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MODIFICATION OF MEMBRANE LIPIDS

PHENETHYL ALCOHOL-INDUCED ALTERATION OF LIPID COMPOSITION IN *TETRAHYMENA* MEMBRANES

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Summary

Tetrahymena pyriformis NT-I cells in the early-logarithmic phase were incubated with phenethyl alcohol (2-phenylethanol) and effects on the lipid composition were examined in various membranes.

1. There was a marked modification in phospholipid head, as well as fatty acyl group composition in pellicles, mitochondria and microsomes of the phenethyl alcohol-treated cells. Compared with membranes of the control cells, the membranes from phenethyl alcohol-treated cells were found to contain a higher level of phosphatidylcholine content with the compensating decrease in phosphatidylethanolamine, while 2-aminoethylphosphonolipid showed only a slight decrease in these membranes. The acyl group profile of membrane phospholipids in the presence of phenethyl alcohol was also modified so that a profound elevation of the content of polyunsaturated fatty acids, linoleic and γ-linolenic acids. The major monounsaturate, palmitoleate decreased. Such lipid alteration is a reversible process, and therefore upon removal of phenethyl alcohol the modified lipid composition returned to normal.

2. By freeze-fracture electron microscopy in combination with temperature quenching, the outer alveolar membrane of the phenethyl alcohol-treated cell was observed to reveal less aggregation of intercalated-membrane particles, as compared with the control membrane. The quantitative analysis of the thermotropic lateral movement of membrane particles provided evidence that the membrane in the phenethyl alcohol-treated cell became more fluid. Such fluidizing effects may result from an increase in the acyl group unsaturation and also in the phosphatidylcholine content.

3. With regard to the mechanism responsible for the marked decrease in

palmitoleate in membrane phospholipids, there was found a depressed conversion of the palmitate to palmitoleate in the phenethyl alcohol-treated cells. It was further suggested that the drug may have an inhibitory effect on the synthesis of palmitoyl-CoA desaturase involving the (16 : 0 → 16 : 1) conversion. Also, it was demonstrated that the increase in a precursor-product fashion of phosphatidylcholine with the corresponding decrease in phosphatidylethanolamine was not due to transformation of phosphatidylethanolamine to phosphatidylcholine through stepwise methylation.

Introduction

In order to shed light on the problem of the structure-function relationship in biomembranes, one useful approach has been to manipulate either quantitatively or qualitatively the membrane lipid composition of cells under the defined growth conditions, and to examine functions in the membranes with altered lipid composition. Although various methods for modifying membrane lipids have been available [1,2], one of the most commonly used procedures is to supplement cells with different substances. The majority of studies of membrane lipid manipulation has largely involved alterations in the phospholipid acyl chain composition in bacteria and mycoplasma [1,3,4]. However, these manipulation techniques have recently been extended to animal cells growing in tissue cultures for altering not only fatty acid [5—7] but also polar head group [8—12] composition of membrane phospholipids. Indeed, since Vagelos' group has developed techniques to manipulate the phospholipid composition of mouse fibroblasts in tissue culture with choline analogues [8], they have extensively investigated effects of lipid alterations upon physical properties and enzyme activities of membranes [13—16]. Since *Tetrahymena* cells have a proven ability to modify their membrane lipid composition in response to growth conditions, this cell also is a promising system for better understanding the roles which lipids play in membrane-associated activities in the cell [17]. Actually, supplementation with sterols [18,19], fatty acids [20,21], glyceryl ether [22], choline analogues (unpublished data) has been applied to *Tetrahymena* cells.

Recently, Nunn and co-workers [23—25] have shown that phenethyl alcohol induces a drastic alteration in the lipid composition in *Escherichia coli.* We applied this drug to *Tetrahymena* cells for modifying the membrane lipid composition. In this communication, we describe the phenethyl alcohol-induced alteration in lipid composition of various membranes, and also the mechanisms for lipid alterations by the drug will be discussed.

Materials and Methods

Cell growth and membrane isolation. Culture conditions for strain NT-I of *Tetrahymena pyriformis* were described in a previous paper [26]. Cells were grown at 39.5°C in an enriched proteose-peptone medium in the shaking incubator at 90 cycles per min. Cell growth was monitored by counting cell number by a hemocytometer. When cell density reaches the early logarithmic

phase, $20 \cdot 10^4$ cells/ml, phenethyl alcohol (2-phenylethanol, $C_6H_5CH_2CH_2OH$, Nakarai Kagaku Chemicals Co., Kyoto) was added dropwise into the culture medium to a final concentration of 5.5 mM and then incubation was further continued. Isolation of various membrane fractions from control and phenethyl alcohol-treated cells was performed according to the procedure of Nozawa and Thompson [27].

Lipid extraction and analysis. Lipids were extracted from whole cells and various membrane fractions by the method of Bligh and Dyer [28], and individual phospholipids were separated on silica gel H plate. After charring the developed plate with 50% H_2SO_4, the bands corresponding to individual phospholipids were scraped off the plate and their phosphorus contents were determined by the Rouser's method [29]. The phospholipid fraction was separated from neutral lipids by a silicic acid-Hyflo Super Cel column chromatography as previously described [26]. Total phosphorus in extracted lipids was determined by the method of Bartlett [30], as modified by Marinetti [31]. Quantitative analysis of fatty acids was carried out by gas-liquid chromatography as previously described [26].

