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ESCHERICHIA COLI B MEMBRANE STABILITY RELATED TO CELL GROWTH PHASE

MEASUREMENT OF TEMPERATURE DEPENDENT PHYSICAL STATE CHANGE OF THE MEMBRANE OVER A WIDE RANGE

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***Escherichia coli* B cytoplasmic and outer membrane from cells in different growth phases showed different chemical compositions. In freezing, logarithmic phase cells showed a marked permeability increase in the outer as well as the cytoplasmic membrane. Whereas, in the stationary phase cells no such change in membrane permeability was observed. Cooling of phospholipids and lipopolysaccharides with *trans*-parinaric acid showed a distinct fluorescence increase from room temperature to far below 0°C. In the outer membrane the fluorescence similar to that of lipopolysaccharides was shown. The outer membranes of cells in different growth phases showed similar temperature-dependent fluorescence changes. The cytoplasmic membrane exhibited a temperature-dependent fluorescence similar to that of the phospholipids. The onset temperature of the increase in fluorescence differed with the cells at different growth phases. The presence of EDTA and $MgCl_2$ modified the fluorescence changes in the membranes from cells in logarithmic phase. Whereas, in the membranes from cells in stationary phase no such effect was observed. These results suggest that the organizational stability of the membranes from cells in stationary phase is a fundamental basis of the membrane's resistance to the freezing damage.**

Introduction

Freezing damage to the outer and cytoplasmic membranes of *Escherichia coli* cells has been studied. Part of the injury sustained is assumed to be the result of structural changes of membranes or membrane lipids occurring at subzero temperatures [1]. Experiments with extrinsic fluorescent probes have been reported in the studies of lipid phase changes in *E. coli* [2,3], Mycobacterium [4] and protein free phospholipid systems [5–7]. However, these studies were primarily concerned with membrane characters at physiological temperature ranges. Measurement of temperature dependent changes in the physical state of the lipids and/or membranes over a wide range of temperatures may be important to elucidate the problems described above.

The fluorescence of *trans*-parinaric acid as a function of the temperature indicates a transition of the physical state of the lipids in bovine heart mitochondrial inner membranes at temperatures below 0°C [8]. The phase transition temperature of *E. coli* membrane is known to differ with respect to membrane fatty acid composition which has been related to their growth temperatures [9–12] and their growth phases [13,14]. Using *trans*-parinaric acid as a fluorescent probe, a study of the physical state change of *E. coli* cell membranes of different growth phases at a range of tempera-

tures from physiological to subzero was made. This paper will report that there were differences in the stability of membrane organization in the cells of different growth phases.

Materials and Methods

Cultivation of bacteria. A 15-h culture of *Escherichia coli* B in trypticase soy broth medium containing 0.3% yeast extract was introduced into a 10-times vol. of the same medium and incubated at 37°C with shaking. Logarithmic phase cells were harvested 2 h after transfer into new medium at an absorbance of 0.8 at 600 nm, near the end of the logarithmic growth phase. Stationary phase cells were obtained by incubation for three additional hours after the growth curve reached a plateau.

Preparation of membrane fraction. Logarithmic and stationary phase cells were washed twice with 10 mM Tris-HCl buffer (pH 7.5) and then were treated for 30 min with lysozyme in 0.25 M sucrose containing 3.3 mM Tris-HCl at 4°C [15]. Sonication for 2 and 3 min, at 200 W in a Kubota KMS-250 ultrasonic generator was required for the adequate disintegration of membranes of logarithmic and stationary cells, respectively. The outer and cytoplasmic membrane fractions were separated on a 35–60% linear sucrose density gradient. Centrifugation was carried out at 27000 rpm for 40 h in a SW 27-1 rotor in a Spinco ultracentrifuge. Cytoplasmic and outer membrane fractions were obtained as clearly visible bands at the top and near the bottom of the gradient, respectively. The fractions were washed twice with 10 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. Buoyant density for each fraction was determined by the same method for density gradient centrifugation. The suspensions were kept either at 4°C or −25°C untill used. Sample storage at temperatures above or below zero degree had no observable effect.

