IMMISCIBILITY IN PHOSPHATIDYLCHOLINE–CHOLESTEROL MIXTURES

A. G. LEE

Department of Physiology and Biochemistry, University of Southampton, Southampton S09 3TU, England

Received 7 January 1976

1. Introduction

Cholesterol is an amphiphilic molecule present in the membranes of all mammalian cells, and constitutes about 40% of the total lipid in myelin and erythrocyte membranes [1]. Despite its obvious importance, the nature of its association with the phospholipid components of the membrane is not well understood. It is known that hydrated phosphatidylcholines in the lamellar phase will take up cholesterol to a molar ratio of 1:1, beyond which pure cholesterol separates out [2]. It is also known that addition of a 1:1 molar ratio of cholesterol to a phosphatidylcholine in the liquid crystalline phase results in a reduction of the long range swinging motions of the lipid fatty acid chains, but with relatively little effect on the small-angle rotations about C–C bonds [3,4]. The situation becomes confusing however at molar ratios of cholesterol less than 1:1. On the basis of studies using X-ray diffraction [5] and differential scanning calorimetry [6] it has been proposed that in mixtures of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol containing less than 33 mole per cent cholesterol, two phases are present; a pure lecithin phase and a mixed phase of lecithin and cholesterol at a molar ratio of 2:1. These conclusions have been criticized by Phillips and Finer [7], who, on the basis of proton n.m.r. data, suggest that in mixtures containing less than equimolar amounts of cholesterol, discrete regions of lipid:cholesterol, 1:1, complex separate out. Since they observed that addition of cholesterol to sonicated dispersions of phosphatidylcholine caused a progressive decrease in amplitude of the n.m.r. signal due to the lipid fatty acid protons with no increase in line width, they concluded that the exchange rate between complexed and uncomplexed lecithin was slower than 0.03 sec. However we [3] and others [8] have found that addition of cholesterol does produce a steady increase in line widths in proton n.m.r. spectra. Finally Shimshick and McConnell [9] have studied the partition of the spin label 2,2,6,6-tetra-methylpiperidine-1-oxyl (TEMPO) into phosphatidylcholine–cholesterol mixtures and concluded that no complexes are formed.

Here we report some studies using chlorophyll *a* as a fluorescent probe. It has been shown elsewhere that the aggregation of chlorophyll *a* in lipid bilayers is sensitive to lipid packing and that on formation of gel phase lipid, the proportion of aggregated, non-fluorescent, chlorophyll *a* increases [10,11].

2. Materials and methods

Dipalmitoyl phosphatidylcholine (DPPC) and dimyristoyl phosphatidylcholine (DMPC) were obtained from Koch-Light and dioleoyl phosphatidylcholine (DOPC) from P-L Biochemicals, chlorophyll *a* was purified by column chromatography using the method of Strain and Svec [12]. Cholesterol was recrystallised from methanol. Samples were dried down from chloroform solution and shaken with buffer (0.01 M Tris-HCl, pH 7.2; NaCl 0.1 M) on a Vortex mixer. For all samples, the lipid:chlorophyll molar ratios were 400:1 and contained 1.6×10^{-9} mole chlorophyll *a* in 4 ml suspension. Fluorescence measurements were made on an Aminco Bowman SPF Fluorimeter, temperatures being measured by a thermocouple inserted into the sample curette. Fluorescence was excited at 420 nm and recorded at 670 nm.

3. Results

Fig.1 shows plots of the fluorescence intensity of chlorophyll *a* as a function of temperature, when incorporated into mixtures of DPPC and cholesterol. Clearly, with increasing mole fraction of cholesterol, there is a decrease in the fluorescence intensity at high temperatures. It has previously been observed [10] that there is a marked concentration quenching of fluorescence for chlorophyll *a* incorporated into lipid bilayers, so that if the amount of chlorophyll *a* is kept constant, a reduction in the molar ratio of chlorophyll–lipid results in a decrease in fluorescence (fig.2). In the experiments with cholesterol, the total amount of lipid (DPPC + cholesterol) was maintained constant, so that an increase in cholesterol meant a reduction in DPPC. However, the decrease in

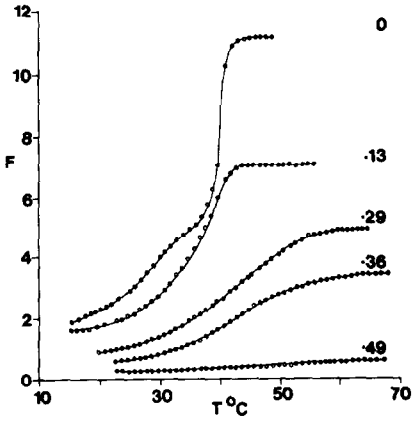

Fig.1. Fluorescence intensity vs temperature for chlorophyll *a* incorporated into liposomes of DPPC plus cholesterol, at the given molar ratio of cholesterol.

