COMPLEX AND CLUSTER FORMATION IN MIXED LECITHINCHOLESTEROL BILAYERS. COOPERATIVITY OF MOTION IN LIPID SYSTEMS

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1. Introduction

Although several workers have studied the lecithin/cholesterol/water system by a range of techniques in the last few years, there have been very few new findings reported since the work of Ladbrooke et al. [1]. These authors showed that cholesterol inhibits the chain motion of lecithin above its gel-liquid crystal transition temperature (T_c), completely removing the transition when present in equimolar quantities. The average chain mobility in the equimolar mixture is intermediate between those characteristic of the gel and liquid crystalline phases. Only spectroscopic methods are capable of providing more detail on the molecular level, as opposed to information about bulk properties provided by such techniques as calorimetry. The ESR spin label method [2] and deuteron NMR [3] have provided the additional information that the lecithin chain motional restriction is less at the free nonpolar ends of the chains than in the region near the glycerol backbone. Quantitative interpretation of these results suffers from the drawback that the presence of the label (either the paramagnetic group for ESR or the isotopic substitution for NMR) must itself produce local perturbations of the molecular environment, especially in regions where tight molecular packing occurs. Until the full significance of these perturbations is understood, NMR studies of unlabelled molecules have the distinct advantage that quantitative results obtained from them undoubtedly give an accurate measure of biologically relevant molecular mobilities.

An early proton NMR study of sonicated aqueous lecithin/cholesterol dispersions also tentatively reached the conclusion that cholesterol inhibits the chain motion of lecithin above its T_c [4]. We have now carried out a full investigation of this system using both high resolution NMR spectroscopy on sonicated dispersions and wide line NMR spectroscopy on unsonicated mixtures. Our results confirm the findings mentioned above, and in addition show that lecithin and cholesterol form an equimolar complex with a lifetime longer than about 30 msec. The complex is bound by Van der Waals interactions between the steroid molecule and the first ten methylene groups in each lecithin chain, severely restricting the motions of this region of the chains while leaving their terminal methyl ends relatively free. In addition, there is a hydrogen bond between the cholesterol OH and the lecithin phosphate group, helping to locate the molecules in the complex. The complex is insensitive to temperature changes up to about 20° above the lecithin T_c, but then becomes less rigid as the temperature is raised further. Another important finding is that the compelx is not distributed evenly within the bilayers in a codispersion where the cholesterol is present in less than equimolar amounts, but forms clusters; this effect is due to the cooperative nature of the chain motions in lecithin bilayers.

2. Materials and methods

The lecithins used were chromatographically pure

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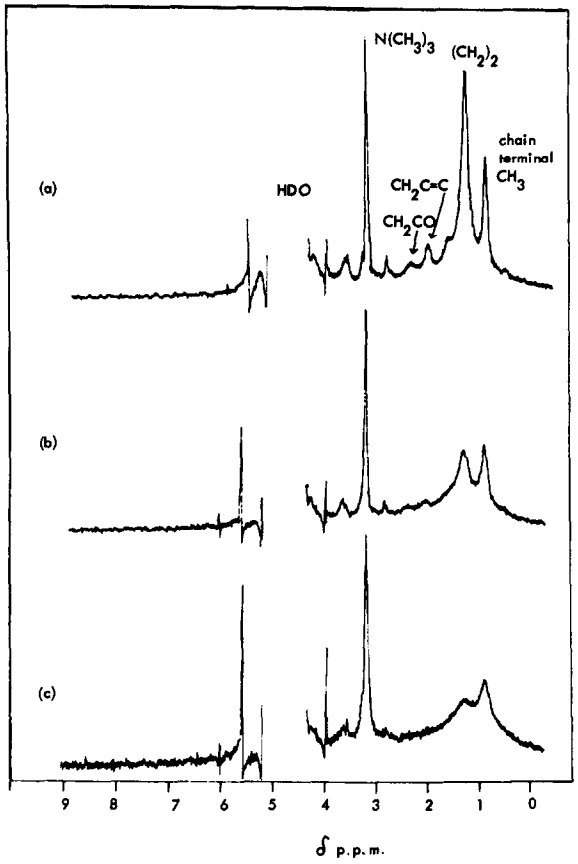

Fig. 1. 220 MHz spectra of sonicated egg yolk lecithin/cholesterol codispersions (5% lecithin in D_2O, 20°). Molar ratios: (a) 1:0, (b) 2:1, (c) 1:1. The intense HDO peak has been omitted.

dipalmitoyl lecithin, egg yolk lecithin and dimyristoyl lecithin with perdeuterated chains (> 98%). Samples for high resolution NMR studies were sonicated under conditions which avoided chemical degradation, and samples for wide line NMR studies were homogenised by centrifugation backwards and forwards through a constriction in a glass tube. D_2O was used for all preparations (added to the lipid mixture after evaporation of organic solvent used for mixing) except for that used for deuterium NMR, when H_2O was used. High resolution proton NMR spectra were recorded at 60 MHz and 220 MHz on Perkin Elmer R10 and Varian HR220 instruments respectively, and wide line and deuteron spectra were recorded on a Varian wide line spectrometer operating at 60 MHz and 8 MHz. Spectra were accumulated on a time-averaging computer where necessary. Wide line spectra were decomposed and integrated by standard methods [5–8], the accuracy of intensity measurements being within ± 10%. Further experimental details are given elsewhere [9].

