

Tris(hydroxymethyl)aminomethane Buffer Modification of *Escherichia coli* Outer Membrane Permeability

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The effect of tris(hydroxymethyl)aminomethane (Tris) buffer on outer membrane permeability was examined in a smooth strain (D280) and in a heptose-deficient lipopolysaccharide strain (F515) of *Escherichia coli* O8. Tris buffer (pH 8.00) was found to increase outer membrane permeability on the basis of an increased V_o of whole-cell alkaline phosphatase activity and on the basis of sensitivity to lysozyme and altered localization pattern of alkaline phosphatase. The Tris buffer-mediated increase in outer membrane permeability was found to be dependent upon the extent of exposure to and concentration of the Tris buffer. The Tris buffer effects were demonstrated not to be due to allosteric activation of cell-associated alkaline phosphatase and were specific for Tris buffer. Exposure of cells to Tris resulted in the release of a limited amount of cell envelope components. Investigators utilizing Tris buffer are cautioned that Tris is not physiologically inert and that it may interact with the system under investigation.

Tris buffer has been widely used in studies concerning the gram-negative cell envelope, with the tacit assumption that the buffer is physiologically inert, although Voss (16) presented evidence that Tris is not. We have investigated the effect of Tris buffer on the outer membrane of *Escherichia coli*, particularly in terms of outer membrane permeability.

E. coli synthesizes alkaline phosphatase (EC 3.1.3.1) under phosphate-limiting conditions (3). The phosphatase is exclusively localized in the periplasmic space of wild-type *E. coli* (2, 8), although both cell surface localization and periplasmic localization are found in deep-rough (heptose-deficient lipopolysaccharide [LPS]) strains (6, 8, 9). Whole-cell alkaline phosphatase activity is "cryptic" (4, 12, 16) owing to the limited penetrability of *p*-nitrophenyl phosphate through the outer membrane (4, 12). Hassan (4) has utilized the crypticity of cell-associated alkaline phosphatase as a monitor of outer membrane permeability changes in a marine pseudomonad.

We utilized whole-cell alkaline phosphatase activity as a monitor of outer membrane permeability changes due to exposure of cells to Tris buffer. These changes were detected after exposure of whole cells to Tris buffer. It is thus concluded that Tris buffer is not physiologically inert.

MATERIALS AND METHODS

The bacterial strains utilized were *E. coli* O8 strain D280 (11), which possesses a wild-type LPS, and strain

F515 (14), which possesses a heptose-deficient, chemotype Re LPS and bears the genetic markers *pro his met mtl Str^r F⁻*. Strain F515 was indirectly obtained from strain D280 through successive mutagenesis. Both strains were the generous gift of Günter Schmidt, Max-Planck-Institut für Immunbiologie, Freiburg, West Germany.

Cells were grown in phosphate-limiting medium that consisted of 0.05 M Tris-hydrochloride buffer, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH₄Cl, 0.003 M Na₂SO₄, 0.01 M MgCl₂, 2×10^{-4} M CaCl₂, 2×10^{-6} M ZnCl₂, and 0.5% (wt/vol) peptone (Difco Laboratories), adjusted to pH 7.5 and supplemented with 50 µg each of L-proline, L-methionine, and L-histidine per ml, 10^{-5} M inosine, and 0.5% glucose as a carbon source.

Single colonies of the strains under investigation were used to inoculate 10 ml of phosphate-limiting medium in an Erlenmeyer flask (50 ml) and were grown at 35°C in a G25 controlled-environment rotary shaker-incubator (New Brunswick Scientific Co.) at 150 rpm for 12 h. This culture provided a 2% inoculum for the experimental culture, which was grown under identical conditions for 8.5 h before harvesting.

