

Surface Localization of *Escherichia coli* 5'-Nucleotidase by Electron Microscopy

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The 5'-nucleotidase of *Escherichia coli* was shown to be located at the cell wall surface by histochemical techniques utilizing the deposition of inorganic phosphate. Penetration of the 5'-nucleotidase in the periplasmic space was seen only in cells treated with ethylenediaminetetraacetic acid (EDTA)-tris(hydroxymethyl)aminomethane (Tris). The 3'-nucleotidase of *E. coli* was also found to have a surface location, and periplasmic precipitation of inorganic phosphate was seen only after EDTA-Tris-sucrose exposure.

The 5'-nucleotidases (uridine diphosphate sugar hydrolases) of various members of the *Enterobacteriaceae* have been extensively studied in this laboratory (7-10). Studies utilizing the technique of osmotic shock (12, 13) have shown that this enzyme in *Escherichia coli* could be considered to be located at the surface of the cell (7). Although studies of the localization of alkaline phosphatase in *E. coli* (1, 3) have placed alkaline phosphatase in the periplasmic space, no studies have been done on the other degradative enzymes discovered in this location by Neu and Heppel (13). The purpose of this paper is to document the histochemical electron microscopic location of the 5'-nucleotidase of *E. coli* at the cell surface.

MATERIALS AND METHODS

Organisms and culture conditions. *E. coli* K37₂ and *E. coli* K-12 were grown in the previously described phosphate medium (12) to approximately 5×10^8 cells. *E. coli* U24 (alkaline phosphatase-deficient) was grown in a tris(hydroxymethyl)aminomethane (Tris)-based medium (12).

Osmotic shock. The previously described (11) technique of osmotic shock for exponential-phase cells was employed.

Enzyme assays. Assays for 5'-nucleotidase, 3'-nucleotidase, and inorganic pyrophosphatase have been published (7).

Enzyme incubation. Washed *E. coli* was incubated for 30 min in a mixture of the following composition: 5 μ g of 5'-adenosine monophosphate (AMP) per ml, 0.08 M Tris-maleate (pH 7.2), 60 mg of PbNO₃ per ml, and 0.01 M MgSO₄ in a final volume of 50 ml. After incubation, cells were washed with Tris-maleate buffer and fixed as noted.

Electron microscopy. The methods for fixation and embedding were those described by Kellenberger et al.

(2) and modified by Margaretten et al. (6). However, phosphate buffers were replaced with Tris-maleate. Cells were incubated with substrate and then fixed in 1% glutaraldehyde. The specimens were washed four times with Tris-maleate buffer (pH 7.2) and then immersed in 1% osmium tetroxide-Veronal acetate (pH 7.35) for 3 hr at 4 C. The specimens were removed and washed four times with Tris-maleate buffer; 2% agar was added to each specimen as a matrix. Specimens were chilled, dehydrated by 10-min passages through increasing concentrations of alcohol, and finally immersed in propylene oxide-Epon mixture (1:1) and embedded. Variations in the technique of preparation are noted below, since it was essential to determine whether differences in technique would affect the location of the 5'-nucleotidase. The specimen blocks were cut on a Porter-Blum automatic microtome (model MT-2), and affixed to Formvar-treated copper grids. The grids were stained with uranyl acetate alone and with uranyl acetate and lead citrate. For the electron photomicrographs, we used a Siemens IA microscope at 80 kv.

RESULTS

General observations. Prefixation of bacteria with 1% glutaraldehyde prevented the hydrolysis of 5'-adenosine monophosphate (AMP) so that precipitation of lead phosphate was not obtained. This contrasts with the studies of Voelz (15) on adenosine triphosphatase, in which the activity of adenosine triphosphatase was unaffected by prefixation with glutaraldehyde. For this reason, samples were incubated with substrate and then fixed.

