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CHOLESTEROL-LIPID INTERACTIONS IN MEMBRANES

THE SATURATION CONCENTRATION OF CHOLESTEROL IN BILAYERS OF VARIOUS LIPIDS

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Summary

1. The integration of cholesterol in a lipid bilayer can be visualized by changes in the fluorescence properties of the probe *N*-phenyl-1-naphthylamine (NPN). An increasing cholesterol content in the lipid phase corresponds to a decreasing fluorescence intensity of NPN and a short wave shift of the emission spectrum.

2. Equilibrium constants for the partition of NPN between water and the various lipid phases are reported. An increasing cholesterol content in a bilayer decreases the solubility of NPN in the bilayer.

3. The saturation concentration of cholesterol in bilayers of various lipids prepared by ultrasonication is determined using the fluorescence probe NPN. The maximal molar ratio of cholesterol : lipid is 2 : 1 for sphingomyelin or egg phosphatidylcholine and 1 : 1 for cerebroside, dipalmitoyl phosphatidylcholine, or dipalmitoyl phosphatidylethanolamine.

4. The comparison of the maximal molar ratio of cholesterol : lipid with the number of proton donor and proton acceptor sites in the lipid moiety is used for a discussion of the polar interactions of cholesterol within a lipid bilayer.

Introduction

The influence of cholesterol on the structure of bilayer membranes is markedly dependent on certain cholesterol : lipid ratios. At a molar ratio of 1 : 2

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Abbreviation: NPN, *N*-phenyl-1-naphthylamine.

(cholesterol : lipid), several properties of the bilayer are changed. For example, the size of vesicles [1,2], the asymmetric partition of cholesterol over the bilayer-halves [1,2] and the mobility of the polar head groups [3] are altered, and the phase transition disappears [4]. The thickness [5] and hydration [1,6,7] of the bilayer depend on the mol fraction of cholesterol in a non-linear function. A molar ratio of 1 : 1 (cholesterol : lipid) is found preferentially in the isolated bilayer particles (vesicles, liposomes) if a cholesterol : lipid mixture with a molar ratio ranging from 1.1 : 1 to 1.9 : 1 is sonicated [8]. For a few lipids, a molar ratio of 2 : 1 (cholesterol : lipid) is found in isolated bilayers if the excess of cholesterol in the sonicated mixture is greater than 2-fold [8—10].

The main questions which arise from the above-mentioned data are: (1) Are all cholesterol molecules at the different molar ratios in an equivalent position with respect to the lipid molecules? (2) Is there a dominant specific molecular interaction of cholesterol with the lipids? Brockerhoff [11] has hypothesised that a hydrogen bond network in the lipid bilayer is formed by a specific hydrogen bond between the β-hydroxyl group of cholesterol and the ester carbonyl of the lipids. In a preliminary report [12] and a recently published review [38], some of the arguments against the importance of such a specific hydrogen bond are presented. (3) From which properties of a lipid molecule does the maximal cholesterol content in the lipid bilayer depend? This question should be approached by first making a systematic comparison of bilayers containing lipids differing in both polar and hydrophobic parts as well as in the back-bone, glycerol or sphingosine.

In the literature, cholesterol to lipid ratios are reported mostly as integral values [7—10,13—15]. But these values depend on the method of bilayer preparation. The optimal temperature and time of sonication depend on the lipid, and must be determined for each lipid separately. Furthermore, it is necessary to ascertain that the excess of free cholesterol is separated quantitatively during the preparation. These conditions were not met in some of these investigations. For this reason, some doubt about the validity of the published values has been expressed by several authors, especially with regard to values of the cholesterol : lipid ratio exceeding unity [8,14,16]. For this reason the validity of the integral values and the maximal ratio of cholesterol : lipid in bilayers had to be examined.

The fluorescence probe *N*-phenyl-1-naphthylamine (NPN), which is incorporated into the hydrocarbon interior of the membrane [17] indicates the integration of cholesterol in the lipid bilayer. This probe is used in the present study to elucidate the saturation concentration of cholesterol in these bilayers without a questionable separation of free cholesterol.

