Quantitative Contributions of Cholesterol and the Individual Classes of Phospholipids and Their Degree of Fatty Acyl (Un)Saturation to Membrane Fluidity Measured by Fluorescence Polarization†

Wim J. van Blitterswijk,* B. Wieb van der Meer,‡ and Henk Hilkmann

Division of Cell Biology, The Netherlands Cancer Institute (Antoni van Leeuwenhoek-Huis), 1066 CX Amsterdam, The Netherlands

Received August 7, 1986; Revised Manuscript Received November 10, 1986

ABSTRACT: Steady-state fluorescence polarization (P) measurements, using the probe 1,6-diphenyl-1,3,5-hexatriene, in a large variety of well-defined liposomes at 25 °C allowed a quantitative description of the contributions of cholesterol, sphingomyelin, and (un)saturation of fatty acyl groups in the various phospholipids to the structural order (or the mutual affinity) of membrane lipids. The P values for liposomes prepared from lipid extracts of natural (purified) membranes of various origins could be more or less predicted (calculated) from the relative contributions of the individual lipid components. In all cases, the polarization varied with the cholesterol/phospholipid molar ratio (C/PL) according to the equation $P = P_{plat} - (P_{plat} - P_{zero}) \exp(-\alpha C/PL)$, in which P_{zero} refers to the polarization without cholesterol and P_{plat} is a maximal plateau value, reached at a high C/PL (>1). The "cholesterol-ordering coefficient" α of the phospholipids was found to increase with the fraction of sphingomyelin or dipalmitoylphosphatidylcholine molecules, indicating that the susceptibility of phospholipids to be ordered by cholesterol is increased by these compounds. P_{zero} increases curvilinearly with the fraction of either of these molecules. P_{plat} increases linearly with the fraction of saturated acyl chains for most phospholipids. Highly unsaturated fatty acyl chains (e.g., 20:4 and 22:6) strongly depress P_{plat} but not P_{zero}. The results suggest that such phospholipids are unlikely to associate with cholesterol and may thus create extremely fluid membrane domains. The disproportionation of cholesterol in the cell can be understood by the differing composition of the phospholipids in plasma membranes and endomembranes and their ordering susceptibility (affinity) toward cholesterol.

The fluidity of the lipid bilayer component of biological membranes has been shown by many studies to influence a variety of membrane functions (Stubbs & Smith, 1984; Schachter, 1984; Spector & Yorek, 1985; Van Blitterswijk, 1985). It has been defined operationally by a variety of techniques. The easiest and most frequently used method to measure fluidity is steady-state fluorescence depolarization of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] (Shinitzky & Barenholz, 1978). The measured data (P values) predominantly reflect the structural order of the membrane lipids (Kinosita et al., 1984; Stubbs & Smith, 1984) and in fact allow calculation of an order parameter, when one makes use of an empirical relationship (Van Blitterswijk et al., 1981; Pottel et al., 1983; Van der Meer et al., 1986). In this context, membrane fluidity may be considered as the reciprocal of the structural order (Van Blitterswijk et al., 1981) or of the packing or the mutual affinity of the various apolar lipid chains.

It is well established now that lipid structural order in membranes at physiological temperatures is largely determined by cholesterol and sphingomyelin content and by the degree of saturation of the phospholipid acyl chains (Kawato et al., 1978; Van Blitterswijk et al., 1981; Stubbs & Smith, 1984; Barenholz, 1984; Yeagle, 1985). However, the contributions of each of these three determinants have hardly been assessed individually in quantitative terms, due to the complexity of the systems. Some of the complicating factors and uncertainties involved are the following: First, cholesterol has a condensing effect (ordering) on the packing of phospholipids in the liquid-crystalline (fluid) state, i.e., above their phase transition temperature (Demel & De Kruyff, 1976; Kawato et al., 1978). However, this condensing effect has been shown to depend on the molecular species of the lipids involved (Demel et al., 1977) as well as on their degree of fatty acyl unsaturation (Demel et al., 1972). Further, the studies of Cooper et al. (1978) and Hoffmann et al. (1981) have indicated that such ordering by cholesterol levels off at high cholesterol/phospholipid (C/PL) molar ratios, but until now, it has not been ascertained which structural factors determine these plateau levels. Second, sphingomyelin not only exerts a rigidifying (lipid ordering) effect in biomembranes but also has been shown to interact preferentially with cholesterol (Demel et al., 1977; Lange et al., 1979; Barenholz, 1984), which may possibly amplify the ordering effect or may promote lipid phase separation into more and less ordered membrane

† This investigation was supported in part by the Queen Wilhelmina Foundation for Cancer Research in The Netherlands.

‡ Present address: Thrombosis/Hematology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; C/PL, cholesterol/phospholipid molar ratio; Sph, sphingomyelin; PC, PE, PS, and PI, phosphatidylcholine, -ethanolamine, -serine, and -inositol, respectively; LPC, lysophosphatidylcholine; DPPC, DOPC, and DLPC, dipalmitoyl-, dioleoyl-, and dilinoleoylphosphatidylcholine, respectively; POPC, PLPC, and PAPC, 1-palmitoyl-2-oleoyl-PC, -2-linoleoyl-PC, and -2-arachidonoyl-PC, respectively; POPE, 1-palmitoyl-2-oleoyl-PE; endo-PC and endo-PE, PC and PE, respectively, purified from rat liver endomembranes; P, fluorescence polarization; P_{zero}, polarization at zero cholesterol content; P_{plat}, polarization plateau value reached at high C/PL ratio; α, cholesterol-ordering coefficient; Δ, cholesterol-ordering susceptibility of membrane lipids (containing a certain amount of cholesterol); Δ_0, cholesterol-ordering susceptibility of phospholipids (at zero cholesterol content); PBS, phosphate-buffered saline.

0006-2960/87/0426-1746$01.50/0

domains (Van Blitterswijk et al., 1981, 1982).

Third, there is some inconsistency in the literature regarding the effects of differences or alterations in the content of unsaturated acyl chains on the lipid fluidity of biological membranes, as discussed by Stubbs and Smith (1984) and Storch and Schachter (1985). There is also some disagreement in the literature as to what the relevant unsaturation parameter is. Cossins and Prosser (1978), for example, noted positive correlations between P values of DPH and the ratios of saturated/unsaturated fatty acyl chains in the choline and ethanolamine phosphoglycerides of synaptosomal membranes of various fish species. Kinosita et al. (1984), however, concluded from P measurements in well-defined liposomes that neither the average number of double bonds nor the fraction of unsaturated chains but rather the fraction of unsaturated phospholipid *molecules* in the membrane is correlated with the P value.

This paper represents a more systematic study to resolve some of the above questions and inconsistencies. DPH fluorescence polarization is measured in a large variety of liposomes of well-defined compositions at a constant temperature (25 °C). The effect of cholesterol on the P value (structural order) of a membrane can be calculated by the C/PL molar ratio using a simple exponential equation in which the P value at zero cholesterol content (P_{zero}) and the limiting plateau value (P_{plat}), at high cholesterol content, appear. These two extreme values can be predicted from the phospholipid class composition and the fractions of saturated phospholipid molecules and of saturated acyl chains, respectively. For various liposomes as well as biological membranes, the calculated values based on chemical compositions are compared to measured fluorescence polarization data and appear to be in reasonably good agreement.

This study also contributes to an important issue in cell biology, namely, the topogenesis of the plasma membrane. The nonhomogeneous distribution of cholesterol among cell membranes is an important phenomenon to understand. Here we show that membrane (phospho)lipid composition may dictate preferences for cholesterol incorporation.

Materials and Methods

Chemicals. The following lipids were obtained from Sigma in the highest available purity (98–99%): synthetic DPPC, POPC, PLPC, DOPC, DLPC, and POPE, egg yolk derived PC, lyso-PC and sphingomyelin, PS from bovine brain, and PI from soybeans. Synthetic PAPC was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). The purity of these compounds was confirmed by thin-layer chromatography and gas–liquid chromatography, as described below. Commercial PS was subjected to an additional thin-layer chromatographic purification to remove traces of PE and lyso compounds. Lipids susceptible to oxidation were stored under nitrogen at −20 °C in chloroform with 0.01% butylated hydroxytoluene added as an antioxidant. Cholesterol and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Koch-Light Laboratories Ltd. (Colnbrook, U.K.).

Isolation of Biological Membranes. Purification of plasma membranes from rat (R/A) liver (Emmelot et al., 1974) and from the transplanted murine leukemic GRSL ascites cell line and of ascites extracellular vesicles from these GRSL cells (Van Blitterswijk et al., 1979, 1982) was performed according to standard methods in our laboratory. An "endomembrane" preparation from rat liver was obtained as described previously (Van Hoeven et al., 1979) from the low-speed supernatant of a standard liver plasma membrane isolation in 10^{-3} M $NaHCO_3$ by precipitation at 105000g for 1 h. Plasma membranes (ghosts) of human and murine blood erythrocytes were purified according to Hanahan and Ekholm (1974).

