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THE EFFECT OF TOLUENE ON THE STRUCTURE AND PERMEABILITY OF THE OUTER AND CYTOPLASMIC MEMBRANES OF *ESCHERICHIA COLI*

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Summary

The effect of toluene on *Escherichia coli* has been examined. In the presence of Mg^{2+}, toluene removes very little protein, phospholipid, or lipopolysaccharide from *E. coli*. In the absence of Mg^{2+}, or in the presence of EDTA, toluene removes considerably more cell material, including several specific cytoplasmic proteins such as malate dehydrogenase (EC 1.1.1.37). In contrast, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glutamate dehydrogenase (EC 1.4.1.4) are not released at all under the same conditions.

Cells treated with toluene in the presence of Mg^{2+} remain relatively impermeable to pyridine nucleotides, while cells treated with toluene in the presence of EDTA become permeable to these compounds. Freeze-fracture electron microscopy shows that toluene causes considerable damage to the cytoplasmic membrane, while the outer membrane remains relatively intact. These results indicate that the permeability characteristics of toluene-treated cells depend at least partly on the state of the outer membrane after the toluene treatment.

Introduction

Toluene renders bacteria permeable to low molecular weight compounds [1,2] and several macromolecules [2—5]; at the same time, toluenized cells generally remain impermeable to proteins larger than 50000 daltons [5]. Treatment of bacterial cells with toluene has therefore been used to make the cell cytoplasm accessible to exogenous substrates; it is a successful method for assaying a variety of intracellular enzymes [1,6—8] and for studying complex cellular processes in vitro, such as the synthesis of heteroribonucleotides [9],

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ribonucleic acid [10,11], peptidoglycan [12,13] and the repair synthesis of deoxyribonucleic acid [14—20].

Several studies have confirmed that the cell envelope of gram-negative bacteria is damaged by toluene [2,21], and that the effect of toluene on a cell depends on the treatment conditions [2,5], but it is not clear to what extent each of the layers of the gram-negative cell envelope is affected. We have therefore examined the effect of toluene on the outer and cytoplasmic membranes of *Escherichia coli.*

Materials and Methods

Bacterial strain and growth conditions. E. coli JC411 (*leu⁻ his⁻ arg⁻ met⁻ lac⁻ mal⁻ xyl⁻ mtl⁻ str⁻*) was grown in a minimal medium at 37°C, as described previously [22]. Late exponential phase cells were harvested at a density of 0.5 mg/ml; under these growth conditions stationary phase cells reached a density of 1.5—1.8 mg/ml [22]. Cell densities (mg cell dry mass/ml) were determined as described previously [23].

Harvesting of bacteria and toluenization procedure. Cells were centrifuged (5000 × *g*, 10 min) at 0°C and suspended to 10 mg/ml, at 2°C, in 100 mM Tris · HCl (pH 8.0 at 20°C), which contained either 10 mM $MgCl_2$ (final concentration), 5 mM EDTA (final concentration), or neither; these are referred to as Tris/Mg^{2+}-treated, Tris/EDTA-treated, and Tris-treated cells, respectively. Each of these suspensions was divided in two portions; to one portion toluene was added to a final concentration of 1% or 10% (v/v) as indicated, while the other portion was the untoluenized control. Both portions were shaken (320 rev./min) for 15 min at 32—34°C, incubated for 1 h at 0°C, and centrifuged (5000 × *g*, 10 min) at 0°C. The resulting supernatants and pellets (resuspended in the original suspension buffers to 20 mg/ml) were stored at −80°C, unless stated otherwise.

Isolation of the cell envelope, cytoplasmic material, cytoplasmic membrane and outer membrane. Late exponential phase cells were converted to spheroplasts [22], and lysed in a French press (Aminco, Silver Spring, Md.) at 10000 lb/inch^2 [24]. After centrifugation (5000 × *g*, 10 min) to remove unlysed spheroplasts, two portions of the supernatant were centrifuged at 176 000 × *g* for 2 h. The supernatants, containing the cytoplasmic material, were frozen and stored. The pellets, containing the envelope material, were washed by suspension in a buffer containing 10 mM Tris · HCl and 1 mM EDTA (pH 8.0) and centrifuged as above. One pellet was resuspended in the same buffer (total envelope fraction). The second pellet was used to isolate the cytoplasmic and outer membranes [22,24]. All samples were stored at −80°C.

Chemical analyses. Protein determinations were performed by the biuret method [25] using bovine serum albumin as the standard. To prevent interference of buffer components with the determination, the protein was precipitated with 20% trichloroacetic acid and dissolved in 1 M NaOH. Phospholipids were extracted and quantitated [26]. For fatty acid analyses, portions of the phospholipid extracts were esterified with 5% H_2SO_4 in methanol; the fatty acid esters were analysed by gas-liquid chromatography [27]. Lipopolysac-

charides were quantitated by determination of 3-deoxy-D-mannooctulosonic acid as described by Osborn et al. [28].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis on slab gels was performed at 30 mA in Studier's apparatus [29], using Laemmli's discontinuous gel system [30], except that the separation gel contained 13% acrylamide and 0.2% sodium dodecyl sulphate. Protein samples contained final concentrations of 0.0625 M Tris · HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue and 0.8—1.2 mg/ml protein. The following proteins (0.1 mg/ml of each) were used as references: horse cytochrome *c* (11700), egg white lysozyme (14000), chymotrypsinogen (25000), aldolase (40000), ovalbumin (43000), and bovine serum albumin (68000). All samples were boiled for 5 min before electrophoresis. After electrophoresis, the gels were stained with 0.1% Fast green FCF in 45% (v/v) methanol/10% (v/v) acetic acid/45% (v/v) deionized water, and destained in the same solution without dye. Stained gels were photographed and the negatives were scanned with a flying spot densitometer, after which absorbance profiles were generated by a computer program [26].

