to localize tumor is in fact the μ-peroxo dicobalt complex, and 1 molecule binds for every 2.9 base pairs of DNA.

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Phospholipid Polar Head Group Manipulation Modulates Concanavalin A Agglutinability of LM Fibroblasts†

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ABSTRACT: The effect of phospholipid polar head group manipulation in LM fibroblasts on fluoresceinylconcanavalin A binding, concanavalin A mediated hemadsorption, and concanavalin A mediated agglutination was determined. Choline analogues (*N,N*-dimethylethanolamine, *N*-methylethanolamine, and ethanolamine) were rapidly incorporated into LM plasma membrane phospholipids (35.1, 33.2, and 41.0 mol %, respectively). There were no changes in phospholipid fatty acid composition, sialic acid content, or membrane biophysical properties (characteristic break temperatures in Arrhenius plots and rotational correlation time of 1,6-diphenyl-1,3,5-hexatriene). The binding affinity of one of the two concanavalin A binding sites was doubled by all analogues tested. In addition, the concanavalin A mediated hemadsorption of sheep red blood cells and the concanavalin A mediated self-agglutinability of LM cells were doubled by choline analogue supplementation. Arrhenius plots of concanavalin A mediated agglutinability showed temperature break points for choline-fed cells at 24.3 and 33.1 °C. In contrast, *N,N*-dimethylethanolamine-, *N*-methylethanolamine-, and ethanolamine-supplemented cells had the following sets of break points: 18.3 and 27.6 °C, 10.7 and 20.1 °C, and 5.3 and 17.4 °C, respectively. The data are thus consistent with a direct correlation between lectin agglutinability characteristics and plasma membrane phospholipid polar head group composition. In addition, since the temperature break points of concanavalin A agglutinability were decreased by choline analogue supplementation while the biophysical temperature break points were not, one could hypothesize that the concanavalin A receptors may reside in specialized fluid domains within the LM plasma membrane.

Plant lectins bind to specific glycoprotein and glycolipid receptors of the cell surface. This interaction can lead to a wide variety of effects including lymphocyte stimulation (Andersson & Melchers, 1976), induction of cytotoxic effector T cells Bevan, 1976), and preferential agglutination of transformed cells (Horwitz et al., 1974; Marikovsky et al., 1974). Although the molecular basis for these effects is not well understood, several features important to lectin agglutinability of cells have emerged: plasma membrane fluidity, cell surface charge, and the cytoskeleton (McCaleb & Donner, 1981). Plasma membrane fluidity is the rate-limiting step affecting lateral diffusion of lectin receptors (Rule et al., 1979). Membrane lipids such as fatty acids, sterol/phospholipid ratio, and lysophospholipids all modulate membrane fluidity and concanavalin A agglutinability of cells (Horwitz et al., 1974; Rule et al., 1979; Rittenhouse & Fox, 1974; Hampton et al., 1980; Tombaccini et al., 1980; Ruggieri & Fallani, 1973; Bergelson & Dyatlovitskaya, 1973; Rittenhouse et al., 1974a;

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Marshall et al., 1979; Weltzien, 1975). These lipids determine the phase transition temperatures of lipids as well as characteristic break-point temperatures in lectin agglutinability of cells. However, the role of plasma membrane phospholipid polar head group composition in lectin binding and agglutinability of cells has not been reported. Phospholipid polar head groups also regulate lipid phase transitions as well as surface charge (Vaughan & Keough, 1979). Preliminary data of other investigators using artificial liposomal membranes containing glycolipids and a variety of phospholipids with different polar head groups indicated that concanavalin A agglutinates negatively charged but not neutral or positively charged liposomes (Hampton et al., 1980). Whether this correlation exists in biological membranes as well as artificial liposomes has not been tested and is the subject of this investigation. The present work demonstrates the results of alterations in polar head group composition on concanavalin A (Con A) agglutinability of LM cells at four levels: (1) plasma membrane phospholipid and structural alterations; (2) fluoresceinyl-ConA receptor binding properties; (3) heterotypic agglutination changes; and (4) homotypic agglutination alterations.

Materials and Methods

Choline and choline analogues (*N*,*N*-dimethylethanolamine, *N*-methylethanolamine, and ethanolamine) were obtained from Eastman Organic Chemicals, Rochester, NY. Concanavalin A (Con A) and fluoresceinyl-Con A (FITC-Con A) were from Miles Laboratories, Elkhart, IN. Ethanol, pentobarbital, colchicine, cytochalasin B, methyl α-D-mannoside, sucrose, D-galactose, and L-xylose were obtained from Sigma Chemical Co., St. Louis, MO. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard (Pentex; Miles Laboratories Inc., Kankakee, IL).

Cell Culture and Growth Conditions. A strain of mouse fibroblasts, LM cells, was obtained from the American Type Culture Collection (CCL 1.2). The cells were first grown in monolayer and then adapted to growth in suspension culture. Cells were grown at 37 °C and kept in suspension by being shook in 500-mL (40–300-mL culture volumes) serum bottles at 140 rpm on a New Brunswick gyrotory shaker (Model G-10, New Brunswick, NJ). Serum bottles with Teflon caps were obtained from Gibco, Grand Island, NY. All glassware used was disposable (obtained from Kimble, Toledo, OH) with the exception of the serum bottles, which were washed with Microsolv detergent (Microbiological Associates, Bethesda, MD) and reused. The lipid-free, protein-free medium of Higuchi (1970) was used throughout and modified to contain the following: 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer, pH 7.4 (Schroeder et al., 1976a); 1 g/L methylcellulose, obtained from Fisher Scientific Co., Fair Lawn, NJ (the methylcellulose was prepared as a concentrated stock solution, 2% w/w, in distilled water by stirring overnight, and undissolved material was removed by centrifugation at 12000*g* in a Sorvall RC2-B centrifuge with a GSA rotor, Sorvall Inc., Norwalk, CT); 250 units each of penicillin and streptomycin (International Scientific Co., Gary, IL); and, unless otherwise stated, 10 μg/mL sodium dextran sulfate. Methylcellulose, sodium dextran sulfate, and buffer were combined with distilled water and autoclaved for 20 min at 121 °C. All other parts of the medium were filtered aseptically as previously described (Higuchi, 1970). In all cases, control medium contained choline, and data obtained with cells cultured in the presence of choline are considered as controls.

