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Studies on the Anomalous Thermotropic Behavior of Aqueous Dispersions of Dipalmitoylphosphatidylcholine–Cholesterol Mixtures†

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ABSTRACT: Examination of the thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine–cholesterol mixtures by high-sensitivity scanning calorimetry has revealed that the phospholipid gel to liquid-crystalline phase transition consists of two components. One, a relatively sharp transition centered at 39.6–40.7 °C, exhibits a transition enthalpy change which decreases linearly with increasing cholesterol content, approaching zero at a cholesterol content of about 25 mol %. The other, a broad, lower intensity transition centered at approximately 41.5 °C for cholesterol concentrations of 20 mol %, displays an enthalpy change which is maximal at about 20–25 mol % cholesterol and which decreases as the cholesterol content decreases to zero or increases above 25 mol %. The origin of these two transitions is discussed in terms of a separation of these lipid mixtures into cholesterol-rich and cholesterol-poor domains.

Since the discovery by Leathes (1925) that cholesterol causes an apparent condensation of phospholipid monolayers, the interaction of this steroid with phospholipids has been the subject of considerable research (for recent reviews, see Demel and de Kruyff 1976; Jain, 1975). In the last decade this research has increasingly taken the form of physical studies aimed at elucidating the thermodynamic properties of cholesterol–phospholipid mixtures. Ladbrooke et al. (1968) initiated this approach with a scanning calorimetric study, demonstrating that the addition of increasing amounts of cholesterol to bilayer arrays of phosphatidylcholines concomitantly diminishes the apparent enthalpy change of the gel to liquid-crystalline phase transition. Subsequent studies have essentially confirmed this result, although a consensus on the

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exact mole fraction of cholesterol required to abolish completely the appearance of this transition has yet to be achieved (Darke et al., 1972; Shimshick and McConnell, 1973a; Engelman and Rothman, 1972; Hinz and Sturtevant, 1972a).

Experiments utilizing magnetic resonance techniques have indicated the phospholipid–cholesterol interaction may be more subtle than a simple mixing process. Darke and coworkers (1972) found that NMR spectra of aqueous suspensions of less than equimolar ratios of cholesterol to dipalmitoylphosphatidylcholine appeared to consist of superimposed broad and narrow resonances. These resonances were interpreted as arising from clusters of an equimolar phospholipid–cholesterol complex and domains of free phospholipid, respectively; that is, cholesterol is not uniformly distributed throughout mixed bilayers with dipalmitoylphosphatidylcholine but exists as aggregrates of 1:1 phospholipid–cholesterol complexes. Shimshick and McConnell (1973a) used the electron spin resonance probe Tempo[1] to construct phase diagrams for aqueous dispersions of cholesterol mixed with dipalmitoylphosphatidylcholine and with dimyristoylphosphatidylcholine. They demonstrated that cholesterol did not mix ideally with either of these phospholipids. The deviation from ideality was such that a lateral phase separation of lipids into cholesterol-rich and cholesterol-poor domains was postulated. Below 35 °C and with less than 20 mol % cholesterol, one of the separate phases was thought to be pure phospholipid. In addition, there was evidence of a phase boundary at 20 mol % cholesterol which could be detected by a spin-labeled phosphatidylcholine.

In view of the profound implications of lateral phase separation for biological systems and the discrepancies which have arisen from previous experiments, a reexamination of dipalmitoylphosphatidylcholine–cholesterol mixtures was initiated with a recently constructed high-sensitivity scanning calorimeter. This publication presents the results of these measurements which are consistent with the coexistence of two phases over a finite range of temperature and composition in this system. These results are in good agreement with those from an independent study of a similar nature on dimyristoylphosphatidylcholine–cholesterol mixtures recently reported by Mabrey et al. (1977).

