glucose-6-phosphate (0.001 M), or bis(*p*-nitrophenyl)phosphate (0.01 M) from Sigma Chemical Co., St. Louis, Mo.; or with uridine 2′,3′-cyclic phosphate (0.01 M) from Schwarz BioResearch, Inc., Orangeburg, N.Y. In some instances, the Veronal buffer was replaced by 0.1 M Tris-hydrochloride. The *p*H of each medium was adjusted with either 0.1 N HCl or 0.1 N NaOH. After incubation, most of these specimens were washed in cold distilled water, immersed in 2% lead nitrate for 5 min at 4 C (50), and rinsed once more in water.

Incubation media employing lead as a capture reagent were generally formulated by the method of Wachstein and Meisel (46), but Tris-hydrochloride, maleate-hydrochloride, acetate, and Veronal buffers were occasionally substituted for Tris-maleate. The following substrates were used at the indicated final concentrations: ethanolamine phosphate (0.02 M), β-glycerophosphate (0.04 M), glucose-6-phosphate (0.016 M), bis(*p*-nitrophenyl) phosphate (0.012 M), or uridine 2′,3′-cyclic phosphate (0.01 M). The *p*H of each medium was adjusted with either 0.1 N HCl or 0.1 N NaOH. Trace amounts of cobalt (0.001 M final concentration) and magnesium (0.01 M final concentration) were included in the incubation media for cyclic phosphodiesterase localization.

Control procedures included omission of substrate and immersion of cryostat sections in boiling distilled water for 2 min prior to incubation.

In several experiments 40-μm frozen sections of clotted glutaraldehyde-fixed bacterial pellets were incubated in phosphate buffer containing malt diastase or in the same buffer without diastase (4).

All cytochemical preparations were postfixed at 4 C for 45 min in phosphate-buffered osmium tetroxide (29).

After final fixation, all tissue samples were rinsed briefly in cold distilled water, dehydrated through a graded ethyl alcohol series, and embedded in Maraglas (41). Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with glass or diamond knives and collected on copper grids coated with a carbonized collodion film. Thin sections were stained with uranyl acetate (48) and lead citrate (43); however, most cytochemical preparations were examined without staining. Sections were viewed with either an RCA EMU-2A or an RCA EMU-3G electron microscope.

Relatively long phosphatase incubations (90 min at 37 C) were employed to ensure reaction-product deposition in sites of enzyme activity, despite an increased risk of nonspecific precipitation (30); shorter incubation times generally reduced the quantity of the precipitate without affecting its distribution. Most reactive tissue showed widespread localization of precipitate, at least in the cells on the surfaces of the cryostat section.

Seven separate cytochemical experiments were performed and the combined results were tabulated. Each "specimen" was unique in its class in at least one of the following respects: (i) it was derived from a separate experiment, (ii) it represented a different bacterial strain or growth phase, (iii) it was prepared with a different fixation procedure, or (iv) it was incubated in a different enzyme reaction mixture.

Biochemical assays. Some effects of fixation on biochemically measurable enzyme activity were studied with suspensions of intact cells and cells which were repeatedly frozen and thawed or treated for 90 sec with a Branson Sonifier, model LS75; phosphatase activity was assayed as previously described (6, 13). Cyclic phosphodiesterase activity was assayed in the presence of excess purified alkaline phosphatase added to the reaction mixture (*see* reference 6). Enzyme activities were reported in terms of international units (1 IU = 1 μmole/min).

RESULTS

Cellular morphology. The fine structure of C-90 (Fig. 1), U-7 (Fig. 2), and K-37 (Fig. 3, 4) strains of *E. coli* generally resembled published descriptions of this species (5, 11, 17, 23, 31). The cell wall, periplasmic space, plasma membrane, ribosomes, nuclear regions, and occasional mesosomes were observed with each fixation procedure. The presumed peptidoglycan layer of the cell wall (11, 47, 49) was not always evident in these preparations.

Dense composite particles measuring approximately 70 μm in diameter were abundant in the cytoplasm of most stationary phase cells which had been fixed in glutaraldehyde (Fig. 4, 5) or in phosphate-buffered osmium tetroxide alone (Fig. 3). Electron-lucent spaces suggestive of extracted particles were encountered in some of these specimens (*compare* reference 8), but no trace of these particles was usually evident in cells fixed by the method of Kellenberger and Ryter (23). When present, these particles resembled glycogen as observed in metazoan cells (9); they did not resemble the larger, homogeneous, spherical polymetaphosphate granules described by others (19). In preliminary experiments, these particles were more readily extracted by diastase treatment than by incubation in buffer lacking this enzyme.

Polar caplike enlargements of the periplasmic space appeared in virtually every cell with each of several different fixatives (Fig. 1–4). This configuration has generally been attributed to shrinkage of the protoplast by hypertonic solutions (10). The ends of the protoplast generally appeared blunt or indented, and the resulting polar spaces were continuous with the narrower lateral periplasmic space (Fig. 1–6). The periplasmic space and notably the polar caps often contained considerable flocculent material of moderate density (Fig. 1–4); however, in most unreactive cytochemical specimens (Fig. 6), the periplasmic space appeared relatively empty.