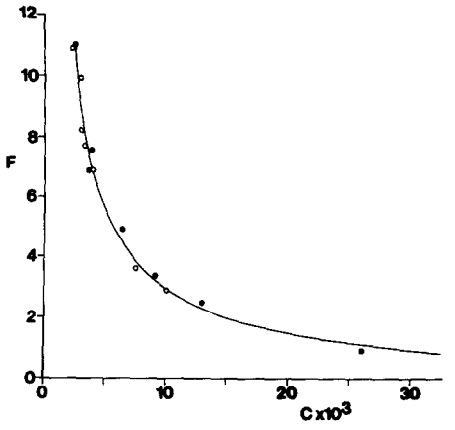

Fig.2. Fluorescence intensity vs 'free DPPC'–chlorophyll molar ratio 1:c in DPPC–cholesterol mixtures (see text). (○) Data for DPPC–cholesterol mixtures; (●) Data for DPPC alone, with the amount of chlorophyll *a* constant at 1.6×10^{-9} mol.

chlorophyll *a* fluorescence intensity does not correspond simply to the decrease in amount of DPPC present. Rather, the observed data can be fitted if it is assumed that a 1:1 DPPC–cholesterol interaction occurs, and that this interaction excludes monomeric chlorophyll *a*. The fluorescence intensity will then reflect the instantaneous proportion of DPPC not involved in interaction with cholesterol, and the curve of fluorescence intensity against the calculated molar ratio of 'free' lipid to chlorophyll agrees well with the previous data (fig.2).

Since interaction of DPPC with cholesterol excludes chlorophyll *a*, the fluorescence plots shown in fig.1 can be used to define the properties of the

Fig.3. Temperatures of onset and completion of solid lipid formation and of the low temperature end of the pre-transition in (a) DPPC–cholesterol mixtures and (b) DMPC–cholesterol mixtures.

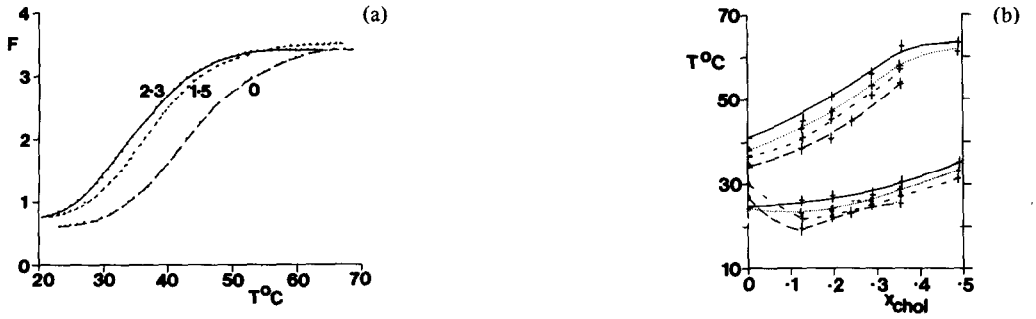

Fig.4. The effect of *n*-octanol on mixtures of DPPC and cholesterol. (a) Fluorescence intensity for a molar ratio of 0.29 cholesterol at the given concentrations (mM) of octanol and (b) the temperatures of onset and completion of gel phase lipid formation and of the low temperature end of the pre-transition: solid line, zero octanol; dotted line, 0.8 mM octanol; dashed line, 1.5 mM octanol; broken line, 2.3 mM octanol.

lipid in the bilayer not involved in interaction with cholesterol. In particular, breaks can be recognised in the slopes of the fluorescence plots corresponding to the temperatures of onset and completion of gel (solid) phase formation. Such an analysis is, however, complicated by the pre-transition observed in pure DPPC and centered at approx. 29°C. Addition of cholesterol up to a mole fraction of at least 0.19 appears not to abolish the pre-transition, since three distinct breaks in slope can be recognised in the fluorescence plots, the one of lowest temperature representing the start of the pre-transition. Beyond a cholesterol molar ratio of 0.2, however, only two distinct breaks appear in the plot. These temperatures are plotted in fig.3a for mixtures of DPPC and cholesterol and the corresponding data for mixtures of DMPC and cholesterol are plotted in fig.3b.

