Thermotropic Lipid Clustering in Tetrahymena Membranes†

Frank Wunderlich,* Adam Ronai, Volker Speth, Joachim Seelig, and Alfred Blume

ABSTRACT: The effect of temperature on the core structure of endoplasmic reticulum membranes has been visualized directly in cells of the poikilothermic eukaryote *Tetrahymena pyriformis* by freeze-etch electron microscopy. Moreover, the effect of temperature on the smooth microsomal membrane vesicles isolated from these cells, as well as on the extracted membrane lipids, has been examined by fluorescence probing, electron spin resonance, proton nuclear magnetic resonance, and calorimetry. Freeze-etch electron microscopy of *T. pyriformis* cells, equilibrated at different temperatures between 28 and 5°, reveals the emergence of smooth areas on the fracture faces of endoplasmic reticulum membranes at temperatures below ~17°. In this temperature range, we also find discontinuities in the glucose 6-phosphatase activity, in the fluorescence intensity of 8-anilino-1-naphthalenesulfonate, in the partition of 4-doxyldecane, and in the separation of the outer hyperfine extrema of 5-doxylstearic acid in the microsomal membranes. These membranes apparently contain at least two lipid environments of different fluidity as indicated by the 12-doxylstearic acid spin-label. Proton nuclear magnetic resonance of the extracted membrane lipids indicates an abrupt change of the fatty acid chain mobilities at temperatures below ~17°. This, however, is not due to a true thermal liquid crystalline ⇌ crystalline phase transition. Calorimetric measurements also support this conclusion. The thermotropic alterations observed within the membranes are interpreted to be due primarily to a clustering of "rigid" liquid crystalline lipid environments which exclude membrane-intercalating proteins.

Temperature has a crucial influence on the structure and function of biomembranes. At physiological temperatures, biomembranes are envisaged as "fluid", i.e., the fatty acyl chains of the membrane bilayer phospholipids are disordered and in rapid motion. This liquid crystalline state has been suggested to account for the diffusion of functional components within the membranes (cf. current membrane models: Singer and Nicolson, 1972; Wallach, 1972; Bretscher, 1973; Capaldi, 1974; Sheetz and Singer, 1974). At low temperatures, however, the fatty acids become more ordered and less mobile. At some critical temperature a liquid crystalline → crystalline phase transition may occur. Such lipid phase transitions (for review, see Steim, 1968; Ladbrooke and Chapman, 1969) have been demonstrated in membranes of different cell types by physical methods such as X-ray diffraction (Engelman, 1970, 1971), calorimetry (Steim et al., 1969; Blazyk and Steim, 1972; DeKruyff et al., 1972), electron spin resonance (Eletr et al., 1973; Linden et al., 1973; Shimshick and McConnell, 1973) and fluorescent dye probing (Overath and Träuble, 1973).

Recently, freeze-etch electron microscopy has revealed temperature-induced lateral separations of particle-depleted areas from particle-bearing areas on the fracture faces of membranes of both prokaryotes (Verkleij et al., 1972; James and Branton, 1973) and eukaryotes (Speth and Wunderlich, 1973; Wunderlich et al., 1973a,b; 1974a,b). Evidence accumulates that, in prokaryotes, these thermotropic separations are induced by a thermal liquid crystalline → crystalline lipid phase transition. In eukaryotes, however, these thermotropic separations await still an exact analysis.

Accordingly, the effect of temperature on the membranes of endoplasmic reticulum is investigated directly in the unicellular eukaryote *Tetrahymena pyriformis* by freeze-etch electron microscopy. Moreover, the effect of temperature is studied on smooth microsomal membrane vesicles isolated from the same cell type as well as on the extracted microsomal lipids by fluorescence probing, electron spin resonance, proton nuclear magnetic resonance (^{1}H NMR), and calorimetry. Also, we examine the temperature response of the glucose 6-phosphatase bound to the isolated membranes.

