Biochimica et Biophysica Acta, 598 (1980) 217—236

BBA 78701

DISCONTINUOUS THERMOTROPIC RESPONSE OF *TETRAHYMENA* MEMBRANE LIPIDS CORRELATED WITH SPECIFIC LIPID COMPOSITIONAL CHANGES

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(Received August 30th, 1979)

Key words: Growth temperature; Lipid composition; Thermotropic response; (Tetrahymena)

Summary

Steady-state fluorescence polarization measurements of 1,6-diphenyl-1,3,5-hexatriene in microsomal lipids from *Tetrahymena pyriformis* cells grown at 39 or 15°C revealed discrete slope discontinuities in plots of polarization vs. temperature. Two well-defined 'break points' were present in the 0—40°C temperature range examined and their precise location was dependent upon the growth temperature of the cells. By mixing phospholipids from cells grown at different temperatures, the break points at 17.5 and 32°C in 39°C-lipid multilayer preparations were shown to correlate with the breaks at 12 and 27°C, respectively, in similar preparations from 15°C-grown cells. The discrete break points were also present, but at slightly different characteristic temperatures, in a phosphatidylcholine fraction and a phosphatidylethanolamine plus 2-aminoethylphosphonolipid fraction purified from the phospholipids and in total microsomal lipids (phospholipids plus the sterol-like triterpenoid, tetrahymanol). However, catalytic hydrogenation of the phospholipid fatty acids or mixing the non-hydrogenated phospholipids with increasing proportions of synthetic dipalmitoyl phosphatidylcholine eliminated the break points. We interpret this discontinuous thermotropic response in microsomal lipids as signalling a lipid phase separation of importance in regulating physiological events.

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Abbreviation: DPPC, dipalmitoyl phosphatidylcholine.

Introduction

For a number of years investigators of biological membrane phenomena have observed characteristic non-linear changes in certain functional activities of membranes when measured over a wide temperature range. Many of these abrupt alterations in the rates of transport and other physiological activities appear to be triggered by changes in the fluid state of the membrane lipids [1]. Similar non-linear responses in enzymatic activity are sometimes seen at constant temperature in situations where changes in pH or cation concentration can modify membrane physical properties. These phenomena suggest that some regulatory actions may be mediated by the membrane's physical state as opposed to being a direct effect of environment on a given enzyme per se.

The study of such regulatory mechanisms requires that the physiological responses of a given membrane be correlated with its physical state. We have sought to make such a correlation in cells of *Tetrahymena pyriformis* during their acclimation to low-temperature stress [2]. In this organism a wide variety of evidence strongly implicates membrane fluidity changes, particularly in microsomal membranes, as the most important initiators of temperature acclimation processes. Yet, the detailed mechanism which couples the physical state of microsomal lipids to the activity of constituent enzymes is not understood.

In order to shed more light on the regulatory role of microsomal membranes, we have undertaken a detailed analysis of their physical properties. This communication describes certain discontinuous changes in the fluorescence polarization of microsomal lipids and intact membranes subjected to decreasing temperature. The characteristics of these changes suggest that a phospholipid phase separation may be influential in triggering the alterations in lipid metabolism responsible for temperature acclimation.

Materials and Methods

Materials

Dipalmitoyl phosphatidylcholine, purchased from Serdary (London, Ontario), revealed a single spot at the expected position on heavily-loaded thin-layer chromatographic plates and was not further purified. Dilauroyl phosphatidylcholine was a gift from Dr. C. Wade (University of Texas, Austin, TX). Its purity was confirmed by both gas-liquid chromatography and thin-layer chromatography. 8-Anilino-1-naphthalene-sulfonic acid, purchased from Eastman Kodak Co. (Rochester, NY) and 1,6-diphenyl-1,3,5-hexatriene, purchased from Aldrich (Milwaukee, WI), were used without further purification.

Methods

Organism. T. pyriformis, strain NT-1, was cultured in a complex medium previously described by Fukushima et al. [3] at either 15 or 39°C.

Lipid isolation and analysis. Microsomes were isolated by using the procedure of Nozawa and Thompson [4], except that in most instances they were sedimented by centrifugation at 100 000 × g for 90 min instead of 60 min and the lipids extracted according to the method of Bligh and Dyer [5]. Phospho-

lipids were separated from neutral lipids and, in some cases, into different classes by silicic acid column chromatography as described by Thompson [6]. Procedures for thin-layer and gas-liquid chromatography have been described [3]. Total lipid phosphorus was estimated by the method of Bartlett [7] as modified by Marinetti [8]. Lipids were hydrogenated, when required, with a platinum catalyst as described by Appleqvist [9].

Fluorescence measurements. Multibilayer vesicles were prepared from microsomal lipids as described by Martin and Thompson [10]. The fluorescent probes employed were 1,6-diphenyl-1,3,5-hexatriene and, in a few cases, 8-anilino-1-naphthalenesulfonic acid. The probe was added to a chloroform solution of lipids in a molar ratio of 1 probe to 500 phospholipids. After removal of hydrophobic solvents from the lipids by evaporation under N_2, any traces of the solvents were removed by re-dissolving the lipids in methanol and re-evaporating the sample, first under a stream of N_2, and then in vacuo from a N_2-flushed container. The lipids were suspended in 15% sucrose/50 mM KCl at a concentration of 1 mM and were sonicated for 1—2 min in a sonicator bath at approx. 40°C or, in the case of DPPC and other saturated lipids, several degrees above the phase transition temperature. Negative staining of sonicated DPPC preparation revealed vesicles of fairly uniform size having an average diameter of 500—1000 Å. Before commencing polarization vs. temperature scans, the lipid preparations were chilled slowly over approximately 2 h to facilitate the coalescence of any small radius of curvature vesicles that might have been created during sonication [11]. Because of the low concentrations of lipids used, effects of light scattering on fluorescence intensities were insignificant.

Steady-state polarization measurements and fluorescence intensity measurements were made using the custom-made device described by Martin and Thompson [10]. The instrument was modified by interfacing the digital voltmeters with a PET 2001 series computer (Commodore, Palo Alto, CA). The computer controlled the simultaneous locking of the voltmeters, thereby eliminating fluctuations in lamp intensity from polarization calculations, and also averaged and recorded voltmeter output. Parallel and perpendicular fluorescence emission intensities were measured 50 times for each data point and mean polarization (P) values were calculated according to the formula:

$$P = \frac{I_{/\!/} - I_{\perp}}{I_{/\!/} + I_{\perp}}$$

where $I_{\parallel}$ is the fluorescence intensity parallel to the excitation beam and $I_{\perp}$ is the intensity perpendicular to the beam.

