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# Fluorescence polarization studies on *Escherichia coli* membrane stability and its relation to the resistance of the cell to freeze-thawing. I. Membrane stability in cells of differing growth phase

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**Physical properties of *Escherichia coli* membrane lipids in logarithmic- and stationary-phase cells were studied by measuring the fluorescence polarization change of *cis*- and *trans*-parinaric acid as a function of temperature. In aqueous dispersions of phospholipids extracted from cytoplasmic and outer membranes of cells of differing growth phase, a similar polarization increase was observed over the range from physiological temperature to below 0°C, and nearly the same transition ratios were obtained in all samples. The cytoplasmic membrane of both of the growth-phase cells showed a higher polarization ratio above the transition temperatures, compared to that in the aqueous dispersion of phospholipids. The polarization ratios below the transition temperatures of these specimens were lower than the value obtained with the lipids, especially in the stationary-phase specimens. The outer membrane specimens showed a similar polarization change but the transition temperature ranges were considerably higher both in the logarithmic- and the stationary-phase specimens, compared to those in the cytoplasmic membrane specimens. Freeze-thawing of logarithmic-phase cells showed the emergence of activity of certain enzymes which are known to be located in the membranes. The stationary-phase cells did not suffer from any such deleterious effect and maintained a high level of cell viability in a similar treatment. These results indicate that in the stationary-phase cell membranes lipids are in a highly ordered state, and the lipid state causes a membrane stability which results in the high resistance of the cell to freeze-thawing.**

## Introduction

Freezing damage to living cells varies depending on the freezing rates, freezing temperatures or the kind of protective substances used, etc. The physical property of the membranes has also been assumed to be one of the deciding factors in the resistivity of the cell to freezing. For example, exponentially growing cells of *Escherichia coli* B that had suffered from freezing damage were found to release considerable amounts of membrane proteins, lipopolysaccharides and phospholipids as well as certain enzymes which are known to be located in the cytoplasm. Viability of the cells in such a specimen appeared to be reduced considerably [1].

The cell envelope of Gram-negative bacteria consists of three layers: cytoplasmic membrane, a peptidoglycan layer and the outer membrane [2]. Among these layers, the outer membrane and cytoplasmic membrane in exponentially growing cells

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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are well defined and have apparently different compositions, with regard to phospholipid fatty acids [2–5] or ratios of protein, phospholipid and lipopolysaccharide, to those of the membranes in the stationary-phase cells [6]. The compositional differences of the membrane constituents might result in the different organization of the membranes, especially in the lipid state.

It is well documented that in lipid samples which contain coexisting solid and fluid phases, *trans*-parinaric acid preferentially partitions into the solid phase, while *cis*-parinaric acid distributes nearly equally in both phases [7–10]. It was thought that the measurement of physical properties of membrane lipids by the use of these probes in tandem might reveal the fine difference in membrane organization of the cells of differing growth phase, especially below the lipid phase transition temperatures.

In the present study, it was demonstrated that immobility of the lipid was increased in the stationary-phase cell membranes. An increase of the protein-to-lipid ratio in the stationary-phase cell membranes was also manifested, suggesting reinforcement of lipid-protein interactions which may cause a stabilizing effect on the membranes. The stability of the membrane was shown to be increased in accordance with the growth phase of the cells, commensurate with the restoration of cell viability after freezing-thawing.

## Materials and Methods

*Materials.* *cis*- and *trans*-Parinaric acid was purchased from Molecular Probes, Inc. (Junction City, OR and Plano, TX, respectively). Dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylcholine (DOPC), palmitoyloleoylphosphatidylcholine (POPC) and egg phosphatidylcholine were products of Sigma Chemical Co. (St Louis, MO).

*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 at 600 nm. Early stationary-phase cells were harvested after approx. 3 h incubation, at an absorbance of 0.9. Stationary-phase cells were obtained by incubation for 2 additional h after the growth curve reached a plateau.