Materials and Methods

Cells. Axenic cultures of the ciliate protozoan *T. pyriformis* (amicronucleate strain GL) were statically grown at 28° in the logarithmic phase (~50,000 cells/ml) in a medium consisting of 0.75% proteose peptone, 0.75% yeast extract, 1.5% glucose, 0.1 m*M* ferric citrate, 0.05 m*M* $MgCl_2$, and 1 m*M* $MgSO_4$ (Leick and Andersen, 1970).

Freeze-Etch Electron Microscopy. The *T. pyriformis* cultures grown at 28° were divided in several aliquots, which were incubated at 28, 24, 20, 15, 10, and 5°. After a 10-min equilibration, the cells were fixed at these temperatures with 2% glutaraldehyde buffered with 0.05 *M* sodium cacodylate (pH 7.2). The cells were then twice washed with the cacodylate buffer, before they were glycerinated, i.e., cells suspended within the cacodylate buffer were made 5% with respect to glycerol, and then in periods of 20 min, the glycerol concentration was in 5% steps raised up to 25%. In this final glycerol concentration the cells were kept for 40 min. The cells were then frozen on cardboard discs in Freon 22 cooled by liquid nitrogen. Fracturing, etching (1 min at −100°), and replication were carried out on a Balzers freeze-etch device (Model BA 360M). The replicas were examined in a Siemens Elmiskop Ia. Shadowing direction is

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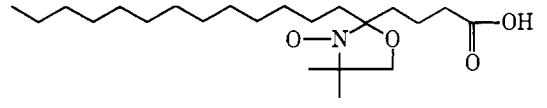
indicated by the arrows in the right corner on the electron micrographs.

Membrane Isolation. Smooth-surfaced membrane vesicles (average diameter, ~200 nm) were isolated from *T. pyriformis* using step sucrose gradients according to a modified method of Bergstrand and Dallner (1969) as described recently (Ronai and Wunderlich, 1975). These membranes are rich in smooth endoplasmic reticulum and reveal a phospholipid/protein ratio of 0.52 (for further characterization see Ronai and Wunderlich, 1975).

Glucose 6-Phosphatase Assay. Glucose 6-phosphatase in the membrane vesicles (~0.15 mg of protein/sample) was measured at V_{max} as in Baginski et al. (1970). The phosphate release was linear both at the highest and lowest temperatures.

Fluorescent Dye Probing. 8-Anilino-1-naphthalenesulfonate was used as probe. The samples contained 10^{-4} *M* 8-anilino-1-naphthalenesulfonate, 0.2 *M* NaCl, 5 m*M* Tris-HCl (pH 7.2), and membrane vesicles (~2.0 mg of lipid). We used a Zeiss PMQ II spectrometer with a ZMF 4 fluorescence accessory and a second monochromator. The excitation and emission wavelengths were 380 and 480 nm, respectively. The temperature was recorded in the reference cuvet (10^{-4} *M* 8-anilino-1-naphthalenesulfonate in methanol) using a copper–constantan thermocouple.

Electron Spin Resonance. The membrane vesicles were labeled with either (I) 5-doxylstearic acid, (II) 12-doxylstearic acid, or (III) 4-doxyldecane (doxyl refers to the 4′,4′-dimethyloxazolidine-*N*-oxyl ring), which were synthesized according to Keana et al. (1967). The molar ratio of membrane phospholipid per label was ≥100:1 in the samples. The paramagnetic spectra were measured at 9.36 GHz with a Varian E-9 spectrometer. The temperature was recorded by mounting a thermistor above the measuring cavity.



