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RAPID MEMBRANE RESPONSE DURING LOW-TEMPERATURE ACCLIMATION

CORRELATION OF EARLY CHANGES IN THE PHYSICAL PROPERTIES AND LIPID COMPOSITION OF *TETRAHYMENA* MICROSOMAL MEMBRANES

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When *Tetrahymena pyriformis* cells grown at 39°C were chilled to 15°C, a rapid desaturation of microsomal phospholipid-bound fatty acids was observed. A concurrent but even more rapid change in the physical properties of the microsomal lipids was detected by steady-state fluorescence polarization measurements of the probe 1,6-diphenyl-1,3,5-hexatriene in lipid multilamellar vesicles. Whereas polarization vs. temperature plots of lipids from 39°C-grown cells showed discrete break points (abrupt slope changes thought to indicate altered phase separation rates) at characteristic temperatures, plots made using lipids from equivalent cells chilled to 15°C for 15 or 20 min lacked such clearly defined break points. The sharp break points reappeared in plots of microsomal lipids from cells maintained at 15°C for 30 min or longer, but in these curves the temperature of each break point was several degrees lower than in 39°C-cell lipids and nearly the same as in cells fully acclimated to low temperature (48 h or more at 15°C). Fluorescence polarization studies on mixtures of natural lipids from 39°C-cells and cells shifted to 15°C or on mixtures of natural and synthetic lipids revealed that each of the two break points in a polarization vs. temperature plot can respond to changes in lipid composition independently of the other. It is concluded that the expeditious desaturation of certain key fatty acids, perhaps coupled with limited retailoring of phospholipid molecular species, leads to pronounced physical changes in *Tetrahymena* microsomal membranes as the first step of low temperature acclimation.

Introduction

Current theory of membrane structure specifies that at normal physiological conditions structural lipids may be present in two coexisting states, one a fluid or liquid-crystalline phase and the other a more rigid gel phase [1]. While it has been possible to detect these two separate phases in simple lipid mixtures (e.g., Refs. 2–4), the complexity of naturally occurring lipid mixtures and intact membranes has made it difficult to interpret their physical properties. Recent advances in physical chemical technology (for example, in nuclear magnetic resonance spectrometry [5]) are at last allowing pertinent data to be gathered using native membranes.

Towards this same goal, our laboratory has recently reported that microsomal lipids and intact microsomal membranes from *Tetrahymena pyriformis* yield very characteristic and reproducible curves of fluorescence polarization versus temperature when analyzed with the probe 1,6-diphenyl-1,3,5-hexatriene [6,7]. Each curve revealed two abrupt changes in slope, or 'break points', in the physiological temperature range, and the characteristic temperatures of the break points were totally dependent upon the previous culture temperature of the cells. We postulated that the shape of these curves was dictated by interactions between two coexisting lipid phases and that the break points, rather than representing the onset or completion of a simple phase separation, sig-

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nified an abrupt change in the rate of a broad phase separation already in progress.

In the present communication we employ these fluorescence polarization techniques to record the specific physical changes in *Tetrahymena* microsomal membrane lipids during the initial stages of low temperature acclimation. We also discuss other treatments which alter the shape of polarization versus temperature plots in such a way as to shed light on the underlying molecular interactions involved.

Materials and Methods

Materials

Dipalmitoyl, dimyristoyl and dilinoleoyl phosphatidylcholines were purchased from Serdary (London, Ontario). The purity was confirmed by thin-layer chromatography and gas-liquid chromatography, and they were then used without further purification. 1,6-Diphenyl-1,3,5-hexatriene was purchased from Aldrich (Milwaukee, WI).

Methods

Culture conditions. T. pyriformis, strain NT-1, was cultured in a complex medium previously described by Fukushima et al. [8]. The cells were grown either isothermally at 39 or 15°C or, in some cases, at 39°C followed by a shift in growth temperature to 15°C. The shifting procedure routinely used was to cool exponentially growing cultures ($1.5 \cdot 10^5$ cells/ml) from 39 to 15°C over 5 min by slowly swirling the flask in an ice bath. The temperature of each culture flask was constantly monitored to assure a linear cooling rate. The flask thus shifted would then be incubated at 15°C for a prescribed time, usually 1 h.