Light microscopic histochemistry of alkaline

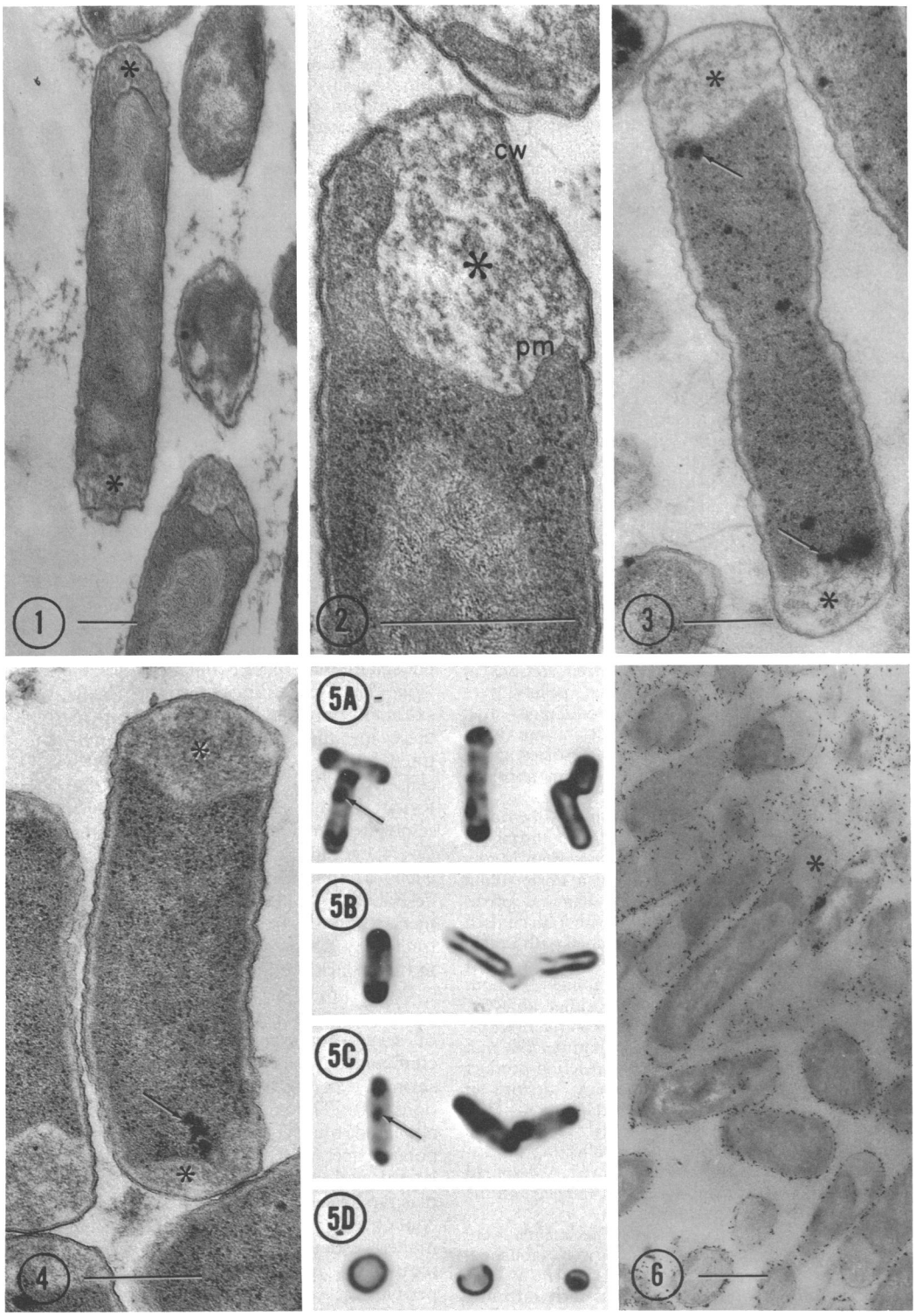

FIG. 1–6

phosphatase. Intact cells containing alkaline phosphatase (strains C-90 and induced K-37) displayed a remarkably uniform staining pattern with the three different methods (calcium-cobalt, lead, and naphthol-AS) for localizing this enzyme, with or without Formalin or acetone fixation and whether reacted on glass slides or in suspension; bovine fibrinogen was not added to any of these preparations. The cells were outlined by reaction product and most cells showed prominent deposits of precipitate at each pole (Fig. 5A–5C). A few cells also contained discrete deposits of precipitate along their lateral surfaces, and some cells lacked the enlarged polar deposits. Occasionally, long filaments were seen in stationary-phase cultures and deposits of precipitate were regularly distributed along their length as well as in the polar caps. Penicillin spheroplasts made from log phase C-90 cells and incubated for alkaline phosphatase localization were generally outlined by a circumferential rim of precipitate, although some rims were incomplete and eccentric deposits of reaction product, suggestive of persisting polar caps, were seen in some individual cells (Fig. 5D). Cells lacking alkaline phosphatase (U-7 and repressed K-37), penicillin spheroplasts made from these cells, and specimens incubated without substrate were all unstained by these methods.

Cytochemical localization of alkaline phosphatase. Of 108 specimens of *E. coli* (strains C-90 and K-37), 101 displayed dense deposits of reaction product after cytochemical incubation for alkaline phosphatase activity (Table 1). Several organic phosphate esters served comparably as substrates in these cytochemical experiments; reaction product was consistently produced over a wide *p*H range between 6.0 and 9.2.

The deposits of alkaline phosphatase reaction product occupied the periplasmic space of cells in some specimens (Fig. 7, 9, 11), but in other specimens the precipitates were restricted to the surface of the cell (Fig. 8, 10). In general, only these two patterns of alkaline phosphatase reaction product deposition were observed, although in nearly all reactive specimens occasional cells displayed some precipitate in their cytoplasm.

