The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine

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Abstract In order to investigate the structure and stability of cholesterol-enriched dispersions of phosphatidylcholine (PC), cholesterol/dipalmitoyl phosphatidylcholine (DPPC) mixtures with molar ratios of 4 ± 0.5/1 to 1/1 were dispersed in water by sonication. These dispersions comprise liposomes and unilamellar vesicles with diameters in the range 200–1800 Å. The bilayers which have a repeat distance of 66 Å in these particles at 20°C can contain up to 4 mol cholesterol/mol PC when DPPC is used and about half this ratio with egg PC. These dispersions are metastable in that storage at either 4 or 20°C leads to aggregation and precipitation of vesicles; in addition, there is a decrease in the cholesterol/PC molar ratio in the particles and formation of cholesterol monohydrate crystals. Cholesterol is released slowly and PC dispersions containing more than equimolar amounts of cholesterol can be stable for several months. The amount of free cholesterol was determined by differential scanning calorimetry from the heat associated with the transition at 157°C from smectic liquid crystal to liquid cholesterol. Under conditions of gentle mixing, the maximum solubility of cholesterol in DPPC bilayers is 1.0 ± 0.1 mol/mol PC when the mixture initially contains less than about 3 mol cholesterol/mol DPPC. This is consistent with published equilibrium phase diagrams which show that equimolar PC/cholesterol bilayers are stable in water.—**Collins, J. J., and M. C. Phillips.** The stability and structure of cholesterol-rich codispersions of cholesterol and phosphatidylcholine. *J. Lipid Res.* 1982. **23:** 291–298.

Supplementary key words cholesterol/phospholipid • membranes • vesicles • liposomes • phase behavior

Cells whose plasma membranes have cholesterol/phospholipid molar ratios (C/P) appreciably greater than the normal value of approximately 1/1 occur in some pathological states. Notably, the normal red blood cell cholesterol/phospholipid ratio of 1.0 increases to 1.3 in cholesterol-fed dogs (1) or to 1.6 in humans with hepatocellular disease (2). In vitro, incubation of cholesterol-loaded phosphatidylcholine/cholesterol dispersions with either red blood cells (3–5), or hepatoma cells (6) and lipoproteins (7), gives enrichment. The physical state of cholesterol in enriched cell or model bilayer membranes is not well understood. Especially unclear is whether or not these systems are truly stable (i.e., at thermodynamic equilibrium) or metastable (i.e., under kinetic control).

In model systems, phase diagrams obtained under equilibrium conditions show a maximum C/P = 2/1 in dry systems (8,9). The situation is more confused for hydrated systems since maximum equilibrium stoichiometries of C/P = 1:2 or 1:1 have been reported (9–12). The discrepancies arise in large part because it is very easy to incorporate high levels of cholesterol into aqueous phospholipid dispersions. Thus, since 1971, dilute phosphatidylcholine (PC) dispersions enriched in cholesterol to levels of 2–3 mol cholesterol per mol PC have been prepared by sonication under a variety of conditions (3, 13–18). Althogh some of these preparations are known to be metastable (15), these systems have not been characterized in detail despite their widespread use for modifying the cholesterol content of many cell systems (19). It is the purpose of this paper to report a physical characterization of the dipalmitoyl phosphatidylcholine (DPPC)/cholesterol system with C/P > 1. Vesicular structures containing PC/cholesterol bilayers exist which, on standing, fuse with concomitant release of crystalline cholesterol. At equilibrium, and in the absence of a large excess of cholesterol, the preferred form of association is bilayers containing equimolar amounts of PC and cholesterol.

MATERIALS AND METHODS

Cholesterol (Sigma grade: 99 + %, Sigma Chemical Company, St. Louis, MO) was confirmed to be free of degradation products by gas–liquid chromatography and by thin-layer chromatography in which silica gel G plates loaded with 75 μg were developed in benzene–ethyl acetate 60:40 (v/v). Cholesterol was assayed by GLC (20)

Abbreviations: DSC, differential scanning calorimeter; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; C/P, cholesterol/phospholipid molar ratio; PC, phosphatidylcholine; DPPC, 1,2 dipalmitoyl-3-*sn*-phosphatidylcholine.

or by the Liebermann-Burchard procedure. The purity of synthetic 1,2 dipalmitoyl-3-*sn*-phosphatidylcholine (Calbiochem-Behring Corporation, La Jolla, CA) was verified by thin-layer chromatography using silica gel G plates developed in chloroform–methanol–water 95:35:4 (v/v). DPPC was assayed by phosphorus content using the method of Sokoloff and Rothblat (21).

