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STUDIES ON *TETRAHYMENA* MEMBRANES:

TEMPERATURE-INDUCED ALTERATIONS IN FATTY ACID COMPOSITION OF VARIOUS MEMBRANE FRACTIONS IN *TETRAHYMENA PYRIFORMIS* AND ITS EFFECT ON MEMBRANE FLUIDITY AS INFERRED BY SPIN-LABEL STUDY

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SUMMARY

The fatty acid distribution pattern of lipids extracted from different subcellular components of *Tetrahymena pyriformis* was found to be significantly different from one type of membrane to another.

The growth-temperature shift caused alterations in fatty acid composition. The ratio of palmitoleic to palmitic acid, especially, showed a sharp linear decline with increase of temperature in all of the membrane fractions.

The spin labels were rapidly incorporated into *Tetrahymena* membranes. The order parameter of 5-nitroxide stearate spin label incorporated into various membrane fractions was found to be different for the different membrane fractions, suggesting the following order of the fluidity; microsomes > pellicles > cilia.

The fluidity of the surface membranes, cilia and pellicles isolated from *Tetrahymena* cells grown at 15 °C was noticeably higher than that of the membranes from cells grown at 34 °C but was not so different with microsomal fractions.

The motion of the spin label in the pellicular membrane was more restricted than in its extracted lipids, thus indicating the assumption that in *Tetrahymena* membranes the proteins influence the fluidity.

It was also suggested that a sterol-like triterpenoid compound, tetrahymanol, which is principally localized in the surface membranes, would be involved in the membrane fluidity.

INTRODUCTION

There is now substantial evidence that lipids, especially phospholipids, are essential structural components in the biological membranes and the distribution of lipids is not uniform among various membranes but different from one type of membrane to another (See, for example, the reviews by Korn [1] and Finean [2]). This may suggest that the lipids must have the functional roles in biomembranes. In fact,

there are some recent reports indicating that the "fluidity" of membrane lipids is closely associated with the enzyme activities [3–9]. Also, it is generally assumed that the phase transition for the biological membranes may reflect the transition in the fatty acid portion of the membrane phospholipids, suggesting that the hydrocarbon chains would be one of the principal factors which are involved in regulating the membrane fluidity [10]. In recent years, a variety of physico-chemical means have become available for detecting the membrane organization such as X-ray diffraction, infrared spectroscopy, NMR, thermal analysis and fluorescence. In addition, since 1968 the powerful physical technique of electron spin resonance (ESR) spectroscopy has been introduced and applied to membranes first by Hubbell and McConnell [11], there have been a number of studies by ESR on biological membranes such as erythrocyte [12], *Neurospora* [13], *Saccharomyces* [14], *Mycoplasma* [15], sarcoplasmic reticulum [16], etc. which have been reviewed recently by Keith et al. [17].

We chose *Tetrahymena pyriformis* as a useful model system for the ESR studies regarding effects of lipid composition on the membrane fluidity, because the *Tetrahymena* cells can be spin labeled quite rapidly and easily compared with other cell systems, together with some other advantages such as rapid bacterial-like growth and the presence of highly developed subcellular organelles similar to mammalian cells. On the other hand, from earlier studies [18–22] we have obtained much interesting information about biosynthesis and lipid composition of *Tetrahymena* membranes and showed that 2-aminoethylphosphonolipids, glyceryl ether-containing phospholipids and a sterol-like compound, tetrahymanol, are chiefly localized in the surface membranes. However, the biological significance of these specific lipids in membranes still remains unclear.

As a first step towards alteration of lipid compositions in *Tetrahymena* membranes, we made efforts to change the fatty acid composition of membrane lipids. For this purpose, two methods are available; (1) supplementation of exogeneous fatty acid and (2) growth-temperature shift. In this study Method 2 was used. It is well known that for a variety of microorganisms [24–28] the shift of growth temperature induces alteration in fatty acid composition and, in general, as the growth temperature decreases the degree of unsaturation of fatty acids increases. We found that the lipids of *Tetrahymena* membranes isolated from cells grown at higher temperature (34 °C) contain a greater percentage of saturated fatty acids than membrane lipids from cells grown at lower temperature (15 °C).

Subsequently, we measured the ESR spectra of various membrane fractions labeled with 5-nitroxide stearate and estimated the order parameter of the spin label incorporated into the membranes. The results from these studies demonstrate that the fluidity varies from one membrane to another and that the motion of the spin-label is less immobilised in the membranes from 15 °C cells than in the membranes from 34 °C cells; "cold" membranes are more fluid than "hot" membranes.