Enzyme assays. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed in a solution containing 0.45 mM NADP, 1.1 mM glucose-6-phosphate, 11 mM $MgCl_2$, and 55 mM Tris · HCl (pH 7.5) [31]. Glutamate dehydrogenase (EC 1.4.1.4) was assayed in this same buffer containing 100 mM glutamate instead of glucose-6-phosphate [31]. Malate dehydrogenase (EC 1.1.1.37) was assayed in a solution containing 0.1 mM NADH, 1 mM oxalacetate, and 50 mM Tris · HCl (pH 7.5) [32]. NADH oxidase activity was determined in this solution except that oxalacetate was omitted. The NADH oxidase activity was never more than 1—3% of the malate dehydrogenase activity, which was therefore not corrected for the NADH oxidation which occurred in the absence of oxaloacetate. All assays were performed at 37°C.

Cell permeability to pyridine nucleotides. The relative permeability of cells to pyridine nucleotides was determined by examining the accessibility of several intracellular dehydrogenases and the NADH oxidase system associated with the cytoplasmic membrane. Although oxalacetate, glucose-6-phosphate and glutamate also need to gain access to the intracellular dehydrogenases if enzyme activity is to be detected, it was assumed that in these experiments the permeability to pyridine nucleotides was limiting, because the nucleotides are larger and more charged than the other substrates. Toluenized and control cells were maintained as suspensions (10 mg/ml) at 2°C. Extracellular enzymes were removed by centrifugation immediately before the permeability measurement, and a portion of the resuspended cells was sonicated for 5 min with a Branson B-12 sonifier (Branson Sonic Power Company, Danbury, Conn.). The temperature of the samples never exceeded 20°C, and from 90 to 95% of the cells were broken. Relative permeabilities were expressed as the activity of whole cells, compared to the activity of an equivalent amount of sonicated cells.

Two of the enzyme assay buffers contained 11 mM $MgCl_2$, which did not however alter the permeability of cells originally suspended in Tris/EDTA despite the fact that the enzyme activities were 20—30% lower when Mg^{2+} was removed from these assay buffers. Accordingly, all permeability measurements were carried out with the assay buffers described above. There was no change

in permeability during storage of treated cell suspensions for up to 48 h, and cell permeabilities were therefore routinely determined for cells which had been stored at 2°C for 22—48 h.

Electron microscopy. Freeze-fracturing, the preparation of replicas, and electron microscopy have been described [27].

Results

Characterization of the material released by cells under different toluenization conditions

Chemical composition. Table I shows that 1% toluene removed very little material from *E. coli* JC411 in the presence of 10 mM Mg^{2+}. Toluene was considerably more effective in the absence of Mg^{2+}, or in the presence of 5 mM EDTA. EDTA alone was also effective; in agreement with the observations of Leive [33], considerable amounts of protein, phospholipid and lipopolysaccharides were released from control cells suspended in Tris/EDTA. When such cells were treated with 1% toluene (in the presence of EDTA) an additional 10.2% of the total proteins and 6% of the total phospholipid was removed. Toluene removed relatively little additional lipopolysaccharides (8%) from these cells, especially when compared with the amount (31%) which was removed by toluene from cells suspended in Tris · HCl. This was probably due to the fact that there is an intrinsic limit to the amount of lipopolysaccharides which can easily be removed from gram-negative cells [34].

Origin of the proteins removed by toluene. The proteins removed from *E. coli* by toluene treatment were compared to membrane and cytoplasmic proteins by means of sodium dodecyl sulphate-polyacrylamide slab gel electrophoresis (Fig. 1). Scans were generated for each of the protein fractions tested and these were compared to determine the source of each of the protein bands released by the various treatments (Fig. 2). Since some of the envelope proteins removed from *E. coli* by toluene could have originated either in the outer

TABLE I

REMOVAL OF PROTEINS, PHOSPHOLIPIDS AND LIPOPOLYSACCHARIDES FROM *E. COLI* JC411 UNDER DIFFERENT TOLUENIZATION CONDITIONS

Treatment	Protein (%) *	Difference due to toluene (%) **	Phospho-lipid (%) *	Difference due to toluene (%) **	Lipopoly-saccharide (%) *	Difference due to toluene (%) **
Tris Mg^{2+}	2.2 ± 0.1	2.0	1.1 ± 0.2	1.1	1.0 ± 0.1	9.9
Tris Mg^{2+} + 1% toluene	4.2 ± 0.1		2.2 ± 0.2		10.9 ± 0.4	
Tris	3.1 ± 0.3	6.5	7.6 ± 1.6	7.4	10.3 ± 2.9	31
Tris + 1% toluene	9.6 ± 0.3		15.0 ± 3.6		41 ± 1	
Tris EDTA	17.7 ± 0.3	10.2	20.5 ± 1.1	6.0	32 ± 2	8
Tris EDTA + 1% toluene	27.9 ± 0.3		26.5 ± 0.7		40 ± 1	

* Results are expressed as percentages relative to the amount of each component present in untreated cells. The values show the average value ± S.D., derived from four to six measurements.