Preparation and analysis of dispersions

Appropriate amounts of cholesterol and DPPC were weighed into a 25-ml glass tube, and 4 mg of sodium azide (0.02% w/v) and 0.26 mg of α-tocopherol (0.1% w/w lipid) were also added to prevent bacterial growth and autoxidation of the cholesterol. One hundred and sixty mg of cholesterol plus 80 mg of DPPC were used to obtain the most highly cholesterol-enriched codispersions; in order to obtain less enriched codispersions, 47 mg of cholesterol and 60 mg of DPPC were weighed out. The lipids in the tube were mixed by dissolution in a small volume of redistilled chloroform which was then removed by evaporation at 50°C under a stream of nitrogen. Twenty ml of distilled water (pH 7), which was further purified by passage through columns of activated carbon and ion exchange resin and deoxygenated (by bubbling nitrogen through the water while it was brought to the boil and then cooled), was added and the mixture was then sonicated for 40 min using the microtip of a Branson S-125 Sonifier at a power setting of 7. Throughout sonication the sample was held under a stream of nitrogen in a water bath at 50°C. After sonication the codispersion was spun at 20,000 rpm in a type 40 aluminum rotor (27,000 *g*) for 30 min at 4°C to remove fragments of titanium and any poorly dispersed lipid. After centrifugation the supernatant was filtered through a 0.45-μm Millipore filter and the pH of the filtrate was readjusted to 7.0 with 0.1 N NaOH. Aliquots of the sample were taken for electron microscopic examination and chemical analysis. The lipids were extracted by the method of Bligh and Dyer (22) using chloroform–methanol–water 2:2:1.8 (v/v). The codispersion was then sealed under nitrogen and stored in the dark at either 20 or 4°C.

Several days after their initial preparation, the stored codispersions were reexamined by electron microscopy. The precipitate which had formed was collected by spinning the sample at 15,000 rpm in a type 40 aluminum rotor (15,000 *g*) for 30 min at 4°C and filtering the supernatant through a 0.45-μm Millipore filter. The filtrate was readjusted to pH 7.0 with 0.1 N NaOH and, after removing aliquots for electron microscopy and chemical analysis, it was sealed under nitrogen and stored in the dark at 20°C. After various further intervals of storage, less material came out of solution and this was removed by filtration alone; both the precipitate and filtrate were analyzed for cholesterol and DPPC content. Furthermore, the physical states of both were monitored by electron microscopy and the precipitate was subjected to analysis by differential scanning calorimetry.

The particles present in the codispersions or the precipitates were visualized in a Zeiss 10 transmission electron microscope operating at 80 K volts after negative staining with 2% w/v sodium phosphotungstate (pH 7.0) on formvar-coated 200 mesh copper grids. The grids were electrostatically charged in a Denton DV-502 vacuum evaporator prior to applying the sample (0.6 μl of a solution containing 0.25 mg solid/ml) and the stain in order to facilitate spreading over the grid.

Differential scanning calorimetry

Appropriate amounts of cholesterol crystals with or without added DPPC were weighed, using a Cahn Gram Electrobalance, directly into 75-μl stainless steel sample pans for the Perkin-Elmer DSC-2. Fifty μl of redistilled chloroform was then pipetted into each pan and the pan was vibrated gently until all the lipid dissolved. The pans were placed in a vacuum oven at 42°C overnight to attempt to remove all of the solvent. DSC studies of the gel to liquid crystal transition of pure DPPC dispersions showed that as long as the pans were held in the vacuum oven for 1.5 hr no effects of residual chloroform could be detected. Fifty μl of distilled, deionized water was added, the pans were hermetically sealed and then placed in an oven at 50°C for 4 hr. Twice during this incubation the pans were vortexed for 30 sec. The pans were then loaded into the differential scanning calorimeter and heated at 10°C/min from 20 to 185°C against an identical reference pan containing distilled water. The calorimeter was calibrated with samples of indium, hydrated dipalmitoyl phosphatidylcholine, cyclohexane, and water. The heat absorbed in transitions of known amounts of hydrated crystals of cholesterol was measured and used to construct a standard curve, the slope of which gave the heat of transition. The amount of free, hydrated cholesterol present in a sample after incubation with DPPC was determined by measuring the heat absorbed during the fusion of hydrated cholesterol and using the above standard curve. The amount of cholesterol that had associated with the DPPC was then calculated by difference.

