Biochimica et Biophysica Acta, 406 (1975) 248–263

BBA 77077

STUDIES ON *TETRAHYMENA* MEMBRANES

MODIFICATION OF SURFACE MEMBRANE LIPIDS BY REPLACEMENT OF TETRAHYMANOL BY EXOGENOUS ERGOSTEROL IN *TETRAHYMENA PYRIFORMIS*

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(Received April 1st, 1975)

SUMMARY

Tetrahymena pyriformis WH-14 cells were grown in the medium supplemented with ergosterol (1 mg/100 ml) and the effects of replacement of tetrahymanol by ergosterol upon the lipid composition in the surface membranes (cilia and pellicles) were examined.

1. By scanning and freeze-etch electron microscopy it was suggested that exogenous ergosterol would be inserted into the lipid regions in the surface membranes. Although freeze-etched faces of filipin-treated membranes containing the native tetrahymanol showed a random distribution of 85-Å protein particles, the ergosterol-replaced membranes after the same polyene treatment revealed the marked ultrastructural alterations on the fracture faces.

2. The replacement of tetrahymanol in membranes by ergosterol induced a profound alteration in the phospholipid class composition and a marked increase in phosphatidylethanolamine with a compensatory decrease in phosphatidylcholine and 2-aminoethylphosphonolipid.

3. There are significant and quantitative but not qualitative changes in the fatty acid composition of total lipids from the ergosterol-replaced membranes. There are also increases in saturated and decreases in unsaturated fatty acids. Phosphatidylethanolamine acyl chains particularly become more saturated, as compared with two other phospholipids, in ergosterol-replaced pellicles. This increase in saturation is due to an appreciable increase in $C_{14:0}$, $C_{16:0}$ and iso-$C_{17:0}$, and a decrease in $C_{18:1}(\Delta^9)$, $C_{18:2}(\Delta^{9,12})$ and $C_{18:3}(\Delta^{6,9,12})$.

4. These results suggest that profound alterations in phospholipids as well as in their fatty acyl chains are required to modify the overall membrane lipid composition for the maintenance of proper membrane fluidity. Our data would also support the thesis that polar head groups are involved in the membrane lipid organization and that sterols interact selectively with phospholipid molecules containing the appropriate fatty acyl chain composition in biological membranes.

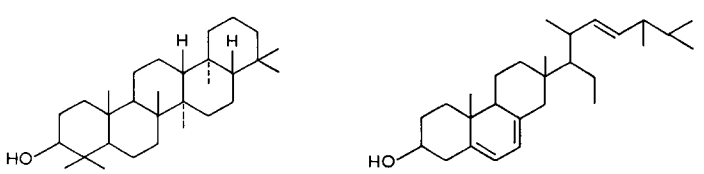
INTRODUCTION

Since the concept has been accepted that the biological membranes are a kind of two dimensional dynamic solution of proteins dispersed in the fluid lipid matrix [1], there is now increasing evidence indicating that membrane fluidity is closely related to the various important functions (e.g. transport, hormone receptor and enzyme activity) taking place in the biological membranes, as recently reviewed by Singer [2]. The membrane lipid especially plays a crucial role in controling the membrane fluidity and there are several factors which are involved in the maintenance of proper fluidity; the acyl chain [3–6] (unsaturation, length, branching) and polar head group of phospholipid [7–10], sterols [11, 12] and carotenoid [13].

In order to obtain direct evidence for the considerable dependence of the membrane fluidity upon the physical state of lipids in biological membranes, we chose the ciliated protozoan, *Tetrahymena pyriformis* cell as an excellently useful model system since various membrane fractions are readily labeled with spin probes and their membrane lipids can be easily modified by changing the growth conditions, such as growth temperature-shift, exogenous supplementation of fatty acids or sterols and starvation. In fact, we have shown, in an earlier paper [5], by spin labeling various membrane fractions isolated from the temperature-shifted *Tetrahymena* cells, that change in growth temperature induces a marked alteration in the fatty acyl composition of the membrane lipids and the degree of unsaturation affects the membrane fluidity; the membrane lipids from cells grown at low temperature with a high level of unsaturated fatty acids are in a "fluid" state and those with a lower content of unsaturated fatty acids from cells grown at high temperature are in a "less fluid" state. It was also suggested that a sterol-like triterpene alcohol, tetrahymanol, which is principally localized in the surface membranes, would be involved in ordering or disordering of membrane phospholipids in the protozoan membranes.

Ferguson et al. [14] have shown in the preliminary work that the ergosterol supplementation to the growth medium induces replacement of tetrahymanol by that sterol and causes small but significant compensatory changes in the fatty acid composition of *Tetrahymena* cells. We have extended the sterol-replacement experiment to investigate the effect of the sterol-manipulation upon the *Tetrahymena* membrane lipid composition.

In the present communication, data are presented showing that the replacement of the native tetrahymanol (Fig. 1A) in the surface membranes (cilia and pellicles) by ergosterol (Fig. 1B) causes a marked alteration in the phospholipids as well as in their fatty acyl composition, an increase of phosphatidylethanolamine with a con-



Tetrahymanol (A) Ergosterol (B)

Fig. 1. Chemical structures of tetrahymanol and ergosterol.

current decrease of phosphatidylcholine plus 2-aminoethylphosphonolipid and an increase of saturated acyl chains associated with a decrease of unsaturated ones. These results would suggest that *Tetrahymena* cells modify their membrane lipid composition to maintain the proper membrane fluidity, thus indicating that the membrane sterols should interact selectively with the phospholipid molecules containing the appropriate polar head group and fatty acyl chain composition.

