EQUILIBRIUM STUDIES OF LECITHIN-CHOLESTEROL INTERACTIONS

I. STOICHIOMETRY OF LECITHIN-CHOLESTEROL COMPLEXES IN BULK SYSTEMS

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ABSTRACT The maximum molar ratio of lecithin:cholesterol in aqueous dispersions has been reported to be 2:1, 1:1, or 1:2. The source of the desparate results has been examined in this study by analyzing (*a*) the phase relations in anhydrous mixtures (from which most dispersions are prepared) and (*b*) various methods of preparing aqueous dispersions, with the purpose of avoiding the formation of metastable states that may be responsible for the variability of the lecithin-cholesterol stoichiometry. Temperature-composition phase diagrams for anhydrous mixtures of cholesterol (CHOL) with dimyristoyl (DML) and with dipalmitoyl (DPL) lecithin were obtained by differential scanning calorimetry (DSC). Complexes form with molar ratios for lecithin:CHOL of 2:1 and 1:2; they are stable up to 70°C. When x(CHOL) < 0.33, two phases coexist: complex (2:1) plus pure lecithin; when 0.33 < x(CHOL) < 0.67 complexes (2:1) and (1:2) coexist as separate phases. The corresponding phase diagram in water for these mixtures was determined by DSC and isopycnic centrifugation in D_2O-H_2O gradients. Aqueous dispersions were prepared by various methods (vortexing, dialysis, sonication) yielding identical results except as noted below. The data presented supports the following phase relations. When x(CHOL) < 0.33, two lipid phases coexist: pure lecithin plus complex (2:1) where the properties of the lecithin phase are determined by whether the temperature is below or above T_c, the gel-liquid crystal transition temperature. Therefore, complex (2:1) will coexist with gel state below T_c and with liquid crystal above T_c. The densities follow in the order gel > complex (2:1) > liquid crystal. The density of complex (2:1) is less sensitive to temperature in the range 5°–45°C compared to the temperature dependence for DML and DPL where large changes in density occur at T_c. When x(CHOL) > 0.33, CHOL phase coexists with complex (2:1); anhydrous complex (1:2) is apparently not stable in H_2O. The results are independent of the method and temperature used for preparing the lipid dispersions. However, when dispersions are prepared by sonication or with solvents at $T > T_c$, an apparent 1:1 complex is formed. Evidence suggests the 1:1 complex is metastable.

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INTRODUCTION

The study of interactions in mixtures of cholesterol with synthetic or naturally occurring lecithins as dispersions in water has largely involved determination of the maximum amount of cholesterol that can be incorporated into a lecithin matrix, and of the changes in physical state that accompany the formation of the mixture. However, the significance of these studies is somewhat circumscribed by the diverse and sometimes contradictory results obtained. The maximum molar ratio of lecithin: cholesterol in anhydrous systems has been reported to be 1:2 from infrared spectroscopy (1) and X-ray crystallography (2, 3). For aqueous dispersions the maximum ratio is 1:2 by sedimentation (4), 1:1 by sedimentation (5, 6), calorimetry (7), nuclear magnetic resonance (NMR; 8, 9), and X-ray (2) studies, and 2:1 by calorimetry (10) and X-ray diffraction (11). These simple molar ratios have been the principal evidence to support proposals that complexes are formed by cholesterol and lecithin. However, on the basis of electron spin resonance (ESR) studies, it was concluded that no particularly unique stoichiometry need occur in mixtures of lecithin and cholesterol (12).

The phase relations deduced from these studies are also uncertain. Implicit in some studies (4–6) is the assumption that cholesterol forms a homogeneous phase with lecithin in water until the solubility limit of cholesterol in the lecithin phase is attained. Other proposals suggest a more complicated phase behavior: "clustering" of lecithin:cholesterol 1:1 complex in a homogeneous lecithin phase (8, 9); lecithin: cholesterol 2:1 complex coexisting with lecithin in the gel state (11); coexisting solid and liquid phases, each with varying composition of lecithin and cholesterol, at temperatures that exceed T_c (the gel-liquid crystal transition temperature), whereas at temperatures below T_c coexisting solid phases for cholesterol concentrations < 20 mol% but forming a solid solution with increasing cholesterol concentrations (12); two coexisting phases of different composition when cholesterol exceeds 20 mol% at temperatures $< 80°C$ (13).

The basis for the disparate results in aqueous dispersions, particularly with supposedly identical systems, is not apparent. Because lipids readily form metastable states (14, 15), and lecithin dispersions in some instances have demonstrable instabilities (3, 16), it is reasonable to suggest that the systems differ by the method in which the lipid dispersions are prepared. Preparation of lipid dispersions in water is inherently difficult because the spontaneous mixing processes are extremely slow. Consequently, various techniques have been employed to prepare dispersions in which the lipid components are both homogeneously mixed and uniformly dispersed in water. Typically, the anhydrous lipids are first dissolved in an organic solvent; the aqueous dispersions are formed either by injecting the solution directly into water or by evaporating the solvent and then dispersing the dry lipids in water by a mechanical method (e.g. vortexing, sonication). Each of these procedures may impart properties to the final state that are characteristic of the method of preparation.

To avoid these potential difficulties in the following study, a variety of standard

procedures for forming dispersions were tested for equilibrium criteria; some of the procedures are shown to introduce history-dependent properties to the final state. The study begins with an analysis of the phase diagram of anhydrous mixtures of lecithin and cholesterol and continues with an examination of aqueous dispersions of these mixtures. It will be demonstrated that the properties of aqueous dispersions are related to those of the anhydrous mixtures, and that many of the current uncertainties of the aqueous systems can be resolved by understanding the phase relations in anhydrous mixtures.

EXPERIMENTAL METHODS

Materials

All lipids used in this study were obtained from Applied Science Labs, Inc., State College, Penn.) and used without further purification. Cholesterol (CHOL) melted sharply at 149°C; L-α-dimyristoyl lecithin (DML), L-α-dipalmitoyl lecithin (DPL), and L-α-dioleoyl lecithin (DOL) were tested for purity by thin layer chromatography (solvent system: chloroform:methanol:water, 65:25:4); 200-μg samples were used, and only one spot was detected, suggesting that the samples were at least 99% pure. Whenever a second spot in the chromatogram was detected, that batch of lipid was discarded and another batch used.

Water was distilled three times, the last two distillations from quartz. D_2O, obtained from ICN Pharmaceuticals Inc. (Cleveland, Ohio), was filtered (2-μm filter; Millipore Corp., Bedford, Mass.) before use.

Differential Scanning Calorimetry

The Perkin-Elmer differential scanning calorimeter (DSC-2; Perkin-Elmer Corp., Norwalk, Conn.) was used to obtain latent heats and temperatures for phase transitions of pure lecithins, cholesterol, and their mixtures. The theory for obtaining these parameters from DSC has been described (17). Thermogram temperatures were calibrated with a series of pure lipid standards that covered the entire range of the study. In principle, as a result of thermal lag in the system, equilibrium transition temperatures are slightly lower than the temperature of the peak of the endotherm. The correction for thermal lag was empirically determined from the thermogram for the melting of a pure sample of indium, and was found to be about 0.2°C. Because the precision for measuring transition temperatures is usually $\pm$ 0.3°C, the peak of the endotherm was routinely chosen as the equilibrium temperature for the lipid phase transitions. Sample weights were kept about 1 mg lipid to minimize corrections for thermal lag in the sample pan.

