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Changes in chemical structure and function in *Escherichia coli* cell membranes caused by freeze-thawing. I. Change of lipid state in bilayer vesicles and in the original membrane fragments depending on rate of freezing

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The effect of different rates of freezing on the character of lipids in unilamellar lipid bilayer vesicles and in the original membrane fragments of *Escherichia coli* B cells was investigated by measuring the temperature-dependent fluorescence polarization ratio changes of *cis*- and *trans*-parinaric acids. In lipid bilayer vesicles, both slow and rapid freezing brought about significant alterations in fluorescence polarization ratios in the specimens derived from both logarithmic and stationary-phase cells. In the original membrane fragments derived from logarithmic-phase cells, slow freezing gave rise to a similar alteration in fluorescence polarization ratio change, but no such alteration was found in the case of rapid freezing. Logarithmic-phase cells suffered from a membrane permeability change during slow freezing, which subsequently resulted in low cell viability. The cells suffered only slight impairment in membrane function during rapid freezing, and maintained higher viability. These results suggest that the primary site of damage due to freezing of the cells is the cellular membranes, and this destruction is due to a lipid state change in the membranes brought about by freezing.

Introduction

Freezing of living cells means, in a sense, in addition to low-temperature stress, the removal of water molecules from the cellular materials due to ice crystal formation. This occurs either inside (intracellular freezing) or outside (extracellular freezing) the membranes, so the cellular envelope membranes can be envisaged as the primary site of such ice crystal formation [1–4]. This implies that the sensitivity of membrane structure to freeze-induced dehydration stress is highly relevant for the sensitivity or tolerance of the cells to freezing damage.

The behavior of the native membrane reflects to some extent the behavior of its individual components. Of the membrane constituents, phospholipids are considered to be most fundamentally involved in the temperature-dependent structural change of the membranes leading to freezing injury. The lipid composition of natural membranes varies greatly in the differing growth phases or growth environments [5–8], each exhibiting its own unique thermotropic or lyotropic behavior. The studies concentrate on the characteristics of the isolated lipid specimens and their original membranes, which are important in understanding how the cellular membrane responds to environmental stresses such as freezing. In our previous studies using fluorescence polarization changes of *cis*- and *trans*-parinaric acids in tandem [9–12], it was demonstrated that *Escherichia coli* membrane lipids undergo a relatively wide-range thermotropic phase transition from physiological temperatures to below 0 °C. Those portions of the lipids related to the phase transition are repressed considerably compared to those in aqueous dispersions of lipids of similar chemical composition [13].

In the present study, thermotropic phase transitions of the lipid in membranes, or in unilamellar lipid bilayer vesicles prepared from the membrane phospholi-

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pids, were estimated before and after freezing at different rates. It was observed that in the lipid bilayer vesicles, also, certain portions of the lipid motion were repressed. Slow freezing induced the release of repression of lipid motion both in the membrane specimens and in lipid bilayer vesicles. Rapid freezing caused a certain release of repression of lipid motion in bilayer vesicles, but it did not bring about any changes in the membranes' lipid state. The different rates of freezing also gave rise to different effects on membrane function and cell viability. The implications of the differences in freezing rate and chemical composition between the lipid vesicles and the membrane fragments are discussed here in relation to freezing damage of living cells.

Materials and Methods

Materials

cis- and *trans*-Parinaric acids were purchased from Molecular Probes, (Junction City, OR and Plano, TX, respectively).

Cultivation of bacteria

A 15 h culture of *E. coli* B in trypticase soy broth medium containing 0.3% yeast extract was introduced into a 10-fold volume of the same medium and incubated at 37°C with shaking. Logarithmic-phase cells were harvested approx. 2 h after transfer into new medium at an absorbance of 0.6 and wavelength of 600nm. The stationary-phase cells were obtained by incubation for an additional 2 h after the growth curve had reached a plateau at an absorbance of 1.0 in an approx. 3 h incubation. The harvested cells were washed twice with ice-cold 10 mM Tris-HCl buffer (pH 7.5) and were used further in suspension in the same buffer at 4°C.