Membrane Isolation. Plasma membranes, microsomes, and mitochondria were isolated according to the procedure of Schroeder et al. (1976a). Enzymatic plasma membrane markers were determined as follows: ouabain-sensitive (Na^+,K^+)-ATPase, 5′-nucleotidase, liberated phosphate, NADPH-dependent and succinate-dependent cytochrome *c* reductase, glucose-6-phosphatase, and inosine diphosphatase were assayed as previously described (Schroeder et al., 1976a).

Lipid Determinations. All organic solvents were glass distilled and all glassware was sulfuric acid/dichromate-washed before use. While cells or membrane fractions were suspended in 1.0 mL of the phosphate-buffered saline solution mentioned above and extracted by the method of Bligh & Dyer (1959) as described earlier (Schroeder et al., 1976a). Neutral lipids and phospholipids were separated by silicic acid column chromatography. Unisil (100–200 mesh, Clarkson Chemical Co., Williamsport, PA) was washed with methanol, activated at 100 °C overnight, and placed in a 3 cm × 0.5 cm column. Just prior to use, each column was eluted with 5 mL of chloroform, 4 mL of methanol, and finally with 5 mL of chloroform. The phospholipid composition of whole cells and membrane fractions was determined by exposing the cells to 2.0 μCi of [^{32}P]phosphate/mL of medium (New England Nuclear Corp., carrier free) as described by Schroeder et al. (1976a). The phospholipids were separated by two-dimensional thin-layer chromatography on silica gel G plates (250-μM thick, Analtech Inc., Newark, DE) that had been preactivated with acetone. The phospholipid components were visualized by autoradiography (Schroeder et al., 1976a), scraped, and counted in 5 mL of Budget Solve (Research Products International Corp., Elk Grove Village, IL) to which 0.5 mL of distilled water was added and then mixed. The relative mobility values and identity of the phospholipids were determined by chromatography in two solvent systems as described previously (Schroeder et al., 1976a): (A) chloroform/methanol/water (65:25:4); (B) 1-butanol/glacial acetic acid/water (6:2:2). The following phospholipid standards were also used for cochromatography and identification of unknown phospholipids: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylinositol, phosphatidylserine, lysophosphatidylethanolamine, cardiolipin, and phosphatidic acid from Serdary Research Labs, London, Ontario, Canada; phosphatidylglycerol and sphingomyelin from Applied Science Labs, State College, PA; lysophosphatidylcholine from Supelco, Bellefonte, PA; phosphatidyl-*N*,*N*-dimethylethanolamine and phosphatidyl-*N*-methylethanolamine from Dr. M. Glaser, Department of Biochemistry, University of Illinois, Urbana, IL 61801.

Agglutination of LM Cells by Concanavalin A. Agglutination of LM cells by Con A was conducted in 3-mL plastic disposable cuvettes according to the procedure of Murphree et al. (1976). Three-milliliter cuvettes were necessary since 1-mL cuvettes were too narrow and the walls of the cuvette inhibited precipitation of cells. For each determination, 32 × 10^6 cells were harvested and pelleted. The cells were then resuspended in 2 mL of phosphate-buffered saline, and 1.62 mL was then transferred to the 3-mL cuvette and resuspended. Then, 0.98 mL of concanavalin A (4.82 μM) was added. The final concentrations were 10 × 10^6 cells/mL and 1.82 μM concanavalin A unless otherwise stated. The cuvettes were then inverted 3 times, and the decrease in absorbance at 546 nm was monitored as a function of time.

Concanavalin A Mediated Hemadsorption to LM Cells. The concanavalin A mediated hemadsorption assay procedure was basically that of Rittenhouse et al. (1974a,b). Cells were cultured in 24-well Costar multiwell dishes for 3 days to 80–100% confluency. The cells were preincubated for 10 min at 25 °C with phosphate-buffered saline, pH 7.3, and washed

2 times with 1.0 mL of 0.9% saline, pH 7.2. Each well was then incubated with 2 mL of Con A (0–2.27 μM) in phosphate-buffered saline containing 0.1 g of $CaCl_2$ and 0.1 g of $MgCl_2 \cdot 6H_2O$ per L, pH 7.3, for 20 min at 25 °C. The cells were then washed 3 times with 1 mL of 0.9% saline, followed by incubation with sheep red blood cells (2% v/v) in phosphate-buffered saline for 20 min. The wash procedure was repeated, and the attached red blood cells were solubilized in 1 mL of 2% sodium dodecyl sulfate (w/v). The hemoglobin content was assayed spectrophotometrically at 418 nm. The number of LM cells in each well was also determined after solubilizing control cells from the dish, determining protein content, and correlating to cell number. All absorbances at 418 nm were corrected for number of LM cells attached. Each well contained approximately 0.5 × 10^6 attached cells.

Thiobarbituric Acid Assay of Sialic Acid. Sialic acid content of cells and plasma membranes was assayed by the method of Warren (1959).

Fluoresceinylconcanavalin A Binding Assay. Fluoresceinyl-Con A (FITC-Con A) binding to LM cells was determined as described by Monsigny et al. (1979). Before use, the FITC-Con A was purified by affinity chromatography on a Sephadex G-50 column. The concentration of lectin in the eluants was determined according to $A_{1cm}^{1\%}$ = 11.4 at 280 nm for Con A. The concentration of FITC-Con A was determined on the basis of the ratio of absorbance at 495 and 280 nm. Since this ratio is 3 for fluoresceinylthiocarbamylethanolamine (Monsigny et al., 1979), the concentration of FITC-Con A can be determined as the basis of the protein contribution of the 280-nm absorbance according to (Monsigny et al., 1979)

$$A_{\text{protein}}^{280\text{nm}} = A_{\text{protein}}^{280\text{nm}} - (1/3)A_{\text{lectin}}^{495\text{nm}}$$

The number of binding sites was measured by binding FITC-Con A to exponentially growing LM cells at 2 × 10^6 cells/mL of phosphate-buffered saline. Cells were incubated with 0.0045–0.9 μM FITC-Con A for 1 h at 4 °C. At the end of incubation, the cells were sedimented at 1000*g* for 10 min, and the concentration of free FITC-Con A in the supernatant was determined by measurement of absorbance-corrected fluorescence as described below. The labeled cells were washed 3 times with phosphate-buffered saline and then incubated with 1 mL of 0.3 M methyl α-D-mannopyranoside for 1 h at 4 °C to determine specifically bound lectin. The cells were then again sedimented, and displaced FITC-Con A, representing specifically bound Con A, was determined in the supernatant. The apparent number of binding sites per cell was then evaluated from a Scatchard plot (1949), and the K_Ds were determined from double-reciprocal plots of (absorbance-corrected fluorescence^{-1} vs. (FITC-Con A)$^{-1}$. Binding of fluoresceinyl-Con A to LM fibroblasts was completed within 30 min at 4 °C. All of the binding experiments were conducted at 4 °C to prevent capping and endocytosis, which occurred if the cells were incubated at 25 and 37 °C (Noonan & Burger, 1973).

