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AN X-RAY DIFFRACTION STUDY ON PHASE TRANSITION TEMPERATURES OF VARIOUS MEMBRANES ISOLATED FROM *TETRAHYMENA PYRIFORMIS* CELLS GROWN AT DIFFERENT TEMPERATURES

HARUTO NAKAYAMA [a], MASAKO GOTO [b], KAZUO OHKI [b], TOSHIO MITSUI [a] and YOSHINORI NOZAWA [b]

[a] *Department of Biophysical Engineering, Faculty of Engineering Science. Osaka University, Toyonaka, Osaka 560 and* [b] *Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500 (Japan)*

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Mitochondrial, microsomal and pellicular membranes were isolated from *Tetrahymena* cells grown at 39°C or 15°C, and phospholipids, in turn, were separated from total lipids extracted from these membranes. The effect of growth temperature on their solid-to-fluid phase transition temperature was examined by wide-angle X-ray diffraction. The transition temperatures of phospholipids from mitochondria, microsomes and pellicles were 21, 19 and 26°C for cells grown at 39°C and −8, −3 and 6°C for cells grown at 15°C, respectively. All phospholipids were found in a completely fluid state at these growth temperatures. From a comparison between the phospholipids and total lipids from pellicles of cells grown at 39°C, a triterpenoid alcohol, tetrahymanol, caused the transition temperature to increase. The alignment of tetrahymanol in membranes was examined with pellicle's total lipid oriented in a sample holder.

Introduction

Most cellular functions are associated with membranes, e.g., transport, transmembrane signalling and exocytosis. It is also known that the physical state of the membrane is closely related to various functions of membranes through interaction between proteins and lipids [1]. For instance, phase transitions observed in membrane lipids often correspond to the breaks in Arrhenius plot of some membrane-bound enzymes [2,3]. In order to survive in variable environments, living organisms are endowed with a regulatory process to keep the physical state of the membrane constant within certain limits. In *Bacillus stearothermophilus*, the gel to liquid-crystalline phase transition of membrane lipids is seen just below the temperature of growth in the range of 42–65°C [4]. A constant fluidity of membranes is maintained in *Escherichia coli* grown at different temperatures [5]. Homeostasis in membranes of these procaryotes is usually attained through alterations in membrane lipid composition. Adaptive modification of membrane lipids was also observed in some eucaryotes, and *Tetrahymena*, a unicellular eucaryote, is a useful model cell for biochemical and physicochemical studies of acclimation of membrane lipids [6]. Various techniques have been introduced to investigate the physical state of membranes; e.g. spin labeling, differential scanning calorimetry, NMR and fluorescent probe. X-ray diffraction also is a powerful method to detect the phase transition of membrane lipids, and we have been able to obtain the phase transition temperatures of membrane lipids from thermally acclimated *Escherichia coli* [7] and *Anacystis nidulans* [8]. As for *Tetrahymena*, there are two reports using lipids [9,10], in which small-angle diffraction was used. Here we employed wide-angle diffraction in the present study.

Biological membranes contain sterols as one lipid constituent; cholesterol in higher animals, stigmasterol in higher plants, ergosterol in fungi and tetrahymanol in protozoa. Although extensive studies have been carried out on cholesterol [11], the precise physiological role of membrane sterols is still unclear. The same situation is also true with, a triterpenoid compound, tetrahymanol in *Tetrahymena* which is rich in the surface membranes, pellicles and cilia. Little information is available which suggests that this sterol-like component might affect membrane fluidity [12].

The present communication demonstrates that the transition temperature of the gel to liquid-crystalline state inferred by X-ray diffraction for different membrane fractions of *Tetrahymena* are closely related to the growth temperature of cells. Moreover, the effect of tetrahymanol on physical properties of surface membranes will be discussed in a comparison with cholesterol.