Whereas the measurements reported here were made by raising chilled liposomes through a series of equilibrated temperature points, representative samples measured over the same temperature range but in an order of descending temperature gave essentially the same results providing they were previously conditioned by a cycle of chilling and warming.

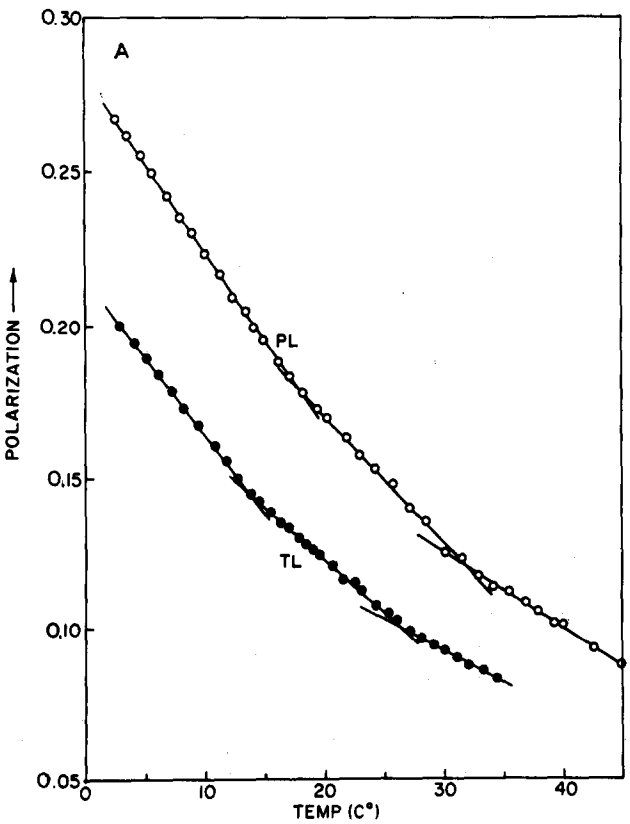
When anilinonaphthalenesulfonate was used as the fluorescent probe, the configuration of the steady-state device was changed to a ratio-recording mode so that total fluorescence of the probe could be measured relative to fluorescent standards.

Results

The observation of abrupt slope changes in polarization vs. temperature plots

The recent observation of discontinuous slope values (break points) in polarization vs. temperature plots of *Tetrahymena* microsomal lipid multibilayers [10] suggested that distinctive changes in membrane lipid physical properties might be discernible by measurements of this type. We have repeated these and related experiments numerous times and confirmed the existance of two abrupt slope changes over a 5—40°C temperature range.

Fig. 1A shows the presence of these break points in the polarization vs. temperature plot of diphenylhexatriene in total microsomal lipids and microsomal phospholipids extracted from *Tetrahymena* grown at 39°C. The phospholipid and total lipid curves differed in two ways. First, the polarization of the total lipid preparations was always lower than that of the phospholipids at any given temperature, implying a greater fluidity for the total lipids. This is due in part to the fluidizing effect of small amounts of triglycerides [12] in some of the preparations and of the small amount of tetrahymanol present (the tetrahymanol/phospholipid molar ratio in microsomal lipids of 39°C-grown cells is 0.078, enough to cause a slight lowering of polarization (Dickens, B.F., unpublished observations)). Whereas the absolute polarization value of phospho-



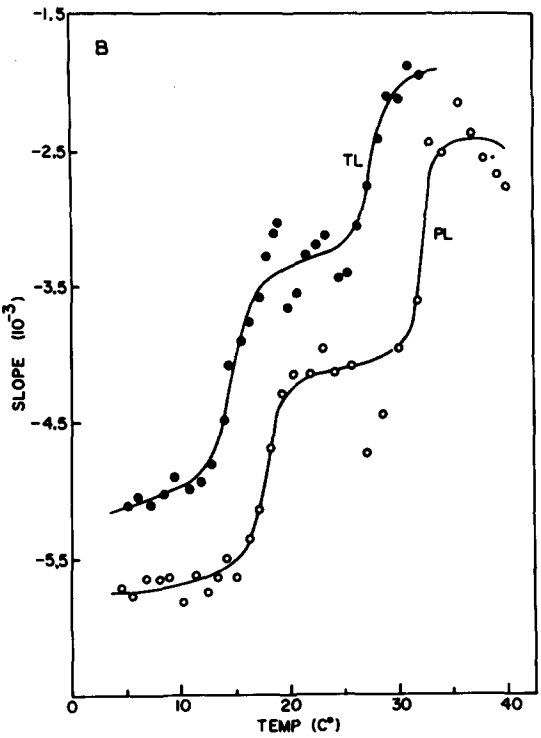


Fig. 1. The influence of temperature on diphenylhexatriene polarization in multibilayer vesicles of microsomal total lipids (TL) and phospholipids (PL) from 39°C-grown *Tetrahymena*. (A) Polarization vs. temperature plots. (B) Slope vs. temperature plots, obtained as follows. Beginning at one end of each curve in Fig. 1A, segments of five adjacent data points were analyzed by the least-squares method for the slope of the straight line best fitting them. After determining the slope of points, 1—5, the analysis was repeated for points, 2—6, and for all subsequent groups of five consecutive data points. The slope of each segment was then plotted against the temperature at the middle data point of that segment.

lipid multibilayers at a certain temperature remained the same from preparation to preparation, the values for total lipids decreased considerably if more than a trace of triglycerides was present. The total lipids of which the polarization curve appears in Fig. 1A contained amounts of triglycerides that were barely detectable by thin-layer chromatography.

The second difference between total lipid and phospholipid plots lay in the position of the break points. Total lipid samples always displayed characteristic break points near 15 and 28°C whereas the corresponding temperatures in phospholipid preparations were 17.5 and 32.5°C.

Although the break points in these curves seemed relatively easy to locate by eye, it seemed highly desirable to eliminate any unconscious operator bias in determining their placement. For this reason, a computer program was conceived for the purpose of detecting the regions of most pronounced slope change in each curve. Fig. 1B illustrates a computer plot in which the slopes of lines connecting each sequential 5-point segment of the curves in Fig. 1A are plotted against the temperatures of the midpoint of that segment. The steepest parts of the curves represent the most abrupt changes in slope. Curves of this

TABLE I

REPRODUCIBILITY OF BREAK POINT TEMPERATURES IN POLARIZATION VS. TEMPERATURE PLOTS OF DIPHENYLHEXATRIENE IN MICROSOMAL PHOSPHOLIPIDS FROM 39°C-GROWN *TETRAHYMENA*.

