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EFFECTS OF CHOLESTEROL ON ACYL CHAIN DYNAMICS IN MULTILAMELLAR VESICLES OF VARIOUS PHOSPHATIDYLCHOLINES

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Key words: Phosphatidylcholine vesicle; Lipid bilayer; Acyl chain dynamics; Cholesterol; Fluorescent probe; Phase modulation fluorescence

Phase modulation fluorescence spectroscopy was used to investigate the influence of cholesterol (0 to 50 mol%) on acyl chain dynamics in multilamellar vesicles of phosphatidylcholine. Four different phosphatidylcholines (DPPC, DOPC, POPC, and egg PC) and six different fluorescent probes (diphenylhexatriene and five anthroyloxy fatty acids) were employed. We found that: (1) Increased cholesterol content had only slight effects on fluorescence lifetimes of the six probes. (2) Increased cholesterol content increased the steady-state fluorescence anisotropy (r) of all the probes except 16-anthroyloxy palmitate (16-AP) in each of the four phosphatidylcholines. (3) Added cholesterol tended to limit the extent of probe rotation (as reflected by r_∞, the infinite-time anisotropy) to a much greater extent than it altered the rate of probe rotation. (4) The tendency for cholesterol to order the structure of the bilayer was greatest in the proximal half of the acyl chains and diminished toward the center of the bilayer. (5) In some phosphatidylcholines the rotation rates of probes located near the bilayer center (diphenylhexatriene and 16-AP) were apparently increased by increasing levels of cholesterol. (6) In several respects dipalmitoylphosphatidylcholine (DPPC) vesicles responded differently to increased cholesterol than vesicles of the other three phosphatidylcholines. (7) A single second-order equation described the relationship between r_∞ and r for the five anthroyloxy fatty acid probes in the four different phosphatidylcholines over a wide range of cholesterol content. The data for diphenylhexatriene in the different phosphatidylcholines could not be fit by a single equation.

Introduction

The interaction of cholesterol with phospholipids in model and biological membranes has been studied by a number of investigators (for reviews, see Refs. 1 and 2). The view that has emerged is that below the phase transition temperature (T_c) cholesterol hinders the phospholipid acyl chains in achieving the quasi-crystalline order that characterizes the gel state and above T_c cholesterol interferes with the lateral motions of the acyl chains that characterize the liquid-crystalline state [3]. High levels of cholesterol may thus tend to put the acyl chain region of the membrane into a state that is intermediate in some respects between the gel and liquid-crystalline states.

The steroid nucleus is more rigid and bulkier than the side-chain (or tail) of the cholesterol molecule. A number of studies suggest that the part of the phospholipid acyl chain (the first 10 carbons or so) that are overlapped by the steroid nucleus of cholesterol are strongly affected by

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; POPC, 1-palmitoyl-2-oleyl-phosphatidylcholine; egg PC, egg phosphatidylcholine; *n*-AS, *n*-(9-anthroyloxy)-stearate; 16-AP, 16-(9-athroyloxy)-palmitate; T_c, phase transition temperature.

cholesterol in the ways described above, but the portion of the acyl chain that lies deeper in the membrane are less affected by cholesterol [4–6]. The mobility of the acyl chain segments that are adjacent to the tail of cholesterol may not be altered or may even be increased by the presence of cholesterol [4,6].

Unsaturation of the fatty acyl chains of phospholipids may strongly influence their interactions with cholesterol. In studies with phospholipid monolayers [7] addition of cholesterol to certain phosphatidylcholines had a condensing effect, a decrease in the mean area per molecule in the monolayer. Monolayers of certain phosphatidylcholines with one saturated and one unsaturated acyl chain were condensed by cholesterol. Monolayers of phosphatidylcholines with two saturated or two unsaturated acyl chains did not show the condensing effect when cholesterol was added. A model of cholesterol/phospholipid interactions that is consistent with these observations has been proposed [8] in which the β surface of the steroid nucleus interacts preferentially with the 'pocket' above the $\Delta_{9,10}$ double bond of a typical unsaturated fatty acid of animal origin, while the α face of the steroid nucleus interacts preferentially with fully saturated acyl chains.

Time-resolved fluorescence spectroscopy of diphenylhexatriene (DPH) in phosphatidylcholine multilamellar vesicles (MLV) suggested that addition of cholesterol diminished the extent of rotation of diphenylhexatriene to a greater extent than it altered the rate of diphenylhexatriene rotation [9,10].

The major purpose of the present study is to investigate the influence of phospholipid acyl chain unsaturation on cholesterol/phospholipid interactions in lipid bilayers. The effects of cholesterol in multilamellar vesicles of phosphatidylcholines (PC) with two saturated acyl chains (dipalmitoyl PC), two unsaturated acyl chains (dioleyl PC), one saturated and one unsaturated acyl chain (1-palmitoyl-2-oleyl PC), and mixed acyl chain composition (egg PC) were investigated. The anthroyloxy fatty acids, 2-, 7-, 9-, and 12-anthroyloxy stearate and 16-anthroyloxy palmitate and diphenylhexatriene were used as probes of cholesterol's effects of different levels in the lipid bilayer. Phase modulation fluorescence spectroscopy was employed to resolved effects of cholesterol on structural constraints to probe rotation from effects on viscous resistance to probe rotation. Our results with diphenylhexatriene are consistent with the large body of data on diphenylhexatriene in lipid vesicles, with and without cholesterol. Our results with the anthroyloxy fatty acids are new and contribute to our understanding of the interaction of cholesterol with neighboring fatty acyl chains at various depths in the bilayer.

Materials and Methods

Chemicals

The phosphatidylcholines used were purchased from Avanti Polar Lipids (Birmingham, AL). Some of the egg PC used was obtained from Makor Chemicals, Ltd. (Jerusalem, Israel). All the phosphatidylcholines gave single spots on heavily-loaded silica gel chromatograms developed with chloroform/methanol/water (65 : 25 : 4, v/v). Cholesterol was obtained from Calbiochem-Behring Corp. (San Diego, CA). Stock solutions of cholesterol (10 mg/ml) were prepared in 'lipopure' chloroform from Applied Science Laboratories (State College, PA). 1,6-Diphenyl-1,3,5-hexatriene was obtained from Koch-Light Laboratories (Colnbrook, U.K.) and from Aldrich Chemical Co. (Milwaukee, WI). Stock solutions of diphenylhexatriene were prepared with tetrahydrofuran from J.T. Baker Chemical Co. (Phillipsburg, NJ). The *n*-(9-athroyloxy)-stearates), where $n =$ 2, 7, 9 and 12 and 16-(9-anthroyloxypalmitate) were obtained from Molecular Probes, Inc. (Junction City, OR). Stock solutions of the anthroyloxy fatty acids (0.5 mM) were made in 'lipopure' methanol. All the fluorescent probes gave single spots on heavily-loaded silica gel thin-layer chromatograms developed with chloroform/methanol/water (25 : 20 : 1, v/v), chloroform/methanol (95 : 5, v/v) and hexane/chloroform/methanol (5 : 5 : 1, v/v).

