On the nature of dilute aqueous cholesterol suspensions

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Abstract Cholesterol is commonly supposed to form a micelle in aqueous solution. We have reassessed this finding using a variety of techniques. Centrifugation sedimented cholesterol at all concentrations above 10^{-8} M. Density gradient analysis of more concentrated solutions revealed two bands whose densities corresponded to crystalline anhydrous cholesterol and crystalline cholesterol monohydrate. The monohydrate was characterized by light microscopy and phase transition. This band is also claimed to contain micelles, but no enhancement of perylene or diphenylhexatriene fluorescence could be detected. Some crystals of monohydrate could pass through 0.7-μm filters but not through 1.5-nm filters. Crystals of monohydrate were detected on the latter filters when solutions with cholesterol concentrations as low as 2.6×10^{-8} M were filtered. Thus, under all conditions where micelles might be expected, we have detected micro-crystals of cholesterol monohydrate and we can find no independent evidence to support the existence of a cholesterol micelle.—**Renshaw, P. F., A. S. Janoff, and K. W. Miller.** On the nature of dilute aqueous cholesterol suspensions. *J. Lipid Res.* 1983. **24:** 47–51.

Supplementary key words anhydrous cholesterol • cholesterol monohydrate • micelles

The thermotropic behavior of the binary system excess cholesterol–water has recently been studied in great detail (1). Unfortunately, however, the concentration-dependent behavior of this system has never been unambiguously characterized. Early work employing filtration of aqueous cholesterol solutions suggested that crystalline cholesterol monohydrate began to appear at concentrations around $6–7 \times 10^{-8}$ M (2). On the other hand, based on rates of dialysis in water, later investigators deduced the occurrence of a cholesterol micelle with a critical micelle concentration of $2–4 \times 10^{-8}$ M (3). A recent study also claimed the existence of a cholesterol micelle with a CMC between $1.3–6.5 \times 10^{-8}$ M based on water–hexane partitioning (4).

Although the existence of a cholesterol micelle is widely accepted (for example, see a recent review by Coleman and Lavietes (5)), the exact nature of this aggregate remains in doubt. In this study, we have employed centrifugation, visible light microscopy, filtration, and spectrofluorimetric techniques to re-examine the nature of the phase in suspension in aqueous solution at total concentrations somewhat greater than 10^{-8} M.

EXPERIMENTAL PROCEDURES

Cholesterol solutions

Crystalline cholesterol (>98% pure) was purchased from Sigma Chemical Co., St. Louis, MO, and was recrystallized three times from methanol. The resulting crystals were used to prepare anhydrous cholesterol and cholesterol monohydrate by further recrystallization from methanol or 95% ethanol–water, respectively, after the method of Igimi and Carey (6). [1,2-^{3}H(N)]Cholesterol (40.7 Ci/mmol) in benzene was purchased from New England Nuclear, Boston, MA. The purity of this stock solution was assayed weekly using the thin-layer chromatography systems: hexane–ethyl ether–acetic acid 80:20:1.5 (v/v/v) and cyclohexane–ethyl acetate 3:2 (v/v) on Silica Gel paper (ITLC SG, Gelman Sciences, Inc., Ann Arbor, MI). Whenever the purity was determined to be less than 99%, purification was achieved by elution from TLC paper into toluene. All organic cholesterol solutions were stored in the dark under nitrogen at 4°C.

Aqueous cholesterol solutions were typically prepared by adding anhydrous cholesterol dissolved in toluene, together with an appropriate amount of the labeled compound, to acid-washed glassware. The solvent was evaporated with dry nitrogen and the crystalline material was redissolved in distilled water. Since extensive adsorption of cholesterol onto the surface of the glassware was always observed, the exact concentration of each solution was determined by counting a small aliquot in a Beckman LS 8100 scintillation counter. The aliquots were collected with glass micropipettes and added (with extensive washing to remove adsorbed cho-

Abbreviations: CMC, critical micelle concentration; TLC, thin-layer chromatography.

lesterol) to 6 ml of scintillation fluid (Liquiscint, National Diagnostics, Somerville, NJ). All samples were counted to 2% standard deviation with an average counting efficiency of 26% after correcting for background.

