Cholesterol–Phosphatidylcholine Interactions in Vesicle Systems. Implication of Vesicle Size and Proton Magnetic Resonance Line-Width Changes†

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ABSTRACT: Vesicular structures composed of phosphatidylcholine (PC) and varying amounts of cholesterol or phosphatidylethanolamine (PE) have been prepared and examined with respect to their inherent vesicle size and resultant proton magnetic resonance spectra. The PC–PE system, which should have little variation in the nature of hydrocarbon interactions as a function of PE content, shows a simple monotonic increase of hydrocarbon chain proton line width and a concomitant increase in vesicle size on increasing the mole per cent PE. The PC–cholesterol system shows a more complex line-width and size behavior. At low cholesterol content both size and line width increase in a manner similar to that observed in the PC–PE system but beyond 30 mol % cholesterol induced effects become much more pronounced. The results suggest that although overall chain motion is slowed in vesicles of low cholesterol content, chain conformations are not restricted much more than in PC–PE vesicles of comparable size. At higher cholesterol concentrations, the nature of the cholesterol–PC interaction must change to a more restrictive one suggesting a change in the mode of phospholipid–cholesterol interaction well below the 1:1 stoichiometry suggested for multilayer systems. The observed changes in vesicle size at low cholesterol content are interpreted on the basis of a thermodynamic model which ascribes major perturbations to a variation in the dependence of enthalpy on vesicle radius.

Phospholipid vesicles, small closed spherical bilayers formed by sonication of aqueous dispersions of lipids such as lecithin (Huang, 1969), have been employed extensively as models in the elucidation of the properties of biological membranes (Levine, 1972). The homogeneity and easy characterization of this system make it ideal for quantitative physical studies. The observed physical properties of these vesicles, namely thermal phase transitions, permeability to ions and solvent, and the fluidity of the hydrocarbon chains, have been used to infer characteristics of the lipid bilayer regions found in biological membranes.

A frequent criticism of these studies is that the vesicle's small size, approximately 300 Å diameter, gives them a radius of curvature quite atypical of most biological membranes. The physical properties of vesicles mentioned above differ in some instances from those of membranes of larger radius of curvature (Sheetz and Chan, 1972; Finer *et al.*, 1972). This puts the validity of applying the conclusions derived from measurements on vesicles to biological membranes in doubt. Although this criticism is justified in some cases, it is ironic that the point from which it stems, the radius of curvature of the vesicle, may have far reaching implications for the properties of bilayers in natural membranes.

Upon examination of the available data it is apparent that vesicle size is not simply a characteristic of the method of preparation. We have found that variation of sonication frequency, intensity or duration, for example, does not vary the average vesicle size, and similarly sized vesicles can be prepared without resort to sonication at all (Batzri and Korn, 1973). Size has, on the other hand, been shown to be a well-defined function of composition in the case of vesicles containing cholesterol and phosphatidic acid (Johnson, 1973). A possible explanation of these facts is that vesicle size is determined by a local minimum in the free energy *vs.* radius curve. As such it is a thermodynamic property dependent on the composition, temperature, and pressure of the dispersion from which the vesicle is prepared. If this suggestion is valid vesicle size and the variation of size with thermodynamic parameters have relevance for the interaction of components in biological membranes composed of similar material.

Assuming that vesicle size is thermodynamically controlled, and that vesicle size can change in a continuous manner during the sonication process, the equation that determines the equilibrium vesicle radius is

$$\frac{\partial G(r)}{\partial r} = \frac{\partial H(r)}{\partial r} - T\frac{\partial S(r)}{\partial r} = 0 \qquad (1)$$

The functional dependence of free energy, G, on radius, r, is in principle accessible through a number of experimental techniques. We choose here to focus on one contribution to $\partial G/\partial r$, namely, the hydrocarbon chain contribution to $\partial S/\partial r$, which can be evaluated under special circumstances by an analysis of lipid proton magnetic resonance (pmr) line widths as a function of vesicle size. The relationship between entropy, S, and pmr line width is not a general one but we believe it is justified here. As such, it is possible to analyze the effect of various membrane constituents on vesicle size in terms of the contributions being largely entropy or enthalpy derived. In cases where line-width changes are not easily related to entropy, or in cases where vesicle size is not a thermodynamic parameter, line width is still a valuable parameter for analyzing induced restrictions of hydrocarbon chain motion and for analyzing interactions of various membrane constituents.

