Biochimica et Biophysica Acta, 769 (1984) 563–570
Elsevier

BBA 72007

THERMAL ADAPTATION OF *TETRAHYMENA* MEMBRANES WITH SPECIAL REFERENCE TO MITOCHONDRIA

II. PREFERENTIAL INTERACTION OF CARDIOLIPIN WITH SPECIFIC MOLECULAR SPECIES OF PHOSPHOLIPID *

KAZUO OHKI, MASAKO GOTO and YOSHINORI NOZAWA

Department of Biochemistry, Gifu University School of Medicine, 40, Tsukasamachi, Gifu 500 (Japan)

(Received June 29th, 1983)

Key words: Cardiolipin; Membrane fluidity; Temperature acclimation; Fluorescence polarization; Spin label; Mitochondria; (Tetrahymena)

A specific effect of cardiolipin on fluidity of mitochondrial membranes was demonstrated in *Tetrahymena* cells acclimated to a lower temperature in the previous report (Yamauchi, T., Ohki, K., Maruyama, H. and Nozawa, Y. (1981) Biochim. Biophys. Acta 649, 385–392). This study was further confirmed by the experiment using fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). Anisotropy of DPH for microsomal and pellicular total lipids from *Tetrahymena* cells showed that membrane fluidity of these lipids increased gradually as the cells were incubated at 15°C after the shift down of growth temperature from 39°C. However, membrane fluidity of mitochondrial total lipids was kept constant up to 10 h. This finding is compatible with the result obtained using spin probe in the previous report. Additionally, the break-point temperature of DPH anisotropy was not changed in mitochondrial lipids whereas those temperatures in pellicular and microsomal lipids lowered during the incubation at 15°C. Interaction between cardiolipins and various phospholipids, which were isolated from *Tetrahymena* cells grown at 39°C or 15°C and synthesized chemically, was investigated extensively using a spin labeling technique. The addition of cardiolipins from *Tetrahymena* cells grown at either 39°C or 15°C did not change the membrane fluidity (measured at 15°C) of phosphatidylcholine from whole cells grown at 39°C. On the other hand, both cardiolipins of 39°C-grown and 15°C-grown cells decreased the membrane fluidity of phosphatidylcholine from *Tetrahymena* cells grown at 15°C. The same results were obtained for phosphatidylcholines of mitochondria and microsomes. Membrane fluidity of phosphatidylethanolamine, isolated from cells grown at 15°C, was reduced to a small extent by *Tetrahymena* cardiolipin whereas that of 39°C-grown cells was not changed. Representative molecular species of phosphatidylcholines of cells grown at 39°C and 15°C were synthesized chemically; 1-palmitoyl-2-oleoylphosphatidylcholine for 39°C-grown cells and dipalmitoleoylphosphatidylcholine for 15°C-grown ones. By the addition of *Tetrahymena* cardiolipin, the membrane fluidity of 1-palmitoyl-2-oleoylphosphatidylcholine was not changed but that of dipalmitoleoylphosphatidylcholine was decreased markedly. These phenomena were caused by *Tetrahymena* cardiolipin. However, bovine heart cardiolipin, which has a different composition of fatty acyl chains from the *Tetrahymena* one, exerted only a small effect.

* For Part I in this series, see Ref. 22.

Introduction

Most parts of cell functions are associated with biological membrane, e.g., ATP synthesis, hormonal regulation, neural regulation, immunological response and transport of biological substances. Much attention has been paid to the relationship between functions and structures of membranes [1–5]. In many cases, physical properties of membrane lipids play an important role in the membrane functions. Membrane fluidity affects activity of membrane bound enzyme [6], endocytosis [7] and activation of adenylate cyclase [8–10]. Activity of membrane enzymes was also modulated by surface charge [11] and phase transition of membrane lipid [12]. Additionally, arrangement and organization of lipid molecules were supposed to play a central role in the mechanism of membrane fusion [13–15].