Experimental Procedures

Materials. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine was synthesized and purified as described previously (Suurkuusk et al., 1976). Cholesterol was purchased commercially (J. T. Baker and Co.) and further purified by the bromination procedure of Schwenk and Werthessen (1952) and by extensive washing in a two-phase solvent system similar to that of Folch et al. (1957). In the latter treatment, the lower phase, containing steroid, was washed four times with a synthetic upper phase made from an aqueous solution containing 50 mM EDTA and 50 mM EGTA. This was followed by four washes with the upper phase containing no chelating agents. Cholesterol was then recovered from the lower phase by evaporation, redissolved in spectral grade chloroform, and filtered through a 0.2-μm pore size Teflon membrane (Millipore Corp.). The resulting solution was stored at −20 °C under argon until use. Aqueous solutions were prepared from KCl (Extra Pure, Heico, Inc.) and water which was deionized and twice distilled from wholly glass apparatus, the first distillation being from alkaline $KMnO_4$.

Preparation of Phospholipid Suspensions. Dispersions of phospholipid both with and without cholesterol were prepared by mixing appropriate aliquots of chloroform solutions of both lipid types. A few milliliters of additional spectral grade chloroform were added to produce a thin film upon solvent evaporation. Chloroform was removed at 30 °C on a rotary evaporator and the resulting paste frozen in an ethanol bath. After at least 4 h under vacuum, the sample was warmed to 45 °C and enough 50 mM KCl solution of the same temperature added to give a phospholipid concentration of about 10 mM. The sample was vortexed until all lipids were suspended and then stirred at 45 °C for 2 h. This dispersion was then loaded into the calorimeter cell for thermal analysis.

Calorimetry. Scanning calorimetry at ambient pressure was performed as outlined by Suurkuusk et al. (1976) on a prototype instrument of the heat-conduction type. Experiments at elevated pressure were performed with the same instrument utilizing a specially designed thick-walled stainless-steel cell connected via a stainless-steel capillary tube to a source of compressed helium (Mountcastle et al., 1978). The sample volume used in the high-pressure studies was reduced to approximately 0.4 mL because of the increased wall thickness of the stainless-steel vessel, but the total amount of phospholipid present was nearly equal to that used in other experiments (~7 μmol).

The actual data (voltage–time curves) were analyzed as described previously (Suurkuusk et al., 1976), except that curves were "smoothed" by a 20-point least-squares fit outside the main transition region and a 5-point fit within the transition region. This procedure produced a sharper transition than previously reported (Suurkuusk et al., 1976) but did not affect the estimates for ΔH and T_m. The reported heat-capacity functions are now accurate representations of the true function and are in good agreement with the results recently reported by Mabrey and Sturtevant (1976) for pure DPPC multilamellar liposomes. Our previously reported results for the main transition were artifactually broadened by the analytical procedures used, but in no way are the conclusions drawn from those studies altered (Suurkuusk et al., 1976; Barenholz et al., 1976).

Gel to liquid-crystalline phase-transition temperatures were taken to be the maximum value of the heat-capacity function, C_p. Transition enthalpy changes were calculated as the integral of C_p over a temperature range encompassing the interval where the heat capacity deviates significantly from that of the buffer. It was assumed that the baseline over the interval of integration could be well approximated by taking the average of the C_p functional values at the interval end points.

After the completion of calorimetric experiments samples were quantitatively recovered by removing them from the instrument cell with a syringe, rinsing the cell three or four times with 2:1 (v/v) chloroform–methanol, and combining the rinses with the original sample. All solvents were removed by evaporation under a stream of dry nitrogen using 4:1 (v/v) benzene–ethanol to hasten the removal of water. The lipid residue was dissolved in exactly 1.0 mL of glacial acetic acid and stored under argon until analyses could be performed.

Lipid Analyses. Lipid samples recovered from the calorimeter were analyzed for phospholipid content by determining total phosphate via the Bartlett (1959) procedure. Cholesterol was analyzed by a procedure modified from that of Courchaine et al. (1959). Triplicate samples and standards containing 0.01 to 0.05 mg of cholesterol were made up to 0.6 mL in glacial acetic acid. To these was added 0.4 mL of ferric chloride re-

[1] Abbreviations used are: Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DPPC, dipalmitoylphosphatidylcholine.