** Difference between the amount of material removed from toluene-treated cells and control cells.

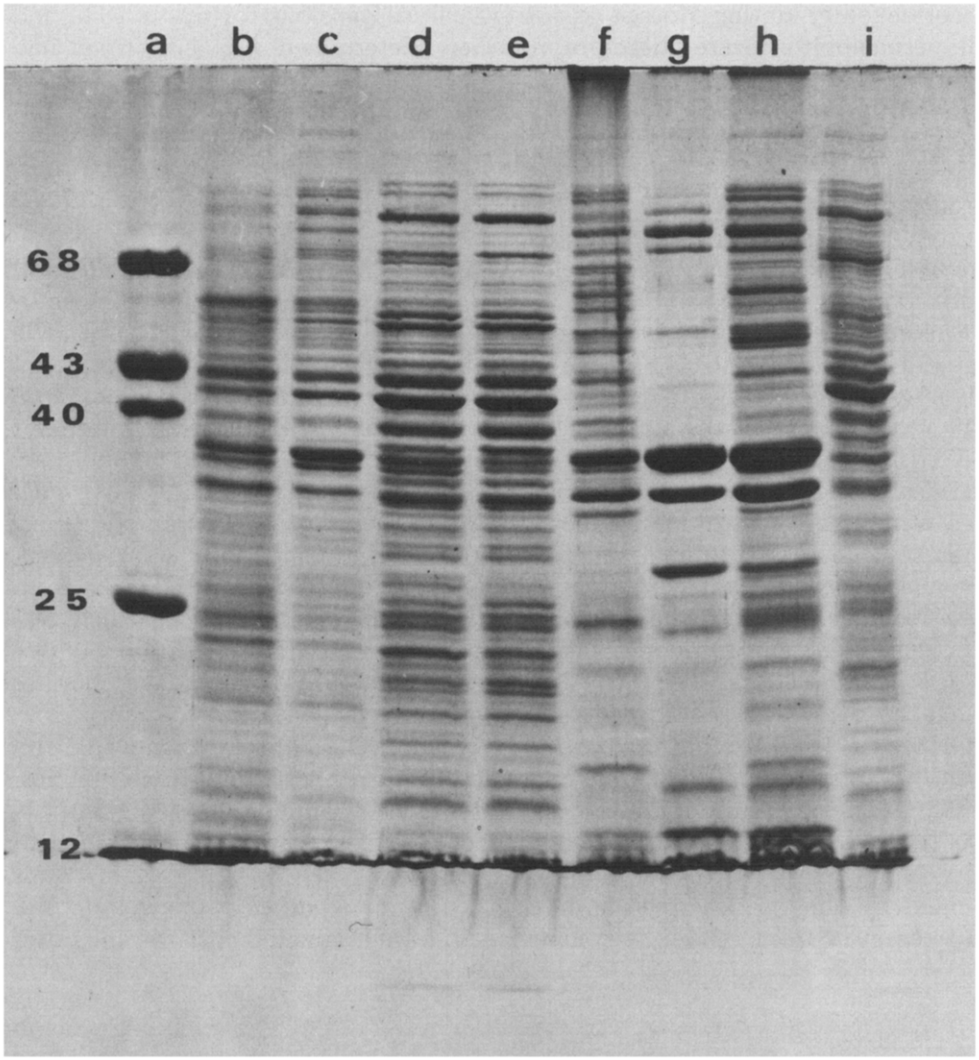

Fig. 1. Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis of the envelope and cytoplasmic proteins of *E. coli* and of the proteins removed from *E. coli* by various treatments with 1% toluene. All samples contained 40 μg protein. (a) Molecular weight standards (kilodaltons). (b) Proteins removed from Tris/Mg^{2+}-treated cells. (c) Proteins removed from Tris/Mg^{2+}-toluene-treated cells. (d) Proteins removed from Tris/EDTA-treated cells. (e) Proteins removed from Tris/EDTA-toluene-treated cells; (f) Cytoplasmic membrane proteins. (g) Outer membrane proteins. (h) Cell envelope proteins. (i) Cytoplasmic proteins. Samples f—i were isolated from untreated cells.

or in the cytoplasmic membrane (see Fig. 1), Fig. 2 shows only whether a given peak corresponds to a cell envelope protein or a cytoplasmic protein. Similar results were obtained with the slab gel system described by Lugtenberg et al. [35].

Fig. 2A shows that in the presence of Mg^{2+}, toluene removed mainly envelope proteins; one of these has an apparent molecular weight of 37 000 and

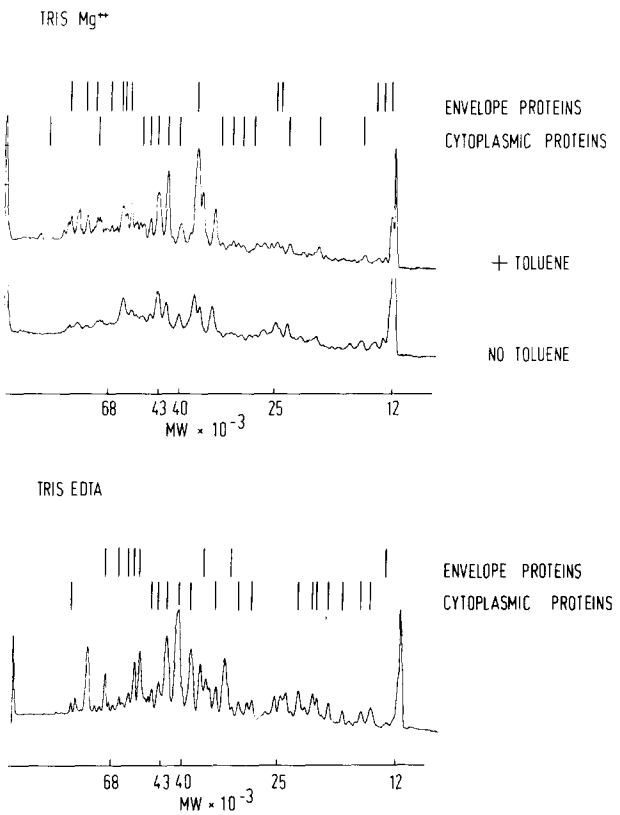

Fig. 2. Absorbance profiles obtained after sodium dodecyl sulphate polyacrylamide slab gel electrophoresis of the proteins removed from control cells and toluene-treated cells. A, Tris/Mg^{2+}-treated cells; B, Tris/EDTA-treated cells. Although in the presence of EDTA more protein is released by toluene-treated cells than by control cells, their absorbance profiles are essentially identical. The vertical marks indicate where each of the protein peaks originated from.

corresponds to the matrix protein * described by Rosenbusch [36].

