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STUDIES ON THE DAMAGE TO *ESCHERICHIA COLI* CELL MEMBRANE CAUSED BY DIFFERENT RATES OF FREEZE-THAWING

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Summary

Freeze-thawing of *Escherichia coli* cells caused a release of cell membrane components such as protein, phospholipids and lipopolysaccharides. A greater amount of release and a lesser extent of cell survival were seen in slow freeze-thawing than in rapid freeze-thawing. Several dehydrogenases in the cells were also freed. The mode of release was also dependent on the rate of freeze-thawing.

The materials released by slow freeze-thawing were found to be mostly composed of outer membrane components, whereas the materials released by rapid freeze-thawing contained cytoplasmic as well as outer membrane components. The chemical composition of these fragments differed significantly from that of the original membranes. The relative content of cytoplasmic membrane-bound enzymes in these fragments also differed from that of the cytoplasmic membrane.

The fragmentation was assumed to have resulted mainly from the crystallization of external water. In slow freeze-thawing, it was considered that the phase separation of the membrane phospholipid bilayer increased the possibility of outer membrane fragmentation. Rapid freeze-thawing caused cytoplasmic membrane damage to the cells as well as to the outer membrane. In rapid freeze-thawing, the effect of phase separation appeared to be small because of rapid passage through the transition temperatures.

The presence of 10% glycerol completely inhibited the release of cellular materials and enzymes. Cell survival was maintained at a high level in the glycerol-treated samples whether freeze-thawed slowly or rapidly.

Introduction

Freezing injury of *Escherichia coli* consisted of various sites of damage to the cells involving the cell wall [1,2], cell membrane [3,4], ribosomes [5] and deoxyribonucleic acid [6]. Several studies have suggested that the freezing of *E. coli* over a wide range of freezing rates reveals functional and constructional changes of lipopolysaccharide [7,8], suggesting that the outer layer of the cell wall is susceptible as the site of damage. It has been demonstrated that the cell survival after freeze-thawing of *E. coli* decreases with increasing freezing rates [9,10]. A comparative study of aerated and non-aerated cultures indicates that the cellular death associated with rapid freezing bears a close relationship to the permeability of cell walls to water molecules [9].

The cell envelope of gram-negative bacteria consists of three layers: cytoplasmic membrane, a peptidoglycan layer and the outer membrane [11]. Among these layers, the outer membrane is known to allow the passage of sugars and other water-soluble components up to a molecular weight of 1000, and is thought to be highly permeable to water molecules [12—14]. The possible barrier to water permeability is the cytoplasmic membrane [15—17]. Although little is known about the relationship between the sites of cellular damage and the rate of freeze-thawing, it is conceivable that slow freezing might cause damage to the outer membrane only, whereas rapid freezing might cause injury to the cytoplasmic membrane, as well as damage to the outer membrane. In this paper, the sites of damage brought about by slow and rapid freeze-thawing were investigated, and the possible mechanism of injury of the sites is disussed on the basis of membrane architecture.

Materials and Methods

Growth and harvesting of bacteria. E. coli B cultures were kindly donated by Dr. M.L. Speck, North Carolina State University, Raleigh. 0.1 vol. of a 15 h culture of bacteria was introduced into trypticase soy broth medium and incubated at 37°C for 2 h in a shaker incubator. The cells were collected with centrifugation (2000 × *g* for 10 min). With the exception of the special experiments which are described in the text, the pellets were washed four times with distilled water (at 5°C) and finally resuspended in distilled water to a concentration of approx. 1 g wet cells per 6 ml. In the experiments in which the effect of buffer was examined, the cells were divided into two portions. One portion was washed and resuspended in distilled water as described above. Another portion was treated similarly with 0.01 M Tris-HCl buffer (pH 7.5). The succeeding experiments were carried out using distilled water or 0.01 M Tris buffer, respectively.

Equilibration with glycerol. Usually, a 4 ml portion of 25% glycerol in distilled water or in 0.01 M Tris buffer (pH 7.5) was gently mixed with 6 ml of cell suspension either in distilled water or in Tris buffer, respectively. This procedure results in a glycerol concentration of 10%, holding the buffer concentration constant. The cell suspensions, either glycerol-treated or untreated, were held at 0°C for 20 min. No difference in glycerol effect was seen between the samples which were held for 10 and 30 min after mixing with glycerol.

Procedure and rates of freeze-thawing. Two freeze-thawing rates were used in this experiment. The rates of freeze-thawing were measured with a 30-gauge copper-constantan thermocouple connected to a Hitachi 056 recorder. For slow freeze-thawing of samples not treated with glycerol, 10 ml of cell suspension in an aluminum petri dish (5.0 cm in diameter) were cooled on ice. After 10 min, the suspension was seeded with a small ice crystal. In glycerol-treated samples, the same amount of cell suspension in a similar but taller aluminum container was transferred into an ethanol bath which was previously cooled to −4°C. After a few minutes equilibration at that temperature, the suspension was seeded in procedure similar to that described above.