Radioisotope labeling. For determination of lipid synthesis, 20-ml aliquots of the growth culture were withdrawn at several times during incubation with phenethyl alcohol and labeled with 5 μCi of [^{14}C]acetate (NEN, 58.3 Ci/mol) for 30 min before extraction. The incorporation of [^{14}C]acetate into the individual lipid fractions was measured by counting the radioactivities in areas corresponding to lipid fractions separated on silica gel H plate using a Beckman LS-8000 scintillation counter. The desaturation rate of palmitate to palmitoleate was measrued according to the procedure described previously [32]. Samples (20 ml) were taken at desired intervals from the control and phenethyl alcohol-treated cultures, and pulse-labeled with 0.25 μCi of [^{14}C]palmitate (NEN, 50.2 Ci/mol) for 20 min. Furthermore, the rate of conversion of phosphatidylethanolamine into phosphatidylcholine was estimated by incorporation of [^{14}C]ethanolamine into phosphatidylcholine fraction. The 200-ml culture was labeled with 5 μCi of [^{14}C]ethanolamine (The Radiochemical Centre, Amersham, 55 Ci/mol) for 165 min, and labeled cells were harvested by centrifugation at $164 \times g$ for 5 min and the resuspended in the fresh medium. The culture was divided into two portions and to one of them phenethyl alcohol was added to a concentration of 5.5 mM. Lipids were extracted from both cultures at different intervals during labeling. The radioactivities in individual phospholipids separated on the thin-layer plate were measured as above.

Freeze-fracture electron microscopy. Freeze-fracture electron microscopy has been proved to be an useful technique for the quantitative analysis of physical states of membrane lipids. Detailed procedures have been described in the previous paper [33]. The particle density index was used, which can be calculated from the equation $(X - A/B - A) \times 100$, where A, B and X is the particle number of 39.5°C-, 0.5°C-fixed membranes and membrane to be examined at a given temperature, respectively. Cells grown at 39.5°C in the presence and absence of phenethyl alcohol were cooled to the desired temperatures over 4 min. After 5 min incubation at each temperature samples were fixed by glutaraldehyde at a final concentration of 1%. The fixed samples were freeze-fractured in Hitachi device and the obtained replicas were examined

with a JEM 100U electron microscope. The protoplasmic fracture face of the outer alveolar membrane was used to monitor distribution of membrane-intercalated particles for determination of the particle density index.

Results

Effects of phenethyl alcohol on growth and lipid synthesis in T. pyriformis NT-I

Phenethyl alcohol at different concentration (0.6—9.8 mM) was added to the culture in the early logarithmic phase. There was a somewhat dose-dependent inhibitory effect on the cell growth. When the cells were incubated with 5.5 mM phenethyl alcohol, their shape and motility was quite normal. Fig. 1 depicts the growth curve of *T. pyriformis* NT-I in the presence and absence of 5.5 mM phenethyl alcohol. After addition of the drug to growing cultures, a gradual decrease in the rate of growth occurs. However, upon transferring phenethyl alcohol-treated cells into the drug-free medium, cells caused a resumption of growth at a normal rate after a certain lag for approx. 30 min. This may indicate that phenethyl alcohol effects are exerted in a reversible fashion. In order to examine phenethyl alcohol's influence on lipid synthesis, cells taken at different intervals during phenethyl alcohol treatment were pulse-labeled with [^{14}C]acetate for 30 min, and the incorporation of the radioactivity into total lipids, phospholipid and triacylglycerol fractions was measured. The relative specific activity (cpm/lipid phosphorus) of 6-h phenethyl alcohol-treated cells was 82% of the control, non-treated cells, indicating a mild inhibition of lipid synthesis. Following phenethyl alcohol addition there was a marked reduction in [^{14}C]acetate incorporation into phospholipids (82.5 → 69.0%), accompanied by a concomitant increase in the triacylglycerol fraction

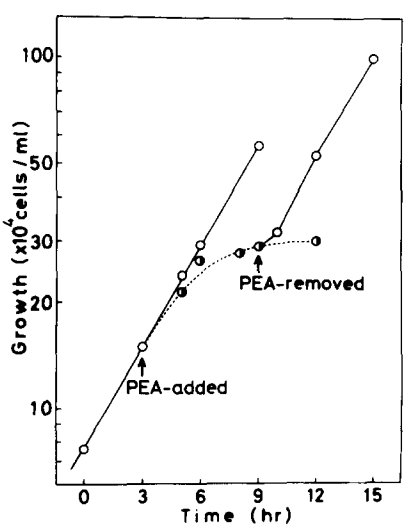


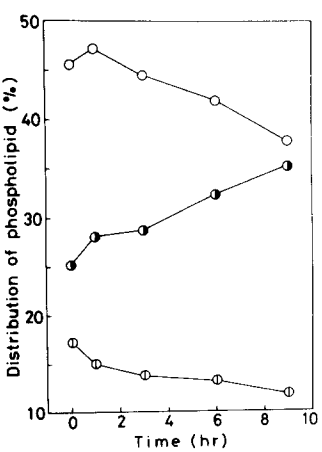
Fig. 1. Effects of phenethyl alcohol on the cell growth. Phenethyl alcohol (5.5 mM) was added and removed as indicated. ○, in the absence of phenethyl alcohol (PEA); ●, in the presence of phenethyl alcohol.