Preparation of multilamellar vesicles and labeling them with fluorescent probes

Multilamellar vesicles (MLV) were prepared according to Bangham et al. [11]. Chloroform solutions of phosphatidylcholine and cholesterol were added to round-bottomed glass tubes with 3–4

glass beads (4 mm diameter). The chloroform was evaporated under a stream of nitrogen. Futher drying was achieved by keeping the tubes in a vacuum dessicator overnight at 4°C. Deionized water was added to each tube to bring the final phosphatidylcholine concentration to 0.15 mM. The cholesterol concentration was varied from 0 to approx. 50 mol%. The tubes were warmed to 45°C and multilamellar vesicles were formed by mixing the lipid/water suspensions vigorously with a vortex mixer three times for one minute each time. The suspension was maintained near 45°C by placing the tubes back in the 45°C water bath between the mixing peroids. The desired probe was added to the vesicles to a probe/lipid molar ratio of less than 1/300. The vesicles were incubated at 45°C for 2 h to allow ful hydration of the lipids and distribution of the probe among the lamellae. The lipid phosphorous content of the multilamellar vesicles was determined by a modification of the method of Bartlett [12] and the cholesterol content by using cholesterol oxidase [13].

Fluorescence measurements

Steady-state fluorescence anisotropy (r), fluorescence lifetimes (τ), and differential polarized lifetimes ($\Delta\tau$) * were measured with a SLM 4800S phase-modulation spectrofluorometer (SLM Instruments, Inc., Urbana, IL) essentially as described by Lakowicz et al. [14]. Lifetime measurements were obtained with the exciting light ampitude-modulated at 18 MHz by a Debye-Sears modulator. Excitation wavelengths were 384 nm for the AS probes and 16-AP and 368 nm for diphenylhexatriene. The emitted light passed through a 418 nm sharp cut-off filter (Schott KV 418) and then through a Glan-Thompson polarizer oriented 55° to the vertical to eliminate the effects of Brownian motion [15]. The phase shift and demodulation of the emitted light relative to a reference of known fluorescence lifetime were determined and used to compute the phase lifetime (τ_p) and the modulation lifetime (τ_m) of the sample [16]. POPOP (1,4-bis(5-phenyloxazol-2-yl)benzene) in ethanol was used as the standard. It has a fluorescence lifetime of 1.35 ns [17,18]. The differential polarized lifetime ($\Delta\tau$), the difference between the lifetimes of the parallel and perpendicular components of the emitted light, was determined [14]. Steady-state anisotropies were measured with the Debye-Sears modulator turned off.

Limiting fluorescence anisotropy (r_0) was measured in a Perkin-Elmer 650-10S spectrofluorometer. Each probe was dispersed in glycerol and the fluorescence anisotropy measured at -5 to -10°C [19]. The limiting anisotropies measured using the excitation wavelengths listed above were 0.386 for diphenylhexatriene, and 0.311 for the anthroyloxy fatty acid probes.

Analysis of fluorescence data

When a fluorescent molecule dissolved in an isotropic solvent is excited by a brief pulse of polarized light, the anisotropy of fluorescence (r) decays in time, exponentially approaching zero. When the rotation of the fluorophore is hindered by an anisotropic environment, the anisotropy decays to a finite value (r_∞). A modified Perrin equation [20] takes this into account:

$$r = r_\infty + ((r_0 - r_\infty)/(1+6R\tau)) \quad (1)$$

where R is the probe rotation rate. R and r_∞ can be considered to reflect different limitations on probe rotation. R, the rate of rotation, depends primarily on the local resistance to rotation (local microviscosity). r_∞, the infinite-time anisotropy, reflects principally the limitation that the local environment of the fluorophore places on the extent of probe rotation.

To separate the effects of cholesterol on R from its effects of r_∞, we used the methods described by Lakowicz et al. [14], in which R is estimated from measurements of r, τ, and the differential polarized lifetime ($\Delta\tau$) according to the theory developed by Weber [21].

* Quantities used to characterize probe dynamics: r, steady-state anisotropy of fluorescence; r_0, limiting steady-state anisotropy of fluorescence as the viscosity of the surrounding medium becomes very large; r_∞, infinite-time anisotropy (time-resolved anisotropy at long times after pulse excitation); τ, fluorescence lifetime; τ_p, fluorescence lifetime determined from phase shift of emitted light relative to exciting light; τ_m, fluorescence lifetime determined from modulation of emitted light relative to exciting light; $\Delta\tau$, differential polarized lifetime: difference between phase lifetimes of parallel and perpendicular components of emitted light; $\tan\Delta = \omega\Delta\tau$; R, apparent rotation rate of fluorophore.

It should be mentioned that we have examined DPPC multilamellar vesicles at 47°C (5 to 7 deg. C above their phase transition temperature), while our experiments with the other phosphatidylcholines were done at 25°C (much farther above their phase transitions). The data (see Fig. 2, for example) suggest that this difference probably does not qualitatively affect our results.

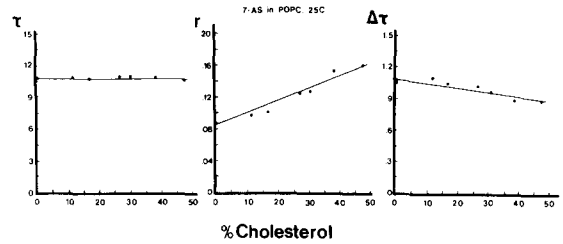

Fig. 1. Samples of our data. Shown are experimentally determined values of fluorescence lifetime (τ), anisotropy of fluorescence (r), and differential lifetime ($\Delta\tau$) for 7-athroyloxystearate in multilamellar vesicles of POPC at 25°C as functions of mol% cholesterol. τ and $\Delta\tau$ are in ns.

Results

Fluorescence anisotropies

The steady-state anisotropies of the AS probes in our phosphatidylcholine/cholesterol multilamellar vesicles increased approximately linearly with the cholesterol concentration (Fig. 1). The plots of anisotropy vs. cholesterol concentration were fit with least-squares lines and the equations of the lines were used to calculate anisotropies at 0, 10, 20, 30, 40, and 50 mol% cholesterol. Anisotropies computed in this way for 2-, 7-, 9-, and 12-AS and for 16-AP in multilamellar vesicles of the four different phosphatidylcholines are shown in Fig. 2. plotted as a function of the carbon number of the fatty acid to which the anthroyloxy moiety is attached.

