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Fluorescence polarization studies on *Escherichia coli* membrane stability and its relation to the resistance of the cell to freeze-thawing. II. Stabilization of the membranes by polyamines

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The effects of polyamines, spermine, spermidine and putrescine on the stabilization of the membrane organization of *Escherichia coli* cells were studied using measurements of fluorescence polarization change of extrinsic fluorescence probes in membrane specimens as a function of temperature. The effects of the polyamines on the restoration of the cell viability after freeze-thawing were also investigated. In logarithmic-phase membrane specimens, polyamines depressed the polarization ratio increase below the transition temperatures in a dose-dependent manner. The physiologically relevant concentration of polyamines repressed the ratios to the same levels as are obtained with the stationary-phase specimens. In the stationary-phase specimens, no effect of polyamines on repression of the polarization increase was observed. A preliminary exposure of logarithmic-phase cells to polyamines protected the cells from the reduction of viability in freeze-thawing. However, a considerably high concentration and a certain length of preincubation time were required in order to an effect to be exerted. These results indicate that the intracellular polyamines could stabilize the membrane organization of logarithmic-phase cells to the same extent as in the stationary-phase cell membranes. It is conjectured that the membrane stability which is mediated by the polyamines results in cellular resistance to freeze-thawing, as it is attained by increasing the growth phase of the cells.

Introduction

The aliphatic polyamines are contained in various amounts in many bacteria and eukaryotic cells [1–6], and are known, due to their cationic character at physiological pH, to bind with nucleic acids and the other negatively charged macromolecules in the cells [1–3,7,8]. While the roles of polyamines in vivo are still uncertain, numerous in vitro stabilization effects on various cellular components have been reported [1–3,9,10]. For example, spermine or related amines protect the osmotically fragile *Escherichia coli* spheroplasts [11–13] and isolated mitochondria [14] from lysis, indicating a direct stabilizing effect on the cell wall or cell membrane.

In the preceding paper [15], it was demonstrated that *E. coli* B logarithmic-phase cells have a higher participation of membrane phospholipids in the phase transition and lower resistance to freeze-thawing, while the stationary-phase cells have a lower participation in lipid phase transition and higher resistance to the freezing injury. The results suggest that membrane stability plays extremely important role in the cells in contributing

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

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to the resistance to freezing.

Recently it was reported that polyamines preferentially bind to ribosomal particles or membrane fractions of *E. coli* cells [16] or modify the properties of renal brush border membrane vesicles [17]. Although many basic compounds can be considered to stabilize membrane structure, it is of interest that *E. coli* cells naturally contain a high concentration of polyamines, putrescine and spermidine [3]. In the present work, the stabilizing effects of exogenous polyamines on *E. coli* membranes were investigated by the measurement of fluorescence polarization using extrinsic fluorescence probes. The relationship between membrane stability and the restoration of cell viability after freeze-thawing was also studied.

Materials and Methods

Materials. *cis*- and *trans*-Parinaric acids were purchased from Molecular Probes, Inc. (Junction City, OR or Plano, TX, respectively). 1,6-Diphenyl-1,3,5-hexatriene and polyamines (putrescine, spermidine and spermine) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cultivation of bacteria. In most cases *E. coli* B cells were grown to the logarithmic and stationary phases with the same procedures described in the preceding paper [15]. For the cultivation of cells in the presence of polyvalent cations, a 15 h culture of *E. coli* B cells in normal culture medium was transferred into a 10-fold volume of the same medium containing 0.5 mg/ml of polyamines or $MgCl_2$. The presence of this concentration of cations did not give any effect on the growth rate of the cells. The logarithmic-phase cells were harvested approx. 2 h after transfer into new medium at an absorbance of 0.6 at 600 nm.

Fluorescence measurement. For the measurement of fluorescence polarization, the membrane samples or lipid dispersions were prepared from the cells cultivated without an addition of polyamines or $MgCl_2$. The membrane samples or the lipid dispersions were added with the concentrated solutions of polyamines or $MgCl_2$ to final concentrations of 0–250 μM, in a volume of 0.9 ml. The samples were mixed with 0.5 ml of medium consisting of mannitol, sucrose and Hepes buffer and then with 1.5 ml of ethylene glycol. The samples were flushed with N_2 and incubated at 37°C for 30 min. Parinaric acids and hexatriene probes were added to the sample just before the fluorescence measurement. The lipid/probe molar ratios were 100 : 1 and 1000 : 1 with parinaric acids and diphenylhexatriene, respectively, in all samples. After an equilibration of probes with sample mixture, the fluorescence versus temperature was measured with the method of Waring et al. [18] as slightly modified as described in the preceding paper [15].