Fluorescence measurement. An aliquot of the outer and cytoplasmic membrane suspension or lipopolysaccharide suspension was diluted to 0.9 ml with distilled water. When the presence of EDTA or $MgCl_2$ in the sample was required, the sample suspensions were diluted to 0.8 ml and 0.1 ml of those chemicals in adequate concentration was added. To the membrane suspensions were added 0.5 ml of 675 mM manitol/225 mM sucrose/60 mM phosphate buffer (pH 7.4). The mixture was then diluted to 50% with ethylene glycol to prevent freezing. Liposomes of the extracted phospholipids or phospholipid-lipopolysaccharide mixture were prepared as follows: Phospholipid dissolved in $CHCl_3$ was evaporated with dry nitrogen and mixed with distilled water or lipopolysaccharide suspension, respectively. The resulting mixture was sonicated with an Artek sonic dismembrator. The liposomes and the liposome-lipopolysaccharide mixtures were also mixed with the manitol/sucrose/phosphate buffer and then ethylene glycol in a manner similar to that of the membrane fractions and lipopolysaccharides. *trans*-Parinaric acid was diluted from an ethanol stock solution (0.5 mg/ml) immediately prior to use and a 0.1 ml of probe at 10 μg/ml was added to the sample which was maintained at the starting temperature (usually 35°C).

The fluorescence measurement was carried out according to the method of Waring et al. [8], using a Shimadzu RF-502 spectrofluorophotometer. The maximum emission at a wave length of 410 nm (excitation 320 nm) versus temperature was continuously recorded. The sample temperature was regulated by a low temperature cuvette holder connected to a refrigerated circulating bath (Neslab endcal LT-50). The sample temperature was recorded continuously by a Hitachi 056 recorder, using a 30 G copper-constantan thermocouple. All procedures were carried out under a dry nitrogen atmosphere.

Freeze-thawing of the cells. Washed cells were suspended in 0.01 M Tris-HCl buffer (pH 7.5), at a concentration of approx. 0.1 g wet cells per ml. A 0.5-ml aliquot of either logarithmic and stationary cells was dispensed into a glass freezing tube (15 × 150 mm). Tubes were transferred into an alcohol bath which had been cooled to −1°C. After equilibration at that temperature (approx. 5 min), the suspensions were inoculated with ice by being touched with the tip of a frosted tungsten wire. Inoculated samples were held an additional 10 min at −1°C to allow equilibration with respect to ice crystallization, then the temperature of the cooling bath was lowered to −5°C at the rate of 1 K/min. Ten minutes after the bath temperature reached

−5°C, samples were removed and thawed in a 30°C water bath with shaking.

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; this measurement represents the amount of enzyme which was liberated from the cells during freeze-thawing. 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].

Extraction and chemical analyses of membrane component. Total lipids were extracted with a $CHCl_3/CH_3OH$ (2:1, v/v) mixture and washed by the method of Folch et al. [16]. Lipopolysaccharides were extracted with 45% phenol and purified following the method of Osborn et al. [17]. Total protein was determined by the method of Lowry et al. [18] using bovine serum albumin as the standard. The amount of phospholipids was measured by using the procedure of Shibuya et al. [19] assuming that the quantity of lipids is 25 times that of the amount of phosphorus. 2-Keto-3-deoxyoctonic acid was estimated either directly with the membrane fragments or with extracted lipopolysaccharides according to the method of Weissbach and Hurwitz [20].

Results

Chemical composition of isolated membranes

The chemical composition of *E. coli* B cytoplasmic and outer membrane fractions from different growth phases is shown in Table I and coincides with previous publications [1,15,21,22]. The outer membrane fraction exhibited low NADH-oxidase activity, whereas the lipopolysaccharide content was high. The enzyme NADH oxidase has been shown to be located in the cytoplasmic membrane [21,22]. The cytoplasmic membrane fractions contained low levels of lipopolysaccharide and high NADH-oxidase activity.

The relative phospholipid content of cytoplasmic membranes was apparently higher than that of the outer membranes (Table I). In a comparison of cells from the two growth phases membranes from cells in logarithmic phase contained higher amount of phospholipid. The lipopolysaccharide content in the outer membrane also differed in specimens from different growth phases. The outer membrane of stationary phase cells contained a higher amounts of protein than that of the logarithmic phase cells. In addition, the cytoplasmic membrane from stationary phase cells exhibited a moderately higher protein content than that of the logarithmic phase cells. The results of buoyant density measurements agreed with these observations.

Tolerance of the cell membranes to freeze-thawing

After freeze-thawing of logarithmic phase cell

TABLE I

CHARACTERISTICS OF THE MEMBRANE FRACTIONS OF *E. COLI* B CELLS AT DIFFERENT GROWTH PHASES

Component	Cytoplasmic membrane		Outer membrane	
	Logarithmic	Stationary	Logarithmic	Stationary
Protein [a]	64.8±1.0	70.3±2.0	69.0±0.5	77.5±0.7
Lipopolysaccharide [a]	2.1±0.2	1.7±0.1	17.6±1.3	14.1±1.5
Phospholipid [a]	33.4±1.3	28.0±1.8	13.4±1.2	9.8±1.3
NADH-oxidase activity [b]	1.20	–	0.01	0.01
Buoyant density (g/cm^3)	1.16	1.17	1.24	1.25

[a] Per cent of total.