In contrast to the results obtained for Tris/Mg^{2+}-treated cells, Tris/EDTA-treated cells released the same set of proteins before and after treatment with 1% toluene, although more protein was removed from the toluenized cells (28% of the total cell protein) than from the control cells (18% of the total cell protein). Fig. 2B shows that these proteins were mainly cytoplasmic.

Distribution of the fatty acids. Table II shows the fatty acid composition of the phospholipids found in the supernatants of toluenized and control cells. Tris/EDTA-treated cells, whether treated with toluene or not, released phospholipids which resembled the total cell phospholipids with respect to fatty acid composition. Tris/Mg^{2+}-treated cells released only 1.1% of their phospholipids before, and another 1.1% after, toluene treatment, but there appeared to be some specificity in this release since these phospholipids were enriched significantly with myristic acid.

* This protein is identical to protein b of Lugtenberg et al. [35] and protein Ia of Schmitges and Henning [38].

TABLE II

COMPARISON OF THE FATTY ACIDS IN THE TOTAL PHOSPHOLIPIDS OF *E. COLI* JC411 AND THE PHOSPHOLIPIDS REMOVED BY VARIOUS TREATMENTS

The results are expressed as percentages of the total fatty acids in each preparation.

Fatty acid	Whole cell phospholipids	Phospholipids removed by treatment with			
		Tris/Mg^{2+}	Tris/Mg^{2+} and 1% toluene	Tris/EDTA	Tris/EDTA and 1% toluene
$C_{14:0}$	3	36	24	4	8
$C_{16:0}$	47	28	35	44	43
$C_{16:1}$	29	19	24	30	32
$C_{17:cy}$	1	2	3	3	1
$C_{18:1}$	18	13	12	17	15
$C_{19:cy}$	1	2	2	2	1

Material removed by 10% toluene. Supernatant from cells treated with 1% toluene consisted of a single phase. However, treatment of cells with 10% toluene resulted in a two-phase supernatant, with material at the interface and in the aqueous phase. The toluene phase contained only trace amounts of fatty acids and a protein with a molecular weight of about 9500 on sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Morphology of toluene-treated cells

Tris/Mg^{2+}-treated cells almost always showed fracture planes through the cytoplasmic membrane (Fig. 3A), while Tris/EDTA-treated cells (which had lost 32% of their lipopolysaccharides) showed fracture planes through the outer, as well as the cytoplasmic, membrane (Fig. 3D).

Toluene treatment damaged the cytoplasmic membrane; the extent of this damage increased with the toluene concentration used and was greater for Tris/EDTA-treated than for Tris/Mg^{2+}-treated cells. Toluene treatment caused fracture planes through the cytoplasmic membrane to disappear (Figs. 3C, 3E and 3F); this suggests that toluene probably disorganized the bilayer by removing phospholipids from the cytoplasmic membrane. At the same time, toluene caused phospholipid vesicles to appear extracellularly (Tris/EDTA-treated cells, Fig. 3E) or intracellularly (Tris/Mg^{2+}-treated cells, Fig. 3B). In the latter case these vesicles sometimes adjoined bare patches of the cytoplasmic membrane, forming multilamellar structures reminiscent of liposomes. This is illustrated clearly in the stereo electron micrograph of Fig. 4 (which shows a phospholipid-rich membrane fragment [27] adjoining the inner surface of the cytoplasmic membrane) and in the stereo electron micrograph of Fig. 5 (which shows several intracellular vesicles in detail). Such additional intracellular membranes were never seen in cells not treated with toluene.

In contrast to the cytoplasmic membrane, the outer membrane did not disintegrate upon treatment with 1% or 10% toluene, and there were no observable changes in cross-sections of the outer membrane. The number and size of the fracture planes through the outer membrane did change, however: while they were hardly found in cells suspended in Tris/Mg^{2+} (Fig. 3A), and then