The above interpretation can be tested by observing the effects of addition of *n*-octanol. If the decrease in fluorescence intensity at high temperatures caused by the addition of cholesterol is due to a 1:1 cholesterol–lipid interaction excluding chlorophyll *a*, then addition of *n*-octanol would be expected to have little effect on these intensities: as shown in fig.4a, this is what is observed. Further, addition of *n*-octanol to mixtures of lipids lowers the temperatures of onset and completion of gel phase formation [13]. This also occurs for mixtures of DPPC and cholesterol as shown by the data in fig.4a. The decrease in transition temperature with aqueous concentration of *n*-octanol is linear within experimental error, the slope being similar to that observed with other lipid mixtures [13]. In most of these plots, only two transitions are clearly visible, corresponding to the onset of gel phase formation and the low temperature end of the pre-transition, and these are shown in fig.4b. For pure DPPC, the pre-transition is abolished beyond 1 mM octanol, and only the temperature for completion of gel phase formation is clearly defined, and so these temperatures are also shown in fig.4b.

Finally, it is possible to study the effects of cholesterol on mixtures of saturated and unsaturated lipids. De Kruyff et al. [14] have shown using differential scanning calorimetry that cholesterol interacts preferentially with unsaturated lipid in lipid mixtures. The data shown in fig.5 shows the same

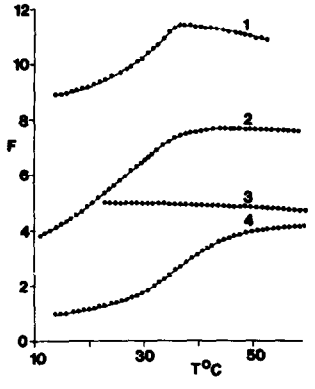

Fig.5. Fluorescence intensity vs temperature for (1) DPPC–DOPC, 120:30; (2) DPPC–DOPC–cholesterol, 100:25:25; (3) DOPC–cholesterol, 100:50; (4) DPPC–DOPC–cholesterol, 80:20:50.

effect. Addition of cholesterol to a DPPC: DOPC mixture at a molar ratio of 80:20 causes the fluorescence plots to change from that of the phosphatidylcholine mixture (curve 1) towards one typical of a mixture of free DPPC and phosphatidylcholine–cholesterol 1:1 complex (curve 4, cf. fig.1).

Conclusions

The results of this study can be summarised as follows.

(1) There is a 1:1 lipid–cholesterol interaction.

(2) The 1:1 lipid–cholesterol complex is immiscible with free lipid in the gel phase.

(3) The 1:1 lipid–cholesterol complex is immiscible with free lipid in the liquid-crystalline phase in mixtures containing more than approx. 35 mole percent cholesterol.

(4) In mixtures of the 1:1 lipid–cholesterol complex with free lipid, the temperature of onset of gel phase lipid formation is raised.

(5) Addition of alcohol lowers the temperatures of onset and completion of gel phase lipid formation.

(6) In mixtures of saturated and unsaturated lipid, cholesterol interacts preferentially with the unsaturated lipid, as shown previously by De Kruyff et al. [14].

Although the experiments reported here strongly suggest the presence of a 1:1 lipid–cholesterol interaction, they provide no evidence as to its nature – the 1:1 stoichiometry could merely reflect the appropriate geometrical arrangement for optimum packing in the mixed bilayer, or it could represent long-lived complex formation, as suggested by Phillips and Finer [7].

Since the 1:1 interaction with cholesterol leads to exclusion of chlorophyll *a*, the fluorescence of chlorophyll *a* in these lipid–cholesterol mixtures reflects the properties of free lipid, uninvolved in interaction with cholesterol. In DPPC–cholesterol mixtures containing less than 20 mole percent cholesterol, three distinct breaks are observed in temperature plots, corresponding to the onset and completion of gel phase lipid formation, and to the start of the pre-transition. Beyond 20 mole percent of cholesterol, only two breaks are observed in the temperature plots, probably due to broadening of the main gel to liquid crystalline phase transition. These results are in agreement with the differential scanning calorimetric data of Ladbrooke et al. [15] which also shows a broadening of the main transition, but with relatively little effect on the pre-transition (this is shown by the data of figure 1 of ref. [15], although Rand et al. [16] have later claimed that the pre-transition is abolished). The horizontal portion of the diagram of fig.3a for the completion of gel phase formation is indicative of immiscibility between DPPC and the DPPC–cholesterol complex. The continued presence of the pre-transition is also indicative of immiscibility. The pre-transition has been attributed to a change in the lipid fatty acid chains from being tilted with respect to the plane of the bilayer to being oriented perpendicular to the bilayer plane [16] thus allowing rotation about their long axes [3]. The pre-transition is abolished when a variety of molecules partition into the bilayer [13], but will obviously be free to occur if there is total immiscibility.