[b] Marker enzyme of cytoplasmic membrane. The enzyme activity is shown in arbitrary values of mg protein.

suspension to −5°C, 35 to 40% of total activities of NADH oxidase, malate dehydrogenase and succinate dehydrogenase in the cells was apparent. In unfrozen cells the activities of these enzymes could not be demonstrated in logarithmic and stationary phase cells. This demonstrates that in logarithmic phase cells the permeability of the outer membrane to the substrates of these enzymes increased with freeze-thawing. Approx. 17% of the total glucose-6-phosphate dehydrogenase activity was liberated from the cells, showing that the cytoplasmic membrane was also damaged. Less than 10% of the logarithmic phase cells survived freezing and thawing. In contrast stationary phase cells exhibited very low membrane bound enzyme activity and no liberation of the glucose-6-phosphate dehydrogenase was observed after the same freezing treatment. In stationary phase specimens more than 95% of the cells were survived freezing and thawing.

Temperature profiles of trans-parinaric acid fluorescence

A. Phospholipids. When the liposomes of *E. coli* B membrane phospholipids containing *trans*-parinaric acid were cooled from 35 to −35°C, fluorescence of the dye increased significantly with a lowering the sample temperature. As is shown in Fig. 1, the four samples, extracted from different membrane fractions showed different onset temperature for fluorescence increase ranging from 22 to 10°C. However, the maximum increase in fluorescence was attained at the same temperatures in almost all of the samples. In these samples, the fluorescence increase was very low just after the onset temperatures, then it turned gradually to