Lipid isolation and analysis. Microsomes were isolated using the fractionation procedure of Nozawa and Thompson [9] except that the centrifugation at $100\,000 \times g$ was 90 min instead of 60 min. The lipids were extracted by the method of Bligh and Dyer [10], and their fatty acid composition was determined by gas-liquid chromatography as previously described [8]. Total lipid phosphorus was estimated by the method of Bartlett [11] as modified by Marinetti [12].

Fluorescence measurements. Multibilayer vesicles were made using 1 μmol samples of microsomal lipids, mixtures of microsomal lipids, or mixtures of microsomal lipids plus a synthetic lipid at the level of 1 mol%, according to the method described by Martin and Thompson [13]. Lipids were always dissolved in chloroform prior to vesicle preparation to insure uniform mixing. The lipid to probe ratio was routinely adjusted to equal 500 phospholipid molecules per probe molecule.

Polarization measurements were made using the custom-made device described earlier [6]. The polarization values were not corrected for light scattering since the liposomes prepared from the microsomal lipids routinely give samples of remarkably low turbidity [13]. Whereas the measurements reported here were made by raising chilled liposomes through a series of equilibrated temperature points, representative samples measured over the same range but in order of descending temperature gave the same results, providing the liposomes were previously conditioned first by slow chilling.

The fit of straight lines to the experimentally determined polarization vs. temperature curves was tested by subjecting selected curves to the mathematical tests and statistical treatments utilized previously [7].

Results

We have previously shown significant differences between polarization versus temperature plots of purified phospholipids, total lipids, and intact membranes of microsomes from 39°C-grown *Tetrahymena* and equivalent preparations from 15°C-grown cells [6,7]. These differences appear to result from differences in the degree of unsaturation of the phospholipid fatty acids, since there is a pronounced increase in unsaturation at the lower growth temperature. However, there is also a temperature-induced change in the polar headgroup distribution of the phospholipids [8] which could also contribute to the altered physical behavior.

In the present communication we compare lipids from 39°C-grown cells and from cells shifted from 39 to 15°C and maintained at the lower temperature for 1 h or less. Even within 1 h after such a shifting procedure the microsomal phospholipid fatty acid composition changes abruptly towards the pattern found in cells fully acclimated to 15°C (Table I). However, the polar headgroup distribution has been reported

TABLE I

FATTY ACIDS OF MICROSOMAL PHOSPHOLIPIDS FROM *TETRAHYMENA* GROWN AT 39 OR 15°C OR GROWN AT 39°C AND THEN SHIFTED TO 15°C FOR THE INDICATED TIMES BEFORE HARVEST

The values are average weight percentages ± S.D. of three analyses of one experiment involving, in columns 1–4, cells from the same batch of cells grown at 39°C. The data are representative of two additional experiments. Fatty acids undergoing the greatest change following the temperature shift are underlined.

