Fluidity of LM Cell Membranes with Modified Lipid Compositions as Determined with 1,6-Diphenyl-1,3,5-hexatriene†

Reid Gilmore, Nina Cohn, and Michael Glaser*

ABSTRACT: The fluorescence polarization and lifetime of 1,6-diphenyl-1,3,5-hexatriene (DPH) were determined from 5 to 40 °C in mitochondrial, microsomal, and plasma membranes isolated from LM cells grown in tissue culture. The rotational relaxation times of DPH calculated from these measurements were highest in plasma membranes and lowest in mitochondrial membranes. Approximately the same ratios of the rotational relaxation times for the three membranes were maintained throughout the temperature range. The phospholipid polar head group composition of the membranes was altered by substituting ethanolamine for choline in the growth medium. Likewise, the fatty acid composition was altered by adding linoleate to the growth medium. These changes in the lipid composition produced an increase and a decrease, respectively, in the rotational relaxation times of DPH in all three membrane fractions. Nevertheless, approximately the same ratios of the rotational relaxation times of DPH were maintained between the different membranes. This suggests that this parameter is closely regulated. Examination of the rotational relaxation times of DPH in whole lipid and phospholipid dispersions prepared from the isolated membranes indicated that the observed differences could be explained by differences in the sterol, fatty acid, and polar head-group compositions. Changes in the sterol content and the phospholipid composition both contributed substantially to the changes produced in the rotational relaxation times.

The fluidity or viscosity of biological membranes has been implicated in a variety of membrane functions (Singer, 1974). The systematic modification of the lipid composition of cells in vivo has proved to be a valuable approach in trying to understand the role of individual lipid components in membrane structure and function (Silbert, 1975).

One animal cell system that has received considerable attention has been LM cells. Systematic modifications can be made in the phospholipid polar head-group composition (Glaser et al., 1974; Blank et al., 1975), or the fatty acid composition (Wisnieski et al., 1973; Williams et al., 1974; Ferguson et al., 1975; Doi et al., 1978), or both simultaneously (Glaser et al., 1974). The changes in the phospholipid composition occur in all the membrane fractions of the cell (Schroeder et al., 1976c). Changes in the phospholipid polar head-group and fatty acid composition in these cells have been shown to alter adenylate cyclase activity (Engelhard et al., 1976), stearoyl-CoA desaturase activity (Blank et al., 1976), and lectin interactions (Rittenhouse et al., 1974). In addition, the level of sterol present in the membrane can be altered and this can influence endocytosis (Heiniger et al., 1976) and Rb^+ transport (Chen et al., 1978).

In order to fully interpret the changes observed in the functional parameters of the membrane caused by altering the lipid composition, it is important to understand the changes in the structural organization and viscosity of the membrane. The effects of modifying the phospholipid polar head-group composition in LM cell membranes has been studied using the fluorescent probes, β-parinaric acid and 8-anilino-1-naphthalenesulfonic acid (Schroeder et al., 1976b). The binding properties of the probes were altered, but the "characteristic temperatures" for mitochondrial, microsomal, and plasma membranes were all the same and it was concluded that the cells maintained constant physical properties of the membranes despite the differences in the lipid composition. The effects of alterations in the fatty acid composition have been studied in many different types of cells. For example, electron spin resonance studies have shown differences in the plasma membrane properties of Ehrlich ascites tumor cells grown in tumor-bearing mice fed different diets (King et al., 1977). The fluorescence intensity of parinaric acid has also been used to show a change in the thermal behavior of phospholipids isolated from CHO cells when they are enriched in palmitic acid (Rintoul & Simoni, 1977).

The fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] has been extensively used to study the properties of liposomes and biological membranes (Shinitsky & Barenholz, 1974; Shinitzky & Inbar, 1974; Shattil & Cooper, 1976; Andrich & Vanderkooi, 1976; Suurkuusk et al., 1976; Stubbs et al., 1976). It has excellent spectral properties (Shinitsky & Barenholz, 1974) and partitions equally between gel and liquid-crystalline phases (Lentz et al., 1976). In a previous study, differences in the plasma membrane of LM cells with altered phospholipid compositions were observed using DPH (Esko et al., 1977). It was also found that tissue culture cells contained appreciable quantities of nonmembranous triacylglycerols and alkyldiacylglycerols that had very low polarization values (Esko et al., 1977; Pessin et al., 1978). Since DPH partitioned rapidly into these lipids, they significantly interfered with the measurements. Since this is likely to be a general problem whenever a hydrophobic probe is used in intact cells or impure membrane preparations, it needs to be evaluated in studies of this type.

In this study the rotational relaxation time of DPH was determined in purified mitochondrial, microsomal, and plasma membranes from LM cells with different phospholipid compositions. The results showed differences in the rotational relaxation time of DPH between the various membrane fractions. These results were consistent with the differences in the lipid composition.

Materials and Methods

Cell Culture and Lipid Modification. Mouse LM cells were grown in suspension culture in Higuchi's medium with different

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received August 29, 1978; revised manuscript received December 13, 1978.* This work was supported by National Institutes of Health Grant GM 21953. R.G. was a predoctoral trainee supported by National Institutes of Health Grant GM 07283. M.G. was supported by National Institutes of Health Research Career Development Award GM 00193.

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; CL, cardiolipin; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/79/0418-1042$01.00/0

supplements as previously described (Glaser et al., 1974; Esko et al., 1977). In order to alter the phospholipid polar head-group composition, cells were grown in medium supplemented with ethanolamine in place of choline at a concentration of 40 μg/mL and harvested after 84 h. In order to modify the fatty acid composition, the medium (containing choline) was supplemented with 40 μg/mL of linoleate (complexed to bovine serum albumin) 18 h prior to harvest. Cell phospholipids were uniformly labeled with [^{32}P]phosphate by the addition of 0.5 μCi/mL [^{32}P]phosphate (carrier free) several generations prior to the experiment and maintained in medium containing the same specific activity throughout the experiment.