*Preparation of membrane fraction.* 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 a 35–60% sucrose density gradient, with the centrifugation at 27000 rpm for 40 h in an SW 27-1 rotor in a Spinco ultracentrifuge. Cytoplasmic and the outer membrane fractions were obtained as clearly visible bands at the top and near the bottom of the gradient, respectively. The fractions were collected and washed twice with 10 mM Tris-HCl buffer, and were kept by suspending in the same buffer at 4°C until used.

*Aqueous dispersion of phospholipids.* Phospholipids were extracted from cytoplasmic and outer membrane fractions with $CHCl_3/CH_3OH$ mixture (2 : 1). The extracts were washed and purified after the method of Folch et al. [11]. Aqueous dispersions of authentic phosphatidylcholines or extracted phospholipids were prepared by drying the lipids with $N_2$ and suspending in 1 ml of 10 mM Hepes buffer (pH 7.4) above the transition temperatures of the lipids. The samples were blended with a Vortex mixer and completely dispersed by brief sonication.

*Fluorescence measurement.* Fluorescence polarization measurement was carried out with the slightly modified method of Waring et al. [12]. The membrane suspensions or lipid dispersions 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 7.4). The samples were mixed with 1.5 ml of ethylene glycol, flushed with $N_2$, then were incubated above the transition temperature of the lipids for 30 min. *cis*- or *trans*-Parinaric acid (0.1 ml of 10 $\mu$g/ml solution) was added to the sample just before the fluorescence measurement. The molar ratio of the probe to phospholipid was approx. 1 : 100 in all systems.

Fluorescence change was continuously recorded with the recorder equipped to a Shimadsu RF-502 spectrofluorophotometer at the emission wavelength 410 nm (excitation at 320 nm). The intensities both parallel and perpendicular to the verti-

cally 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 [13]. Temperature scans were performed by cooling or warming the cuvette holder connected to a Neslab Endcal RT 50 cooling bath. Sample temperature was monitored by a 40 gauge copper-constantan thermocouple placed in the sample just above the excitation beam. Decreasing temperature, mostly at the rate of 0.5 Cdeg/min, was recorded by YEW Type 3066 pen recorder. All procedures were carried out under a dry nitrogen atmosphere.

*Equilibration of cell with glycerol.* Usually, a 4 ml portion of 25% glycerol in 10 mM Tris-HCl buffer (pH 7.5) was gently mixed with 6 ml of cell suspension in tris-HCl buffer. This procedure results in a glycerol concentration of 10%, holding the buffer concentration constant. The cell suspensions were held at 0°C for 20 min, to allow full equilibration of glycerol in the cell. No effect of glycerol on the viability of the cells or enzyme activities was observed in an unfrozen specimen.

*Freeze-thawing of the cells.* Washed cells were suspended in 10 mM Tris-HCl buffer (pH 7.5) at a concentration of approx. 0.5 g wet cells per ml. A 0.5 ml aliquot of cell suspension was dispensed into a glass freezing tube (15 × 150 mm). 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 −10°C at the rate of 1 Cdeg/min. 30 min after the bath temperature reached −10°C, samples were thawed in a 35°C water bath with shaking. Glycerol-treated cells were frozen and thawed in a similar manner, to −30°C with an inoculation of ice at −4°C.

*Enzyme assays.* All enzyme assays were performed at 25°C using a Beckman DB-GT spectrophotometer. The whole cell suspension was employed as enzyme source for the measurement of NADH oxidase, malate dehydrogenase and succinate dehydrogenase activities. The activity of glucose-6-phosphate dehydrogenase was measured in the supernatant of centrifuged cell suspension. All enzyme activities were presented as a percentage of total activities which were obtained with a 2 min sonication of unfrozen cell suspensions. The assay methods have been published elsewhere [1].