MATERIALS AND METHODS

Growth of T. pyriformis cells

T. pyriformis WH-14 was grown at 25 °C in an enriched proteose-peptone medium as described previously [15].

For the ergosterol-replacement experiment the cells were grown in a medium supplemented with exogenous ergosterol. Ergosterol (2 mg/200 ml of culture) was added to the growth medium at 50 °C as a sterile ethanolic solution before inoculation, and thus could be dissolved homogeneously in the medium. The culture medium was inoculated with a 4-day-old cell culture which had already been adapted to ergosterol supplementation. Cells were harvested in the mid-logarithmic phase of growth.

Isolation of surface membranes from T. pyriformis cells

Surface membrane fractions were prepared from the native and ergosterol-replaced cells essentially 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 previously described [15, 16].

Lipid extraction and analysis

Lipids were extracted from individual membrane fractions by the method of Bligh and Dyer [17] and the resultant lipid solutions were stored in chloroform/methanol (6 : 1, v/v) at −20 °C.

Individual phospholipids were separated on a silica gel G thin-layer plate and the corresponding bands were scraped off the plate into centrifuge tubes. For elution of phospholipids from silica gel powder a mixture of formic acid/methanol/chloroform (2 : 1 : 1, v/v) was added and the tubes were shaken well and left to stand overnight. After centrifugation, aliquots of supernatant were removed for phosphorus analysis by the method of Bartlett [18], modified by digestion with perchloric acid according to Marinetti [19].

For analysis of fatty acids by gas-liquid chromatography, methyl esters were prepared by transmethylation 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 into a 200×3 cm glass column packed with 15 % diethyleneglycolsuccinate supported on Chromosorb W (80–100 mesh, Gaschro Kogyo Co., Tokyo). The column temperature was 185 °C and the pressure of the carrier gas, N_2, was 0.5 kg/cm^2. The amounts of individual fatty acid were determined by triangulation. Fatty acids were identified by comparison of their retention times with those of authentic standards and, in some cases, by gas chromatography-mass spectrometry for which an Hitachi RMU-6MG+002 system was used. Acceleration potential and ionizing voltage were 3200 V and 20 eV, respectively.

Quantitative analysis of tetrahymanol and ergosterol was performed essentially following the method described previously [20], using cholesterol as an internal standard. Care was taken not to expose ergosterol-containing samples too long to light and air.

Radioisotope labeling of T. pyriformis cells with [^{14}C]acetate

For determination of incorporation of [^{14}C]acetate into tetrahymanol and individual phospholipids, the cells in the mid-log phase were labeled with sodium [1-^{14}C]acetate (50 Ci/mol, Daiichi Pure Chemicals Co., Tokyo). Radioactivity measurement was performed in a Beckman liquid scintillation spectrometer using toluene scintillation liquor [21].

Scanning and freeze-etch electron microscopy

The surface ultrastructure of native and ergosterol-replaced *T. pyriformis* cells, which were treated with a polyene antibiotic, filipin, was examined by a scanning electron microscope (JSM-U3). The samples washed with 0.85 % NaCl were fixed for 1 h with 0.75 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Dried cells after dehydration in acetone were shadowed with carbon-gold [22].

For freeze-etch electron microscopy, membrane preparations were prefixed for 20 min at 0 °C with 2 % glutaraldehyde in 50 mM cacodylate buffer, pH 7.4, and then transferred to 40 % glycerol in 0.85 % NaCl. The specimens were placed on a brass holder and were immediately frozen in liquid Freon 22 at the temperature of liquid nitrogen. Subsequently, the frozen specimens were freeze-fractured, etched and shadowed with platinum-carbon in an Hitachi HFZ-I freeze-etching device [23]. The platinum-carbon replicas were floated on distilled water, cleaned by adding a hypochlorite solution, and soaked in 75 % H_2SO_4 for 24 h. After the cleaning procedure the replicas were rinsed three times with distilled water prior to mounting on 400-mesh grids. The ultrastructure of the membranes was examined in a JEM-1000 V electron microscope.

Chemicals

Ergosterol was kindly provided by Dr N. Ariga (Department of Chemistry, Faculty of General Education, Gifu University, Japan). The purity was examined on silica gel G plates with a petroleum ether/diethyl ether/acetic acid (70 : 30 : 1, by vol.) solvent system and also checked by gas-liquid chromatography. When necessary the sterol was recrystallized twice from ethanol. A polyene antibiotic, filipin, was kindly provided by Dr G. B. Whitfield (The Upjohn Company, Kalamazoo, Michigan, U.S.A.) and was used as dimethylsulfoxide solution (20 mg/ml).

RESULTS

Replacement of tetrahymanol in cilia and pellicle membranes by exogenously supplemented ergosterol

As we have shown in the previous studies [20, 24] on tetrahymanol, the ciliated protozoan *T. pyriformis* synthesizes a saturated, pentacyclic triterpenoid as their most abundant neutral lipid, instead of the usual sterols, and this sterol-like compound is principally localized in the surface membrane fractions; the cilia and pellicles. However, the

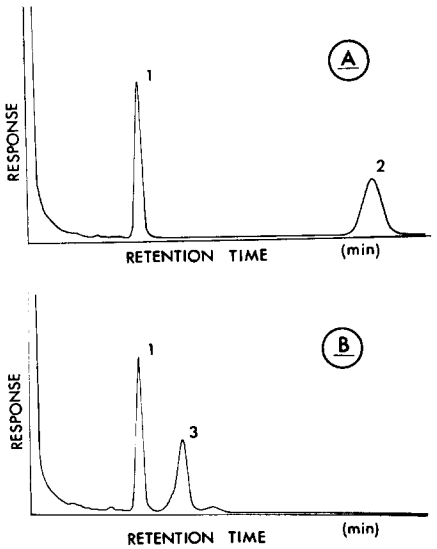


Fig. 2. Gas-liquid chromatograms of the non-saponifiable lipid fractions from the native (A) and the ergosterol-replaced (B) *T. pyriformis* pellicle membranes. Peaks 1, 2 and 3 represent cholesterol (internal standard), tetrahymanol and ergosterol, respectively.