Membrane Preparation. Isolation of mitochondrial, microsomal (endoplasmic reticulum), and plasma membrane fractions from 500 mL of cells at a density of 3–4 × 10^6 cells/mL was accomplished by a modification of the method of Esko et al. (1977). After hypotonic lysis the cells were sheared in 10 mL of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.2, and then diluted into 160 mL of the same buffer. After removal of nuclei by centrifugation at 3000*g* for 10 min, the supernatant fraction was centrifuged for 15 min at 18000*g* to obtain a pellet enriched primarily in mitochondrial and plasma membranes. The supernatant from this fraction was centrifuged at 100000*g* for 60 min to obtain a pellet enriched in microsomal membranes. The 18000*g* pellet was resuspended in 40 mL of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.2, and centrifuged in one tube at 18000*g* for 30 min to concentrate the membranes prior to resuspension in 3 mL of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.2. This material was layered on a discontinuous sucrose gradient containing 0.5 mL of 55% (w/w) sucrose, then 2.0 mL each of 40, 35, 32, and 29% sucrose, and finally 1.7 mL of 20% sucrose. The 100000*g* pellet was resuspended in 2 mL of 1 mM Tris-HCl, pH 7.2, before layering on a discontinuous sucrose gradient consisting of 6.0 mL of 55% sucrose, 2.0 mL of 20% sucrose, and 2.0 mL of 10% sucrose. Both gradients were centrifuged at 41 000 rpm in a SW-41 rotor for 150 min. The bands forming at the interfaces of the sucrose solutions were collected and diluted to 9 mL with 1 mM Tris-HCl, pH 7.2, and centrifuged at 100000*g* for 60 min. The bands at the 29–32% and 35–40% interfaces in the first gradient corresponded to the plasma membrane and mitochondrial membranes, respectively. The band at the 20–50% interface on the second gradient corresponded to the microsomal fraction.

Enzyme Assays. Oubain-sensitive (Na^+,K^+)ATPase was assayed as described by Esko et al. (1977) with minor modifications. The concentration of oubain was increased to 10 mM in the assay solution. Samples were alowed to incubate at 4 °C for 60 min prior to the addition of ATP (Boehringer-Mannheim). NADPH-dependent cytochrome *c* reductase and succinate-dependent cytochrome *c* reductase activities were assayed spectrophotometrically by the method of Sottocasa et al. (1967).

Lipid Extractions and Analysis. Lipids were extracted from whole cells or membranes by the method of Bligh & Dyer (1959) as described by Ames (1968). Membranes and whole cells were diluted with PBS (137 mM NaCl, 2.7 mM KCl, 12.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2) prior to extraction to prevent the formation of emulsions. Phospholipids were separated from neutral lipids by silicic acid chromatography on 5-cm Unisil columns (Clarkson Chemical Co.) (Ferguson et al., 1975). The phospholipid compositions of whole cells and membranes were determined by two-dimensional thin-layer chromatography on silica gel G (250 μm, Analtech, Inc.) as previously described (Esko et al., 1977). Phosphatidylserine and phosphatidylinositol were resolved by one-dimensional thin-layer chromatography on Redi-coat-2D (250 μm, Supelco, Inc.) using $CHCl_3$:CH_3OH:NH_4OH (65:25:5) as the solvent system. Phospholipid phosphate was determined after extraction of membranes by the ashing procedure of Ames (1966). Desmosterol concentration was determined by gas–liquid chromatography on a 1.5-m column of 3% SE-30 on 80–100 Gas Chrom Q (Supelco, Inc.) using 5α-cholestane as an internal standard. The amount of triacylglycerols and alkyldiacylglycerols in purified membrane fractions was measured by the procedure of Snyder & Stephens (1959) after separation from phospholipids by Unisil columns.

Fatty acid methyl esters were prepared by the NaOH:CH_3OH method as described previously (Ferguson et al., 1975). The methyl esters were separated on a 1.5-m column of 10% SP-2340 on 100–120 Chromasorb W AW (Supelco, Inc.). A temperature program was used with 10 min of chromatography at 160 °C, followed by a 2 °C/min gradient until a temperature of 190 °C was reached, with a carrier gas flow rate of 25 mL/min. All gas–liquid chromatography was conducted on a Varian 3700 gas chromatograph with a CDS-111 data analysis system and flame ionization detector.

Preparation of DPH-Labeled Samples. The basic procedure used for labeling samples has been descril ed previously (Esko et al., 1977; Pessin et al., 1978). Membrane samples were labeled by the addition of 1.0–1.5 mg of membrane protein to 40–60 mL of 2 μM DPH in PBS. After 40 min at 37 °C, this solution was centrifuged at 100000*g* for 1 h and the pellet was diluted to 1.0 mL with PBS. Whole lipids and phospholipids were labeled for fluorescence measurements by the addition of DPH in chloroform to 0.3–0.5 μmol of lipid phosphate in $CHCl_3$ at a ratio of 1000:1 (phospholipid:DPH). After drying this solution under a stream of nitrogen, 0.5 mL of PBS was added and the lipids were allowed to swell in an argon atmosphere at 37 °C. The suspension was sonicated in a Branson bath sonicator for 30 s to disperse the lipid. Desmosterol–phospholipid dispersions were labeled by the same method after the addition of desmosterol (Applied Science Laboratories) in chloroform to phospholipids isolated from LM cell membranes. Aliquots were removed from these stock solutions of labeled membranes and lipid dispersions for fluorescence polarization and lifetime measurements. Sepharose 4B chromatography of the lipid dispersions showed that all the lipid was excluded indicating that single-lamellar vesicles were not present.

