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Preparation and Properties of Spin-Labeled Lecithin-Cholesterol Liposomes

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Summary. Lecithin-cholesterol vesicles of various compositions containing membrane-bound spin-labeled cholestane can be prepared by appropriate choice of initial concentrations of components during sonication. Increasing incorporation of spin label increases incorporation of cholesterol and decreases incorporation of lecithin, with the result that liposomes with cholesterol-lecithin molar ratios larger than 2 can be obtained. Besides associating with cholesterol-lecithin complexes in the liposome, the spin label seems to associate with cholesterol. Changes of the paramagnetic resonance spectrum of the liposome-bound spin label due to changes in liposomal cholesterol and spin label mole fractions – assessed by three parameters – can be used in cell-liposome interaction studies.

Key words spin-labeled liposomes · lecithin-cholesterol vesicles · liposome-cell interaction · membrane-bound spin label · cholestane spin label · lecithin-cholesterol dispersions

Introduction

The presence of cholesterol in biological membranes has elicited extensive investigations at different organizational levels of biological systems and in model membranes. In monolayers prepared from mixtures of cholesterol and phospholipids, cholesterol has been shown to decrease the molecular surface area of phospholipids, the so-called condensing effect [7, 8, 38, 46]. In multibilayers, both a fluidizing and a condensing effect of cholesterol has been described, according to whether the temperature is respectively below or above the phase transition temperature of the bilayer lipids [10, 40, 54]. ESR[1] studies have shown that, in the fluid state, the presence of cholesterol in the bilayer inhibits the rotational motion of spin probes and the chain motion of phospholipids [27, 28]. It has thus been suggested, from these studies, that cholesterol would regulate membrane fluidity by preventing the formation of crystalline gel areas below the transition temperature of the bilayer lipids and by inhibiting the motion of hydrocarbon chains above this temperature. In oriented multibilayers, prepared from cholesterol-phosphatidylcholine mixtures, a marked increase in the lateral diffusion of fluorescent lipid probes has been observed at cholesterol mole fractions equal to or larger than 0.2 [48]. At this cholesterol concentration a phase transition, similar to that observed with the pure phospholipid [34, 36, 47, 53, 57], has been observed in these mixtures. The effect of cholesterol on the physical state of the membrane lipids has been shown to have a pronounced effect on the binding of specific antibodies against haptens present in lecithin-cholesterol liposomes[2]; an enhancement in antibody binding occurring at cholesterol mole fractions equal to or larger than 0.2 [4, 41]. Enhancement of antibody binding would be due to increased lateral mobility of the haptens and to a steric effect of cholesterol on the bilayer structure [32], associated with increased exposure of the hapten to the antibody sites.

In aqueous dispersions of lecithin and cholesterol, the cholesterol-lecithin molar ratios obtained have been shown to be 1:4 [34, 36, 47, 53, 57], 1:2 [16, 19, 25, 47], 1:1 [10, 37, 38, 44], or 2:1 [18, 19, 26, 39], depending on the conditions used to prepare these dispersions. The maximum cholesterol-solubilizing capacity of lecithin has thus been shown to be 2:1. Some of these dispersions have been found to be metastable [18, 19, 37].

Cholesterol has been incorporated into liposomes used in interaction studies with cells and organisms. In certain cases this has been done with the purpose of studying the role of cholesterol on cellular regulation mechanisms. An example of this approach is studies on the effect of cholesterol on the immune

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[1] Abbreviations: ESR, electron spin resonance; doxyl, 2,2-dimethyl-N-oxyl-oxazolidine; LCC-liposomes, lecithin-cholesterol-3-doxyl-cholestane liposomes; SL, spin label.

[2] The terms liposome and vesicle are used as synonyms in the present work.

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responses of lymphocytes [2, 9, 14, 23]. In other cases the purpose has been to improve carrier properties of liposomes such as permeability and stability. In such studies, cholesterol has been shown to reduce the permeability of the liposome membrane to different solutes [11, 20, 22] and to increase liposome stability in blood plasma [22]. In these cell-liposome interaction studies it has been show that the characteristics of this interaction depend partly on the physicochemical properties of the liposomes, which in turn depend on their composition [44, 45]. It is therefore essential to define the conditions under which liposomes can be reproducibly prepared.

