Use of β-Parinaric Acid, a Novel Fluorimetric Probe, to Determine Characteristic Temperatures of Membranes and Membrane Lipids from Cultured Animal Cells*

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A naturally occurring fluorescent compound, β-parinaric acid, was employed as a probe to measure the effects of temperature changes on plasma membranes, microsomes, and mitochondria and on their respective lipids after isolation from LM cells grown in suspension culture. A computer-centered spectrofluorimeter simultaneously measured the absorbance, absorbance-corrected fluorescence, and relative fluorescence efficiency of β-parinaric acid incorporated into the membranes or isolated membrane lipids. These parameters were measured as a function of temperature. The probe revealed five characteristic breaks or changes in slope with both the plasma membranes as well as their extracted lipids. These discontinuities occurred at approximately 18, 23, 31, 38, and 43°. The other isolated subcellular organelles, microsomes, and mitochondria, as well as their respective isolated lipids, exhibited approximately the same characteristic temperatures (±1°) as plasma membranes. Thus, these data negate one criterion of the theory that an asymmetric distribution of characteristic temperatures exists across the membranes of LM cells.

Investigators attempting to ascertain the physical states of lipids or the conformation of proteins in membranes have utilized probe molecules (electron paramagnetic resonance, nuclear magnetic resonance, or fluorescence) that report on the membrane environment through their spectroscopic properties (1-3). Any external probe introduced into a biological or artificial membrane system should satisfy at least two major criteria: (a) the probe should produce a minimum perturbation of its environment, and (b) the nature of the environment in which the probe is located should be ascertainable. Many probes in use today are synthetic molecules that often do not satisfy the first criterion (1, 3-6). Commonly, the second requirement proscribing a knowledge of the probe’s location has been indirectly inferred from solvent or model membrane studies, or both (3). Such data can be taken as qualitative evidence only. Recently, Sklar et al. (Ref. 7 and Footnotes 1 to 4) reported the discovery of a naturally occurring fluorescent compound, β-parinaric acid, which can readily be used as a probe that can satisfy both of the above requirements. β-Parinaric acid is a linear transconjugated polyene fatty acid with four double bonds and 18 carbon atoms. These investigators characterized the behavior of the molecule in artificial lipid systems and found that β-parinaric acid (either the free acid, or esterified to the position 2 of phosphatidylcholine) was incorporated into artificial lipid membranes. Factors favoring the use of β-parinaric acid as a natural fluorescent probe are: (a) the hydrophobic nature of β-parinaric acid is typical of other lipid membrane constituents; (b) the molecule is approximately the same size and shape as other fatty acids and would not be expected to alter its environment in a manner differing from that of other fatty acids (For example, it has been observed to interact with serum albumin as do other fatty acids' (3-9); (c) β-parinaric acid can be biosynthetically incorporated into Escherichia coli and rat liver lipids (3-8); (d) the fluorescence of the molecule is sensitive to the chain length of membrane fatty acids as well as the type of polar head group of phospholipids (3-7); (e) characteristic temperatures of phospholipids reported by β-parinaric acid are identical to those reported by differential scanning calorimetry (3-9). In the present study we have
extended the use of \( \beta \)-parinaric acid to investigations of the physical properties of membranes and membrane lipids obtained from cultured animal cells. The computer-centered spectrofluorimeter designed by Holland et al. (10, 11) was used for simultaneous measurements of absorbance, absorbance-corrected fluorescence, and relative fluorescence efficiency of \( \beta \)-parinaric acid located in plasma membranes, microsomes, mitochondria, and their isolated lipids. This instrumental system has been used in similar studies to investigate the interactions of steroids with polyene antibiotics, another class of naturally occurring fluorescent probes (12, 13), and those of cryoprotectants with proteins or amino acids that have intrinsic fluorescence (14).