Fatty acid *	Cell treatment				
	39°C-grown	39 → 15°C (15 min)	39 → 15°C (30 min)	39 → 15°C (60 min)	15°C-grown
12 : 0	1.8 ± 0.05	0.6 ± 0	1.0 ± 0.15	1.2 ± 0.06	0.8 ± 0.23
14 : 0	14.6 ± 0.50	13.6 ± 0.21	11.7 ± 0.22	11.4 ± 0.27	11.7 ± 0.94
ai 15 : 0	4.7 ± 0.13	4.3 ± 0.10	4.4 ± 0.16	4.2 ± 0.10	1.6 ± 0.11
15 : 0	2.0 ± 0.05	2.0 ± 0.06	2.4 ± 0.06	2.2 ± 0.05	tr
16 : 0	12.7 ± 0.24	12.5 ± 0.17	12.7 ± 0.13	10.4 ± 0.05	8.4 ± 0.27
16 : 1	12.5 ± 0.59	11.7 ± 0.17	13.9 ± 0.21	14.6 ± 0.12	8.6 ± 0.33
16 : 2 + 17 : 0	5.7 ± 0.05	5.7 ± 0.10	6.8 ± 0.06	5.2 ± 0.27	5.2 ± 0.13
unk	1.2 ± 0.05	1.1 ± 0.05	1.0 ± 0.05	1.1 ± 0.05	2.1 ± 0.18
18 : 0	0.6 ± 0.10	0.7 ± 0.05	0.8 ± 0.17	0.7 ± 0.06	tr
18 : 1	6.5 ± 0.13	7.0 ± 0.18	5.6 ± 0.14	5.4 ± 0.10	8.1 ± 0.31
18 : 2, $\Delta^{6,11}$	2.0 ± 0.14	2.2 ± 0.10	0.9 ± 0.05	0.7 ± 0	6.2 ± 0.22
18 : 2, $\Delta^{9,12}$	11.9 ± 0.42	13.0 ± 0.13	13.4 ± 0.33	13.6 ± 0.19	16.5 ± 0.75
18 : 3	21.2 ± 0.40	22.9 ± 0.30	22.8 ± 0.21	25.7 ± 0.30	28.5 ± 0.68
Unsaturation index **	1.26	1.33	1.34	1.41	1.62

* The number preceding the colon represents the number of carbon atoms, while that following the colon is the number of double bonds present.

** Calculated average number of double bonds per fatty acid (considering 17 : 0 to be a negligible fraction of the 16 : 2 + 17 : 0 peak).

not to change to its characteristic 15°C pattern until several hours after the temperature shift [14,15], and we have confirmed that there is no significant alteration in the headgroup distribution by 1 h following the shift to 15°C (unpublished data). Therefore interpretation of the physical alterations brought about by the shift is simplified.

Polarization versus temperature curves for microsomal total lipids of the isothermally grown and temperature shifted cells were determined experimentally. Following the 39 to 15°C shift, which was executed over a 5-min period, there was a gradual decrease in polarization at all temperatures. More remarkable was the rapid change in shape of the polarization vs. temperature curves. A typical family of curves, displaced on the ordinate scale sufficiently to make the configuration of each curve clear, is shown in Fig. 1. By 30 min following the shift (curve D) the shape was nearly identical to that found in fully acclimated 15°C cells (curve F).

The break-point temperatures were routinely found to approximate those of either isothermal 39°C-grown cells (15 and 28°C) or 15°C-grown cells (8 and 20°C), except for some cases involving a 15-min shift (curve B) and in the single 20-min shift experiment (curve C). Here we observed a complex pattern which revealed hints of the break points characteristic of 15°C preparations and 39°C preparations, all in the same curves. These latter experimental curves were too complex for us to confirm the presence of distinct break points by our statistical methods, because these methods require relatively long straight line segments between the breaks. However, curves B and C were unquestionably different in shape from any obtained using isothermally-grown cells. Similar patterns of changes were observed using purified phospholipids and intact membranes (data not shown), except that the break point temperatures for these preparations (and those from fully-acclimated cells) were slightly different, in agreement with