precise function of tetrahymanol in the membrane is not yet known. In this work, for the modification of the *Tetrahymena* membrane lipid composition, the native tetrahymanol was replaced by exogenously added ergosterol. The proteose-peptone growth medium was supplemented with ergosterol (2 mg/200 ml of culture) and was inoculated with a stock culture which has been adapted to the ergosterol medium. The growth of the ergosterol-supplemented *Tetrahymena* cells is relatively slow compared with that of unsupplemented cells, but there were no differences in the cell shape, size or mobility. The cell number must be kept under about 20×10^5 cells/ml culture, otherwise some cells can restore the ability to synthesize tetrahymanol. As can clearly be seen in the gas-liquid chromatogram of the nonsaponifiable lipid component from the native and ergosterol-replaced pellicle membranes (Fig. 2), no peak of tetrahymanol is observed in the latter membranes. Table I represents the results of gas chromatographic analysis of tetrahymanol or ergosterol in the surface membranes without or with ergosterol supplementation, respectively, indicating that tetrahymanol is completely replaced by ergosterol. Moreover, the molar ratio of the two alcohols to phospholipid phosphorus is the same in the cilia membranes, and in good accordance with the data of Conner et al. [25]. It should be noted here that there is no significant difference in phospholipid phosphorus content between these two types of cell. This may imply that ergosterol molecules would be incorporated into the sites where the native tetrahymanol was present. However, this is not the case with pellicle membranes. Although a satisfactory explanation for such an over-high content of ergosterol is not immediately apparent, one possibility cannot be ruled out that extra ergosterol may be inserted into the alveolar membranes attached to the outermost membrane of the pellicle.

This complete replacement of tetrahymanol by ergosterol can be easily ex-

TABLE I

REPLACEMENT OF TETRAHYMANOL BY ERGOSTEROL IN SURFACE MEMBRANES OF *T. PYRIFORMIS*

Surface membrane fractions (cilia and pellicles) were isolated according to Nozawa and Thompson's procedure [15] from the ergosterol-supplemented and unsupplemented *T. pyriformis* cells. Tetrahymanol was analyzed by gas-liquid chromatography and phospholipid phosphorus was determined by the modified Bartlett method [18]. The numbers in parentheses represent the number of different experiments. Values are means ±S.D.

Tetrahymanol or ergosterol	Whole cells	Cilia	Pellicles
Native*			
Tetrahymanol			
(mol/mol P)	0.0.57	0.303	0.084
(g/g P)	0.790	4.201	1.170
Ergosterol-replaced			
Ergosterol			
(mol/mol P)	0.175±0.015 (11)	0.306±0.042 (9)	0.359±0.057 (5)
(g/g P)	2.247±0.198 (11)	3.934±0.553 (9)	4.606±0.737 (5)

* Data of Thompson et al. [20].

plained by the potent inhibition of tetrahymanol biosynthesis in *Tetrahymena* cells grown in the presence of ergosterol (Table II). However, the precise nature of the inhibition remains obscure, although there appear to be two steps to the inhibition; (1) between acetate and mevalonate, and (2) between mevalonate and tetrahymanol [26].

TABLE II

EFFECT OF ERGOSTEROL-REPLACEMENT UPON [^{14}C]ACETATE INCORPORATION INTO TETRAHYMANOL IN *T. PYRIFORMIS* CELLS

Tetraymena cells were grown in the ergosterol-supplemented medium (10 μg/ml). When the cells reached the mid-logarithmic phase, they were labeled with sodium [1-^{14}C]acetate and then at different intervals aliquots were withdrawn to measure the radioactivity in tetrahymanol. For thin-layer chromatographic separation of tetrahymanol the solvent system, petroleum ether/ether/acetic acid (70 : 30 : 1, v/v), was used. Figures are the averages of three different experiments.

	Radioactivity in tetrahymanol (cpm/10^6 cells)		
Incubation time:	10 min	30 min	90 min
Native cells	2350	4430	9820
Ergosterol-replaced cells	170	290	480
Inhibition (%)	93	94	95

Evidence for ergosterol insertion into the surface membranes: scanning and freeze-etch electron microscopy

The question arises as to whether the ergosterol molecules are actually inserted into the surface membranes and not just absorbed on to them. The unsupplemented and ergosterol-supplemented *Tetrahymena* cells are incubated for 1 h at 28 °C with a polyene antibiotic, filipin which reacts specifically with sterols and are then subjected to scanning electron microscopy. It is revealed from electron micrographs that filipin-treated cells supplemented with ergosterol exhibit drastically damaged surfaces and the native pyriform cell shape can no longer be maintained (Fig. 3A). No filipin-induced effect is observed in unsupplemented cells (Fig. 3B). This demonstrates that the polyene reacts with ergosterol but not with tetrahymanol in surface membranes, implying therefore that ergosterol may play a crucial role in the maintenance of surface membrane integrity.