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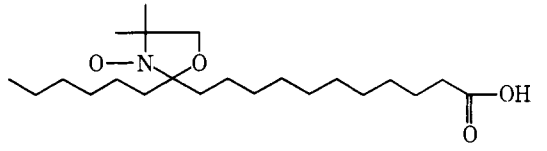

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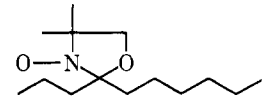


III

The membrane-aqueous environment partition of 4-doxyldecane was determined according to Lee et al. (1974; see also Shimshick and McConnell, 1973). The amplitudes *B* and *F* of the high-field signals (see Figure 1 in Lee et al., 1974) were evaluated, which are approximately proportional to the amount of label dissolved in the membranes and the aqueous environment, respectively. The ratio *B*/*F* has been termed spectral parameter α.

Lipid Extraction. The lipids of the membrane vesicles were extracted three times with chloroform–methanol (2:1) at 0–4° according to Folch et al. (1957) and evaporated to dryness under nitrogen. They have been intensely characterized elsewhere (Ronai and Wunderlich, 1975). The major membrane phospholipids are phosphatidylethanolamine (30%), its phosphono analog glyceride aminoethylphosphonate (23%), phosphatidylcholine (18%), and ceramide aminoethylphosphonate (14.2%). The neutral lipid fraction constituting ~17% of the total membrane lipids contains free fatty acids, diglycerides, an unknown compound, triglycerides, and no cholesterol. The total membrane fatty acids (mainly even-numbered ranging between C_{12} and C_{18}) contain 63% unsaturated acids, 68.7% of which are contributed by about equal portions of the octadecadienoic and octadecatrienoic acid.

Proton Nuclear Magnetic Resonance. The dried lipids (~22 mg/ml) were dissolved in D_2O and sonicated in a Branson sonifier for 3 min. The measurements were performed at 90 MHz with a Bruker HX-90-FT spectrometer equipped with a variable temperature unit.

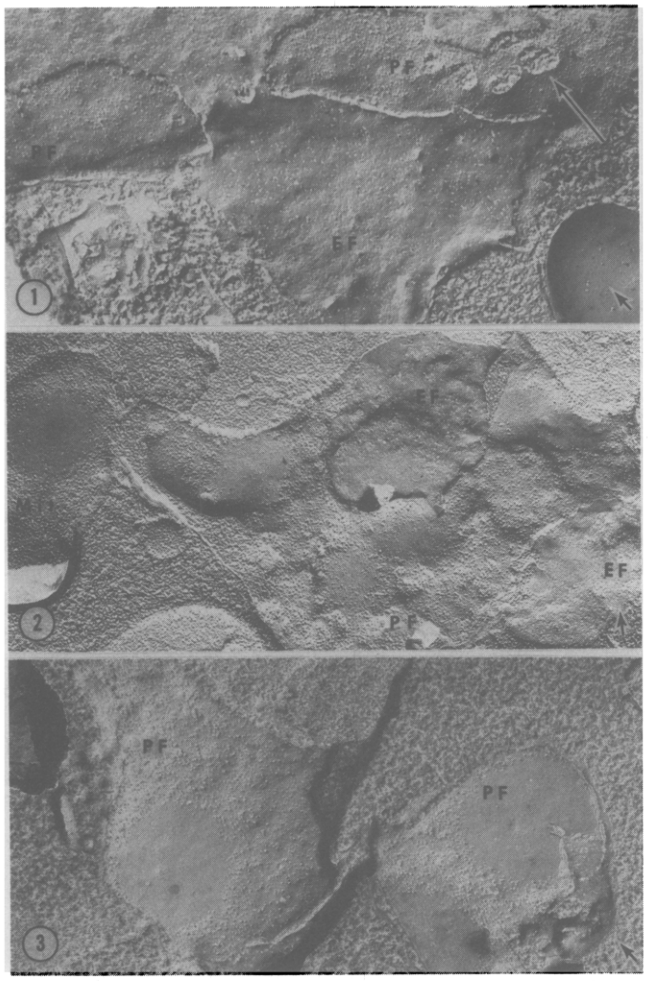
Calorimetry. The calorimetric measurements were carried out in an adiabatic differential scanning calorimeter; a detailed description of its construction and sensitivity has been given by Grubert and Ackermann (1974). This apparatus works with a gold-plated double cell containing in its two compartments 25 ml of the sample and 25 ml of the solvent as a reference, respectively, and can detect, e.g., the phase transition of dipalmitoylphosphatidylcholine at the minimal concentration of 5 mg/25 ml.