Effect of Tris buffer on whole-cell alkaline phosphatase activity. A cell suspension was prepared by harvesting 500 ml of an 8.5-h culture by centrifugation ($5,000 \times g$ for 30 min at 4°C) and suspending the cell pellet in cold (4°C) water. The cell suspension was split into two aliquots and pelleted by centrifugation ($12,000 \times g$ for 10 min at 4°C), and the water was aspirated off. The cell pellets were then suspended in cold (4°C) Tris buffer (pH 8.00) containing 50 µg of chloramphenicol per ml. Whole-cell alkaline phosphatase activity was then determined directly (within 30 s of exposure to the Tris buffer), after a 3-h exposure to Tris buffer, and after an 18-h exposure to Tris buffer at 4°C in the presence of chloramphenicol. The time-dependent nature of Tris buffer effects on the activity was determined in 0.1 M Tris buffer (pH 8.00) containing 50 µg of chloramphenicol per ml.

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Whole cells were suspended in cold (4°C) Tris buffer, and alkaline phosphatase activity was determined every 5 min over a period of ~3 h.

Effect of Tris buffer on pure alkaline phosphatase and outer membrane-associated alkaline phosphatase. Purified alkaline phosphatase (Sigma Chemical Co.) and outer membrane-associated alkaline phosphatase (R. T. Irvin et al., manuscript in preparation) were diluted into cold (4°C) Tris buffer (pH 8.00) of various concentrations containing 50 µg of chloramphenicol per ml. Alkaline phosphatase activity was then determined directly.

Effect of Tris buffer on lysozyme sensitivity of the smooth strain. Portions (10 ml) of an 8.5-h culture of *E. coli* O8 strain D280 grown in phosphate-limiting medium were pelleted by centrifugation (12,000 × *g* for 10 min at 4°C), and the cells were suspended in 10 ml of either water or 0.1 M Tris buffer (pH 8.00). To each of these cell suspensions 10 mg of lysozyme (Sigma) was added, and after a 20-min incubation fractions were examined by phase microscopy for spheroplasting. The lysozyme-cell-water suspension was then split into two 5-ml samples, and 0.5 ml of 1 M Tris buffer (pH 8.00) was added to one sample and 0.5 ml of water was added to the other. The extent of spheroplasting was determined by phase microscopy.

Assays utilized. Alkaline phosphatase activity was determined at room temperature by following the production of *p*-nitrophenol at 420 nm, using a Unicam SP 1800 double-beam spectrophotometer equipped with a Unicam AR 25 recorder. The assay mixture utilized was that of Garen and Levinthal (3), except that the Tris buffer concentration was 0.1 M rather than 1 M.

Protein concentration was determined by the method of Lowry et al. (7), employing bovine serum albumin (Sigma) as a standard.

LPS was determined spectrophotometrically by assaying for 2-keto-3-deoxyoctonate, employing the thiobarbituric assay of Weissbach and Hurwitz (18) and using 2-keto-3-deoxyoctonate (Sigma) as a standard.

Electron microscopy. Samples were prepared for transmission electron microscopy as previously described (5) and were examined with an AEI 801 electron microscope operating at an accelerating potential of 60 kV.

RESULTS

Previous investigation had revealed that whole-cell alkaline phosphatase activity of *E. coli* O8 maintained in 0.1 M Tris buffer (pH 8.00) increases over the time course of the experiment (10). The effect of Tris buffer (pH 8.00) on whole-cell alkaline phosphatase activity was therefore investigated.

E. coli O8 strains D280 (wild type) and F515 (heptose-deficient LPS) were grown under phosphate-limiting conditions to derepress the synthesis of alkaline phosphatase (3) and were harvested in the late log phase by centrifugation when the cell-associated alkaline phosphatase

specific activity of both strains was maximal (8). The time-dependent activation of whole-cell alkaline phosphatase activity due to exposure of cells to Tris buffer was examined by suspending harvested cells in cold (4°C) 0.1 M Tris buffer (pH 8.00) containing 50 µg of chloramphenicol per ml and subsequently following whole-cell alkaline phosphatase activity as a function of time after exposure to Tris buffer. The activity for both strain D280 (smooth) and strain F515 (deep-rough) increased in a time-dependent manner after exposure to 0.1 M Tris buffer (pH 8.00). The rate of increase of the whole-cell alkaline phosphatase activity of strain F515 (deep-rough) plateaued after ~1.5 h, whereas that for strain D280 (smooth) did not plateau until after ~2.5 h (Fig. 1).