Materials and Methods

Chemicals and equipment were obtained from the following sources: Sigma Chemical Co. (dipalmitoyl phosphatidylcholine, synthetic; cerebroside from bovine brain, 98% pure; sphingomyelin from bovine brain); Fluka (dipalmitoyl-L-α-phosphatidylethanolamine, synthetic; cholesterol, 3 × recrystallized from methanol); Merck (anthrone); Eastman Kodak Co. (*N*-phenyl-1-naphtylamine); Calbiochem AG (enzymatic assay mixture for cholesterol); Aminco Bowman

(Fluorometer SPF with the photomultiplier P 21, which excludes the registration of scattered light of a wavelength <300 nm). Phosphatidylcholine and phosphatidylethanolamine were prepared from egg yolk [18,19].

The purity of the lipids was checked routinely by thin-layer chromatography on silica gel plates. The solvent system used was chloroform/methanol/25% ammonia, in the ratio of 100 : 15 : 1 (by vol.) for cholesterol, 65 : 15 : 1 for cerebroside and 65 : 30 : 3 for phospholipids and for mixtures of these components. The spots were identified according to Dittmer and Lester [20].

Preparation of lipid-cholesterol bilayers

Sonication procedure. Ultrasonication of aqueous suspensions of lipids were carried out with a "Branson sonifier", equipped with a standard tip. The sonication was performed at the energy level 6–7 (50–60 W).

Solutions of lipids and cholesterol in chloroform/methanol (1 : 1, v/v) in the desired molar ratio were added into a 10 ml sonication vessel, and the solvent was completely evaporated under a stream of nitrogen. A 10 ml portion of degassed water was added followed by sonication in a nitrogen atmosphere. The sonication vessel was maintained at a constant temperature above the phase transition temperature of the lipid. The optimal time of sonication was determined by the maximal fluorescence intensity of NPN in a sample with standard conditions as given below. The times are given with the corresponding experimental data.

The sonication of cholesterol/lipid mixtures at temperatures above 30°C had the disadvantage that a unrepreducible amount of cholesterol adsorbed at the surface of the titanium tip. After sonication the suspension was centrifuged at 2000 × *g* for 10 min at room temperature to remove the titanium which originated from the sonication tip. No other components of the suspensions sedimented during this process. Only suspensions with an absorbance smaller than 0.02 (at 385 nm, 1 cm light path) were used for analysis. This liminiation is necessary if cholesterol is present in the suspension. The unbuffered suspensions had a pH value of approx. 5.0. The supernatant was stored under nitrogen at room temperature and used for analysis within 5 h.

Phospholipid concentrations. Determined according to Eibl and Lands [21], using the reagent kit from Serva.

Cholesterol concentrations. Determined using the enzymatic assay kit of Calbiochem. The sample, either in aqueous suspension or dissolved in 50 μl acetone was diluted to 0.5 ml with water, 0.5 ml of the undiluted reagent mixture was added, and the mixture was incubated at 37°C for 30 min. An excess of lipids occasionally caused slight turbidity, and in this case, the solution was centrifuged. The absorption at 500 nm does not change for at least 20 min at room temperature. The cholesterol concentration (M) of the original lipid suspension was calculated using the molar extinction coefficient at 500 nm: $\epsilon = 6830\ cm^{-1} \cdot M^{-1}$.

Quantitative determination of glycolipids

The cerebroside sample is solubilized in 100 μl chloroform/methanol (4 : 100, v/v). A 2 ml portion of anthrone reagent [22] was added, mixed and incubated at 100°C for 12 min. The insoluble components in the incubation mixture (e.g.

cholesterol and fatty acids) were extracted from the hot incubation mixture with 1 ml cyclohexane. The possible contribution of an unspecific anthrone reaction with the unsaturated fatty acids was calculated from the ratio of the absorbances at two different wavelengths (620 and 560 nm). Triplicate samples were used for each determination as well as six blanks in each set of measurements. The estimated values for samples and blanks were averaged and subtracted.