Absorption-corrected fluorescence was determined as described previously (Schroeder et al., 1976b) with the computer-centered spectrofluorometer of Holland et al. (1973, 1977) or a SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Champaign-Urbana, IL). Fluorescence parameters (absorbance-corrected fluorescence, CO; relative fluorescence efficiency, RFE; and corrected fluorescence emission, CFE) and absorbance, AB, were determined with a unique computer-centered fluorometer (Holland et al., 1973, 1977) as described earlier (Schroeder et al., 1976b). This instrument corrects fluorescence for instrumental and inner filter effect variables. AB, CO, and CFE are fluorophore concentration dependent parameters; in contrast, RFE is directly proportional to quantum yield and is a fluorophore concentration independent parameter. Light scattering was corrected by using narrow band-pass and cut-off filters (Schott Optical Co., Duryea, PA). Membrane samples of 50 μg of protein/mL of phosphate-buffered saline solution were excited at 495 nm, and emission was measured at 530 nm. Fluorescence values were calculated by an interfaced PDP 8E computer (Digital Equipment Co., Maynard, MA). The computer corrects for inner filter effect up to absorbance of 1.80 absorbance units. Two other fluorophore concentration independent fluorescence parameters (polarization, P, and fluorescence lifetime, τ) were determined at 6, 18, and 30 MHz with the T format SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Champaign-Urbana, IL) equipped with movable Glan-Thompson polarizers. Since membrane vesicles have some turbidity, light scattering effects may cause artifacts in polarization and lifetime measurements with the SLM spectrofluorometer. For circumvention of these potential problems, sample absorbances were maintained below 0.15 OD, and light scattering was corrected by using narrow band-pass and nonfluorescent cut-off filters (Schott Optical Co., Duryea, PA). Polarization values represent limiting polarization values at 0 absorbance since each sample was diluted several times, polarization was measured, and limiting polarization was obtained by extrapolation to 0 absorbance (Lentz et al., 1979). Light scattering artifacts as well as legitimate factors may influence lifetime determination, possibly producing erroneous values as multiple lifetimes. A 35° polarizer was placed in the excitation path during lifetime measurements to compensate for rotational motion (Spencer & Weber, 1970). Fluorescence lifetimes were simultaneously measured relative to a reference solution of 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (Me_2POPOP) in absolute ethanol as described previously (Lakowicz et al., 1980). This solution, rather than the usual light scattering solution, minimizes wavelength and geometry-dependent time responses of the photomultiplier tubes. The fluorescence lifetime of Me_2POPOP in ethanol at 24 °C was 1.45 ns. Because of this lifetime, the phase angle (θ_R) of Me_2POPOP lags behind the exciting light by 3.13° at 6 MHz, 9.31° at 18 MHz, and 15.29° at 30 MHz. Phase angles can be corrected for these shifts and are therefore absolute phase angles relative to the phase of the modulated excitation. Lifetimes were average values calculated from phase and modulation measurements at 18 MHz. These values should not differ more than 1 ns for a single lifetime. In addition, lifetimes were determined by phase and modulation at 6, 18, and 30 MHz and subjected to heterogeneity analysis (SLM lifetime analysis program no. 4). All measurements were at 24 °C unless otherwise specified.

Alterations in polarization were converted to rotational correlation times, $\bar{P}$, calculated from the Perrin equation (Shinitzky & Inbar, 1976):

$$\frac{1}{r} - \frac{1}{3} = \left(\frac{1}{r_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\bar{P}}\right)$$

In this equation, r is the anisotropy [the anisotropy is equal to $2P/(3 - P)$ where P is the polarization], τ is the lifetime of the excited state, and r_0 is calculated from the polarization in the absence of rotational motion (r_0 = 0.362 for 1,6-diphenyl-1,3,5-hexatriene; Shinitzky et al., 1971). However, it should be noted that the steady-state anisotropy, which is calculated according to the above equation, depends on both the rate and range of the wobbling motion of the probe molecule and on the fluorescence lifetime (Kinosita et al.,

Table I: Phospholipid Composition (%) of Plasma Membranes from LM Fibroblasts Cultured with Choline Analogues[a]

	supplement			
	choline	N,N-dimethyl-ethanolamine	N-methyl-ethanolamine	ethanolamine
phosphatidylcholine	35.6 ± 1.7	11.4 ± 2.7	13.1 ± 1.7	14.1 ± 2.5
phosphatidyl-N,N-dimethylethanolamine		35.1 ± 3.1		
phosphatidyl-N-methylethanolamine			33.2 ± 2.4	
phosphatidylethanolamine	31.3 ± 4.4	19.3 ± 2.1	16.4 ± 1.5	41.0 ± 3.2
phosphatidylinositol + phosphatidylserine	10.4 ± 1.6	13.4 ± 1.2	14.0 ± 3.5	15.1 ± 1.9
sphingomyelin	4.6 ± 1.1	7.6 ± 1.8	9.1 ± 1.6	11.2 ± 2.6
phosphatidylglycerol	7.1 ± 1.3	3.8 ± 1.1	3.1 ± 2.0	14.6 ± 2.8
other	11.0 ± 3.0	9.4 ± 2.8	11.2 ± 3.5	14.6 ± 2.8
phosphatidylethanolamine/ phosphatidylcholine	0.87	1.69	1.25	2.91

[a] LM fibroblasts were cultured for 3 days in ^{32}P-containing medium with analogues of choline, plasma membranes were isolated, and phospholipid composition was determined as described under Materials and Methods.