Fluorescence Measurements and Instrumentation. Fluorescence polarization measurements were performed on a T-format instrument as described previously (Weber & Bablouzian, 1966; Esko et al., 1977). Scattered light was reduced to less than 2% by the use of a 2 M $NaNO_2$ solution and Corning 3-72 cutoff filters. Fluorescence lifetimes were measured on a cross-correlation phase fluorometer as described previously (Spencer & Weber, 1969) with updated electronics by SLM Instruments, Inc. Lifetime measurements were made with the excitation light modulated at 18 MHz. The average of the phase and modulation measurements were used for all calculations. Rotational relaxation times were calculated as described previously for DPH (Pessin et al., 1978) using the Perrin equation (Weber, 1953)

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{\rho}}\right)$$

where p is the polarization, τ is the lifetime of the excited state,

Table I: Specific Activities and Recoveries of Marker Enzyme Activities in Membrane Fractions Isolated from Choline-Supplemented Cells[a]

fraction	protein[b]	(Na^+,K^+)ATPase	succinate cyto *c* reductase	NADPH cyto *c* reductase
particulate lysate	108.4 (100%)	25.8 (100%)	51.7 (100%)	15.2 (100%)
plasma membrane	1.61 (1.5%)	298.0 (17.4%)	28.1 (0.8%)	31.1 (3.1%)
mitochondrial membrane	10.0 (9.2%)	13.3 (4.8%)	323.8 (57.5%)	13.3 (8.0%)
microsomal membrane	7.6 (7.0%)	25.7 (7.0%)	29.8 (4.1%)	38.5 (17.8%)

[a] Cells grown for 84 h with choline were fractionated as described in Materials and Methods. Enzyme activities are expressed as nmol min^{-1} (mg of protein)$^{-1}$. Assays were performed on the particulate fraction after centrifugation for 1 h at 100000*g*. The total activities in the particulate lysate of the (Na^+,K^+)ATPase, succinate-dependent cytochrome *c* reductase, and the NADPH-dependent cytochrome *c* reductase were 2.79, 5.61, and 1.65 μmol min^{-1}, respectively. Numbers in parentheses represent the percent recovery of the particulate lysate. [b] Milligrams of particulate protein.

Table II: Phospholipid Composition of Membranes Isolated from Cells Supplemented with Choline, Ethanolamine, and Linoleate[a]

supplement	membrane fraction	phospholipid composition (% phosphate) PC	PE	PI	PS	Sph	CL	other[b]	μmol of phospholipid/ mg of protein	mol of sterol/ mol of phospholipid
choline	PM	40.7	29.0	6.2	9.2	11.4	1.2	2.3	0.87	0.31
	MicM	51.7	22.6	6.2	6.2	8.0	1.1	4.2	0.77	0.22
	MitM	52.9	26.2	5.5	0.9	0.5	12.2	1.8	0.55	0.024
ethanolamine	PM	15.2	40.7	7.0	10.6	20.6	0.7	5.2	0.88	0.31
	MicM	18.6	40.5	7.0	8.0	13.4	0.6	11.9	0.82	0.29
	MitM	28.1	45.2	7.9	2.2	3.0	11.4	2.2	0.55	0.054
linoleate	PM	33.6	29.6	6.0	11.5	13.4	1.9	4.0	0.88	0.32
	MicM	49.3	24.2	6.6	6.5	9.2	1.2	3.0	0.75	0.21
	MitM	52.3	26.9	6.2	1.4	1.3	9.8	2.1	0.51	0.033

[a] Membranes were isolated from cells grown with [^{32}P] phosphate and supplemented with choline, ethanolamine, and/or linoleate as described in Materials and Methods. PM, plasma membrane; MicM, microsomal membrane; MitM, mitochondrial membrane for this and following tables. [b] This category consists of minor phospholipids including phosphatidylglycerol, lysophosphatidylcholine, lysophosphatidylethanolamine, and lysobisphosphatidic acid.

and $\bar{\rho}$ is the rotational relaxation time.[2] $P_0 = 0.460$ for DPH (Shinitzky & Inbar, 1974) and it is the polarization in the absence of rotational motion. This equation applies to nonspherical fluorophores when the absorption and emission oscillators are parallel (Shinitzky et al., 1971). This is true for DPH when it is excited in the last absorption band. In this case the rates of rotation in the plane and out of the plane of the molecule contribute equally to the observed depolarization. $\bar{\rho}$ represents the harmonic mean of the rotational relaxation times for the in-plane and out-of-plane motions. If the viscosity of the surrounding medium is increased, then the relaxation time will increase.

Results

Isolation of Membranes. The membrane preparation used in these experiments yielded three membrane fractions which were enriched in marker enzyme activities for mitochondrial, microsomal, and plasma membranes. Data from a representative preparation are shown in Table I. Plasma membranes that were collected from the interface between the 29 and 32% sucrose solutions were enriched 12-fold in (Na^+,K^+)ATPase as compared with the particulate lysate fraction with an overall yield of 17% of the total activity. Mitochondrial membranes were obtained from the interface between the 35 and 40% solutions of the sucrose gradient. This membrane fraction was six- to eightfold enriched in succinate-dependent cytochrome *c* reductase activity over the particulate lysate with an overall yield of between 50% and 60%. Microsomal membranes were obtained from the interface between the 20% and 50% sucrose layers in the microsomal gradient. Purification of this membrane fraction varied between 2.5- and 4.5-fold with approximately a 20% yield of the total NADPH-dependent cytochrome *c* reductase activity. This purification method was relatively quick and it provided a good reproducible purification compared with other methods. The purification of the three membrane fractions from ethanolamine-supplemented or linoleate-supplemented cells was not significantly different from the purification from choline-supplemented cells as judged by membrane marker enzymes. In addition, all membrane fractions contained less than 2 mol % triacylglycerols and alkyldiacylglycerols which would not significantly interfere in the fluorescence measurements.

Lipid Compositions of the Membrane Fractions. Significant differences were found in the lipid compositions of the mitochondrial, microsomal, and plasma membrane fractions. Cardiolipin was present primarily in the mitochondrial membranes, while phosphatidylserine, sphingomyelin, and desmosterol were practically absent from this fraction (Table II). Mitochondrial membranes also had the lowest ratio of phospholipid to protein. Plasma membranes contained the highest amount of sphingomyelin, phosphatidylserine, phosphatidylethanolamine, and desmosterol, and the lowest amount of phosphatidylcholine. Microsomal membranes resembled plasma membranes except for a higher amount of phosphatidylcholine and a reduced level of the other lipids. The ratio of phospholipid to protein for microsomal membranes was intermediate between that for the mitochondrial and plasma membranes. The phospholipid analysis was quite reproducible and gave a standard deviation of less than 2% total phosphate for all phospholipid classes from four separate preparations of choline-supplemented cells. A 2% standard deviation was also true for the fatty acid analysis.

