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Molecular Control of Membrane Properties During Temperature Acclimation. Fatty Acid Desaturase Regulation of Membrane Fluidity in Acclimating *Tetrahymena* Cells†

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ABSTRACT: This is a study of the molecular mechanisms employed by *Tetrahymena pyriformis* to change the lipid composition and thereby the fluidity of its various membranes during temperature acclimation. By quantitatively measuring the intramembrane particle aggregation using freeze-fracture electron microscopy, membrane physical properties in 39.5 °C grown cells shifted to 15 °C were found to be correlated with the degree of phospholipid fatty acid desaturation. Alteration of the phospholipid polar head group distribution from that of 39.5 °C-grown cells to the significantly different pattern of 15 °C grown cells appeared not to be of critical importance in the acclimation process. Changes in fatty acid desaturation during acclimation from high to low temperatures and vice versa were analyzed using normal cells and cells fed large amounts of polyunsaturated fatty acids. Fatty acid desaturase activity corresponded to the degree of membrane fluidity but not to the cell temperature. All evidence was compatible with the hypothesis that membrane fluidity is self-regulating, with the action of fatty acid desaturases being modulated by the physical state of their membrane environment.

The matrix of biological membranes appears to exist as a bilayer of mobile lipids, the relative motion of which determines the fluidity or viscosity of the membrane interior (Singer and Nicolson, 1972; also see review by Singer, 1974). The fluidity of the membrane, primarily determined by its lipid composition, is apparently of considerable importance to the organism in that it affects the activity of membrane-bound enzymes (Esfahani et al., 1971; Mavis and Vagelos, 1972; Kimelberg and Papahadjopoulos, 1972), membrane transport (Shechter et al., 1974; Thilo and Overath, 1976; Linden et al., 1973), and the mobility of membrane proteins (Horwitz et al., 1974). These observations indicate that optimal fluidities may exist to maintain the proper function of cell membranes and that cells may therefore have need to regulate their membrane lipid compositions to conform to a given temperature and to other environmental parameters. This would be especially true of poikilothermic organisms, which sometimes encounter wide ranges of growth temperature. The fact that these organisms do undergo changes in membrane lipids has been shown in a number of cases (Fukushima et al., 1976; Wunderlich et al., 1973; Marr and Ingraham, 1962; McElhaney, 1974). Furthermore, Nozawa et al. (1974) have demonstrated by the use of spin-label probes that the lipid changes induced by low temperature do have a fluidizing effect on the membranes of *Tetrahymena*.

In a previous publication, we have described the adaptive ability of *Tetrahymena pyriformis*, strain NT-1, which responds to varying temperatures by altering not only the degree of fatty acid unsaturation in its membrane phospholipids but also the relative proportions of the phospholipid species themselves (Fukushima et al., 1976). Another way to produce rapid and drastic lipid alterations in the membranes of this strain is by feeding large amounts of unsaturated fatty acids to exponentially growing cells. We found it instructive to compare the adaptive process in cells whose membranes had been perturbed by one or both of the above methods, since this would seem to provide an opportunity for discriminating between the roles of temperature per se and membrane fluidity in the regulation of the adaption process.

In this communication, we describe the effects of altered membrane lipid composition on acclimation by cells grown at high temperatures and shifted to low growth temperatures and vice versa. The following paper in this issue (Kasai et al., 1976) describes the adaptive changes which occur isothermally following even greater dietary modification of the membranes.

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Materials and Methods

Palmitic acid (hexaecanoic acid), referred to hereafter as 16:0, was obtained from Eastman Organic Chemicals; linoleic acid (*cis,cis*-9,12-octadecadienoic acid) (18:2) was from Supelco, Inc., and γ-linolenic acid (all *cis*-6,9,12-octadecatrienoic acid) (18:3) was from Serdary Research Labs. Sodium [1-^{14}C]acetate (56 mCi/mmol) was purchased from Schwarz-Mann and 1-^{14}C-18:2 (50 mCi/mmol) from New England Nuclear.

Cell Growth, Lipid Isolation, and Analysis. Culture conditions for strain NT-1 of *Tetrahymena pyriformis* were described in a previous paper (Fukushima et al., 1976). Cell densities were measured with a Coulter Counter, Model B. Cells were harvested during midlogarithmic phase by centrifugation and cell fractionations were performed by the procedure of Nozawa and Thompson (1971). Lipid extractions were carried out according to Bligh and Dyer (1959). Thin-layer chromatographic separation and analytical determination of phospholipid species, as well as gas-liquid chromatography of fatty acid methyl esters, have also been previously described (Fukushima et al., 1976). In some experiments, lipids were directly extracted from aliquots of the growth medium in order to account for all of the exogenously supplied fatty acids. In experiments where radioactive fatty acid methyl esters were separated and collected by gas-liquid chromatography, sufficient amounts of the methyl esters were used to ensure that a minimum of 4200 dpm was collected for scintillation counting. Total phosphorus in extracted lipids was determined by the method of Bartlett (1959), as modified by Marinetti (1962).

Fatty Acid Supplementation and Temperature Shifts. Cells for the temperature shift experiments were grown at 39.5 °C in 200-ml culture medium in 500-ml erlenmeyer flasks. Fatty acid supplements of either 16:0 (3.2 μmol) or a mixture of 18:2 (1.3 μmol, sometimes containing 1-^{14}C-18:2) and 18:3 (1.9 μmol) were prepared as 3-ml sonicated emulsions in either sterile distilled water or inorganic medium (Hamburger and Zeuthen, 1957). When the cultures reached a density of 75 000 cells/ml, the supplements were added dropwise into the growth medium with shaking over a 10-min period. Following addition of the fatty acids, the culture was cooled to 15 °C over a 30-min period by swirling the growth flask in an ice-water slurry. The temperature drop was essentially linear with time and was monitored by placing a sterile thermometer directly into the medium. The temperature reduction caused a temporary inhibition of cell division. The length of the lag period was dependent upon the cooling method, with generally longer periods observed in a few experiments where cells were cooled rapidly over a period of 2–4 min. In the following experiments, we chose to cool the cells slowly at a constant rate over a 30-min period to produce consistent growth lags of 7–8-h duration.

Acetate Labeling Experiments. The sodium [1-^{14}C]acetate used in these experiments was adjusted to a specific activity of 2 mCi/mmol with sodium [^{12}C]acetate prior to use. Rates of acetate incorporation into lipids were measured at the experimental temperature by incubating 10-ml aliquots of the growth cultures with 2 μCi of [^{14}C]acetate for 30 min. Preliminary experiments showed that, under the conditions employed, the incorporation of acetate was linear over that time period. Incorporation of acetate was stopped by chilling the cells in ice, followed by two washes with ice-cold inorganic medium using a clinical centrifuge to pellet the cells. In experiments involving lipid analyses, 50-ml aliquots of the cultures were incubated for the appropriate times with 10 μCi of [^{14}C]acetate. The uptake of labeled acetate in these experiments was quenched by a 250-fold excess of sodium [^{12}C] acetate, followed by rapid lipid isolation. Extracted lipids from these experiments were washed two times, according to the method of Folch et al. (1957), in order to remove unincorporated acetate. Radioactive lipids were assayed using a Packard Tricarb scintillation counter having a counting efficiency of 85%.