Density gradient centrifugation

Linear sucrose density gradients (from 2.0 to 20% sucrose) were prepared in 13.2-ml nitrocellulose tubes. Either they had unlabeled crystalline anhydrous cholesterol or crystalline cholesterol monohydrate layered on top, or they had fixed concentrations of labeled anhydrous cholesterol uniformly distributed throughout. Before making the gradients, these sucrose solutions were purged with nitrogen for 30 min to minimize the effect of auto-oxidation (7, 8). Gradients containing uniformly distributed labeled cholesterol were centrifuged in an SW 41 rotor at 201,000 *g* at 20°C for 72 hr. Gradients onto which large amounts of crystalline cholesterol had been layered were centrifuged for 8 hr. After centrifugation, the cholesterol was assayed for purity by TLC; in every case, only small amounts (<2%) of oxidation products were detected. Gradient fractions of approximately 0.2 ml were collected from the bottom of the gradient with a peristaltic pump and were assayed for cholesterol concentration by counting 0.1-ml aliquots in a liquid scintillation counter. Fractions were observed by polarizing light microscopy (200×) in direct light and between crossed Nicol prisms. The phase behavior of these cholesterol solutions was also studied between 0° and 95°C at 40× magnification.

Aqueous solution centrifugation

In order to determine the aqueous cholesterol concentration in equilibrium with cholesterol aggregates, the problem of adsorption had first to be overcome. Most plastic centrifuge tubes were found to adsorb a high proportion of the cholesterol in solution with a long time course. In stainless steel tubes, however, after an initial phase of 3 hr during which 15% of the cholesterol was adsorbed, little further adsorption was detectable. By working in equilibrium with this adsorption, reproducible results were obtained.

Cholesterol solutions were stirred under nitrogen for at least 24 hr and up to 2 weeks. Equilibration was carried out in two ways. First, suspensions were equilibrated in 10.0-ml stainless steel centrifuge tubes and then centrifuged in situ. Second, suspensions were equilibrated in acid-washed glassware before being transferred to 0.25-ml stainless steel centrifuge tubes for the final 24 hr of equilibration. These equilibrated solutions of cholesterol were centrifuged in the stainless steel tubes for 1 hr at 105,000 *g* in either a Sorval OTD Ultracentrifuge or a Beckman Airfuge. Sampling was done immediately before and after centrifugation to account for losses due to adsorption. The tubes were washed with chloroform–methanol 2:1 between runs to remove adsorbed cholesterol. Large stainless steel centrifuge tubes were from Beckman; those for the airfuge were especially manufactured by Research Design, Winchester, MA.

Spectrofluorimetry

Spectrofluorimetry was performed on a Perkin-Elmer 203 spectrometer with an excitation wavelength of 250 nm and a detection wavelength of 450 nm. The cholesterol and phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) solutions used in fluorescence studies were sonicated for 10 min and then stirred with glass beads onto which perylene (Sigma Chemical Co.) had previously been adsorbed from a chloroform solution, after the method of Mast and Haynes (9). The final concentration of perylene was on the order of 1 ppm (w/v) in each case. When 1,6-diphenylhexatriene (Aldrich Chemical Co., Milwaukee, WI) was used, it was dried onto acid-washed glassware from chloroform under a stream of N_2 and vortexed vigorously with aqueous cholesterol solutions.

Aqueous solution filtration

Solutions were filtered either through Whatman GFF glass fiber filters on a Millipore manifold or through Amicon PM 10 filters in an Amicon ultrafiltration apparatus.

