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Effects of Growth at Different Temperatures on the Physical State of Lipids in Native Microsomal Membranes from *Tetrahymena*†

Benjamin F. Dickens‡ and Guy A. Thompson, Jr.*

ABSTRACT: Fluorescence measurements of the probe 1,6-diphenyl-1,3,5-hexatriene in native *Tetrahymena pyriformis* microsomal membranes revealed characteristic "break points" in curves of polarization vs. temperature. In the 5–35 °C range, membranes from cells grown at 39 °C exhibited two break points, one at 11.6 ± 0.6 °C and another at 23.1 ± 1.6 °C. Membranes from 15 °C grown cells also showed two break points, one at 8.0 ± 1.7 °C and another at 17.7 ± 1.7 °C. Complementary measurements of turbidity (absorbance at 360 nm) vs. temperature revealed break points at approximately the same temperatures as observed with the fluorescent probe, thus strengthening the likelihood that the break points signify the onset or termination of lipid phase separations or some other significant structural alteration of lipids. In general, break points measured in the native membrane samples occurred at slightly lower temperatures than did break points in lipids extracted from comparable membranes. This suggests two possible types of protein–lipid interaction. First, there may be a selective withdrawal of relatively highly saturated phospholipid molecular species from the bulk lipid phase and into the protein annulus regions. Alternatively, the configuration of the hydrophobic core of certain key membrane proteins may be such that nonspecific interactions with the lipids stabilize the liquid-crystalline phase.

The protozoan *Tetrahymena pyriformis* is widely used as a model system for investigating molecular mechanisms involved in cellular acclimation to environmental temperature extremes (Thompson & Nozawa, 1977; Thompson, 1980). It is now well established that *T. pyriformis*, like many other plant and animal cells, is capable of adapting to temperature changes by rapidly adjusting certain metabolic pathways. What is perhaps the key metabolic alteration results in a modification of the membrane lipid composition, which in turn offsets the pronounced rigidifying or fluidizing effects of sudden exposure to low or high temperatures, respectively.

One of the most important of these lipid changes associated with temperature acclimation involves fatty acid desaturase enzymes that occur as integral membrane proteins of the endoplasmic reticulum. We have postulated (Kasai et al., 1976) that one or more of the desaturases can in some fashion sense a change in the fluidity of its own immediate membrane environment and respond by increasing or decreasing its activity relative to the rates of other lipid-forming enzymes. Such a self-regulating mechanism could maintain membrane fluidity relatively constant at an optimal level despite sharp temperature fluctuations.

Recent work in this laboratory showed that temperature-induced changes in the physical properties of *T. pyriformis*

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microsomal (endoplasmic reticulum) lipids can be detected with great sensitivity by fluorescence polarization measurements (Dickens et al., 1980). Nonlinear changes (break points) in the slope of polarization vs. temperature plots of diphenylhexatriene in membrane lipids were reproducible under constant conditions but plainly different when cells were grown at different temperatures. A variety of data from several laboratories suggests that these break points represent the initiation or termination of extensive lipid phase separations which could, if they occur in native membranes, have significant effects on fatty acid desaturase activity (Dickens et al., 1980; Wunderlich et al., 1978).

In the present paper we have extended our observations to intact microsomal membrane vesicles isolated from *T. pyriformis* grown at either 39 or 15 °C. The characteristic membrane changes are still clearly detectable and are generally similar to those noted in isolated lipids. However, certain distinctive differences suggest that protein–lipid interactions can be influential in regulating the physical state of this membrane.

Experimental Procedures

Materials

Dipalmitoylphosphatidylcholine, 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. Dilauroylphosphatidylcholine was a gift from Dr. C. Wade (University of Texas, Austin, TX). Its purity was confirmed by both thin-layer chromatography and gas–liquid chromatography of derived methyl esters. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was used without further purification. Tetrahydrofuran was purchased from Fisher Scientific Co. (Pittsburgh, PA) and was further purified by fractional distillation prior to use.

Organism. *T. pyriformis*, strain NT-1, was cultured in a complex medium previously described by Fukushima et al. (1976) at either 15 or 39 °C and routinely harvested at cell densities of 1.5×10^5–2.0×10^5 cells/mL.

Methods

Lipid Isolation. Microsomal membranes were isolated by the procedure of Nozawa & Thompson (1971), except that they were sedimented by centrifugation at 100000*g* for 90 min instead of 60 min. The isolated microsomal membrane pellets were either used immediately or frozen at −70 °C for subsequent use as a source of intact microsomal membranes. In experiments requiring isolated microsomal lipids, these lipids were extracted from freshly prepared membranes by the procedure of Bligh & Dyer (1959). Lipids could be extracted from membranes after 2 days of freezing without affecting the polarization vs. temperature curves, but only data from extracts of fresh microsomes are included in this paper. Total lipid phosphorus was estimated by the method of Bartlett (1959) as modified by Marinetti (1962).

Fluorescence Measurements. Lipid multibilayer vesicles were prepared from microsomal lipids or synthetic lipids as described earlier (Martin & Thompson, 1978). Diphenylhexatriene was dissolved in organic solvents containing lipids to give a probe to lipid molar ratio of 1:500. The solvents were evaporated under N_2, and any trace of the solvent was then removed by redissolving the lipids in methanol and reevaporating the sample, first under 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 0.5 mM and sonicated for 1–2 min in a sonicator bath at ~40 °C. Before commencing polarization vs. temperature scans, the lipid preparations were chilled slowly over ~2 h to facilitate the coalescence of any small radius of curvature vesicles that might have been created during sonication (Larrabee, 1979) and to generally condition the lipids. This slow cooling was essential in order to obtain completely reproducible polarization vs. temperature plots.