*Viable count of the cells.* The thawed samples were serially diluted in sterile distilled water and plated in trypticase soy agar, containing 0.3% yeast extract. 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. [14] using bovine serum albumin as a standard. Content of lipopolysaccharide was estimated directly with the membrane fragments according to the method of Osborn et al. [15]. Phospholipid contents were determined by the method of Shibuya et al. [16], assuming that it corresponds to 25-times the phosphorus content. The phospholipid composition was determined after a separation of the lipid classes by thin layer chromatography on a Kieselgel 60 plate (Merck). Fatty acid composition of the phospholipid was determined using a Hitachi K-53 gas chromatograph.

## Results

### *Fluorescence polarization change of parinaric acids in phospholipids as a function of temperature*

#### *Synthetic and egg phosphatidylcholine*

The thermal phase transitions monitored by *trans*-parinaric acid in aqueous dispersions of various synthetic and egg phosphatidylcholine are shown in Fig. 1. Phospholipid of saturated fatty acyl residues (DPPC) showed a definite transition at 38°C. Above and below the main transition, polarization ratios were weakly dependent on temperature. Phospholipid of unsaturated fatty acyl residues (DOPC) also showed a steep increase of polarization ratio with its maximum around −22°C. The transition temperatures are in close agreement with the values reported earlier [10,17]. With the phospholipid which has saturated and unsaturated fatty acyl residues in its molecule (POPC), a weak pretransition followed by a definite polarization increase was observed in the temperature range from 30 to −15°C. Egg phosphatidylcholine showed a gradual polarization change over a wide temperature range, as may be predicted for a sample of a mixture of saturated and unsaturated phosphatidylcholine [8,10]. Both

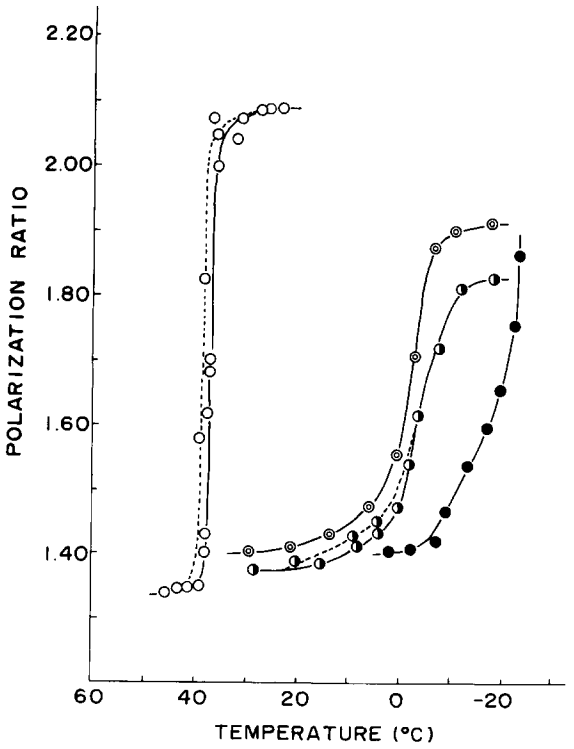


Fig. 1. Fluorescence polarization of *trans*-parinaric acid in aqueous dispersions of synthetic and egg phosphatidylcholine as a function of temperature. The aqueous dispersions of lipid samples were prepared as described under Materials and Methods. The samples were kept just above (below) the scanning temperature ranges for 30 min, then cooled, (———) or warmed (- - - - - -) at the rate of 0.5 Cdeg/min. ○, DPPC; ●, DOPC; ◎, POPC; ◑, egg phosphatidylcholine.

DPPC and egg phosphatidylcholine showed a cooling- and heating-dependent hysteresis effect when this was examined.

*Phospholipids extracted from E. coli cell membranes*

In aqueous dispersions of *E. coli* outer membrane phospholipids, fluorescence polarization of *trans*-parinaric acid in two specimens of differing growth phase showed an apparent increase in a similar temperature range from 35 to 0°C. In the specimens of cytoplasmic membrane phospholipids, stationary-phase specimens showed a polarization increase from 20 to −5°C, whereas logarithmic-phase specimens showed an increase from 10 to −10°C. In spite of the different temperature dependency, which resulted from the different lipid compositions, the absolute polarization ratios of these four phospholipid specimens were almost the same; approx. 1.40 above and 1.95 below the transition temperatures were obtained for all samples (Fig. 2).