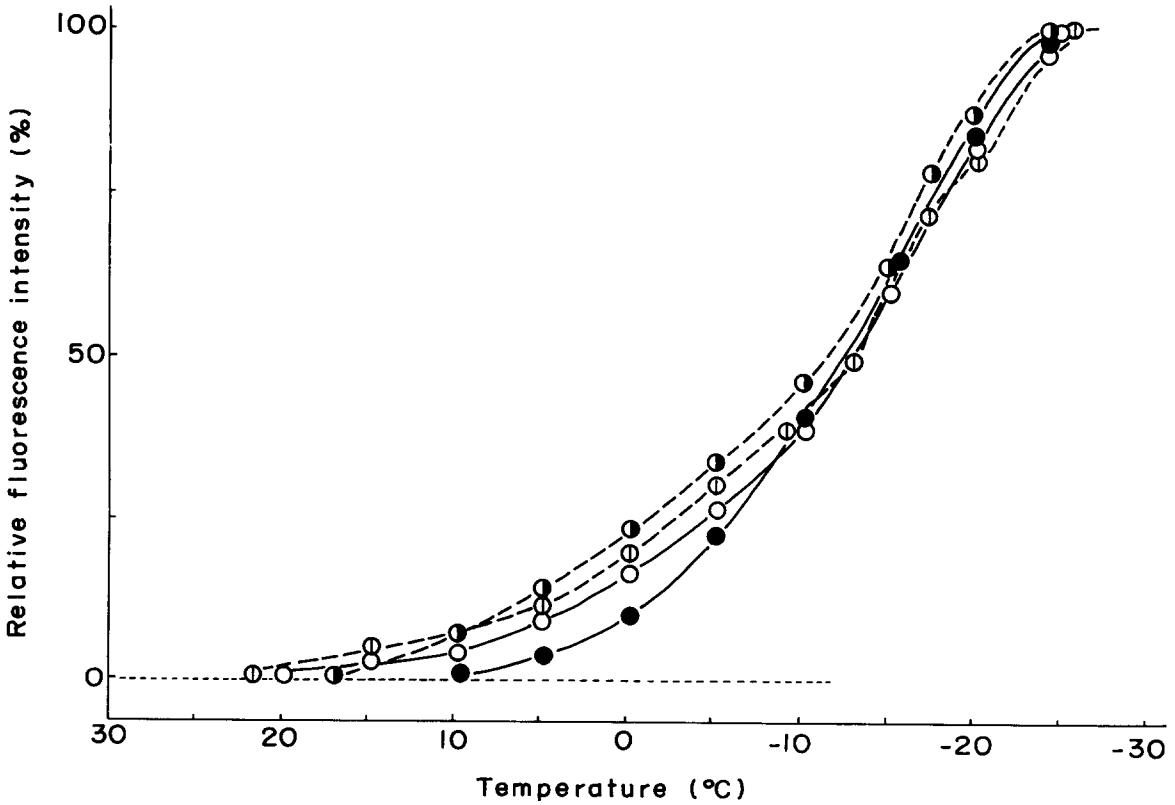

Fig. 1. Temperature dependency of the fluorescence in *E. coli* B membrane phospholipids. The phospholipids were extracted from the outer and cytoplasmic membranes of both growth phases. Then the samples were cooled from 35 to −35°C at a rate of 0.3 K/min, and the fluorescence intensity and the sample temperature were recorded simultaneously. ●——●, log cytoplasmic; ○——○, stationary cytoplasmic; ◑——◑, log outer; ⊙------⊙, stationary outer membrane phospholipids.

higher rates. Approx. 10 and 20% of the observed maximum fluorescence was observed at 0°C in logarithmic and stationary phase cells, respectively. A 90% maximum intensity change was attained at −20 to −22°C in all samples.

B. Lipopolysaccharides. The fluorescence change of the dye in the lipopolysaccharide fractions extracted from logarithmic and stationary cell membranes were observed at similar but somewhat extended temperature ranges. The fluorescence increase was initiated at a 4 degrees higher temperature in the lipopolysaccharide fraction from stationary phase than that of logarithmic phase cells. However, the degree of fluorescence increase at each temperature was practically the same with the logarithmic and stationary phase cells in the region below 15°C and the maximum fluorescence was attained at the same temperature in both samples (Fig. 2, Table II). The increase in fluorescence was almost linear with decreasing temperatures except that an abrupt increase in the fluorescence was observed between −15 and −17°C. Approx. 25% of the total fluorescence was ob-

TABLE II

ONSET TEMPERATURES OF INCREASING FLUORESCENCE IN MEMBRANES FROM *E. COLI* B CELLS AT DIFFERENT GROWTH PHASES

L, logarithmic; S, stationary.

Fraction	Cytoplasmic		Outer	
	L	S	L	S
Membrane	15.0	21.0	30.0	30.0
Phospholipid	10.0	20.0	17.5	22.0
Lipopolysaccharide	–	–	21.0	25.0