Materials and Methods

Cells. Tetrahymena pyriformis NT-1, a thermotolerant strain, was grown at 39°C or 15°C with constant shaking (90 strokes/min) in an enriched medium; 2% proteose-peptone (Difco), 0.5% glucose (Nakarai), 0.2% yeast extract (Difco) and 90 μM Fe^{2+}-EDTA complex. Pellicular, microsomal and mitochondrial fractions were isolated according to the method of Nozawa and Thompson [13] and lipids were extracted by the method of Bligh and Dyer [14], and then phospholipids were separated from tetrahymanol by silicic acid column chromatography [13].

X-ray diffraction. The extracted lipid were first dried under nitrogen gas stream, then in vacuo for 2–3 h. The dried lipid was hydrated by 1–2 μl of buffer (50 mM NaCl/10 mM Tris-HCl, pH 7.5, deoxygenated by nitrogen replacement) per mg of lipid. The mixture was incubated under nitrogen in a sealed bottle on ice overnight. The swollen sample was transferred to a plastic holder of 1 mm thickness and wrapped by thin aluminium foil just before measurement. All the procedures were performed under nitrogen gas flow and on ice. The measurement of X-ray was carried out according to the previous report [7]. X-rays were obtained from a Rigaku rotating-anode microfocus generator RU-100, and nickel-filtered CuKα radiation (wavelength 1.542 Å) was used. The distance from sample to detector was adjusted to 80–90 mm. For estimation of peak width, a position-sensitive proportional counter (Rigaku Denki Co.) was operated in delay line method with the spatial resolution of 0.16 mm on wire. The collected data of intensity were sent directly to and processed by PDP11/34 minicomputer. Temperature of the specimen was controlled to within ± 0.1°C from desired temperature by a stream of nitrogen gas through a regulating heater. Two-dimensional X-ray pattern from oriented samples were recorded on Sakura cosmic-ray film using the same generator and optical instruments.

Results

Effect of growth temperature on phase transition temperature of different membrane fractions

Fig. 1a shows the wide-angle X-ray diffraction patterns of total lipids extracted from pellicles of cells grown at 39°C. The abcissa is an inverse of the spacing: $1/d = 2 \sin \theta/\lambda$, where 2θ is the diffraction angle and λ is the wavelength (1.542 Å). Typical features were observed in this specimen; a sharp peak centred at 4.2 Å ($1/d = 0.24$ Å^{-1}) was increased and broad peak centred around 4.6 Å ($1/d = 0.22$ Å^{-1}) was decreased as the temperature was lowered. These two peaks were also observed in pellicular phospholipids (Fig. 1b) and appeared in all samples (total lipids extracted from pellicles, mitochondria and microsomes of cells grown at 39°C or 15°C, and their phospholipids fraction) used in the present experiment. The 4.2 Å peak indicates the existence of solid phase of hydrocarbon chains in lipid bilayer [15]. In order to characterize the phase behaviour more clearly, profiles of 4.2 Å peaks were extracted according to the following procedure: Background intensity from optics was corrected for absorption by samples (calculated absorption coefficient was 0.36) and subtracted from all the patterns of the series (Fig. 2). To eliminate the diffraction from the aqueous solvent, the amount of solvent in sample was estimated from the difference of diffraction intensities at 0.320–0.330 Å^{-1} between the normal sample and the frozen sample. After the subtraction of solvent diffraction, resultant patterns at