Preparation of membrane fragments

Washed cells were treated with lysozyme in 0.75 M sucrose containing 10 mM Tris-HCl buffer (pH 7.5), at 4°C for 45 min. The outer and cytoplasmic membrane fractions were separated on six stepwise layers of 35–60% sucrose density gradient, with centrifugation at 27000 rpm for 40 h in an SW 27-1 rotor in a Spinco ultracentrifuge. Cytoplasmic and outer membrane fragments, which were fractionated as clearly visible layers above 35 and 60% sucrose, respectively, were collected. The other fractions, distributed over the intermediate step layers, were discarded. The collected fractions were washed twice with 10 mM Tris-HCl buffer, and were kept in suspension in the same buffer at 4°C until used.

Preparation of reverse-phase evaporation lipid vesicles

Phospholipids were extracted from cytoplasmic and outer membrane fragments with $CHCl_3/CH_3OH$ (2 : 1). The extracts were washed and purified by the method of Folch et al. [14]. The reverse-phase evaporation vesicles were prepared according to the method of Szoka and Papahadjopoulos [15], as follows. The extracted phospholipid was added to a 20 ml round-bottom flask, and the solvent was removed under reduced pressure by a rotary evaporator. The system was then purged with nitrogen, and the lipids were redissolved in $CHCl_3/CH_3OH$ (2 : 1) and were then added to the same amount of diethyl ether. One third volume of 10 mM Tris-HCl buffer (pH 7.5) was added and the resulting two-phase system was sonicated until the mixture became a homogeneous opalescent dispersion. The mixture was then placed on the rotary evaporator and the organic solvent was removed under reduced pressure with an aspirator at 25°C. As most of the solvent was removed, excess buffer was added and the suspension was successively evaporated for an additional 15 min at 25°C to remove traces of solvent. The preparations were used without further treatment to remove nonencapsulated material or residual organic solvent.

Fluorescence measurements

Fluorescence polarization measurements were carried out by the slightly modified method of Waring et al. [16]. The suspensions of lipid bilayer vesicles or the membrane fragments were made to a final volume of 0.9 ml, then were mixed with 0.5 ml of medium containing 675 mM mannitol/225 mM sucrose/40 mM Hepes buffer (pH 7.3). The samples were mixed with 1.5 ml of ethylene glycol to prevent freezing and then were incubated at 37°C for 30 min. *cis*- or *trans*-Parinaric acid (0.1 ml of 10 μg/ml solution) was added to the sample in a molar ratio of the probe to phospholipid of approx. 1 : 100. The temperature scanning was started after a few minutes' incubation to equilibrate the probe in the whole specimen.

Fluorescence polarization change was continuously recorded with the recorder equipped to a Shimadsu RF-502 spectrofluorophotometer at an emission wavelength of 410 nm (excitation at 320 nm). The intensities both parallel and perpendicular to the vertically polarized excitation beam were measured. The polarization ratio is defined as $I_{\parallel}/(I_{\perp} \times H_H/H_V)$, where H_H/H_V is a correction factor for instrumental anisotropy [17]. Temperature scans were performed by cooling the cuvette holder connected to a Neslab Endcal RT-50 cooling bath. The sample temperature was monitored by a 40-gauge copper-constantan thermocouple positioned just above the excitation beam in the sample. Decreasing temperature, at the rate of 0.5 C°/min, was recorded by a YEW Type 3066 pen recorder. All procedures were carried out under a dry nitrogen atmosphere.

Freeze-thawing

A 0.3 ml aliquot of suspension of the specimens in 10 mM Tris-HCl buffer (pH 7.5) was dispensed into a glass

freezing tube (15 × 150 mm). In slow freezing, the samples were cooled in an alcohol bath at −1°C for 5 min, then were inoculated with ice by being touched with the tip of a frosted tungsten wire. Approx. 15 min after inoculation, the temperature of the cooling bath was lowered to between −10 and −15°C at the rate of 1 C°/min. In rapid freezing, the same amount of the specimen in the freezing tube was directly immersed into liquid nitrogen. The specimens were thawed in a 35°C water-bath with shaking.

Enzyme assays

All enzyme assays were performed at 25°C, using a Shimadsu UV-200 double-beam spectrophotometer. The whole cell suspension and cell-free medium were employed as enzyme sources for the measurement of NADH oxidase, malate dehydrogenase and succinate dehydrogenase activities [18]. The activity of glucose-6-phosphate dehydrogenase was measured in the cell-free medium [19]. All enzyme activities are presented as a percentage of total activities which were obtained with a 2 min sonication of the unfrozen cell suspension.