The specimens were frozen by transferring the vessels stepwise into a −25°C and a −78°C freezer, and finally they were cooled to liquid N_2 temperature. The rates of cooling in both samples in the −25°C and −78°C freezers and in liquid N_2 were approx. 1, 5 and 50°C per min, respectively. Warming was carried out by reversing the order of the freezing steps, with final thawing in a refrigerator. The warming rates in each step were approx. 10, 5 and 3°C per min in the −78°C and −25°C freezers and in the refrigerator, respectively. In rapid freeze-thawing, the same volumes of cell suspension in the aluminum containers (5.0 cm in diameter) were immersed directly into liquid N_2. They were held there for more than 10 min, then thawed in a 30°C water bath.

The cooling rates either in glycerol-treated or untreated samples showed some heterogeneity above −60°C. At the periphery on the container, it was approx. 60°C/min in both glycerol-treated or untreated samples. At the centre-top of the samples, the untreated sample showed some delay in freezing, then the temperature dropped abruptly (approx. 200°C/min). In glycerol-treated samples, the temperature was lowered gradually to near −30°C (approx. 30°C/min), then it dropped similarly, as shown in the untreated samples. From −60°C to liquid N_2 temperature, the rate of cooling was homogeneous in all samples. It was approx. 200°C/min. The warming rates of all samples were similar for nearly the entire temperature range and it was calculated to be approx. 400°C/min.

Isolation of membrane fragments after freeze-thawing. The thawed samples were centrifuged at $5000 \times g$ for 10 min. The supernatant was centrifuged at $105\,000 \times g$ for 30 min to collect membrane fragments. The pellet of the $5000 \times g$ centrifugation was resuspended in water and a portion thereof was sonicated at 60 W for 2 min with an ARTEK sonic dismembrator. Both the cell-free membrane fragments and the sonicated cell fraction were washed twice with 0.1 M NaCl solution and used for chemical analyses and assays for membrane enzyme activities. For the assay of soluble enzyme activities, the supernatant of a $18\,500 \times g \cdot 20$ min centrifugation was employed.

Chemical analyses. Total protein was determined by using the method of Lowry et al. [18] using bovine serum albumin as the standard. 2-Keto-3-deoxyocturosonic acid was estimated directly with the membrane fragments according to the method of Osborn et al. [17]. Phospholipids were extracted with a 2 : 1 $CHCl_3/CH_3OH$ mixture, according to the method of Folch et al. [19]. The total phosphorus was measured by using the procedure of Shibuya et al. [20]. Phospholipid contents were calculated as 25-times the phosphorus amount. The phospholipid subclasses were separated by thin-

layer chromatography on plates of Kieselgel H(Merck). The spots were visualized with iodine vapour, and the phospholipid in each spot was extracted by using the method of Skipski et al. [21]. The amount of phospholipid in each spot was determined in the same manner as that previously mentioned.

Sodium dodecyl sulphate polyacrylamide slab gel electrophoresis. The electrophoresis of membrane proteins was performed with 8.0% acrylamide and 1% sodium dodecyl sulphate (SDS). Protein samples were incubated at 100°C for 2 min in a mixture of 1% SDS, 5% sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 40 mM dithiothreitol and 10 μg/ml Pyronin Y. The clear solution was placed on a slab gel of 2 mm thicknes and a current of 15 mA/cm^2 was employed. After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue in 25% isopropanol, 10% acetic acid and water. The following proteins were used as molecular weight markers (molecular weight in parentheses): bovine plasma albumin (66 000), ovalbumin (45 000), pepsin (34 700), trypsinogen (24 000), β-lactoglobulin (18 400) and lysozyme (14 300).

Enzyme assays. All enzyme assays were performed at 25°C using a Shimadzu UV-200 double beam spectrophotometer. Succinate dehydrogenase, D-lactate dehydrogenase and NADH oxidase activities were measured according to the procedure of Osborn et al. [17]. Malate dehydrogenase activity was measured in a mixture containing 0.1 mM NADH, 1 mM oxaloacetate and 50 mM Tris-HCl (pH 7.5) [22]. Usually, malate dehydrogenase activity is more than 4-times greater than the NADH oxidase activity in our sample. Therefore, a minimum amount of membrane sample was used in the assay mixture to minimize the NADH oxidation which occurred in the absence of oxaloacetate. Glucose-6-phosphate dehydrogenase activity was measured in a mixture containing 0.45 mM NADP, 1.1 mM glucose 6-phosphate, 11 mM $MgCl_2$ and 55 mM Tris-HCl (pH 7.5) [23]. Glutamate dehydrogenase activity was measured in a similar mixture containing 100 mM glutamate instead of glucose 6-phosphate [23].