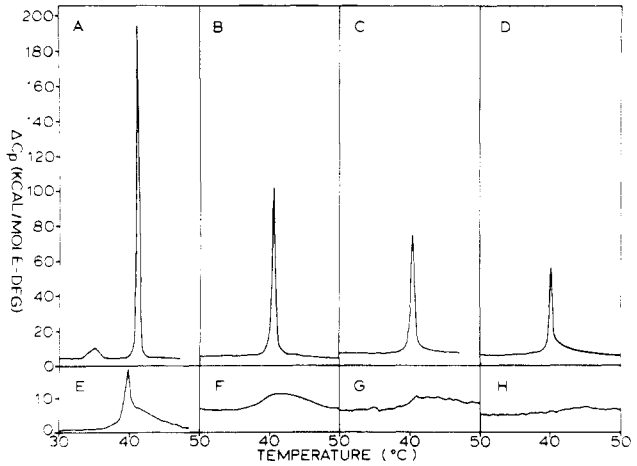

FIGURE 1: Calorimetric scans of aqueous dispersions of dipalmitoylphosphatidylcholine–cholesterol mixtures at a scanning rate of approximately 15 °C/h. Phospholipid concentration was approximately 10 mM in all samples. Heat-capacity values were calculated per mole of phospholipid. Samples contained: (A) 0.0, (B) 6.6, (C) 11.7, (D) 14.8, (E) 20.9, (F) 24.2, (G) 31.0, and (H) 34.8 mol % cholesterol.

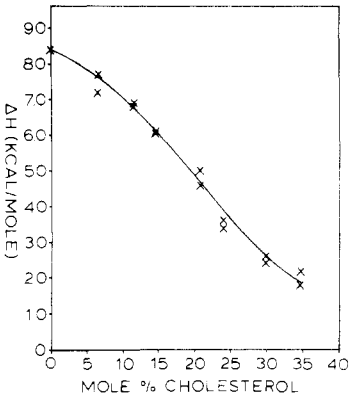

FIGURE 2: Effect of cholesterol on the total enthalpy (ΔH) per mole of phospholipid of the gel to liquid-crystalline phase transition of dipalmitoylphosphatidylcholine. Pairs of values at the same mol % cholesterol represent duplicate runs on the same sample. These data were calculated from the integral of heat-capacity curves such as those of Figure 1.

agent prepared as given in the publication mentioned above. All samples were gently shaken and then placed in a boiling water bath for 1 min. After a 20-min interval to allow complete color development, the absorbance at 550 nm was measured against a blank. In making these readings, care was taken to pipet solutions gently into clean, dry cuvettes so as to avoid bubble formation and light scattering from inhomogeneously mixed solutions. In solutions containing very high ratios of phospholipid to cholesterol, it was sometimes necessary to warm the cuvettes containing the samples over a steam bath to disperse entrapped bubbles. The 1-min heating in boiling water was found to triple the rate of color development and disperse bubbles formed in the mixing process. The final absorbance value attained after completion of the color reaction was stable for at least 90 min and probably much longer. Heating for 2 min or longer, however, was detrimental, resulting in a decreased final absorption value which became smaller the greater the period of heating.

Possible lipid breakdown during the experimental procedure was assessed by thin-layer chromatography of representative samples recovered from the calorimeter. Lipids were separated on silica gel plates (Q-1, Quantum Industries) in 65:35:4 (v/v) chloroform–methanol–water or 40:50:2:0.2 (v/v) ethyl ether–benzene–ethanol–acetic acid and visualized by iodine staining. These analyses indicated that lipid integrity was maintained during repeated calorimetric scans, since no degradation products could be detected.

