Membrane Organization at Low Cholesterol Concentrations: A Study Using 7-Nitrobenz-2-oxa-1,3-diazol-4-yl-Labeled Cholesterol†

Sushmita Mukherjee‡ and Amitabha Chattopadhyay*

Centre for Cellular & Molecular Biology, Uppal Road, Hyderabad 500 007, India

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ABSTRACT: Cholesterol is most often found distributed nonrandomly in the plane of the bilayer, giving rise to cholesterol-rich and -poor domains. Many of these domains are thought to be crucial for the maintenance of membrane structure and function. However, such well-characterized domains generally occur in the membranes that contain relatively large amounts of cholesterol. Cholesterol organization in membranes containing very low amounts of cholesterol has not been investigated extensively. Recent evidence from differential-scanning calorimetric studies suggests that cholesterol may not form uniform monodisperse solutions, as assumed earlier, in the membranes even at very low concentrations. Fluorescent cholesterol analogues, when chosen carefully, offer a powerful approach for studying the distribution and organization of cholesterol in membranes at low concentrations. In this paper, we have studied the organization of cholesterol in membranes at very low concentrations (up to 5 mol % of the total lipid) using a fluorescent cholesterol analogue (NBD-cholesterol) which is labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group at the flexible acyl chain, without any alteration in the structural features necessary for proper membrane incorporation. Our results show that NBD-cholesterol exhibits local organization even at very low concentrations. This is consistent with the recently suggested model of cholesterol organization in membranes at low concentrations, involving the formation of transbilayer, tail-to-tail dimers [Harris, J. S., Epps, D. E., Davio, S. R., & Kezdy, F. J. (1995) *Biochemistry 34*, 3851−3857]. The implications of such local cholesterol organization in membranes that have very low cholesterol content *in vivo*, such as the endoplasmic reticulum and the inner mitochondrial membrane, open up interesting possibilities.

The fluid mosaic model for biological membranes defines the membrane as an oriented, two-dimensional, viscous solution of proteins and lipids in instantaneous thermodynamic equilibrium (Singer & Nicolson, 1972). Although the basic tenets of this model have stood the test of time, one of its implicit assumptions, that the lipid molecules merely provide the milieu for the membrane proteins and that they are distributed uniformly throughout the bilayer, has been found to be not quite true. In fact, current evidence points out that both transverse and lateral regionalization, which can be described in terms of micro- and macrodomains, are common features of many biological membranes (Curtain *et al*., 1988; Edidin, 1992; Tocanne, 1992; Glaser, 1993; Jacobson *et al*., 1995).

Cholesterol is one of the membrane components that is found, more often than not, distributed nonrandomly in structural and kinetic domains or pools in both biological and model membranes (Yeagle, 1985; Gennis, 1989; Schroeder *et al*., 1991; Liscum & Underwood, 1995). The reason for this lies in the rather unusual chemical structure of cholesterol that can by no means be considered a representative component of the "bulk" membrane (see Figure 1). The unusual shape of cholesterol also causes a phase separation in membranes at relatively high concentrations, resulting in the formation of cholesterol-rich and -poor domains that are structurally and compositionally so distinct that they can be observed histochemically and isolated physically from epithelial cell plasma membranes (Schroeder *et al*., 1991).

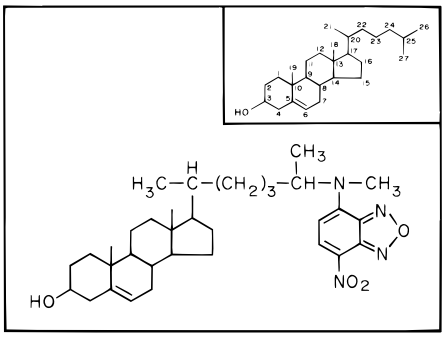

FIGURE 1: Chemical structure of NBD-cholesterol. The structure of cholesterol is shown in the inset for comparison.

Although a large body of literature exists about the behavior of cholesterol in model membranes at relatively high cholesterol concentrations, very little is known about its organization in the membrane when the cholesterol

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* Address correspondence to this author. Telephone: +91-40-672241. Fax: +91-40-671195. E-mail: amitabha@ccmb.uunet.in.

‡ Present address: Room 15-420, Department of Pathology, College of Physicians and Surgeons, Columbia University, 630 West 168th Street, New York, NY 10032.

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content is very low (<5 mol %). It has been assumed that cholesterol is distributed uniformly in the bilayer at these concentrations, although the evidence for it is quite tenuous (Estep *et al*., 1978; Vist & Davis, 1990; McMullen *et al*., 1993).

Very few techniques exist that can detect the intrinsic properties of cholesterol-induced structures in membranes. Techniques such as differential-scanning calorimetry and electron microscopy provide only bulk and static information. Further, ^{1}H and ^{13}C NMR spectra of cholesterol in membranes are generally too broad to obtain much meaningful information (Schroeder, 1984). The use of fluorescent sterols thus constitutes a powerful approach for studying cholesterol behavior in membranes due to their high sensitivity, time resolution, and multiplicity of measurable parameters. One class of probes commonly used for such studies is sterols that are chemically linked to extrinsic fluorophores (Schroeder, 1984). The advantage of this class of probes is that one has a choice of the fluorescent label to be used, and thus, specific probes with appropriate properties can be designed for specific applications.

One of the extrinsic fluorescent moieties that has been widely used for biophysical, biochemical, as well as cell biological applications is the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)[1] group [for a review, see Chattopadhyay (1990)]. This compound possesses some of the most desirable properties for serving as an excellent probe for both spectroscopic as well as microscopic applications (Kobayashi & Pagano, 1988; Pagano *et al*., 1989). It is very weakly fluorescent in water. Upon transfer to a hydrophobic medium such as membranes, it fluoresces brightly in the visible range (Lin & Struve, 1991; Fery-Forgues *et al*., 1993; Mukherjee *et al*., 1994) and shows a large degree of environmental sensitivity (Chattopadhyay & London, 1988; Rajarathnam *et al*., 1989; Chattopadhyay, 1991; Chattopadhyay & Mukherjee, 1993; Fery-Forgues *et al*., 1993; Mukherjee *et al*., 1994). It is relatively photostable, and lipids labeled with this group mimic endogenous lipids in studies of intracellular lipid transport (Lipsky & Pagano, 1983, 1985; Pagano & Sleight, 1985; van Meer *et al*., 1987; Pagano & Martin, 1988; Koval & Pagano, 1990; Ting & Pagano, 1990).