Samples of membranes were prepared by adding diphenylhexatriene in tetrahydrofuran directly to a concentrated aqueous suspension (15% sucrose–50 mM KCl) of microsomal membranes resuspended after centrifugation by gentle hand homogenization in an all-glass homogenizer. The probe to lipid molar ratio was approximately 1:150–200. At this ratio fluorescence emissions by natural fluorophores in the membrane were small (≤1%) when compared to probe emissions; therefore, no corrections were made to compensate for these spurious emissions. The membrane–probe suspensions were typically adjusted to an absorbance of 0.20 ± 0.01 at 360 nm for use in polarization measurements. The membrane-containing samples were maintained at 0–4 °C during isolation and until polarization measurements were begun. In some cases membrane preparations were diluted to give several samples of various absorbances but with a uniform probe to lipid ratio. These were used for investigating the influence of light scattering on polarization. In a further control experiment, quenching of DPH fluorescence by native membranes was tested for by keeping probe levels constant and adding additional membranes. In this case, no loss of total fluorescence intensity was noted over the range of membrane concentration discussed in this report, and it was therefore unnecessary to correct polarization values for quenching.

Steady-state polarization measurements were made with the custom-made device described by Martin & Thompson (1978), modified by interfacing with a PET 2001 series computer (Commodore, Palo Alto, CA) as previously described (Dickens et al., 1980). Temperature regulation was with a jacketed cuvette and a circulating water bath as previously described (Dickens et al., 1980). Polarization values were recorded only after equilibrating the sample at the desired temperature for at least 3–4 min. Although stirring was not routinely employed, the data gained from time-equilibrated samples were indistinguishable from control data recorded after stirring the cuvette thoroughly by hand at each experimental temperature. In most cases the samples were measured by raising chilled liposomes through a series of equilibrated temperature points. If the temperature of samples warmed in this way was subsequently lowered in a similar stepwise fashion, the polarization behavior could be shown to be reversible.

In some experiments a special cooling device was employed for chilling samples to subzero temperatures. Dry nitrogen gas was passed through a copper coil immersed either in liquid nitrogen or hot water. This gas was then blown upward through holes near the base of the cuvette onto all four faces of the sample cuvette located within a custom-made chimney with appropriate holes cut to allow passage of excitation and emission radiation. Temperature was not equilibrated for these measurements.

In all polarization experiments, parallel and perpendicular fluorescence emission intensities for each temperature were automatically measured 10 times and averaged. Mean polarization (P) values were calculated from five of these averaged measurements at each temperature according to the formula

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ represent the fluorescence intensities parallel and perpendicular to the excitation beam, respectively. The standard deviation of P was less than ±0.001 for all data points presented.

The following computer analysis (Dickens et al., 1980) was performed in order to identify regions of greatest slope change in the polarization vs. temperature curves. Beginning at one end of each curve, segments of five adjacent points were analyzed by the least-squares method for the slope of the straight line best fitting them. After determination of 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 of the middle point of that segment.

Absorbance Measurements. The effect of temperature on the optical density of multibilayer liposomes of synthetic lipids and lipids isolated from *T. pyriformis* microsomes and in microsomal membrane vesicles was recorded continuously at 360 nm with a Beckman Model UV 5260 spectrophotometer and a 1 cm path length cuvette. The liposomes were prepared by the same technique used for fluorescence studies except that the lipid concentration was higher and no fluorescent probe was added. Synthetic lipid samples were prepared at a final concentration of 2 mg/mL, while microsomal lipids were utilized at a concentration of 2 μmol of lipid phosphorus/mL. The intact membrane suspensions were typically prepared to give a convenient absorbance for the chart recorder used and contained 0.13–0.20 μmol of lipid phosphorus/mL. The samples were heated (~1 °C/min) in a jacketed cuvette connected to a circulating water bath, and the temperature was monitored with a thermistor placed directly into the sample. Aliquots of microsomal lipid multibilayer preparations removed at 4.5 and 30 °C for negative-staining electron microscopy revealed no obvious change in vesicle size attributable to temperature-induced fusion during an absorbance scan. Regions of greatest slope change were identified, as described above, by computer, utilizing absorbance values recorded at 0.5 °C intervals.

Results

Differences between Physical Behavior of Diphenylhexatriene in Lipid Bilayers and in Native Membranes. Polarization vs. temperature plots of diphenylhexatriene (DPH) in resuspended membrane preparations were made in order to correlate the previously determined (Dickens et al., 1980) physical behavior of liposomes prepared by using total lipids extracted from *T. pyriformis* microsomes with the behavior of intact microsomal membranes. Figure 1A compares lipids and intact membrane vesicles derived from microsomes from 39 °C grown cells, and Figure 1B compares similar preparations from 15 °C grown cells. In both cases the abrupt slope changes, or break points, shown earlier (Dickens et al., 1980) to occur very reproducibly at fixed temperatures in extracted lipid samples were also clearly discernible in membrane samples. The precise location of each break point was confirmed by a computer-assisted plot of slope vs. temperature (see insets in Figure 1). Although the slope vs. temperature plots reveal that the regions of the curves on either side of break point are not absolutely straight, we have joined them by straight lines as a convenience in making comparisons.

Because there has been some uncertainty in the literature and in our minds as to how experimental curves of this nature should be interpreted, we submitted representative data to several additional graphical and statistical tests (Willcox & Patterson, 1979; Wolfe & Bagnall, 1979; Potter & Ross, 1979). By all these diverse criteria, the experimental data of