In general, when unicellular organisms are exposed to a new environment, lipid composition of the membranes changes by some adaptation mechanism. Even in multicellular organisms, lipid composition of membranes could be modified by diet [10,16]. In order to study the role of lipids in biological membrane, a method used widely is to alter the membrane lipid composition by changing the growth temperature of certain microorganisms [17] and then to examine the effects on membrane functions [18]. Experiments have been designed to study the relationship between the physical properties of membranes and their lipid composition in *Tetrahymena* cells acclimated to a new growth temperature. Exposure of *Tetrahymena* grown at 39°C to a lower growth temperature (15°C) caused a marked and relatively rapid alteration of fatty acid composition of phospholipid, i.e. and increase of unsaturated fatty acid content. Spin labeling technique revealed that the membrane fluidity was increased as a consequence of unsaturated fatty acid increase [19–21]. In *Tetrahymena* cells acclimated to the lower growth temperature, membrane fluidities of cilia, pellicles and microsomes were raised as the content of unsaturated fatty acids increased during the incubation at 15°C. However, membrane fluidity of mitochondria was kept constant in spite of the increased level of unsaturated fatty acids. This relative constancy of mitochondrial physical state would be considered to be attributed to cardiolipin which is known to be exclusively localized in mitochondrial inner membranes [22].

In the present paper, our previous study was further extended by employing the fluorescent probe DPH, and the effect of cardiolipin on membrane fluidity was also studied for isolated and synthetic phospholipids. The results obtained by the fluorescence technique was found to be consistent with those by the spin probe study. Moreover, by an extensive spin labeling study, cardiolipin has been proved to interact preferentially with phosphatidylcholine possessing two unsaturated fatty acyl chains, and cardiolipin was observed to prefer phosphatidylcholine rather to phosphatidylethanolamine and 2-aminoethylphosphonolipid.

Materials and Methods

Cells. Tetrahymena pyriformis, NT-1, a thermotolerant strain, was cultured with constant shaking at 39°C in an enriched medium; 2% proteose-peptone (Difco), 0.5% glucose (Nakarai), 0.2% yeast extract (Difco) and 90 μM Fe^{2+}-EDTA complex. After 24-h incubation the growth temperature was lowered to 15°C over a period of half an hour. In order to adjust the cooling rate to 0.8 K/min, the temperatures were monitored by a sterile thermometer placed in the medium [23]. After the growth temperature was lowered to 15°C, cell division was not observed before 10 h passed.

Cilia, pellicles, mitochondria and microsomes were isolated from *Tetrahymena* cells at various periods of incubation according to the previous method [24].

Extraction and isolation of Tetrahymena's lipids. Total lipids were extracted from individual membrane fractions or whole cells according to the method of Bligh and Dyer [25]. The extracted lipid dissolved in chloroform was applied to the silicic acid (Mallinckrodt, 100 mesh) column and eluted with three bed volumes of chloroform to remove neutral lipids. Cardiolipin, phosphatidylethanolamine/2-aminoethylphosphonolipid and phosphatidylcholine were eluted by step-wise gradients of chloroform/methanol; 98 : 2 (v/v), 90 : 10 (v/v), 80 : 20 (v/v), 70 : 30 (v/v), 60 : 40 (v/v), 50 : 50 (v/v), 40 : 60 (v/v), 20 : 80 (v/v) and 0 : 100 (v/v).

Every 2 ml was collected, and a portion was developed on Silica gel G plate by chloroform/methanol/acetic acid/water (50 : 30 : 8 : 4, by vol.) to identify the phospholipid species. Individual fractions of cardiolipin, phosphatidylethanolamine/2-aminoethylphosphonolipid and phosphatidylcholine were collected and stored in chloroform/methanol (6 : 1, v/v) at −20°C.

Synthesis of 1-palmitoyl-2-oleoylphosphatidylcholine and dipalmitoleoylphosphatidylcholine. 1-Palmitoyl-2-oleoylphosphatidylcholine and dipalmitoleoylphosphatidylcholine were synthesized according to the method of Robles and Van den Verg [26]. Dipalmitoylphosphatidylcholine (Sigma) was converted to 1-palmitoyllysophosphatidylcholine by phospholipase A_2 (*Naja naja*, Sigma). The 1-palmitoyl-2-oleoylphosphatidylcholine was formed by addition of oleic anhydride, and isolated with chloroform extraction followed by diethyl ether washing. Dipalmitoleoylphosphatidylcholine was synthesized from glycerophosphorylcholine and palmitoleic anhydride, and isolated as described above.