In experiments 7 and 8, 8-anilino-1-naphthalenesulfonate was employed as the probe replacing diphenylhexatriene and fluorescence intensity was measured instead of polarization.

Experiment	Lower break point (B1)	Higher break point (B2)
1	17.5	31.5
2	17.2	32.5
3	17.2	30.0
4	18.2	33.0
5	17.5	30.0
6	17.2	31.2
7	17.0	33.5
8	17.2	31.2

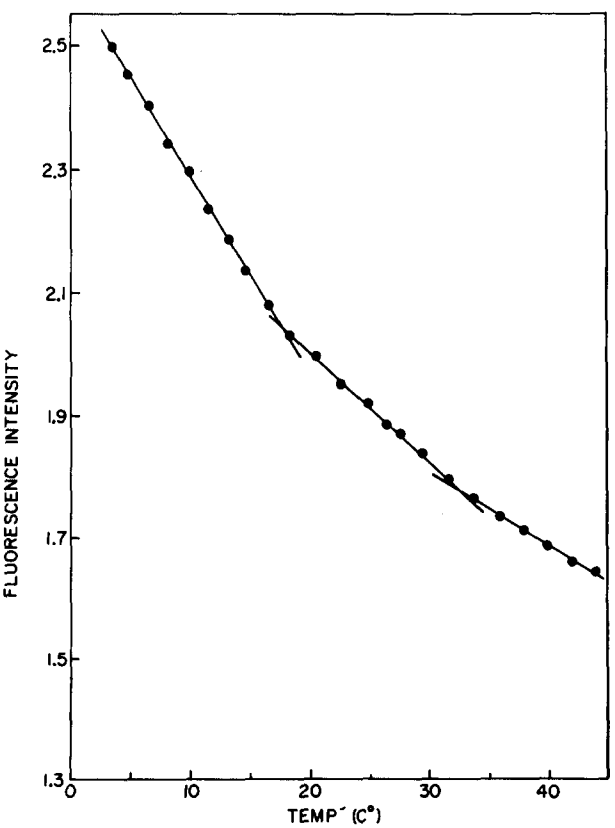
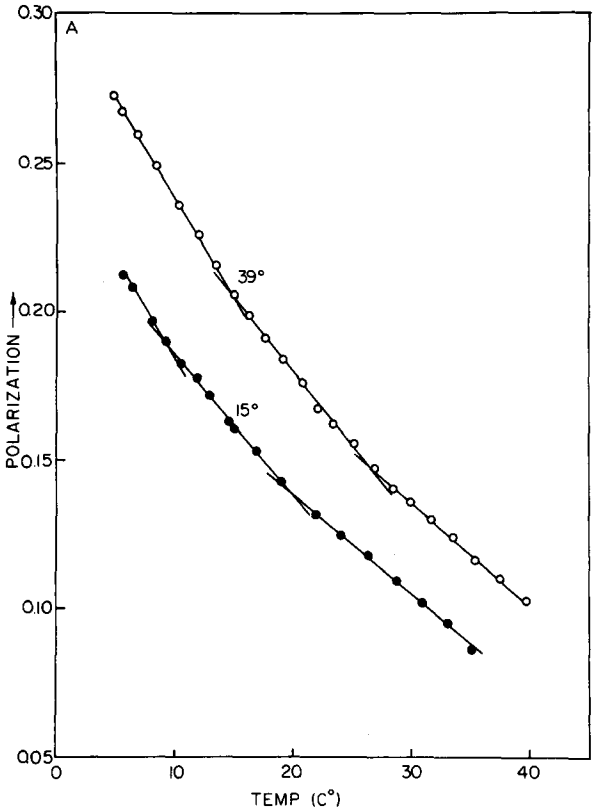


Fig. 2. The influence of temperature on the fluorescence intensity of anilinonaphthalenesulfonate in microsomal phospholipid multibilayers from 39°C-grown *Tetrahymena*. The fluorescence intensity is given on the ordinate in relative intensity units.



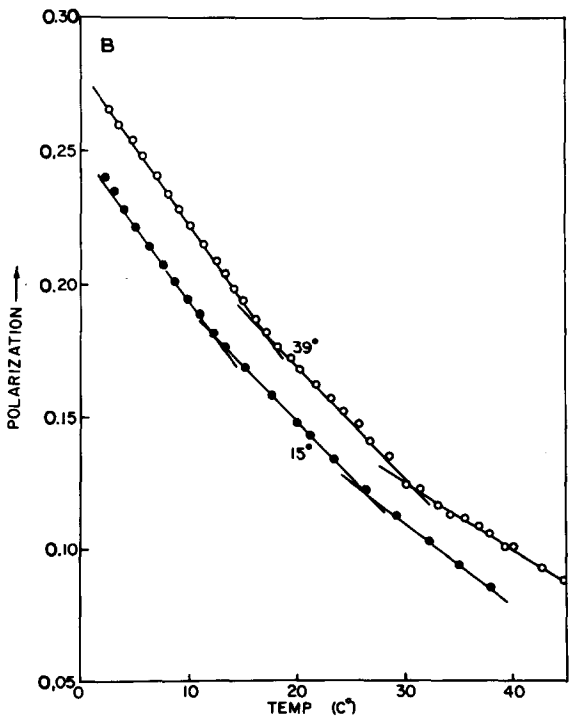


Fig. 3. The influence of temperature on diphenylhexatriene polarization in multibilayer vesicles of microsomal lipids from *Tetrahymena* grown at 39 and 15°C. A, total lipids; B, phospholipids.

type were generated for many P vs. T plots to confirm the accuracy of assigned break point temperatures.

Repeated tests with many different microsomal lipid preparations confirmed that the break points were real and highly reproducible. Table I summarizes the results of several experiments involving microsomal phospholipids from 39°C-grown cells. All curves were virtually equal in shape to the phospholipid curve in Fig. 1A. The range of variation with respect to break point temperatures was small. On rare occasions, the degree of slope change at the break points was less pronounced than shown in Fig. 1 (the control sample of 39°C-phospholipids (lower curve) in Fig. 7 is an extreme example of this response).