In addition, a possible involvement of tetrahymanol in the ordering or disordering of membrane lipids was also suggested.

MATERIALS AND METHODS

Growth of T. pyriformis cells

T. pyriformis, WH-14 was grown at 25 °C in an enriched proteose–peptone

medium as previously described [18]. Cultures of 200 ml were harvested after 37–38 h when the cells reached the logarithmic phase of growth. For the temperature-shift experiment the cells grown at 25 °C for 24 h were transferred to the shakers set at 34 °C or 15 °C.

Isolation of membrane fractions from T. pyriformis

Various subcellular fractions such as cilia, pellicles, mitochondria, microsomes and post-microsomal supernatant were isolated according to the method using a high-phosphate buffer (0.2 M K_2HPO_4–0.2 M KH_2PO_4–3 mM EDTA–0.1 M NaCl, pH 7.2) as described in the previous paper [18].

Lipid extraction and analysis

Lipids were extracted from individual membrane fractions by the method of Bligh and Dyer [29], and the resultant lipid solutions were stored in chloroform–methanol (6 : 1 v/v) under −20 °C. Phospholipid phosphorus was determined by the method of Bartlett [30] modified by digestion with 70 % perchloric acid according to Marinetti [31].

For analysis of fatty acids by gas–liquid chromatography, methyl esters were prepared by interesterification of lipid samples for 4 h at 100 °C with 5 % anhydrous HCl–methanol, then extracted with petroleum ether and examined with a JEOL Model JGC-1100 gas chromatograph. The samples were injected onto a 200 × 3 cm glass column packed with 15 % diethyleneglycolsuccinate supported on Chromosorb W, 80–100 mesh (Gaschro Kogyo Co., Tokyo). Column temperature was 185 °C and the pressure of carrier gas, N_2, was 0.5 kg/cm^2. Peaks of individual fatty acids were quantified by triangulation and identified by comparison of retention times with those of authentic standards.

Quantitative analysis of tetrahymanol was carried out essentially following the method described previously [23].

ESR spectroscopy

The label, *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid was synthesized according to the procedure of Waggoner et al. [32]. For spin labeling of whole cells or different subcellular membrane fractions, each sample was transferred to a small test tube, the bottoms of which had been coated with a thin film of the spin label, and incubated at 29 °C for 10 min. For preparation of spin-labeled lipid dispersion, lipid extracts in chloroform–methanol were mixed with spin label in ethanol and solvents were evaporated under a stream of N_2. To this mixture small amounts of a Tris buffer (50 mM Tris–HCl–150 mM NaCl, pH 7.5) were added. The mixture was subsequently sonicated for 10 min with a T-A-4201 sonifier (Kaijo Denki Co., Tokyo).

The spin-labeled membranes and extracted lipid dispersions were taken into a cylindrical quartz tube or glass capillary tube and their ESR spectra were measured at various temperatures using a commercial X-band spectrometer (JEOL ME-2X) equipped with a variable temperature control. All the spectra can be well interpreted with an axially symmetrical spin Hamiltonian. The parallel ($T_{\parallel}$) and perpendicular ($T_{\perp}$) principal values of the hyperfine tensor were estimated from the spectra and the order parameter S was calculated using the relation $S = (T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx})$ where T_{zz} (32.9 G) and T_{xx} (5.9 G) are the hyperfine principal values of the nitroxide radical.

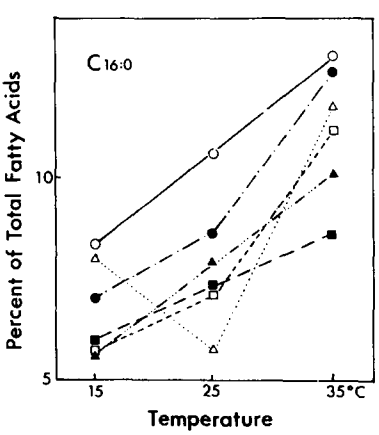
The order parameter measures the mean value of $(3\cos^2\theta - 1)/2$ where θ is the angle between one of the principal axes of the nitroxide moiety and the axis of the axial symmetry. The smaller S becomes the greater the spread in θ or the amplitude of rapid anisotropic motion. In the present paper, we use the order parameter of the fatty acid spin label incorporated into the membranes for the discussion of the fluidity.