Table II: Fatty Acid Composition of Plasma Membrane Phospholipids of LM Cells Cultured with Analogues of Choline[a]

	fatty acid composition (%)							
supplement	14:0	15:0	16:0	16:1	18:0	18:1	>18	unsaturation (%)
choline	0.6	0.5	14.7	2.5	13.1	48.9	19.7	65
N,N-dimethylethanolamine	1.0	0.3	12.5	6.0	18.8	38.9	22.5	60
N-methylethanolamine	0.8	0.1	13.8	5.3	19.0	38.7	22.3	63
ethanolamine	0.4	0.6	15.0	6.1	16.4	39.8	21.7	61

[a] Phospholipids were extracted from plasma membranes of LM cells cultured with choline analogues for 3 days, methyl ester derivatives were prepared, and the fatty acid composition was determined by gas–liquid chromatography as described under Materials and Methods. Values represent the mean of three determinations with individual values deviating less than 10% from the mean. The nomenclature refers to the carbon chain length:number of double bonds.

Table III: Effect of Choline Analogue Supplementation on Structure of LM Fibroblast Plasma Membranes As Determined by 1,6-Diphenyl-1,3,5-hexatriene Fluorescence Properties[a]

supplement	polarization (P)	lifetime (ns)	rotational correlation coeff (ns)	characteristic temp break points in Arrhenius plots (°C)
choline	0.327 ± 0.005	9.7 ± 0.3	61 ± 4	17, 24, 32, 37, 41
N,N-dimethylethanolamine	0.308 ± 0.006	9.8 ± 0.2	51 ± 3	19, 23, 32, 36, 42
N-methylethanolamine	0.323 ± 0.005	9.5 ± 0.3	57 ± 4	18, 24, 34, 37, 41
ethanolamine	0.315 ± 0.004	10.0 ± 0.3	56 ± 3	17, 22, 33, 37, 43

[a] Plasma membranes were obtained as described in the legend to Table I. Rotational correlation times ($\bar{P}$) were calculated from $r_0/r = 1 + 3\tau/\bar{P}$ where r_0, r, and τ are the limiting anisotropy, measured anisotropy, and lifetime. $r = 2P/(3 - P)$. The limiting anisotropy of diphenylhexatriene is $r_0 = 0.362$ (Shinitzky et al., 1971).

1981). The steady-state anisotropy, r^s, can be expressed as the sum of a kinetic term $r^\delta(t)$ and a static term, r_∞. In biological membranes, r^s reflects mainly the range of motion rather than the rate, and membrane perturbants such as cholesterol and proteins primarily alter the range of motion rather than rate (Kinosita et al., 1981). Thus, although the measured polarization represents a steady-state value, alterations in P or $\bar{P}$ are likely to be due to changes in range of motion of the probe.

Results

Effect of Choline Analogue Supplementation on Lipid Composition and Structure of LM Fibroblast Plasma Membranes. When LM fibroblasts were cultured for 3 days in medium containing choline analogues such as N,N-dimethylethanolamine, N-methylethanolamine, or ethanolamine, these bases were incorporated into plasma membrane phospholipids (Table I). The analogue containing phospholipids replaced primarily phosphatidylcholine and to a lesser extent phosphatidylethanolamine in the plasma membranes such that the ratio of phosphatidylethanolamine/phosphatidylcholine increased from 0.87 to 1.69, 1.25, and 2.91 for cells supplemented with N,N-dimethylethanolamine, N-methylethanolamine, and ethanolamine, respectively. During the 3-day growth period, the choline analogues had no adverse effect on cell growth or on the activity of membrane surface marker enzymes such as (Na^+,K^+)-ATPase or 5′-nucleotidase. In addition, the choline analogues did not affect the fold purification of the plasma membranes (data not shown). The latter results were consistent with those of earlier findings from this laboratory (Schroeder et al., 1976a) and others (Esko et al., 1977). As demonstrated in Table II, the fatty acid composition of the plasma membrane phospholipids was not altered by choline analogue supplementation.

Model system data (Vaughan & Keough, 1979) in which the phospholipid polar head group composition of artificial membranes was varied from choline to N,N-dimethylethanolamine, N-methylethanolamine, and ethanolamine indicated that the phase transition temperature decreased linearly over 20 °C with increasing methylation of the polar head groups. Thus, the LM fibroblast plasma membrane lipid compositional data obtained herein indicate that considerable alterations in plasma membrane biophysical properties might be expected upon choline analogue supplementation. However, the LM fibroblast plasma membrane structural properties measured with 1,6-diphenyl-1,3,5-hexatriene were not significantly different between control (choline) and analogue-supplemented cells (Table III). Fluorescence polarization,

Table IV: Concanavalin A Binding Sites and Their Association Constants in LM Fibroblasts Cultured with Choline Analogues[a]

	high-affinity binding site			low-affinity binding site		
supplement	FITC-Con A bound (pmol/mg of protein)	sites/cell $\times 10^{-6}$	assocn constant $\times 10^{-6}$ (1×10^{-1})	FITC-Con A bound (pmol/mg of protein)	sites/cell $\times 10^{-6}$	assocn constant $\times 10^{-6}$ (1×10^{-1})
choline	24.4 ± 6.5	3.0 ± 0.4	0.77 ± 0.15	430 ± 51	53.1 ± 6.1	0.013 ± 0.002
N,N-dimethylethanolamine	19.1 ± 4.2	2.4 ± 0.3	0.61 ± 0.22	137 ± 47*	16.7 ± 3.4*	0.025 ± 0.004*
N-methylethanolamine	16.4 ± 4.3	2.1 ± 0.3	0.96 ± 0.20	146 ± 35*	18.9 ± 2.6*	0.028 ± 0.006*
ethanolamine	22.6 ± 6.8	2.8 ± 0.4	1.05 ± 0.21	142 ± 27*	17.9 ± 4.0*	0.030 ± 0.005*

[a] The number of binding sites per cell and values of apparent association constants were determined from Scatchard plots as described under Materials and Methods. The molecular weight of fluoresceinylconcanavalin A at pH 7.2, 4 °C, was 1.1×10^5. Values represent the mean ± SEM ($n = 3$). An asterisk (*) refers to $p < 0.05$ (by Student's t test) as compared to the choline-supplemented samples.