The whole-cell alkaline phosphatase activity of both strain D280 and strain F515 was found to be activated in a concentration-dependent manner by Tris buffer (pH 8.00) (Table 1). The Tris buffer activation of whole-cell alkaline phosphatase activity of strain D280 (smooth) was quite significant, as whole-cell alkaline phosphatase activity was increased ~33-fold by 1.5 M Tris buffer (pH 8.00) relative to that of cells maintained in water and ~25-fold relative to that of cells exposed to 0.1 M Tris buffer (pH 8.00) (Table 1). The whole-cell alkaline phosphatase activity of strain F515 (deep-rough) was increased ~57-fold by 1.5 M Tris buffer (pH 8.00) relative to that of cells maintained in water and ~33-fold relative to that of cells exposed to 0.1 M Tris buffer (pH 8.00) (Table 1).

The data contained in Table 1 may be plotted as a modified double-reciprocal plot, i.e., $1/V_0$ of the whole-cell alkaline phosphatase activity versus $1/\text{Tris buffer (pH 8.00) concentration}$. The

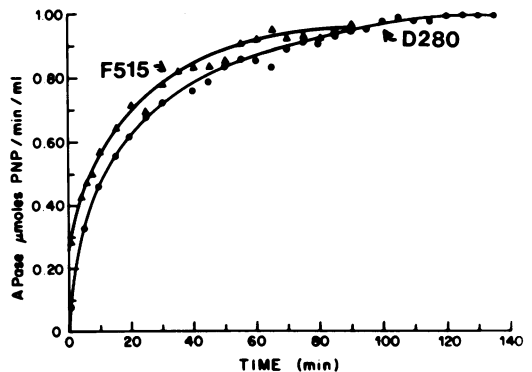


FIG. 1. Whole-cell alkaline phosphatase activity of *E. coli* O8 strains D280 and F515 as a function of time of exposure to cold (4°C) 0.1 M Tris buffer (pH 8.00) containing 50 µg of chloramphenicol per ml. PNP, *p*-Nitrophenol.

TABLE 1. Dependence of whole-cell alkaline phosphatase activity of *E. coli* O8 strains D280 and F515 on Tris buffer concentration

[Tris] ^a (mol/liter)	Whole-cell alkaline phosphatase activity ^b	
	Strain D280	Strain F515
0 ^c	0.03	0.04
0.1	0.04	0.07
0.5	0.13	0.15
1.0	0.83	1.49
1.5	1.00	2.28

^a Tris, Cold (4°C) Tris buffer, at pH 8.00, containing 50 μ g of chloramphenicol per ml.

^b Whole-cell alkaline phosphatase activity was determined immediately after suspension of cells in the appropriate Tris buffer (assayed <1 min after original exposure to Tris). Expressed in micromoles of *p*-nitrophenol produced per minute per milliliter.

^c Water containing 50 μ g of chloramphenicol per ml and maintained at 4°C.

modified double-reciprocal plot may then be utilized to determine the concentration of Tris buffer at which there is half-maximal activation of whole-cell alkaline phosphatase activity, i.e., the apparent K_a for Tris buffer. The modified double-reciprocal plot indicates that the apparent K_a of Tris buffer is ~1 M for strain D280 (smooth) and ~1.2 M for strain F515 (deep-rough) (Fig. 2). The modified double-reciprocal plots for both strains have a break in linearity at ~1 M Tris concentrations (Fig. 2).

Outer membrane-associated and pure alkaline phosphatase were slightly activated by Tris buffer (pH 8.00), with apparent K_a values of 0.35 and 0.40 M, respectively (Fig. 3 and 4). However, neither the outer membrane-associated alkaline phosphatase nor the pure alkaline phosphatase demonstrated an upward break at the 1 M Tris buffer concentration in $1/V_o$ versus $1/[Tris]$ plots (Fig. 3 and 4), but both exhibited a downward break in the $1/V_o$ versus $1/[Tris]$ plots which the whole-cell alkaline phosphatase did not demonstrate (cf. Fig. 2 with Fig. 3 and 4).

