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Changes in chemical structure and function in *Escherichia coli* cell membranes caused by freeze-thawing. II. Membrane lipid state and response of cells to dehydration

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Glycerol and spermine prevented the freeze-induced alteration of lipid character in membrane fragments derived from *Escherichia coli* B logarithmic-phase cells, protecting against loss of membrane function and subsequently maintaining higher cell viability. The membrane specimens derived from the stationary-phase cells exhibited high resistance to freezing-induced alteration of the membrane lipid character, and to freezing injury. Freeze-drying of membrane fragment specimens from both logarithmic- and stationary-phase cells gave rise to alterations in lipid state similar to those shown in freeze-thawing of logarithmic-phase cell membrane specimens. Freeze-drying brought about reduction of cell viability in both growth phase specimens. It is suggested, therefore, that the fundamental cause of the injury induced by freezing of living materials is dehydration of lipid-rich systems such as cellular membranes.

Introduction

The motional freedom of the lipids in *Escherichia coli* cell membrane fragments is repressed considerably as compared to that in aqueous dispersions of comparable phospholipids, indicating that certain portions of the membrane lipids exist in an ordered state [1]. In logarithmic-phase cells, slow freezing of the membrane fragments results in a significant change of lipid state from order to disorder, concurrently with the loss of the membrane functions and reduction of the cell viability [2].

Aliphatic polyamines are reported to bring about a repression of lipid motion in the membranes of *E. coli* logarithmic-phase cells, as well as to maintain higher viability of the cells upon freezing [3]. The observations indicate that the repression of lipid motional freedom in the membranes may contribute to the stability of the membrane structure. Polyamines are found in many bacteria and eukaryotic cells [4–9] and are known to bind to negatively charged molecules in the cells [4–6,10,11]. Together with important in vivo functions, numerous effects of polyamines on membrane properties have been reported [12–16].

In contrast to the action of polyamines, glycerol, one of the best-known substances protecting living materials against freezing, is also known to prevent loss of membranes function in the same logarithmic-phase cells [17]. The protective effect of glycerol is probably exerted by modifying the water encompassing the biological systems.

In the stationary-phase cells, which exhibit stable membrane function and high viability in freezing, the immobility of the membrane lipids has been demonstrated to be of great importance [1]. The stability of membrane structure can be explained by the higher protein-to-lipid ratios in the stationary-phase cell membranes. The reinforcement of lipid–protein interactions in protein-rich membranes, which increases the immobility of the lipids in the membranes, may also increase the structural stability of the membranes.

The common factor in the high viability of the cells in freezing is protection of the membrane functions. In the present study, we have investigated the relationship between the stability of membrane lipid state and resistance of the cells to freezing, mediated by the various factors. The effect of dehydration of membrane fragments was also investigated to surmise the mechanisms related to the injury of living materials by freezing.

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Materials and Methods

Materials

cis- and *trans*-Parinaric acids were purchased from Molecular Probes (Junction City, OR and Plano, TX, respectively). Spermine was obtained from Sigma Chemical Co. (St. Louis, MO).

Bacterial cultivation and membrane fragmentation

E. coli B cells were grown to their logarithmic and stationary-phases with the procedures described in the preceding paper [2]. The membrane fragments were prepared from cells in both growth phases also according to the method described in Ref. 2.

Glycerol equilibration and freeze-thawing of the cells and the membrane fragments

Usually, a 0.4 ml portion of 25% glycerol in 10 mM Tris-HCl buffer (pH 7.5) was gently mixed with 0.6 ml of cell or membrane fragment suspension in the same buffer. This procedure results in a glycerol concentration of 10%, holding the buffer concentration constant. The cell suspension was held at 0 °C for 20 min before freezing, to allow full equilibration of glycerol in the cells. The glycerolated specimens were frozen and thawed as described in the preceding paper, except that, in slow freezing, lowering of the temperature was continued to −30 °C with ice inoculation at −4 °C. Enzyme activities and cells viability were measured in the presence of glycerol. No effect of glycerol on the viability of the cells or on the enzyme activities was observed in unfrozen cell specimens.

