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Heat-Induced Blebbing and Vesiculation of the Outer Membrane of *Escherichia coli*

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Thermal damage to the outer membrane of *Escherichia coli* W3110 was studied. When *E. coli* cells were heated at 55°C in 50 mM Tris-hydrochloride buffer at pH 8.0, surface blebs were formed on the cell envelope, mainly at the septa of dividing cells. Membrane lipids were released from the cells during the heating period, and part of the released lipids formed vesicle-like structures from the membrane. This vesicle fraction had a lipopolysaccharide to phospholipid ratio similar to that of the outer membrane of intact cells, whereas it had a lower content of protein than the isolated outer membrane. After heating bacterial cells at 55°C for 30 min, the resulting leakage from the cells of a periplasmic enzyme, alkaline phosphatase, amounted to 52% of the total activity, whereas no release of a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, was detected. The results obtained suggest that surface blebs formed by heat treatment almost completely consist of the outer membrane and that the blebs may be gradually released from the cell surface into the heating menstruum to partially form vesicles.

Several possible targets for the thermal inactivation of bacteria, such as DNA, RNA, protein, and membranes, have been proposed (15, 41, 45). In particular, the membranes have many functions essential for cell growth and survival; e.g., the maintenance of cell integrity, substrate transport, and the activities of membrane-associated enzymes. Membrane damage from heating may therefore significantly affect the survival of cells. In fact, several researchers have reported that the lipid fluidity of bacterial membranes is closely related to the heat resistance of bacterial cells (1, 2, 22, 48). Studies on the nature of the membrane injury due to heat stress, therefore, contribute to the understanding of the mechanisms of thermal death of bacterial cells.

Heat damage to bacterial membranes has been confirmed by many workers. Destruction of the permeability barrier in the cell envelope causes the leakage of intracellular materials, such as UV-absorbing substances, carbohydrates, ninhydrin-positive substances, and magnesium and potassium ions (16, 17, 34, 36, 39), and sensitization to an antibiotic, tylosin (42). Moreover, some workers have presented evidence of damage to substrate transport systems due to heat (10, 32).

From the viewpoint of morphological changes in bacterial cells, few investigations have been done. According to de Petris (6), who studied the ultrastructure of *Escherichia coli* envelopes, electron micrographs of heated *E. coli* B cells at extremely high temperatures (75 to 100°C) indicated the formation of large blebs of the outer membrane. Scheie and Ehrenspeck (35) observed blebs with *E. coli* B/r and B_{s-1} under more mild heat stress (49 to 55°C) by using phase contrast microscopy and suggested that the bleb formation was closely related to the thermal death of cells, although they provided no direct evidence of damage to the outer membrane.

In addition, membrane components such as protein (33) and lipopolysaccharide (LPS) (13, 33) are known to be released from bacterial cells during heating. However, the release of lipids does not seem to have been confirmed. For gram-negative bacteria including *E. coli*, the following questions may be asked. Is a substantial amount of membrane lipid released by heat? If so, from which layer is it released, the outer or cytoplasmic or both membranes?

In this paper, we report morphological changes in the outer membrane and the chemical properties of vesicles released from heated *E. coli* cells.

MATERIALS AND METHODS

Organism and culture conditions. *E. coli* W3110 was grown at 37°C in EM9 medium (8.8 g of Na_2HPO_4, 1.2 g of KH_2PO_4, 5.0 g of NaCl, 1.0 g NH_4Cl, 1.0 g of

vitamin-free Casamino Acids [Difco Laboratories], and 0.25 g of $MgSO_4 \cdot 7H_2O$ per liter [pH 7.0]) supplemented with 0.2% glucose. Bacteria were harvested by centrifugation at 3,000 × *g* for 5 min during the logarithmic-growth phase (3×10^8 to 4×10^8 cells/ml), washed twice with 50 mM Tris-hydrochloride buffer (pH 8.0), and then suspended in fresh buffer. These operations were performed at room temperature.

Heat treatment. The cell suspension was incubated at 0°C for 30 min. After this preincubation, portions of the cell suspension were withdrawn and immediately added in 10-fold dilution to a flask containing, unless otherwise stated, an appropriate amount of Tris buffer which had been preheated to 61.1°C. At this temperature, a final temperature of 55°C was immediately obtained on rapid heating. After mixing, heat treatment at 55°C was continued in an incubator with shaking at 100 strokes per min.