Fig.4a and 4b show that addition of cholesterol raises the temperature for the onset of gel phase formation for the free lipid not involved in direct 1:1 interaction with cholesterol, up to approx. 35 mole percent cholesterol, beyond which there is no further change. This is indicative, of partial miscibility in the fluid phase. Interestingly, other studies also show discontinuities at approx. 35 mole percent cholesterol. Thus Newman and Huang [17] have shown that sonicated vesicles become non-spherical at this point, and that there is a change in water-binding, and Lecuyer and Dervichian [2] have shown that the condensing effect of cholesterol on lipid molecular area decreases beyond 35 mole percent cholesterol. Finally, Jain [1] observed a marked decrease in the water permeability of liposomes beyond a 35 mole percent of cholesterol.

For the reasons discussed elsewhere [11,13] care has to be taken in interpreting diagrams such as figs. 3 and 4b as 'phase' diagrams. Nevertheless, the plots of fig.4b do clarify the effects of addition of alcohols. Since addition of cholesterol to DPPC raises the temperature of onset of gel phase formation of the DPPC not involved in interaction with cholesterol, the amount of lipid in a fluid phase at some intermediate temperature will be decreased. This effect can, however, be partially reversed by addition of *n*-octanol, since this lowers the temperature for the onset of gel phase formation. Addition of octanol does not, however, affect the amount of lipid involved in interaction with cholesterol.

Finally, the results reported here disagree with those of Shimshick and McConnell [9], who studied the partitioning of TEMPO into lipid–cholesterol mixtures. One possible reason for the disagreement suggested elsewhere [11] is that TEMPO partitions into the glycerol backbone region both of the free lipid and of the lipid–cholesterol complex, so that it is reporting partly on both: the chlorophyll *a* fluorescence experiments, on the other hand, appear only to report on the state of the free lipid.

References

[1] Jain, M. K. (1975) in: Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.) pp. 1–57, Academic Press, Inc., New York.
[2] Lecuyer, H. and Dervichian, D. G. (1969) J. Mol. Biol. 45, 39–57.
[3] Lee, A. G., Birdsall, N. J. M. and Metcalfe, J. C. (1974) in: Methods in Membrane Biology, Vol. 2 (Korn, E. D., ed.) pp. 1–156. Plenum Press, New York.
[4] Godici, P. E. and Landsberger, F. R. (1975) Biochemistry 14, 3927–3933.
[5] Engelman, D. M. and Rothman, J. E. (1972) J. Biol. Chem. 247, 3694–3697.
[6] Hinz, H-J. and Sturtevant, J. M. (1972) J. Biol. Chem. 247, 3697–3700.
[7] Phillips, M. C. and Finer, E. G. (1974) Biochim. Biophys. Acta 356, 199–206.
[8] Gent, M. P. N. and Prestegard, J. H. (1974) Biochemistry 13, 4027–4033.
[9] Shimshick, E. J. and McConnell, H. M. (1972) Biochem. Biophys. Res. Commun. 53, 446–451.
[10] Lee, A. G. (1975) Biochemistry 14, 4397–4402.
[11] Lee, A. G. (1975) Biochim. Biophys. Acta 413, 11–23.
[12] Strain, H. H. and Svec, W. A. (1966) in: The Chlorophylls (Vernon, L. P. and Seely, G. R., eds.) Academic Press, New York.
[13] Lee, A. G. (1976) Biochemistry, submitted for publication.
[14] De Kruyff, B., Demel, R. A., Stotboom, J. A., Van Deenen, L. L. M. and Rosenthal, A. F. (1973) Biochim. Biophys. Acta 307, 1–19.
[15] Ladbrooke, B. D., Williams, R. M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340.
[16] Rand, R. P., Chapman, D. and Larsson, K. (1975) Biophys. J. 15, 1117–1124.
[17] Newman, G. C. and Huang, C-H. (1975) Biochemistry 14, 3363–3370.