(4.8 → 15.6%), suggesting that phospholipid synthesis would be fairly inhibited. However, removal of phenethyl alcohol from the medium was shown to lead to restoration of acetate incorporation into phospholipids to the normal level.

Alterations by phenethyl alcohol in lipid composition of whole cells

Changes in the phospholipid polar head group composition in cells exposed to 5.5 mM phenethyl alcohol for up to 9 h are shown in Fig. 2. Since there was no change in the amount of lipid phosphorus in the cell (0.65—0.70 μg phosphorus/10^5 cells) during this incubation period, values shown in Fig. 2 may reflect the proportional composition based on the net amounts of phospholipids in a cell. The content of phosphatidylcholine increases gradually with time at the expense of phosphatidylethanolamine. In contrast small changes are seen for 2-aminoethylphosphonolipids.

The presence of phenethyl alcohol was found to result in a profound change in the phospholipid fatty acyl group composition (Fig. 3). The proportion of polyunsaturated fatty acids, linoleic (18 : 2) and γ-linolenic (18 : 3 (6, 9, 12)) acids increases progressively with time. In contrast, the level of palmitoleic acid (16 : 1) exhibits a considerable decrease depending on length of the incubation period. As for the saturated fatty acids, there is a slight increase in the percentage of palmitic acid (16 : 0), whereas the decline of myristic acid (14 : 0) content is seen. Furthermore, it was demonstrated that the phenethyl alcohol treatment caused a great increase in the unsaturation index at an almost linear rate. These results indicate that phospholipids of phenethyl alcohol-treated cells have more double bonds in one molecule, and also that phenethyl alcohol might exert certain effects on desaturation of fatty acids. In particular, the conversion of palmitic into palmitoleic acid seems to be depressed, which was confirmed by data of [^{14}C]acetate incorporation into these two fatty acids (not shown). Upon removal of phenethyl alcohol from the medium the composition of phospholipid and fatty acid was observed to restore to that of the control.



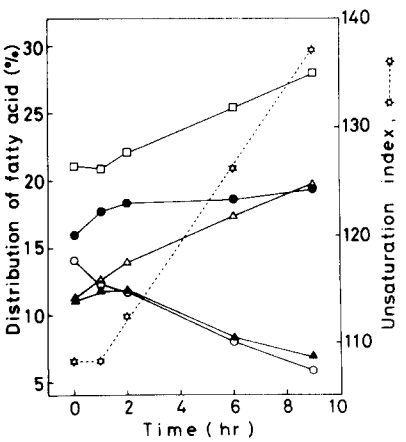


Fig. 2. Alterations in the phospholipid head group composition after addition of phenethyl alcohol. ○, phosphatidylethanolamines; ●, phosphatidylcholines; ⊕, 2-aminoethylphosphonolipids.

Fig. 3. Alterations in the phospholipid acyl group composition after addition of phenethyl alcohol. □, 18 : 3 (6, 9, 12); △, 18 : 2; ●, 16 : 0; ○, 16 : 1; ▲, 14 : 0.

TABLE I

PHENETHYL ALCOHOL-INDUCED ALTERATION IN PHOSPHOLIPID CLASS COMPOSITION OF VARIOUS MEMBRANE FRACTIONS IN *T. PYRIFORMIS* NT-I

Tetrahymena cells were grown in the absence of phenethyl alcohol at 39.5°C. When the culture reached logarithmic phase, phenethyl alcohol was added into the growth medium with shaking at a final concentration of 5.5 mM. After cells were treated by phenethyl alcohol for 6 h, cell fractionation was performed using the procedure of Nozawa and Thompson [27]. Lipids were extracted from various membrane fractions by the method of Bligh and Dyer [28]. Phospholipids were separated on a silica gel H thin layer plate in a solvent system. chloroform/acetic acid/methanol/water (75 : 25 : 5 : 2.2, v/v). For estimation of the phospholipid class composition, the areas corresponding to individual phospholipids were scraped off the plate, and their phosphorus contents were measured by a minor modification of the method of Rouser et al. [29]. The results are averages of two experiments for the control and five experiments for the phenethyl alcohol-treated cells, and are expressed as percentage of total phospholipids. PEA, phenethyl alcohol-treated cells.

Phospholipids	Whole cell		Pellicles		Mitochondria		Microsomes	
	Control	PEA	Control	PEA	Control	PEA	Control	PEA
Phosphatidylcholine	25.5 ± 0.8	32.7 ± 2.4	19.0	26.7 ± 2.4	27.6	34.3 ± 2.5	28.5	34.5 ± 2.2
Lysophosphatidylcholine	2.0 ± 1.2	1.8 ± 1.2	0.1	2.8 ± 1.0	1.0	1.9 ± 1.3	3.2	3.8 ± 1.2
Phosphatidylethanolamine	45.5 ± 1.4	42.2 ± 1.1	49.6	42.3 ± 1.1	45.8	42.4 ± 0.7	44.1	38.7 ± 0.8
2-Aminoethylphosphonolipid	16.7 ± 1.0	13.1 ± 1.1	23.3	18.0 ± 1.2	12.6	9.4 ± 0.9	18.0	14.6 ± 1.4
Lysophosphatidylethanolamine plus lyso-2-aminoethylphosphonolipid	—	—	—	—	—	0.1 ± 0.2	0.4	0.9 ± 0.5
Cardiolipin	6.1 ± 1.2	7.0 ± 0.9	2.5	4.1 ± 1.0	10.2	10.4 ± 1.3	1.7	3.3 ± 0.9
Unknown	2.9 ± 0.6	2.6 ± 1.0	5.3	5.6 ± 0.3	1.8	0.8 ± 1.0	3.7	4.2 ± 1.2