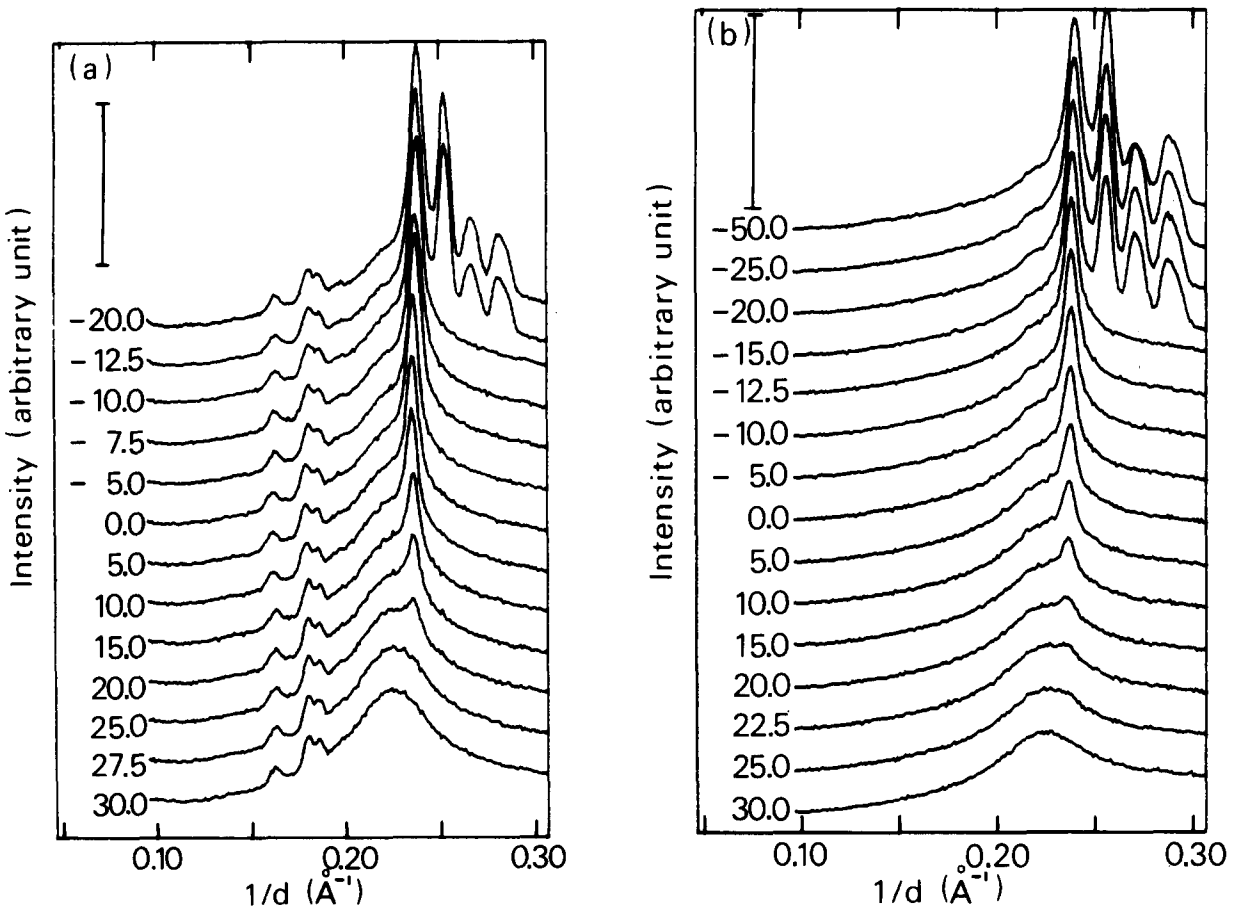

Fig. 1. Wide-angle X-ray diffraction patterns. Background X-rays from optics, after absorption correction, were subtracted from original intensity data. Abscissa: $1/d = 2 \sin\theta/\lambda$ (2θ, diffraction angle; λ; wavelength of X-rays (= 1.542 Å). Ordinate: intensity (bar, $2 \cdot 10^4$ counts in full length). The numbers at the left edge of the patterns are temperatures (°C) of measurement. Three sharp peaks at 0.26–0.28 $Å^{-1}$ which appear at lower temperatures are from ice. (a) Patterns of a total lipid fraction of pellicles of cells grown at 39°C. (b) Patterns of a phospholipid fraction of pellicles of cells grown at 39°C.

high angle region contain only high-angle reflections due to lipids, namely a broad 4.6 Å peak and a sharp 4.2 Å peak in most cases and only a broad 4.6 Å peak at high temperatures. For the estimation of phase transition temperature, the 4.2 Å peak was extracted from each diffraction pattern. The diffraction pattern at the highest temperature contained the pure 4.6 Å peak. Using this pattern, the 4.6 Å peak was substracted from the diffraction patterns at various temperatures. Resultant patterns included only the 4.2 Å sharp peak. Fig. 3 shows the temperature dependence of integrated intensity of the 4.2 Å peak. We defined the solid-to-fluid phase transition temperature, T_f, as the temperature at which the 4.2 Å peak disappeared by raising the temperature. Therefore T_f gave the upper limit of broad transition, i.e. the transition temperature from mixed state of solid and fluid phases to the complete fluid phase. The temperature-dependence curves of integrated intensity of 4.2 Å peaks did not saturate at lower temperatures until -15°C to -20°C except for total lipids of pellicular fraction (Fig. 3), indicating that the transition undergoes gradually, as usually seen with the lipid mixture from biological membranes.