Freeze-thawing and a viable count of the cells. The cells harvested from the normal culture medium were washed twice with 10 mM Tris-HCl buffer (pH 7.5) and then were suspended in 10 ml of the same buffer containing 0–1 mg/ml of polyamines or $MgCl_2$, at a concentration of 0.05 mg wet cells per ml. The suspensions were incubated at 37°C for 0–30 min with vigorous shaking to prevent precipitation. The cells were collected, washed once with 10 mM Tris-HCl buffer and resuspended in the same buffer to the concentration of approx. 0.5 g wet cells per ml. The cells harvested from polyamine-containing culture medium were washed once with 10 mM Tris-HCl buffer and resuspended in the same buffer in a similar manner to that described above. The same freeze-thawing and viable counting procedures described in the preceding paper were employed with both specimens.

Results

Effect of polyamines on fluorescence change in the membranes or in the aqueous dispersions of phospholipids

Membranes from logarithmic-phase cells

The membranes of *E. coli* B logarithmic-phase cells showed a higher polarization ratio at low temperatures compared to that in the membranes of stationary-phase cells, indicating the higher participation of the membrane lipids in phase transition [15].

With an incubation of cytoplasmic membrane of logarithmic-phase cells with spermine, the increase in polarization with *cis*- and *trans*-parinaric acids was considerably repressed in the region below the transition temperatures compared to the

values obtained with the membrane samples without spermine treatment. The repression of the polarization increase with *cis*-parinaric acid was considerably greater than that observed with the *trans* isomer (data is not shown). As it was demonstrated in the preceding paper [15], repression of the polarization change in the membrane sample indicates the reduction of lipid portions which are thermally immobilized below the transition temperatures, although the polarization ratio above the transition is not likely to be affected.

The effects of spermine on lipid immobilization were dose-dependent (Fig. 1). The membrane fraction in the presence of as little as 10 μM spermine resulted in a consistently detectable polarization change repression. The extent of the repression increased at higher spermine concentration up to 50 μM. In this concentration, the increase of the polarization ratio was repressed to approx. 1.80, namely the value comparable to that shown in the stationary-phase cell membranes without polyamine. No additional increase of repression was observed above this concentration of spermine, except for an addition of very high concentration (over 150 μM) which caused aggregation of membrane particles during the preincubation. In order to discriminate the possible artificial interactions of positively charged polyamine with negatively charged fatty acid probes, the experiments were reproduced using 1,6-diphenyl-1,3,5-hexatriene. A similar dose-dependent polarization repression was observed upon using this non-charged probe (inset in Fig. 1). Two other physiologically important polyamines, spermidine and putrescine, also repressed the polarization ratio increase below the transition temperature region in a dose-dependent fashion but were found to be less potent than spermine. For instance, incubation of the membrane with 100 μM spermidine caused the repression of the polarization increase to 1.80, while over 150 μM putrescine repressed the increase to 1.82 (Fig. 2). No further effects of these polyamines above these concentrations were observed.