RESULTS

Alteration in fatty acid composition by temperature shift

It is well established that numerous microorganisms change fatty acid composition with growth temperature [24–28]. The ciliate *Tetrahymena* cells have also been known to induce alteration in fatty acid composition in response to temperature [33, 34]. However, no information has been available about fatty acid changes of the lipids from different *Tetrahymena* membrane fractions. Table I summarizes the fatty acid composition of the lipids of various subcellular components isolated from cells grown at the different growth temperatures. It demonstrates that the fatty acid pattern of the non-membraneous fraction, the post-microsomal supernatant, was found to be considerably different from that of other membrane fractions and that there are some significant variations in the fatty acid distribution between various membrane fractions with different biological functions. It is also shown that the major unsaturated fatty acids are oleic, linoleic and γ-linolenic acids while myristic and palmitic acids are present as the main saturated acids. Also, it appears that in the fractions, except for cilia, as the growth temperature increases the ratio of unsaturated to saturated fatty acids decreases.

The percentage of the major saturated fatty acid, palmitic acid ($C_{16:0}$) increases with the rise in growth temperature (Fig. 1) while a concurrent decrease of



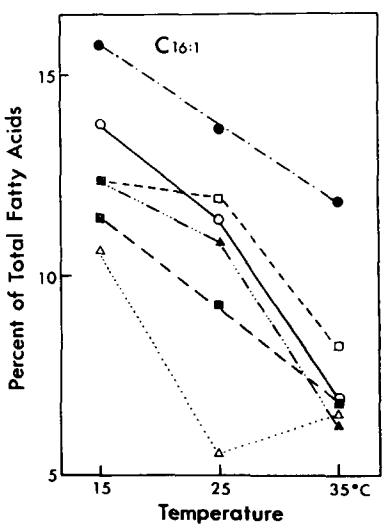


Fig. 1. Changes in the percentage of the principal saturated fatty acid, palmitic acid ($C_{16:0}$) in total lipids extracted from different *Tetrahymena* membrane fractions. ▲, whole cells; △, cilia; ○, pellicles; ■, mitochondria; □, microsomes; ●, post-microsomal supernatant.

Fig. 2. Changes in the percentage of palmitoleic acid ($C_{16:1}$) in total lipids of different membrane fractions. The symbols are the same as used in Fig. 1.

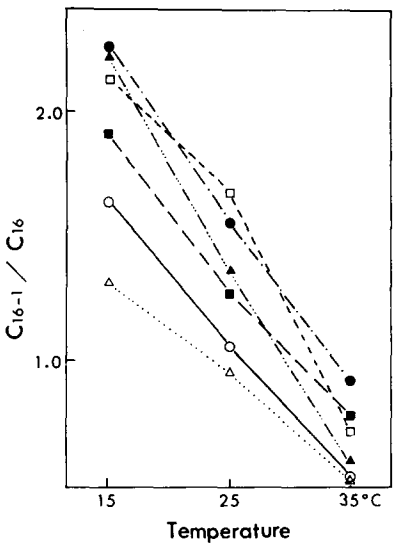
TABLE I

FATTY ACID COMPOSITION IN LIPIDS OF WHOLE CELLS AND SUBCELLULAR FRACTIONS FROM *T. PYRIFORMIS*

The cells grown at 25 °C for 24 h were transferred to 15 °C or 34 °C for the temperature shift. All membrane fractions were isolated according to Nozawa & Thompson's procedure [18]. Total lipids were extracted by the method of Bligh and Dyer [29], saponified and analysed by gas–liquid chromatography. Each figure, expressed as wt %, is the average of 3–4 experiments.