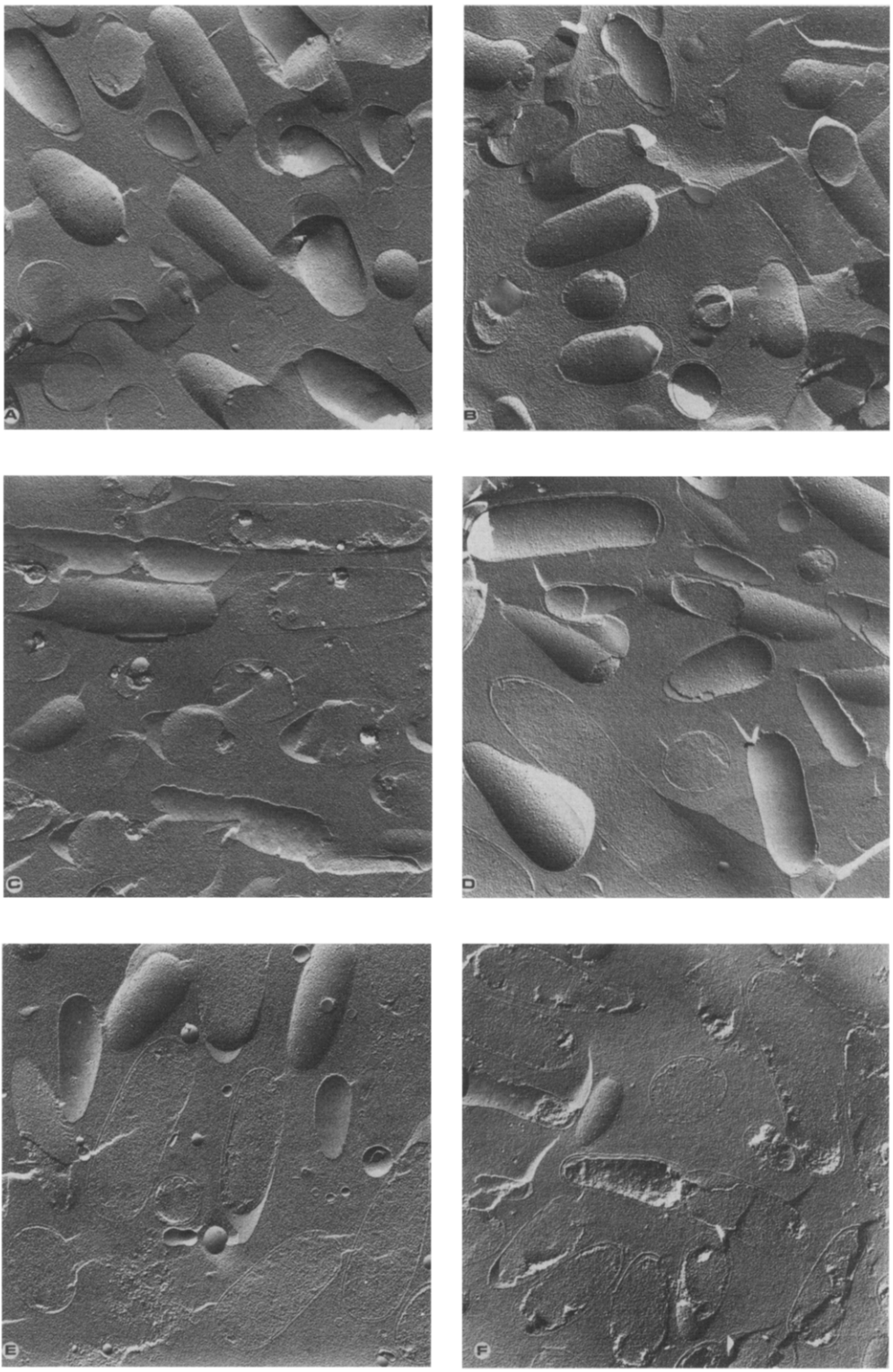

Fig. 3. Freeze-fracture electron microscopy of control cells and toluene-treated cells. The direction of shadowing was from below or lower left, and shadows are white. Magnification is × 15030. (A) Control cells in Tris/Mg^{2+}. (B) Cells treated with 1% toluene in Tris/Mg^{2+}. (C) Cells treated with 10% toluene in Tris/Mg^{2+}. (D) Control cells in Tris/EDTA. (E) Cells treated with 1% toluene in Tris/EDTA. (F) Cells treated with 10% toluene in Tris/EDTA.

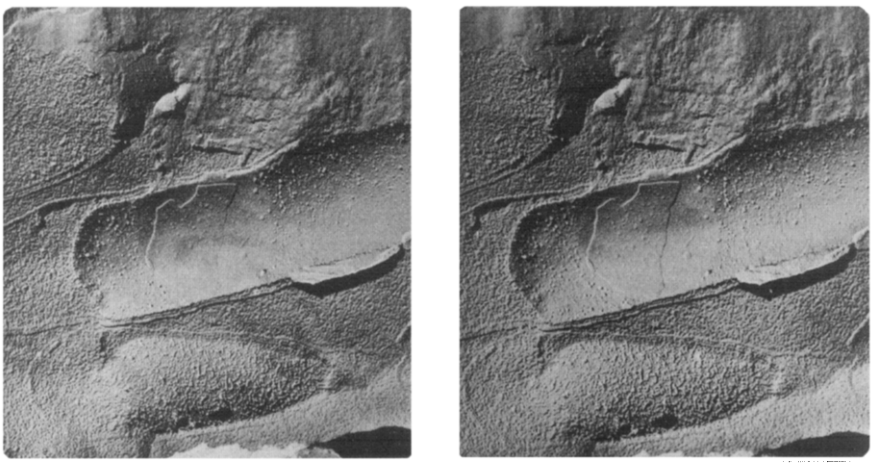

Fig. 4. Stereo pair of freeze-fractured *E. coli* JC411 cells, suspended in Tris/Mg^{2+} and treated with 1% toluene. The large cell was fractured mainly through the cytoplasmic membrane, exposing the exoplasmic fracture face (for nomenclature see Branton et al. [48]). The right half and the left-most portion of this fracture face show rather large particles. The center portion, which contains few particles, shows an additional fracture face, which must be part of a large intracellular phospholipid bilayer or vesicle which adheres to the inner surface of the cytoplasmic membrane. The direction of shadowing was from the top, and shadows are black. Magnification is X 34000.

treated with 1% toluene (Fig. 3B), they occurred frequently when the toluene concentration was raised to 10% (Fig. 3C). Morphologically identical outer membrane fracture faces were seen in cells suspended in Tris/EDTA, both before and after treatment with toluene (Figs. 3D—3F). It appears therefore that both EDTA alone and 10% toluene alone alter the outer membrane sufficiently to significantly increase the frequency of fractures through the outer membrane.