Treatment of cells and membrane fragments with spermine

Logarithmic-phase cells were harvested and washed twice with 10 mM Tris-HCl buffer. The cells of 100 mg wet base were suspended in 10 ml of distilled water containing 1 mg/ml of spermine, and the suspension was incubated at 37 °C for 20 min with vigorous shaking to prevent precipitation. The cells were collected, washed once with distilled water to remove excess spermine, and resuspended in 0.3 ml of the Tris-HCl buffer. The cell suspension was freeze-thawed and enzyme activities and viability of the cells were measured. In the membrane fragment specimens, the suspension was added with the concentrated solution of spermine to a final concentration of approx. 50 μM, and freeze-thawed by the usual procedure in the presence of spermine. No effect of spermine on the enzyme activity or on fluorescence polarization was observed in these treatments.

Drying of the cells and the membrane fragments

In freeze-drying, a 0.3 ml portion of either cell or membrane fragment suspension was dispensed into a round-bottomed drying ampoule and then frozen rapidly with liquid nitrogen, rotating the tilted ampoule by hand. The ampoules were fitted to the freeze-drier equipped with liquid nitrogen trap and dried in a vacuum. Vacuum drying without prefreezing was carried out as follows. A 0.3 ml portion of membrane fragment suspension in the drying ampoule was fitted to a rotary evaporator, and the bulk of water was removed under reduced pressure with water aspirator at 25 °C. The specimen was vacuum-dried for an additional 60 min with a freeze-drier equipped with a liquid nitrogen trap. The dried specimen was rehydrated with 0.3 ml of distilled water in room temperature.

Miscellaneous procedures

The procedures for fluorescence polarization measurements, enzyme activity measurements and the chemical analyses were as described in the preceding paper [2]. Viable counts on thawed or rehydrated cell specimens were carried out also as per Ref. 2.

Results

Effect of protective additives on membrane and/or cells response to freezing

Glycerol

Slow freezing of logarithmic-phase cell membrane fragments in the presence of 10% glycerol brought about a slight increase in the release of membrane components, as compared that observed in non-glycerolated specimens. The sample definitely exhibited no aggregation of membrane fragments in freezing. The chemical compositions of cytoplasmic as well as outer membranes were also unaffected.

The protective effect of glycerol on the freezing-induced modification of fluorescence polarization ratios of the logarithmic-phase membrane fragments was monitored by the use of *cis*- and *trans*-parinaric acids. As has been shown in the preceding paper [2], slow freezing of cytoplasmic membrane fragments in the absence of glycerol produced a typically altered polarization ratio change. However, the same slow freezing of the specimens in the presence of glycerol showed no alteration of phase-transition ratios as compared to the unfrozen specimens (Fig. 1a). The same freezing protocol for the outer membrane specimen showed a typical increase of polarization ratio below the main transition temperature region, or no alteration of the ratios in the absence or presence of glycerol, respectively. A sharper increase of the polarization ratio in the main transition-temperature region was observed in the specimens (Fig. 1b).

The protective effect of glycerol on freezing injury to the cells and to the membrane functions was also investigated with viability counting and measurements of