The K_m for *p*-nitrophenyl phosphate of the whole-cell alkaline phosphatase activity of strain F515 (deep-rough) maintained in water was 21 mM, whereas that for cells of strain F515 (deep-rough) maintained in 0.1 M Tris buffer (pH 8.00) was 5.6 mM. The K_m for *p*-nitrophenyl phosphate of pure alkaline phosphatase was found to be 2.4 mM when the enzyme was maintained in 0.1 M Tris buffer (pH 8.00) and 0.8 mM when the enzyme was maintained in 2.0 M Tris buffer (pH 8.00).

Gomori end-product localization of alkaline phosphatase in strain D280 (smooth) revealed that before exposure to 0.1 M Tris buffer (pH 8.00), alkaline phosphatase was exclusively lo-

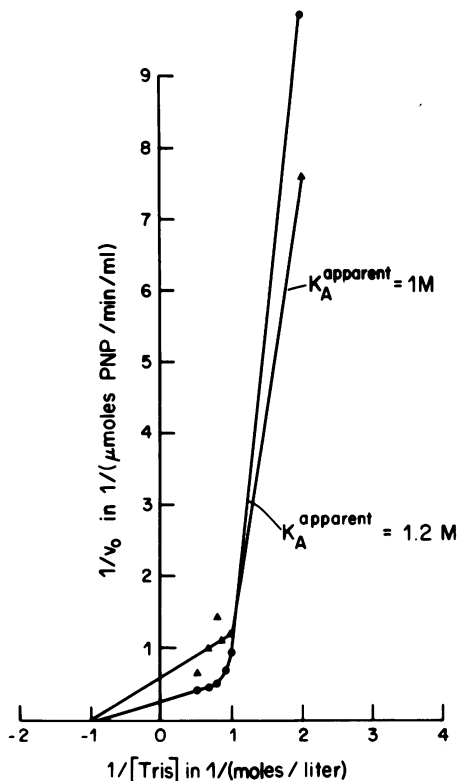


FIG. 2. Modified double-reciprocal plot of Tris buffer (pH 8.00) activation of whole-cell alkaline phosphatase activity of *E. coli* O8 strains D280 (●) and F515 (▲). The plot is the reciprocal of the V_o of the whole-cell alkaline phosphatase activity (determined <1 min after exposure to Tris buffer) versus the reciprocal of the Tris buffer (pH 8.00) concentration. PNP, *p*-Nitrophenol.

calized within the periplasmic space (Fig. 5 and 7) but that subsequent to exposure to 0.1 M Tris buffer (pH 8.00) for 20 min, alkaline phosphatase was localized on the cell surface and within the periplasmic space (Fig. 6 and 7). This alteration in localization pattern of alkaline phosphatase was accompanied by an increased sensitivity to lysozyme. Cells of strain D280 (smooth) maintained in a lysozyme-water solution were insensitive to the lysozyme, whereas cells maintained in 0.1 M Tris buffer (pH 8.00) were sensitive to lysozyme and formed spheroplasts. Cells of strain F515 (deep-rough) were found to be sensitive to lysozyme even when maintained in water, confirming previous results (10).

Exposure of cells to 0.1 M Tris buffer (pH 8.00) releases protein, LPS, and alkaline phosphatase from the cell envelope of strain D280 (smooth) and strain F515 (deep-rough) in the absence of cell lysis (no cell lysis as determined