Two principal types of NBD-labeled steroids have been synthesized and used in membrane studies. In one of these, the NBD group is esterified to the 3β-OH of cholesterol *via* a relatively long hydrophilic spacer (Rando *et al*., 1982). The features that have been found to be necessary for a biologically active cholesterol analogue are an intact alicyclic chain, a free 3β-OH, a planar $\Delta^{5(6)}$ double bond, angular methyl groups, and a branched seven-carbon alkyl chain at the 17β-position (see Figure 1) (Schroeder, 1984; Ranadive & Lala, 1987). The 3β-OH-labeled analogue of cholesterol is thus expected to show serious discrepancies with cholesterol since the 3β-OH here is esterified to the NBD group, and consequently, the molecule has to anchor to the membrane rather unusually *via* its NBD group. The other class of cholesterol analogues constitute those where the NBD group is covalently attached to the branched alkyl chain of cholesterol (Craig *et al*., 1981). In this derivative, the NBD group is attached to carbon position 22 of the cholesterol moiety. It thus almost completely eliminates the seven-membered alkyl chain at the 17β-position of native cholesterol that has been found to be important for cholesterol function. In fact, previous studies suggest that the alkyl side chain of cholesterol is quite rigid up to the carbon 23 position (Oldfield *et al*., 1978) and that the addition of a polar group at carbon 22 of cholesterol which alters the configuration of the side chain severely affects its interaction with the bilayer (Vincent & Gallay, 1983). Another related study has shown that a carbon chain length of at least four to five carbons at the 17β-position is necessary for the optimal activity of certain antibiotics that are known to interact with cholesterol (Nakamura *et al*., 1980). This NBD-labeled cholesterol derivative was found to be 40% as effective as cholesterol as a substrate for lecithin−cholesterol acyltransferase and about 2 times more efficient in suppressing the action of 3-hydroxy-3-methylglutaryl coenzyme A reductase (Craig *et al*., 1981). The above derivative of cholesterol has also been very recently analyzed with regard to its partitioning properties into lateral phases of pure and mixed cholesterol/phospholipid monolayers (Slotte, 1995; Slotte & Mattjus, 1995). It was found that this compound did not mimic cholesterol in either pure or mixed monolayers and was excluded from lateral phases with high packing densities in both cases.

The NBD-cholesterol used by us (see Figure 1) differs from all the analogues discussed above. It is expected to mimic native cholesterol better than any of the above analogues since it fulfills all the criteria for an ideal cholesterol analogue, with a free 3β-OH, and the NBD group attached at the end of the branched alkyl chain, leaving most of the chain intact. A detailed characterization of the spectroscopic and ionization properties as well as the specific location of this molecule in membranes has previously been carried out by one of us (Chattopadhyay & London, 1987, 1988). In model membrane systems, the NBD group of this molecule has been found to be localized deep in the hydrocarbon region of the membrane, approximately 5−6 Å from the center of the bilayer (Chattopadhyay & London, 1987). Unlike the NBD-labeled phospholipids, the NBD group in NBD-cholesterol does not loop back to the membrane interface in spite of its polar nature, probably due to the stereochemical rigidity of the sterol ring (Chattopadhyay, 1990). This unique orientation of NBD-cholesterol offers a convenient handle for localization of a polar and potentially reactive group deep inside the membrane. In fact, the unique position of this probe in the membrane has been exploited in an energy transfer study of the spatial relationships of specific sites on chloroplast coupling factor to the bilayer surface in reconstituted vesicles (Mitra & Hammes, 1990).

In this paper, we have utilized NBD-cholesterol as a cholesterol analogue to study the distribution and organization of this rather unique molecule in the membrane at low concentrations. Our results show that NBD-cholesterol exhibits local organization in the membrane even at very low concentrations and may in fact form transbilayer, tail-to-tail dimers. These results are in agreement with the recent observations from differential-scanning calorimetric studies using the DPPC/cholesterol system at comparable cholesterol concentrations (Harris *et al*., 1995).

[1] Abbreviations: DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; GPI, glycosylphosphatidylinositol; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD-cholesterol, 25-[*N*-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino]-27-norcholesterol; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TLC, thin layer chromatography; ULV, unilamellar vesicle.

MATERIALS AND METHODS

Materials. DPPC and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-cholesterol was from Molecular Probes (Eugene, OR). DPPC was checked for purity by TLC on silica gel plates in chloroform/methanol/water (65:35:5, v/v/v) and was found to give one spot with a phosphate-sensitive spray and on subsequent charring (Dittmer & Lester, 1964). TLC of NBD-cholesterol was done using the same solvents but in a slightly different proportion (65:35:4, v/v/v), and it was found to be pure when detected by its color or fluorescence. Lipid concentration was determined by phosphate assay subsequent to total digestion by perchloric acid (McClare, 1971). Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Preparation of Liposomes. All experiments involving absorption and steady state fluorescence were done using unilamellar vesicles (ULV) of DPPC containing 0.1−2.0% (mol/mol) NBD-cholesterol prepared by the ethanol injection method (Batzri & Korn, 1973; Kremer *et al*., 1977). For this, 640 nmol of DPPC and 0.64−12.8 nmol of NBD-cholesterol were taken together in methanol, a few drops of chloroform were added and mixed well, and the samples were dried under a stream of nitrogen while being warmed gently (∼35 °C). After the lipids were dried further under a high vacuum for at least 3 h, they were dissolved in ethanol, to give a final concentration of about 40 mM lipid in ethanol. This ethanolic lipid solution was then injected into 10 mM sodium phosphate, 150 mM sodium chloride, and pH 7.1 buffer while vortexing to give a final concentration of 0.43 mM lipid in the buffer. The buffer was always maintained at a temperature higher than the phase transition temperature of DPPC (*i.e*., >41 °C) while the injections were made. In experiments where cholesterol was required, it was added from a methanolic stock solution along with DPPC and NBD-cholesterol in the organic phase, and all three components were dried together and injected together as ethanolic solutions. Background samples were prepared the same way except that the probes were omitted. For experiments in which fluorescence lifetimes were measured, ULVs were prepared as described above, except that the concentrations of both the lipid and the probe were doubled in order to improve signal to noise ratio, while their relative concentrations were the same as those used for steady state experiments. All experiments in gel phase membranes were done at 23 °C. Experiments involving fluid phase membranes were performed at 54 °C.

Measurement of Absorption Spectra. The absorption spectra were obtained using a Hitachi U-2000 UV−visible absorption spectrophotometer. Quartz cuvettes with a path length of 1 cm were used. Background samples without the fluorophore were used for base line corrections.

Steady State Fluorescence Measurements. Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal band-pass of 5 nm were used for all experiments. Background intensities of samples in which NBD-cholesterol was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. All experiments were done with multiple sets of samples, and average values of fluorescence and polarization are shown in the figures. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation (Chen & Bowman, 1965)

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer vertically oriented and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is equal to I_{HV}/I_{HH}. In significantly absorbing samples, the inner filter effect was corrected for by using the following equation (Lakowicz, 1983):

$$F_{correct} = F_{apparent} \times 10^{(A_{ex}+A_{em})/2} \quad (2)$$

where A_{ex} and A_{em} are the measured absorbances of the sample at the excitation and emission wavelength, respectively.

Time-Resolved Fluorescence Measurements. Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon-counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (17 ± 1 in. of mercury vacuum) and is run at 22−25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox as the scatterer. To optimize the signal to noise ratio, 5000 photon counts were collected in the peak channel. The excitation wavelength used was 337 nm which corresponds to a peak in the spectral output of the nitrogen flash lamp. Emission wavelength was set at 522 or 539 nm. All experiments were performed using excitation and emission slits with a nominal band-pass of 2 nm. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. The data stored in a multichannel analyzer were routinely transferred to an IBM personal computer for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \quad (3)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered using a nonlinear least squares iterative fitting procedure based on the Marquardt algorithm (Bevington, 1969). The program also includes statistical and plotting subroutine packages (O'Connor & Phillips, 1984). The goodness of the fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals (Lampert *et al*., 1983), and the autocorrelation function of the weighted residuals (Grinvald & Steinberg, 1974). A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value (not more than 1.4). Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times

and preexponential factors using the following equation (Lakowicz, 1983):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \quad (4)$$

Global Analysis of Lifetimes. The primary goal of the nonlinear least squares (discrete) analysis of fluorescence intensity decays discussed above is to obtain an accurate and unbiased representation of a single fluorescence decay curve in terms of a set of parameters (*i.e.*, α_i, τ_i). However, this method of analysis does not take advantage of the intrinsic relations that may exist between the individual decay curves obtained for the same system under different conditions. A condition in this context refers to temperature, pressure, solvent composition, ionic strength, pH, excitation/emission wavelength, or any other independent variable which can be experimentally manipulated. This advantage can be derived if multiple fluorescence decay curves, acquired under different conditions, are simultaneously analyzed. This is known as the global analysis in which the simultaneous analyses of multiple decay curves are carried out in terms of internally consistent sets of fitting parameters (Knutson *et al*., 1983; Beechem, 1989, 1992; Beechem *et al*., 1991). Global analysis thus turns out to be very useful for the prediction of the manner in which the parameters recovered from a set of separate fluorescence decays vary as a function of an independent variable and helps distinguish between models proposed to describe a system.