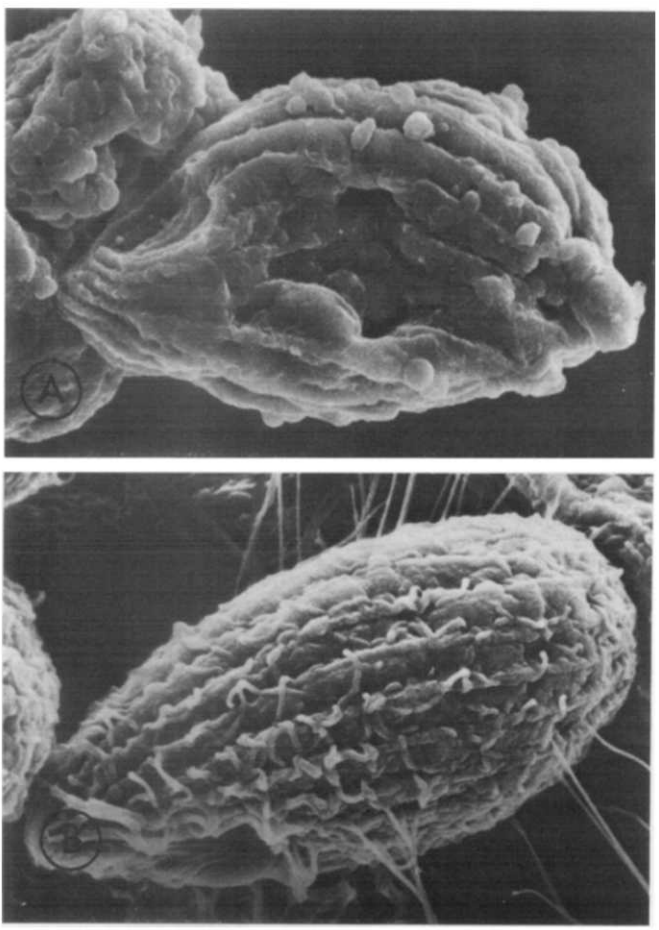


Fig. 3. Scanning electron microscopy of the ergosterol-replaced and the native *T. pyriformis* cells which were treated with filipin. A; ergosterol-replaced cell, B; native cell containing tetrahymanol. Concentration of filipin is 76.4 μM.

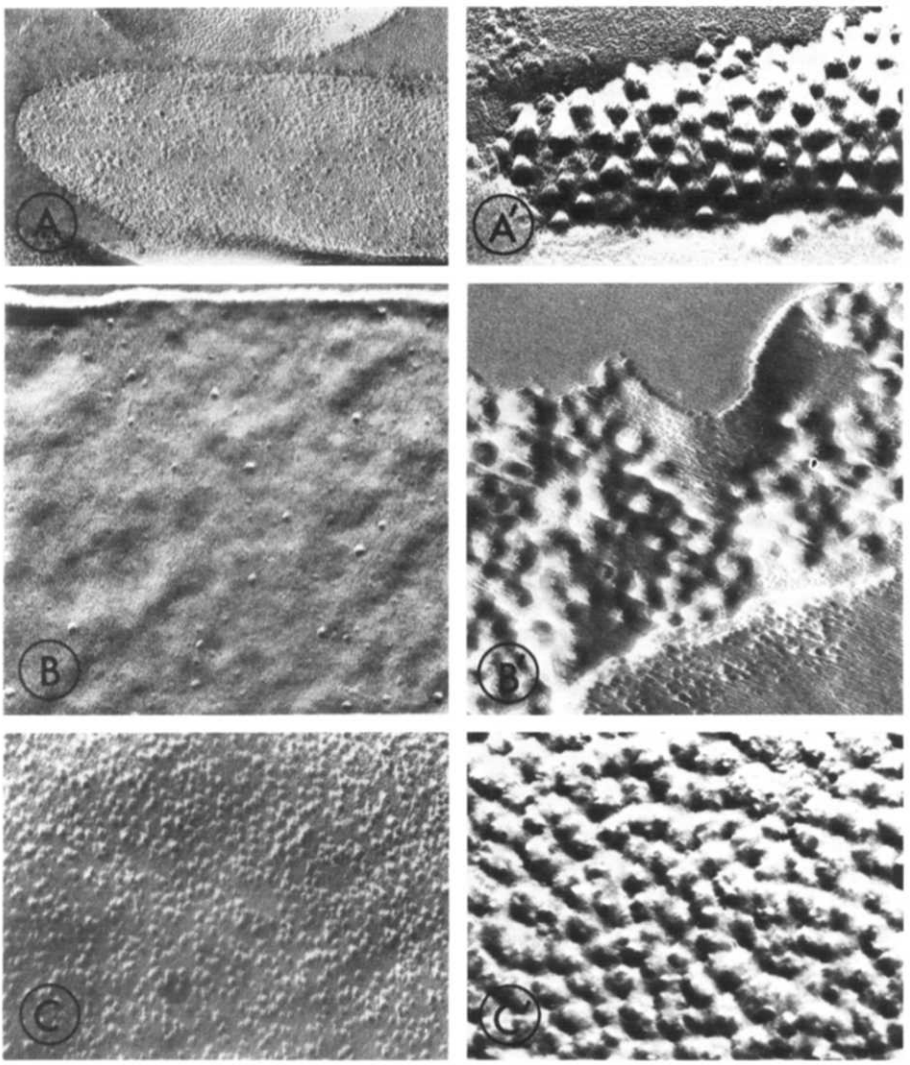


Fig. 4. Freeze-etch electron microscopy of the ergosterol-replaced cilia and pellicle membranes of *T. pyriformis*, and *E. floccosum* plasma membranes which were treated with filipin. A and A′; ergosterol-replaced cilia membranes untreated (A) and treated (A′) with filipin; inner fracture face. B and B′; ergosterol-replaced pellicle membranes untreated (B) and treated (B′) with filipin; outer fracture face. C and C′; *E. floccosum* plasma membranes untreated (C) and treated (C′) with filipin; inner fracture face. Concentration of filipin is 76.4 μM. Magnification; × 80 000.