Lipid Purification and Analysis. Total lipids were extracted from the membrane preparations with chloroform/methanol (2:1 v/v) followed by partition according to Folch et al. (1957). Phospholipids were isolated by thin-layer chromatography on precoated silica gel plates (Merck) using n-hexane/diethyl ether/acetic acid (85:15:2 v/v). For further analytical or preparative purposes, phospholipids were separated by two-dimensional thin-layer chromatography on silica gel plates, using the solvent systems chloroform/methanol/0.88 M ammonia (60:60:5 v/v) and chloroform/methanol/acetic acid/H_2O (50:30:8:4 v/v), as described in detail previously (Van Blitterswijk et al., 1982). The phospholipids were quantitated by phosphate analysis (Morrison, 1964). Cholesterol was determined enzymatically (Van Blitterswijk et al., 1982). Purified phospholipids from membranes and other sources were analyzed for their fatty acid composition after transesterification with H_2SO_4/methanol followed by capillary gas–liquid chromatography on an open tubular glass column, 25 m × 0.21 mm, wall-coated with Silar 5 CP, as described before (Van Blitterswijk et al., 1982). The phospholipids purified from rat liver endomembranes (endo-phospholipids) and other sources, to be used for the present experiments, were stored in chloroform solution in the presence of butylated hydroxytoluene under nitrogen at −20 °C, to prevent oxidation.

Fluorescence Polarization in Liposomes and Biological Membranes. The degree of fluidity in membrane lipids was determined by steady-state fluorescence polarization of the apolar probe DPH at one constant temperature of 25 °C. The degree of polarization (P value) was measured in an Elscint apparatus, Model MV-1A (Elscint Ltd., Haifa, Israel), as described previously (Van Blitterswijk et al., 1977, 1979). The steady-state measurement senses a combination of both the angular range (orientational constraint) and the rate of motions of DPH in the membrane, of which the former contribution dominates the polarization, above a P value of about 0.170 (Van Blitterswijk et al., 1981; Stubbs & Smith, 1984). This range contribution can be quantified with the use of an empirical relationship, thus allowing the calculation of structural order parameters (Van Blitterswijk et al., 1981; Pottel et al., 1983; Van der Meer et al., 1986). In the present study, however, it suffices to present the P values as read directly from the instrument, noting that they mainly reflect the lipid structural order.

Native membranes, in an amount corresponding to about 100 μg of lipid, were labeled for 30 min at 37 °C with 10^{-6} M DPH dispersed in 0.02 M phosphate-buffered saline, pH 7.3 (PBS). Artificial membranes (multilamellar liposomes) of various compositions were labeled with DPH during their preparation as follows: A total amount of 50 μg of (various combinations of) phospholipids plus appropriate amounts of cholesterol was taken from their chloroform stock solutions, mixed, and evaporated to dryness under nitrogen. Then, 2.5 mL of the DPH dispersion of PBS was added, and sonication was performed under nitrogen, first for 30 s with a Branson sonifier fitted with a standard probe (50 W) and subsequently in a bath sonifier for 10 min. Sonications were performed with ice cooling, except when the sample contained a high amount of DPPC or sphingomyelin, in which case sonication was performed above the phase transition temperature. The presence of a trace of butylated hydroxytoluene (from the stock solutions) did not affect the measured P values.

Data Analysis. Polarization data were fitted to an exponential or a linear function or to a ratio of quadratic functions.

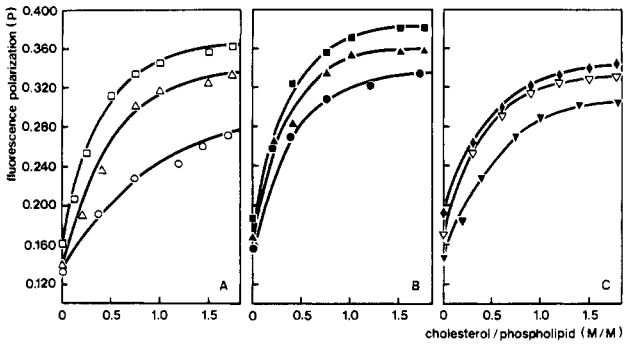

FIGURE 1: Some typical fluorescence polarization measurements, P, at 25 °C, using the probe DPH in various liposomes as a function of the C/PL molar ratio. Computer best-fitting curves according to eq 1 (see text) are shown. The lowest point of each curve is denoted as P_{zero} (at zero cholesterol content). At high cholesterol content, the curves reach a plateau value (P_{plat}). The slope of the curves is determined by the coefficient α. (A) DLPC (○), PAPC (△), and POPC (□), yielding P_{plat} = 0.295, 0.344, and 0.368, α = 1.11, 1.84, and 2.30, and χ_r^2 = 1.6, 2.8, and 1.0, respectively. (B) Same phospholipids as in panel A, but with 20% sphingomyelin (closed symbols), yielding P_{plat} = 0.337, 0.359, and 0.385, α = 2.36, 2.97, and 2.69, and χ_r^2 = 1.0, 4.9, and 1.2, respectively. (C) Total phospholipids isolated from natural membranes: endomembranes (▼) and purified plasma membranes (▽) from rat liver, and human erythrocyte ghosts (♦), yielding P_{plat} = 0.318, 0.332, and 0.344, α = 1.56, 2.61, and 2.20, and χ_r^2 = 1.0, 0.1, and 0.5, respectively. Correlation coefficients were in all cases ≥0.98.

In all cases, the number of adjustable parameters was 2. The best fit was determined by minimizing χ^2 (Bevington, 1969), defined as

$$\chi^2 = \sum_{i=1}^{N} (P_{ic} - P_{io})^2/\sigma^2$$

where the summation index, i, runs over the N data points and P_{ic} denotes a calculated and P_{io} an observed polarization value; σ is the estimated average standard deviation of the measured polarization value and is here fixed to σ = 0.005. The reduced χ^2, χ_r^2 [minimal value of $\chi^2/(N-2)$], is a measure for the goodness of fit; the factor $N-2$ is the number of degrees of freedom left after fitting N data points to two parameters (Bevington, 1969). If χ_r^2 is less than or close to unity, the fitting function can be considered appropriate. Values of χ_r^2 that are significantly greater than unity (e.g., $\chi_r^2 > 10$) indicate that the assumed function is probably inadequate to explain the data.

RESULTS

The effects of cholesterol on the DPH fluorescence polarization (P) at 25 °C were studied in a large variety of liposomal membranes containing one or more synthetic or natural phospholipid classes with various degrees of unsaturation in their fatty acids, or containing the total phospholipids isolated from various natural membranes. In all cases, the change in polarization as a function of cholesterol content is pronounced at low cholesterol concentration but levels off at high concentration. Empirically, this variation with the cholesterol/phospholipid molar ratio (C/PL) in each case can well be described by the exponential equation:

$$P = P_{plat} - (P_{plat} - P_{zero}) \exp(-\alpha C/PL) \qquad (1)$$

where P_{plat} is the plateau value reached at high cholesterol content, usually at C/PL > 1; P_{zero} refers to the polarization of the liposome without cholesterol; α is a parameter describing the variation of P by cholesterol and may be called the "cholesterol ordering coefficient". Experimental data for various liposomes were fitted to eq 1. In this procedure, α and P_{plat} were chosen such that χ^2 was at a minimum (see Materials and Methods), whereas P_{zero} was set equal to the measured value for the polarization at zero cholesterol concentration.

Table I: Parameters Describing the Effect of Cholesterol on the DPH Fluorescence Polarization at 25 °C in Liposomes of Individual Phospholipids[a]

phospholipid	$P_{zero} \times 10^3$	$P_{plat} \times 10^3$ fitted	$P_{plat} \times 10^3$ measured	α	χ_r^2
POPC	160 ± 3	369 ± 4	364 ± 3	2.45 ± 0.23	0.8
PLPC	141 ± 7	366 ± 1	359 ± 3	1.84 ± 0.05	3.4
PAPC	132 ± 5	342 ± 8	333 ± 4	1.88 ± 0.23	4.0
PC (egg)	143 ± 4	365 ± 1	355 ± 1	1.93 ± 0.01	2.7
endo-PC[b]	140 ± 4	290 ± 10	278 ± 12	1.90 ± 0.25	0.7
DOPC	136 ± 3	328 ± 4	315 ± 9	1.76 ± 0.28	6.4
DLPC	134 ± 2	291 ± 5	271 ± 2	1.33 ± 0.18	2.2
lyso-PC	205	nd[c]	400	nd	nd
POPE	230 ± 5	355 ± 1	353 ± 2	3.35 ± 0.02	1.2
endo-PE[b]	172 ± 1	283	250	1.61	2.2
PS (brain)	185 ± 3	350 ± 9	341 ± 5	1.66 ± 0.30	0.7
PI (soybean)	170 ± 4	355 ± 7	344 ± 6	1.84 ± 0.29	3.9
DPPC	428 ± 8	nd	409 ± 4	nd	nd
Sph (egg)	427 ± 8	nd	414 ± 4	nd	nd

[a] P_{zero} is the polarization at zero cholesterol content; P_{plat} is the plateau value reached at high cholesterol content, and the cholesterol-ordering coefficient α results from fitting fluorescence polarization data at various cholesterol concentrations to eq 1. χ_r^2 is a measure for the goodness of fit (see Methods and Methods). Measured values of P_{plat} refer to the actually measured data at C/PL = 1.5–2.0. Data are means ± SD of at least three experiments. [b] Endo-PC and endo-PE refer to phospholipids extracted from rat liver endomembranes and purified by thin-layer chromatography (see Materials and Methods). [c] nd, not determined.