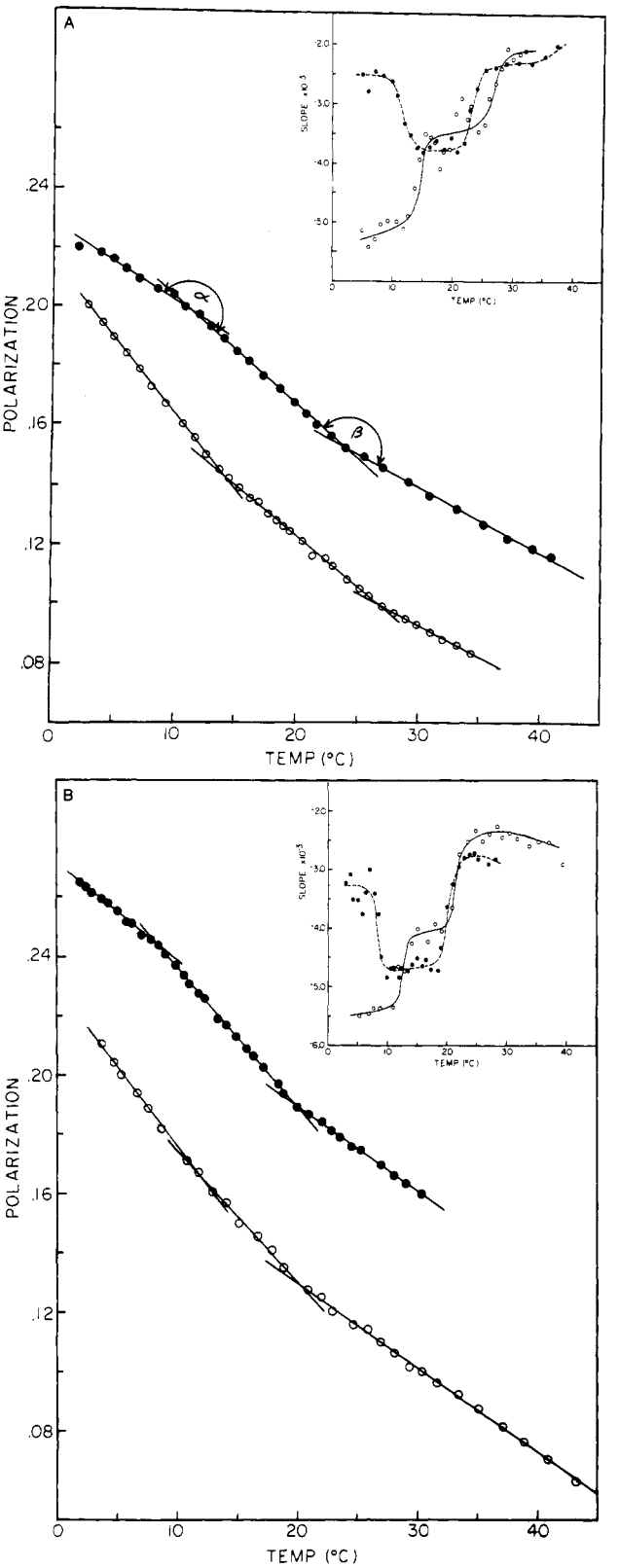

FIGURE 1: Influence of temperature on DPH polarization in multibilayer vesicles of microsomal lipids (○) and in microsomal membrane vesicles (●). (A) Samples from 39 °C grown cells. (B) Samples from 15 °C grown cells. (Insets) Slope vs. temperature plots of microsomal lipids (○) and microsomal membrane vesicles (●) derived from the associated polarization vs. temperature plots as described under Methods.

Figure 1 are fit better by three straight lines than by a single smooth curve.

Variation in the break-point temperatures was small, with the breaks in the 15 °C membranes ranging from 5.0 to 9.7 °C (average 8.0 ± 1.7 °C) for the low break point and from 15.0 to 20.0 °C (average 17.7 ± 1.7 °C) for the high break point in seven separate experiments. One plot of 15 °C membranes gave anomalous values of 10 and 28 °C. Some error may have been involved in establishing slopes of this latter curve since an atypically low number (16) of experimental points was available. In 39 °C membranes the breaks ranged from 11 to 12.5 °C (average 11.6 ± 0.6 °C) for the low break point and from 21.5 to 25.2 °C (average 23.1 ± 1.6 °C) for the high break point in 11 experiments. Here, too, one curve was anomalous, giving values of 12 and 29 °C.

As a supplemental criterion of reproducibility, the angles formed at each break point were compared for replicate experiments. This approach was feasible only because the experimental polarization values on either side of the breaks could be roughly approximated by straight lines in all cases. The angle of the low-temperature break point of 39 °C membranes (angle α, Figure 1A) was 189.0 ± 2.2°, while the high-temperature angle (angle β, Figure 1A) averaged 166.4 ± 3.1°. The corresponding values for 15 °C membrane curves (Figure 1B) were angle α = 190.5 ± 3.6° and angle β = 163.3 ± 3.6°. In all other aspects, e.g., absolute slope values, the replicate curves were also remarkably similar.

A comparison of the membrane curves with the corresponding lipid curves (Figure 1), which were equally reproducible (Dickens et al., 1980), reveals the following differences. First, the polarization values for membranes were always higher than those for lipids at any given temperature, reflecting the additional constraint on motional freedom of the DPH probe exerted by the membrane proteins. Secondly, the break points in the membrane preparations always appeared at temperatures which were at least slightly lower than those observed in lipid preparations, even when both samples were prepared from the same batch of cells. And, finally, there was a striking difference in the slopes of the membrane and the lipid curves at temperatures below ~10 °C. As compared with the >180° angle α for intact membrane samples reported above, angle α in the 39 °C lipid preparations was 170.8 ± 0.96° and in 15 °C lipid preparations was 169.8 ± 1.3°. Thus the inflection of the low-temperature membrane break point was consistently in the opposite direction to that observed in the protein-free liposomes. In contrast, the high-temperature break point (angle β) was 168.8 ± 1.3° in 39 °C lipids and 168.0 ± 3.9° in 15 °C lipids—nearly the same as described above for membranes.