Phase-contrast microscopy. After the heated cell suspension was cooled to room temperature, samples (1 drop) were withdrawn and placed on glass slides, covered with glass cover slips, and viewed in positive phase contrast with a Nikon Labophot microscope with a ×100 objective and ×15 oculars. In quantifying bleb formation, at least 300 cells were scrutinized for the presence of blebs.

Freeze-fracturing and electron microscopy. Freeze-fractured specimens were prepared as follows. The cell suspension was cooled to 37°C after heat treatment and then sedimented, without a change in temperature, by low-speed centrifugation at 500 × *g* for 10 min. For the fraction released by heat treatment, heated cell suspension was cooled to 0°C and sedimented by centrifugation (3,000 × *g* for 5 min, twice) in the cold. The resulting supernatant was resedimented by high-speed centrifugation (200,000 × *g* for 1 h) at 0°C. The pellet was prepared on a specimen holder without a change in temperature and then quickly frozen in liquid Freon 22 by the sandwich method (28). The frozen specimens were freeze-fractured at −100°C, etched for 1 to 3 min, and shadowed with platinum-carbon in a vacuum evaporator (type JEE 4AS, Japan Electron Optics Laboratory Co., Ltd.). The replicas were obtained by standard procedures and studied under a JEM-100C electron microscope (Japan Electron Optics Laboratory) at ×20,000 or ×26,000 magnification.

In preparing ultrathin section specimens, 4 ml of 2.5% glutaraldehyde–25 mM phosphate buffer (pH 7.2) was added to 1-ml samples including cell suspension cooled to 37°C after heating, and then the samples were allowed to stand at room temperature for 100 min. Fixed cells were centrifuged at 1,000 × *g* for 10 min. The cell pellets were then mixed with about 0.1 ml of 2% agar. After the agar had solidified, it was divided into portions of about 1 mm^3, washed twice with 25 mM phosphate buffer (pH 7.2), and postfixed with 1% osmic acid–25 mM phosphate buffer (pH 7.2) for 1.5 h. Specimen cores were dehydrated by passage through increasing concentrations of ethanol and embedded in Epon (Shell Chemical Co.). Sections were cut with a glass knife on a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc.), mounted on 300-mesh copper grids, stained with uranyl acetate and lead, and examined under a JEM-100C electron microscope.

Measurement of lipids. For the measurement of lipids released by heat treatment, cells were labeled with 1 mM sodium [2-^{14}C]acetate (0.1 μCi/ml) added to the growth medium. After heat treatment, the cells were cooled to 0°C and sedimented by centrifugation (3,000 × *g* for 5 min) in the cold. The resulting supernatant was recentrifuged at 12,000 × *g* for 10 min. Lipids were extracted from the resulting supernatant by the method of Bligh and Dyer (3) by using commercial phosphatidylcholine (Sigma Chemical Co.) at a concentration of 1 mg per ml of chloroform as a carrier lipid. The lipids extracted were dissolved in chloroform, and the solution was absorbed on a glass filter (AP25, 1.5 by 5 cm; Millipore Corp.). After drying the filter, radioactivity was measured in a Beckman LS-250 liquid scintillation counter. For the determination of total lipids, extraction was from the cell suspension, and the radioactivity was measured as described above.

Isolation of outer and cytoplasmic membranes. Following the procedure of Osborn et al. (31), we separated the outer and cytoplasmic membranes. EDTA-lysozyme spheroplasts were lysed by sonication in an ice bath. After centrifugation, the membrane pellet was suspended in 30% sucrose (wt/wt) in 3.3 mM Tris buffer (pH 7.5) containing 5 mM EDTA and then layered on top of a continuous sucrose gradient from 35 to 55% (wt/wt) containing 5 mM Tris and 5 mM EDTA (pH 7.5). The gradient was centrifuged at 190,000 × *g* in a Hitachi RPS-40T rotor at 0°C for 16 h and fractionated by collecting drops from the top of the gradient with an automatic Densi-Flow II C (Buchler Instruments Div., Nuclear-Chicago Corp.) and measuring absorption at 280 nm with a UV monitor. For chemical assays of the outer and cytoplasmic membranes, fractions corresponding to each band were collected, diluted in 20 ml of 50 mM Tris buffer (pH 8.0), and then washed by centrifugation (200,000 × *g* for 2 h, 0°C). The resultant pellet was suspended in 2 ml of distilled water.