Several details of the cytochemical procedure influenced the distribution of reaction product (Table 1). Of 39 specimens fixed with glutaraldehyde and processed with lead as a capture reagent, 32 displayed precipitate in the periplasmic space of the bacteria (Fig. 7, 9, 11). After Formalin fixation, precipitate was restricted to the cell surface in more than half of the reactive specimens (e.g., Fig. 10), and the remainder also showed periplasmic staining. With calcium as a capture reagent, 43 of 46 specimens displayed only surface localization (Fig. 8), and no periplasmic staining was seen.

When these procedures were carried out on enzyme-deficient strains of *E. coli* (U-7, or K-37

FIG. 1–6. *Electron micrographs of thin sections of fibrin-embedded pellets of E. coli in stationary growth phase, except the light microscope images of this organism in Fig. 5 as noted below. Except as otherwise indicated, cells were fixed with glutaraldehyde and phosphate-buffered osmium tetroxide, cytochemical specimens were incubated for 90 min, and thin sections were stained with a sequence of uranyl and lead salts. Markers represent approximately 0.5 μm. (Fig. 1) Cells of strain C-90 fixed by the method of Kellenberger and Ryter (23). The three bacterial strains used in this study (C-90, U-7, and K-37) appear similar when comparably fixed (compare Fig. 2 and 4). Virtually every cell possesses polar enlargements of the periplasmic space (*) between the cell wall and the plasma membrane; these polar caps are most evident in longitudinal sections. The periplasm contains abundant flocculent material of considerable density. × ~17,500. (Fig. 2) Portion of a cell of strain U-7 fixed by the method of Kellenberger and Ryter (23). At this higher magnification, the cell wall (cw), the plasma membrane (pm), and the periplasmic space (*) with its flocculent contents can each be traced continuously from the polar region to the sides of the cell. × ~61,000. (Fig. 3) Cells of strain K-37 fixed in phosphate-buffered osmium tetroxide alone. The periplasmic space with flocculent contents is evident all around the protoplast; both polar enlargements of the space are included in this longitudinal section. The dense composite particles approximately ~70 μm in diameter seen in the cytoplasm of these cells (arrows) bear a superficial resemblance to glycogen (9). Such particles are evident in some specimens of each strain prepared in this way or fixed with a glutaraldehyde-osmium tetroxide sequence (Fig. 4), but they have not been seen after fixation by the method of Kellenberger and Ryter [(23) Fig. 1, 2]. × ~28,000. (Fig. 4) Cells of strain K-37 fixed in a glutaraldehyde-osmium tetroxide sequence. The periplasmic space, including both polar caps, is filled with a flocculent substance of moderate density. Note the glycogen-like particles (arrow). × ~37,000. (Fig. 5) Light micrographs of cells of strain C-90 fixed in Formalin without the use of bovine fibrinogen and incubated for localization of alkaline phosphatase. × 1,000. (A) Calcium-cobalt method. Dense polar staining is typical in these cells, and occasionally discrete lateral deposits are also evident (arrow); a few cells display a uniform rim of stain with this method (right). (B) Lead method. Precipitate is typically concentrated in polar caps (left), although some cells may be outlined with a relatively uniform rim of stain (right). (C) Naphthol-AS method. Staining is typically evident in the polar caps of each cell and occasionally in discrete lateral foci (arrow). (D) Penicillin spheroplasts incubated for alkaline phosphatase activity with the calcium-cobalt method. Most individuals show a complete circumferential rim of reaction product (left), but in some cells the rim is incomplete (center), or displays eccentric foci reminiscent of polar caps (right). (Fig. 6) Cells of strain C-90 incubated in a substrate-free medium at pH 9.0 with calcium as the capture reagent. A light precipitate is seen along the cell wall and, to a lesser extent, on the plasma membrane of each cell; polar caps are void of deposit. × ~ 11,500.*

TABLE 1. *Tabulation of cytochemical specimens incubated for the localization of alkaline phosphatase*[a]

Substrate	Fixation	Strain constitutive for alkaline phosphatase (C90) with						Strains deficient in alkaline phosphatase with[b]	
		Lead cation			Calcium cation			Lead cation	Calcium cation
		No. of reactive specimens		Total no. of specimens	No. of reactive specimens		Total no. of specimens	Total no. of specimens, all unreactive	Total no. of specimens, all unreactive
		PPS	CS		PPS	CS			
Ethanolamine phosphate	G	7	3	12[c]	0	12	12	3[b]	2[b]
	F or BHA	0	7	8	0	11	11	2	2
Glycerophosphate	G	1	0	1	0	1	1	1	1
	F or BHA	0	1	1	0	1	1	1	1
p-Nitrophenyl-phosphate	G				0	4	6		1
	F or BHA				0	3	3		1
Glucose-6-phosphate	G	24	1	26	0	9	9	1[e]	4[e]
	F or BHA	8	5	14[d]	1	2	3		1[e]
Totals	G	32	4	39	0	26	28	5	8
	F or BHA	8	13	23	1	17	18	3	5
Grand totals		40	17	62	1	43	46	8	13

[a] Abbreviations: PPS, periplasmic space; CS, cell surface; G, glutaraldehyde; F, Formalin, and BHA, β-hydroxyadipaldehyde. Variation in the *p*H of the incubation media over the range of *p*H 6.0 to 9.2 produced no significant differences in the localization of precipitate among the reactive specimens, with the exceptions cited in footnotes *c* and *d*.

[b] Strain U7 was used for all of these experiments except two of the specimens fixed with glutaraldehyde and incubated with ethanolamine phosphate; one specimen each of those incubated with lead or calcium consisted of repressed cells of strain K37, and they, too, were unreactive.

[c] Two out of four of these specimens incubated at *p*H 6.0 showed staining principally of the cell surface (and one with reactive PPS), whereas only one out of eight specimens incubated at higher *p*H (6.7 to 9.0) showed staining principally of the cell surface (and six with reactive PPS).

