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AGE-DEPENDENT MODIFICATIONS IN MEMBRANE LIPIDS

LIPID COMPOSITION, FLUIDITY AND PALMITOYL-CoA DESATURASE IN *TETRAHYMENA* MEMBRANES

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Summary

The membrane lipid composition of *Tetrahymena pyriformis* NT-I was observed to change in a manner markedly dependent on the progress of culture age. The pellicular, mitochondrial and microsomal membranes were isolated from cells harvested at various growth phases (I, early exponential; II, mid-exponential; III, late exponential; IV, early stationary; V, late stationary) and their lipid composition was analyzed by thin-layer and gas-liquid chromatography. Although the phospholipid composition varied somewhat among membrane fractions, the most general age-dependent alteration was a considerable decrease in the content of phosphatidylethanolamine accompanied by a small increase in phosphatidylcholine. The 2-aminoethylphosphonolipid, enriched in the surface membrane pellicle, did not undergo a consistent change. As for fatty acid composition the most notable variation occurred in unsaturated fatty acids; a great increase in oleic and linoleic acids and a compensatory decrease in palmitoleic acid. This resulted in an augmented unsaturation of the overall phospholipid fatty acid profile of the aged membranes.

The age-associated drastic decline in the palmitoleic acid content in membrane phospholipids could be accounted for by the markedly lowered activity of palmitoyl-CoA desaturase. The microsomes from the early exponential phase cells possess a 4-fold higher activity of the desaturase as compared to that of the late stationary phase microsomes. The decreased desaturase activity associated with the culture age was also reflected in the corresponding decrease in the conversion rate of [^{14}C]palmitate to [^{14}C]palmitoleate in cells labelled in vivo.

The ESR spectra of the spin-labeled phospholipids extracted from the

pellicular and microsomal membranes have led to the suggestion that these types of membrane would become more fluid with the age of growth.

Introduction

Aging or senescence is a ubiquitous biological phenomenon of living organisms and a variety of altered biochemical properties of tissues or cells have been observed to be associated with progressing age. However, the molecular mechanisms by which cellular aging is caused remain poorly understood, despite numerous proposed hypotheses in the past several years. Recently, some attempts have been made to interpret the age-dependent deteriorations in cell functions in view of the case of membrane lipids. For example, in *Mycoplasma* [1—3] and plants [4—6], the lipid composition and physical properties of their aging membranes were examined in detail, and the results provided evidence that profound modifications occurred in the quantitative profile and the fluidity of membrane lipids. A free-living eukaryote, *Tetrahymena pyriformis* possesses several advantages which can be used: for example, bacteria-like rapid growth, the presence of well defined organelles, ease of isolating different membranes and prompt metabolic response to environmental changes, and therefore has been employed as an excellent model cell for various membrane studies [7,8].

In the present study we have examined the lipid composition and physical states of the pellicular, mitochondrial and microsomal membranes, which were isolated from cells harvested at different growth phases. The data showed that considerable changes in phospholipid polar head (decrease in phosphatidylethanolamine) as well as fatty acyl (increase in linoleic acid with concomitant decrease in palmitoleic acid) group composition was accompanied with advancing age of culture, and also that the fluidities as indicated by decreasing order parameters were greater in the aged membranes.

Materials and Methods

Growth of cells. A thermotolerant strain of *T. pyriformis* NT-I was grown at 34°C in an enriched proteose/peptone medium in a shaking incubator (90 cycles/min) as described previously [9], until the cells reached the desired phase of growth. Cell growth was monitored by counting cell number using a hemocytometer. Special care was taken to obtain a reproducible growth curve by maintaining consistently the same culture conditions.

Isolation of various membrane fractions. Cultures of 200-ml were harvested at different growth stages, and subcellular membrane fractions, pellicles, mitochondria and microsomes were isolated according to the procedure of Nozawa and Thompson [10]. The harvested cells were washed in a potassium phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/2 mM EDTA/0.1 M NaCl, pH 7.2), and homogenized in a hand glass homogenizer. The pellicle ghosts were spun down at $1020 \times g$ for 5 min by centrifugation of the homogenate, and then purified by discontinuous density gradient centrifugation in sucrose (0.34, 1.0 and 1.72 M). Mitochondria and microsomes were prepared from the

particulate fraction by differential centrifugation at 19 600 × *g* for 20 min and at 105 000 × *g* for 60 min, respectively.

Lipid extraction and analysis. Total lipids were extracted from isolated membrane fractions according to the method of Blight and Dyer [11] with cloroform/methanol. Phospholipid phosphorus content was determined by using the method of Bartlett [12] with the modification of decomposition in 70% perchloric acid as described by Marinetti [13]. Individual phospholipids were separated on silica gel H thin-layer plate. After charring the developed plate with 50% H_2SO_4, the areas corresponding to individual phospholipids were scraped off the plate and their phosphorus content was determined by using the method of Rouser et al. [14]. The phospholipid fraction was separated from neutral lipids by silicic acid-Hyflo Super Cel column chromatography. The neutral lipids were eluted first using $CHCl_3$, and phospholipids were then eluted with $CHCl_3/CH_3OH$ (1 : 1, v/v) followed by $CHCl_3/CH_3OH$ (1 : 9, v/v). Quantitative analysis of fatty acids was carried out by using gas-liquid chromatography as previously described [9].