Intact bacteria. Figure 1 shows that the hydrolysis of 5'-AMP, as evidenced by the precipitation of lead phosphate, is limited to the cell wall. Lead precipitation was specific for 5'-nucleoti-

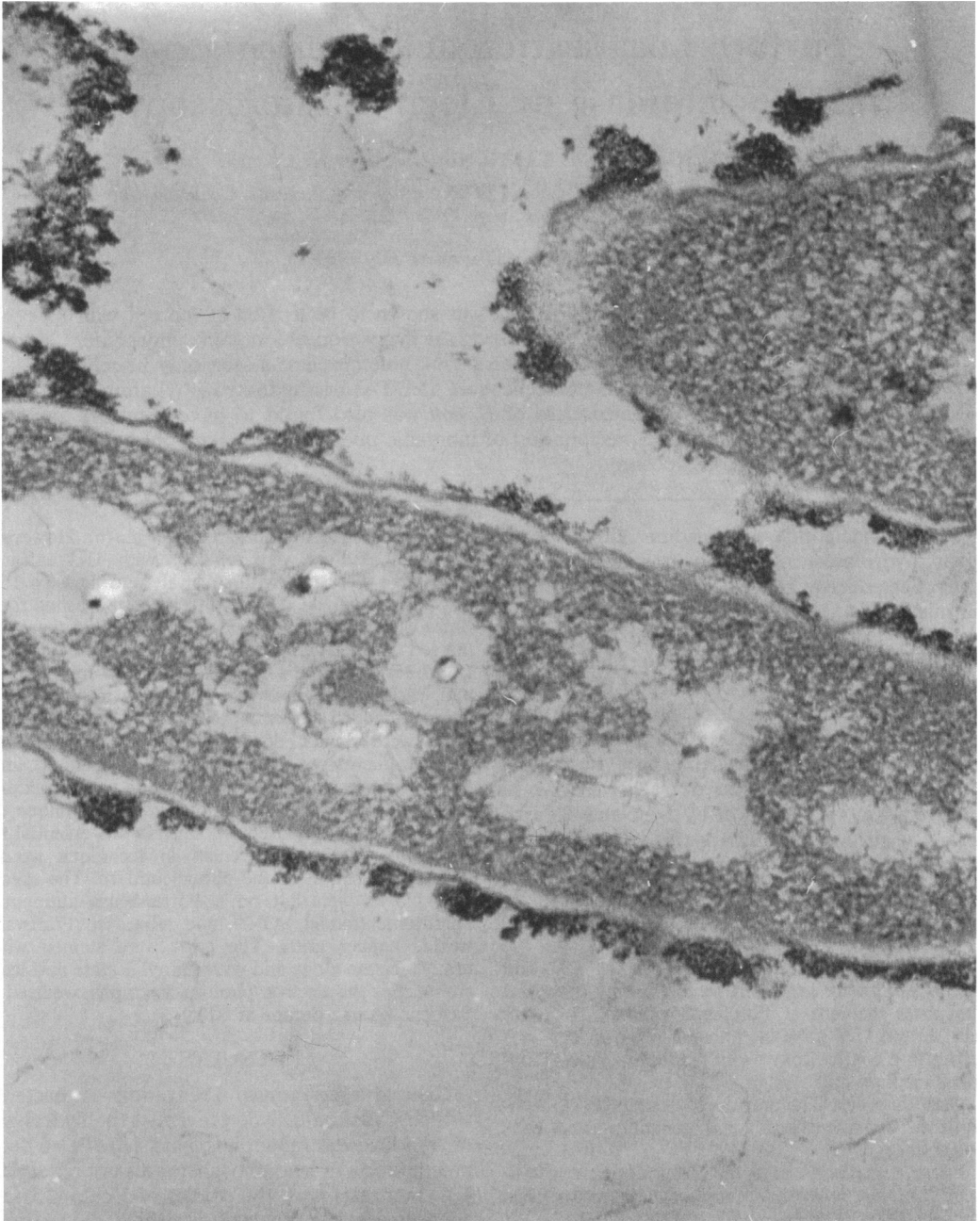


FIG. 1. Precipitation of lead phosphate at the cell wall of *E. coli* U24 (mutant lacking alkaline phosphatase). $\times 80,000$.

dase, since incubation of cells with lead nitrate alone did not result in deposits on the surface of the bacteria.