The purpose of the present work is, first, to find out the conditions for reproducible preparation of spin-labeled lecithin-cholesterol liposomes. Secondly, to describe the effect of liposome composition on liposome structure and stability. Thirdly, to investigate the effect of concentration of spin label and cholesterol on the liposomal paramagnetic resonance spectrum, with the aim that such concentration-dependent effects be used in interaction studies with cells. With this purpose in mind, the lipid-soluble spin-labeled steroid 3-doxyl-cholestane, that is readily incorporated into lipid bilayers [6, 30], has been used. The paramagnetic resonance spectrum of this molecule changes both with the cholesterol and its own mole fraction in the bilayer. As pointed out above, changes of the paramagnetic resonance spectrum due to changes of the cholesterol mole fraction are due to restriction of the rotational motion of the spin probe and/or increase in order of the bilayer lipids [27–31]. Spectral changes due to spin probe concentration are mainly the result of spin exchange and electron-electron dipole-dipole interactions [12, 13, 50–52]. Such changes have been used to study lateral diffusion of lipids in natural and artificial membranes [12, 13, 52].

Materials and Methods

Chemicals

Lecithin was purchased from Lipids Products, London. Its phosphate content was determined and found to be 4.20% (wt/wt). Cholesterol was purchased from Sigma Chemical Co., St. Louis, Mo., and recrystallized three times from methanol. The spin label used, 3-doxyl-cholestane (I), was prepared using the method of Keana, Keana, and Beethan [33], with some modifications, by Lars Mittermaier, at this laboratory. Sepharose-4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

I

Chemical Determinations

Phosphate was determined with the method of Bartlett [3]. Cholesterol was determined either with the method of Hanel and Dam [24] or the method of Zlatkis and Zak [58], according to Rudel and Morris [49]. Both methods gave similar results, but the latter was preferred due to its higher sensitivity.

Preparation of Liposomes

A chloroformic solution of lipids, including the spin label, was dried first under nitrogen and next under vacuum. The mixture was suspended in 10 ml 0.15 M NaCl, 2.5 mM phosphate (pH 7.40) to yield a 25 mM suspension of lipid, and sonicated 1 hr under nitrogen in a cylindrical vial 2 cm in diameter in an ice bath by means of a Branson sonifier model W-350, using a microtip, at a power setting of 4 and 50% effective time. The suspension was centrifuged 2 hr at 40,000 × *g* at 2–4 °C and the supernatant, containing the vesicles, kept at 4 °C. The lipids used were either lecithin or lecithin-cholesterol mixtures in various molar ratios, commonly 1:3. The molar concentration of spin label was 2% of the total lipid or higher. The possibility that lipid oxidation occurred during preparation of the liposomes was routinely investigated by UV spectroscopy [35]. No oxidation was detected. Chromatography of liposomes was performed on Sepharose-4B by Dr. Jiri Prejza at this laboratory.

ESR Measurements

ESR measurements were performed at 23 °C with a JE ME-1X spectrometer (Japan Electron Optics Laboratories Co., Ltd.). The sample was contained in a 50 µl glass capillary tube. Treatment of the spectral data described in the text was performed with a Varian 620/i computer. Spin-label concentration was determined by double integration of the ESR spectrum and comparison with a Cu(II)-EDTA standard after *g*-value correction [1].

Results

Preparation and Composition of Liposomes

Results from a representative preparation of spin-labeled lecithin-cholesterol liposomes are shown in Table 1. The low yield of cholesterol obtained is not only due to the excess cholesterol used during the preparation since the same yields were obtained for all suspensions, even for those in which the cholesterol-lecithin molar ratio was lower than 2.

Within the range of concentrations used, the lipid content of the liposomes increased linearly with the initial amounts of lipids. The molar fractions of lecithin and cholesterol in the liposome increased linearly with their respective initial molar fractions[3]. The molar fraction of 3-doxyl-cholestane in the liposome increased also linearly with the initial molar fraction and it was not affected by the initial cholesterol-leci-

[3] The concentrations and molar fractions of the lipids used during sonication will be referred to, in the text and in the Figures, as initial concentrations and molar fractions, respectively. Those of the dispersion obtained after sonication and centrifugation will be referred to as concentrations and molar fractions of the liposome(s).