These differences $A_{620\,nm}$ and $A_{520\,nm}$ were inserted into Eqn. 1 thus, giving the corrected absorbance of the hexose moiety, $A'_{620\,nm}$. This calculation is based on the differences in the absorption ratios ($A_{560nm} : A_{620nm}$) of hexose ($H = 0.555$) and the oxidation products of unsaturated fatty acids ($U = 1.20$). This corrected value ($A'_{620\,nm}$) was then inserted into Eqn. 2 to determine the cerebroside content of the original sample. V is the solution volume of the original sample in μl. The constant (0.44) corresponds to the determined extinction coefficient at 620 nm (for a galactosyl ceramide).

$$A'_{620\,nm} = \frac{A_{620\,nm} \cdot U - A_{560\,nm}}{U - H} \tag{1}$$

$$[\text{Cer}] = A'_{620\,nm} \cdot 0.44 : V\,[\text{M}] \tag{2}$$

Fluorescence measurements. To 2.5 ml of a lipid suspension ($4 \cdot 10^{-5}$ M) in the fluorescence cuvette were added 5 μl of a solution of $2 \cdot 10^{-3}$ M *N*-phenyl-1-naphthylamine in methanol. The suspension was equilibrated at 20°C and stirred continually during fluorescence measurement. The stirring of the suspension renders a slow photochemical reaction negligible. Quinine sulfate (0.2 mg/100 ml 0.05 M H_2SO_4) was used as a fluorescence standard before and after each lipid sample. The excitation wavelength was 320 nm. For each sample, both the absorption and fluorescence emission spectra without and with NPN were measured. With these data the measured fluorescence intensity was corrected. The difference of the emission with and without NPN was calculated and then corrected for the inner filter effect using the measured absorbance. These values of fluorescence intensity were standardized by referring to a distinct value of fluorescence intensity of the quinine sulfate standard. The relatively small contribution of the fluorescence of the free NPN in water was then subtracted. The concentration of the free NPN was calculated using the known equilibrium constants (Table I). In all other cases the approximation, free NPN in water equals total NPN, was used. By this way the reported relative fluorescence intensities in arbitrary units were determined. The standard deviation of four determinations for one bilayer preparation was smaller than 2%. The standard deviations of the values for different bilayer preparations are remarkable (±8% for four values), depending on the sonication procedure with its rather poor reproducibility. The equilibrium constants in Table I were determined by the fluorescence measured for systems with different ratios of lipid to NPN concentration. From the values of ten different ratios the two unknown constants, the equilibrium constant K, Eq. 3, and the relative quantum yield of NPN in the corresponding phase, NPN-Lip in Eqn. 4, were calculated by the method of least squares with an interation procedure for Eqn. 3.

$$K = \frac{[\text{NPN}]_{eq} \cdot [\text{Lip}]_{eq}}{[\text{NPN-Lip}]} = \frac{[\text{NPN}_{tot} - H_\lambda \cdot i_\lambda][\text{Lip}_{tot} - H_\lambda \cdot i_\lambda]}{H_\lambda \cdot i_\lambda} \quad (3)$$

$$[\text{NPN-Lip}] = H_\lambda \cdot i_\lambda \quad (4)$$

H_λ is the amplitude of the corrected fluorescence spectrum at a constant wavelength, i_λ is the proportionality factor between H_λ and the absolute quantum yield. i_λ depends on the amplitude/area ratio of the spectrum and on the arbitrary amplification factors used for measurement of H_λ. The best fit for K for all lipids investigated was found with Eqn. 3, as no correlation of fluorescence intensity with a higher order of the concentration of NPN nor that of the lipids was found.

The condition that H_λ is linearly proportional to [NPN-Lip] is fulfilled in some of the cases. In other cases, especially for the lipids with a low affinity for NPN, the approximation of $[\text{NPN}]_{free} = [\text{NPN}]_{total}$ is acceptable for the correction of the fluorescence data. Regardless, this correction is rather small anyway.

The fluorescence measurements are recorded at 20°C for all lipid suspensions. This is below the phase transition temperature of cerebroside (62°C), sphingomyelin (42°C), dipalmitoyl phosphatidylethanolamine (60°C) and dipalmitoyl phosphatidylcholine (40°C). This has to be kept in mind for the comparison of the equilibrium constants. For the valid interpretation of the cholesterol-dependent change in fluorescence intensity, the temperature should not be consequential because one has already sonicated above the phase transition temperature. The integration of cholesterol in the bilayer was checked by the disappearance of the phase transition.