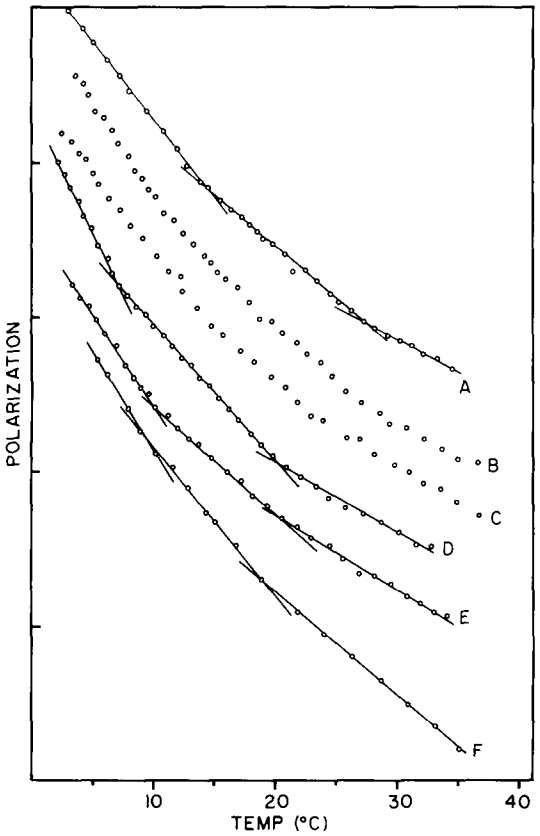

Fig. 1. Changes in the polarization of diphenylhexatriene in microsomal total lipids during low temperature acclimation in vivo. Lipids were isolated from microsomes of 39°C-grown cells (curve A) or from cells chilled from 39 to 15°C and maintained at 15°C for 15 min (curve B) *, 20 min (curve C), 30 min (curve D), or 60 min (curve E). Curve F depicts the pattern found in cells grown for several days at 15°C. Polarization values were calculated by the usual equation: $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ [6]. The absolute P value at any given temperature decreased only slowly with increasing time following the shift from 39 to 15°C (e.g. P at 20°C was in the range of 0.13–0.16 for all curves). To facilitate examination of the curve shape, the curves have been displaced on the y axis so that they do not overlap. Thus while each unit on the y axis represents a ΔP of 0.05 units, the absolute value for each curve differs slightly.

* A few of the 15 min, curves resembled more closely curve A, indicating that 15 min at the low temperature must be the minimal time needed to detect a physical change.

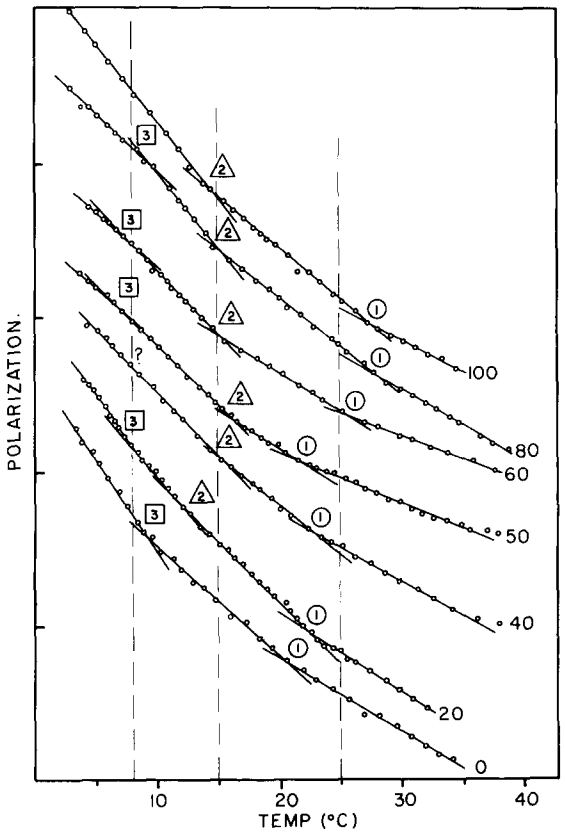

Fig. 2. The physical effects of mixing total lipids from microsomes of 39°C-grown cells (curve 100) with differing proportions of total lipids from microsomes of cells shifted from 39 to 15°C for 60 min. The mol% of 39°C-lipid is indicated near the right end of each curve. Polarization units are as described in Fig. 1. See text for discussion of the numbered break points.

the previously reported findings [6,7]. In several independent analyses of microsomal total lipids, phospholipids, and intact membranes isolated during the first 30 min after the cells were chilled to 15°C, the usual two discrete break points never appeared at temperatures intermediate between the 39°C and the 15°C patterns.