Phenethyl alcohol-induced alteration in lipid composition of various subcellular membrane fractions

Phospholipid polar head group. As some considerable changes was found to be induced by phenethyl alcohol treatment in phospholipid head groups as well as acyl group composition, subcellular fractionation was carried out to see how the lipid composition is altered in various membrane fractions. In general, it is of importance to note the common trend that the presence of phenethyl alcohol causes a decrease in the ethanolamine-containing phospholipids with an increase in phosphatidylcholines. The similar or more profound trends of lipid changes observed for the whole cells are reflected in all membrane fractions tested (Table I). The surface membrane, pellicle is most remarkable in alteration of phospholipid composition.

Phospholipid acyl group. Table II shows the fatty acid composition of phospholipids from pellicles, mitochondria and microsomes of the control and phenethyl alcohol-treated cells. The most profound alterations occurred with 16 : 0, 16 : 1, 18 : 2, 18 : 3 and large differences in the ratio of 16 : 0/16 : 1 between membranes from the control and phenethyl alcohol-treated cells, especiially in microsomal fraction. In the three membrane fractions isolated from the phenethyl alcohol-treated cells, there was a marked elevation

TABLE II

PHENETHYL ALCOHOL-INDUCED ALTERATION IN PHOSPHOLIPID FATTY ACID COMPOSITION OF VARIOUS MEMBRANE FRACTIONS IN *T. PYRIFORMIS* NT-I

Lipids were extracted from various isolated membrane fractions. Phospholipids were separated from neutral lipids using silicic acid-Hyflo Super Cel column chromatography [26], and fatty acid composition of phospholipid was determined by gas-liquid chromatography. The results are averages of 2—3 different experiments and are expressed as weight percentage of total fatty acids. The error range is less than 5% of each figure. PEA, phenethyl alcohol-treated cells.

Fatty acids	Pellicles		Mitochondria		Microsomes	
	Control	PEA	Control	PEA	Control	PEA
12 : 0	5.7	2.8	0.8	1.8	1.0	0.9
14 : 0	13.1	10.0	7.8	6.5	11.2	7.7
15 : 0 (iso)	4.8	4.0	3.1	2.4	4.6	3.5
15 : 0	1.8	2.6	1.2	1.7	1.7	2.5
16 : 0	15.9	19.8	11.8	15.1	14.6	18.9
16 : 1 (9)	12.6	8.0	14.6	8.3	15.7	8.1
16 : 2 (?) *	5.8	4.0	6.5	4.1	7.5	4.3
18 : 0	2.2	2.1	2.5	2.2	2.5	2.0
18 : 1 (9)	3.8	4.3	3.4	3.0	4.0	3.9
18 : 2 (6, 11)	2.3	2.2	2.3	2.0	2.2	2.3
18 : 2 (9, 12)	8.8	15.2	13.3	18.7	10.3	17.2
18 : 3 (6, 9, 12)	18.4	21.2	28.1	30.1	20.0	24.6
16 : 0/16 : 1	1.26	2.48	0.81	1.82	0.93	2.33
18 : 2 + 18 : 3	27.2	36.4	41.4	48.8	30.3	41.8
U/S **	1.10	1.29	2.48	2.23	1.56	1.66
U.I. ***	93.8	110.7	133.5	143.0	104.7	124.8

* Contains 17 : 0.

** Ratio of total unsaturated to total saturated fatty acids.

*** U.I., unsaturation index, which is calculated from [percentage of each unsaturated fatty acid] × [number of double bond] × 100, expresses double-bonds per 100 acyl groups.

(8—11%) of the content of polyunsaturated acids, linoleic (18 : 2) and γ-linolenic (18 : 3 (6, 9, 12)) acids.

Whereas the ratio of total unsaturated to total saturated fatty acids was not considerably altered by phenethyl alcohol-treatment, the unsaturation index was observed to increase to a great extent in response to the presence of the drug, indicating a higher degree of fatty acid unsaturation.

In order to determine which phospholipid plays a dominant role in modifying the membrane acyl group composition in the phenethyl alcohol-treated cells, we have analyzed the fatty acid constituents of major phospholipids in pellicles and microsomes (Table III). Each phospholipid has a characteristic

TABLE III

FATTY ACYL CHAIN COMPOSITION OF MAJOR PHOSPHOLIPIDS FROM CONTROL AND PHENETHYL ALCOHOL-TREATED MEMBRANES OF *T. PYRIFORMIS* NT-I

Various membrane fractions were prepared as described in the legend for Table I. Lipids were extracted from various membrane fractions of control and phenethyl alcohol-treated cells by the method of Bligh and Dyer [28]. Individual phospholipids were separated on a silica gel H thin-layer plate by developing in a solvent system containing chloroform/acetic acid/methanol/water (75 : 25 : 5 : 2.2, v/v), and the corresponding areas were scraped off the plate into the centrifuge tubes. Each phospholipid fraction was extracted from silica gel with chloroform/methanol (1 : 1, v/v), and chloroform/methanol (1 : 9, v/v). The quantitative analysis of its fatty acids was carried out by gas-liquid chromatography [26]. The results are averages of two different experiments and expressed as weight percentage of total fatty acids. The values in parentheses express the absolute amount of each fatty acid in the total cell phospholipid (μmol/mg P_i). PEA, phenethyl alcohol-treated cells.