Preparation and isolation of membrane fractions. Essentially, the method of Hasin et al. [24] was used. The cells, harvested and washed with distilled water, were treated by lysozyme (at 4°C for 30 min), followed by sonic disintegration in 0.25 M sucrose containing 3.3 mM Tris-HCl (pH 7.5). The cell envelope fraction was layered on 15 ml of a 35—60% linear sucrose density gradient (about 10 mg protein to each tube), then centrifuged at 27 000 rev./min for 20 h in an SW 27-1 rotor in a Spinco ultracentrifuge. Two clearly visible bands (one at the top and one near the bottom of the gradient), which corresponded to cytoplasmic and outer membrane, respectively, were collected separately. The fractions were washed twice with distilled water and resuspended in cold water. The suspensions were kept at —80°C until used. The buoyant densities for membrane fractions and cell-free fragments were determined by a procedure of density gradient centrifugation similar to that described above.

Freeze-fracture electron microscopy. A drop of sample was quenched from 0°C with liquid Freon which was pre-cooled to its freezing point with liquid N_2, and fractured with a freeze-etching apparatus (JEE-AFE-01) at a temperature of about —90°C. The fractured surfaces were replicated with platinum-palladium and carbon at the same temperature. The replicas were washed

with distilled water and observed under the JEM-100 C electron microscope. In the special experiments, the samples were frozen rapidly or slowly as indicated in the method of freezing. Other treatments were similar to the method mentioned above.

Survival of frozen E. coli cells. Samples were serially diluted in sterile distilled water and plated in 1.0 or 0.5 ml portions in trypticase soy agar, containing 0.3% yeast extract. The plates were incubated at 37°C for 24 h and colonies were counted. The extent of cellular death was determined from the differences in colony-forming units in the samples before and after freezing.

Results

Release of cellular materials and survival of the cells depending upon the rate of freeze-thawing

When untreated *E. coli* cell suspensions were freeze-thawed, a considerable amount of membrane components including proteins, phospholipids and lipopolysaccharides were released into the media (Table I). Slow freeze-thawing induced a release of 5.5 and 7.4% of phospholipids and lipopolysaccharides from the cells, respectively. The release of protein was rather low, compared to other materials, and it was shown that only 2.3% of the membrane protein was released. When the specimen was freeze-thawed rapidly, a similar amount of protein was released, but the release of phospholipids and lipopolysaccharides were reduced to approx. 75 and 71% of the amount released by slow freeze-thawing, respectively.

Several dehydrogenases located in the periplasm, as well as in the cytoplasm, were found to be released by freeze-thawing of untreated sample (Table II). In slowly freeze-thawed specimens, approx. 56% of malate dehydrogenase was released into the medium. Glutamate dehydrogenase and glucose-6-phosphate dehydrogenase (cytoplasmic marker enzyme [25]) were almost negligible in the supernatant and in the whole cell suspension, suggesting that the cell lysis was not a result of slow freeze-thawing. When the specimen was rapidly freeze-thawed, a considerable amount of glutamate dehydrogenase and glucose-6-phosphate dehydrogenase were freed from cells into the suspending medium,

TABLE I

RELATIONSHIP BETWEEN THE RELEASE OF MEMBRANE COMPONENTS AND SURVIVAL OF GLYCEROL-TREATED AND UNTREATED *E. COLI* CELLS AT DIFFERENT RATES OF FREEZE-THAWING

Results are presented as the mean ± S.D. of five (—glycerol) or three (+glycerol) specimens.

	Released as cell-free fragments (%)				
	Unfrozen	Slow freeze-thawing		Rapid freeze-thawing	
		—Glycerol	+Glycerol	—Glycerol	+Glycerol
Protein	trace	2.3 ± 0.4	0.8 ± 0.1	2.4 ± 0.5	1.1 ± 0.2
Phospholipid	0	5.5 ± 0.3	0.03 ± 0.01	4.1 ± 0.3	0.03 ± 0.01
Lipopolysaccharide	0	7.4 ± 0.5	1.3 ± 0.1	5.3 ± 0.4	0.6 ± 0.1
Cell survival	100	0.5	89.5	33.8	85.6

TABLE II

RELEASE OF SEVERAL DEHYDROGENASES FROM *E. COLI* CELLS AND ITS PREVENTION BY GLYCEROL AT DIFFERENT RATES OF FREEZE-THAWING

Results are presented as the mean ± S.D. of five specimens.