Table I summarizes the T_f values of phospholipids from mitochondria, microsomes and pellicles of *Tetrahymena* grown at 15°C and 39°C. The T_f

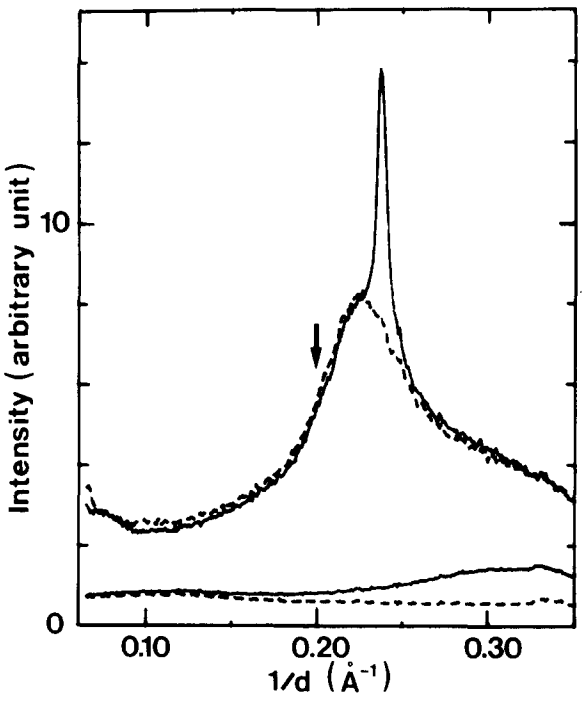

Fig. 2. Procedure of data analysis. Ordinate: intensity. Abscissa: $1/d$ (Å^{-1}). Pattern was from pellicular phospholipid of cells grown at 39°C. Upper solid line: observed intensity profile (I_{obs}). Upper dashed line: intensity profile of the same sample at the temperature higher than T_f, normalized at 0.195–0.205 Å^{-1} (arrow) to I_{obs}. Lower solid line: intensity profile of buffer solution at the same temperature with I_{obs}, normalized at 0.320–0.340 Å^{-1} (see text). Lower dashed line: parasitic scattering from optics multiplied by absorption coefficient (0.36).

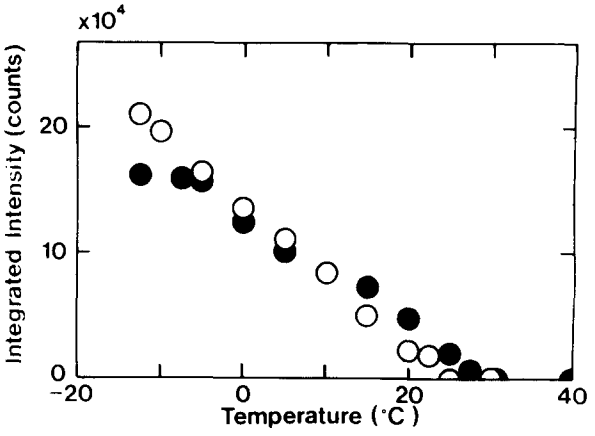

Fig. 3. Temperature dependence of the relative intensity of the 4.2 Å peak. Abcissa: measurement temperature (°C). Ordinate: integrated intensity of the 4.2 Å peak (arbitrary unit). Arrow: the temperature at which peak (arbitrary unit). Arrow: the temperature at which the 4.2 Å peak disappeared in the case of pellicular total lipid cells grown at 39°C, judged by the profiles of the patterns. ●, pellicle total lipid from 39°C-grown cells; ○, pellicle phospholipid from 39°C-grown cells.