Radioisotopic labeling. For determination of the rate of in vivo desaturation of palmitate, cells were labeled with [1-^{14}C]palmitic acid. 30-ml aliquots of the growth culture were taken at different intervals during aging and labeled with 0.25 μCi [1-^{14}C]palmitate for 20 min before lipid extraction. In order to investigate the distribution of ^{14}C radioactivity in individual fatty acid radioactive total lipids and phospholipids were methylated with BF_3/CH_3OH and the methylesters separated by gas-liquid chromatography were collected into glass tubes (5 × 150 mm) with two open ends and eluted from the tubes with toluene into glass vials. The radioactivity was then measured in a liquid scintillation spectrometer using a toluene liquor [15]. The recovery of the eluted radioactivity was over 95%.

Assay of palmitoyl-CoA desaturase. Desaturation of [1-^{14}C]palmitoyl-CoA was determined as previously described [16]. The composition of 1 ml standard reaction mixture was 40 μM [1-^{14}C]palmitoyl-CoA (1 Ci/mol)/0.1 mM NADH/0.7 mg microsomal protein in 0.1 M potassium phosphate buffer (pH 7.2). The assay tubes were preincubated for 1 min at 34°C prior to addition of the enzyme solution to initiate the reaction and then incubated for 3 min at the same temperature with continuous shaking. The methyl esters of released free fatty acids were separated by thin-layer chromatography on silica gel G plates impregnated with $AgNO_3$ by developing in ether/hexane (1 : 9, v/v). The spots located by spraying with 0.2% 2,7-dichlorofluorescein were scraped into vials and the radioactivity measurement was performed as above. The specific activity of the desaturase was calculated as nmol palmitoleate formed/min per mg microsomal protein. Protein content was assayed by using the procedure of Lowry et al. [17], using bovine serum albumin as standard.

Electron spin resonance spectroscopy. The label, *N*-oxyl-4,4′-dimethyloxazolidine derivative of 5-ketostearic acid (5-NS), was synthesized according to the procedure of Waggoner et al. [18]. Spin labeling and spectrometry were carried out as described previously [19]. Lipids were extracted from pellicular and microsomal membranes isolated from whole cells harvested at various stages of growth, and were mixed with the spin probe in ethanol. Solvents

were evaporated under a stream of N_2 and to this mixture small amounts of a Tris buffer (50 mM Tris-HCl/150 mM NaCl, pH 7.5) were added. This mixture was subsequently sonicated for 10 min under a stream of N_2 with a tip-type sonifier (TA-4201, Kaijo Denki Co., Tokyo). The spin-labeled lipid dispersion was taken into a cylindrical quartz tube and its ESR spectra were measured at 27 and 34°C using a commercial X-band spectrometer (JEOL FE-2X) equipped with a variable temperature control. The parallel ($T'_{\parallel}$) and perpendicular ($T'_{\perp}$) principal values of the hyperfine tensor of an axially parameter, S, was calculated using the relationship:

$$S = \frac{T'_{\parallel} - T'_{\perp}}{T_{zz} - (T_{xx} - T_{yy})/2} \cdot \frac{a}{a'} ,$$

where T_{zz} (32.9 G), T_{xx} (5.9 G) and T_{yy} (5.9 G) were the hyperfine principal values of the nitroxide radical. a/a' was the polarity correction factor, where $a' = (T'_{\parallel} + 2T'_{\perp})/3$ and $a = (T_{zz} + T_{xx} + T_{yy})/3$ (= 14.9 G).

Chemicals. Palmitoyl-CoA was purchased from PL Biochemicals and NADH from Kyowa Fermentation Co. (Tokyo). [1-^{14}C]Palmitoyl-CoA (56 Ci/mol) and [1-^{14}C]palmitic acid (40—60 Ci/mol) were obtained from New England Nuclear (Boston). [1-^{14}C]Palmitoyl-CoA was added to the unlabeled palmitoyl-CoA in acetate buffer (pH 6.0) in order to adjust to 1 Ci/mol. The boron trifluoride-methanol complex was purchased from Nakarai Chemicals Co. (Kyoto).

Results

Growth phases of T. pyriformis

A thermotolerant strain (NT-I) of *T. pyriformis* employed in the present study has a maximum growth temperature of 40—41°C, whilst other usual strains are unable to grow above 35°C. In order to obtain reproducible growth rates, care was taken to maintain culture flasks shaking constantly and to use the same size of inoculum for inoculation. Fig. 1 shows the growth curve at 34°C of *T. pyriformis* NT-I in an axenic medium containing 2% proteose/peptone, 0.2% yeast extract, 0.5% glucose and $9 \cdot 10^{-3}$ M EDTA-Fe^{3+} complex. The culture reached the stationary phase after approx. 55 h of incubation at 34°C. Several representative growth stages, which are indicated by arrows, are the early exponential (I), mid-exponential (II), late exponential (III), early stationary (IV) and late stationary (V) phases. Samples of the culture were withdrawn at these selected intervals for analyses of composition and physical properties of membrane lipids. There was little or no significant change in the phospholipid content per cell, 4.3—4.8 mg phospholipids/10^6 cells through these phases.

