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Selective Release of Proteins from *Spirillum itersonii* by Tris(hydroxymethyl)aminomethane and Ethylenediaminetetraacetate

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Treatment of *Spirillum itersonii* with tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetate (EDTA) results in the quantitative release of alkaline phosphatase and ribonuclease into the surrounding medium. At the same time, about 90% of the total cellular soluble cytochrome *c* is liberated. This process occurs within 1 min of treatment at both 24 and 4 C. Release of these proteins by Tris-EDTA treatment is highly selective, since only 9% of the total cell protein is liberated, concomitantly with less than 5% ribonucleic acid, deoxyribonucleic acid, and malate dehydrogenase. Different sigmoidal curves are obtained for release of proteins as a function of EDTA concentration. The order of liberation with increasing EDTA is as follows: alkaline phosphatase, protein, soluble cytochrome *c*, and ribonuclease. Treatment of cells with Tris-EDTA under conditions which cause extensive loss of alkaline phosphatase, soluble cytochrome *c*, and ribonuclease results in cell death, with cessation of protein and ribonucleic acid synthesis. Cells treated with EDTA in phosphate buffer (in the absence of Tris) liberate a large portion of their soluble cytochrome *c*, but negligible amounts of alkaline phosphatase and ribonuclease. Addition of Tris to cells pretreated with phosphate-buffered EDTA releases high levels of alkaline phosphatase, but not ribonuclease. These results suggest that a common surface alteration is not solely responsible for release of periplasmic proteins. More likely, each protein of the periplasm is bound in an independent and specific manner.

Several techniques have been developed which selectively and quantitatively release a group of hydrolytic enzymes from *Escherichia coli*. When *E. coli* is converted into spheroplasts in a hyperosmolar solution containing Tris(hydroxymethyl) aminomethane (Tris), ethylenediaminetetraacetate (EDTA), and lysozyme, a number of degradative enzymes are liberated into the environment (33-35). A similar class of enzymes is released from cells by "osmotic shock," a process whereby Tris-EDTA-treated bacteria are subjected to an abrupt osmotic transition (35, 36). In addition to hydrolytic enzymes, several factors involved in active transport are liberated by the latter procedure (2, 3, 20, 37, 38, 43). These and other observations suggest that certain gram-negative bacterial enzymes are located in the periplasmic space (6, 11, 12), a region between the cytoplasmic membrane and the cell wall layers (29).

Treatment of *E. coli* with Tris-EDTA, without osmotic transition, does not result in significant release of periplasmic enzymes (21, 35). This technique, however, does liberate 30 to 50% of the cell wall lipopolysaccharide (22, 25) and results in a transient nonspecific increase in permeability (21, 24). Both Tris-EDTA treatment and "osmotic shock" do not significantly damage cellular metabolism, since treated cells remain viable (24) and regrow after a brief lag (4).

While investigating hemoprotein synthesis in *Spirillum itersonii*, it was noticed that a soluble form of cytochrome *c* was readily released from cells by Tris-EDTA treatment. Since studies on the selective release of proteins from gram-negative bacteria and resulting physiological alterations caused by such treatments have been limited to the *Enterobacteriaceae* (31), it seemed worthwhile to study these relationships in the unrelated gram-negative bacterium, *S. itersonii*.

MATERIALS AND METHODS

Materials. Sodium α-naphthyl acid phosphate and Fast Blue RR were obtained from Dajac Laboratories, Philadelphia, Pa. *E. coli* soluble ribonucleic acid was purchased from Schwarz Bioresearch Inc., Van Nuys,

Calif. Uracil-^{3}H (G), L-arginine-^{14}C (U), and ethylenediaminetetraacetic-2-^{14}C acid were purchased from New England Nuclear Corp., Boston, Mass.

Growth of cells. Stock cultures of *S. itersonii* were maintained as previously described (9). The standard medium (GGS + KNO_3) contained glutamate, glycine, and succinate as the carbon sources, with 40 μM iron citrate and 20 mM KNO_3 (9). *S. itersonii* cultures (0.5%, v/v, inocula) were grown at 30 C in flasks filled to 80% their capacity with shaking at 200 rev/min. *E. coli* B was grown in Hershey broth with vigorous aeration at 37 C. Growth was monitored at 540 nm by using a Zeiss M4 QIII spectrophotometer (Carl Zeiss, Inc., New York, N.Y.). Cells were harvested at the end of exponential growth by centrifugation at 4,000 $\times$ *g* for 15 min at 24 C.