Preliminary studies indicated that heating cholesterol in air at its melting point resulted in oxidation of the sample; repetitive runs lowered the melting point and broadened the endotherm. Oxidation was eliminated by sealing all samples in "volatile" sample pans (Perkin-Elmer Corp.) under an atmosphere of nitrogen. Anhydrous lecithins, when heated above their thermotropic mesomorphic transition temperatures (about 100°C; 18), slowly undergo irreversible transformation as noted by progressive lowering of the transition temperature and broadening of the endotherm. The transition temperature decreased about 1°C for each hour of heating at 100°C; the decrease was minimized by keeping the samples at elevated temperatures for no longer than necessary (about 30 min).

Initial attempts to prepare anhydrous mixtures without the use of solvents were unsuccessful. Anhydrous mixtures were prepared by weighing each component into a sample pan; the sealed pan was then heated above the melting point of cholesterol (149°C) to allow the com-

ponents to mix in the liquid state. Unfortunately, the time required to achieve complete mixing in the melt was excessive and resulted in erratic behavior either because of incomplete mixing or thermal decomposition of the lipids. Therefore, chloroform solutions of known concentration of each lipid were prepared, and mixtures of cholesterol and lecithin were made by combining small aliquots of each component lipid solution in a small vial to form a chloroform solution of the two components for each desired mole fraction. This solution was then put into the sample pans where the bulk of the solvent was removed in a stream of nitrogen with final drying in a vacuum oven at room temperature for about 18 hr. At this stage the anhydrous sample was either sealed in the sample pan under nitrogen, or, if an aqueous preparation was desired, water was added and the sample pan then sealed under nitrogen. For aqueous systems, approximately 2 mg water was added to the 1-mg lipid sample.

DSC thermograms were obtained using various heating and cooling rates (2.5°–10°C/min). No significant differences in heats and temperatures of transitions were observed under these conditions, therefore most of the studies were at a rate of 10°C/min. The samples were initially heated to 10°C above the phase transition of interest, and maintained at that temperature until no further change in DSC output was observed, usually no longer than 30 min. The sample was then cooled and heated repeatedly over the temperature range of interest until consecutive heating thermograms were identical; usually a total of three or four cycles was required. Anhydrous molten cholesterol supercools about 20°C, and in lecithin mixtures supercooling was also observed. Therefore only heating curves are presented in this study.

Lipid Densities by Isopycnic Centrifugation

Linear density gradients of H_2O-D_2O were prepared by standard methods. The gradients were calibrated by measuring the refractive index (33-45-26, Bausch & Lomb Inc., Rochester, N.Y.) of 0.5-ml fractions collected with the Buchler Auto-Densi-Flo IIC fraction collector (Buchler Instruments Div., Searle Diagnostics Inc., Fort Lee, N.J.). A previously determined calibration of refractive index vs. density from known H_2O-D_2O mixtures allowed conversion of the gradient fractions into densities. For calibration the gradient was formed, centrifuged 16–18 h at 200,000 *g*, and fractionated at room temperature (21–22°C). The following density (grams/milliliter)-volume (milliliter) relation is obeyed: $d = 6.2 \times 10^{-3} V + 1.0043$. At values of $d < 1.015$ precision of the gradient is poor. When temperatures other than that of calibration (21–22°C) were used, the standard curve was adjusted by using the change in density of water at the experimental temperature to correct all values of the standard curve by the same amount. To test for possible perturbation of the D_2O-H_2O gradient by the dispersion, the refractive index of gradients containing dispersed lipid was measured after centrifugation; the gradient was unchanged by the dispersion.

Lipid was uniformly dispersed in the aqueous phase (see next section), and the gradient then formed with pure D_2O. The resulting gradient has lipid distributed throughout the entire tube, approximately duplicating the composition gradient of water. Centrifugation results in particles of lipid moving to the isopycnic position from all parts of the tube. Control experiments in which lipid was dispersed in D_2O yielded identical results at equilibrium under identical centrifugation conditions. Because the gradient changes slowly with time, these centrifugation conditions were chosen to allow the lipids to reach their equilibrium positions in the gradient while the gradient remained reasonably constant. Shorter centrifugation times (10 h) gave essentially the same results except that the bands of lipid were somewhat more diffuse. Rotor speed was varied to test whether the gravitational field affected lipid densities; in the range of 80×10^3–200×10^3 *g*, the density of the lipid dispersions was unchanged.

The Beckman L5-65 ultracentrifuge and SW-40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) were used with standard polyallomer tubes (12.6-ml vol). Temperature

control was ± 1°C in the range 5–43°C. Immediately after the completion of the centrifugation run, the limits of each band and the position of the meniscus were marked by scratches on the side of the centrifuge tube. The density was obtained from a calibration of volume vs. tube length; reproducibility was ± 0.001 g/ml. Fractions of the entire contents of the tube were collected and analyzed for phosphorus (19) and cholesterol (20). Generally, the concentration peaks coincided with the positions of the bands in the centrifuge tubes.

Preparation of Aqueous Dispersions and Criteria for Equilibrium

The following criteria of equilibrium were used for aqueous dispersions: (*a*) The number of bands (phases) present in the gradient shall not exceed those allowed by the phase rule. (*b*) The system is invariant with time, tested by sampling the dispersion periodically over a number of days and determining the density of each sample. (*c*) Thermal history does not influence the state. (*d*) The state of the lipid suspension is independent of the method of preparation of the dispersion. If any of these criteria is violated, the system is not at equilibrium.

Four methods were tested for preparing lipid dispersions in water using DML as a typical lecithin. The first three are mechanical methods: shaking-by-hand, vortexing (Vortex-Genie, Scientific Industries, Inc., Springfield, Mass.), and bath sonication (Sonogen, model AP-10, Branson Sonic Power Co., Danbury, Conn.); the fourth method combines use of methanol as a solvent for the lipids and dialysis against water to disperse the lipids. T_c for DML is about 23.5°C (7, 10), which is experimentally convenient for studying the effects of thermal cycling on the gel-liquid crystal transition of lecithin. DML dispersions were prepared below (5°C) and above (35°C) T_c to test for thermal reversibility. Specific details for each method follow.

MECHANICAL METHODS For the mechanical methods of forming dispersions, water was added to anhydrous DML or DML-cholesterol mixtures; the latter were formed by evaporating chloroform solutions of the lipids to dryness under nitrogen followed by final drying in a vacuum oven at room temperature. (*a*) Shaking by hand and (*b*) vortexing: Dispersions in water did not form readily at 5°C by these methods; therefore, the preparations were heated first to 35°C, shaken by hand for 30 min, and then cooled to 5°C when either vortexing or shaking by hand was continued for 30 min. (*c*) Bath sonication: Water was added to anhydrous lipids in Erlenmeyer flasks and sonicated for 1 h at either 5° or 35°C. Temperatures were monitored continuously, and whenever the temperature increased more than 5°C, sonication was interrupted and the bath water was changed.