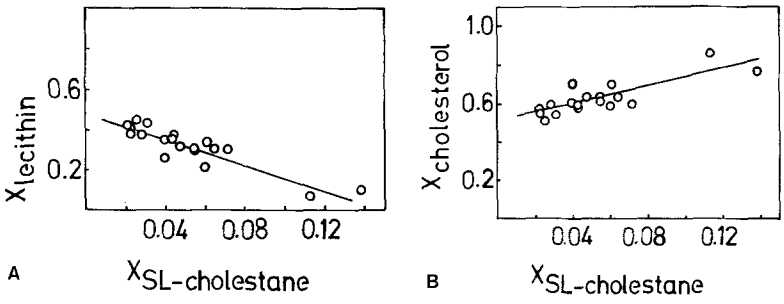

Fig. 1. Effect of 3-doxyl-cholestane on the incorporation of lecithin and cholesterol into LCC-liposomes. Initial lipid concentration = 25 mM

Table 1. Preparation of LCC-Liposomes[a]

Component	Initial amount[b] (mg)	Final amount[b] (mg)	
		Sediment	Liposomes
Lecithin	40.0	16.9	23.6
Cholesterol	60.0	34.1	25.1
SL-cholestane	9.5	7.2	2.7

[a] The results shown are from a representative preparation. Details of the preparation procedure are given in the text. Volume of the suspension = 10 ml

[b] See footnote 3, p. 146

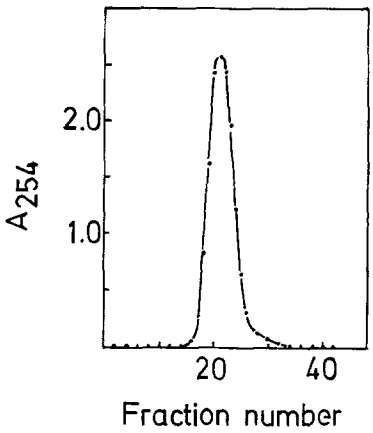

Fig. 2. Chromatography of LCC-liposomes on Sepharose-4B (column of 3 × 65 cm) at 4 °C. The adsorption of lipid from the sample was avoided by presaturating the column with lipid of the same preparation used in the run

thin molar ratio. The whole composition of the liposome was strongly influenced by the 3-doxyl-cholestane content. Increasing the mole fraction of 3-doxyl-cholestane of the liposome increased its cholesterol mole fraction and decreased its lecithin mole fraction (Fig. 1). Thus cholesterol-lecithin molar ratios larger than 2, the highest ratio reported in the literature for lecithin-cholesterol dispersions [18, 21, 26, 39, 42], could be obtained.

The elution profile of lecithin-cholesterol liposome preparations from Sepharose-4B was constituted by a single, fairly symmetrical peak (Fig. 2).

Lack of Soluble Complexes in the Absence of Lecithin

One question that arose in the course of this work was whether or not all the cholesterol and the spin label present in the liposome suspension were incorporated into the vesicles. This question was particularly important in connection with the possibility of using these liposomes in interaction studies with cells. Although the elution profile of these liposomes on Sepharose-4B, constituted by a single symmetrical peak, suggested that cholesterol, 3-doxyl-cholestane, or mixed complexes, were absent in the liposome suspension, the possibility that small amounts of cholesterol or spin label were present in the suspension and reached undetectable levels by sample dilution or by adsorption on the Sepharose-4B matrix could not be excluded by this method. This question was further investigated by sonicating mixtures of cholesterol and 3-doxyl-cholestane under identical conditions as those used for the preparation of liposomes. The results obtained showed that the supernatant, that normally contains the liposomes, did not contain cholesterol, nor had any detectable ESR-signal. Cholesterol was instead completely recovered in the sediment. The sediment exhibited also a broad one-line ESR-signal owing to the high concentration of spin label.

Stability of Liposome Preparations

Two criteria were chosen to judge the stability of a spin-labeled lecithin-cholesterol liposome preparation. These were: precipitate formation and ability to interact with living cells [14]. The system was found to be unstable. Precipitate formation, even when small in certain preparations, was present in all liposome preparations studied. It occurred to a larger extent in preparations with a higher cholesterol content. However, the removal of the sediment from preparations that were three weeks old, showed that the re-

maining liposomes retained the ability to interact with lymphocytes to the same extent as that of fresh preparations (G. Dresdner, L. Hammarström and C.I.E. Smith, *unpublished results*).

ESR Measurements

The type of ESR measurements sought on spin-labeled lecithin-cholesterol liposomes were those that could find applicability in the study of cell-liposome interaction.

