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THE USE OF THE FLUORESCENT PROBE α-PARINARIC ACID TO DETERMINE THE PHYSICAL STATE OF THE INTRACYTOPLASMIC MEMBRANES OF THE PHOTOSYNTHETIC BACTERIUM, *RHODOPSEUDOMONAS SPHAEROIDES*

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Summary

α-Parinaric acid has been used to determine the degree of ordering of the hydrocarbon region of purified intracytoplasmic membranes of *Rhodopseudomonas sphaeroides*. The usefulness of α-parinaric acid as a probe of membrane fluidity was established by comparison of its fluorescent properties in phosphatidylcholine vesicles with those of the more commonly used fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene. Both fluorescent probes were shown to monitor similar environments in the phosphatidylcholine vesicles when the phospholipids were maintained at temperatures above their phase transition temperature.

The rotational mobility of α-parinaric acid in the intracytoplasmic membranes was determined from 0 to 50°C, a region where no phase transitions were detectable. The rotational mobility of α-parinaric acid dissolved in vesicles formed from total extracted intracytoplasmic membrane phospholipids, was 2—3-fold greater than that measured in the intact intracytoplasmic membranes; demonstrating that the presence of protein greatly reduces the mobility of the phospholipid acyl chains of the intracytoplasmic membranes. Due to the high protein content of these membranes, the perturbing effect of protein on acyl chain mobility may extend to virtually all the intracytoplasmic membrane phospholipid.

Introduction

The use of fluorescent probes to investigate the physical state of artificial and biological membranes is well documented [1—9]. Although the data

Abbreviation: DPH, diphenylhexatriene.

obtained using extrinsic fluorescent probes generally agrees with data obtained from NMR, calorimetric and X-ray diffraction studies, specific criticisms of the technique include: (1) possible probe-mediated membrane perturbations, and (2) uncertainty concerning the location and distribution of the probe in the membrane. Recently, a new class of naturally occurring fluorescent compounds, α-parinaric acid (*cis-trans-trans-cis*) and β-parinaric acid (*trans-trans-trans-trans*) has been introduced [10—13] which appears to both minimize the problem of membrane perturbation and reduce the uncertainty concerning the location of the probe within the membrane. Since the structures of the parinaric acid isomers are very similar to naturally occurring fatty acids, the perturbing effects of these molecules on biological membranes should be minimal. This fact has been substantiated by Tecoma et al. [14], who demonstrated that both parinaric acid isomers could be incorporated directly into the cellular phospholipids of an *Escherichia coli* fatty acid auxotroph. Phase transitions detected in *E. coli* membranes containing the esterified parinaroyl-phospholipid probes were identical to those determined with the free fatty acid probes.

In the present paper, α-parinaric acid is used to determine the physical state of the hydrocarbon region of the intracytoplasmic membranes of a carotenoidless mutant of the photosynthetic bacterium, *Rhodopseudomonas sphaeroides*. Although the intracytoplasmic membranes of this organism have effectively served as a model system for the study of bacterial photochemistry [15—19] and membrane biogenesis [20—24], the comparison of this membrane system with other membrane systems has been precluded by the absence of information concerning its physical state. Since this investigation represents the first application of α-parinaric acid for the determination of membrane fluidity in the photosynthetic bacteria, and because the strong absorption by bacteriochlorophyll at 380 nm prevented the use of other fluorescent probes in the intracytoplasmic membrane , the results obtained with α-parinaric acid in determining the fluidity of sonicated phosphatidylcholine vesicles have been compared with those using the more familiar fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene.

Materials and Methods

Organism, media and growth. R. sphaeroides, R_{26} (a carotenoidless strain derived from the wild-type strain, 2.4.1) was kindly supplied by W.R. Sistrom (University of Oregon). The organism was routinely grown photoheterotrophically in a succinic acid minimal salts medium as previously described [21]. A General Electric Model 213 light meter was used to determine light intensities.

Preparation of intracytoplasmic membranes and phospholipid vesicles. Late logarithmic phase cultures ($1.8 \cdot 10^9$ cells/ml) were harvested by centrifugation (10 min at 12 000 × *g*) and the cells were resuspended in 5.0 ml of 0.1 M sodium phosphate buffer, pH 7.0, containing 0.05 M ethylenediaminetetraacetate, 5 mM 2-mercaptoethanol acid and 5 mM sodium azide and disrupted by sonication as previously described [21,22]. The intracytoplasmic membrane fraction was obtained and purified by discontinuous sucrose gradient centrifugation as previously described [21,22].

Total phospholipids were extracted from purified intracytoplasmic membrane vesicles by the method of Folch et al. [25]. Neutral and polar lipid fractions were obtained as described by Gray [26] employing chromatography on silicic acid H equilibrated in chloroform/methanol (49 : 1, v/v). Neutral lipids were removed by elution of the column with 20 volumes of chloroform/methanol (49 : 1, v/v) and total phospholipids were eluted with 10 column volumes of methanol. The phospholipid fraction was taken to dryness under reduced pressure, resuspended in 0.1 ml of methanol and precipitated with 4.9 ml of cold acetone to remove residual bacteriochlorophyll [27]. The phospholipid precipitate was separated from the acetone solution by centrifugation (10 min at 12 000 × *g*). The procedure was repeated and the phospholipids were stored in chloroform at −20°C.

Sonicated phospholipid vesicles were prepared essentially as described by Huang and Thompson [28]. 20 mg of total intracytoplasmic membrane phospholipid (or pure phosphatidylcholine) dissolved in chloroform was evaporated under nitrogen and redissolved in 0.75 ml of Spectro-grade benzene and lyophilized to dryness. The lyophilized phospholipid was then resuspended in 0.75 ml of 0.05 M phosphate buffer (pH 7.0) and subjected to sonication with a Branson W-350 Sonifier Cell Disruptor for 30.0 min at maximum power (power output — 7) in a water bath maintained at 48—50°C. Titanium particles were removed by centrifugation in a Beckman Microfuge B for 10.0 min. The vesicle suspensions were then centrifuged (150 000 × *g* for 1.5 h at 4°C) to remove the larger Bangham-type vesicles from the preparation.