Fluorescence measurement. Lipids were dispersed by vortexing with glass beads in Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 6.8) as a final concentration 65 μM, and 1,6-diphenyl-1,3,5-hexatriene (DPH, Aldrich) was added to the dispersion at 1/500 as a molar ratio of DPH to lipids. The steady-state fluorescence intensity and the anisotropy were measured with a self-constructed instrument which was described previously [27,28]. The excitation wavelength was selected at 360 nm and all fluorescence above 420 nm was collected with cut off filters (Hoya L-39 and L-42 filters). Every lipid dispersion scattered and absorbed about 15% of excitation light of 360 nm. The error in the emission anisotropy values was estimated to be within 1% in all experiments. Nanosecond time-resolved fluorescence investigation revealed two components of DPH fluorescence in lipid dispersions. The life time of the major components was 7-10 nsec and that of minor component was 1–3 ns. DPH anisotropy of steady-state measurement reflected mainly the major component.

ESR measurement. Lipid (10 mM) and 0.1 mM of stearic acid spin probe (*N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-keto stearic acid, Syva Associates) were dispersed homogeneously in Tris-buffered saline by a Branson sonifier (B-12). ESR spectra were measured at various temperatures using a commercial X-band spectrometer (JEOL FE-1X) equipped with a temperature controller. The parallel ($T'_{\parallel}$) and perpendicular ($T'_{\perp}$) principal values of the hyperfine tensor of an axially symmetrical spin Hamiltonian were estimated from the ESR spectra and the order parameter, S, was calculated using the following relation.

$$S = \frac{a(T'_{\parallel} - T'_{\perp})}{a'\left(T_{zz} - \frac{1}{2}(T_{xx} + T_{yy})\right)}$$

where $a = (T_{xx} + T_{yy} + T_{zz})/3$, $a' = (T'_{\parallel} + 2T'_{\perp})/3$, $T_{xx} = T_{yy} = 5.9$ G and $T_{zz} = 32.9$ G are the hyperfine principal values of the nitroxide radical. In the *Tetrahymena* membranes and their extracted lipids, the order parameter of the stearate spin probe was a good indication of the membrane

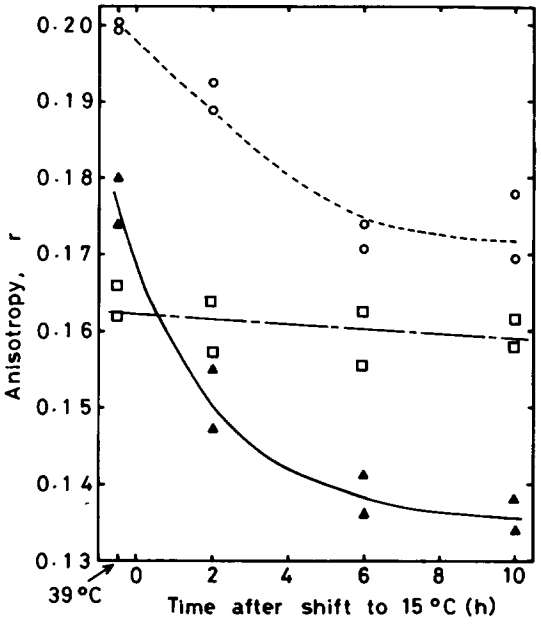

Fig. 1. Change in anisotropy of DPH in mitochondria, pellicles and microsomes from *Tetrahymena* cells during acclimation to 15°C. *Tetrahymena* cells grown at 39°C for 24 h were incubated at 15°C. Total lipids were extracted from isolated membrane fractions, and anisotropy of DPH was measured in the dispersions of these lipids at 15°C. □-·-□, Mitochondrial; ○------○, pellicular and ▲———▲, microsomal lipids. Data from two experiments were plotted in the figure.

fluidity [19–22]. And the term 'fluidity' was used as an expression of order parameter in this paper.

Results

In the previous paper [22], ESR study using stearic acid spin probe revealed that the membrane fluidity of *Tetrahymena*'s mitochondria was not changed during acclimation to a lower growth temperature, despite the increase in unsaturation of the fatty acyl chains. Another method was introduced in the present study. Measurement of the membrane fluidity by a fluorescence technique is widely used as well as an ESR method. Therefore, a fluorescence study was carried out on the same lipid system as that studied by ESR. Fig. 1 shows the results of the experiment using a fluorescent probe, DPH. *Tetrahymena* cells were grown at 39°C for 24 h, and eight generations passed during the incubation periods. The growth temperature was lowered to 15°C over a period of 0.5 h. Mitochondria, pellicles (surface membranes of the cells) and microsomes were isolated at 0.5 h before, and 2, 6 and 10 h after the temperature shift.

