LATERAL PHASE SEPARATIONS IN BINARY MIXTURES OF CHOLESTEROL AND PHOSPHOLIPIDS

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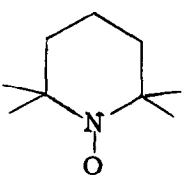
SUMMARY

The phase diagrams of binary mixtures of cholesterol and dipalmitoyl phosphatidylcholine, and cholesterol and dimyristoyl phosphatidylcholine in the presence of excess water have been investigated using spin labels.

It has been noted that the addition of cholesterol to dispersions of phosphatidylcholine causes the phase transition of the phospholipid to "disappear" at sufficiently high cholesterol concentrations.[1,2,3,4] It has also been noted that cholesterol reduces the fluidity of the lipids above the transition temperature of the phospholipid.[5,6,7] Such behavior is readily interpretable in terms of the phase equilibria of a cholesterol-phosphatidylcholine mixture and the lateral phase separations of the components of such a mixture into regions composed of fluid and solid lipids.

We have determined the phase diagrams of binary mixtures of cholesterol and L-α-dimyristoyl phosphatidylcholine (DMPC), and cholesterol and L-α-dipalmitoyl phosphatidylcholine (DPPC) dispersed in excess buffer. The method used has been described previously and is based on the application of the spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl or TEMPO (I) as a probe of the "fluidity" of the membrane.[8]



(I)

EXPERIMENTAL

The DPPC was purchased from Schwarz-Mann and was used without

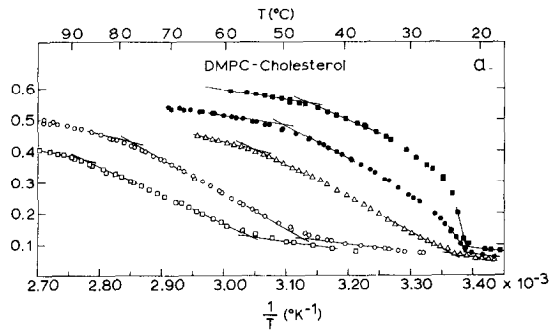
further purification. The cholesterol (Matheson, Coleman & Bell) was twice recrystallized from ethanol. The DMPC was synthesized by the method of Cubero Robles and van den Berg.[9]

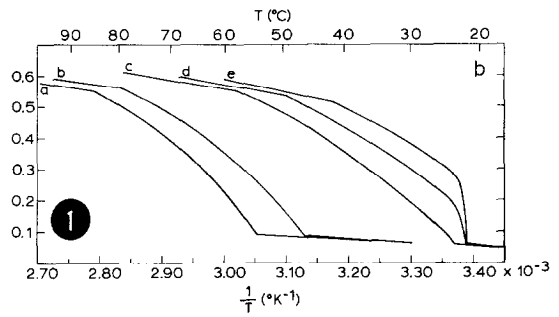
The interior of a 10 ml flask was coated with a thin film of lipids by the evaporation to dryness under vacuum of a chloroform solution containing 30 micromoles of the desired mixture of cholesterol and phosphatidylcholine. 200 microliters of a 0.01M sodium phosphate buffer at pH 7.0 and a glass bead were then added to the flask. The lipids were dispersed with the aid of a vortex mixer at a temperature of 65-75°C. 20 microliters of a 5×10^{-3}M TEMPO solution were added to the lipid dispersion which was then transferred to a 50 microliter pipet which was sealed and used as a sample cell. In other experiments, 0.28 micromoles of the 7,6 phosphatidylcholine spin label were added to 30 micromoles of the appropriate cholesterol-DPPC mixture in $CHCl_3$, which was then dried and dispersed in 225 microliters of buffer. All measurements were made on a Varian E-12 spectrometer at X-band with the microwave cavity in a horizontal configuration. The temperature was controlled with a Varian variable temperature accessory and measured with a Smith-Florence potentiometric microvoltmeter and a copper-constantan thermocouple in thermal contact with the sample cell.

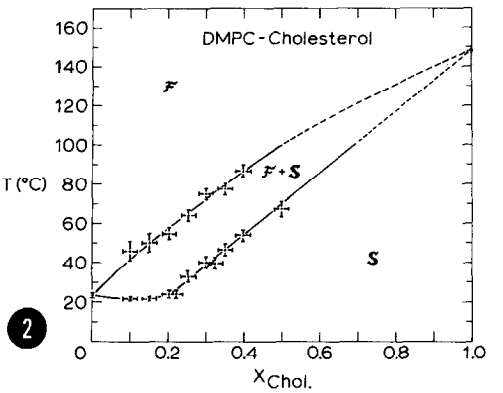
In these experiments, spectra were obtained as a function of temperature with heating and cooling rates of about 10°C per hour.