Supplementation with ethanolamine resulted in an increase in phosphatidylethanolamine and a decrease in phosphati-

[2] The rotational relaxation time is the time it takes for molecules to rotate through an angle θ, such that $\overline{\cos\theta} = e^{-1}$.

Table III: Fatty Acid Composition of Membrane Phospholipids from Cells Supplemented with Choline, Ethanolamine, and Linoleate[a]

supplement	membrane fraction	fatty acid composition (wt %) 14:0	16:0	16:1	18:0	18:1	18:2	odd chain[b]	>C-18[c]	% saturated fatty acids
choline	PM	1.6	10.1	2.4	15.2	55.3		8.4	10.8	32.9
	MicM	1.5	10.5	3.1	10.9	59.6		8.8	9.2	28.3
	MitM	1.2	10.5	3.4	11.0	62.2		9.2	5.3	27.7
ethanolamine	PM	1.9	9.1	4.0	11.1	56.8		8.9	14.0	27.3
	MicM	1.6	6.2	5.0	8.8	61.2		10.5	11.6	22.8
	MitM	1.9	8.4	5.9	9.2	60.7		9.9	7.1	24.5
linoleate	PM	1.0	8.9	2.1	14.2	30.9	26.6	6.2	13.3	28.2
	MicM	0.8	9.0	1.7	12.5	31.2	31.2	4.1	10.9	27.2
	MitM	0.7	8.4	2.4	11.6	32.2	32.7	4.3	8.6	25.7

[a] Membranes were isolated from cells supplemented with choline, ethanolamine, and/or linoleate as described in Materials and Methods. [b] Includes odd chain fatty acids up to C-23. No fatty acids >C-24 were found. No polyunsaturated fatty acids were found except on supplementation with linoleate. [c] Includes fatty acids from C-19 to C-24. The increase in long chain fatty acids on linoleate supplementation was due primarily to elongation of linoleate to 20:2.

dylcholine in all three membrane fractions. There was also an increase in the amount of sphingomyelin in plasma and microsomal membranes and an increase in the desmosterol content in microsomal membranes. The increase in "other" phospholipids (Table II) which occurred primarily in microsomal membranes was due to an increase in lysobisphosphatidic acid, which is a positional isomer of phosphatidylglycerol rather than a phospholipid hydrolysis product (Gilmore et al., 1979).

Supplementation with linoleate caused a selective decrease in the amount of phosphatidylcholine in plasma membranes while there were only small changes in the concentrations of the other lipid species. Lysophosphatidylcholine and lysophosphatidylethanolamine did not exceed 1% in any supplement or membrane type.

Changes in the fatty acid composition between the different cell membranes were less pronounced than the changes in the phospholipid polar head-group composition or desmosterol content. Plasma membranes contained more 18:0 and long-chain fatty acids (>18 carbons) and less 18:1 than microsomal or mitochondrial membranes (Table III). Linoleate supplementation resulted in incorporation of approximately the same amount of linoleate in both microsomal and mitochondrial membranes (31.2 and 32.7%, respectively). Incorporation of linoleate into plasma membranes was slightly less (26.6%). The incorporation of linoleate did not result in a decrease in saturated fatty acids because linoleate incorporation was paralleled by a reduction in the oleate concentration. This resulted in an increase in the number of double bonds per fatty acid. Mitochondrial membranes from choline-supplemented cells, for example, contained 71.8 double bonds per 100 fatty acid chains, while mitochondrial membranes from linoleate-supplemented cells contained 106.6 double bonds per 100 fatty acid chains.

Fluorescence Lifetimes of DPH. In order to calculate the rate of rotation of a fluorophore, it is necessary to measure both the fluorescence polarization and lifetime. As noted previously, lifetimes deduced from measurements of the fluorescence intensity did not always give reliable values (Esko et al., 1977). Consequently, lifetimes were measured directly on a cross-correlation phase fluorometer.

The fluorescence lifetime of DPH is changed not only by temperature, but also by the structure and composition of the lipid bilayer. Some representative lifetime values are given in Tables IV and V. The fluorescence lifetime of DPH in a given sample showed less than 0.5 ns variation between different preparations. Increasing the desmosterol content causes an increase in the fluorescence lifetime (Table IV). The

Table IV: Effect of Desmosterol Content on the Fluorescence Lifetimes of DPH in Phospholipid-Desmosterol Vesicles[a]

mol of desmosterol/mol of phospholipid	fluorescence lifetime (ns) 8 °C	26 °C	40 °C
0	9.4	8.9	8.4
0.11	9.6	9.3	8.6
0.21	9.8	9.5	9.0
0.25	9.9	9.6	9.2
0.32	10.1	9.7	9.4
0.42	10.2	10.0	9.7

[a] Fluorescence lifetimes of mixtures of desmosterol with microsomal phospholipids isolated from choline-supplemented cells. Measurements were performed as described under Materials and Methods.

lifetime in intact membranes showed less variation with temperature than the lifetime in whole lipid extracts (Table V). When phospholipids were separated from desmosterol on a Unisil column, the lifetimes became shorter as expected from Table IV. Mitochondrial membranes and mitochondrial whole lipid extracts, which have little desmosterol, also had shorter DPH lifetimes than the corresponding fractions from plasma and microsomal membranes. Supplementation with ethanolamine resulted in slightly longer lifetimes, while supplementation with linoleate resulted in slightly shorter lifetimes for membranes and lipid dispersions.

Rotational Relaxation Times of DPH in Different Membranes. The fluorescence polarization and fluorescence lifetime of DPH were measured as a function of temperature between 5 and 40 °C in different membranes. Rotational relaxation times were calculated from these data and the results are shown in Figure 1 for cells supplemented with choline, ethanolamine, and linoleate. There were no consistent breaks or discontinuities in these plots which could be interpreted as a sharp phase transition or "characteristic temperature". At all temperatures the rotational relaxation time of DPH increased in going from mitochondrial to microsomal to plasma membranes. Supplementation of the cells with ethanolamine resulted in an increase in the rotational relaxation times of DPH in all three membrane fractions, while supplementation with linoleate caused a decrease in all three membrane fractions. For both supplements, the ratio of the rotational relaxation time for plasma membranes to mitochondrial membranes or for microsomal membranes to mitochondrial membranes remained approximately the same.

