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R. S. Khare [a] & C. R. Worthington [a]
[a] Departments of Biological Sciences and Physics, Carnegie-Mellon University, Pittsburgh, PA, 15213
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An X-Ray Diffraction Study of Sphingomyelin–Cholesterol Interaction in Oriented Bilayers

R. S. KHARE and C. R. WORTHINGTON

Departments of Biological Sciences and Physics, Carnegie-Mellon University, Pittsburgh, PA 15213

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Oriented samples of sphingomyelin (SM), cholesterol (CHOL) and their mixtures in SM : CHOL molar proportions of 4 : 1, 3 : 1, 2 : 1, 1 : 1 and 1 : 2 were studied by x-ray diffraction. These samples were in multilayered form such that a series of discrete series of x-ray reflections were recorded from a well-defined lamellar repeating distance. Samples with SM : CHOL molar ratio of 2 : 1 had a phase different from the SM bilayers alone. Samples with SM : CHOL ratio less than and greater than 2 : 1 appeared to have separate SM and CHOL phases. An x-ray analysis of the diffraction pattern of the 2 : 1 SM : CHOL complex is presented.

INTRODUCTION

Amphiphilic molecules such as phospholipids and sterols are major lipid constituents of biomembranes[1] and there is considerable evidence that many biomembranes have as their basis a bilayer of phospholipid and cholesterol.[2] In recent years the molecular interactions of phospholipids and steroids in the lamellar gel and liquid crystalline phases have been studied by a variety of physical techniques and the effects of cholesterol on the structural and dynamical properties of phospholipid bilayers and biomembranes are becoming better understood.[3] It is now well documented[4] that addition of cholesterol to dispersions of lecithin and other lipids causes the thermal phase transition of these lipids to disappear at sufficiently high cholesterol (about 50 percent) molar concentrations. It has also been noted that cholesterol reduces the fluidity of the lipid hydrocarbon chains above the transition temperature and increases the fluidity below the transition temperature.[4] Explanation of these effects have ranged from the postulation of the formation of molecular complexes[5–8] to one based on the conclusion that there is no interaction between lipids and cholesterol.[9,10]

Calorimetric, x-ray and NMR studies indicate that lecithin and cholesterol interact to form a molecular complex at stoichiometric ratios of 1 : 1[11,12] and also at 2 : 1[13,14] and 1 : 2.[32] There is evidence that cholesterol and lysolecithin combine in equimolar ratio.[15] But knowledge on the kind of phases or the kind of complexes between phospholipids and cholesterol in other than equimolar ratios is meagre and this situation remains controversial.[16–19] Most of the earlier investigations have emphasized the effect of cholesterol on the apolar regions of the lipid bilayers. Recently the importance of hydrogen bond formation by the 3β-OH group of the cholesterol with the polar portions of phospholipids has attracted attention,[20,21] but it is not known which atomic group of the phospholipid is involved in the hydrogen bond formation. Evidence for hydrogen bond formation at the phosphate oxygen,[22,23] with ester carbonyl groups[24,25] or hydroxyl group in ceramides[26,27] has been reported and the quaternary ammonium group via ionic or hydrogen bonding has been postulated.[5,7]

Most of studies on phospholipid–cholesterol bilayers have been made with hydrated samples, mostly lecithins or lipid mixtures derived from biological membranes. Oriented multilayers are not very suitable for studying phospholipids in excess water and therefore most of the work has been done with lecithin–cholesterol codispersions. There are a number of diffraction studies on lecithin–cholesterol interaction[11,13,17,28–32] and a detailed structural study has been recently reported.[33,34] Sphingomyelin is one of the major lipid constituents of most mammalian biomembranes[1] and has a thermal liquid–crystalline transition near the physiological temperature range.[35] The sphingomyelin–cholesterol interaction is of intrinsic interest for in many animal tissues and membranes there appears to be a correlative relationship in the amounts of cholesterol, sphingomyelin and total lipids present.[36,37] Possible molecular complexes between sphingomyelin and cholesterol have been suggested for nerve myelin[2,7,38,39] but only a few studies have been made. However, studies on sphingomyelin–cholesterol interaction using titration,[40] ESR,[41,42] NMR[43] and calorimetric[43,44] techniques have been reported. There is no x-ray diffraction study on sphingomyelin–cholesterol interaction in the literature, although Shipley *et al*[45] has reported on the phase behavior in sphingomyelin–cholesterol–lecithin–water mixtures. It is commonly assumed that the interaction of sphingomyelin with cholesterol is very similar to that of lecithin with cholesterol,[7,46] however, x-ray evidence for such an assumption has not been reported and our present x-ray results (to be described) indicate that it is, in fact, different.