Figure 1 shows some representative best-fitting exponential curves on liposomes containing various unsaturated phosphatidylcholines without (Figure 1A) or with 20% egg sphingomyelin (Figure 1B), or containing the total phospholipids isolated from various biological membranes (Figure 1C). The results for single-phospholipid liposomes are compiled in Table I. A reasonably good fit to eq 1 was generally obtained (see also Table VI). In these liposomes, α varied in the phosphatidylcholines from 1.3 for DLPC to 2.5 for POPC and reached in POPE a level of 3.4. The P_{plat} values obtained by computer fitting were generally somewhat higher than the values that were actually measured at a C/PL ratio of around 1.8. Only in two cases (DLPC and endomembrane-derived PE), this difference was rather large, indicating that here the plateau level of polarization was not yet reached at this C/PL ratio. It should be noted that in liposomes containing >10% sphingomyelin or DPPC the measured and fitted P_{plat} values were much closer ($\Delta P < 0.007$; only shown in Figure 1B,C).

Table I furthermore shows high P_{zero} values (around 0.428) for DPPC and sphingomyelin that are in the gel phase at 25 °C, whereas the other phospholipids show much lower P_{zero} values, typical for the liquid-crystalline (fluid) state. In the latter liposomes, the addition of cholesterol increases the P values toward $P_{plat} > P_{zero}$ (condensing or rigidizing effect), but in DPPC and Sph liposomes, cholesterol causes some disordering ($P_{plat} < P_{zero}$; fluidizing effect). Experimental data points in the latter case were rather scattered, so that no reliable α values could be obtained.

Effect of Sphingomyelin or DPPC. It can be seen in Figure 1B, in comparison to Figure 1A, that sphingomyelin at 25 °C increases both P_{zero} and P_{plat} as well as the cholesterol-ordering coefficient α of PC's containing at least one unsaturated fatty acyl chain. Similar results were obtained with the disaturated DPPC, that is also in the gel phase at this temperature. These

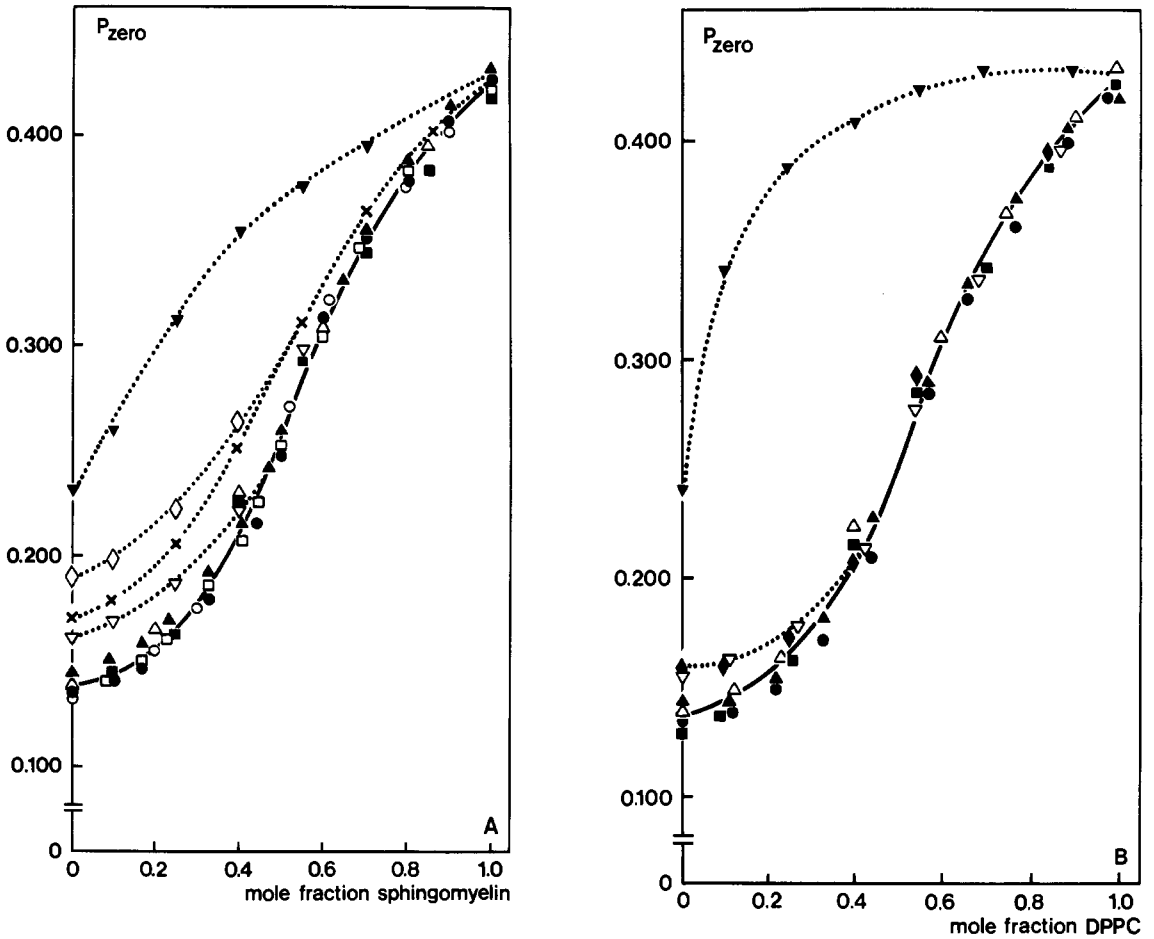

FIGURE 2: Fluorescence polarization of various liposomes at zero cholesterol content (P_{zero}) at 25 °C as a function of the mole fraction of sphingomyelin (panel A) or DPPC (panel B). A best-fitting sigmoidal curve according to eq 2 has been drawn (solid line) through the measured data of egg yolk PC (▲), DOPC (●), DLPC (○), PLPC (□), PAPC (■), and PC purified from rat liver endomembranes (endo-PC; △). Other phospholipids (dotted lines) do not or only partly fit to eq 2. These are POPC (▽), POPE (▼), endo-PE (×), bovine brain PS (◇), and soybean PI (◆).

effects can be quantitatively described for the various phospholipids as follows:

As regards P_{zero} (cholesterol absent), Sph and DPPC exert a similar ordering effect on the various unsaturated phospholipids investigated, as shown in Figure 2A,B. The relation between P_{zero} and the mole fraction of Sph or DPPC, to be denoted as x, generally follows a sigmoidal curve. This may reflect an isothermal liquid-crystalline to gel phase transition determined by the chemical composition, since $x = 0$ (unsaturated phospholipids at 25 °C) corresponds to the liquid-crystalline phase and $x = 1$ (Sph and DPPC at 25 °C) to the gel state. All phosphatidylcholines studied, except POPC, follow exactly the same (solidly drawn) curve with Sph (Figure 2A) as well as with DPPC (Figure 2B). This curve can be accurately described by the function

$$P_{zero} = 0.137 + 0.271x^2/(1 - 1.54x + 1.47x^2) \quad (2)$$

where x is the mole fraction of Sph or DPPC or the fraction of saturated phospholipid molecules (see below and Discussion). This function was constructed as follows: P_{zero} was expressed as a ratio of two quadratic functions of x, so that three conditions were satisfied: (1) $P_{zero} = 0.137$ at $x = 0$, which corresponds to the average value measured for egg PC, DLPC, DOPC, PLCP, PAPC, and endo-PC. (2) $P_{zero} = 0.428$ at $x = 1$, which corresponds to the average value measured for DPPC and sphingomyelin. (3) The slope of P_{zero} at $x = 0$ approaches zero, as is suggested by the data. In accordance with these three conditions, two adjustable parameters are left, and these were determined by minimizing χ^2 (see Materials and Methods). To check whether x (the fraction of saturated phospholipid molecules) or y (the fraction of saturated fatty acyl chains) is the relevant parameter, the same fitting procedure was applied to the data of P_{zero} as a function of y instead of x. This resulted in a curve similar to the one plotted in Figure 2 (solid line), but with a fit worse than the one of eq 2: P_{zero} vs. x gave $\chi_r^2 = 2.0$, whereas P_{zero} vs. y yielded $\chi_r^2 = 85.6$.

It should be noted that eq 2 is valid for the PC's with one or two unsaturated acyl chains, measured for up to four double bonds. Only POPC shows a higher P_{zero} at a fraction of Sph or DPPC below 0.4. Other phospholipids, i.e., PI, PS, and PE, also show higher P_{zero} values than would follow from eq 2, especially at a low amount of Sph or DPPC. POPE deviates most in this respect, especially in combination with DPPC (Figure 2B). The isothermal phase transition for the various PC's could be taken to occur at $x = 0.57$ where the (solid) curves of P_{zero} vs. x (Figure 2A,B) have a point of inflection where the second derivative changes sign.