The last two lines in Table I report data from experiments in which the fluorescent probe 8-anilino-1-naphthalenesulfonate was utilized instead of diphenylhexatriene. Temperature-induced changes (Fig. 2) in the fluorescence intensity of this probe, which differs markedly from diphenylhexatriene in its physical properties and orientation in the lipid bilayer [13], provided good evidence that the observed break points are characteristic of the associated membrane lipids and do not merely reflect a molecular property of the probe itself.

The effects of growth temperature on microsomal lipid properties

Whereas the characteristic temperatures of the break points in P vs. T plots of total lipids and phospholipids of microsomes of 39°C-grown cells were found to be quite reproducible, they were clearly different from the break point temperatures for the equivalent lipids from 15°C-grown cells. The latter lipids contain more highly unsaturated fatty acids than those from 39°C-cells [3]. The total lipid multibilayer preparations from 15°C-microsomes exhibited break points at 8 and 20°C vs. 15 and 28°C in 39°C-grown cells) and phospholipid multibilayers showed break points at 12 and 27°C (vs. 17.5 and 32.5°C in 39°C-grown cells) (Fig. 3). The values for the low-temperature-grown cell lipids were found to be as consistent from experiment to experiment as those reported above (Table I) for 39°C-grown *T*etrahymena.

A number of studies were carried out in an effort to establish the nature of the observed break points and to learn what factors determine their characteristic temperatures. In most of these studies, phospholipids freed from tetrahymanol and other neutral lipids were employed. This was done in part because we anticipated that the use of purified phospholipids might simplify theoretical interpretations. Most importantly, we wished to avoid any effects due to triglycerides [12] which were occasionally present in small amounts in the microsomes.

Interactions between lipids produced at different growth temperatures

It seemed probable that the break point at 12°C in 15°C-microsomal phospholipids was caused by the same physical interactions responsible for the 17.5°C break point in 39°C-grown microsomes. Likewise, the two higher-temperature break points, 27 and 32.5°C, might be similarly related. This question was investigated by analyzing phospholipids from 39°C and 15°C-microsomes mixed in varying proportions. By following the gradual shift in break point temperatures as the amount of 15°C-lipids was increased, it was possible to correlate the break points of the parent phospholipids.

TABLE II

ALTERATION OF THE BREAK POINT TEMPERATURES IN POLARIZATION VS. TEMPERATURE PLOTS OF DIPHENYLHEXATRIENE IN 39°C-MICROSOMAL PHOSPHOLIPIDS AS A RESULT OF ADDED 15°C-MICROSOMAL PHOSPHOLIPIDS.

mol% 15°C-lipid present	Lower break point temperature (°C)	Upper break point temperature (°C)	Polarization at 10°C
0	17.6	32.2	0.210
25	16.6	28.7	0.199
50	16.7	29.6	0.192
75	16.3	29.1	0.170
100	12.6	29.2	0.172

Table II indicates that increasing proportions of 15°C-lipids in experimental mixtures did lead to a decrease in the break temperatures and a concurrent decrease in polarization values. It is noteworthy that a significant shift in the first break point (B1) towards lower temperatures required a fairly high pro-

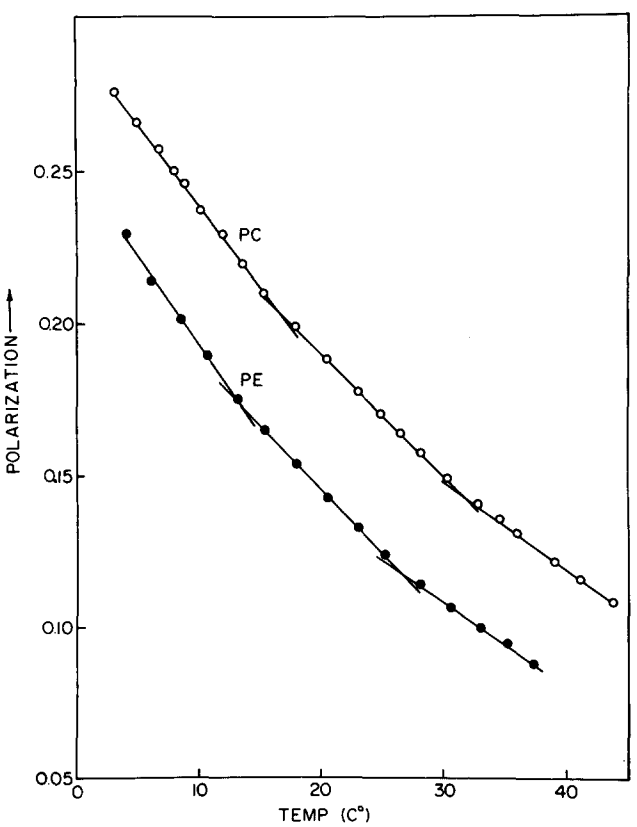


Fig. 4. The influence of temperature on diphenylhexatriene polarization in multibilayer vesicles of purified phospholipid classes isolated from microsomal lipids of 39°C-grown *Tetrahymena*. PC, phosphatidylcholine; PE, mixed fraction containing phosphatidylethanolamine and 2-aminoethylphosphonolipid.

portion of 15°C-phospholipids, whilst a sizable downward temperature shift for the second break point (B2) was already apparent when only 25% of the mixture consisted of 15°C-phospholipids.

Contribution of different phospholipid moieties to the physical properties of lipid mixtures

The elements of phospholipids most likely to affect their physical properties are the polar head-groups and the component fatty acids. Three major head-group classes are present in *Tetrahymena* microsomal phospholipids. When grown at 39°C, choline phosphoglycerides constitute 32% of the total lipid phosphorus whilst ethanolamine phosphoglycerides make up 44% and 2-aminoethylphosphonolipids account for 15% [3]. Since it is possible to achieve a quantitative chromatographic separation of the choline phosphoglycerides from those containing ethanolamine and its close structural analog, 2-aminoethylphosphonate [6], the physical properties of these two preparations were studied.