MATERIALS AND METHODS

Cell Culture—A strain of mouse fibroblasts, LM cells, was obtained from the American Type Culture Collection and adapted to growth in suspension culture as previously described. The cells were cultured at 37 \(^\circ\)C in a lipase-free, chemically defined culture medium described by Higuchi (15), modified as stated elsewhere. \( ^5 \)

Membrane Isolation—Plasma membranes, microsomes, and mitochondria were isolated as described by Schimmel et al. (16) with minor modifications. \( ^5 \)

Membrane Lipid Isolation—Purified plasma membranes, microsomes, and mitochondria were resuspended in phosphate-buffered saline (PBS) made up without calcium or magnesium (17). The PBS consists of \( \frac{1}{4} \) g of NaCl, \( \frac{1}{4} \) g of KCl, \( \frac{1}{3} \) g of Na,PO\(_4\), 2H\(_2\)O, and 0.2 g of KH\(_2\)PO\(_4\)/liter distilled H\(_2\)O; pH was adjusted to 7.4. Lipids were isolated from 1 mg of membrane protein (as protein) by the method of Bligh and Dyer (18) as described by Ames (19). Contamination of the lipid extract by protein was determined by the method of Lowry et al. (20) and by measurement of fluorescence due to aromatic amino acids. Both methods indicated negligible contamination of the lipid extracts by protein.

Incorporation of \( \beta \)-Parinaric Acid into LM Subcellular Membranes and Lipids—The fluorescence probe, \( \beta \)-parinaric acid, was stored in hexane (3 mg/ml) as described by Sklar et al. (1). Working solutions of the probe were prepared fresh daily by dilution in ethanol (1:100). Aliquots of the working solution were placed in acid-washed, Teflon-capped, Pyrex test tubes and the solvent was evaporated with a gentle stream of nitrogen. A 1.5 ml aliquot of membrane suspension (40 \( \mu \)g of protein/ml of PBS, pH 7.4) was added to the tube. The sample tube was flushed with nitrogen, capped, and blended at maximum speed on a Vortex Genie mixer for 3 min. Membrane lipid dispersions were treated similarly except that the isolated lipids were redissolved in chloroform:methanol (2:1) and an aliquot was added to the sample tube. The solvent was evaporated with nitrogen and 1.5 ml of PBS was added. The sample was then blended on a Vortex mixer as above. The final concentration of lipid in the sample tube was equivalent to that extracted from 50 \( \mu \)g of membrane protein/ml of PBS. Unless otherwise specified, the molar ratio of \( \beta \)-parinaric acid probe to lipid was in all cases between 1:500 to 1:lOO. All of the above procedures with \( \beta \)-parinaric acid were carried out under \( \frac{1}{2} \)-lumen and reduced light.

Instrumentation and Spectroscopy—The computer-centered spectrofluorimeter was used in the excitation mode to measure absorption (A), absorption-corrected fluorescence (CO) which corrects for the inner filter effect, the primary absorption effect, and the instrumental variables. In addition, during excitation scans it outputs RFE, a quantity which is directly related to the total fluorescence efficiency of a fluorophore, thereby eliminating the necessity for using two scans on different instruments to measure fluorescence efficiency. The recorded values along the scan axis of each of the above quantities (A, CO, corrected fluorescence emission, and RFE) represent the average of 40 measurements taken by the computerized system in several milliseconds. Each value deviated less than 0.2\% in repeat scans. In each repeat scan an additional 40 measurements were taken with the same sample at a given temperature. Data were taken every 0.25 nm during a scan (10, 11). The sample temperature was continuously monitored with a thermocouple. Data were automatically obtained by the computer every 1° change during temperature scans over the range 13 to 50°. The sample and reference temperatures were controlled by a water-jacketed cuvette holder and the temperature was varied at a rate of 2°/min. Unless otherwise stated, samples were equilibrated at the lowest temperature for at least 30 min before increasing temperature scanning. Plots of CO\(_{334}\) of \( \beta \)-parinaric acid versus temperature in solvents, such as ethanol, indicated an exponential decay with increasing temperatures, but no discontinuities or characteristic temperatures were found. Thus the characteristic temperatures determined under “Results” do not appear to be a systematic instrumental artifact. In addition, others (7) have demonstrated similar exponential decreases in fluorescence of \( \alpha \)-parinaric acid in decane; also, no discontinuities were apparent. Quantum yields were determined relative to ANS (Pierce Chemical Co.) in ethanol (quantum yield, 0.37 for ANS according to Stryer (22)).