The plateau value of the fluorescence polarization, P_{plat}, for liposomes at high cholesterol content (see Figure 1) correlates with the fraction of saturated fatty acyl chains, denoted as y, as shown in Figure 3. Phosphatidylcholine from egg yolk as well as the synthetic DOPC, POPC, and PLPC showed in this respect a linear relation with y (the solid line in Figure 3A):

$$P_{plat} = 0.331 + 0.083y \quad (3)$$

The fraction of saturated fatty acyl chains, y, can be varied by the amount of DPPC or Sph to be added to a given (com-

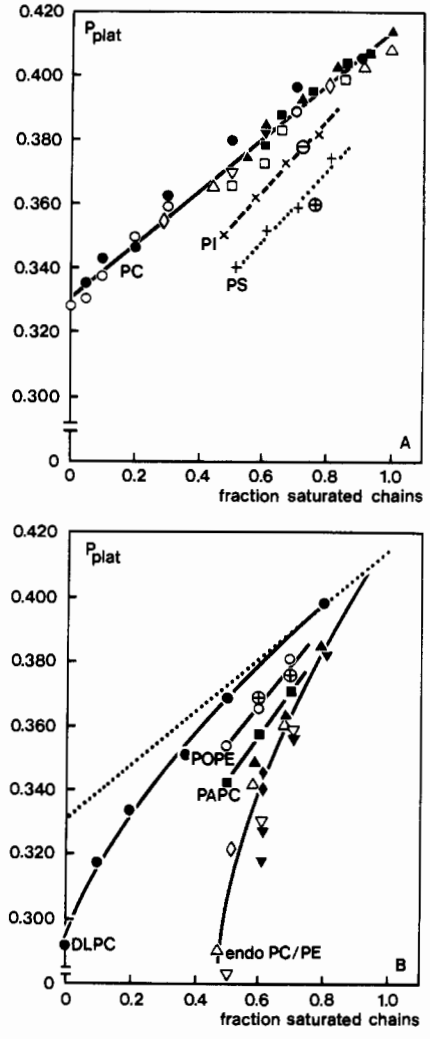

FIGURE 3: P_{plat} vs. the fraction of saturated fatty acyl chains. P_{plat} is the fluorescence polarization plateau value reached at high cholesterol content, following from fitting data points to eq 1. The fraction of saturated chains in the liposomes was varied by the content of DPPC or Sph. The percentage of saturated chains in nonsynthetic phospholipids is indicated in Table V. The Sph (from egg yolk) molecule was approximated to have two saturated chains (see Discussion). (A) A best-fitting straight line (solid) according to eq 3 (see text) has been drawn through the data for egg yolk PC (△, ▲), DOPC (○, ●), PLPC (□, ■), POPC (▽, ▼), and 1:1 DOPC/PLPC (◇). Closed symbols refer to the various phospholipids mixed with Sph in various ratios. Open symbols refer to the phospholipids with or without DPPC. A different line (dashed) has been drawn through the data for PI with or without DPPC (×) or with Sph (⊖) and another one through the data for PS (dotted line) with or without DPPC (+) or with Sph (⊕). (B) Other phospholipids, with a higher degree of acyl chain polyunsaturation, not fitting the straight line according to eq 3 (shown here dotted): DLPC (●) and PAPC (■) with or without Sph; endo-PC (△, ▲), endo-PE (▽, ▼), and the total phospholipids purified from rat liver endomembranes (◇, ◆) mixed with or without DPPC (open symbols) or with Sph (closed symbols). POPE mixed with or without Sph (○) or with DPPC (⊕).

bination of) phospholipid(s). DPPC and Sph (from egg yolk, containing 97% saturated fatty acyls) were both assumed to represent two saturated chains (see Discussion). The fraction of saturated chains in the natural phospholipids is indicated in Table IV. Equation 3 follows from fitting 34 data points to a linear function, minimizing χ^2. Here y is the relevant parameter (gives the better fit) rather than x (=fraction of saturated molecules); the χ_r^2 between P_{plat} and y equals 1.4, whereas that between P_{plat} and x amounts to 6.4.

Phosphatidylcholines with a higher degree of unsaturation (DLPC, PAPC, and PC purified from rat liver endomembranes) appear no longer to fit to eq 3, as is shown in Figure 3B (the eq 3 curve is shown dotted here). Especially endo-PC, endo-PE, or the total endophospholipids show extremely low P_{plat} values. POPE (Figure 3B) and PI and PS (Figure 3A) also show lower P_{plat} levels than the PC's described by eq 3. There is no striking difference between the effects of DPPC and Sph on the P_{plat} values of the various phospholipids. For a few PC's, there is only a tendency of slightly higher P_{plat} values in combination with Sph.

Table II: Cholesterol-Ordering Coefficients (α) at 25 °C in Liposomes without and with Various Contents of Sphingomyelin or Dipalmitoylphosphatidylcholine[a]

phospholipids	without Sph or DPPC	with sphingomyelin 20%	30%	40%	50%	with DPPC 20%	30%	40%
POPC	2.45	2.85						
egg PC	1.93	2.93	3.29		3.74			
endo-PC	1.90	2.32		4.38		2.16		4.36
PLPC	1.84	2.89			3.46	2.83	2.83	
PAPC	1.88	3.16		5.14				
total endo-PL[b]	1.77	2.95						
DOPC	1.76	2.60	3.19		3.61	2.72	2.51	
PS (brain)	1.66	2.79						
endo-PE	1.61	3.17		4.92		3.08		3.24
DLPC	1.33	2.46						

[a] α results from fitting fluorescence polarization data at various cholesterol concentrations to eq 1, by which it is defined. The goodness of fit is in all cases similar to that shown in Table I. [b] Endo-PL is the total phospholipids extracted and purified from rat liver endomembranes.

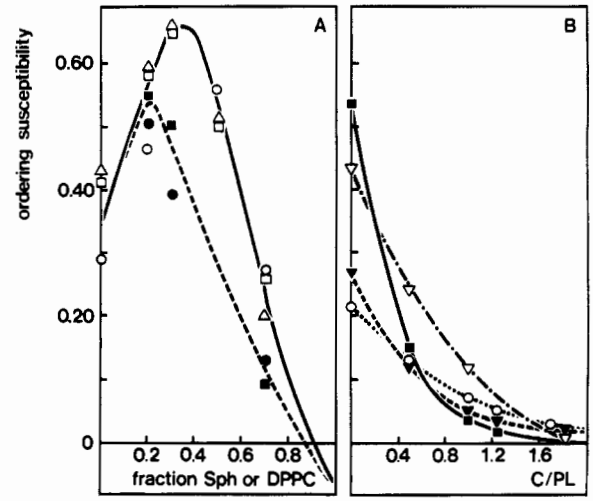

FIGURE 4: Cholesterol-ordering susceptibilities Δ_0 and Δ (defined by eq 4a and 4b, respectively) of various lipid compositions. (A) Δ_0 as a function of the mole fraction of Sph (open symbols; solid line) or DPPC (closed symbols; dashed line): DOPC (○, ●), PLPC (□, ■), and egg yolk PC (△). (B) Δ as a function of the C/PL molar ratio. Symbols denote the same phospholipid compositions as in Figure 1: (■—■) 8:2 POPC/sphingomyelin; (○···○) DLPC; (▽-·-▽) lipids from rat liver plasma membranes; (▼---▼) lipids from rat liver endomembranes.

Table II shows how the cholesterol-ordering coefficients α (defined by eq 1) of unsaturated phospholipids are increased by adding Sph or DPPC. With 20% of these compounds, the α values generally increased at least 1 unit, except for POPC and endo-PC where the elevation was found to be moderate. It furthermore appears that the level of α increases with increasing Sph content (measured up to 50%). With DPPC, the maximal level of α seems, in most cases, to have been reached already at a content of 20% (see also below and Figure 4A).

Cholesterol-Ordering Susceptibility. Cholesterol orders the lipid bilayer in the liquid-crystalline phase and fluidizes it in

the gel phase (Kawato et al., 1978; see above, Figure 1, and Table I). This dual effect of cholesterol is implicit in eq 1 as can be shown as follows. Taking the derivative in eq 1 to C/PL, we have for C/PL → 0:

$$\Delta_0 = \alpha(P_{plat} - P_{zero}) \quad (4a)$$

where Δ_0 represents the initial cholesterol-induced polarization change (starting at zero cholesterol content) and may be called the "ordering susceptibility" of the phospholipids toward cholesterol. In most cases, P_{plat} is larger than P_{zero} (Table I), so that Δ_0 is positive, which means that cholesterol has an ordering effect. However, for phospholipids in the gel state, P_{plat} is smaller than P_{zero}. Hence, Δ_0 is negative, which means that cholesterol has a fluidizing effect. This is the case for Sph or DPPC liposomes at 25 °C (see Table I). Figure 4A shows how the ordering susceptibility, Δ_0, varies with the fraction of Sph or DPPC in liposomes. At a low content of Sph (≤30%) or DPPC (≤20%), Δ_0 increases due to the increase in α (Table II). However, at a higher content of these compounds, Δ_0 decreases again because of a decreasing $P_{plat} - P_{zero}$ value and becomes negative at about 90% Sph or DPPC. The exact values of Δ_0 at these high contents of saturated phospholipids are not known, because of the uncertainty in α (see above).