Either ~200 mg of dried lipids or ~200 mg of dried phospholipids extracted from the *T. pyriformis* membranes were ultrasonically dispersed in 25 ml of a 1:1 mixture of glycol and 25 m*M* Tris-HCl (pH 7.5) at room temperature. In order to avoid a possible broadening of the phase transition by too extensive sonication it was performed for only 5–30 sec. The phospholipids were separated from total lipids as described previously (Ronai and Wunderlich, 1975). The lipid dispersions were heated from 0 to 30° with a rate of 14°/hr.

Results

Freeze-Etch Electron Microscopy. During freeze-fracturing, the membranes of the endoplasmic reticulum of *T. pyriformis* are internally cleaved (cf. PintodaSilva and Branton, 1970; Tillack and Marchesi, 1970). This exposes two fracture faces: the PF face is oriented to the cytoplasm, while the EF face borders the cisternal space (Figure 1). At the optimal growth temperature of 28°, the PF faces always display uniformly distributed, intramembranous particles (Figure 1) ranging between 3 and 12 nm (mean ~7.5 nm). The EF faces, however, show fewer particles, but numerous, uniformly distributed depressions (Figure 1). This appearance of the two fracture faces is also revealed in cells equilibrated at 24 and 20°. At 15°, however, smooth areas with a diameter of at least 100 nm can be detected on both fracture faces (Figure 2). Still larger smooth-faced areas are regularly seen in cells at 10°, and at 5° they can reach diameters of up to 1 μm (Figure 3). Importantly, the particles in the particle-bearing areas on the PF faces are not aggregated, even at 5°. This temperature effect on membrane core structure is reversible. Upon rewarming the cells from 5 to 28° within 30 sec the smooth-faced areas disappear, and the particles are again uniformly distributed.

Glucose 6-Phosphatase. In order to test the effect of temperature on a function associated with endoplasmic reticulum membranes, we measured the activity of the typical endoplasmic reticulum marker enzyme, glucose 6-phosphatase, in the isolated membrane vesicles. The Arrhenius plot in Figure 4 shows that the glucose 6-phosphatase activity



FIGURES 1–3: Survey electron micrographs of freeze–fractured endoplasmic reticulum membranes of *T. pyriformis* cells incubated at different temperatures. Figure 1: At 28°, the membrane-intercalated particles are uniformly distributed on the PF faces. Arrow indicates a cisternal pore. Figure 2: At 15°, small smooth areas can be seen on both fracture faces as well as on a freeze–fractured mitochondrial membrane (Mit). Figure 3: At 5°, large smooth areas are seen on the PF faces. Note that particles are not aggregated in the particle-bearing areas! Magnification: ×33,000.

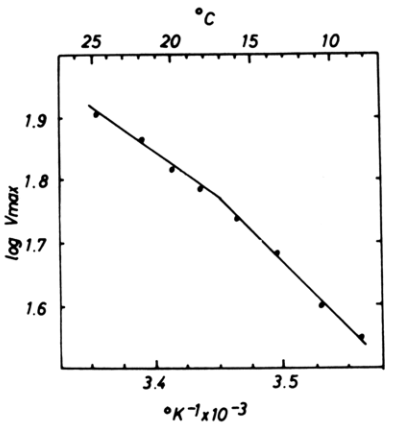


FIGURE 4: Glucose 6-phosphatase activity measured at V_{max} in the *T. pyriformis* membrane vesicles as a function of temperature. Note the discontinuity at ~17°.