In this paper, we have obtained fluorescence decays as a function of probe concentration at two different monitoring wavelengths. The physical model under investigation is one where the population of fluorophores centered at one of the monitoring wavelengths (namely, 539 nm) represents a population of interacting fluorophores, as against the uniformly distributed population centered at 522 nm. The global analysis, in this case, thus assumes that the lifetimes are linked among the data files (*i.e*., the lifetimes for any given component are the same for all decays) but the corresponding preexponentials are free to vary. This is accomplished by using a matrix mapping of the fitting parameters in which the preexponentials are unique for each decay curve while the lifetimes are mapped out to the same value for each decay. All data files are simultaneously analyzed by the least squares data analysis method using the Marquardt algorithm (as described above) utilizing the map to substitute parameters appropriately while minimizing the global χ^2. The program used for the global analysis was obtained from Photon Technology International (London, Western Ontario, Canada).

RESULTS

Figure 2 shows the fluorescence emission spectra of NBD-cholesterol in different concentrations in unilamellar vesicles of DPPC in gel and fluid phases. The spectra are quite smooth and homogeneous in both gel and fluid phases when the NBD-cholesterol concentration is extremely low (0.1 mol % of the total lipid), with the maximum of fluorescence emission around 522 nm in both cases. As the NBD-cholesterol concentration increases, the shape of the fluorescence emission spectrum starts differing between the gel and the fluid phase membranes. Whereas the spectrum

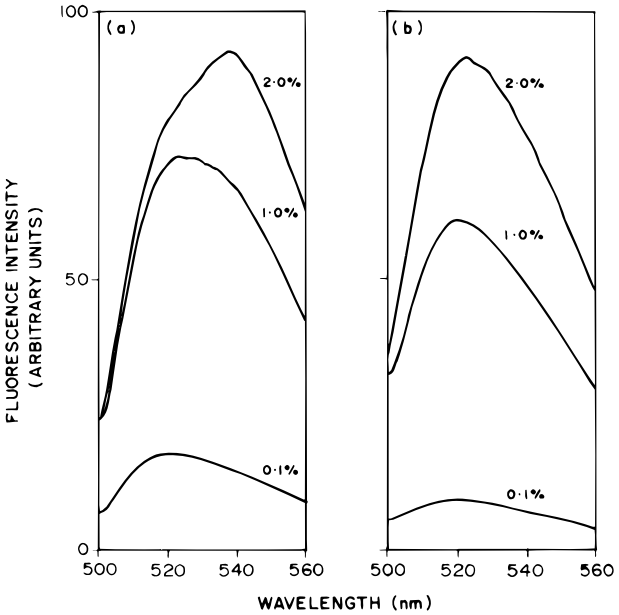

FIGURE 2: Fluorescence emission spectra of NBD-cholesterol in unilamellar vesicles of DPPC in the (a) gel phase (23 °C) and (b) fluid phase (54 °C). The concentration of DPPC was 0.43 mM in all cases, whereas the NBD-cholesterol concentrations in the samples were 0.43 μM (0.1 mol %), 4.3 μM (1.0 mol %), and 8.6 μM (2.0 mol %). The excitation wavelength was kept fixed at 485 nm. See Materials and Methods for other details.

remains quite smooth with the maximum of emission constant at 522 nm in the fluid phase membranes till the highest probe concentration investigated (*i.e*., 2 mol %) (Figure 2b), the spectra start showing deviations in the gel phase with increasing NBD-cholesterol concentration (Figure 2a). A new peak centered around 539 nm appears in these cases so that the spectra become broad and inhomogeneous, and at 2 mol % of the probe, the maximum of emission is clearly shifted from 522 nm and is centered at 539 nm. We argued that the inhomogeneity, and the resultant deviations observed in the gel phase spectra, could arise from two different populations of fluorophores. The population whose fluorescence is centered at 522 nm and is observed at low probe concentrations could represent a component of the fluorophores that are uniformly distributed throughout the bilayer. On the other hand, the new population arising only at relatively high probe concentrations and exclusively in the gel phase represents a population of fluorophores that are close enough in the membrane to be able to interact with each other during their excited state lifetimes. It is not surprising that such a phase separation is observed only in the gel phase in the concentration range investigated, since cholesterol has recently been shown to be completely immiscible in the highly ordered gel phase (Harris *et al*., 1995).

The above results are represented more quantitatively in Figure 3, where the ratio of fluorescence intensities at 539 and 522 nm is plotted as a function of NBD-cholesterol concentration. These intensities could be affected by inner filter effects. However, inner filter effect corrections were found not to alter the ratios significantly even at higher concentrations. Figure 3 shows that, in gel phase membranes, this ratio increases steadily with increasing concentration of the probe, till the intensity of the 539 nm peak becomes more than that of the original 522 nm peak. In the fluid phase membranes, on the other hand, this ratio increases

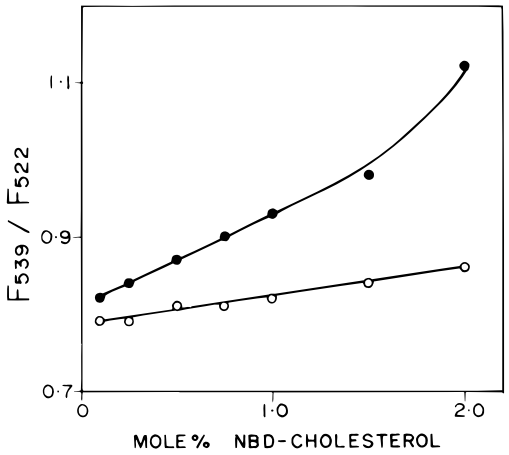

FIGURE 3: Change in the ratio of fluorescence intensities at emission wavelengths 539 and 522 nm as a function of NBD-cholesterol concentration in unilamellar vesicles of DPPC in the gel (●) and the fluid (○) phase. The concentration of DPPC was 0.43 mM in all cases, and the NBD-cholesterol concentration varied between 0.1 and 2.0 mol % of the total lipid. All other conditions are as in Figure 2.