Alternatively, eq 4a can be slightly modified in

$$\Delta = \alpha(P_{plat} - P_{C/PL}) \quad (4b)$$

where $P_{C/PL}$ denotes the polarization at a given cholesterol/phospholipid molar ratio and Δ represents the ordering susceptibility (polarization change) at this C/PL ratio when more cholesterol is added. This follows from eq 1 by taking the derivative to C/PL and eliminating $\exp(-\alpha C/PL)$, using eq 1 rewritten as $\exp(-\alpha C/PL) = (P_{plat} - P_{C/PL})/(P_{plat} - P_{zero})$. From the curves in Figure 1, it can already be seen that the ordering susceptibility of lipids toward cholesterol decreases exponentially with increasing C/PL and approaches zero at a C/PL of 1.5–2.0, according to $\Delta = \Delta_0 \exp(-\alpha C/PL)$. The maximal value of Δ equals Δ_0. For some of the lipid compositions of Figure 1, the data were calculated according to eq 4b and are shown as Δ vs. C/PL plots in Figure 4B. It is clear that 8:2 POPC/Sph liposomes have a relatively high susceptibility to be ordered by cholesterol but that Δ declines sharply at increasing C/PL. In contrast, DLPC liposomes have an extremely low ordering susceptibility (due to low P_{plat} and α values) and show only a shallow decline of Δ with increasing C/PL.

More Complex Mixtures of Lipids: Effect of Endophospholipids. The question can be asked whether more complex liposomes follow the same rules as described above. In other words, can P values of such liposomes (of known composition) be predicted from the behavior of the individual components? Table III shows a variety of phospholipid compositions (chosen rather arbitrarily) for which P_{zero} and P_{plat} were calculated on the basis of data for the individual components (derived from Figures 2 and 3) as weighted averages. These calculated values differed ≤0.008 from the experimental data in all cases, except for the P_{plat} values of liposomes containing endophospholipids, i.e., purified PC or PE from rat liver endomembranes, which contain a high amount of the polyunsaturated fatty acids 20:4 and 22:6 (see Table V). Experimental P_{plat} values for the latter liposomes were 0.018–0.033 below the calculated data. These low experimental values can be explained as a (partial) lateral phase separation in the liposomal membranes in which the endophospholipids create domains that are relatively poor in cholesterol.

Table III: Experimental and Calculated P_{zero} and P_{plat} Values in Liposomes Consisting of Various Mixtures of Unsaturated Phospholipids without or with Sphingomyelin[a]

composition of phospholipids	ratio	P_{zero} exptl	P_{zero} calcd	P_{plat} exptl	P_{plat} calcd
POPC/PAPC/Sph	7:1:2	0.171	0.175	0.379	0.377
POPC/PLCP/Sph	7:1:2	0.177	0.175	0.380	0.380
POPC/PLPC/PAPC	6:3:1	0.159	0.152	0.369	0.369
POPC/PLPC/PAPC/Sph	48:24:8:20	0.173	0.166	0.374	0.378
egg PC/PAPC	1:1	0.142	0.138	0.346	0.353
	8:2	0.143	0.140	0.359	0.360
egg PC/PAPC/Sph	55:25:20	0.163	0.158	0.363	0.367
	3:5:2	0.160	0.158	0.360	0.362
egg PC/DLPC/Sph	55:15:30	0.184	0.180	0.378	0.376
	4:4:2	0.157	0.158	0.359	0.362
endo-PC/egg PC	1:1	0.142	0.140	0.309	0.327
endo-PC/egg PC/Sph	4:4:2	0.152	0.160	0.343	0.362
	5:75:20	0.178	0.173	0.348	0.370
endo-PC/POPE/Sph	5:5:2	0.200	0.203	0.322	0.349
endo-PE/egg PC	2:5	0.158	0.160	0.308	0.341
endo-PE/egg PC/Sph	2:5:2	0.180	0.177	0.341	0.363
	5:75:20	0.182	0.176	0.350	0.369

[a] Calculated values were derived from Figures 2 and 3 as a weighted average of the individual phospholipids with or without sphingomyelin. Experimental values were actually measured (P_{zero}) or fitted according to eq 1 (P_{plat}), like in Figure 1 and Table I.

The significant effect of endophospholipids on the lipid ordering by cholesterol could also be measured more directly by comparing liposomes without and with a small percentage of these endo compounds. For instance, addition of 10% endo-PC to egg PC/cholesterol (0.4 and 1.5 M/M) decreased the P values of the liposomes by as much as 0.032 and 0.045, respectively. Inclusion of as little as 2% endo-PC or endo-PE in various egg PC/Sph/cholesterol liposomes still caused a detectable decrease (≥0.005) in the polarization (data not shown).

Application to Biological Membranes. The results presented above and illustrated in Figures 1–3 can in principle be combined to predict the DPH fluorescence polarization at 25 °C in a biological membrane of known lipid composition, or at least in liposomes prepared from its total lipid extract. By comparing the predicted (calculated) P value with the P value actually measured, we get an idea whether extrapolation of data from relatively simple systems to bulk (or mean) lipid fluidity of a complex membrane is valid. To this end, we need to know for such a membrane P_{zero}, P_{plat}, α, and the C/PL molar ratio, so that the P value can be calculated according to eq 1. For simple liposomes such as egg yolk PC/Sph/cholesterol mixtures, the procedure is illustrated in Figure 5 and can be summarized as follows: (1) Calculate P_{zero} from x (fraction of Sph or DPPC molecules) by using eq 2, or read its value from Figure 2. (2) Calculate P_{plat} from y (fraction of saturated acyl chains) by using eq 3, or read its values from Figure 3. (3) Substituted these P_{plat}, P_{zero}, the known C/PL ratio, and α value (Tables I and II) into eq 1 to obtain the polarization P (illustrated in Figure 5). For complex mixtures of phospholipids, such as in biological membranes, not only are P_{zero} and P_{plat} determined by eq 2 and 3 or the solidly drawn curves in Figures 2 and 3A, respectively, but also the other phospholipids, represented by the other curves in Figures 2 and 3, have a proportional (weighted) contribution.

Table V lists the calculated P_{zero} and P_{plat} values of liposomes of the total phospholipids extracted from six types of biological membranes, making use of the known lipid composition of these membranes, a simplified survey of which is given in Table IV. A few approximations were made (see footnote of Table V), and the calculation was based on only the major membrane phospholipids (PC, PE, PS and Sph). Table V shows that the calculated values are generally in good agreement with the

Table IV: Simplified Survey of Contents of Major Phospholipids of Isolated Biomembranes and Fatty Acids of Individual Phospholipids, as Relevant To Calculate Data in the Present Study

	phospholipids (%)				fatty acids (%)									
					18:1		18:2		20:4		22:6		saturated	
type of membrane	PC	PE	PS	Sph	PC	PE	PC	PE	PC	PE	PC	PE	PC	PE
human erythrocyte ghosts	30	28	16	26	17	16	22	6	5	19	2	9	50	39
mouse erythrocyte ghosts[a]	54	24	7	10	20[a]		10[a]		19[a]		8[a]		53[a]	
rat liver plasma membranes[b]	30	19	15	23	8	7	19	13	17	23	4	7	47	41
rat liver endomembranes	66	25	4	2	6	5	14	10	25	27	5	10	48	51
GRSL plasma membranes	49	34	10	1	19	15	23	19	3	17	2	11	46	27
GRSL extracellular vesicles[c]	39	34	10	9	14	11	14	14	4	22	2	17	60	25
phospholipid														
PC (egg yolk)					26.5		15		4		2		44	
PS (bovine brain)					36						7		51	
PI (soybean)					6		43						47	

[a] Erythrocytes were from mice bearing the GRSL ascites lymphoma; the fatty acid data in this case refer to the total phospholipids. [b] Data obtained from Van Hoeven et al. (1972, 1975). [c] Data obtained from Van Blitterswijk et al. (1982).

Table V: Calculated and Measured P_{zero} and P_{plat} Values of Liposomes Containing Total Phospholipids Extracted from Biomembranes[a]

	P_{zero}		P_{plat}			
type of biomembrane	calcd	measured	calcd	measured (fitted)	α	χ_r^2
human erythrocyte ghosts	0.194	0.192	0.345	0.344	2.20	0.5
mouse erythrocyte ghosts	0.167	0.171	0.334	0.331	2.37	0.6
rat liver plasma membranes	0.182	0.169	0.335	0.332	2.61	0.1
rat liver endomembranes	0.149	0.146	0.300	0.316	1.77	1.0
GRSL plasma membranes	0.156	0.159	0.333	0.323	2.26	2.1
GRSL extracellular vesicles	0.201	0.210	0.337	0.343	2.34	1.7

[a] Measured (fitted) P_{plat} and α values were determined by fitting fluorescence polarization data at various cholesterol concentrations to eq 1, in the same way as shown in Figure 1 and Table I. Calculated P_{zero} and P_{plat} values were obtained from the individual contributions (weighted averages) of only the major membrane phospholipids (PC, PE, PS, and Sph) as an approximation, making use of the data presented in Tables I and V and Figures 2 and 3. Since the contents of the fatty acids 20:4 and 22:6 are important for the level of P_{plat} (see Figure 3B), and PC's from human erythrocytes, GRSL plasma membranes, and extracellular vesicles are almost equal to egg yolk PC in this respect, P_{plat} values of egg yolk PC (following the solid straight line in Figure 3A) were taken as an approximation for the PC's in these membranes. PC's from rat liver plasma membranes and mouse erythrocytes, however, were approximated by P_{plat} values of egg PC/endo-PC in a 1:2 ratio. A percentage of saturated acyl chains of >50% in PC was assumed to represent disaturated (DPPC-like) molecules and was as such taken into account, together with % Sph, to calculate P_{zero}. Contributions of PE and PS were approximated by the P_{zero} and P_{plat} values of endo-PE and bovine brain PS, respectively. χ_r^2 is a measure for the goodness of fit (see Materials and Methods).