3. Results and intepretation

3.1. *Complex formation*

Careful examination of the high resolution spectra obtained from sonicated egg yolk lecithin-cholesterol codispersions at room temperature (e.g. fig. 1) shows that increasing amounts of cholesterol cause a decrease in the intensity of the lecithin hydrocarbon chain proton resonance without a gradual broadening*, i.e. there is a two-state process whereby the lecithin spectrum is changed from the original spectrum to one where the bulk of the chain protons give a peak so broad as to be unobservable on the high resolution scale. The $N(CH_3)_3$ signal is only slightly broadened. The residual spectrum of a 1:1 codispersion arises solely from the lecithin molecules, as is demonstrated by its disappearance on deuteration of the lecithin chains. The residual hump at δ 1 ppm in fact arises from the ends of the chains, showing that these have considerable motional freedom. Similar results are obtained for an equimolar dipalmitoyl lecithin/cholesterol mixture over the temperature range 10–60°, whereas a dipalmitoyl lecithin dispersion only gives a spectrum above its T_c (41°). The two-state process results from the formation of an equimolar temperature-insensitive complex in which the ends of the lecithin chains are fairly mobile. The latter fact is confirmed by saturation-recovery spin-lattice relaxation time (T_1) measurements [10] of the residual hump, which give T_1 = 276 ± 20 msec compared with 307 ± 20 msec for the chain protons in an egg yolk lecithin dispersion. A value of T_1 of 292 ± 20 msec decreasing to 256 ± 20 msec for the $N(CH_3)_3$ protons on the addition of

* There is a slight broadening at a molar ratio of 2:1, where the linewidth increases from 40 Hz to 50 Hz. This is probably due to "edge effects", but if it were due solely to complex dissociation and exchange with free lecithin, the minimum lifetime of the complex can be calculated to be 30 msec [9].

equimolar cholesterol indicates a slight reduction in the mobility of this group.

Wide line NMR spectra of unsonicated non-equimolar codispersions of cholesterol with dipalmitoyl lecithin were extremely complex, owing to the superposition of spectra of the complex and uncomplexed lecithin. With equimolar codispersions four lines (plus a narrow "high resolution" central component) were observed, both with dipalmitoyl lecithin and the perdeuterated lecithin, although the lines had different relative intensities in each case. The linewidths, which were 5.2, 3.4, 1.2 and 0.2 gauss, were almost completely temperature independent over the range 10–60°, again demonstrating the temperature stability of the complex. Using the change in relative intensities after deuteration, the proportion of chain protons contributing to each line was assessed; ten CH_2's on each chain contributed to the two broadest lines, the other CH_2's to the 1.2 gauss line, and the terminal CH_3's to the 0.2 gauss line. In the gel phase of dipalmitoyl lecithin, the chain proton linewidth is 4.0 gauss [11]; thus while most of the chain protons are rendered more mobile by the addition of cholesterol below the lecithin T_c, some are in fact immobilised further. Cholesterol slightly loosens the lecithin glycerol backbone, the protons of which contribute to the 1.2 gauss line (1.4 in pure lecithin liquid crystals), by the insertion of the bulky molecules altering the lecithin packing (originally tightest here [12]). It therefore seems surprising that the $N(CH_3)_3$ mobility is reduced, until one examines a molecular model in which the cholesterol molecule is placed adjacent to the lecithin chains such that the first ten methylene groups are in contact with it while the ends of the chains are free: the OH of the cholesterol lies adjacent to the phosphate of the lecithin, and presumably slightly restricts the $N(CH_3)_3$ mobility by the formation of a hydrogen bond. This is shown in fig. 2. Formation of such a bond has been detected in the dry state [13].

The deuteron spectrum of a 50% w/w H_2O dispersion of an equimolar mixture of cholesterol and dimyristoyl lecithin with perdeuterated chains, recorded at 24° with about 1800 scans on the time averaging computer, shows splittings of 52 ± 1 KHz (similar to that obtained by Oldfield et al. [3]) and 9 ± 1 KHz (not found by Oldfield et al.), with a single central component (width 2 KHz). Thus there