Fatty acids	Pellicles		Microsomes	
	Control	PEA	Control	PEA
Phosphatidylcholine				
14 : 0	8.3 (1.02)	7.0 (1.21)	9.0 (1.66)	6.6 (1.47)
16 : 0	14.9 (1.83)	22.0 (3.79)	15.2 (2.80)	21.6 (4.81)
16 : 1 (9)	13.8 (1.69)	7.7 (1.33)	14.8 (2.72)	8.0 (1.78)
16 : 2 (?) *	8.9 (1.09)	4.3 (0.74)	9.2 (1.69)	4.7 (1.05)
18 : 1 (9)	4.6 (0.56)	2.5 (0.43)	4.4 (0.81)	2.8 (0.62)
18 : 2 (6, 11)	2.7 (0.33)	2.3 (0.40)	2.4 (0.44)	2.8 (0.62)
18 : 2 (9, 12)	10.5 (1.29)	14.6 (2.51)	10.3 (1.89)	15.9 (3.54)
18 : 3 (6, 9, 12)	22.2 (4.68)	27.2 (4.68)	20.2 (3.71)	25.4 (5.65)
Phosphatidylethanolamine				
14 : 0	16.1 (5.15)	12.5 (3.41)	13.4 (3.81)	11.0 (2.75)
16 : 0	19.9 (6.37)	23.1 (6.30)	15.9 (4.52)	18.0 (4.49)
16 : 1 (9)	14.9 (4.77)	9.2 (2.51)	19.5 (5.55)	9.3 (2.32)
16 : 2 (?)	6.0 (1.92)	4.2 (1.15)	8.1 (2.30)	4.6 (1.15)
18 : 1 (9)	5.2 (1.66)	5.2 (1.42)	4.5 (1.28)	4.2 (1.05)
18 : 2 (6, 11)	— (—)	0.7 (0.19)	0.3 (0.09)	1.5 (0.37)
18 : 2 (9, 12)	8.1 (2.59)	15.9 (4.34)	9.6 (2.73)	18.3 (4.57)
18 : 3 (6, 9, 12)	8.7 (2.78)	14.0 (3.82)	12.5 (3.56)	18.8 (4.69)
2-Aminoethylphosphonolipid				
14 : 0	3.8 (0.57)	4.4 (0.51)	4.5 (0.52)	4.3 (0.41)
16 : 0	6.5 (0.98)	11.5 (1.34)	6.8 (0.79)	10.6 (1.00)
16 : 1 (9)	9.1 (1.37)	5.6 (0.65)	10.2 (1.18)	5.4 (0.51)
16 : 2 (?)	5.1 (0.77)	3.3 (0.38)	5.7 (0.66)	3.2 (0.30)
18 : 1 (9)	3.7 (0.56)	3.6 (0.42)	3.7 (0.43)	3.4 (0.32)
18 : 2 (6, 11)	5.8 (0.87)	6.2 (0.72)	5.6 (0.65)	6.2 (0.58)
18 : 2 (9, 12)	10.5 (1.58)	13.2 (1.53)	10.9 (1.27)	14.5 (1.37)
18 : 3 (6, 9, 12)	46.9 (7.05)	42.4 (4.92)	43.3 (5.03)	43.1 (4.06)

* Contains 17 : 0.

fatty acid profile. For example, aminoethylphosphonolipids of the control pellicle has a high content of γ-linolenic acid (46.9%), thereby being the most unsaturated phospholipid, whereas phosphatidylethanolamine contains only 8.7% of the fatty acid. The incubation with 5.5 mM phenethyl alcohol produced a general trend towards an increase of polyunsaturated fatty acids, 18 : 2 and 18 : 3, with the exception of aminoethylphosphonolipids in which no change or even a slight decrease occurred. The absolute concentrations of individual fatty acids in major phospholipids are also included in parentheses. In both pellicles and microsomes of phenethyl alcohol-treated cells, the greatest decrease in the net amounts of palmitoleic acid occurs with phosphatidylethanolamine, while phosphaticylcholine gives rise to the greatest increase in the palmitic acid content. The phosphatidylcholine or phosphatidylethanolamine seems to participate in the increase in polyunsaturates (linoleic and γ-linolenic acids) in pellicles or microsomes, respectively. On the other hand, the aminoethylphosphonolipids tends rather to decrease the polyunsaturated fatty acids. Therefore, it could be concluded that the marked increase in the unsaturation index of phospholipid acyl group composition in various membrane fractions (Table II) results entirely from phosphatidylcholine and phosphatidylethanolamine.

Altered physical states of the lipid modified membranes: A freeze-fracture study

Recently the technique of freeze-fracture electron microscopy has been proved to be a greatly useful tool to monitor the physical state of membranes. Quantitative measurements of lateral movement or translocation of membrane-intercalated particles have been done primarily on the basis of the particle density [33—35].