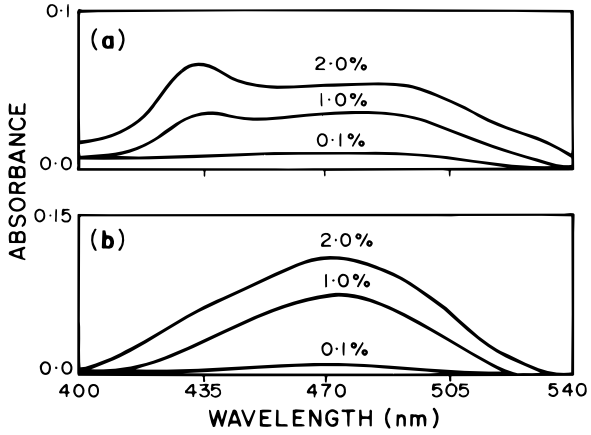

FIGURE 4: Absorption spectra of NBD-cholesterol in unilamellar vesicles of DPPC in the (a) gel phase (23 °C) and (b) fluid phase (54 °C). All other conditions are as in Figure 2.

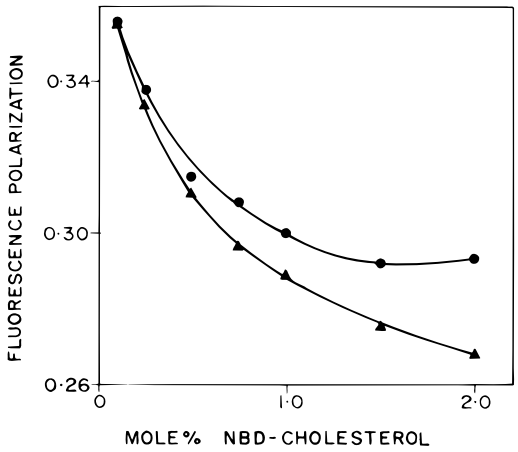

FIGURE 5: Change in the fluorescence polarization at emission wavelengths 539 nm (▲) and 522 nm (●) as a function of NBD-cholesterol concentration in unilamellar vesicles of DPPC in the gel phase. All other conditions are as in Figure 3.

only slightly in this concentration range. It is interesting to note here that, at very low NBD-cholesterol concentrations, this ratio is very similar in both gel and fluid phase membranes. This observation may be interpreted as additional evidence that the population at 522 nm is the one consisting of freely distributed fluorophores, since their concentration at this stage is too low to exhibit any appreciable interprobe interaction in either physical state of the membrane.

The specific association of the NBD-cholesterol molecules in gel phase membranes (as discussed above) could be due to the formation of a ground state complex. Alternatively, it could result from an excited state interaction between the fluorophores. The former possibility is more realistic in membranes since diffusion coefficients for membrane-bound molecules are rather small (Yeagle, 1992) to allow much translational motion (necessary for complex formation) during the excited state lifetime. To distinguish between these two possibilities, absorption spectra were recorded at various concentrations. Since the absorption process is instantaneous, and no physical displacement is allowed in this time scale, the information obtained from an absorption spectrum corresponds to the distribution of the fluorophores as they were in the ground state. Figure 4 shows that a new peak appears in the absorption spectrum in cases of gel phase membranes that contain >0.5 mol % NBD-cholesterol (*i.e.*, concomitantly with the appearance of the new 539 nm peak in the emission spectrum). No such peak is observed in fluid phase membranes in this concentration range (Figure 4b). These results thus indicate the presence of NBD-cholesterol molecules that are associated with each other in the ground state.

Fluorescence polarization provides an additional approach for assessing whether the two fluorescence peaks centered at 522 and 539 nm indeed correspond to two independent fluorophore populations. Figure 5 shows the fluorescence polarization of NBD-cholesterol in gel phase membranes as a function of the probe concentration. The polarization experiments were performed separately at emission wavelengths 522 and 539 nm, keeping the excitation wavelength fixed at 485 nm. The figure shows a decrease in polarization with increasing NBD-cholesterol concentration at both wavelengths. This effect could be attributed to a combination of the following possibilities. Cholesterol has been reported to be quite immiscible in the gel phase membranes, and exhibits a strong tendency to segregate into domains enriched in cholesterol, that will naturally be more fluid than the highly compact bulk of the gel phase membrane (Harris *et al.*, 1995). This segregation of domains rich in NBD-cholesterol is expected to increase with increasing NBD-cholesterol concentration, leading to higher depolarization. On the other hand, since the absorption and emission spectra of NBD-cholesterol overlap, there could be self energy transfer between closely spaced NBD-cholesterol molecules, leading to depolarization. The extent of depolarization, in such a case, would also depend on the NBD-cholesterol concentration. Figure 5 also shows that, although the polarization values are very similar at 522 and 539 nm at low NBD-cholesterol concentrations, they start deviating with increasing probe concentration. Thus, given the same NBD-cholesterol concentration, the reduction in polarization is much more pronounced when emission is monitored at 539 nm as compared to 522 nm. Our results from absorption and fluorescence intensity measurements in gel phase membranes show that NBD-cholesterol forms a ground state complex at concentrations >0.5 mol % that emits at 539 nm. This complex will obviously be larger in size than the

Table 1: Lifetimes of NBD-Cholesterol in Unilamellar Vesicles of DPPC in the Gel Phase as a Function of Concentration at Two Different Monitoring Wavelengths[a]

	emission wavelength 522 nm				emission wavelength 539 nm			
[NBD-cholesterol] (mol %)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
0.50	0.77	1.99	0.23	6.51	0.83	2.23	0.17	7.68
	(0.76)	(1.90)	(0.24)	(6.33)	(0.82)	(2.20)	(0.18)	(7.30)
1.00	0.81	2.17	0.19	6.99	0.84	2.46	0.16	7.83
	(0.77)	(1.90)	(0.23)	(6.33)	(0.82)	(2.20)	(0.18)	(7.30)
1.50	0.80	2.15	0.20	6.97	0.83	2.38	0.17	8.25
	(0.76)	(1.90)	(0.24)	(6.33)	(0.78)	(2.20)	(0.22)	(7.30)
2.00	0.80	2.14	0.20	6.96	0.84	2.61	0.16	8.52
	(0.76)	(1.90)	(0.24)	(6.33)	(0.78)	(2.20)	(0.22)	(7.30)

[a] Numbers in parentheses indicate values for global analysis.

freely distributed monomer that emits at 522 nm. Since the fluidization of gel phase membranes by cholesterol is a direct consequence of its inability to fit in the highly ordered matrix, the relatively large complex is thus expected to fit in the gel phase matrix even more poorly, resulting in greater fluidization of the membrane in its immediate vicinity. Such a behavior is reflected in the enhanced decrease in polarization at 539 nm compared to 522 nm. Additionally, the formation of a complex would also enhance the extent of depolarization resulting from energy transfer between NBD-cholesterol molecules. In such an event, the extent of depolarization observed at 522 nm would reflect the efficiency of energy transfer due to the statistical probability of two NBD-cholesterol monomers coming within the energy transfer range in the plane of the membrane during the fluorescence lifetime of the NBD group. The enhancement of depolarization observed at 539 nm at a given concentration of NBD-cholesterol relative to the corresponding value at 522 nm would then reflect the increase in the efficiency of energy transfer due to the specific interactions between the NBD-cholesterol monomers that bring them close together in space.