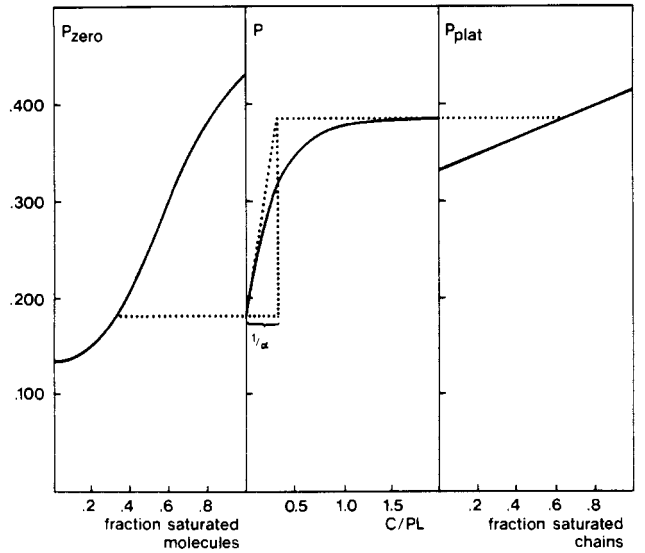

FIGURE 5: Combination of the essential parts of Figures 1–3, showing how the DPH fluorescence polarization in a membrane with a given cholesterol/phospholipid (C/PL) molar ratio is determined. The left-hand panel shows the solid curve of Figure 2, and in the right-hand panel, the solid line of Figure 3A is reproduced. In the middle panel, an example of a P variation with C/PL (such as in Figure 1) is shown, which according to eq 1 can be described by $P = 0.384 - 0.202 \exp(-3.29C/PL)$ and corresponds to a mixture of egg yolk PC/Sph, 7:3. In addition, α, or rather $1/\alpha$, is visualized here.

experimental data. The largest differences are seen for the P_{zero} of liver plasma membranes (0.013) and for the P_{plat} of liver endomembranes (0.016). As already shown for a few cases in Figure 1C, the experimental P values of the total lipids from biomembranes as a function of the C/PL ratio fit very well to eq 1. The α values thus obtained do not differ much among the five plasma membrane derived liposomes listed in Table V (mean value: $\alpha = 2.4$), but for the endomembranes, α is lower, due to the lack of Sph and the high degree of fatty acyl polyunsaturation (see also Tables I, II, and IV).

In Table VI, it is shown that the P values predicted (calculated) for liposomes prepared from the total lipid extracts of the various biological membranes are in reasonably good agreement with the values actually measured. In the calculation according to eq 1, the measured C/PL ratios as listed in Table VI were used, together with the calculated P_{zero}, P_{plat}, and α values listed in Table V. Table VI also shows that the P values measured in the isolated native membranes are in all cases higher than in the total lipid liposomes. This difference, which we attribute to intrinsic membrane proteins, is much larger for the liver endomembranes than for the other (plasma) membranes. The intrinsic membrane proteins of mitochondria and (rough) endoplasmic reticulum are apparently present in sufficiently high amounts to cause this large ordering effect in the endomembrane fraction.

In Table VI, the P values measured in the total lipid liposomes are also compared to the maximal levels (P_{plat}) that would be reached by increasing the cholesterol content, following exponential curves as shown in Figure 1C. In the membrane preparations from erythrocytes and liver, the P values were 0.024–0.035 below P_{plat}. However, in GRSL plasma membranes, the difference amounted to as much as 0.069, which is indicative of the relative deficiency of mem-

Table VI: DPH Fluorescence Polarization (P, at 25 °C) Measured in Various Types of Isolated Native Biomembranes and in Liposomes of Their Total Lipid Extracts, in Comparison with Predicted (Calculated) Values for These Liposomes and Maximal Levels (P_{plat}) Measurable by Increasing the Cholesterol Content[a]

	native membranes		liposomes of lipid extracts		
			P (total membrane lipids)		
type of biomembrane	C/PL	P, measured	measured	calcd	P_{plat}, measured
human erythrocyte ghosts	0.82	0.332	0.318	0.320	0.342
mouse erythrocyte ghosts	0.78	0.313	0.302	0.308	0.328
rat liver plasma membranes	0.65	0.322	0.302	0.307	0.330
rat liver endomembranes	0.10	0.196[b]	0.166[b]	0.173	0.301
GRSL plasma membranes	0.40	0.268	0.251	0.261	0.320
GRSL extracellular vesicles	1.10	0.334	0.328	0.327	0.343

[a] Predicted P values were calculated according to eq 1 by using the calculated P_{zero}, P_{plat}, and α values listed in Table VI. P_{plat} values were actually measured at a C/PL ratio of 1.5–2.0; note that these measured P_{plat} values differ ≤0.003 from the fitted values (Table VI), except in the case of endomembranes. [b] Due to contamination with (mainly) neutral triglycerides which rendered the native endomembranes a somewhat lower P value than published previously (Van Hoeven et al., 1979), the membrane total phospholipids and cholesterol were first purified by thin-layer chromatography (see Materials and Methods) and subsequently mixed for liposome preparation.

brane cholesterol in these intraperitoneally growing (ascites) leukemia cells (Van Blitterswijk et al., 1985). In contrast, extracellular membrane vesicles which were shed from the surface of these GRSL cells into the ascites fluid have a high C/PL ratio (1.10) (Van Blitterswijk et al., 1979, 1982) and show a P value (measured in the liposomes) that is already quite close (0.015) to P_{plat}. Finally, it may be noteworthy that radially from endomembranes → plasma membranes → extracellularly shed membrane vesicles, both P and P_{plat} as well the intrinsic C/PL ratio increase.

Discussion

Steady-state fluorescence polarization using the apolar probe DPH is a rapid and powerful technique to determine the overall lipid structural order in membranes. A high structural order implies a high degree of packing or a high mutual affinity among the lipids, mainly due to relatively strong interactions between the apolar moieties. This paper represents a systematic quantitative study of the lipid features that determine structural order (P values) in membranes composed of various combinations of lipids.

In mixtures of phospholipids, the structural order in the absence of cholesterol (P_{zero}) at 25 °C increases curvilinearly with the fraction of Sph or the disaturated DPPC. All unsaturated phospholipids studied, except POPE, show a similar S-shaped curve (Figure 2) that may be considered as an isothermal phase transition induced by varying the mole fraction of Sph or DPPC. One could argue that the somewhat anomalous behavior of POPE, also with respect to ordering by cholesterol (high α value; Table I), may be possibly related to its existence in a nonbilayer phase, in the absence of, or when sequestrated from other phospholipids (Cullis & De Kruyff, 1979). However, the highly unsaturated endo-PE, which may be expected to have a similar preference for the hexagonal phase, does not show this behavior. Moreover, label studies employing NMR and ESR techniques have revealed little difference in order parameters between bilayer and nonbilayer lipids (Cullis & De Kruyff, 1979).

Cholesterol acts as a buffer to large changes in the bulk physical properties in the membrane imposed by the other lipids. Phospholipids in the gel phase are disordered by cholesterol. In the liquid-crystalline phase, cholesterol has an ordering effect that can be described by an exponential function (eq 1). We have described in detail how this function is determined by the individual phospholipids. The ordering effect of cholesterol is strong at low cholesterol content and levels off at high cholesterol content (C/PL > 1) where the fluorescence polarization reaches a maximum (P_{plat}). Cholesterol-rich codispersions of cholesterol and phospholipids may under certain conditions (storage) be metastable and may release cholesterol, but only very slowly (Collins & Phillips, 1982), so that this effect will not generally influence our results. We only occasionally observed irregularities in the P vs. C/PL curves at the plateau level (not shown). At high C/PL ratios (at P_{plat}), it is likely that cholesterol is clustered in domains (Gibert et al., 1975) in a form that fails to influence membrane fluidity or, alternatively, that may exclude DPH (Cooper et al., 1978). The latter possibility is supported by our observation that the total fluorescence intensity, like the P value, increases exponentially toward a maximal level with increasing cholesterol content (not shown). This would mean that clusters of cholesterol in membranes, occurring at C/PL > 1, are not represented by the motions of the DPH probe. An alternative explanation for the occurrence of P_{plat} is offered by Hoffmann et al. (1981). They suggest that as soon as one (rigid) cholesterol molecule is sufficiently close to a DPH molecule, the motion of this probe is affected drastically, so much that the proximity of more cholesterol molecules becomes irrelevant. P_{plat} is then reached when every DPH molecule is associated with at least one cholesterol molecule.