RESULTS

Stoichiometry and electron microscopy

In order to determine the maximal incorporation of cholesterol into dispersions with DPPC under our method of preparation, codispersions were prepared

starting with molar ratios of cholesterol to DPPC ranging from 2 to 8. The stoichiometry of the resultant dispersion is a function of this initial ratio and the most highly cholesterol-enriched codispersions have C/P = 4 ± 0.5 at a total solids concentration of about 10 mg/ml (cf. 3,4). Under identical conditions but using egg PC, the maximum enrichment is only half this value. Electron micrographs reveal no significant difference between the morphology of equimolar codispersions and cholesterol-enriched codispersions. In either case, the nonaggregated particles in the codispersion appear as spherical particles, ranging from 200 Å to 1800 Å in diameter (for an example, see **Fig. 1a**); normally more than 70% of the particles are between 300 Å and 600 Å in diameter. Examination of the particles individually at magnifications of 80,000× or greater reveals that they are liposome structures with a bilayer repeat distance of 50–70 Å. This is true for codispersions containing roughly equimolar amounts of cholesterol and DPPC, and for codispersions highly enriched in cholesterol (see Fig. 1b). X-ray diffraction measurements on a preparation with initial C/P = 4 gave a lamellar repeat distance of 66 Å at 20°C.[1]

Codispersions that are initially highly enriched in cholesterol throw a precipitate after sitting undisturbed for several days at either 4 or 20°C. In order to investigate this phenomenon, the precipitate was removed by filtration without damaging the integrity of the remaining particles of dispersed lipid. Sedimentation was not employed routinely because exposure of the codispersion to high centrifugal fields (10^5 *g*) induces the formation of spherical structures of about 100 Å in diameter from the multilamellar particles. The data in **Fig. 2** indicate that, as the precipitate is removed, the dispersions become depleted in cholesterol; this leads to a somewhat variable rate of decrease in C/P. Oxidation of the cholesterol is effectively prevented for up to 140 days with 0.1% of α-tocopherol and, under our conditions of preparation and storage, only a very slight degradation of PC (<5%) occurs during this span of time; omission of the preservatives does not affect the colloidal stability of the dispersions (Fig. 2). The temperature and enthalpy of the gel to liquid crystal transition of a 5 mol % cholesterol/DPPC codispersion with and without 0.1% α-tocopherol present were identical, indicating that this level of antioxidant also does not perturb the phase equilibrium significantly. Preparations that initially contain between 1 and 2 mol cholesterol/mol DPPC show no significant change in this ratio over 134 days in spite of repeated filtrations, whereas codispersions with an initial C/P = 3.5 decline to C/P = 1–2. The precipitate contains primarily cholesterol and, in the differential scanning calorimeter, it exhibits three endothermic transitions at 83°C, 123°C, and 157°C on heating from 20° to 185°C.

The precipitate formed from cholesterol-enriched codispersions was also investigated using electron microscopy. A dispersion aged for 4 days at 20°C (see Fig. 2) contains many aggregated particles and needle-shaped and rectangular crystals that are characteristic of cholesterol (Fig. 1c). At high magnification, multilamellar liposomes that appear to have needle-shaped crystals emerging from them can be seen (Fig. 1d). When the precipitate is removed and examined, it contains many needle-shaped and rectangular cholesterol crystals (Fig. 1e) while the codispersion again appears to consist entirely of spherical vesicles (Fig. 1f).

Differential scanning calorimetry

Pure hydrated cholesterol in a closed system exhibits three endothermic transitions on first heating between 20 and 185°C; these occur at 83°, 123°, and 157°C (cf. 23); the two high temperature peaks are in trace 1/0 of **Fig. 3.** On second and subsequent heating runs, the 83°C transition disappears and a small peak appears at 32°C (cf. 23) and furthermore, the 123°C transition is broadened and occurs about 5°C earlier; the 157°C transition is independent of the thermal history of the sample. The transition of anhydrous cholesterol crystals to smectic liquid crystal, which occurs at 123°C, has an enthalpy (ΔH) of 3.3 kcal/mol, while the transition of this mesophase to liquid cholesterol dispersed in water occurs at 157°C with a ΔH of fusion of 2.2 kcal/mol. These values are in good agreement with those of Loomis, Shipley, and Small (23) who reported ΔH = 3.42 ± 0.20 and 2.29 ± 0.14 kcal/mol, respectively.