RESULTS AND DISCUSSION

Complete dissolution of cholesterol solutions, as monitored by simple centrifugation, was observed at concentrations below $1 \pm 2.0 \times 10^{-8}$ M after 5 to 14 days of stirring (**Fig. 1**). This solubility limit is consistent with previous determinations of $2–4 \times 10^{-8}$ M (CMC, ref. 3), $1.3–6.5 \times 10^{-8}$ M (CMC, ref. 4) and 6.7×10^{-8} M (formation of cholesterol monohydrate, ref. 2). This latter value may be somewhat high as no check for cholesterol oxidation was reported by the authors of this study. At concentrations greater than 10^{-8} M, centrifugation consistently pelleted cholesterol even after 2 weeks of stirring. Equilibrium was approached slowly. After 1 hr of stirring, cholesterol was pelleted even from solutions as dilute as 4×10^{-11} M, after 1 day from 4×10^{-10} M, and after 3 days from about 10^{-8} M.

In order to ascertain the nature of the material being pelleted, sucrose density gradients of aqueous cholesterol solutions were run. At average concentrations between 10^{-8} M and 10^{-6} M, three major cholesterol-containing bands were detected (**Fig. 2**). All gradients con-

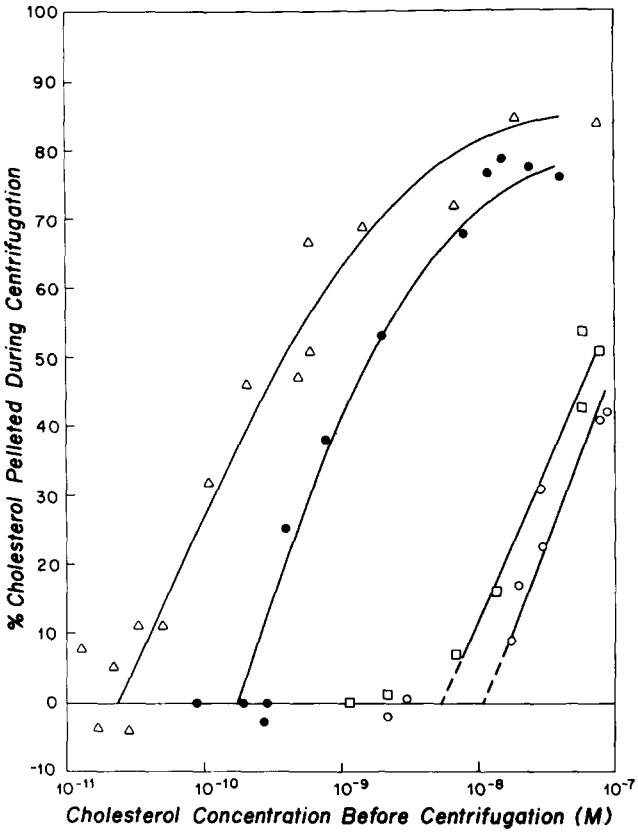

Fig. 1. Determination of cholesterol solubility as a function of time by centrifugation of aqueous [^{3}H]cholesterol solutions at 105,000 *g* for 1 hr at 20°C after equilibration in stainless steel tubes (see text). The solutions were stirred for various times and were sampled immediately before and after centrifugation with glass micropipettes (△, 1 hr of stirring; ●, 1 day of stirring; □, 3 days of stirring; ○, 5 or more days of stirring). The final equilibrium was not reached until after 5 days of stirring and the equilibrium supernatant concentration was then 1.0 ± 2.0 × 10^{-8} M, as determined by extrapolating the least-squares line drawn through these points. Similar results were obtained in 10 mM HEPES, pH 7.4 (not shown).

tained a distinct band at the sucrose–air interface, including those with concentrations less than 10^{-8} M. Calculations show that the amount of cholesterol found in the interfacial fractions is of the same order of magnitude as that expected from previous studies of cholesterol monolayers at the air–wave interface (10–12). Thus, it seems unlikely that these elevated cholesterol levels in the last fractions resulted from a collection artifact.