RESULTS

Spectral Characteristics and Probe Environment—The probe, \( \beta \)-parinaric acid, is nonfluorescent in aqueous solution (7). The spectral parameters of \( \beta \)-parinaric acid in ethanol are shown in Fig. 1, illustrating the type of on-line data obtained with the computerized spectrofluorimeter. Fig. 1, A, B, and C, were simultaneously obtained on an excitation scan and represent absorbance, absorbance-corrected fluorescence, and relative fluorescence efficiency, respectively. These parameters indicated that \( \beta \)-parinaric acid had maxima at 292, 299, and 313 nm as expected for absorbance and uncorrected fluorescence.

\[ \text{Absorbance (A)} \]

\[ \text{Corrected Fluorescence (CO)} \]

\[ \text{Relative Fluorescence Efficiency (RFE)} \]

![Fig. 1. Spectral properties of \( \beta \)-parinaric acid in ethanol. \( \beta \)-Parinaric acid was dissolved in ethanol, 1.08 mg/ml. As described under “Materials and Methods,” absorbance, corrected fluorescence, and relative fluorescence efficiency were determined with emission measured at 415 nm and corrected fluorescence emission was measured with excitation at 313 nm.](http://www.jbc.org/)

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\(^5\) Schroeder, F., Perlmuter, J. F., Glaser, M., and Vagelos, P. R. (1976) J. Biol. Chem., 251, 6015-6026. This paper precedes the present one as part of a series.

\(^6\) The abbreviations used are: PBS, phosphate-buffered saline prepared without calcium or magnesium, A, absorption, CO, absorption-corrected fluorescence, RFE, relative fluorescence efficiency; ANS, 8-anilino-1-naphthalene sulfonic acid; \( \varepsilon \), dielectric constant; LM cells, a clonal-requiring strain of mouse fibroblast cells.

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The shape of the relative fluorescence efficiency curve indicated that the chromophore was also the major fluorophore and little, if any, absorbance due to impurities was present (10, 11). Fig. 1D illustrates a corrected fluorescence emission scan and shows that β-parinaric acid had maximal emission at 415 nm, also as predicted (7). The total quantum efficiency of β-parinaric acid fluorescence in ethanol at 25 °C, using ANS as a standard (22), was 0.083. The absorbance and corrected fluorescence emission spectra needed for this determination were obtained simultaneously with the same instrument. In order for a probe to be informative, its spectral characteristics must be influenced by the environment (3, 23). By comparing the spectral characteristics of a fluorescence probe in a series of solvents of differing hydrophobicity, polarity, dielectric constant, or hydrogen bonding ability with the same parameters measured in membranes or membrane lipids, it is possible to qualitatively ascertain the type of environment in which the probe may be located in the more complex membrane or membrane lipid systems. Both the wavelength of fluorescence emission and the quantum efficiency of fluorescence may be sensitive to the dielectric constant, ε, as is the case for ANS and N-phenyl-1-naphthylamine (3, 25, 24). Such data have been interpreted to correlate with the "polarity" of a probe's environment or binding site. However, such interpretations may be oversimplified since polarity is dependent on at least three factors: dielectric constant of the solvent, dipole-induced dipole interactions between the probe and its solvent environment, and the polarization of solvent molecules induced by the fluorescence probes. As shown in Fig. 2, the spectral parameters of β-parinaric acid were measured as a function of dielectric constant, ε. The dielectric constant was varied by dissolving the acid in a series of dioxane:water mixtures as previously described by ANS (24). In contrast to results obtained with ANS and N-phenyl-1-naphthylamine (24), the wavelength of maximal fluorescence emission of β-parinaric acid was relatively constant over a wide range of ε rather than continuously varying as a function of ε. Similar results have been obtained by Simoni and co-workers (Ref. 7 and Footnotes 1 to 4). The fluorescence of the probe in methanol and ethanol was used to confirm this relationship. The large decrease in AS11 and CO311 with increasing ε (Fig. 2B) indicated that β-parinaric acid became increasingly insoluble in polar environments such as H2O. Water has an ε of 80 (24). Similar solubility problems in aqueous media have been encountered with other polyenes (12, 13). These data indicate that the behavior of β-parinaric acid in aqueous solvents is probably not ideal and that micelles and aggregates may form at high concentrations. It is important to note that the absorbance of β-parinaric acid in water, when added to the water as described under "Materials and Methods" by first coating the sides of the test tube before addition of aqueous solvent, is low. The probe is so poorly soluble that the absorbance is almost negligible (see Fig. 2). However, the large decrease in RFE311, which is independent of fluorophore concentration, with increasing ε, indicates that other factors, e.g. solvent polarizability (3, 25, 26) are affecting the fluorescence efficiency of this fluorophore.