Pure DPPC dispersed in excess water shows no phase transition between 120 and 180°C (Fig. 3, trace 0/1). Similarly, when equimolar amounts of cholesterol and DPPC are codispersed, a decrease in heat capacity occurs at about 145°C but there is no phase transition between 150 and 170°C (Fig. 3, trace 1/1). However, samples with molar ratios of cholesterol to DPPC progressively greater than 1:1 do exhibit a phase transition due to the melting of excess liquid-crystalline cholesterol that is not associated with the DPPC (Fig. 3, traces 1.5/1–2.75/1). When C/P = 1.5–2.1, the peak of the DSC transition occurs at 160°C while when C/P = 2.1–3.3, this temperature is reduced 5°C. The reasons for this are not clear but, since the change in transition temperature is not proportional to C/P, partition of DPPC into the free cholesterol phase cannot be the sole cause. In the presence of DPPC the 123°C transition is broadened and decreased in magnitude on a first heating run (see Fig. 3, trace 1.75/1) and there is a broad peak at 88°C. On second and subsequent scans, the 123°C peak is no longer visible (see Fig. 3, traces 2.5/1 and 2.75/1), and the

[1] Atkinson, D. Personal communication.

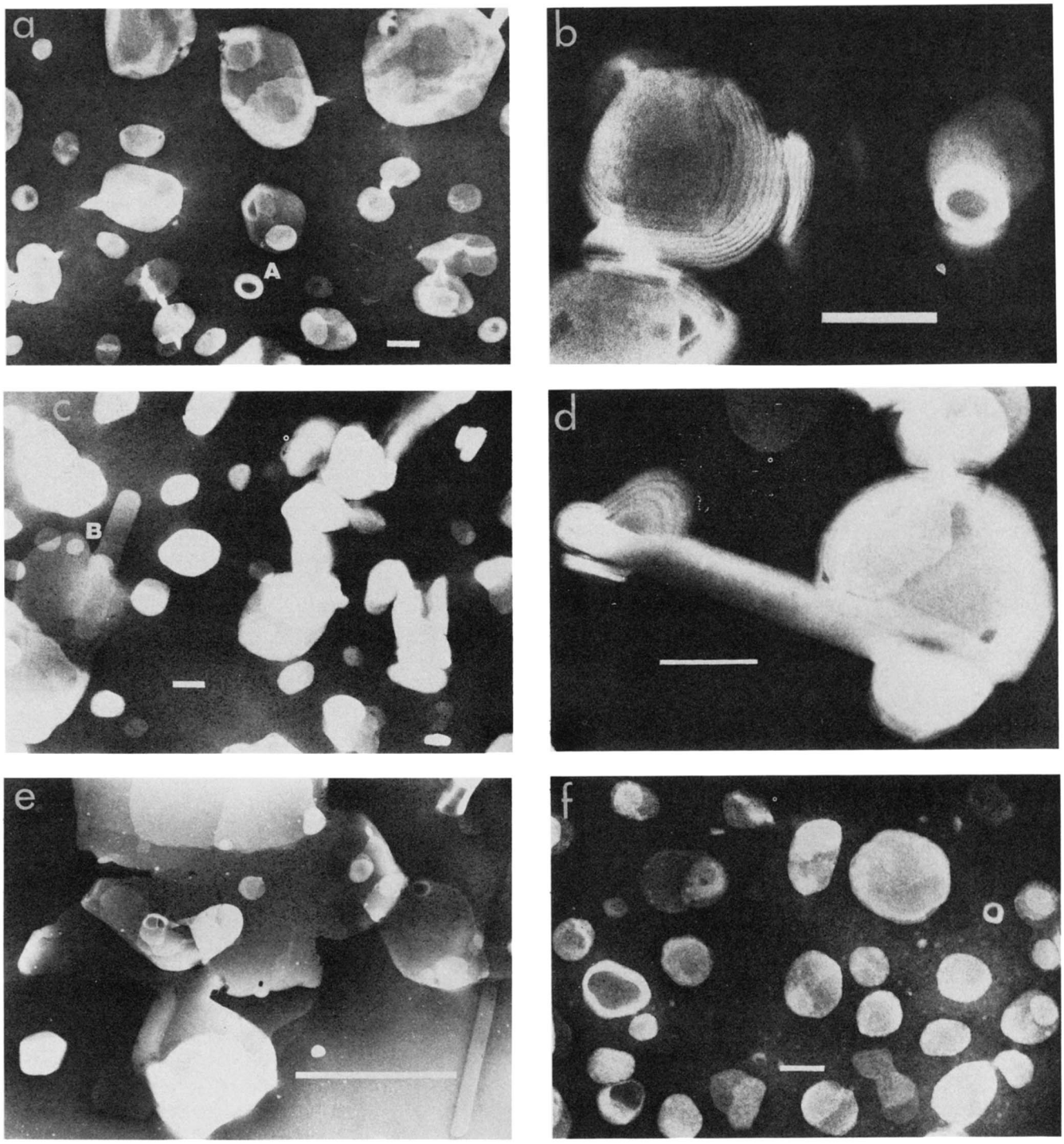

Fig. 1. Electron micrographs of cholesterol/dipalmitoyl phosphatidylcholine mixtures dispersed in water at an initial solids content of about 10 mg/ml. a), Fresh preparation at an initial cholesterol/dipalmitoyl phosphatidylcholine molar ratio of 3.75/1. Heterogeneous spherical particles and some stain-filled vesicles (A) are evident (bar = 10^3 Å). b), Particle with multilamellar repeat distance of 50–70 Å (bar = 10^3 Å). c), Dispersion aged for 4 days showing particle aggregation and a cholesterol crystal (B) (bar = 10^3 Å). d), Needle-like cholesterol crystal apparently growing from multilamellar liposomes (bar = 10^3 Å). e), Precipitate removed by passage through a 0.45-μm filter after 4 days storage at 20°C—large aggregates and cholesterol monohydrate crystls are visible (bar = 10^4 Å). f), Filtrate from step (e) showing presence of spherical unilamellar vesicles (bar = 10^3 Å).