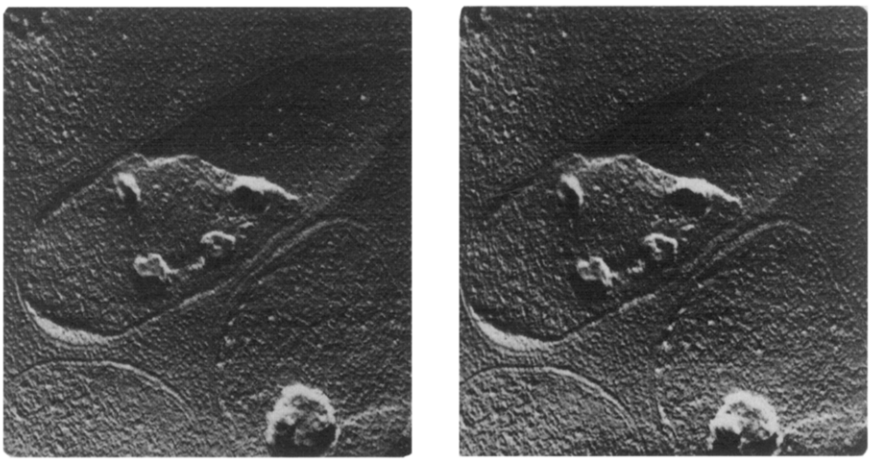

Fig. 5. Stereo pair of freeze-fractured *E. coli* JC411 cells, suspended in Tris/Mg^{2+} and treated with 10% toluene. The right half of the most prominent cell was fractured through the outer membrane, exposing the protoplasmic fracture face. The remainder of the cell was fractured through the cytoplasm and through four intracellular vesicles, which appear as deep pits. A similar large pit can be seen in another cell. The direction of shadowing was from below and shadows are black. Magnification X 54000.

Release and retention of several dehydrogenases

The effect of toluene on the permeability of the cell envelope to specific proteins was determined by examining the release of several dehydrogenases. Each of the enzymes tested remained fully active for at least 1 week when the cells were stored at 2°C, whether the cells had been treated with toluene or not, and whether the enzymes remained intracellular or were released.

Table III shows the effect of storage of cell suspensions at 2°C for 48 h. All of the glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, and cytoplasmic membrane-bound NADH oxidase activity remained associated with the toluenized cells. Since toluene treatment caused the release of significant amounts of cellular material (see Table I), we questioned whether these results could be interpreted to mean that there was no cell lysis. An alternative possibility was that there was cell lysis, but that the released intracellular enzymes aggregated or bound to the remaining unlysed toluenized cells, in which case no enzyme activity would be detected in the cell free supernatant. Accordingly, a sonicated cell suspension (which contained soluble cytoplasmic enzymes) was added to a toluenized cell suspension and stored at 2°C for up to 5 days. It was determined that during this entire storage period, the added glucose-6-phosphate dehydrogenase and glutamate dehydrogenase remained in the cell-free supernatant when the toluenized cells were pelleted at 5000 × *g* for 10 min. Thus, these enzymes do not bind to toluenized cells, and the results of Table III therefore imply that there was no cell lysis during storage for 2 days. Even after storage of the cell suspensions for 7 days, only low levels of glucose-6-phosphate dehydrogenase (4—9%) and glutamate dehydrogenase (3—10%) were found in the supernatants of toluenized as well as control cells, indicating that toluenized cells are quite stable.

In contrast with the above results, malate dehydrogenase was released by toluene treatment in the presence of EDTA, in a time-dependent process as shown in Fig. 6. Control cells suspended in Tris/EDTA lost 19% of their intracellular malate dehydrogenase activity; all of this release occurred within 1 h of storage (Fig. 6). A similar time-dependent release after toluene treatment has been observed for glutathione reductase and for isocitrate dehydrogenase (Witholt, B., unpublished results).

TABLE III

RELEASE OF SEVERAL DEHYDROGENASES FROM TOLUENIZED CELLS OF *E. COLI* JC411

Treated cells were stored at 2°C for 45—48 h, and centrifuged at 5000 × *g* for 10 min. Results are expressed as the percentage of enzyme activity in the supernatant, relative to the total activity of the sonicated cell suspension.

Enzyme	Enzyme activity released (%)			
	Tris/Mg^{2+}	Tris/Mg^{2+} + 1% toluene	Tris/EDTA	Tris/EDTA + 1% toluene
Glucose-6-phosphate dehydrogenase	<1.0	1.3	<1.0	<1.0
Glutamate dehydrogenase	1.6	1.7	<1.0	<1.0
NADH oxidase	<1.0	<1.0	<1.0	<1.0
Malate dehydrogenase	1.5	5.4	19.0	76.7

TABLE IV

PERMEABILITY OF TOLUENIZED CELLS OF *E. COLI* JC411 TO SUBSTRATES OF VARIOUS DEHYDROGENASES

Results are expressed as percent activity in cell suspensions relative to the activity of equivalent sonicated cell suspensions. The values are average values ± S.D., measured after storage for 22 and 48 h at 0°C. The standard deviations have been derived from four values.

Enzyme	Specific activity in sonicates of whole cells * (units/mg cells)	Enzyme activity of unbroken cells (%)				
		Tris/Mg^{2+}	Tris/Mg^{2+} + 1% toluene	Tris/Mg^{2+} + 1% toluene	Tris/EDTA	Tris/EDTA + 1% toluene
Glucose-6-phosphate dehydrogenase	0.17—0.20	5.6 ± 2.9	7.1 ± 1.2	9.2 ± 1.2	6.1 ± 1.4	41.9 ± 4.5
Glutamate dehydrogenase	0.07—0.10	0	6.7 ± 0.4	15.1 ± 0.5	6.7 ± 0.1	41.6 ± 0.0
NADH oxidase	0.10—0.15	0	0	0	0	55.0 ± 6.3
Malate dehydrogenase	5.94 —7.31	7.3 ± 1.2	16.4 ± 1.4	32.0 ± 1.0	4.0 ± 1.2	50.4 ± 11.4

* Range of enzyme activities of control and toluenized cells, after sonication.