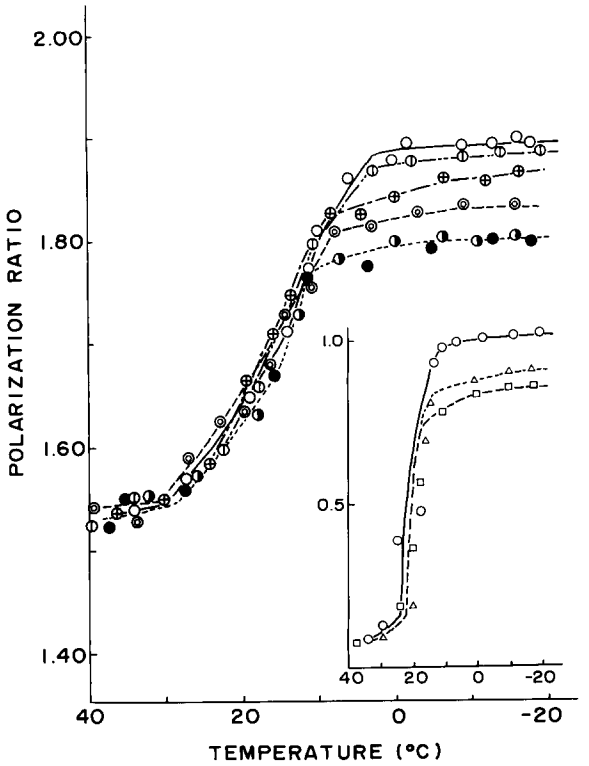

Fig. 1. Effect of spermine concentration on fluorescence polarization change in cytoplasmic membrane of logarithmic-phase cells. Membrane samples were added with various concentrations of spermine and were kept at 37°C for 30 min. Samples were then warmed to 45°C and fluorescence polarization was determined at descending temperature at the rate of 0.5 Cdeg/min. ○, control (no spermine); ⊙, 10; ⊕, 20; ◎, 40; ◑, 50; and ●, 100 μM spermine, with *trans*-parinaric acid. Insert: similar experiments which were carried out with the use of 1,6-diphenyl-1,3,5-hexatriene. ○, control (no spermine); △, 30; and □, 40 μM spermine. The polarization ratio has been scaled between 0 (above the transition temperature) and 1 (below the transition temperature).

The outer membrane of logarithmic-phase cells was also affected with spermine in a similar manner to the cytoplasmic membrane, as it was demonstrated by using parinaric acid and hexatriene probes (Fig. 3, with inset). $MgCl_2$ at concentrations as high as 125 μM did not affect the polarization change in either the cytoplasmic or outer membrane specimens.

Membranes from the stationary-phase cells

Polyamines were found to have a less potent effect on the fluorescence polarization change in the stationary-phase cell membranes. For instance, incubation of the cytoplasmic membrane with 60 μM spermine resulted in a slight repression of the polarization ratio increase below the transition temperatures. No change of the polarization ratio above the transition temperature was observed. The presence of 50 μM spermidine or 150 μM

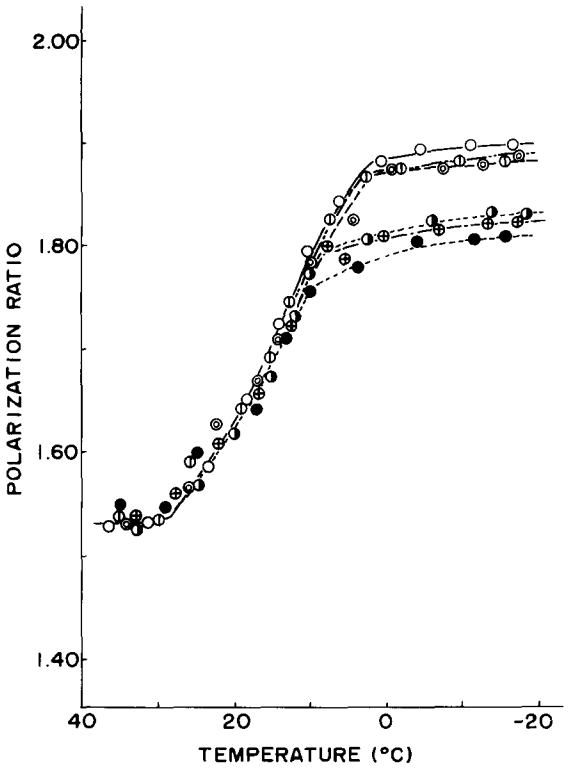

Fig. 2. Effect of spermidine and putrescine concentration on fluorescence polarization change in cytoplasmic membrane of logarithmic-phase cells. Membrane samples were added with spermidine or putrescine of various concentrations. The procedures of sample incubation and fluorescence polarization measurement were similar to those described in Fig. 1. ○, control (no polyamine); ◎, 20; ◑, 50; and ●, 100 μM spermidine; ⊕, 50; and ⊕, 150 μM putrescine. *trans*-Parinaric acid was used as a probe.