values of all membrane fractions were lower than the temperatures at which cells had been grown, suggesting that the compositions of phospholipids and their fatty acyl chains are modified so that the membranes are in fluid phase at growth temperature. In mitochondria and microsomes, T_f values were observed at about 20 K (or degrees celsius) below the growth temperature, while the T_f value of pellicle was about 10 K below the growth temperature. The order of T_f values among the three membrane fractions was mitochondria < microsome < pellicles at both growth temperatures. This finding can be explained by difference in unsaturation of fatty acids [16] and in fluidity measured by the ESR method [12]. The temperature of phase change was obtained by electron microscopic observation as the temperature at which smooth patches devoid of intramembranous particles emerged on freeze-fractured faces of pellicles [17]: the phase change temperatures were around 25°C for cells grown at 39°C and around

TABLE I

PHASE TRANSITION TEMPERATURES OF PHOSPHOLIPIDS FROM VARIOUS *TETRAHYMENA* MEMBRANE FRACTIONS

Transition temperatures were determined by visual inspection of the profiles rather than the plots shown in Fig. 2 falling on zero. The experiments were repeated twice beginning from independent cell cultures. The values of t_f changed from time to time maximally 7 K, but all the relations between fractions were reproduced well. Data shown here are a set from one batch.

	t_f	
Growth temperature	15°C	39°C
Mitochondria	−7.0°C to −9.5°C	approx. 20.5°C
Microsomes	−4.5°C to −2.0°C	18.0°C to 20.5°C
Pellicles		
Phospholipids	approx. 5.5°C	approx. 25.5°C
Total lipids	-	28.0°C to 30.5°C

1°C for cells grown at 15°C. These temperatures obtained by electron microscopy were well compatible with those determined by X-ray diffraction analysis for pellicular phospholipids in the present study. Therefore, the transitions obtained by X-ray diffraction for lipids seem to reflect the transition of intact membranes.

X-ray diffraction of pellicular membrane lipids containing tetrahymanol

The role of tetrahymanol in *Tetrahymena* membrane is presumed to be somehow similar to that of cholesterol in biological membranes of higher animal cells, despite some structural difference in the D ring; the former lacks the aliphatic side chain at C-17 position. To compare the effects of tetrahymanol with that of cholesterol, the authors examined the X-ray patterns from two samples; total lipids of pellicles containing tetrahymanol, and the mixture of cholesterol and pellicle phospholipids in the same proportion of tetrahymanol/phospholipids as found in pellicular membranes (0.098 mol/mol phosphate [16]).

The temperature dependence of the high-angle patterns from cholesterol/phospholipids is shown in Fig. 4. By addition of cholesterol, the 4.2 Å peak at lower temperatures was broadened drastically as was observed in the case of a cholesterol/dipalmitoylphosphatidylcholine mixture [18]. This means that lateral packings of fatty acyl chains in the solid phase were disordered by addition of cholesterol, and is evidence of the dual effect of cholesterol; rigidifying the membrane at higher temperature and fluidizing it at lower temperature. Furthermore the diffracted intensity at the 0.14–0.18 $Å^{-1}$ region increased at lower temperatures, resulting in a very broad peak in this region. This change was reversed when the temperature was raised again. In the case of the tetrahymanol/phospholipid system, the 4.2 Å peak was as sharp as in pure phospholipids prepared from biological membranes, and the peak width was unaffected by the addition of tetrahymanol to the resolution of 0.0015 $Å^{-1}$ (Fig. 5). Consequently, it was suggested that tetrahymanol did not exert any perturbance in packing hydrocarbon chains in the membrane lipid bilayer. Instead, the diffraction patterns of total pellicular lipids containing tetrahymanol showed three minor sharp