DIALYSIS Lipid dispersions were prepared by adding 1 ml of a methanol solution of the lipids to 25 ml of a methanol:water (5:1) solution, followed by dialysis against distilled water at 5° or 35°C for 3 days. The order of adding the lipids to the methanol-water solution does not alter the results for DML-cholesterol dispersions.

Before use the dialysis tubing was rinsed in a solution of methanol:water (5:1). Loss of lipid from the tubing during the dialysis was negligible as indicated by the fact that all the lipid was recovered from the tubing at the completion of the dialysis.

Each preparative technique was tested by measuring the density of the final dispersion by isopycnic centrifugation over the entire range of experimentally available temperatures (5°–43°C). In general, for pure DML dispersions prepared below T_c, i.e. at 5°C, all four methods gave identical results: a single band whose density and temperature dependence were characteristic of DML. Once the dispersions were prepared they could be stored in the refrigerator for several weeks without any significant change in their properties at all temperatures. Moreover, if the preparations were kept at temperatures greater than T_c for several days, no significant change in density was observed over the entire range of temperature. Thus, these preparations conform to the equilibrium criteria.

When the temperature of preparation was 35°C, exceeding T_c, the properties of the lipids depended on the method used to form the dispersion. For the bath-sonicated and dialyzed dispersions of pure DML, two bands appeared in the gradients at all temperatures, indicating the presence of two lipid phases. These bands appeared even after the preparations were allowed to reequilibrate at 5°C for several weeks. According to the phase rule, this system can have two coexisting lipid phases only at a transition temperature (e.g. T_c). It is evident, therefore, that nonequilibrium states are formed when sonicated and dialyzed dispersions are prepared at 35°C. Preliminary studies using a high energy probe sonicator at 35°C yielded similar results. Moreover, this preparation continued to change over a prolonged period (30 days; J. N. Weinstein and N. L. Gershfeld, unpublished results).

In contrast, the hand-shaken and vortexed 35°C preparations retain all the characteristics of the 5°C preparations in conformity with equilibrium criteria.

Lipid mixtures of DML and cholesterol prepared at 5°C by the four methods and by hand-shaking or vortexing at 35°C also conform to equilibrium criteria. However, the use of sonication at 35°C to prepare dispersions of mixed lipids resulted in erratic behavior, showing poor reproducibility with respect to the number of bands formed and their densities. Sometimes more bands were formed than allowed by the phase rule. The dialysis method at 35°C yielded reproducible results but the dispersions that formed were significantly different from the hand-shaked or vortexed 35°C preparations. The properties of the DML-cholesterol dispersions prepared by dialysis at 35°C are of general interest and therefore will be discussed more fully in the main body of the text.

In summary, the preparation of dispersions below T_c yields systems that conform to equilibrium criteria. But when the preparation is at temperatures above T_c, sonication and dialysis form nonequilibrium states. In this study, dispersions were prepared at 5°C by the dialysis method because it was shown to form equilibrium dispersions, and because the bands formed by this method were more sharply defined than with the other procedures. For those lecithins with T_c below 0°C (e.g. dioleoyl lecithin), preparation of equilibrium dispersions is limited to the shaking-by-hand or vortexing procedures.

RESULTS

Phase Relations for Anhydrous Mixtures of Lecithin and Cholesterol

The phase diagram for anhydrous mixtures was constructed from differential scanning calorimeter thermograms. Thermograms for a series of mixtures in which the mole fraction of cholesterol x was varied are shown in Fig. 1. A single endotherm characterizes the behavior of DML, $x = 0$, and represents the thermotropic transition from crystal to liquid crystal (18). As increasing amounts of cholesterol are added to DML, striking changes in the thermograms are noted. A broad endotherm, which begins at approximately 5°C and peaks in the region of 25–45°C (NP, Fig. 1), is closely followed by another at about 50°–60°C (QR, Fig. 1). These transitions are followed by a series of endotherms, in one of which the temperature is independent of composition and in the second of which the temperature varies with composition. When $x = 0.25$, only one of these peaks occurs (B, Fig. 1). Additional endothermal peaks occur at still higher temperatures; these are not presented but will be indicated on the phase diagram in Fig. 2. The temperatures obtained from the DSC thermograms were corrected by calibration with melting point standards, and the corrected peak temperatures were plotted as a function of the cholesterol mole fraction x in Fig. 2.

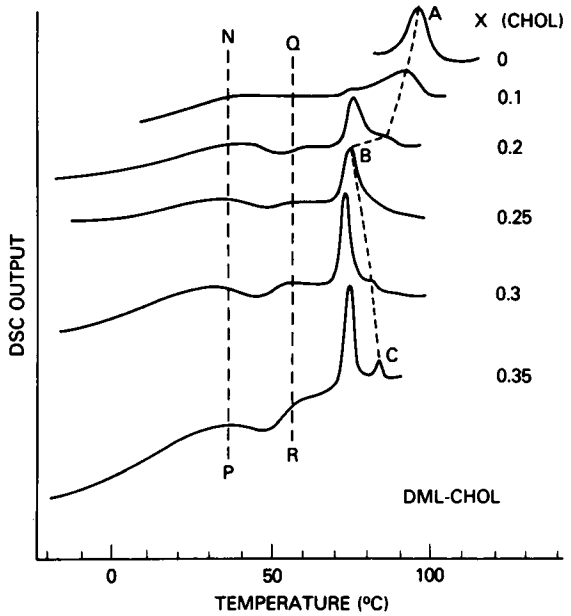

FIGURE 1

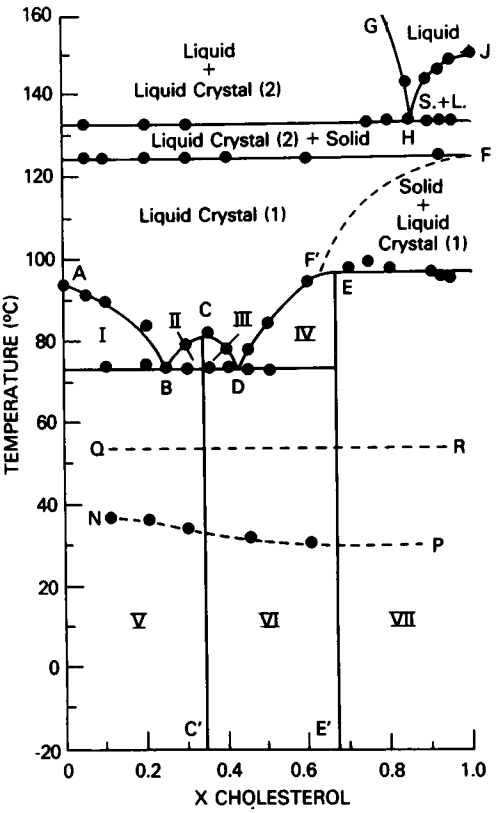

FIGURE 2

FIGURE 1 DSC thermograms of anhydrous DML-cholesterol mixtures. Composition is given as mole fraction of cholesterol, x(CHOL). Temperatures listed are those recorded but not corrected by calibration because the calibration varies with temperature. For corrected temperatures see Fig. 2. Points A, B, and C correspond to the same letters in Fig. 2. For significance of NP and QR, see text.