Fatty acids	Percentage fatty acid composition in total lipids of:																		
	Whole cells			Cilia			Pellicles			Mitochondria			Microsomes			Post-microsomal supernatant		
(°C):	15	25	34	15	25	34	15	25	34	15	25	34	15	25	34	15	25	34
$C_{12:0}$	1.8	1.9	2.7	1.2	0.8	1.7	2.8	3.1	3.3	1.2	1.2	1.8	1.4	1.5	1.8	2.3	2.0	5.0
$C_{14:0}$	4.9	5.2	5.6	4.9	3.1	4.0	6.4	6.5	6.0	4.0	4.0	4.4	5.2	5.2	6.0	6.0	6.4	8.0
$C_{16:0}$	5.5	7.9	10.2	8.0	5.7	11.8	8.3	10.6	13.0	5.9	7.3	8.6	5.7	7.1	11.2	7.0	8.6	12.6
$C_{16:1}$	12.6	10.8	6.2	10.6	5.5	6.5	13.8	11.4	6.9	11.4	9.2	6.7	12.3	11.9	8.2	15.7	13.6	11.8
$C_{18:0}$	0.7	0.6	2.7	1.4	0.8	3.3	0.8	0.5	1.8	0.7	0.8	2.8	0.7	0.8	1.5	1.2	1.6	9.3
$C_{18:1}$	7.6	8.6	13.1	7.6	6.1	11.0	8.9	8.6	14.0	8.0	7.1	10.9	7.4	7.9	13.2	11.7	11.5	15.1
$C_{18:2}$	19.9	19.8	17.7	16.0	16.6	16.1	16.0	16.8	14.9	20.2	21.4	20.7	20.4	21.0	17.5	16.5	18.0	11.8
$C_{18:3}$	32.2	33.6	30.2	31.0	43.2	30.0	26.0	29.2	25.4	34.6	38.3	36.2	33.6	34.1	28.4	24.2	26.0	14.4
$\Sigma U/\Sigma S$	5.6	4.7	3.2	4.2	6.9	3.2	3.5	3.3	2.5	6.3	5.7	4.2	5.7	5.4	3.2	4.1	3.7	1.5

palmitoleic acid ($C_{16:1}$) occurs (Fig. 2), thus producing a drastic linear decline of the ratio of palmitoleic to palmitic acid in all membrane fractions depending upon the temperature rise (Fig. 3). In contrast, the levels of two principal unsaturated acids,



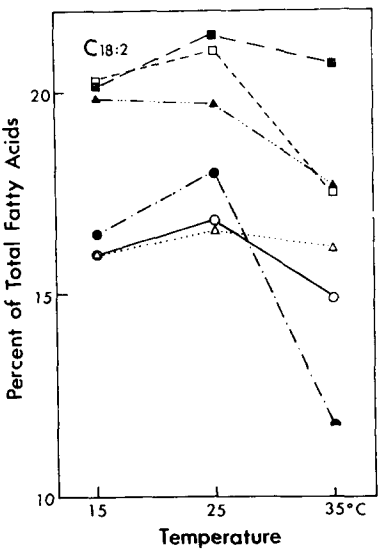
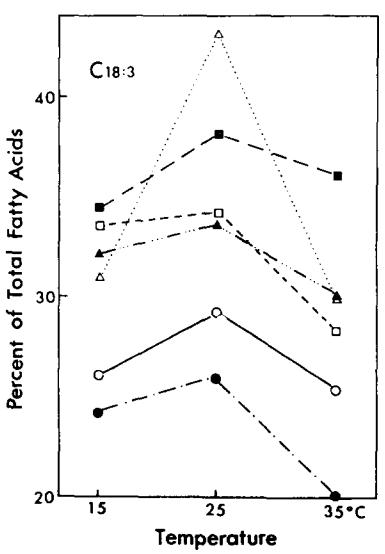


Fig. 3. Changes in the ratio of palmitoleic to palmitic acid in different membranes. The symbols are the same as used in Fig. 1.

Fig. 4. Changes in the percentage of linoleic acid ($C_{18:2}$) in total lipids of different membranes. The symbols are the same as used in Fig. 1.



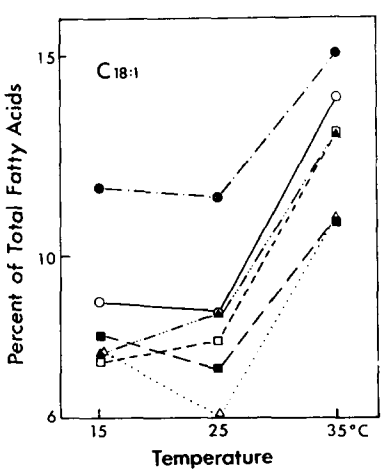


Fig. 5. Changes in the percentage of the most abundant unsaturated fatty acid, γ-linolenic acid ($C_{18:3}$) in different membranes. The symbols are the same as used in Fig. 1.

Fig. 6. Changes in the percentage of oleic acid ($C_{18:1}$) in total lipids of different membranes. The symbols are the same as used in Fig. 1.

linoleic ($C_{18:2}$) and γ-linolenic ($C_{18:3}$) acids, although varying with membrane fractions, are not affected to a marked extent by changing temperature (Figs 4 and 5), except that the percentage of γ-linoleic acid in the cilia is tremendously high at 25 °C and low equally both at 15 °C and 34 °C. The oleic acid ($C_{18:1}$) level remains fairly constant at 15 °C and 25 °C but unexpectedly increases at 34 °C (Fig. 6). This unusual trend was also seen with *Candida* cells [25]. From the data presented here it may be concluded that two fatty acids of palmitic and palmitoleic acids, which show the striking temperature dependency, would be principally involved in adapting *Tetrahymena* membranes to the environmental temperature changes.