The phospholipid compositions of the intact intracytoplasmic membranes and total extracted intracytoplasmic membrane phospholipids were determined on identical membrane preparations from cells grown on media supplemented with 5 μCi/ml [^{32}P]phosphate. Phospholipids were separated by thin-layer chromatography on boric acid-impregnated silica gel G plates, employing the two-dimensional systems of Poorthuis et al. [29]. Phospholipids were located by iodine staining and identified by comparison with authentic standards. Appropriate areas of the plate were scraped into scintillation vials and counted in a toluene-based scintillant using a Nuclear Chicago scintillation counter.

Phosphatidylcholine vesicles. Dipalmitoyl phosphatidylcholine and egg phosphatidylcholine were obtained from Sigma (purity >99%). The purity was confirmed chromatographically on silica gel G plates using two one-dimensional solvent systems (chloroform/methanol/water (65 : 25 : 4, v/v)) and (chloroform/methanol/acetic acid/water (70 : 30 : 10 : 3, v/v)). Visualization was accomplished by charring with 5 M H_2SO_4. Dipalmitoyl phosphatidylcholine and egg phosphatidylcholine vesicles were prepared as described above. The concentration of phospholipid phosphorus was determined according to the method of Bartlett [30].

Fluorescent probes and labeling of phospholipid vesicles. α-Parinaric acid was obtained from Molecular Probes (Roseville, Minn.) and stored in chloroform (−20°C) containing 0.1% butylated hydroxytoluene as an antioxidant. Prior to use, 0.1 ml of the stock solution was layered onto a silica gel H (type 60) column (0.4 × 4 cm) equilibrated in chloroform, and eluted with 10.0 ml of chloroform. Under these conditions, contaminants and degradation products remain absorbed to the column (Haugland, R., personal communication). The

chloroform was removed by evaporation under nitrogen gas and the purified α-parinaric acid was redissolved in 1.0 ml of ethanol containing 1% butylated hydroxytoluene. Aliquots (1—10 μl) of the ethanolic stock were added directly to the vesicle suspension.

1,6-Diphenyl-1,3,5-hexatriene (Aldrich) was of sufficient spectral purity to be used without purification. A 1.0 mM stock (in tetrahydrofuran) was diluted 1000-fold with the vesicle suspension and incubated at 45°C for 2.0 h to ensure equilibration before measurements were made.

All fluorescence measurements were performed at an absorbance of <0.3 at the exciting wavelength for 0.5-cm pathlength cuvettes and <0.1 for 1.0-cm pathlength cuvettes. The molar ratio of probe/phospholipid was less than 1/500 for all measurements in phospholipid vesicles and less than 1/200 for measurements in intact intracytoplasmic membranes.

Fluorescence measurements. Fluorescence polarization measurements were made in a T-format polarization instrument equipped with photon-counting electronics [31]. The sensitivity of the photon-counting technique greatly facilitates measurements where the signal is weak due to high dilution or poor quantum efficiency. The digital nature of the output simplifies the correction for background due to scattered light or solvent impurities. With monochromatic, vertically polarized exciting light, $I_{\parallel}$ and $I_{\perp}$ photon pulses from the sample are collected sequentially for a predetermined time (usually 30 s), where $I_{\parallel}$ and $I_{\perp}$ represent light intensities polarized parallel and perpendicular to the laboratory axis, respectively. The background counts are then collected for the same interval and the corrected polarization is calculated by the equation below:

$$P_{\text{corrected}} = \frac{(I_{\parallel} - I_{\parallel}^{s}) - (I_{\perp} - I_{\perp}^{s})}{(I_{\parallel} - I_{\parallel}^{s}) + (I_{\perp} - I_{\perp}^{s})}$$

where the superscript, s, denotes the background signal. α-Parinaric acid was excited at 326 nm (bandwidth 4.7 nm), the exciting light passed through a Corning 7-54 broad band pass filter, while emission was observed through Corning 0-52 sharpcut filters. Diphenylhexatriene (DPH) was excited at 370 nm (bandwidth 3.6 nm) through the 7-54 filter and observed through Corning 3-75 sharpcut filters. Polarization measurements are accurate to within ±0.002.

Fluorescence lifetimes were determined on the cross correlation phase and modulation fluorimeter of Spencer and Weber [32], equipped with improved electronics from SLM Instruments (Champaign, Ill.). The exciting light was modulated at 10 MHz or 30 MHz and the lifetimes determined by the phase shift and demodulation of the emission relative to a scattering solution of glycogen.

In all measurements, temperature was controlled by a thermostated-circulating water bath, the measured temperature recorded with a thermometer immersed in the bath was identical to that obtained using a thermistor immersed in the cuvette. All the data were obtained from heating scans in which the rate of heating was approx. 0.2°C/min (cooling scans gave identical results showing no hysteresis in the transition and >95% of the original

emission intensity). A comparison of the absorption spectra before and after the heating scans revealed no decomposition of the probe.

Calculations of depolarization, rotational diffusion rates and microviscosity. Fluorescence depolarization was expressed as the average angle ($\langle\theta\rangle$), in degrees, between the direction of the transition moments at the times of absorption and emission according to the equation [33].

$$\frac{\frac{1}{P}-\frac{1}{3}}{\frac{1}{P_0}-\frac{1}{3}}=\frac{2}{3\cos^2\theta-1}$$

where P_0 is the limiting polarization (determined in propylene glycol at −55°C) and P is the measured polarization. $\langle\theta\rangle$ represents the depolarization of fluorescence due both to intrinsic and extrinsic causes.

The polarization and fluorescence lifetimes of α-parinaric acid dissolved in American White Oil No. 58 were measured as a function of temperature. The values of $1/P-1/3/1/P_0-1/3$ were plotted versus $T\tau/\eta$, where T is the absolute temperature (°K), η is the solvent viscosity (poise), τ is the fluorescent lifetime (s × 10^{-9}) and $1/P-1/3/1/P_0-1/3$ refers to the degree of depolarization, to give a standard curve (not shown). The same values were determined for DPH using $P_0 = 0.485$ *. These calibration curves were used to evaluate microviscosities according to the method of Shinitzky et al. [1] using the relationship:

$$\frac{\frac{1}{P}-\frac{1}{3}}{\frac{1}{P_0}-\frac{1}{3}}=1+\frac{RT\tau}{\eta V_0}$$

where V_0 is the effective rotational molar volume of the probe and R is the gas constant. The rotational diffusion constant (or rotational rate), $\bar{R}$, was determined directly from the Perrin relationship [33]:

$$\bar{R}=\frac{\frac{\frac{1}{P}-\frac{1}{3}}{\frac{1}{P_0}-\frac{1}{3}}-1}{6\tau}$$

Results

The application of fluorescent probes to study the biological membranes is frequently complicated by the presence of intrinsic chromophores, such as

* The exceptional rejection ratio of the Glan-Taylor polarizers coupled with the narrow aperture permissible with the sensitivity of photon counting results in a higher value of P_0 than is attainable with many other instruments. Hence, in the instrument employed we measure $P_0 = 0.485$ as compared to previous values of 0.46 [34].