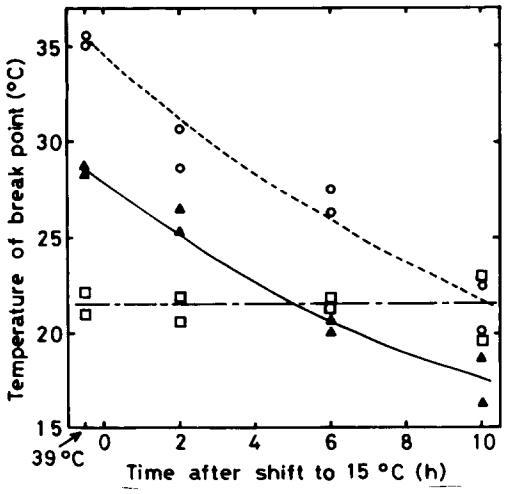

Fig. 2. Changes in break point of plots of $\log[(r_0 - r)/r]$ vs. $1/T$ in mitochondrial, pellicular and microsomal lipid from *Tetrahymena* cells during acclimation to 15°C. Total lipids were extracted from isolated membrane fractions, anisotropy of DPH was measured in the temperature range of 50 to 5°C. values of $\log[(r_0 - r)/r]$ were plotted for $1/T$, and break points were obtained by hand fitting. □-·-□, Mitochondrial; ○------○, pellicular and ▲———▲, microsomal lipids. Data from two experiments were plotted in the figure.

Anisotropy of DPH in liposomes of these membrane lipids decreased in order of pellicles, microsomes and mitochondria; membrane fluidity increased in this order. The lowest fluidity was observed in the ciliary lipids including the highest content of tetrahymanol (data not shown). As for membranes of these organelles, the same order of fluidity was obtained by a spin labeling technique [19]. With the increasing period of incubation at 15°C, anisotropy of DPH in both pellicular and microsomal lipids decreased gradually but anisotropy in mitochondrial lipids showed almost no change up to 10 h. During this period, the content of unsaturated fatty acid in membrane phospholipids increased in these three membrane fractions [22]. However, no increase of fluidity was observed in mitochondrial lipids. This result is identical to that obtained by a spin labeling technique used in the previous paper [22]. The following experiments were designed to gain more insight into the underlying mechanisms of this phenomenon.

The measurement of DPH fluorescence in lipid dispersions was automatically carried out at every temperature from 50°C to 5°C under control of a minicomputer. $\text{Log}[(r_0 - r)/r]$ was plotted against $1/T$ to obtain the temperature of the break point in a curve. Fig. 2 shows changes in break-point temperatures for pellicles, microsomes and mitochondria during acclimation to 15°C. Each break point in the plots can be attributed to the temperature at which the physical properties of the membrane change. Indeed, these break points correspond to the temperatures at which crystalline phase (4.2 Å peak) starts to appear in X-ray diffraction of membrane lipids of *Tetrahymena* [29,30]. Incorporation of double bond into a fatty acyl chain of phospholipid decreases the phase transition temperature of liquid crystalline to crystalline. The results of pellicles and microsomes were in agreement with this effect of a double bond's lowering the phase transition temperature. The break-point temperature of DPH fluorescence in mitochondrial lipids showed a singular phenomenon as observed in the measurement of fluidity; the break-point temperature was sustained constant while the unsaturation of the fatty acyl chain increased gradually.