Chemical analyses. Total protein was determined by the method of Lowry et al. (24) with bovine serum albumin (Sigma) as a standard.

Phosphorus was determined by the procedure of Shibuya et al. (37). Phospholipid content was calculated as 25 times the amount of phosphorus (23).

LPS content was estimated by assaying 2-keto-3-deoxyoctonate by the thiobarbituric acid test (44).

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The electrophoresis of membrane proteins was performed entirely according to Fairbanks et al. (7). Protein samples were incubated at 100°C for 3 min in a mixture of 1% sodium dodecyl sulfate, 10% sucrose, 10 mM Tris buffer (pH 8.0), 1 mM EDTA, and 1% β-mercaptoethanol. The solutions (50 μl) contained 50 to 100 μg of protein and were placed on a slab gel (14 cm length, 1 mm thickness) consisting of 7.5% acrylamide, 0.285% bisacrylamide, 0.1% sodium dodecyl sulfate, 0.025% *N,N,N′,N′*-tetramethylethylenediamine, and 0.15% ammonium persulfate and electrophoresed at a constant current of 60 mA. After electrophoresis, the gel was stained with Coomassie brilliant blue and then destained with 10% acetic acid at 37°C for 2 days. Protein standards (Bio-Rad Laboratories) including (molecular weight in parentheses) phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and lysozyme (14,400) were used for the estimation of molecular weight.

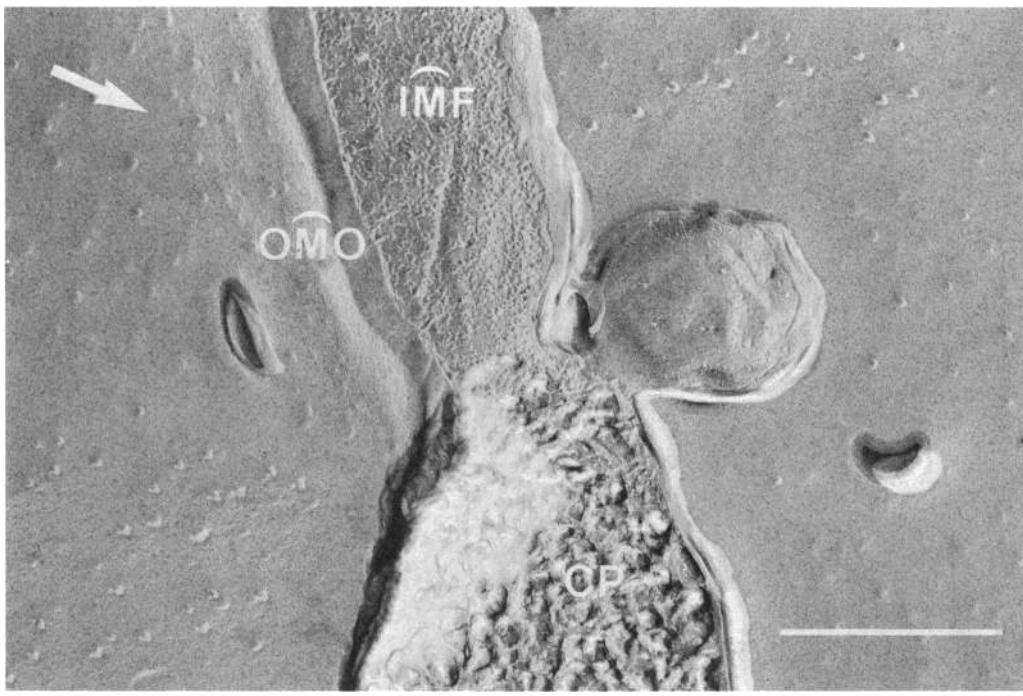

FIG. 1. Freeze-fractured faces of an *E. coli* cell heated at 55°C for 10 min. IMF, inner membrane fracture face; OMO, outer membrane, outer surface; CP, cytoplasm; ⌒, convex aspect. The white arrow at the corner of the micrograph shows the direction of shadowing. The bar represents 0.5 μm.