Fluorescence lifetime serves as a sensitive indicator for the local environment in which a given fluorophore is placed. In order to obtain the lifetimes of the two presumably different populations of NBD-cholesterol that are centered at 522 and 539 nm, fluorescence intensity decays were acquired as a function of NBD-cholesterol concentration at both these wavelengths, with the excitation wavelength kept at 337 nm. Table 1 shows the fluorescence lifetimes of NBD-cholesterol as a function of concentration at 522 and 539 nm. As is seen in the table, the fluorescence decays obtained at both the emission wavelengths fit to biexponential functions. Since the biological membrane offers considerable motional restriction to a fluorophore embedded in it, the slightly larger lifetime components observed at the longer emission wavelength (539 nm) for a given NBD-cholesterol concentration is not surprising. In fact, such an increase in fluorescence lifetime with increasing emission wavelength has previously been reported for fluorophores in environments of restricted mobility (Ware *et al*., 1971; Matayoshi & Kleinfeld, 1981; Lakowicz *et al*., 1983; Demchenko & Shcherbatska, 1985; Chattopadhyay & Mukherjee, 1993; Mukherjee & Chattopadhyay, 1994). The results shown in Table 1 indicate that, whereas the two lifetimes remain more or less constant over the NBD-cholesterol concentration range investigated when the fluorescence decays are monitored at 522 nm, they exhibit a distinct increase with concentration at 539 nm. This increase in lifetime with

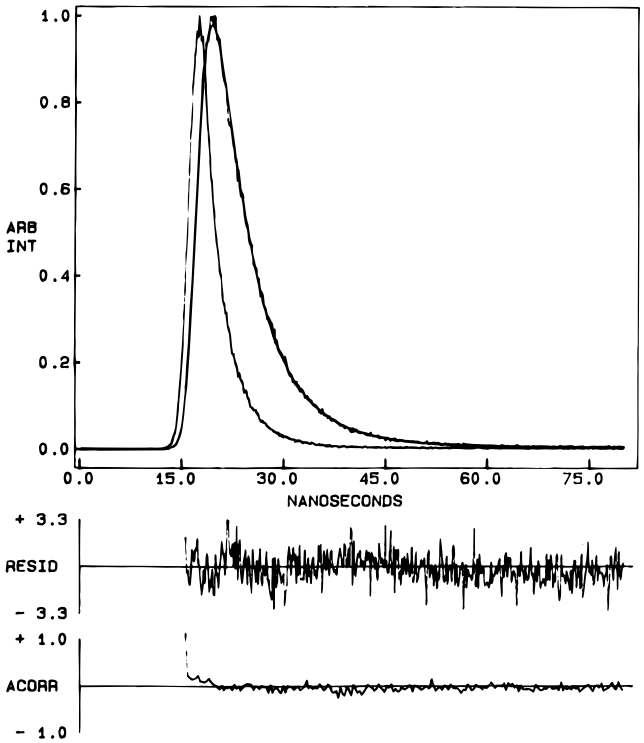

FIGURE 6: Time-resolved fluorescence intensity decay of 1 mol % NBD-cholesterol in unilamellar vesicles of DPPC in the gel phase when excited at 337 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 522 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a biexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. See Materials and Methods for other details.

increasing NBD-cholesterol concentration in the latter case could be interpreted as the buildup of a new population of fluorophores whose fluorescence is centered at 539 nm. A typical decay profile with its biexponential fitting and the various statistical parameters used to check the goodness of the fit is shown in Figure 6.

In order to confirm the above interpretation, the same set of fluorescence decays was subjected to global analysis. The decays were all assumed to be biexponential (on the basis of the results from discrete analysis), with fixed lifetime components whose relative contributions (preexponential factors) were allowed to vary. The results obtained upon analysis are shown in parentheses in Table 1. The fittings of the set of decay profiles analyzed by the global method are presented as a pseudo-three-dimensional plot of intensity *vs* time *vs* increasing file number in Figure 7. The weighted

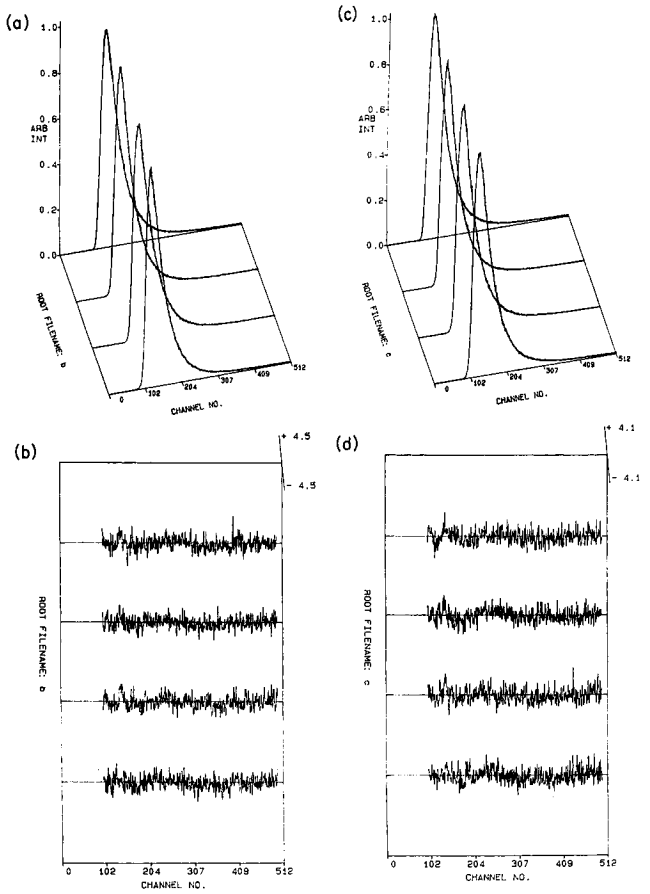

FIGURE 7: Global fittings and the corresponding weighted residuals of the set of decay profiles of NBD-cholesterol at emission wavelengths 522 nm (a and b) and 539 nm (c and d) as a function of NBD-cholesterol concentration in unilamellar vesicles of DPPC in the gel phase. All other conditions are as in Figure 3, except that both the lipid and the probe concentrations were doubled.

residuals corresponding to each of these fittings are also shown. The global normalized χ^2 values obtained were 1.17 and 1.14 for the data sets at emission wavelengths 522 and 539 nm, respectively.

The mean lifetimes, calculated using eq 4, are plotted in Figure 8 for both discrete and global analysis. This figure shows that the mean lifetimes do not exhibit any appreciable change with increasing NBD-cholesterol concentration at 522 nm in the concentration range investigated, irrespective of the method of analysis (discrete or global). On the other hand, an increase is observed at 539 nm, again reflecting the buildup of a new, presumably interacting population of fluorophores whose fluorescence is centered at 539 nm, and has a different fluorescence lifetime.

All the above results thus indicate that the NBD groups in NBD-cholesterol are close enough in gel phase membranes at concentrations greater than 0.5 mol % to be able to interact with each other. To gain further insight into the origin of this effect, we investigated whether it was due to the formation of a specific complex with a unique geometry or whether it was a result of nonspecific aggregation (or phase separation) alone. Two sets of experiments were designed to address this issue. In the first set, ULVs were prepared containing DPPC, 0.1 mol % NBD-cholesterol, and variable amounts of unlabeled cholesterol (0−1.9 mol %) so that the total concentration of "cholesterol-like species" (*i.e.*, NBD-cholesterol + cholesterol) varies between 0.1 and 2.0 mol % of the total lipid. The logic used was that, if the