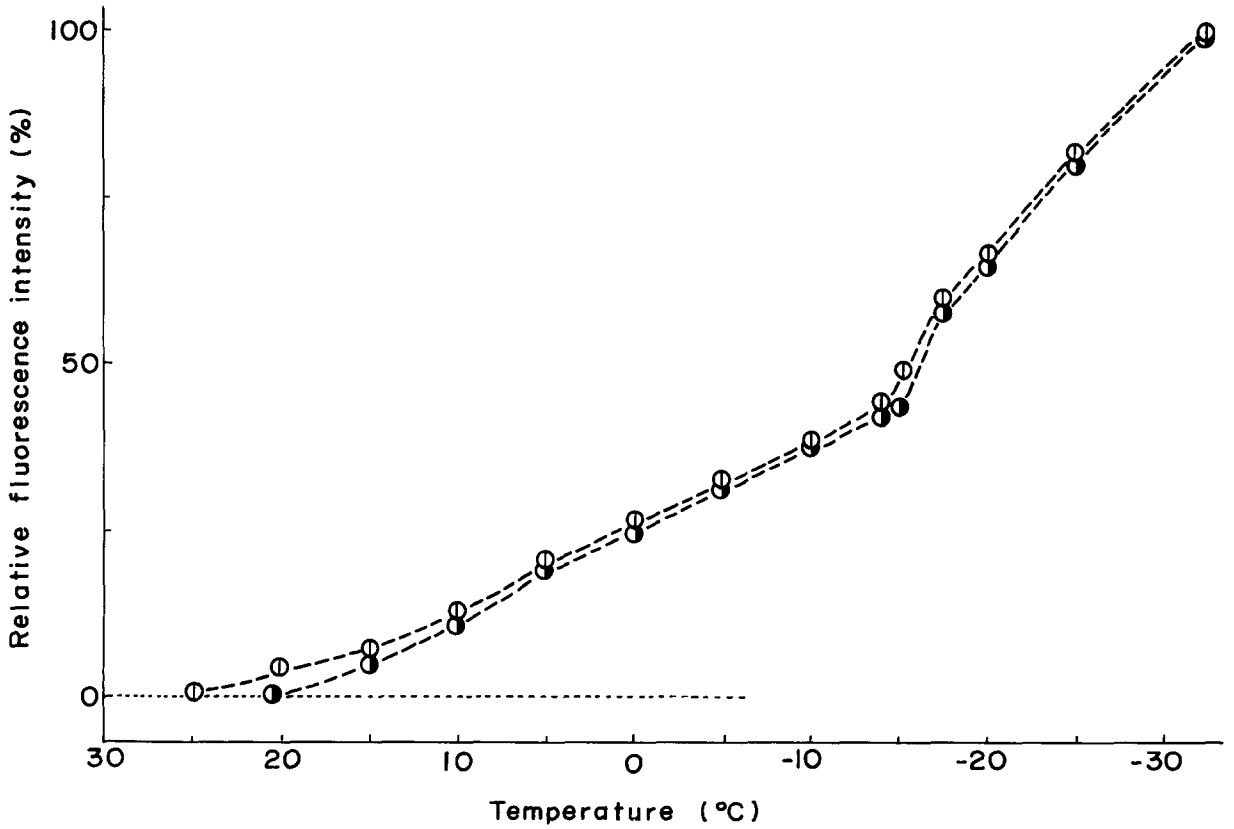

Fig. 2. Temperature dependency of the fluorescence in *E. coli* B membrane lipopolysaccharides. The lipopolysaccharides were extracted from the outer membranes of logarithmic (◑------◑) and stationary phase cells (Ⓓ——Ⓓ). The experimental procedures were as in Fig. 1.

served at 0°C and 90% of the total fluorescence intensity was attained at −27°C in cells from both growth phases.

C. Membrane fractions. When the cell membrane fractions were cooled with *trans*-parinaric acid, fairly different fluorescence changes were seen between the outer and cytoplasmic membranes (Fig. 3 and Table II). In the outer membrane, the fluorescence increase was initiated at 30°C and continued to −28°C. The fluorescence change was almost linear with the decreasing temperature. Approx. 35% of total fluorescence was seen at 0°C, and 90% was attained at −25°C. No difference in degree of fluorescence change was observed between stationary and logarithmic phase cells. In the cytoplasmic membranes, the fluorescence increase started at 21 and 15°C in the stationary and logarithmic phase cells, respectively. The rate of fluorescence increase was low above 5°C, then it turned gradually to higher rates. Consequently, the initiation of fluorescence increase of the cytoplasmic membrane sample was 9 to 15 degrees lower than that of the outer membranes, and 90% of the total fluorescence was attained at a 4 degree higher temperature. Regardless of the different initiation temperatures, the fluorescence over 10% was almost the same in samples from cells in both growth phases. Approx. 28% of the total fluorescence was exhibited at 0°C and 90% fluorescence change was attained at −21°C.

When the cooled samples were rewarmed, the fluorescence decreased to the initial value. Some cooling and heating dependent hysteresis was seen when the temperature scan rate was not sufficiently low (less than 0.3 K/min). If the rewarmed