*cis*-Parinaric acid also manifested a similar phase transition in these lipid samples. Although the temperature ranges of the polarization change were approx. 10 degrees lower compared to those which were obtained with the *trans* isomer, the increment of the polarization ratio was almost the same as that obtained with the *trans* isomer (data is not shown).

*Fluorescence polarization measurement in E. coli cell membranes*

*Cytoplasmic membrane*

The physical state of the lipids in *E. coli* membrane samples were also investigated by using the same probes (Fig. 3). A preliminary experiment demonstrated that the chemical compositions of the lipid in the aqueous dispersions and the original membranes were comparable. In cytoplasmic membrane, the absolute values of the polarization with *trans*-parinaric acid were significantly higher in the regions above the transition temperature in both of the growth phase specimens (1.52). In logarithmic-phase specimens, the polarization increase was initiated at 25°C and terminated around −10°C. The polarization ratio below the transition temperature of this membrane sample did not exceed 1.90. In the stationary-phase specimens, the transition temperatures ranged between 30 and 0°C. The polarization ratio below the transition temperature was less than 1.83 in this membrane sample and no more increase was observed below −20°C.

The polarization ratios observed with *cis*-parinaric acid were approx. 1.40 above the transition temperature in both growth-phase specimens. The transition temperature ranges were 15 to 10 degrees lower compared to those of the *trans* isomer in the stationary- and logarithmic-phase specimens, respectively. The polarization ratio below the transition temperature was 1.87 in logarithmic-phase specimens but the ratio observed with the stationary-phase specimens was very low (1.72).

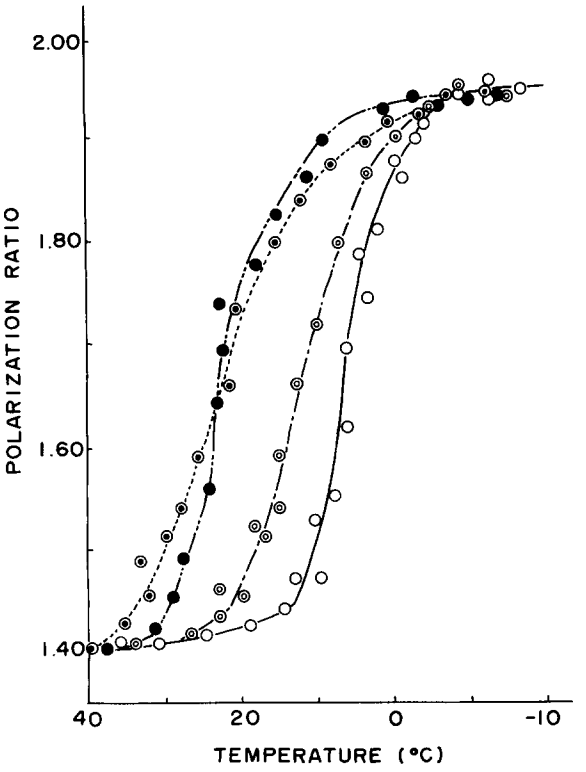


Fig. 2. Fluorescence polarization of *trans*-parinaric acid in aqueous dispersions of phospholipids extracted from *E. coli* cell membranes as a function of temperature. The aqueous dispersions of phospholipids were prepared as described under Materials and Methods. All samples were kept at 45°C for 30 min, then fluorescence polarization was measured in a cooling of the samples at the rate of 0.5 Cdeg/min. Phospholipids extracted from: —○—, cytoplasmic membrane of logarithmic-phase cells; -—◎-—, cytoplasmic membrane of stationary-phase cells; - - —●— - -, outer membrane of logarithmic-phase cells; ----◉----, outer membrane of stationary-phase cells.