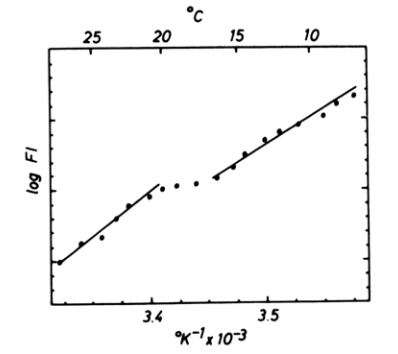


FIGURE 5: Fluorescence intensity FI (relative units) of 8-anilino-1-naphthalenesulfonate in the *T. pyriformis* membranes suspension as a function of temperature. A discontinuity is observed between 20 and 16°.

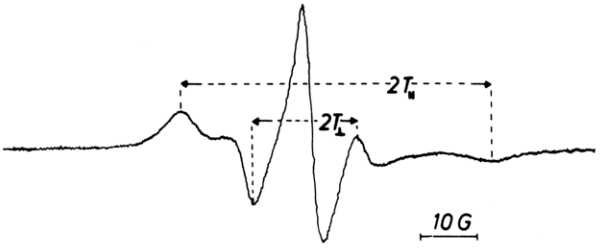


FIGURE 6: Electron spin resonance spectrum of 5-doxylstearic acid incorporated in the isolated *T. pyriformis* membranes at 28°. $2T_{\parallel}$ and $2T_{\perp}$ indicate the separation of the outer and inner hyperfine extrema, respectively.

does not decrease linearly upon temperature lowering, but rather is biphasic with a discontinuity at ~17°.

8-Anilino-1-naphthalenesulfonate Fluorescence. The amphilic fluorescent dye 8-anilino-1-naphthalenesulfonate can be used as a membrane "surface probe". Its polar site, the negative sulfonate group, is assumed to face the aqueous phase, while the aromatic ring system is buried in the hydrophobic membrane region near the membrane surface (Shechter et al., 1971; Radda and Vanderkooi, 1972; Overath and Träuble, 1973). Figure 5 shows that the 8-anilino-1-naphthalenesulfonate fluorescence intensities in the membrane suspension increase with decreasing temperatures. Between 20 and 15°, a discontinuity in the 8-anilino-1-naphthalenesulfonate fluorescence intensity can be seen, i.e., the fraction of 8-anilino-1-naphthalenesulfonate bound to the membrane surfaces becomes dramatically reduced at this temperature range.

Electron Spin Resonance. Figure 6 shows a representative electron spin resonance spectrum obtained with the 5-doxylstearic acid label in the membrane vesicles suspension. This spectrum is typical for a fast anisotropic rotation of the spin-label as is generally observed in lipid bilayers (Seelig, 1970; Hubbell and McConnell, 1971; Seelig and Hasselbach, 1971). From the separation of the outer and inner hyperfine maxima ($2T_{\parallel}$ and $2T_{\perp}$, respectively, in Figure 6) an order parameter $S = 0.63$ and an isotropic hyperfine splitting constant $a_N = 15$ G can be calculated according to Seelig (1970). The maximal separation of $2T_{\parallel}$ and also the line width become larger with decreasing temperatures (Figure 7). This indicates that the motional freedom of the spin-label becomes more restricted at lower temperatures and, at ~17°, a discontinuity is revealed in the decrease of "fluidity" of the *T. pyriformis* membranes (Figure 7).

Several of the spectra obtained at different temperatures with the 12-doxylstearic acid label in the *T. pyriformis* membranes are represented in Figure 8. Conspicuously, the

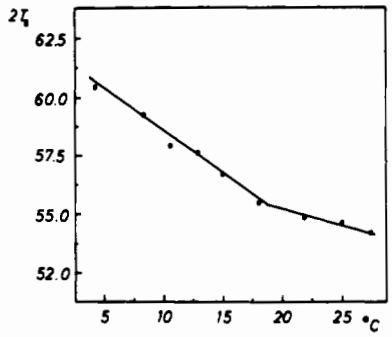


FIGURE 7: The separation of the outer hyperfine extrema ($2T_{\parallel}$) of the 5-doxylstearic acid labeled *T. pyriformis* membrane vesicles as a function of temperature. Note the discontinuity at ~17°.