In the course of the interaction between liposomes and cells, different phenomena such as adsorption, fusion or endocytosis may occur. During fusion, the mixing of membrane components will be followed by lateral diffusion of these components in the plane of the resulting membrane. A monitoring system of cell-liposome interaction should, therefore, be able to provide information about the total mass of the membrane systems before interaction, the resulting mass of the whole system after interaction, and the physical heterogeneity resulting from the association of different membrane systems in various forms as a consequence of phenomena such as adsorption of liposomes on the cell surface, membrane fusion, and endocytosis. If before the onset of interaction one of the membrane systems contains a spin probe, ESR measurements combined with chemical analysis will be able to provide information about the masses of the labeled membranes present before and after the onset of interaction. Besides, as the different environment that the spin probe will have in each of these membrane systems may be reflected in the shape of its paramagnetic resonance spectrum, it may be possible to identify, before and after the onset of interaction, the various membrane systems in which the spin probe is present. Differences in environment may arise for instance from a different composition of the membranes, which may be associated to differences in the motional freedom of the probe. Differences may arise also owing to different concentrations that the spin probe may attain in the composed interacting system of membranes, due for instance to lateral diffusion. Decreased concentration of the probe will be associated with decreased collision frequency of probe molecules and decreased interaction between the nitroxyl groups of these molecules.

The present study is thus an attempt to investigate these possibilities of identification and quantitative determination by ESR measurements the various membrane forms and associations present in an interacting system of liposomes and cells.

The spectral line widths and shapes and the correlation times for spin labels, particularly 3-doxylcholestane, incorporated into multibilayers and liposomes, have been described elsewhere [5, 27–30]. In particular, the restriction on the motion of the spin label and the ordering effect of cholesterol on the bilayer lipids have been demonstrated in these studies. Studies on the changes of the line shapes due to spin-label concentration in model and natural membranes have also been published [12, 13, 50–52, 56]. These concentration-dependent changes of the ESR spectrum have been used to determine the rate of lateral diffusion in lipid membranes [12, 13, 52]. All these changes are, therefore, valuable in studying cell-liposome interaction.

In what follows the reader is referred for greater details to the references quoted in the preceding paragraph.

As studied in the present investigation, the ESR spectra of spin-labeled cholestane at the bilayer membrane of lecithin-cholesterol liposomes (Figs. 4*A* and 5*A*) will be affected by two mechanisms: (1) changes in the rotational motion of the spin probe and (2) electron spin-electron spin interactions between pairs of label molecules. Spin-spin interactions are dependent on the distance between free radicals and therefore on their concentration in the bilayer. When the unpaired electrons of these radicals are sufficiently close, they can interact by one or both of the following mechanisms: dipole-dipole interactions and spin-exchange interactions. Spin-exchange interactions require that the nitroxyl radicals be in van der Waals contact.

Decrease in the rotational motion of the probe in the bilayer will be accompanied by line broadening and larger correlation times. Increased spin-spin interaction will affect the line shapes in different ways. The dipole-dipole interaction will broaden the resonance lines. The spin-exchange interaction at low label concentration will broaden the lines and the field position of the two side resonances will change to the center of the spectrum. As the spin-label concentration increases the resonances will coalesce into a single broad line whose width will decrease further with increasing concentration. Since the contributions of dipole-dipole and spin-exchange interactions to line broadening are temperature-dependent, they can be assessed in each case by ESR measurements performed at various temperatures. At low temperature ($T \lesssim 20$ °C) the dipole-dipole contribution predominates. At high temperature the spin-exchange contribution predominates.

In the system studied here, two main composition variables will affect the shape of the ESR signal of the liposome-bound spin label. One is the dependence on cholesterol concentration; the other is the dependence on spin-label concentration. Increasing cholesterol concentration will decrease the motional free-

dom of the spin probe in the lipid bilayer and will therefore broaden the resonance lines. Increasing spin label concentration will increase the rate of intermolecular collisions and will therefore produce line broadening and the global changes in the shape of the paramagnetic resonance signal described above. Figures 4*A* and 5*A* show the ESR spectra of spin-labeled lecithin-cholesterol liposomes obtained for two concentrations of each of the components referred to above.

In order to assess the effect of these variables, the composition of the system was changed in two steps. In the first step the liposomal spin-label mole fraction was kept within a range of 0.022–0.035 and the concentration of cholesterol varied. Assuming a homogeneous distribution of spin label in the bilayer, the estimated nearest-neighbor distance between spin probe molecules, in the molar fraction range of 0.022–0.035, varied between 55 and 45 Å, respectively (for an explanation *see* the legend of Fig. 6). In the second step the molar fraction of cholesterol was kept high (>0.5–0.6) and the spin-label concentration varied. Liposomal cholesterol molar fractions of 0.6 were about the largest ones obtained when the spin-label mole fraction of the liposome was ≤ 0.035.