In order to investigate further the significance of the observed break points, we mixed together in various proportions microsomal lipids from 39°C-grown cells and equivalent lipids from cells that had been shifted to 15°C for 1 h. In this manner it was possible to manipulate the fatty acid composition artificially while maintaining the phospholipid headgroup proportions constant.

The results of this experiment are shown in Fig. 2. Here, as in Fig. 1, the curves are displaced on the

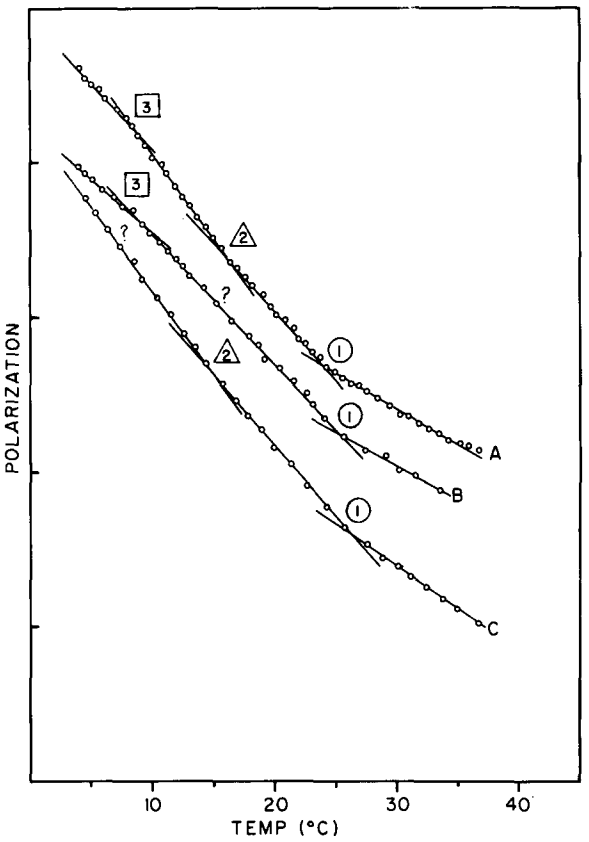

Fig. 3. The physical effects of mixing small amounts (1 mol%, based on phosphorus content) of synthetic phospholipids with *Tetrahymena* microsomal total lipids. Curve A = a 60/40 (mol/mol) mixture of 39°C-grown lipids and 39→15°C chilled (60 min) lipids. The other curves were made using the same 60/40 mixture used for curve A but were altered to contain 1 mol% of dipalmitoyl phosphatidylcholine (curve B) or 1 mol% dimyristoyl phosphatidylcholine (curve C). Polarization units are as described in Fig. 1.

y-axis for clarity. Comparison of the curves reveals that although the high-temperature break point (1, in circle) gradually shifted downwards from 29 to 20°C as the proportion of shifted lipids was increased, the low-temperature break point (2, in triangle) remained relatively constant at 14–15°C but became less and less distinct until it finally disappeared entirely in the pure 15°C-shifted lipids. Furthermore, when as little as 20 mol% of the 15°C-shifted lipids was incorporated into the mixture, a third break point (3, in square), appeared near 8°C. The temperature of this break point varied only slightly with increasing proportions of the shifted lipids, but as the composition of the mixture changed, there was a remarkable increase in the slope of the curves at temperatures below the 8–9°C break point. The slope of this temperature segment of the curve increased so rapidly that when only 40 mol% of the 39°C-lipids remained, the break point in the vicinity of 8°C was entirely eliminated, presumably because the angle joining the adjacent segments expanded to 180°. In mixtures which contained less than 40 mol% of the 39°C-lipids, the break point reappeared, but with the slope of the line defining it being steeper rather than shallower than that of the adjacent line segment.