It was previously shown that the ratio of absorbance peaks may be a sensitive indicator of polyene chromophore conformation and the ratio of fluorescence peaks was a measure of noncovalent interaction with the fluorophore (12, 13). Table I illustrates that the ratio of absorbance maxima (A311/A292) of β-parinaric acid, in various solvents of varying hydrophobicity and ε, was relatively constant. However, the ratio of the fluorescence excitation maxima, especially CO311/CO292 varied as a sensitive function of the fluorophore environment. These data were consistent with the known sensitivity of polyene fluorophores to polarizability and other factors affecting the excited state (25, 26) rather than conformational changes of the chromophore. β-Parinaric acid located in plasma membrane or plasma membrane lipids had low values of CO311/CO292 (1.05 and 1.16, respectively). β-Parinaric acid dissolved in dioxane, methanol, and ethanol had CO311/CO292 values of 1.20, 1.26,

![Table 1](http://www.jbc.org/)

**Table 1**

| Spectral peak ratios of β-parinaric acid in a variety of environments |
|---|
| Samples were prepared for spectral peak ratio determination as described in the legends of Figs. 2 and 3. |
| Solvent or environment of β-parinaric acid | Spectral peak ratios |
|---|---|
| Dioxane, 100%, in H2O | 0.86 | 0.85 | 1.93 | 1.90 |
| Dioxane, 90%, in H2O | 0.88 | 0.86 | 1.25 | 1.25 |
| Dioxane, 80%, in H2O | 0.87 | 0.87 | 1.22 | 1.26 |
| Dioxane, 70%, in H2O | 0.85 | 0.88 | 1.26 | 1.31 |
| Dioxane, 60%, in H2O | 0.88 | 0.90 | 1.27 | 1.38 |
| Dioxane, 50%, in H2O | 0.91 | 1.29 | 1.02 |
| Dioxane, 40%, in H2O | 0.94 | 1.27 | 2.36 |
| Methanol, 100% | 0.88 | 0.88 | 2.76 |
| Ethanol, 100% | 0.87 | 1.27 | 1.38 |
| Plasma membrane | 0.88 | 0.88 | 1.20 | 1.05 |
| Plasma membrane lipids | 0.84 | 0.81 | 0.94 | 1.16 |