Results and Discussion

The measured fluorescence intensity of NPN in bilayers depends on the lipid. In Table I the dissociation constant K is given for different lipids, and indicates that bilayers prepared from different lipids vary in their ability to integrate NPN, whereas the respective fluorescence quantum yields do not change to the same extent (e.g. sphingomyelin and egg phosphatidylcholine).

NPN also indicates time-dependent changes in aged suspensions. The "aging" of bilayers of the unsaturated lipids corresponds with a decrease in the amount of NPN integrated in the bilayer together with a decrease of the fluorescence quantum yield of NPN (egg phosphatidylcholine (2d) in Table I). By these reasons egg phosphatidylcholine bilayers had a reduced relative fluorescence intensity of 10% in 10 h. Bilayer of saturated lipids are rather stable under these conditions. The influence of NPN (<5 mol %) and methanol (<0.2% vol.) on the bilayer structure is negligible [23] under the standard conditions used.

The integration of cholesterol in a membrane decrease the solubility of NPN in this lipid phase and decrease the fluorescence quantum yield as well (Table I).

The corresponding wavelength shift of the emission maximum of NPN as a function of the total cholesterol content in a suspension of cerebroside bilayers is given in Fig. 1a. The lowest wavelength is reached for about 50% (mol frac-

TABLE I

FLUORESCENCE PROPERTIES AND PARTITION OF *N*-PHENYL-1-NAPHTHYLAMINE IN AQUEOUS SUSPENSIONS OF VARIOUS LIPIDS

The relative fluorescence intensity, I, of NPN in the lipid phase, is given in arbitrary units and was calculated from the area of the corrected spectra. From the total fluorescence of the system ($4 \cdot 10^{-5}$ M lipid, suspended by sonication as given in the legends of Figs. 2—4 and $4 \cdot 10^{-6}$ M NPN, 0.2% methanol) the fluorescence of free NPN in water is subtracted. This concentration of free NPN is calculated with the equilibrium constant K, as far as known. In the case where K is undetermined (**) the approximation, free NPN in water equals total NPN, is found to be acceptable. The cholesterol content is given as mol fraction. The equilibrium constant $K = [NPN]_{eq} \cdot [Lip]_{eq} \cdot [NPN—Lip]^{-1}$ is determined at 20°C as described under Materials and Methods. The quantum yield θ refers to quinine sulfate with $\theta = 0.5$.

Lipid	Relative fluorescence (I)	Equilibrium constant (K)	Quantum yield (θ)
None (NPN in water)	0.45	—	0.022 *
Cholesterol	0.53	$4.0 \cdot 10^{-4}$	0.29
Sphingomyelin (bovine brain)	2.2	$9.7 \cdot 10^{-5}$	0.38
Egg phosphatidylcholine	5.1	$3.2 \cdot 10^{-5}$	0.45
Egg phosphatidylcholine (2d) ***	2.2	$6.7 \cdot 10^{-5}$	0.30
Egg phosphatidylcholine + cholesterol (42%)	2.0	$9.5 \cdot 10^{-5}$	0.30
Cerebroside (bovine brain)	2.0	**	—
Dipalmitoyl phosphatidylcholine	1.9	**	—
Dipalmitoyl phosphatidylcholine + cholesterol (23%)	1.3	**	—
Dipalmitoyl phosphatidylethanolamine	0.75	**	—
Dipalmitoyl phosphatidylethanolamine + cholesterol (19%)	0.90	**	—

* The same value as determined by Radda [36].

** The very small amount of NPN integrated in the lipid phase and the non-linear dependence of fluorescence intensity from the NPN concentration does not allow a correct determination by the method described.