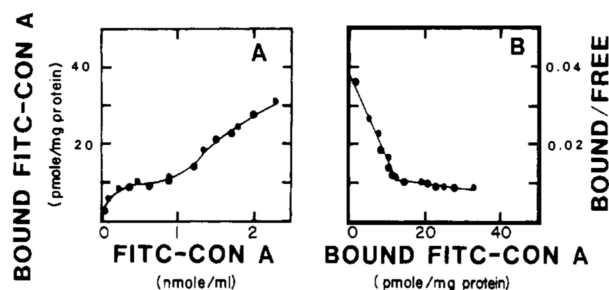

FIGURE 1: Saturation curve (A) and Scatchard analysis (B) of the binding of fluoresceinylconcanavalin A to LM fibroblasts. Data points correspond to the average of duplicates in a representative experiment. LM cells cultured for 3 days with *N,N*-dimethylethanolamine were harvested and incubated (4×10^6 cells/mL) with fluoresceinylconcanavalin A (0–2.27 μM) as for 1 h at 4 °C. Specific binding was determined as described under Materials and Methods.

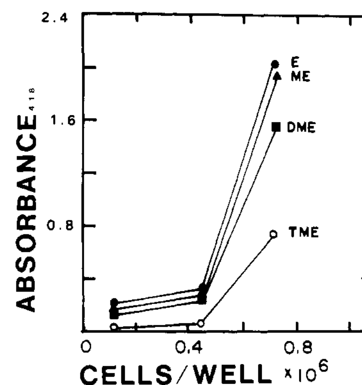

FIGURE 2: Effect of LM cell density on concanavalin A mediated hemadsorption. LM cells were cultured for 3 days with choline or choline analogues (TME, choline; DME, *N,N*-dimethylethanolamine; ME, *N*-methylethanolamine; E, ethanolamine), and concanavalin A hemadsorption was measured as described under Materials and Methods. The cell concentration was varied from 0.12 to 0.73 cell/well. Cells were incubated with the indicated concentration of concanavalin A for 10 min at 24 °C, followed by 20-min incubation with excess sheep red blood cells as described under Materials and Methods. The absorbance of hemoglobin at 418 nm is a measure of hemadsorption.

lifetime, rotational correlation time, and characteristic temperatures in Arrhenius plots of 1,6-diphenyl-1,3,5-hexatriene in LM plasma membranes were not altered significantly by choline analogue supplementation.

Choline Analogue Effects on Fluoresceinylconcanavalin A Interaction with LM Fibroblasts. The results in Table I indicate that the choline analogues reduced the phosphatidylcholine content of plasma membranes by as much as 68%. Saturation curves and Scatchard analysis (1949) of FITC-Con A binding to LM fibroblasts supplemented with choline or choline analogues indicated the presence of two classes of apparent binding sites (Figure 1). The FITC-Con A associated with choline-fed LM cells with two apparent binding sites: a high-affinity, low-capacity ($K_A = 0.77 \times 10^6$ L/mol; 24.4 pmol/mg of protein) and a low-affinity, high-capacity ($K_A = 0.013 \times 10^6$ L/mol; 430 pmol/mg of protein) site (Table IV). After incubation of LM cells with the FITC-Con A, the cells were washed 2 times with phosphate-buffered saline. No appreciable fluorescence was detected in the second wash supernatant, indicating that the bound lectins are not in true equilibrium with the surrounding medium or that the releasing process is very slow. Specific binding of FITC-Con A was determined by releasing bound FITC-Con A with 0.3 M methyl α-D-mannopyranoside and measuring the released fluorescence. Approximately 75–85% of the FITC-Con A was specifically bound to choline or choline analogue supplemented cells under the conditions used herein. Choline analogue supplementation did not affect the high-affinity binding site; neither the K_A nor the number of binding sites were significantly altered (Table IV). In contrast, the low-affinity binding sites were markedly altered: (1) the association constant was increased approximately 2-fold, and (2) the number of binding sites was decreased nearly 3-fold.

Concanavalin A Mediated Hemagglutination of LM Cells Supplemented with Choline Analogues. Con A mediated hemadsorption was maximal in 10 min at 24 °C (data not shown). The degree of Con A mediated hemadsorption was dependent on the Con A concentration and reached maximal values at 1.8–2.7 μM Con A. At all concentrations of concanavalin A tested, the choline analogue supplemented had 2–3-fold greater hemadsorption properties. Increasing the number of LM cells in the assay but keeping the red blood cell and Con A concentrations constant (0.9 μM) increased the hemadsorption. However, in all cases the choline analogue supplemented cells adsorbed more red blood cells (Figure 2). Thus, choline analogue supplementation decreased the content of zwitterionic phospholipid in the LM plasma membrane, increased the low-affinity binding site association constant for FITC-Con A, and increased the heterotypic adsorption of sheep red blood cells to LM fibroblasts mediated by Con A.

Homotypic Agglutination of Choline Analogue Supplemented LM Cells by Concanavalin A. The agglutination of LM cells was monitored spectrophotometrically at 546 nm (Murphree et al., 1976). The absorbance of the LM cells at 546 nm increased with increasing cell concentration over the range 0–28 $\times 10^6$ cells/mL (Figure 3). The change in absorbance vs. cell number appeared to be most sensitive in the exponential region of the curve. Therefore, a concentration of 10×10^6 cells/mL was chosen for the agglutination assay. Cells with higher density did not produce as large an absorbance difference upon agglutination as cells at lower density. The spectrophotometric curves of Con A mediated cell agglutination (Figure 4) indicated that the lag time, defined as the time prior to a decrease in absorbance, was not affected

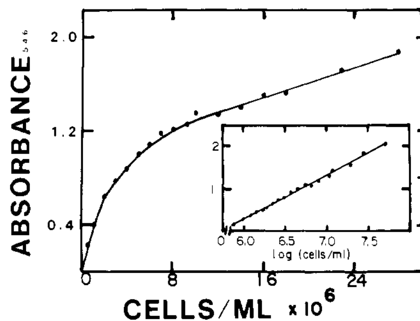

FIGURE 3: Effect of LM cell density on absorbance. Choline-fed LM cells at the indicated cell concentration were resuspended in phosphate-buffered saline, and the absorbance at 546-nm wavelength was determined. Each point represents one determination. Linear regression analysis of the inset revealed $r = 0.9887$; LR = −5.87.