At concentrations higher than 10^{-8} M, gradients also contained a second band at 1.021–1.025 g/ml and a third band peaking in the range 1.054–1.061 g/ml (peak range represents band width at half peak height). The latter band, which became more prominent as the cholesterol concentration increased, had a density close to that (1.050–1.057 g/ml) found when a slurry of cholesterol monohydrate was layered on a similar gradient. Analogously, centrifugation of a freshly prepared slurry of anhydrous cholesterol for 8 hr yielded a primary band at 1.020–1.026 g/ml together with some monohydrate at 1.050–1.057 g/ml. These densities correspond to those reported in the literature of 1.02–1.03 g/ml for anhydrous cholesterol (13), 1.04–1.05 g/ml for the monohydrate (14, 15), and 1.050–1.057 g/ml for cholesterol micelles (3, 16). Previous investigators (1) have noted the complete conversion of anhydrous cholesterol to cholesterol monohydrate in stirred

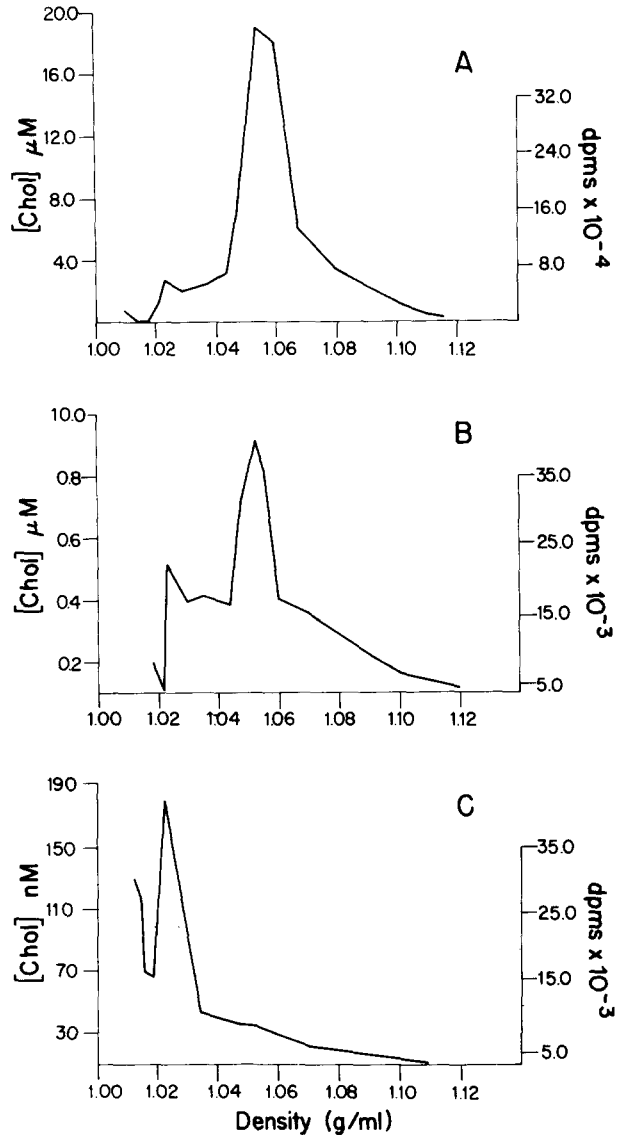

Fig. 2. Sucrose density gradient centrifugation of aqueous [^{3}H]cholesterol solutions. Gradients uniformly layered with cholesterol were spun at 201,000 *g* for 72 hr at 20°C. Sampling was done from the bottom of the gradient with an automated fraction collector. A: final total average concentration = 1.99 × 10^{-6} M, specific activity = 9.9 × 10^{-3} Ci/mmol; B: final total average concentration = 3.5 × 10^{-7} M, specific activity = 5.33 Ci/mmol; C: final total average concentration = 3.3 × 10^{-8} M, specific activity = 9.9 × 10^{-1} Ci/mmol. Similarly prepared gradients with fresh slurries of cholesterol monohydrate and anhydrous cholesterol yielded primary bands at 1.050–1.057 g/ml and 1.020–1.026 g/ml, respectively.

aqueous solution at room temperature. On the gradient, this conversion was very slow and small but significant amounts of anhydrous cholesterol could readily be detected even after 72 hr of non-stirred equilibration.