The results for the different phosphatidylcholines are qualitatively similar in many respects. There is an increase in the anisotropy of each AS probe with increasing cholesterol concentration for each phosphatidylcholine tested. However, increasing cholesterol causes no significant changes in the anisotropy of 16-AP. The anisotropy profiles (Fig. 2) of the anthroyloxy fatty acids are almost linear in the absence of cholesterol and become progressively more concave downwards as cholesterol is added. The shape of the profile for egg PC multilameller vesicles differs slightly from those of the other phosphatidylcholines in having a more pronounced decrease in anisotrophy between 7-AS and 9-AS, especially at higher cholesterol concentrations.

The anisotropy of diphenylhexatriene increased with increasing cholesterol concentration (Fig. 3). The increases in anisotropy with added cholesterol were greatest for DPPC vesicles and least for DOPC vesicles, with POPC and egg PC being approximately equal in this regard.

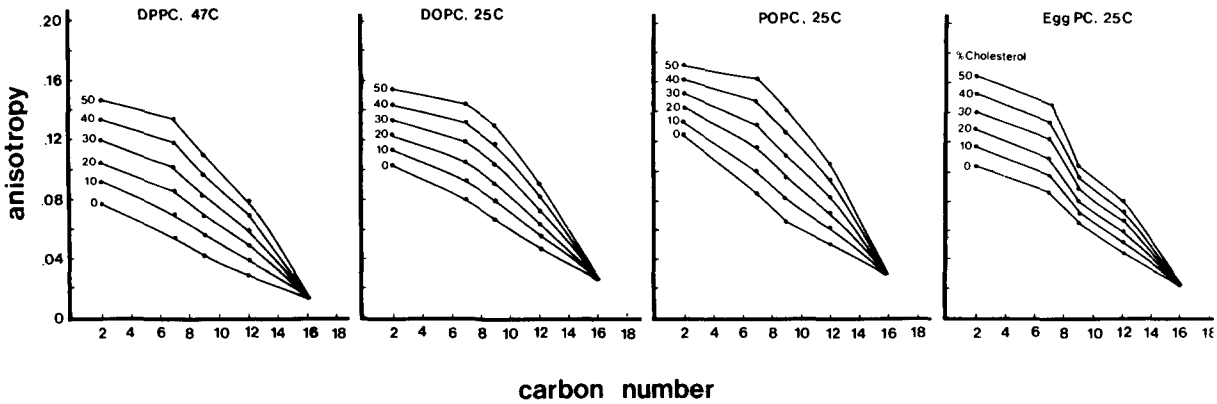

Fig. 2. The fluorescence anisotropies of the five anthroyloxy fatty acids (2-, 7-, 9-, and 12-AS, and 16-AP) in multilamellar vesicles of the five phosphatidylcholines are shown for various cholesterol mol%. See the text for an explanation of how the data were derived. The abscissae indicate the carbon number on the fatty acid to which the fluorophore is attached.

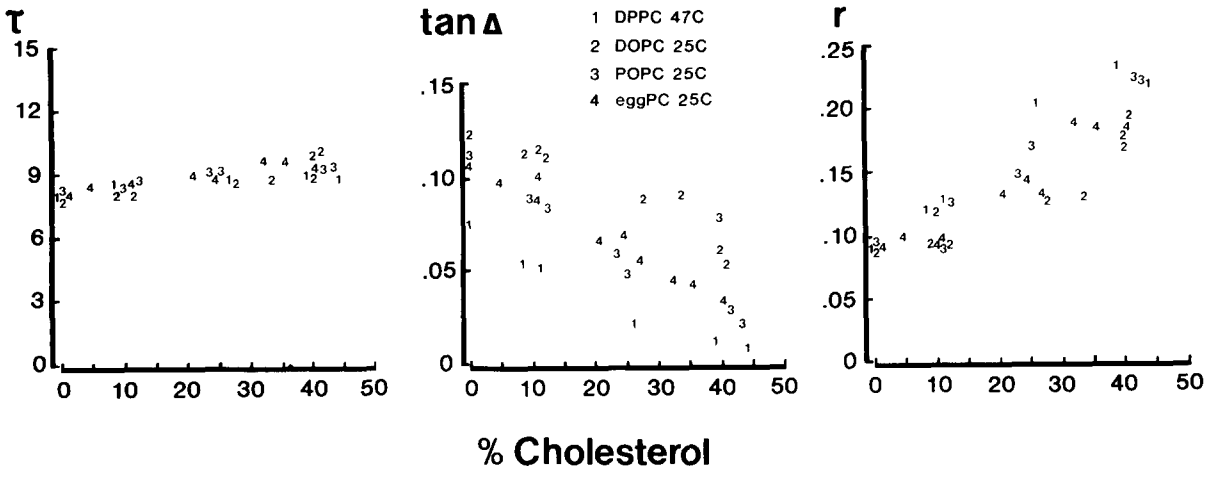

Fig. 3. Fluorescence parameters for diphenylhexatriene in multilamellar vesicles of the four phosphatidylcholines as functions of mol% cholesterol. τ is in ns.

Fluorescence lifetimes

In general fluorescence lifetimes did not change markedly with increasing cholesterol concentration (Figs. 1 and 3). The plots of average lifetime vs. mol% cholesterol were fit by least-squares lines and the equations of the lines used to calculate the lifetimes at 0, 10, 20, 30, 40, and 50 mol% cholesterol.

Fig. 4 shows the lifetimes of the AS probes and 16-AP in multilamellar vesicles of the four phosphatidylcholines.. In egg PC and DOPC the lifetime of only one of the probes depends significantly on cholesterol concentration: 16-AP for DOPC and 9-AS for egg PC. In POPC multilamellar vesicles the lifetimes of 2-AS and 12-AS depend slightly on cholesterol concentration. In DPPC vesicles the 2-AS probes show the most marked dependence of lifetime on cholesterol concentration, while the lifetimes of 7-AS, 12-AS, and 16-AP are less dependent on cholesterol concentration. The tendency for lifetime to increase with increased depth of the anthroyloxy fluorophore in the membrane was reported by Thulborn and Sawyer [22].

The lifetime of diphenylhexatriene in the phosphatidylcholine/cholesterol multilamellar vesicles depends only slightly on cholesterol concentration (Fig. 3) and is essentially independent of the phosphatidylcholine used.