The data presented thus far suggested that changes in the phospholipid molecular species distribution can affect the polarization versus temperature curve fine structure in a variety of ways. To determine what effect a small change in the relative abundance of one specific molecular species might play in this thermotropic response, we utilized three synthetic phospholipids with contrasting physical properties: dilinoleoyl phosphatidylcholine (DLPC), dimyristoyl phosphatidylcholine (DMPC), and dipalmitoyl phosphatidylcholine (DPPC). Each synthetic lipid was tested by adding it in a final concentration of 1 mol% to a 60/40 mixture of 39°C-lipids and 39→15°C-shifted lipids (curve A, Fig. 3; also see curve labeled 60, Fig. 2). The only marked effect of DLPC (whose phase transition occurs well below 0°C) was to decrease the overall polarization without affecting the shape of the curve or the location of the break points (data not shown). Addition of the other synthetic lipids had no effect upon the absolute polarization at most temperatures, but altered the slope of the curves (Fig. 3). As in the earlier figures, these curves have been displaced on the y-axis to assist in the visualization of slope changes. The addition of DMPC (phase transition = 23°C) caused the low-temperature break point (3, in square) to disappear without greatly affecting the rest of the curve (curve C, Fig. 3). In contrast, addition of 1 mol% DPPC (phase transition = 41°C) abolished the mid-temperature break point (2, in triangle) with little change elsewhere in the curve (curve B, Fig. 3).

Discussion

The cause of break points in polarization versus temperature curves such as those described here has

not been firmly established. Janoff et al. [16], after comparing the polarization properties of *Escherichia coli* membranes with those of extracted lipids, concluded that the alterations of break point positions during temperature acclimation were caused not by lipid changes, but rather by changes in a nonlipid outer membrane component. In *Tetrahymena,* on the other hand, the temperature induced modification of the membrane lipid composition seemed primarily responsible for the differences in break point location in membranes of 39°C-grown and 15°C-grown cells [7]. However, since both the fatty acid composition and the polar headgroup distribution differ in 39°C-grown and 15°C-grown *Tetrahymena* [8] considerable uncertainty remained as to what specific property of the lipids was directly involved.

In the experiments described above, we manipulated *Tetrahymena* cells by a brief temperature-shift to induce a marked change in the phospholipid fatty acid composition without altering the polar headgroup pattern. The polarization versus temperature plots obtained using lipids from cells transferred to 15°C for 1 h were not significantly different from those derived using cells fully acclimated to 15°C. The shape of the polarization versus temperature curves and the location of the break points are therefore primarily dictated by the specific fatty acid composition.

When lipids from the 1 h-shifted cells were mixed with the lipids from 39°C-grown cells in different proportions (Fig. 2), a variety of responses was observed. We believe that these responses can be logically explained based upon the following assumptions. First, we assume that both liquid-crystalline and gel phase lipids are present over the 5 to 40°C temperature range considered here. This conclusion is based on our observation that at temperatures which are lower (−10 to −20°C) and higher (40 to 50°C) than illustrated in our figures, the slope of the polarization versus temperature curves are much less pronounced (Ref. 7 and unpublished data). Compared to these 'baseline' regions, the region of the curves having an appreciable slope change must describe a lipid bilayer undergoing a continuous phase transition of at least some molecules, since fluorescence probe polarization values are known to increase sharply during a liquid crystalline to gel transformation. This conclusion is supported by the work of Wunderlich et al. [17] who used X-ray diffraction measurements to show the existence of two distinct phases in *Tetrahymena* lipids at physiological conditions. By this way of thinking, the slope of the polarization versus temperature curves should always be at least approximately proportional to the rate of phase separation if the probe is, as reported [18], equally distributed. It follows that an abrupt increase in slope, such as occurs at the observed break points, signifies not the beginning of a lipid phase separation but merely a sudden escalation in the rate of a phase separation already in progress.

The second basic assumption is that at any given temperature in the 5 to 40°C range, the specific lipid molecules which can undergo a phase separation are determined not only by their own individual molecular properties but also by the nature of the other molecular species associated with them. There is much direct and indirect experimental evidence to support this idea of cooperativity during lipid phase separations [19,20].