Freeze-Fracture Electron Microscopy. In order to visualize the effects of temperature upon cell membranes by freeze-fracture electron microscopy, cells in the growth medium were cooled directly to the appropriate temperatures (33, 30, 27, 24, 21, 18, 15, 12, 10, 9.5, and 0.5 °C) over a 4-min period by shaking the flask in cold water.[1] After incubation of the samples for 5 min at the desired temperatures, they were fixed for 15–20 min by the addition of ⅓ volume of 4% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2) to make a final glutaraldehyde concentration of 1%. The fixative was precooled to the sample temperature and care was taken to control the temperatures to within ±0.5 °C during the fixation. The fixed cells were then exposed to increasing concentrations of glycerol, terminating with a 24–28-h incubation with 30% glycerol in the previously described fixation buffer. The cells were pelleted in a clinical centrifuge and samples were frozen in liquid Freon 12 and transferred to liquid nitrogen before fracturing in a Balzers apparatus (Balzers BA, 360M, Fürstentum, Liechtenstein) at −110 °C. Platinum–carbon replicas were examined with a Hitachi HS-8 electron microscope at 50 kV.

Determination of the Particle Density Index. The PF face (Branton et al., 1975) of the outer alveolar membrane (which, in this case, was the outer fracture face of the membrane viewed from the inside of the cell toward the outside) was used to determine the particle density index. Only those particulated areas of the membrane plane which were located perpendicular to the direction of the electron beam were chosen for the determinations, in order to eliminate errors due to distortion of the replica and inaccurate area measurements caused by angular deviations from the viewing plane. Particles were counted in 200 × 200 nm areas and were expressed as particles/μm^2 (see Figure 1 and the accompanying legend for the counting method and representative areas which were counted for each membrane state). Thirtyfive to fifty areas on 7–10 different platinum–carbon replicas were counted for every sample fixed at a particular temperature. An example of the actual values measured and the variability encountered is shown in Table I. The particle density index (PDI[2]) was obtained by assigning the particle number/μm^2 of 39.5 °C fixed membranes a value of a and that of 0.5 °C-fixed membranes a value of b for cells which had been acclimated to a growth temperature of 39.5 °C. The two values, a and b, represent the extremes in membrane particle distribution that we have observed, with a corresponding to an apparently random distribution, thought to represent an optimally fluid membrane, and b corresponding to a high degree of particle aggregation produced presumably by extensive crystallization of membrane lipids and a corresponding lateral displacement of membrane particles into small regions of the membrane plane. The PDI was then calculated by the following equation with the particle density of a given temperature-fixed membrane as y.

$$\text{PDI (\%)} = \left(\frac{y - a}{b - a}\right)100$$

[1] More rapid (<5 s) cooling produced slightly different freeze-fracture patterns if cells were fixed immediately. However, if these cells were incubated at the low temperature for 30 min, the patterns became similar to those in cells chilled over a 4-min period.

[2] Abbreviation used is: PDI, particle density index.