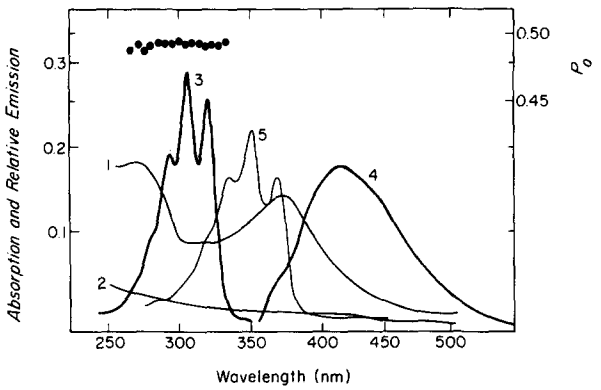

Fig. 1. Absorption spectra of intracytoplasmic membranes (curve 1) and total extracted intracytoplasmic membrane phospholipid vesicles (curve 2) in 10 mM NaH_2PO_4 buffer (pH 7.0), and the absorption (curve 3) and technical emission (curve 4) spectra of α-parinaric acid ($3.5 \cdot 10^{-6}$ M) in methanol. The polarization spectrum of α-parinaric acid (•) in propylene glycol at −55°C and the absorption spectrum of DPH in hexane (curve 5) are also included.

cytochromes [4], retinal [6] and chlorophyll [35] whose absorption spectra overlap the excitation or emission spectra of the fluorophore. Consequently, as a result of color quenching and/or energy transfer, the fluorescence emission from the probe can be greatly diminished. The strong absorption by bacteriochlorophyll at 380 nm in the intracytoplasmic membranes overlaps the absorption and emission spectra of most commonly employed membrane probes and effectively prevents their use in this membrane. This problem is illustrated in Fig. 1, where the overlap of the bacteriochlorophyll absorption with that of DPH is shown. As a result of the reduced DPH emission intensity, an adequate fluorescent signal could only be achieved at probe concentrations of 1 mol DPH/5—10 mol phospholipid. At these relatively high concentrations, Podo and Blasie [36] have demonstrated using NMR techniques the dynamical perturbation of phospholipid molecules induced by the incorporation of fluorescent probes into the bilayer. Importantly, the large Stoke's shift of the absorption and emission spectra of α-parinaric acid minimizes the overlap between the bacteriochlorophyll and α-parinaric acid absorption spectra (Fig. 1) and permits this probe to be used effectively in the intracytoplasmic membrane at concentrations less than 1 mol α-parinaric acid/200 mol phospholipid.

Studies with phosphatidylcholine vesicles

Since a direct comparison of fluidity values measured by α-parinaric acid in the intracytoplasmic membranes with those of another reference probe was precluded by the presence of bacteriochlorophyll in the membrane, the utility of α-parinaric acid as a probe of membrane dynamics was established by comparing the fluidity values determined by α-parinaric acid and DPH in sonicated egg phosphatidylcholine and diplamitoyl phosphatidylcholine vesicles. Egg phosphatidylcholine was chosen because the degree of unsaturation of its acyl groups (1.8 double bonds/phospholipid molecules [6]) should be very similar to that of intracytoplasmic membrane phospholipids [37]. Dipalmitoyl phosphatidylcholine vesicles undergo a sharp phase transition at

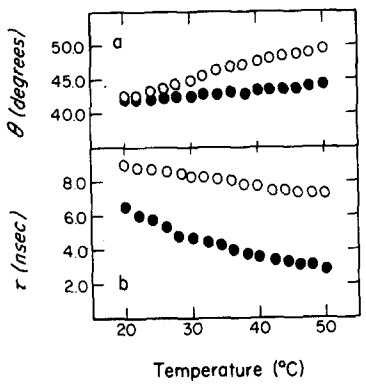

Fig. 2. (a) Temperature dependence of the fluorescence depolarization of α-parinaric acid (●———●) and DPH (○———○) in egg phosphatidylcholine vesicles. (b) Temperature dependence of the excited-state lifetime of α-parinaric acid (●———●) and DPH (○———○) dissolved in egg phosphatidylcholine vesicles. The lifetimes shown represent the average of the values measured by the phase and modulation methods at 10 mHz.

41°C and allows the investigation of the spectral properties of the probe in solid phase lipid. Sklar et al. [13], have shown that the polarization, lifetime, and intensity of α-parinaric acid fluorescence are sensitive indicators of the dipalmitoyl phosphatidylcholine phase transition. These investigators have also established that the α-isomer partitions nearly equally between solid and liquid phase lipid.

The fluorescence lifetime ($\tau_{\alpha\text{-parinaric acid}}$) and depolarization ($\langle\theta\rangle_{\alpha\text{-parinaric acid}}$) of α-parinaric acid dissolved in egg phosphatidylcholine vesicles were determined as a function of temperature between 20 and 50°C (Fig. 2). The depolarization of α-parinaric acid fluorescence is expressed as the average angle, $\langle\theta\rangle$, between the transition moments in absorption and emission, so that a more physical perspective can be placed on polarization measurements. When linear polyenes are excited in the last absorption band, the absorption and emission oscillators are colinear [34], so $\langle\theta\rangle$ is a measure of the angular disorientation of the fluorophore during its lifetime. The values of $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ (Fig. 2a) increased 5.2%, from 42 to 44.2°, over the temperature range studied (20—50°C); whereas $\langle\theta\rangle_{DPH}$ increased 13.7%, from 43.7 to 49.7°, over the same temperature span. The fluorescent lifetimes of the two probes (Fig. 2b) represent the numerical average of the lifetime values measured by the phase and modulation methods at 10 MHz. The lifetimes determined by these two methods are identical in the case of homogeneous emission as discussed by Spencer and Weber [32]. However, if there exists a heterogeneity in the emitting population, the phase and modulation values will differ; specifically, the phase value tends to weight the shorter lifetime components and the modulation value weights the longer lifetime components. The lifetime values for α-parinaric acid dissolved in egg phosphatidylcholine vesicles at three representative temperatures are shown in Table I. A small amount of heterogeneity is apparent, since the difference in the values determined by the phase and modulation methods are greater than the experimental error. The spread between the phase and modulation values gives a qualitative index of the system's heterogeneity. The observed heterogeneity probably results from the