Fluorescence lifetimes were obtained in two

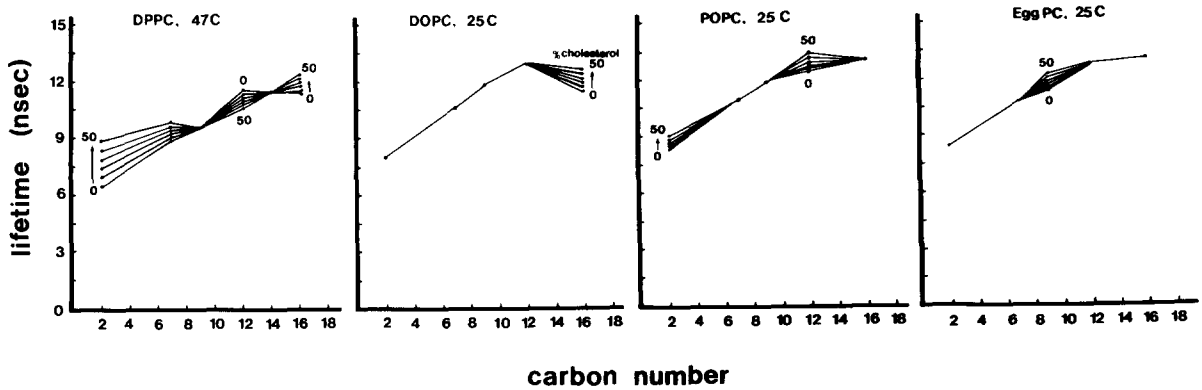

Fig. 4. Fluorescence lifetimes of the five anthroyloxy fatty acid probes in multilamellar vesicles of the four phosphatidylcholines at the indicated mol% cholesterol. See the text for an explanation of how the data were derived.

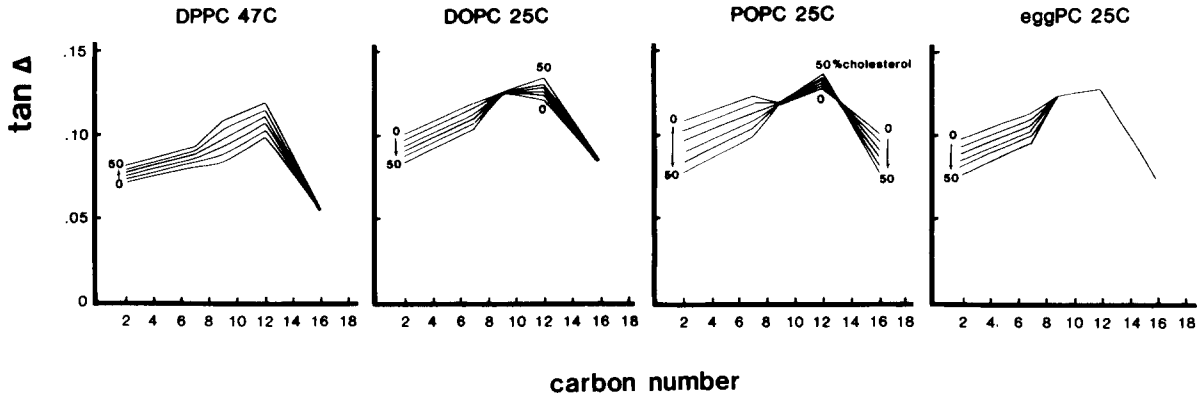

Fig. 5. Differential tangents (tan Δ) for the five anthroyloxy fatty acid probes in multilamellar vesicles of the four phosphatidylcholines at the indicated mol% cholesterol. See the text for an explanation of how the data were derived.

ways: (1) from the phase shift of emitted light relative to the exciting light and (2) from the demodulation of the emitted light relative to the exciting light [21]. For diphenylhexatriene the lifetime obtained from the phase shift (τ_p) was equal, within experimental error, to the lifetime obtained from the change in light modulation (τ_m). This equality of τ_p and τ_m for diphenylhexatriene is consistent with the interpretation that there is a single poole of diphenylhexatriene molecules with the same mode of decay from the excited state [21].

For the anthroyloxy fatty acid probes τ_p tended to be somewhat smaller than τ_m. Typically τ_p was about 1 ns (about 10%) less than τ_m. The values we report are averages of τ_p and τ_m. The fact that τ_m differs from τ_p is consistent with the interpretation that for each anthroyloxy fatty acid there is heterogeneity of the environment of the fluorophore or heterogeneity of the modes of decay from the excited state [21]. The apparent inhomogeneity of the anthroyloxy fatty acid probes limits the interpretation of our data. This problem is considered further in Discussion.

Differential polarized lifetimes

The dependence of differential polarized lifetimes ($\Delta\tau$) of the anthroyloxy fatty acid probes on cholesterol concentration was approximately linear (Fig. 1). The plots of $\Delta\tau$ vs. cholesterol concentration were fit by least-squares lines and the equations for the lines used to calculate tan Δ (tan Δ = $\omega\Delta\tau$) values for 0, 10, 20, 30, 40, and 50 mol% cholesterol. Fig. 5 shows the tan Δ values for the anthroyloxy fatty acids in multilamellar vesicles of all the phosphatidylcholines tested. The profiles of tan Δ tend to increase from 2-AS to 12-AS and then to decrease from 12-AS to 16-AP in multilamellar vesicles of all four phosphatidylcholines. The changes in the tan Δ profile with increasing cholesterol vary somewhat from one lipid to another.

The dependence of tan Δ for diphenylhexatriene on cholesterol concentration is shown in Fig. 3. Tan Δ tends to decrease with cholesterol concentration in all the phosphatidylcholines. The decrease of tan Δ with increasing cholesterol is greatest in DPPC vesicles and least in DOPC

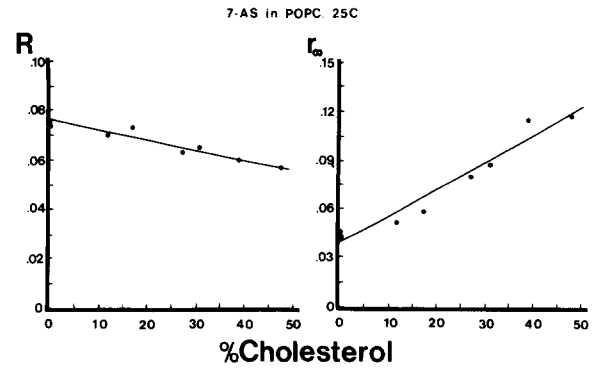

Fig. 6. Calculated values of the apparent rotation rate (R) and infinite-time anisotropy (r_∞) for 7-anthroyloxystearate in multilamellar vesicles of POPC as functions of mol% cholesterol.