Alteration of membrane lipid composition during aging

Phosphilipid polar head group. Individual phospholipids were separated on thin-layer plate (silica gel H) using the developing solvent, $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (75 : 25 : 5 : 2.2, v/v). The phospholipid class composition was determined by the phosphorus content assayed using the method of Rouser et al. [14] of the spot corresponding to each phospholipid. Changes in the com-

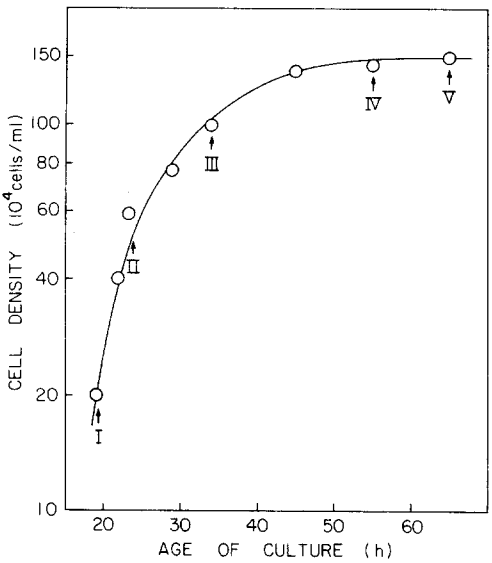

Fig. 1. Cell density of *Tetrahymena pyriformis* NT-I at various phases of growth. Cells were grown, with shaking, in 200 ml of 2% proteose/peptone containing 0.5% glucose, 0.2% yeast extract and $9 \cdot 10^{-6}$ M EDTA Fe^{3+} complex. Five representative stages, indicated by arrows, are the early exponential (I), mid-exponential (II), late exponential (III), early stationary (IV) and late stationary (V) phases.

position of major polar head groups on aging of the culture (19—65 h) are displayed in Fig. 2. Since there was little or no significant change in the amount of lipid phosphorus in the cell during this period of incubation time, the distribution profile of phospholipids may reflect the proportional composition based on the net amounts of individual phospholipids in a cell. Although the phospholipid composition varied somewhat among membrane fractions, the general trend observed, depending on the age of culture, was a profound decrease in the content of phosphatidylethanolamine and a slight increase in phosphatidylcholine. In contrast, the specific phospholipid, 2-aminoethylphosphonolipid, which is rich in the surface membrane (pellicle), did not show a consistent alteration during aging. Cardiolipin was present primarily in the mitochondrial membranes, but its content was almost unchanged throughout the growth cycle. It is of some interest to note the gradual increase up to 6% with age of culture in lysophosphatidylcholine in the pellicular membranes (data not shown). In mitochondria the decrease in phosphatidylethanolamine was compensated by the corresponding increase in phosphatidylcholine, in the fashion of a precursor-product relationship.

Phospholipid acyl group. The phospholipid fatty acid composition also was markedly affected by the age of the culture. Fig. 3 shows the changes in the relative proportion of major fatty acids of whole cell phospholipids. The most pronounced variation occurred in oleic (18 : 1), linoleic (18 : 2), palmitoleic (16 : 1) and myristic (14 : 0) acids. The first two acids increase greatly with a concomitant decrease in the last two acids. Such marked alteration is distinct, especially at the exponential phase of growth from stage I to III. After this period, similar but less profound changes were observed. The stationary phase cells contained larger amounts of C18-unsaturated fatty acids (67%) as com-

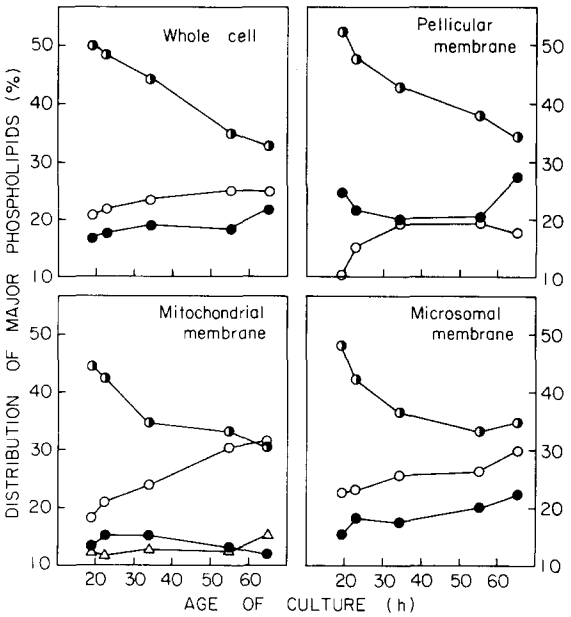

Fig. 2. Age-dependent alterations in the phospholipid polar head group composition of whole cells and various membrane fractions of *T. pyriformis* NT-I. The pellicular, mitochondrial and microsomal membranes were isolated by Nozawa and Thompson [10] from cells harvested at various growth stages. Individual phospholipids were separated by silica gel H thin-layer chromatography. Values are expressed as percentage of the total phospholipid content. ○, phosphatidylcholine; ◑, phosphatidylethanolamine; ●, 2-aminoethylphosphonolipid; △, cardiolipin.