FIGURE 2 Temperature-composition phase diagram for anhydrous mixtures of DML and cholesterol. Each point represents the temperature at the peak of an endotherm (see Experimental Methods). All temperatures have been corrected by melting point standards.

In the temperature-composition phase diagram, Fig. 2, point A represents the transition from crystal to liquid crystalline DML. Points B and D represent eutectics below which two phases coexist. The peak at point C represents the congruent transition temperature of the DML:cholesterol 2:1 complex. At $x = 0.33$ only the DML:cholesterol 2:1 complex is present, and the thermogram normally will not exhibit an endotherm at the temperature of the eutectic (73°C). Thus the presence of a large endotherm at 73°C for $x = 0.35$ seems paradoxical. However, the paradox may be resolved if the large endotherm at 73°C contains contributions from processes other than fusion of a simple eutectic system. For example, if the process that commences at temperatures along QR (Fig. 1) were to end at 73°C accompanied by an endotherm,

both it and the fusion process at the eutectic would be indistinguishable. To separate the two processes, a region in the phase diagram must be selected in which one is absent. When $x = 0.1$, no endotherm is detected in the thermogram at QR (Fig. 1), yet a small but significant peak is observed at 73°C. However, when $x = 0.2$–0.35, the endotherm along QR is present and, concomitantly, there is a disproportionate increase in the peak size at 73°C (Fig. 1). It is therefore assumed that at $x = 0.1$ the endotherm at 73°C represents only the fusion process associated with the eutectic, whereas for $x = 0.2$–0.35 the endotherm at 73°C represents both the eutectic fusion process and the process that commences at QR. Although the interpretation of this aspect of the phase diagram is perhaps speculative, the explanation will be shown to be consistent with equilibrium surface film data presented in the accompanying paper (21). It is recognized that application of other methods (e.g. X-ray, NMR) may further elucidate the phase diagram, and may possibly necessitate a modification of the present interpretation.

Above line ABCDF′, a homogeneous liquid crystalline state exists. Line DF′ approaches a maximum with increasing cholesterol composition in the region where the mole fraction of cholesterol is 0.66, indicating the existence of another complex with the molar ratio of lecithin:cholesterol of 1:2. However, a peritectic point occurs at F′ which obscures the precise location of the maximum and the exact molar ratio of the complex. Inasmuch as independent studies (1, 3) support the existence of a 1:2 anhydrous complex, the phase line EE′ is drawn at $x = 0.66$. The temperature of the peritectic is approximately the same as the liquid crystal transition for pure DML (point A, Fig. 2).

For a two-component system, the maximum number of phases allowed to coexist at constant pressure is three, and for the DML-cholesterol system the eutectic at B, Fig. 2, shows liquid crystal, solid DML, and complex 2:1. Therefore, the dotted line FF′ is drawn to restrict crystalline cholesterol in the region to the right of FF′, thereby avoiding violation of the phase rule. It should be noted that no thermal transitions have as yet been identified for line FF′.

The horizontal line drawn at 125°C (point F) represents the transition from one liquid crystalline state (state 1) to another (state 2). The transition is seen in the thermogram as a small endotherm with a heat < 1 cal/g (data not shown). This region contains two phases: liquid crystal (state 2) and crystalline cholesterol. A similar transition has been reported for other anhydrous phospholipids (22–24).

At temperatures above 130°C another eutectic is observed at H, where above the lines GH and JH isotropic liquid exists. J is the melting point of cholesterol. Along line GH, DML melts; when $x = 0$ (pure DML), the melting temperature is approximately 230°C. Because DML melts with decomposition (18), the details of this region of the phase diagram are omitted.

The phase relations below line ABCDF′ may be conveniently characterized by regions I–VII. Region I: two phases, liquid crystal plus crystalline DML; regions II and III: two phases, DML:cholesterol complex 2:1 in equilibrium with liquid crystal; region IV: two phases, DML:cholesterol complex 1:2 and liquid crystal; region V: two

TABLE I

EQUILIBRIUM TRANSITION TEMPERATURES FOR ANHYDROUS LECITHIN-CHOLESTEROL MIXTURES

Lipid	T_m*	$T_{2:1}$‡	$T_{1:2}$§	T_{eut}‖
	°C	°C	°C	°C
DML	94	82	96	73
DPL	103	88	105	83

*Thermotrophic transition from crystal to liquid crystal (point A, Fig. 2).
‡Transition of lecithin:cholesterol 2:1 complex to liquid crystal (point C, Fig. 2).
§Temperature of peritectic in region of lecithin: cholesterol 1:2 complex (point E, Fig. 2).
‖ Temperature of eutectic at point B, Fig. 2.

phases, DML:cholesterol complex 2:1 plus crystalline DML; region VI: two phases, DML:cholesterol complexes 2:1 and 1:2. Region VII is assumed to contain crystalline cholesterol and DML:cholesterol complex 1:2.

Regions V and VI require further comment because of the peaks noted along lines NP and QR, Fig. 1. These peaks are broad, and therefore it is difficult to assess the nature of these transitions. Because DML alone does not exhibit these transitions, it is reasonable to associate these transitions with the complexes that also exist in these regions. However, the fact that the transitions depend slightly on the relative amounts of each phase (DML and complex 2:1, complex 2:1 and complex 1:2) suggests that some second-order interaction occurs between the two phases. The nature of the transitions along NP and QR, Fig. 1, are presently unknown.

Anhydrous mixtures of DPL with cholesterol exhibited similar phase relations as reported for DML in Fig. 2; hence the phase diagram for DPL is not shown. However, the pertinent temperatures for both DML and DPL-cholesterol mixtures are given in Table I. The DPL-cholesterol endotherms, which correspond to those shown for DML-cholesterol mixtures along line NP (Fig. 1), are about 10°C higher, and are less distinct. Anhydrous DOL did not show a thermotropic transition by DSC, therefore it was not possible to examine the anhydrous phase diagram for this lipid by this method.

Influence of Water on the Lecithin-Cholesterol Phase Relations

The addition of water to anhydrous lecithin-cholesterol mixtures under the conditions of temperature and composition of region V, Fig. 2, would be expected to yield two lipid phases, pure lecithin and complex 2:1, if the intrinsic factors that determine the anhydrous phase relations persist in the presence of water. Thus when the mole fraction of cholesterol $x \leq 0.33$, the gel-liquid crystal transition of pure lecithin should be evident; but when $x = 0.33$, only the properties of complex 2:1 should be observed. Four properties of the lipid dispersions were measured as a function of lipid composi-

tion to examine this concept: the transition temperature T_c, heats of transition, density, and surface tension of the lipid dispersions. The latter is examined in detail in the accompanying paper (21).