Effect of Light Scattering on Polarization Values. All curves shown in Figure 1 are uncorrected for artifacts due to light scattering. We have shown earlier (Martin & Thompson, 1978) that the low optical density of lipid samples precludes the need for corrections, but the much higher turbidity of the membrane suspensions creates potential problems. When experimental data are collected by using polarized light, any scattering of either the excitation or the emission photons can result in an apparent decrease in polarization by changing the orientation of the measured light (Teale, 1969; Lentz et al., 1979). The effect of increasing membrane absorbance on polarization is illustrated in Figure 2. In this experiment DPH was added to a concentrated 15 °C microsomal membrane suspension, and the membrane–probe mixture was then diluted with sucrose–KCl buffer to give a series of samples having different absorbance values but the same probe to membrane

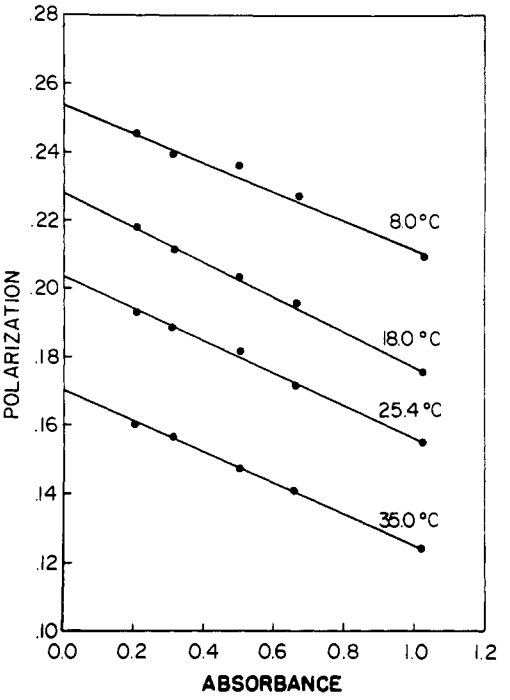

FIGURE 2: Effect of increasing absorbance on polarization values in microsomal membrane vesicles. A concentrated sample of DPH-labeled 15 °C microsomal membranes was diluted to give five samples of decreasing absorbance. Polarization for each sample was measured at a variety of temperatures and plotted vs. sample absorbance.

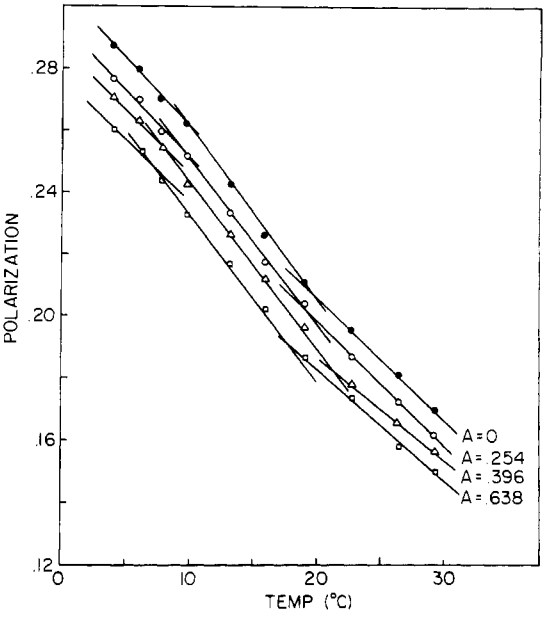

FIGURE 3: Effect of increasing sample absorbance on polarization vs. temperature plots of 15 °C microsomal membranes. A concentrated sample of DPH-labeled 15 °C microsomal membranes was diluted to give several different samples of different absorbances. The polarization of each sample was measured at a series of temperatures. The values for polarization at 0 absorbance were extrapolated from curves of polarization vs. absorbance for each isolated temperature, as drawn in Figure 2.

ratios. A rather marked decrease in polarization with increasing absorbance was noted at all temperatures. Light scattering induced reductions in polarization could be corrected for by extrapolating each curve to 0 absorbance.

From these values and similar ones obtained at other temperatures, it was feasible to construct a family of polarization vs. temperature curves (Figure 3), including one corrected to

0 absorbance (upper curve). After determining the slope of the curves in Figure 2, corrections could be made mathematically as well as graphically by employing the equation

$$\bar{P} = P_a\left(\frac{1}{1 - KA}\right)$$

as described by Lentz et al. (1979). In this formulation $\bar{P}$ is the polarization at 0 absorbance, P_a is the apparent polarization at the experimental absorbance (A), and K is a constant equal to the slope of the polarization vs. optical density curve. It was clear that apart from uniformly raising the polarization values at all temperatures, correction of the curves for light scattering artifacts had no significant effect on the shape of the curves. By routinely avoiding the use of samples having an optical density in excess of 0.25, it was possible to derive accurate information concerning break points and slopes from raw data without the need for correction.

Polarization Values Measured at Subzero Temperatures. Our recent experience in measuring polarization break points in a variety of lipid mixtures (Dickens et al., 1980) strongly suggested that the two break points in each intact membrane curve (upper plots, Figure 1) were casually equivalent to the two break points occurring in each lipid curve (lower plots, Figure 1) at slightly higher temperatures, respectively. However, the tendency of the membrane curves to decrease in slope at lower temperatures raised the possibility that lipid transformation into the gel phase was much more nearly complete here than in curves for the extracted lipids. Accordingly, an effort was made to chart the behavior of both types of samples at even lower temperatures. Using the cooling device described under Methods, we were able to chill preparations suspended in 50% ethylene glycol to subzero temperatures by directing a stream of chilled N_2 gas onto the faces of the sample cuvette. The design of this chilling device dictated that data would necessarily be taken during the slowly increasing or decreasing sample temperature rather than at temperature equilibrium. In the case of 39 °C grown cells, the curves of both native membranes and extracted lipids flattened out between −12 and −16 °C (data not shown), indicating that the presence of the proteins in the lipid bilayer had no marked effect on the low-temperature physical behavior of the lipids. Likewise, equivalent samples from 15 °C grown cells showed no pronounced effect of protein perturbation, since both began to flatten out at −20 to −24 °C. It can be assumed from these findings that the extent of phase separation in lipid vesicles and in membranes is at least approximately the same at any given temperature.