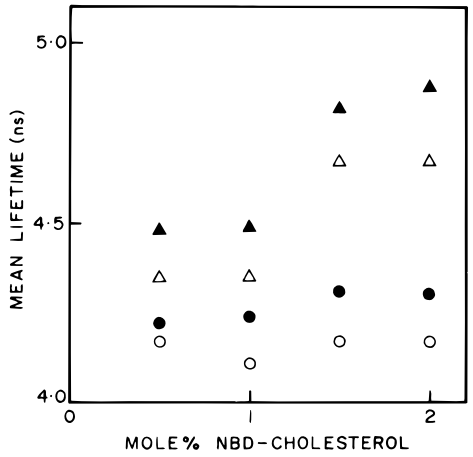

FIGURE 8: Change in the mean fluorescence lifetimes of NBD-cholesterol at emission wavelengths 522 and 539 nm as a function of NBD-cholesterol concentration in unilamellar vesicles of DPPC in the gel phase. The fluorescence intensity decays have been subjected to both discrete analysis [emission wavelength 522 nm (●) and 539 nm (▲)] and global analysis [emission wavelength 522 nm (○) and 539 nm (△)]. All other conditions are as in Figure 7.

interaction observed among NBD-cholesterol molecules was due to an increase in its local concentration (phase separation) alone, then such effects should also be observed with pure cholesterol since cholesterol has been shown to be completely immiscible in the gel phase and to exist as separate solid domains even at very low concentrations (Harris *et al.*, 1995). In such a case, the NBD-cholesterol would act merely as a reporter of cholesterol phase separation, and this would be reflected in a gradual increase in the ratio of the fluorescence intensities at 539 and 522 nm, due to a higher probability of the NBD groups being statistically placed close together in the cholesterol-enriched phase. Figure 9a shows that this is not the case and that NBD-cholesterol, as judged by its fluorescence behavior, is unable to sense the presence of additional cholesterol in the membrane. This points out the formation of a specific complex where the NBD groups of NBD-cholesterol are oriented in a specific geometry relative to each other rather than phase separation alone.

In a variation of the above experiment, unilamellar vesicles of DPPC were prepared containing a total of 2.0 mol % cholesterol-like species (NBD-cholesterol + cholesterol). In each successive sample, however, the concentration of NBD-cholesterol was gradually increased (from 0.1 to 2.0 mol %), and the concentration of cholesterol was correspondingly reduced (from 1.9 to 0 mol %), keeping the total concentration of (NBD-cholesterol + cholesterol) fixed at 2 mol %. In this case, it was expected that, if NBD-cholesterol were reporting cholesterol phase separation, then all these samples should behave identically. Figure 9b shows that this is not the case, and again, the interaction of NBD-cholesterol moieties with each other seemed to be independent of the amount of cholesterol added. The behavior of NBD-cholesterol in gel phase membranes, as reflected by the appearance of a new fluorescence peak at 539 nm, thus appears to be independent of the amount of cholesterol added (see Figure 3 for comparison). Both of the above experiments suggest that the tendency of NBD-cholesterols to interact with each other in gel phase membranes at concentrations above 0.5 mol % involves the formation of a specific complex, with a unique orientation of the NBD groups relative to each other.

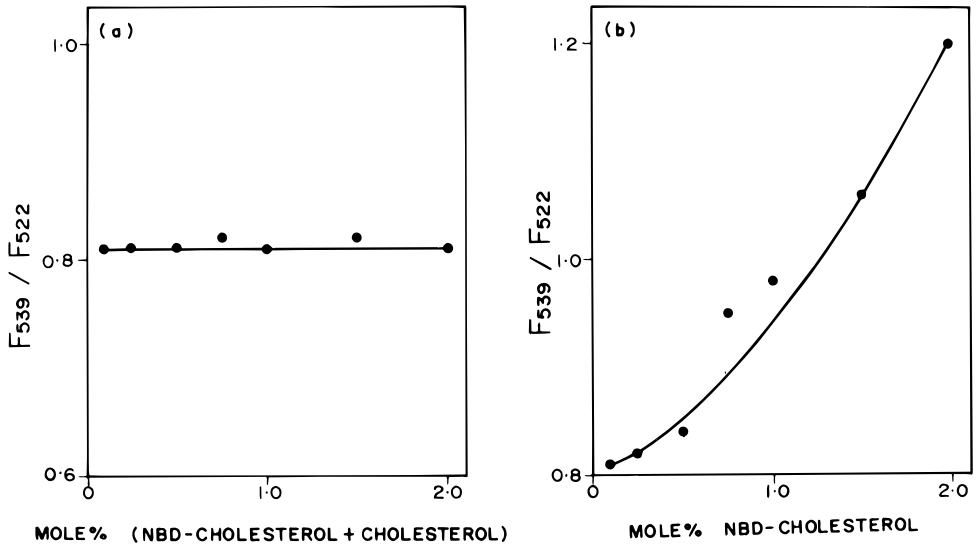

FIGURE 9: Change in the ratio of fluorescence intensities at emission wavelengths 539 and 522 nm as a function of (a) total concentration (in mole percent) of NBD-cholesterol and cholesterol [the concentration of NBD-cholesterol was kept constant at 0.1 mol % in all the samples, the rest of the concentration of (NBD-cholesterol + cholesterol) in each case being made up by unlabeled cholesterol] and (b) mole percent NBD-cholesterol, the total concentration of (NBD-cholesterol + cholesterol) being kept constant at 2 mol % in each case. All other conditions are as in Figure 3.

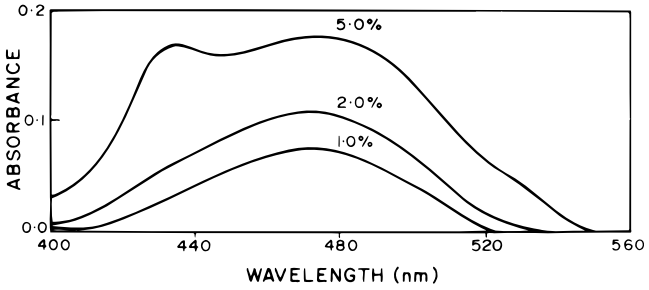

FIGURE 10: Absorption spectra of NBD-cholesterol in unilamellar vesicles of DPPC in the fluid phase (54 °C) at various NBD-cholesterol concentrations. All other conditions are as in Figure 2.

A possible identity of such a specific complex could be the transbilayer, tail-to-tail dimer that has been very recently proposed to occur in fluid phase membranes at cholesterol concentrations above 3 mol % (Harris *et al*., 1995; also see Discussion). It has been previously shown that the NBD group in NBD-cholesterol is located close to the center of the bilayer (Chattopadhyay & London, 1987). It is thus possible that the appearance of the new fluorescence peak that is observed above 0.5 mol % cholesterol in gel phase membranes could result from the transbilayer dimerization of NBD-cholesterol that would bring the two NBD groups of these molecules close together in space. In fact, such a transbilayer dimerization could precede, or accompany, the phase separation of cholesterol in the gel phase membrane at low concentrations. In order to confirm this, we investigated the spectra of 5 mol % NBD-cholesterol in fluid phase membranes. This concentration was chosen since the formation of transmembrane dimers has been suggested previously to occur above 3 mol % cholesterol in the fluid phase (Harris *et al*., 1995). At the same time, this concentration is too low for any other complicating alteration in the phase behavior of these systems (Harris *et al*., 1995). Figures 10 and 11 show the appearance of exactly the same peaks as those observed in gel phase membranes above 0.5 mol % NBD-cholesterol in the absorption (Figure 10) and fluorescence (Figure 11) spectra in this case as well. Since the