TABLE II

LIPID COMPOSITION OF *T. PYRIFORMIS* WHOLE CELLS AT THE DIFFERENT GROWTH TEMPERATURES

The cells were grown at 25 °C for 24 h and then transferred to 34 °C or 15 °C for the temperature shift. Lipids were extracted according to the method of Bligh and Dyer [29]. Tetrahymanol was analysed by gas–liquid chromatography and individual phospholipids were separated on silica gel G thin-layer chromatographic plate. Each figure is the average of 3–4 experiments

Growth temperature (°C)	Tetrahymanol (μg/μg PL P)	Molar ratio (Tetrahymanol/PL P)	Mole % of total phospholipids					
			Lyso-PC	PC	Lyso-AEPL and LysoPE	PE	AEPL	CL
15	0.974	0.070	2.1	27.9	1.8	31.2	32.2	3.2
25	1.050	0.076	1.7	25.0	1.4	34.5	30.0	6.1
34	0.995	0.072	2.7	28.1	1.3	34.2	29.4	3.8

Abbreviations: PL: phospholipids; PC, phosphatidylcholine; AEPL, 2-aminoethylphosphonolipids; PE, Phosphatidylethanolamine; CL, cardiolipin.

In contrast, other lipid compositions of *Tetrahymena* cells do not apparently change and the relative proportions of phospholipids are not affected significantly by changing the growth temperature (Table II) indicating that the fatty acyl chains in phospholipids would play the key role in regulating the fluidity of *Tetrahymena* membranes.

ESR spectroscopy

ESR spectra of various subcellular membrane fractions labeled with 5-nitroxide stearate are shown in Fig. 7. These spectra are indicative of radicals performing a rapid anisotropic motion and the parallel and perpendicular hyperfine principal values are measured as indicated in the figure. The spectral shapes were alike and only the hyperfine splittings were different among the different membrane fractions. A considerably rapid decay, which might be due to reduction of nitroxide moiety, was observed with the mitochondrial fraction. Therefore, this fraction was not available for further experiments which require a long time for measurements. However, it is shown that the *Tetrahymena* membrane system provides a potentially useful model for spin-label studies.

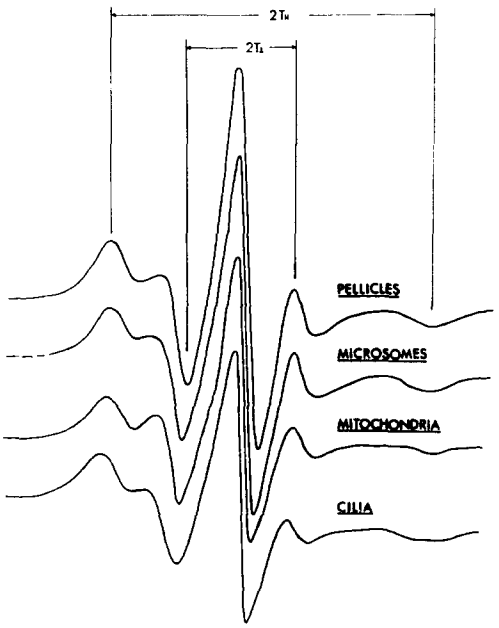


Fig. 7. The ESR spectra of different *Tetrahymena* membrane fractions labeled with 5-nitroxide stearate. The membrane fractions were isolated from cells grown at 25 °C. All spectra measured at 22 °C.

The temperature dependence was investigated for all of the membrane fractions. A representative spectral change in response to temperature is presented in Fig. 8, in which the distance $2T_{\parallel}$ decreases gradually with the rise in temperature.

For comparison of the fluidities of various membrane fractions isolated from cells grown at 25 °C, the order parameters were plotted in Fig. 9 as a function of

TABLE III

LIPID COMPOSITION OF SOME MEMBRANE FRACTIONS FROM *T. PYRIFORMIS* CELLS

	Cilia	Pellicles	Microsomes
Tetrahymanol* (μg/μg of phospholipid phosphorus)	4.164	1.175	0.481
Glyceryl ether* (moles/100 moles of phospholipid phosphorus)	52.6	32.8	18.3
Phospholipids** (%)			
Lysophosphatidylcholine	1	5	1
Phosphatidylcholine	28	25	35
Lysophosphatidylethanolamine	9	3	3
Phosphatidylethanolamine	11	34	34
2-Aminoethylphosphonolipids	47	30	23
Cardiolipin	1	2	1

* Data of Thompson et al. [23].