DSC thermograms of cholesterol mixtures with DML, DPL, or DOL with water in the weight ratios of lipid:water of 1:2 were obtained as a function of the mole fraction of cholesterol x. Fig. 3 gives the temperatures at which the peak of the endotherm occurs, the gel-liquid crystal transition temperature T_c for these systems. Because the thermograms are typical of those reported for these and other systems (18, 25), they are not presented here. For all three systems, T_c is unaffected by the addition of cholesterol. Data points from Hinz and Sturtevant (10) for DML are also shown, and they are in agreement with the data in Fig. 3.[1] These data are restricted to $x \leq 0.2$ because the size of the endotherm decreases with increasing cholesterol content, in agreement with other reports (7, 10). When x exceeds 0.2, the peak height is sufficiently reduced so that it is difficult to make a precise measurement of the peak temperature. However, estimates of the temperature at the peak for $x \geq 0.2$ are consistent with the general conclusion that added cholesterol does not change T_c for pure lecithin in water. Inasmuch as cholesterol does not change the temperature of the gel-liquid crystal transition for lecithin, it follows that cholesterol does not mix with either the gel or liquid crystal states. Thus pure lecithin must be present in the dispersions when $x < 0.33$. This is precisely what is anticipated if the anhydrous phase relations of region V, Fig. 2, persist in the presence of water.

The observation of decreasing heats of transition with increasing cholesterol content while the transition temperature is unchanged is also consistent with the anhydrous phase diagram of region V, Fig. 2. If complex 2:1 forms, it will do so at the expense of free lecithin, and thus as the relative amount of cholesterol in the mixture increases, the amount of lecithin will decrease and concomitantly the heat of transition. It has been reported that the heats of transition for DML and DPL decrease monotonically with increasing cholesterol composition to a limiting value at $x = 0.33$ (10). The same general results have been observed in this study for cholesterol mixtures with DML and DPL and therefore are not presented. However, similar studies with DOL-cholesterol mixtures have not been reported previously, and, therefore, Fig. 4 gives the results of the effect of cholesterol on the transition endotherm for DOL dispersions. When $x \leq 0.33$, the heat of transition decreases to a small value (1–2 kcal/mol) and remains at that level even as x is raised above 0.33. These results are in general agreement with those obtained with DML and DPL and are consistent with the concept that a lecithin:cholesterol 2:1 complex coexists with free lecithin when $x \leq 0.33$.

The third series of studies to test the persistence of the anhydrous phase relations of region V, Fig. 2, in aqueous dispersions are density-composition experiments. If two

[1]The apparent decrease in T_c for DML with increasing cholesterol content must be viewed with caution because no range of errors for each determination has been given (10). More recent studies for pure DML dispersions from the same laboratory report slightly different values for T_c of 23.9° (26), 23.7° (27), and 23.5°C (28). These variations are similar to those reported in Fig. 3 (± 0.3°C).

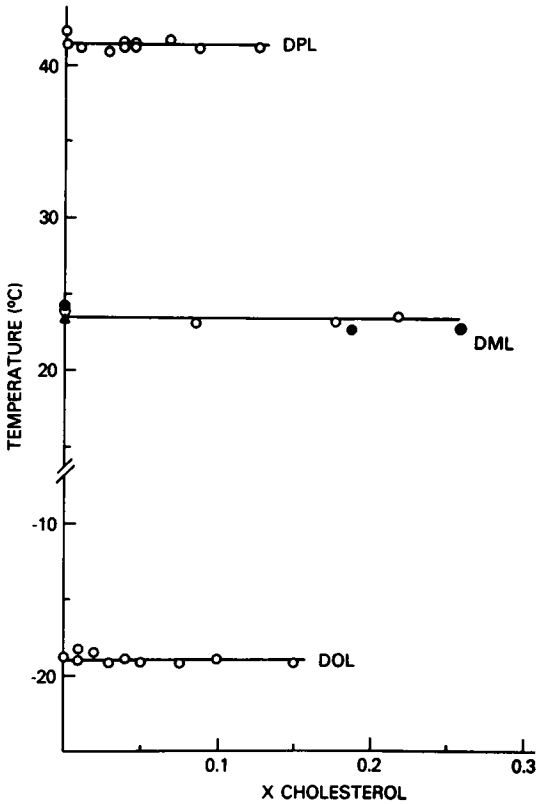

FIGURE 3

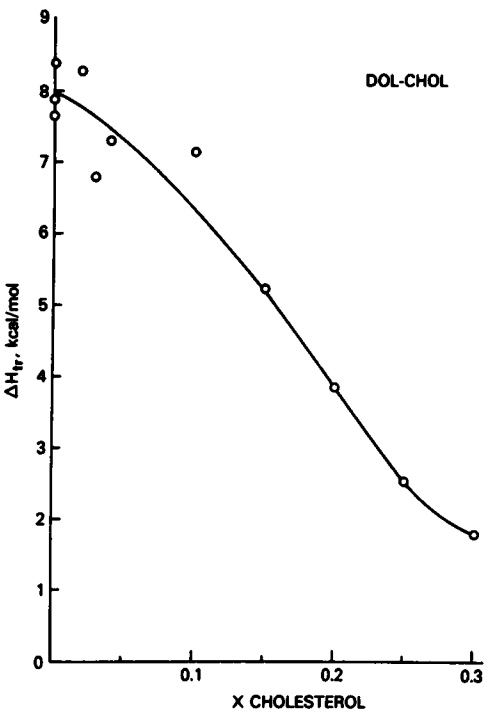

FIGURE 4

FIGURE 3 Gel-liquid crystal transition temperatures for lecithin-cholesterol mixtures in water. Lecithin:water 1:2 wt/wt. ●, data of Hinz and Sturvevant (10). ▲, data of Ladbrooke et al. (7).

FIGURE 4 Transition heats (kilocalorie/mol DOL) obtained from DSC thermogram of DOL-cholesterol mixtures in water as a function of cholesterol content. Heats remain at 1–2 kcal/mol when $x > 0.3$.

phases characteristic of region V are present in water, either two bands with the appropriate composition will appear in the D_2O–H_2O gradient or, if the phases coprecipitate, a single band will appear that will conform to the following density-composition relation:

$$d = d_0 d_c / [d_c + \phi(d_0 - d_c)], \quad (1)$$

where d is the density of the mixture at any mole fraction; 0, and c refer to pure lecithin and complex, respectively, and ϕ is the weight fraction of lecithin in the lipid mixture. Eq. 1 is derived assuming that the masses and volumes of the two phases are additive.[2] Any other behavior would indicate that the anhydrous phase relations of

[2] Ideal mixing of the two components would also lead to the same density-composition relation, but inasmuch as it has already been demonstrated that cholesterol does not mix with the pure lecithin phase in water, this possibility is clearly excluded.