The changes of the paramagnetic resonance spectrum of the cholestane spin label present in LCC-liposomes that occurred as a consequence of changes in liposome composition were assessed by three parameters: parameter H/D, the hyperfine splitting $2T'_{\parallel}$ (Fig. 4*A*), and parameter δ (Fig. 5*A*).

Parameter H/D was chosen as a measure of line broadening or narrowing. H is the amplitude of the center line of the nitroxide ESR spectrum (Fig. 5*A*) and D is the value of the double integral of the spectrum, that is proportional to spin label concentration. H increased nonlinearly with the liposomal mole fraction of 3-doxyl-cholestane. From this concentration dependence was derived the ratio H/D that is instead an intensive parameter. H/D decreased slightly and linearly with the liposomal mole fraction of cholesterol for $X_{\text{chol}} < 0.6$ (Fig. 3*A*). This decrease was accompanied by line broadening (Fig. 3*A*), determined primarily by decrease in the motional freedom of the spin probe [27–30]. Increasing the spin-label concentration of the liposome decreased markedly the value of H/D (Fig. 3*B*) and was accompanied by line broadening (Fig. 3*B*). For molar fractions larger than 0.06–0.07 the value of H/D remained unchanged. The effect of spin-label concentration on H/D may have been in some experiments the result of both increased interaction between label molecules and decreased motional freedom of the label, since increasing liposomal concentration of spin label was accompanied by increasing cholesterol concentration in the liposome (Fig. 1*B*). That the effect of spin-label concentration on H/D conformed, however, to the functional dependence depicted in Fig. 3*B* is supported by the results from those liposome preparations that had a cholesterol mole of 0.5–0.6 and various spin-label mole fractions but nevertheless fitted within the relationship shown in Fig. 3*B*. In contrast, the H/D values of those liposome preparations with a $X_{\text{chol}} \geq 0.5$ did not fit within the relationship depicted in Fig. 3*A*. The effect observed seems, therefore, to be predominantly the result of increased interaction between spin-label molecules.

The hyperfine splitting $2T'_{\parallel}$ (Fig. 4*A*) is a measure of the rotational motion of the spin label and the order of the bilayer lipids. A decrease in the rotational motion of the spin label will increase the value of $2T'_{\parallel}$. The hyperfine splitting is also sensitive to spin-spin interaction and therefore the collision rate of spin-label molecules. In lipid systems, the variation of $2T'_{\parallel}$ with spin-label concentration will be complex. At moderately high concentrations of spin label the dipole-dipole interaction will increase $2T'_{\parallel}$ and the spin-exchange interaction will decrease it [12, 51], the overall effect observed depending on which contribution predominates. Higher spin-label concentration will decrease $2T'_{\parallel}$. In the system studied in the present work $2T'_{\parallel}$ increased more or less linearly with the cholesterol-lecithin liposomal molar ratio (Fig. 4*B*), which indicated decreased mobility of the spin probe, as found earlier by other investigations [27–30]. Increasing the mole fraction of cholestane spin label decreased $2T'_{\parallel}$ in a linear fashion up to a mole fraction of 0.06–0.07 (Fig. 4*C*), after which $2T'_{\parallel}$ remained constant.

Parameter δ was empirically chosen from the changes observed on the spectral line shapes determined by increasing the cholesterol or the spin-label concentration in the liposomes. The zone of the ESR spectrum that was more sensitive to these changes in concentration was chosen to define δ (Fig. 5*A*). It is associated with the line broadening that occurs when the mobility of the spin label in the system decreases or the interaction between nitroxyl radicals in the system increases. It is empirically related to both the average distance between label molecules in the system (Fig. 6*A*) and their exchange frequency (Fig. 6*B*). Further studies, among them spectral simulation, are necessary in order to get a more fundamental explanation of its physical significance. Concentration-dependent line broadening was also observed for the low field resonance, that can also be correlated as δ with the liposomal cholesterol and spin-label concentration. If ESR-difference spectra measurements are sought, measurements of δ are adequate, whereas those performed on the low field resonance