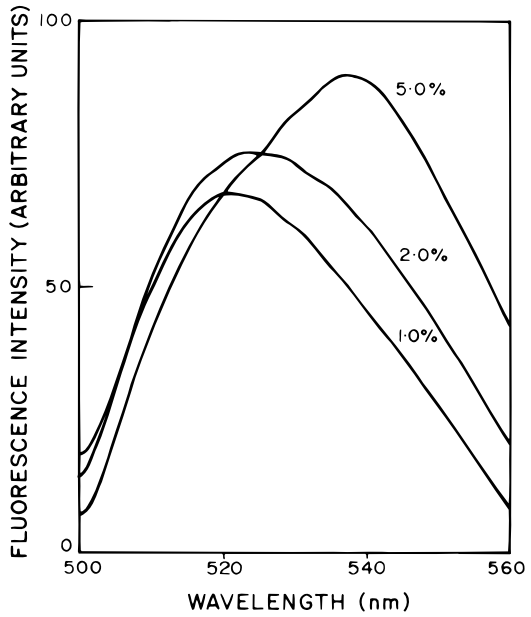

FIGURE 11: Fluorescence spectra of NBD-cholesterol in unilamellar vesicles of DPPC in the fluid phase (54 °C) at various NBD-cholesterol concentrations. All other conditions are as in Figure 2.

only association that cholesterol has been shown to exhibit in the fluid membranes at this concentration is the formation of transmembrane dimers (Harris *et al*., 1995), these results thus suggest that NBD-cholesterol does indeed form transbilayer dimers in the membrane and that these dimers exhibit specific signatures in both the absorption and the fluorescence spectra.

DISCUSSION

The overall goal of this paper has been to assess the behavior of cholesterol as a membrane component at low concentrations, using NBD-cholesterol as a fluorescent analogue. This study is significant in view of the emerging realization that cholesterol may not form uniform monodisperse solutions in the membrane even at very low concentra-

tions (Estep *et al*., 1978; Vist & Davis, 1990; McMullen *et al*., 1993; Harris *et al*., 1995). Since there are various intracellular organelles such as the endoplasmic reticulum and the inner mitochondrial membrane where the cholesterol content is actively maintained at a low level (Yeagle, 1985; Schroeder *et al*., 1991; Bretscher & Munro, 1993), such local organization of cholesterol at low concentrations may have important functional consequences.

Cholesterol is a component of most eukaryotic plasma membranes and, at the relatively high concentrations that are found in these membranes, is rather asymmetrically distributed in the plane of the bilayer, often inducing structurally and functionally important cholesterol-rich and -poor domains. It is interesting to note that, whereas the membranes of some of the cellular organelles contain cholesterol in amounts roughly equimolar to the sum of all other lipids (*e.g*., the plasma membrane), some others, like the endoplasmic reticulum and the inner mitochondrial membrane, have only negligible amounts (Yeagle, 1985, 1987). In fact, 80−90% of the total cellular cholesterol has been found to be present in the plasma membrane (Yeagle, 1985, 1987). Such asymmetric distribution of this molecule both among various organelles and within the bilayer (Yeagle & Young, 1986) raises an interesting possibility that these cholesterol domains may not be only structural in nature but may have functional roles as well.

Two classes of functional roles have been assigned to these domains in cells. On one hand, specific membrane proteins (receptors, transporters, etc.) have been shown to be localized specifically in cholesterol-rich (*e.g*., the nicotinic acetylcholine receptor, human erythrocyte band 3 protein, glycophorin, as well as Na^+,K^+-ATPase) or cholesterol-poor (*e.g*., Ca^{2+}-ATPase) domains, so that the precise structure and composition of these domains, rather than the bulk membrane composition, would affect the functions of these proteins (Yeagle, 1985; Fong & McNamee, 1987; Cornelius, 1995). One situation where such a cholesterol-induced domain is thought to play an important role in cell physiology is the case of the GPI-anchored proteins, in which the carboxy terminal peptide sequence is replaced by a GPI moiety that serves as a membrane anchor. When cross-linked by antibodies (Mayor *et al*., 1994), these molecules have been shown to be clustered into microdomains at the cell surface over specific membrane invaginations called caveolae, often in association with caveolin (a cytoplasmic caveolar coat protein), as well as with cholesterol and glycolipids (Sargiacomo *et al*., 1993; Schroeder *et al*., 1994). There is evidence that such clustering of the GPI-anchored proteins depends upon the presence of cholesterol in the membrane (Rothberg *et al*., 1990). These GPI-anchored proteins, once localized in the caveolae, have been shown to transduce signals to the cytoplasm that result in tyrosine phosphorylation. This transduction event apparently derives from a physical association between GPI-anchored proteins and *Src*-like kinases in the caveolae, a phenomenon of immense importance in cellular transformation (Lisanti *et al*., 1994). In addition to the clustering of the GPI-anchored proteins in the caveolae, cholesterol has also been reported to modulate the transport of various anions, potassium, thymidine, and glucose, as well as the uptake of γ-aminobutyric acid in cells (Yeagle, 1985).

Another class of functional role for the cholesterol-induced membrane domains comes from the kinetic evidence that the transport of cholesterol itself, both through and between membranes, may occur through specific domains (Liscum & Underwood, 1995). The regulation of the size and properties of these domains may control cholesterol transport and accumulation in cells (Schroeder *et al*., 1991; Bretscher & Munro, 1993). For example, intracellular transport of cholesterol from the endoplasmic reticulum to the plasma membrane has been suggested to occur *via* specific domains (Bretscher & Munro, 1993). The retinal rod cells constitute another typical case where cholesterol movement *in vivo* is related to developmental changes. It has been shown (Andrews & Cohen, 1979) that, as the disks move up the rod outer segment (by phagocytosis of the old disks at the top and replacement at the bottom of the stack by new disks), the cholesterol content of the disk membrane decreases progressively. Developmentally, this corresponds to an aging of the disk membrane.

In order to appreciate the role of cholesterol in the above cellular functions, it is thus important to note that cholesterol is generally not present as a monodisperse solution in membranes, and it is this asymmetry in cholesterol distribution that may be important for its function. Detailed phase diagrams of cholesterol in membranes have been constructed by various groups (Lentz *et al*., 1980; Sankaram & Thompson, 1991; Huang *et al*., 1993). In the DPPC/cholesterol system, cholesterol has been proposed to be freely distributed in the bilayer at very low concentrations (<5 mol %). The first change in membrane organization is found to occur above 5 mol %, where a change in the angle of tilt of the fatty acyl chains in the solid PC phase is observed. Sharp changes in the slopes of the phase diagrams are observed at ~22 and 30 mol % cholesterol and have been interpreted to be either due to nonideal mixing of cholesterol phospholipid complexes or due to the appearance of cholesterol−cholesterol lateral dimers (Houslay & Stanley, 1982). Above 50 mol %, a pure cholesterol phase has been suggested to separate out. The first indication that cholesterol could form lateral dimers came from an NMR study of cholesterol in organic solvents at high concentrations (Feher *et al*., 1974). In membranes, such cholesterol dimers have been suggested to interact either through van der Waals interactions between the two β-faces or through close contact between the planar α-faces (Martin & Yeagle, 1978). This model is attractive because, by fixing the number of phospholipids that are required to completely surround a monomer and a dimer of cholesterol in the plane of the membrane, the discontinuities in the phase diagram (that are experimentally observed) can be mathematically predicted.