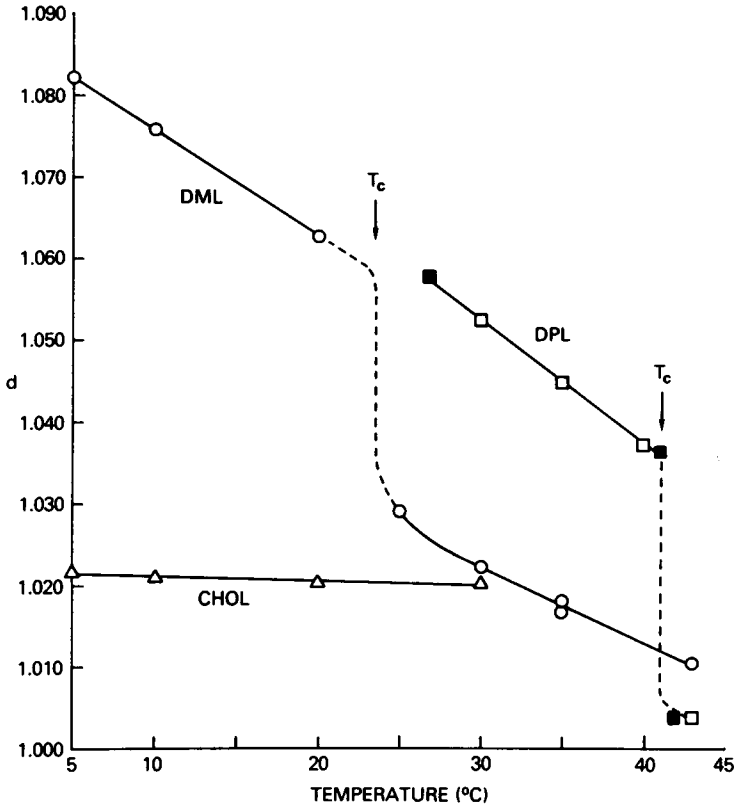

FIGURE 5 Density (gram/cubic centimeter) of lipids in H_2O-D_2O gradients as a function of temperature. Lipid dispersions prepared by dialysis at 5°C (see Experimental Methods). ■, data of Sheetz and Chan (30).

region V, Fig. 2, have been altered in the presence of water. The densities for the pure components and their mixtures are given in Figs. 5 and 6.

The densities of DML, DPL, and cholesterol are shown in Fig. 5 as a function of temperature. Each of these systems was prepared by the dialysis method at 5°C (see Experimental Methods). DOL dispersions were also prepared by vortexing (data not shown); the density at 5°C was 1.018, which decreased with increasing temperature. Because the density gradient technique is poor when the densities fall below this value, the values for DOL at higher temperatures are not presented, though they are < 1.018.

The sharp break in density for DML between 20° and 25°C, and for DPL between 40° and 43°C is characteristic of the gel-liquid crystal transition for these compounds. Moreover, the changes in density at the transition temperatures are in agreement with results obtained by other methods (29–31). Values for DPL obtained by Sheetz and Chan (30) are in agreement with those given in Fig. 5. The value of 1.022 for the density of cholesterol in the range of 5°–30°C is slightly lower than the value of 1.03 reported for the monohydrate (32). The density of anhydrous cholesterol is about

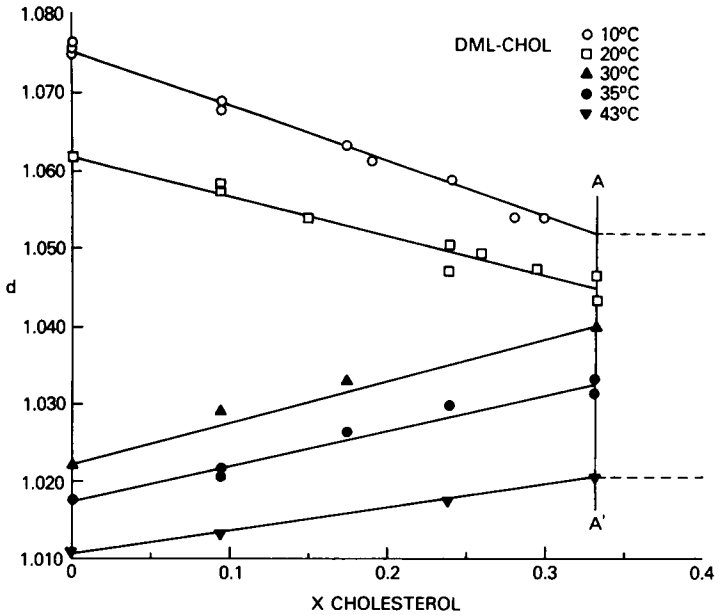

FIGURE 6 Density-composition isotherms for DML-cholesterol dispersions in D_2O–H_2O gradients. Lines are drawn according to Eq. 1. Dotted lines to the right of AA′ signify density limits of broad dispersion of particles at 10°C; these limits vary with temperature as the densities of cholesterol (lower limit) and complex 2:1 (upper limit).

1.05 (32), and it has been observed in this study only when finely ground crystals of cholesterol are added to the gradient.

Isopycnic centrifugation of DML-cholesterol mixtures prepared by the dialysis method (see Experimental Methods) yielded results presented in Fig. 6. For mole fractions of cholesterol < 0.33 only a single band appears in the gradient at all temperatures. The solid lines drawn through these data were calculated from Eq. 1 in which the density of pure DML and the density of the band when $x = 0.33$, i.e., the presumed lecithin:cholesterol 2:1 complex, were used for the values of d_0 and d_c, respectively. The agreement between the data and the theoretical line is consistent with the presence of two phases—pure DML and complex—as a coprecipitate.

When the composition exceeds $x = 0.33$, a broad diffuse dispersion of particles appears located within the limits of the density of cholesterol (about 1.02) and the density of the band for $x = 0.33$, the 2:1 complex. These limits varied with temperature precisely as the densities of cholesterol and the 2:1 complex at each temperature. The limits of the dispersion in the gradient at 10°C when $x = 0.33$ are shown by the dotted lines drawn to the right AA′, Fig. 6. Density measurements of DPL-cholesterol mixtures were equivalent to the results obtained with DML; any differences were reflections of the differences in T_c for the two lecithins.

The temperature dependence of the density of the lecithin:cholesterol 2:1 complex for both DML and DPL are shown in Fig. 7. It is significant that the density of the

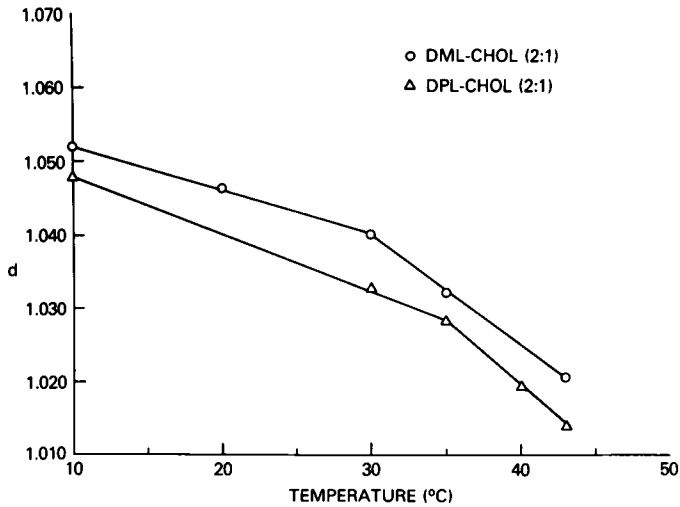

FIGURE 7 Density-temperature relations of DML- and DPL-cholesterol 2:1 complexes. Break in plots at 30° and 35°C, respectively, coincides with anhydrous transitions for these mixtures at temperatures along line NP, Figs. 1 and 2.