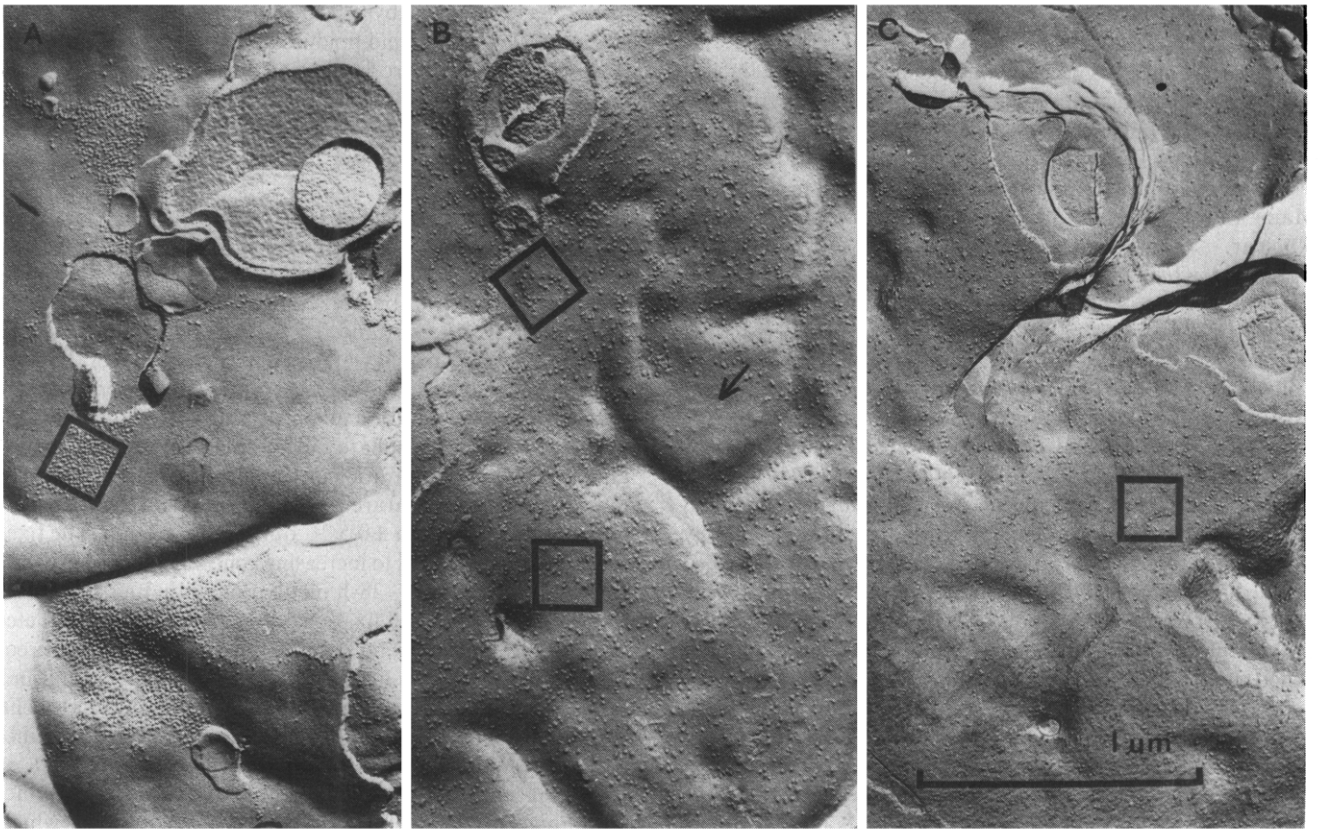

FIGURE 1: Effect of rapidly decreasing temperature on membrane particle distribution on 39.5 °C cells and the method for counting particles to determine the particle density index (PDI). The density of particles on the PF face (outer half of the membrane as viewed from the inside of the cell) of the fractured outermost alveolar membrane, revealed by freeze-etch electron micrographs, is temperature dependent when the temperature of the cells is rapidly shifted downward from a growth temperature of 39.5 °C. The 4×10^4 nm^2 frames show the types of areas used for counting membrane particles. Counts were made in regions where the electron beam direction appeared to be normal to the membrane plane and no membrane distortions were evident. (a) Cells fixed at 0.5 °C, corresponding to 100% PDI (see Materials and Methods), showing extreme particle aggregation. (b) Cells fixed at 30 °C with a PDI of 7.8%. Particles were nearly randomly distributed with some circular particle-free areas (arrow). (c) Cells fixed at the 39.5 °C growth temperature with a PDI of 0%. Particles were randomly distributed and the membrane was devoid of smooth areas. Shadowing direction in these micrographs was from the bottom to the top. Magnification is 36 000×. Actual counting was from prints enlarged to 57 000×.

Results

The aim of the experiments described here was to investigate the mechanisms whereby *Tetrahymena* is able to change its membrane lipid composition from the pattern characteristic of 39.5 °C cells to the very different pattern found at 15 °C (Fukushima et al., 1976). Towards this end, we have carried out a series of experiments in which cells grown at 39.5 °C were chilled gradually over a 30-min period to 15 °C and maintained at this level until the low temperature-induced blockage of cell division was overcome by acclimation. Under these conditions, approximately 8 h elapsed before the chilled cells resumed division. During this time, the fatty acids bound to membrane phospholipids became increasingly more unsaturated, finally approaching the distribution found in 15 °C grown cells.

Accompanying the change in fatty acid pattern following the shift to 15 °C was a pronounced increase in apparent membrane fluidity, as estimated by freeze-fracture electron microscopy, proceeding from a very low level to an optimal level (Fukushima et al., 1976). We have sought to determine whether the increased fatty acid desaturation causing these fluidity changes was triggered by low temperature itself or by the physical change in membrane fluidity stemming from the temperature reduction.

One useful approach has been the dissociation of membrane fluidity from stringent control by temperature. We have compared the changes in membrane fluidity and phospholipid fatty acid distribution following the shift of normal 39.5 °C cells to 15 °C with the equivalent changes in cells fed large amounts of linoleic acid (18:2) and γ-linolenic acid (18:3), the major *Tetrahymena* polyunsaturated fatty acid species.

The exogenously supplied mixture of unsaturated fatty acids was rapidly incorporated into membrane lipids. In a representative experiment, during which a 2:3 molar ratio of 18:2/18:3 (the former labeled with ^{14}C) was administered in an amount equal to 82% of the existing cellular content of free and bound fatty acids, 39% of the added mixture was incorporated into phospholipids during the 30-min cooling period. The remainder was found in triglycerides (38%) and free fatty acids (10%). Incorporation of 18:2 and 18:3 from these latter two pools into phospholipids continued at a slower rate throughout the acclimation period at 15 °C so that, by the end of 8 h, 55% of the supplied fatty acids were found in phospholipids, despite the absence of cell division. Triglycerides in the supplemented cells accounted for approximately 35–40% of the total cellular fatty acids 1 h after the temperature shift and contained primarily 18:2 and 18:3. There appeared to be no preferential incorporation of either of these fatty acids into phospholipids, since the ratio of the two in the neutral lipids was constant throughout the acclimation process and remained identical to that of the administered mixture. In contrast, the triglyceride pool in unsupplemented, temperature-shifted cells was quite small (5% of the total lipid) throughout the experiment and

TABLE I: Effects of Temperature Upon Particle Aggregation in the Outer Alveolar Membrane of *Tetrahymena*.

Temp of Fixation (°C)	Cells Grown at 39.5 °C		Cells Grown at 15 °C		Temp of Fixation	Cells Shifted from 39.5 to 15 °C for 4 h			
						Control cells		Cells fed 18:2 + 18:3	
	Particles/μm^2	PDI[b]	Particles/μm^2	PDI		Particles/μm^2	PDI	Particles/μm^2	PDI
39.5	388 ± 78[a]	0							
30	564 ± 44	7.8 ± 2.0							
27	702 ± 168	14.0 ± 6.2							
24	1681 ± 103	57.8 ± 4.6							
21									
18	2150[c]	78.5			18	986 ± 269	26.6 ± 11.6	642 ± 93	9.9 ± 4.1
15	2347 ± 225	88.4 ± 11.4			15	1161 ± 250	34.2 ± 11.0	683 ± 255	13.2 ± 10.6
12					12	1372 ± 360	43.8 ± 16.2	651 ± 203	11.8 ± 9.1
10					10				
9			446 ± 116	4.6 ± 2.9	9	1856 ± 283	63.2 ± 12.2	972 ± 180	26.0 ± 8.0
5			625 ± 74	10.6 ± 3.3					
0.5	2633 ± 383	100	1530 ± 230	51.2 ± 10.3					

[a] Each value is the average ± standard error of 35–50 measurements on 7–10 different replicas made as described under Materials and Methods. [b] Particle density index, as defined under Materials and Methods. [c] Only one replica counted.

TABLE II: Major Phospholipid Fatty Acid Species from *Tetrahymena pyriformis*, Strain NT-1, Supplemented with Linoleic and Linolenic Acids Followed by a Temperature Shift from 39.5 to 15 °C.[a]

Fatty Acid	39.5 °C Control	Cells Shifted from 39.5 to 15 °C				15 °C Control
		1-h Control	1-h Fed	4-h Control	4-h Fed	
14:0	11.8 ± 0.9	10.6 ± 1.1	7.8 ± 1.1	8.3 ± 1.2	7.0 ± 0.8	6.9 ± 0.7
16:0	14.6 ± 1.9	9.6 ± 1.3	8.9 ± 1.1	9.1 ± 2.5	9.4 ± 2.2	8.9 ± 0.5
16:1	12.9 ± 2.3	17.2 ± 1.8	10.2 ± 1.9	18.2 ± 2.9	9.5 ± 2.9	8.7 ± 0.9
16:2 + 17:0	8.3 ± 1.8	5.4 ± 2.0	2.5 ± 0.4	6.9 ± 2.3	2.2 ± 0.5	2.2 ± 0.2
18:1	5.8 ± 0.7	4.4 ± 2.4	4.3 ± 1.4	5.7 ± 0.7	5.2 ± 1.2	9.6 ± 1.6
18:2	12.9 ± 1.4	13.0 ± 1.4	18.9 ± 1.8	13.4 ± 2.7	19.9 ± 1.2	20.2 ± 1.6
18:3	23.0 ± 1.7	27.3 ± 3.8	38.9 ± 4.9	29.4 ± 4.9	38.6 ± 6.1	31.1 ± 0.9
Double bonds/100 molecules	136	154	187	160	188	176

[a] The temperature shift and the fatty acid supplementation were carried out as described under Materials and Methods. The data represent averages from at least five experiments, except for the 1-h control, where three experiments were averaged. Values for 39.5 and 15 °C control cells are included for comparison, with the 15 °C values being taken from a previous publication (Fukushima et al., 1976). Fatty acid species less than 5% of the total weight of fatty acids are not shown. The complete fatty acid composition has been published previously (Fukushima et al., 1976).

consisted mainly of saturated and monounsaturated fatty acids.