TABLE I

LIFETIME HETEROGENEITY OF α-PARINARIC ACID IN PHOSPHOLIPID AND INTRACYTOPLASMIC MEMBRANE VESICLES

The excitation wavelength for α-parinaric acid was 326 nm. The phospholipid concentration in all samples was adjusted to 0.6—1.0 mM. The concentrations of α-parinaric acid used with vesicles preparations of egg phosphatidylcholine, dipalmitoyl phosphatidylcholine, and total extracted intracytoplasmic membrane phospholipid was 10^{-6} M (1 mol probe/500 mol phospholipid), the concentration used with intact intracytoplasmic membrane vesicles was $3.5 \cdot 10^{-6}$ M (1 mol probe/200 mol phospholipid). Homogeneous emission was not observed for α-parinaric acid dissolved in any of the membrane vesicle systems or in hexane since the values obtained by the phase and modulation methods show differences outside of the experimental error. Heterogeneous emission in the case of hexane may be due to segmental motion of the fluorophore, the formation of more than one type of molecular complex with the solvent or slight contamination by the β-isomer. By obtaining phase and modulation values at two frequencies, one can solve a two-lifetime component system analytically (Weber, G., personal communication and manuscript in preparation). Such an analysis was not done in the present study, but currently this analysis is being employed to investigate phospholipid domain structure and boundary lipid.

Preparation	Phase method *	Modulation method	Average
Egg phosphatidylcholine (30°C)	4.15	4.90	4.52 ± 0.37
Egg phosphatidylcholine (40°C)	3.23	3.76	3.49 ± 0.26
Egg phosphatidylcholine (50°C)	2.47	2.87	2.67 ± 0.20
Dipalmitoyl phosphatidylcholine (30°C)	16.21	20.70	18.45 ± 2.24
Dipalmitoyl phosphatidylcholine (40°C)	10.80	14.60	12.7 ± 1.9
Dipalmitoyl phosphatidylcholine (50°C)	4.54	5.37	4.95 ± 0.42
Intracytoplasmic membrane vesicles (20°C)	3.13	4.76	3.94 ± 0.82
Extracted lipid vesicles	4.82	5.84	5.33 ± 0.96
Intracytoplasmic membrane vesicles (30°C)	2.70	4.19	3.44 ± 0.75
Extracted lipid vesicles	3.47	4.92	4.19 ± 0.73
Intracytoplasmic membrane vesicles (40°C)	2.30	3.80	3.05 ± 0.75
Extracted lipid vesicles	2.59	4.01	3.3 ± 0.71
Intracytoplasmic membrane vesicles (50°C)	2.02	3.54	2.78 ± 0.76
Extracted lipid vesicles	2.14	3.58	2.86 ± 0.72
Hexane (25°C)	3.97	4.40	4.18 ± 0.22
Hexane (40°C)	3.15	3.65	3.43 ± 0.25

* The standard deviation of the phase and modulation measurements was less than 0.04.

inherent segmental flexibility of the probe or from contamination by the β-isomer, since a similar degree of heterogeneity is apparent in the lifetime values for α-parinaric acid dissolved in hexane (Table I). The sharpness of the α-parinaric acid absorption peaks in Fig. 1 would indicate that significant contamination by the β-isomer is unlikely.

Although polarization measurements alone can be used as a relative estimate of hydrocarbon chain disordering, a more precise comparison of the fluidity values measured in egg phosphatidylcholine vesicles by α-parinaric acid and DPH is obtained by expressing the lifetime and polarization data in Fig. 2 as rotational diffusion rates ($\bar{R}$), because lifetime effects on the polarization measurements are eliminated. The limiting polarization, P_0, of the fluorophore must be known in order to determine $\bar{R}$. The P_0 of α-parinaric acid fluorescence determined in propylene glycol at —50°C (Fig. 1), was quite high ($P_0 \sim 0.491$) and constant across the absorption band (265—335 nm). This property eliminates polarization errors resulting from changes in P_0 as a result of small

shifts in the absorption spectrum as well as providing a large dynamic range for polarization measurements. We do not intend to give the impression that $\bar{R}$, calculated in this manner, represents an absolute rotational diffusion constant; but it should be noted the values obtained for $\bar{R}$ in this study are in good agreement with those measured in other membrane systems by a variety of physical techniques [38].

α-Parinaric acid has a higher rotational mobility (13—27%) in the egg phosphatidylcholine vesicles than does DPH, although the relative changes in $\bar{R}$ for the two probes are the same (Fig. 3a). The higher mobility for α-parinaric acid could result from structural differences between the two probe molecules or the results may indicate that α-parinaric acid and DPH are monitoring different environments in the bilayer. To distinguish between these two possibilities, the lifetime and polarization data have been used to calculate microviscosity (η) as described by Shinitzky et al. [1], since differences between the two probes in their molecular shapes or volumes would be normalized by calibration in white oil. The values of η obtained for α-parinaric acid and DPH egg phosphatidylcholine vesicles differed by less than 10% over the entire temperature range (Fig. 3b), indicating that both probes were monitoring similar environments and that the discrepancies observed in the $\bar{R}$ values originate from diffferences