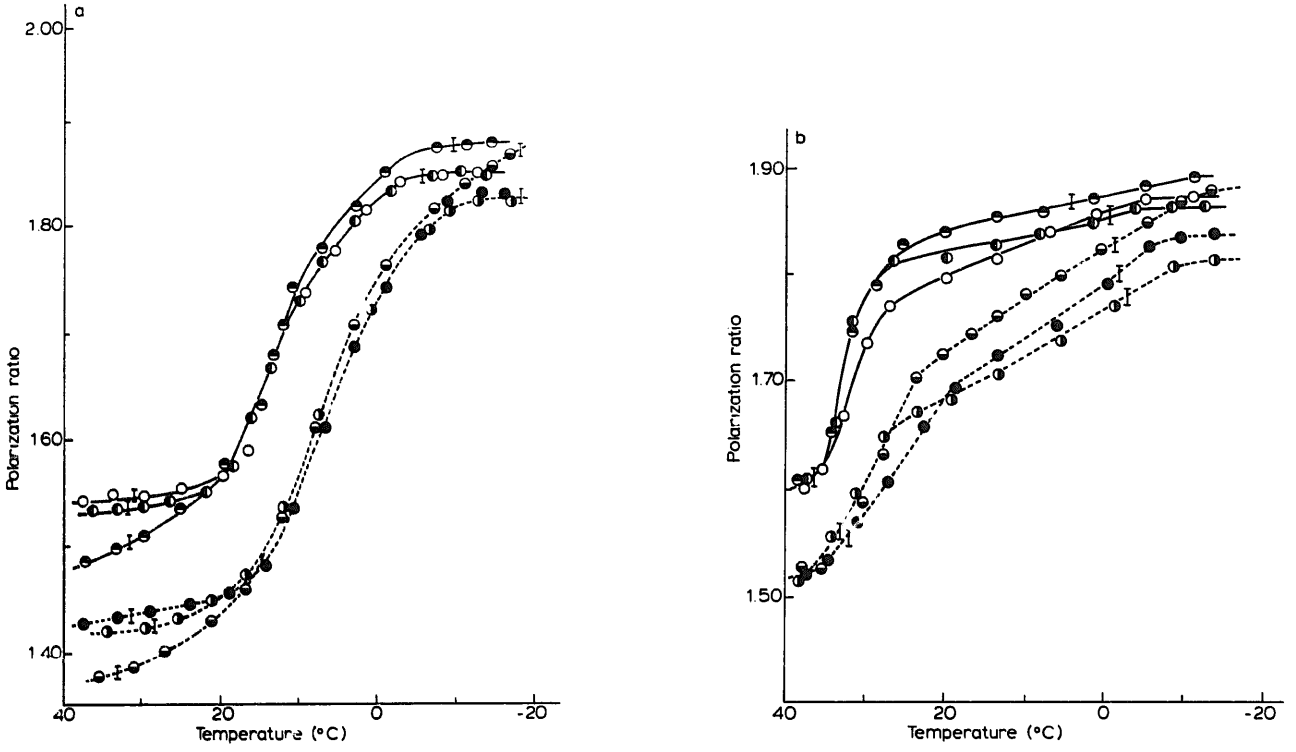

Fig. 1. Protective effect of glycerol on freeze-induced alteration of fluorescence polarization ratio changes in cytoplasmic (a) and the outer (b) membrane fragments derived from logarithmic-phase cells. Both glycerolated and non-glycerolated membrane fragments were frozen slowly and thawed as described under Matrials and Methods. The temperature-dependent fluorescence polarization ratio changes in thawed specimens were compared to the value obtained with non-glycerolated, unfrozen specimens, using (- - - - -), *cis*- and (———) *trans*-parinaric acids. ○ ●, unfrozen control; ◐ ◑, frozen in 10% glycerol; and ⊖ ⊖, frozen without glycerol. Results are presented as an average of three measurements in each treatment.

the cellular enzyme activities (Table I). No increase in NADH · oxidase and malate dehydrogenase activities was revealed by slow or rapid freezing of the glycerolated cells. Succinate dehydrogenase activity, which is stimulated almost 100% even after rapid freezing in the absence of glycerol, was hardly affected, indicating that glycerol prevents the change in permeability of the outer membrane very effectively. No liberation of glucose-6-phosphate dehydrogenase from the cells was observed, suggesting that glycerol more than likely prevents the destruction of cytoplasmic membrane. Glycerol maintained a very high viability of the cells in slow as well as in rapid freezing.