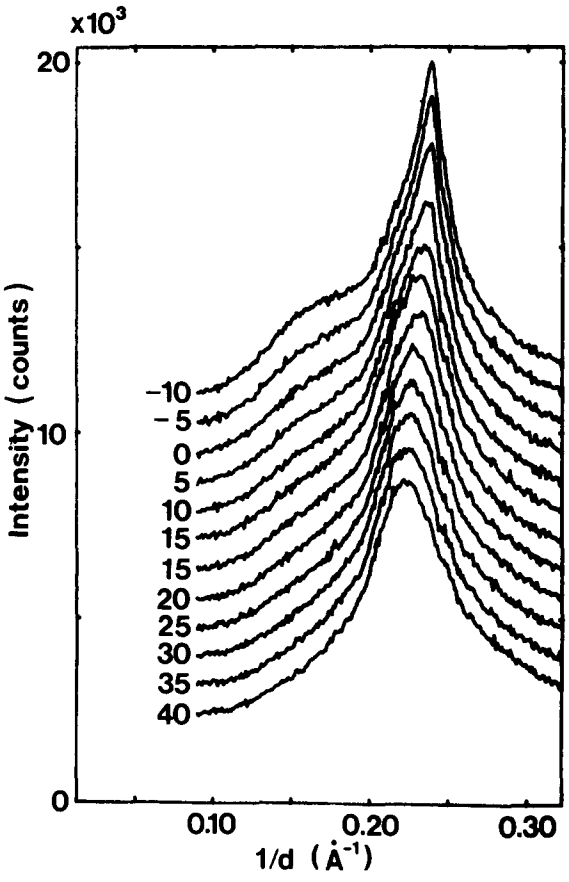

Fig. 4. Wide-angle X-ray diffraction patterns from binary mixture of cholesterol and *Tetrahymena* phospholipids. Cholesterol and pellicular phospholipids of cells grown at 39°C were mixed and swollen with twice weight of buffer solution. The numbers at the left edge of each pattern are temperatures (°C) of the sample. Scale of ordinate is for the intensity of the pattern measured at 40°C. Origins of other patterns have been shifted up with distances of 350 counts between neighbouring patterns.

peaks at the spacings between 0.14 $Å^{-1}$ and 0.18 $Å^{-1}$ in addition to the 4.2 and 4.6 Å peaks (Fig. 1a). The relative intensities of these peaks differed from sample to sample, but were independent of the temperature of measurement. These peaks may indicate an emergence of separated domains of phospholipids and tetrahymanol. However, added tetrahymanol did not seem to form a pure segregated domain, because the T_f value of the total lipid fraction was raised significantly compared to that of the phospholipids (Table I). Thus, in order to know whether any partial or sporadic separation is induced, an X-ray picture was taken for the

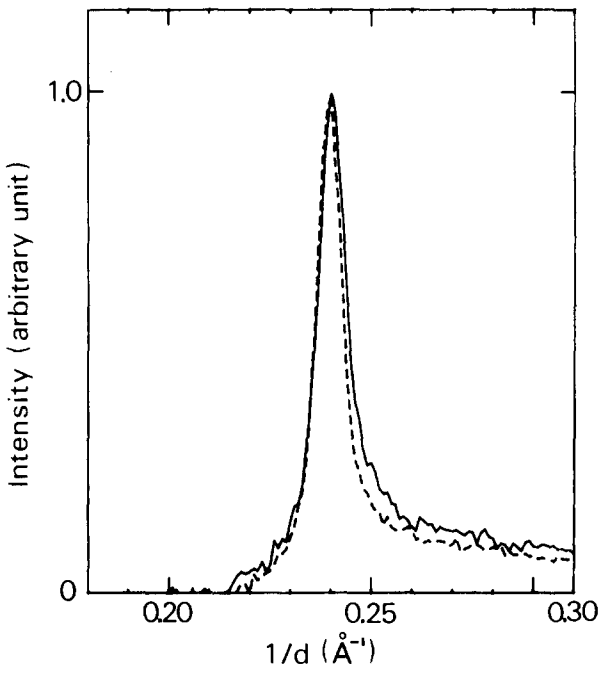

Fig. 5. Comparison of the 4.2 Å peak profiles between phospholipids and total lipids of pellicles. From the original diffraction patterns the 4.2 Å peak profile was extracted by the method explained in the text (Fig. 2). Peak profile of direct beam was not deconvoluted, but both patterns were measured under exactly the same experimental conditions.