The variation of vesicle size and variation of pmr line width with cholesterol content is of particular interest because of the ubiquitous occurrence of this lipid in many biological membranes and because of the well known correlation of bilayer fluidity with cholesterol content (Darke *et al.*, 1972; Butler *et*

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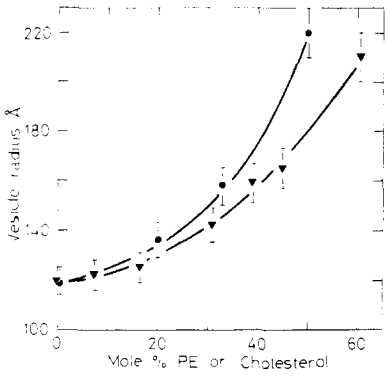

FIGURE 1: Most probable vesicle radius for PC vesicles containing cholesterol (●) or PE (▼).

al., 1970). The fact that cholesterol is known to restrict hydrocarbon chain motion for membrane lipids above their thermal phase transition suggests that the cholesterol induced change in vesicle size could be dominated by the entropy factor. The validity of this assertion will be examined by analysis of lipid pmr line widths as a function of cholesterol induced vesicle size changes. A parallel determination on a mixed soybean lipid (PC-PE)[1] system provides a reference for changes which are believed to be dominated by a variation of the enthalpy rather than variation of the entropy term.

Methods

Egg-yolk PC was purified from fresh egg yolks by the method of Singleton *et al.* (1965). Cholesterol was obtained from Fisher (lot no. 732063), mp 147.5–148.5°, and was used without further purification. PC and PE for mixed phospholipid studies were obtained by silicic acid column separation of crude soybean phospholipids (Calbiochem lot no. 12C 3010) using a chloroform–methanol gradient elution. Neither lipid was isolated completely pure. The PE contained about 10% of a phospholipid migrating at R_F 0.8 on thin-layer chromatographic (tlc) silica gel plates developed with chloroform–methanol–water (65:25:4). The PC contained less than 1% of a lipid migrating at R_F 0.8 by tlc as described above. The impurity is suspected to be phosphatidic acid.

Samples were prepared to be 10% (w/v) total lipid using the lipids described above and a D_2O buffer containing 0.01 M Tris, 0.10 M KCl, and 0.02% NaN_3. The measured pH was 7.5 and can be converted to pD by the relation pD = pH + 0.4 (Wang and Copeland, 1973). For mixed lipids composition is given in mole per cent of total lipid assuming the following molecular weights: PC, 760; PE, 715; cholesterol, 380. Concentrations are accurate to ±2%. Mixed lipids were weighed together and dissolved in chloroform and then dried under vacuum overnight to assure a homogeneous mixture before dispersing in buffer solution by vortexing. Vesicle solutions were prepared from aqueous dispersions by sonication with either a probe type sonicator (Branson S125) using a microtip at the lowest power level and the sample immersed in an ice bath, or with a bath sonicator (Branson Model E) with temperature control, 30° unless otherwise indicated. Both sonications were carried out under nitrogen after degassing and neither method produced detectable chemical degradation. Sonicated samples were found to be indefinitely stable above the lipid phase transition temperature as long as microbial growth was deterred and the percentage of larger particles was small.

[1] Abbreviations used are: PC, L-α-diacylglycerolphosphatidylcholine; PE, L-α-diacylglycerolphosphatidylethanolamine.

Vesicle sizes were determined by analytical gel partition chromatography on Sepharose 2B. Sepharose 2B (Pharmacia lot no. 8522) was equilibrated with column buffer and egg-yolk PC vesicles before packing a 2.6 cm i.d. column to a height of 32 cm. Typically 0.60 ml of a 10% (w/v) vesicle preparation was layered onto the top of the gel bed after mixing it with 3.0 ml of column buffer, and the column was then eluted with the water analog of the D_2O buffer at a rate of 44 ml/hr under a water pressure of 40 cm. Lipids were detected in the eluent using a null detecting differential refractometer (Waters Associates). Except for vesicle radii greater than 200 Å all peaks due to vesicles were symmetrical and with an amplitude consistent with the vesicle concentration. This behavior is in contrast to the distorted profiles obtained from optical density measurements which weight disproportionately the contribution of any large particles present.

The elution volume of a vesicle preparation determined by the maximum in the refractive index curve, relative to the void volume indicated by a peak due to multilayer structures, and the total volume indicated by a peak due to D_2O, gave the vesicle gel partition coefficient K. Vesicle radius, r, was determined from K by using the equation of Ackers (1967) and calibration constants determined with an egg-yolk PC vesicle preparation, r = 120 Å (Huang, 1969), and several proteins of known dimension.

Random errors are estimated at ±5%. Possible systematic errors due to residual column specificity may also exist for PE containing vesicles where K values do not extrapolate to the value of K for pure soybean PC vesicles. K values for PE containing vesicles were corrected by subtracting a small constant, 0.025. If this correction is not applied vesicle sizes will be ~8% smaller than indicated.

Nuclear magnetic resonance (nmr) spectra were taken on a Varian HA-100 spectrometer with variable temperature capability. Unless otherwise stated the temperature was 28 ± 1°. Spectra were generally taken at 500-Hz sweep width using an internal HDO lock. Nmr spectral line widths were analyzed by visually fitting spectra with curves generated by a computer routine that sums Lorentzian lines of variable position, height, and width. The integrated intensities of all the resolvable spectral peaks remained approximately constant over a 50° temperature range and a wide range of lipid compositions indicating that the full intensity is seen in one Lorentzian line.