88°C peak becomes extremely broad (extending from 40 to 100°C) unless the sample is held at −18°C for several days. Presumably, these changes occur because nucleation of cholesterol crystallization on cooling the mesophase is inhibited by the presence of DPPC. Since the 157°C transition is invariant with the number of scans,

relatively insensitive to the presence of DPPC, and large enough to quantitate accurately in samples containing more than 1.8 mol of cholesterol/mol of DPPC, this peak was used to determine the amount of free cholesterol in the mixtures with DPPC. As shown in **Fig. 4,** over a broad range of compositions the mixed bilayers that form have a molar ratio of cholesterol to DPPC of 1.0 ± 0.1 (±1 S.D.). Only when cholesterol is present in large excess (i.e., greater than about 3 mol of cholesterol per mol of DPPC) does the ratio of spontaneous association between cholesterol and DPPC begin to exceed 1:1.

Several of the pans containing cholesterol and DPPC were stored at low temperatures for various lengths of time, then re-scanned in the DSC. As indicated in **Fig. 5,** samples in which the molar ratios of association of cholesterol with DPPC were initially 0.9:1 and 1.6:1 did not change significantly during storage at −18°C for periods of up to 90 days. Inspection of the samples by GLC and TLC after such storage indicated that no detectable chemical decomposition had occurred. Similarly, a sample with an initial ratio of 1.2:1 remained unchanged when stored at 4°C for 10 days. In agreement with the data in Fig. 2, where the relatively dilute dispersions had concentrations in the range 1–10 mg/ml, a sample with an initial molar ratio of association between cholesterol and DPPC of 3:1 and 300 mg total solids/ml also exhibited considerable metastability so that after 11 days storage at 4°C the ratio decreased to 1.7:1.

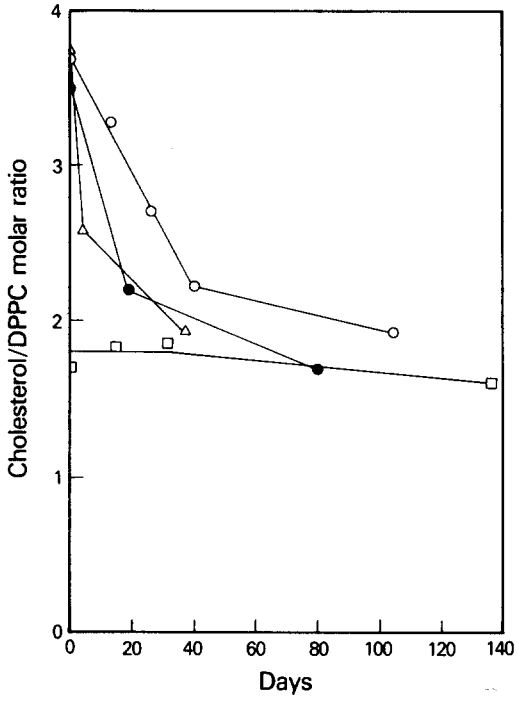

Fig. 2. Change in cholesterol/dipalmitoyl phosphatidylcholine (DPPC) molar ratio for dispersions stored at 20°C. The initial solids content of about 10 mg/ml decreased to 2 mg/ml after two filtrations and to about 1 mg/ml at the end of the experiment. The symbols (○,△,□) represent three separate dispersions containing sodium azide and α-tocopherol to prevent chemical decomposition, whereas preparation (●) did not contain additives. The cholesterol/DPPC molar ratios are accurate to ±0.1.