Spheroplast formation. Cells used for spheroplast conversion were harvested at 24 C and were not washed. The reaction mixture for spheroplast conversion contained the following components: 40 mM Tris (*p*H 8.0), 4 mM EDTA, 0.5 M sucrose, 225 μg of crystalline lysozyme per ml, and 0.5 to 1.0 mg of cell protein per ml. After 2 min of incubation at 30 C, 10 mM $MgCl_2$ was added. Spheroplast formation was complete after an additional 30 min of incubation. Spheroplasts were removed by centrifugation at 12,000 $\times$ *g* for 20 min at 4 C.

Tris-EDTA treatment. Cells harvested at 24 C were washed twice by resuspension to their original culture volume in 20 mM Tris (*p*H 8.0), followed by centrifugation at 4,000 $\times$ *g* for 15 min at 24 C. The resulting pellet was resuspended with 40 mM Tris (*p*H 8.0) to approximately 2 $\times$ 10^9 to 4 $\times$ 10^9 cells per ml, and treated with EDTA (concentrations indicated in text) for 2 min at 24 C. The reaction was terminated by the addition of 5 mM $MgCl_2$-5 mM $CaCl_2$, and cells were removed by centrifugation at 20,000 $\times$ *g* for 15 min at 4 C. The per cent release of an activity is defined as the ratio of the released activity to the total activity present in an equivalent portion of untreated, complete cell extract, times 100. Recovery was 90 to 110% under all conditions employed, as measured by the amount of activity released plus the amount of activity remaining in the treated cells.

Preparation of extracts. Cell suspensions were disrupted by sonic oscillation at 4 C in two 30-sec intervals using a Branson model LS-75 Sonifier (Branson Instruments Inc., Stanford, Conn.). When necessary, unbroken cells and large cell fragments were removed by centrifugation at 20,000 $\times$ *g* for 30 min at 4 C.

Soluble cytochrome c estimation. *S. itersonii* contains both soluble and membrane-bound forms of cytochrome *c*. Membrane-bound cytochrome *c* was removed by centrifugation at 100,000 $\times$ *g* for 3 hr at 4 C. Soluble cytochrome *c* was then estimated in resulting supernatants by measurement of the α band reduced minus oxidized spectrum by use of a Cary model 14 R spectrophotometer (Applied Physics Corp., Monrovia, Calif.) with a 0.1 absorbance full-scale slide-wire (9). Dilute cytochrome *c* preparations were estimated by their Soret difference spectra.

Enzyme assays. Previously published methods were used for the determination of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1; see reference 27); cyclic phosphodiesterase [*N*-ribosyl-purine (pyrimidine)-2',3'-cyclic phosphate *N*-ribosyl-purine (pyrimidine)-3'-phosphate phosphohydrolase, E.C. 3.1.4.d; see reference 35]; 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5; see reference 35); glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9; see reference 35); ribonuclease (32); ribonucleic acid (RNA)-inhibited deoxyribonuclease (36); and nitrate reductase (13). A unit of enzyme activity is defined as the amount of enzyme that converts 1 μmole of substrate per min under the prescribed assay conditions, with the exception of ribonuclease activity. A unit of ribonuclease activity is defined as the amount of enzyme that liberates 1 μmole of nucleoside monophosphate per min from *E. coli* soluble RNA (assuming that 1 μmole of nucleoside monophosphate has an absorbance at 260 nm of 9.8).

The alkaline phosphatase of *S. itersonii* was inactivated by prolonged exposure either to Tris in the absence of divalent cations, or to EDTA. Thus, excess magnesium and calcium ions were added to fractions containing Tris or EDTA, or both, to prevent such inactivation.