Enzyme assay. The following enzymes were assayed: alkaline phosphatase (26) and cyclic phosphodiesterase (29) as periplasmic enzymes; glucose-6-phosphate dehydrogenase (26) and aldolase (5) as cytoplasmic enzymes; and D-lactate dehydrogenase (9) and NADH oxidase (43) as cytoplasmic membrane-associated enzymes. For the determination of total enzyme activities, the cell suspension was sonically treated, centrifuged (6,000 × *g* for 10 min) at 0°C, and then the supernatant fraction was assayed. For the assay of membrane D-lactate dehydrogenase in isolated membranes, cells were grown in nutrient broth. Specific activities of the enzyme in the outer and cytoplasmic membranes isolated were 0.026 and 0.16 U per mg of protein, respectively.

RESULTS

Bleb formation on the surface of heated cells. When a suspension of log-phase *E. coli* W3110 cells which had been kept at 0°C was rapidly heated to 55°C in Tris buffer, blebs appeared on the surfaces of cells. When examined by phase contrast microscopy, visible blebs were mainly (about 80%) located at the septa of dividing cells and partly at or near polar regions. Figure 1 shows a freeze-fracture electron micrograph of a bleb that occurred on a dividing cell heated at 55°C for 10 min. In Fig. 2, an ultrathin section electron micrograph of a cell heated at 55°C for 10 min is presented. The appearance of the convex fracture face and the densely stained layer of the bleb imply that the bleb consists of the outer membrane. In addition, it should be noted that the blebs had a multilayered structure. However, some of the observed blebs were composed of a single layer of the outer membrane, which was identical to the observation of de Petris (6).

Patterns of bleb formation were examined by counting cells having visible blebs under a phase-contrast microscope. A significant percentage (11%) of the total cells were found to have visible blebs on their surfaces immediately after heating (15 s). After that, however, the proportion of bleb-bearing cells decreased with heating time and reached less than 1% after 30 min. This suggests the release of blebs from cells during the heating period. In this experiment, no substantial loss of viability was detected after 15 s of heating at 55°C, although the viability decreased to 0.8% after 5 min. Furthermore, we found that Mg^{2+} added to a final concentration of 10 mM in Tris buffer markedly inhibited bleb formation, which was less than 1% during heating for up to 30 min. No blebs were detected during the incubation period at 0 and 37°C when observed by phase contrast and electron microscopy.