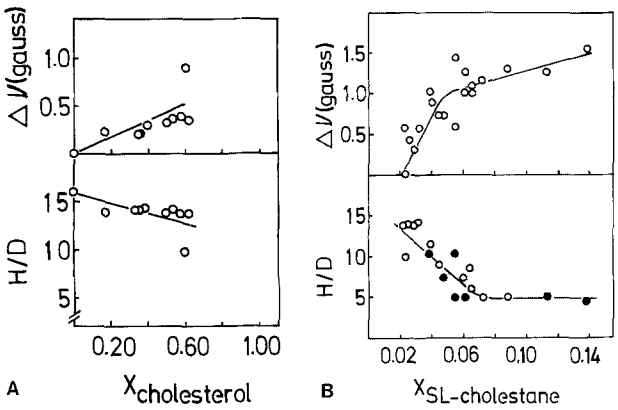

Fig. 3. Variation of ESR-parameter H/D and the linewidth enhancement $\Delta\nu$ with the liposomal mole fraction of cholesterol (*A*) and 3-doxyl-cholestane (*B*). $\Delta\nu$ was measured for the middle resonance and calculated using different conditions in *A* and *B*. The linewidth is defined as the separation in gauss between the maximum and the minimum of the line. *A*. $\Delta\nu$ was calculated by subtracting the width of the middle line of the spectrum at $X_{chol}=0$ and $X_{SL\text{-}chol}=0.022$ from the width of the middle line at a higher value of X_{chol}. The mole fraction of the spin label in the samples varied between 0.022 and 0.035. *B* $\Delta\nu$ was calculated by subtracting the width of the middle line of the spectrum at $X_{chol}=0.58$ and $X_{SL\text{-}chol}=0.023$ from the width of the middle line at a higher value of $X_{SL\text{-}chol}$. The range of the cholesterol mole fraction of the samples was as follows; open circles: 0.50–0.62, closed circles: 0.64–0.85. The two open circles with $X_{SL\text{-}chol}>0.07$ had, in order of increasing SL-cholestance concentration, X_{chol} values of 0.55 and 0.50, respectively. The two closed circles with $X_{SL\text{-}chol}<0.05$ had, in order of increasing SL-cholestane concentration, X_{chol} values of 0.71 and 0.64, respectively

are not, because of a more marked shift of the field position of this line with concentration. The variation of δ with the cholesterol mole fraction of the liposome is shown in Fig. 5*B*. Its value decreased linearly until a cholesterol mole fraction of about 0.5 was reached. After that it remained constant up to cholesterol-lecithin mole ratios of about 2. A further linear decrease of δ at this cholesterol-lecithin molar ratio was obtained by increasing the liposomal mole fraction of 3-doxyl-cholestane (Fig. 5*C*). A change in slope was detected near $X_{SL\text{-}chol}=0.065$, as observed also with H/D and $2T'_{\parallel}$.

Discussion

The results presented here confirm the view sustained by previous investigations [17–19, 38, 39, 44] that lecithin constitutes an essential substrate for cholesterol solubilization. This statement also applies to the spin label 3-doxyl-cholestane, as no cholesterol, 3-doxyl-cholestane, or mixed soluble complexes of these substances could be detected in the dispersion after prolonged sonication. Considering that these liposomes can be used in interaction studies with cells, this result is particularly important [14, 15].

If comparison is made between the single-peak elution profile of these lecithin-cholesterol liposomes in Sepharose-4B (Fig. 3) and that found for lecithin liposomes [31], it becomes apparent that the presence of cholesterol eliminated polydispersity of the suspension. The fraction of lecithin that did not contribute to the building of vesicles became associated with cholesterol in the form of large insoluble aggregates that sedimented easily by centrifugation.

The results presented here on the stoichiometry of sonicated lecithin-cholesterol mixtures and the liposomes formed from them agree in many features with those obtained by other investigators [18, 21, 26, 39], but they also show some differences. This was expected since the system studied here contained a new component: the spin label 3-doxyl-cholestane. Some of the data suggest that, in this system, cholesterol-lecithin complexes with a 1:1 and 2:1 stoichiometry might be formed. In some results the presence of a phase boundary at a cholesterol-lecithin molar ratio of 1:1 is suggested (Figs. 4 and 5). The scatter of the data, does not allow, however, to make a safe statement in this sense. The molar ratios of these lipids found in liposomes with a 3-doxyl-cholestane mole fraction lower than 0.035 support the idea that 2:1 cholesterol-lecithin complexes might be present in the liposomes. The 2:1 cholesterol-lecithin complex has been found to be metastable [18, 26], which leads to vesicle aggregation [39, 42]. This would be reflected in the present results by the formation of precipitate in the liposome suspension. No evidence has been found in the present study, as found in other cases, for a phase boundary at a 1:4 or a 1:2 cholesterol-lecithin molar ratio [16, 19, 25, 34, 36, 47, 55, 57]. It must be pointed out, however, that the evidence shown to support the presence of phase boundaries in binary mixtures of cholesterol and lecithin depends distinctly on both the method used to prepare these mixtures and the method used to study them [18, 19, 26, 34, 36–38, 44, 47, 55, 57].