Results

Vesicle Size Dependence on Composition. Vesicle sizes for egg-yolk PC–cholesterol vesicles of composition 0–50% cholesterol and soybean PC–PE vesicles of 0–60% PE composition are given in Figure 1. The radius is seen to increase monotonically with increasing cholesterol content from 120 Å at 0% to 220 Å at 50% cholesterol with the part of the curve above 30% being noticeably steeper. This is qualitatively the behavior seen by Johnson using sedimentation techniques for size determinations (Johnson, 1973). However, we do not observe the discontinuous decrease noted by Johnson at 10% cholesterol; hence our results differ by 15% at higher concentrations. This difference may be due to the difference in size determination technique or to differences in sample composition. Johnson's samples contained 4% phosphatidic acid, a charged phospholipid.

The PE containing samples, after correction for a suspected column specificity for PE, show a similar monotonic increase in vesicle radius from 120 Å at 0% PE to 210 Å at 60% PE. The effect of PE on size is, however, not as great on a mole per cent basis as is the effect of cholesterol and the curve is less steep at high PE content.

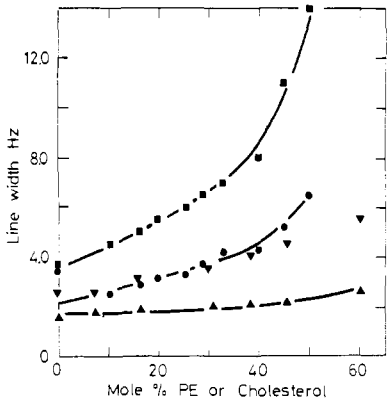

FIGURE 2: Half-line width at half-height for pmr spectra, at 28° and 100 MHz, of PC vesicles containing cholesterol (choline methyl (●); chain terminal methyl (■)) or PE (choline methyl (▲); chain terminal methyl (▼)).

Line-Width Dependence on Composition. Line widths obtained by Lorentzian curve fitting high-resolution spectra of vesicle dispersions *vs.* per cent PE and per cent cholesterol are presented in Figures 2 and 3. All points represent the line width of a single Lorentzian line of constant intensity, with the exception of the methylene resonance. The methylene line width in these figures is a weighted average of broad and narrow Lorentzian lines whose combined intensities remained constant over the series of points. The necessity of using a two-component line for the methylene resonance is not surprising since this represents protons over the entire length of the hydrocarbon chain. ^{13}C T_1 measurements have shown that each methylene group has a different correlation time and line width (Levine *et al.*, 1972). Using two Lorentzian components to approximate the methylene resonance is a necessary simplification.

The data in Figures 2 and 3 show a monotonic increase of line width with increasing cholesterol or PE content. The increase is in general more pronounced for cholesterol containing vesicles. In most cases the data extrapolate to the resonance width in pure PC vesicles at 0% PE or cholesterol. An exception is the choline methyl resonance in cholesterol systems which extrapolates to a line width of 2 Hz rather than the 3.5 Hz observed. The width at 0% is in part due to the fact that inside and outside cholines have different chemical shifts (Kostelnick and Castellano, 1973). A head group rearrangement at approximately 10% cholesterol has been postulated by other authors (Johnson, 1973). Such a rearrangement could diminish the shift difference between inside and outside choline methyls and lead to an apparent line narrowing.

Line-width data are valuable both because of possible application in the evaluation of $T\partial S/\partial r$ and because of potential detection of changes in the mode of interaction between lipid hydrocarbon chains and other lipid molecules in their immediate environment. The latter is particularly important for PC-cholesterol dispersions where there is some difference of opinion regarding interaction stoichiometry; at the bilayer phase transition a stoichiometry of 2:1 has in some cases been found (Engelman and Rothman, 1972; Hinz and Sturtevant, 1972). Other researchers have seen only 1:1 interactions, both at the phase transition temperature and above (Ladbrooke *et al.*, 1968; Darke *et al.*, 1972).

For PC-cholesterol samples the choline methyl, terminal methyl, and methylene line width *vs.* per cent cholesterol plots seem to have a change in slope above 30% cholesterol, while below this point there is a linear relation of line width *vs.* per cent cholesterol. Similar but less pronounced trends are seen

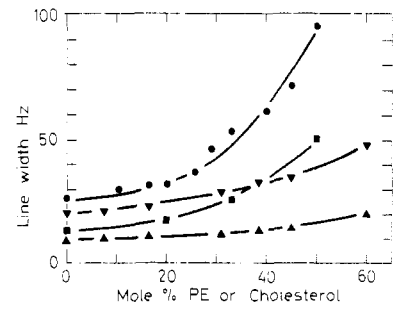

FIGURE 3: Half-line width at half-height for pmr spectra, at 28° and 100 MHz, of PC vesicles containing cholesterol (methylene (●); methylene adjacent to a double bond (■)) or PE (methylene (▼); methylene adjacent to a double bond (▲)).

in the vesicle radius curves. The breaks in these curves are probably not sharp enough to determine an interaction stoichiometry and it is unlikely that it would be well defined in a vesicle system at any rate. The data do, however, suggest a change in the mode of cholesterol interaction well below 1:1 stoichiometry.