The polarization vs. temperature plots of the two purified fractions from 29°C-grown cells are depicted in Fig. 4. The polarization values for the ethanol-

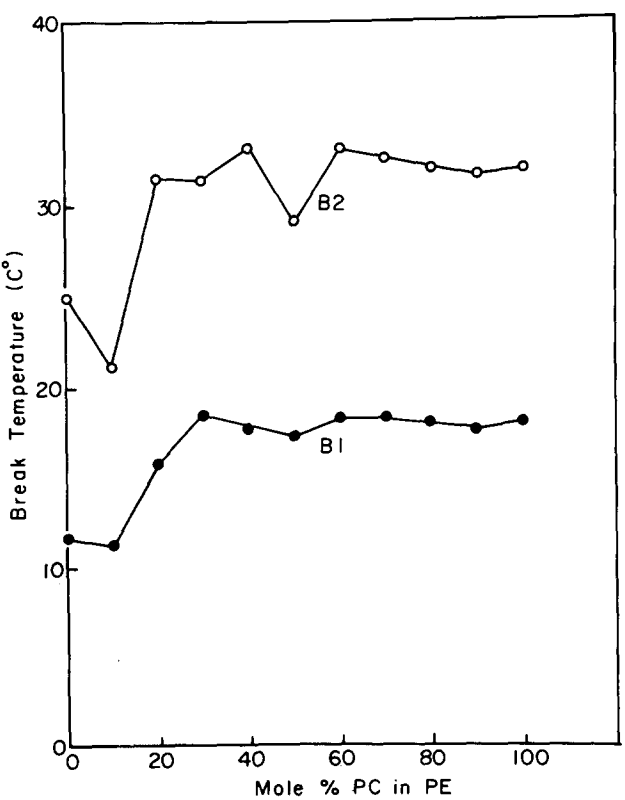
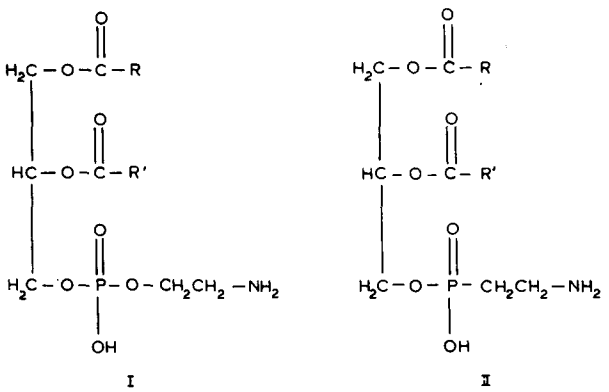


Fig. 5. Response of the lower temperature break point (B1) and the higher temperature break point (B2) of the 39°C-grown microsomal phosphatidylcholine fraction (PC) and the phosphatidylethanolamine plus 2-aminoethylphosphonolipid fraction (PE) when the two fractions were mixed in varying proportions. The lipids were mixed in organic solvents in each case and then made into liposomes in the usual way.

amine and 2-aminoethylphosphonate phospholipid fractions were lower at each temperature than the corresponding values for the choline-containing phospholipids, strongly suggesting that the former fraction was more fluid. That the polarization differences could be due in part to environmentally-induced changes in diphenylhexatriene fluorescence lifetimes has not been rigorously eliminated, but such changes are thought to be small [14].

Fig. 5 illustrates the effect that mixing the two lipid fractions in various proportions had on the temperatures of the break points. Whereas the purified ethanolamine and 2-aminoethylphosphonate lipid fraction consistently exhibited break points several degrees lower than the equivalent values for the choline lipids, addition of as little as 20 mol% of the choline lipids raised the first and second break points to the range of 16—19 and 31—33°C, respectively (reconstituting the two lipid fractions in their natural proportions resulted in a polarization vs. temperature plot identical to that shown in Fig. 1A). The naturally-occurring proportions of the two fractions (a molar ratio of purified ethanolamine-2-aminoethylphosphonate lipid : phosphatidylcholine ratio of approx. 2 : 1) are such that a slight decrease in phosphatidylcholine would lower the break point temperatures whereas a rise in phosphatidylcholine would have little effect.

The comparative polarization properties of diphenylhexatriene in the two purified phospholipid fractions were unexpected, since it is well known that synthetic phosphatidylethanolamines undergo phase transitions at temperatures as much as 20°C higher than do phosphatidylcholines of equivalent fatty acid composition [15]. Because phosphatidylethanolamine and the accompanying 2-aminoethylphosphonolipid differ in the structure of their polar moieties only slightly (see Scheme I), the reversal of the usual physical behavior of the lipid classes was assumed to result from differences in the fatty acids bound to them. Analysis did show significant differences (Table III) which must account for the similarities in break point temperatures of these lipid classes in *Tetrahymena* microsomes. In view of the important role that the constituent fatty acids play in determining the position of break points, it may be that the regulation of fatty acid composition in each phospholipid class is independent and



Scheme I. I, phosphatidylethanolamine; II, 2-aminoethylphosphonolipid.

TABLE III

MAJOR FATTY ACID COMPONENTS OF TOTAL PHOSPHOLIPIDS AND MAJOR PHOSPHOLIPID CLASSES FROM MICROSOMES OF 39°C-GROWN *TETRAHYMENA*

In the shorthand designations of fatty acids, the figure preceding the colon indicates the number of carbon atoms while that following the colon indicates the number of double bonds.

Fraction	Fatty acid								
	14 : 0	15 : 0	16 : 0	16 : 1	16 : 2 + 17 : 0	18 : 0	18 : 1	18 : 2	18 : 3
Total phospholipids	14.3	7.0	12.4	10.1	4.2	1.8	2.8	15.7	31.4
Phosphatidylcholine	19.0	6.0	15.2	9.6	2.8	1.9	2.2	12.7	30.6
Phosphatidylethanol-amine + phosphonolipid	10.6	6.2	12.0	11.3	4.4	1.5	3.1	16.2	34.7

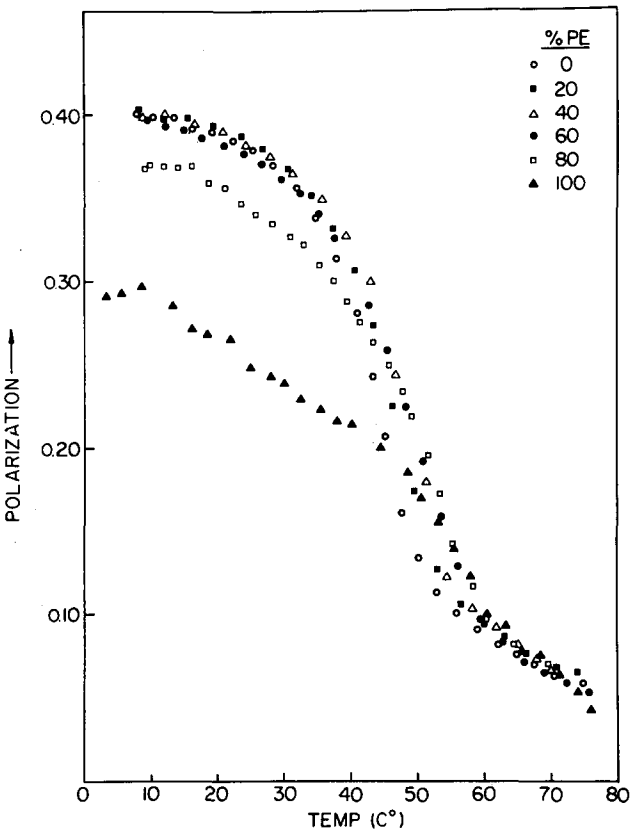


Fig. 6 The effect on diphenylhexatriene polarization vs. temperature plots of mixing the catalytically-hydrogenated phosphatidylethanolamine plus 2-aminoethylphosphonolipid fraction (PE) in varying proportions with hydrogenated phosphatidylcholine. The mol% PE is indicated by the different symbols as shown. Lipids were mixed as described in Fig. 5.

keyed in some fashion to the physical properties of the resulting molecules.