Table V: Fluorescence Lifetimes of DPH in Membranes, Whole Lipid Dispersions, and Phospholipid Dispersions[a]

membrane fraction & supplement	fluorescence lifetimes (ns)								
	membranes			whole lipids			phospholipids		
	8 °C	25 °C	40 °C	8 °C	25 °C	40 °C	8 °C	25 °C	40 °C
PM									
choline	10.4	10.2	9.5	10.6	10.0	9.5	9.3	8.8	8.0
ethanolamine	10.5	10.2	9.7	*b*	*b*	*b*	9.4	9.0	8.3
linoleate	10.3	10.0	9.5	10.6	10.0	9.4	9.3	8.8	8.0
MicM									
choline	9.8	9.6	9.0	10.2	10.0	9.2	9.4	8.9	8.4
ethanolamine	10.2	10.0	9.5	10.9	10.6	10.0	9.8	9.4	8.7
linoleate	9.7	9.3	8.9	10.1	9.7	9.0	9.1	8.8	8.0
MitM									
choline	8.5	8.0	7.4	8.8	8.5	7.8	9.0	8.4	7.8
ethanolamine	8.8	8.2	7.8	9.4	8.9	8.4	9.3	8.8	8.2
linoleate	8.2	7.9	7.3	8.7	8.3	7.6	8.9	8.6	8.0

[a] The fluorescence lifetime measurements were performed on membranes and on whole lipid and phospholipid dispersions prepared from the membranes, as described in Materials and Methods. [b] Values for these points were not determined in this experiment.

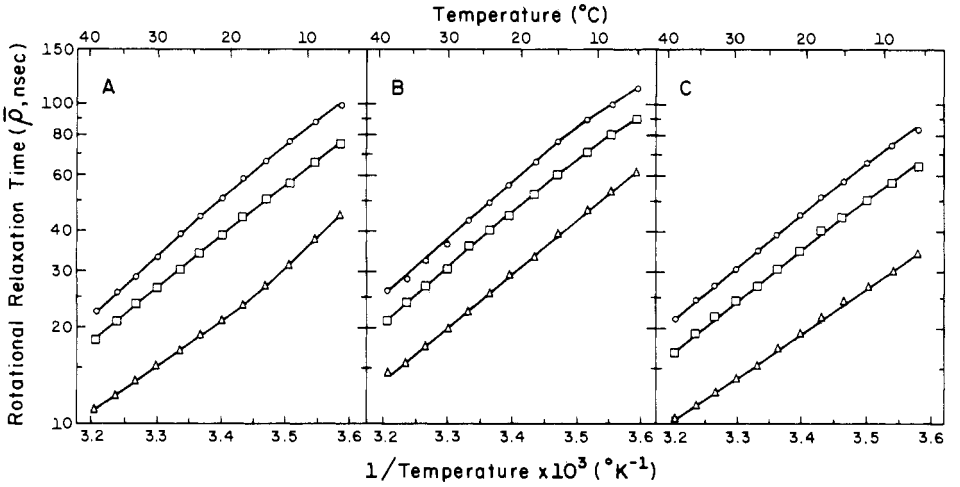

FIGURE 1: The temperature dependence of the rotational relaxation time of DPH in (O—O) plasma membranes, (□—□) microsomes, and (Δ—Δ) mitochondria from (A) choline-supplemented cells, (B) ethanolamine-supplemented cells, and (C) linoleate-supplemented cells. The procedures for membrane isolation and fluorescence measurements are described in Materials and Methods.

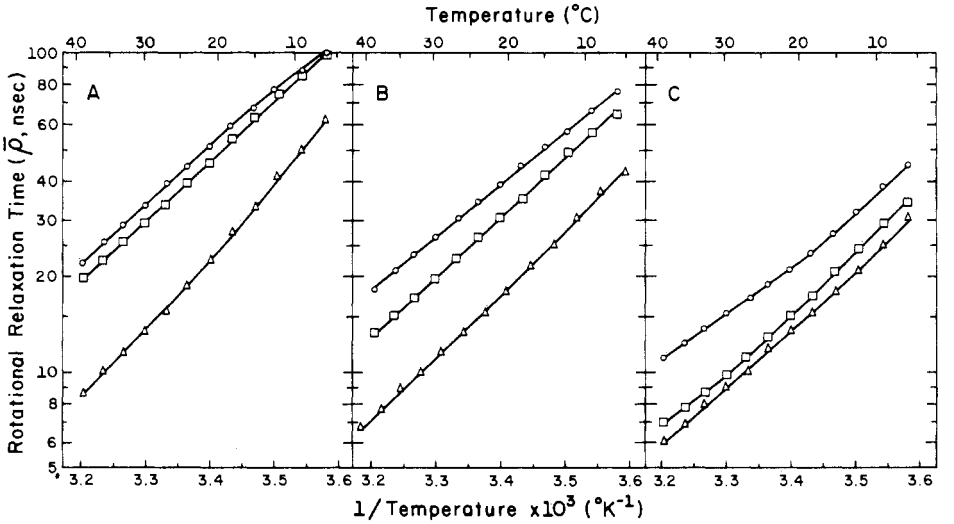

FIGURE 2: The temperature dependence of the rotational relaxation time of DPH in (O—O) membranes, (□—□) whole lipid dispersions, and (Δ—Δ) phospholipid dispersions. (A) Plasma membranes, (B) microsomal membranes, and (C) mitochondrial membranes isolated from choline-supplemented cells. The procedures for lipid dispersion and fluorescence measurements are described in Materials and Methods.

The extent of incorporation of ethanolamine or of the fatty acid greatly affected the extent of the change in the rotational relaxation time for the different membranes (data not shown). In choline-supplemented cells, where the lipid composition did not change significantly, the standard deviation of the measurements was less than 10% (four preparations). For a repetition of a measurement on the same sample, the agreement was within 3%.