*** 2 days kept at room temperature, under nitrogen.

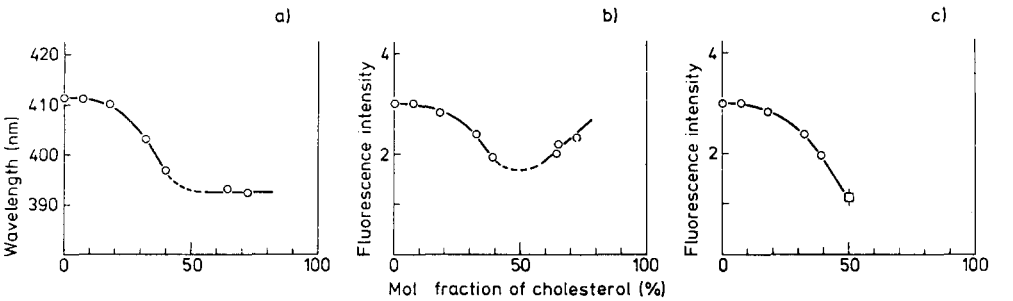

Fig. 1. Influence of cholesterol on the fluorescence properties of *N*-phenyl-1-naphthylamine in the cerebroside cholesterol bilayer. The fluorescence data were corrected for the inner filter effect and for the fluorescence intensity of free NPN in water according its partition in the phases. (a) Wavelength shift of the emission maximum for increasing cholesterol content. (b) Relative fluorescence intensity (arbitrary units) measured at a constant wavelength (420 nm). The mol fraction is calculated for the total amount of cholesterol in the suspension (cholesterol in the lipid phase plus cholesterol in water). (c) the same data as in a and b. Values above 50 mol % cholesterol (suggested saturation concentration of cholesterol in the lipid phase) were corrected for the NPN-cholesterol fluorescence. ϕ, calculated average with standard deviation of the corrected values.

tion) cholesterol. In Fig. 1b the same data as presented in Fig. 1a are evaluated in an other way: The relative fluorescence intensity is measured as the amplitude of the spectrum at a constant wavelength. Instead of the plateau seen in Fig. 1a for the saturation concentration of cholesterol, one observes a minimum for the curve. This method allows a more accurate evaluation especially because of the greater relative difference of the values compared with the pure wavelength shift (Fig. 1a). Fluorescence intensity at a constant wavelength is a more complicated function, which depends on (a) wavelength shift, (b) change in quantum yield and (c) change in equilibrium constant of NPN in the lipid phase. a and b are often coupled functions depending on the environmental constraint and the polarity of environment [36]. From data in Table I it is clear that an increasing concentration of cholesterol decreases the amount of NPN in the lipid phase. If the fluorescence is measured at a wavelength $\lambda > \lambda_{max}$, all three functions (a—c) contribute with the same sign to the intensity, an increasing cholesterol concentration in the bilayer induced a decrease in the relative fluorescence intensity.

Above the saturation concentration the increase of the concentrations of free cholesterol lead to an increase in fluorescence intensity. This is due to the high quantum yield of the cholesterol · NPN complex. With the known distribution of NPN between the different phases (Table I) it is possible to generate a mathematical fit for a distinct saturation value. As for the cholesterol : lipid ratios integral numbers are suggested [8—10], the fluorescence values were fitted for these two possibilities 1 : 1 (50 mol %) or 2 : 1 (66 mol %). The fluorescence values of mixtures above the suggested saturation concentration of cholesterol are corrected by subtraction of the possible contribution of the pure cholesterol · NPN complex. The best fit for cerebroside is given in Fig. 1c; and for sphingomyelin, both fits are shown in Figs. 2b and 2c. Fig. 2b shows the divergence in the corrected values, whereas in Fig. 2c, the average of all corrected values indicates an optimal convergence for a molar ratio of 2 : 1 for sphingomyelin.