Table II presents the fatty acid distribution patterns in phospholipids from unsupplemented and supplemented cells (and control cells grown isothermally at 39.5 and 15 °C). While the change of the pattern in shifted unsupplemented cells proceeded steadily towards the 15 °C values, unsaturation in the supplemented cells increased to a level even higher than that of 15 °C grown control cells.

These compositional alterations are reflected in the freeze-fracture data. Particle aggregation, thought to represent phase separations in the membrane lipid bilayer (Shimshick and McConnell, 1973; Wunderlich et al., 1975; Kleeman and McConnell, 1976), was quantified by the method described under Materials and Methods. The standard measurement consisted of estimating the particle aggregation in the PF face of the outer alveolar membrane, one of three membranes making up the pellicle, or outer envelope of the cell. Many replicas of other membranes were also observed and it was established that, while each functionally different membrane had its own characteristic initiation temperature for phase separations, all membranes reacted to temperature changes in the same general way as did the outer alveolar membrane.

In order to demonstrate that the changes in particle aggregation resulted from the fluidizing effect of increasing unsaturation rather than some less specific action, e.g., a detergent effect of the fatty acids, cells were, in one case, supplemented with palmitic acid (16:0) prior to the temperature shift. Aliquots of these cells, analyzed 4 h after the shift, had not sustained a lowering of the particle density index, suggesting that it is, indeed, the degree of unsaturation which controls fluidity.

Figure 2 shows particle density index curves for 39.5 and 15 °C isothermally grown cells, as well as 18:2 plus 18:3 supplemented cells and unsupplemented cells examined at three time intervals during acclimation to 15 °C. Whereas both kinds of acclimating cells show progressive increases in fluidity with time, the supplemented cells are consistently more fluid, particularly at the 4-h periods.

The Pattern of Fatty Acid Change Within the Cell. While the degree of particle aggregation in the fatty acid supple-

TABLE III: The Distribution of Major Fatty Acids in Cell Fractions of 18:2- plus 18:3-Fed Cells 1-h and 4-h after Shifting from 39.5 to 15 °C.[a]

	Cell Fraction								
	Cilia			Pellicles			Microsomes		
Fatty Acid	39.5 °C Control	1 h	4 h	39.5 °C Control	1 h	4 h	39.5 °C Control	1 h	4 h
14:0	6.7	5.9	5.9	8.9	11.5	8.9	7.3	8.6	5.6
16:0	16.8	17.4	15.7	16.4	16.2	12.8	13.2	12.0	8.0
16:1	8.3	12.8	7.9	7.1	8.7	7.4	8.7	9.0	6.7
16:2 + 17:0	2.3	2.2	2.2	4.0	2.0	2.3	4.9	3.0	2.1
18:1	11.7	15.0	13.6	12.6	4.9	4.7	13.9	4.8	4.3
18:2	8.1	11.4	11.8	10.5	14.0	17.4	13.0	16.2	18.7
18:3	20.7	20.7	29.8	18.5	31.7	37.1	21.4	37.3	48.0

[a] Fatty acid compositions of 39.5 °C grown cell membranes are included for comparison; data from Fukushima et al. (1976).

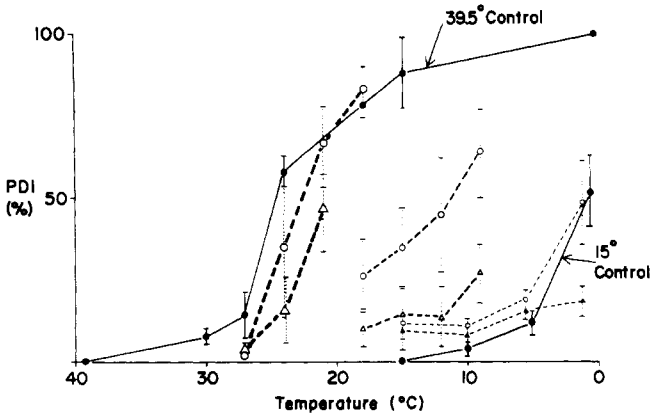

FIGURE 2: The effects of reducing the growth temperature from 39.5 to 15 °C and supplementing polyunsaturated fatty acids on the fluidity of *Tetrahymena* outer alveolar membranes. The particle density index (PDI, see Figure 1) is plotted vs. the temperature at which the cells were fixed with glutaraldehyde after chilling from their growth temperature over a 4-min period. The filled circles (●) and solid lines indicate the curves of control cells grown isothermally at 39.5 and 15 °C. The circles (○) and broken lines represent the fluidity curves at 0.5 h (thick lines), 4 h (medium thickness lines), and 8 h (thin lines) after initiation of the temperature shift of unsupplemented cells. The open triangles (△) represent the PDI curves at the equivalent times for cells supplemented with 18:2 plus 18:3 (see Materials and Methods). The fluidizing effect of the fatty acid supplement can be estimated by comparing PDI's at a specific temperature on two broken lines of the same thickness.

mented, acclimating cells was very much less than in their unsupplemented counterparts by 4 h (Figure 2), the difference was less striking in the 0.5-h cells. This seemed rather surprising in view of the greatly heightened level of unsaturation already attained by this time (Table II).

Because earlier work in our laboratories had shown that fatty acids in *Tetrahymena* are incorporated into phospholipids only at microsomal sites and then move slowly outward to the other cell membranes (Nozawa and Thompson, 1971), it seemed possible that the newly ingested 18:2 and 18:3 would be nonuniformly distributed within the cell. Therefore, we fractionated supplemented cells at 1 and 4 h following the temperature shift and separately analyzed the phospholipids isolated from the cilia, pellicles, and microsomes for their fatty acid composition. Table III shows that, by 1 h into the acclimation phase, the most striking change in membrane fatty acids occurs in the microsomes, with an unusually large increase in 18:3. The level of this fatty acid continues to increase in the microsomes until by 4 h approximately one-half of the phospholipid fatty acids are 18:3. At both points, the level of polyunsaturated fatty acids (18:2 and 18:3) was much higher than that found in the microsomes of 15 °C acclimated cells.