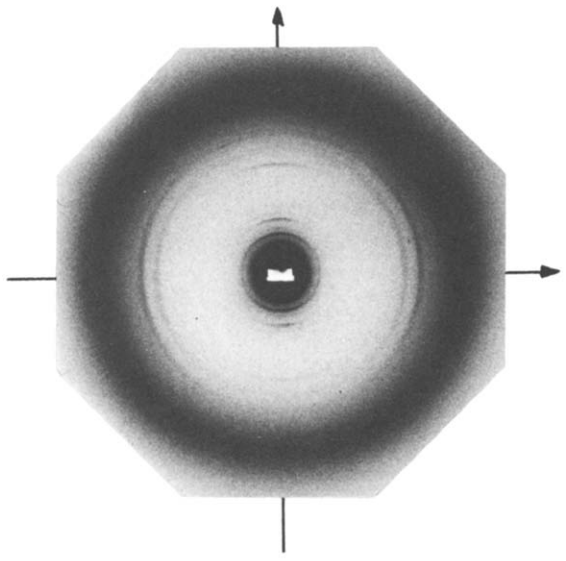

Fig. 6. X-ray diffraction picture from oriented specimen of pellicular total lipid of cells grown at 39°C. The normal of a plane on which specimen was dried and oriented is parallel to the ordinate. Distance from specimen to film was 48.6 mm. The specimen was kept at room temperature. Exposure time was 2.5 h. Order of lamellar stackings of the membrane gives sharp arcs concentrated in the direction of the ordinate, and its intramembranous order of hydrocarbon chains gives 0.225 and 0.238 $Å^{-1}$ arcs in the direction of the abscissa. Two sharp arcs can be seen at 0.160 and 0.180 $Å^{-1}$ in the direction of the abscissa (one more arc is weak and overlapped with the second).

pellicular total lipid whose molecular axes were oriented perpendicularly to the incident beam by partial drying (Fig. 6). Consequently, (i) three sharp peaks were observed at 5–6 $Å^{-1}$ in the direction parallel to the membrane, and (ii) sharp peaks at the lower angle region (0.14–0.18 $Å^{-1}$) were aligned in the direction perpendicular to the membrane. The result indicated that tetrahymanol was oriented in the membrane lipid bilayer. The lack of powder patterns, which were observed for the purified tetrahymanol (data not shown), and the absence of any peak other than the above-mentioned peaks suggested that tetrahymanol was not present in a crystalline form outside the membrane lipid bilayer. These results may provide possible evidence that cocrystallization of tetrahymanol and phospholipid would occur independently of α or β conformation of hydrocarbon chains of phospholipids.

Discussion

The present study, using X-ray diffraction, demonstrated that phase transition temperatures of various membranes of *Tetrahymena* were changed in response to cell growth temperature. The obtained results agreed with those described in the previous reports of membranes of temperature-acclimated *Tetrahymena* cells with spin probe [12,19,20], fluorescent probe [21,22] and freeze-fracture electron microscopy [17]. The increase in fluidity of membranes and membrane lipids of *Tetrahymena* cells grown at a lower temperature, which was shown in our earlier experiments, seems to correspond to the decrease of phase transition temperature observed by X-ray diffraction in the present study. The formation of the crystalline phase at 10–20 K below the growth temperature agreed with the electron micrscopic observations that intramembranous particles were aggregated

by the cooling membranes. Changes in the phase transition temperature caused by decreasing the growth temperature can be explained by alterations in lipid composition as well as changes in fluidity of the membranes. Indeed, it was shown that smooth-fractured face (intramembranous particle-free area) was mainly composed of phospholipids with saturated fatty acids [23].