Rotational Relaxation Times of DPH in Membranes, Whole Lipid Extracts, and Phospholipids. In order to ex-

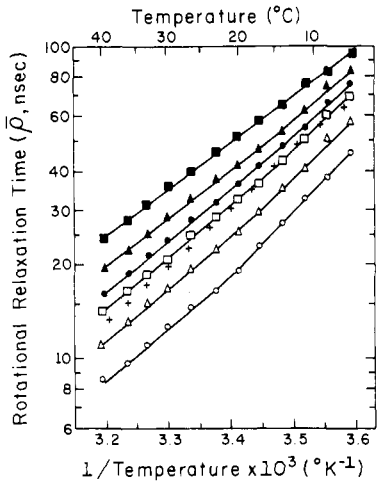

FIGURE 3: The temperature dependence of the rotational relaxation time of DPH in mixed phospholipid–desmosterol dispersions. Mole ratio of desmosterol to phospholipid derived from microsomal membranes of choline-supplemented cells: (O—O) 0; (Δ—Δ) 0.1; (□—□) 0.21; (●—●) 0.25; (▲—▲) 0.32; (■—■) 0.42. Whole lipid extracts from microsomal membranes of choline-supplemented cells (+—+). Lipid dispersions were prepared as described in Materials and Methods.

amine the contribution of different lipid classes to the rotation rates of DPH, whole lipid and phospholipid dispersions were prepared from mitochondrial, microsomal, and plasma membranes. Figure 2 shows the rotational relaxation times of DPH in these fractions derived from cells supplemented with choline. Whole lipid dispersions had lower rotational relaxation times than the intact membrane. The largest difference was in mitochondria while the smallest difference was in the plasma membrane. Although a number of things could contribute to this difference (e.g., loss of phospholipid asymmetry on extraction, nature of the lipid dispersion, etc.), one cause may be the influence of the membrane proteins. The mitochondrial membranes had the highest protein to lipid ratio, while plasma membranes had the lowest protein to lipid ratio.

When phospholipids were isolated from the whole lipid extracts by removing neutral lipids on a Unisil column, there was a large decrease in the rotational relaxation times of DPH in the fractions from microsomal and plasma membranes. The decrease was much smaller in the fractions derived from mitochondrial membranes, since this membrane fraction contained little desmosterol (Table II).

Rotational Relaxation Times of DPH in Phospholipids with Varying Levels of Sterol. The effect of the desmosterol content is illustrated in Figure 3. Increasing the desmosterol content in phospholipids derived from microsomal membranes caused a continuous increase in the rotational relaxation time of DPH. This is similar to the results obtained with cholesterol (Shinitzky & Inbar, 1976).

When the amount of desmosterol eqivalent to the amount found in microsomal lipids was added to the phospholipids, the rotational relaxation times of DPH were similar to the values found in whole lipid extracts. Consequently, the differences between the whole lipid extract and the phospholipids can be entirely ascribed to the desmosterol content and there was negligible contamination with other neutral lipids such as triacylglycerols or alkyldiacylglycerols.

Rotational Relaxation Times of DPH in Phospholipids Derived from Different Membranes. The rotational relaxation times of DPH in phospholipids isolated from mitochondrial, microsomal, and plasma membranes are shown in Figure 4. The same pattern observed for the intact membranes (Figure 1) or for the whole lipid extracts (Figure 2) was observed for the isolated phospholipids. That is, the rotational relaxation time of DPH increased in the order mitochondrial membranes to microsomal membranes to plasma membranes. This was true for the phospholipids derived from cells supplemented with choline, ethanolamine, or linoleate.

Activation Energies for the Different Membrane Fractions. The activation energies were calculated from the temperature dependence of the rotational relaxation times of DPH (Table VI). Small differences in the activation energies were observed for the different membranes and whole lipid extracts, but in general they were quite similar. Microsomal and plasma membrane phospholipids had a higher activation energy than their respective membranes or whole lipid extracts which correlates with the removal of desmosterol. When the ratio of desmosterol to phospholipid was increased (0, 0.1, 0.21, 0.25, 0.32, 0.42), the activation energy decreased (8.4, 8.1, 7.9, 7.7, 7.3, to 6.8 kcal/mol, respectively) (Figure 3). Supplementation with linoleate gave a decreased activation energy in most cases.

Discussion

Membrane fluidity depends on a number of factors including the lipid composition and temperature. Eucaryotic cells have

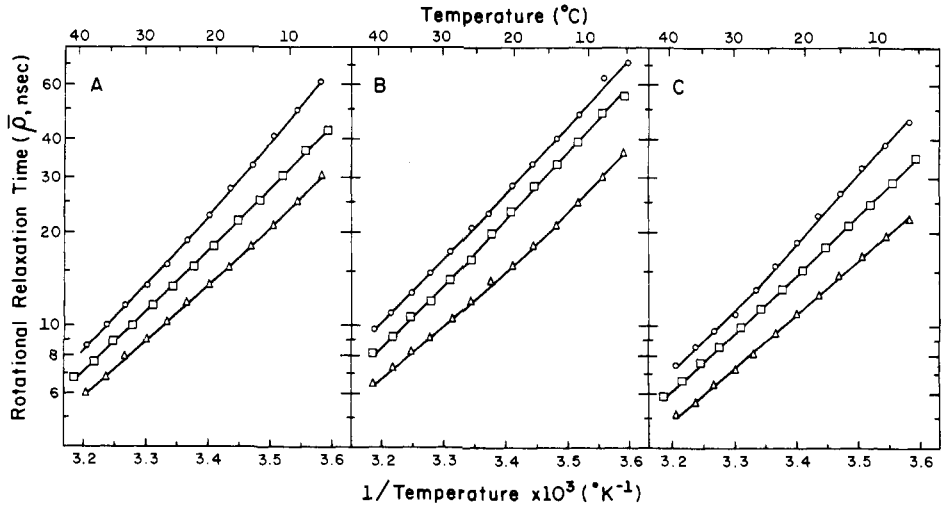

FIGURE 4: The temperature dependence of the rotational relaxation time of DPH in phospholipid vesicles prepared from (O—O) plasma membranes, (□—□) microsomes, and (Δ—Δ) mitochondria isolated from (A) choline-supplemented cells, (B) ethanolamine-supplemented cells, and (C) linoleate-supplemented cells. Phospholipid isolation, vesicle preparation, and fluorescence measurements were performed as described in Materials and Methods.