Viable count of the cells

The freeze-thawed samples were serially diluted in trypticase soy broth medium containing 0.3% yeast extract and plated in the same medium containing agar. The plates were incubated at 37°C for 24 h and colonies were counted.

Analytical procedures

Total protein was determined following the method of Lowry et al. [20] using bovine serum albumin as a standard. Phospholipid content in the membrane fragments was determined with the extracted lipid specimens following the method of Shibuya et al. [21], assuming that it corresponded to 25-times the phosphorus content. The phospholipid composition was determined after a separation of the lipid into the individual lipid classes by thin-layer chromatography on a Kieselgel 60 plate (Merck). The fatty acid composition of the phospholipid was determined using a Hitachi K-53 gas chromatograph.

Results

Effect of freezing on the chemical composition of the membranes or lipid bilayer vesicles

In either slow or rapid freezing of *E. coli* logarithmic-phase cell membrane preparations, hardly any release of membrane proteins or phospholipids was observed. After thawing, cytoplasmic membrane samples showed an increase in turbidity, suggesting that certain portions of the membrane fragments had aggregated during the freeze-thaw process. Indeed, significant portions of the membrane fragments became precipitable under low-speed centrifugation. The outer membrane fragments showed a slight aggregation in the same freeze-thaw procedures. No compositional difference in the protein-to-phospholipid ratio between the precipitated and non-precipitated membrane fractions was observed (Table I). No difference was observed in phospholipid class composition or fatty acid composition before and after freezing of the membrane specimens. Similarly, the lipid bilayer vesicle specimens prepared from the membrane phospholipids of either logarithmic- or stationary-phase cells showed no change in chemical composition after freezing (data not shown).

Effect of the different rates of freezing on fluorescence polarization ratios in the lipid bilayer vesicles or membrane fragments

Lipid bilayer vesicles. The thermal phase transition of the lipids in unilamellar lipid bilayer vesicles before and after freezing was monitored by *trans*-parinaric acid fluorescence polarization changes. As shown in Fig. 1, with logarithmic-phase specimens, the lipid vesicle specimens showed a minimal polarization ratio of approx. 1.4 around physiological temperature, and a maximal ratio of approx. 1.9 below 0°C, before freezing. These ratios were quite similar in the vesicle specimens which were derived from cytoplasmic and the outer

TABLE I

The effect of freezing on the chemical composition and the membrane aggregation of E. coli logarithmic-phase membrane fragments

The percentages were calculated on the bases of the protein amounts as the average value of three measurements on three different cultures.

	Released (%)	Precipitated		Non-precipitated	
		(%)	protein : PL	(%)	protein : PL
Cytoplasmic membrane					
Unfrozen	0	0	-	100	64.6 : 35.4
Slow freezing	0.7	55.3	64.4 : 35.6	44.0	64.9 : 35.1
Rapid freezing	1.0	41.2	62.8 : 37.2	57.8	65.5 : 34.5
Outer membrane					
Unfrozen	0	0	-	100	81.4 : 18.6
Slow freezing	0.2	11.5	77.4 : 22.6	88.3	81.2 : 18.8
Rapid freezing	0	12.8	77.5 : 22.5	87.2	81.8 : 18.2