The rapid incorporation of added fatty acids into microsomal phospholipids was reflected in the electron microscopy patterns. Freeze-fracture replicas from both fed and unfed cells fixed at the 15 °C growth temperature 30 min after the onset of cooling showed phase separations in the endoplasmic reticulum. However, after warming aliquots from both cultures to 21 °C over a 4-min period, only unfed cells showed phase separations. Unfortunately, the lack of distinguishable lateral particle movement in these membranes (Wunderlich, 1973) precludes measurements of a PDI, and we can say only that the apparent fluidizing effect of 18:2 plus 18:3 on the endoplasmic reticulum was large. We are currently gathering detailed data on the physical properties of the membranes by electron spin resonance and fluorescence polarization experiments.

Although the pellicles also showed large changes in their fatty acids at these times (Table III), the alterations were neither as extensive nor as rapid as those in the microsomes. Thus, the delayed changes of alveolar PDI in the early acclimation period (Figure 2) do not accurately reflect the physical state of the other cellular membranes.

Ciliary membrane lipids were the last to show the effects of temperature and supplementation. This is, presumably, a reflection of their remote location on the cell surface.

Phospholipid Distribution During Temperature Acclimation. In addition to the altered fatty acid patterns, large differences exist in the phospholipid composition of cells adapted to high and low temperatures (Fukushima et al., 1976). The phospholipid composition of both fed and unfed cells at 1 and 4 h into the acclimation period reflect the composition of cells growing at high temperatures. No significant differences were found between the supplemented and unsupplemented cells at either time. Some changes occurred between membranes of supplemented cells, however (Table IV).

Early in the lag period the relative amount of aminoethylphosphonolipid increased sharply in the microsomal membranes, accompanied by a drop in this species in the pellicles. By 4 h the situation was reversed. Complementary changes in choline glycerophosphatides were seen at these times, while ethanolamine glycerophosphatides remained relatively con-

TABLE IV: Major Phospholipids of *Tetrahymena* Microsomal and Pellicle Membranes During Temperature Adaptation After 18:2 plus 18:3 Supplementation.[a]

	Microsomes				Pellicles			
	39.5 °C Control[b]	1 h (18:2 + 18:3)	4 h (18:2 + 18:3)	15 °C Control[b]	39.5 °C Control[b]	1 h (18:2 + 18:3)	4 h (18:2 + 18:3)	15 °C Control[b]
Choline glycerophosphatides	32.4	23.6	38.3	30.0	23.5	31.6	22.8	18.9
Ethanolamine Glycerophosphatides	43.9	43.4	45.3	34.1	49.2	45.7	42.1	33.2
2-Aminoethyl phosphonolipid	14.7	22.8	9.6	22.9	19.1	12.0	20.0	30.8
Cardiolipin	1.9	5.3	6.5	2.7	1.7	6.1	4.3	1.3

[a] Results are expressed as % of total recovered lipid phosphorus. [b] Control data from Fukushima et al. (1976).

stant. Since there appeared to be no change in the relative proportions of the total cellular phospholipids, it is likely that these changes were due to the intracellular movement of lipids from one membrane type to another.

In comparison with cells grown at the two extreme temperatures, changes in the phospholipid distribution during the acclimation period appear to be minor, indicating that rapid adaptation to low temperatures in this strain is accomplished primarily by the alteration of phospholipid fatty acid compositions.

Activity of Fatty Acid Desaturases During Acclimation. To ascertain the role of lipid synthesis and desaturation in the adaptive process, the incorporation of [^{14}C]acetate into fatty acids was followed. [^{14}C]acetate incorporation was depressed to a level well below that of 15 °C grown cells when the 39.5 °C cells were chilled to 15 °C (Figure 3). Incorporation by the fatty acid supplemented cells was only 50–75% of the level utilized by unsupplemented cells, possibly due to feedback inhibition. By 6 h after the onset of cooling, fatty acid synthesis in both cultures had risen to the level found in 15 °C grown cells.

Synthetic patterns were determined by measuring ^{14}C incorporation into individual fatty acid species at five intervals throughout the acclimation period. Two such experiments are summarized in Figure 4. ^{14}C incorporation into phospholipid fatty acids of unsupplemented cells changes markedly when the cells are chilled from 39.5 to 15 °C. Whereas 32% of the fatty acid radioactivity in isothermal 39.5 °C cells was found in the major saturated fatty acids (14:0 and 16:0) after 2 h (see later graphs for details), only 12% was in those species in cells recently shifted to 15 °C. Unsupplemented cells also exhibited a slightly higher rate of desaturation (mainly into 16:1) than isothermally-grown 15 °C cells during the first few hours (Figure 4A). By 6 h, the patterns for temperature-shifted cells and isothermal 15 °C cells differed very little (Figure 4B).

Figure 4 also depicts the differences between [^{14}C]acetate incorporation into fatty acids of the 18:2 and 18:3 supplemented and the unsupplemented cells, both shifted from 39.5 to 15 °C. Incorporation of exogenous 18:2 and 18:3 into the cells dramatically altered the desaturation of newly synthesized fatty acids. This was signified by reduction of the label in 16:1, 18:2, and 18:3 and increases in labeled saturated fatty acids (Figure 4A,B). Extremely low levels of ^{14}C-18:2 and 18:3 indicated that strong inhibition of 18-carbon fatty acid desaturation occurs even in the latter part of the acclimation period (Figure 4B).

The relative amounts of label incorporated into major phospholipid fatty acids throughout the acclimation period is

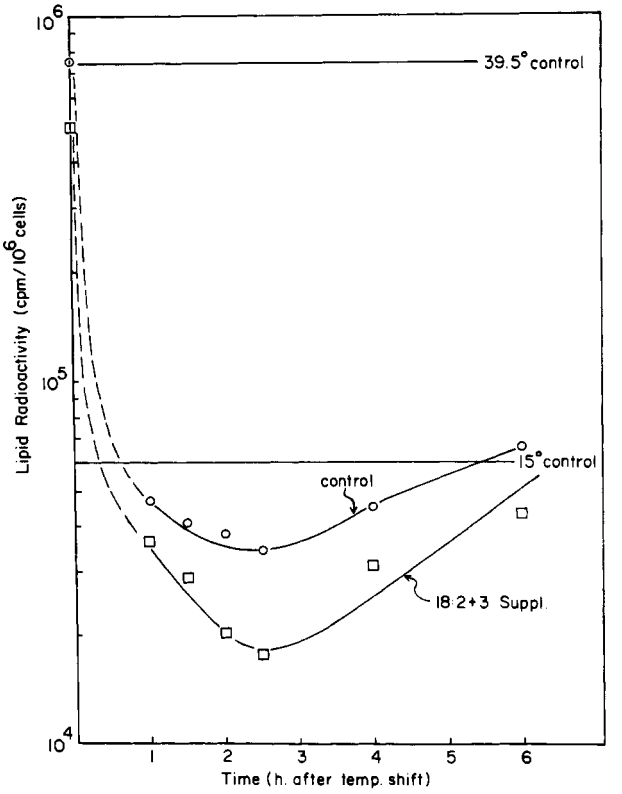

FIGURE 3: The incorporation of [^{14}C]acetate into total lipids of temperature-shifted cells with (□) or without (○) 18:2 plus 18:3 supplementation, as compared with that of isothermally growing cells at 39.5 and 15 °C (upper and lower horizontal lines, respectively). All controls, as well as fatty acid enriched cells, were incubated with [^{14}C]acetate for 30 min prior to lipid extraction at the times indicated on the abscissa.

summarized in Figure 5. The major effect of supplementation is to promote the accumulation of newly synthesized fatty acids as saturated rather than unsaturated species. Overall desaturation in fed cells over the first 7 h of the acclimation period was ⅙ that found in the temperature-shifted, unfed cells, with approximately 70% of the fatty acid radioactivity remaining in saturated species, as opposed to only 30% in the unfed cells.