The steady-state association of cholesterol with biomembranes probably also involves sphingomyelin and this association is thought to be important for myelin structure. It is therefore of interest to examine the association of

cholesterol with sphingomyelin by x-ray diffraction and to draw comparisons between the cholesterol–lecithin complexes. The purpose of this communication is to present the results of our preliminary x-ray study on sphingomyelin–cholesterol interaction in oriented bilayers.

MATERIALS AND METHODS

Bovine brain sphingomyelin (SM) and cholesterol (CHOL) were purchased from Sigma Chemical Company and were used without further purification. Oriented samples of SM, CHOL and their mixtures in various molar ratios were obtained by slow evaporation of their solution in chloroform in glass capillaries (1.0 mm diameter). The inner surfaces of the glass capillaries were coated with a monolayer of the silane surface coupling agent, N,N-dimethyl-N-octadecyl-1-1, 3-amino-propyl-trimethoxysilyl chloride (DMOAP) (Dow Corning #XZ 2-2300). The silane coupling agent constrains the SM and CHOL molecules to align fairly uniformly normal to the surface of the capillary as shown by their x-ray diffraction patterns.

X-ray diffraction patterns were recorded using Phillips microfocus x-ray source. For obtaining better resolved lower order reflections, an optically focusing x-ray camera[47] and Elliot Rotating anode generator was used. Most patterns were recorded using a point focus collimation, which was obtained by using only one mirror and a guard slit collimation. X-ray patterns were recorded on Ilford Industrial G x-ray films. The patterns were usually recorded using a specimen-to-film distance of about 7.5 cm or about 10 cm. Exposure times varied from $\frac{1}{2}$ hr to 10 hrs. Spacings and relative intensities of the reflections were measured on microdensitometer (Joyce–Loebl MK-IIIC). The background curve was subtracted from the tracing in the usual way. Spacings (d) were calculated from Bragg's equation $2d \sin \Theta = h\lambda$, where h is the diffraction order. The discrete intensities $I(h)$ of lamellar reflections[48] on the meridian were obtained by measuring the area under the diffraction peaks and then multiplied by h^2. All experiments were carried out at room temperature (20 ± 2°C). In the x-ray analysis of lamellar reflection, we use the notation[49] that $t(x)$ is the electron density of the unit cell of width d, and $T(X)$ is the Fourier transform of $t(x)$, where x and X are real and reciprocal distances. If the SM + CHOL bilayers have a regular lamellar repeating unit, then discrete reflections at $X = h/d$, where h is an integer, are recorded. The observed Fourier transform for these bilayers is related to the integrated intensities by the relation

$$T(h) = [h^2 I(h)]^{1/2}.$$

The low-angle x-ray data show a minimum Bragg spacing of d/h_{max} and the x-ray data have a resolution of $d(2h_{max})^{-1}$.

RESULTS

X-ray diffraction patterns from oriented bilayers of SM and CHOL are shown in Figure 1A and B respectively. It is evident from these patterns that these specimens have an oriented lamellar structure. The x-ray pattern from