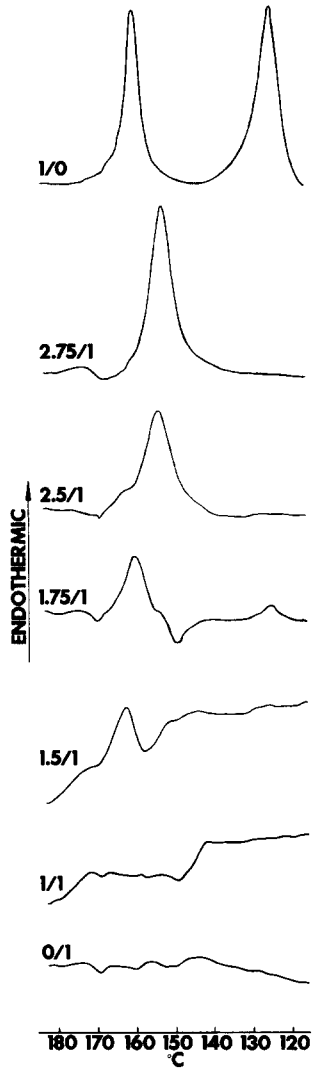

Fig. 3. Differential scanning calorimeter heating scans at high temperatures for cholesterol/dipalmitoyl phosphatidylcholine (DPPC) dispersions in excess water. Trace 1.75/1 is a first heating scan and the remainder are second heating scans. 15 mg of total solids was dispersed in 50 μl of distilled water and heated at 10°C/min. The cholesterol/DPPC molar ratios are shown against each trace.

DISCUSSION

Initial stoichiometry

Electron micrographs and X-ray diffraction measurements of cholesterol-enriched liposomes at 20°C show 50–70 Å and 66Å repeat distances, respectively, which are similar to the 63 Å bilayer repeat spacing of pure gel-phase DPPC in excess water at 20°C (24) and to the equivalent figure of 64 Å for equimolar DPPC/cho-

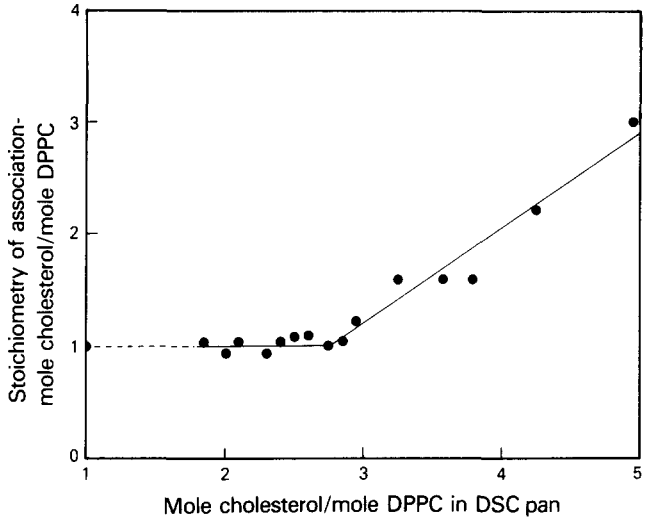

Fig. 4. Dependence of the stoichiometry of molecular association on the relative amounts of cholesterol and dipalmitoyl phosphatidylcholine (DPPC) originally present and mixed gently in the differential scanning calorimeter pan. The dotted line represents the region where the endotherm due to the fusion of free cholesterol monohydrate is too small to be quantitated accurately. The cholesterol/DPPC molar ratios are accurate to ±0.1.

lesterol mixtures at 25°C (11). This indicates that the lamellar phase prevails from pure PC up to C/P ratios of 4/1, and that the periodicity of these hydrated systems would not be expected to alter as C/P changes in the range 1–3; this is in contrast to anhydrous systems where a decrease in lamellar repeat distance was observed on ageing (15). As with pure PC, sonication induces fragmentation and formation of small unilamellar vesicles with diameters in the range 300–600 Å for our DPPC cholesterol preparation, which is similar to the size of equimolar egg PC/cholesterol vesicles as prepared by Forge, Knowles, and Marsh (25). Their overall morphology is sperical except that spurs protruding from some particles are evident (Fig. 1a); these are analogous to the protrusions that appear on the cholesterol-enriched red blood cells occurring in spur cell anemia, suggesting that the defect occurs in cholesterol-enriched lipid bilayer regions of the membrane (cf. 3,5).