In order to determine whether micelles might be present in the third fraction, we turned to the use of the fluorescent probe perylene, which has been shown to detect micelle formation accurately in aqueous solution due to its dramatically increased quantum yield upon incorporation into environments of low polarity (9). In toluene, an increase in relative intensity could be detected at probe concentrations as low as 10^{-10} M. Incorporation of perylene (1 ppm) into phosphatidylcholine vesicles gave a signal to noise ratio of 2 at 10^{-9} M lipid. Nonetheless, we were unable to detect any perylene incorporation at concentrations of cholesterol from 5×10^{-10} to 5×10^{-5} M, which is up to three orders of magnitude greater than the reported critical micelle concentration of cholesterol. Similar results were obtained with the fluorescent probe diphenylhexatriene at 10^{-8} M in 2.5×10^{-6} M cholesterol solutions.

Having failed to demonstrate the presence of micelles at any concentration, we returned to the question of what separated cholesterol phase is present above 10^{-8} M. One liter of 1.4×10^{-7} M cholesterol was stirred in a brown bottle under nitrogen for 3 weeks and filtered rapidly on a GFF glass fiber filter (98% retention of 0.7 μm particles). Many crystals were apparent by light microscopy. They had the appearance and phase transition (88–90°C) characteristic of cholesterol monohydrate (1, 6). However, the filtrate concentration was 1.1×10^{-7} M (i.e., above the saturation limit of 10^{-8} M), consistent with the claimed 100-nm size of the putative cholesterol micelle (3). In another typical experiment, 1 liter of 5×10^{-7} M and 3.5 liters of 2.7×10^{-8} M and of 3.1×10^{-9} M solutions were equilibrated for at least 10 days and then filtered on GFF filters. Monohydrate crystals were detected and characterized only on the first two filters. Filtrate concentrations were 3.8×10^{-7}, 2.6×10^{-8}, and 3.1×10^{-9} M, respectively. Further filtration on Amicon PM 10 filters (molecular weight cutoff of 10,000, approximate pore size of 1.5 nm) revealed crystals of monohydrate at the two highest concentrations, but not at the lowest. Adsorption on the PM 10 filters was serious and the concentration of the filtrate could not, therefore, be determined. Nonetheless, this experiment demonstrates the presence of crystalline cholesterol monohydrate in a solution whose total concentration is close to the saturation limit of 10^{-8} M.

Thus, both filtration and density gradient experiments reveal microcrystals of cholesterol monohydrate to be present under all conditions for which the cholesterol micelle has been proposed to exist. Under similar conditions, no fluorescence enhancement, such as might be expected in the presence of fluid micellar structures, could be detected by perylene or diphenylhexatriene, thus confirming previous work with the probe hemin (3). If some separate cholesterol phase does exist in equilibrium with monomer and monohydrate, the most likely candidate would be a gel phase bilayer analog of the liquid crystalline phase detected at much higher temperatures by Loomis, Shipley, and Small (1). It seems unlikely, however, that such a phase would not aggregate further to form the monohydrate.

In conclusion, we find the sedimentable phase present in aqueous cholesterol solutions above 10^{-8} M to be crystals of cholesterol monohydrate. Although it would be difficult to entirely rule out the presence of some micellar material, it is clearly more economical to regard 10^{-8} M as a simple solubility limit, rather than as a critical concentration for hypothetical cholesterol micelle formation. These observations should place the interpretation of experiments both on cholesterol exchange between membranes (17, 18) and on cholesterol solubilization (19) on a firmer foundation.

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