** Data of Nozawa and Thompson [18].

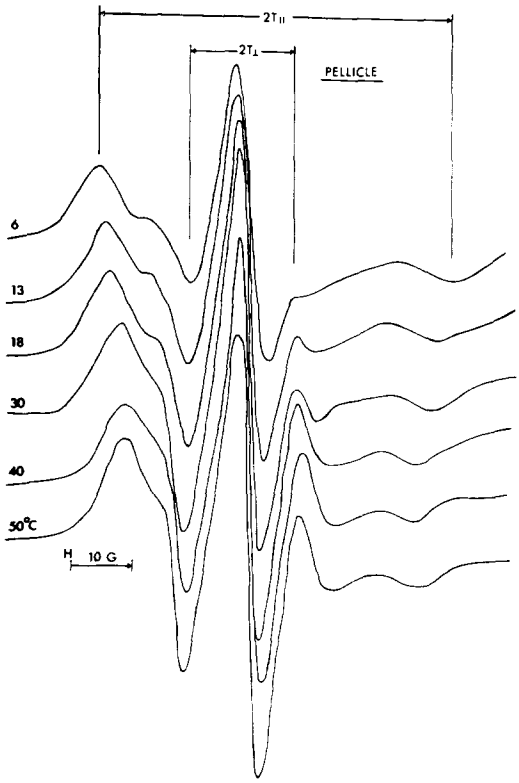


Fig. 8. Temperature dependence of the ESR spectra of 5-nitroxide stearate label incorporated in the pellicular membrane.

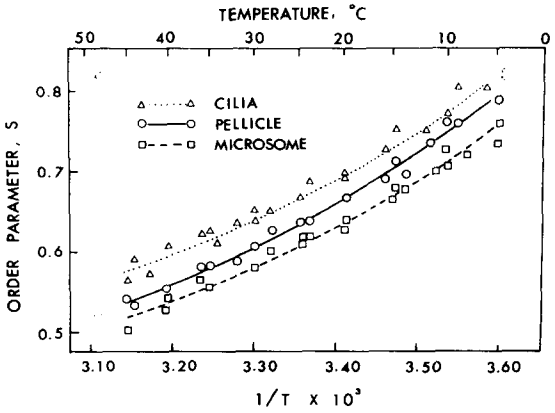


Fig. 9. The order parameter of 5-nitroxide stearate label in various *Tetrahymena* membranes as a function of the reciprocal of the absolute temperature. Membrane fractions were isolated from cells grown at 25 °C.

temperature. It is shown that there are significant differences in the fluidity between the membranes in the temperature range; they are more fluid in the order of micro-

somes > pellicles > cilia. It would be of great interest to note that various membranes with different biological functions have their own proper fluidities. On the other hand, the functionally distinct membranes within a particular cell have been