The phenomenon in mitochondrial lipids has

been proved to be due to cardiolipin [22]. In order to examine the effect of cardiolipin on membrane lipid in detail, the extracted lipids from cells grown at 39°C or 15°C were separated into the major phospholipid fractions. *Tetrahymena pyriformis*, NT-1, possesses phosphatidylcholine, phosphatidylethanolamine and 2-aminoethylphosphonolipid as their phospholipids. In addition, cardiolipin is present in mitochondrial membrane in *Tetrahymena* as well as in other animal cells. Although there were compositional differences among the fatty acyl chains of these phospholipids, the same tendency was observed in the change of fatty acid composition induced by the acclimation to a lower growth temperature [21]. Furthermore two types of phosphatidylcholine molecules having defined fatty acyl chains were synthesized chemically. Analysis of phospholipid species in *Tetrahymena* cells revealed that 1-palmitoyl-2-oleoylphosphatidylcholine and dipalmitoleoylphosphatidylcholine were representative molecular species of cells grown at 39°C and 15°C, respectively [31]. Membrane fluidities of these synthetic phosphatidylcholines with and without cardiolipin were measured at various temperatures using stearate spin probe. In Fig. 3, the order parameters

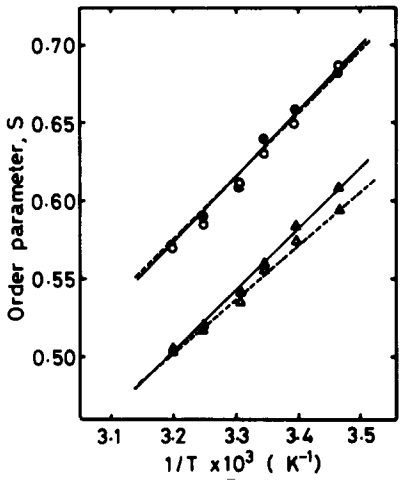

Fig. 3. Order parameter vs. $1/T$ of synthetic phosphatidylcholines with and without cardiolipin. 1-Palmitoyl-2-oleoylphosphatidylcholine (○, ●) and dipalmitoleoylphosphatidylcholine (△, ▲) were synthesized chemically according to the method of Robles and Van den Berg [26]. The order parameter of the stearate spin probe was calculated from the ESR spectrum of these phosphatidylcholines with (●, ▲) and without (○, △) cardiolipin from *Tetrahymena* cells grown at 15°C.

of stearate spin probe in 1-palmitoyl-2-oleoylphosphatidylcholine and dipalmitoleoylphosphatidylcholine liposomes were plotted for $1/T$ (K^{-1}). Also the values were plotted for liposomes containing cardiolipin. Order parameters were obtained at six different temperatures. Phase transition temperatures of these phospholipids were below 0°C (t_m of 1-palmitoyl-2-oleoylphosphatidylcholine is −5°C), and no break was detected by an ESR measurement in curves of order parameter between 50°C and 4°C. Theoretically, the order parameter has a linear correlation with an inverse of absolute temperature [32]. Therefore, straight lines in the figure were obtained statistically by linear regression of these points. And order parameter at 15°C was determined from the line. This method was applied to determination of the order parameters of extracted lipids from *Tetrahymena*. The order parameter of 1-palmitoyl-2-oleoylphosphatidylcholine was larger than that of dipalmitoleoylphosphatidylcholine by 0.075, and there were parallel variations in the parameters as a function of temperature between these two phospholipids. Addition of cardiolipin altered the temperature dependency of the order parameter in dipalmitoleoylphosphatidylcholine but not in 1-palmitoyl-2-oleoylphosphatidylcholine. The effect of cardiolipin on membrane fluidity was supposed to depend on the fatty acyl composition of the membrane lipids.

To assess in more detail the effect of cardiolipin on membrane fluidity, phosphatidylcholine, phosphatidylethanolamine (including 2-aminoethylphosphonolipid) and cardiolipin were isolated from *Tetrahymena* cells grown at 39°C or at 15°C to a late logarithmic phase. 2-Aminoethylphosphonolipid includes ethylamine moiety instead of ethanolamine and is expected to show a similar physicochemical property to phosphatidylethanolamine. Indeed, the phase transition temperatures of dipalmitoylphosphatidylethanolamine and dipalmitoyl-2-aminoethylphosphonolipid were 61.2°C and 61.4°C, respectively, by ESR measurement using TEMPO as spin probe. A mixture of these two phospholipids was used in the present experiment without further separation. The order parameters at 15°C were obtained according to the method described above for various combina-