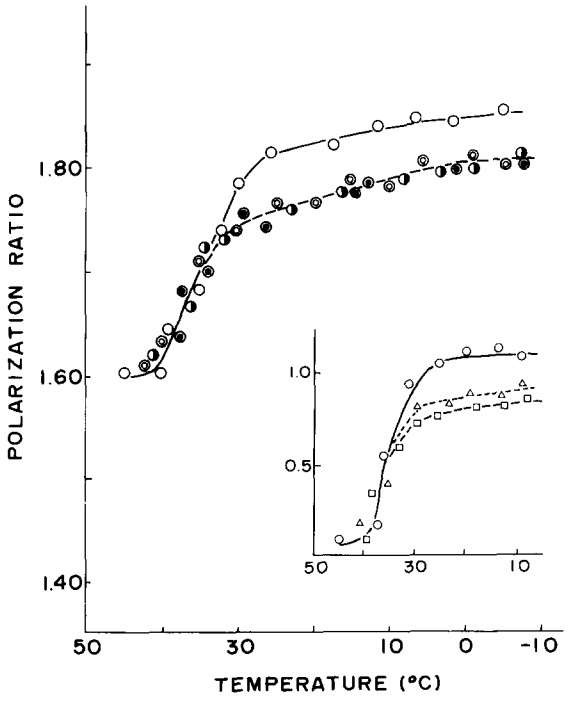

Fig. 3. Effect of spermine on fluorescence polarization change in the outer membrane of two different growth-phase cells. Membrane samples were added with spermine and were kept at 37°C for 30 min. Samples were then warmed to 50°C and fluorescence polarization was determined at descending temperature at the rate of 0.5 Cdeg/min. ○, outer membrane of logarithmic-phase cells with no spermine; ◑, with 50 μM spermine; ◎, outer membrane of the stationary-phase cells with no spermine; ◉, with 50 μM spermine, measured with the use of *trans*-parinaric acid. Insert: similar experiments which were carried out with the use of 1,6-diphenyl-1,3,5-hexatriene. ○, outer membrane of logarithmic-phase cells with no spermine; △, with 30 μM; □, with 50 μM spermine. The polarization ratio has been scaled between 0 (above the transition temperature) and 1 (below the transition temperature).

putrescine did not have any effect on changing the polarization ratios over the entire temperature range measured (Fig. 4). No effect of 50 μM spermine on the polarization change in the outer membrane was observed (Fig. 3). The addition of more than 150 μM spermine also resulted in aggregation of the membrane particles both in cytoplasmic and outer membrane samples.

From the results, it was conjectured that in the logarithmic-phase cell membranes, the fraction of lipid resistant to ordering at low temperature was augmented by the presence of polyamines. However, in the membranes of the stationary-phase cells, the lipid state change was not considered likely to be affected with polyamines.

Aqueous dispersions of membrane phospholipids

Unlike the results obtained with the membrane samples, the fluorescence polarization of parinaric acid in aqueous dispersions of cytoplasmic and outer membrane phospholipids derived from the logarithmic-phase cells did not show any change upon incubation with up to 50 μM spermine or 125 μM $MgCl_2$ (Fig. 5). The results suggested that an increased disordering of lipids in the membranes of logarithmic-phase cells in the presence of polyamines was not due to the lipid state change but resulted from the change of lipid-pro-

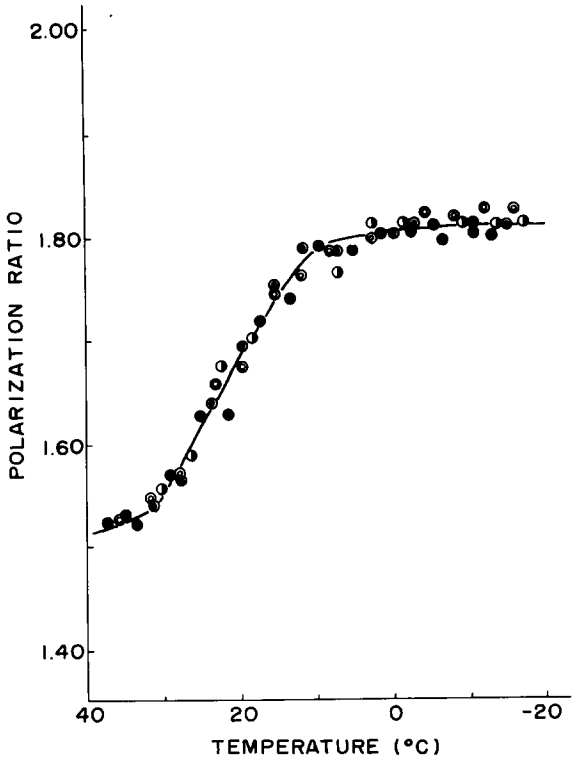

Fig. 4. Effect of various polyamines on fluorescence polarization change in cytoplasmic membrane of stationary-phase cells. Membrane samples were added with various polyamines at various concentrations. The procedures of sample incubation and fluorescence polarization measurement were similar to those described in Fig. 1. ◎, cytoplasmic membrane of stationary-phase cells (control, no polyamine); ●, 60 μM spermine; ◉, 50 μM spermidine; ◑, and 150 μM putrescine. *trans*-Parinaric acid was used as a probe.