Although differences in the phospholipid fatty acid compositions were present in these fed, unfed, and 15 °C acclimated cells, the total number of double bonds was almost identical in each case. There were 1.4 and 1.7 mol of double bonds/mol of phospholipid fatty acids, respectively, in the

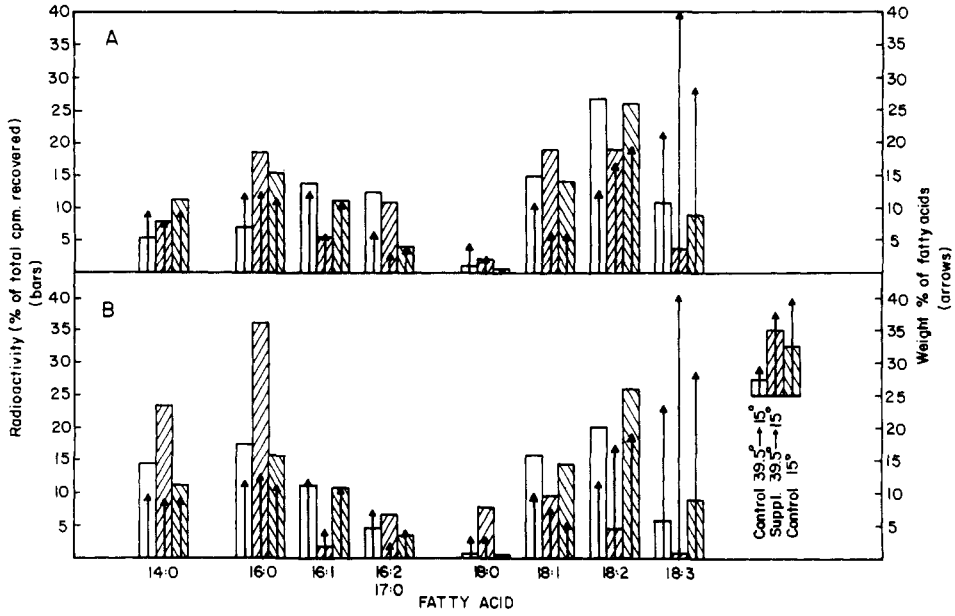

FIGURE 4: [^{14}C]Acetate incorporation (bars) and fatty acid composition (arrows) are shown for phospholipids 3 (A) and 6 h (B) after temperature shift. Cells have been incubated with [^{14}C]acetate for 2 h prior to lipid extraction. Control temperature-shifted cells (left-hand bars) are compared with temperature-shifted, 18:2- plus 18:3-supplemented cells (middle bar) and with cells growing isothermally at 15 °C (right-hand bar).

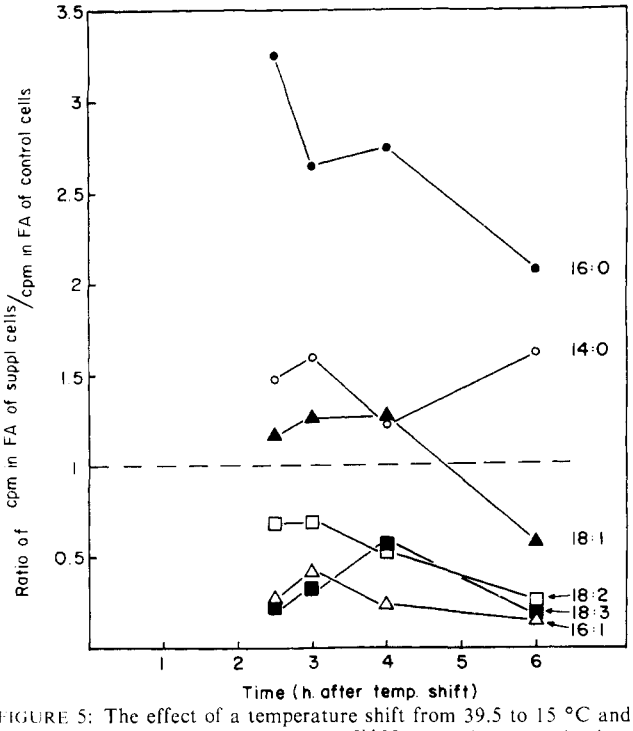

FIGURE 5: The effect of a temperature shift from 39.5 to 15 °C and of 18:2 plus 18:3 supplementation upon [^{14}C]acetate incorporation into fatty acids of phospholipids, using data from Figure 4 and similar data obtained at other time intervals. The percent of recovered radioactivity in a particular fatty acid of supplemented cells was, in each case, divided by the equivalent percent in the same fatty acid of control cells extracted at the identical time after temperature shift. This ratio, expressed on the ordinate, indicates the effect of feeding upon the pattern of ^{14}C incorporation.

unfed and fed cells at the end of the 8-h lag period. Cells grown isothermally at 15 °C contained 1.6 mol of double bonds/mol of phospholipid fatty acids. The similarities are due to large reductions in the monounsaturates of supplemented cells and is another indication of the extreme inhibition of nascent fatty acid desaturation produced by supplementation.

The decreased activity of fatty acid desaturases in the temperature-shifted cells as a result of 18:2 and 18:3 uptake might be induced by the excessive amounts of these fatty acids present either in the free state or as triglycerides. These possible complications have been minimized by conducting a different type of experiment in which cells grown at 15 °C were rapidly shifted to 39.5 °C. Such cells possessed a higher percentage of unsaturated fatty acids in membrane phospholipids than 39.5 °C grown cells and, presumably, had membranes of superoptimal fluidity. But in contrast to the 18:2 plus 18:3-supplemented cultures, they contained very few free fatty acids or triglycerides. Examination of [^{14}C]acetate incorporation, under these conditions, showed a strong inhibition of 18:2 desaturation and a somewhat weaker inhibition of 16:0 and 18:1 desaturation (Figure 6) in comparison to 39.5 °C control cells.

Although neutral lipids constituted only a small fraction of the total lipids in temperature-shifted and 15 °C isothermal control cells, a significant amount of radioactivity from [^{14}C]acetate was found in this fraction after a 2-h labeling period. The amount of incorporated acetate in triglycerides in these two cultures ranged from 11 to 16% during the acclimation period. More label occurred in this fraction of the supplemented cells, averaging about 21% of the total incorporated radioactivity in the short-term-labeling experiments.