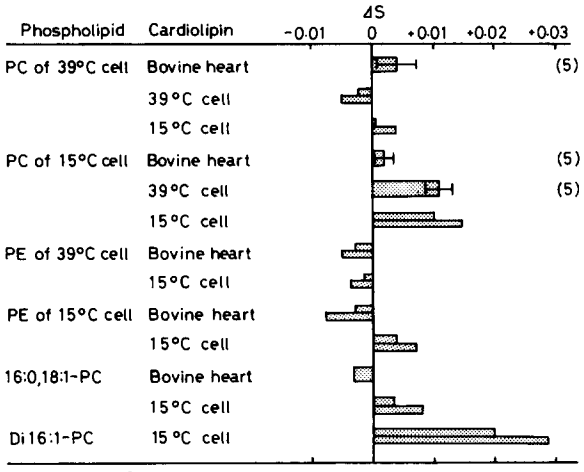

Fig. 4. Effects of cardiolipin on the order parameter of various phospholipid dispersions. Phosphatidylcholine and phosphatidylethanolamine (including 2-aminoethylphosphonolipid) were isolated from *Tetrahymena* cells grown at 39°C or 15°C. Figures in parentheses indicate the number of experiments.

tions of cardiolipin and phosphatidylcholine (or phosphatidylethanolamine/2-aminoethylphosphonolipid) from whole cells grown at 39°C or 15°C. Fig. 4 summarizes the effects of added cardiolipin on membrane fluidity of various phospholipids. Cardiolipins used in the present experiment were from various sources; bovine heart, *Tetrahymena* cells grown at 39°C and 15°C. The changes in order parameter caused by 7.5 mol% of cardiolipin, which was equivalent to the cardiolipin content in *Tetrahymena* mitochondria, were shown in the figure. A positive ΔS indicates a decrease in membrane fluidity. When cardiolipin from each source was added to phosphatidylcholine isolated from *Tetrahymena* cells grown at 39°C, only small changes in order parameter were observed. However, cardiolipin from both 39°C- and 15°C-grown cells increased the order parameter for phosphatidylcholine in 15°C-grown cells. Bovine heart cardiolipin scarcely affected the order parameter. Similar experiments were carried out for phosphatidylcholine purified from mitochondria of 15°C- and 39°C-grown cells. A decrease in fluidity was observed only when cardiolipin was added to phosphatidylcholine from mitochondria of 15°C-cells (data not shown). As for phosphatidylethanolamine, the addition of cardiolipin caused a slight effect on the order parameter in all tested combinations of cardiolipin and phosphatidylethanolamine. However, when the effects of added cardiolipin were compared between phosphatidylethanolamine from 39°C-grown cells and from 15°C-grown cells, there was a significant increase in order parameter, induced by *Tetrahymena* cardiolipin, in phosphatidylethanolamine isolated from 15°C-grown cells. Phospholipids of cells grown at 15°C were specifically affected by the addition of *Tetrahymena* cardiolipin, suggesting evidence that unsaturated fatty acids might play an important role in the interaction between phospholipid and cardiolipin. Indeed, a more remarkable effect of cardiolipin was observed in a synthetic phosphatidylcholine. Cardiolipin from *Tetrahymena* source was found to increase the order parameter of dipalmitoleoylphosphatidylcholine to a much greater extent than that of 1-palmitoyl-2-oleoylphosphatidylcholine. Phospholipid which has an unsaturated fatty acid at 1-position of glycerol is characteristic of *Tetrahymena* cells adapted to 15°C [31]. There was also a marked difference between the effects of *Tetrahymena* cardiolipin and bovine heart cardiolipin on membrane fluidity. This might be due to the fatty acid composition of these cardiolipins; 87% of fatty acid of bovine heart cardiolipin is linoleic acid, and *Tetrahymena* cardiolipin comprises linoleic acid (37%) and linolenic acid (44%) [22]. Therefore, the fatty acid species of cardiolipin and phospholipid were considered to take a key part in the interaction.