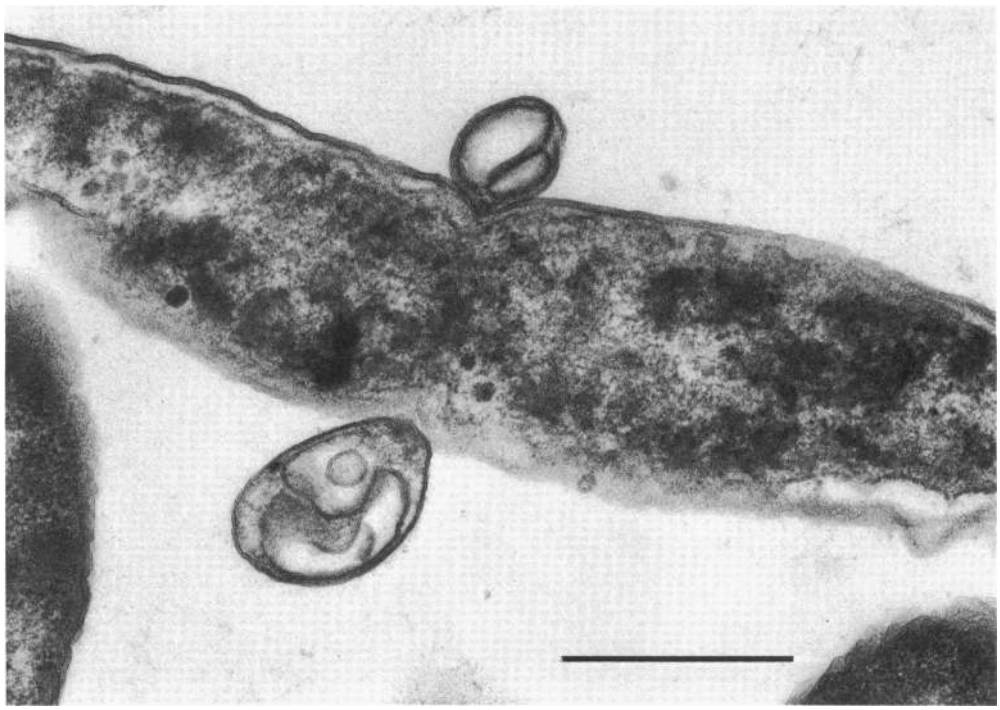

FIG. 2. Ultrathin section of *E. coli* cell heated at 55°C for 10 min. Blebs are located at the septa of dividing cell. The bar represents 0.5 μm.

Heat-induced release of membrane lipids. Since it was suggested that blebs formed are detached from the cells during heating, we examined whether membrane lipids are released from *E. coli* cells on heating. When the cells labeled with sodium [2-^{14}C]acetate were heated rapidly from 0°C to 55°C in Tris buffer, a significant amount of lipids, estimated from the radioactivity, leaked out from cells (Fig. 3). The pattern of the release showed two stages: a rapid initial (15 s) release caused by heat shock treatment and a secondary release at a slower rate during the subsequent heating period. The released amounts were 3.3 and 9.0% of total cellular lipids after 15 s and 30 min, respectively. No substantial release of lipids was detected during the incubation at 0 and 37°C, even after 30 min (<1%).

Chemical composition of the released vesicle fraction. The fraction released from cells heated for 30 min was centrifuged at 200,000 × *g* for 1 h, and then a freeze-fractured specimen of the sedimented fraction was prepared for electron microscopic observation. Figure 4 shows an electron micrograph which evidently indicates the existence of membrane-structured vesicles of various sizes (less than about 0.5 μm in diameter).

We therefore attempted to analyze the biochemical properties of the released vesicles and compare them with those of the outer and cytoplasmic membranes of intact cells. First, we

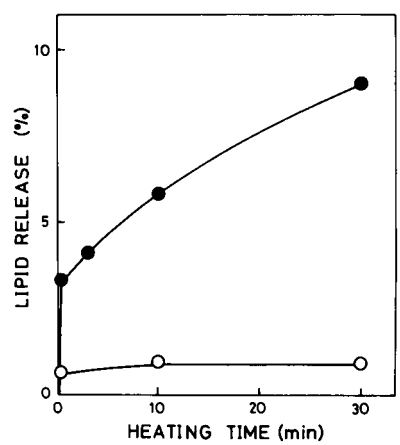

FIG. 3. Lipid release from heated *E. coli* cells. Cells labeled by growing in sodium [2-^{14}C]acetate were kept at 0°C for 30 min and then heated at 55°C (●) and 37°C (○) in 50 mM Tris buffer at pH 8.0. Heated cell suspension was centrifuged after heating, and lipids were extracted from the resulting supernatant, as described in the text. Then, the radioactivities of the chloroform layer were counted. The results are the average value of two independent determinations.

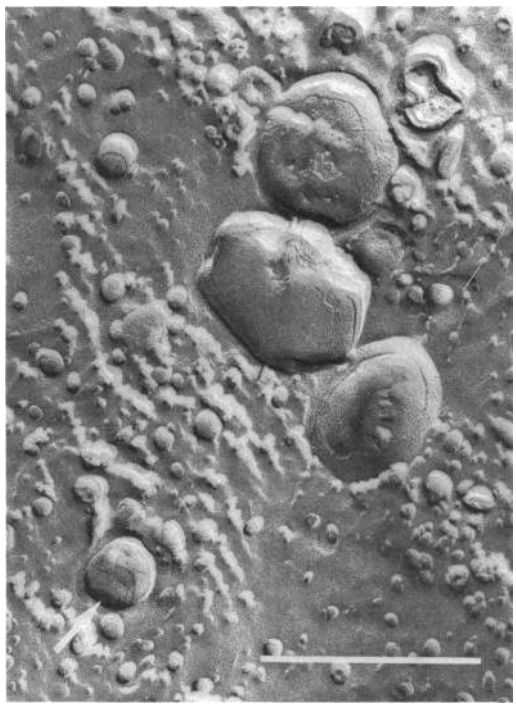

FIG. 4. Electron micrograph of vesicles released from freeze-fractured *E. coli* cells heated at 55°C. The released fraction was centrifuged at 200,000 × *g* for 1 h, and then the freeze-fracture specimen was prepared from the pellet. The white arrow at the corner of the micrograph shows the direction of shadowing. The bar represents 0.5 μm.