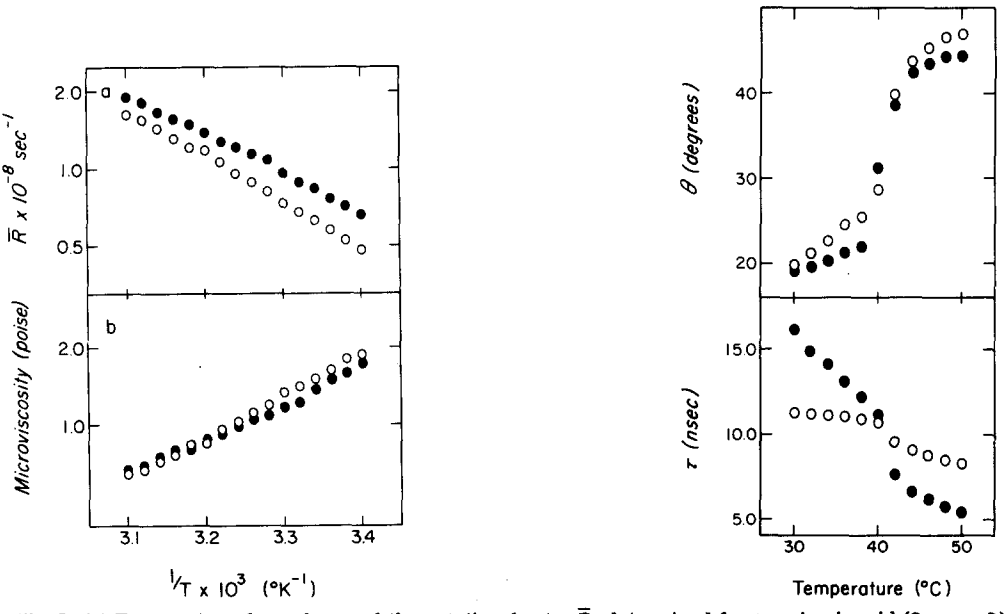

Fig. 3. (a) Temperature dependence of the rotational rate, $\bar{R}$, determined for α-parinaric acid (●———●) and DPH (○———○) dissolved in egg phosphatidylcholine vesicles, presented as log $\bar{R}$ versus $1/T$. (b) Plot of the log of the microviscosity versus $1/T$ for egg phosphatidylcholine vesicles measured by α-parinaric acid (●———●) and DPH (○———○).

Fig. 4. (a) Temperature dependence of the fluorescence depolarization of α-parinaric acid (●———●) and DPH (○———○) in dipalmitoyl phosphatidylcholine vesicles. (b) Temperature dependence of the excited-state lifetime of α-parinaric acid (●———●), and DPH (○———○) dissolved in dipalmitoyl phosphatidylcholine. The lifetimes represent the average of the values measured by the phase and modulation methods at 10 MHz.

in the hydrodynamic properties of the two dye molecules. These values for η are in excellent agreement with values previously determined for egg phosphatidylcholine vesicles by Cogan et al. [2], using perylene, 9-vinylanthracene and 2-methylanthracene.

The fluorescence lifetimes and polarization of α-parinaric acid and DPH dissolved in dipalmitoyl phosphatidylcholine vesicles plotted against temperature are shown in Fig. 4. The values of $\langle\theta\rangle$ for both probes remained relatively constant in the regions above and below the dipalmitoyl phosphatidylcholine phase transition (Fig. 4a), but increased sharply from about 22 to 44° during the transition region. The two probes reported identical values of 41.0° for the dipalmitoyl phosphatidylcholine phase transition, which is in good agreement with those values determined by non-perturbing techniques. Although the lifetimes of both probes showed abrupt discontinuities at the phase transition, $\tau_{\alpha\text{-parinaric acid}}$ was much more temperature dependent than τ_{DPH} (Fig. 4b). τ_{DPH} in solid phase lipid approached the value for τ_0 (11.4 ns) given by Shinitzky and Barenholtz [34], whereas the τ_0 of α-parinaric acid is approx. 100 ns [13]. This major difference between the two polyene probes results from differences in the number of double bonds and their influence on the changes in the geometry of the nuclear framework which occurs upon excitation [39]. The lifetimes determined for α-parinaric acid in dipalmitoyl phosphatidylcholine vesicles by the phase and modulation methods were more heterogeneous than those determined in egg phosphatidylcholine vesicles (Table I). Sklar et al. [13], have suggested the lifetime heterogeneity may reflect the coexistence of stable domains of dipalmitoyl phosphatidylcholine vesicles having different fluidities. Recent ESR investigations [40] of dipalmitoyl phosphatidylcholine vesicles have shown the heterogeneity of "fluidity" to be associated with the region near the polar head groups, since heterogeneity is not detected with probes which are further removed from the head group region. Lee [41] has discussed the possibility that conformational changes of the polar head groups may be partially responsible for the pretransition and defects observed in the structure of gel phase phosphatidylcholines. It is possible that α-parinaric acid fluorescence, by virtue of the interaction of its carboxyl group with polar head groups, could be influenced by changes in the orientation of both the fatty acid chains and the polar head groups of the phospholipids.

The rotational rates calculated for α-parinaric acid dissolved in dipalmitoyl phosphatidylcholine vesicles are compared to those determined for DPH (Fig. 5). The rotational mobility of α-parinaric acid was 15—20% greater than that of DPH when the membrane is in the liquid state (as was found in the egg phosphatidylcholine vesicles), but below the dipalmitoyl phosphatidylcholine phase transition, the values of $\bar{R}$ for α-parinaric acid were 40—50% less than those determined for DPH. It should be pointed out that below the dipalmitoyl phosphatidylcholine phase transition, $\tau_{\alpha\text{-parinaric acid}}$ is increasing in a region where $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ is remaining relatively constant; a result which would be expected if the range of $\langle\theta\rangle$ over which α-parinaric acid can freely rotate in solid phase lipid is restricted, as has been indicated by Chen et al. [42], for DPH. Increasing the lifetime in the absence of corresponding polarization changes, would then result in arbitrarily lower values of $\bar{R}$ measured for

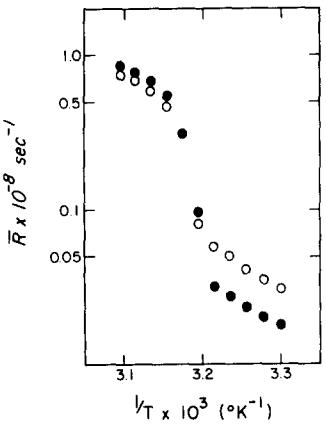

Fig. 5. Temperature dependence of the rotational rate, $\bar{R}$, determined for α-parinaric acid (●———●), and DPH (○———○) dissolved in dipalmitoyl phosphatidylcholine vesicles, presented as log $\bar{R}$ versus $1/T$.