RESULTS AND DISCUSSION

A TEMPO solubility parameter, f, approximately equal to the mole fraction of spin label dissolved in the fluid hydrophobic regions of the lipids, was measured from the partially resolved high field nitroxide hyperfine line as a function of temperature. This TEMPO parameter is plotted as a function of 1/T for DMPC-cholesterol dispersions containing different mole fractions of cholesterol in Fig. 1a. These curves were completely reversible and contain points obtained while both heating and cooling. Abrupt changes in the slope of the f versus 1/T curves (indicated by the intersections of straight lines) were used to define points on the fluidus and solidus curves of the phase diagram of the binary lipid mixture. These phase diagrams are illustrated in Fig. 2 for DMPC-cholesterol and Fig. 3 for DPPC-cholesterol. The TEMPO solubility curve for DMPC-cholesterol was calculated from the







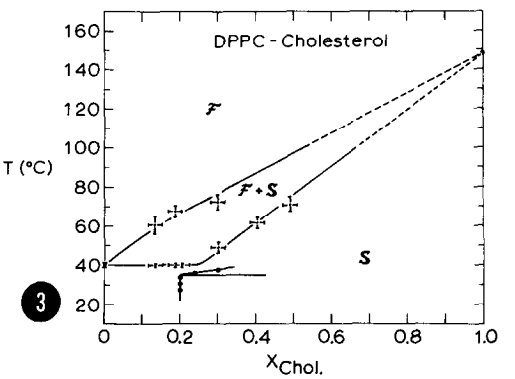


Fig. 1 (a) TEMPO solubility parameter, f, as a function of 1/T for aqueous dispersions of DMPC-cholesterol mixtures containing: □ 40 mole % cholesterol; ○ 35%; △ 20%; ● 15%; and ■ 10%.

(b) Calculated TEMPO solubility curves for: (a) 40 mole % cholesterol; (b) 35%; (c) 20%; (d) 15%; and (e) 10%.

Fig. 2 Phase diagram of DMPC-cholesterol dispersed in excess water. The regions in which the Fluid phase, F, the Solid phase, S, and an equilibrium mixture of Fluid and Solid phases, F + S, are stable are indicated.

Fig. 3 The phase diagram of DPPC-cholesterol dispersed in excess water.

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experimentally determined phase diagram as described previously[8] and is illustrated in Fig. 1b. The agreement is semi-quantitative.

The variation in S, the order parameter[8], of the 7,6 phosphatidylcholine spin label as a function of cholesterol mole fraction for DPPC-cholesterol mixtures at several temperatures is illustrated in Fig. 4. The breaks in the slope of the S versus cholesterol mole fraction plot indicate a possible solid phase transformation which is indicated by the filled circles in Fig. 3.

Both phase diagrams are similar to one another and exhibit solid phase immiscibility. The solidus curves are approximately horizontal up to 20-25 mole % cholesterol. Beyond this point the solidus curve can be extrapolated to the melting point of cholesterol. This behavior resembles that seen in binary mixtures of DMPC-dipalmitoyl phosphatidylethanolamine (DPPE) and DPPC-DPPE.[8] A change in the slope of the solidus curve usually indicates the intersection of another phase boundary with the solidus curve at that point.

Calorimetric, X-ray, and NMR studies indicate that phosphatidylcholine and cholesterol interact at stoichiometric ratios of 1:1[2,10] or 2:1.[1,11] It has been suggested that this represents complex formation.[10] A complex would be represented by a vertical line on the phase diagram extending below the melting point of the phosphatidylcholine at the composition of the complex. We see no evidence for complex formation at these concentrations. However, we do see a phase boundary with the phospholipid label at approximately 20 mole % cholesterol extending from 25°C to 35°C. The slope of this boundary changes at 35°C, which corresponds to the temperature at which pure cholesterol undergoes a polymorphic phase transition.[12] This observation is consistent with complex formation in which the area to the left of the vertical line is an equilibrium mixture of pure phosphatidylcholine + complex while the region to the right is an equilibrium mixture of

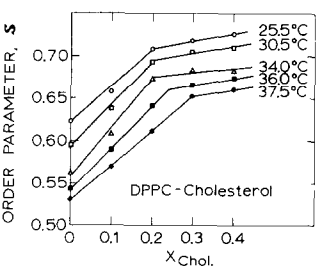

Fig. 4 Order Parameter, S, as a function of the mole fraction of cholesterol in DPPC-cholesterol dispersions for several temperatures.

complex + pure cholesterol. However, it is also possible that the observed phase boundary is only an indication of solid phase immiscibility and not necessarily complex formation. This interpretation does not require that the ratios of phosphatidylcholine and cholesterol at the abrupt change in slope of the solidus curve be the ratio of small whole numbers.