[d] Three out of four of these specimens incubated at *p*H 6.0 or 6.7 showed staining principally of the cell surface (and one with reactive PPS), whereas only two out of ten specimens incubated at higher *p*H (7.0 to 9.0) showed staining principally of the cell surface (and seven with reactive PPS).

[e] The control incubations of alkaline phosphatase-deficient bacteria (strain U7), using glucose-6-phosphate as substrate, were carried out at *p*H 9.0 at which point hexose monophosphatase is inactive (*compare* Table 2).

repressed by growth on high phosphate medium), definite reaction product was absent from the cells (e.g., Fig. 12). Omission of substrate from the media (Fig. 6), incubation at *p*H 9.0 with bis-*p*-nitrophenylphosphate (which is not hydrolyzed by alkaline phosphatase), or preincubation exposure of specimens to boiling water for 2 min each yielded negative results with cells which contained alkaline phosphatase (C-90, induced K-37).

Glucose-6-phosphatase localization. Strains of *E. coli* which lacked the nonspecific alkaline phosphatase due to genetic deletion (U-7) or to repression during growth (K-37) showed reaction product indicative of acid hexose monophosphatase. Such activity was observed in 13 of 16 specimens (Table 2). Nearly all of the specimens fixed in glutaraldehyde and processed with lead as the capturing ion displayed staining of the periplasmic space and the surface of the bacteria (Fig. 13, 15). On the other hand, all of those fixed in formaldehyde showed only surface staining after similar treatment (Fig. 14). When specimens of the alkaline phosphatase-deficient strains (U-7) were incubated at *p*H 9.0 (at which point glucose-6-phosphatase is inactive) or without substrate, the cells failed to show reaction-product deposition.

Cyclic phosphodiesterase localization. Three-fourths of those specimens incubated at an acid *p*H with substrates hydrolyzed specifically by cyclic phosphodiesterase (2'3'-uridine cyclic phosphate or bis-*p*-nitrophenylphosphate) were reac-

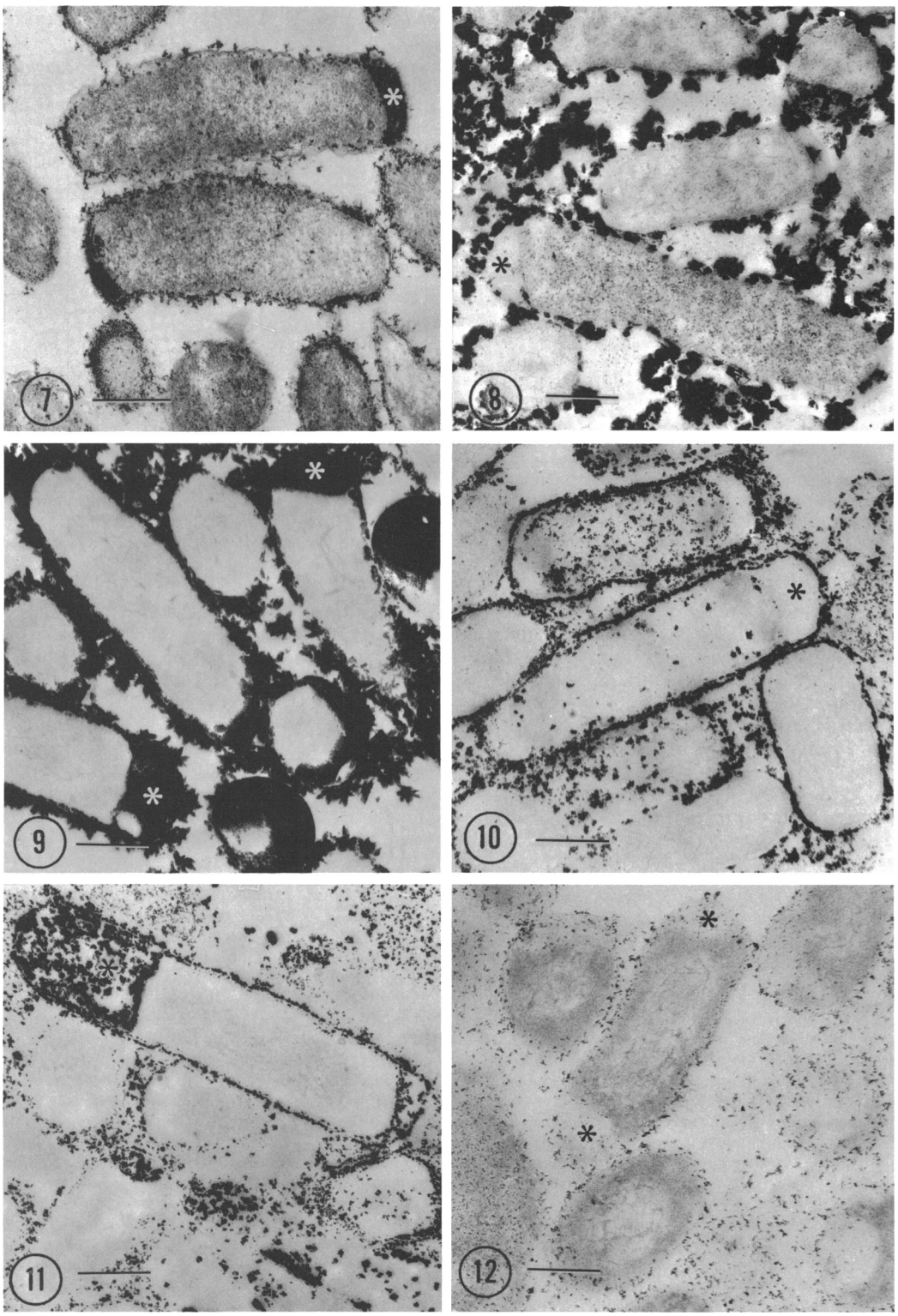

FIG. 7-12

TABLE 2. *Tabulation of specimens prepared for the cytochemical localization of hexose monophosphatase*[a]