α-parinaric acid in solid phase lipid. This effect would not be apparent in the R values measured by DPH, since $\tau_{DPH} = \tau_0$ in solid phase lipid. This anomalous behavior could also, in part, reflect the redistribution of DPH molecules in the bilayer, as suggested by Lentz et al. [43], to energetically more favorable positions, such that the average environment measured by DPH is now more fluid than that measured by α-parinaric acid. Alternatively, changes in surface charge of polar head group orientation during the phase transition may sufficiently influence α-parinaric acid fluorescence to result in the lower values of η.

A limitation of α-parinaric acid as a membrane probe is its relatively small quantum yield at higher temperatures. The quantum yield of α-parinaric acid relative to DPH in dipalmitoyl phosphatidylcholine vesicles at 30°C is approx. 0.2 and drops to 0.04 at higher temperatures (data not shown). This decrease is an important experimental consideration when using α-parinaric acid at higher temperatures as the scattering contribution to the fluorescent signal can become significant.

Studies with purified intracytoplasmic membrane vesicles

The comparisons of α-parinaric acid and DPH incorporated into egg phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles indicate that α-parinaric acid is a reliable probe for determining the degree of ordering of the hydrocarbon regions of phospholipid bilayers and thus facilitates its use as a fluidity probe of the intracytoplasmic membranes of *R. sphaeroides*. The intracytoplasmic membrane forms and extensive internal membrane system in which the photochemical complexes of the cell are localized. Following cell disruption, the intracytoplasmic membrane fragments and reseals to form spherical vesicles, approx. 550—600 Å in diameter, which function photochemically and retain numerous enzymatic activities [44,47]. These vesicles have been purified and characterized by several laboratories [42—50], and have been shown to be comprised by weight, of protein (70—80%), phospholipid (20—25%), and photopigments (3—5%). The major membrane protein components consist of 5—7 polypeptides of which four are thought to be

involved in primary or secondary photochemical conversions [51—54]. The bulk of the membrane phospholipids are comprised by phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, and cardiolipin [55], while the predominant fatty acid (70—99%) found in this membrane is vaccenic acid (*cis*-Δ11, 18 : 1 [37]). All measurements were performed with intracytoplasmic membrane samples prepared from the carotenoidless strain, R_{26}, as attempts to make similar measurements in carotenoid-containing strains were unsuccessful because of reduced signal strength; this was presumably due to energy transfer between α-parinaric acid and carotenoids.

Fluorescence measurements employing intact R_{26} intracytoplasmic membrane vesicles and vesicles formed from total extracted R_{26} intracytoplasmic membrane phospholipid were made to assess the extent to which protein and bacteriochlorophyll influenced the physical state of the membrane. The absorption spectra of the intact R_{26} intracytoplasmic membrane total extracted phospholipids are shown in Fig. 1. The total extracted intracytoplasmic membrane phospholipid vesicles were virtually free of bacteriochlorophyll and protein contamination and displayed phospholipid compositions similar to that of intact intracytoplasmic membranes *. This strongly suggests that the differences cannot be attributed to altered phospholipid composition; although, the possible influences that phospholipid asymmetry [56], vesicle size [9], or other minor lipid components [57] may exert on membrane fluidity cannot be eliminated.

The fluorescent lifetimes and depolarization of α-parinaric acid dissolved in intact R_{26} intracytoplasmic membrane and total extracted phospholipid vesicles are shown in Fig. 6. The values of $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ in intact intracytoplasmic membrane vesicles were about 25% less than those determined for $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ in total extracted phospholipid vesicles (Fig. 6a). Similar changes in DPH polarization values have been observed between intact bovine retinal rod outer segment disk membranes and total extracted disk phospholipid vesicles [6] and have been interpreted as demonstrating that rhodopsin hinders the mobility of the hydrocarbon chains of the disk phospholipids.

$\tau_{\alpha\text{-parinaric acid}}$ in intact R_{26} intracytoplasmic membrane vesicles were less than the lifetimes measured in extracted phospholipid vesicles (Fig. 6b and Table I). The different slopes for the two curves probably result from viscosity effects on the processes which deactivate the excited state; as the efficiency of these processes in polyenes is reduced by increasing the viscosity of the environment [39]. The lifetime values determined by the phase and modulation methods in the intracytoplasmic membranes and extracted phospholipid vesicles were more heterogeneous than those observed for the phosphatidylcholine vesicles. Since the heterogeneity observed in the lifetime measurements of both the extracted phospholipid and intracytoplasmic membrane vesicles was similar, it cannot be mediated by the effects of protein or other membrane components on bilayer structure or probe fluorescence. Rather, the greater heterogeneity must be intrinsic to the mixed phospholipid species in the

* The requirement for the complete removal of photopigments prior to spectrofluorometric analysis, necessitated the addition of an acetone precipitation step in the purification of intracytoplasmic membrane phospholipids (Materials and Methods). It may be noted that some reduction in the percentage of phosphatidylcholine was observed following this treatment.

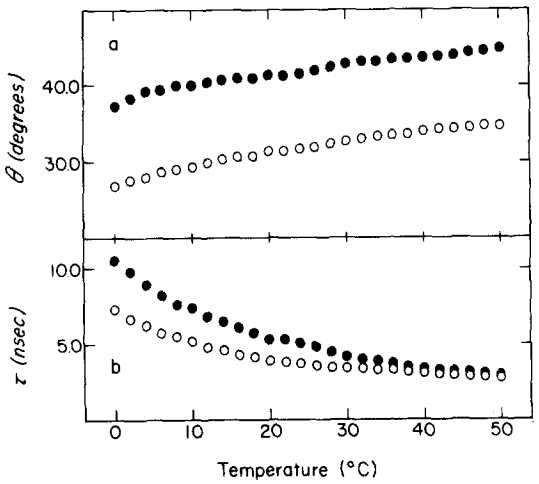

Fig. 6. (a) Temperature dependence of the fluorescence depolarization of α-parinaric acid in intact intracytoplasmic membrane vesicles (○———○) and total extracted intracytoplasmic membrane phospholipid vesicles (●———●). (b) Temperature dependence of the excited state lifetime of α-parinaric acid dissolved in intracytoplasmic membrane vesicles (○———○) and total extracted phospholipid vesicles (●———●). The lifetimes represent the average of the values measured by the phase and modulation methods at 10 MHz.