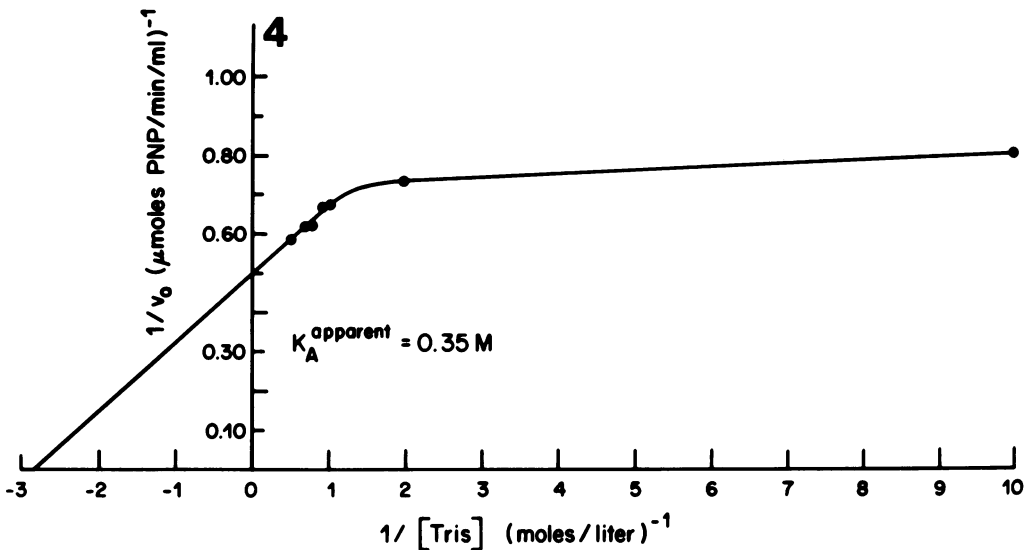
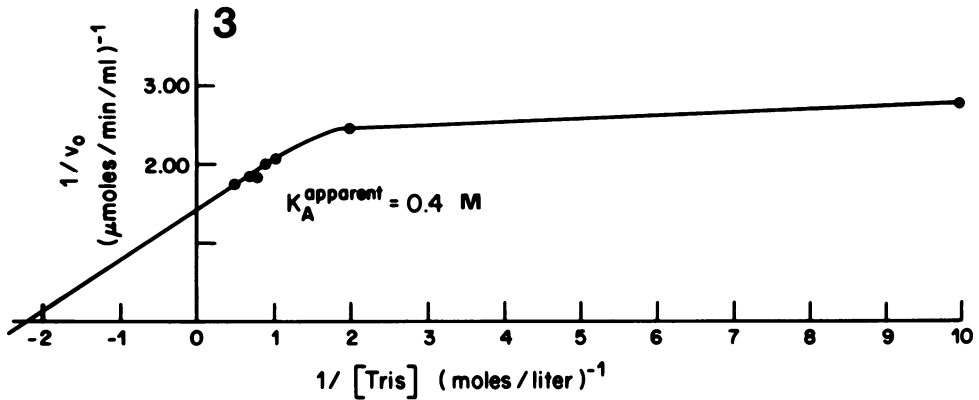


FIG. 3. Modified double-reciprocal plot of Tris buffer (pH 8.00) activation of purified alkaline phosphatase.

FIG. 4. Modified double-reciprocal plot of Tris buffer (pH 8.00) activation of an outer membrane component-associated alkaline phosphatase. The apparent activation constant for Tris buffer (pH 8.00) was found to be 0.35 M Tris.

by absence of glucose-6-phosphate dehydrogenase activity, phase microscopy, and electron microscopy). Strain F515 (deep-rough) was found to release 10-fold-more alkaline phosphatase, 2-fold-more LPS, and equivalent amounts of protein as compared with strain D280 (smooth) (Table 2).

DISCUSSION

We investigated the effects of Tris buffer (pH 8.00) on the permeability of the outer membrane in a smooth strain and in a deep-rough (heptose-deficient LPS) strain of *E. coli* O8, utilizing the cryptic nature of whole-cell alkaline phosphatase activity (4, 10, 12, 15). We have previously demonstrated that both strains of *E. coli* O8 utilized in the present investigation have inactive monomers of alkaline phosphatase present in their cell envelopes (9). Experimental conditions were chosen such that dimerization of inactive monomers to form active alkaline phosphatase (3, 13) would be highly unlikely (4°C and short exposure times [<1 min]). Chloramphenicol was added (50 $\mu\text{g}/\text{ml}$) to the buffers to inhibit *de novo* synthesis of alkaline phosphatase, as both strains of *E. coli* O8 have previously been shown to be very sensitive to chloramphenicol (10). Thus, it is highly unlikely that the

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reported activation of whole-cell alkaline phosphatase activity was due to dimerization of inactive monomers to form active alkaline phosphatase or due to de novo synthesis of alkaline phosphatase.