Bacterial strain[b]	Fixation	Incubation at or below *p*H 7 with lead cation			Incubation at *p*H 9 with	
		No. of reactive specimens		Total no. of specimens	Lead cation	Calcium cation
		PPS	CS		Total no. of specimens, all unreactive	Total no. of specimens, all unreactive
U7 (genetic deletion for alkaline phosphatase)	G	8	1	12	1	4
	F	0	3	3		1
K37 (repressed for alkaline phosphatase)	G	1	0	1		
Totals		9	4	16	1	5

[a] Abbreviations: PPS, periplasmic space; CS, cell surface; G, glutaraldehyde; F, Formalin; and BHA, β-hydroxyadipaldehyde. Glucose-6-phosphate was utilized as substrate in all of these experiments.

[b] Alkaline phosphatase-deficient bacterial strains (U7 and repressed K37) were used in these experiments to avoid ambiguous hydrolysis of this substrate. (Note the lack of precipitate after incubation at *p*H 9.)

tive (Table 3). After fixation in glutaraldehyde, most of the reactive specimens showed staining of the periplasmic space (e.g., Fig. 17, 18), and the remainder showed only surface staining. After fixation in Formalin (Fig. 16) or β-hydroxyadipaldehyde, however, the precipitate was restricted to the cell surface in all of the reactive specimens.

The cyclic nucleotide is specifically hydrolyzed by cyclic phosphodiesterase, but the resulting phosphomonomer intermediate is also hydrolyzed by alkaline phosphatase (*see* reference 15). Thus, the final reaction product in cells of strain C-90 (which contain alkaline phosphatase) could represent the site of either enzyme. However, the reactive specimens of strains which lacked alkaline phosphatase (U-7 and repressed K-37) gave unambiguous evidence for the localization of cyclic phosphodiesterase in the periplasmic space. Specimens incubated with bis-*p*-nitrophenylphosphate above *p*H 9.0 (at which point cyclic phosphodiesterase is inactive) or without substrate were all unreactive.

FIG. 7–12. *Electron micrographs of thin sections of fibrin-embedded pellets of E. coli in stationary growth phase. Except as otherwise indicated, cells were fixed with glutaraldehyde and phosphate-buffered osmium tetroxide, cytochemical specimens were incubated for 90 min, and thin sections were stained with a sequence of uranyl and lead salts. Markers represent approximately 0.5 μm. (Fig. 7) Cells of strain C-90 incubated 15 (rather than 90) min for alkaline phosphatase hydrolysis of ethanolamine phosphate at pH 9.0 with lead as the capture reagent. Precipitate is concentrated in the polar enlargements of the periplasmic space. Deposits also occur in the lateral periplasmic space, on the outer surface of the cell wall, and lightly scattered in the cytoplasm. × ~24,000. (Fig. 8) As in Fig. 7, except with calcium as the capture reagent. The calcium-engendered precipitate is principally localized to the outer surface of each bacterium and scattered among the cells. Some precipitate occurs on the plasma membrane, and a light deposit is seen in the cytoplasm of some cells; however, the polar caps (*) are generally free of precipitate. The thin section has not been stained. × ~22,000. (Fig. 9) As in Fig. 7, except for incubation at pH 6.7. Precipitate is strikingly localized to the polar caps (*) and the lateral periplasmic space and is unevenly distributed along the outer surface of the cell wall. The cytoplasm is essentially free of precipitate in this specimen. The thin section has not been stained. × ~22,000. (Fig. 10) As in Fig. 7, except for fixation in Formalin and the use of glycerophosphate as substrate. In contrast to glutaraldehyde-fixed specimens (Fig. 7, 9), precipitate is principally restricted to the outer surface of the cell wall (compare Fig. 14 and 16); scattered deposits occur on the plasma membrane and in the cytoplasm of some bacteria. The periplasmic space (*) is generally free of precipitate in this specimen. The thin section has not been stained. × ~22,000. (Fig. 11) As in Fig. 9, except with glycerophosphate as substrate. Alkaline phosphatase activity is evident in the periplasmic space, especially in a large polar cap (*) and on the outer surface of the cell. The cytoplasm is generally free of precipitate in this specimen. The thin section has not been stained. × ~22,000. (Fig. 12) Cells of strain U-7 incubated with glucose-6-phosphate as substrate at pH 9.0 with lead as the capture reagent. The absence of all but a light scattering of precipitate (compare the substrate-free control, Fig. 6) presumably reflects the lack of phosphatase activity in this specimen. This strain (U-7) possesses negligible alkaline phosphatase which would attack this substrate, and the constitutive hexose monophosphatase (compare Fig. 13 and 14) is inactive at this pH (9.0). The thin section has not been stained. × ~22,000.*