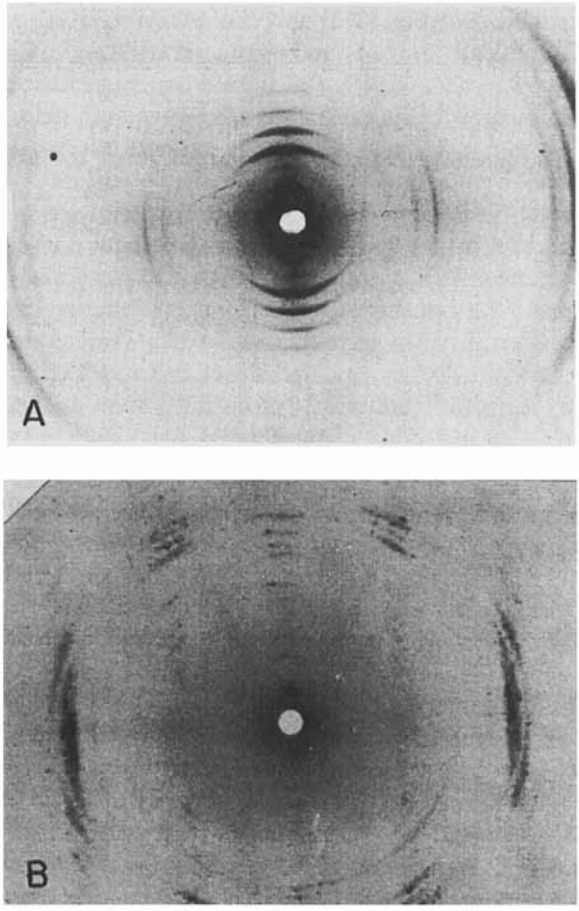

FIGURE 1 A: Low-angle x-ray diffraction pattern from sphingomyelin bilayers. Seven orders of a lamellar repeat of $d = 68.5$ Å are present in the reproduction while fourteen orders are visible on the original negative. A strong 4.2 Å reflection in the equatorial plane is also visible. B: Low-angle x-ray diffraction pattern from cholesterol bilayers. A series of lamellar reflection of a 34 Å repeat, many reflections clustered around 5 to 6 Å in the equatorial plane and other crystalline reflections are present.

SM bilayers (Figure 1A) contained fourteen orders of diffraction of a 68.5 Å repeat and a strong 4.2 Å reflection in the equatorial plane was present. The x-ray pattern from CHOL bilayers (Figure 1B) contained a series of lamellar reflections of a 34 Å repeat, together with reflections clustered around 5 Å to 6 Å in the equatorial plane and other crystalline reflections. X-ray diffraction patterns from SM + CHOL mixtures are shown in Figures 2A, B, C, D and 3A, B, C, D and the results are summarized in Table I. From Table I and from Figure 2 it is evident that a well-defined series of lamellar reflections was obtained from the 2 : 1 SM : CHOL mixture whereas only a few and not well oriented lamellar reflections were obtained from the other mixtures with different molar ratios. We therefore concentrate our attention on the x-ray data from the 2 : 1 mixture.

TABLE I

Summary of x-ray diffraction spacings in Å for sphingomyelin (SM) and cholesterol (CHOL) mixtures

	SM alone	SM : CHOL 4 : 1	3 : 1	2 : 1	1 : 1	1 : 2	CHOL alone
Lamellar diffraction	Orders of 68.5	68.5 s 17 ms	68.5 s 17 ms	Orders of 68.5	68.5 s 50 s 17 w	68.5 ms 50 s	Orders of 34
Equatorial diffraction	4.2 vs	4.2 s	4.2 s	4.6 s	4.6 ms		5–6 s
Unoriented diffraction			10	11	5.5 12	5.5 12	

Intensity scale: vs, very strong; s, strong; ms, medium strong; m, medium; w, weak.

The appropriate Patterson function[49] is defined as follows:

$$P(x) = \frac{2}{d} \sum_{h=1}^{h_{max}} |T(h)|^2 \cos 2\pi hx/d.$$

Patterson functions were computed for SM alone and for SM + CHOL 2 : 1 mixture using the first six lamellar reflections in each case and are shown in Figure 4. The intensities were normalized so that the origin values of the Patterson functions were both unity. Differences in the Patterson functions of SM and 2 : 1 SM : CHOL is apparent. The curve for the 2 : 1 mixture shows less detail although both curves have the same kind of fall-off with increase in x.