The specific properties of the two phospholipid fractions responsible for their physical behavior cannot be deduced from a consideration of the fatty acid composition alone. This is because the presence of a saturated ether-linked side-chain in 20% or more of the *Tetrahymena* phospholipid molecules [3] also might be expected to contribute significantly to the physical properties of the lipid bilayers. In order to test the effects of fatty acid unsaturation directly, samples of the two fractions were individually hydrogenated prior to fluorescence analysis. The position and shape of the curves were markedly altered (Fig. 6) giving a pattern in which the midpoint of the transition, i.e., the midpoint of the region of the steepest slope for choline-containing lipids was now several degrees Celsius lower than that for the mixed ethanolamine and 2-aminoethylphosphonate lipids. At temperatures below 45°C, the polarization of the latter fraction remained lower, perhaps due to an irregular packing of the phosphorylethanolamine head-group with its phosphonate analog. When the two fractions of hydrogenated lipids were mixed together in different proportions, analysis showed that the fluorescence properties of diphenyl-

hexatriene were strongly influenced by the choline-containing lipids. However, a steady shift of the transition midpoint to higher temperatures was found with increasing amounts of the phosphatidylethanolamine and phosphonolipid fraction.

In all cases, except possibly the 100% ethanolamine aminoethylphosphonate lipids, the discrete easily-detected break points in the plots of non-hydrogenated lipids gave way to smooth transition curves exhibiting the general shape characteristic of pure dipalmitoylphosphatidylcholine and other similar fully-saturated phospholipids.

Effect of a single phospholipid molecular species on the physical properties of a phospholipid mixture

Tetrahymena microsomes contain three major phospholipid classes and fifteen different fatty acids in significant amounts [3]. Thus, some 675 distinct phospholipid molecular species can theoretically exist in these membranes. The data presented above suggest that the break points characterizing each different membrane lipid preparation are determined by specific interactions among certain of these molecular species, especially those containing unsaturated fatty acids. In view of the heterogeneity present in the membrane lipids, it is of inter-

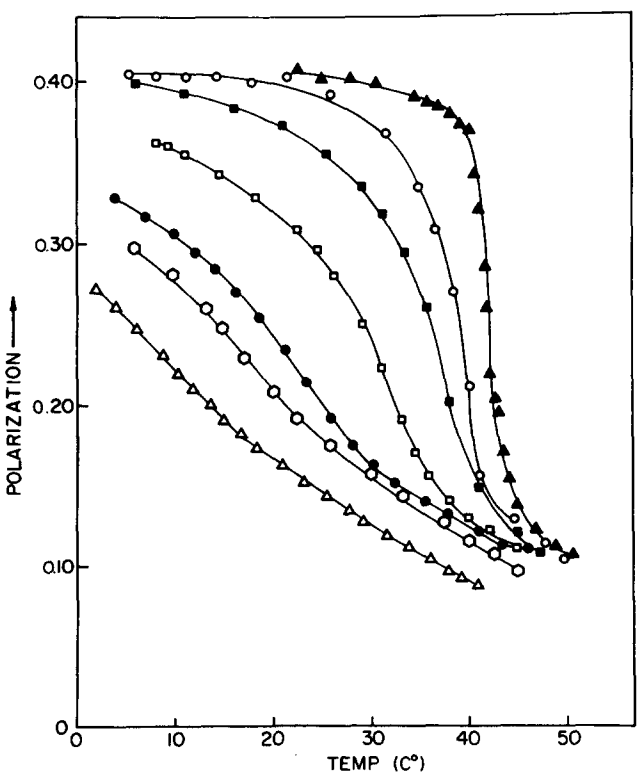


Fig. 7. The effects on diphenylhexatriene polarization vs. temperature plots of mixing phospholipids from 39°C-microsomes with dipalmitoylphosphatidylcholine in varying proportions. The mol% values of dipalmitoyl phosphatidylcholine are represented as follows: △, 0%; ○, 10%; ●, 25%; □, 50%; ■, 75%; ○, 90%; ▲, 100%.

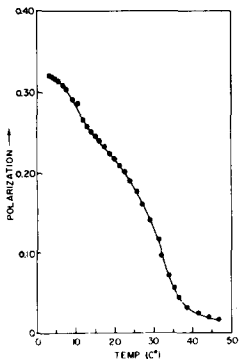


Fig. 8 The influence of temperature on diphenylhexatriene polarization in multibilayer vesicles composed of equimolar proportions of dipalmitoyl phosphatidylcholine and dilauroyl phosphatidylcholine.

est to determine the contribution of a given molecular species to the fluidity of the bilayer. It would be particularly informative to determine how much variation in a given species can be tolerated before significant changes in the properties of the bilayer occur. In the following series of experiments, we have investigated the effect of artificially-altered levels of a fully saturated phospholipid upon the thermotropic properties of microsomal phospholipid bilayers.

Fig. 7 depicts the polarization vs. temperature curves of phospholipids from 39°C-microsomes mixed in varying proportions with dipalmitoyl phosphatidylcholine. As the mol% of dipalmitoyl phosphatidylcholine decreased, the polarization values at any given temperature fell, implying a general increase in average fluidity. Break points could not be discerned in the preparations highly enriched with dipalmitoyl phosphatidylcholine. In curves containing 25% dipalmitoyl phosphatidylcholine or less, it was possible to locate with certainty the break points identifiable in the natural mixture.