Interestingly, the low-temperature scans, which were generally extended upward to 25 °C or higher, did not reveal the characteristic breaks that are so evident in Figure 1. Initially, it was thought that the absence of these discrete breaks might be due to the presence of 50% ethylene glycol in the samples, but testing ethylene glycol containing preparations by the standard, stepwise equilibration method revealed breaks at the usual temperatures. Further investigation established that despite a relatively slow heating rate of ~0.5 °C/min, entrance of the chilled N_2 gas near the base of the cuvette containing the viscous 50% ethylene glycol mixture could cause it to develop a considerable temperature gradient from top to bottom (as much as 10 °C at the lowest temperatures), thereby obliterating the fine structure detectable by using the more sensitive equilibration technique.

Light Scattering Induced Absorbance Changes as Indications of Phase Changes. During the course of our experimentation to correct polarization measurements for light scattering artifacts, we examined the possibility of utilizing absorbance at 360 nm as an indicator of lipid phase changes. Light scattering affords a sensitive means of detecting the changes in lipid refractive index and birefringence that accompany phase transitions (Yi & MacDonald, 1973). The sensitivity of our spectrophotometer for making these measurements was assessed by noting the effect of temperature on the absorbance of light at 360 nm by dipalmitoylphosphatidylcholine multibilayers. A sharp (0.08 absorbance unit) drop of light scattering in samples warmed to 41 °C coincided precisely with the phase transition of this lipid as determined by others (Chapman, 1975; Janiak et al., 1976). The technique also appears capable of detecting phase separations, such as that induced by changing the temperature of multibilayer liposomes made from an equimolar mixture of dipalmitoylphosphatidylcholine and dilauroylphosphatidylcholine (Figure 4). Although much less conspicuous than the change observed in the one-component dipalmitoylphosphatidylcholine sample described above, we were able to reproducibly obtain regions of slightly increased slope at approximately 5 and 25 °C, corresponding roughly to the temperature ranges for the two transitions as detected previously by fluorescence polarization (Dickens et al., 1980) and calorimetry (de Kruijff et al., 1974).

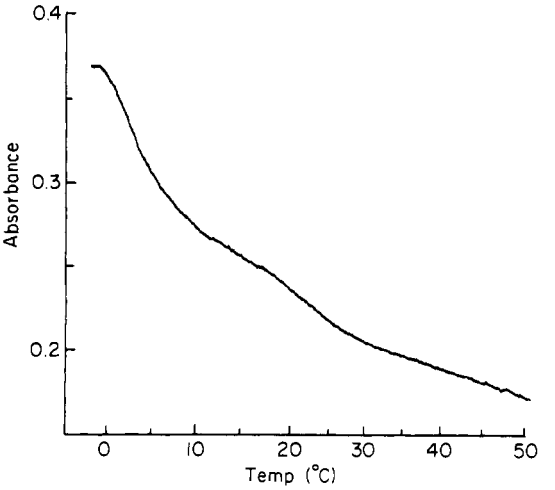

FIGURE 4: Effect of temperature on absorbance of multibilayer vesicles made by using equal weights of dipalmitoylphosphatidylcholine and dilauroylphosphatidylcholine. The absorbance was measured at 360 nm with a heating rate of ~1 °C/min. Note that the abscissa for this spectrophotometer tracing is not precisely linear, due to slight variations in the heating rate.

Preparations of microsomal lipid liposomes and of intact microsomal membrane vesicles were examined for light-scattering changes. Parts A and B of Figure 5 show typical absorbance vs. temperature curves of total lipid samples and membrane vesicle samples, respectively, from 39 and 15 °C grown microsomal membranes. In this figure the slow and slightly variable cooling rate has been linearized on the temperature axis and the data have been replotted from the direct scan to illustrate that break points in the absorbance vs. temperature curves coincide almost exactly with the polarization break points shown in Figure 1. At present we have no explanation for the rise in polarization at the higher temperatures in Figure 5B (>40 °C in the upper curve and >27 °C in the lower curve). The possibility that this represents the effect of protein denaturation became less likely when the high-temperature rise was found to be reversible.

It must be emphasized that the absorbance changes displayed by these microsomal samples are very small—much smaller than those considered earlier (Figures 2 and 3) as