Results

The thermotropic behavior of aqueous dispersions of dipalmitoylphosphatidylcholine mixed with various amounts of cholesterol is illustrated by the calorimetric scans in Figure 1. Several points in this sequence are notable. The first is that a relatively small mole fraction of cholesterol is required to completely suppress the pretransition seen in pure dipalmitoylphosphatidylcholine samples at 33–36 °C. From other scans (not illustrated) the ratio of cholesterol sufficient to accomplish this is found to be as low as 3.6 mol %. This is in contrast to previous work which indicated that the pretransition persists at a cholesterol content in excess of 5.0 mol % (Ladbrooke et al., 1968). Other work in this laboratory has demonstrated that gaseous anesthetics also reduce the midpoint and increase the width of the low-temperature transition until it can no longer be observed. The significance of this effect is not clear, since this transition is kinetically slow compared to the calorimetric scanning rate used in these studies (Suurkuusk et al., 1976; B. R. Lentz, private communication). Tsong and Kanehisa (1977) have also demonstrated that the pretransition in dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine dispersions exhibits severe hysteresis when the transition is continuously monitored by cooling or heating. The observed effect of cholesterol on this transition may be the manifestation of changes in the thermodynamics or the kinetics of the transition or both. Clarification of this point requires further study.

Secondly, the presence of cholesterol diminishes the total enthalpy change of the main phospholipid gel to liquid-crystalline phase transition. This is shown in Figure 2 where the total enthalpy of transition is plotted vs. mol % cholesterol. In reducing this transition enthalpy change, cholesterol also produces a marked asymmetry in the shape of the heat-capacity function. This is exemplified by the scans in Figure 1 with 14.8 and 20.9 mol % cholesterol. The asymmetry appears to be caused by the existence of two superimposed components, one a sharp transition occurring at a temperature (39.6–40.7 °C) slightly below that for pure dipalmitoylphosphatidylcholine (41.3 °C) and the other a broad transition centered at approximately 41.5 °C for samples containing 20 mol % cholesterol and moving to higher temperatures as more steroid is added.

The calorimetric scans of Figure 1 were resolved into two parts corresponding to the sharp and broad components. This was accomplished by assuming that the line shape of the sample containing 24.2 mol % cholesterol was characteristic of the broad component and that this component exhibits the same form at lower cholesterol levels. The high-temperature "tail" of the transition at low cholesterol levels was used to scale the intensity of the broad component, since the sharp component should not contribute to this part of the overall peak. The area of the broad component was then subtracted from that of the total transition to obtain the area of the sharp component. While this procedure is undoubtedly somewhat arbitrary, the results are internally consistent and represent a reasonable rationalization for the observed transition peak shapes. The enthalpy changes of the sharp and broad components resolved by this procedure are given as a function of cholesterol content in Figure 3.

It appears that the sharp component decreases in intensity in a linear fashion with an increasing mole fraction of choles-

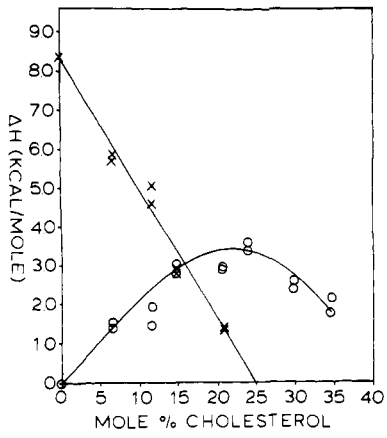

FIGURE 3: Resolution of the total transition enthalpy data of Figure 2 into sharp (×) and broad (○) components according to the procedure outlined under Results. The ΔH values were calculated per mole of total phospholipid. When summed together, the two curves of this figure give a plot identical to that of Figure 2.