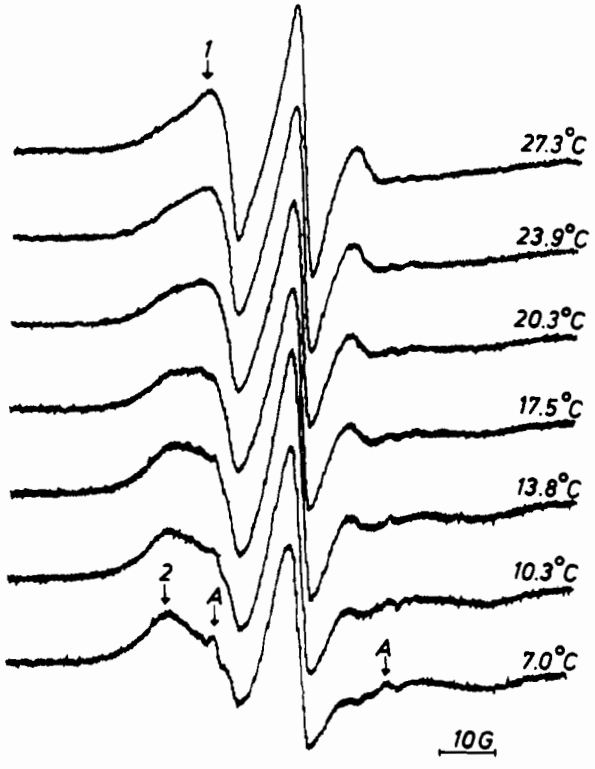


FIGURE 8: Electron spin resonance spectra of *T. pyriformis* membrane vesicles labeled with 12-doxylstearic acid at different temperatures. The low-field line separates in two peaks. At higher temperatures, peak 1 predominates, while peak 2 is prevailing at low temperatures. A indicates non-membrane-bound label.

low-field line is split into two peaks, which have the same height at ~17°(cf. Figure 8). At lower temperatures, the outer peak predominates, while the inner peak is prevalent at higher temperatures (Figure 8). Although an exact analysis is not possible, our experience with computer-simulated spin-label spectra (Schindler and Seelig, 1973; Scandella et al., 1974) indicates that the spectra in Figure 8 comprise contributions from at least two spin-label environments characterized by different order parameters and different rates of motion. One environment seems to enlarge at the expense of the other as a function of temperature.

In Figure 9, the logarithm of the spectral parameter is plotted as a function of temperature. Upon temperature lowering, α decreases, i.e., the 4-doxyldecane label becomes "squeezed out" from the membranes into the aqueous environment. This "squeeze out" process is dramatically enhanced at ~17°.

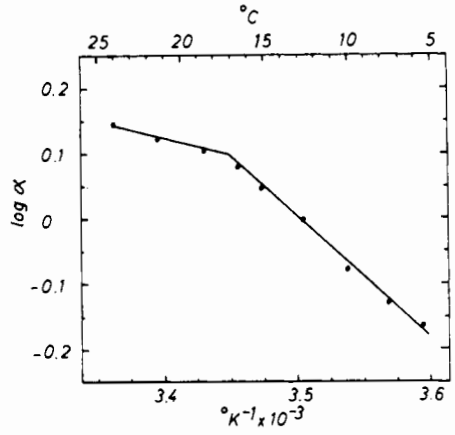


FIGURE 9: Spectral parameter (for definition see Material and Methods) of the 4-doxyldecane label in the *T. pyriformis* membrane suspension as a function of temperature. Note the discontinuity at ~17°.