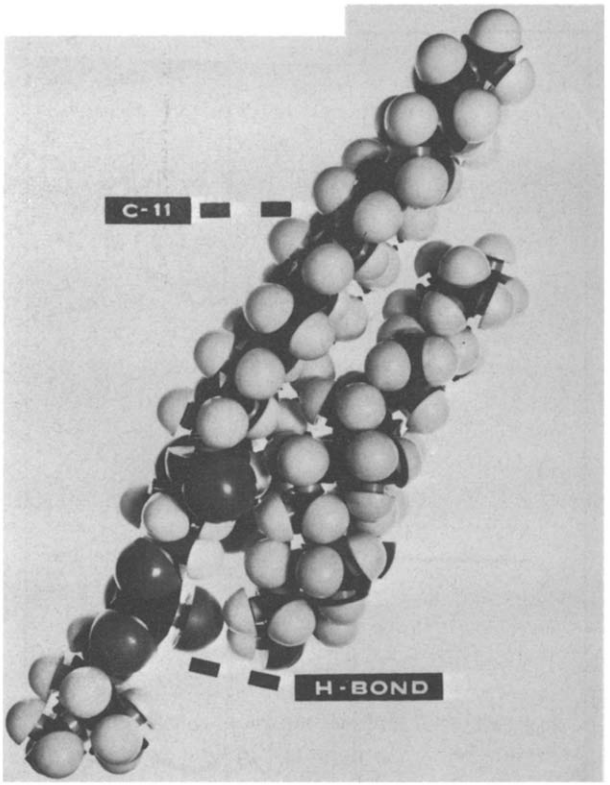

Fig. 2. Photograph of space-filling models of dipalmitoyl lecithin and cholesterol, arranged so that the first ten lecithin chain methylene groups are in contact with the cholesterol and thereby sterically hindered. The probable hydrogen bond is indicated.

are at least three regions of mobility, in agreement with the proton NMR results. It is probable that the very widely split component from regions more rigid than the gel phase would not be observable. Furthermore, it should be noted that a distribution of splittings giving overlapping absorption doublets would give a first derivative spectrum indistinguishable from that observed, and it is therefore not possible from the deuteron spectra to put an upper limit on the number of regions of chain mobility.

3.2. *Cluster formation and cooperativity of chain motions*

The fact that spectra of intermediate molar ratios appear to be superimposed spectra of complex and free lecithin indicates that the complex present does not affect the spectrum of the free lecithin. Since

the mobility of lecithin chains depends on the highly cooperative motions of the array, it would be expected that the introduction of immobile complex molecules into a lecithin array would severely restrict that mobility, especially at molar ratios of 4:1 lecithin: complex (at which there is no broadening at all of the residual lecithin spectrum). We conclude that the complexed molecules are not randomly interspersed throughout the lecithin array, but rather are localised in clusters. This might be expected, since it will be entropically favourable for the lecithin sheets to reject the rigid complex molecules in order to maintain their cooperative chain motions (see below). Localisation of cholesterol has been observed in biological membranes [14, 15]. Clustering, which we envisage as being only short-lived, would not show up in small angle X-ray scattering of multilamellar systems because the multilamellar repeats would only show an average environment. However, temperature-jump studies by Träuble [16] have provided further evidence for the existence of clusters. The relaxation of the gel to liquid crystal transition of dipalmitoyl lecithin in excess water has been measured by temperature jump techniques and found to be highly cooperative, with a time of the order of 1 sec. Since this is approximately 10^{10} times longer than molecular relaxation times, it is striking further evidence of the highly cooperative nature of the hydrocarbon chain motions in lipid bilayers. Despite the increase in packing density and presence of rigid molecules, addition of 25 mole% cholesterol to dipalmitoyl lecithin causes the gel to liquid crystal transition to occur roughly 10^2 times faster. This increase points to a reduction of the cooperativity between lecithin molecules. This is readily understood in terms of clustering of the lecithin/cholesterol complex. The domains of hydrocarbon chains which have to undergo the transition cooperatively are smaller in area and hence the overall rate is more rapid.

The cooperative nature of hydrocarbon chain motions and the tendency for clustering within lipid bilayers is of great biological significance. When bilayers contain different types of lecithin molecules the gel to liquid crystal transition is broad [17] and whenever the environmental temperature is not above that of the transition range clustering of molecules occurs. The T_c range for the isolated lipids of several membranes encompasses their usual *in vivo* temperature. This indicates that clustering exists within the bilayers of the isolated lipids of some membranes and may occur in the intact membranes [17]. Such clustering of phospholipids of different chain lengths and of the lecithin/cholesterol complex would lead to variations in permeability and other properties along the planes of bilayers or membranes. The cooperative nature of the motions of the chains within lipid arrays also indicates a mechanism whereby the interaction of a small molecule or protein side chain could affect the packing over a large area by an allosteric type of mechanism. The observation that one molecule of the cyclic peptide alamethicin can change the packing of about 600 phosphatidylserine molecules may be an indication of such effects [18]. Any lateral propagation of motion within the plane of a bilayer, by a cooperative motion of the hydrocarbon chains, will be rather slow and will lead to an apparent bending of the chains when observed over a short time (e.g. 10^{-8} sec) if the motion is a wave-type. Such a bending has been observed by an ESR spin-label study [19]. A slow motion which might be due to such a wave has been observed by $T_{1\rho}$ measurements [20], which showed that there is a motion of lecithin chains at 100° which has a correlation frequency of only 34 kHz. This corresponds to a propagation velocity of the order of 10^{-3} cm sec^{-1} at room temperature.

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