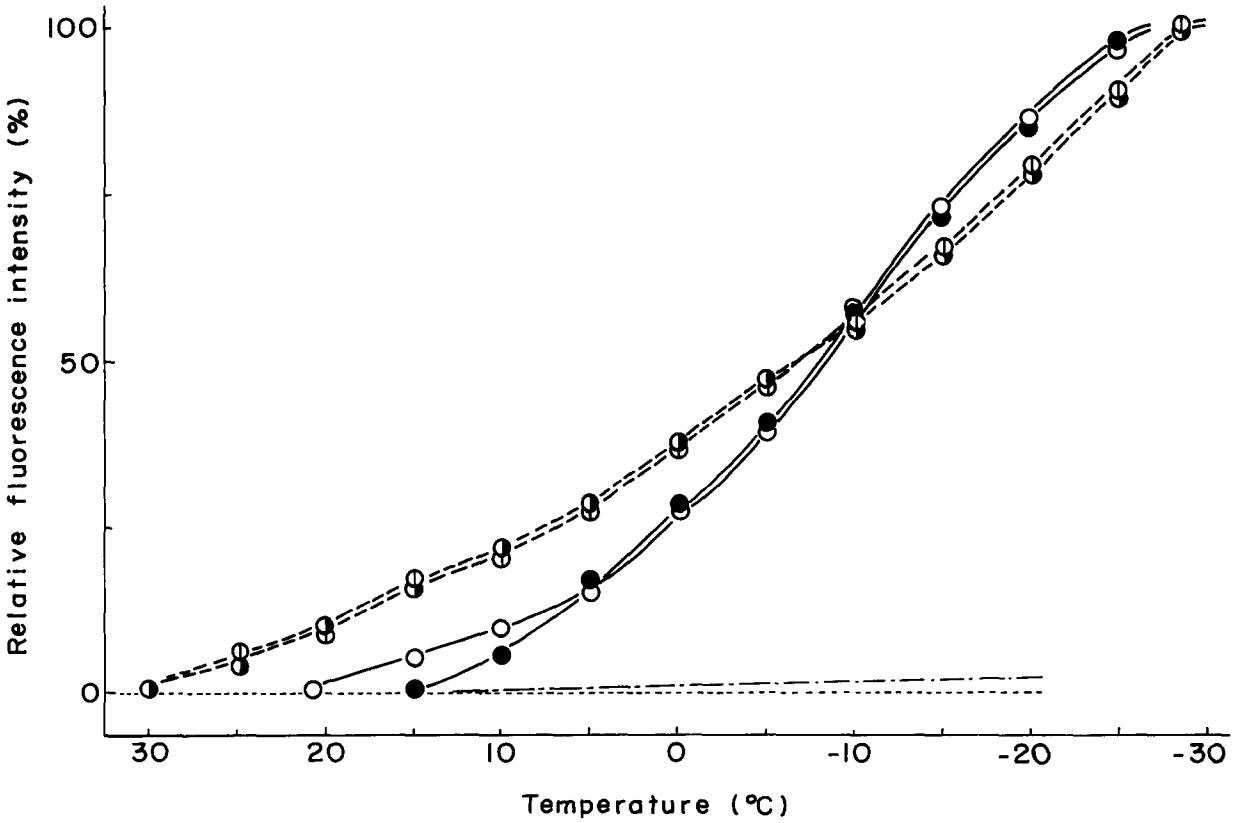

Fig. 3. Temperature dependency of the fluorescence in *E. coli* B membrane fractions. The experimental procedures were as in Fig. 1. ●———●, log cytoplasmic; ○———○, stationary cytoplasmic; ◑------◑, log outer; ⦶------⦶, stationary outer membranes; ·-·-·, probe and buffer mixture without membrane fraction; ·····, membrane and buffer mixture without probe.

sample was subsequently recooled, the temperature dependent fluorescence increase was reduced to 30% of the initially observed maximum (data not shown). This was common in all samples including extracted phospholipids and lipopolysaccharides.

Cooling of *trans*-parinaric acid and a 50% ethylene glycol-buffer mixture without membrane fraction or its components showed very little temperature dependent increase in fluorescence, indicating minimal probe-solvent interaction (Fig. 3).

Resistibility of the membrane to chemical modifications

The fluorescence intensity changes in the original membranes showed a significant differences with those of their component lipids. The physical state of lipids is known to be affected by divalent cations. The addition of EDTA or $MgCl_2$ to the membrane samples from cells at different growth phases apparently induced changes in the fluorescence of these samples. In membrane preparations from cells in the logarithmic growth phase the addition of EDTA shifted the initiation of fluorescence increase lower temperatures. The initiation of the fluorescence increase was reduced 5 degrees in the outer membrane and 3 degrees in the cytoplasmic membrane, respectively. Consequently, the fluorescence reduced several per cent, particularly in the temperature range above $-10°C$. As a result of these changes, the fluorescence curve for the outer membrane coincides with that of phospholipid-lipopolysaccharide mixture. The fluorescence curve for the cytoplasmic membrane resembles that of the phospholipid liposome fraction. The addition of $MgCl_2$ to the membrane prepara-