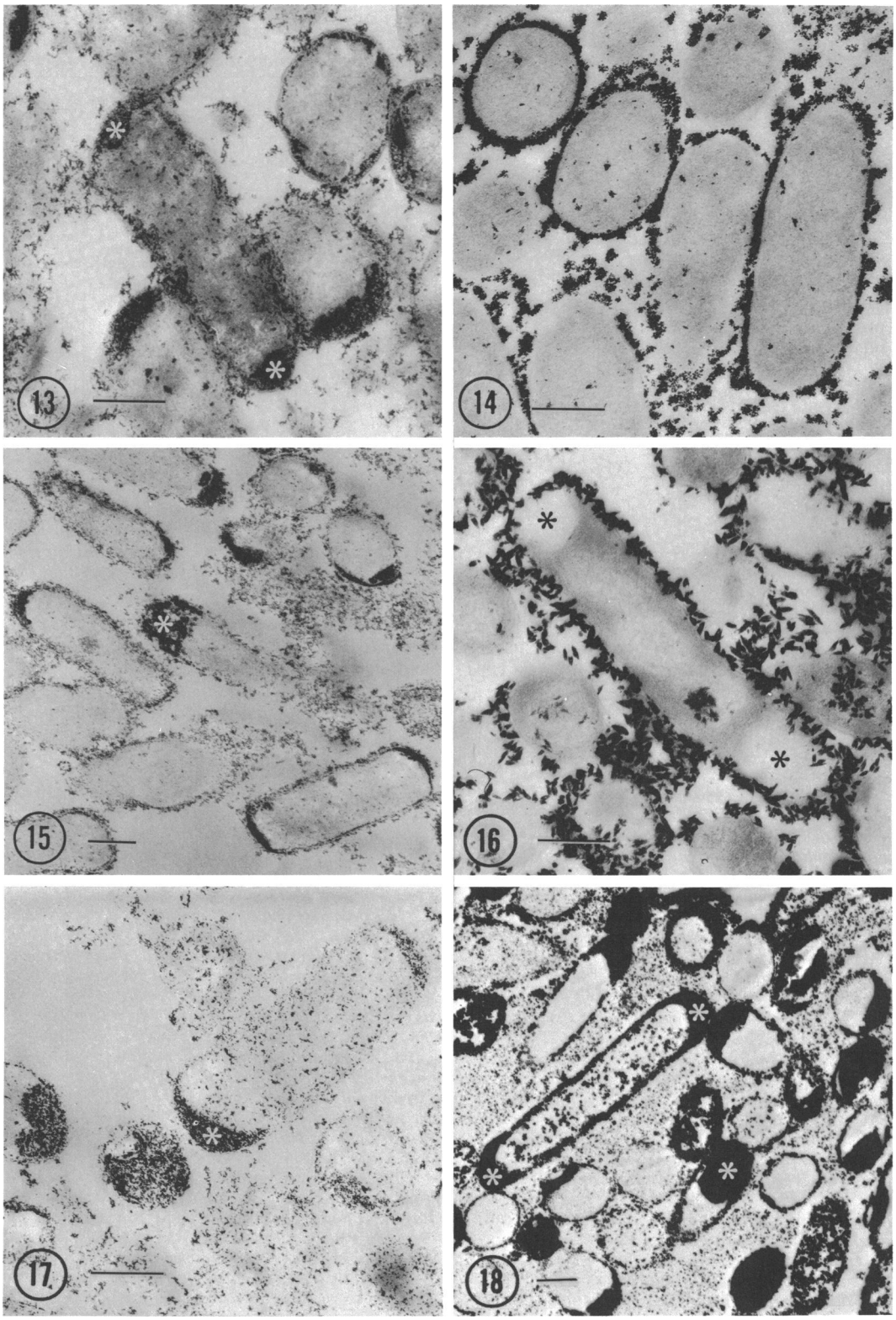

FIG. 13–18

TABLE 3. *Tabulation of specimens prepared for the cytochemical localization of cyclic phosphodiesterase*[a]

Substrate	Bacterial strain	Fixation	Incubation at or below pH 7 with						Incubation at pH 9.0 with	
			Lead cation			Calcium cation			Lead cation	Calcium cation
			No. of reactive specimens		Total no. of specimens	No. of reactive specimens		Total no. of specimens	Total no. of specimens, all unreactive	Total no. of specimens, all unreactive
			PPS	CS		PPS	CS			
Bis-*p*-nitrophenyl phosphate	C90	G	3	3	10	0	0	1	1	2
		F	0	4	4					
	U7	G	1	1	6					2
	K37	G	1	0	1					
Uridine 2',3'-cyclic phosphate	C90	G	11	3	16					
		F or BHA	0	2	2					
	U7	G	0	1	3					
		F	0	1	1					
Totals			16	15	43	0	0	1	1	4

[a] Abbreviations: PPS, periplasmic space; CS, cell surface; G, glutaraldehyde; F. Formalin; and BHA, β-hydroxyadipaldehyde.

Effects of fixation on enzyme activity. Phosphatase activity was assayed biochemically in suspensions of intact and ultrasonically treated cells without prior fixation and after suspension in 10% Formalin for 25 or 75 min with subsequent washings (Table 4). In nearly all cases, ultrasonically treated cells showed higher values than identically reacted whole cells in suspension, especially in unfixed preparations. Remarkably, Formalin fixation often had little effect on the values obtained with whole cells in suspension. Furthermore, substantial enzyme activity (approximately 50% of unfixed preparations) was still evident in ultrasonically treated cells previously fixed for 75