Analytical procedures. Nucleic acids were fractionated by a modification of the Schmidt-Thannhauser-Schneider method (39). RNA was assayed after hydrolysis in 0.3 N KOH for 18 hr at 37 C by absorbance measurements at 260 nm. Deoxyribonucleic acid (DNA) was estimated at 260 nm after hydrolysis in 5% $HClO_4$ for 15 min at 100 C. Protein was determined by the method of Lowry et al. (26), with crystalline bovine serum albumin used as the standard. Particulate fractions were digested for 7 min at 100 C in 1.0 N NaOH for determination of their protein content. Acid-soluble material (soluble in cold 3% $HClO_4$) was estimated at 260 nm. Inorganic phosphate was determined by the method of Sumner (41).

Unchelated EDTA was estimated in Tris-EDTA supernatants by titration with magnesium ions at *p*H 10.0, with Eriochromeschwartz T used as an indicator for free magnesium (7). Binding of ^{14}C-labeled EDTA was measured in 4-ml reaction mixtures at 24 C by the addition of 0.4 μCi of ^{14}C-labeled EDTA (1.84 mCi/mmole) to each Tris-EDTA solution before addition of cells (2 $\times$ 10^9 per ml). Radioactivity of Tris-EDTA supernatants and treated cells was counted in a thin-window Nuclear-Chicago gas-flow counter.

Polyacrylamide gel electrophoresis. Electrophoresis was conducted at 4 C by the method of Hjertén et al. (18), by using the standard buffering systems of Davis (10) at *p*H 9.5 or 4.3. Cytochrome was localized by a modification of the benzidine staining technique described by Sherman et al. (40). Alkaline phosphatase activity was detected by incubation of gels at 24 C in 50 mM boric acid buffer (*p*H 8.5) containing 1 mg of sodium α-naphthyl acid phosphate and 1 mg of Fast Blue RR per ml. Protein was stained with 1% Amido Black in 7% acetic acid.

RESULTS

Selective release of proteins by spheroplast conversion and Tris-EDTA treatment. Preliminary experiments demonstrated that alkaline phospha-

tase, soluble cytochrome *c*, and ribonuclease were selectively released from cells, in high yield, during spheroplast formation and by Tris-EDTA treatment (Table 1). The latter result was unexpected since significant release of periplasmic proteins from the *Enterobacteriaceae* normally requires osmotic transition in addition to Tris-EDTA treatment (21, 35). However, preferential release of soluble cytochrome *c*, alkaline phosphatase, and ribonuclease from *S. itersonii* occurred during Tris-EDTA treatment, by using cells harvested from early and mid-exponential growth phases, as well as the stationary phase. Similar results were also obtained when cells were grown in complex medium (GGS plus KNO_3 plus 0.1% yeast extract) or in GGS plus KNO_3 containing 3 mM magnesium.

Significant amounts of alkaline phosphatase and ribonuclease were not detected in culture supernatants, nor were these enzymes liberated from cells during normal handling and washing procedures. However, about 16% of the total soluble cytochrome *c* was found in culture supernatants, and an additional 12 to 20% of this cytochrome was released from cells by washing once with Tris buffer. Further washes, however, did not liberate additional cytochrome *c*. Washing cells with 1 M NaCl did not release significant amounts of soluble cytochrome *c*, although washing with similar salts has been used successfully to elute a *c* type cytochrome from *Micrococcus denitrificans* (L. Smith and P. B. Scholes, Fed. Proc. **27**:778, 1968).

Effects of time, temperature, and EDTA concentration. Parameters affecting the selective release of proteins were examined using cells harvested at the end of exponential growth and washed twice (unless otherwise stated) with 20 mM Tris (*p*H 8.0) at 24 C. After resuspension to about 2×10^9 cells per ml in 40 mM Tris (*p*H 8.0), the effect of time, temperature, and EDTA concentration on selective release of proteins was tested.

Liberation of soluble cytochrome *c*, alkaline phosphatase, and ribonuclease was complete after 1 min of treatment with 0.3 mM EDTA at both 4 and 24 C (Fig. 1). Although rapid, the rate of ribonuclease release was slower than the rates of either phosphatase or cytochrome liberation. About 9% of the cellular protein and 5% of the malate dehydrogenase were released after 2 min of treatment.