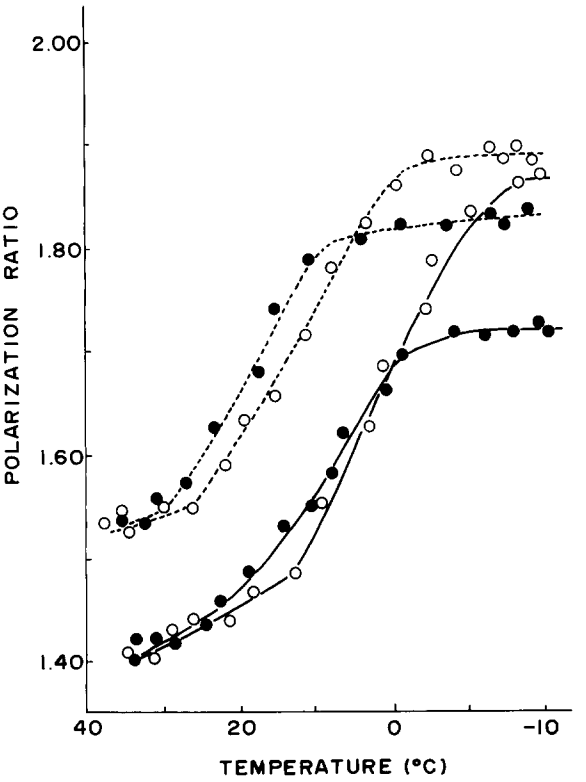


Fig. 3. Fluorescence polarization of *cis*- and *trans*-parinaric acids in cytoplasmic membranes of cells of differing growth phase as a function of temperature. The cytoplasmic membrane samples were prepared as described under Materials and Methods. The membrane suspensions were kept at 45°C for 30 min, then fluorescence polarization was determined in a decreasing temperature at the rate of 0.5 Cdeg/min. —○—, logarithmic-phase specimen; —●—, and stationary-phase specimen with *cis*-parinaric acid; ----○----, logarithmic-phase specimen; ----●----, and stationary-phase specimen with *trans*-parinaric acid.

*Outer membrane*

In the outer membrane, the polarization ratios in the region above the transition temperature in both growth-phase specimens were higher compared to those of cytoplasmic membranes (Fig. 4). In the logarithmic-phase specimens, the polarization increase of *trans*-parinaric acid was initiated at 40°C and terminated above 10°C. The temperature range of the polarization increase was approx. 15°C higher than that of the cytoplasmic membrane of the same growth phase. The maximum polarization value of this sample was lower than 1.86. In the stationary-phase specimen, the temperature range of polarization increase was similar to that of the logarithmic-phase specimen, while the maximum polarization ratio was lower than the value observed in the logarithmic-phase specimen (approx. 1.81).

*cis*-Parinaric acid showed the polarization increase in a temperature range approx. 10 degrees lower than that of the *trans* isomer. The increment of the polarization ratio in the stationary-phase membrane was considerably lower (1.74) than that of the logarithmic-phase specimen (1.85).

It has been shown previously that the absolute values of parinaric acids polarization ratios were