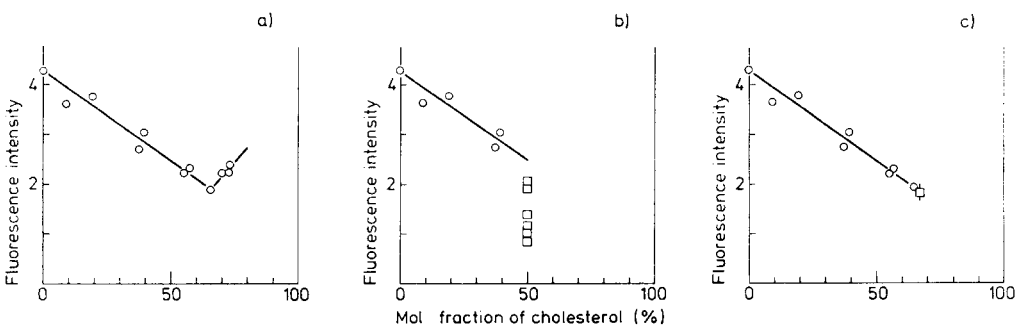

Fig. 2. Fluorescence intensity of *N*-phenyl-1-naphthylamine for different cholesterol concentrations in a sphingomyelin bilayer. The suspensions ($1 \cdot 10^{-4}$ M in sphingomyelin) were sonicated at 75°C for 2 h and diluted to $4 \cdot 10^{-5}$ M sphingomyelin for fluorescence analysis. (a) Relative fluorescence intensity (in arbitrary units) measured at 410 nm, standardized, corrected for the inner filter effect and the fluorescence of the NPN in water. (b, c) The data of a are corrected for the fluorescence of NPN with the free cholesterol, with the supposition that the cholesterol saturation is (b) 50 mol % or (c) 66.6 mol %. □ in b, corrected single values with obvious divergence. ⊕, in c, average with standard deviation of all corrected values.

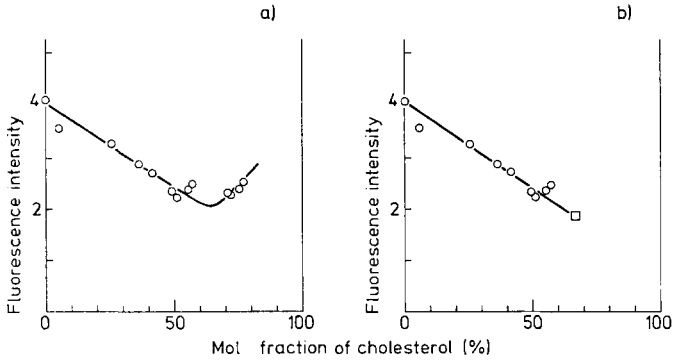

Fig. 3. Influence of cholesterol on the fluorescence intensity of NPN in an egg phosphatidylcholine bilayer. The suspensions ($1 \cdot 10^{-4}$ M in phospholipid) were sonicated at 60°C for 30 min. Alternatively, the sonication at 20°C for 1 h does not alter the results. Also centrifugation with 100 000 × *g*, 3 h does not change the qualitative results. (a) See legend to Fig. 2a. (b) Data corrected for the excess of cholesterol with a best fit for 66 mol % cholesterol in the lipid phase. ϕ, average with standard deviation of the corrected values.

For the cholesterol/lipid mixtures investigated (Figs. 1–4), two types of evaluation are reported: the relative fluorescence intensity as a function of the total cholesterol concentration, and secondly, the best fit for an integral mol ratio. The fit for an integral mol ratio is satisfying for all lipids investigated. The calculated standard deviation of the averaged values corrected by two different ways in Figs. 2b and 2c are ±34% (n = 7) and ±8% (n = 4) respectively.