The amount of EDTA required to release alkaline phosphatase, soluble cytochrome *c*, and ribonuclease was proportional to cell concentration, and varied with growth conditions and with the number of times cells were washed before treatment. Sigmoidal curves for the liberation of these proteins as a function of EDTA concentration were obtained (Fig. 2). Alkaline phosphatase was most easily released, followed by cytochrome *c* and ribonuclease. The profile of protein liberation fell between those of alkaline phosphatase and cytochrome *c* release.

It was not clear why cells, when treated with low concentrations of EDTA, released only a portion of their periplasmic components. The possibility that EDTA was limiting at low concentrations due to saturation by cellular divalent cations was tested (Table 2). Cells were treated with various concentrations of EDTA, and supernatants were assayed for unchelated EDTA by titration with magnesium ions. Free EDTA was not detected in supernatants from cells initially treated with 0.06 or 0.10 mM EDTA. Since ^{14}C-EDTA was not appreciably bound by cells, the lack of detection of free EDTA was apparently due to its saturation by divalent cations. It is of interest that about 0.12 μmole of EDTA was complexed per 2×10^9 cells over a wide range of excess initial EDTA concentrations, and that this amount was required to liberate the bulk of the periplasmic protein in this experiment.

Effect of preexposure to Tris or EDTA. To explore the relationship between Tris buffer and EDTA in promoting selective release of proteins, cells were treated with only one of these agents, and subsequently exposed to the other (Table 3). Significant release of ribonuclease occurred only in the presence of both Tris and EDTA. In con-

TABLE 1. *Selective release of proteins by spheroplast conversion and Tris-EDTA treatment*[a]

Treatment	Soluble cytochrome *c* (nmoles/ml)[b]	Alkaline phosphatase (units/ml)[c]	Ribonuclease (units/ml)[c]	Malate dehydrogenase (units/ml)[b]	Protein (μg/ml)[b]
Spheroplast conversion	0.37 (88)	1.10 (97)	1.09 (107)	0.26 (10)	42 (10)
Tris-EDTA	0.39 (93)	1.08 (96)	1.07 (105)	0.15 (6)	37 (9)
Tris wash (control)	0.06 (15)	0.02 (2)	(0)	0.05 (2)	19 (4)
Sonic extract, untreated cells	0.42 (100)	1.13 (100)	1.02 (100)	2.70 (100)	441 (100)

[a] Equal portions of unwashed cells (2×10^9 per ml) were treated as described.
[b] Figures in parentheses indicate percentages.
[c] Figures shown to be multiplied times 10^{-2}. Figures in parentheses indicate percentages.

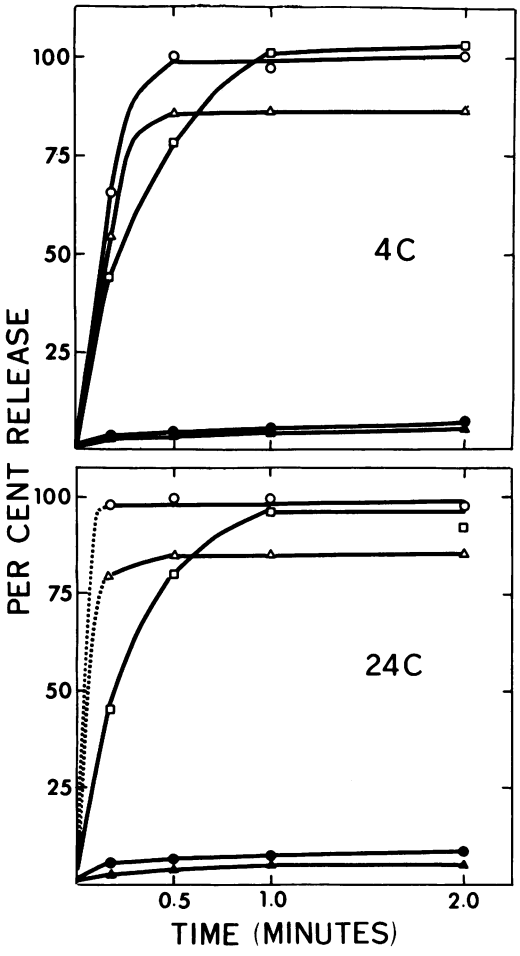

FIG. 1. *Rate of release of soluble cytochrome c, enzymes, and protein during Tris-EDTA treatment. Equal portions of cells (2 × 10⁸ per ml) were incubated at either 4 or 24 C with 40 mM Tris (pH 8.0)-0.3 mM EDTA, and 5 mM $MgCl_2$-5 mM $CaCl_2$ was added at the indicated times. After centrifugation, supernatants were assayed for various activities. Symbols:* ○, *alkaline phosphatase;* △, *soluble cytochrome c;* □, *ribonuclease;* ●, *protein;* ▲, *malate dehydrogenase.*