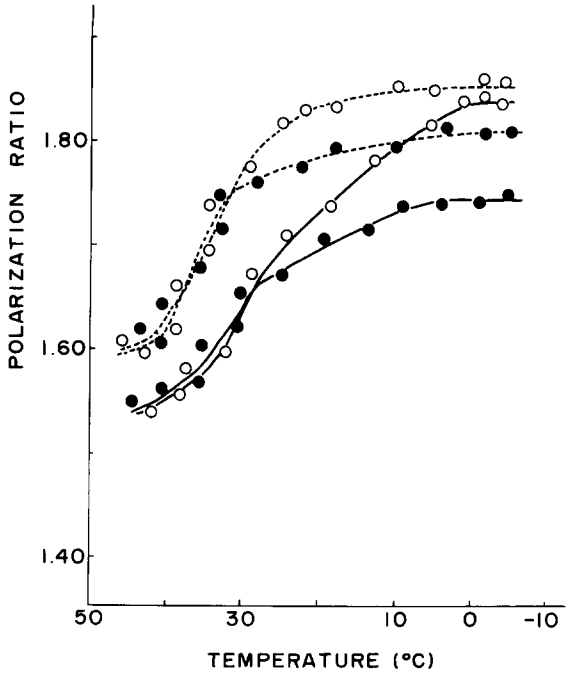


Fig. 4. Fluorescence polarization of *cis*- and *trans*-parinaric acids in the outer membrane of cells of differing growth phase as a function of temperature. The outer membrane samples were prepared as described under Materials and Methods, and kept at 50°C for 30 min. Then fluorescence polarization was determined at descending temperature at the rate of 0.5 Cdeg/min. —○—, logarithmic-phase specimen; —●—, and stationary-phase specimen with *cis*-parinaric acid; ----○----, logarithmic-phase specimen; ----●----, and stationary-phase specimen with *trans*-parinaric acid.

correlated with the amount of solid-phase lipid [8]. In the present experiments, the polarization ratios which were observed with membranes below the phase transition temperatures were lower, especially in the stationary-phase samples, compared to the values obtained with lipid dispersions. The result indicates that, in the membranes, certain portions of lipids remained below the transition temperatures without participation in the phase transition. Furthermore, the non-participating lipid portions were considered to be greater in the cells of increased growth phases, compared to logarithmic-phase cells.

*The relationship between membrane stability and the resistance of the cells to freeze-thawing*

An increased amount of ordered-state lipids in a membrane suggests a high degree of organization of the membrane constituents. in order to certify this supposition, membrane protein concentration in different growth-phase cells was investigated. Table I shows a higher protein-to-lipid ratio in the stationary-phase cell membranes compared to those in logarithmic-phase cell membranes, suggesting a possible increase of protein-protein or protein-lipid interactions which might cause the stability of stationary-phase cell membranes.

The increased stability of the stationary-phase cell membranes was demonstrated by the following experiments: In freeze-thawing of logarithmic-phase cells, approx. 36% of malate dehydrogenase, 40% of NADH oxidase, 33% of succinate dehydrogenase and 20% of glucose-6-phosphate dehydrogenase activities were revealed, indicating the extent of serious damage to the cytoplasmic membrane as well as to the outer membrane. The stationary-phase cells showed no such deleterious effect in a comparable experiment, nor did the logarithmic-phase cells frozen in the presence of 10% glycerol. Moreover, a higher level of viability of the cells after freeze-thawing was obtained in the cells of increased growth phases, or with loga-

TABLE I

CHEMICAL COMPOSITION OF *E. COLI* MEMBRANE FRACTIONS DERIVED FROM THE CELLS OF DIFFERING GROWTH PHASE

| | Logarithmic-phase cell | | Stationary-phase cell | |
|---|---|---|---|---|
| | cytoplasmic membrane | outer membrnae | cytoplasmic membrane | outer membrane |
| mg Phospholipid/mg protein | 0.62 | 0.22 | 0.51 | 0.15 |
| μmol KDO/mg protein | – | 0.12 | – | 0.09 |
| μmol KDO/mg phospholipid | – | 0.56 | – | 0.57 |

KDO, 2-keto-3-deoxyoctulosonic acid as a representative of lipopolysaccharide.

TABLE II

RESTORATION OF VIABILITY AFTER FREEZE-THAWING OF *E. COLI* CELLS OF DIFFERENT GROWTH PHASES IN THE PRESENCE OR ABSENCE OF GLYCEROL

| Time of cultivation (h) | Viability of the cell after freeze-thawing (%) | | |
|---|---|---|---|
| | unfrozen | − glycerol | + glycerol |
| 2 | 100 | 5.4 | 89.5 |
| 3 | 100 | 52.3 | 93.7 |
| 5 | 100 | 93.9 | 97.4 |

rithmic-phase cells which were frozen in the presence of 10% glycerol (Table II). The results indicate that the cells of increased growth phases have a stable membrane organization, and the stability of the membranes has a direct relation to the resistance of the cells to freezing. The relation between membrane damage and cell viability is also shown by the results that, in the freeze-thawing of logarithmic-phase cells with 10% glycerol, the release of the cellular enzyme activity was prevented completely and cell viability was restored to high levels.