Enzyme	Per cent activity in suspending medium				
	Unfrozen	Slow freeze-thawing		Rapid freeze-thawing	
		—Glycerol	+Glycerol	—Glycerol	+Glycerol
Malate dehydrogenase	trace	56.4 ± 4.2	0.4 ± 0.01	27.3 ± 2.3	0.3 ± 0.01
Glucase-6-phosphate dehydrogenase	0	trace	0	10.6 ± 2.2	trace
Glutamate dehydrogenase	0	trace	0	10.1 ± 1.7	trace

while the release of malate dehydrogenase was considerably lower than that of slowly freeze-thawed specimens. It is believed that malate dehydrogenase is not closely associated with the cytoplasmic membrane but is located close to the cell periphery [26]. Thus, it was hypothesized that slow freeze-thawing affected mainly the outer membrane, whereas rapid freeze-thawing induced considerable damage to the cytoplasmic membrane, although the damage to the outer membrane was considered to be somewhat lower than with slow freeze-thawing. As shown in Table I, the survival of cells in slowly freeze-thawed specimens was considerably lower than that of rapidly freeze-thawed specimens.

The presence of 10% glycerol completely prevented the release of cellular materials and enzymes, and maintained the cell survivals at a very high level, either in slowly or rapidly freeze-thawed samples.

The sensitivity to freezing of the cells which were washed and suspended in distilled water or the cells which were washed by and suspended in 0.01 M Tris-HCl buffer (pH 7.5) were compared with that of the cells which were not washed. No difference in cell viabilities or cellular enzyme release following the freeze-thawing was observed between washed and non-washed cells. The effect of buffer on the release of membrane materials induced by freeze-thawing was also not detected (Table III).

TABLE III

CHEMICAL COMPOSITION OF CELL MEMBRANE FRACTIONS AND CELL-FREE FRAGMENTS THE FREEZE-THAWING IN BUFFERED AND NON-BUFFERED MEDIUM

Results are presented as the mean ± S.D. of three specimens.

Component	Released as cell-free fragments (%)			
	Slow freeze-thawing		Rapid freeze-thawing	
	Buffered	Non-buffered	Buffered	Non-buffered
Protein	2.5 ± 0.4	2.5 ± 0.4	2.5 ± 0.5	2.3 ± 0.4
Phospholipid	5.6 ± 0.3	5.0 ± 0.3	3.7 ± 0.2	4.5 ± 0.2
Lipopolysaccharide	7.6 ± 0.5	7.0 ± 0.4	5.3 ± 0.3	5.7 ± 0.3

Characterization and differentiation of released materials

The cell-free supernatants, which contain the membrane components released by either slow or rapid freeze-thawing, were collected and separated on Sephadex G-200 columns. After elution with 0.1 M ammonium acetate (pH 8.0), the materials in each sample were separated into two fractions. For both the slowly and rapidly freeze-thawed specimens, the first fractions appeared in the void volumes. The turbidity of these fractions suggested that the materials were highly aggregated and complexed with each other.

The buoyant densities of the main bands of these materials overlapped the band of the outer membrane fraction, but these showed relatively broad bands, suggesting that they have some heterogeneity in their composition. In rapidly freze-thawed samples, a small amount of low-density material was observed. This material had a buoyant density similar to that of a cytoplasmic-membrane fraction, suggesting that the materials resulted from the cytoplasmic membranes which were contained in this fraction (Table IV). The lower molecular weight fractions following the first fractions were composed mainly of protein and were thought to be a mixture of cytoplasmic proteins and certain nucleotides. Further investigation of these fractions was not carried out.

Table IV shows the overall chemical composition of membrane fractions and released fragments. The chemical compositions of both cytoplasmic and outer membrane fractions are similar to those in previously published work, showing that the outer membrane has a higher protein-to-phospholipid ratio than the cytoplasmic membrane, and that the lipopolysaccharides are located exclusively in the outer membrane [17,24,27–29]. The materials released both by slow and rapid freeze-thawing contained a large amount of lipopolysaccharide, suggesting that the outer membrane is one of the origins of these materials. However, in both fractions the ratio of protein to lipopolysaccharide and to phospholipid was considerably lower than that in the outer membrane. On the contrary, the ratio of phospholipid to lipopolysaccharide in these fragments was higher than that in the outer membrane. When comparing the two fractions, the ratio of protein and phospholipid to the lipopolysaccharide was higher in rapidly freeze-thawed samples than in slowly freeze-thawed samples.

TABLE IV

CHEMICAL COMPOSITION OF CELL MEMBRANE FRACTIONS AND CELL-FREE FRAGMENTS RELEASED BY FREEZE-THAWING

KDO, ketodeoxyoctulosonic acid.

Chemical composition	Cytoplasmic membrane	Outer membrane	Fragments released by freeze-thawing	
			Slow	Rapid
mg protein/μmol KDO	76.9	5.88	2.86	4.00
mg phospholipid/μmol KDO	42.3	1.76	2.65	3.16
mg protein/mg phospholipid	1.82	3.33	1.08	1.27
				1.16 *
Buoyant density	1.16	1.236	~1.24	~1.24

* Small amount of the fraction at the top of the gradient.