Spermine

Spermine is known to be effective in protecting the osmotically fragile membranes from lysis, and it has also been reported to repress the fluorescence polarization ratio changes in *E. coli* logarithmic-phase membranes in a dose-dependent fashion [3]. The relationship

TABLE I

The protective effect of glycerol on the loss of membrane function and viability of E. coli logarithmic phase cells on freezing [a]

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

	Unfrozen	Slow freezing		Rapid freezing	
		−glycerol	+glycerol	−glycerol	+glycerol
NADH oxidase (whole cell suspension)	6.5±1.1	76.2±5.3	5.4±2.6	31.3±3.5	5.0±1.3
Malate dehydrogenase (whole cell suspension)	7.7±1.2	69.1±4.3	5.2±1.3	45.3±2.2	4.5±1.3
Succinate dehydrogenase (whole cell suspension)	44.4±3.3	100.0±4.8	57.2±6.3	100.0±3.2	48.0±2.5
Glucose-6-phosphate dehydrogenase (cell-free medium)	0	32.7±2.7	0	10.3±1.3	0
Cell survival (%)	100	5.3±3.0	70.7±4.3	14.9±3.5	90.2±4.5

[a] Cells were cultivated in trypticase soy broth (TSB) medium, which does not contain yeast extract.

TABLE II

Effect of spermine treatment on the protection of membrane function and viability of E. coli logarithmic phase cells on freezing

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

	Non-treatment		Spermine treatment	
	unfrozen	freeze-thawed	unfrozen	freeze-thawed
Whole cell suspension				
NaDH oxidase	3.5±1.8	24.3±2.1	3.5±1.5	9.4±1.7
Malate dehydrogenase	9.9±1.4	43.3±3.6	7.6±1.5	17.9±2.4
Cell-free medium				
Glucose-6-phosphate dehydrogenase	0.7±0.3	15.2±0.4	0.8±0.6	7.9±1.3
Malate dehydrogenase	14.1±1.3	55.6±3.3	6.3±1.3	20.8±2.5
Cell survival (%)	100	8.9±4.4	100	70.5±3.0

of the repression of fluorescence polarization ratio changes to the resistance of the membrane to freezing-induced damage was investigated by measuring the fluorescence polarization of the parinaric acids. As predicted from the previous work [3], the polarization ratios of

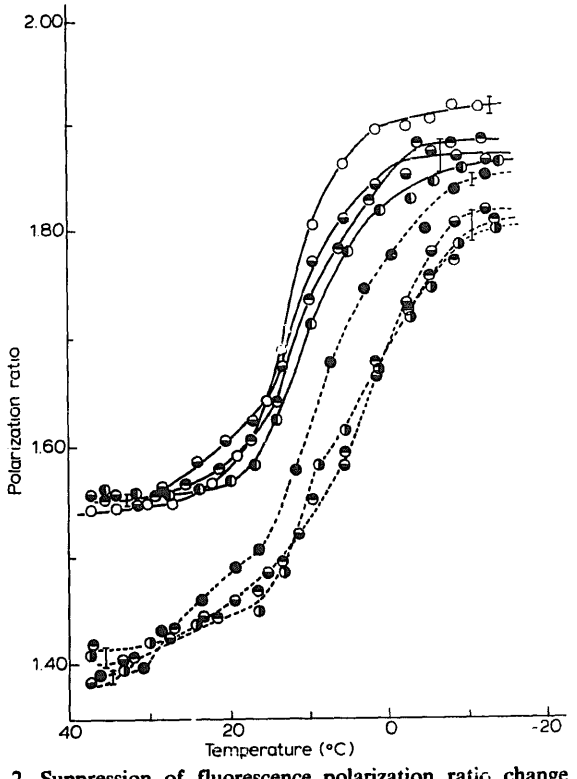

Fig. 2. Suppression of fluorescence polarization ratio change and prevention of freeze-induced alteration of the polarization ratio change with spermine, in cytoplasmic membrane fragments derived from logarithmic-phase cells. The temperature-dependent fluorescence polarization ratio changes were measured before and after freeze-thawing of the specimen in the presence or absence of spermine. The measurements were carried out with (- - - - - -), *cis*- and (———) *trans*-parinaric acids. ○ ●, unfrozen control without spermine; ⊖, unfrozen specimen in the presence of spermine; ◐ ◑, slowly; and ⊖, rapidly frozen then thawed in the presence of spermine. Results are presented as an average of three measurements in each treatment.