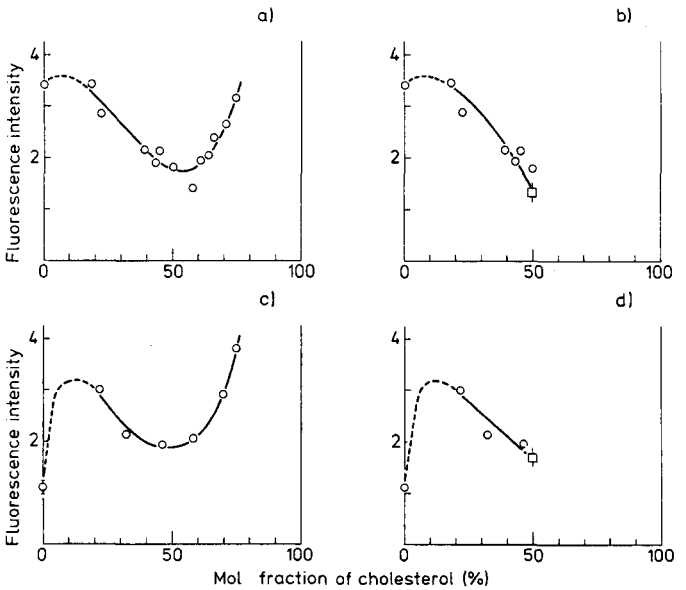

Fig. 4. Influence of cholesterol on the fluorescence intensity of NPN in a dipalmitoyl phosphatidylcholine bilayer (a, b) and a dipalmitoyl phosphatidylethanolamine bilayer (c, d). The suspensions ($1 \cdot 10^{-4}$ M in phospholipid) were sonicated for the phosphatidylcholine at 60°C, 30 min and for the phosphatidylethanolamine at 90°C for 30 min. For fluorescence measurement diluted to $4 \cdot 10^{-5}$ M phospholipids. (a, c) See legend to Fig. 2a. (b, d) Data corrected for the excess of cholesterol with a best fit for 50 mol % cholesterol in the lipid phases. ϕ, average and standard deviation of the corrected values.

This second value, corrected for the integral mol ratio 2 : 1 (cholesterol : lipid) gives the best convergence of the data. The same is true for the data given in the investigations reported in Figs. 3 and 4, if corrected for integral values. These data thus give further evidence for integral mol ratios of cholesterol/lipid bilayers under saturation conditions in an ultrasonicated preparation.

Additionally it is clear that there is a significant difference between the maximal concentration of cholesterol in bilayers of dipalmitoyl phosphatidylcholine (Fig. 4) and egg phosphatidylcholine (Fig. 3) with a mol ratio of 1 : 1 and 2 : 1, respectively.

By this method the values for maximal cholesterol/lipid ratios were determined (Figs. 1—4) and summarized in Table II. These values in Table II confirm some of the values reported previously [5,8—10,13—16]. The maximal concentration of cholesterol in a cerebroside bilayer with 50 mol % is smaller than obtained by the conventional method [15]. This discrepancy depends on various aspects: The integration of cholesterol in lipid bilayer preparations increases the density and size of the particles. This leads to severe difficulties in the usual preparation procedure: the excess of free cholesterol cannot be separated quantitatively by centrifugation or column chromatography on Sepharose (4B/6B) or controlled pore glass (CPG 10, 3200 Å pore diameter). The only exception is seen for egg phosphatidylcholine bilayers, which have a lower density than free cholesterol and the corresponding vesicles with a cholesterol content smaller than 50 mol % are small enough to be separated [1].

These analytical problems for the determination of the cholesterol saturation are solved by the fluorescence method described in this paper. Nevertheless, there is a lack of a satisfying more reproducible method for the preparation of cholesterol-lipid bilayers containing high concentrations of cholesterol.

Interaction of cholesterol and lipids in the bilayer

Egg phosphatidylcholine with its unsaturated fatty acids is able to integrate 2 mol of cholesterol per mol of lipid in the bilayer (Fig. 3 and Table II),

TABLE II

MAXIMAL MOLAR CHOLESTEROL CONTENT AND THE NUMBER OF PROTON SITES IN THE BILAYER OF VARIOUS LIPIDS

The given numbers of acceptor and donor sites of the various lipids refer to the sites at the ester groups and the hydroxyl groups of the fatty acids and do not include the sites at the headgroup moiety. The maximal molar ratios of cholesterol in the lipid bilayers are determined as shown in Figs. 1—4. The lipid/cholesterol systems are prepared by ultrasonication as described.

Lipid	Maximal molar ratio (cholesterol : lipid)	Proton acceptor sites	Proton donor sites
Egg phosphatidylcholine	2 : 1	2	0
Dipalmitoyl phosphatidylcholine	1 : 1	2	0
Dipalmitoyl phosphatidylethanolamine	1 : 1	2	0
Sphingomylein	2 : 1	1	1
Cerebroside	1 : 1	1	2 *

* Average for cerebrosides from bovine brain [37].

whereas the corresponding lipid with saturated fatty acids integrates only 1 mol of cholesterol per mol of lipid (Figs. 4a and 4b, and Table II). One possible explanation for this dependence of the cholesterol affinity for unsaturated phosphatidylcholine, suggests a dominant importance of the cholesterol-lipid interactions in the hydrophobic part of the bilayer [24]. This observation cannot be generalized for all unsaturated lipids, as the unsaturated lipid egg phosphatidylethanolamine has a low affinity for cholesterol [14] in the bilayer. Additionally there was neither an exact correlation of the cholesterol affinity with the phase transition temperature (see ref. 25 and Table II) nor with the condensing effect [13].