TABLE V

COMPARISON OF THE PHOSPHOLIPID COMPOSITION OF CELL MEMBRANE FRACTIONS AND CELL-FREE FRAGMENTS RELEASED BY FREEZE-THAWING

Results are expressed as per cent.

Phospholipid	Outer membrane	Fragments released by freeze-thawing		Cell envelope after freeze-thawing		Cytoplasmic membrane
		Slow	Rapid	Slow	Rapid	
Cardiolipin	1.9	2.0	3.5	3.0	3.1	3.6
Phosphatidylethanolamine	82.5	81.9	82.4	76.7	76.2	77.9
Phosphatidylglycerol	7.8	12.0	12.8	14.3	14.4	16.4
Lysophosphatidylethanolamine	6.9	2.5	2.4	4.9	5.5	2.1

The phospholipid and protein composition

The phospholipid compositions of membrane fractions and the fragments released by freeze-thawing are shown in Table V. In good agreement with previous work, our results demonstrate that the outer membrane is relatively rich in phosphatidylethanolamine, while the cytoplasmic membrane is enriched in phosphatidylglycerol and cardiolipin [17,30]. The fractions released either by slow or rapid freeze-thawing contained amounts of phosphatidylethanolamine similar to those of the outer membrane. The content of phosphatidylglycerol in these fractions, however, was at a level intermediate between that in the outer and cytoplasmic membranes. Regarding the content of phosphatidylglycerol and cardiolipin, it seems likely that the materials released by rapid freeze-thawing contain more cytoplasmic membrane phospholipid than the materials released by slow freeze-thawing. The low content of lysophosphatidylethanolamine in the released fractions indicated that phspholipase A was not present in these fractions, although the enzyme is known to be located in the outer membrane [17,31,32].

Protein profiles in the fragments released either by slow or rapid freeze-thawing and the cell envelope, outer and cytoplasmic membranes were compared by means of SDS-polyacrylamide slab gel electrophoresis. The gels showed that the fragments released by slow freeze-thawing were composed mostly of outer membrane proteins. The proteins which dominated the outer membrane at a molecular weight of approx. 37 000 was also distinguishable in the fragments. However, the smallest molecular weight protein in the fraction was lacking completely, and the content of some other lower molecular weight proteins was apparently low. The fraction released by rapid freeze-thawing seemed to be composed of a mixture of outer membrane and cytoplasmic membrane proteins. Some of the outer membrane proteins, which were lacking or reduced in the slowly freeze-thawed fragments, were also lacking or reduced in these fragments (Fig. 1).

Content of several dehydrogenases in the released fragments

The activities of NADH oxidase and succinate and D-lactate dehydrogenases in released fragments were investigated, since these enzymes are known to be