The difference in the line-width behavior for cholesterol containing vesicles in the 0–30 and 30–50% regions can be most easily depicted by examining individual narrow and broad components of the methylene resonance. In the 0–30% region spectra can be fit reasonably well with a two-component line with a narrow resonance of fixed width, 13 Hz, and broad resonance, 50 Hz. Only the relative intensities need to be varied. As depicted in Figure 4, such a treatment leads to zero intensity in the narrow component at approximately 30% cholesterol. Beyond 30%, an additional broad component line, ~100 Hz, must be introduced.

The line-width plots for PE containing samples do not have as sharp a break in the 30–40% concentration region. If PC-PE methylene widths are fit with two constant width component resonances as described above for PC-cholesterol mixtures, the narrow component intensity does not fall to zero until well beyond 60% PE, the highest concentration studied. This points to the fact that there is a marked difference in PC-cholesterol and PC-PE interaction.

Line-Width Dependence on Vesicle Size. Line width is known to vary as a function of vesicle size. That the difference between the effect of cholesterol and PE on line width extends beyond simple induced increases in the size of vesicles can be seen by plotting line width *vs.* radius in each case. A plot for the methylene resonances is presented in Figure 5. Cholesterol is seen to produce a more pronounced increase in line width for a given radius than does PE. The corrections made in the PC-PE vesicle radius calculations predict line width *vs.* radius plots

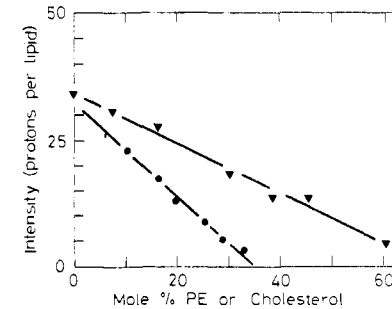

FIGURE 4: Intensity of the narrow component used in Lorentzian curve fitting of the methylene resonance with narrow and broad constant width components. The spectra simulated are from PC vesicles containing cholesterol (●) and PE (▼).

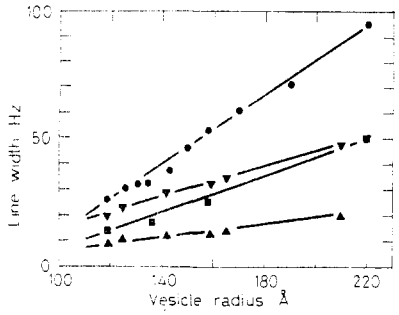

FIGURE 5: Half-line width at half-height *vs.* the most probable vesicle radius for PC vesicles containing cholesterol (methylene (●); methylene adjacent to a double bond (■)) or PE (methylene (▼); methylene adjacent to a double bond (▲)).

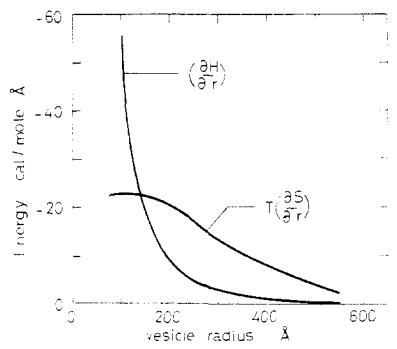

FIGURE 6: $\partial H/\partial r$ and $T(\partial S/\partial r)$ curves *vs.* vesicle radius that would predict the observed vesicle size distribution.

that extrapolate smoothly to the values at 0% PE. This lends confidence to the procedures adopted to correct for column affinity. The fact that these plots show no abrupt variation at 30% cholesterol suggests that any changes in interaction at this composition affect both line width and vesicle size.

Discussion

A Thermodynamic Argument for the Existence of Vesicle Structures. The results presented above demonstrate clearly that lipid composition has a profound influence on both vesicle size and on the nmr line width for lipids in those vesicles. It is also apparent that the induced effects are quite different for lipid membranes containing cholesterol and those containing PE. A complete description on a molecular basis of the factors which contribute to a determination of vesicle size is beyond the slope of this study. The contribution of lipid chain packing enthalpy and hydrocarbon chain configurational entropy can, however, be evaluated and compared. These contributions are of particular interest since the rigid cholesterol molecule may well perturb the ability of adjacent chains to respond to changes in vesicle curvature.

Assuming vesicle radius is a thermodynamic parameter, the way in which a radius dependent entropy term may contribute to a determination of vesicle size can be seen from the thermodynamic equation governing an equilibrium among vesicles of slightly different sizes (eq 2). The equation is conceptually use-

$$\frac{\partial H(r)}{\partial r} = T\frac{\partial S(r)}{\partial r} \qquad (2)$$

ful in the sense that equilibrium size is determined by the intersection of two radius-dependent curves, one $\partial H/\partial r$ *vs.* r which can be discussed primarily on the basis of intermolecular interaction energies, and one $T(\partial S/\partial r)$ *vs.* r which expresses a radius-dependent disorder in the system.