Under our conditions of preparation, besides being a function of the C/P ratio in the initial mixture, the stoichiometry of the fresh codispersions is a function of the PC hydrocarbon chain composition. Thus, the disaturated DPPC system can incorporate more cholesterol than egg PC which has as its main component palmitoyloleoyl PC (cf. 4). In addition, McCabe and Green (16) have established that varying the phospholipid polar group also affects the degree of incorporation of cholesterol, with only choline-containing phospholipids being able to incorporate up to 2/1 mol cholesterol/mol phospholipid. Other investigations have suggested that lipid concentration and temperature are also critical, with dilute suspensions and high temperature favoring enhanced C/P ratios (4). We find that at 300 mg total solids/ml a mixture of 4/1 mol/mol cholesterol/DPPC gives an initial C/P = 2/1 even with gentle mixing, so that the early reports (13, 14) of less than 6 mg/ml being limiting for cholesterol enrichment are not valid. The stoichiometry of the final hydrated cholesterol/PC mixed bilayers must be limited by the maximum C/P ratio of the dry cholesterol/PC mixed bilayers originally deposited from organic solvent. Changing the organic solvent can alter the stoichiometry (15) presumably because of differences in the relative solubilities of PC and cholesterol leading to differential rates of deposition. In support of this, anhydrous cholesterol/egg PC bilayers have a maximum stoichiometry of 2/1 (8) which corresponds to the maximum stoichiometry of the hydrated cholesterol/egg PC bilayer. At temperatures below 100°C, anhydrous mixtures containing more than 2 mol cholesterol/mol DPPC consist of 2/1 cholesterol/DPPC complex coexisting with crystalline cholesterol when the system is at equilibrium (9). Thus, the higher C/P ratios of up to 4/1 achieved with DPPC compared to egg PC are not due to a higher equilibrium solubility of cholesterol in anhydrous bilayers of the former lipid. Kinetic effects must permit the greater retention of cholesterol by DPPC during the mixing, hydration, and sonication procedures. Consistent with this concept, studies in this laboratory with unilamellar vesicles of either DPPC or egg PC and cholesterol have shown that cholesterol molecules desorb into the aqueous phase (26) more rapidly from the unsaturated egg PC bilayers than from the saturated DPPC bilayers,[2] which may contribute to the lower initial C/P ratios observed with egg PC.

Stability

Regardless of total lipid concentration in the range 10–300 mg/ml, the C/P ratio of codispersions decreases with time (Fig. 2, 5) indicating that cholesterol crystallizes from both unilamellar vesicles and multilamellar liposomes. Dispersions with C/P = 2–4/1 are metastable and the C/P ratio decreases relatively rapidly into the

[2] McLean, L. R., and M. C. Phillips. Unpublished work.

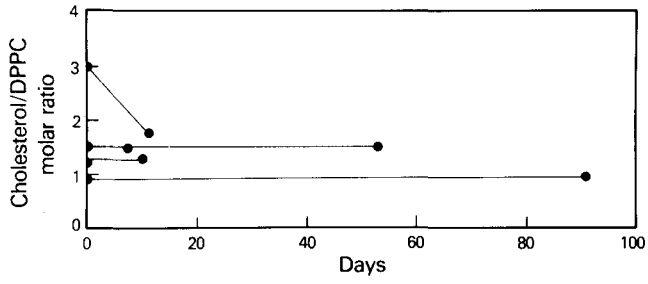

Fig. 5. Change in stoichiometry of association on storage at low temperature of the cholesterol/dipalmitoyl phosphatidylcholine codispersions described in Fig. 4. See text for further details.

range of 1–2/1; preparations with initial C/P = 1–2/1 do not exhibit detectable changes in the initial stoichiometry when stored for 3–4 months at either 4 or 20°C, although some precipitation occurs. Any decrease in C/P ratio is associated with the occurrence of bilayers containing less cholesterol and formation of a pure cholesterol phase. The needle-like and rectangular structures that are apparent in the electron micrographs are characteristic of cholesterol crystals. Furthermore, studies of the X-ray diffraction spacings and intensity distribution (cf. Fig. 9 in ref. 27) of the precipitates confirm the presence of cholesterol monohydrate crystals;[3] the thermotropic behavior of the precipitate is also consistent with the occurrence of cholesterol monohydrate crystals. This precipitation of cholesterol monohydrate is accompanied by some general aggregation and precipitation of vesicles or liposomes so that the overall solids content of the filtered dispersion progressively decreases. This fact, coupled with the slow changes in composition, only allowed about four time points to be accumulated over 3 months so that detailed analyses of the kinetics of cholesterol release are not possible. Presumably, the rate of growth of cholesterol monohydrate crystals is lower at low cholesterol concentrations, which is the condition when the dispersion is dilute and when C/P is not much greater than one, so that under these conditions the overall stability of the preparation is enhanced.

In summary, it is apparent that the cholesterol-enriched codispersions studied here are metastable with both their formation and stability being under kinetic control. The precise method of preparation influences the initial C/P ratio and lipid concentration achieved. PC dispersions containing more than equimolar amounts of cholesterol can be stable for at least 4 months.