Furthermore, the presence of ergosterol within the membrane is also confirmed by freeze-etch electron microscopy. It shows that abundant projections of 250–300 Å in diameter are visible at the freeze-fractured faces of cilia and pellicles (Fig. 4A′ and B′). Such filipin-induced alterations are more drastically observed in the plasma membrane of a human pathogenic fungus, *Epidermophyton floccosum* which contains ergosterol as the principal membrane sterol (Fig. 4C′), as being comparable with the

filipin-treated *Acholeplasma laidlawii*, erythrocyte and liposomal membranes containing cholesterol [27], thereby indicating that ergosterol is incorporated into the hydrophobic region of the membrane.

Alteration of phospholipid composition in the ergosterol-replaced cilia and pellicle membranes

The influence of ergosterol supplementation on the phospholipid composition in *Tetrahymena* membranes has not been reported and we examined it in this study. The phospholipid distribution pattern of cilia and pellicle membranes isolated from cells grown in the absence or presence of ergosterol is presented in Table III. It should be stressed that replacement of tetrahymanol in membranes by ergosterol induces a profound alteration in phospholipid class composition, and a marked increase of phosphatidylethanolamine (8–9 %) with a compensating decrease of phosphatidylcholine plus 2-aminoethylphosphonolipid. Phosphatidylcholine decreases in cilia more than in pellicles. The total amount of phospholipids does not change markedly in these two cell types.

In order to elucidate the possible mechanism of induction of such marked changes in membrane phospholipids, we have performed preliminary radioisotope-labeling experiments. Ergosterol-supplemented and unsupplemented *T. pyriformis* cells were labeled with sodium [1-^{14}C]acetate and the radioactivity incorporated

TABLE III

ALTERATIONS INDUCED BY ERGOSTEROL-REPLACEMENT IN PHOSPHOLIPID COMPOSITION OF SURFACE MEMBRANES OF *T. PYRIFORMIS*

Lipids were extracted from the isolated membranes according to the method of Bligh and Dyer [17]. Individual phospholipids were separated on silica gel G thin-layer chromatographic plates and analyzed by the modified Bartlett's method [18]. The numbers in parentheses represent the number of different experiments. Values are means ±S.D.

Phospholipids	Mol percentage of phospholipid of:					
	Whole cells		Cilia		Pellicles	
	Native (4)	Ergosterol-replaced (7)	Native (3)	Ergosterol-replaced (3)	Native (3)	Ergosterol-replaced (3)
Lysophosphatidylcholine	3.2±1.7	2.1±0.9	1.6±0.2	2.2±0.9	4.7±1.2	4.0±1.7
Phosphatidylcholine	30.8±1.2	26.8±1.2	33.3±1.9	26.5±2.0	22.9±2.9	19.6±0.8
Lysoaminoethylphosphonolipid and lysophosphatidylethanolamine	2.1±1.0	3.0±1.0	2.6±1.8	2.4±0.3	1.9±0.8	1.1 ±0.4
Phosphatidylethanolamine	32.4±1.9	37.7±3.0	10.9±0.8	18.2±3.3	33.8±2.9	42.6±1.3
2-Aminoethylphosphonolipid	24.5±0.2	22.9±0.9	48.8±0.8	46.6±4.1	33.1±1.9	28.8±1.8
Cardiolipin	5.1±1.6	5.9±0.7	0.9±0.4	1.0±0.3	1.4±0.9	1.3±0.8

TABLE IV

INCORPORATION OF [^{14}C]ACETATE INTO MAJOR PHOSPHOLIPIDS OF NATIVE AND ERGOSTEROL-REPLACED *T. PYRIFORMIS* CELLS

T. pyriformis cells were grown in the presence or absence of ergosterol to the mid-logarithmic phase. The cells were labeled with sodium [1-^{14}C]acetate and then at intervals aliquots were withdrawn to measure the radioactivity in major phospholipids. Individual phospholipids were separated on silica gel G plates using the solvent system chloroform/acetic acid/methanol/water (75 : 25 : 5 : 2.2, v/v). Figures are the averages of three different experiments.

Incubation time (min)	Percentage of total incorporation of [^{14}C]acetate in:					
	Phosphatidylcholine		Phosphatidylethanolamine		2-Aminoethylphosphonolipid	
	Native	Ergosterol-replaced	Native	Ergosterol-replaced	Native	Ergosterol-replaced
30	33.1	27.3	23.1	28.6	11.5	8.6
90	30.1	28.6	24.6	34.3	18.9	13.6
210	26.4	24.2	24.0	33.3	23.0	17.1

TABLE V

FATTY ACID COMPOSITION OF TOTAL LIPIDS FROM NATIVE AND ERGOSTEROL-REPLACED SURFACE MEMBRANES OF *T. PYRIFORMIS*

Lipids were extracted according to the method of Bligh and Dyer [17] from the membranes from cells grown in the presence or absence of ergosterol. For fatty acid analysis the lipids were saponified, methylated and examined by gas-liquid chromatography. Numbers in parentheses represent the number of different experiments. Peaks of $C_{16:1}$ and $C_{17:0}$ were overlapped and therefore estimated by dividing them tentatively.