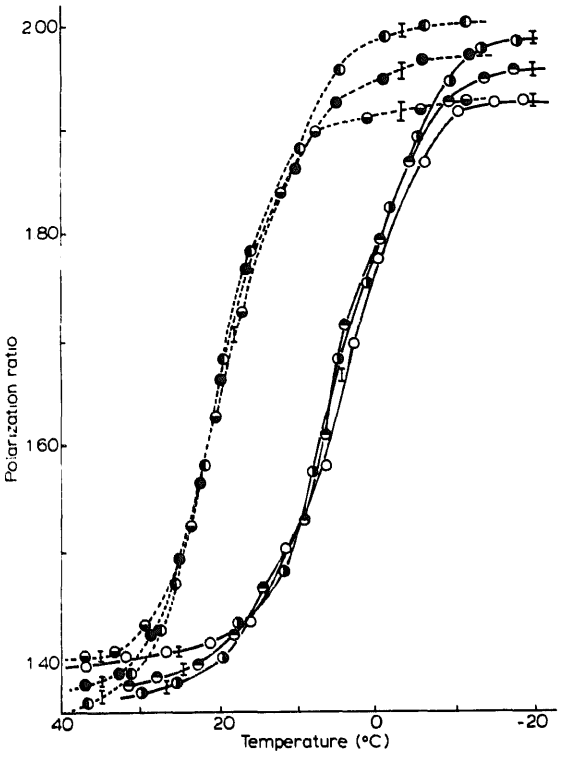

Fig. 1. The effect of different rates of freezing on fluorescence polarization ratio changes in the lipid bilayer vesicles derived from the membranes of logarithmic-phase cells. The unilamellar lipid bilayer vesicles, which were prepared as described under Materials and Methods, were frozen either slowly or rapidly. The temperature-dependent fluorescence polarization ratio changes in frozen and unfrozen specimens were compared using *trans*-parinaric acid. The vesicles were prepared from cytoplasmic (———) and the outer membrane (- - - - - -) phospholipids. ○, ⊖, unfrozen control; ◐, ◑, slowly freeze-thawed; and ⊖, ●, rapidly freeze-thawed specimens. Each line represents an average of three measurements.

membranes of either logarithmic- or stationary-phase cells. The maximal ratios attained with these specimens seemed considerably lower as compared with the ratios which have been observed with aqueous dispersions of comparable phospholipid samples (approx. 2.0).

The specimens frozen slowly to −10°C and then thawed exhibited significantly decreased and increased polarization ratios as compared unfrozen specimens, at physiological and subzero temperatures, respectively. Rapid freezing of the specimens brought about much less alteration in the polarization ratio, as compared with the results obtained with slowly frozen specimens. The polarization ratio changes modified by slow or rapid freezing in the stationary-phase specimens were almost the same as those of the logarithmic-phase specimens, regardless of the different individual lipid compositions in the original membranes.

Membrane fragments. The effect of freezing on the lipid character in logarithmic-phase membrane fragments was also investigated measuring the fluorescence polarization ratio with *cis*- and *trans*-parinaric acids. In a comparison of the ratios obtained with unfrozen specimens, slowly freeze-thawed cytoplasmic membrane fragments exhibited a definite decrease and increase in polarization ratios above and below the main transition temperature ranges, respectively. A slight upward shift of the main phase transition temperature range was exhibited, suggesting a lower moisture content in the specimens [22]. It seems likely that the *cis* isomer was involved more in shifting the transition temperature range and the *trans* isomer in altering the ratios. Rapid freezing of the specimens hardly affected the polarization ratio changes over the entire temperature range measured with both isomeric probes (Fig. 2a).

In the outer membrane fractions, slow freezing induced an increase in the polarization ratios in lower temperature regions, but, at the same time, there was no apparent alteration in the physiological temperature region. A shift in the main phase transition range to higher temperature regions was also observed in the measurements with *cis* isomers. The rapid freezing of the outer membrane samples brought about no appreciable alterations in polarization ratio change nor shift of transition temperature over the entire temperature range measured using the same probes (Fig. 2b).

It is shown in Table I that slow freezing of cytoplasmic membrane specimens produced more membrane aggregation as compared to the results obtained with rapid freezing. In order to discriminate a possible artificial effect of membrane fragment aggregation on the polarization changes, further investigations were carried out with the samples, which were separated into precipitable and non-precipitable portions. Both precipitated and non-precipitated membrane fractions resulting from slow freezing showed a similar altered polarization ratio change and an upward shift of the main transition temperatures (Fig. 3). No difference in polarization ratio change was seen between the precipitated and non-precipitated specimens, although these two fractions showed a definite difference in turbidity. A similar measurement performed with the specimens after rapid freezing demonstrated unmodified polarization ratio changes as compared to the results obtained with unfrozen specimens.

The results indicated that none of the alterations in fluorescence polarization ratio changes appearing after slow freezing of the membrane fragments was caused by the superficial configurational modification of the membrane fragments, such as aggregation, but rather that they resulted from the intrinsic lipid-state changes in the membranes.