Whole-cell alkaline phosphatase activity is clearly activated in a time-dependent manner by 0.1 M Tris buffer (pH 8.00) in both the smooth strain and the deep-rough strain (Fig. 1). The differences in the time course of activation in the wild-type strain and in the heptose-deficient LPS strain may be due, in part, to differences in the localization of alkaline phosphatase activity within the cell envelope (8).

The extent of activation of whole-cell alkaline phosphatase activity in both strains was demonstrated to be dependent upon the Tris buffer concentration (Table 1). The activation for *E. coli* O8 strain D280 (smooth) by 1.5 M Tris buffer (pH 8.00) was 33-fold (relative to that of cells maintained in water), whereas that for strain F515 (deep-rough) was activated 57-fold by 1.5 M Tris buffer (relative to that of cells maintained in water) (Table 1). Modified double-reciprocal plots of $1/V_o$ of the whole-cell alkaline phosphatase activity versus $1/\text{Tris}$ buffer concentration revealed that the apparent K_a of Tris buffer for the wild-type strain (D280) was ~ 1 M Tris and for the heptose-deficient LPS strain (F515) was ~ 1.2 M Tris (Fig. 2) and also exhibited an upward break in the plot at ~ 1 M Tris for both strains (Fig. 2). To determine whether the observed activation of whole-cell alkaline phosphatase activity was due to an unreported allosteric activation of alkaline phosphatase when associated with the cell envelope, the effect of Tris on purified alkaline phosphatase (Sigma) and on outer membrane component-associated alkaline phosphatase obtained from strain F515 (R. T. Irvin, Ph.D. thesis, University of Calgary, Calgary, Alberta, Canada, 1977) was investigated. Both the purified alkaline phosphatase and the outer membrane component-associated alkaline phosphatase were slightly activated by Tris buffer, with apparent K_a values of ~ 0.4 and ~ 0.35 M Tris, respectively (Fig. 3 and 4). More significantly, the modified double-reciprocal plots for both pure alkaline phosphatase and outer membrane component-associated alkaline phosphatase were linear through the 1 M Tris buffer concentration region but demonstrated a downward break in the plot at ~ 1.5 M Tris, an observation not noted for cell-associated alkaline phosphatase (Fig. 2 to 4). Clearly, the activation of whole-cell alkaline phosphatase activity is not wholly due to allosteric activation of the enzyme by Tris, as the

kinetics for both purified and outer membrane-associated alkaline phosphatase are radically different.

Several investigators have utilized K_m values for periplasmically localized enzymes obtained in whole-cell assays for establishing outer membrane permeability differences (1, 4, 12, 16). The K_m of whole-cell alkaline phosphatase activity of *E. coli* O8 strain F515 for *p*-nitrophenyl phosphate was substantially reduced by exposing cells to Tris buffer ranging from 21 to 5.6 mM. To ensure that the decreased K_m reflected a change in outer membrane permeability, the K_m of purified alkaline phosphatase for *p*-nitrophenyl phosphate was determined in various Tris buffer concentrations. Tris did alter the K_m of alkaline phosphatase, but the drastic alteration of the whole cell-alkaline phosphatase K_m by relatively low Tris concentrations could not be accounted for by allosteric modification of alkaline phosphatase by Tris.

The alteration of the V_o and K_m of cell-associated alkaline phosphatase activity by Tris buffer was accompanied by an alteration in the localization pattern of the alkaline phosphatase. Some of the previously periplasmically localized alkaline phosphatase of strain D280 (smooth) was subsequently found on the cell surface, and a very minor amount was found as cell-free enzyme after exposure to 0.1 M Tris buffer (pH 8.00) (Fig. 5 to 7; Table 3). The apparent outer membrane permeability to alkaline phosphatase correlated well with the observed sensitivity to lysozyme when cells of strain D280 (smooth) were maintained in 0.1 M Tris buffer (pH 8.00) and the insensitivity when cells were maintained in water. These obvious outer membrane permeability changes caused by exposure to 0.1 M Tris buffer (pH 8.00) were correlated with the release of a small amount of protein, LPS, and alkaline phosphatase from the cell envelope (Table 2). The morphological studies and the relatively minor quantities of cell envelope components released from the cell envelope argue against a release of large sections of outer membrane by Tris buffer.