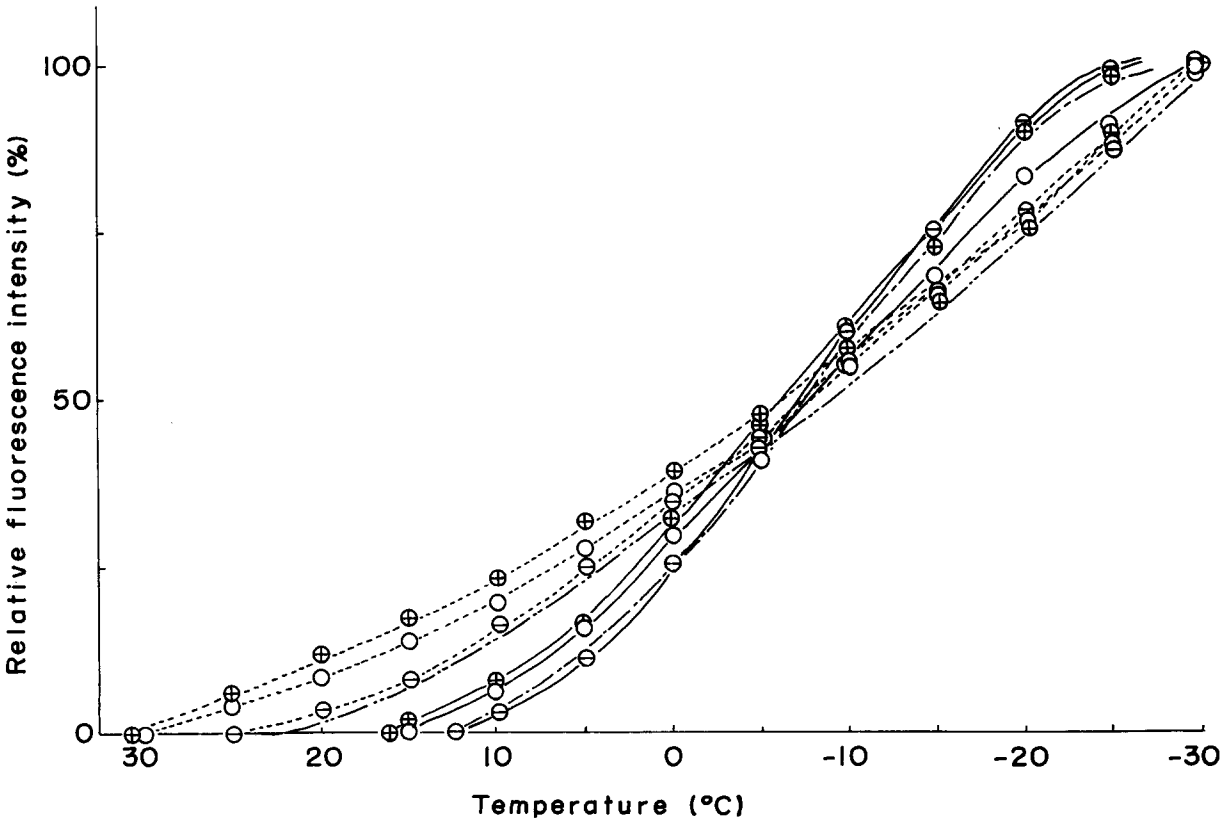

Fig. 4. Effect of EDTA and $MgCl_2$ on the temperature dependent fluorescence in logarithmic phase cell membranes. The experimental procedures are as in Fig. 1. ·····, Outer membrane; ———, cytoplasmic membrane; ○, control; ⊖, +0.7 mM EDTA; ⊕, +0.8 mM $MgCl_2$; — - - —, outer membrane phospholipid-lipopolysaccharide mixture; -——-, cytoplasmic membrane phospholipid liposome.