Discussion

Living organisms have the ability to adapt to their environments. Especially for unicellular organisms, the adaptation mechanism is essential for survival of changes in environment, since they suffer extracellular stimuli directly. Temperature is one of most important factors for cell growth. And alteration of the membrane lipid composition is an important mechanism of thermal adaptation. Indeed, *Tetrahymena* cells exposed to a lower growth temperature could recover cell division [33], lateral diffusion of membrane proteins [23,33], and swimming velocity [18] by increasing unsaturation in the fatty acyl chains of the membrane phospholipids. On the other hand, *Bacillus stearothermophilus* could live in a higher growth temperature by

increasing the ratio of saturated to unsaturated fatty acids [34]. Temperature acclimation of cells is attained by alteration of the lipid composition, especially by regulating the content of double bonds in fatty acyl chains. Incorporation of double bonds could recover the membrane fluidity that had been decreased by lowering growth temperature [19,21]. However, in mitochondrial lipids, increase of membrane fluidity was not observed by spin labeling technique, whereas unsaturation of fatty acyl chains occurred [22]. It was claimed that the spin labeling technique using fatty acid spin probe did not always reflect the fluidity of membranes [35,36]. Therefore, a fluorescent probe was used for the same samples as used in the previous study. From comparing the time course of DPH anisotropy with that of the stearate spin probe order parameter, it was demonstrated that these two results from different techniques were essentially identical. The extended experiment revealed that cardiolipin of *Tetrahymena* cells preferentially interacted with specific phospholipids, phosphatidylcholines of *Tetrahymena* cells grown at 15°C. In *Tetrahymena* cells acclimated to a lower growth temperature, characteristic feature of altered lipid composition was the incorporation of unsaturated fatty acid into the 1-position of the glycerol backbone of phospholipids [31]. This tendency was observed in all phospholipid species of individual membrane fractions [37]. Incorporation of unsaturated fatty acid into 1-position of glycerol moiety modified the physical properties of phospholipid. In 1-stearoyl-2-polyunsaturated phosphatidylcholine, the increase of unsaturation at 2-position of glycerol raised the phase transition temperature. On the other hand, dioleoylphosphatidylcholine which contained monounsaturated fatty acyl chain at 1-position showed a lower temperature of phase transition than these phosphatidylcholines [38]. Also phase transition temperature of 1-oleoyl-2-palmitoylphosphatidylcholine was lower then that of 1-palmitoyl-2-oleoylphosphatidylcholine [39]. Cardiolipin is known to form a non-bilayer structure (hexagonal II phase) in the presence of Ca^{2+} [40–42]. Lipid molecules were classified into three types from their shapes; cone (smaller polar headgroup), cylinder and inverted cone (larger polar headgroup). According to the molecular shape, each type of lipid molecule tends to form hexagonal II, planer bilayer and micelle, respectively [43]. Incorporation of double bond into fatty acyl chain converts the moleculer shape into cone type (relatively smaller polar headgroup). And the lipids forming bilayer could be incorporated into hexagonal II phase [44]. Therefore, it is plausible to think that in mitochondria newly synthesized phospholipids, which comprise unsaturated fatty acid at the 1-position of glycerol, would form a hexagonal II structure with cardiolipin. Thus apparently any increase in membrane fluidity was not observed for mitochondrial phospholipids.

Acknowledgement

The authors would like to express their sincere thanks to Dr. Kazuhiko Kinosita, Jr. and Dr. Akira Ikegami of The Institute of Physical and Chemical Research for kind permission to use a fluorescence instrument and valuable discussions. This study was in part supported by the Grant-in-Aid from the Ministry of Education, Culture and Science, Japan.

References

1 Singer, S.J. and Nicolson, G.L. (1972) Science 175, 720–731
2 Cullis, P.R. and De Kruijff, B. (1971) Biochim. Biophys. Acta 559, 399–420
3 Farias, R.N. (1980) Adv. Lipid Res. 17, 251–282
4 Klingenberg, M. (1981) Nature 290, 449–454
5 Sandermann, H., Jr., (1978) Biochim. Biophys. Acta 515, 209–237
6 Bukland, R.M., Radda, G.T. and Wakefield, L.M. (1981) Biochim. Biophys. Acta 643, 363–375
7 Schroeder, F. (1981) Biochim. Biophys. Acta 649, 162–174
8 Hanski, E., Rimon, G. and Levitzki, A. (1979) Biochemistry 18, 846–853
9 Atlas, D., Volsky, D.J. and Levitzki, A. (1980) Biochim. Biophys. Acta 597, 64–69
10 Castuma, C.E. and Brenner, R.R. (1983) Biochim. Biophys. Acta 729, 9–16
11 Clancy, R.M., Wissenberg, A.R. and Glaser, M. (1981) Biochemistry 20, 6060–6065
12 Brasitus, T.A., Schachter, D. and Memouneous, T.G. (1979) Biochemistry 18, 4136–4144
13 Van Venetie, R. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 692, 397–405
14 Rand, R.P., Reese, T.S. and Miller, R.G. (1981) Nature 293, 237–238
15 Hope, M.J. and Cullis, P.R. (1981) Biochim. Biophys. Acta 640, 82–90