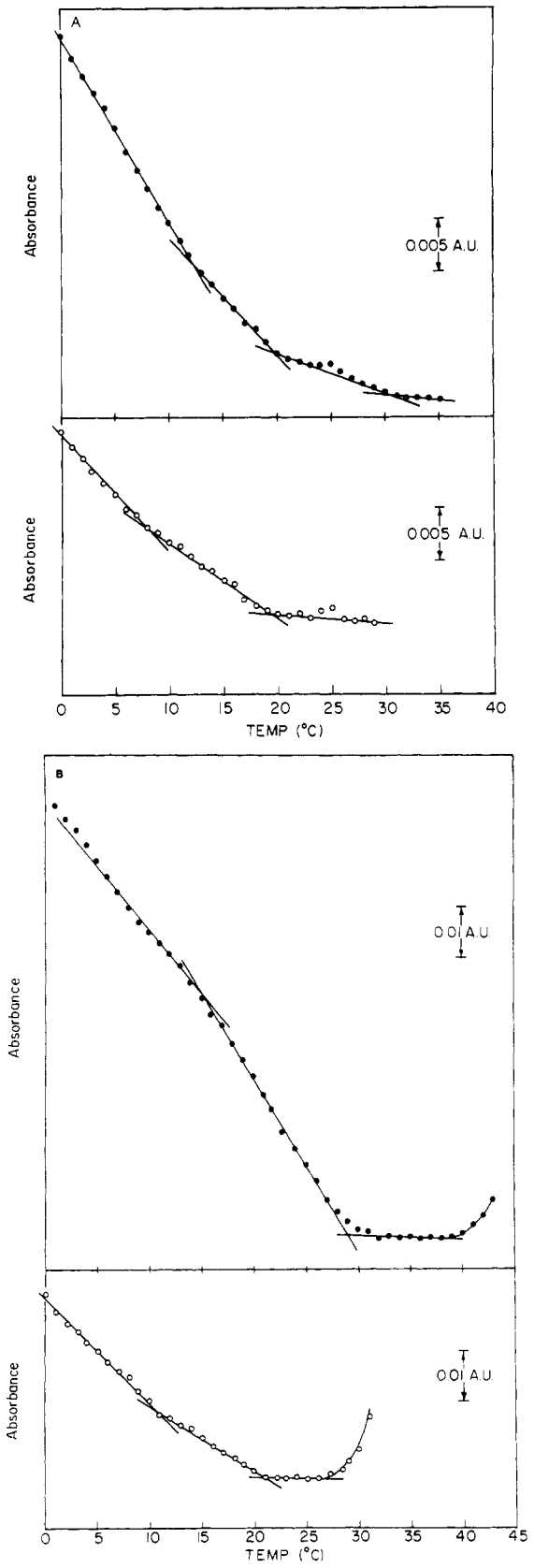

FIGURE 5: Effect of temperature on absorbance of multibilayer microsomal lipid vesicles (A) and microsomal membrane vesicles (B). The absolute absorbance of the intact microsomal membranes at 360 nm was 3–4 times higher than that for the isolated lipids. The absorbance was measured at 360 nm with a heating rate of ~1 °C/min and replotted to give a linear abscissa. Upper curves (●), preparations from 39 °C cells; lower curves (○), preparations from 15 °C cells.

indirectly affecting polarization values. It can easily be calculated that these small absorbance differences would have no detectable influence on the polarization of DPH in similar samples (no DPH was present in the preparations used for making Figure 5). If an effect could be detected, it would be expected to accentuate rather than diminish the sharpness of the polarization break points.

Discussion

It is now well established that the physical properties of membrane lipids are influential in controlling the activity of membrane-bound proteins (Sanderman, 1978). A wide variety of studies have sought to characterize the precise way in which lipids and proteins interact in membranes (Longmuir et al., 1977; Birrell et al., 1978; Marsh & Barrantes, 1978; Chapman et al., 1979). The complexities of molecular organization in a multicomponent natural membrane are so formidable that most analyses have been restricted to simpler mixtures of synthetic lipids and proteins.

We have recently shown that the physical behavior of native *T. pyriformis* microsomal lipids can be sensitively monitored by studying the depolarization of lipid-associated diphenylhexatriene (Dickens et al., 1980). This probe has been found to associate tightly with the hydrophobic lipid bilayer core and to distribute itself into both the liquid-crystalline and the gel phases when these two phases are present together (Lentz et al., 1976). Measurements of polarization vs. temperature obtained on temperature-equilibrated lipid samples by using a computer-balanced steady-state device disclosed very reproducible, characteristic slope changes for different preparations, depending upon the growth temperature (and therefore lipid composition) of the cells from which they were derived. Certain trivial explanations of the abrupt slope changes, e.g., that they are artifacts due to a nonlinear effect of temperature on the lifetime of DPH fluorescence, have been eliminated (B. F. Dickens and G. A. Thompson, unpublished experiments), but the precise cause of slope changes remains to be established. Considering the polarization data together with X-ray diffraction measurements (Wunderlich et al., 1978), freeze–fracture electron microscopy analyses (Wunderlich & Ronai, 1975; Martin et al., 1976), Arrhenius plots of membrane-bound enzyme activity (Wunderlich & Ronai, 1975), and the present light-scattering results, it is logical to conclude that each break in a polarization vs. temperature curve might represent a marked change in the rate of lipid phase separation. On the basis of these varied findings, we have proposed (Dickens et al., 1980) that a sudden rate change of this type might represent the beginning or end of a phase transformation involving a particularly large fraction of phospholipid molecular species having very similar physical properties. In a recent study of LM-cell plasma membranes, Rintoul et al. (1979) concluded that similar nonlinear thermotropic responses would be triggered by a specific cooperative phase transformation of phosphatidylcholine–sphingomyelin complexes. On the other hand, our data are not sufficiently clear-cut to rule out alternative explanations, such as the transformation of some lipids to a type of nonbilayer conformation (Cullis & de Kruijff, 1979).

In the present paper we have extended our previous work with extracted lipids to intact microsomal membrane vesicles. We find that the break points, though shifted to slightly different temperatures, are still clearly discernible in the membrane preparations and are still characteristic of the cells' growth temperature. The correspondence of these break points to structural reorientation of lipids is supported by light-scattering measurements of both extracted lipids and intact

membranes. It would seem highly probable that living *T. pyriformis* cells subjected to the rapid reduction in temperature (39 °C → 15 °C) that we impose during temperature acclimation studies (Martin & Thompson, 1978) would experience those changes in membrane physical properties. Our goal is to determine whether one or more of the specific physical alterations is directly responsible for the temperature-induced change in membrane-bound fatty acid desaturase activity shown to occur in *T. pyriformis* (Martin & Thompson, 1978).

Polarization measurements of membrane vesicles such as those reported here are subject to artifacts caused by light scattering if the optical density of samples is high. After making such corrections for our preparations (Figure 3), we found that by utilizing low (~0.2 OD) membrane concentrations for polarization studies, the only significant changes in the uncorrected membrane curves (upper curves in Figure 1A,B) would be to increase each polarization value slightly. In the ensuing discussion, we have chosen to utilize uncorrected values for membrane polarization.