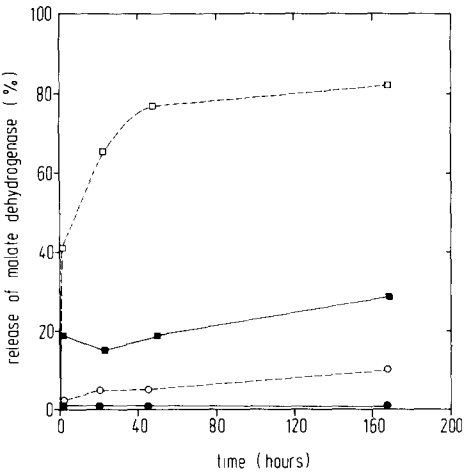

Fig. 6. Time-dependent release of malate dehydrogenase from toluene-treated cells of *E. coli* JC411. Late exponential phase cells were treated as described in Materials and Methods, and the resulting suspensions were stored at 2°C. At different times, portions were taken from these suspensions and centrifuged at 5000 × *g* for 10 min. The release of malate dehydrogenase is expressed as the percent activity in the supernatant after centrifugation, relative to the total activity present in sonicates of the cell suspension before centrifugation. Cells were suspended in Tris/Mg^{2+} (●———●); and treated with 1% toluene (○— — —○), or they were suspended in Tris/EDTA (■———■), and treated with 1% toluene (□— — —□).

Effect of toluene on cell permeability to pyridine nucleotides

Table IV shows that cells treated with 1% toluene in the presence of Mg^{2+} were only slightly more permeable than the corresponding control cells. Except for malate dehydrogenase, no more than 7% of the dehydrogenase activities could be detected in unbroken cells in the presence of extracellular substrates; even treatment with 10% toluene, which completely disrupted the cytoplasmic membrane (Fig. 3C), did not result in significantly higher enzyme activities, again with the exception of malate dehydrogenase.

The permeability of control cells in Tris/EDTA was about as low as that of control cells in Tris/Mg^{2+}, despite the fact that EDTA caused the release of considerable amounts of material from *E. coli* (Table I). An increase in relative cell permeability was observed only when the toluene treatment was carried out in the presence of EDTA, in which case 40—60% of the intracellular dehydrogenase and oxidase activities could be detected.

Discussion

Freeze-fracture electron microscopy showed that treatment of *E. coli* with 1% toluene in the presence of EDTA caused considerable damage to the cytoplasmic membrane. Fracture planes through the bilayer no longer occurred; remnants of the cytoplasmic membrane were seen only in cross-section, and probably represent aggregates of hydrophobic integral membrane proteins [37]. In the presence of Mg^{2+}, 1% toluene also removed phospholipids from the cytoplasmic membrane, but the damage was less extensive and fracture faces through the cytoplasmic membrane were still seen. When the toluene concentration was raised to 10% however, the cytoplasmic membrane was disorga-

nized completely, in the presence of Mg^{2+} as well as EDTA. The cytoplasmic membrane phospholipids extracted by toluene were released from Tris/EDTA-treated cells but not from Tris/Mg^{2+}-treated cells, where they appeared to form intracellular protein-poor vesicles [27] (Figs. 3—5).

The outer membrane was much less susceptible to toluene, as shown by its appearance after freeze-fracturing. This was true even for cells treated with toluene in the presence of Tris/EDTA, in spite of the fact that these cells lost 40% of their outer membrane lipopolysaccharides.

Release of cellular components by toluene

The effect of toluene depended on the presence of Mg^{2+} and EDTA (Table I). Exposure of Tris/EDTA-treated cells to 1% toluene enhanced the well known disruptive effects of EDTA [33,34], and caused the release of additional lipopolysaccharide, phospholipid and protein from *E. coli.*

The presence of 10 mM Mg^{2+} stabilized the cell envelope to such an extent that there was almost no removal of cellular material by toluene. The small amount of material which was released originated largely in the outer membrane. Thus, several of the proteins removed by toluene from Mg^{2+}-stabilized cells appear to have been derived from the outer membrane, including a major outer membrane protein of 37000 daltons [35,38] which has been shown to be closely associated with the murein layer [36]. Similarly, toluene removed about 10% of the outer membrane lipopolysaccharides. Toluene failed to remove phospholipids however, indicating that they may be less accessible than the lipopolysaccharides [39].

When neither Mg^{2+} nor EDTA was present, the lipopolysaccharides were evidently not bound to other outer membrane components very effectively, because the addition of toluene to cells suspended in Tris caused the release of as much lipopolysaccharide (41%) as was released in the presence of both EDTA and toluene (Table I). There was no similar extensive release of phospholipid, possibly because phospholipids are localized largely in the inner monolayer of the outer membrane [39] and in the cytoplasmic membrane, while lipopolysaccharides are located in the outer layer of the outer membrane [40].

Permeability of toluenized cells to small molecules

In the absence of toluene treatment, Tris/EDTA-treated cells were about as impermeable to pyridine nucleotides as Tris/Mg^{2+}-treated cells, which correlates well with the finding that the cytoplasmic membrane was relatively unaffected by EDTA as seen by freeze-fracture electron microscopy. Nevertheless, Tris/EDTA-treated cells did release some cytoplasmic proteins. These paradoxical results are not necessarily contradictory however, because there is no direct quantitative relationship between the release of intracellular compounds and the entry of extracellularly added substrates. The sensitivity ranges of these two measuring techniques differ considerably, that is, the permeability coefficients necessary to allow significant leakage of intracellular compounds are several orders of magnitude lower than those necessary for the full detection of intracellular dehydrogenases with extracellularly added substrates, as illustrated in the Appendix.