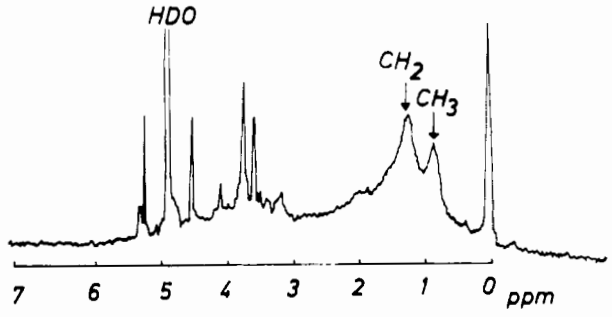


FIGURE 10: ^{1}H NMR spectrum of total lipids extracted from the *T. pyriformis* membrane vesicles at 9°.

Calorimetry and Proton Nuclear Magnetic Resonance. In order to test whether the lipids in the *T. pyriformis* membranes can undergo a liquid crystalline → crystalline phase transition, these were extracted from the membranes and subjected to ^{1}H NMR and calorimetry. These are valuable methods to detect lipid phase transitions without introducing a "probe" into the sample. Typical for a liquid crystalline → crystalline lipid phase transition is an enthalpy change which can be measured by calorimetry (for review, see Steim, 1968; Ladbrooke and Chapman, 1969). Similarly a true liquid crystalline → crystalline lipid phase transition can be detected by means of ^{1}H NMR, where the characteristic change is the complete disappearance of the $-CH_2$ and $-CH_3$ signals in the crystalline state (Lee et al., 1972).

Figure 10 shows a typical ^{1}H NMR spectrum of the extracted membrane lipids at 20°. The height of the $-CH_2$ and $-CH_3$ signals of the total fatty acids of total lipids are plotted against temperature in Figure 11. At ~17° one observes a discontinuity, but the $-CH_2$ and $-CH_3$ signals do not disappear at lower temperatures. In accord, we cannot detect any significant endothermic transitions either in the total lipids or in the phospholipids between 0 and 30° by calorimetry (cf. in this context the lipid composition in Material and Methods under lipid extraction).

Discussion

Our freeze-etch results demonstrate that, at temperatures below ~17°, particle-depleted areas emerge on the membrane fracture faces of the endoplasmic reticulum in intact *T. pyriformis* cells. This phenomenon has been pre-

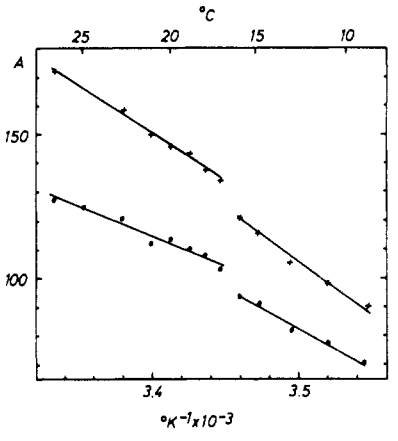


FIGURE 11: The height (A) of the CH_2 (+) and CH_3 (●) signals of Figure 10 are plotted against temperature in relative units.