both cytoplasmic and the outer membrane fragments were repressed with 50 μM spermine, to the level of those observed in the stationary phase specimens. In the case of either slow or rapid freezing of the cytoplasmic membrane specimen in the presence of the same concentration of spermine, the repressed polarization ratios seemed unlikely to be affected (Fig. 2). A similar polarization ratio change which was unaffected was also observed in the outer membrane specimens frozen by the same protocol in the presence of spermine.

The effect of spermine on protection against freezing injury to membrane function was investigated by measurements of enzyme activity in the cells. The enzyme activities after freezing of spermine-treated cells after freezing were about one-half to one-third of the untreated specimen values (similarly frozen), indicating that both the outer and cytoplasmic membranes were well protected against damage by the treatment of the cells with spermine (Table II). Similar treatment of the cells also resulted in very high cell viability, as is shown in the same table. The results indicate that the repression of the membrane lipid motion induced by spermine contributed to the increase in cell resistance to freezing.

Membrane stability and freezing resistivity of stationary-phase cells

In contrast to the results obtained in the freezing of logarithmic-phase membrane specimens, approx. 85 and 52% of the outer membrane fragments aggregated during either slow or rapid freezing, respectively, and were precipitated in low-speed centrifugation. Approx. 33 and 42% of the cytoplasmic membrane fraction aggregated during slow and rapid freezing, respectively. Chemical analysis of the stationary-phase membrane specimens showed a considerably higher protein-to-lipid ratio, both in cytoplasmic (68.3 : 31.7) and outer membrane fragments (87.7 : 12.3), compared to the ratios observed in logarithmic-phase membrane specimens [2]. The chemical compositions of the two membrane specimens were hardly affected by freezing.