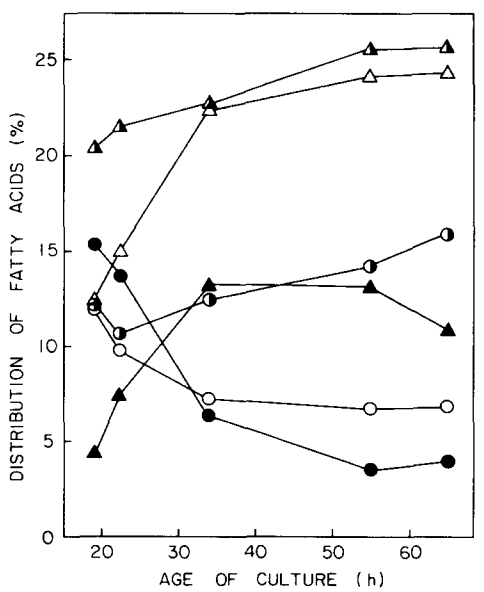

Fig. 3. Age-dependent alterations in the fatty acyl group composition of total phospholipids from whole cells. Phospholipids were separated from neutral lipids by silicic acid-Hyflo Super Cel column chromatography. The fatty acids were methylated by boron trifluoride/methanol and their methyl esters were analyzed by gas-liquid chromatography. Values are expressed as relative percentage of the major fatty acids. Other minor components included 12 : 0, 15 : 0, iso-15 : 0, 16 : 2 (plus 17 : 0), 18 : 0, 18 : 2(6,11). ○, myristic acid (14 : 0); ◑, palmitic acid (16 : 0); ●, palmitoleic acid (16 : 1); △, linoleic acid (18 : 2); ▲, linolenic acid (18 : 3); ▲, oleic acid (18 : 1).

pared to early exponential phase cells (40%). This resulted in a great increase on aging in the unsaturation index, which is calculated from (percentage of each unsaturated fatty acid) × (number of double bond). The indices for phases I, III and IV, were 110, 140 and 150, respectively. The similar or more marked trends of acyl group changes observed with whole cells were reflected also in pellicular, mitochondrial and microsomal membranes (Table I). In general, the proportion of oleic (18 : 1) and linoleic (18 : 2) acids increased progressively with time, whereas the level of palmitoleic acid (16 : 1) exhibited a considerable decrease. As for saturated fatty acids, there was a slight increase in the percentage of palmitic acid (16 : 0) and a great reduction in myristic acid (14 : 0) content. The principal polyunsaturated fatty acid, γ-linolenic acid (18 : 3 (6, 9, 12)), was greatest in amount in the mitochondrial phospholipids, but it did not show drastic age-dependent changes as observed with pellicular and microsomal membranes. Large differences in the unsaturation index were found between the early exponential (stage I) and late stationary (stage V) phases; 91 (stage I) to 140 (stage V), 138 to 177 and 106 to 147 for pellicular, mitochondrial and microsomal membranes, respectively. Another parameter, the ratio of the unsaturation index to the percentage of saturated fatty acids, also revealed changes characteristic of different types of membrane.

In order to determine which phospholipid plays a dominant part in profound changes of the membrane acyl group composition, we have analyzed the fatty acid constituents of major phospholipids derived from cells harvested at various growth phases. The example of the early stationary phase is shown in Table II including also data of the early exponential phase for comparison. Each phospholipid has its own typical fatty acid profile. For instance, aminoethylphosphonolipids contained larger amounts of γ-linolenic and cilienic (18 : 2 (6,11)) acids than phosphatidylcholine and phosphatidylethanolamine. Upon aging of cells, the fatty acid composition was to a varying degree affected quantitatively in all phospholipids. The general trends observed in total membrane phospholipids (Table I), an increase in oleate and linoleate and a decrease in palmitoleate and myristate, were well reflected in each phospholipid of different membranes. Whereas the γ-linoleate content was increased both in phosphatidylcholine and phosphatidylethanolamine in the pellicular membrane, and in phosphatidylcholine in the microsomal membrane, little change was noted in any particular phospholipid in the mitochondrial membrane. It was interesting that despite no change in γ-linoleic acid the unusual fatty acid, cilienic acid (18 : 2 (6,11)), increased by 2-fold in aminoethylphosphonolipid at stage IV. Also this membrane phospholipid derived from the aged cells became almost completely occupied by C18-unsaturated fatty acids. This resulted in a higher unsaturation as inferred by the unsaturation index, implying that the phosphonolipid contained approx. 200 double bonds per 100 fatty acid chains. The total content of mono-unsaturates was well balanced regardless of growth stages: the decreased proportion of palmitoleate was compensated by the increase in oleate.