Observed Differences between Polarization Curves of Lipids and Membranes. It is informative to compare the polarization vs. temperature curves of membranes with those of the membrane-derived lipids, from both 39 °C cells (Figure 1A) and 15 °C cells (Figure 1B). In both cases, three types of differences could be discerned. First, polarization values, both uncorrected and corrected, at any given temperature were considerably higher for intact membrane preparations than for dispersed lipids. Second, the "break temperatures" observed in membranes were lower than the corresponding break temperatures in the extracted lipids. Third, the slope of the polarization vs. temperature curves of membranes experienced a decrease at temperatures below the lowest break point (12 °C in Figure 1A and 8 °C in Figure 1B), while slopes of the lipid curves became greater below their lowest break point. All three of these changes can be logically explained in the light of existing experimental or theoretical findings.

Effect of Membrane Proteins on Absolute Polarization Values. Let us consider first the general increase in polarization values of membrane samples throughout the temperature range examined. There is considerable evidence in the literature (Marsh & Barrantes, 1978; Birrell et al., 1978; Longmuir et al., 1977) indicating that lipids tend to associate with integral membrane proteins in such a way as to reduce the mobility of the bound lipid molecules. Thus those DPH probe molecules present in such a lipid annulus would contribute toward a greater average polarization. Although DPH has been shown to distribute itself fairly uniformly in a mixture of liquid-crystalline and gel phase lipids (Lentz et al., 1976), its physical behavior is not well enough understood for us to try and estimate the extent of the protein-immobilized lipids on the basis of our steady-state measurements.

Effect of Membrane Proteins on Characteristic Break-Point Temperatures. The second observed type of change was the shift of each characteristic break temperature to a lower value in membrane preparations as compared with lipid preparations extracted from identical membranes. Break points in extracted microsomal total lipid vesicles, analyzed as described by Dickens et al. (1980), were as follows: from 39 °C grown cells, 15 and 28 °C (vs. 11.6 and 23.1 °C shown in the present paper for native membrane vesicles), and from 15 °C grown cells, 8 and 20 °C (vs. 8.0 and 17.7 °C for membrane vesicles).

Two rather different kinds of protein–lipid interaction might be invoked to account for this reduction in the characteristic temperatures. The first envisions a selective attraction of certain phospholipid species into the lipid annulus surrounding each protein molecule. If these molecular species were bound tightly enough to the proteins to restrict their free diffusion among the bulk-phase lipids of the bilayer, the net effect would be a depletion of those species from the bulk phase. Pink & Chapman (1979) have predicted on theoretical grounds that T_c, the phase-separation temperature in the bulk lipid phase, could either decrease or increase, depending upon whether the proteins exhibited a preferential binding for saturated or unsaturated lipid species, respectively. Experimental findings seemingly in support of this prediction have recently been published by Verma et al. (1980). Even the small change in bulk-phase lipid composition resulting from a protein-induced depletion could have a detectable effect. Wilkinson & Nagle (1979) confirmed that a significant elevation of the liquid-crystalline to gel transition temperature of dimyristoylphosphatidylcholine could be induced by adding <5 mol % of distearoylphosphatidylcholine, and Rintoul et al. (1979) detected large changes in phase-separation temperatures of natural phosphatidylcholine mixtures when relatively small additions of natural sphingomyelin were made. In the latter study, slope changes in fluorescence polarization vs. temperature curves resembled in many respects those reported here and in our previous work with *T. pyriformis* microsomal lipids.

An alternative explanation for the lowering of break-point temperatures through lipid–protein interaction is based on the recent theoretical study of Owicki & McConnell (1979). These authors devised a mathematical model whereby the effects of integral membrane proteins on bulk-phase lipids may be estimated without assuming an annulus of specific molecular species. The basis for this treatment is an order parameter μ, which is proportional to membrane thickness. The value of μ for the lipid bilayer may vary from 1, when the lipids are in the gel phase, to 0, when the lipids are warmed to the liquid-crystalline phase, thereby decreasing the bilayer thickness by about 20–30% (Janiak et al., 1976). The hydrophobic core of the membrane proteins is assumed not to undergo these temperature-induced fluctuations in thickness but rather to remain at a constant thickness of μ_0. The influence of the proteins upon lipid phase changes would be dependent upon μ_0; a value of 1 for μ_0 would stabilize the lipid gel phase by approximating the thickness of the gel phase bilayer, whereas a value of <0.5 for μ_0 would favor protein–lipid interactions stabilizing the liquid-crystalline phase and, in the process, lowering the phase-transition temperature.

Following strictly the Owicki–McConnell model, we might explain our findings by assuming that the average μ_0 value of *T. pyriformis* microsomal membrane proteins is <0.5. By encouraging the lipids to remain in the liquid-crystalline state, this would drive the phase separation temperatures lower, as observed. On the other hand, if one considers the complex mixture of phospholipid species present in the natural membrane (a condition not dealt with by Owicki and McConnell) a much *higher* value of μ_0 might also account for our experimental results. In this case the thicker hydrophobic protein core would be expected to attract the more saturated phospholipid species into its annulus, thereby raising the unsaturation index in the remaining bulk phase and reducing phase separation temperatures as discussed above.

Effect of Membrane Proteins on the Slope of Polarization vs. Temperature Curves. Establishing the presence or absence of a lipid annulus in the *T. pyriformis* microsomes would simplify interpretation of the observed changes in physical properties described above. Some insight into this question may be gained from a consideration of the polarization values of membranes at low temperatures (0–10 °C, Figure 1). Here

can be seen a third difference between lipid and membrane curves. In this temperature range the slope of the curves is much less than that observed in isolated lipid preparations. This might be due to a heightened influence of proteins on lipid phase changes brought about by an ever-increasing concentration of proteins crowding into the lipids remaining liquid crystalline at these temperatures (Pink & Chapman, 1979). However, our freeze–fracture electron microscopy observations (Kitajima & Thompson, 1977) indicated that in this particular membrane little protein enrichment by lateral diffusion occurs at low temperature. For this reason it would seem more probable that the attenuated response of polarization at low temperature is caused not by an increasing amount of interacting proteins but rather by a more pronounced effect exerted by the proteins that are present. Ample experimental evidence exists to show that the size of a lipid annulus may increase rapidly at low temperatures (Curatolo et al., 1977; Longmuir et al., 1977). But even though the accretion of more phospholipid molecules to an annulus would decrease their mobility under these conditions, the immobilization influenced by proteins would not be as pronounced as in a transition from the liquid-crystalline to the true gel phase, such as observed in the protein-free lipid specimens (Figure 1, lower curves). This interpretation is in agreement with data obtained by using proteins interacting with synthetic lipids (Gómez-Fernández et al., 1979).