The outer membrane permeability changes mediated by Tris buffer were independent of cell age (all studies reported were carried out on late-log phase cells because of the higher alkaline phosphatase activity and cell density). Outer membrane permeability could not be altered by use of cacodylate buffer (pH 8.00), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.00), borate buffer (pH 8.00), increased pH (pH 8.00 water), high salt (NaCl), or high osmotic strength (20% sucrose)

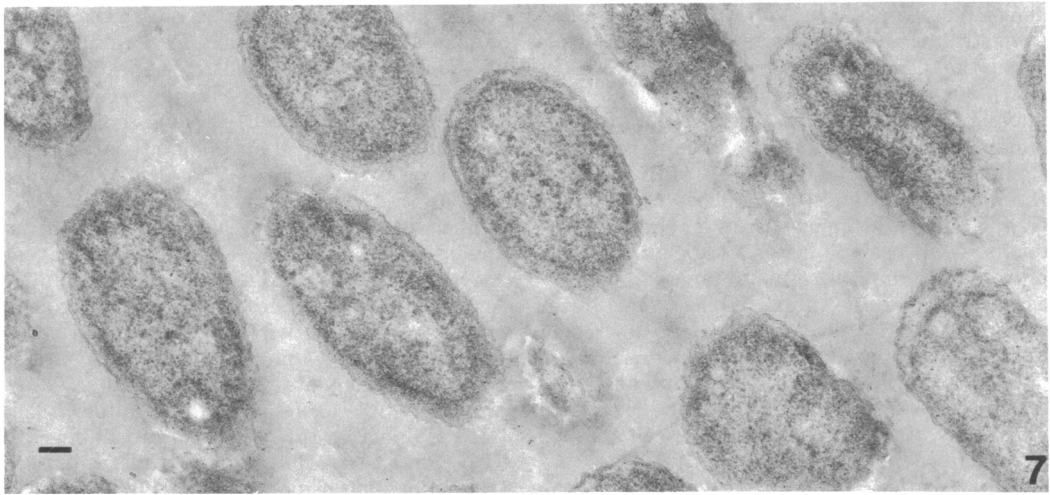
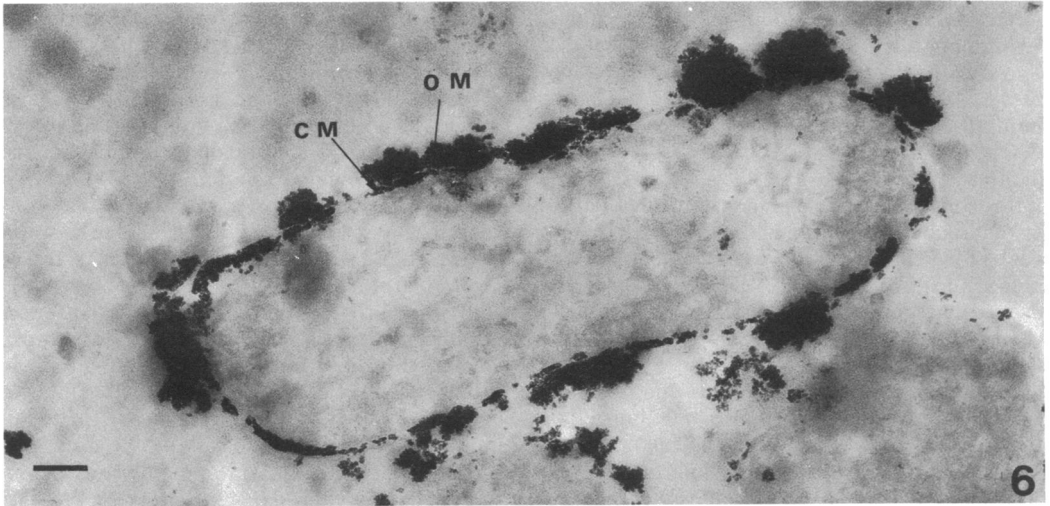
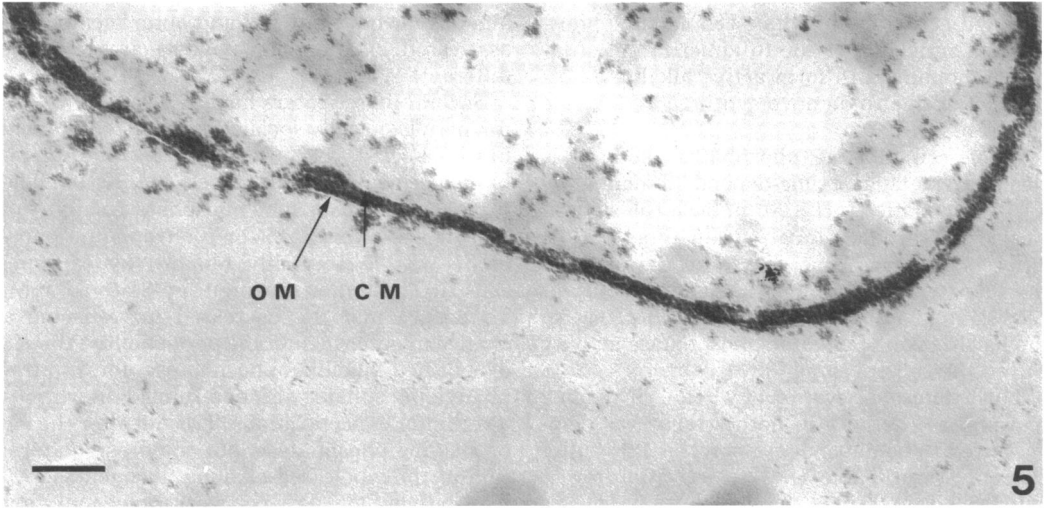