Penetration of nucleotides into intact bacteria does not occur (11). Treatment with ethylenediaminetetraacetic acid (EDTA) in the presence of

Tris buffer alters cell permeability (5, 11) without loss of viability of the organisms. *E. coli* K37₂ was exposed to EDTA in the following manner. Washed bacteria, 10^9 organisms, were suspended in 0.01 M Tris-hydrochloride (pH 7.2), 0.1 mM EDTA at 37 C for 4 min. The cells were rapidly

centrifuged, and the pellet of cells was washed with 1 mM $MgCl_2$ to prevent chelation of the lead. As Fig. 2 demonstrates, hydrolysis of 5'-AMP occurred only at the surface of the cell. A variety of treatments of cells after EDTA exposure all

produced the same location of enzymatic activity (Fig. 3).

Bacteria after exposure to sucrose-Tris-EDTA. Studies from our laboratory (12) have demonstrated that 5'-nucleotidases and 3'-nucleotidases



FIG. 2. Unstained preparation of *E. coli* K-12 showing precipitation of lead phosphate around the cell. No penetration has occurred. $\times 60,000$.

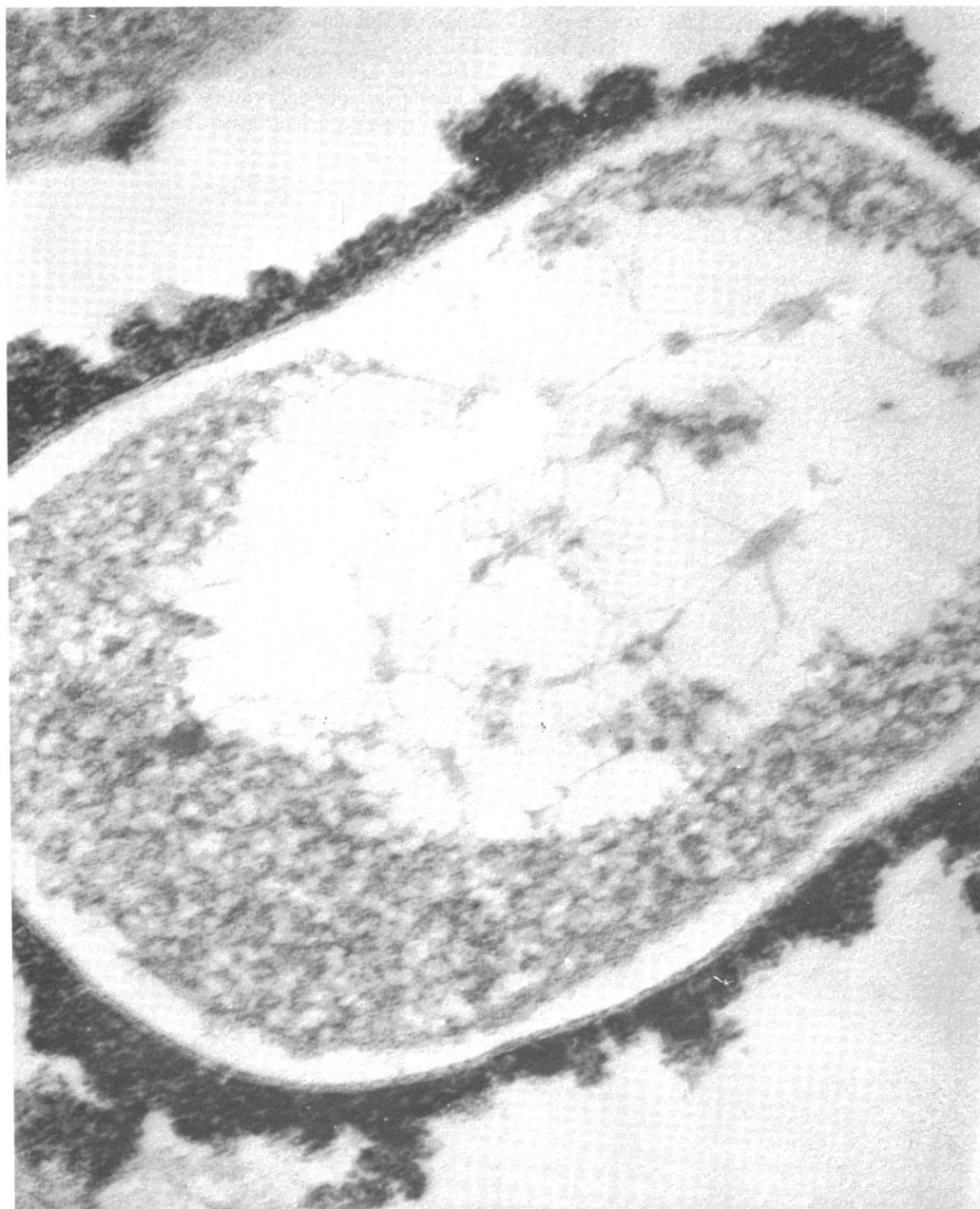


FIG. 3. *E. coli* U24 treated with EDTA before incubation with substrate and then incubated with 5'-AMP. No precipitation is seen in the periplasmic space or within the cell.

are not released from cells during incubation in 0.5 M sucrose in the presence of EDTA and Tris. An osmotic transition is required. Electron photomicrographs of *E. coli*, after the sucrose-Tris-EDTA, exposure, revealed some cells with precipitate beneath the cell wall but external to the

plasma membrane (Fig. 4). No cytoplasmic penetration of precipitate was seen.

Cells after osmotic shock. After osmotic shock in which most of the 5'-nucleotidase was released (Table 1), virtually no precipitation of lead phosphate occurred, indicating the absence of the