The most distinctive feature of the labeled fatty acids in this fraction was the accumulation in the supplemented cells of nascent saturated and monounsaturated fatty acids in a pattern resembling that found in the phospholipid fraction (data not shown).

Effects of Altered Membrane Fluidity Upon Cell Division. We showed earlier (Fukushima et al., 1976) that cell division ceased for several hours after cultures acclimated to 39.5 °C were shifted down to 15 °C. It was during this period that the lipid composition and the membrane fluidity were changing towards that found in 15 °C grown cells. We were, naturally, interested in determining whether suboptimal membrane fluidity was the sole factor blocking growth of the cultures.

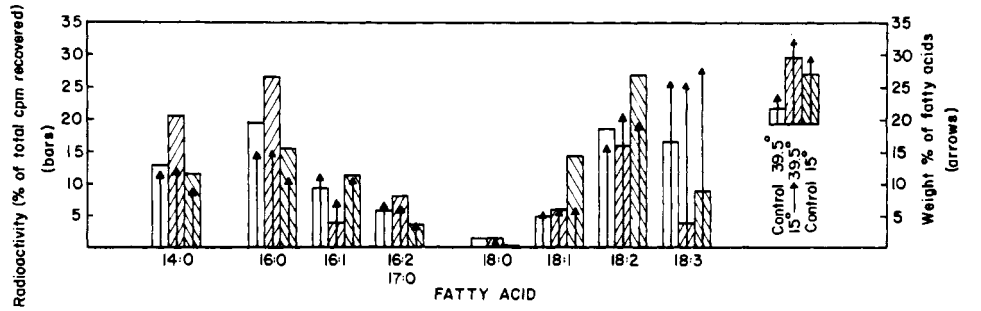

FIGURE 6: [^{14}C]Acetate incorporation (bars) and fatty acid composition (arrows) are shown for phospholipids of 15 °C grown cells (right-hand bars), 15 to 39.5 °C temperature-shifted cells (middle bars), and 39.5 °C grown cells (left-hand bars). Initial cell density in each case was approximately 75 × 10^3 cells/ml, and [^{14}C]acetate was administered for 2 h (starting in the case of temperature shift immediately after the higher temperature was attained).

Therefore, we have carried out several experiments in which the resumption of cell division was compared in 18:2 and 18:3 supplemented and unsupplemented cells following the downward shift in temperature.

There was no consistent reduction of the growth lag, due to the fluidizing effect of added fatty acids. Typical results resembled those shown in Figure 7. In this particular experiment, an additional test was conducted. One hundred-milliliter aliquots of both fed and unfed cultures were raised to 20 °C after 4 h in the hope that the shortened period of acclimation might magnify any small effects of altered fluidity. Differences in the times of growth inhibition were again slight, however, and of doubtful significance despite the increased fluidities which should be present in the supplemented cells at this higher temperature.

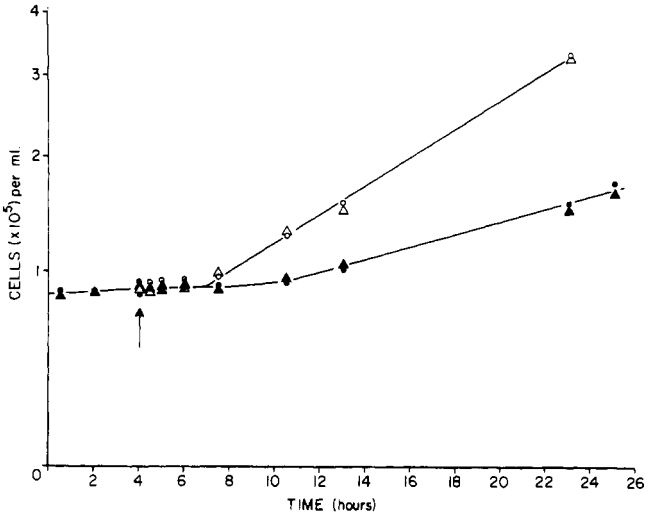

FIGURE 7: The effects of 18:2 plus 18:3 supplementation upon cell division of *Tetrahymena* cultures chilled from 39.5 to 15 °C. (●) unfed cells, (▲) 18:2/18:3 fed cells. (○) unfed cells raised from 15 to 20 °C after 4 h (arrow), (△) 18:2/18:3 fed cells raised from 15 to 20 °C after 4 h. The abscissa indicates time following completion of the 39.5 to 15 °C shift.

Discussion

In a previous report, we described the differences in lipid composition of *Tetrahymena pyriformis,* strain NT-1, when grown at 39.5 and 15 °C (Fukushima et al., 1976). These were among the most pronounced temperature-induced changes ever recorded for any organism and included not only large variations in fatty acid saturation but also marked differences in the proportions of the phospholipid classes. Increased fatty acid unsaturation is generally associated with increasing membrane fluidity in biological systems (Oldfield and Chapman, 1972; McElhaney, 1974; Sinensky, 1971, 1974). Less is known concerning the effects of the phospholipid polar head groups in membranes, although it is apparent from work on artificial systems that varying the proportions of the head groups can markedly affect the liquid-crystalline to crystalline phase transition temperature (Chapman et al., 1975).

Since the physical state of the membrane is largely determined by its constituent lipids, the differences we see in *Tetrahymena* apparently represent the mechanism whereby this organism maintains an optimal membrane fluidity at a particular growth temperature. This assumption is strongly supported by the electron microscopic data showing temperature-induced fluidity changes and by the previous electron spin resonance observations with a closely related *Tetrahymena* strain (Nozawa et al., 1974) in which the fluidities of ciliary, pellicle, and endoplasmic reticulum membranes became greater at lower growth temperatures. Temperature is known to be capable of inducing an actual transformation of membrane lipids from a rigid gel phase to a liquid-crystalline phase, with mixed phases occurring under intermediate conditions (Baldassare et al., 1976). We must emphasize, however, that the freeze-fracture data utilized in the present study provide direct information about the state of the membrane only under those conditions where particle reorganization (and, presumably, lipid phase separation) actually take place. As with certain physical-chemical techniques, such as differential scanning calorimetry, one can only infer what the physical state of the membrane might be under other conditions. We are currently using electron spin resonance and fluorescence polarization measurements in order to gain quantitative information on fluidity changes.

An increasing body of evidence suggests that the fluidity of cell membranes may be critical to the optimal function of membrane-bound enzymes and membrane-mediated processes (Schecter et al., 1974; Linden and Fox, 1973; Kimelburg and Papahadjopoulos, 1972; Thilo and Overath, 1976; Esfahani et al., 1971). Thus, the need for regulation of membrane fluidity to counter perturbing environmental changes is of great importance, especially to poikilothermic organisms. In order to gain insight into the regulatory mechanisms which govern membrane fluidity, we have turned to an examination of the molecular events by which cells alter their membranes in response to temperature stress.