Fatty acids	Percentage fatty acid composition of total lipids from:					
	Whole cells		Cilia		Pellicles	
	Native (4)	Ergosterol-replaced (7)	Native (6)	Ergosterol-replaced (7)	Native (6)	Ergosterol-replaced (5)
$C_{12:0}$	1.7	2.4	0.7	2.1	3.6	5.2
$C_{14:0}$	5.3	7.3	4.3	7.5	7.7	10.5
$C_{15:0}$ (iso)	1.3	1.3	0.8	1.2	2.1	1.6
$C_{15:0}$	0.8	0.8	1.2	1.6	1.0	1.3
$C_{16:0}$ (iso)	0.5	0.6	0.9	1.1	0.5	1.0
$C_{16:0}$	9.0	9.4	10.4	12.9	11.8	13.5
$C_{16:1}$ (Δ^9)	3.2	1.0	trace	trace	3.9	1.1
$C_{17:0}$ (iso)	7.8	13.2	7.3	10.9	8.2	11.9
$C_{17:0}$	1.2	2.4	0.9	2.0	1.2	2.3
$C_{17:1}$	1.6	2.7	1.4	2.1	1.2	1.9
$C_{18:0}$	1.1	0.8	2.9	3.7	1.5	1.2
$C_{18:1}$ (Δ^9)	8.2	6.4	10.0	9.4	10.7	7.6
$C_{18:2}$ ($\Delta^{6,9}$)	3.8	5.5	6.8	7.5	4.2	5.3
$C_{18:2}$ ($\Delta^{9,12}$)	20.2	17.0	14.7	11.2	15.2	12.4
$C_{18:3}$ ($\Delta^{6,9,12}$)	34.6	29.6	39.1	28.7	27.4	23.6
Total unsaturated	71.6	62.2	72.0	58.9	62.6	51.9
Total saturated	28.7	38.2	29.4	43.0	37.6	48.5
Total unsaturated/total saturated	2.5	1.6	2.5	1.4	1.7	1.1

into the three major phospholipids was measured. The results shown in Table IV demonstrate a preferential incorporation of [^{14}C]acetate into phosphatidylethanolamine rather than into phosphatidylcholine and 2-aminoethylphosphonolipid, in the ergosterol-replaced cells. From the data presented here it can be strongly suggested that the newly inserted ergosterol does somehow affect certain biosynthetic sites of phospholipids and induces adequate modification of membrane phospholipids.

Effect of ergosterol replacement upon fatty acyl composition in the surface membrane lipids

The results presented above on membrane phospholipid-composition changes prompted us to investigate the influence of ergosterol-replacement upon fatty acid composition in surface membrane lipids. There are significant and reproducible quantitative but not qualitative changes in the fatty acid composition (Table V). Specifically, an overall tendency is shown for increase in saturated and decrease in unsaturated fatty acid in the ergosterol-replaced cells and surface membranes. This lowers the ratio of total unsaturated to saturated fatty acid, e.g. from 2.5 to 1.4 for cilia and from 1.7 to 1.0 for pellicles. These marked changes are principally due to a decrease of two major unsaturated, linoleic ($C_{18:2}$, $\Delta^{9,12}$) and γ-linolenic ($C_{18:3}$, $\Delta^{6,9,12}$) acids, together with a compensating increase of myristic ($C_{14:0}$), palmitic ($C_{16:0}$) and isomargaric acids ($C_{17:0}$).

Alteration of the fatty acyl composition in individual phospholipids of ergosterol-replaced membranes

The effects of ergosterol-replacement on the degree of saturation of the principal phospholipid acyl chains are given in Tables VI and VII. There is a common trend towards increased saturation and decreased unsaturation in all three phospholipids of the ergosterol-replaced cells. Such a significant decrease in the unsaturation of acyl chain composition is mainly due to a decrease in palmitoleic ($C_{16:1}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$) and γ-linolenic ($C_{18:3}$) acids. It is worth noting that the most abundant fatty acid, γ-linolenic acid ($C_{18:3}$), is exclusively present in phospholipids, especially 2-aminoethylphosphonolipid (about 50 %). Little is seen in the neutral lipid fraction (Table VI). It should be noted, furthermore, that the ratio of total unsaturated to saturated acyl chains becomes lower in the order phosphatidylethanolamine < phosphatidylcholine < 2-aminoethylphosphonolipid. This indicates that phosphatidylethanolamine acyl chains are more saturated as compared with other phospholipids.

These observations are, however, more prominent with the pellicle membrane phospholipids (Table VII). Especially in the phosphatidylethanolamine of the ergosterol-replaced pellicle membranes, the ratio of total unsaturated to total saturated acyl chains goes down from 1.1 to 0.6. The noticeable increase in myristic and palmitic, and decrease in oleic ($C_{18:1}$, Δ^{9}), linoleic ($C_{18:2}$, $\Delta^{9,12}$) and γ-linolenic ($C_{18:3}$, $\Delta^{6,9,12}$) acids are involved in such a considerably lower degree of unsaturation. A marked decrease in unsaturated acyl chains is also observed in the 2-aminoethylphosphonolipid but an increase of unusual fatty acid, γ-linoleic acid ($C_{18:2}$, $\Delta^{6,9}$) should be noted. Unlike phosphatidylethanolamine, myristic and palmitic acids are present in much smaller amounts in the 2-aminoethylphosphonolipid and do not increase even by ergosterol replacement.

TABLE VI

FATTY ACYL CHAIN COMPOSITION OF MAJOR PHOSPHOLIPIDS FROM NATIVE AND ERGOSTEROL-REPLACED *T. PYRIFORMIS* CELLS

Lipids were extraced according to the method of Bligh and Dyer [17]. Individual phospholipids were separated on silica gel G thin-layer plates and eluted. For fatty acid analysis each phospholipid fraction was saponified, methylated and examined by gas-liquid chromatography. The numbers in parentheses represent the number of different experiments.