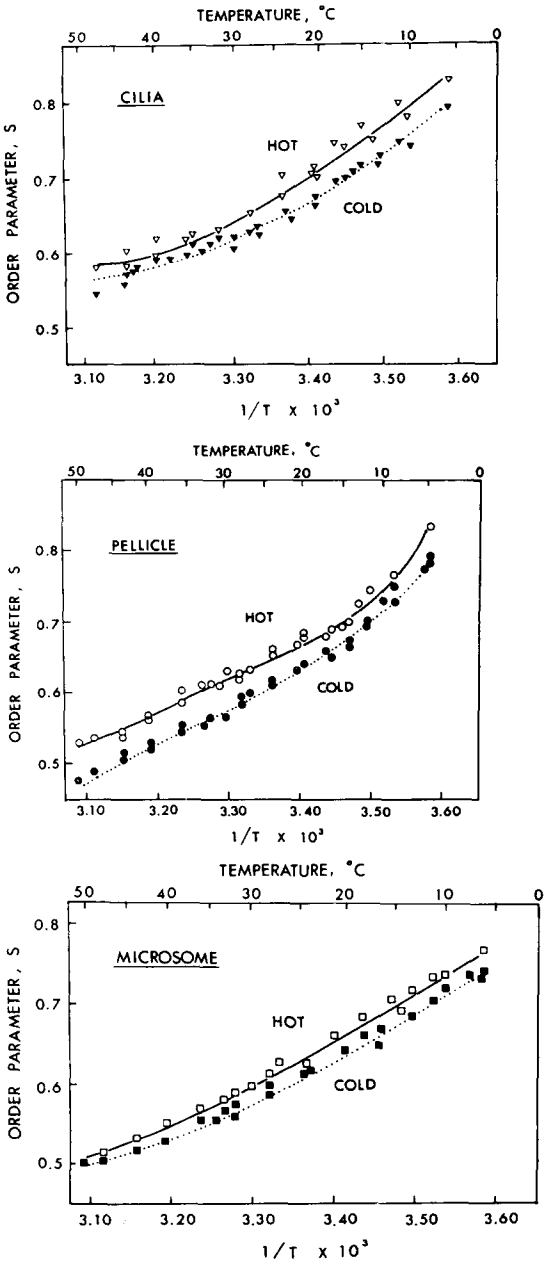


Fig. 10. The order parameter of 5-nitroxide stearate label incorporated in cilia (a), pellicles (b) and microsomes (c). The hot or cold membrane was isolated from cells grown at the shifted temperatures of 34 °C or 15 °C.

known to show different lipid compositions. In fact, we have reported earlier that the localization of certain lipids is striking in particular membrane fractions of *Tetrahymena* cells (Table III). It is noteworthy that the content of a sterol-like triterpene, tetrahymanol, becomes higher in such order as cilia > pellicles > microsomes and the membranes become less fluid following the same order. The effects of tetrahymanol on the membrane fluidity will be discussed later.

From the results of the temperature-shift experiments presented in Fig. 10 a, b and c, it appears that, in general, the membranes isolated from cells grown at 15 °C are more fluid than those from cells at 34 °C. This might be related to temperature-induced alterations in fatty acid composition as shown in Table I. The surface-membrane pellicles show greater difference in the order parameter depending on the growth temperature than do cilia and microsomes. This might lead us to think that the pellicle membrane must be fairly flexible in adjusting the fluidity to the environmental changes.

As a comparison between the membranes and the extracted lipid dispersions, Fig. 11 shows the temperature dependence of the order parameter for the pellicles.

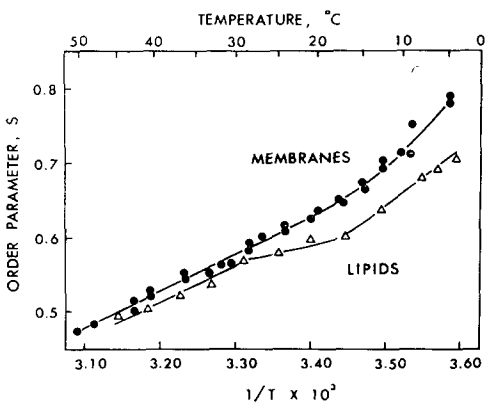


Fig. 11. The order parameter of 5-nitroxide stearate label in the pellicle membrane and in aqueous dispersion of lipids extracted from the membrane.

Interestingly, there is a definite difference in the freedom of motion of the spin label, especially at lower temperatures < 28 °C. This difference may be most likely due to packing effects by membrane proteins, as would be compatible with the results of *Mycoplasma* [15, 35] and *Halobacterium* [36] membranes.

The question arises whether tetrahymanol is involved in the membrane fluidity or not. As a preliminary experiment to clarify this question, we have measured the order parameters for the dispersions of the total lipids containing tetrahymanol (0.076 mole/1 mole phospholipid phosphorus) and of the phospholipids extracted from *Tetrahymena* whole cells (Fig. 12). It appears that the tetrahymanol-containing lipid dispersion is more and less fluid at temperatures below and above the phase transition point 27 °C. Hence, this result would suggest that in *Tetrahymena* membranes tetrahymanol may have the "dual" effects as observed with cholesterol [37–39]; rigidifying at higher temperature and fluidizing at lower temperature.