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Effect of solvent dielectric constant, ε, on spectral properties of β-parinaric acid. Absorbance, A311 ( ), absorbance-corrected fluorescence, CO311 ( ), absorbance-corrected RFE311 ( ), and relative fluorescence efficiency, RFE311 ( ), were measured at 311 nm with emission maintained at 415 nm as described under "Materials and Methods." The emission wavelength maxima ( ) were measured from corrected fluorescence emission spectra with excitation at 313 nm as described in Fig. 1. The values of these parameters for β-parinaric acid dissolved in methanol or ethanol were indicated on each curve by X and Δ, respectively. ε values were taken from previously published data for methanol, ethanol, and 0 to 100% dioxane:H2O (24). The concentration of β-parinaric acid was approximately 2.0 mg/ml.
and 1.31, respectively. Thus, in the membrane or membrane lipid, \( \beta \)-parinaric acid appeared to be located in an environment more nonpolar and of lower \( \epsilon \) than any of the solvents tested.

**Spectral Characteristics of \( \beta \)-Parinaric Acid Interaction with LM Membranes and Lipids**—The interaction of LM cell plasma membranes with \( \beta \)-parinaric acid is shown in Fig. 3. Excitation and emission wavelengths were chosen to minimize the possibility of interference from the protein aromatic amino acids, tryptophan, tyrosine, and phenylalanine. As indicated by the dashed lines, the LM plasma membranes exhibited a negligible contribution of these fluorophores to the measurements made. In the presence of \( \beta \)-parinaric acid excited at 313 nm, fluorescence emission occurred at 415 nm (Fig. 3D). Maxima in the excitation spectra (Fig. 3, A, B, and C) appeared at 292, 299, and 313 nm. Similar spectral characteristics were obtained when plasma membranes were interacted with \( \beta \)-parinaric acid at probe to lipid molar ratios over the range of 1:40 to 1500. In the presence of other absorbing chromophores, the relative fluorescence efficiency curve of a fluorophore tends to resemble the absorbance curve (10, 11).

This relationship is demonstrated when the RFE\(_{313} \) curves of Figs. 1 and 3 are compared. The plasma membranes contain chromophores which absorb at 313 nm (the excitation wavelength) and do not fluoresce at 415 nm. The RFE\(_{313} \) spectrum of \( \beta \)-parinaric acid in the plasma membranes more closely approximated the absorbance curve (compare Fig. 3C with Fig. 1A) than in ethanol (compare Fig. 1C with Fig. 1A).

The following comparisons between the spectral characteristics of \( \beta \)-parinaric acid in the plasma membrane versus the solvent studies may be made. (a) The fluorescence efficiency of \( \beta \)-parinaric acid in the LM cell plasma membrane was higher which is to be expected if the probe were located in a nonaqueous or hydrophobic environment. The quantum efficiency, relative to ANS, of \( \beta \)-parinaric acid fluorescence was 0.286 in the plasma membrane, 0.274 in pure dioxane, and 0.083 in pure ethanol as compared with almost zero in water or PBS buffer. (b) The RFE\(_{313} \) and CO\(_{313} \) values of the probe in the plasma membrane correlated with an \( \epsilon \) of approximately 2 (see Fig. 2), which is in agreement with reported estimates of the interior of a lipid bilayer or membrane as having a polarity corresponding to an \( \epsilon \) from 2 to 10 (24). (c) The CO\(_{313} \)/CO\(_{313} \) for \( \beta \)-parinaric acid in the plasma membrane versus the \( \beta \)-parinaric acid in the plasma membranes at 24\(^\circ\) (Table I) had values that were similar to those obtained with isolated plasma membrane lipids (Table II) and with isolated plasma membranes and lipids within 3 to 5 min, at 24\(^\circ\). Both CO\(_{313} \) and RFE\(_{313} \) had maximal values at this point. Increased time or temperature of incubation did not increase the value of