The overall profile of the age-associated changes in major unsaturated fatty acids is demonstrated in a simplified figure in which the increase in total C18-unsaturates including 18 : 1, 18 : 2 (6,11), 18 : 2 (9,12) and 18 : 3 (6,9,12), and the decrease in 16 : 1 of main phospholipids extracted from the

TABLE I

CHANGES DURING AGING IN THE FATTY ACYL GROUP COMPOSITION OF TOTAL PHOSPHOLIPIDS FROM VARIOUS MEMBRANE FRACTIONS OF *T. PYRIFORMIS* NT-I

The pellicular, mitochondrial and microsomal membranes were prepared according to the method of Nozawa and Thompson [10] from cells collected at different stages of growth. Phospholipids were separated from neutral lipids by silicic acid - Hyflo Super Cel column chromatography, and their fatty acid composition was determined by gas-liquid chromatography. The results are averages of two separate experiments and are expressed as relative percentage of total fatty acids. The error range was less than 5% of each value. Other minor components included 12:0, 15:0, iso-15:0, 16:2 (plus 17:0), 18:0, 18:2 (6,11). Unsaturation index (UI) indicating the number of double bonds per 100 fatty acids was calculated from (percentage of each unsaturated fatty acid) X (number of double bond). SFA, saturated fatty acid.

Membrane fraction	Age of culture (h)	Fatty acid composition (wt.%)								UI	UI/SFA
		14:0	16:0	16:1	18:1	18:2	18:3	18:1 + 18:2 + 18:3	SFA		
Pellicular	19	15.9	14.4	13.1	4.6	9.8	16.2	30.6	45.5	91.0	2.0
	23	12.5	13.9	12.5	6.6	13.0	18.0	37.6	38.9	104.5	2.6
	34	7.9	15.7	6.6	13.1	20.8	20.1	54.0	32.1	128.2	3.9
	55	8.1	16.6	3.4	14.3	21.3	22.5	58.1	32.9	136.6	4.1
	65	7.8	17.0	3.8	11.9	21.8	24.0	57.7	33.2	139.9	4.2
Mitochondrial	19	8.7	8.9	14.8	3.9	16.2	27.4	47.5	25.1	138.5	5.5
	23	7.5	9.6	13.7	5.5	17.7	26.2	49.4	24.2	140.2	5.7
	34	5.8	12.8	6.3	10.5	23.8	26.8	61.1	25.7	149.4	5.8
	55	4.3	11.6	3.4	12.4	28.0	27.2	67.6	21.5	160.0	7.4
	65	4.0	11.6	3.1	10.4	30.1	32.5	73.0	19.8	177.2	8.9
Microsomal	19	12.5	11.9	16.7	4.4	12.4	18.5	35.3	34.5	106.2	3.0
	23	10.9	12.6	14.7	7.4	13.9	17.3	38.6	33.3	107.8	3.2
	34	8.2	14.7	6.7	13.0	21.9	20.2	55.1	30.3	131.1	4.3
	55	7.4	14.8	3.1	14.9	24.7	23.7	63.3	27.8	147.5	5.3
	65	7.5	15.7	2.8	12.2	24.2	24.6	61.0	29.5	146.6	4.9

TABLE II

FATTY ACID COMPOSITION OF MAJOR PHOSPHOLIPIDS FROM VARIOUS MEMBRANE FRACTIONS OF EARLY STATIONARY PHASE CELLS

Lipids were extracted from the pellicular, mitochondrial and microsomal membranes prepared from cells at the early stationary phase (stage IV). Major phospholipid fractions, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and 2-aminoethylphosphonolipid (AEPL) were separated by thin-layer chromatography on silica gel H plate, and their corresponding areas were scraped off for determination of the fatty acid composition by gas-liquid chromatography. Other minor components not listed in the table were 12:0, 15:0, iso-15:0 and 16:2 (plus 17:0). The results are averages of two separate experiments and are expressed as relative percentage of total fatty acids. The error range was less than 5%. For comparison, the results of a single experiment for the early exponential phase cell (stage I) are also included in parentheses. SFA, saturated fatty acid, UI, unsaturation index.

Membrane and phospholipid	Fatty acid composition (wt.%)									UI	UI/SFA
	14:0	16:0	16:1	18:1	18:2 (6,11)	18:2 (9,12)	18:3	18:1 + 18:2 + 18:3	SFA		
Pellicular											
PC	4.6	14.8	2.3	13.7	6.0	22.2	30.5	72.4	24.0	163.9	6.8
	(13.9)	(14.9)	(15.3)	(5.7)	(2.9)	(10.5)	(16.2)	(35.3)	(37.8)	(96.3)	(2.6)
PE	11.1	20.7	3.0	15.6	—	23.9	14.1	53.6	42.8	108.7	2.5
	(20.2)	(14.8)	(15.0)	(5.8)	(—)	(8.6)	(8.5)	(22.9)	(53.4)	(63.5)	(1.9)
AEPL	2.5	7.0	1.6	11.9	14.2	19.3	39.9	85.3	12.6	200.2	15.9
	(7.4)	(8.7)	(10.9)	(5.3)	(7.5)	(10.6)	(36.5)	(59.9)	(21.8)	(161.9)	(7.4)
Mitochondrial											
PC	3.9	14.9	3.8	13.5	5.7	22.8	29.0	71.0	23.6	161.3	6.8
	(10.0)	(10.2)	(13.6)	(4.3)	(4.2)	(11.3)	(27.6)	(47.4)	(26.1)	(131.7)	(5.0)
PE	5.3	10.1	3.0	15.1	—	34.0	28.2	77.2	19.0	170.6	9.0
	(11.2)	(8.9)	(19.3)	(3.8)	(1.8)	(13.1)	(22.7)	(41.4)	(28.6)	(121.0)	(4.2)
AEPL	2.7	7.5	2.4	11.4	15.0	20.2	36.7	83.3	13.6	194.3	14.4
	(5.8)	(7.9)	(10.7)	(4.8)	(7.4)	(11.2)	(38.5)	(61.9)	(18.7)	(171.2)	(9.2)
Microsomal											
PC	4.0	11.3	2.9	16.5	6.1	24.6	29.4	76.6	19.5	169.0	8.7
	(14.6)	(12.2)	(16.1)	(4.3)	(2.7)	(11.1)	(17.9)	(36.0)	(34.9)	(101.7)	(2.9)
PE	9.5	15.5	3.9	17.2	—	31.6	16.1	64.9	30.5	132.6	4.4
	(13.6)	(10.0)	(20.1)	(4.3)	(1.9)	(12.6)	(15.8)	(34.6)	(33.1)	(100.8)	(3.0)
AEPL	3.2	6.9	2.3	10.4	14.2	20.5	38.5	83.6	13.6	197.6	14.5
	(7.6)	(7.8)	(12.7)	(4.4)	(6.4)	(11.6)	(35.5)	(57.9)	(20.6)	(159.6)	(7.7)