Table VI: Activation Energies for the Rotational Relaxation Times of DPH in Membranes, Whole Lipid Extracts, and Phospholipids[a]

membrane fraction & supplement	activation energy (kcal/mol) membranes	whole lipid extracts	phospholipids
PM			
choline	8.1	8.7	10.1
ethanolamine	8.0		10.0
linoleate	7.4	8.6	10.0
MicM			
choline	7.5	8.5	9.0
ethanolamine	7.8	8.2	9.8
linoleate	7.0	7.9	8.7
MitM			
choline	6.5-8.6	8.7	8.4
ethanolamine	7.8	8.0	8.1
linoleate	6.3	7.8	8.0

[a] Activation energies were calculated from the Arrhenius plots in Figures 1, 2, and 4 assuming the best straight line fit. The standard deviation was less than 0.3 kcal/mol for choline-supplemented cell membranes (four preparations).

several different types of intracellular membranes which carry out specialized functions, and each type of membrane has its own complex lipid composition. Changes in the sterol content, the phospholipid polar head-group composition, and the fatty acid composition provide three potential levels of control over membrane fluidity. The main objective of this study was to examine the fluidity of the mitochondrial, microsomal, and plasma membranes of LM cells and to determine the contribution of the individual lipid species to the overall membrane fluidity.

Fluorescence spectroscopy offers a very sensitive and direct method for studying the motion of molecules in membranes (Shinitzky et al., 1971; Cogan et al., 1973). The Perrin equation can be used to calculate the microviscosity of the fluorophore's environment from the measured values of the fluorescence polarization and lifetime. Recently, evidence has been presented that the motion of DPH in a phospholipid vesicle, especially below the phase transition temperature, may not be the same as the motion in an isotropic reference oil and the calculation of the microviscosity using a reference oil may not be valid (Andrich & Vanderkooi, 1976; Dale et al., 1977; Kawato et al., 1977; Hare & Lussan, 1977; Lakowicz & Prendergast, 1978). The calculation of a purely phenomenological parameter, the rotational relaxation time, appears to be the most appropriate way to characterize the motions of DPH in membranes and to compare the motions of DPH in similar membrane preparations. The rotational relaxation time is a very sensitive indicator of phase transitions and provides a relative measure of the rate of molecular motion.

The results presented in this paper or in earlier studies with DPH in LM cell membranes (Barenholz et al., 1976; Esko et al., 1977) did not give any indication of a sharp phase transition or a discrete region of a lateral phase separation in the temperature region that was studied (5–40 °C). The motions of DPH in membranes were intermediate between the motions in the gel phase and the liquid-crystalline phase as shown by a pure phospholipid (Esko et al., 1977). The most likely interpretation of the data is that there is a continuous change in the motion of DPH and the fatty acid chains in this temperature range. This is contrary to the conclusion reached by electron spin resonance studies (Wisnieski et al., 1974) and the fluorescence studies using parinaric acid and 8-anilino-1-naphthalenesulfonic acid (Schroeder et al., 1976a) in which "characteristic temperatures" were observed. It does not seem likely that a sharp phase transition will occur in membranes that are highly unsaturated, very heterogeneous, and have a high content of sterol. Further analysis of individual phospholipid classes shows that their phase transition temperatures are practically all below 0 °C (except for sphingomyelin, a minor membrane component) (Gilmore et al., 1979).

The desmosterol content and the phospholipid polar head-group composition showed considerable variation between the different membrane fractions. Differences in composition between the membrane fractions have been described in a number of cells (Rouser et al., 1968) including LM cells (Schroeder et al., 1976c; Schroeder & Vagelos, 1976). The compositions reported here differ in several respects from that reported by Schroeder et al. (1976c), but this may be due to differences in the cells, growth conditions, and the method of membrane preparation.

The effect of sterol content on the rotational relaxation time of DPH is shown by the difference between the whole lipid and the phospholipid dispersions. In mitochondrial membranes, which had little sterol, there was only a small difference between whole lipids and phospholipids, while in plasma membranes, which had the most sterol, the difference was large. The magnitude of the difference depended on the temperature, and for plasma membrane lipids the presence of desmosterol increased the rotational relaxation times 1.6- to 2.3-fold over the temperature range studied for choline-supplemented cells.

Differences between the whole lipid extracts from the various membranes reflect both the desmosterol content and the phospholipid composition. The differences in the rotational relaxation times between mitochondrial whole lipid extracts and plasma membrane whole lipid extracts were 2.8- to 2.9-fold in choline-supplemented cells, for example. The contribution of the phospholipid composition can be seen by comparing the isolated phospholipids. The differences in the phospholipids from mitochondrial and plasma membranes were 1.5- to 2.0-fold in choline-supplemented cells. Thus, the differences in the phospholipid composition were responsible for a substantial part of the change in the rotational relaxation times as compared with the changes produced by desmosterol.

The change in the rotational relaxation time produced by adding a given amount of desmosterol depended on the initial rotational relaxation time. Consequently, the effect varied for each phospholipid composition and temperature.

The changes observed in the rotational relaxation times of DPH in isolated phospholipids could be due to changes in either the polar head group or fatty acid composition. Supplementation with a fatty acid or a polar head-group analogue resulted in major modifications of one part of the molecule with minimal modification of the other. This allowed the effects of each part of the molecule to be investigated independently. Supplementation with ethanolamine produced an increase in the rotational relaxation time in all three membrane fractions. The increases were also present in the phospholipids isolated from the membranes indicating that changes in the polar head-group composition were responsible for the changes in the rotational relaxation time. Supplementation with ethanolamine primarily caused an increase in phosphatidylethanolamine and a decrease in phosphatidylcholine in all the membrane fractions. There was also an increase in the amount of sphingomyelin and phosphatidylserine in microsomal and plasma membranes.