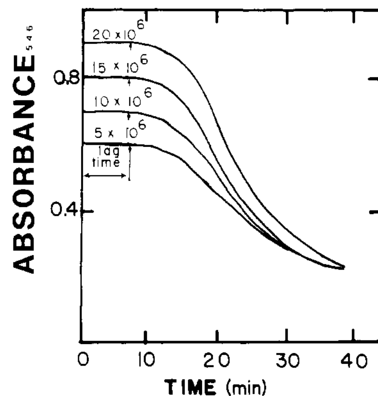

FIGURE 4: Effect of LM cell density on concanavalin A agglutinability. The agglutination of LM fibroblasts cultured for 3 days with choline by concanavalin A (1.8 μM) was monitored as a function of time. The cell density in the assay was varied from 5 to 20 × 10^6 cells/mL.

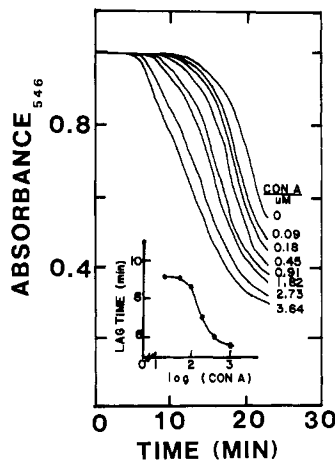

FIGURE 5: Dose–response curve for concanavalin A mediated agglutination of LM cells. Choline-fed LM cells were resuspended at a final concentration of 10 × 10^6 cells/mL, together with 0–3.6 μM concanavalin A. The decrease in absorbance at 546 nm was then monitored as a function of time.

by the cell density. In contrast, the slope or rate of agglutination was highly dependent on the starting cell density. Lastly, varying the concentrations of Con A from 0 to 3.6 μM had little effect on the rate of cell settling in the cuvette but reduced the lag time for agglutination from 11.3 to 4.6 min. The dose–response curve (inset in Figure 5) indicates that the Con A concentration eliciting half-maximal decrease in lag time for agglutination was approximately 1.8 μM. This concentration was chosen for all subsequent assays. The specificity of the effect of concanavalin A on LM cell agglutination lag time was determined by use of competing displacing sugars (Table V). As expected, methyl α-D-mannoside and sucrose both effectively competed with the LM cells for Con A, while D-galactose and L-xylose did not effectively compete.

Table V: Effect of Displacing Sugars on Concanavalin A Mediated Agglutination of LM Cells[a]

saccharide	concn (M)	Con A (μg/mL)	lag time (min)
none	0	0	11.30 ± 0.29
none	0	200	7.37 ± 0.28*
inhibitors			
methyl α-D-mannoside	0.05	200	9.70 ± 0.36*
methyl α-D-mannoside	0.10	200	10.73 ± 0.35
sucrose	0.10	200	10.21 ± 0.32
noninhibitors			
D-galactose	0.10	200	6.50 ± 0.49*
L-xylose	0.10	200	8.01 ± 0.35*

[a] Cells were cultured in choline-containing medium, and concanavalin A mediated agglutinability was determined as described under Materials and Methods at 24 °C. Values refer to the mean ± SEM ($n = 3$). An asterisk (*) refers to $p < 0.05$ as compared to zero saccharide and zero concanavalin A added.

Table VI: Effect of Several Drugs on Concanavalin A Mediated Agglutinability of LM Cells[a]

drug	concn (M)	Con A (μg/mL)	lag time (min)
none	0	0	10.95 ± 0.24
none	0	200	7.88 ± 0.19*
ethanol	0.13	200	4.81 ± 0.18*,‡
pentobarbital	0.25 × 10^{-3}	200	5.03 ± 0.21*,‡
colchicine	10^{-5}	200	5.91 ± 0.11*,‡
cytochalasin B	0.06 × 10^{-6}	200	6.05 ± 0.18*,‡

[a] Cells were cultured in choline-containing medium, incubated for 30 min with the indicated drug, and concanavalin A agglutinability was determined. Values represent the mean ± SEM. An asterisk (*) signifies $p < 0.05$ as compared to zero drug and zero concanavalin A; a dagger (‡) signifies $p < 0.05$ as compared to zero drug and 1.8 μM concanavalin A.

The effect of microtubule and microfilament disrupting drugs and anesthetic agents on Con A mediated LM cell agglutinability was tested (Table VI). Both anesthetics (ethanol and pentobarbital) that fluidize membranes (Harris & Schroeder, 1981, 1982) and microtubule disrupting drugs such as colchicine (Rittenhouse et al., 1974b) enhanced agglutinability of LM cells by shortening the lag time. Longer exposure of cells to colchicine (1.25–24 h at 0.1–10 × 10^{-6} M) also resulted in shortened lag times (data not shown). Cytochalasin B had less effect.

Effect of Choline Analogue Supplementation on Concanavalin A Mediated Agglutination of LM Cells. Alterations in LM cell plasma membrane lipid composition by choline analogue supplementation reduced the lag time for agglutination of LM cells by concanavalin A from 7.88 ± 0.24 min to 3.34 ± 0.25, 2.40 ± 0.20, and 2.04 ± 0.22 min (Figure 6). These differences were not due to arrest of analogue-supplemented cells in different parts of the cell cycle. Growth of choline- and analogue-supplemented cells was identical for the first 3 days of culture. In addition, the cell density during culture did not affect the lag time of concanavalin-mediated agglutinability (data not shown).

It is possible that these differences in agglutination upon choline analogue supplementation were not due to polar head group changes but rather to sialic acid changes. Sialic acid is negatively charged and alterations in cell surface sialic acid content have been correlated with differences in agglutinability of cells (Raz et al., 1980). However, the sialic content of LM

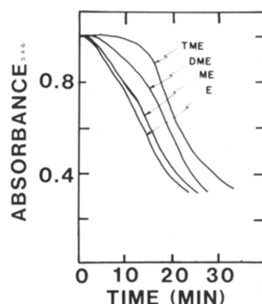

FIGURE 6: Effect of choline analogue supplementation on LM cell agglutination by concanavalin A. LM cells were grown in the presence of choline (TME), dimethylethanolamine (DME), methylethanolamine (ME), or ethanolamine (E) for 3 days. The agglutination by concanavalin A was then monitored as a function of time as described under Materials and Methods.