pellicular and microsomal membranes were shown to become more distinct with the age of growth (Fig. 4). The general pattern was more or less similar between two membrane fractions, but the reduction in the palmitoleate content was more pronounced in the microsomal membranes, especially in phosphatidylethanolamine. It should be noted, furthermore, that regardless of its original content the proportion of palmitoleate declines to the low level of less than 5% in all membrane phospholipids from the stationary phase cells. Whereas palmitate (16 : 0) showed rather small changes within 5% in all the phospholipids of the pellicular and microsomal membranes, the level of myristate (14 : 0) displayed a marked decrement with the culture age which was especially predominant in phosphatidylcholine.

Age-dependence of the fatty acid desaturation activity

The most striking alteration in phospholipid acyl group composition with aging was an increase in C18-unsaturated fatty acids, especially linoleic acid and a decrease in palmitoleic acid (Table I, Fig. 4). Therefore, the activity of fatty acid desaturation would be expected to alter, depending on the stage of growth. There exist two main pathways in *Tetrahymena* for unsaturation of a fatty acid: palmitoleate and stearate pathways. The former route participates in the conversion of palmitic to palmitoleic acid and the latter in the transformation of palmitic to γ-linoleic acid through the sequence, 16 : 0 → 18 : 0 → 18 : 1 → 18 : 2 → 18 : 3. We have examined the influence of aging upon the palmitoleate pathway. Cells collected at various growth stages were labeled for 20 min with [1-^{14}C]palmitic acid and lipids were extracted for measurements of the radioactivity in palmitoleic acid separated by gas-liquid chromatography. The results of labeling experiments are shown in Fig. 5, indicating a drastic reduction with age in the conversion rate of [^{14}C]palmitate to [^{14}C]palmitoleate. This suggests a corresponding decrease in palmitoyl-CoA desaturase

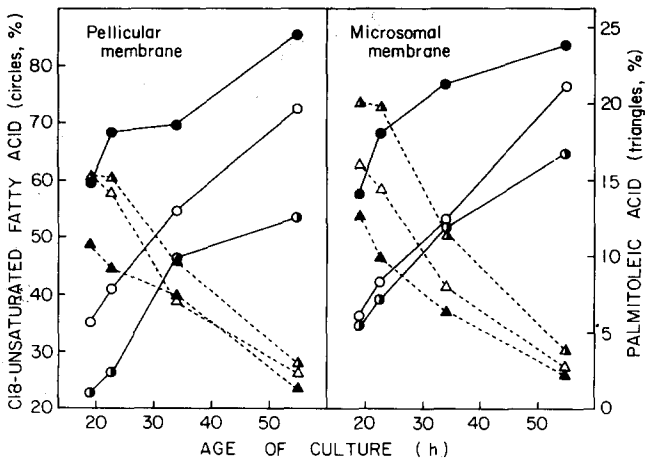

Fig. 4. Age-dependent changes in C18-unsaturated fatty acids and palmitoleic acid in major phospholipids from the pellicular and microsomal membranes. The membrane isolation and lipid analysis were carried out as described in the legend for Table II. C18-unsaturated fatty acids included in the figure were oleic (18 : 1), linoleic (18 : 2(9,12)), cilienic (18 : 2(6,11)), and γ-linolenic (18 : 3) acids. ○ and △, phosphatidylcholine; ◑ and ▲, phosphatidylethanolamine; ● and ▲, 2-aminoethylphosphonolipid.