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| Fraction | Temperature | \( K_\text{D} \) | Minimum number of lipid molecules/binding site
|---------|-------------|----------------|-------------------------------------------|
| Plasma membrane | 20\(^\circ\) | 1.0 | 13 |
| | 30 | 1.1 | 12 |
| | 40 | 1.2 | 11 |
| | 50 | 1.2 | 10 |
| Plasma membrane lipids | 20 | 1.4 | 12 |
| | 30 | 1.4 | 11 |
| | 40 | 1.5 | 10 |
fluorescence measurements of β-parinaric acid can be used to monitor the phase transitions of artificial bilayer lipid membranes. We have extended the studies to animal (LM) cell membranes. Figs. 4 to 6 show plots of CO₁₁₃, a concentration-dependent parameter, and RFE₁₁₃, a quantum yield-dependent parameter, versus temperature in plasma membranes, microsomes, mitochondria, and their isolated lipids. Both parameters indicated characteristic temperatures of plasma membranes (Fig. 4), microsomes (Fig. 5), and mitochondria (Fig. 6) at approximately 18, 23, 31, 38, and 43°C. The characteristic temperatures of the isolated lipids were in agreement ±1°C. These same characteristic temperatures (±1°C) were noted at four different probe concentrations (20-fold range) and in descending as well as ascending temperature scans (data not shown) for LM plasma membranes, microsomes, mitochondria, and their isolated lipids. Very little probe decomposition appears to have occurred since almost full fluorescence intensity returned after the second scans.

As indicated in Figs. 4 to 6, CO₁₁₃ and RFE₁₁₃ decreased by 70 to 80% with increasing temperature for both membranes as well as lipids. Part of this decrease is due to a decrease in fluorescence efficiency at elevated temperatures (3). A second possibility was that large alterations in probe binding ability as a function of temperature may have occurred. Similar behavior has been noted with ANS (27). As indicated by CO₁₁₃ and RFE₁₁₃, in the previous section, large alterations in binding ability of β-parinaric acid as a function of temperature did not occur at the probe concentrations employed.

Accessibility of Plasma Membrane Lipids to β-Parinaric Acid—It is possible that β-parinaric acid may interact with only a small group of lipids in the plasma membrane or with membrane proteins. It can interact with proteins such as serum albumin (7). As shown by Table II, the maximal relative fluorescence efficiencies (RFE) of β-parinaric acid in plasma membranes and plasma membrane lipids were very similar in value (within approximately 10%). The increase of approximately 10% in the isolated lipids could indicate that the extent of the probe-lipid interaction may be obstructed slightly by protein or other membrane components, or both. Thus removal of the protein appears to have slightly increased the ability of the probe to interact with the plasma membrane lipids. The protein may, therefore, sequester some lipid and prevent its interaction with β-parinaric acid. In these studies, the probe variation in the absorbance of β-parinaric acid in water was very low (Fig. 2), the absorbance of unbound probe would not be expected to contribute significantly to RFE₁₁₃ of probe incorporated into membranes or lipids. The binding characteristics of β-parinaric acid with plasma membranes and plasma membrane lipids of LM cells are shown in Table III. The dissociation constant, Kₛ, increased only slightly (10%) with increasing temperature. Concomitantly the number of lipid molecules/β-parinaric acid binding site decreased by approximately 10 to 15%. In addition the CO₁₁₃ of β-parinaric acid in the plasma membrane or plasma membrane lipids was found to increase in hyperbolic fashion reflecting an increase in unbound probe as saturation is approached while RFE₁₁₃ remained independent of probe concentration. Because of the low solubility and probability of micelle formation of fatty acids in water, the accuracy of the Kₛ values may be suspect. However, we have attempted to partially circumvent this problem by coating the probe fatty acid on the side of the tube and then blending on a Vortex mixer with membranes or lipids in buffer (see "Materials and Methods") and this would not require a high concentration of probe in the aqueous medium once equilibrium of β-parinaric acid was established between the glass wall, the aqueous buffer, and the membrane vesicles. The membrane or lipid vesicles would absorb the probe from the side of the reaction vessel in a binding process that may reflect saturation better than if the fatty acid probe were simply added in ethanol solution at concentrations possibly much higher than the critical micelle concentration. We have not, however, determined the critical micelle concentration of β-parinaric acid in aqueous buffer solution. Extensive binding studies with model lipids indicated similar characteristics and values of the same order of magnitude with β-parinaric acid as noted here.