The effect of freezing on membrane function and viability of logarithmic-phase cells

The effects of freezing on the membrane functions in *E. coli* logarithmic-phase cells are shown in Table II. When slowly freeze-thawed cell suspensions were incubated with external enzyme substrates, the cells ex-

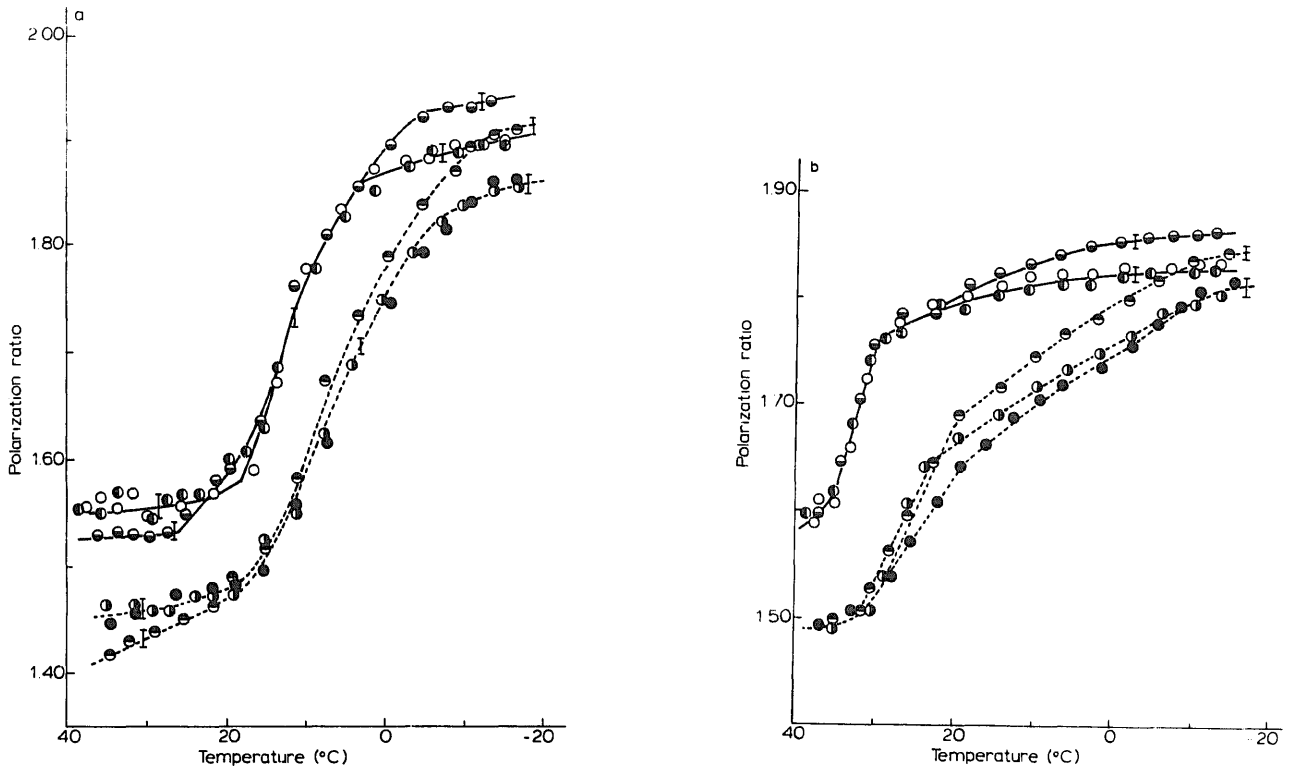

Fig. 2. The effect of different rates of freezing on fluorescence polarization ratio changes in cytoplasmic (a) and the outer (b) membrane fragments prepared from logarithmic-phase cells. The membrane fragment specimens which were prepared as described under Materials and Methods were frozen either slowly or rapidly. The temperature-dependent fluorescence polarization ratio changes after and before freezing of the specimens were measured by the use of (- - - - -), *cis*- and (———) *trans*-parinaric acids. ○, ●, unfrozen control; ⊖, ⊜, slowly freeze-thawed; and ◐, ◑, rapidly freeze-thawed specimens. Results are presented as an average of three measurements for each treatment in each of three different cultures.

TABLE II

The effect of different rates of freezing on revelation of enzyme activity and viability of E. coli logarithmic-phase cells

The enzyme activities are shown as percent of the total activities which were obtained by sonifying the same amount of the cells with the average ± S.D. of three measurements each in three different cultures.