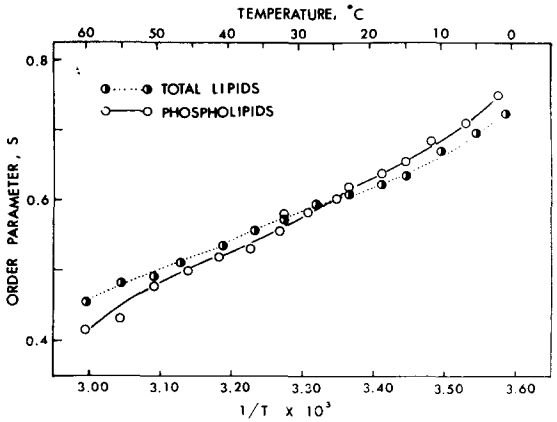


Fig. 12. The order parameter for the dispersions of spin-labeled total lipids and phospholipids extracted from *Tetrahymena* whole cells grown at 25 °C. Total lipid dispersion contains tetrahymanol at a molar ratio of 0.076 (tetrahymanol/phospholipid phosphorus). Phospholipids were separated from total lipids by silicic acid column chromatography.

DISCUSSION

In the present studies we have shown that alterations in the fatty acid composition of *Tetrahymena* membranes occur by changing the environmental temperature. Since several lines of evidence are presented to show that a variety of microorganisms change the fatty acid composition with the growth temperature [24–28], the ciliated protozoan *Tetrahymena* is no exception. However, all of the studies have been performed with the whole cells and no information is available for fatty acid changes in different membranes within a particular cell.

In general, the decrease of growth temperature results in the higher degree of unsaturation of fatty acids. In fact, we have found that in *Tetrahymena* membranes the level of palmitic and palmitoleic acid changed remarkably with the temperature; decrease of palmitic acid with a corresponding increase of palmitoleic acid at 15 °C and vice versa at 34 °C (Fig. 1). Although several possible explanations have been available for the mechanism of the temperature-induced change in fatty acid composition, little is as yet understood. It might be possible for different desaturases with different temperature optima to be involved in synthesizing different unsaturated fatty acids. In addition, recently the direct desaturation mechanism for fatty acyl chains in the intact phospholipid molecule has been demonstrated [40–42]. Pugh & Kates indicated that *Candida lipolytica* contains a membrane-bound desaturation system capable of desaturating directly phospholipids [41].

On the other hand, numerous observations have been established which demonstrate the profound effect of hydrocarbon chains on the physical state or the fluidity of the membrane lipid phase which is known to be closely associated with the membrane functions. For example, the results of several experiments indicated that fatty acids affect markedly some enzyme activities such as glycerol-3-phosphate acyltransferase [8], Na^+, K^+ATPase [4], Mg^{2+}ATPase [43], phosphorylcholine-glyceride transferase [44] etc. Recently, Tsukagoshi and Fox [45] observed in the experiments using

an unsaturated fatty acid auxotroph of *E. coli* that β-galactoside transport is primarily influenced by the average fatty acid composition of the membrane phospholipids, and they also concluded that this transport system is likely to be associated with the coordinated insertion of newly synthesized lipids and proteins.

In addition to fatty acid chains, some other factors are known to affect the physical state of biomembranes. Although sterol compounds, e.g. cholesterol in mammalians or ergosterol in fungi, are the major constituents of membrane lipids and are found to influence activities of some membrane-bound enzymes [46–48], their physiological roles are not well clarified at this stage. However, it should be noted that cholesterol has the dual function on phospholipids of being liquidified below the phase-transition temperature phospholipid layers and rigidified above the transition point [37]. Therefore, one may consider that sterol molecules would act as a membrane stabilizer to keep the fluidity proper. Our preliminary data presented in this investigation would suggest a similar effect of tetrahymanol on *Tetrahymena* membrane lipids (Fig. 12). But further detailed experiments are necessary for a better understanding of the physiological role of tetrahymanol in membranes.

Since the membrane lipids have been known to be closely related with membrane functions, it would not now be unreasonable to suppose that the functional lipids are present in biological membranes, though, up to this date, lipids have been exclusively thought to be structural components. Furthermore, according to some recent studies of organization of lipids in membranes, it is shown that the heterogeneous or patch-like distribution [49, 50] and the lateral phase separations [51–54] of lipids would be relevant for maintaining the appropriate functions in biological membranes. Shimshick and McConnell [52] have pointed out that the lateral phase separations could facilitate the insertion of newly synthesized membrane components without changing the original membrane area. In the earlier studies we have shown that certain specific lipids such as 2-aminoethylphosphonolipids and tetrahymanol are primarily localized in the particular membrane fractions, cilia and pellicles of *Tetrahymena* cells. Also the experimental results obtained from the present study have demonstrated that the *Tetrahymena* membrane system is convenient for study by spin labels. Therefore it will be hopeful to obtain much useful information about relationships of these specific membrane lipids with the fluidity and the function of various membranes in the *Tetrahymena* cell.

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