With *Tetrahymena* membranes, extensive work has been conducted by Wunderlich and his colleagues [36], indicating that thermotropic lateral separations of membrane protein particles may be due to a temperature-induced transition from the liquid crystalline to the crystalline lipid phase. Also, data of freeze-fracture studies were found to be fairly well correlated with those obtained using physical techniques, e.g. fluorescence, ESR, NMR or X-ray diffraction [37,38]. Furthermore, we have established a convenient method with freeze-fracture electron microscopy for estimating the membrane fluidity in *Tetrahymena*, which was described in detail in a previous paper [35]. Since it was expected that the membranes of which the lipid composition was modified by phenethyl alcohol treatment would be altered in their physical state, we examined the thermotropic profiles of the lateral movement of membrane-intercalated particles of both control and phenethyl alcohol-treated cells. For this purpose, we chose the outer alveolar membrane in the pellicle, since the membrane is most sensitive to temperature changes and its particles (~115 Å) are larger than those of other membranes (~85 Å). Some typical examples are displayed in Fig. 4. When cells grown at 39.5°C in the presence and absence of phenethyl alcohol were chilled to desired temperatures (33, 27, 15, 0°C) over 4 min, various degrees of lateral movement of membrane particles are observed. In general, compared with the control membrane, the phenethyl alcohol-membrane revealed less aggregation of intramembrane particles. The data of the

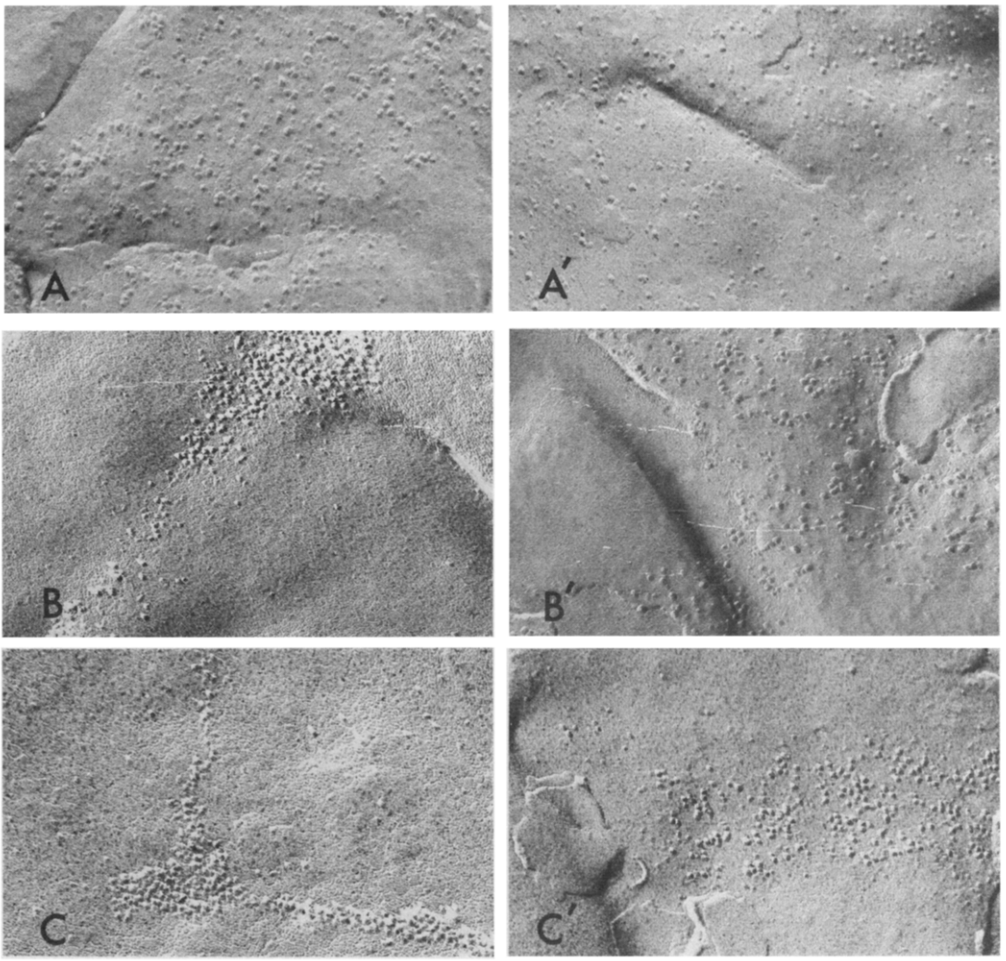


Fig. 4. Freeze-fracture electron microscopy of the outer alveolar membrane in the control (A, B, C) and the phenethyl alcohol-treated (A', B', C') cell fixed at different temperatures. A, A', 27°C; B, B', 21°C; C, C', 15°C. Magnification, ×60 000.

degree of particle aggregation of the control and phenethyl alcohol-membranes at different temperatures revealed that there was considerable difference in the particle aggregation between membranes of the control and phenethyl alcohol(6 h)-treated cells. But the distribution of membrane particles of phenethyl alcohol(1 h)-treated cells with little lipid modification was shown to be almost identical with that of the control. This indicates that such different thermotropic profiles of lateral movement of membrane particles may not be caused by a direct effect on the membrane by phenethyl alcohol itself. For more convenience to visualize the altered lateral movement of membrane particle in the phenethyl alcohol-treated cells, this particle movement was quantified using the particle density index as previously described [35]. Fig. 5 depicts particle density index curves for the outer alveolar membrane in cells grown in the presence and absence of phenethyl alcohol (5.5 mM). The particle density index of the control cells increases progressively up to 82% with lowering