isolated the outer and cytoplasmic membranes from *E. coli* cells by centrifugation (190,000 × *g* for 16 h) in a sucrose gradient (30 to 55%) by the method of Osborn et al. (31). Figure 5 depicts the separation pattern and shows two bands. The heavier band contained much LPS (Table 1), whereas the lighter band contained much membrane D-lactate dehydrogenase, as described above, thus indicating the outer and cytoplasmic membranes, respectively.

The released vesicle fraction obtained by high-speed centrifugation of a sample heated at 55°C for 30 min was assayed similarly. As shown in Fig. 5, this fraction was found to consist of a single band after centrifugation in a sucrose gradient and to have an intermediate buoyant density of 1.21 g/cm^3 (Table 1). The vesicle fraction contained much LPS. Table 1 indicates that the LPS/phospholipid ratio of the released vesicle fraction was quite similar to that of the intact outer membrane, whereas the LPS/protein and phospholipid/protein ratios were approximately twice as large as those of the outer membrane.

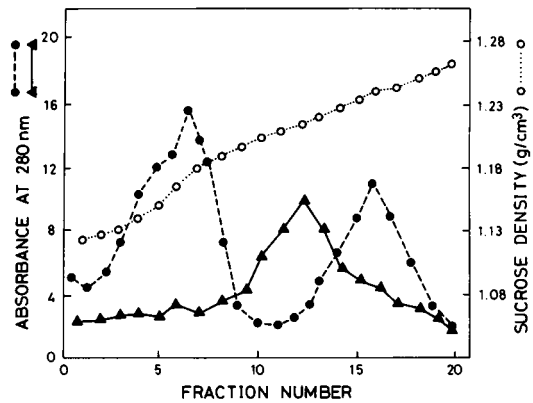

FIG. 5. Sucrose gradient centrifugation of membrane fractions isolated from *E. coli* cells (●) and the vesicle fraction released from cells heated at 55°C for 30 min (▲). Spheroplasts formed by treating cells with EDTA-lysozyme were sonicated, washed, and placed on a sucrose gradient, as described in the text. The vesicle fraction was prepared as described in the legend to Fig. 4. ○, Buoyant density.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the vesicle proteins. In Fig. 6, sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of proteins contained in the vesicle fraction released from *E. coli* cells after heating at 55°C for 15 s and 30 min are shown together with those of the proteins of the outer and cytoplasmic membranes. The indicated pattern of the vesicle proteins resembled that of the outer membrane rather than that of the cytoplasmic membrane. Bands corresponding to two major outer membrane proteins, having apparent molecular weights of 39,000 and 28,500,

TABLE 1. Chemical compositions of membrane fractions and the released vesicle fraction[a]

Fraction	mg of LPS[b] per mg of protein	mg of LPS[b] per mg of phospholipid	mg of Phospholipid per mg of protein	Buoyant density (g/cm^3)[c]
Cytoplasmic membrane	0.07	0.16	0.47	1.17
Outer membrane	0.36	1.06	0.32	1.23
Vesicle	0.64	0.97	0.66	1.21

[a] Membranes and the vesicle fraction were prepared as described in the text. The vesicle fraction was the sample from cells heated at 55°C for 30 min. The results are each the average value of two independent determinations.

[b] LPS determined as 2-keto-3-deoxyoctonate.

[c] Buoyant density indicates the value at the peak of each fraction.

were found in both vesicle fractions (samples after 15 s and 30 min heating). It should be noted, however, that other bands of outer membrane proteins with apparent molecular weights of 18,000 and 16,500 appeared much less clearly, and one band with an apparent molecular weight of 11,000 was lacking in samples of the vesicle fraction.