viously detected in other endomembranes of *T. pyriformis* such as those of the alveolar sacs, nuclei, mitochondria, peroxisomes, etc. (Speth and Wunderlich, 1973; Wunderlich et al., 1973a,b; R. Müller, V. Speth, and F. Wunderlich, unpublished). Unlike the situation observed with the alveolar membranes of *T. pyriformis* (Speth and Wunderlich, 1973) as well as with plasma membranes of several prokaryotes such as Acholeplasma (Verkleij et al., 1972; James and Branton, 1973) and *Escherichia coli* (Haest et al., 1974; Kleemann and McConnell, 1974; Shechter et al., 1974; see, however, Tsien and Higgins, 1974), however, the particles in the particle-bearing areas are not aggregated on the endoplasmic reticulum fracture faces. This means that the total number of particles decreases on the fracture faces as was recently also demonstrated in freeze-etched nuclear membranes of several different lymphoid cells (Wunderlich et al., 1974b). We have suggested, therefore, that in the region of the smooth-faced areas, the intramembranous particles become squeezed out from the membrane core in the direction of the membrane surface (Wunderlich et al., 1974b). This implies alterations at and near the membrane surface not detectable within the cells by freeze-etch electron microscopy. Indeed, this suggestion is compatible with the data obtained from the isolated *T. pyriformis* membranes using the electron spin resonance and 8-anilino-1-naphthalenesulfonate fluorescence probing techniques. Thus, spin-label molecules dissolved in the membrane lipid core are squeezed out from these hydrophobic environments into the aqueous environments at temperatures below ~17° (Figure 9). In the same temperature range, alterations at and near the membrane surfaces are indicated by the displacement of 8-anilino-1-naphthalenesulfonate bound to the membrane surface (Figure 5) and by the discontinuity observed in the hyperfine extreme separation of the 5-doxylstearic acid incorporated into the membranes (Figure 6). These structural changes correlate with changes in membrane functions as indicated by the thermotropic discontinuity of the glucose 6-phosphatase activity (Figure 4).

Such temperature-induced alterations in membrane structure and function are generally ascribed to liquid crystalline → crystalline lipid phase transitions (e.g., Eletr et al., 1973; Grisham and Barnett, 1973; Inesi et al., 1973; Linden et al., 1973; Overath and Träuble, 1973; Shimshick and McConnell, 1973). Specifically, we suggested previously that the smooth areas on membrane fracture faces represent crystalline lipid domains separating laterally from the liquid crystalline ones (Speth and Wunderlich, 1973). However, no evidence for a lipid phase transition is provided by the present measurements with proton nuclear magnetic resonance and calorimetry of the extracted membrane lipids of *T. pyriformis*.

Nevertheless, it is still very likely that a lipid reorientation within the *T. pyriformis* membranes is primarily responsible for the thermotropic alterations, since proton nuclear magnetic resonance indicates an impairment of the fatty acid chain mobilities below ~17° (Figure 11). In this context, the recent suggestion of Lee et al. (1974), that thermotropic membrane alterations such as we observed in *T. pyriformis* may not be induced by true lipid phase transitions, but rather by cluster formation of "quasicrystalline" lipids in biological bilayer membranes, is noteworthy. The term quasicrystalline cluster is used to indicate a short-lived, more densely packed arrangement of adjacent liquid crystalline lipid molecules within an environment of freely dispersed liquid crystalline lipid molecules. Interestingly, our spectra obtained from the 5-doxylstearic acid labeled membranes (together with the fact that the membranes are internally cleaved along the center of their hydrophobic core upon freeze-etching) indicate that the major portion of the lipids in the *T. pyriformis* membranes is arranged in bilayer domains. In this bilayer there might be at least two environments of different fluidity as indicated by the spectra of the 12-doxylstearic acid labeled membranes. In accord, freeze-etch electron microscopy also indicates at least two different lipid environments within *T. pyriformis* membranes, one of which excludes the membrane-intercalating protein particles. With reference to Lee et al. (1974) we would suggest at present the following straightforward explanation for the thermotropic alterations in the *T. pyriformis* membranes. At the optimum growth temperature of 28°, "rigid" liquid crystalline lipid environments are randomly dispersed within a predominately "fluid" liquid crystalline environment. When the temperature is lowered, the rigid environments enlarge at the expense of the fluid environments, but remain randomly dispersed. At ~17°, however, the "rigid" lipids reach a critical concentration so that the two lipid environments separate laterally, i.e., the rigid lipids cluster. Below 17°, the clusters extend two dimensionally at the expense of the fluid environments. At the cluster boundary the protein particles are displaced normally to the membrane plane in the direction of the membrane surface, at least so far until they disappear from the fracture plane.

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