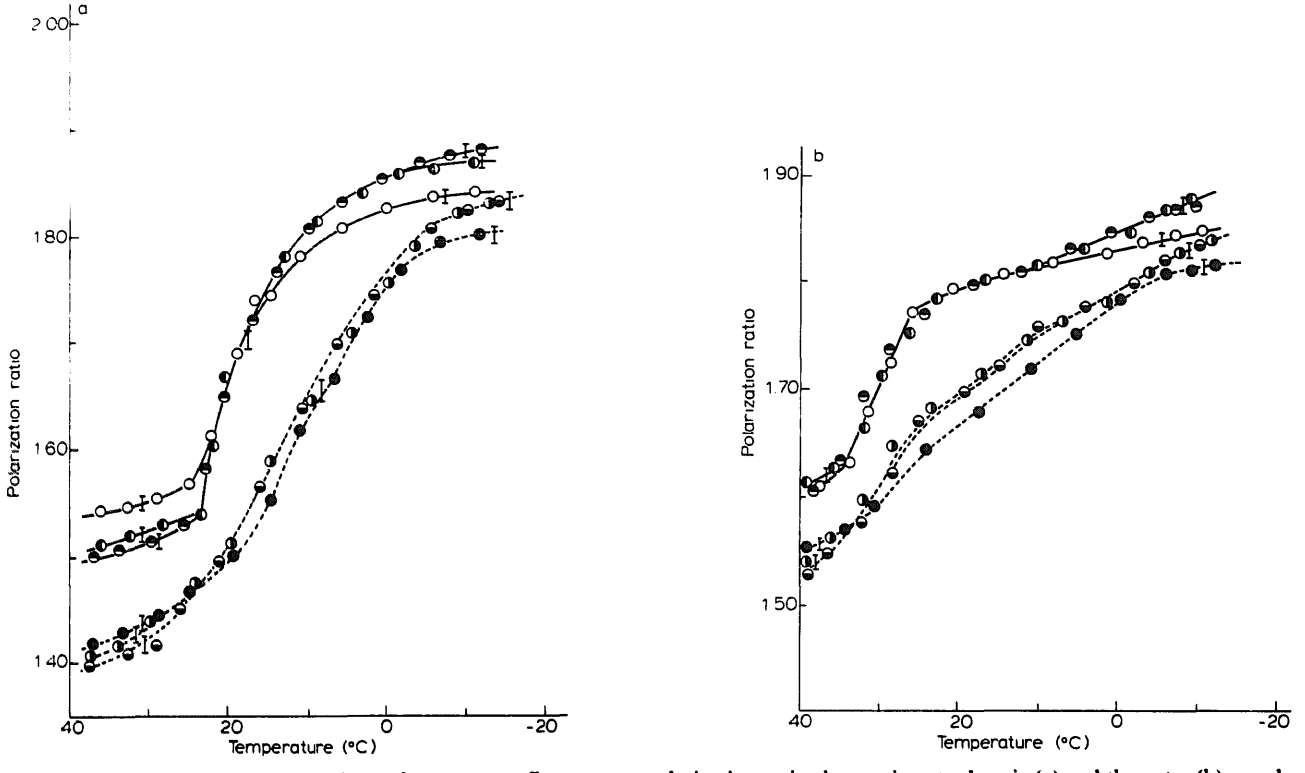

Fig. 3. The effect of dehydration of membrane fragments on fluorescence polarization ratio changes in cytoplasmic (a) and the outer (b) membranes derived from stationary-phase cells. The specimens were either freeze-dried or vacuum-dried as described under Materials and Methods. The temperature-dependent fluorescence polarization ratio changes in rehydrated specimens were compared to the ratio obtained with non-dehydrated specimens, using (- - - - -), *cis*- and (———) *trans*-parinaric acids. ○ ●, non-dehydrated control; ⊖ ⊖, freeze-dried; and ◐ ◑, vacuum-dried specimens. Results are presented as an average of three measurements for each treatment each in three different cultures.

No change in the fluorescence polarization ratios was observed during freezing of the membrane fragments, whether slowly or rapidly. Further experiments, carried out after separation of freeze-thawed specimens into precipitable and non-precipitable portions, also showed little difference between specimens.

The results supported the conjecture that the lipid state in the membranes was hardly affected by change in the configurational modification of the membrane such as aggregation [2].

The effect of freezing on membrane function of the stationary-phase cells was investigated by measurement of the membrane-located enzyme activities. Succinate dehydrogenase activity, which was revealed to be most susceptible to freezing in logarithmic-phase cells, was not seen to be affected by the freezing at either rate. Approx. 8 and 5% of NADH oxidase and malate dehydrogenase activities, respectively, were exhibited upon freezing the cells by direct immersion in liquid nitrogen, but a very slight increase in these enzyme activities was induced by slow freezing. No activity of glucose-6-phosphate dehydrogenase was revealed after slow freezing. The cells maintained 95% survival during slow freezing and 91% survival during rapid freezing by direct immersion in liquid nitrogen.

The effect of dehydration on membrane lipid character and cell viability

Freeze-drying or vacuum desiccation of cytoplasmic as well as outer membrane fragments of logarithmic-phase specimens exhibited an alteration in fluorescence polarization ratios similar to those observed in slow freezing (data not shown). The stationary-phase membranes, which appeared to be unaffected by freezing, were affected after dehydration similarly to logarithmic-phase specimens (Figs. 3, a and b). No significant differences between freeze-dried and vacuum-dried specimens were seen in any of the specimens.

The modification of polarization ratio changes caused by drying of membrane fragments from both growth phases is quite similar to that resulting from slow freezing of logarithmic-phase specimens. This result indicates that the effect of drying on the lipid character is similar to that of freezing, except that, compared to freezing, the dehydration caused by drying should have a more drastic effect on the biological membranes, since the stationary-phase membranes were also affected by drying.