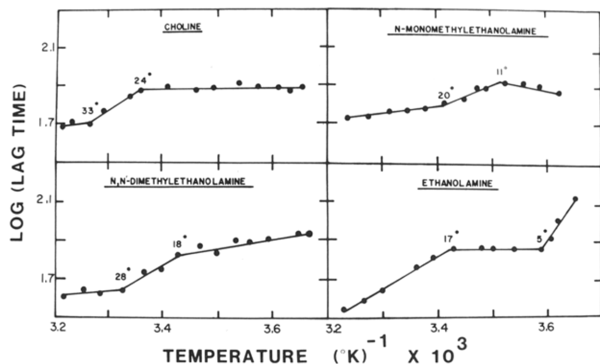

FIGURE 7: Temperature dependence of LM fibroblast concanavalin A agglutinability. LM cells were cultured for 3 days in choline or choline analogue containing medium. The concanavalin A agglutinability was measured as described under Materials and Methods with a Gilford spectrophotometer. The spectrophotometer was water jacketed, and the temperature was varied with an Endocal-Exocal (Neslab Instr., Portsmouth, NH) heating–cooling system. The cells were stirred and equilibrated at each temperature for 5 min prior to addition of concanavalin A (1.8 μM). The temperature was monitored with a thermocouple, inserted within a few millimeters of the light beam, and a digital thermometer. Values represent the mean of three determinations. Individual values varied less than 10% from the mean.

fibroblasts was 2.71 ± 0.23, 1.90 ± 0.42, 2.14 ± 0.57, and 3.09 ± 0.45 μmol/mg of protein for cells cultured in the presence of *N*,*N*-dimethylethanolamine, *N*-methylethanolamine, or ethanolamine, respectively, not significantly different.

The agglutinability of LM cells supplemented with choline analogues as a function of temperature is shown in Figure 7. Cells supplemented with choline had break points at 24.3 ± 2.2 and at 33.1 ± 1.8 °C. These break points occurred at similar temperatures as two of the characteristic temperatures detected by the fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (Table III) and may therefore represent a sensitivity of the Con A receptor to phase transitions or alterations in lipid microdomains. Such break points for Con A agglutinability have also been noted by others (Rittenhouse & Fox, 1974; Rittenhouse et al., 1974a,b) and are sensitive to the lipid phase transition. The break points of Con A agglutinability of LM cells supplemented with *N*,*N*-dimethylethanolamine, *N*-methylethanolamine, or ethanolamine were at 18.3 ± 1.6 and 27.6 ± 2.1, 10.7 ± 1.1 and 20.1 ± 1.8, and 5.3 ± 1.0 and 17.4 ± 2.5 °C, respectively (Figure 7).

Discussion

It has been reported by several investigators that the free movement of Con A receptors in the plane of the cell surface mambrane is required for Con A stimulated cell agglutination (Horwitz et al., 1974; Rittenhouse & Fox, 1974; Rittenhouse et al., 1974a,b; Rosenblith et al., 1973). Major determinants of membrane fluidity are fatty acid composition, sterol content, and phospholipid polar head group composition. The effect of the former two has been investigated in detail (Horwitz et al., 1974; Rittenhouse & Fox, 1974; Rittenhouse et al., 1974a,b; Marshall et al., 1979). However, the effect of changing phospholipid polar head group composition has been measured only with a model liposomal system (Hampton et al., 1980). The effect of phospholipid polar head group composition on lectin agglutinability has not previously been documented in living cells.

Supplementation of LM fibroblasts with choline analogues drastically altered phospholipid polar head group composition but did not significantly change the phospholipid fatty acid composition. These results are in agreement with earlier data from this laboratory and others (Schroeder et al., 1976a; Esko et al., 1977). However, the structural properties (e.g. fluorescence polarization, lifetime, rotational correlation time, and characteristic break temperatures of fluorescence probe molecules) of the plasma membranes were not altered, also in agreement with earlier findings (Schroeder et al., 1976b,c; Schroeder, 1980, 1978a,b). A reasonable explanation for this lack of expected structural alterations (Vaughan and Keough, 1979) was that the LM fibroblast "homeoviscously adapted" (Schroeder, 1978a). The primary mechanism for this adaption was redistribution of fatty acyl chains between the phospholipid species and synthesis of choline analogue containing phospholipids with intermediate fatty acid composition. There was no net change in the total phospholipid fatty acid composition. Alternate lipid-mediated causes for changes in Con A response noted here must be considered. Methylation of phospholipids may mediate hormone action, thereby leading to altered cell metabolism, including rate of synthesis of receptors capable of binding Con A. However, elsewhere we have demonstrated that the phosphatidylethanolamine methylation pathway is not only lacking in LM fibroblasts (Feller et al., 1982) but also is not responsive to receptor binding by benzodiazepines, prostaglandins, or to choline analogues. Thus, possible effects secondary to altered polar head groups are not mediated via the phospholipid methylation pathway in LM fibroblasts.

Altered surface interactions in LM fibroblasts may also be due to possible modification in plasma membrane protein components upon choline analogue supplementation. Using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, Engelhard et al. (1976) did not obtain alterations in plasma membrane protein composition of choline analogue supplemented cells. This observation was confirmed in our laboratory (R. N. Fontaine and F. Schroeder, unpublished data). Lastly, the data presented here also indicate that the sialic acid content, the primary cell surface charge determinant, was not altered in LM cells supplemented with choline analogues. The majority of sialic acid residues are components of glycoproteins and to a lesser extent glycolipids.

Choline-supplemented LM cells demonstrated two binding sites for FITC-Con A. The affinity constants were 0.77 × 10^6 and 0.013 × 10^6 L/mol; at saturation 24.4 pmol were specifically bound per mg of protein, and 430 pmol were specifically bound per mg of protein by the two sites, respectively. Approximately 75–85% of the bound FITC-Con A was displaced by methyl α-D-mannoside, indicating specific binding. In a related cell line, the L cell cultured in a defined medium, other investigators have shown two binding affinities for [I^{125}]iodoCon A (Marshall et al., 1979). At saturation, a total of 500 pmol of [I^{125}]iodoCon A was bound per mg of L cell protein, and 75% was specifically bound. Thus, the L cell and LM cell exhibited similar binding parameters. Supplemen-

tation of LM fibroblasts with choline analogues did not alter the high-affinithy binding site but doubled the affinity constant of the low-affinity binding site. Thus, although the fluidity of the LM plasma membrane as measured by the rotational correlation time of 1,6-diphenyl-1,3,5-hexatriene was not altered by choline analogue supplementation, the binding of FITC-Con A was changed 2-fold. Using model phospholipid systems, Hampton et al. (1980) demonstrated that the content of zwitterionic phospholipids (phosphatidylcholine) dramatically affected the binding of Con A to liposomes containing glycolipids; Con A did not agglutinate neutral vesicles composed of phosphatidylcholine or a mixture of phosphatidylcholine and phosphatidylethanolamine. Similarly, in the present work choline analogues reduced LM cell phosphatidylcholine content and increased FITC-Con A affinity.