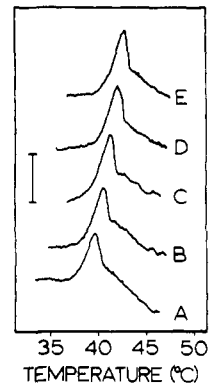

FIGURE 4: Calorimetric scans of an aqueous dispersion of dipalmitoylphosphatidylcholine plus 16.0 mol % cholesterol at various pressures. The vertical bar represents 1 kcal mol^{-1} deg^{-1}. Phospholipid concentration was approximately 10 mM. Experimental pressures of helium were (A) 15 (1 atm), (B) 620 (42 atm), (C) 1020 (69 atm), (D) 1480 (101 atm), and (E) 1950 (133 atm) psi.

terol, approaching zero at approximately 25 mol %. It does not appreciably broaden or change shape upon cholesterol addition, although the heat-capacity maximum does shift to slightly lower temperatures. This shift increases in magnitude with increasing cholesterol content, amounting to 1.5–2.0 °C at 15.0–25.0 mol % steroid.

The broad component increases in intensity with added cholesterol until a maximum is reached at 20.0–25.0 mol %. Higher mole fractions of cholesterol result in a decrease in the magnitude and broadening of this component. However, the exact limiting enthalpy change approached at high concentrations of cholesterol could not be ascertained.

In order to further examine the nature of the two components comprising the dipalmitoylphosphatidylcholine–cholesterol transition, calorimetric scans were run on the same sample under various pressures. The heat capacity vs. temperature curves so obtained are displayed in Figure 4 for a sample with 16 mol % cholesterol. While baseline curvature inherent in the pressure-cell scans causes the data to be less reproducible than that from the standard cell, it is clear that the primary effect of increased pressure is to shift both components of the transition to a higher temperature, leaving the overall shape unchanged. The shift of the sharp component transition maximum with increasing pressure is plotted in Figure 5 along with the values from a similar study with pure dipalmitoylphosphatidylcholine. For the mixed lipid sample, the sharp component transition temperature varies linearly with pressure in a manner similar to that of phospholipid alone. Since the overall line shape remains unaltered, the change in the broad component transition temperature with pressure must also parallel that of pure dipalmitoylphosphatidylcholine. The decrease in ΔH for the sharp transition with increasing cholesterol appears to reflect only the *number* of phospholipid monomers involved in the transition. Since $\Delta S = \Delta H/T_m$ and since T_m is only slightly changed in the presence of cholesterol, it follows the ΔS per mole of phospholipid in the sharp transition is approximately constant.

At the transition temperature, the Gibb's free energy change is equal to zero. Therefore, it follows that the slope of the transition temperature vs. pressure plot is equal to $\Delta V/\Delta S$, where ΔV and ΔS are the volume change and entropy change per mole of phospholipid involved in the transition (Mountcastle et al., 1978). Thus, assuming that the ΔS (= 27.3 cal/(K mol)) is unchanged by the presence of cholesterol, a ΔV of 23.6

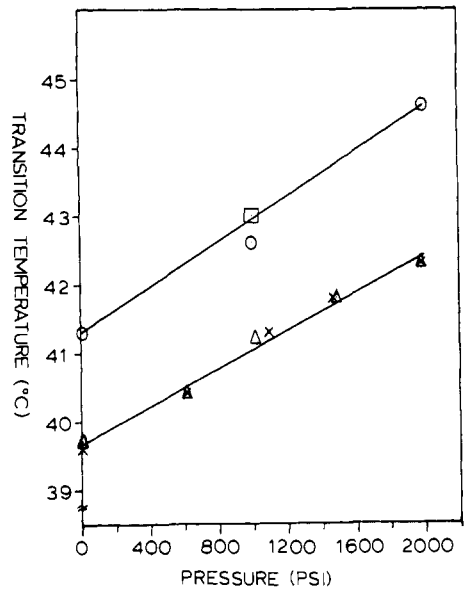

FIGURE 5: Comparison of transition temperature vs. pressure plots for aqueous dispersions of (□,○) pure dipalmitoylphosphatidylcholine and (×, △) the sharp component of a mixture of dipalmitoylphosphatidylcholine plus 16.0 mol % cholesterol. The various symbols identify experiments performed with different preparations of liposomes.