membrane [56] or the presence of phospholipid domain structure [58]. Due to the variability of $\tau_{\alpha\text{-parinaric acid}}$ within different environments, the extent to which the lifetimes determined in the intracytoplasmic membrane might be decreased by bacteriochlorophyll quenching cannot be unambiguously determined. An approach employed to assess the effect of bacteriochlorophyll on α-parinaric acid fluorescence considers the observation of Worden and Sistrom [59], that the specific level of bacteriochlorophyll (μg Bchl/mg protein) in the intracytoplasmic membrane is inversely proportional to the light intensity under which the cells are grown. By growing strain R_{26} at light intensities between 200 and 7500 lux, the specific level of bacteriochlorophyll in the

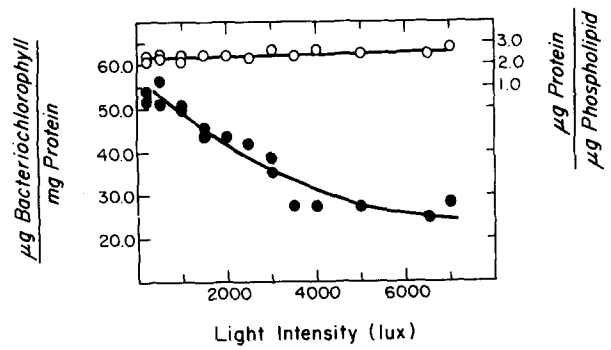

Fig. 7. Variations in the composition of purified intracytoplasmic membranes from cells grown at different incident light intensities. The cultures grown at the various light intensities were harvested at a cell density less than $7.5 \cdot 10^8$ cells/ml to avoid the possibility of self-shading [19]. The ratios of protein to phospholipid and bacteriochlorophyll to protein in the intracytoplasmic membrane samples were determined as previously described [21,22]. ●———●, μg protein/μg phospholipid; ○———○, μg bacteriochlorophyll/mg protein.

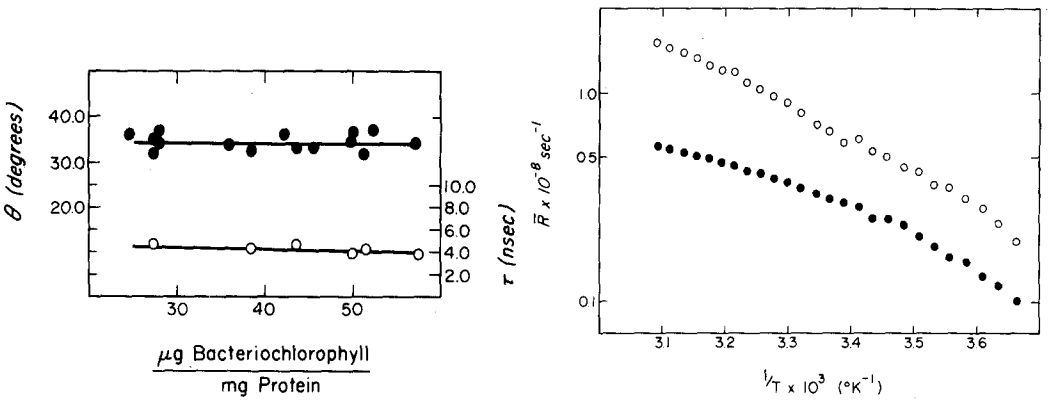

Fig. 8. Dependence of the depolarization (●———●) and lifetime (○———○) of α-parinaric acid fluorescence on the specific level of bacteriochlorophyll in the intracytoplasmic membrane. The intracytoplasmic membrane samples from cells grown at different light intensities were adjusted to the same phospholipid concentration (0.5 mg/ml) and the polarization of lifetime measurements were performed at 20°C. The lifetimes represent the average of the values measured by the phase and modulation methods at 10 MHz.

Fig. 9. Temperature dependence of the rotational rate, $\bar{R}$, of α-parinaric acid dissolved in intact intracytoplasmic membrane vesicles (●———●) and total extracted intracytoplasmic membrane phospholipid vesicles (○———○).

intracytoplasmic membrane could be altered approximately 3-fold (Fig. 7), while the protein-to-phospholipid ratio remained constant. The values of $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ and $\tau_{\alpha\text{-parinaric acid}}$ determined in these membranes at 20°C are plotted versus the specific level of bacteriochlorophyll (Fig. 8). Although the experimental scatter is greater among the individually prepared samples, it is apparent that neither $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ or $\tau_{\alpha\text{-parinaric acid}}$ are greatly influenced by the 3-fold change in the specific level of bacteriochlorophyll in the intracytoplasmic membrane. The lack of dependence of $\tau_{\alpha\text{-parinaric acid}}$ on the increasing bacteriochlorophyll concentration indicates that dipole-dipole resonance energy transfer between α-parinaric acid and bacteriochlorophyll is not occurring. Investigations of the quenching of α-parinaric acid fluorescence in organic solvents by extracted, free bacteriochlorophyll confirm the results of the in vivo experiments by demonstrating that $\tau_{\alpha\text{-parinaric acid}}$ does not change upon the addition of bacteriochlorophyll at concentration 10-fold greater than that used in the in vivo experiments. A reduction in the fluorescence intensity is observed at these high concentrations which is due to inner filter effects that occur when bacteriochlorophyll is added.

The rotational rates determined for α-parinaric acid dissolved in intact R_{26} intracytoplasmic membranes and total extracted phospholipid vesicles are shown in Fig. 9. The rotational mobility of α-parinaric acid in the extracted phospholipid vesicles was nearly three times greater than that measured in the intact intracytoplasmic membrane and clearly indicates that the presence of protein in the intracytoplasmic membrane greatly reduces the mobility of the hydrocarbon chains of the phospholipids. The rotational rates for α-parinaric acid in the total extracted intracytoplasmic membrane phospholipid vesicles were nearly identical to those determined for egg phosphatidylcholine vesicles (Fig. 3).