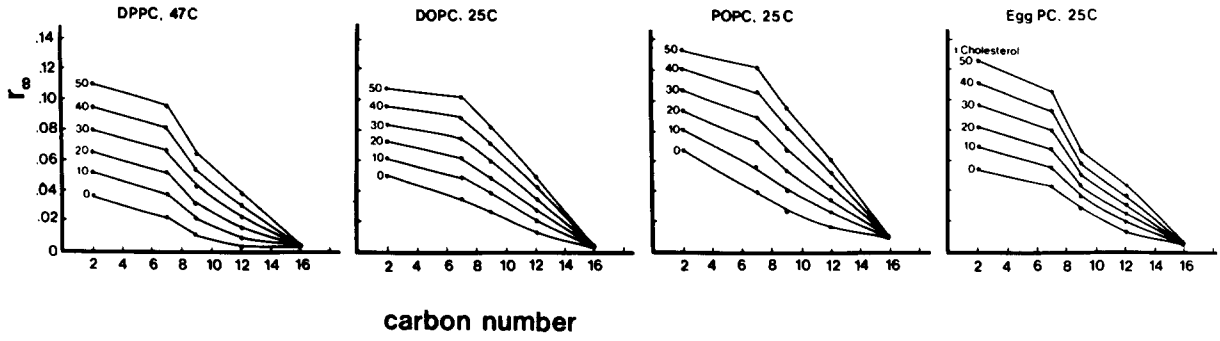

Fig. 7. Calculated values of the infinite-time anisotropy of the five anthroyloxy fatty acid probes in multilamellar vesicles of the four phosphatidylcholines at the mol% cholesterol indicated. See the test for an explanation of how the data were derived.

vesicles. Tan Δ values of diphenylhexatriene in egg PC and POPC are approximately equivalent.

Calculated values of r_∞ and R

Plots of values of the infinite-time anisotropy (r_∞) and rotation rate (R) vs. mol% cholesterol were approximately linear (Fig. 6). Least-squares lines were fitted to these data and the equations for the lines used to calculated r_∞ and R values for 0, 10, 20, 30, 40, and 50 mol% cholesterol. Fig. 7 shows the r_∞ profiles and Fig. 8 shows the R profiles for the anthroyloxy fatty acid probes in the various phosphatidylcholine/cholesterol multilamellar vesicles. The r_∞ profiles resemble the profiles on the steady-state anisotropy (Fig. 2). Adding cholesterol had no significant effect on the r_∞ of 16-AP. For the AS probes the increases in r_∞ with increased cholesterol tend to increase toward the carbonyl group, so that 2-AS and 7-AS show the largest increases in r_∞ when cholesterol is added. The profiles of r_∞ tend to become concave downward as cholesterol is added. This tendency is most pronounced for DOPC, in which the r_∞ of 7-AS becomes almost as great as the r_∞ of 2-AS as the cholesterol level approaches 50 mol%. For all the As probes (in all the phosphatidylcholines except DPPC) the relative increases in r_∞ with increasing cholesterol are generally greater than the relative increases in R with increasing cholesterol.

The profiles of rotation rates (R) of the anthroyloxy fatty acid probes with increasing cholesterol are shown in Fig. 8. The responses of the DPPC multilamellar vesicles to increased cholesterol are qualitatively different from those of the other phosphatidylcholines. In the absence of cholesterol

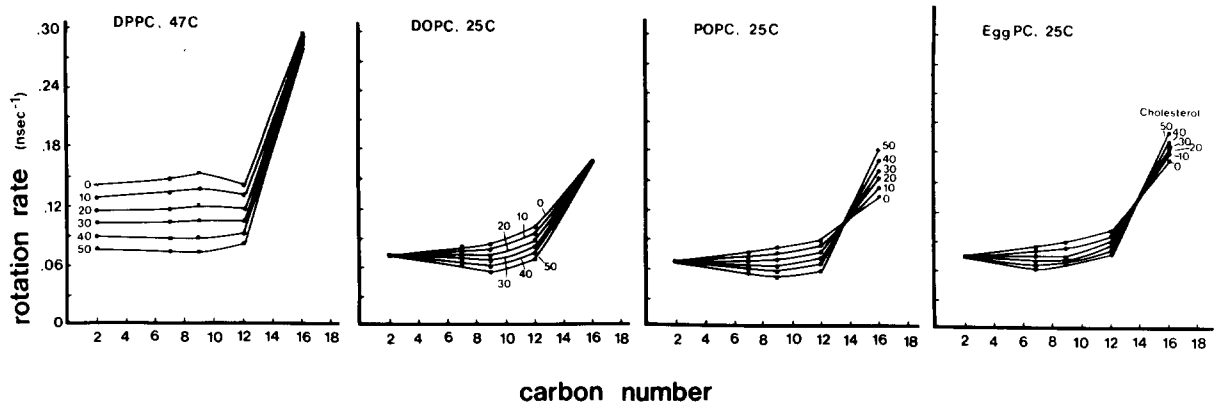

Fig. 8. Calculated values of the apparent rotation rates of the five anthroyloxy fatty acid probes in multilamelar vesicles of the four phosphatidylcholines at the indicated mol% cholesterol. See the text for an explanation of how the data were derived.

the rotation rates of 2-, 7-, 9-, and 12-AS in a given phosphatidylcholine are rather similar, but 16-AS has a somewhat higher R value. With addition of cholesterol to the multilamellar vesicles, there is no significant change in the R value of 2-AS in DOPC, POPC, or egg PC multilamellar vesicles. In DPPC multilamellar vesicles, however, adding cholesterol produces a large decrease in the R value of 2-AS. For 7-, 9-, and 12-AS, adding cholesterol decreases R in all the phosphatidylcholines; the decreases in R are considerably larger in DPPC than in the other three phosphatidylcholines.