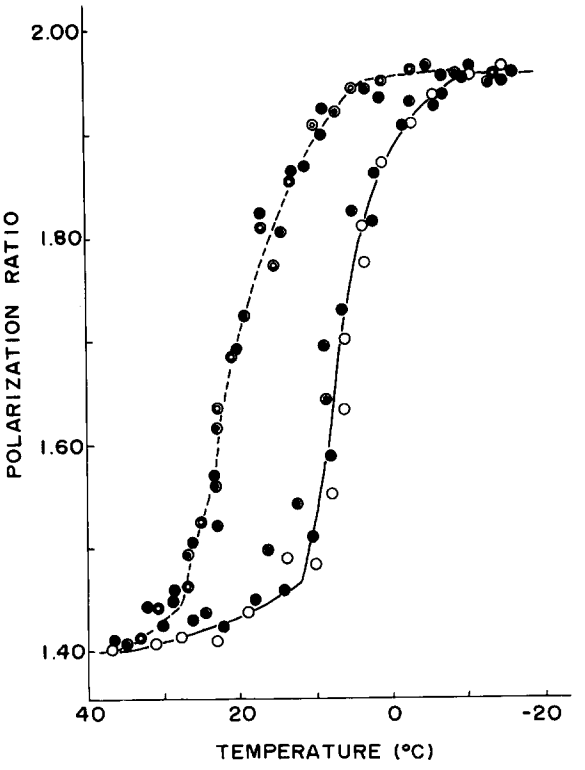

Fig. 5. Effect of spermine and $MgCl_2$ on fluorescence polarization change in aqueous dispersions of phospholipids extracted from the cytoplasmic and outer membranes of logarithmic-phase cells. Lipid dispersions which were added with spermine or $MgCl_2$ were incubated at 37°C for 30 min. Fluorescence polarization was determined in a similar procedure as described in Fig. 1. ○———○, phospholipid extracted from cytoplasmic membrane (control); ●, with 50 μM spermine; ◉, with 125 μM $MgCl_2$; ◎— — —◎, phospholipid extracted from the outer membrane (control); ●, with 50 μM spermine; ◉, with 125 μM $MgCl_2$. *trans*-Parinaric acid was used as a probe.

tein or protein-protein interactions mediated by the action of polyamines.

Effect of polyamines on cell viability in freeze-thawing

In contrast to the potent effect on the polarization change in membrane preparations, polyamines improved the recovery of the cells very slightly in freeze-thawing of intact normally cultivated cells in their solutions in concentrations which gave the membrane sufficient stability.

When the cells which were exposed to polyamines were freeze-thawed in polyamine-free buffer solution, their protective effects varied depending on whether the treatment of preliminary exposure of the cells to these compounds, namely cultivation of the cells in 0.5 mg per ml spermine, spermidine or putrescine, was effective. When the cells grown in normal culture medium were incubated in the same concentration of polyamines at 37°C for 15 min, no effect was seen, while the cells which were incubated for 30 min showed high viability. A similar incubation of the cells in higher polyamine concentration (1 mg/ml) showed a remarkably high viability after 15 min incubation. $MgCl_2$ did not show any effect on the viability recovery in a comparable experiment (Table I).