mL/mol for the sharp transition is calculated. This value is in good agreement with the ΔV calculated from the pressure dependence of the transition temperature of the main transition of pure dipalmitoylphosphatidylcholine liposomes and a direct evaluation of ΔV by Wilkinson and Nagle (1977). Because the shape of the superimposed transitions in the presence of cholesterol is unchanged, the $\Delta V/\Delta S$ is the same for both the sharp and broad transitions. However, since we cannot estimate the number of lipids involved in the broad transition, the enthalpy change and, hence, the entropy change per mole of lipid involved in the transition cannot be calculated. Therefore, ΔV for the broad transition cannot be estimated.

Discussion

While reaffirming previous observations that increasing cholesterol content reduces the apparent enthalpy change associated with the dipalmitoylphosphatidylcholine gel to liquid-crystalline phase transition, the data presented in this

publication indicate this process is more complex than originally thought (Labrooke et al., 1968; Hinz and Sturtevant, 1972a). The fact that two transitions are present with moderate (0–0.25) mole fractions of cholesterol is consistent with proposals that mixed systems of this nature consist of two thermodynamically distinct phases at temperatures less than 40 °C (Shimshick and McConnell, 1973a). By virtue of the fact that the sharp transition closely resembles that of pure dipalmitoylphosphatidylcholine with regard to transition temperature, line shape, and behavior with respect to pressure, we suggest that this component does reflect a gel to liquid-crystalline transition from a relatively pure phosphatidylcholine domain. On the basis of its enthalpy change with respect to composition, it appears that this domain exists only at mole fractions of cholesterol less than about 0.25. In this regard, this interpretation agrees with the conclusions of Shimshick and McConnell (1973a) derived from ESR experiments.

It should be noted that the T_m of the sharp transition decreased monotonically as the cholesterol content increased. Furthermore, at very high cholesterol (>15 mol %) the heat-capacity curve tends to broaden. These results are consistent with the suggestions that either cholesterol tends to contaminate the phospholipid domains or that as the cholesterol is increased the average size of the pure phospholipid domains decreased. It is not clear which is the case. More likely, both perturbation mechanisms are at work in this system.

The origin of the broad component is less certain, but the most plausible explanation is that it is related to the existence of an interfacial region between pure phospholipid and cholesterol-containing domains. It is not possible to describe the exact nature of such an interfacial region. Our thinking is that it is a relatively small region of phospholipid which contains a nonstoichiometric amount of cholesterol which is preferentially located at the boundaries of the region and that the thermodynamic characteristics of this region are distinct from either pure phospholipid domains or cholesterol rich domains. Such regions would be expected to arise and increase in extent as more cholesterol-rich phase is formed. A point would eventually be reached when no pure phospholipid phase would remain and the bilayer would consist solely of cholesterol-rich domains and boundary regions between these domains. This is thought to be the situation when dipalmitoylphosphatidylcholine is combined with 25 mol % cholesterol. Although these boundary regions do not necessarily contain a large mole fraction of cholesterol, the phospholipid molecules in them could nevertheless be perturbed due to the proximity of solid-like cholesterol-rich areas. One result of this proximity, coupled with the limited size of any particular contiguous boundary region, could be a reduction in the cooperativity of melting of the boundary phospholipids. This would cause a broadening of the transition peak as is experimentally observed.

As the cholesterol content is increased the cholesterol-rich phase grows at the expense of the pure phosphatidylcholine phase, ultimately resulting in the disappearance of any so-called interfacial region. This should correspond to the abolition of any *detectable* transition, above about 40 mol % cholesterol. Thus, the observed dependence of the enthalpy change of the broad transition on cholesterol content is reasonable if this component reflects the extent of boundary domains. In this model it is assumed that the cholesterol-rich phase does not undergo a transition in the temperature range examined in the calorimetric experiments. It is conceivable that the broad transition extensively broadens as the cholesterol content is increased until we can no longer observe it. If this is the case, then any statement about it not existing above a particular concentration is nebulous at best.