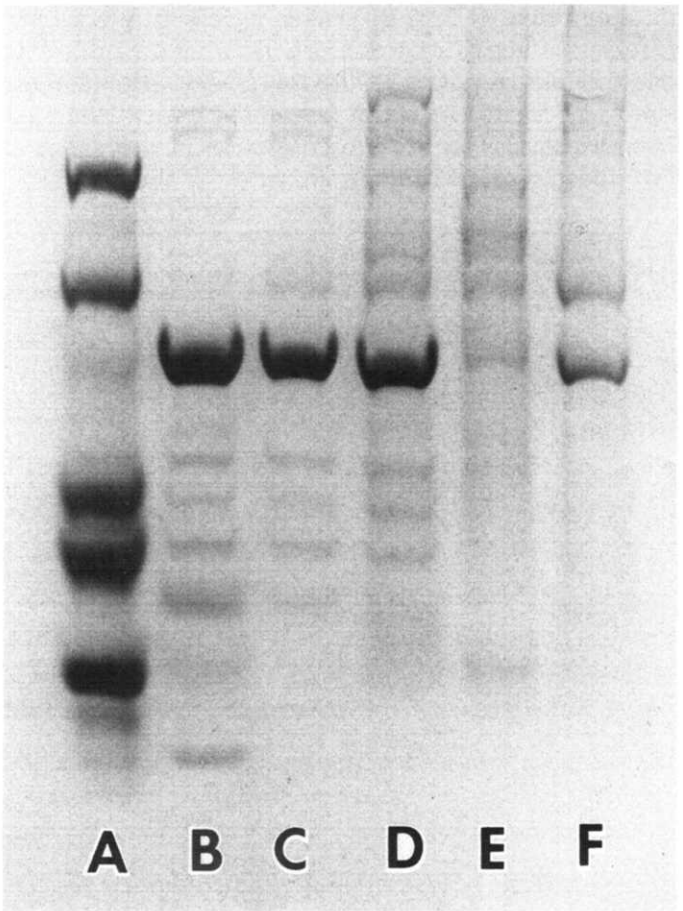

Fig. 1. SDS-polyacrylamide slab gel electrophoresis of the cell evelope proteins of *E. coli* B after freeze-thawing or separation to the outer and cytoplasmic membranes. (A) Molecular weight markers. (The molecular weights from the top are 66 000, 45 000, 34 700, 24 000, 18 400 and 14 300). (B) Outer membrane proteins. (C) Proteins in cell-free fragment released by slow freeze-thawing. (D) Proteins in cell-free fragment released by rapid freeze-thawing. (E) Cytoplasmic membrane proteins. (F) Proteins in cell envelope.

TABLE VI

CYTOPLASMIC MEMBRANE ENZYME AND CYTOPLASMIC ENZYME ACTIVITIES IN THE MEMBRANE FRACTIONS AND CELL-FREE FRAGMENTS RELEASED BY FREEZE-THAWING

Enzyme activities are shown in arbitrary values per mg protein. — Experiment was not run in these samples.

Enzyme	Cytoplasmic membrane	Outer membrane	Fragments released by freeze-thawing		Cell envelope after freeze-thawing	
			Slow	Rapid	Slow	Rapid
NADH oxidase	1.20	0.01	0.02	0.12	1.21	1.04
Succinate dehydroganse	2.17	0.004	0.014	0.10	0.50	0.53
D-Lactate dehydrogenase	0.48	0.004	0.004	0.043	0.12	0.15
Glucose-6-phosphate dehydrogenase	0	0	0	0	—	—
Malate dehydrogenase	0	0	—	0	—	—

located in the cytoplasmic membrane [17,27,33]. Malate dehydrogenase and glucose-6-phosphate dehydrogenase activities were also examined (Table VI). As expected from several previous reports [17,28,34], very little activity was seen in the outer membrane fraction. In the fragments released by slow freeze-thawing, enzyme activities similar to those of the outer membrane fraction were seen. The activity levels of membrane enzymes in the fragments