The $\partial H/\partial r$ term as well as the $\partial S/\partial r$ term contain, in principle, contributions from forces other than those involved in simple hydrocarbon chain interactions, hydrophobic forces, for example. We cannot rigorously dismiss these factors as being unimportant in determining vesicle radius. However, in the interest of clarity we will confine our discussion here to lipid–lipid interactions. This simplification is not completely unjustified since the large volume changes expected on changes in hydrophobic interaction (Friedman and Scheraga, 1965) are not seen in vesicle systems. In fact no change is observed in going from a vesicle of 150 Å radius to a multilayer of radius $\gg$ 2000 Å (Sheetz and Chan, 1972).

In terms of interlipid interactions, $\partial H/\partial r$ can be qualitatively discussed as the sum of van der Waals and electrostatic interactions. van der Waals interactions depend strongly on interchain distance (Salem, 1962). At the multilayer limit a typical hydrocarbon chain having three to four β-coupled gauche bonds (Nagle, 1973) fills a volume which on the average is cylindrical in shape. Slight bending of the multilayer and the resultant tapering of the average shape can be accommodated by a simple redistribution of these bonds along the chain to make it thicker or thinner near the interface. This ensures maintenance of complete interchain contact at little cost in conformational enthalpy and $\partial H/\partial r$ will be zero at large radii. As a vesicle radius approaches 100 Å the ability of a lipid to fill an increasingly tapered effective volume while maintaining maximum van der Waals interaction will decrease, because simple redistribution of β-coupled gauche bond sequences that retain a linear chain profile can do no more than create a slight thickening at one part of the chain. Thus, $\partial H/\partial r$ will become increasingly negative at smaller r. Assuming electrostatic interactions to be attractive and noting that two-thirds of the lipids are on the outer surface of a vesicle, there will be an additional negative contribution to $\partial H/\partial r$ if surface expansion occurs. Qualitatively the $\partial H/\partial r$ *vs.* r curve should therefore behave as indicated in Figure 6.

$T(\partial S/\partial r)$ per mole of lipid for fixed T is potentially dependent on variation in the number of accessible configurations of lipid hydrocarbon chains and on translational entropy contributions coming from the production of larger and larger numbers of small vesicles with decreasing r. Because of the large number of molecules per vesicle even at 150 Å radius (~5000) the translational contribution to $\partial S/\partial r$ on a per mole lipid basis can be neglected.

Intrachain entropy contributions to $\partial S/\partial r$ could on the other hand be substantial. At large radius a tendency toward hexagonal close packing of the hydrocarbon chains restricts their angular excursions from a normal to the bilayer. The number of accessible conformational states is small and can be described by conformers having combinations of coupled gauche and trans bonds such that a linear chain configuration is maintained (Seiter and Chan, 1973). Small increases in bilayer curvature can be accommodated by redistribution of these coupled gauche pairs. Therefore, $\partial S/\partial r$ will be small. As the radius decreases, noncoupled gauche bonds that create a nonlinear chain profile will occur due to disruption of the orderly intrachain packing, and S will increase rapidly. At a very small radius, conformational contributions to S must reach a limit characteristic of an isolated lipid molecule so $\partial S/\partial r$ cannot become indefinitely large but must reach some maximum value. The expected behavior of $T(\partial S/\partial r)$ has also been depicted in Figure 6. The point at which $\partial H/\partial r$ crosses $T(\partial S/\partial r)$ gives an equilibrium vesicle radius.

Equilibrium radius can be changed by shifting either $\partial H/\partial r$ or $T(\partial S/\partial r)$. If a membrane constituent such as cholesterol made $T(\partial S/\partial r)$ less negative through a restriction in the num-

ber of uncoupled gauche bonds which could be added to a hydrocarbon chain on decreasing vesicle radius, it is clear from Figure 6 that the point of intersection would move toward larger r. Cholesterol is in fact observed to increase equilibrium vesicle size.

The minimum change in $T(\partial S/\partial r)$ needed to produce a significant variation in vesicle radius can be estimated by assuming the width of the size distribution found by Sepharose chromatography or sedimentation measurements to be thermodynamically controlled. A mean square deviation in size of 50 Å then corresponds to a difference in free energy of about 1 kcal between equilibrium size vesicles and those 50 Å larger. This value can be equated with the area between $\partial H/\partial r$ and $T(\partial S/\partial r)$ curves in Figure 6.

Preparation at temperatures ranging from 10 to 70° of vesicles containing various amounts of cholesterol have shown only minor (<10%) changes in vesicle size. This suggests that the slope of the $\partial H/\partial r$ line is large compared to that of the $T(\partial S/\partial r)$ line. In accord with this description let us assume that near equilibrium $\partial H/\partial r$ is linear in r and $\partial S/\partial R$ is independent of r. Integration from equilibrium radius to a radius 50 Å larger or smaller leads to the conclusion that $T(\partial S/\partial r)$ must shift by 40 cal/(mol Å) to produce a 50-Å change in vesicle radius. Whether or not such a shift in $T(\partial S/\partial r)$ occurs on adding cholesterol can be tested by an examination of pmr line-width and relaxation time data.