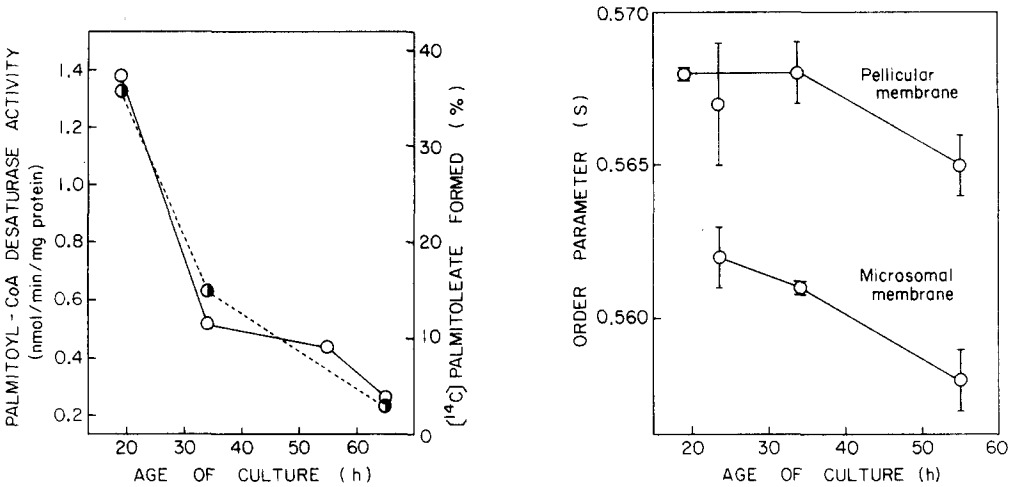

Fig. 5. Changes in the activities of conversion of [^{14}C]palmitate to [^{14}C]palmitoleate in whole cells and palmitoyl-CoA desaturase in microsomal membranes. Cells taken at various growth stages were labeled with [1-^{14}C]palmitic acid for 20 min prior to lipid extraction. The radioactive fatty acids of extracted lipids were methylated with boron trifluoride/methanol, separated by gas-liquid chromatography, and the radioactivity associated with each peak was measured in a scintillation counter. The palmitoyl-CoA desaturase activity was measured for microsomes isolated from cells harvested at different stages of growth. Details for the enzyme assay are described in Materials and Methods. ○, palmitoyl-CoA desaturase; ●, conversion rate of [^{14}C]palmitate to [^{14}C]palmitoleate.

Fig. 6. Changes in the fluidity of pellicular and microsomal membranes from cells at different stages of growth. The pellicular and microsomal membranes were isolated from cells at several growth phases, and lipids were extracted from these membranes. The phospholipid fraction was separated by silicic acid-Hyflo Super Cel column chromatography, and the phospholipid dispersion was labeled with a spin probe (5-NS) for electron spin resonance spectrometry. The order parameter, S, was calculated by using the equation described in Materials and Methods. Bars represent the range of values obtained from two to three experiments.

involving the transformation of 16 : 0 to 16 : 1. The activity of this enzyme was eventually measured for microsomes and found to be well correlated with the formation rate of [^{14}C]palmitoleate in [^{14}C]palmitate-labeled whole cells (Fig. 5). Such strikingly depressed activity of palmitoyl-CoA desaturase could accound for the very low content of palmitoleate in membrane phospholipids from aged cells.

Changes in membrane fluidity during aging

Since profound changes in phospholipid polar head as well as acyl group composition of various membranes were found to occur during progress in the culture age, it was expected that the physical state of membrane lipids was affected by the markedly altered lipid composition. Therefore, we have examined by ESR spectrometry the molecular motions of the extracted phospholipids of pellicular and microsomal membranes which were isolated from cells at different stages of growth. As a parameter for estimating the relative fluidities of the two types of membrane, the order parameter, S, was employed. The values obtained using 5-NS reflect mainly the frequency of *trans-gauche* isomerization which occurs between the first and the fifth carbon atoms of the fatty acid chains attached to the spin probe and its neighboring phospholipids. In the

membranes having a higher frequency of isomerization, i.e. a smaller S value, the molecules undergo more rapid motion. The order parameters measured at 34°C were plotted as a function of culture age (Fig. 6). The lower values for microsomal membranes would indicate that this membrane may be more fluid than the pellicular membranes. In general, these two membranes showed similar patterns of change in S values during the age of growth. Whereas the parameter decreased progressively with age in the microsomal membranes, it remained mostly unchanged from stage I to III and then decreased in the pellicular membranes. These results suggest that the aged membranes have greater fluidities than the young membranes, thus reflecting a good correlation between the increased fluidity and the enhanced unsaturation of fatty acids. But the possibility cannot be excluded that the alterations in phospholipid polar head group composition would be involved in the increased membrane fluidity.

Discussion

Age-related modification, either quantitative or qualitative, has been known to occur in structures and/or functions of membranes in various types of cell including mammalian cells. However, the detailed mechanisms of deteriorative reactions are hardly understood. As it is now widely accepted that diverse physiological functions of biological membranes are closely associated with physical states of their lipids, one would expect that certain age-dependent impairments of membrane functions may be accounted for by the altered lipid composition occurring upon aging. As for mammalian cell membranes, however, only sparse information is available on the age-dependence of lipid composition. A typical example has been reported by Rouser et al. [20] on human brain myelin, showing some conspicuous lipid modifications with age. On the other hand, in many microorganisms such as bacteria, *Mycoplasma* and fungi, altered lipid compositions have been observed to accompany the progressing age of culture. But detailed lipid analysis has not been performed on membranes of these cells. It was shown in *Mycoplasma hominis* that on aging of the culture the plasma membrane became less fluid as indicated by electron spin resonance spectrometry [2]. The recent extensive studies on bean cotyledons by McKersie and Thompson [6] have revealed that, by X-ray diffraction analysis, both rough and smooth microsomes of *Phseolus vulgaris*, cotyledons have increasing proportions of gel-phase lipids as senescence precedes. They argued also the possible involvement of neutral lipids in undergoing lateral phase separation of the polar lipids in the aged membranes.