Fatty acyl chains	Percentage fatty acid composition of major phospholipids							
	Phosphatidyl-choline		Phosphatidyl-ethanolamine		2-Aminoethyl-phosphonolipid		Neutral lipids	
	Native (3)	Ergosterol-replaced (2)	Native (3)	Ergosterol-replaced (2)	Native (3)	Ergosterol-replaced (2)	Native (3)	Ergosterol-replaced (2)
$C_{12:0}$	0.7	trace	2.3	3.0	0.4	trace	3.7	2.8
$C_{14:0}$	3.7	3.3	7.0	8.9	2.4	2.7	6.7	10.3
$C_{15:0}$ (iso)	1.0	0.8	1.7	1.7	0.6	trace	2.9	3.4
$C_{15:0}$	0.6	0.6	1.0	0.7	0.4	0.4	1.4	1.8
$C_{16:0}$ (iso)	trace	trace	trace	trace	trace	trace	1.6	1.8
$C_{16:0}$	9.1	8.8	10.1	11.7	5.1	4.9	11.8	17.8
$C_{16:1}$ (Δ^9)	4.1	1.2	3.3	1.6	1.1	trace	7.0	trace
$C_{17:0}$ (iso)	7.4	12.3	10.4	15.5	4.1	7.2	13.1	19.1
$C_{17:0}$	1.7	4.5	1.2	2.5	0.7	1.6	2.6	3.9
$C_{17:1}$	1.6	1.9	1.8	3.4	1.0	1.5	2.5	2.9
$C_{18:0}$	1.5	1.4	0.5	0.5	0.6	0.7	7.4	5.4
$C_{18:1}$ (Δ^9)	11.0	8.2	9.8	7.7	6.8	5.2	16.7	9.6
$C_{18:2}$ ($\Delta^{6,9}$)	4.7	6.5	2.8	2.6	8.1	14.9	3.4	2.6
$C_{18:2}$ ($\Delta^{9,12}$)	19.2	17.1	21.0	17.8	18.0	14.2	10.0	6.6
$C_{18:3}$ ($\Delta^{6,9,12}$)	34.0	33.2	27.3	22.5	50.9	46.5	9.6	10.4
Total unsaturated	74.6	68.1	66.0	55.6	85.9	82.3	49.2	32.1
Total saturated	25.7	31.7	34.2	44.5	14.3	17.5	51.1	66.3
Total unsaturated/ total saturated	2.9	2.2	1.9	1.3	6.0	4.7	1.0	0.5

TABLE VII

FATTY ACYL CHAIN COMPOSITON OF MAJOR PHOSPHOLIPIDS FROM NATIVE AND ERGOSTEROL-REPLACED PELLICLE MEMBRANES OF *T. PYRIFORMIS*

Lipids were extracted according to the method of Bligh and Dyer [17] from pellicle membranes from cells grown in the presence or absence of ergosterol. Individual phospholipids were separated on thin-layer plate and eluted. For fatty acid analysis each phospholipid fraction was saponified, methylated and examined by gas-liquid chromatography. The numbers represent the number of different experiments.

Fatty acyl chains	Percentage fatty acid composition of major phospholipids					
	Phosphatidyl-choline		Phosphatidyl-ethanolamine		2-Aminoethyl-phosphonolipid	
	Native (3)	Ergosterol-replaced (2)	Native (3)	Ergosterol-replaced (2)	Native (3)	Ergosterol-replaced (2)
$C_{12:0}$	1.0	0.4	5.8	7.5	trace	trace
$C_{14:0}$	4.9	4.9	9.7	13.9	2.3	3.6
$C_{15:0}$ (iso)	1.1	0.5	2.4	2.7	0.7	trace
$C_{15:0}$	0.7	0.4	0.9	1.6	trace	trace
$C_{16:0}$ (iso)	0.6	0.5	trace	trace	trace	trace
$C_{16:0}$	12.3	12.4	15.8	19.1	4.4	4.9
$C_{16:1}$ (Δ^9)	5.0	1.5	4.9	1.4	0.6	trace
$C_{17:0}$ (iso)	9.5	14.8	11.0	15.1	4.1	8.1
$C_{17:0}$	2.0	5.6	1.6	3.1	0.7	1.8
$C_{17:1}$	1.4	2.0	1.5	2.6	1.0	1.5
$C_{18:0}$	2.6	2.0	0.9	0.7	0.4	0.6
$C_{18:1}$ (Δ^9)	13.3	9.2	12.6	8.2	6.7	4.8
$C_{18:2}$ ($\Delta^{6,9}$)	3.7	5.0	2.8	2.4	9.8	12.8
$C_{18:2}$ ($\Delta^{9,12}$)	16.6	15.3	16.0	11.3	16.9	13.2
$C_{18:3}$ ($\Delta^{6,9,12}$)	26.5	25.9	13.8	10.5	52.6	47.8
Total unsaturated	65.5	59.9	51.6	36.4	87.6	80.1
Total saturated	34.7	40.5	48.1	63.7	12.6	19.9
Total unsaturated/ total saturated	1.9	1.5	1.1	0.6	7.0	4.0

From the results presented here indicating the marked alteration in phosphatidylethanolamine acyl chain composition, taking into consideration that the amount of this phospholipid increases considerably by ergosterol replacement, it can be concluded that the phosphatidylethanolamine molecules would play an important role in structure or function of the surface membrane.