The apparent rotation rate of 16-AP is larger than that of any of the AS probes, The increased rotation rate of 16-AP is most pronounced in DPPC multilamellar vesicles. As cholesterol is added to DPPC multilamellar vesicles the R of 16-AP doesn't change, but the change in R between 12-AS and 16-AP increases greatly due to the large decrease in R of 12-AS with increased cholesterol. In DOPC multilamellar vesicles the rotation rate of 16-AP also does not change with increasing cholesterol. In egg PC and POPC multilamellar vesicles, however, adding cholesterol increases the rotation rate of 16-AP.

the effects of added cholesterol on the r_∞ and R values of diphenylhexatriene in multilamellar vesicles of the four phosphatidylcholines are shown in Fig. 9. In each phosphatidylcholine the r_∞ of DPH increases with added cholesterol. The largest increase in r_∞ occurs in DPPC multilamellar

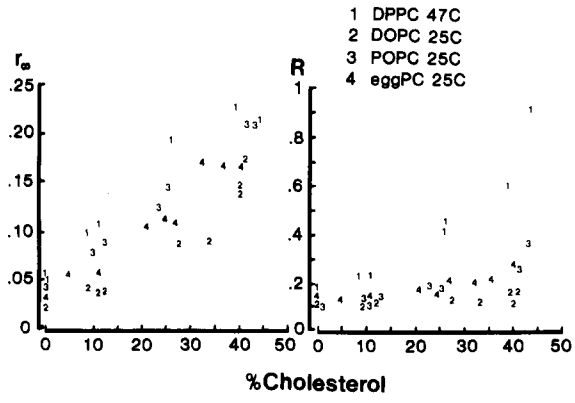

Fig. 9. Calculated values of the infinite-time anisotropy and the apparent rotation rate of diphenylhexatriene in multilamellar vesicles of the four phosphatidylcholines as functions of mol% cholesterol.

vesicles, the smalles increase in r_∞ occurs in DOPC, while POPC and egg PC are intermediate and roughly equivalent in this regard.

The changes in apparent rotation rate (R) of DPH in DOPC, POPC, and egg PC multilamellar vesicles with increased cholesterol are relatively small. In DOPC multilamellar there is essentially no change in the R of diphenylhexatriene as cholesterol levels increase. In DPPC multilamellar vesicles, however, the rotation rate of diphenylhexatriene increases some 4-fold as cholesterol increases toward 50 mol%.

Discussion

Effects of cholesterol on phosphatidylcholine bilayers as reported by diphenylhexatriene

The structure and motion of the acyl chains surrounding a fluorescent probe in a membrane influence and are influenced by the presence of the probe. Certain of the fluorescence parameters of the probe reflect the dynamics of the probe in the membrane and from measuring these parameters we make inferences about the dynamics of the lipid molecules in the membrane. The perturbing effects of the probes on the lipids should be kept in mind when making these inferences [23–26].

There is good evidence that diphenylhexatriene is preferentially located near the center of the lipid bilayer [27,28]. However, its orientation and precise location within the membrane cannot be precisely determined. Diphenylhexatriene is about 13 Å long and is relatively unrestricted in its location in the bilayer. The motion of the diphenylhexatriene molecules is greatly influenced by the surrounding lipid molecules as illustrated by the fact that the anisotropy of diphenylhexatriene changes more than that of any other fluorescent probe yet reported in lipid bilayers undergoing phase transitions.

Changes in the fluorescence lifetime of diphenylhexatriene with increasing cholesterol concentration (Fig. 3) were small, suggesting that the much larger changes in anisotropy of fluorescence are due to changes in the motional properties of diphenylhexatriene in the lipid bilayer. The increases in the steady-state anisotropy values suggest that addition of cholesterol to the phosphatidylcholine multilamellar vesicles results in increased struct-

ural constraint of motions of the diphenylhexatriene molecules.

The values of infinite-time anisotropies (r_∞) and apparent probe rotation rates (R shown in Fig. 9 suggest that for all the phosphatidylcholines, except DPPC, the major effect of adding cholesterol is to increase r_∞, with comparatively little effect on R. Other investigators [9,10] also concluded that changes in steady-state anisotropy of diphenylhexatriene in egg PC multilamellar vesicles containing cholesterol are due mainly to changes in r_∞. Our calculated values of r_∞ and R of diphenylhexatriene in multilamellar vesicles of egg PC as a function of cholesterol concentration are quantitatively similar to the values obtained by Hildenbrand and Nicolau [9] with time-resolved emission spectroscopy under comparable experimental conditions. In DPPC multilamellar vesicles a large increase in R of diphenylhexatriene with increasing cholesterol is also observed along with a large inceasse in r_∞. The large increases in r_∞ when cholesterol is added suggest that a major effect of cholesterol in multilamellar vesicles of all four phosphatidylcholines studied is to increase the structural order of the acyl chains that surround diphenylhexatriene and limit the extent of its rotation. The small effects of cholesterol on the apparent rotation rate of diphenylhexatriene in multilamellar vesicles of DOPC, POPC, and egg PC suggest relatively slight effects of added cholesterol on the 'microviscosity' that limits the rate of diphenylhexatriene rotation.

The magnitude of the increase in r_∞ with added cholesterol can be related to the proportion of the phospholipid acyl chains that are unsaturated. In multilamellar vesicles of DOPC, with all the acyl chains unsaturated, the influence of cholesterol on r_∞ is smallest. In multilamellar vesicles of DPPC, with all the acyl chains saturated, the ordering effect of cholesterol is greatest. In multilamellar vesicles of lipids with one saturated and one unsaturated acyl chain (POPC and egg PC) the effect of cholesterol on r_∞ is intermediate. These results suggest that increased unsaturation of the acyl chains enhances the ability to resist the ordering influences of cholesterol on the segments of the acyl chains that affect the motion of diphenylhexatriene.

The effect of cholesterol on the apparent rotation rates (R) of diphenylhexatriene in DPPC are qualitatively different from the effects of cholesterol on R in multilamellar vesicles of the other phosphatidylcholines tested. Cholesterol has little effect on R in multilamellar vesicles of DOPC, POPC, and egg PC, but adding cholesterol dramatically increases R of diphenylhexatriene in DPPC multilamellar vesicles.

Effects of cholesterol on phosphatidylcholine bilayers as reported by the anthroyloxy fatty acids

Taken together, the data for the anistropies and lifetimes of the AS probes (Figs. 2 and 4) suggest that addition of cholesterol significantly decreases of the motional freedom of all the AS probes in all the phosphatidylcholines tested. In the very center of the bilayer, 16-AP reports no effect of adding cholesterol.

The apparent heterogeneity of the lifetimes of the anthroyloxy fatty acids was mentioned above. Each population of fluorophores in a heterogeneous mixture may have a different fluorescence lifetime. The arithmetic mean of τ_m and τ_p that we use in our calculations is not, in general, equal to the weighted average of the lifetimes of the fluorophores present. For this reason the values of r_∞ and R that we calculate are also not proper weighted averages over all the fluorophores present, but merely approximations to the rigorously correct values of r_∞ and R. Nevertheless, we feel that the comparison of the r_∞ and R values of the different probes in the same membrane type and the ways that r_∞ and R are altered by adding cholesterol yields useful information.