Characteristic Temperatures of Membranes and Membrane Lipids Indicated by β-Parinaric Acid—Fluorescent molecules have been used as probes of the physical state of lipids in membranes, and alterations or transitions in the behavior of the probes have been correlated to physiologically important parameters (3, 23, 24, 27). Sklar et al. (7) have shown that fluorescence measurements of β-parinaric acid can be used to...
**DISCUSSION**

The data presented here indicate that \( \beta \)-parinaric acid may be an ideal fluorescence probe for measuring changes in the physical properties of the isolated membranes and extracted membrane lipids from LM cells grown in suspension culture. \( \beta \)-Parinaric acid interacted quickly at room temperature with membranes of lipids and maximal interaction occurred in less than 3 to 5 min. Other probes require lengthy incubation times and often elevated temperatures (20, 24, 32). \( \beta \)-Parinaric acid appeared to satisfy the two major criteria for a good probe molecule (3) set forth in the introduction: (a) it is a natural molecule that is sensitive to the nature of its environment and (b) its environmental location in LM membranes or lipids can be qualitatively ascertained. In the intact membranes the \( \beta \)-parinaric acid interacted with approximately 90% of the plasma membrane lipids, indicating that about 10% of the lipids were inaccessible to the probe. This would tend to support the sequestering of lipid by protein in the intact membrane as the major cause for the difference in the relative fluorescence efficiency between the isolated lipids and the intact membrane. However, the magnitude of this trend may be suspect. These results may be interpreted as being consistent with the Singer membrane model (33) which predicts that at least some of the lipids may be tightly bound by proteins and that a microheterogeneity of lipids may exist within membranes of mammalian cells. Such a microheterogeneity is indicated by a multiplicity of characteristic temperatures (20, 34).

As shown here, characteristic temperatures in LM cell membranes (plasma membranes, microsomes, and mitochondria) were located at 18, 23, 31, 38, and 43\(^\circ\). The precision of the individual data points used to determine these characteristic temperatures was \( \pm 0.2\% \). These data may indicate a close similarity in physical properties of the subcellular organelles of an animal cell line (LM suspension cells) despite considerable variation in the composition and subcellular distribution of sterols, phospholipids, and ether-linked glycerides. However, the changes in slope were not always in the same direction as one temperature for separate samples (see Fig. 6). This indicates that the "breakpoints" may appear similar, but the behavior of the samples is not the same. Using electron spin resonance (ESR) probes, it was shown that L suspension cell microsomes and mitochondria had the same fluidity in their membranes (35). These workers also showed that considerable differences in fluidity existed between different cell types such as L suspension cells, lymphocytes, and erythrocytes (L suspension cell membranes were the most fluid). The large number (rather than the expected one or two) and location of characteristic temperatures found in the isolated suspension cultured LM cell plasma membranes, microsome, and mitochondria were very similar to those noted with ESR by Fox and co-workers (20, 34) in plasma membranes and microsomes from monolayer LM cells and membranes of New Castle Disease viruses propagated in embryonated chick eggs. However, these investigators found that isolated lipids from LM monolayer cell microsomes and New Castle Disease viruses revealed only two characteristic temperatures. LM Monolayer cell plasma membranes were compared with New Castle Disease virus lipids (20) and the data were interpreted to indicate the existence in the membranes of two hydrocarbon compartments (inner and outer monolayer of a bilayer) with different sets of characteristic temperatures. Thus an asymmetry of characteristic temperatures was proposed. Several assumptions appear to be implicit in this deduction. First, LM monolayer cell plasma membranes, microsomes and New