The smoothness of the broad melting curves of dipalmitoyl phosphatidylcholine containing moderate amounts of 39°C-microsomal phospholipids suggested a strong co-operative effect of the many different *Tetrahymena* phospholipid molecular species on the dipalmitoyl phosphatidylcholine phase transition. In this respect, the physical behavior was quite distinct from that observed using the simple system, 50% dipalmitoyl phosphatidylcholine: 50% dilauroyl phosphatidylcholine (Fig. 8). In the latter case, polarization changes were more pronounced at 5 and at 33°C, indicating a semi-independent physical response of the two phospholipids with a low degree of co-operativity. The two separate phase transitions seen in this system are in close agreement with earlier differential-scanning calorimetry measurements [16].

Discussion

Tetrahymena typifies a wide variety of poikilothermic organisms in having the ability to acclimate to a change of environmental temperature by altering its membrane lipid composition. The biochemical basis for this metabolic response in *Tetrahymena* has been examined in some detail [2] and the evi-

dence indicates that physical properties of the microsomal lipids exert a key regulatory action on the membrane-bound fatty acid desaturases responsible for acclimation. The experiments described in the present communication constitute our initial effort to analyze in greater detail the physical changes occurring in the temperature range of greatest physiological interest, 5–45°C.

Most of the experiments utilized membrane phospholipids freshly-separated from neutral lipids to ensure a more easily interpretable system. Because of the atypically low content of sterol-type compounds found in *Tetrahymena* microsomes, the properties of the phospholipids should (and do, see Fig. 1) resemble fairly closely the patterns observed with total membrane lipids.

Our experiments revealed that decreasing the temperature of microsomal phospholipid preparations causes non-linear changes in their physical properties as reported by fluorescent probes (Figs. 1A, 2). The changes were very reproducible in different lipid preparations produced under identical growth conditions and they were also very similar when measured with probes having quite different structures and hydrophobicities (Table I). We have analyzed the experimental data in such a way as to minimize observer bias and demonstrate clearly the reality of the break points (Fig. 1B). In all cases, the observed break point joins two segments of the polarization vs. temperature plot which, considered separately, would form a straight or nearly straight line. For the time being, we can offer no theoretical reason why these lines should be straight. If the experimental points are plotted in the usual Arrhenius format (ln polarisation vs. 1/K), slope discontinuities are still apparent, but segments that are virtually straight in our P vs. T plots are slightly curved. The format we have chosen facilitates comparison of the nearly-straight segments with the intersections having much more pronounced slope changes.

The most logical explanation for these break points is that they represent some type of structural reorientation within the lipid bilayer. The alternative explanation, that the polarization vs. temperature plot slope changes represent a direct effect of temperature on inherent properties of the probe molecule, is rendered unlikely by: (1) the finding that diphenylhexatriene and anilinonaphthalenesulfonate, two probes having very different structures and hydrophobicities, reported the same break points and (2) the fact that diphenylhexatriene polarization measured in completely gel-phase preparations of dipalmitoyl phosphatidylcholine [17] or in completely liquid-crystalline phase preparations of dilauroyl phosphatidylcholine (Dickens, B.F. and Thompson, G.A. Jr., unpublished observations) yielded continuous smooth curves with no abrupt slope changes over the temperature range (5—35°C) where *Tetrahymena* lipids exhibited break points.

Our present results may be fruitfully compared with the findings of Wunderlich et al. [18]. Based on small- and wide-angle X-ray diffraction data, these workers calculated that a small fraction (≈11%) of 28°C-grown *Tetrahymena* microsomal lipids existed in a highly-ordered form even when measured at the growth temperature. Likewise, nearly 30% of 18°C-grown microsomal lipids also appeared to exist in ordered domains at 18°C. These ordered domains abruptly began to be transformed into an apparently more rigid and extensive gel phase when chilled further to approx. 16°C in the case of lipids from 28°C-cells. This transition began at nearly the same temperature in total lipids and

in the purified phospholipids. In both the 28°C- and the 18°C-grown cells, the transitions in lipid properties coincided with definite breaks in the slopes of Arrhenius plots of glucose-6-phosphatase (EC 3.1.3.9), an endoplasmic reticulum-bound enzyme [19,20]. The presence of some highly-ordered lipids at the growth temperature has recently been reported in intact membranes of prokaryotic organisms by Kang et al. [21] and Smith et al. [22] using ^{2}H-NMR. Preliminary experiments from our laboratory have shown that freshly-isolated intact *Tetrahymena* microsomal membranes labeled with diphenylhexatriene exhibit break points at the same temperatures as we have described in the present communication using extracted total lipid multilamellar vesicles. This finding indicates that the changes we have described may also be manifested in the living cells.

Whereas the precise physical changes causing the break points found in the present study are not obvious, we favor the following interpretation which is based mainly upon various data obtained in our laboratory and extrapolations from the findings of Wunderlich et al. [18]. We propose that the break points represent temperatures at which sub-populations of molecular species within the membrane fairly abruptly begin a transition from a disordered to an ordered arrangement in lipids being chilled or vice versa in preparations being warmed (as in most of our experiments). Although we have not established whether the separation is triggered by interactions of polar head-groups or hydrophobic side-chains, current physical chemical evidence would favor the growth of molecular assemblages having similar fatty acyl moieties [23,24]. Whether the ordered arrangement is a gel phase in the classical sense or a somewhat less-rigid domain of closely-interacting lipid species, which is technically liquid-crystalline yet immiscible with the bulk lipid phase, is not clear. The latter state could constitute the 'clusters' described by Wunderlich. An example of two phospholipid species co-existing as immiscible fluids has been documented by Wu and McConnell [25].

The proposed effects upon microsomal membranes of chilling cells below their growth temperature may be visualized by considering the information assembled in Fig. 9. Cells growing at 39°C already have some small populations of highly-organized lipid domains. As the cells are chilled past 27°C, certain relatively saturated classes of phospholipid molecular species reach their point of immiscibility and begin to separate from the bulk phase. The enhanced rate of polarization increase with decreasing temperature commencing at this point (Fig. 3A) is good evidence that new, more cohesive molecular associations are forming. The rapid growth of these more-rigid domains by accretion ultimately leads to the large-scale exclusion of proteins, giving rise to phase separations visible at the electron-microscopic scale. The fact that the microscopically-discernible growth of gel domains in microsomal membranes is accompanied by an apparent-perpendicular protein movement rather than a lateral movement [19,26] is consistent with the idea that the gel domains grow simultaneously from many small nucleation centers.