When cells were treated with toluene the cytoplasmic membrane was disrupted, but this disruption was not necessarily accompanied by a vastly increased permeability to pyridine nucleotides. When the toluene treatment was carried out in the presence of Mg^{2+}, which stabilizes the outer membrane [41,42] the cells remained relatively impermeable to pyridine nucleotides; they became more permeable to these compounds only when the toluene treatment was carried out in the presence of EDTA, which destabilizes the outer membrane [34]. Since pyridine nucleotides have molecular weights of 663 and 743, our results are in accord with several recent studies which show that the outer membranes of *Salmonella typhimurium* and *E. coli* are impermeable to oligosaccharides larger than 600 daltons [43,44]; moreover, the negative charges of pyridine nucleotides probably also contribute to their inability to penetrate through a stabilized outer membrane.

Thus, it appears possible to alter the permeability characteristics of the outer and cytoplasmic membranes independently to some extent, at least for molecules larger than 600 daltons. EDTA increases the permeability of the outer membrane but has less effect on the cytoplasmic membrane, while in the presence of Mg^{2+}, toluene disrupts the cytoplasmic membrane but leaves the outer membrane relatively intact. Cells toluenized in the presence of Mg^{2+} might therefore prove useful in exploring the permeability characteristics of, and possible transport processes associated with, the outer membrane.

Release of cellular enzymes from toluenized cells

Cells treated with toluene in the presence of EDTA lost substantial amounts of some intracellular proteins, including most of their malate dehydrogenase. This was not due to cell lysis since other cytoplasmic proteins such as glucose-6-phosphate dehydrogenase and glutamic dehydrogenase were not released at all. It was also not due to obvious differences in the localization of malate dehydrogenase compared to classical cytoplasmic enzymes; malate dehydrogenase does not behave as a typical periplasmic protein (Feenstra, A. and Witholt, B., unpublished results), nor is it likely to be closely associated with the cytoplasmic membrane [45]. Nevertheless, given its easy release from *E. coli*, and its ready accessibility to extracellularly added substrates, it remains possible that malate dehydrogenase is localized close to the cell periphery.

The release of specific intracellular proteins could also be based on the ability of the murein layer or the modified outer membrane to discriminate between different proteins on the basis of their size or shape. In that case, there must be a well defined upper limit to the size of possible gaps in the murein layer [42] or the holes created in the outer membrane by EDTA/toluene treatment. Alternatively, proteins which are retained may bind to the murein layer, while proteins which are released do not.

Regardless of the mechanism responsible for this phenomenon, the highly specific release of several intracellular proteins by toluene suggests that toluene treatment of cells suspended in Tris/EDTA may be a useful first step in the isolation and purification of such proteins. In addition, since complex enzymatic systems such as those responsible for cell wall synthesis depend on numerous different proteins, it might be useful to compare the activities of these systems in cells toluenized in the presence of EDTA and Mg^{2+}. Reduced enzymatic

activities in EDTA/toluene-treated cells could be due to the specific loss of one or more proteins (or other cofactors) involved in the synthetic machinery; such proteins should be present among those released from these cells.

Appendix

Permeability can be quantitated by means of the permeability coefficient $P_x = J_x/\Delta c_x$, where J_x is the flux of solute x through a membrane or envelope due to a concentration differential Δc_x [46].

The permeability coefficient of the cell envelope for intracellular proteins P_p can be estimated as follows. The protein flux J_p from the cells equals the quantity of protein which leaks from a given number of cells in a given amount of time or

$$J_p = \frac{fr \cdot c_p \cdot v \cdot m}{\Delta t \cdot m},$$

where fr = fraction of the intracellular proteins which is released; c_p = intracellular protein concentration (mmol protein/ml); v = intracellular volume per cell dry mass; for bacteria $v \cong 3$ ml/g [47]; m = cell dry mass (g); and Δt = time interval during which protein release is measured (min). The protein concentration differential Δc_p is close to, or equal to c_p, because the extracellular protein concentration is negligible compared to c_p. Thus,

$$P_p = \frac{J_p}{\Delta c_p} = \frac{fr \cdot v}{\Delta t}.$$

When cells are treated with Tris/EDTA, the amount of protein which is released in 1 h is about 20% of the total protein (see Table I). Hence, $fr = 0.2$, $\Delta t = 60$ min and $v = 3$ ml/g which yields $P_p = 10^{-2}$ ml · min^{-1} · g cells^{-1}. This is an average for all intracellular proteins; P_p for individual proteins might be greater or smaller. In addition, the observed release of intracellular proteins may have occurred in less than 1 h, in which case P_p would be higher than calculated above. The above estimate therefore only indicates the order of magnitude of P_p for Tris/EDTA-treated cells.

The permeability coefficient of the cell envelope for extracellularly added NADP, P_N, can be estimated as follows. The NADP flux into a given number of cells must be equal to, or exceed, the rate at which NADP is reduced in these cells by intracellular enzymes. For cells treated with Tris/EDTA, Table IV shows activities of about 6% for glucose-6-phosphate dehydrogenase and glutamate dehydrogenase, which are present intracellularly with activities of about 0.1 unit/mg cells. Thus, $J_N > 0.1$ μmol (NADP reduced) · min^{-1} · mg cells^{-1} × 6% = 6 nmol · min^{-1} · mg cells^{-1} = 6 μmol · min^{-1} · g cells^{-1}.

The concentration differential of NADP, Δc_N, must be less than 0.45 mM, the concentration of the extracellular NADP used in the dehydrogenase assays, and hence

$P_N > 6$ μmol · min^{-1} · g cells^{-1}/0.45 μmol · ml^{-1} = 13 ml · min^{-1} · g cells^{-1}.

Thus, for cells treated with Tris/EDTA, P_N exceeds P_p by about three orders of magnitude; that is, the envelope of these cells is about 10^3 times more permeable to NADP than to intracellular proteins.

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