trast, about 60% release of soluble cytochrome *c* occurred upon exposure to EDTA alone. The most striking results obtained by these experiments were concerned with release of alkaline phosphatase. Nearly complete release of this enzyme was observed when cells pretreated with EDTA were subsequently exposed to Tris buffer. Furthermore, release of the phosphatase did not occur when the order of exposure to these reagents was reversed.

Nature of components released by Tris-EDTA treatment. Tris-EDTA treatment released 9% of the cellular protein, 2.5% RNA, 3% DNA, 66% of the acid-soluble material, and less than 8% of the soluble, cellular nitrate reductase activity (13). The average molecular weight of Tris-EDTA-released proteins was 65,000 as determined by sucrose gradient centrifugation (28). Although large amounts of soluble cytochrome *c* were liberated by Tris-EDTA treatment, membrane-bound cytochromes *b* and *c* (9) were not solubilized.

The possibility that other hydrolytic enzymes might be released by Tris-EDTA treatment was explored. Three enzymes which are liberated from *E. coli* by osmotic shock of Tris-EDTA-treated cells, RNA-inhibited deoxyribonuclease

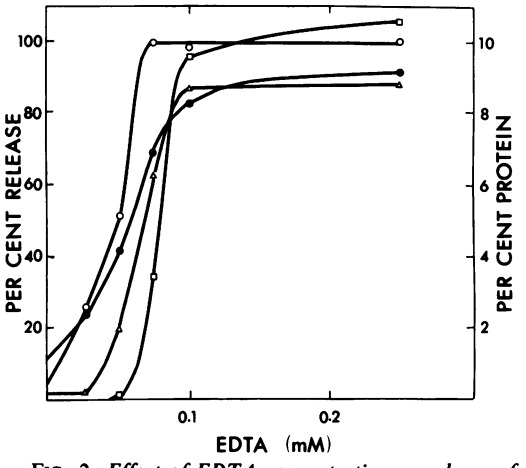

FIG. 2. *Effect of EDTA concentration on release of soluble cytochrome c, enzymes, and protein. Equal portions of cells (2 × 10⁸ per ml) previously washed three times were incubated at 24 C with 40 mM Tris (pH 8.0) and EDTA at the indicated concentrations. After 2 min, 5 mM $MgCl_2$-5 mM $CaCl_2$ was added. Cells were removed by centrifugation, and supernatants were assayed for various activities. Symbols:* ○, *alkaline phosphatase;* △, *soluble cytochrome c;* □, *ribonuclease;* ●, *protein.*

TABLE 2. *Measurement of unchelated EDTA in Tris-EDTA supernatants*[a]

Unchelated EDTA (mM)			Cell-bound ^{14}C-EDTA (%)		Protein release (%)
Initial	Final	Apparent loss	Unwashed cells	Washed cells	
0					1.0
0.06	<0.01	>0.05	1.4	0.05	6.7
0.10	<0.01	>0.09	1.3	0.05	8.4
0.25	0.14	0.11	1.4	0.04	8.7
0.50	0.38	0.12	1.0	0.04	8.5
1.00	0.89	0.11	2.1	0.05	8.5
5.00	4.86	0.14	2.2	0.04	9.3

[a] Equal portions of cells (2 × 10⁸ per ml) previously washed once were treated with EDTA solutions (containing 0.1 μCi per ml of ^{14}C-EDTA) in 40 mM Tris (*p*H 8.0) at 24 C. After centrifugation, supernatants were titrated for unchelated EDTA. Pellets were assayed for radioactivity before and after washing with 20 mM Tris (*p*H 8.0).