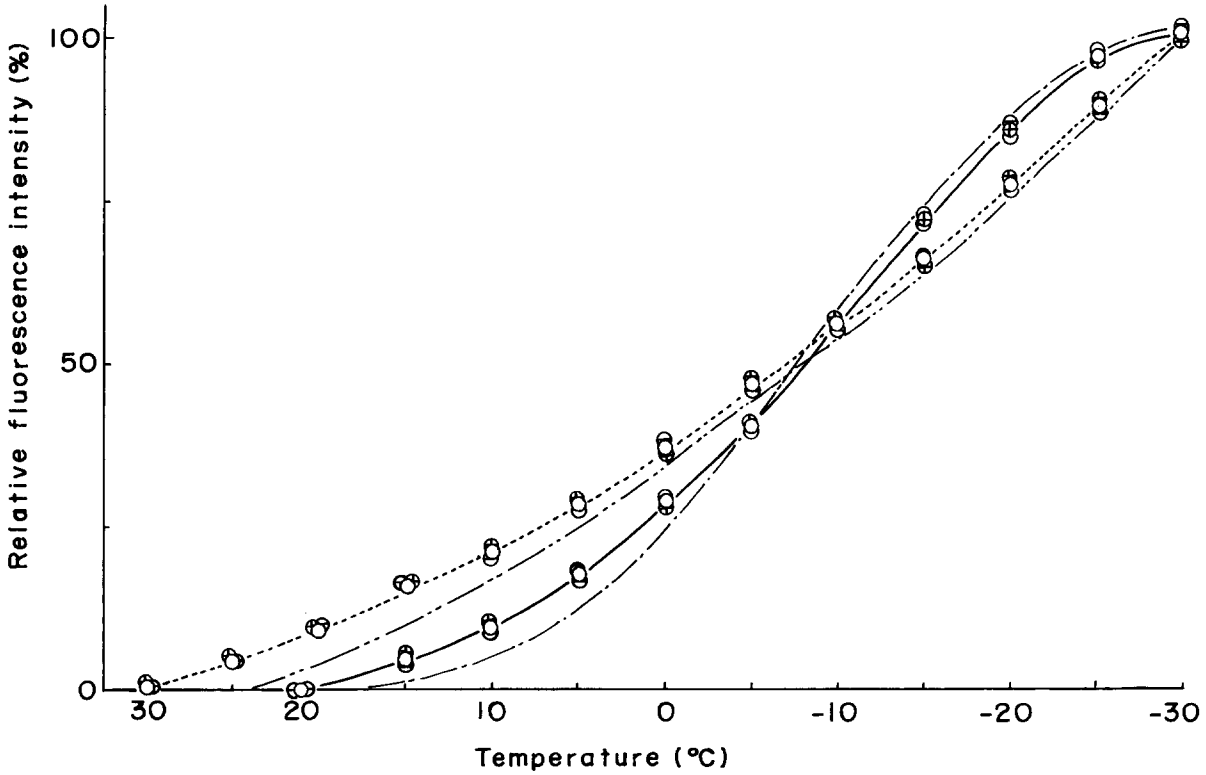

Fig. 5. Effect of EDTA and $MgCl_2$ on the temperature dependent fluorescence in stationary phase cell membranes. The experimental procedures and symbols in the figure are as presented in Fig. 4.

tions yielded the curves slightly increased relative fluorescence in the temperature range similar to those of samples treated with EDTA (Fig. 4).

The addition of EDTA or $MgCl_2$ to membrane preparation from cells in stationary phase exhibited no changes in the fluorescence versus temperature curves, or their initiation temperatures, when compared to the original samples. Thus, a slight difference in the fluorescence between the membranes and the component lipids was not changed (Fig. 5).

Discussion

Changes in *trans*-parinaric acid fluorescence are known to result from lipid phase transition [23,24] and indicate marked differences in stability of the membranes at reduced temperatures. In this study, the temperature range at which the fluorescence increased extended from physiological temperatures to −25°C. Fluorescence changes below zero degree cannot be directly compared to known phospholipid phase transition temperature ranges of *E. coli* cells [25,26]. The subzero-temperature dependent changes in fluorescence might in part reflect enthalpy changes of the membranes, as reported in rat liver mitochondria membrane, monitored by differential scanning colorimetry [27].

Shechter et al. [28] demonstrated that in a fatty acid auxotrough supplemented with unsaturated fatty acids, the fluid phase persists at low temperatures. The observed shift of fluorescence increase to the lower temperatures in the present study may result in part from the presence of unsaturated fatty acids (data not shown) and ethylene glycol in the experimental systems. Ethylene glycol appears to lower the lipid transition temperatures by several degrees [8,27], when compared to differential scanning colorimetry data in an aqueous medium [29].

Nakayama et al. [30] employed X-ray diffraction techniques to study the phase transition tem-

perature of *E. coli* B membrane and membrane components, grown at different temperatures. The temperatures at which all lipid bilayers in the membrane turn into a fluid state upon elevation of temperature are in close agreement with the initiation temperatures of fluorescence increase in the present study. Therefore, the observed change in fluorescence could be related to the proportion of solid state in the lipid bilayer.

In cytoplasmic membranes from stationary phase cells, the onset temperature of the fluorescence increase was higher than that of membranes from logarithmic phase cells, showing good agreement with the data for cytoplasmic phospholipids. This observation, together with the overall shape of the fluorescence curves, suggests that the changes in the physical state of the cytoplasmic membranes are dominated by the changes of component phospholipids. Good agreement is also seen between the fluorescence curves of the outer membrane and those of the phospholipid and lipopolysaccharide mixtures. Lipopolysaccharides are known to form bilayer in water [31] and have their phase transition point around 25°C [30,32]. The increasing fluorescence in the outer membrane might be dominated by the change of both lipopolysaccharides and phospholipids present in the membrane. The observed differences in fluorescence between the cytoplasmic and outer membrane and those of their component lipids are possibly due to the different physical state of the lipids in the membranes and that in the artificial liposomes.

It is known that the physical state of lipopolysaccharides [32] and phospholipids [33] are affected strongly by the presence of divalent cations such as Ca^{2+} and Mg^{2+}. In membranes from logarithmic phase cells, EDTA and $MgCl_2$ could affect the structure of the membranes by decreasing or increasing divalent cations, resulting in a change in fluorescence from that of the original membranes. In membranes from cells in stationary phase, stable organization of the membrane may resist to the action of EDTA and $MgCl_2$. As a result, the fluorescence would be similar to those of the original membranes.

The results suggest that the stability of membrane organization is different between two phase cells. In this study the structural stability of the membranes paralleled the tolerance of *E. coli* cells to freezing. The membrane stability are positively assumed to be responsible for the cell's viability.

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