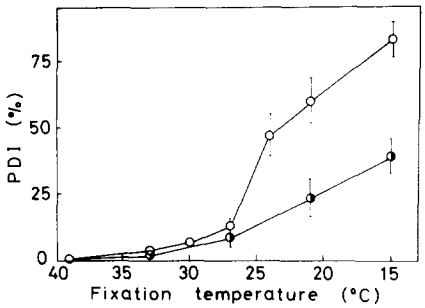


Fig. 5. Particle density index (PDI) curve of the outer alveolar membrane in the control and the phenethyl alcohol(6 h)-treated cell. ○, control; ●, phenethyl alcohol-treated.

temperatures, especially above 27°C, whereas the phenethyl alcohol-treated cells show only a slight, even linear increase reaching at most 39% particle density index at 15°C. Since such abrupt increase in particle density index is thought to be presumably due to a thermal lipid phase transition (liquid crystalline to gel), it would be reasonable to conclude that the membranes of phenethyl alcohol-treated cells with a significantly modified lipid composition do not undergo a sharp lipid phase transition and are rather more fluid than the control membranes.

Mechanisms of membrane lipid alteration

In order to obtain information regarding the mechanisms by which such lipid modification is produced, we have performed some preliminary experiments. First, with regard to the altered fatty acyl group composition, cells were labeled with [^{14}C]acetate after addition of phenethyl alcohol (5.5 mM) and then some aliquots were withdrawn at different intervals to extract lipids. As expected, the distribution pattern of the radioactivity was observed to reflect alterations in fatty acid composition; decrease in [^{14}C]14 : 0, [^{14}C]16 : 1 and increase in [^{14}C]18 : 2, [^{14}C]18 : 3. As for reduction of the palmitoleate level, we further examined by labeling cells with [^{14}C]palmitate, the rate of conversion from [^{14}C]16 : 0 to [^{14}C]16 : 1, and found that there was a great decrease in the radioactivity in palmitoleate, thereby indicating the decreased desaturation activity of 16 : 0 → 16 : 1. Thus, the ratio of [^{14}C]16 : 1/[^{14}C]16 : 0 (control; 0.27) was strikingly reduced to 0.12, 0.05, 0.03 in the 2-, 3- and 6-h-phenethyl alcohol-treated cells, respectively. In the previous papers [31,32,35], we have provided evidence that there are two possible factors to regulate the enzyme, palmitoyl-CoA desaturase involving the transformation of 16 : 0 to 16 : 1: membrane-fluidity and enzyme synthesis. The former might be excluded since as described above there was found no significant direct effects by phenethyl alcohol itself on the physical state of membranes. Therefore, in order to test involvement of the second factor, the influence of this drug upon protein synthesis was examined. After addition of 5.5 mM phenethyl alcohol, cells were labeled with [^{3}H]leucine and its incorporation into proteins was measured at different incubation periods by Byfield and Scherbaum's method [39]. It was found that protein synthesis was depressed to about 60% of the control. Thus, the plausible explanation for decrease in palmitoleate content in

TABLE IV

TRANSMETHYLATION OF PHOSPHATIDYLETHANOLAMINE INTO PHOSPHATIDYLCHOLINE

After cells were labeled with [^{14}C]ethanolamine for 165 min and harvested by centrifugation, cells were resuspended in the fresh culture medium and divided into two portions. Both cultures were further continued to grow in the presence and absence of phenethyl alcohol (5.5 mM), respectively. During the incubation period, several samples were taken at different intervals and then lipids were extracted by Bligh and Dyer's method [28]. The radioactivities in individual phospholipids separated on a silica gel plate were measured.

Specific radioactivity	Control				Phenethyl alcohol-treated		
	0 h	1 h	3 h	6 h	1 h	3 h	6 h
Phosphatidyl[^{14}C]ethanolamine (A)	317.7	300.0	189.0	128.9	296.5	218.5	129.3
Phosphatidyl[^{14}C]choline (B)	119.7	151.3	187.2	200.6	145.8	136.0	111.7
$\frac{B}{A+B}$	0.274	0.335	0.498	0.609	0.330	0.384	0.463

the membrane phospholipids would be the reduced level of palmitoyl-CoA desaturase. Furthermore, this concept was supported by another experiment of temperature shift-down. Whereas the maximal rate of conversion of [^{14}C]-16 : 0 → [^{14}C]16 : 1 was 64.7% in the control cells shifted from 39.5°C to 15°C, only 28.1% of [^{14}C]16 : 0 was transformed to [^{14}C]16 : 1 by desaturation in the phenethyl alcohol-treated cells.