16 King, M.E., Stavens, B.W. and Spector, A.A. (1977) Biochemistry 16, 5280–5285
17 Thompson, G.A., Jr. and Nozawa, Y. (1977) Biochim. Biophys. Acta 427, 55–92
18 Goto, M., Ohki, K. and Nozawa, Y. (1982) Biochim. Biophys. Acta 693, 335–340
19 Nozawa, Y., Iida, H., Fukushima, H., Ohki, K. and Ohnishi, S. (1974) Biochim. Biophys. Acta 367, 134–147
20 Iida, H., Maeda, T., Ohki, K., Nozawa, Y. and Ohnishi, S. (1978) Biochim. Biophys. Acta 508, 55–64
21 Ohki, K., Kasai, R. and Nozawa, Y. (1979) Biochim. Biophys. Acta 558, 273–281
22 Yamauchi, T., Ohki, K., Maruyama, H. and Nozawa, Y. (1981) Biochim. Biophys. Acta 649, 385–392
23 Nozawa, Y. and Kasai, R. (1978) Biochim. Biophys. Acta 529, 54–66
24 Nozawa, Y. and Thompson, G.A., Jr. (1971) J. Cell Biol. 49, 712–721
25 Bligh, E.G. and Dyer, W. (1959) Can. J. Biochem. Physiol. 37, 911–917
26 Robles, E.C. and Van den Berg, D. (1969) Biochim. Biophys. Acta 187, 520–526
27 Kinosita, K., Jr., Mitaku, S., Ikegami, A., Ohbo, N. and Kunii, T.L. (1976) Jap. J. Appl. Phys. 15, 2433–2440
28 Kinosita, K., Jr., Kataoka, R., Kimura, Y., Gotoh, O. and Ikegami, A. (1981) Biochemistry 20, 4270–4277
29 Nakayama, H., Goto, M., Ohki, K., Mitsui, T. and Nozawa, Y. (1983) Biochim. Biophys. Acta 730, 17–24
30 Nakayama, H., Ohki, K., Mitsui, T. and Nozawa, Y. (1984) Biochim. Biophys. Acta 769, 311–316
31 Watanabe, T., Fukushima, H. and Nozawa, Y. (1980) Biochim. Biophys. Acta 620, 133–141
32 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
33 Wunderlich, F., Speth, V., Batz, W. and Kleinig, H. (1973) Biochim. Biophys. Acta 298, 39–49
34 McElhaney, R.N. and Souza, K.A. (1976) Biochim. Biophys. Acta 443, 348–359
35 Schreier, S., Polnaszek, C.F. and Smith, I.C.P. (1978) Biochim. Biophys. Acta 515, 395–436
36 Poon, R. and Clark, W.R. (1982) Biochim. Biophys. Acta 689, 230–240
37 Maruyama, H., Banno, Y., Watanabe, T. and Nozawa, Y. (1982) Biochim. Biophys. Acta 711, 229–244
38 Coolbear, K.P., Berde, C.B. and Keough, K.M.W. (1983) Biochemistry 22, 1466–1473
39 Santarén, J.F., Rico, M., Guilleme, J. and Ribera, A. (1982) Biochim. Biophys. Acta 687, 231–237
40 Cullis, P.R., Verkleij, A.J. and Ververgaert, P.H.J.T. (1978) Biochim. Biophys. Acta 513, 11–20
41 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
42 De Kruijff, B., Verkleij, A.L., Leunissen-Bijvelt, J., Van Echteld, C.J.A., Hille, J. and Rijnbout, H. (1982) Biochim. Biophys. Acta 693, 1–12
43 Wieslander, A., Christiansson, A., Rilfors, L. and Lindblom, G. (1980) Biochemistry 19, 3650–3655
44 Vasilenko, I., De Kruijff, B. and Verkleij, A.J. (1982) Biochim. Biophys. Acta 685, 144–152