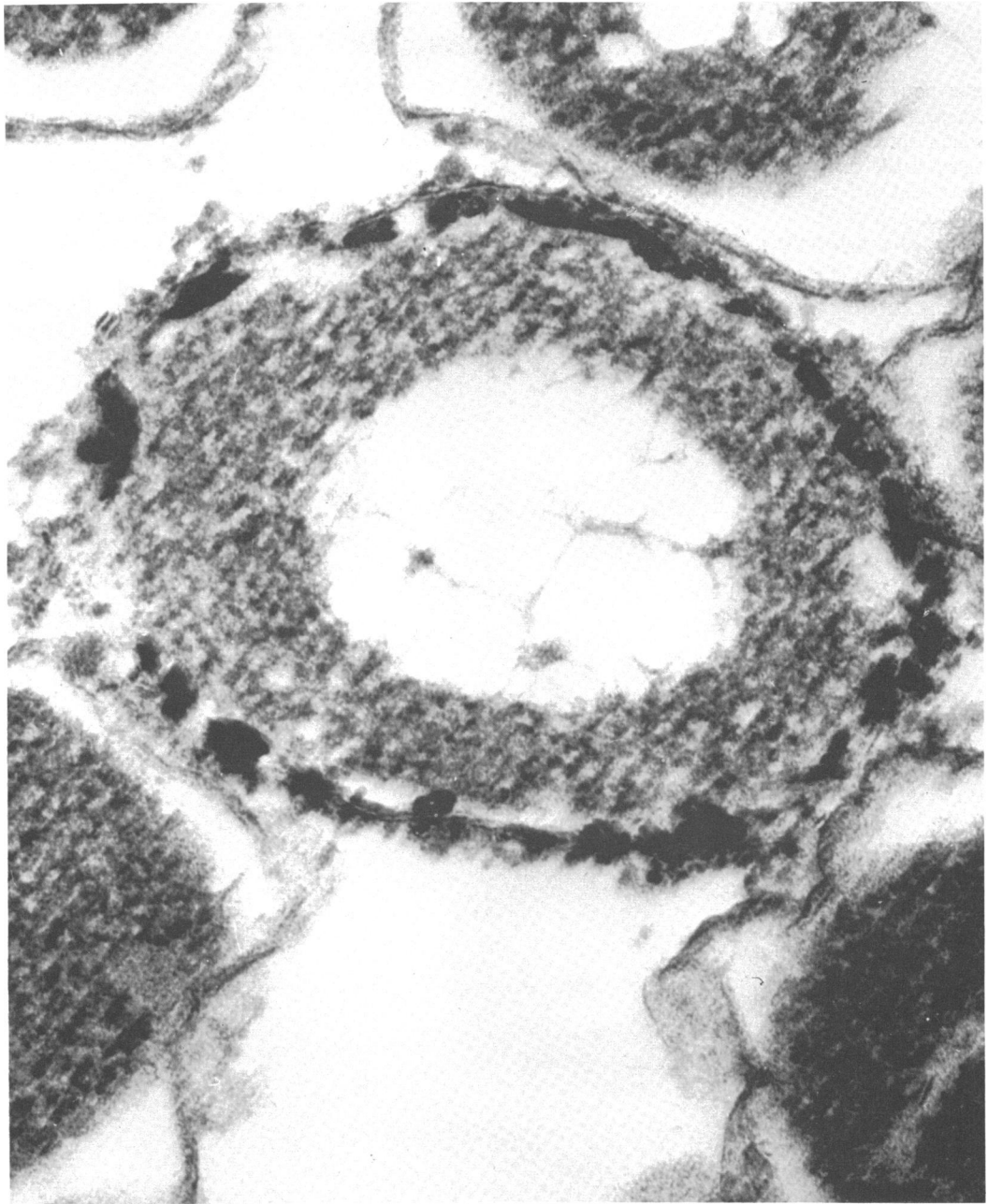


FIG. 4. *E. coli* K37 treated with EDTA-Tris-sucrose. Precipitation of lead phosphate is within the periplasmic space. $\times 100,000$.

TABLE 1. Release of 5'-nucleotidase from *E. coli* by osmotic shock^a

Material assayed	5'-Nucleotidase (units/ml)	3'-Nucleotidase (units/ml)	Inorganic pyrophosphatase (units/ml)	A ₂₆₀ ^b	Survival (%)
Sonic-treated cells.....	47	28	468		
Sucrose-Tris-EDTA.....	4	27	12	.285	92
Shock fluid.....	61	26	10	.327	91

^a *E. coli* K37₂ was subjected to osmotic shock by the usual technique. Enzyme assays were performed on a sonic extract of cells, sucrose-Tris-EDTA supernatant material, and the shock fluid.

^b Absorbancy at 260 nm.

enzyme (Fig. 5). About 5% of the cells showed a reaction indicative of 5'-nucleotidase activity, of which some showed the enzyme, as in Fig. 4, beneath the cell wall. This amount of activity is consistent with the enzyme left in cells which were not fully shocked.

Penicillin spheroplasts of *E. coli*. Spheroplasts

of *E. coli* U24 were prepared with penicillin by Lederberg's method (4). By using a technique of Voelz (14) to fix the cells, we stabilized the cells in 20% sucrose during incubation and treatment. The 5'-nucleotidase remained surface-localized with some penetration in cell wall defect areas. This is consistent with the failure of penicillin