The presence of 3-doxyl-cholestane introduced a new component in the system, which differentiated these dispersions from lecithin-cholesterol dispersions. The first apparent difference is the fact that the incorporation of 3-doxyl-cholestane into the liposome favored, in relative terms, the incorporation of cholesterol and decreased the incorporation of lecithin (Fig. 1), with the result that liposomes with cholesterol-lecithin molar ratios larger than 2 – which are the highest ratios reported in the literature for cholesterol-lecithin dispersions [18, 26, 38, 39] – could be obtained. The slopes of the curves in Fig. 1 show that, for each mole of 3-doxyl-cholestane incorporated, the liposome contained in relative terms two additional moles of cholesterol and one less mole

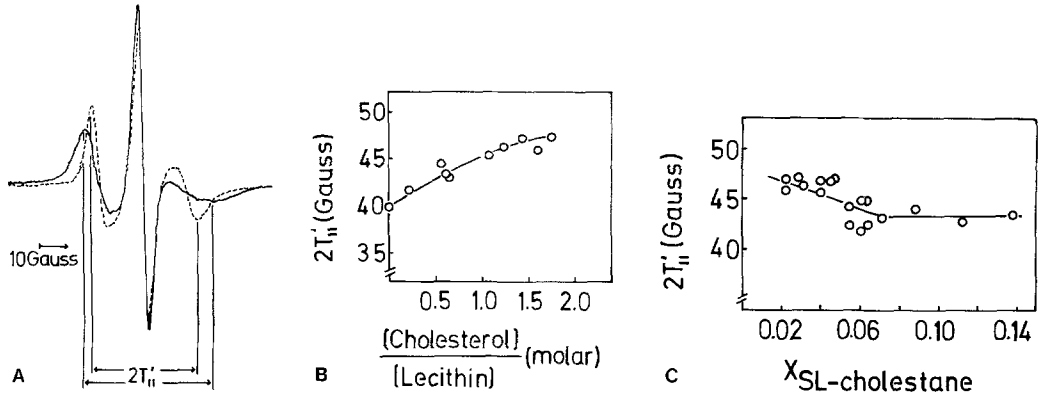

Fig. 4. Variation of the hyperfine splitting $2T'_{II}$ of LCC-liposomes with the liposomal cholesterol-lecithin molar ratio (*B*) and the liposomal 3-doxyl-cholestane mole fraction (*C*). In *B* the value of $X_{SL\text{-}chol}$ was 0.022–0.035. In *C* the value of cholesterol/lecithin (molar) $\geqq$ 1.0

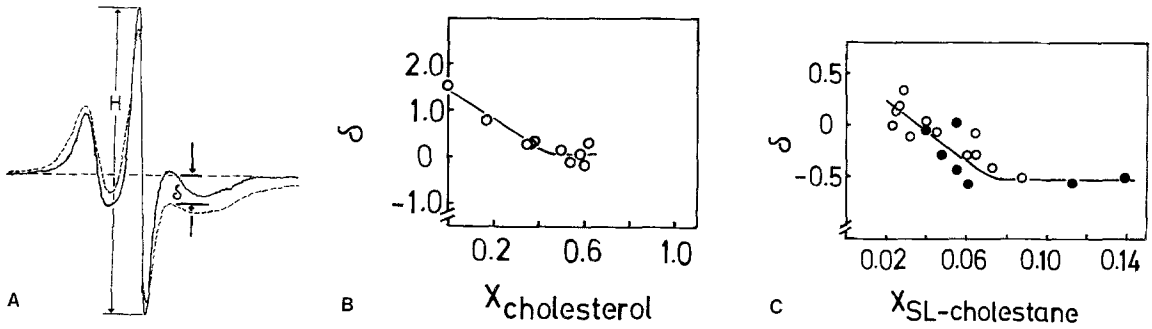

Fig. 5. Variation of ESR-parameter δ with the liposomal mole fraction of cholesterol (*B*) and 3-doxyl-cholestane (*C*). δ values are positive above the baseline and negative below it. Before calculation the ESR spectra were normalized by assigning to the double integral of the spectra the same arbitrary value. In *B* the value of $X_{SL\text{-}chol}$ was 0.022-0.035. In *C* the values of X_{chol} were as described in Fig. 3*B*