2:1 complex is greater than either the liquid crystal state of the pure lecithin or the hydrated cholesterol (cf. Figs. 5 and 7). Moreover, the complex does not show the dramatic change in density observed for the pure lecithins at the gel-liquid crystal transition temperature. A change in slope does occur at about 35°C with DPL and 30°C with DML, which may indicate a higher order phase transition for the complexes at these temperatures (see Discussion). Finally, Fig. 7 indicates that the 2:1 complex for DML and DPL persist well below the respective gel-liquid crystal transition temperatures for these lipids, indicating that the complex may coexist with either the gel or liquid crystal state of lecithin.

The DML-cholesterol disperions used for the studies reported in Figs. 6 and 7 were prepared at 5°C (below T_c). When the dispersions are prepared by dialysis at 35°C, the properties of the system are dramatically altered. The results are presented in Fig. 8, curve A, where the density is given as a function of composition at 35°C. For comparison the densities of the samples prepared at 5°C and centrifuged at 35°C are also shown in curve B. Pure DML dispersions prepared at 35°C show two bands in the density gradient; the majority of the lipid (about 90%) was in a band at d 1.040, whereas the remainder was at d 1.032. In contrast, the DML dispersion prepared at 5°C showed a single band at d 1.018. Cholesterol-lecithin dispersions prepared at 35°C by dialysis (curve A) formed a single band whose density decreased with increasing cholesterol concentration; the density decreases continuously to a limit equivalent to a molar ratio of DML: cholesterol of 1:1 ($x = 0.5$). When $x > 0.5$, free cholesterol appears along with the band with composition $x = 0.5$ and d 1.030. In contrast, mixtures prepared at 5°C form dispersions in which the particle density increases with cholesterol composition to a limiting ratio of lecithin:cholesterol of 2:1 (curve B,

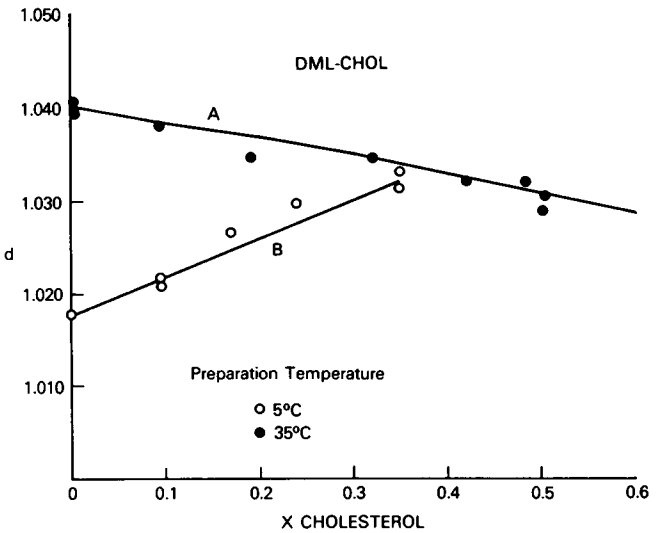

FIGURE 8 Density (gram/milliliters)-composition 35°C isotherms for DML-cholesterol dispersions in H_2O–D_2O gradients. Influence of preparation temperature. (A) Dispersions formed by dialysis at 35°C. For pure DML two bands appear simultaneously: d (g/ml) 1.040 (90%) and 1.032 (10%); only 1.040 band shown. (B) Dispersions formed by dialysis at 5°C (same as Fig. 6). Lines are drawn according to Eq. 1; curve A drawn assuming either complex 1:1 plus DML as coprecipitate or hydrate cholesterol plus DML as coprecipitate. Curve B drawn assuming DML and complex 2:1 as coprecipitate.

Fig. 8). When the 35°C preparations of DML-cholesterol mixtures are kept at 5°C for several days, the density-temperature relations revert to those of the 5°C preparations (Fig. 6), where further cycling of the temperature is now consistent with equilibrium criteria. Similar behavior was observed for dispersions sonicated at 35°C, and is characteristic of metastable states which are usually history-dependent.

DISCUSSION

Equilibrium Dispersions of Lipids in Water

One of the principal goals of this study was the establishment of experimentally feasible criteria for distinguishing equilibrium dispersions of lecithin and lecithin-cholesterol mixtures in water, thereby avoiding arbitrary choices for experimental conditions. The methods in the study were chosen to represent as broad a spectrum as possible of those reported in the literature, to establish some rationale for choosing among the various disparate results obtained for the lecithin-cholesterol stoichiometries. From the density gradient studies it has been established that all methods used for dispersing the lipids below the gel-liquid crystal transition temperature yield equilibrium dispersions. However, when the temperatures at which the dispersions are prepared exceed T_c, it was shown that only the mildest forms of agitation are allowed (shaking by hand or vortexing) if equilibrium conditions are to prevail. When either

solvents (as for the dialysis method) or sonication are used in the preparation of the dispersions at temperatures exceeding T_c, the systems have been shown to violate the phase rule, to be irreversible with respect to temperature cycling, and to change with time (3, 16). These nonequilibrium dispersions often exhibit properties that depend on the method of preparation and therefore pose a serious dilemma for the experimenter in the choice of establishing variables to represent the dispersed state.

In the case of DML dispersions prepared at 35°C (above T_c), that two phases (bands) are seen in the gradient does not necessarily indicate that only one of them is the nonequilibrium state. On the contrary, both may be nonequilibrium states, as indicated by the fact that the densities of the two phases are different from the density of the equilibrium state (Fig. 8). Similar density effects have been noted by Sheetz and Chan (30). It may be anticipated that other solvent methods (33) used to form dispersions at temperatures greater than T_c will similarly produce nonequilibrium dispersions.

It is premature to examine the question of metastability of lecithin dispersions; however, it should be noted that metastable states in colloids are typically associated with extremely small particle sizes as in the case of supersaturated solutions (34). Thus, it is reasonable to suggest that the very small particles formed by sonication of lecithin (35) are at least in part responsible for the metastable properties of these dispersions: the observations that sonicated dispersions change with time (16) are also consistent with this contention.

Lecithin-Cholesterol Complexes

The phase diagram for anhydrous mixtures of cholesterol with DML or DPL indicates the presence of complexes with molar ratios of 2:1 and 1:2 (Fig. 2, regions V and VI). These complexes are stable over a wide range of temperatures from below 0°C to above 70°C. It is important to recognize that these complexes are stable in the same temperature interval that aqueous dispersions are formed. Thus it is natural to suggest that the complexes may persist in the presence of water.