DISCUSSION

In recent years much attention has been paid to the cells of the ciliate *Tetrahymena* from the ultrastructural as well as biochemical points of view [28]. Particularly, *T. pyriformis* is a potentially excellent model system for membrane studies, since this organism has several favorable advantages. (1) It is a large, unicellular organism without a rigid cell wall. (2) It shows bacteria-like rapid growth in axenic culture, with (3) easy isolation of different subcellular organelles and (4) enrichment

with unusual membrane lipids i.e., C-P-containing phospholipids (phosphonolipids) and a pentacylic triterpene alcohol (tetrahymanol). Our series of studies using *T. pyriformis* membranes have produced much useful information regarding membrane biogenesis [29, 30].

In addition, it should be of great interest to note that this organism can undergo considerable modification of membrane lipids in response to the growth environment. This may indicate that such environmentally induced alteration in biological membrane lipids would be responsible for maintaining the suitable membrane fluidity for proper functions. It is now widely recognized that the lipids play a major role in controlling the fluidity of biomembranes [2].

In our recent ESR experiments [5], we have examined the effects of temperature-induced alteration in fatty acyl composition on the membrane fluidity. It was demonstrated that the membrane becomes more fluid in the order of microsomes > pellicles > cilia and that membranes from cells grown at 34 °C are less fluid than those from 15 °C-grown cells, thus indicating the predominant role of the degree of unsaturation in acyl chains.

In the present study, as another approach to the lipid modification in *Tetrahymena* membranes, we used the ergosterol-replacement technique by which the native tetrahymanol can be readily replaced by exgenous supplementation of ergosterol [25]. Observations from scanning and freeze-etch electron microscopy suggest that the newly inserted ergosterol molecules would be present in lipid bilayer regions and be reponsible for membrane lipid organization. The lesions induced by filipin in ergosterol-replaced cilia and pellicle membranes are very similar, if not identical, with those observed in fungal cell membranes containing ergosterol as the native principal sterol. Unsupplemented tetrahymanol-containing membranes are not affected by the polyene at the concentrations tested. This might be due to the difference in chemical structure between ergosterol and tetrahymanol.

By incorporation of ergosterol in *Tetrahymena* surface membranes, noticeable alterations occur in the composition of the phospholipid as well as its fatty acyl chains. First, for the phospholipid class composition change it should be stressed that phosphatidylethanolamine increases markedly in ergosterol-replaced cilia and pellicle membranes. In contrast, there is a compensatory decrease in phosphatidylcholine and 2-aminoethylphosphonolipid. The precise mechanism for and significance of such an increase of phosphatidylethanolamine is not known. However, it could be possible that ergosterol replacement produces some inhibitory effects on stepwise methylation of phosphatidylethanolamine to form phosphatidylcholine. As several reports have shown [8–10] that the polar head group is also an important factor in regulating the membrane fluidity, it might be that increase of phosphatidylethanolamine in ergosterol-containing *T. pyriformis* surface membranes would compensate for the sterol change.

Moreover, besides such changes in the polar head group of membrane phospholipids, alterations in fatty acyl compositions of individual phospholipids are significantly observed. Although there is an overall tendency for lowering the level of unsaturated acyl chains in ergosterol-replaced pellicle membrane phospholipids the phosphatidylethanolamine, in particular, shows the less unsaturated acyl chain composition (36 %), when compared with phosphatidylcholine (59 %) and 2-aminoethylphosphonolipid (80 %). This noticeable decrease in unsaturation is accomplished

mainly by the long chain unsaturated fatty acids; oleic, linoleic and γ-linolenic. In addition, there is a corresponding increase in myristic and palmitic acids. The mechanism for this acyl composition change is not clear, but one might expect probable impairment of chain elongation and/or desaturation reactions.

As clearly demonstrated by the data of the present study, the ergosterol-replaced *Tetrahymena* surface membranes contain larger amounts of phosphatidylethanolamine and its acyl chains are more saturated than those of the native membranes. The two other phospholipids associated with less saturated acyl groups, on the other hand, are decreased. These changes should lead to a considerable modification of the overall membrane phospholipid as well as acyl chain composition, and it is therefore not unreasonable to assume that such alterations are required to accomodate the newly incorporated ergosterol molecules so that the proper membrane fluidity can be maintained. Ergosterol is a molecule similar, though not identical, with tetrahymanol. The latter has extra methyl groups at C-4 and C-14, and also cyclization of its side chain instead of ergosterol's free paraffin chain. Therefore, ergosterol would not be able to interact properly with phospholipids without altering the fatty acyl composition. This may suggest evidence for selective interaction of sterols with phospholipids in biological membranes [31]. It could also be that tetrahymanol is involved in the regulatory mechanism of the fluidity in the *Tetrahymena* membrane, and that this sterol-like compound plays some important roles in membrane functions. Recently, Beedle et al. [26] has proposed, to explain tetrahymanol biosynthesis inhibition by the inclusion of cholesterol, a working hypothesis that membrane-bound enzymes responsible for tetrahymanol biosynthesis would become inactive by their conformational changes. But this idea is still not beyond the speculative stage. More recently, in preliminary experiments by ESR, we have obtained data demonstrating that the ergosterol-replaced surface membranes are less fluid than the native membranes, which contain the naturally occurring tetrahymanol. The detailed data will be published elsewhere in the near future. Although much further research is needed, this membrane lipid modification system in *T. pyriformis* cell would hopefully be able to provide useful information regarding the phospholipid-sterol interaction in the biological membranes.

ACKNOWLEDGEMENTS

The authors wish to express thanks to Dr G. B. Whitfield for kindly supplying filipin, to Mr N. Akimori for helping us with gas chromatography-mass spectrometry analysis and also to Mr T. Sekiya for technical assistance in electron microscopy. This work was supported in part by grant No. 968022 from the Ministry of Education in Japan and also by grant No. 5R070 under the Japan-USA Co-operative Science Research Program from the Japan Society for the Promotion of Science.

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