FIG. 13–18. *Electron micrographs of thin sections of fibrin-embedded pellets of E. coli in stationary growth phase. Except as otherwise indicated, cells were fixed with glutaraldehyde and phosphate-buffered osmium tetroxide, cytochemical specimens were incubated for 90 min, and thin sections were stained with a sequence of uranyl and lead salts. Markers represent approximately 0.5 μm. (Fig. 13) Cells of strain U-7 incubated for hexose monophosphatase hydrolysis of glucose-6-phosphate at pH 6.0 with lead as the capture reagent. Precipitate is concentrated in the polar enlargements of the periplasmic space (e.g., *); scattered deposits are also seen on the outer surface of the cell wall and in the cytoplasm. This strain (U-7) lacks alkaline phosphatase which, if present, would account for most of the hydrolysis of this substrate under these conditions (also see Fig. 14 and 15). × ~22,000. (Fig. 14) As in Fig. 13, except the specimen was fixed in Formalin. In contrast to glutaraldehyde-fixed specimens (Fig. 13), precipitate is principally restricted to the surface of most cells (compare Fig. 10 and 16); scattered deposits sometimes occur in the cytoplasm. The thin section has not been stained. × ~22,000. (Fig. 15) Cells of strain K-37 with their alkaline phosphatase activity repressed by growth on a high phosphate medium (see Table I, footnote b); these cells have been incubated only 15 min for hexose monophosphatase hydrolysis of glucose-6-phosphate at pH 6.0 with lead as the capture reagent. Precipitate is concentrated in the polar enlargements of the periplasmic space (*) and scattered along the outer surface of the cells. The cell wall often appears as a negative image between these deposits (e.g., lower right). The thin section has not been stained. × ~14,500. (Fig. 16) Cells of strain C-90 fixed in Formalin and incubated for cyclic phosphodiesterase hydrolysis of uridine 2',3'-cyclic phosphate at pH 6.7 with lead as the capture reagent. In contrast to glutaraldehyde-fixed specimens (Fig. 17 and 18), precipitate indicative of this enzyme is almost exclusively localized to the outer surface of the cell (compare Fig. 10 and 14); occasional deposits also occur in the cytoplasm. The polar enlargements of the periplasmic space (*) appear void of precipitate. × ~22,000. (Fig. 17) Cells of strain U-7 incubated for cyclic phosphodiesterase hydrolysis of bis(p-nitrophenyl) phosphate at pH 6.7 with lead as the capture reagent. Precipitate is concentrated in the polar enlargements of the periplasmic space (*), with scattered deposits in the cytoplasm and on the cell surface. This strain lacks alkaline phosphatase which also could attack the phosphomonoester intermediate in this reaction. The thin section has not been stained. × ~22,000. (Fig. 18) Cells of strain C-90 incubated for cyclic phosphodiesterase hydrolysis of uridine 2',3'-cyclic phosphate at pH 6.7 with lead as the capture reagent. Precipitate is heavily concentrated in the periplasmic space and on the surface of each cell; the stained polar caps are especially prominent (*). Scattered precipitate is also seen in the fibrin-rich intercellular material and in the cytoplasm of some cells. The thin section has not been stained. × ~12,000.*

TABLE 4. *Effects of formalin fixation on biochemical assays of enzyme activity in suspensions of whole cells and sonically treated extracts*[a]

Enzyme tested	Substrate	Cell suspension	Minutes in calcium acetate-Formalin for *E. coli* C90			Minutes in calcium acetate-Formalin for *E. coli* K37			Minutes in calcium acetate-Formalin for *E. coli* U7		
			0	25	75	0	25	75	0	25	75
Alkaline phosphatase at *p*H 9.0	*p*-Nitrophenyl phosphate[b]	Intact	0.162	1.551	1.503						
		Sonically treated extracts[c]	2.970	2.460	2.094						
	2′-Adenosine monophosphate	Intact	0.217	0.358	0.403						
		Sonically treated extracts[c]	0.258	0.232	0.192						
Cyclic phosphodiesterase at *p*H 6.7	bis(*p*-Nitrophenyl) phosphate	Intact	0.307	0.307	0.290	0.135	0.153	0.142	0.083	0.110	0.103
		Sonically treated extracts[c]	0.422	0.407	0.322	0.165	0.178	0.173	0.130	0.128	0.118
	Uridine 2′,3′-cyclic phosphate[b]	Intact	0.300	0.303	0.172	0.085	0.087	0.083	0.092	0.077	0.078
		Sonically treated extracts[c]	0.535	0.362	0.312	0.403	0.323	0.263	0.312	0.218	0.182

[a] Values are expressed in international units of micromoles of inorganic phosphate formed per minute at 37 C.
[b] Values represent an average of three experiments.
[c] Fixed cell suspensions were treated for 90 sec with a Branson Sonifier LS 75.

min in Formalin; somewhat lower values would be expected after the 90-min fixation used routinely for cytochemical studies.

Enzyme activity was also measured in dispersed cryostat sections of fixed cells prepared exactly as for cytochemical studies; these values were consistently lower than those obtained with suspensions of unfixed whole cells which had been simply frozen and thawed (Table 5).

In other experiments, intact cells were fixed in various ways and sonically treated extracts were subsequently assayed for enzyme activity. Fixation in calcium acetate-Formalin for 90 min reduced acid hexose monophosphatase activity by 25% and cyclic phosphodiesterase activity by 35% as compared with sonic-treated material of unfixed cells. On the other hand, fixation for 90 min in Tris-buffered glutaraldehyde reduced acid hexose monophosphatase activity by 60%, and cacodylate-buffered glutaraldehyde caused a 70% reduction. Glutaraldehyde fixatives caused nearly complete reduction of the cyclic phosphodiesterase activity which appeared in the sonic extracts.

TABLE 5. *Biochemical comparison of enzyme activity in suspensions of fresh cells and cryostat sections*[a]

Enzyme	Strain	Unfixed frozen and thawed cells in suspension	Suspended cryostat sections of fixed pellets	
			Calcium acetate-formalin (90 min)	Cacodylate-glutaraldehyde (90 min)
Alkaline phosphatase (ethanolamine phosphate)	C90	56	42.67	4.27
Cyclic phosphodiesterase (uridine 2′,3′-cyclic phosphate)	C90	26.67	13.33	
	U7	11.67	2.50	

[a] Values are expressed in international units of 1 μmole of inorganic phosphate formed per minute at 37 C per gram (wet weight) of cells.