TABLE 3. *Effect of preexposure to Tris or EDTA on selective release of proteins*[a]

Treatment	Soluble cytochrome *c* (nmoles/ml)[b]	Alkaline phosphatase (units/ml)[c]	Ribonuclease (units/ml)[c]	Protein (μg/ml)[b]
Expt I				
Tris-EDTA	0.54 (88)	2.73 (98)	1.82 (103)	39 (8)
Expt II				
Step 1: Tris	0.11 (18)	0.05 (2)	(0)	20 (4)
Step 2: $3H_2O$ washes	0.05 (8)	0.05 (2)	0.04 (2)	15 (3)
Step 3: EDTA	0.21 (34)	0.13 (5)	0.07 (4)	39 (8)
Expt III				
Step 1: EDTA	0.35 (58)	0.28 (10)	0.23 (13)	29 (6)
Step 2: $3H_2O$ washes	0.07 (12)	0.13 (5)	0.04 (2)	15 (3)
Step 3: Tris	0.04 (6)	2.17 (78)	0.02 (1)	5 (1)
Expt IV				
Sonic extract, untreated cells	0.61 (100)	2.78 (100)	1.77 (100)	490 (100)

[a] Equal portions of unwashed cells (2×10^9 per ml) were incubated for 2 min at 24 C in the presence of either 40 mM Tris (*p*H 8.0), 40 mM Tris (*p*H 8.0)-4 mM EDTA, or 50 mM potassium phosphate buffer (*p*H 7.0)-4mM EDTA. EDTA was neutralized by addition of 5 mM $CaCl_2$-5 mM $MgCl_2$. After washing, pellets were suspended to their original volume in either 40 mM Tris (*p*H 8.0) or 50 mM potassium phosphate buffer (*p*H 7.0)-4 mM EDTA. EDTA was neutralized as above, and cells were removed by centrifugation.

[b] Figures in parentheses indicate percentages.

[c] Figures shown to be multiplied times 10^{-2}. Figures in parentheses indicate percentages.

(34), glucose-6-phosphatase (35), and 5'-nucleotidase (35), could not be detected in either crude extracts or Tris-EDTA fractions of *S. itersonii*. Control experiments with extracts of *E. coli* B confirmed the validity of the assays. Cyclic phosphodiesterase, an enzyme also released from *E. coli* by the "osmotic shock" process (35), was readily detected in extracts of *S. itersonii*. Less than 10% of this enzyme was released from *S. itersonii* by Tris-EDTA treatment, or during spheroplast conversion. The cyclic phosphodiesterase of *S. itersonii* was found to be soluble, and thus appears to be an intracytoplasmic enzyme.

Disc electrophoresis of Tris-EDTA-released proteins, conducted at *p*H 9.5, revealed at least 13 protein bands, with two major components (Fig. 3). Both major components were apparently quantitatively liberated by Tris-EDTA treatment, since electrophoresis of an extract prepared from cells previously treated with Tris-EDTA did not reveal these bands. Neither phosphatase activity nor hemoprotein-positive material was associated with these major components. Alkaline phosphatase activity, however, was detected in two minor protein bands which migrated slightly slower than the slowest major component. Soluble cytochrome *c* did not migrate toward the anode at *p*H 9.5, since it is a basic protein (8). When electrophoresis of a Tris-EDTA fraction was conducted at *p*H 4.3, eight protein bands were observed, and cytochrome *c* was the fastest component migrating toward the cathode.

Physiological alterations caused by Tris-EDTA treatment. Cell viabilities were severely affected by Tris-EDTA treatment. After 2 min of treatment of 10^9 cells per ml with various concentrations of EDTA, a rapid loss in viability occurred at the concentration known to release the bulk of the periplasmic protein (Fig. 4). Cells treated in Tris buffer with the minimal concentration of EDTA required to completely release alkaline phosphatase, soluble cytochrome *c*, and ribonuclease also failed to regrow, and incorporated ^{3}H-uracil and ^{14}C-L-arginine into acid-insoluble material at less than 1% the rate of untreated cells. Attempts to reverse killing caused by Tris-EDTA treatment, by addition of released components, divalent cations, or 0.1% yeast extract, were not successful.