A eukaryotic single cell, *Tetrahymena*, which has proved to be a useful model system for various membrane studies [7,8], was employed to investigate the relationship between age and membrane lipid composition. Although it has already been observed that the lipid composition of this cell changes in a quantitative manner with age of culture, lipid analysis has not been performed in detail on various membrane fractions isolated from aging cells.

In the present experiments we have isolated different major membrane fractions, pellicular, mitochondrial and microsomal membranes, from *T. pyriformis* NT-I cells at several stages of growth, and then analyzed their lipid composition by thin-layer and gas-liquid chromatography. The results obtained here indi-

cated that profound modifications in phospholipid polar head as well as fatty acyl group were apparent in all aging membranes examined. The most noticeable alteration in phospholipid class composition was a considerable decrease in phosphatidylethanolamine. Although at the present moment, there is no direct experimental evidence to explain such a marked decline of phosphatidylethanolamine associated with an increase in phosphatidylcholine, either one or both of two mechanisms should be involved; sequential methylation of phosphatidylethanolamine to transfer phosphatidylcholine and the cytidine diphosphate-ethanolamine (choline) pathway. In order to determine which of these mechanisms actually contributes to the altered phospholipid profile, the activities of enzymes involving the above pathways are required to be measured and are under examination in our laboratory. As for the fatty acids, the most notable variation occurred in unsaturated fatty acids; a drastic increase in linoleic acid and a corresponding decrease in palmitoleic acid. The recent findings of the age-related change in fatty acid constituents in *Paramecia aurelia* cells exhibited a great increase in linoleic and arachidonic acids, accompanied by a striking decrease in oleic acid [21].

The progressive decline of palmitoleic acid content in membrane phospholipids of *Tetrahymena* would be assumed to be due to the lowered activity of palmitoyl-CoA desaturase, catalyzing transformation of palmitate to palmitoleate (16 : 0 → 16 : 1). As shown clearly in Fig. 5, the desaturase activity in microsomes was found to decline to a much lower level. However, the decreased amount of palmitic acid could not account for the increased relative proportion of C18-unsaturates in the overall fatty acid composition, suggesting that the other unsaturation pathway (16 : 0 → 18 : 0 → 18 : 1 → 18 : 2 → 18 : 3) may be operating at an increased rate. Since palmitoyl-CoA desaturase and the enzyme catalyzing the elongation reaction (16 : 0 → 18 : 0) use the same substrate, palmitate, it is possible that a decrease in the palmitate desaturation may evoke an increase in palmitate elongation [22]. Stearates thus formed are transformed into oleic, linoleic and linoleic acids by a sequential desaturation reaction, leading to the marked increase in C18-unsaturated fatty acids.

The above-mentioned modifications in membrane lipid composition during aging of *Tetrahymena* cells prompted us to investigate the physical properties of the lipid-altered membranes. It was demonstrated from data of electron spin resonance spectrometry that the pellicular and microsomal membranes from aged cells had increased fluidities as inferred by decreasing order parameters. Such enhanced fluidity could result primarily from a marked increase in the degree of unsaturation of the fatty acid chains. Our previous observations have shown that the membranes from cells grown at low temperatures were rich in unsaturated fatty acids and greater in fluidity, as compared to the membranes from high temperature-grown cells [19,23]. In addition, a decrease in phosphatidylethanolamine with an increase in phosphatidylcholine also might participate in the increase in membrane fluidity, because the former phospholipid has greater packing effects on the lipid bilayer than the latter [24]. These results show that the higher fluidities in aged membranes are not in accordance with those observed for *Mycoplasma* [2,3] and plants [4—6]. The fluidities of plasma membranes of *Mycoplasma*, microsomal membranes of bean cotyl-

edons, and protoplast plasmalemma of rose petals were reported to decrease with advancing age. The discrepancy may be due in part to differences in cell species. However, the possibility cannot be excluded that protein changes may be involved in the increased fluidity in our experiments, since proteins are known to affect, usually, a reduction in membrane fluidity in a concentration-dependent manner [19,25].

The age-associated changes in composition and the physical state of membrane lipids would affect some functions taking place in membranes of *Tetrahymena* cells. For example, adenylate cyclase activity, which has been found to be entirely associated with the surface pellicular membrane, showed a tendency to increase with age of cell growth [26]. It is assumed that marked alterations in the pellicular membrane lipids may be responsible for the rise in the adenylate cyclase activity. Another interesting example is the effect of aging upon endocytotic activity. Aged cells have reduced capacity of food vacuole formation in both *Tetrahymena* [27] and *Paramecia* [28]. However, no plausible explanation is now available for this loss of endocytotic ability.

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