Heterotypic agglutination of LM cells with sheep red blood cells was dramatically enhanced by prior supplementation of the LM cells with choline analogues. Others have shown that this agglutination was sensitive to fatty acid composition and therefore fluidity (Rittenhouse & Fox, 1974; Rittenhouse et al., 1974a,b). The results herein indicate that not only fluidity but also polar head group composition needs to considered as modulators of heterotypic lectin agglutinability.

Homotypic agglutination of LM cells with Con A was specific, relatively independent of culture density parameters, enhanced by anesthetics, and enhanced by microtubular and microfilament disruptors. The temperature break points of Con A mediated agglutinability of LM cells were markedly reduced by choline analogue supplementation. This finding was very surprising as 1,6-diphenyl-1,3,5-hexatriene temperature break points were not altered in the plasma membrane. If they had been altered, then they should have been increased, not reduced (Vaughan & Keough, 1979). Possible explanations for these unexpected findings might be that (1) Con A receptors are specifically localized in fluid microdomains in the LM plasma membrane or (2) the asymmetric distribution of lipids across the membrane bilayer (Fontaine & Schroeder, 1979) and the associated fludity gradient (Schroeder, 1978b, 1980, 1981a; Hale & Schroeder, 1982) may affect the transbilayer linkage of the Con A receptor to the cytoskeleton.

In conclusion, our measurements in a biological system are consistent with phospholipid model system data (Hampton et al., 1980). Phospholipid polar head group composition of cell membranes must be considered as a potential parameter affecting lectin agglutinability of living cells as well as associated endocytic processes (Schroeder, 1981b, 1982; Kier & Schroeder, 1982).

Acknowledgments

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Unifying Description of the Effect of Membrane Proteins on Lipid Order. Verification for the Melittin/Dimyristoylphosphatidylcholine System†

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ABSTRACT: The effect of melittin on lipid order in dimyristoylphosphatidylcholine bilayers is investigated by means of Raman spectroscopy and fluorescence anisotropy using diphenylhexatriene as fluorescence probe. In the fluid lipid phase, the Raman results indicate a slight increase in the conformational order of the lipid chains, and the fluorescence anisotropy results indicate a considerable increase in the rigid-body orientational order of the lipid chains. These results are contrasted with the reported decrease of the deuterium magnetic resonance order parameter. A consistent interpretation of the complete set of experimental data is presented according to which proteins induce a tilt of the preferred axes of lipid orientation and increase the orientational order with respect to these axes. The values of the tilt angle and the orientational order parameter at the surface of proteins are determined from the experimental data within a continuum model of lipid–protein interaction. The same values are obtained for melittin, Ca/Mg-ATPase, and cytochrome *c* oxidase, suggesting that different membrane proteins affect the lipid order in the same way.

A large body of experimental data on the effect of proteins on lipid order in membranes has been accumulated during the past few years. However, no consensus has been attained as to whether proteins in a fluid membrane cause an ordering or a disordering of the lipids. The confusion arises from the disagreement of results obtained by different experimental techniques. On the one hand, electron spin resonance (ESR),[1] fluorescence anisotropy (FA), and Raman spectroscopy indicate that proteins increase the lipid order. On the other hand, deuterium magnetic resonance (^{2}H NMR) indicates that proteins decrease the lipid order.

A consistent interpretation of these experimental results has been proposed previously (Jähnig, 1979), requiring that proteins induce a tilt of the preferred axes of lipid orientation and increase the lipid orientational order with respect to these tilted axes. In the present paper, this concept is elaborated quantitatively within the framework of a continuum model of lipid–protein interaction (Owicki et al., 1978; Jähnig, 1981). Here a protein molecule imposes at its surface boundary conditions upon the tilt angle of the preferred axes and the lipid orientational order parameter. With increasing distance from the protein the perturbation of lipid order falls off exponentially with a characteristic length given by the coherence length of lipid order. Within this model, the experimental data representing different averages of the spatially varying lipid order can be evaluated for the boundary values of the tilt angle and the orientational order parameter.

The determination of the boundary order parameter is exemplified by the system melittin in dimyristoylphosphatidylcholine bilayers on the basis of Raman and FA results presented. If FA data from the literature for two other proteins, Ca/Mg-ATPase and cytochrome *c* oxidase, are evaluated in the same way, a striking similarity among different proteins is found. The boundary tilt angle is determined for Ca/Mg-ATPase and cytochrome *c* oxidase, for which the necessary ^{2}H NMR data are available. The result again indicates a similarity amont different proteins in their effect on lipid order.

Materials and Methods

Chemicals. DMPC was purchased from Fluka. No impurities were detected by thin-layer chromatography. Melittin was from Mack (Illertissen, BRD) and was purified by gel filtration through a Sephadex G-100 column in the presence of 10^{-3} M HCl. Diphenylhexatriene (DPH) was obtained from EGA-Chemie (Steinheim, BRD).

Sample Preparation for X-ray and Raman Measurements. A 10-mg sample of DMPC and an appropriate amount of melittin were dissolved in 100 μL of freshly distilled 2-chloroethanol. After addition of 2 mL of benzene, the mixture was freeze-dried. Buffer (10^{-3} M Tris-HCl and 10^{-4} M EDTA, pH 7.4) was added to the dry samples to obtain a water/lipid weight ratio of 1/1. The membrane preparations were incubated for 2 h at 35 °C, then transferred to thin-walled glass capillaries, and centrifuged for 10 min at 50000*g*

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[1] Abbreviations: ^{2}H NMR, deuterium magnetic resonance; ESR, electron spin resonance; FA, fluorescence anisotropy; DPH, diphenylhexatriene; tPnA, *trans*-parinaric acid; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; P(PO)PC, 1-palmitoyl-2-palmitoleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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