The interactions in the polar part of the membrane were looked at as well, and were characterized by hydration [5–7, 26–28], electrostatic interactions of the polar headgroups [3] or by general hydrogen bonds [29]. Cholesterol has been found to be localized in the bilayer near the ester carbonyl of the lipid [26].

Sphingolipids and glycerolipids have different number of proton acceptor and proton donor sites (Tabel II). The donor and acceptor positions at the headgroups can be neglected in this case, since it has been shown that no direct interaction between the phosphate moiety and the hydroxyl of cholesterol occurs [3,26]. From the data in Table II, it is obvious that the maximal concentration of cholesterol in the lipid bilayer does not correlate with the number of proton acceptor position. Phosphatidylcholine, with its two proton acceptor sites, "binds" two molecules of cholesterol per lipid molecule; but sphingomyelin, a phosphosphingolipid which has only one equivalent strong proton acceptor site per lipid molecule, also binds two cholesterol molecules per lipid molecule as well. This lack of correlation between the number of carbonyl ester groups and the number of cholesterol molecules bound in the membrane is in reasonable agreement with the finding of Seelig and Seelig [30] that the two ester carbonyl groups of a glycerolipid in the liquid crystalline phase of a bilayer have different positions.

These observation give strong arguments against the general validity of a specific hydrogen bond between the oxygen of the ester carbonyl and 3-β-hydroxyl of cholesterol. If the ester group is replaced by an ether in the lipid [31], there is no influence observed on the condensing effect by cholesterol. Nevertheless, the presence of the ester group is important for a interrelation between cholesterol and lipid indicated by the partial specific volume [32]. There is possibly but not necessarily a contradiction in both of these results [31,32]: no change in the mean molecular area, but a change in partial specific volume. A possible interpretation which would explain both results could come from a change of thickness of the hydrate layer, but this argument is not yet confirmed because of the ambiguous results previously reported in the literature [1,5,6,28]. Although the ester carbonyl oxygen is suggested to be necessary for some of the effects of cholesterol in the membrane, there is no necessity to characterize the function of the carbonyl oxygen as a specific hydrogen bond acceptor of a hydrogen bridge with the β-hydroxyl of cholesterol.

From the data and the arguments presented above it appears more plausible to look at the general influence of the polar groups on the hydrate structure

in the polar region of the membrane. The polar headgroups, the ester carbonyl groups, the α-hydroxyl group of some fatty acids, and the 3-β-hydroxyl of cholesterol (each of them is hydrated) contribute to the structure of the aqueous polar region of the membrane surface. The known difference in the hydration of ethanolamine and choline in the headgroup of the lipids [27,33], as well as the known instabilities of bilayer vesicles formed from phosphatidylethanolamines [34] contribute to this point of view.

In Fig. 4c a 3-fold increase in fluorescence intensity is observed if 20 mol % of cholesterol are sonicated together with the dipalmitoyl phosphatidylethanolamine. What does this mean? Since the corresponding effect for phosphatidylcholine bilayer (Fig. 4a) is very small, the influence of cholesterol on these lipid membranes should depend on the interactions in the polar, aqueous part of the bilayer. Differences in the hydration as a function of the headgroups could provide the explanation for the differences in the amount of cholesterol integrated into a glycosphingolipid (cerebroside) and a phosphosphingolipid (sphingomyelin) bilayer (Table II).

The reported stereospecificity of the hydroxyl group of cholesterol in its influence of the permeability of non-electrolytes [35] could be explained by different states of hydration of the α- and the β-hydroxyl as well. Of course the 3-β-hydroxyl group represents only one of three structural essentials [38] for the membrane functions of cholesterol. The influence of the hydrophobic moiety of cholesterol in its interactions with lipids and respective membrane functions should not be overlooked. There is no contradiction with the statement that both, the hydrophobic and the polar part of the lipid structure are the limiting conditions for a maximal integration of cholesterol (66 mol %) in the membrane.

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