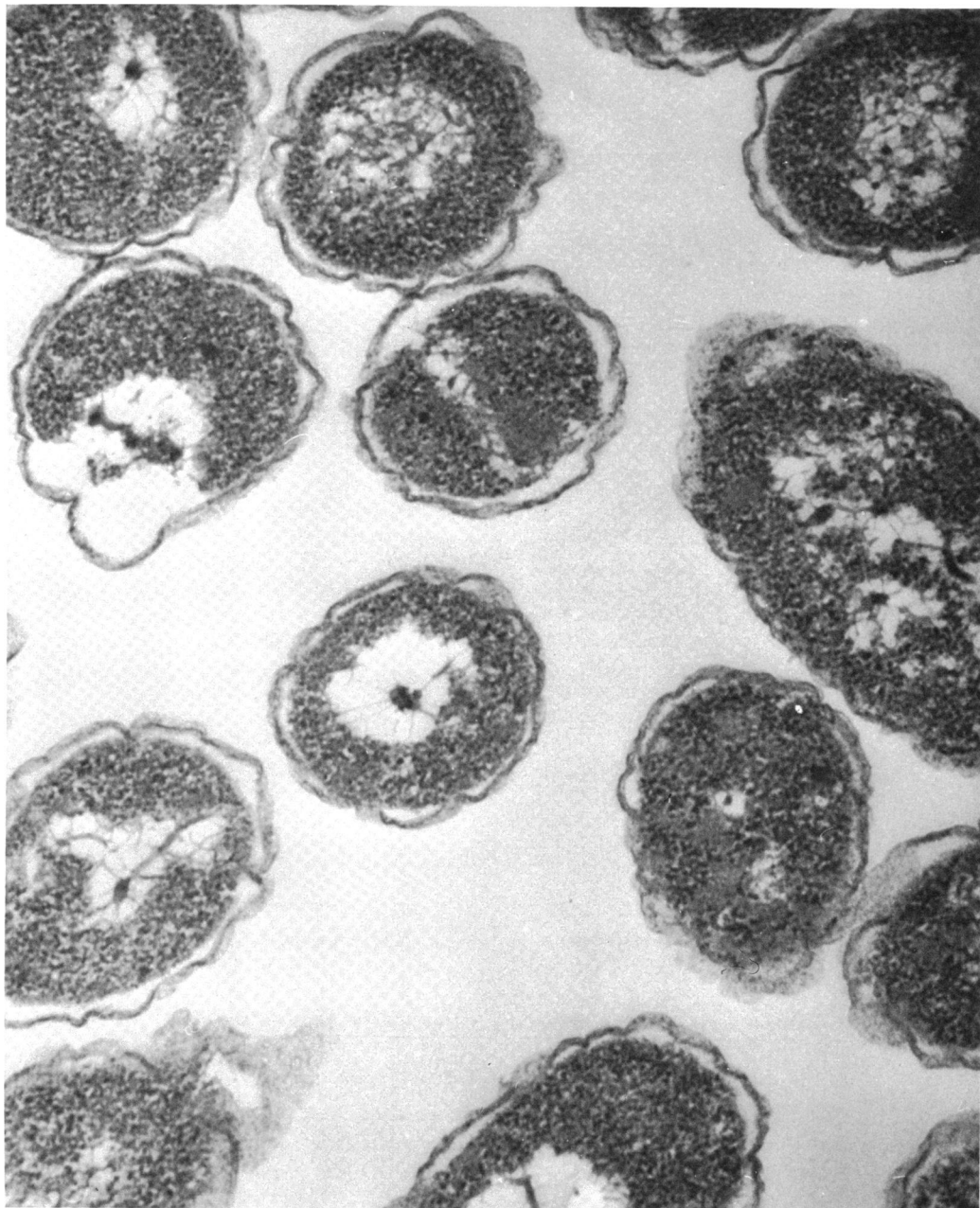


FIG. 5. Cells after osmotic shock treatment. Note absence of lead staining. $\times 60,000$.

protoplasts of *E. coli* to release 5'-nucleotidase into the stabilizing medium (7).

Experiment with 3'-nucleotidase. The enzyme cyclic phosphodiesterase (3'-nucleotidase; 10) was studied by techniques identical to those for 5'-nucleotidase by using 3'-AMP as the substrate. Figure 6 demonstrates surface precipitation of lead phosphate in intact cells and the internal penetration after exposure to sucrose-Tris-EDTA. As with the 5'-nucleotidase, no enzymatic activity was seen after osmotic shock.

Cryostat sections. Spicer, Wetzel, and Heppel (Fed. Proc. 25: 539, 1966) have used cryostat sections of bacteria in their localization of *E. coli* alkaline phosphatase and cyclic phosphodiesterase. We prepared cryostat sections and looked for both 5'-nucleotidase and 3'-nucleotidase localization. In both cases in untreated cells, the major activity was localized at the cell surface. We could not show localization of enzyme in the periplasmic space in cells that had not been exposed to EDTA.

DISCUSSION

Although the accumulated biochemical data suggest that alkaline phosphatase (1, 3, 13), 5'-nucleotidases (7, 9), and cyclic phosphodiesterases (3'-nucleotidases; 10) are so-called "surface enzymes," histochemical, electron microscopic

data have been published only for alkaline phosphatase (1, 3). The precise localization of alkaline phosphatase, however, is still not absolutely clear. The enzyme could be bound to the cell wall, in the cell wall, in the periplasmic space, or bound to the cytoplasmic membrane. Each group of investigators has made different observations, so that it remains unclear whether alkaline phosphatase is bound to the outer or inner aspect of the cell wall.