The cholesterol-ordering coefficient α, defined by eq 1, was shown to increase with increasing Sph or DPPC (Table II), which means that the ordering capacity of cholesterol increases by addition of these compounds, or that in lipid bilayers in the fluid state cholesterol has a relatively high affinity to Sph and DPPC. The latter conclusion is in agreement with published results of calorimetric and cholesterol transfer (equilibrium) studies (Demel et al., 1977; Lange et al., 1979; Nakagawa et al., 1979). Further support for this conclusion is obtained from the following reinterpretation of the probability theory of Hoffmann et al. (1981):

Hoffmann et al. (1981) have constructed a theory based upon the assumption that only one cholesterol molecule needs to be adjacent to a DPH molecule (or a phospholipid acyl chain) to have a large effect on its motion. The change in DPH motion (polarization) then depends upon the probability p_c of all positions next to a fatty acyl chain being free of cholesterol molecules. It was calculated that $p_c = \exp(-Mq)$, in which q is related to cholesterol concentration and M is the number of DPH molecules (or fatty acyl chains) that cholesterol is able to accommodate around its circumference. This exponential expression shows some similarity to our eq 1 and gives a good fit to the experimental data of Hoffmann et al. (1981). From their formulas 1, 3, and 5, we calculate (see Appendix) Δ_0, the derivative of the polarization to C/PL, for C/PL → 0:

$$\Delta_0 = M(P_{plat} - P_{zero})(3 - P_{zero})/2(3 - P_{plat}) \quad (5)$$

From comparison with our eq 4a, it is evident that M is related to α according to

$$M = 2\alpha(3 - P_{plat})/(3 - P_{zero}) \approx 1.875\alpha \quad (6)$$

The latter approximation was obtained by substituting the P_{plat} and P_{zero} values for all types of liposomes presently studied. It appears that $M/\alpha = 1.875 \pm 0.026$. As a result, M follows the trend displayed for α in Table II and is not a constant, in contrast to the prediction of Hoffmann et al. (1981) that $M \approx 6$. We find, for example, that M is small for DLPC (2.5) and endophospholipids (3.3), larger for POPC (4.5), and largest (>5) for phospholipids in the presence of Sph or DPPC. Considered in the context of this theory, the high M values in the presence of Sph or DPPC may indicate that cholesterol associates strongly with Sph or DPPC molecules to form a larger entity that can interact with more DPH molecules (or fatty acyl chains). This interpretation is consistent with the ordering susceptibility concept, according to which the susceptibility Δ_0 (defined by eq 4a) of phospholipids to be ordered by cholesterol was shown to increase by Sph and DPPC. This increases of Δ_0, however, reached a maximum at 20% DPPC or at 30% Sph and then decreased to zero and even to a negative value (disordering or fluidization by cholesterol) above 90% of these compounds, where the lipid dispersion is in the gel phase at 25 °C (Figure 4A).

The sphingomyelin used in the present study was purified from egg yolk and was found to contain about 97% saturated fatty acids (24:0, 22:0, and 16:0) and 3% nervonic acid (24:1). In mammalian cell membranes, the amount of 24:1 is much higher (up to 55%; Barenholz, 1984). We have examined the possibility that such types of Sph might behave differently in the present type of studies. Sph from bovine brain, containing 31% 24:1, gave the same P_{plat} values as egg Sph, whereas P_{zero} values below 50% Sph were also the same as with egg Sph. Only above 50% brain Sph (in combination with egg PC), we found P_{zero} values 0.020–0.035 below those obtained with egg Sph (results not illustrated). The cis-double bond of the nervonic acyl chain is located between carbon atoms 15 and 16, apparently too far from the polar head group region to affect ("feel") the interaction with cholesterol (Darke et al., 1972). Furthermore, the sphingosyl residue has a trans-double bond between carbon atoms 4 and 5 and has a similar cross-sectional area and lipid-packing property as the saturated acyl chains of DPPC. It can thus be understood that Sph and DPPC behave similar in the lipid ordering of fluid membranes as presently demonstrated (Figures 2 and 3).

In the absence of Sph and DPPC, the susceptibility Δ_0 of the various individual phospholipids to be ordered by cholesterol can be calculated (by using eq 4a and data of Table I) and appears to decrease in the order POPC ($\Delta_0 = 0.59$) > egg PC (0.43) > PLPC, POPE (0.42) > PAPC (0.39) > DOPC, soybean PI (0.34) > endo-PC (0.28) > brain PS (0.27) > DLPC (0.21) > endo-PE (0.18). Our results confirm and extend the early findings of Demel et al. (1977) on the preferential interaction (affinity) of cholesterol with (to) different classes of phospholipids. Phospholipids from rat liver endomembranes have relatively large amounts of 20:4 and 22:6 fatty acyl chains, presumably located in the *sn*-2 position (Table IV). These endophospholipids appear to have a relatively low ordering susceptibility (affinity) toward cholesterol. This finding as well as the low Δ_0 value for DLPC is in agreement with the notion that addition of cholesterol to 16:0/22:6 PC or to PC's with two polyunsaturated fatty acyl chains neither induces a substantial lipid condensation nor alters liposome permeability (Stubbs & Smith, 1984; Demel et al., 1972). Stubbs and Smith (1984) have reviewed the effects of increasing the number of cis-double bonds on the packing and motions of the phospholipid acyl chains in model membranes. Their computer modeling study suggests a possible conformation of the 18:0/22:6 PC in which the polyunsaturated chain is approximately helical and its effective length is markedly reduced. It is conceivable that such a conformation has a minimal affinity to the rigid sterol nucleus.

The very existence of preferential associations of cholesterol with certain phospholipids while less with others would suggest that, in biomembranes, phase separation into (micro)domains of distinct lipid composition is likely to occur. Lentz et al. (1976) have demonstrated that the DPH probe weights equally fluid and less fluid regions. Therefore, the steady-state P values only represent the overall fluidity and do not resolve possible domains. Nevertheless, our results have indirectly demonstrated the existence of extremely fluid microdomains devoid of cholesterol in liposomes containing the highly polyunsaturated endophospholipids (see Results, Table III). Such phospholipids, containing 20:4 and 22:6 at the *sn*-2 position, are also important components of the plasma membrane. Arachidonoyl (20:4) PC (and possibly also 22:6 PC) is a preferential substrate for the membrane-bound phospholipase A_2 in situ (Van den Bosch, 1981; Kannagi et al., 1981; Irvine, 1982; Storch & Schachter, 1985). It may be suggested that this selectivity is (perhaps partly) based on the high lipid fluidity or lipid disarrangement (Kannagi et al., 1981) in the microdomain of the substrate, in which the active site of the enzyme may penetrate more easily to reach the ester bound. This possibility is supported by monolayer studies [reviewed by Verger & de Haas (1976)] showing that the penetration and activity of phospholipase A_2 are inversely related to the tightness of phospholipid packing. Once the enzymatic reaction in the membrane has taken place and the free polyunsaturated fatty acid has subsequently been released, the remaining lyso compound (having much higher P_{zero} and P_{plat} values; see Table I) will lead to rigidization of the domain, as has indeed been shown experimentally (Storch & Schachter, 1985).

Given the structural complexity of lipids in biomembranes (lateral heterogeneity, asymmetry, etc.), the predicted overall fluidity (P values) in their total lipid liposomes, on the basis of the presently described behavior of the individual lipids, is in surprisingly good agreement with the measured data (Tables V and VI). It is also remarkable that, given the rather extreme P_{zero} and P_{plat} values that can be reached in simple artificial systems, the overall structural order in the various types of plasma membrane studied here and elsewhere (Schachter, 1984; Van Blitterswijk, 1985) falls within a rather narrow range of values, apparently optimal for proper functioning.

Plasma membranes are generally characterized by a high C/PL molar ratio relative to the cellular endomembranes. Cholesterol is synthesized on intracellular membranes where membrane cholesterol is low and transported to the plasma membrane where cholesterol content is high. This transfer is a unidirectional process (Lange & Matthies, 1984). How and why this occurs have not yet been understood. Yeagle and Young (1986) have recently tackeled this problem and have suggested that membrane lipid composition may play a role in the distribution of cholesterol among the membranes of a cell. Our results confirm this suggestion and provide a quantitative explanation for the preferential association of cholesterol with the plasma membrane. We base this on the differing affinities (ordering susceptibilities, Δ_0) of the phospholipids of plasma membranes and endomembranes toward cholesterol, given the fact that equilibration of cholesterol

in and between membranes is much faster than of phospholipids (Demel & De Kruyff, 1976; Nakagawa et al., 1979; Yeagle, 1985). For rat liver plasma membranes and endomembranes, Δ_0 was calculated (from data of Table V, using eq 4a) to be 0.43 and 0.27, respectively. Our results further indicate that this preference of cholesterol for the plasma membrane is entirely due to its higher sphingomyelin content (Table IV), since an amount of Sph in endomembranes similar to that in the plasma membrane would augment the former's Δ_0 value to about 0.50. We suggest that the disproportionation of cholesterol in the cell is driven and maintained by intracellular segregation (sorting) of sphingomyelin (and probably also glycosphingolipids) into vesicles having a distinct destination in the cell. In other words, in the topogenesis of the plasma membrane, cholesterol would be "dragged along", rather than "targeted" specifically. Segregation of cholesterol has also been shown to occur during the formation (shedding) of extracellular vesicles at the surface of leukemic GRSL cells (Van Blitterswijk et al., 1979, 1982). Here, however, the enrichment of cholesterol in these vesicles relative to the plasma membrane (Table IV) cannot be ascribed to a difference in cholesterol-ordering susceptibility of the phopholipids (Δ_0 = 0.31 and 0.36, respectively) but may be induced by intrinsic viral glycoproteins, as discussed previously (Van Blitterswijk et al., 1979).

In this paper, we have given an extensive description of how the bulk structural order of membrane lipids, as measured by DPH fluorescence polarization, is determined by the molar composition. Our results may explain inconsistencies noted by others as to whether or not certain alterations in phospholipid fatty acyl composition may affect the bulk membrane fluidity. Following this quantitative description of parameters of bulk membrane fluidity, future research should be directed at establishing new methods to detect and quantitate compositionally different membrane domains.