The results of our experiments provide an empirical record of discrete physical changes in native *T. pyriformis* microsomal membranes. They also promise a rare opportunity to interpret the mechanism for temperature-induced fluidity change in these natural membranes. Experiments utilizing time-resolved fluorescence polarization techniques are now under way to refine the values for lifetimes of fluorescence decay obtained earlier with *T. pyriformis* lipids (Martin & Foyt, 1978) and to quantify the extent of different microdomains under specified conditions. It is important to confirm whether temperature change can induce discontinuous physical responses that might alter the activity of fatty acid desaturase residing in the membranes. Modifying the proportions of various molecular species populations by feeding experiments (Nozawa & Thompson, 1979) and by selective in vivo hydrogenation (Restall et al., 1979) should permit the identification of the principal lipid species involved in each break-point transition.

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Characterization of Collagens of Diseased Human Gingiva†

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ABSTRACT: In the gingiva and other connective tissues, alteration in the collagens is primarily responsible for their functional impairment during disease. To study the collagen alterations, we extracted diseased human gingival tissue with neutral and acidic solvents and then with pepsin. The pepsin extract was separated into proteins soluble in 2.5 and 1.5 M NaCl and proteins insoluble in 1.5 M NaCl. By the criteria of solubility behavior in NaCl solutions, elution from (carboxymethyl)cellulose (CM-cellulose) columns, sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis, CNBr peptide pattern, and amino acid composition, the collagens of acidic and neutral solvent extracts and 1.5 M soluble fraction of pepsin extract were characterized as type I collagen and the 1.5 M NaCl insoluble collagen as type III. The 2.5 M NaCl fraction contained α1, A, and B chains. The α1 chains resembled α1[I] in amino acid composition, and, since α2 chains were lacking, it appeared that these chains derived from type I trimer collagen. The A and B chains were purified from the 2.5 M NaCl fractions by salting out at acidic pH. The final (A plus B) chain fraction was resolved into two major and one minor protein peaks by phosphocellulose chromatography. The major peaks were characterized as A and B chains on the basis of amino acid composition and CNBr peptide patterns. The minor peak had electrophoretic mobility slightly less than B chains, and the amino acid composition was different. Analysis of the proportion of different collagen types extracted indicated that type III collagen, which is the second major fraction in other connective tissues, is only a minor constituent in the gingiva. More interestingly, A and B chains accounted for a greater proportion than type III. Unlike the fibroblast cultures, the type I trimer formed only a small proportion of collagens of diseased gingival tissue.

The connective tissue component of normal human gingiva is made up predominantly of collagen and proteoglycans, and these components are responsible for the tensile strength, tooth supporting property, and stability of the gingiva. The gingival collagen has an unusually high degree of structure, and it is organized into distinct structural and functional fiber groups (Goldman, 1951; Arnim & Hagerman, 1953; Page et al., 1974). Analysis of CNBr digests indicated that the gingival connective tissue contains predominantly type I collagen together with small amounts of type III (Ballard & Butler, 1974; Dabbous & Brinkley, 1977), but, in contrast to skin, the gingival collagen turns over at an inordinately high rate, even in adulthood (Page & Ammons, 1974). So far systematic characterization studies to identify additional collagen chains, such as A and B,[1] in the gingiva have not been attempted, and whether the various fibers are made up of the same or different collagens remains unknown.

The collagenous component of the gingiva undergoes severe quantitative and qualitative alterations during the development of gingivitis and periodontitis, and these alterations result in functional impairment of the gingival tissues [for reviews, see Page & Schroeder (1973, 1976)]. At an early stage, ~70% of the collagen immediately adjacent to the junctional epithelium is lost, and the resident fibroblasts manifest features of cytopathic alteration. As the disease progresses, fibrosis and scarring may occur. Fairly extensive studies have been done with cultured fibroblasts obtained from explants of normal and periodontally diseased human gingiva. Cells from normal tissues produce type I and III collagens in approximately the same proportion as they are found in most tissues. Fibroblasts derived from the diseased tissue appear to produce normal amounts of collagenous proteins, and type I and III collagens are made. However, in addition these cells synthesize a new collagen identified as type I trimer[2] (Narayanan & Page, 1976; Narayanan et al., 1978). Whether or not the type I trimer collagen is present in diseased tissues is not known. Nor is it known if additional collagens such as A and B, which were only recently described (Burgeson et al., 1976; Rhodes & Miller, 1978), occur in diseased tissues. In order to answer some of these questions, we have isolated and characterized the constituent collagens of diseased human gingiva.

Experimental Procedures

Materials

The gingival tissue used consisted of surgical specimens obtained from patients with chronic periodontitis of varying severity. Standard type I and III collagens were obtained from human fetal skin by methods described by Epstein (1974) and Chung & Miller (1974). Pepsin (sp act. 2500 units/mg) was the product of Worthington Biochemical Corp., Freehold, NJ. Ion-exchange celluloses were the products of Whatman Biochemicals Ltd., Maidstone, Kent, U.K. P-2, Bio-Gel A-5M (200–400 mesh) and the chemicals for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, CA. All

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[1] Because neither the stoichiometry of the A and B chains nor their relationship has been clearly resolved, we refer to these simply as A and B chains. These chains may originate from collagens of composition A_3, B_3, or AB_2 or from collagens containing additional chains designated as αC (Brown & Weiss, 1979; Sage & Bornstein, 1979).

[2] This term represents the homotrimer of α1[I] chains.

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