Supplementation of cells with linoleate resulted in an altered fatty acid composition and a decrease in the rotational re-

laxation time of DPH in all membrane fractions. The ratio of the rotational relaxation times between one membrane fraction and another, or one phospholipid extract to another, remained approximately constant on supplementation with linoleate. This was also true for supplementation with ethanolamine and suggests that the cell regulates the lipid composition in the different membranes to maintain these differences.

The effects of supplementation with ethanolamine and linoleate showed that both the phospholipid polar head group and fatty acid composition were responsible for the differences in the rotational relaxation times of DPH between the different membrane fractions. Due to the complexity of the lipid composition it was difficult, however, to discern the contributions from each component. The accompanying paper (Gilmore et al., 1979) deals with the purification of individual phospholipid classes from the different membrane fractions of LM cells and their physical properties.

Acknowledgments

We thank Dr. Gregorio Weber and his research group for advice and for the use of their fluorescence instrumentation (supported through National Institutes of Health Grant GM 11223). In particular, we thank Dr. Fenton Williams for his advice and help with the fluorescence lifetime measurements.

References

Ames, B. N. (1966) *Methods Enzymol. 8*, 115.
Ames, G. F. (1968) *J. Bacteriol. 95*, 833.
Andrich, M. P., & Vanderkooi, J. M. (1976) *Biochemistry 15*, 1257.
Barenholz, Y., Moore, N. F., & Wagner, R. R. (1976) *Biochemistry 15*, 3563.
Blank, M. L., Piantadosi, C., Ishaq, K. S., & Snyder, F. (1975) *Biochem. Biophys. Res. Commun. 62*, 983.
Blank, M. L., Lee, T.-C., Piantadosi, C., Ishaq, K. S., & Snyder, F. (1976) *Arch. Biochem. Biophys. 177*, 317.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.
Chen, H. W., Heiniger, H.-J., & Kandutsch, A. A. (1978) *J. Biol. Chem. 253*, 3180.
Cogan, U., Shinitzky, M., Weber, G., & Nishida, T. (1973) *Biochemistry 12*, 521.
Dale, R. E., Chen, L. A., & Brand, L. (1977) *J. Biol. Chem. 252*, 7500.
Doi, O., Doi, F., Schroeder, F., Alberts, A. W., & Vagelos, P. R. (1978) *Biochim. Biophys. Acta 509*, 239.
Engelhard, V. H., Esko, J. D., Storm, D. R., & Glaser, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4482.
Esko, J. D., Gilmore, J. R., & Glaser, M. (1977) *Biochemistry 16*, 1881.
Ferguson, K. A., Glaser, M., Bayer, W. H., & Vagelos, P. R. (1975) *Biochemistry 14*, 146.
Gilmore, R., Cohn, N., & Glaser, M. (1979) *Biochemistry* (following paper in this issue).
Glaser, M., Ferguson, K. A., & Vagelos, P. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4072.
Hare, F., & Lussan, C. (1977) *Biochim. Biophys. Acta 467*, 262.
Heiniger, H.-J., Kandutsch, A. A., & Chen, H. W. (1976) *Nature (London) 263*, 515.
Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1977) *Biochemistry 16*, 2319.
King, M. E., Stavens, B. W., & Spector, A. A. (1977) *Biochemistry 16*, 5280.
Lakowicz, J. R., & Prendergast, F. G. (1978) *Science 200*, 1399.
Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4529.
Pessin, J. E., Salter, D. W., & Glaser, M. (1978) *Biochemistry 17*, 1997.
Rintoul, D. A., & Simoni, R. D. (1977) *J. Biol. Chem. 252*, 7916.
Rittenhouse, H. G., Williams, R. E., & Fox, C. F. (1974) *J. Supramol. Struct. 2*, 629.
Rouser, G., Nelson, G. J., Fleischer, S., & Simon, G. (1968) in *Biological Membranes* (Chapman, D., ed.) p 5, Academic Press, New York.
Schroeder, F., & Vagelos, P. R. (1976) *Biochim. Biophys. Acta 441*, 239.
Schroeder, F., Holland, J. F., & Vagelos, P. R. (1976a) *J. Biol. Chem. 251*, 6739.
Schroeder, F., Holland, J. F., & Vagelos, P. R. (1976b) *J. Biol. Chem. 251*, 6747.
Schroeder, F., Perlmutter, J. F., Glaser, M., & Vagelos, P. R. (1976c) *J. Biol. Chem. 251*, 5015.
Shattil, S. J., & Cooper, R. A. (1976) *Biochemistry 15*, 4832.
Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652.
Shinitzky, M., & Inbar, M. (1974) *J. Mol. Biol. 85*, 603.
Shinitzky, M., & Inbar, M. (1976) *Biochim. Biophys. Acta 433*, 133.
Shinitzky, M., Dianoux, A.-C., Gitler, C., & Weber, G. (1971) *Biochemistry 10*, 2106.
Silbert, D. F. (1975) *Annu. Rev. Biochem. 44*, 315.
Singer, S. J. (1974) *Annu. Rev. Biochem. 43*, 805.
Snyder, F., & Stephens, N. (1959) *Biochim. Biophys. Acta 34*, 244.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., & Bergstrand, A. (1967) *J. Cell. Biol. 32*, 415.
Spencer, R. D., & Weber, G. (1969) *Ann. N.Y. Acad. Sci. 158*, 361.
Stubbs, G. W., Litman, B. J., & Barenholz, Y. (1976) *Biochemistry 15*, 2766.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393.
Weber, G. (1953) *Adv. Protein Chem. 8*, 415.
Weber, G., & Bablouzian, B. (1966) *J. Biol. Chem. 241*, 2558.
Williams, R. E., Wisnieski, B. J., Rittenhouse, H. G., & Fox, C. F. (1974) *Biochemistry 13*, 1969.
Wisnieski, B. J., Williams, R. E., & Fox, C. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3669.
Wisnieski, B. J., Huang, Y. O., & Fox, C. F. (1974) *J. Supramol. Struct. 2*, 593.