In the absence of cholestrol a roughly linear order profile is observed with r_∞ greatest for 2-AS and least for 16-AP. Order profiles determined with NMR methods show a plateau region of high order to approximately the level of the 9th or 10th carbon, beyond which the order decreases toward the center of the bilayer [29]. In the absence of cholesterol we do not observe a plateau in the order profile in the multilamellar vesicles lipid bilayer. This may be partly due to the size of the fluorophore and the motion of its fatty acid backbone. The anthroyloxy fluorophore spans about six methylene groups and reports on the average order across that segment of the bilayer. In addition, the fatty acid backbone of an AS probe

undergoes *cis-trans* isomerizations just like the acyl chains of the neighboring phospholipids. For these reasons, the order profile reported by the anthroyloxy fatty acid probes lacks the resolution of the NMR-determined order profiles.

Comparison of values of r_∞ and R for the anthroyloxy fatty acid probes (Figs. 7 and 8) shows that in general cholesterol addition has much greater effects on r_∞ than on R. As was the case for diphenylhexatriene, cholesterol appears to increase the structural constraints that limit the extent of rotation of the anthroyloxy probes to a much greater extent that it alters the microviscosity that determines the rate of probe rotation.

The patterns of increase of r_∞ with added cholesterol are qualitatively similar among the different phosphatidylcholines used in (Fig. 7). The r_∞ of 16-AP was not changed by increased cholesterol in multilamellar vesicles of any of the phosphatidylcholines we tested. The largest increases in r_∞ with added cholesterol occurred in DPPC multilamellar vesicles, where the increase in r_∞ of 2-AS was about 3-fold, increasing to about 8-fold for 12-AS. In multilamellar vesicles of the other phosphatidylcholines adding cholestrol produced changes in r_∞ that were qualitatively similar, but of smaller magnitude. In all the phosphatidylcholines tested, a plateau region with similar r_∞ values for 2-AS and 7-AS develops as cholestrol is added. The r_∞ values then decrease steeply to 16-AP. The overall change in order from the proximal region of the acyl chains to the center of the membrane increases dramatically with the addition of cholesterol. Our results suggest that cholesterol increases structural order in all regions of the bilayer except near the bilayer center. The greatest increase in order is observed in the region occupied by the planar ring structure of cholesterol, with the largest increase in r_∞ occurring near the middle of the acyl chains. The greatest increases in r_∞ induced by increased cholesterol were observed in DPPC multilamellar vesicles, suggesting that the fully-saturated acyl chains of DPPC less readily accommodate cholesterol than those of phosphatidylcholines with one or more unsaturated acyl chains.

The apparent rotation rates of the anthroyloxy fatty acids change relatively little with increased cholesterol (Fig. 8). The R values are relatively constants from 2-AS to 12-AS, but the R values of 16-AP are considerably greater (1.5- to 2.1-times) than the R values of 12-AS. While the effective 'microviscosity' is probably at a minimum in the very center of the bilayer, the large R values for 16-AP may be partly due to differences between 16-AP and the other anthroyloxy fatty acids used. The fluorophore of 16-AP is attached to the terminal methyl group of the fatty acid chain, thereby greatly diminishing the stearic hindrance that the fatty acyl chain exerts on fluorophore rotation. This results in 16-AP having larger R values in isotropic solvents than the AS probes [30].

The effects of cholesterol on the apparent rotation rates of the anthroyloxy fatty acids in DPPC multilamellar vesicles stand out from the responses to cholesterol of R in the other phosphatidylcholines. Only in DPPC is the R values of 2-AS decreased By adding cholesterol. In multilamellar vesicles of DPPC the R value of 2-AS is reduced about 50% by 50 mol% cholesterol. In DPPC multilamellar vesicles the R values of 2-, 7-, 9-, and 12-AS at any level of cholesterol are approximately equal. This may reflect a high degree of cooperativity between the two fully saturated palmitoyl chains of DPPC. The R values of 16-AP are not changed by adding cholesterol to multilamellar vesicles of DPPC or DOPC, but the R values of 16-AP are increased by adding cholesterol to POPC or egg PC. Perhaps this effect requires a proponderance of phospholipids with one saturated and one unsaturated acyl chain.

Comparison of results with diphenylhexatriene and 12-AS

The average location of diphenylhextriene in the membrane [31,32] is similar to the average location of 12-AS [33]. It is interesting to compare our results using diphenylhexatriene and 12-AS.

Both the emission and absorption transition dipoles of diphenylhexatriene are parallel to the long axis of the molcule [27] and its average orientation is parallel to the bilayer normal. Thus rotations of diphenylhexatriene about axes perpendicular to the plane of the bilayer do not depolarize its fluorescence. The main mode of depolarizing motion of diphenylhexatriene in membranes appears to be a wobbling of diphenylhexatriene about axes that lie in the plane of the membrane bilayer [34].

For the AS probes the absorption dipole lies in the plane of the athracene ring system and is perpendicular to the long axis of the ring system. The emission dipole is also in the plane of the ring system and at an angle of about 30° to the absorption dipole [35]. ^{14}C-NMR studies suggest that there is little motion of the anthracene ring system relative to the fatty acyl backbone of AS probes incorporated into phospholipid bilayers [36]. For the AS probes the most effective motion in depolarizing fluorescence will be rotation about the long axis of the acyl chain (the type of motion that is 'invisible' to diphenylhexatriene). Wobbling motions of the probe will depolarize fluorescence, but much less effectively.

It may be useful to consider that to a first approximation depolarization of diphenylhexatriene fluorescence tells us about wobbling motions about axes in the plane of the bilayer (and gives no information about spnning about axes parallel to the normal to the bilayer) and that the AS probes tell us primarily about rotations about axes normal to the plane of the bilayer and to a much lesser extent about wobbling motions.

In the absence of cholesterol R values of diphenylhexatriene and 12-AS are quite similar in all the lecithins studied with R_{DPH} being about 10 to 35% greater than $R_{12\text{-}AS}$. There are larger differences among r_∞ values. The r_∞ of diphenylhexatriene is roughly twice as large as r_∞ for 12-AS in the unsaturated phosphatidylcholines and 10-times larger in DPPC. In terms of the analysis presented above this implies that the structure of the acyl chain region of the bilayer presents more structural constraint to the wobbling of diphenylhexatriene than to the spinning of 12-AS about the bilayer normal. The high resistance to wobbling is especially pronounced in DPPC.

Incorporating cholesterol to 50 mol% has different effects on the rotation rates of diphenylhexatriene and 12-AS. When cholesterol is added, the R values for diphenylhexatriene increase (except in DOPC), the increase being about 100% in POPC and egg PC and about 500% in DPPC. As suggested by Rothman and Engelman [6] adding cholesterol apparently structures the part of the bilayer occupied by the steroid nucleus but disorders the center of the bilayer. Our results are consistent with the interpretation that diphenylhexatriene can wobble within the disordered central core of the bilayer that is deeper than the cholesterol nucleus penetrates. The R values of 12-AS are diminished in the presence of 50 mol% cholesterol by 25 to 50%. This is consistent with the interpretation that rotation of the 12-AS about the stearic acid long axis is inhibited by increased order and microviscosity in the part of the bilayer occupied by the cholesterol steroid nucleus (since this part of the 12-AS molecule must rotate as well as its deeper parts).