The temperature-dependence curve of solid phase (Fig. 3) usually did not saturate at lower temperatures up to −15°C to −20°C, therefore the 4.2 Å peak intensities at various temperatures divided by that at the lowest temperature are the same or more than true percentage of solid phase in whole lipid at the respective temperatures. In a comparison with the percentages of particle-free area observed by electron microscopy, the increase in solid phase by lowering the temperature is drastically less in the case of estimation by the 4.2 Å peak. At 15°C, for example, 85% of the outer alveolar membrane of cells grown at 39°C are occupied by particle-free area, while the solid-phase area was only 40% of the pellicular total lipids. However, the agreement between two transition temperatures obtained by X-ray diffraction and electron microscopy indicates at least the occurrence of the solid phase as a nucleus or a core of particle-free area. By extensive studies on microsomal membranes of *Tetrahymena* Wunderlich and his colleagues proposed a hypothesis that clustering of the 'rigid' fluid (quasi-crystalline) state might be responsible for discontinuities in temperature dependence of various parameters denoting membrane physical states [9,24]. More recently, detailed analysis of the physical properties of microsomal membranes of *Tetrahymena* was done by Dickens et al. [21], using a fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). Compared to our present results with microsomal phospholipids by X-ray diffraction, it was considered that the break points in the plot of fluorescence polarization vs. temperature might account for the separation of the more 'rigid' fluid domain ('cluster' as described by Wunderlich et al. [24]) which is immiscible with the bulk lipid surrounding the domain, rather than the phase transition of liquid-crystalline to crystalline states, since the discontinuity was not seen in the boundary of solid phase but in the fluid phase or mixed state of fluid and solid phases.

Wunderlich et al. [9] observed temperature-dependent changes in relative intensities of lower angle X-ray diffraction peaks. Their 'solid' peaks at lower angle disappeared at far higher temperatures then the T_f values obtained in our experiments, and the 4.2 Å peak at higher angles disappeared still at the growth temperature. In their case the samples were directly transferred from the vacuum pump. The solid-to-fluid phase transition is generally known to shift to the higher temperatures when lipids are dried over critical concentration [15]. For example, when we directly transferred a dried sample from the vacuum pump to the sample holder without adding buffer solution, the 4.2 Å peak persisted over the growth temperature and disappeared at 55–60°C for the pellicle phospholipid of cells grown at 39°C, and by addition of twice the weight of buffer solution to this sample t_f shifted down to 27.5°C. It is thus explicable that the 4.2 Å peak for our sample with excess water disappeared at lower temperatures, compared to their experimental results. In this study the authors used samples with excess water because membranes in vivo are surrounded by excess water.

The present results of the solid to fluid phase transition are based on, if partially, existence of lamellar phase. The presence of large amounts of phosphatidylethanolamine and its phosphonate derivative, 2-aminoethylphosphonolipid in *Tetrahymena* membranes tempted us to ascertain whether its extracted lipids produce the hexagonal phase. Recently, with the use of ^{31}P-NMR, Jarrell et al. [25] and Ferguson et al. [10] have studied the phase behaviours of phospholipids with or without 2-aminoethylphosphonolipids, and of natural lipid extracts from *Tetrahymena*, indicating evidence that they had a bilayer organization below 20°C, but the single component of either 2-aminoethylphosphonolipid or phosphatidylethanolamine alone showed a tendency to form hexagonal structure. For phospholipid and total lipid from each fraction, the authors observed at least one series of sharp peaks at lower angle region at all the temperatures (−20°C to 35°C), which could be indexed as lamellar pattern series. It permits us to discuss about solid-to-fluid thermal transition in lipid bilayer.

Tetrahymanol was found to raise the phase-

transition temperature of pellicular membrane lipids. In our earlier studies, the effect of tetrahymanol on phospholipids was examined by spin labeling. This sterol-like membrane component was shown to exert a decreasing influence on fluidity and a dual effects on phase transition [12]. Hence tetrahymanol is considered to have similar effects as cholesterol on the physical properties of membranes. Further extensive investigations deserve to be preceeded for the better understanding of the true role of tetrahymanol in the surface membrane of *Tetrahymena*.

Recently, Funk et al. [26] reported that the 4.2 Å reflection on microsomal membrane isolated from *Tetrahymena* grown at 28°C showed 'break point' at approx. 18°C.

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