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\[ AC_{\text{Lim}} \] is determined near two possible transitions for plasma membranes and plasma membrane lipids as previously described (24). The accessible fraction is defined (24) as the value of \( AC_{\text{Lim}} \) in the plasma membrane/the value in the plasma membrane lipids.

| Cell fraction       | \( AC_{\text{Lim}} \) near possible transition: |
|--------------------|---------------------------------------------|
|                    | 24-30\(^\circ\) | 37-43\(^\circ\) |
| Plasma membrane    | 16.0          | 2.3            |
| Plasma membrane lipids | 17.0          | 2.6            |
| Accessible fraction | 0.94          | 0.88           |

\*The limiting corrected fluorescence at 313 nm (\( AC_{\text{Lim}} \)) was determined near two possible transitions for plasma membranes and plasma membrane lipids as previously described (24). The accessible fraction is defined (24) as the value of \( AC_{\text{Lim}} \) in the plasma membrane/the value in the plasma membrane lipids.
Castle Disease virus membranes have a vertical asymmetry of phospholipid distribution. Secondly, the isolated lipids from these membranes reassemble randomly both vertically and horizontally in a bilayer when dispersed in aqueous solutions. No data were presented to confirm either of these assumptions in this system. Also, it is possible that differences in the horizontal asymmetry rather than vertical asymmetry of phospholipids in intact members versus isolated membrane phospholipids may give rise to some of these observations. Contrary to the results of these workers, we have found that the isolated lipids from all three LM cell membrane fractions exhibited approximately the same characteristic temperatures as did the intact membranes. Therefore, the data presented here negate one of the three criteria for membrane physical asymmetry of characteristic temperatures presented by Wisniewski et al. (20). Two asymmetric sets of phase transitions, one for the inner monolayer and another for the outer monolayer, of the membranes were not detected. The reason for this discrepancy is not known; however, it may be due to differences in the degree of entry of the probe into the hydrophobic environment of the isolated lipids. Several other major differences in the experimental procedures may be pointed out. (a) ESR and fluorescence probes measure very different parameters. (b) Intrinsic differences exist in the types of probes used (2,2-dimethyl, 4-butyl, P-pentyl, N-oxyloxazolidine, an unnatural probe, versus β-parinaric acid, a naturally occurring probe). (c) Different buffers were used for resuspension of membranes and lipids (triethanolamine versus PBS). (d) Other methods of physical dispersion were employed (sonication versus blending on a Vortex mixer). (e) Cell culture (monolayer versus suspension) and media (partly defined versus chemically defined) were different. The chemical composition and biological behavior of monolayer and suspension cultured LM cells are very dissimilar (36). In addition, the lipid composition of the three types of membranes isolated from suspension cultured LM cells is quite different. (f) In this work LM plasma membrane characteristic temperatures were compared with LM plasma membrane lipid characteristic temperatures. However, Wisniewski et al. (20) compared membrane and lipid characteristic temperatures from two different sources, LM cells versus New Castle Disease viruses. Future investigations should resolve these differences.

Since the characteristic temperatures of the three types of membranes and lipids are approximately the same but the lipid compositions vary, it appears that the LM suspension cell can maintain a constancy of characteristic temperatures in all three membranes by a variety of combinations of lipid classes as shown previously. This is not an unreasonable compensation mechanism to be expected from a homeothermic living cell. Elsewhere we have reported such compensation has been observed after induced perturbations of phospholipid compositions of plasma membranes, microsomes, and mitochondria.

The significance or function of the multiplicity of characteristic temperatures noted here and elsewhere (20, 34), rather than the one or two transitions shown in prokaryotic systems, is not known. Two hypotheses may be presented. (a) Such characteristic temperatures may reflect transitions in the lipid phase of membranes from gel to liquid crystalline (20, 34). The transitions can often be correlated with similar characteristic temperatures or breaks in Arrhenius plots of enzyme activities.

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