It is apparent that the solid phase behavior of these cholesterol mixtures is complex. This may account for the fact that Hinz and Sturtevant[1] see a vanishing of the transition enthalpy for the phosphatidylcholine at 30-35 mole % cholesterol. It is possible that they are measuring, in part, a solid phase transformation of the phosphatidylcholine-cholesterol mixture, especially since their observed transition width appears to increase in an exponential manner beyond 20 mole % cholesterol. At 20°C, Engelman and Rothman[11] see the disappearance of a sharp X-ray diffraction line associated with the DPPC gel phase at 30-33 mole % cholesterol which they attribute to the existence of a phase boundary. However, this is 5°C lower than any of our measurements, and it is quite likely that other solid phase changes can occur at these temperatures. The spin labels we have used have been able to detect both the fluidus and solidus curves and a solid phase boundary. Additional probes could be designed that are more sensitive to the other solid phase transformations that occur in this system.

The "disappearance" of the phase transition of the phospholipid upon the addition of the cholesterol can be understood in terms of the shape of the phase diagram. Since the solidus curve is nearly horizontal up to approximately 20 mole % cholesterol, the fraction of the phosphatidylcholine remaining in the fluid phase at a temperature slightly above the transition temperature of the phospholipid, as determined by material balance, is approximately equal to $1 - aX^\circ$ where X° is the overall mole fraction of cholesterol in the lipid mixture and a is the reciprocal of the cholesterol mole fraction at which the change in slope of the solidus curve occurs. As the cholesterol concentration is increased, the apparent intensity of the phospholipid transition decreases and vanishes at about 20 mole % cholesterol. Beyond this point, where the solidus curve is no longer horizontal, a transition associated with the pure phospholipid can no longer be observed.

The decrease in fluidity above the transition temperature of the phospholipid as the cholesterol content is increased can also be understood by means of the phase diagram. As cholesterol is progressively added to the phospholipids, the system changes from one which is entirely in the fluid phase to one which is a mixture of both fluid and solid phases and eventually to one in which all lipids are in the solid phase.

In biomembranes, it is quite likely that cholesterol acts to provide a

mixture of fluid and solid phases at physiological temperatures. Under these conditions, the lateral compressibility of the membrane would be expected to be enhanced. Such a high lateral compressibility could facilitate the insertion of proteins into the membrane and enhance the activity of transport systems.[13]

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REFERENCES

1. Hinz, H. J. and Sturtevant, J. M., J. Biol. Chem., 247, 3697, (1972).
2. Ladbrooke, B. D., Williams, R. M., and Chapman, D., Biochim. Biophys. Acta., 150, 333, (1968).
3. Oldfield, E. and Chapman, D., FEBS Letters, 23, 285, (1972).
4. Branton, D. and Deamer, D. W., 1972. Protoplasmatologia, Vol. II E1.
5. Cogan U., Shinitzky, M., Weber, G. and Nishida, T., Biochem., 12, 521, (1973).
6. Hubbell, W. L. and McConnell, H. M., J. Amer. Chem. Soc., 93, 314, (1971).
7. Oldfield, E., Chapman, D. and Derbyshire, W., FEBS Letters, 16, 102, (1971).
8. Shimshick, E. J. and McConnell, H. M., Biochem. (in press).
9. Cubero Robles, E. and van den Berg, D., Biochim. Biophys. Acta, 187, 520.
10. Darke, A., Finer, E. G., Flook, A. G. and Phillips, M. C., J. Mol. Biol., 63, 265, (1972).
11. Engelman, D. and Rothman, J., J. Biol. Chem., 247, 3694, (1972).
12. Spier, H. L. and van Senden, K. G., Steroids, 6, 871, (1965).
13. Linden, C., Wright, K. L., McConnell, H. M., and Fox, C. F., Proc. Nat. Acad. Sci. (in press).