Relationship between Pmr Line-Width and Lipid Configurational Entropy. That a relation between a kinetic parameter, such as line width, $\Delta\nu_{1/2}$, or the transverse relaxation time as determined from line width $T_2 = 1/\pi\Delta\nu_{1/2}$, and a thermodynamic parameter, such as entropy, exists is certainly not a general phenomenon. That a relation exists under certain circumstances which we believe to be applicable here can be demonstrated *via* an analysis of transverse, T_2, and longitudinal, T_1, relaxation times for protons on the hydrocarbon chains.

T_1 and T_2 for vesicles are observed to be relatively long but still not equal to one another as expected in a fluid. T_1 is approximately equal to $10T_2$ (Horowitz *et al.*, 1972). Differences between T_1 and T_2 can arise from several sources, but recent studies of proton relaxation rates in vesicles and other membrane systems restrict these possibilities to factors which originate in dipole–dipole interactions (Finer *et al.*, 1972; Chan *et al.*, 1971). Both intramolecular and intermolecular dipole interactions make important contributions to methylene resonance proton relaxation times (Lee *et al.*, 1973). Intermolecular contributions, however, depend on lateral diffusion rates of lipids in bilayers. The rate of diffusion in vesicle and multilayer systems is identical within experimental error (Lee *et al.*, 1973; Devaux and McConnell, 1972; Träuble and Sackmann, 1972) suggesting that the resonance line broadening seen as vesicle radius increases is not due to a change in the intermolecular contribution to relaxation. Therefore, the line broadening must be due to an intramolecular relaxation phenomenon, such as an increase in the degree of anisotropy of motion of the dipole–dipole interaction vector of geminal methylene protons. Relaxation due to this effect has been postulated for multilayer systems (Seiter and Chan, 1973), and it is probable that a certain degree of anisotropy of motion remains in vesicle preparations.

The origin of the anisotropy at a molecular level can be seen by considering possible hydrocarbon chain isomerizations. One class involves β-coupled pairs of trans–gauche rotations in a single chain. These rotate the dipole–dipole vector with a minimum of chain interaction with nearest neighbor chains since an overall linear chain profile is maintained. This motion should have a short correlation time, $\tau_c(a)$, but will not rotate the dipole–dipole vector over all angles. A second class involves uncoupled gauche–trans isomerizations. These motions will distort the chain from a linear profile. Substantial interactions with nearest neighbor chains ensure a slow correlation time, $\tau_c(i) \gg \tau_c(a)$, but the average motion will be isotropic. Overall vesicle tumbling might be considered a third class of motion. However, an estimate of its correlation time shows that it could not lead to the observed narrowing of nmr lines, and this fact has been confirmed by experiment (Sheetz and Chan, 1972). Hence, $\tau_c(i)$ must be the shorter and dominant isotropic correlation time.

As long as fast motion, as measured by $\tau_c(a)$, is appreciably anisotropic, the slow isotropic correlation time, $\tau_c(i)$, will make the dominant contribution to T_2, and T_2 will be proportional to $1/\tau_c(i)$. Either motion can dominate in determining T_1. Because of our assumptions regarding the motions characterized by $\tau_c(i)$ and $\tau_c(a)$, however, it is unlikely that $\tau_c(a)$ will vary with vesicle size while it is quite probable that $\tau_c(i)$ will. Results show T_2 to vary with size, a fact which is consistent with its dependence on $\tau_c(i)$. T_1, on the other hand, shows little variation in going from multilayer to vesicle (Seiter and Chan, 1973; Gent and Prestegard, 1974). Hence we shall take T_1 proportional to $1/\tau_c(a)$ and the relative rates of uncoupled gauche rotations to β-coupled gauche rotations will be measured by T_1/T_2.

Under certain assumptions changes in the relative rate of coupled to uncoupled gauche isomerizations can be related to variation of hydrocarbon conformational entropy. Qualitatively the relation exists for systems in which each motion stems from the same set of initial states and is characterized by equivalent activation energies for reaching intermediate states; in this case about 3 kcal/mol (Horowitz *et al.*, 1972). Rates then depend on the ratio of partition functions for intermediate conformers of each motion. These intermediate conformers can be taken as the set of coupled gauche rotamers in the case of anisotropic motion and the set of uncoupled gauche rotamers in the case of isotropic motion. Since the rate of coupled gauche rotation does not change with vesicle radius, a change in the T_2/T_1 ratio is a direct measure of the variation of the part of the hydrocarbon chain partition function describing uncoupled gauche conformers and hence is a qualitative measure of $\partial S/\partial r$. A more exact consideration of the relation of τ_c to T_1 or T_2 and the partition function to entropy leads to an estimated maximum value of 1 cal/(mol Å) for $T(\partial S/\partial r)$ if T_1 remains constant and line width changes from 20 to 35 Hz with a 50-Å change in vesicle radius.