Leakage of enzymes. The loss of permeability control in the cell envelope was examined. Table 2 shows the leakage of a periplasmic enzyme, alkaline phosphatase, and a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, during the heating period at 55°C. Neither enzyme showed a loss of activity during heating at 55°C for at least 30 min. The amount of alkaline phosphatase in the fraction released from cells increased with heating time, amounting to 52% of the total cell activity after 30 min. Another periplasmic enzyme investigated, cyclic phosphodiesterase, also was released, 31% after 15 min, although this enzyme was inactivated by heat, and the total activity decreased to 70%. On the contrary, no glucose-6-phosphate dehydrogenase activity was detected in the released fraction even after 30 min (Table 2). For other enzymes tested, a cytoplasmic enzyme, aldolase, and two cytoplasmic membrane-associated enzymes, D-lactate dehydrogenase and NADH oxidase, no activity was detected in the heating menstruum, although these enzymes were inactivated to a large extent during the heating period.

DISCUSSION

Surface blebs were observed on heated *E. coli* cells by phase contrast and electron microscopy, in accord with the observations of de Petris (6) and Scheie and Ehrenspeck (35). These blebs, as illustrated in Figs. 1 and 2, seemed to be derived from the outer membrane, as judged by microscopic observations of ultrathin sections and freeze-fractured specimens.

Tris buffer has recently been reported to alter outer membrane permeability, resulting in the release of a limited amount of cell envelope components (20). In our investigation, however, the rapid formation and the subsequent release of blebs in 50 mM Tris buffer at pH 8.0 were not due to the combined effects of heat and the harmful action of the Tris buffer, but due to the heat itself, since the proportion of bleb formation after 15 s of heating was found to be affected neither by higher concentrations of Tris buffer (up to 200 mM) nor by the replacement of Tris buffer by deionized water adjusted to pH 8.0, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), or barbital buffer at 50 mM and pH 8.0 (data not shown).

The pattern of the lipid loss (Fig. 3) seemingly corresponded to that of bleb formation; in particular the secondary release of lipids seemed closely related to the reduction in bleb-bearing cells. The heat-induced loss of membrane lipids from *E. coli* cells may reflect the release of at least part of the outer membrane blebs produced on the cell surface. With electron microscopy, in fact, vesicles of various sizes were found in the pellet obtained on high-speed centrifugation (200,000 × *g* for 1 h) of the fraction released by heat treatment (Fig. 4).

The similarity of the relative phospholipid and LPS contents in the released vesicle fraction to those of the intact outer membrane supports the hypothesis that the vesicles may be derived from the surface blebs consisting of the outer membrane. However, the vesicle fraction was found to have a relative protein content markedly lower than that of the outer membrane isolated from intact cells. This may be responsible for the lower density (1.21 g/cm^3) of the vesicles compared to the outer membrane (1.23 g/cm^3) (Table 1).

The *E. coli* outer membrane has been reported to contain several major proteins (11, 30). Some of these proteins were also observed with the outer membrane isolated in this study. Among them, a protein band with an apparent molecular weight of 39,000, which may be a set of major proteins (30), clearly appeared in the released-vesicle fraction. However, proteins showing lower molecular weights appeared less clearly, and the protein with an apparent molecular weight of 11,000 was lost from vesicle fractions (Fig. 6). This suggests that vesicles with a larger relative amount of the protein with an apparent molecular weight of 39,000 were released selectively from cells, leaving other outer membrane proteins on the cell surface, in accord with the

TABLE 2. Release of enzymes from *E. coli* cells during heating at 55°C[a]

Heating time (min)	Activity	
	Alkaline phosphatase	Glucose-6-phosphate dehydrogenase
0	0.0	0.0
0.25	2.3	0.0
15	44	0.0
30	52	0.0

[a] Cells were heated at 55°C for the indicated times in 50 mM Tris buffer (pH 8.0) and then centrifuged. The resulting supernatants were assayed for alkaline phosphatase and glucose-6-phosphate dehydrogenase. During the heating period tested, no loss of total activity of either enzyme was detected. The results are the average value of two independent determinations.