On the other hand, as we have shown in Fig. 2 and Table I, the phospholipid head group composition was markedly modified by growing cells in the presence of 5.5 mM phenethylalcohol. In particular, the phosphatidylcholine content increases greatly with time by phenethyl alcohol treatment at the expense of phosphatidylethanolamine (Fig. 2), suggesting an augmented activity of methylation of phosphatidylethanolamine to form phosphatidylcholine. Therefore, the conversion rate of phosphatidylethanolamine into phosphatidylcholine was measured in both types of cultures. The total [^{14}C]-ethanolamine taken up by the cells and the amount incorporated into the phosphatidyl[^{14}C]ethanolamine and phosphatidyl[^{14}C]choline were determined in the phenethyl alcohol-treated cells and compared with the control cells under identical incubation conditions. The results are shown in Table IV, in which the pathway of phosphatidylethanolamine to phosphatidylcholine is observed to operate with the high activity in both cultures. However, against our expectation, the rate of synthesis of phosphatidylcholine from phosphatidylethanolamine was never higher or rather slightly lower in the phenethyl alcohol-treated cells than in the control cells. Thus, the rise in phosphatidylcholine in the phenethyl alcohol-treated cells cannot be explained by the activity of methylation of phosphatidylethanolamine.

Discussion

The concept has been well accepted that the physical state of membrane lipids exerts substantial influence on important biological activities occurring in membranes in the cell. The manipulation of the membrane lipid composition

has been applied mainly for bacteria and LM cells as a potential approach to yield further insight into the lipis' role in membrane functions [17]. As we have demonstrated in the previous communication [19,21,22] an eukarytic cell, *Tetrahymena* is though to be a useful model system since its membrane lipid profile can be with ease modified, by either thermal change or supplementation of ergosterol, hexadecyl glycerol, fatty acids, etc. It was also shown that lipid-manipulated *Tetrahymena* membranes were altered in the physical properties as well as enzyme activities, e.g. desaturase, ATPase, adenylate cyclase (refs. 19 and 21, and unpublished data).

In the present study, phenethyl alcohol (2-phenylethanol) was used to manipulate the membrane lipid composition in *Tetrahymena* cell. This drug has been known as the inhibitor of protein and nucleic acid synthesis in the past several years, but recently evidence was presented that phenethyl alcohol exerts a strong inhibitory effect on lipid synthesis in *E. coli* [23—25]. The results of the present experiments demonstrated that although the de novo synthesis of lipids was not greatly inhibited, the lipid (phospholipid and fatty acid) composition was markedly modified in various membranes. There was a decrease in palmitoleate with a compensating increase in linoleate and linolenate, leading to a large enhancement of unsaturation index. This decrease in palmitoleate was found to be probably due to the depressed desaturation of 16 : 0 to 16 : 1, which would result from the lowered level of palmitoyl-CoA desaturase content rather than the decreased activity of the pre-existing enzyme through membrane fluidity [35]. The reason for this thesis is that phenethyl alcohol does inhibit protein synthesis but not affect the membrane physical properties. At the moment therefore, we prefer the preferential inhibition of palmitoyl-CoA desaturase synthesis as a plausible explanation for decrease in palmitoleate in membrane phospholipids. On the other hand, quite recently Nunn and his colleagues [24,25] have drawn a conclusion from in vivo and in vitro experiments in *E. coli* that the site of phenethyl alcohol effect on phospholipid synthesis is at the level of *sn*-glycerol-3-phosphate acyltransferase. The possibility cannot completely excluded in our case that the acyltransferase system would be involved in the alteration of the fatty acid profile in membrane phospholipids. However, this seems unlikely because acyltransferases were observed not to be the primal factor for determining the phospholipid acyl group composition in *Tetrahymena* [40,41]. Further detailed experiments including the activity assay of acyltransferases should be necessary before we propose the molecular mechanism responsible for modification by phenethyl alcohol of phospholipid fatty acid composition.

With regard to phospholipid head group composition, in phenethyl alcohol-treated cells there was an increase in phosphatidylcholine and a decrease in phosphatidylethanolamine in a precursor-product. This finding simply prompted us to think of activated phosphatidylcholine synthesis by stepwise methylation of phosphatidylethanolamine, and we then examined the rate of conversion, phosphatidylethanolamine → phosphatidylcholine by labeling with [^{14}C]ethanolamine. The results did not support the theory that the augmentation in the methylation activity may contribute to the increase in phosphatidylcholine content in phenethyl alcohol-treated cell membranes. An alternative explanation is the preferential utilization of diacylglycerol to form phosphati-

dylcholine rather than phosphatidylethanolamine, indicating that phenethyl alcohol has somehow affect activities of CDPcholine or CDPethanolamine: 1,2-diacylglycerol cholinephosphotransferase.

The modification induced by phenethyl alcohol treatment in the fatty acyl and phospholipid group composition in membranes was expected to alter the physical state (fluidity) of membrane lipids. The results from freeze-fracture electron microscopy demonstrated that the pellicles from phenethyl alcohol-treated cells were found to be more fluid as compared with those from the control cells. This changed membrane fluidity can be interpreted by increase in unsaturation index of phospholipid fatty acids and also probably by increased level of phosphatidylcholine content. The phosphatidylcholine which possess a quaternary trimethylamine is known to be packed less tightly than phosphatidylethanolamine [42,43]. Thus, the increase in phosphatidylcholine with the corresponding decrase in phosphatidylethanolamine would participate fairly in fluidizing membrane lipids in phenethyl alcohol-treated cells. Since lipid-modified membranes can be easily prepared from *Tetrahymena*, this system is of great advantage to use to investigate the effects of the altered lipid composition upon various membrane functions, i.e. cell division, membrane-bound enzymes, transport, and some of them are under progress in our laboratory.

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