We are ultimately interested in a possible shift in $T(\partial S/\partial r)$ on addition of cholesterol. To detect such a shift we must evaluate $T(\partial S/\partial r)$ in the absence of cholesterol. Attempts at preparation of a series of vesicles of different size by varying temperature have failed. Instead we varied the radius by changing composition in a way unlikely to change the hydrocarbon contribution to $\partial S/\partial r$.

For the PC–PE dispersions the fatty acid composition of the lipids does not change as the PE concentration increases. The dependence of chain conformational entropy should, therefore, be independent of the PE concentration, and it is assumed that any changes in vesicle size are due to a shift in the $\partial H/\partial r$ curve.

The line width *vs.* radius curves for PC–PE systems, depicted in Figure 5, show a constant slope of 0.25 Hz/Å. If T_1 remains constant, this means that the $T(\partial S/\partial r)$ *vs.* r curve is qualitatively consistent with that sketched in Figure 6. The value of $T(\partial S/\partial r)$ at 25° under this same assumption is ~1 cal/(mol Å). This value is small suggesting that hydrocarbon configurational entropy may not be the only contribution to $T(\partial S/$

∂r). Addition of cholesterol will have to change the hydrocarbon portion of $T(\partial S/\partial r)$ by more than an order of magnitude to be a primary cause of the induced change in vesicle size.

A qualitative measure of $T(\partial S/\partial r)$ for cholesterol containing vesicles is not easily obtained. Adding increasing amounts of cholesterol presumably gives both new $\partial H/\partial r$ and $T(\partial S/\partial r)$ curves. It is, however, unlikely that $T(\partial S/\partial r)$ shifts to more negative values with increasing cholesterol content, so assuming the $T(\partial S/\partial r)$ curve is fixed and examining line widths in a series of cholesterol containing vesicles will give a lower limit to $T(\partial S/\partial r)$.

Figure 5 shows that the slope of the line width *vs.* radius curve is greater for cholesterol containing vesicles than for PC-PE vesicles. Assuming that T_1 is unchanged, the slope is not sufficiently different to shift the hydrocarbon contribution to $T(\partial S/\partial r)$ by more than 2-3 cal/(mol Å).

T_1 is, however, not likely to remain constant. An examination of chain terminal methyl and choline methyl line widths as a function of cholesterol concentration, Figure 3, shows that, unlike the PC-PE system, the widths of these two resonances increase rapidly as a function of cholesterol. Since cholesterol is believed to dissolve in the hydrocarbon part of the bilayer adjacent to the first ten methylenes of the fatty acid chains, the line width of the terminal methyl and the choline methyl is not expected to increase due to restriction solely of isotropic motions. Therefore, coupled gauche motions must also be slowed down, and it is probable that T_1 decreases in proportion to T_2. Proton T_1 measurements (Lee *et al.*, 1972) on cholesterol containing systems confirm the slowing of T_1 determining motions. We therefore conclude that changes in the hydrocarbon contribution to $T(\partial S/\partial r)$ are not the cause of cholesterol induced changes in vesicle size.

If vesicle radius is thermodynamically controlled, it is obvious that any effect cholesterol has on vesicle size must be explained in terms of a shift in the $\partial H/\partial r$ curve. If vesicle size is kinetically controlled, the point of sudden increase in $\partial H/\partial r$ is also likely to be the determining factor. In the qualitative discussion of $\partial H/\partial r$, it was postulated that the abrupt increase in $\partial H/\partial r$ occurred at a small radius when a redistribution of coupled gauche conformers in the lipid chains could no longer adapt to the increasingly conical sector allowed each chain while still maintaining maximum interchain contact. In a cholesterol containing vesicle, some of the sectors will be occupied by cholesterol which can do nothing in terms of conformational changes to adjust to a noncylindrical environment. Hydrocarbon chains adjacent to a cholesterol molecule will compensate to some extent by further distortion from a cylindrical shape. Because of their expanded role, the ability of these chains to adjust to bilayer curvature will be exhausted at a larger radius. The predicted result, one that is consistent with observation, will be a $\partial H/\partial r$ curve that becomes sharply negative at a larger radius. Thus, cholesterol induces changes in bilayer membrane flexibility by affecting intermolecular lipid packing rather than by affecting the dependence of the isotropy of motion of individual lipid chains on vesicle radius.

Conclusion

Results indicate that the effect of cholesterol on hydrocarbon chain configuration and on vesicle size, for systems above the thermal phase transition, must be considered separately for the concentration regions above and below 33% cholesterol. The majority of our data refers to the lower concentration region. Here we can say that if vesicle size is a thermodynamic function, then cholesterol-induced restriction of hydrocarbon chain configurations does not play a significant role in changing vesicle size. Cholesterol does restrict the motion of lipid hydrocarbon chains, but in such a way that for equivalent sized vesicles, the isotropy of motion of individual chains is not very different in cholesterol containing *vs.* PE containing systems. This is consistent with a picture in which restrictions in motion, due to adjacent rigid molecules such as cholesterol, are compensated by an increase in the conical character of the average occupation volumes of the neighboring nonrigid hydrocarbon chains. An induced shift in the $\partial H/\partial r$ curve to larger radius is the most probable cause for a change in vesicle size.