	Unfrozen	Slow freezing		Rapid freezing	
		just after thawing	24 h after thawing	just after thawing	24 h after thawing
NADH oxidase					
cell suspension	3.7 ± 1.2	28.1 ± 1.1	57.0 ± 3.8	17.8 ± 1.8	43.0 ± 1.1
cell-free medium	0	0.3 ± 0.1	1.5 ± 0.4	0.9 ± 0.7	2.0 ± 0.5
Malate dehydrogenase					
cell suspension	3.5 ± 1.2	40.2 ± 5.8	66.0 ± 5.6	12.3 ± 5.9	22.0 ± 3.2
cell-free medium	0	45.6 ± 6.1	60.0 ± 2.8	9.1 ± 2.7	22.4 ± 2.2
Succinate dehydrogenase					
cell suspension	35.9 ± 2.3	81.2 ± 5.8	74.4 ± 3.2	76.8 ± 6.2	60.9 ± 6.7
cell-free medium	0	0.9 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.3
Glucose-6-phosphate dehydrogenase					
cell-free medium	0	16.0 ± 0.3	40.0 ± 2.0	0.4 ± 0.1	3.5 ± 1.4
Cell survival (%)	100	4.6 ± 3.5	–	53.1 ± 5.3	–

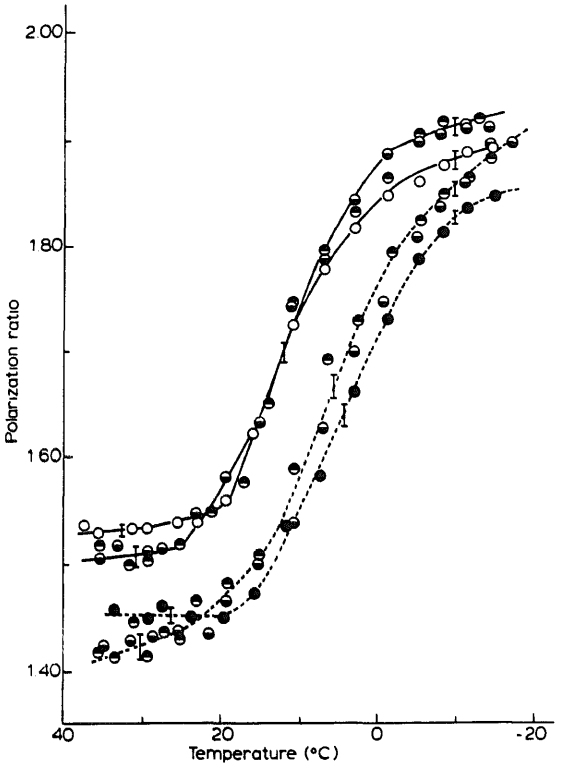

Fig. 3. The effect of freeze-induced membrane fragment aggregation on fluorescence polarization ratio changes after slow freezing of cytoplasmic membrane fragments derived from logarithmic-phase cells. Thawed specimens were centrifuged at 1500 × *g* for 15 min, and resulting precipitates were resuspended in 10 mM Tris-HCl buffer solution. The temperature-dependent fluorescence polarization ratio changes in either precipitated or non-precipitated fractions were compared with those in unfrozen specimens, using (------), *cis*, and (———) *trans*-parinaric acids. ○, ●, unfrozen control; ⊖, precipitated fraction; and ⊖, non-precipitated fraction. Results are presented as an average of three measurements for each treatment in each of three different cultures.

hibited a considerably stimulated activity of those enzymes which are known to be located in the periplasm or embedded in the cytoplasmic membrane. The activities of these enzymes in the cell-free medium were very low, except for the activity of malate dehydrogenase, which is known to be located in the periplasm. The results indicated that slow freezing of the cells brought about an increase in the permeability of the outer membrane to the substrates approaching the enzymes, but that no breakdown and/or leakage of the cytoplasmic membrane debris from the cells were induced. In the cell-free medium, on the other hand, a significant activity of glucose-6-phosphate dehydrogenase, which is known as an *E. coli* cytoplasmic marker enzyme, appeared together with the malate dehydrogenase activity. This indicates that cytoplasmic membranes as well as the outer membranes had become permeable to such macromolecules. The leakage or release of activity of the enzymes increased upon storage of the thawed cells at 4°C for 24 h, suggesting that slow freezing of the cells resulted in an irreversible disturbance of the membrane organization.