Acknowledgments

We thank Dr. R. P. van Hoeven for his continuous interest and stimulating discussions and Dr. H. L. Ploegh and Dr. D. M. Jameson (Dallas) for reading the manuscript and providing useful comments. We also thank G. G. H. Meijerink for typing the manuscript.

Appendix

Derivation of Equation 5. We can express the DPH fluorescence polarization in terms of the steady-state anisotropy, r (Van Blitterswijk et al., 1981), as

$$P = 3r/(2 + r) \quad \text{and}$$
$$r = r_\infty + (r_0 - r_\infty)/(1 + K) = (r_0 + Kr_\infty)/(1 + K) \quad \text{(A1)}$$

in which r_0 is the theoretically maximal and r_∞ the limiting hindered fluorescence anisotropy, whereas K is the ratio of the fluorescence lifetime to the rotational correlation time, assuming that the anisotropy is governed by a single exponential decay. We can write

$$P = 3(r_0 + r_\infty K)/[2 + r_0 + (2 + r_\infty)K] \quad \text{(A2)}$$

We assume that K is a constant, which is in full agreement with the results of Hoffmann et al. (1981). Then we can derive the cholesterol-ordering susceptibility Δ_0 of phospholipids (see Results) by taking the derivative of eq A2 to C/PL for C/PL $\rightarrow$ 0. By subsequently substituting eq A1, and $r = r_{zero}$ at C/PL $\rightarrow$ 0, we obtain

$$\Delta_0 = \frac{6K}{(1 + K)(2 + r_{zero})^2}\left(\frac{dr_\infty}{d(C/PL)}\right)_{C/PL\rightarrow 0} \quad \text{(A3)}$$

We can write eq 3 of Hoffmann et al. (1981) as

$$r_\infty(q) = r_{\infty_{zero}}p_c(M,q) + r_{\infty_{plat}}[1 - p_c(M,q)] \quad \text{(A4)}$$

in which $p_c(M,q)$ is the probability that there are no cholesterol molecules adjacent to a DPH molecule in a homogeneous phase with the mole fraction of cholesterol c = (C/PL)/[(C/PL) + 1], and $q = c/(2 - c)$, the fraction of lattice sites occupied by cholesterol molecules, compared to the fraction occupied by fatty acyl chains in the lipid bilayer; M is the number of DPH molecules (or fatty acyl chains) that cholesterol is able to accommodate around its circumference. We write eq 5 of Hoffmann et al. (1981) as

$$p_c(M,q) = (1 - q)^M = \left[\frac{2}{(C/PL) + 2}\right]^M \quad \text{(A5)}$$

$$\left[\frac{dp_c}{d(C/PL)}\right]_{C/PL\rightarrow 0} = -M \times 2^M\left[\frac{1}{(C/PL) + 2}\right]^{M+1} = -M/2 \quad \text{(A6)}$$

From eq A3, A4, and A6, we derive

$$\Delta_0 = 3MK(r_{\infty_{plat}} - r_{\infty_{zero}})/(1 + K)(2 + r_{zero})^2 \quad \text{(A7)}$$

Using eq A1 and subsequently the relationship $r = 2P/(3 - P)$, we finally obtain

$$\Delta_0 = M(P_{plat} - P_{zero})(3 - P_{zero})/2(3 - P_{plat}) \quad \text{(A8 = eq 5)}$$

Registry No. C, 57-88-5; DPPC, 2644-64-6; DOPC, 10015-85-7; DLPC, 6542-05-8; POPC, 6753-55-5; PLPC, 6931-84-6; PAPC, 6931-56-2; POPE, 10015-88-0; oleic acid, 112-80-1; linoleic acid, 60-33-3; arachidonic acid, 506-32-1; docosahexaenoic acid, 32839-18-2.

References

Barenholz, Y. (1984) in *Physiology of Membrane Fluidity* (Shinitzky, M., Ed.) Vol. I, pp 131–173, CRC Press, Boca Raton, FL.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Collins, J. J., & Phillips, M. C. (1982) *J. Lipid Res. 23*, 291–298.

Cooper, R. A., Leslie, M. H., Fischkoff, S., Shinitzky, M., & Shattil, S. J. (1978) *Biochemistry 17*, 327–331.

Cossins, A. R., & Prosser, C. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2040–2043.

Cullis, P. R., & De Kruyff, B. (1979) *Biochim. Biophys. Acta 559*, 399–420.

Darke, A., Finer, E. G., Flook, A. G., & Phillips, M. C. (1972) *J. Mol. Biol. 63*, 265–279.

Demel, R. A., & De Kruyff, B. (1976) *Biochim. Biophys. Acta 457*, 109–132.

Demel, R. A., Geurts van Kessel, W. S. M., & Van Deenen, L. L. M. (1972) *Biochim. Biophys. Acta 266*, 26–40.

Demel, R. A., Jansen, J. W. C. M., Van Dijck, P. W. M., & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta 465*, 1–10.

Emmelot, P., Bos, C. J., Van Hoeven, R. P., & Van Blitterswijk, W. J. (1974) *Methods Enzymol. 31*, 75–90.

Folch, J., Lees, M., & Sloane-Stanley, G. H. (1957) *J. Biol. Chem. 226*, 497–509.

Gibert, D. B., Tanford, C., & Reynolds, J. A. (1975) *Biochemistry 14*, 444–448.

Hanahan, D. J., & Ekholm, J. E. (1974) *Methods Enzymol. 31*, 168–172.

Hoffmann, W., Pink, D. A., Restall, C., & Chapman, D. (1981) *Eur. J. Biochem. 114*, 585–589.

Irvine, R. F. (1982) *Biochem. J. 204*, 3–16.
Kannagi, R., Koizumi, K., & Masuda, T. (1981) *J. Biol. Chem. 256*, 1177–1184.
Kawato, S., Kinosita, N., Jr., & Ikegami, A. (1978) *Biochemistry 17*, 5026–5031.
Kinosita, K., Jr., Kawato, S., & Ikegami, A. (1984) *Adv. Biophys. 17*, 147–203.
Lange, Y., & Matthies, H. J. G. (1984) *J. Biol. Chem. 259*, 14624–14630.
Lange, Y., D'Alessandro, J. S., & Small, D. M. (1979) *Biochim. Biophys. Acta 556*, 388–398.
Lentz, B. R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4529–4537.
Morrison, W. R. (1964) *Anal. Biochem. 7*, 218–224.
Nakagawa, Y., Inoue, K., & Nojima, S. (1979) *Biochim. Biophys. Acta 553*, 307–319.
Pottel, H., Van der Meer, B. W., & Herreman, W. (1983) *Biochim. Biophys Acta 730*, 181–186.
Schachter, D. (1984) *Hepatology (Baltimore) 4*, 140–151.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367–394.
Spector, A. A., & Yorek, M. A. (1985) *J. Lipid Res. 26*, 1015–1035.
Storch, J., & Schachter, D. (1985) *Biochim. Biophys. Acta 812*, 473–484.
Stubbs, C. D., & Smith, A. D. (1984) *Biochim. Biophys. Acta 779*, 89–137.
Stubbs, C. D., Kouyama, T., Kinosita, K., Jr., & Ikegami, A. (1981) *Biochemistry 20*, 4257–4262.
Van Blitterswijk, W. J. (1985) in *Membrane Fluidity in Biology* (Aloia, R. C., & Boggs, J., Eds.) Vol. 3, pp 85–159, Academic Press, New York.
Van Blitterswijk, W. J., Emmelot, P., Hilkmann, H. A. M., Oomen-Meulemans, E. P. M., & Inbar, M. (1977) *Biochim. Biophys. Acta 467*, 309–320.
Van Blitterswijk, W. J., Emmelot, P., Hilkmann, H. A. M., Hilgers, J., & Feltkamp, C. A. (1979) *Int. J. Cancer 23*, 62–70.
Van Blitterswijk, W. J., Van Hoeven, R. P., & Van der Meer, B. W. (1981) *Biochim. Biophys. Acta 644*, 323–332.
Van Blitterswijk, W. J., De Veer, G., Krol, J. H., & Emmelot, P. (1982) *Biochim. Biophys. Acta 688*, 495–504.
Van Blitterswijk, W. J., Damen, J., Hilkmann, H., & De Widt, J. (1985) *Biochim. Biophys. Acta 816*, 46–56.
Van den Bosch, H. (1980) *Biochim. Biophys. Acta 604*, 191–246.
Van der Meer, B. W., Van Hoeven, R. P., & Van Blitterswijk, W. J. (1986) *Biochim. Biophys. Acta 854*, 38–44.
Van Hoeven, R. P., & Emmelot, P. (1972) *J. Membr. Biol. 9*, 105–126.
Van Hoeven, R. P., Emmelot, P., Krol, J. H., & Oomen-Meulemans, E. P. M. (1975) *Biochim. Biophys. Acta 380*, 1–11.
Van Hoeven, R. P., Van Blitterswijk, W. J., & Emmelot, P. (1979) *Biochim. Biophys. Acta 551*, 44–54.
Verger, R., & De Haas, G. H. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 77–117.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.
Yeagle, P. L., & Young, J. E. (1986) *J. Biol. Chem. 261*, 8175–8181.