Equilibrium stoichiometry

Although phase diagrams for fully hydrated egg PC (10, 12) and DPPC (11) studied under equilibrium conditions show equimolar cholesterol as the maximum incorporation for both systems, the ability to make cholesterol-enriched dispersions throws doubt on this figure as the true equilibrium cholesterol-saturation value for PC bilayers. Since our storage tests indicate that the enriched dispersions are metastable, it becomes crucial to know the C/P value to which the systems relax. Unfortunately, it is difficult to measure this parameter because of the very long times involved and the problem of separating vesicles and cholesterol microcrystals, especially when the C/P ratio is in the range 1–2/1.

In order to avoid this problem, we have attempted to prevent the overincorporation of cholesterol caused by vigorous mixing (sonication) and have mixed systems gently and allowed them to equilibrate before measuring the free cholesterol by DSC. This information on the free cholesterol concentration coupled with knowledge of the total cholesterol content of the system allows us to estimate by difference the stoichiometry of phospholipid-cholesterol association. Earlier DSC studies (11) have shown that, at less than equimolar amounts of cholesterol, DPPC/cholesterol bilayers undergo a gel to liquid crystal transition at 41°C; we have shown that in these systems no further transitions occur up to 180°C, indicating that there is no free cholesterol phase in these hydrated mixtures. The mesomorphic transitions due to hydrated cholesterol become detectable when there are more than equimolar amounts of cholesterol present and only become large enough to quantify at C/P $\geqslant$ 1.8/1. It is clear that the maximum solubility of cholesterol in DPPC bilayers is 1.0 $\pm$ 0.1 mol/mol PC when the initial C/P ratio is less than about 3, which is consistent with the published equilibrium phase diagrams. Also in agreement with this is the observation[4] that the gel to liquid crystal transition of DPPC is only eliminated when cholesterol is incorporated in the bilayer at an equimolar level. Equimolar cholesterol/PC bilayers are the preferred form of association until a more than 3-fold excess of cholesterol is present, at which point the data in Fig. 4 suggest that incorporation of somewhat higher levels of cholesterol can occur, even with relatively gentle mixing conditions. When PC dispersions are prepared at temperatures above the gel to liquid crystal transition, non-equilibrium dispersions are easily formed (9) so that these preparations with C/P = 3 are metastable.

In summary, under normal conditions and in the absence of large excesses of cholesterol, the hydrated DPPC bilayer becomes saturated with cholesterol at equimolar levels. Excess cholesterol is present as cholesterol monohydrate crystals.

Physiological significance

The cholesterol in enriched bilayer membranes is at high chemical potential so that it can transfer to other membrane systems and load them with cholesterol. For example, incubating liposomes with cholesterol/DPPC molar ratios of 3/1 with red blood cells in the presence of serum increases the C/P ratio in the cells from one to as high as 2.7 (4). Such cholesterol enrichment leads to spur cell appearance when produced either in vivo or in vitro. Although they are metastable, PC/cholesterol bilayers containing more than equimolar levels of free cholesterol can maintain C/P ratios in the range of about 2–1/1 for at least 4 months at 4 or 20°C. This suggests that enriched red blood cells in the absence of suitable cholesterol-acceptors would maintain the extra cholesterol load for periods comparable to their 120-day life-

[3] Shipley, G. G. Personal communication.

[4] Forte, M., and M. C. Phillips. Unpublished results.

time in the circulation of humans. This is consistent with the occurrence of red blood cells with C/P increased from the normal level of about 1 to 1.3 in cholesterol-fed dogs (1) or up to 1.6 in patients with liver disease (2), although in these situations the continuous oversupply of cholesterol to red blood cells from lipoproteins is probably the main cause of the enrichment.

Recent investigations by Katz and Small (28) of the composition of the lipid phases of human atherosclerotic plaques indicate that the phospholipid bilayer phase isolated from plaques has C/P ~ 1.4; this suggests that metastable cholesterol/phospholipid phases similar to those described here might exist in atherosclerotic plaques.

We are indebted to Dr. Joseph Leighton, Department of Pathology, The Medical College of Pennsylvania, for providing the electron microscope facilities and to Mr. James Diven for expert guidance when required. We also thank Dr. Donald Small, Boston University Medical Center, for helpful comments and Drs. David Atkinson and G. Graham Shipley for their generosity in making X-ray diffraction measurements for us. This work was supported by The National Heart, Lung, and Blood Institute Grant PPG HL-22633.

Manuscript received 26 January 1981, in revised form 14 August 1981, and in re-revised form 28 September 1981.

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