DISCUSSION

In this study, more than 86% of 167 cytochemical specimens which meet the biochemically established criteria (appropriate strain, substrate, *p*H) for expected phosphatase activity display definite reaction product in the periplasmic space or on the cell surface. On the other hand, the 32 specimens expected to be negative because of the bacterial strain or the incubation conditions all lack definite reaction-product deposition. This close correlation with biochemical data supports the validity of the cytochemical procedures.

It is difficult to interpret the apparent interference of Formalin fixation and calcium capture with periplasmic staining in specimens for electron microscopy. Although greater enzyme activity is evident biochemically after Formalin than after glutaraldehyde fixation, Formalin treatment results in less precipitate in the periplasmic space. One could postulate that Formalin immobilizes the enzymes only on the surface, whereas those in the periplasm are lost from the cells. Glutaraldehyde fixation is more drastic and could retain the periplasmic enzymes for cytochemical purposes, although masking them somehow from biochemical assay. Alternatively, the greater surface activity which survives Formalin fixation may exhaust a reagent from the medium at the cell wall before it can diffuse into the periplasmic space, or the cell wall may simply be less permeable to trapping reagent or substrate after Formalin fixation.

The rest of the specimens that were unreactive despite biochemically propitious conditions probably represent technical inconsistencies (e.g., samples taken from the unreactive centers of cryostat sections). Similarly, procedural differences may account for disparate results from different laboratories. For example, the cell wall may represent a barrier (26, 38) to cytochemical reagents, especially in fresh unfixed cells; this could explain the predominantly surface localization of phosphatases reported in *E. coli* by Kushnarev and Smirnova (24) and by Nisonson *et al.* (34). Indeed, no current account (including the present one) of phosphatase cytochemistry in intact bacteria can distinguish between authentic surface localization of the enzymes and artifactual adsorption or accumulation of precipitate along the physical barrier of the cell wall. By the same argument, the lack of convincing reaction product deposition in the bacterial cytoplasm does not rule out the presence of these "surface" enzymes within the plasma membrane, particularly since positive cytochemical evidence localizing the internal phosphatases of *E. coli* by these procedures is not yet available.

The results of the present study focus attention on the nature and possible significance of the periplasmic space in gram-negative bacteria. Most accounts of the normal fine structure of these organisms (11, 15) indicate close apposition of the plasma membrane against the cell wall even at the poles of the cell, delimiting a very small periplasmic space. The polar enlargements of this space depicted in the present study more than double the size of this compartment. In our experience, such polar caps are evident, at least occasionally, in most routine fine structural preparations of normal gram-negative bacteria. However, since polar vacuoles are known to appear in cells exposed to hypertonic solutions (10), the converse is generally assumed—that enlargement of the periplasm necessarily indicates artifactual plasmolysis—and such specimens are commonly discounted. If this assumption were correct, one would expect all polar caps to appear as "empty" vacuoles with attenuated periplasmic enzyme activity. Instead, flocculent substance of considerable density fills these polar caps in many specimens, and striking depots of cytochemical reaction product appear there whenever the periplasmic space is reactive.

With such substantial concentrations of dense substance and "surface" enzymes, the refractive index of the polar caps of normal bacteria could well approach that of the cytoplasm; this would account for the fact that polar caps are invisible by phase-contrast microscopy unless they are altered experimentally (as in plasmolysis). Polar caps can be observed by light microscopy in fresh cells incubated directly for alkaline phosphatase localization. Thus, one must consider the possibility that the polar caps exist in vivo, despite their evident susceptibility to extraction and swelling or to obliteration due to swelling of the protoplast. The demonstration of ubiquitous polar caps and polar localization of surface enzymes argues strongly for this possibility, although a relatively high incidence of polar caps could reflect either unusual preservation or accentuation of the terminal periplasm during tissue preparation. The use of fibrinogen, for example, could influence the appearance of the polar caps. (Preliminary comparisons indicate similar results with 3% fibrinogen and reduced prominence without fibrinogen.) However, fibrinogen has been omitted from all of our light microscopy preparations and, therefore, could not be responsible for the consistent polar localization of alkaline phosphatase observed under a variety of conditions. No interpretation of this phenomenon can be entirely conclusive until the nature of the periplasmic space and its response to environmental changes (particularly during tissue preparation) are understood.

The authenticity of the polar caps is further supported by studies of a mutant strain of *E. coli* which buds off small, spherical terminal segments termed "minicells" (1). The minicells show higher specific activities of the "surface" enzymes than typical rod-shaped cells, and in each minicell the periplasmic space is enlarged and cytochemically reactive for these enzymes, resembling the polar caps of rod-forms (14). The minicells thus acquire shares of periplasmic space and periplasmic enzymes which are disproportionately large compared to the entire rod-forms, but are commensurate with the polar caps; the direct incorporation of preexisting polar caps with their enzyme concentrations into the terminal minicell buds seems a likely explanation of these findings.

Thus, the periplasmic space of gram-negative bacteria may represent a discrete compartment for the sequestration of the "surface" enzymes and binding proteins. Presumably, the bacterium can selectively modify the contents of this compartment by active transport and secretion across the plasma membrane (40). It is possible that the partially impermeable cell wall surrounding this space could normally retain even soluble substances and help maintain a controlled external milieu for the protoplast.

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