The break point occurring at 15°C marks the beginning of yet another accentuation in the low-temperature-induced rise of polarization values (Fig. 3A), implying a further intensification of rate of disorder to order transition involving sub-populations of phospholipids. Quantitative data derived from

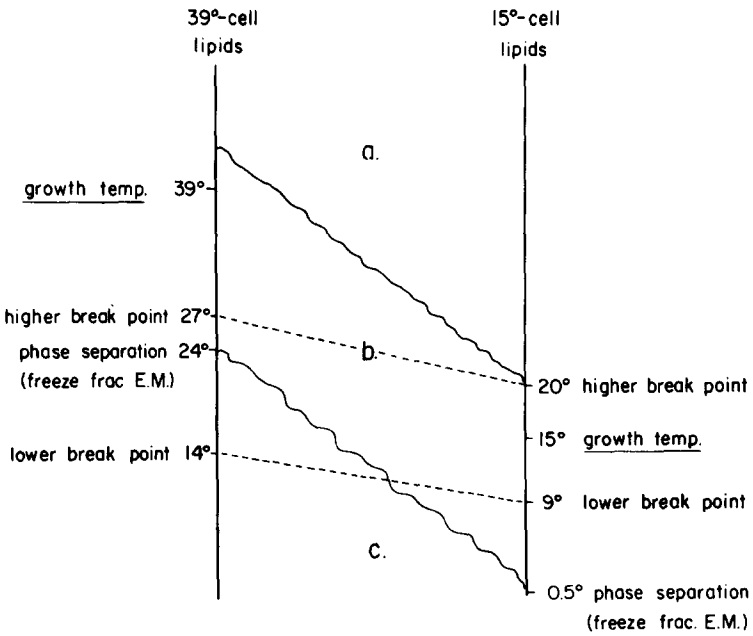


Fig. 9. A rationalization of the observed physical properties of 39°C- and 15°C-grown *Tetrahymena* microsomes and microsome-derived lipids. The left vertical axis depicts the physical behavior of preparations from 39°C-grown cells while the right vertical axis represents the same features of preparations from 15°C-grown cells. The lines connecting equivalent events on the two axes are not meant to imply that linear responses would necessarily be found in cells grown at intermediate temperatures. Wavy lines divide the system roughly into three regions: (a) region of fully-miscible lipids present in the liquid-crystalline phase; (b) region characterized by the presence of many relatively small liquid-crystalline and/or gel-phase molecular assemblages that are immiscible in the bulk lipid phase. The dashed line connects those temperatures at which the first sizable discrete population of phospholipid molecular species begin undergoing a liquid-crystalline to gel transition upon cooling; (c) region featuring a rapid increase in the extent of highly-ordered gel domains, effecting a microscopically-detectable reorientation of membrane integral proteins. The dashed line representing the second observed break point connects temperatures at which another discrete group of phospholipids commences undergoing a phase transition. Although the break points are detectable by fluorescence polarization, the impact of the phase transition on the overall structure of the membrane depends, as with the higher break point, upon the percentage of available phospholipid molecules that participate.

freeze-fracture electron microscopy [26,27] of a closely-related membrane within the *Tetrahymena* pellicle confirm that the rate of lipid phase separation (as inferred from the rate of protein particle aggregation) is, indeed, a non-linear process with respect to temperature.

A similar pattern is proposed for the 15°C-cells. In this case, however, the difference in lipid composition generated during acclimation to the lower temperature [3] creates quantitative (and perhaps qualitative) changes in the phospholipid molecular species population. Thus, the sub-population separating from the bulk phase at 20°C (Fig. 3A), although shown to be the same as that effecting the 27°C break point and subsequent detectable protein reorientation in 39°C-cells (Table II), appears quantitatively inadequate to produce such a dramatic result in the 15°C-cells. Instead, the co-operativity neccessary for the phase separation microscopically visible at 0.5°C seems to require the participation of a second and major sub-population of phospholipid molecular species

joining the ordered phase at 9°C and below.

Our most telling evidence that polarization break points can, in some cases, represent relatively modest structural transitions comes from two sources. The first is the observation that break points clearly occur in 15°C-microsomal lipids at temperatures far above those which trigger a microscopically-indicated phase separation. The second is the demonstrated identity between the break point in 39°C-microsomal phospholipids at 32.5°C and the break point in 15°C-microsomal phospholipids at 28°C. The implication of the latter is that physical transitions involving a given population of molecular species can have quite different consequences depending upon the proportion of those molecular species present and/or the nature of the lipids associated with them.

Other experimental manipulations of the microsomal phospholipids reported in the present communication are in general agreement with the interpretation proposed above. Mixtures made using phospholipid classes separated chromatographically (Fig. 5) indicate that the break points can be made to shift, but over a limited temperature range, by altering fatty acid composition and the relative proportions of different polar head-groups. The imposition of a 'phosphatidylcholine-like' polarization pattern in mixtures containing as little as 30 mol% of phosphatidylcholine reflects the higher degree of fatty acid saturation present in this fraction (Table III).

The addition of increasing amounts of a single molecular species, dipalmitoyl phosphatidylcholine, to 39°C-phospholipids caused initially a shift of the break points to higher temperatures and finally, a complete disappearance of the usual type of break points (Fig. 7). This provides a semi-quantitative impression of how small increments of one saturated molecular species affect a complex natural phospholipid mixture and vice versa.

Despite the highly reproducible nature of the polarization data, it is not presently possible to translate them quantitatively into units of microviscosity. This extrapolation would require that the steady-state polarization values first be corrected for any temperature-induced changes in the fluorescence lifetime of the bilayer-associated diphenylhexatriene. Although our laboratory has measured such changes in microsomal lipids of 39°C-grown *Tetrahymena* at two temperatures, 39 and 15°C [28], the complexity of the decay kinetics precludes correction of polarization values without extensive additional work.

In order to gain more information on the two or more physically-distinct microenvironments which appear to co-exist in the microsomal lipids at different temperatures [28], we are currently proceeding with time-resolved measurements of fluorescent probe lifetimes. Refinements in the lifetime apparatus have now made it feasible to obtain precise and rapid measurements of lipid multilayer preparations and also to measure the thermotropic properties of intact microsomes. Hopefully, additional detailed information on the physical changes in microsomal lipids can be meaningfully correlated with equally detailed data concerning temperature-regulated fatty acid desaturase activity.

Acknowledgments

This study was supported in part by grants from the National Institute of General Medical Sciences (GM20148), the National Cancer Institute (1 T32

CA09182), the Robert A. Welch Foundation (F-350 and F-365) and the National Science Foundation (OIP74-22029 and PCM-7824205). B.F.D. and C.E.M. were National Cancer Institute Post-Doctoral Trainees.

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