These results suggested that polyamines gave

TABLE I

EFFECT OF POLYAMINE OR $MgCl_2$ ON VIABILITY OF *E. COLI* CELL IN FREEZE-THAWING

		Cell viability after freeze-thawing (%)				
		control (no additive)	$MgCl_2$	putrescine	spermidine	spermine
Freezing of the cell in the presence of polyamine (0.05 mg/ml)		7.0	–	7.3	11.0	10.9
Cultivation of the cell in the presence of polyamine or $MgCl_2$ (0.5 mg/ml)		7.2	10.0	9.0	7.0	9.2
Pre-incubation of the cell in the presence of polyamine or $MgCl_2$ (0.5 mg/ml) at 37°C for (min)	0	11.0	–	14.4	15.4	11.6
	15	7.1	–	10.7	16.2	19.3
	30	7.3	10.6	29.6	32.7	33.2
Pre-incubation of the cell in the presence of polyamine or $MgCl_2$ (1.0 mg/ml) at 37°C for (min):	0	7.1	–	11.5	10.3	14.6
	15	7.5	–	28.8	34.4	35.4
	30	7.2	10.5	36.5	39.7	38.6

rise to the stability of membrane organization and the cell resistance to freezing. The requirement of a high concentration or prolonged incubation for exerting the effect of cellular resistance might be a result of the low permeability of cytoplasmic membrane to these polyamines. Taken together, these findings indicate that it is the intracellular polyamines that exert their effect on membrane stability and the resistance of the cells to freeze-thawing.

Discussion

The present study indicated that polyamines reduced thermal immobility of lipids in the membranes, probably by altering the protein-protein or protein-lipid interactions in lipid bilayer membranes. Such interaction between membrane constituents may result in the stabilization of membrane organization and hence raising of freezing resistance of *E. coli* cells.

The membrane-stabilizing effect of polyamines can be explained by considering them as polyvalent cations. Polyamines may exert a direct effect in bridging protein and lipid carboxyl groups present in the lipid bilayer of the membranes. The effect on protein-protein or protein-lipid interactions may have a strong influence on the membrane stabilization, because the change of properties of the lipid-lipid interaction was not clearly detected in the present study. A similar conclusion was reached in a study with erythrocytes, in which polyamines were shown to inhibit the lateral mobility of erythrocyte membrane proteins [19]. The effect was not due to changes in the membrane lipid state but was attributed to the restriction of the lateral mobility of membrane protein as a result of the interaction of polyamines with the cytoskeletal proteins [20]. Due to its four positively charged moieties at physiological pH, spermine will have an appreciable chance for bridging negatively charged groups most effectively [21,22]. Spermidine and putrescine may have a less potent efficiency with their lesser cationic sites, as seen in the present experiment.

In contrast to the polarization change in logarithmic-phase cell membranes, the change in the stationary-phase cell membranes was not affected by polyamines. The difference in lipid nature in different growth-phase cell membranes might result from the different protein-to-lipid ratios in these two growth-phase specimens [15,23]. A possibility that membrane turbidity changes which were caused by the presence of polyamines could cause light-scatter-induced polarization changes can be excluded, because the polarization change in the stationary-phase membrane samples was not affected by the addition of polyamines; re-

gardless, turbidity changes comparable to those in logarithmic-phase specimens occurred in the samples.

For the viability restoration of freeze-thawed cells, a high concentration of polyamine was required, in contrast to a very low concentration for stabilizing the membrane organization. This would be attributed to low permeability of the cytoplasmic membrane to polyamines. Ballas et al. [24] demonstrated that the polyamines entrapped in resealed ghosts of human erythrocytes exert a membrane-stabilizing effect at concentrations that are relevant to the erythrocytes (10–15 μM). In contrast, when intact washed erythrocytes were incubated with 5 mM polyamines at 37°C for up to 5 h, there was no change in erythrocyte membrane stability. They explained the result that it is the intracellular polyamines that exert the effect on membrane stabilization.

The requirement of a high concentration of spermine in the protection of osmotic lysis of *E. coli* protoplasts has been observed previously [12]. When the protoplasts were suspended in a 1 mM solution of spermine instead of water, the protoplasts were completely protected from lysis, while lower concentrations were ineffective. The concentrations of spermine or spermidine which protects the osmotic lysis of protoplasts were comparable to the concentrations required to prevent the freezing injury of the cells in the present experiment. It may be considered that part of the added polyamine may penetrate the membrane to exert its effect on membrane stabilization. Thus, the effect appeared in a time- and dose-dependent manner.

Although the present studies were carried out with externally added polyamines, it seems likely that the stabilizing of the membrane organization may be an important aspect of resistance of the cells to freeze-thawing, and these stabilizing effects might in part be the physiological functions of polyamines. Within this concept, it is very important that the membrane-stabilizing effect of polyamines appeared with concentrations comparable to the physiological levels of normally growing cells [25]. Thus, it is conceivable that the polyamines serve as modulators of membrane physical properties in other types of cells.

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