The concept of an interfacial region between cholesterol- and phospholipid-rich phases was previously advanced by Phillips and Finer (1974) on the basis of NMR and calorimetric studies (Ladbrooke et al., 1968; Darke et al., 1972; Hinz and Sturtevant, 1972a; Phillips and Finer, 1974). In considering the calorimetric data these authors deduced that domains of dipalmitoylphosphatidylcholine which exhibit melting behavior similar to that of the pure phospholipid could not exist in the presence of more than 30 mol % cholesterol, a figure in reasonable agreement with the results of the present study. Kleeman and McConnell (1976) have also embraced this concept in the interpretation of freeze-fracture electron microscopic and ESR data from several cholesterol-containing systems. It therefore appears that explanation of the calorimetric studies in these terms is consistent with the conclusions derived from other independent studies.

It should be noted that freeze-fracture electron micrographs of dipalmitoylphosphatidylcholine mixed with 0–20 mol % cholesterol do not exhibit discernible multiple domain structure, although such domain structure is observed in micrographs of immiscible phospholipid mixtures (Verkleij et al., 1974). As has been mentioned previously, this may be due to a lack of contrast between cholesterol-containing and phospholipid domains or to the domain size being less than the resolution of the technique (Opella et al., 1976). The freeze-fracture experiments do, however, indicate the presence of a phase boundary at a composition of 25 mol % cholesterol, since this quantity of steroid is just sufficient to completely abolish the banding pattern seen in micrographs of pure solid phospholipid bilayers (Verkleij et al., 1974).

An alternative explanation for the present results is that the broad transition arises directly from a cholesterol-rich phase. This would require the existence of two types of cholesterol-containing domains at 25 to 40 mol % steroid because of the disappearance of the broad component at high cholesterol concentrations. It must then be assumed that if boundary regions exist their contributions to the observed changes in C_p are negligible. We consider this interpretation less attractive because of the probable existence of a boundary region which is distinguishable from coexistent bulk phases and because the data is adequately explained by the presence of a single type of cholesterol-rich region.

A weaker objection to this alternative arises from the results of the pressure experiments. The fact that both transition components display similar thermal behavior under varying pressure implies that the volume changes associated with the phases giving rise to these components are comparable. In general, one would suppose it more likely that the boundary-region lipids would display a volume change similar to that of one of the bounded phases rather than lipids in a completely distinct domain. For this reason it is felt the pressure data is more consistent with the broad transition being assigned to boundary-region lipids.

A third possibility is that both components of the dipalmitoylphosphatidylcholine–cholesterol transition arise from a single phase. Pure saturated phosphatidylcholine liposomes in excess water give rise to two distinct transitions but the complex behavior of the two components of dipalmitoylphosphatidylcholine–cholesterol mixtures seems unprecedented within the framework of a one-phase hypothesis (Chapman et al., 1967; Hinz and Sturtevant, 1972b). On the other hand, there is a growing literature supporting the idea that lateral-phase separation may occur in binary lipid mixtures (Phillips et al., 1970; Shimshick and McConnell, 1973b; Ververgaert et al., 1973; Mabrey and Sturtevant, 1976). Saturated phosphatidylcholines differing by more than four carbons in hy-

drocarbon chain length display a readily discernible solid-phase immiscibility (Mabrey and Sturtevant, 1976). The structural differences between steroids and phospholipids are certainly more pronounced than those between these immiscible phosphatidylcholines so that the occurrence of a lateral-phase separation in dipalmitoylphosphatidylcholine-cholesterol mixtures should not be surprising.

It is interesting to note that a nonuniform lateral distribution of cholesterol has been detected in red blood cell membranes (Murphy, 1965). This provides another precedent for the interpretation of the calorimetric results presented above and indicates that the observed separation into phases of distinct composition may be of some biological relevance. The real significance of separation into compositionally different domains must, however, be established by determination of its ubiquity in a number of membrane systems.

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