If these assumptions are correct, then a logical explanation of Fig. 2 can be put forth. For example, the high temperature break point (1, in circle) moves to a lower temperature when lipids from 15°C-shifted cells are added because the sizable sub-population of phospholipid molecular species which suddenly joins the ongoing phase separation process at 29°C in normal 39°C-grown lipids is now enriched with more unsaturated but still compatible lipid species, thereby lowering the temperature at which the cooperative liquid-crystalline to gel transformation of this subpopulation begins.

In a different sort of change, the addition of 20 mol% or more of lipids from 15°C-shifted cells induced the appearance of a break point (3, in square) where none existed before. This situation could arise through the introduction of particular molecular species capable of preventing many of the remaining liquid-crystalline lipids from undergoing phase separations by sequestering them into new associations. The dramatic increase in the slope of the lowest temperature line segment (below break point 3) as more 15°C-shifted lipids were added could merely signify an increase in the absolute quantity of that subpopulation of molecular species which undergoes phase separation at 9°C or slightly below.

The changes in the thermotropic response pro-

duced by the addition of 1 mol% of synthetic lipid species demonstrate that even small increments of a given molecular species can have a pronounced effect, depending upon its ability to interact with the other lipid species in the mixture. The very fluid DLPC had no modifying effect on phase transformations in the 5 to 40°C range, while DMPC altered the behavior of lipids in the vicinity of 10°C (Fig. 2). DPPC, which in pure form undergoes a phase transition at 41°C, obviously interacts with more saturated lipid subpopulations in the mixture.

It would be premature to assume that the details of fine structure in these curves made using total microsomal lipids are due entirely to interactions among the phospholipids and are not secondarily influenced by the sterol-like tetrahymanol molecules present. Tetrahymanol may affect cooperative binding between members of a particular lipid sub-population. Earlier experiments [6], in which we mixed in differing molar ratios purified phospholipids from microsomes of cells fully acclimated to 39 and 15°C, also revealed abrupt changes in the break-point temperatures. However, no atypically shaped curves, such as curves B and C in Fig. 2, were detected. Unfortunately, these experiments are not easily compared with the ones reported here, since in addition to the absence of tetrahymanol, the 15°C cells employed earlier had been at the low temperature long enough to alter their pattern of polar headgroups. Some differences could be due to the fine tuning induced by changes in the phospholipid headgroups [8]. In retrospect, it is also possible that some structural details of the polarization versus temperature curves in this earlier work were overlooked because fewer experimental points were collected.

Our experiments with mixtures of natural lipids (Fig. 2) and natural lipids plus synthetic lipids (Fig. 3) provide convincing evidence that the location of break points in polarization versus temperature curves is a function of the phospholipid molecular species distribution. This conclusion may be used to interpret the physical changes observed in the native lipids of *Tetrahymena* microsomes during temperature acclimation. In nature there would appear to be only a limited number of compatible lipid sub-populations, each of which responds to temperature change nearly independently of others. The pattern of phospholipid molecular species biosynthesis and transformation following a shift from 39 to 15°C gives rise to a polarization versus temperature curve of the 15°C type even more rapidly than the change in fatty acid composition (Table I) or our artificial mixing experiments (Fig. 2) might suggest. This is because acclimation in vivo, which proceeds mainly by the desaturation of preexisting phospholipids [21], entails the simultaneous depletion of certain molecular species classes and the enrichment of others. Furthermore, after desaturation there may be extensive retailoring of certain molecular species by deacylation-reacylation reactions [22].

The identification and quantification of the particular molecular species belonging to each of the discrete phospholipid populations are underway in our laboratory, but the work has not yet been completed. Nor have the physical manifestations of the significant change in the proportions of polar head groups that eventually follows a temperature shift been adequately measured (although physical studies indicate that the kind of P vs. T curves shown above should be influenced to some extent by polar head group alterations [23]). Such information is essential for characterizing physical changes in the local environment of microsomal fatty acid desaturases, which are so crucial to the acclimation process. The *Tetrahymena* microsomal system affords a promising opportunity to correlate membrane structure and function during the response of cells to environmental stress.

Acknowledgments

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