Rapid freezing of the cells brought about a slight increase in the activity of the membrane-located enzymes, with the exception of succinate dehydrogenase. The succinate dehydrogenase activity, which is high in the intact cells, appeared to be increased by rapid freezing. A slight amount of malate dehydrogenase was also released, but the release of glucose-6-phosphate dehydrogenase from the cells was very low as compared the release of malate dehydrogenase. The results indicate that the outer membrane was damaged to some extent, but the permeability of cytoplasmic membrane was not affected by rapid freezing.

The viability of the cells after freeze-thawing at different rates is also shown in Table II. Slow freezing of the cells resulted in a very low viability; in contrast, it was shown that a much higher viability was observed by rapid freezing of the cells.

Discussion

Fluorescence polarization studies in *E. coli* B cell membranes indicated that a certain portion of lipids in the membrane systems did not participate in the thermotropic phase transition and were considered to be in an ordered state. The presence of an ordered state of lipids in the membranes of Gram-negative bacteria has also been demonstrated by the experiments with X-ray diffraction [23], ^{2}H-NMR measurements [24–26], differential scanning calorimetry [27,28] and fluorescence spectroscopy [29]. The studies indicated that less than 40% of the outer membrane lipids participated in the phase transition, and the percentage is twice as high in cytoplasmic membranes. The lipids in an ordered state in cytoplasmic membrane were considered to result from the lipid–protein interaction, which represses lipid motional freedom in the systems [30–34]. In the outer membranes the lipid motion will be more strongly restricted by the presence of lipopolysaccharides, as well as by the presence of proteins [35,36].

The fluorescence polarization measurements in unilamellar lipid bilayer vesicles also demonstrated the existence of portions of lipid which did not participate in the phase transition during cooling of the vesicles from physiological temperatures to below 0°C. Also in a time-resolved X-ray diffraction study on lipid bilayer vesicles, the existence was demonstrated of lipids for which low temperature alone may not be sufficient for the phase transitions [37]. From these results it is surmised that the interactions between lipid molecules can also induce some forces to repress the lipid motion.

In freeze-thaw experiments, slow freezing gave rise to a significant lipid-state change both in the unilamellar lipid bilayer vesicles and in membrane specimens. However, rapid freezing resulted in a less intensive change

and no change, respectively, of the lipid state in the unilamellar lipid vesicles and in the membrane specimens. The results suggest that the disturbance of the bilayer organization, both in lipid vesicles and in membrane specimens, will presumably be caused by dehydration of the hydrophilic moieties of the lipids, in accordance with the crystallization of water molecules. This is because slow freezing must effectively cause such a dehydration process [38], in contrast to the very rapid freezing protocol which would fix the system in less dehydrated states. If the lipid-rich systems in living materials are exposed to low temperatures, the systems can be in a position to resist or to tolerate such a stress by virtue of their lipid composition. However, when a freeze-induced dehydration is imposed, in addition to low-temperature stress, the lamellar liquid crystalline state may no longer be able to withstand the stress. An upward shift of the main phase transition temperature ranges in freeze-thawed specimens suggests that the moisture content of the specimen is not fully recovered after thawing.

In the lipid bilayer vesicles, both slow and rapid freezing brought about an irreversible lipid-state change. At the same time, in the membrane fragments, slow freezing resulted in a similar lipid-state change but rapid freezing did not give rise to any such effect. This indicates that the interactions between lipid molecules are highly sensitive to freeze-induced dehydration when cooperation of the other interactions of the membrane constituents can not be expected. The effect of slow freezing on the change of chemical characteristics of lipid liposomes has previously been reported from NMR and ESR studies [39]. The interactions between lipid and protein and/or lipid and lipopolysaccharide may have a stabilizing effect on lipid bilayer structures in the membrane systems. It has been reported that some portions of the lipid in *E. coli* membranes were very strongly restricted in their motional freedom by interaction with proteins [35,36,40].

These results imply that the primary site of freezing damage to the cells will be in the lipid bilayer sheets in the membranes, where the destruction is caused by dehydration-induced lipid-state changes that arise from the crystallization of water molecules. In accordance with the lipid-state changes, the cells suffered from membrane permeability change which subsequently resulted in cell death. The irreversible change in membrane permeability after freezing of the cells is in good agreement with the results showing that the changes of the lipid state in the membranes are at least a partly irreversible reaction.

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