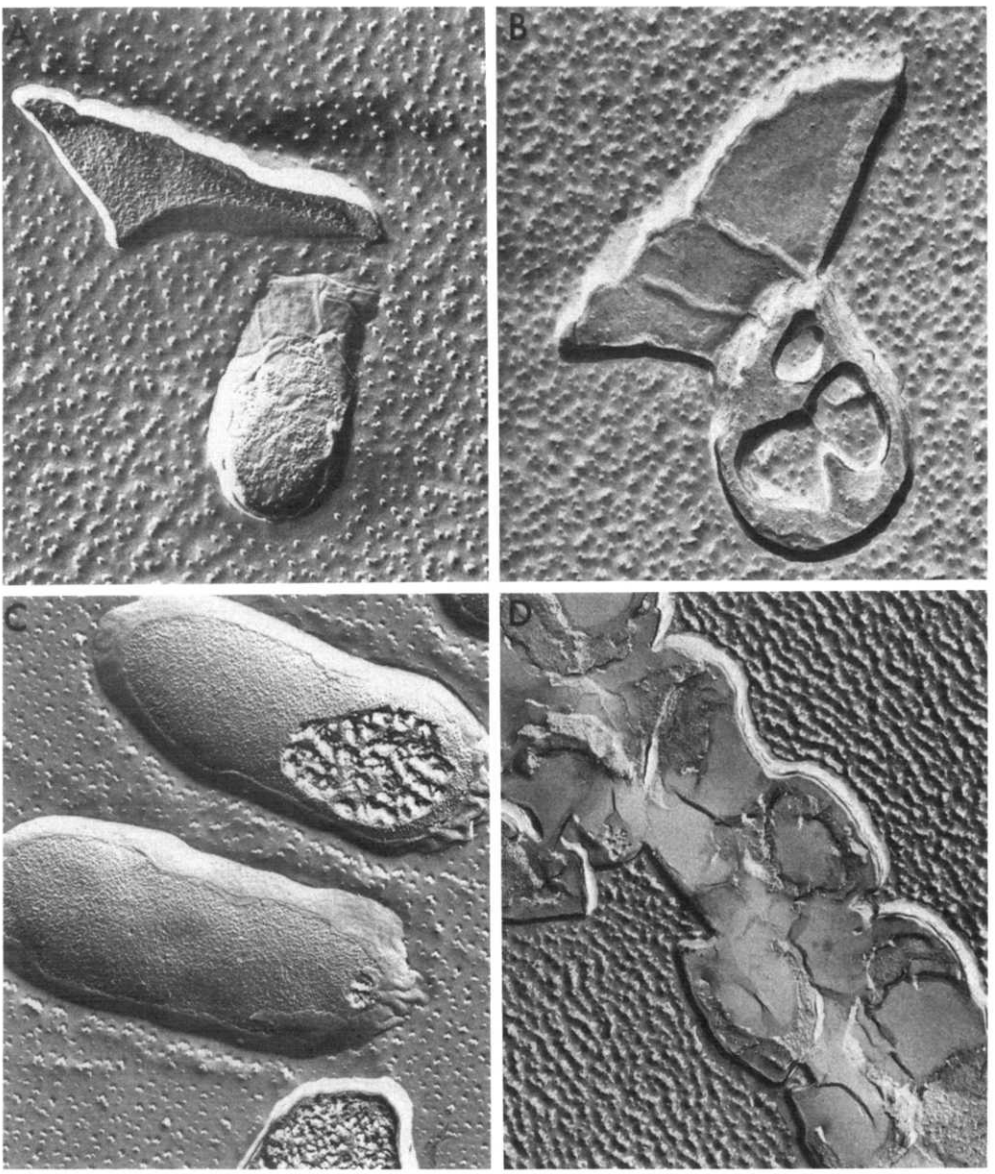

Fig. 2. Freeze-fracture electron microscopy of *E. coli* cells frozen rapidly in the absence of glycerol or slowly in the presence of 10% glycerol. (A and B) Cells frozen rapidly with liquid N_2 as described in the text. (C) Control cells quenched very rapidly with liquid Freon. (D) Cells frozen slowly in the presence of 10% glycerol.

released by rapid freeze-thawing were found to be considerably higher than those of the outer membrane or in the fragments released by slow freeze-thawing. However, the content of these enzymes was still very low compared to that in the cytoplasmic membrane or cell envelope. Moreover, the relative content of the enzymes in the fraction was obviously different from that of the original membrane. No glucose-6-phosphate dehydrogenase or malate dehydrogenase activity was found in either of the fractions. These results suggest that the enzymes in the membrane fragments released by either slow or rapid freeze-thawing are not derived from contamination of isolated membrane enzymes or cytoplasmic contents, but the components of the cytoplasmic membrane which had different composition from that of original membrane are contained in the fragments. The content of cytoplasmic membrane in the fragements released by slow freeze-thawing was thought to be very low as suggested by the results of chemical analyses.

Morphological appearance of the frozen cells

The cells frozen with liquid N_2 (200°C/min) without glycerol shrank mostly, and on their outer surfaces, a number of folds which were considered to have resulted from the shrinkage of the cell were often seen. In their inner membrane fracutre faces, partly aggregated intramembrane particles were seen (Fig. 2A). These cells seemed to be devoid of intracellular ice crystals. Very rarely, cells with a trace of intracellular ice crystals were observed (Fig. 2B). The control cells which were quenched very rapidly with liquid Freon showed a very smooth outer surface, and homogeneously distributed intramembrane particles on their inner membrane fracture face. In these very rapidly quenched cells, many small intracellular ice crystals were seen (Fig. 2C). In the specimens frozen slowly with or without glycerol, many cells were seen packed together and no individual cells could be defined (Fig. 2D).

Discussion

Experimentation showed that freeze-thawing caused a release of membrane components into the medium as cell-free fragments, with cellular death following. The release of cell membrane materials from gram-negative bacteria has been observed by several authors. Rothfield and Pearlman-Kothencz [35] and Knox et al. [36,37] demonstrated the excretion of a lipopolysaccharide-phospholipid-protein complex from cells growing under amino acid-limiting conditions. Recently, Hoekstra et al. [28] reported that the outer membrane fragments, which have chemical compositions very similar to those of outer membranes, were released from the cells, and the protein found in the medium amounted to 0.3—0.5% of the total cell proteins. In these studies, the membrane components, whether from normal cells or from cells affected with antibiotics [35] or phages [38], were released as a result of metabolic activity.

In the present study, it was shown that the materials were released only after the specimens were freeze-thawed. The amount and chemical composition of released materials and the loss of cellular functions show a close relationship, varying with the rate of freeze-thawing and the presence or absence of glycerol. It is considered here that the materials were released not as a result

of natural processes such as cell growth or the lysis of cells during the processes before freezing, but resulted, rather from the freeze-thawing processes. The repeating washing of the cells, which is necessary to remove the outer membrane components released during the cultivation [28], might have resulted in the deleterious effect on the cell surfaces. However, it appeared to be negligible, as the cell viability and the quantity of the enzymes released from the washed cells after freeze-thawing were not different from those of the cells which were not washed.