Acclimation of *Tetrahymena pyriformis,* strain NT-1, to an extreme drop in growth temperature coincides with a prolonged lag in growth, during which membrane fluidity is progressively altered toward the low-temperature state. Although

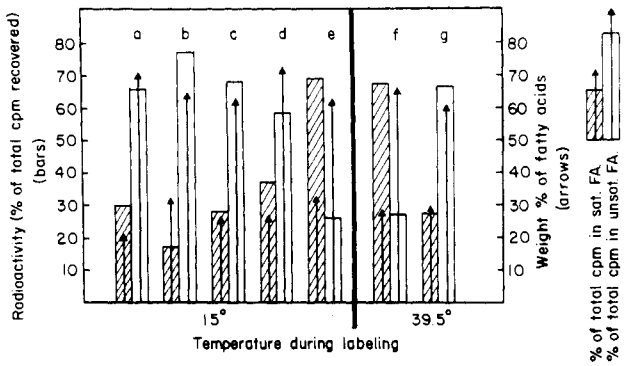

FIGURE 8: The amount of [^{14}C]acetate incorporation into unsaturated phospholipid fatty acids (open bars) is compared with that of saturated fatty acids (hatched bars). The weight % of individual fatty acids are shown by arrows in: (a) 15°-grown cells; (b) 39.5 to 15 °C temperature-shifted cells 3 h after beginning shift. (c) 39.5 to 15 °C temperature-shifted cells 7 h after beginning shift. (d) 39.5 to 15 °C temperature-shifted cells (18:2 plus 18:3 supplemented cells) 2.5 h after beginning shift. (e) 39.5 to 15 °C temperature-shifted cells (18:2 plus 18:3 supplemented cells) 7 h after beginning shift. (f) 15 to 39.5 °C temperature-shifted cells 2 h after beginning shift. (g) 39.5 °C grown cells. The experiments were conducted as described under Materials and Methods, and [^{14}C]acetate was administered for 2 h, except in c and e where incubation time was 6 h before the lipids were extracted.

both the degree of fatty acid saturation and the relative proportions of the phospholipids differ in cells grown at 39.5 and 15 °C (Fukushima et al., 1976), it is only the former parameter that can be seen to change appreciably during the period of growth inhibition. The resumption of normal growth of the temperature-shifted cells, in spite of a minimal alteration in the distribution of phospholipid head groups, indicates that the pattern of polar head groups does not play a crucial role in temperature adaptation. Therefore, the primary mechanism for altering membrane fluidity in response to temperature change appears to be the adjustment of the degree of phospholipid fatty acid unsaturation. Recent tests (Skriver and Thompson, 1976) have indicated that this adjustment is not controlled by the level of dissolved O_2 as it seems to be in some other organisms (Brown and Rose, 1969; Harris and James, 1969). Our attention has turned to an examination of temperature and fluidity itself as regulatory factors.

Using *Tetrahymena* as a test organism, it is easy to alter two important membrane properties: (1) fluidity and (2) lipid composition. While the two properties cannot always be varied independently, fatty acid supplementation and temperature manipulation allow their dissociation in some cases. The combined use of these tools has provided us with an insight into the process of temperature acclimation.

Feeding large amounts of linoleic (18:2) and γ-linolenic (18:3) acids to cells prior to shifting them from 39 to 15 °C produced large alterations in the membranes of the acclimating cells. These fatty acids were chosen since they were the species which showed the greatest increase during temperature acclimation and, presumably, have a rapid fluidizing effect on cell membranes. Early in the acclimation period, the incorporated acids were found primarily in the microsomes, followed by a net movement to other membranes over a period of hours. Our freeze-fracture evidence supports this conclusion, showing that supplementation immediately lowers the temperature at which particle-free areas are produced in the microsomes, while marked reduction in outer membrane fluidity by fed 18:2 and 18:3 is not apparent until midway through the acclimation period.

This intracellular movement appears to be the same process as that previously described for the synthesis and intracellular movement of membrane phospholipids in another strain of *Tetrahymena* (Nozawa and Thompson, 1971), in which phospholipids are formed and incorporated in the microsomes, followed by a relatively slow movement of the nascent lipids to the peripheral cell membranes. Thus, it is likely that most major alterations in membrane lipids are executed in this fashion.

It was also possible to induce a state of membrane superfluidity by growing cells at 15 °C and raising them quickly to 39.5 °C. Cell growth and [^{14}C]acetate incorporation into fatty acids immediately assumed the 39.5 °C rates following the upward temperature shift, and an alteration of membrane fluidity back towards the optimal level was achieved by reduced desaturase activity.

By using cells induced by the above-mentioned procedures to have either suboptimal or superoptimal membrane fluidities, it was possible to infer the role of membrane fluidity in the control of fatty acid metabolism.

The degree of saturation in membrane phospholipids is normally determined by the relative rates of two processes: de novo fatty acid synthesis, which provides a continuous supply of saturated acids to the membranes, and desaturation, which maintains the proper level of unsaturated acids against this influx. The rate of fatty acid catabolism in growing cells is too low to play a significant role (Borowitz and Blum, 1976). Regulation of membrane fatty acid composition could also occur by selective transfer of fatty acids into triglycerides; however, the relatively small size of this pool in unsupplemented cells indicates that this is of minor importance.

Thus, one looks, primarily, at the rates of fatty acid synthesis and desaturation during a period of acclimation. When cells were chilled from 39.5 to 15 °C, there was a pronounced drop in the level of [^{14}C]acetate incorporation into total lipids (Figure 3). It is, perhaps, not unexpected that the incorporation should fall below the level observed in 15 °C grown cells because of the temperature shock. A further inhibition is seen in 18:2- plus 18:3-supplemented cells, possibly due to feedback inhibition resulting from a high internal concentration of free fatty acids.

Under these non-steady-state conditions, we observed that following the shift fatty acid desaturation increased, with respect to fatty acid synthesis, in unsupplemented cells and did not increase in supplemented cells (Figures 4 and 5). Increasing desaturation was, therefore, correlated with decreasing fluidity, not with decreasing temperature.

Figure 8 summarizes the various experiments described above, emphasizing, once more, the closer correlation of enzymatic desaturation with apparent fluidity than with temperature.

It is possible that cell division may require increased membrane fluidity at certain times in the cell cycle, such as cytokinesis. Indeed, we assumed that the extensive phase separations in temperature-shifted cells would be the primary and, perhaps, the sole cause for the growth lag that occurred during low-temperature adaptation. However, since supplementation by unsaturated fatty acids failed to shorten the lag period, it seems likely that other factors may also be involved in retarding growth. The reactivation of other physiological responses during acclimation is currently being tested.

The data provided in this communication strongly implicate membrane fluidity as the major factor regulating the activity of fatty acid desaturases. Further evidence will be presented in the following paper of this issue (Kasai et al., 1976).

Acknowledgments

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