According to our studies, 5'-nucleotidase appears to be bound to the surface of the cell in intact organisms. It is only when the permeability barrier of the cell wall is altered by EDTA that it is possible to observe the enzyme in the periplasmic space.

This enzyme could not be recognized within the cell by the histochemical technique because of the presence of the intracellular protein inhibitor of 5'-nucleotidase activity (8).

Preliminary studies on the location of the cyclic phosphodiesterase (3'-nucleotidase) suggest that it is also located at the cell surface. Spicer, Wetzel, and Heppel (Fed. Proc. 25: 539, 1966) have stated that this enzyme is located in the periplasmic space. We have not been able to confirm this. The absence of an intracellular inhibitor should have allowed us to observe the enzyme within the cell after EDTA alteration of permeability. Since this did not occur and since

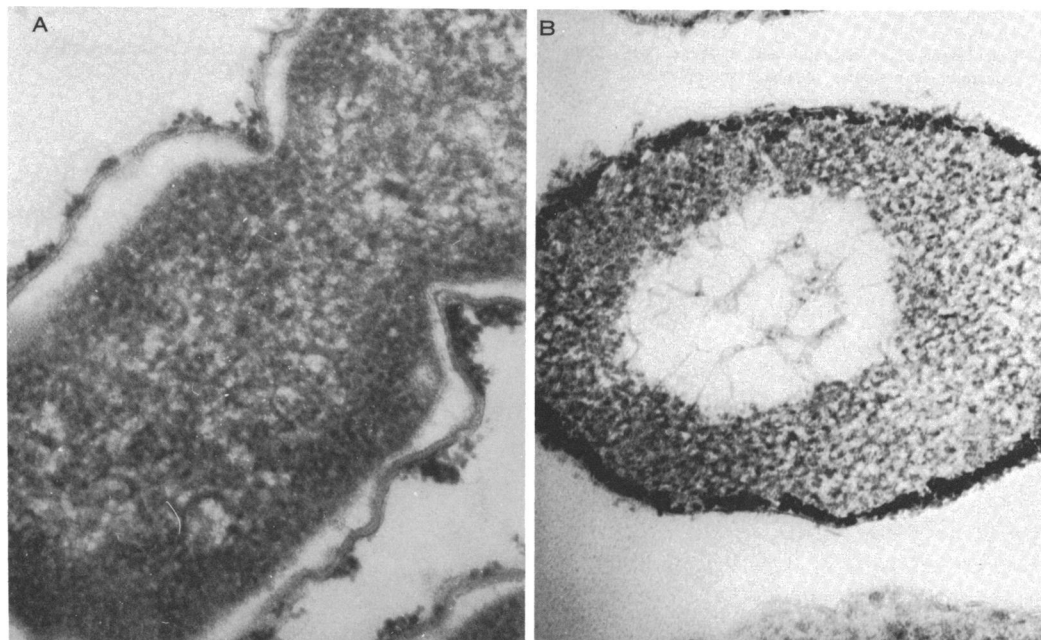


FIG. 6. *E. coli* U24. (A) Surface precipitation of lead phosphate after incubation with 3'-AMP. (B) Penetration of lead phosphate into the periplasmic space after exposure to EDTA, Tris, and sucrose before osmotic shock. $\times 60,000$.

only selected cells showed the enzyme in the periplasmic space and never within the cell, we conclude that bacterial nucleotidases are truly surface enzymes.

The mechanism by which these enzymes remain cell-bound remains obscure. The forces cannot be purely cationic since the enzymes are not released by simple chelation. Attempts to elute these enzymes with high ionic strength buffers have also been unsuccessful (12).

Currently we are attempting to prepare a ferritin-labeled antibody which may offer further insight into the precise location of the cell surface. But here again penetration of the antibody below the cell wall is unlikely in view of findings in *Salmonella* (14).

This study suggests that 5'- and 3'-nucleotidases are attached to the outer aspect of the cell wall and can enter the periplasmic space if permeability of the cell is altered, but the enzymes do not enter the interior of the cell.

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