The deleterious effect of freeze-drying compared to freeze-thawing was investigated by comparing the resistivity of the cells in differing growth phases. The

viability of the cells after freeze-thawing increased more than 90%, in accordance with the progress of cell-growth phases. Meanwhile, the viability of the cells after freeze-drying did not exceed 40%, even after their growth state had reached the stationary phase.

Discussion

The present study showed that glycerol or polyamine prevented freezing-induced alteration of membrane lipid character in logarithmic-phase specimens, at the same time protecting against loss of membrane function and maintaining a high cell viability. Under the same freezing conditions, but in the absence of any protective agent, the stationary-phase specimens exhibited a very high resistance to alteration of the membrane lipid character and to the reduction of the viability of the cells. Freeze-drying of the stationary- as well as logarithmic-phase specimens resulted in alterations of membrane lipid character and reduction of cells viability in a manner similar to that obtained by freezing of logarithmic-phase specimens.

Glycerol is believed to protect the biological substances from freezing injury by modifying the activity of water in the systems. On the other hand, the membrane-stabilizing effect of polyamine can be explained by considering this to comprise polyvalent cations which exert a direct effect on the membrane organization by bridging the negatively charged groups in the lipid bilayer of the membranes [18–21].

The membrane constitution of logarithmic- and stationary-phase cells varies in lipid fatty acid composition [22–25] and protein-to-lipid ratios [1,26], etc. The membrane stability in the stationary-phase specimens may have resulted from higher protein-to-lipid ratios in the membrane constituents. The effect of protein concentration on the repression of lipid motion in synthetic bilayer membranes [27,28], and in *E. coli* cell membranes [29–31] has been previously reported. In this respect, the chelating activity of the Tris buffer used could not be totally eliminated. However, the stability of the membrane varied largely depending on cell-growth conditions, indicating that the effect of tris buffer would not be so significant.

The factor common to these diverse modes of protective effects or the resistance of the cells to freezing seems to be the suppression of the mobility of the bilayer lipids in the membranes. If the freezing process caused alterations of the membrane specimens' lipid state it invariably resulted in loss of membrane function and a reduction in cell viability. Interestingly, the same qualities of lipid-state change and reduction of cell viability was brought about by freeze-drying of the specimens, suggesting that the modifications of the lipid character in the freezing process might also be caused by the dehydration of the membrane systems. It has been reported that, in a variety of lipid systems, low moisture favorably stabilizes non-bilayer states [32,33].

As indicated in the preceding paper, rapid freezing did not give rise to any changes in the lipid state of the membrane fragments, or in the membrane functions in the logarithmic-phase cell specimens. Thus, with freeze-drying, which is performed by very rapid prefreezing, the lipid nature seems unlikely to be affected during the prefreezing processes. Vacuum desiccation of the membrane, which does not include any freezing process but induced a quite similar change in the membrane lipid character, appears to support this conclusion. The stationary-phase specimens, which were not affected by any rate of freezing, were influenced by drying similarly to the logarithmic-phase specimens. This indicats that the drying process described leads to a more drastic dehydration than freezing. A lyotropic bilayer to non-bilayer transition similar to the freeze-drying of living material may be possibly introduced by freeze-induced dehydration in accordance with the crystallization of water molecules. In either cases, the lipid mesomorphic phase change is likely to influence the membrane structure and the functions thereof.

As a result, it is suggested that the primary source of the injury caused by freezing of living material is dehydration of lipid-rich systems, such as organisms, subcellular organelles and membrane systems, etc. The well-known effect of the size of ice crystals or their configuration on extra- or intracellular ice crystal formation might be explained by the fact that these factors depend on the rate of freezing. The rate of freezing giving rise to the most effective dehydration from the systems varies extensively from organism to organism and tissue to tissue, by virtue of lipid composition or interactions with other membrane constituents. Thus the injury due to freezing in individual species will vary widely, depending on the rate of freezing.

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