Morphology. Visible differences in cell morphology caused by Tris-EDTA treatment were not detected by phase-contrast microscopy. No spherical or disfigured cell forms were observed.

DISCUSSION

Since the release of soluble cytochrome *c*, alkaline phosphatase, and ribonuclease by Tris-EDTA treatment occurred in high yield, and was selective, these proteins are assumed to be localized in the surface layers of the cell. The amount of periplasmic protein of *S. itersonii* can be estimated by correcting for contamination of Tris-EDTA-released protein by cytoplasmic protein due to partial cell lysis during treatment. About 9% of the cell protein and 5% of the malate dehydrogenase are released by Tris-EDTA treatment,

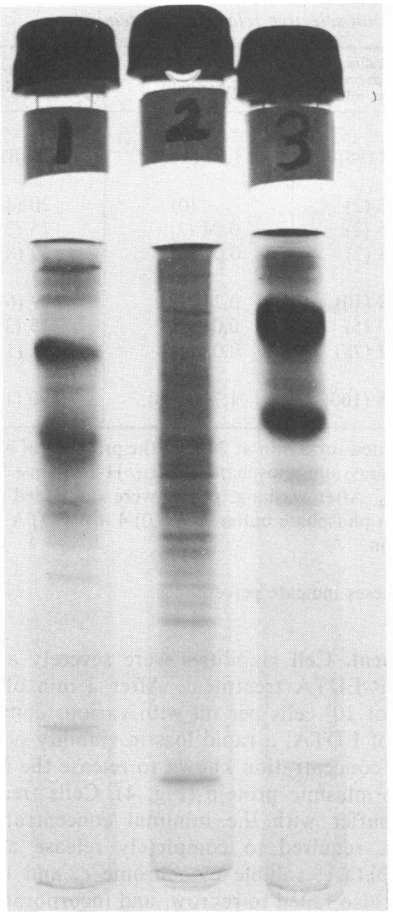

FIG. 3. *Polyacrylamide gel electrophoresis of protein released by Tris-EDTA treatment. Electrophoresis was conducted at pH 9.5. Gels were stained for protein with Amido Black. The fastest migrating band (bottom) is tracking dye. Gel numbers: (1) 200 μg of complete extract protein; (2) 200 μg of protein of an extract prepared from Tris-EDTA-treated bacteria; (3) 200 μg of Tris-EDTA-released protein.*

under conditions in which 65% of the total cell protein is soluble. Thus, periplasmic protein would comprise 0.09 – (0.05) (0.65), or about 6% of the total protein. Under growth conditions which favor maximum production of soluble cytochrome *c*, this hemoprotein comprises 1% of the total cell protein (W. T. Garrard and J. Lascelles, *unpublished data*), and therefore about one-sixth of the periplasmic protein.

The liberation of cytochrome, phosphatase, and nuclease by Tris-EDTA treatment was rapid, and appears to be nonenzymatic, since release was complete after 1 min of incubation at either 24 or 4 C. Similar rapid, temperature-independent kinetics have been observed for permeability alterations (23) and lipopolysaccharide release (25) mediated by Tris-EDTA treatment of *E. coli*. In addition, release of 5'-nucleotidase, acid hexose phosphatase, and cyclic phosphodiesterase by cold osmotic shock of Tris-EDTA-treated *Shigella sonnei* has been shown to be complete within several minutes (31).

The release of periplasmic proteins from *S. itersonii* by Tris-EDTA treatment was irreversible under the experimental conditions employed. Addition of excess magnesium and calcium ions after 2 min of treatment did not lead to an apparent reassociation of released components. Experiments in several laboratories suggest that various transport factors liberated from *E. coli* by the "osmotic shock" process can reassociate with "shocked cells," resulting in restoration of transport capacity (2, 20, 43). In this regard, a study of the requirements necessary to obtain reattachment of transport proteins and other periplasmic components offers another approach to elucidate the nature of interactions at the cell surface.