of lecithin. At liposomal 3-doxyl-cholestane mole fractions larger than 0.05, liposomes with cholesterol-lecithin molar ratios larger than 2 could be obtained. Beyond this point, both the molar fractions of cholesterol and 3-doxyl-cholestane continued to increase and that of lecithin continued to decrease. Since, under the conditions of stability of these dispersions, cholesterol is practically insoluble in water, it is suggested from these results that, after the maximal cholesterol-solubilizing capacity of lecithin in the dispersion has been reached, 3-doxyl-cholestane must be the cholesterol-stabilizing factor in the dispersion. The hyperfine splitting $2T'_{II}$ remained constant for molar fractions of 3-doxyl-cholestane larger than 0.065 (Fig. 4), which suggested that the attainment of this ratio might be related to a phase boundary where a new phase containing exclusively cholesterol might be formed. But as the value of $2T'_{II}$ is increased by decreased motional freedom of the spin probe and decreased by increased collision rate of the probe molecules it is not possible, with the composition that the system has at this mole fraction of 3-doxyl-cholestane, to determine the effect of the liposomal cholesterol mole fraction alone on $2T'_{II}$ and ascertain whether or not such phase is formed. The scatter

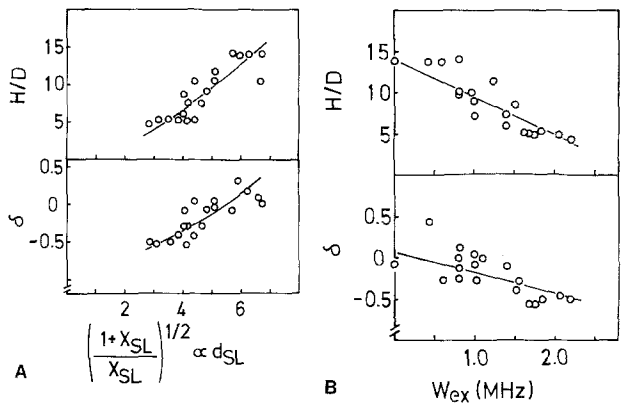

Fig. 6. Parameters *H*/*D* and δ as a function of the distance between spin-label molecules in the liposome bilayer and as a function of their exchange frequency. The distance between label molecules d_{SL} is directly proportional to $((1+X_{SL})/X_{SL})^{\frac{1}{3}}$ where X_{SL} is the mole fraction of spin label in the bilayer [56]. The exchange frequency W_{ex} is given by $W_{ex}=\Delta\nu/2$, where $\Delta\nu$ is the linewidth enhancement due to spin exchange as defined in Fig. 3*B* [51]

of the data in Figs. 3*B*, 4*B* and 5*C* could also be agreeable with other than the linear plots shown there. For liposomal $X_{SL\text{-}cholestane} > 0.05$ the molar ratio cholesterol-(3-doxyl-cholestane) increases and approaches the value of 5–6 and the lecithin-(3-doxyl-

cholestane) molar ratio approaches 0. In order to explain the (transient) stability of cholesterol in these dispersions some sort of specific interaction between cholesterol and 3-doxyl-cholestane must be accepted. It is possible that the presence in 3-doxyl-cholestane of the bulky nitroxyl group at position 3 of the cholestane molecule, and near the hydrophilic region of the bilayer, may play a role in the assembly, packing, and stoichiometry of these molecules to constitute liposomes.

The choice of the ESR spectral parameters for the spin probe 3-doxyl-cholestane incorporated into liposomes, described in the present work, has been done based on their dependence on cholesterol and spin-label concentration. Parameters H/D and δ are associated with spectral line broadening or narrowing due to changes in the motional freedom of the spin probe or to spin-spin interactions. It is expected, therefore, that these parameters will be convenient in monitoring cell-liposome interaction [14]. An application of these results is shown in the accompanying paper [15].

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