The results are applicable to bilayer regions in biological membranes in that the same $\partial H/\partial r$ and $T(\partial S/\partial r)$ dependences should exist. These functions will determine membrane flexibility when local convolutions approach the radius of curvature seen in vesicles.

The technique of examining nmr line width in parallel with determination of vesicle size will be useful for analyzing the effect of other membrane active agents on the structure of vesicles and biological membranes.

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Variable Region Sequence of the Heavy Chain from a Phosphorylcholine Binding Myeloma Protein†

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ABSTRACT: The variable region sequence of the heavy chain from McPC 603, a phosphorylcholine binding myeloma protein, has been determined primarily by use of the automated sequencer. The variable region of this protein contains methionine residues at positions 34 and 83. Three cyanogen bromide fragments were isolated from cleaved heavy chains and pepsin Fab's which accounted for this entire sequence. The sequence of this protein outside the hypervariable regions shows considerable homology to the variable regions of other mouse as well as human proteins suggesting a conservation of genes coding for heavy chains.

Mouse myeloma proteins with antigen binding specificity for a wide variety of antigens such as phosphorylcholine (Potter and Leon, 1968; Potter and Lieberman, 1970; Sher *et al.*, 1971), β-(1→6)-D-galactan (Potter *et al.*, 1972; Jolley *et al.*, 1973; Rudikoff *et al.*, 1973), α-(1→3)-dextran (Leon *et al.*, 1970; Weigert *et al.*, 1970), α-(1→6)-dextran, β-(2→1)-fructosan (Cisar *et al.*, 1974), and dinitrophenol (Eisen *et al.*, 1968; Jaffe *et al.*, 1969) have been previously described. These proteins provide excellent models for studying antibody structure as well as for exploring structure-function relationships among proteins binding the same hapten.

We have begun to explore in depth the structural and functional properties of a group of five phosphorylcholine binding proteins (M603, M167, T15, S107, and H8) all of which originated in the highly inbred BALB/c strain of mice. Many of the questions relating to the structure of binding sites depend upon a determination of both three-dimensional and primary structures. A systematic attempt has been made to crystallize the pepsin Fab fragments from our collection of phosphorylcholine binding proteins. Crystals suitable for X-ray diffraction studies were obtained from the Fab fragment of M603 and have been previously described (Rudikoff *et al.*, 1972). Padlan *et al.* (1973) have extended these initial observations and recently described the three-dimensional structure of this molecule at 4.5-Å resolution.

In the present study we have determined the variable region sequence from the heavy chain of M603. We propose to use this sequence in the construction of a three-dimensional model of the M603 Fab and as a prototype in comparative studies with the other phosphorylcholine binding proteins described above.

Materials and Methods

Protein Purification. Plasmacytoma McPC 603 (IgA, κ) has previously been described by Potter and Leon (1968) and Leon and Young (1971). The protein (M603) was purified by affinity chromatography on Sepharose-phosphorylcholinecolumns as described by Chesebro and Metzger (1972).

Heavy Chain Preparation. M603 protein (20–30 mg/ml) was dialyzed against 0.15 M Tris-HCl–0.15 M NaCl–2 mM Na_2EDTA and was reduced with 10 mM dithiothreitol for 2 hr at room temperature, followed by alkylation for 15 min with 20 mM iodoacetamide (Bridges and Little, 1971). The partially reduced and alkylated protein was dialyzed overnight against 6 M urea–1 M acetic acid, and heavy and light chains were separated by chromatography on a Sephadex G-100 column (5 × 100 cm) equilibrated in 6 M urea–1 M acetic acid.

Pepsin Fragments. Pepsin Fab's were prepared as previously described (Rudikoff *et al.*, 1972). Protein, partially reduced and alkylated as described above, was dialyzed against 0.1 M sodium acetate (pH 4.5) and digested with pepsin (Worthington) at a weight ratio of 1:100 (enzyme:protein) for 6 hr at 37°. The digestion was stopped by adjusting the pH to 8.6 by the addition of 2 M Tris and the Fab was separated by chromatography on Sephadex G-100 columns equilibrated in borate-buffered saline (pH 8.0).

Cyanogen Bromide Cleavage. Proteins were dissolved in 70% formic acid and CNBr was added at a 4:1 weight ratio (CNBr:protein). The reaction mixture was allowed to stand overnight at 4° and was then diluted with water and lyophilized. Fragments derived from cleavage of heavy chains will be denoted as Cn while those derived from cleavage of the pepsin Fab will be designated Cn′.

Sequence Determination. Amino acid compositions were determined on a Beckman 119 amino acid analyzer equipped with high sensitivity cuvets and recorder following hydrolysis of peptides in 6 N HCl for ~18 hr in evacuated and sealed tubes. Automated sequence determinations were performed on a Beckman Model 890C sequencer using the standard dimethylallylamine peptide program. Sequencer fractions obtained after each degradation cycle were converted to phenylthiohydantoin derivatives (Pth)[1] as previously described (Rudi-

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[1]Abbreviation used is: Pth, phenylthiohydantoin.