Although a large body of literature exists about the phase behavior of cholesterol in model membranes, very little is known about its organization in the membrane when the cholesterol content is very low (<5 mol %). It has been assumed that cholesterol is distributed uniformly in the bilayer at these concentrations, although subtle deviations from the predicted behavior of uniform solutions have been previously reported in this concentration range (Estep *et al*., 1978; Vist & Davis, 1990; McMullen *et al*., 1993; Harris *et al*., 1995). Very recent differential-scanning calorimetric studies on DPPC liposomes show that there is in fact a sharpening of the phase transition by cholesterol at very low concentrations (0−5 mol %) instead of the broadening expected if the chain melting were accompanied by continuously changing compositions of the two phases (Harris *et*

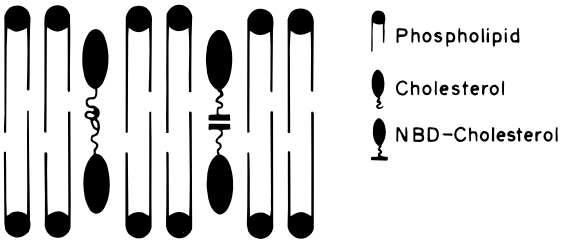

FIGURE 12: Schematic diagram of the DPPC bilayer showing the transbilayer dimers of NBD-cholesterol. The transbilayer tail-to-tail dimer of cholesterol proposed by Harris *et al*. (1995) is also shown for comparison.

al., 1995). Further analysis of these results shows that, whereas cholesterol is completely immiscible in the gel phase membrane and exists as separate solid domains, it forms monodisperse solutions below 2 mol % in the fluid phase. However, above a concentration of 3 mol %, the cholesterol molecules have been shown to exist as transbilayer, tail-to-tail dimers. This is different from the lateral dimerization that has been proposed to occur at relatively high concentrations due to interaction of the sterol rings. The rather deep localization of cholesterol that will be required for the transbilayer dimer formation has been previously proposed (Sankaram & Thompson, 1990, 1991). The above results suggest that the cholesterol molecules are not distributed randomly in the plane of the bilayer even at relatively low concentrations.

Fluorescent cholesterol analogues offer one of the very few experimental handles available for studying the distribution and dynamics of cholesterol in membranes. The NBD moiety represents one such fluorescence probe that has found wide application in various branches of biology (Chattopadhyay, 1990). In this paper, we have discussed the behavior of the NBD-labeled cholesterol analogue where the NBD group is covalently attached to the flexible hydrocarbon tail of cholesterol, keeping all the structural features for proper membrane incorporation of the cholesterol molecule intact. Our results show that NBD-cholesterol forms a monodisperse solution in the gel phase membranes only at very low concentrations (up to ~0.5 mol % NBD-cholesterol). Above this concentration, these molecules seem to specifically interact with each other, giving rise to deviations in their fluorescence behavior, which may be interpreted as an altered local environment experienced by the interacting fraction of the population. No such deviations are observed in fluid membranes in this concentration range of NBD-cholesterol (0−2 mol %).

It has been previously shown that the NBD group in NBD-cholesterol is located close to the center of the bilayer (Chattopadhyay & London, 1987). Since the NBD label in NBD-cholesterol is placed at the end of the flexible hydrocarbon chain of cholesterol [*i.e*., the same parts of the molecule which interact with each other near the center of the bilayer to form the transbilayer, tail-to-tail dimer that has been suggested to occur at very low cholesterol concentrations (Harris *et al*., 1995)], such dimerization of NBD-cholesterol will be favorable (see Figure 12). This would bring the two NBD groups of the interacting molecules close together in space, thereby imparting to them the unique absorption and fluorescence properties.

Concentration dependent self-quenching of the NBD group is quite well-characterized (Nichols & Pagano, 1981; McIntyre *et al*., 1993; Prieto *et al*., 1994) and constitutes the basis of a large number of assays in membrane biology, such as the phase separation of lipids (Hoekstra, 1982; Wiener *et al*., 1985), phospholipid transfer between mixed micelles (Nichols, 1988), and phospholipase A_2 activity (Meyuhs *et al*., 1992). However, what we observe in this paper is not nonspecific quenching of fluorescence. Rather, we find the gradual appearance of a new population of fluorophores with a different emission maximum and fluorescence lifetime with increasing concentration of NBD-cholesterol in the gel phase (Figures 2, 3, 5, and 8). We interpret this to be due to a specific geometry of the NBD groups in the transbilayer dimer. Further, self-quenching occurring merely due to an increase in the local concentration of the fluorophore would not be expected to affect the absorption spectrum of the fluorophore, since in this case, the fluorophores absorb as usual but are deactivated preferentially *via* nonradiative pathways. In this paper, we report the appearance of a new peak in the absorption spectra concomitantly with the alteration in the fluorescence spectra (Figure 4). This strongly argues for the formation of a specific ground state complex at these concentrations, and not nonspecific aggregation.

It is interesting to note that the spectral signatures observed in the gel phase membranes above 0.5 mol % NBD-cholesterol also appear in the fluid phase at 5 mol % NBD-cholesterol. This is in excellent agreement with the observations of Harris *et al*. (1995) of the formation of transbilayer cholesterol dimers in fluid phase membranes at low concentrations (>3 mol %). This reinforces our proposal of the formation of transbilayer NBD-cholesterol dimers at low concentrations in both gel and fluid phases. This also explains the deviations in the fluorescence behavior being exhibited exclusively by the labeled cholesterol, and not by pure cholesterol. All our results thus point out that NBD-cholesterol forms transbilayer dimers in membranes at low concentrations and that these dimers exhibit specific signatures in both the absorption and the fluorescence spectra. We propose that the formation of such transmembrane dimers could constitute the first step in the ultimate separation of a pure cholesterol domain in gel phase membranes at low concentrations. One factor that could contribute to the stability of such dimers is aromatic−aromatic interaction between the NBD rings.

If such transbilayer dimers are indeed present in the membrane, it will be of interest to find out the minimum number of NBD-cholesterol molecules that will be required per vesicle to form these dimers. The "molecular weight" of DPPC ULVs prepared by the ethanol injection method, when the concentration of DPPC in ethanol is ~40 mM, has been previously determined to be 7.14×10^7 g mol^{-1} (Kremer *et al*., 1977). Given the molecular weight of the DPPC monomer to be 734 g mol^{-1}, the total number of lipids that constitute each of these ULVs will be $\sim 10^5$ (Huang & Mason, 1978). Assuming that the NBD-cholesterol molecules are uniformly distributed between vesicles, each vesicle, on the average, will have ~100 NBD-cholesterol molecules for samples containing 0.1 mol % NBD-cholesterol. For 1.0 mol % NBD-cholesterol samples, the corresponding number will be ~1000. Our results thus indicate that, whereas a total of more than 500 NBD-cholesterol molecules per vesicle (composed of $\sim 10^5$ DPPC molecules) is required to give rise to dimer formation in the

gel phase, close to 5000 of these molecules are required in the fluid phase to do so. These numbers are rather interesting since the formation of such dimers could very well signify the initiation of the phase separation of cholesterol in the membrane.

The results presented in this paper confirm that NBD-cholesterol molecules exhibit local organization in the plane of the membrane even at very low concentrations. In molecular terms, this organization could very well correspond to the formation of transbilayer, tail-to-tail NBD-cholesterol dimers, although the possibility of other types of aggregation cannot be ruled out. It will be interesting to determine the implications of such local cholesterol organization in membranes that have very low cholesterol concentrations *in vivo*. Such studies will be especially relevant in the case of the endoplasmic reticulum which, in spite of being the intracellular site for cholesterol biosynthesis, actively maintains the cholesterol concentration in its own membrane at a very low level.

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