In an earlier study[50] we had determined the electron density profile for SM bilayers and the most probable phase choice for the first six reflections,

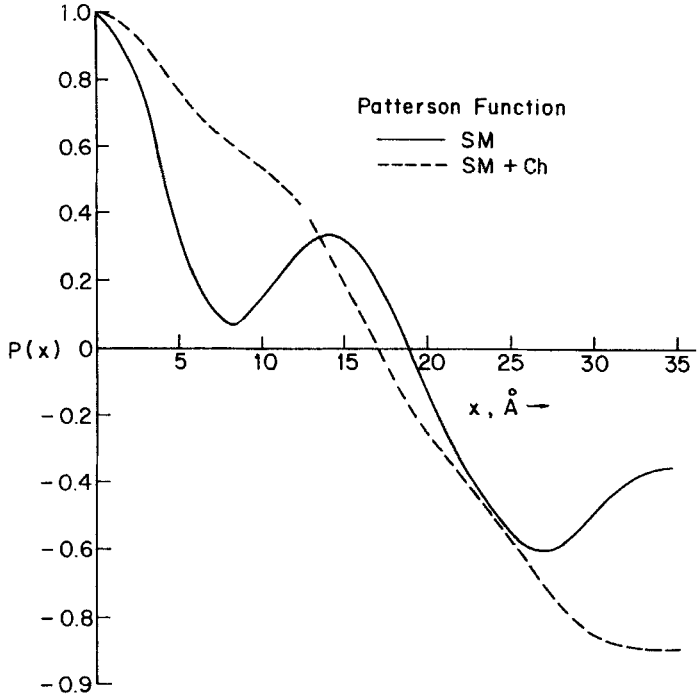

FIGURE 4 Patterson functions for sphingomyelin (solid line) and sphingomyelin–cholesterol, 2 : 1 molar complex (dotted line). The Patterson functions were computed using the first six reflections of $d = 68.5$ Å.

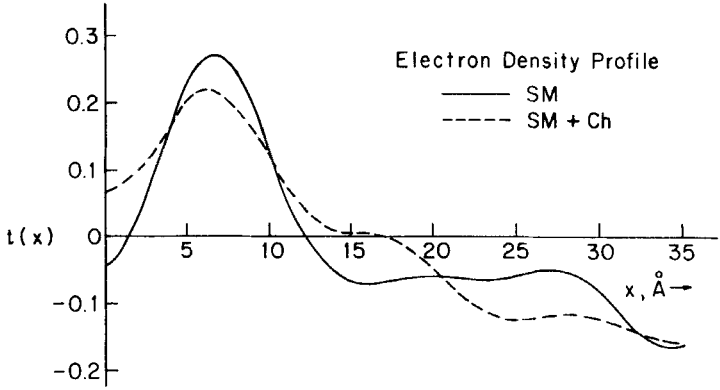

FIGURE 5 Fourier syntheses (electron density profiles) for sphingomyelin (solid line) and sphingomyelin–cholesterol 2 : 1 molar complex (dotted line). The first six reflections of $d = 68.5$ Å were used in computing these profiles and they have a resolution of about 6 Å.

SM bilayers. Using this phase choice, electron density profiles, $t(x)$, defined as

$$t(x) = \frac{2}{d} \sum_{h=1}^{h=h_{max}} \{\pm\} |T(h)| \cos 2\pi hx/d,$$

where $\{\pm\}$ is the phase information, were computed for SM and the 2 : 1 SM : CHOL mixture. The first six orders of the lamellar repeat from SM and the 2 : 1 mixture were used in the computation. The lamellar repeat for the 2 : 1 molar ratio was the same as for SM alone and the $h = 6$ orders of $d = 68.5$ Å corresponds to a resolution of about 6 Å. The electron density profiles for SM and for the 2 : 1 SM : CHOL mixture are shown in Figure 5. A comparison of the electron density profiles in Figure 5 indicates that in the 2 : 1 mixture the nucleus of the cholesterol molecule is near the polar region of the sphingomyelin.