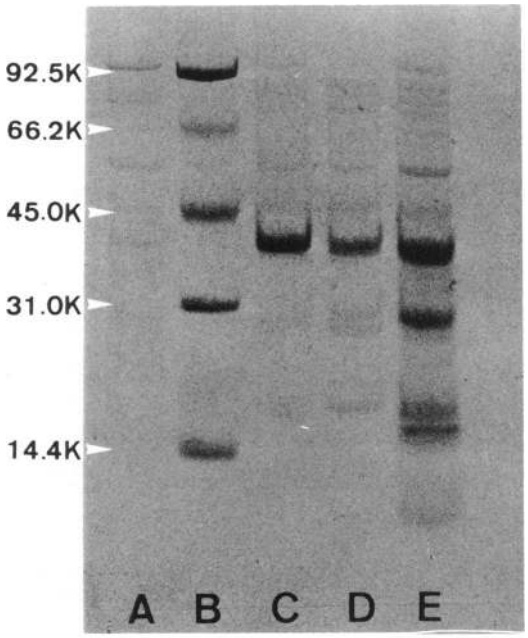

FIG. 6. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of the cell envelope proteins of *E. coli* cells and proteins of the vesicle fraction released from cells heated at 55°C. Lane A, cytoplasmic membrane; lane B, molecular-weight marker proteins; lanes C and D, vesicle fractions from samples after heating for 15 s and 30 min, respectively; lane E, outer membrane. The molecular-weight markers used and the procedure for electrophoresis are described in the text.

result showing reduced protein content of the vesicle fraction.

The lipids in the outer membrane of gram-negative bacteria have in general been reported to contain much more phosphatidylethanolamine (21, 25, 31) and saturated fatty acids (21, 25, 47) than the cytoplasmic membrane. We have observed by chromatographic analyses that the lipid fraction released from heated cells was significantly enriched in phosphatidylethanolamine (including lysophosphatidylethanolamine which formed during heating period) and in saturated fatty acids, as compared with the lipids in intact cells (data not shown). This fact, therefore, may support the hypothesis that the released lipids are substantially derived from the outer membrane.

The above hypothesis was strongly supported by the results of the experiment on enzyme release. When *E. coli* was heated at 55°C, a periplasmic enzyme, alkaline phosphatase, was found to leak out of cells to a large extent, whereas a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, did not at all (Table 2), thus indicating the selective destruction of the outer membrane structure in the sense that internal macromolecules were maintained. In addition, the lack of leakage of the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, reveals there was no cell lysis for at least 30 min of heating at 55°C.

The mechanisms of bleb and vesicle formation due to heat remain to be investigated. To form blebs and vesicles, part of the outer membrane should be released from the underlying peptidoglycan by breakage of the interaction between these layers. Several proteins have recently been shown to be involved in this interaction, including murein lipoprotein (4, 18) and peptidoglycan-associated lipoproteins (27, 46). One can suppose, therefore, that the loss of this association induces the abnormality in bacterial envelopes. In fact, Suzuki et al. (40) and Fung et al. (8) found that *E. coli lpo* mutants with defects in the lipoprotein produced single-layered blebs of the outer membrane and that the bleb formation was prevented by adding Mg^{2+} to the growth medium. These mutants were also reported to release periplasmic enzymes during growth (8, 12, 40). Interestingly, these events markedly coincide with our results. If these lipoproteins are detached from the outer membrane matrix due to heat treatment, they should not be released into the heating menstruum. This might explain the decrease in or loss of proteins with lower molecular weight in the released vesicles in this study.

A similar phenomenon of the partial release of the outer membrane from gram-negative bacteria has been recently observed with other stressed cells, freeze-thawed cells of *E. coli* B (38) and cells of an *E. coli* LPS-deficient strain treated with citrate-Tris (19). It was also shown that these stressed cells released periplasmic enzyme(s) in agreement with our results. Moreover, even normally growing cells of *E. coli* have been reported to release a small amount of outer membrane fragments (0.3 to 0.5% of the total cell protein) into the medium (14).

No substantial death was detected 15 s after heating, in spite of the formation of visible blebs on 11% of the total cells. This seems to show that the bleb formation itself is not the direct cause of thermal death, in contrast to the proposal of Scheie and Ehrenspeck (35). Although Hitchener and Egan (13) also suggested that outer membrane damage does not contribute to the thermal death of *E. coli*, further studies are required to elucidate the relation of the outer membrane damage to bacterial death due to heat.

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