The membrane fragmentation on freezing might result from lysis caused by ice crystal formation. If this were the case, cytoplasmic enzymes or cytoplasmic membrane components, as well as outer membrane components, would be found in the medium. However, the membrane fragment fractin after slow freeze-thawing did not contain any cytoplasmic membrane-bound enzymes, and no evidence of release of glucose-6-phosphate dehydrogenase and glutamate dehydrogenase activities from the cells was found. A slight cell lysis might have occurred in rapidly freeze-thawed specimens, because the supernatant of the sample contained cytoplasmic enzymes. However, the relative content of cytoplasmic membrane marker enzymes in the released fragments was considerably lower than that found in whole-cell membrane fractions. This suggests that even in rapidly freeze-thawed samples, the membrane fragmentation resulting from cell lysis was not great. Therefore, mechanisms other than mere cell lysis appear to be responsible for the release of membrane materials in both the slow and rapid freeze-thawing.

Chemical analysis of the cell-free fraction indicated that the fragments after slow freeze-thawing resulted from the outer membrane because they contained a signficant amount of ketodeoxyoctulosonic acid, and their phospholipid composition and protein profiles were similar to those of the outer membrane. In the fraction of rapidly freeze-thawed specimens, a certain amount of cytoplasmic membrane fragments was accompanied by outer membrane fragments. However, the overall chemical composition and the relative ratio of membrane-bound enzymes presented in the fragments were obviously different from those of the original membranes. One possibility as to how these fragment might have been formed is presumably the result of phase separation of the lipid bilayer. It is known that both the outer and inner membrane fracture faces of *E. coli* and other gram-negative bacteria have intramembrane particles on their surfaces. At temperatures above the transition point, the intramembrane particles are distributed randomly throughout the whole surface. When the cells are cooled below the transition temperature, certain particle-free domains appear [39,40]. The process apparently induces protein-lipid segregation in the membrane, and final lysis of the cells leads to membrane fragments with different amounts of intramembrane particles per unit area. In the fragment which had no visible particles, the phospholipid-to-protein ratio is 5-times greater than that of the original membrane, and the relative contents of membrane marker enzymes are all different from those in the normal membrane [33]. Similar results have also been observed with erythrocyte membranes [41].

Freeze-fracture electron microscopy of our specimens showed that phase separation and cell shrinkage had occurred even in rapidly frozen cells, and

dissociation of inner and outer membrane is seen in some parts. In the process of slow freezing, the domains which have different chemical compositions, especially those that have lower content and different composition of proteins from the original membranes, will thus be formed. The structural change of the membrane which is the consequence of the phase separation might be responsible for the sites at which association of membranes was weakened. The slow freezing should cause the cells to be shrunken more than the rapid freezing, and this will force the dissociation of two membranes. In certain parts of the outer membrane, where association of the membranes is weakened, they might split off as a result of the stress owing to ice crystal formation during freezing of external water. Glycerol will prevent the splitting off of the outer membrane by changing the conformation of ice crystals around the cells and allow the cells to survive.

Structural changes appear to occur throughout the entire cell surface because almost 60% of malate dehydrogenase is freed from the cells. Although the temperature of phase separation of this organism is not known, some change did occur at sub-zero temperatures because several particle-free domains were seen in the cytoplasmic membrane fracture face, even in rapidly frozen specimens. The domains which are never seen on the cells were quenched very rapidly from room temperature to —150°C.

Rapid freeze-thawing would have a similar but less damaging effect on the outer membrane. The vesicles from rapidly freeze-thawed cells contain protein and phospholipid derived from the cytoplasmic membrane, but the amount of lipopolysaccharides is lower than that of slowly freeze-thawed samples: because, by passing rapidly through the transition temperatures, lipid will crystallize without allowing for a typical rearrangement of intramembranous protein particles. Besides, a lesser shrinkage of the cells in rapid freezing will reduce the percentage of dissociation of the membranes. The lesser amount of malate dehydrogenase released in rapidly freeze-thawed samples as compared to slowly freeze-thawed samples indicated that the extent of surface structural changes is rather small in rapidly freeze-thawed samples. The deleterious effect of rapid freeze-thawing will be restricted around the cytoplasmic membrane. Mazur [42] demonstrated that the minimal cooling velocity for formation of intracellular ice is approx. 200°C/min. The cooling rate we employed in this experiment is somewhat lower than that. Indeed, mostly the cells shrank considerably and did not seem to have any intracellular ice crystals. Considerable amounts of cellular water freeze extracellularly in a very short time. A rapid movement of a significant amount of water through the membrane might cause damage to the cytoplasmic membrane. On the other hand, the outer membrane of *E. coli* and other gram-negative bacteria is known to be highly permeable to water molecules, so much so that the velocity of water movement will not cause any effect on the membrane.

The possibility that intracellular ice crystal formation is a cause of cellular damage cannot be excluded, since intracellular ice crystals were observed in a few cells in a rapidly frozen specimen. But, it cannot explain such a low survival rate with so few intracellularly frozen cells.

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