The following is supporting evidence for the existence of the lecithin:cholesterol 2:1 complex in water: (*a*) The apparent decrease in latent heat of transition with increasing cholesterol composition (Fig. 4) is consistent with the sequestering of 2 mol of lecithin for each mole of cholesterol added as suggested by Hinz and Sturtevant (10). (*b*) The mean heat absorption for DML- and DPL-cholesterol dispersions in water has recently been resolved into two components, one sharp and the other broad. The sharp component corresponds to the midpoint of the transition of the pure lecithin component which completely disappears when x (CHOL) > 0.3. The broad transition is present only in the cholesterol-containing dispersions and occurs at a higher temperature than T_c for the pure component (28). (*c*) The isopycnic centrifugation studies indicate that lecithin and the 2:1 complex are codispersed when $x < 0.33$, but that 2:1 complex coexists with cholesterol when $x > 0.33$. In the former case, pure lecithin behaves independently with respect to temperature, forming gel below T_c and liquid crystal above T_c (Fig. 6). Verification of the existence of the 2:1 complex in aqueous dispersions has been obtained from surface tension studies (21). (*d*) The

density of lecithin:cholesterol 2:1 complex is greater than that of either cholesterol or the liquid crystal state of lecithin (cf. Figs. 5 and 7).

Less direct, but nevertheless significant, supporting evidence for the similarity of the anhydrous and aqueous states of lecithin-cholesterol mixtures are the following: (*a*) The transition temperatures for DML, DPL, and DOL in water are independent of and unchanged by the presence of cholesterol when $x < 0.33$, indicating the presence of two phases, one of which must be pure lecithin (Fig. 3). Similar phase considerations apply to the anhydrous system (Fig. 2, region V). (*b*) In the anhydrous phase diagram (Fig. 2) where lecithin-cholesterol 2:1 complex is stable, a small endothermal transition occurs along line NP, with a peak between 30–35°C for DML, and about 10°C higher for DPL-cholesterol mixtures. The endotherm occurs only when cholesterol is present, and persists with mole fractions of cholesterol > 0.5. It is significant that Maybrey et al. (28) have also observed a broad endotherm in cholesterol-containing aqueous dispersions of DML and DPL which occurs at a higher temperature than T_c for the pure lecithin, and is still present at mole fractions > 0.5. At approximately 30°C for DML and at about 35°C for DPL-cholesterol 2:1 complexes in water, the densities indicate the presence of a phase transition (Fig. 7). The temperature dependence of the Raman spectrum for DML-cholesterol dispersions also indicates the presence of a transition near 32°C (36). (*c*) Raman spectra of anhydrous DPL-cholesterol mixtures and aqueous dispersions were noted to be "strikingly similar," and adding cholesterol to aqueous dispersions of lecithin was considered similar to removing water from the hydrated lecithin (37). On the basis of these similarities between the anhydrous and aqueous states, it is believed that the lecithin:cholesterol 2:1 complexes are essentially the same with perhaps a second-order perturbation of the lamellar structure introduced by the addition of water.

Electron spin resonance (ESR) studies are not consistent with these observations. No complexes are reported, and though the presence of a two-phase domain is noted at temperatures exceeding T_c for the pure lecithin, each of the phases is of variable composition (12). It should be noted, however, that the use of a molecular probe introduces another component into a complex heterogeneous system, and the ESR observations may represent only the properties of the local environment of the probe molecule and not the macroscopic system (38).

Recent NMR studies report the existence of a phase line when $x(\text{CHOL}) \geq 0.2$ in which two phases appear containing different amounts of cholesterol (13). This observation conflicts with ESR data which report two solid phases when $x(\text{CHOL}) < 0.2$, and solid solution when $x(\text{CHOL}) > 0.2$ (12). The difference between the ESR and NMR data are presently not resolvable, particularly as no phase transition at $x(\text{CHOL}) = 0.2$ has been observed by X-ray diffraction (2, 3, 11), by calorimetry (28), or by the density gradient results of the present study.

Aqueous dispersion of lecithin-cholesterol mixtures with molar ratios of 1:1 are generally noted with preparations that have been sonicated above T_e. These preparations have been shown to be metastable. It is of interest to note that when Eq. 1 is applied to the density data for these metastable systems (Fig. 8, curve A) the conclu-

sions with respect to the nature of the metastable mixture are not clear. The solid line of curve A, Fig. 8, may be calculated by assuming either of two distinct models for the mixture: either that a 1:1 complex has coprecipitated with pure DML, or that pure cholesterol (d = 1.02) has coprecipitated with the DML dispersion. Therefore it is uncertain whether a metastable 1:1 complex has formed.

Hydrated lecithin-cholesterol aggregates with molar ratios of 1:2 have generally been observed with sonicated preparations (4, 39). However, this ratio was not stable (3) but degenerated to aggregates with molar ratios of 1:1, in agreement with the metastable sonicated dispersions in this study (Fig. 8, curve A). No evidence has been found in the present study for the existence of a stable aqueous lecithin-cholesterol complex with molar ratio 1:2..

The coexistence of a lecithin and lecithin-cholesterol 2:1 complex in water is consistent with the molecular mobility interpretation of NMR studies. The addition of cholesterol to lecithin dispersions generally increases the hydrocarbon chain "fluidity" when the lecithin is in the gel state, but decreases the hydrocarbon chain "fluidity" when the liquid crystal state is present (40). Under the conditions of the majority of these studies (x = 0.33), it is primarily the 2:1 complex whose spectra are obtained. If "fluidity" of the hydrocarbon chains may be considered a property inversely reflecting the density of the material, and because the densities of DML-cholesterol mixtures follow as gel > 2:1 complex > liquid crystal (Fig. 6), it follows that the "fluidity" of the 2:1 complex will be greater than the gel state but less than the liquid crystal state of pure lecithin. Therefore the formation of the 2:1 complex can also account for the "fluidizing" properties of cholesterol; depending on the temperature, either below or above T_c, the addition of cholesterol will give the appearance of either enhancing or diminishing the "fluid" character of the dispersion. In reality the 2:1 complex forms over the entire range of temperatures where this effect has been reported (41).

X-ray diffraction studies at temperatures below T_c are consistent with the phase relations reported here (11). However, studies of lecithin-cholesterol dispersions at temperatures greater than T_c do not indicate the presence of two phases when the mole fraction of lecithin is < 0.33 (2, 3). If the domain size is small enough, it has been suggested that the phases would not be detected by low-angle X-ray scattering (42).

The studies presented are generally consistent with the formation of a lecithin-cholesterol 2:1 complex; the dispersion is heterogeneous when either lecithin or cholesterol is present in excess of this stoichiometry. A rigorous thermodynamic study that utilizes an equilibrium surface tension method provides direct evidence for these phase relations (21). However, it may be anticipated that more subtle aspects of the phase relations remain to be uncovered. Indications of the complexities of this system are the unresolved transitions along lines NP and QR (Fig. 2) for the anhydrous system, which may also appear in the aqueous dispersion (Fig. 7). The resolution of these complexities will likely depend on spectroscopic methods (12, 13, 43).

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