TABLE 2. Compositional analysis of material released from *E. coli* O8 strains D280 and F515 by exposure to 0.1 M Tris (pH 8.00) for 10 min at room temperature

Strain	Material released by Tris buffer		Alkaline phosphatase activity (μmol of PNP ^b /min per ml)
	Protein ($\mu\text{g}/\text{ml}$)	LPS (nmol of KDO ^a /ml)	
D280	168	5	0.017
F515	153	11	0.173

^a KDO, 2-Keto-3-deoxyoctonate.

^b PNP, *p*-Nitrophenyl.

in place of Tris. Thus, as previously indicated by Voss (17), the effect of Tris on the outer membrane appears to be highly specific. It seems likely that Tris alters outer membrane permeability by disrupting ionic interactions, as described by Schindler and Teuber (14).

ACKNOWLEDGMENTS

This investigation was supported by National Research Council of Canada grant A5731.

We greatly acknowledge the technical assistance of J. Lam and R. Chan.

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FIG. 5. Gomori end-product localization of alkaline phosphatase in cells of *E. coli* O8 strain D280 (smooth) that have not been exposed to Tris buffer. Note that the alkaline phosphatase is localized exclusively within the periplasmic space. OM, Outer membrane; CM, cytoplasmic membrane. The bar in this and subsequent micrographs represents 0.1 μm .

FIG. 6. Gomori end-product localization of alkaline phosphatase in cells of *E. coli* O8 strain D280 (smooth) that had been exposed to 0.1 M Tris buffer (pH 8.00) for 20 min. Note that the alkaline phosphatase is localized in the periplasmic space and on the surface of the outer membrane. OM, Outer membrane; CM, cytoplasmic membrane.

FIG. 7. Cells of *E. coli* O8 strain D280 that were incubated in Gomori end-product incubation mixture lacking *p*-nitrophenyl phosphate (the substrate for alkaline phosphatase). Note the absence of lead salts.