Discussion

Comparison of α-parinaric acid and DPH incorporated into egg phosphatidylcholine and dipalmitoyl phosphatidylcholine vesicles indicate that α-parinaric acid can be used reliably for determining the degree of ordering of the hydrocarbon region of phospholipid bilayers. The use of the free (non-esterified) form of the probe in these studies to determine membrane fluidity is supported by the incorporation experiments with *E. coli* fatty acid auxotrophs [14], which showed that phase transitions detected in *E. coli* membranes by the esterified parinaroyl-phospholipid probe were identical to those determined with free parinaric acid. Similarly, NMR studies [60] have revealed a strong similarity in the deuterium order parameters obtained with free deuterated fatty acid and phospholipid probes in phosphatidylcholine vesicles. However, due to the limitations accompanying the use of any spectroscopic probe as well as the specific assumptions involved in determining the rates of motion in membranes by fluorescent probes [42,61], the term "fluidity" is used to connote qualitative differences in the disorder of hydrocarbon regions of phospholipid vesicles.

The results of the initial studies with intracytoplasmic membrane vesicles indicate that α-parinaric acid can be used to monitor the fluidity of the hydrocarbon regions of these photosynthetic membranes with only minimal interference by bacteriochlorophyll or other membrane chromophores. The constancy of $\langle\theta\rangle_{\alpha\text{-parinaric acid}}$ and $\tau_{\alpha\text{-parinaric acid}}$ in intracytoplasmic membrane preparations in which the specific bacteriochlorophyll levels have been altered, suggest that bacteriochlorophyll exerts only minimal influence on bilayer structure and probe fluorescence. This observation is also consistent with spin label studies [62], which indicate that the macrocyclic ring of the chlorophyll molecule is localized outside of the hydrocarbon region of the bilayer.

As might be predicted from the high percentage of unsaturated fatty acids which comprise the membranes of *R. sphaeroides*, no phase discontinuities were detected in either the intracytoplasmic membrane or total extracted phospholipid vesicles in the biologically significant temperature region from 0 to 50°C. However, a broad phase transition below 0°C (T_c near −10°C; unpublished results) generates the curvature observed in the rotational rate plots (Fig. 9) at the lower temperature values. Phase transitions, occurring between 0 and −15°C, have also been observed in mitochondrial membranes [63]; and in the membranes of *E. coli* unsaturated fatty acid auxotrophs [64] in which the degree of fatty acid unsaturation is very similar to that normally present in *R. sphaeroides*.

The rotational mobility of α-parinaric acid in the R_{26} intracytoplasmic membrane was 2—3-fold less than that determined in total extracted phospholipid vesicles. Cossins [65] has similarly found that the rotational mobility of DPH in goldfish synaptosomal membrane is less than half the value determined for total extracted synaptosomal phospholipid vesicles. These findings are consistent with numerous other investigations [4,5,6,66,67] which suggest that the presence of proteins in membranes decreases acyl chain mobility. The effects of protein and cholesterol on membrane fluidity have been discussed [68] in

terms of a condensation effect which controls the amplitude of acyl chain motion. Whether the perturbing effect of protein extends to the bulk of the membrane phospholipid or is exerted only on a narrow annulus of boundary lipid is yet unclear. In the R_{26} intracytoplasmic membrane which has a relatively high protein-to-phospholipid ratio, (μg protein/μg phospholipid $\approx$2.6), these two effects may be difficult to distinguish, as the amount of lipid in association with protein can be expected to be quite high. An estimate of the amount of boundary phospholipid present in the intracytoplasmic membranes can be made based on the calculations by Jost et al. [66] for cytochrome oxidase in phospholipid vesicles. They have shown that 0.17 mg of phospholipid per mg protein can be accommodated in the first layer around a protein complex of molecular weight 210 000. Assuming the protein complexes in the R_{26} intracytoplasmic membrane are of similar size, 44% of the intracytoplasmic membrane phospholipid would be expected to be in direct association with protein. In the intracytoplasmic membrane of carotenoid-containing strains, which have an even higher protein content (μg protein/μg phospholipid $\sim$3.7 [21]), it would be expected that at least 62% of the phospholipid is boundary phospholipid. It is important to emphasize these values as very likely to be underestimates since greater than 40% of the membrane protein in the intracytoplasmic membrane is comprised by a single 15 000 dalton polypeptide [69], which would greatly increase the surface area available for phospholipid/protein contact.

Since α-parinaric acid has been shown to partition nearly equally between solid and liquid phase lipid [13], it would be expected that if large lateral gradients in fluidity existed between motionally restricted annular phospholipid and free fluid phospholipid, the heterogeneity of the α-parinaric acid lifetimes measured by the phase and modulation methods would be much greater in intracytoplasmic membrane vesicles than those determined in total extracted phospholipid vesicles. A comparison of the values in Table I shows little difference in the heterogeneity of the lifetime measurements and the data are consistent with the interpretation that large lateral gradients in fluidity do not exist in the intracytoplasmic membrane and that instead the motional properties of the bulk of the membrane phospholipids are influenced by proteins. A similar conclusion has been reached by Stubbs et al. [6] concerning the perturbation of acyl chain mobility caused by the presence of rhodopsin in bovine rod outer segment disk membranes, which appears to extend to essentially all the disk phospholipid. It should be noted that recent proton NMR experiments [70] with rod outer segment membranes have indicated the existence of fluid and less-fluid phospholipid domains and that a rapid exchange of phospholipid occurs between them. The apparent disparity between the NMR and fluorescence results may originate from differences in the nature and timescale of the motions measured by the two techniques.

It has been suggested that a reduction in the mobility of membrane lipids due to a decrease in temperature results in an even greater reduction in the lateral mobility of membrane proteins [71,72]. If the reduction of membrane phospholipid mobility by protein perturbation, in turn, causes a similarly pronounced decrease in the lateral mobility of membrane proteins, the overall motional properties of the intracytoplasmic membrane would be much less

than that predicted on the basis of the high degree of unsaturation of the fatty acids alone. As a result, the extent to which protein lateral diffusion can freely occur in the intracytoplasmic membrane might be sufficiently reduced that macromolecular diffusion may account, in part, for time delays observed in electron transport [73] as was originally suggested by Chance et al. [74].

Finally, these results are consistent with our earlier proposal that changes in the extent of protein-lipid interactions, as a result of the variation in the protein-to-lipid ratio of the intracytoplasmic membrane during the cell cycle [22], could result in fluidity changes which might serve as a regulatory mechanism for membrane synthesis.

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