Differential release of alkaline phosphatase, soluble cytochrome *c*, and ribonuclease occurred when *S. itersonii* was treated in Tris buffer with increasing concentrations of EDTA. Thus, Tris-EDTA-mediated release of these proteins is probably not associated with a single common surface alteration, such as lipopolysaccharide release, but requires specific interactions at various independent sites. Neu and Chou (31) suggested that periplasmic enzymes are probably loosely bound to the cytoplasmic membrane by divalent cations. Differential release of surface proteins from *S. itersonii* could be due to the differential affinity of EDTA for various cations involved in this association.

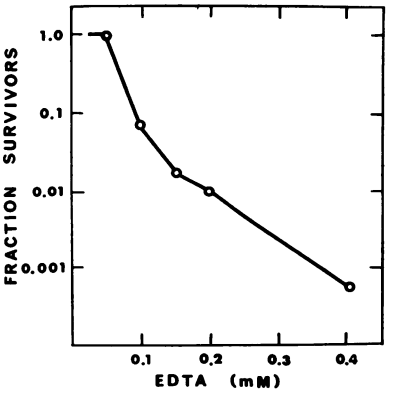

FIG. 4. *Effect of EDTA concentration on viability. Equal portions of cells (10⁸ per ml) previously washed twice were incubated for 2 min at 24 C with 40 mM Tris (pH 8.0) and EDTA at the indicated concentrations. After addition of 5 mM $MgCl_2$-5 mM $CaCl_2$, cells were serially diluted and plated in duplicate.*

The partial release of periplasmic proteins observed during Tris-EDTA treatment at low EDTA concentrations was apparently due to the complete saturation of unchelated EDTA by divalent cations. In addition, radioactive EDTA was not appreciably bound to cells over a wide range of concentrations. Dvorak (12) also reported the absence of appreciable EDTA binding to *E. coli*.

The presence of Tris buffer during EDTA treatment, and the order of addition of Tris and EDTA were important factors in determining what proteins were released from *S. itersonii*. Tris buffer has also been shown to be a necessary component for the EDTA-mediated permeability change in *E. coli* (23), the liberation of lipopolysaccharide from *Haemophilus parainfluenzae* (42), and the release of several hydrolytic enzymes from *E. coli* (30, 35) and *S. sonnei* (31) by the "osmotic shock" process. Goldschmidt and Wyss (14) found that Tris buffer had a pronounced effect on the nature of the divalent cations sequestered from cysts of *Azotobacter vinelandii* by EDTA. The influence of Tris buffer in such systems is most likely due to its ability to chelate various metals (1, 16), and act in a cooperative fashion with EDTA, resulting in an amplification of the chelating potential of the solution.

Studies with Tris-EDTA-treated *S. itersonii* indicated that conditions which caused extensive loss of alkaline phosphatase, soluble cytochrome *c*, and ribonuclease resulted in cell death. In contrast, *E. coli* regrows and maintains high viability when subjected to Tris-EDTA treatment (24) or to "osmotic shock" (4). However, other members of the *Enterobacteriaceae* appear to be more sensitive to such treatments (31). In addition, *Pseudomonas aeruginosa* (15, 23) and *A. vinelandii* (14) have been reported to be extremely sensitive to killing mediated by Tris-EDTA.

In contrast to *S. itersonii*, release of periplasmic proteins from *Enterobacteriaceae* requires a two-step process, Tris-EDTA treatment followed by an abrupt osmotic transition (35). Tris-EDTA treatment of *E. coli* is known to liberate 30 to 50% of the cell wall lipopolysaccharide, and lipopolysaccharide comprises 85 to 90% of the total material released by this procedure (25). Such treatment would also appear to weaken the association of periplasmic enzymes with the cell surface, such that osmotic transition would supply the force necessary to expel these proteins from the cell (17). Since osmotic transition is not required to release periplasmic proteins from *S. itersonii*, the cell wall of this organism apparently offers less restriction for enzymes to diffuse into the surrounding environment. Neu and Chou (31) found that several members of the *Enterobacteriaceae* released periplasmic enzymes during Tris-EDTA treatment if the cells were previously grown under conditions of low magnesium. Since it is believed that divalent cations play an important role in cell wall architecture (5, 19), this observation supports the idea that the basic difference between periplasmic enzyme release in *Enterobacteriaceae* and *S. itersonii* is the porosity of the cell wall after Tris-EDTA treatment.

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