DISCUSSION

The SM bilayers show an equatorial reflection at 4.2 Å and this reflection corresponds to a hexagonal packing of hydrocarbon chains. From Table I and Figure 2 it is evident that the addition of cholesterol disorients and weakens this reflection. Samples with SM : CHOL with the molar ratio of 2 : 1 and 1 : 1 gave a reflection at about 4.6 Å but, with further increase in the cholesterol concentration the 4.6 Å reflection was no longer observed. Thus, increasing amounts of cholesterol produces increasing fluidity in SM bilayers and this is consistent with the previous observations on phospholipid–cholesterol mixtures where cholesterol increases the fluidity of lipids below their transition temperature.[4]

In mixtures with SM more than 2 : 1 molar ratio the lamellar reflections were insufficiently resolved to say whether these reflections originated from the SM alone of from the 2 : 1 SM + CHOL complex or from any other phase.

A reflection of 50 Å (Figure 3C and D) is probably due to cholesterol, since its relative intensity is dependent on the relative amounts of cholesterol present in the sample and it was only observed in molar ratios of 1 : 1 and 1 : 2. Cholesterol does not appear in our x-ray patterns as a condensed phase as has been observed with lecithins.[17,32]

The wide-angle diffuse reflections around 5.5 Å and 12 Å observed with 1 : 1 and 1 : 2 molar ratios are probably due to cholesterol molecules, since a spacing of 5.5 Å is close to the small cluster of the strongest lines in the x-ray powder pattern of crystalline cholesterol[51] (Figure 1B).

A summary of x-ray results from lecithin–cholesterol mixtures (PC : CHOL) with similar molar ratios as in Table I is presented in Table II. Comparison of Tables I and II indicates that there are differences in the SM : CHOL and the PC : CHOL mixtures. For instance, in the case of PC mixtures the 4.6 Å reflection appears with 20% cholesterol[13] whereas a 33% cholesterol concentration is required in the case of SM. The size of the lamellar repeat remains remarkably constant for SM : CHOL mixtures. The situation with PC + CHOL mixtures is unclear but with higher concentrations of cholesterol[32] the lamellar repeat is smaller (45 Å) than for PC alone (56 Å). The SM : CHOL 2 : 1 mixture has a single lamellar repeat whereas the same molar ratio for PC : CHOL has a number of different lamellar repeats.[17]

TABLE II

Summary of x-ray diffraction spacings[a] in Å for lecithin (PC) and cholesterol (CHOL) mixtures

	PC alone	PC : CHOL				
		4 : 1	3 : 1	2 : 1	1 : 1	1 : 2
Lamellar diffraction	Orders of 56 and 48.2 ms	Order of 55 and 40.8 s		56 s 49.2 w 45 m 42.4 s 39.6 ms 35 s	50 s or orders of 34	Orders of 45 or 34
Equatorial diffraction	4.15 s	4.15 s 4.7 vw	4.15 s 4.7 w	4.7 s	4.7 s	4.8 s

[a] Taken from Refs. 13, 17 and 32.
Intensity scale: s, strong; ms, medium strong; m, medium; w, weak; vw, very weak.

Earlier observations on molecular models[6,7] suggested that a complex with a 1 : 1 ratio will pack in a hexagonal array and 1 : 2 and 1 : 3 ratios will probably form a linear array. No evidence of any such organization in the plane of SM : CHOL bilayers has been obtained in the present studies.

Our x-ray results indicate that SM and CHOL probably forms a molecular association when mixed in a molar ratio of 2 : 1. The diffraction pattern of this molar ratio does not contain any diffraction line proper to the crystal of cholesterol. We note that there is no change in the lamellar repeat value of 68.5 Å, which is same as observed with SM bilayers. The observation of the 4.6 Å reflection instead of the 4.2 Å reffection indicates the existence of a different phase from that of the SM bilayers alone. The difference between the electron density profiles for SM and the 2 : 1 SM + CHOL bilayers can

be interpreted by assuming that part of the steroid nucleus is in the polar region of the SM bilayers. This interpretation is similar to that suggested by Rand and Luzzati[30] for an erythrocyte lipid–cholesterol mixture, which also contained some sphingomyelin.

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