For both diphenylhexatriene and 12-AS the structural constraint to depolarizing rotations increases on the addition of cholesterol to 50 mol%. The values of r_∞ for both probes are approx. 5- to 10-fold greater in the presence of 50 mol% cholesterol than in the absence of cholesterol. The ordering by cholesterol of the outer parts of the acyl chain region of the bilayer thus imposes roughly similar structural constraints to depolarizing motions of both diphenylhexatriene and 12-AS. The differential effects of cholesterol on r_∞ and R for diphenylhexatriene appear contradictory: the rate of diphenylhexatriene motion is apparently increased but the extent of diphenylhexatriene motion decreased. This result might be explained if part of one of the phenyl rings of diphenylhexatriene resides in the region occupied by the steroid nucleus and the rest in the more disordered region in the bilayer center. In the presence of high cholesterol the diphenylhexatriene molecule might find itself in the position of having one end anchored in a highly structured region and the other end in a region of low order and microviscosity. It might then undergo wobbling motions of increased frequency, but decreased amplitude. This effect is greatest in DPPC where the inflexible saturated acyl chains are least able to fully occupy the increased volume in the core of the bilayer produced by the presence of cholesterol.

Relationship between r and r_∞

The significance of the relationship between the infinite-time anisotropy (r_∞) and the steady-state anisotropy (r) has previously been considered [9,31,32,37,38,39]. Jähnig [32] used a variant of Eqn. 1 and experimentally-determined values of r, r_0, and τ for diphenylhexatriene to obtain: $r_\infty = 1.125r - 0.05$. This equation fits r_∞ values for di-

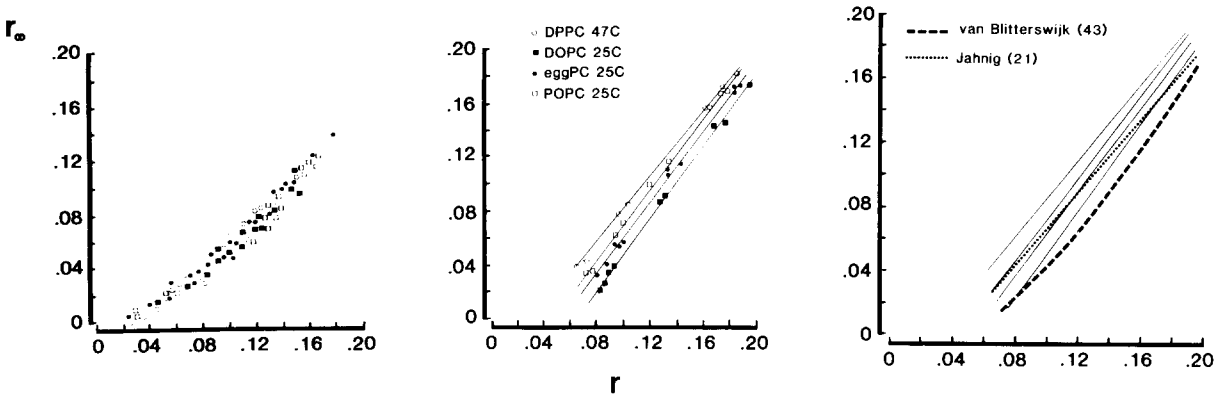

Fig. 10. Calculated values of r_∞ (the inifinite-time anisotropy) plotted vs. experimental values of r (steady-state anisotropy). Left-hand panel: our data for the five anthroyloxy fatty acid probes in multilamellar vesicles of the four different phosphatidylcholines over the entire range of cholesterol concentration. Middle panel: our data for diphenylhexatriene in multilamellar vesicles of the four different phosphatidylcholines over the range of cholesterol concentrations. Right-hand panel: our data for diphenylhexatriene (solid lines) are compared with theoretical predictions (...., Jähnig [32] and - - -, Van Blitterswijk et al. [39]).

phenylhexatriene in DPPC membranes obtained from 10 to 60°C [14,19]. Fulford and Peel [38] used data from Ref. 9 for diphenylhexatriene in several artificial and natural membranes to obtain: $r_\infty = 1.324r - 0.099$. Heyn [31] and Hildebrand and Nicolau [9] found that r approaches r_∞ as r increases. Engel and Prendergast [37] and Van Blitterswijk et al. [39] found that the relationship between r_∞ and r for diphenylhexatriene was better described by a curvilinear equation. Their equations fit the data obtained with diphenylhexatriene in biological membranes and various model membrane systems.

Fig. 10 shows our data for r_∞ vs. r for the anthroyloxy fatty acids and diphenylhexatriene in multilamellar vesicles of the four phosphatidylcholines we used. The data for the anthroyloxy fatty acids appear to fall along a single curve. The slope of the relationship between r_∞ and r increases monotonically and the relationship can be approximated by the 2nd-order polynomial: $r_\infty = -0.036 + 0.298r + 2.86r^2$. It is noteworthy that the data from five different anthroyloxy fatty acids in multilamellar vesicles of four different phosphatidylcholines, over a wide range of cholesterol concentrations, are reasonably well-described by a single relationship.

Our data for r_∞ vs. r for diphenylhexatriene (Fig. 10, center panel) are fit quite well by straight lines that are close to being parallel, but differ in position from one phosphatidylcholine to another. For the same r value, r_∞ is largest in DPPC and smalles in DOPC. This is consistent with the interpretation that the contribution of structural constraint to the anisotropy of diphenylhexatriene is largest in DPPC and smallest in DOPC. In the right-hand panel of Fig. 10 we compare our experimental data with the predictions of the equations of Jähnig [32] and Van Blitterswijk et al. [39]. Neither of the theoretical relationships predicts our data very closely, but the slopes of our linear relationships are similar to the slope of the linear part of the Van Blitterswijk equation.

Engel and Prendergast [37] urged caution in using theoretical models to estimate r_∞ from r until the models can be adequately tested. Our results do not support the interpretation that a single equation can represent the relationship between r_∞ and r for all experimental conditions. We feel that the relationship between r_∞ and r may depend on the fluorescent probe, the structure and composition of the membrane studied, and certain experimental conditions. At the present time it appears unwise to extrapolate beyond the particular conditions for which an equation relating r_∞ and r has been experimentally verified.

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