## Discussion

The present study demonstrated that *E. coli* membrane lipids undergo a relatively wide-range phase transition, from physiological temperature to below 0°C, and their properties are considerably modified in the membranes. The wider temperature range of transitions of the membrane phospholipids compared to those observed with phosphatidylcholine samples may have been predicted from the complicated phospholipid composition of the membranes. Also, the modifications of lipid properties in the membranes are to some extent expected from the previous studies. Thermotropic order-disorder phase transition in the membrane of gram-negative bacteria have been previously measured with X-ray diffraction [18], $^2$H-NMR [19–21], differential scanning calorimetry [22,23] and fluorescence spectroscopy [24]. The temperature of the phase transition ranges over 20 Cdeg and it is approx. 7°C higher in the outer membrane than that of the cytoplasmic membrane [21]. The higher transition temperature range of the outer membrane can be explained by the higher degree of saturation of fatty acids of the phospholipids and the lower phosphatidylglycerol content of the outer membrane [21]. Our present data coincide with these results, suggesting that the use of parinaric acid probes is an excellent means of investigating differences in the physical state of lipids in cellular membranes as well as in lipid liposomes.

The application of these probes to the study of the physical properties of membrane revealed that the polarization ratios of the membranes in the physiological temperature region were significantly higher than those for vesicles of corresponding membrane phospholipids. The presence of membrane protein could produce such higher polarization ratios in the physiological temperature region by decreasing lipid mobility. The effect of protein on lipid mobility has been observed previously by fluorescence polarization using diphenylhexatriene as a probe, and it has been explained that it can produce higher polarization ratios, decreasing the amplitude of acyl chain motions [25–29].

In contrast, our present work revealed that maximum polarization ratios in the outer and cytoplasmic membranes were lower compared to that of aqueous dispersions of phospholipids, indicating that certain portions of the lipids in the membrane remained in unordered state in the region below the transition temperature. Similar results have been observed by X-ray diffraction study and it is indicated that only 25–40% of the outer membrane lipids participate in the phase transition. This percentage is twice as high in the cytoplasmic membrane [18]. It is also documented that some parts of the lipids in *E. coli* membranes were very strongly restricted in their motional freedom by interaction with proteins [30,31]. In the same growth-phase specimens, the outer membrane lipids showed lower participation in phase transition than the cytoplasmic membrane phospholipids. The result could be explained by the presence of lipopolysaccharides. The restricted motional freedom of *E. coli* outer membrane lipid by the presence of lipopolysaccharide has also been reported [32,33].

In logarithmic-phase cell membranes, both *cis*

and *trans* isomer yield comparable values at low temperature, and it has previously been interpreted to mean that most of lipid becomes in an ordered state. The loss of membrane stability on freeze-thawing might therefore be a consequence of too much solid lipid, which causes a phase separation of proteins. In the stationary-phase cell membranes, our present study demonstrated an increase of the protein-to-phospholipid ratio, indicating that the increasing protein concentration is likely to be responsible for the repression of the lipid phase transition. The effect of protein concentration on the repression of lipid motion in synthetic bilayer membranes [34,35] and in *E. coli* cell membranes [36] has been studied by the spin label technique, and it was shown that at high protein-to-phospholipid ratios the lipid spin label was highly immobilized.

It should be emphasized that an increased amount of protein in the stationary-phase cell membranes could reinforce the protein-protein and presumably protein-lipid interactions that result in the reduction of lipid motion in the membrane, and such residual disordered lipid might stabilize the membrane structure. The resistance of the cells to freezing damage in the stationary-phase cells may be basically attributed to the stable membrane structures.

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