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Self-association of Cholesterol in Aqueous Solution

(micelles/critical micelle concentration)

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ABSTRACT **Cholesterol has a maximum solubility in aqueous solutions of 1.8 μg/ml or 4.7 μM. It undergoes a thermodynamically reversible self-association with a critical micelle concentration of 25 to 40 nM at 25°. The cholesterol micelle is heterogeneous in size, probably rod-like in shape, and stabilized by an unusually high interaction energy between the aggregated monomers.**

A critical problem in biochemistry today is the elucidation of the molecular organization of lipid and protein components in biological membranes and serum lipoproteins. Not only is a definition of the structure itself essential, but also an explanation in thermodynamic terms for its existence, i.e., free energies of association among the various components. However, any such investigation requires prior knowledge of the physical properties of the components themselves in aqueous solvents. Lipids, for example, are amphiphiles that undergo monomer–aggregate equilibria. Just as it was essential to define these equilibria in thermodynamic terms in order to interpret binding of simple detergents to serum albumin (1, 2), so the same information is required to determine the modes of association of biological lipids with membrane proteins and serum lipoproteins.

Numerous structural investigations of pure phospholipid bilayers and of the incorporation of cholesterol into these bilayers have been published (3–5). However, only a single phospholipid system, dipalmitoyl phosphatidylcholine, has been studied from the standpoint of monomer–micelle equilibrium (6). Cholesterol–water solutions have been completely neglected despite the ubiquitous nature of this neutral lipid in eukaryotes.

We report here the critical micelle concentration of cholesterol in water and the formation of a heterogeneous self-aggregate in which the forces of interaction are stronger than those usually found in micellar systems.

EXPERIMENTAL PROCEDURE

Crystalline cholesterol (99+% pure) was obtained from Applied Science Laboratories and stored dessicated under reduced pressure at 10°. [1α,2α-^{3}H]Cholesterol, 30 Ci/mol, was purchased from Amersham Searle Corp. and stored in benzene as a 1.1 μg/ml solution at 10°. [4-^{14}C]Cholesterol, 50 Ci/mol and 99% radiochemically pure, was obtained from Applied Science.

Chemical and radiochemical purities of the cholesterol were examined once a month. The unlabeled compound (20–150 μg) gave a single spot when chromatographed by single elution on a silica-gel thin-layer plate (Supelco, Inc.) with benzene–ethyl acetate 90:10 and by double elution on a thin-layer plate impregnated with 5% silver nitrate, with chloroform–acetone 98:2 as the ascending solvent. The tritiated compound (0.1–0.2 μCi) gave a single peak that chromatographed at the same R_F value as the unlabeled compound and contained >98% of the radioactivity. The radiochemical purity was reported to be >98% by the supplier, using additional thin-layer chromatography systems: cyclohexane–ethyl acetate 6:4 on silica gel, 90% acetic acid–paraffin 100:1 on silica gel, and benzene–diethyl ether 7:3 on alumina. Aqueous solutions of cholesterol were likewise examined at the conclusion of several experiments and were 99% chemically and radiochemically pure. No oxidation products of cholesterol were present. Identical behavior of solutions from various carrier dilutions of cholesterol was observed throughout the studies. [4-^{14}C]Cholesterol, 50 Ci/mol, was judged to be 99% radiochemically pure by the above criteria.

We prepared aqueous solutions of cholesterol by adding the compound dissolved in benzene to a glass volumetric flask, thoroughly evaporating the solvent with a nitrogen stream, and dissolving the crystalline material in an appropriate volume of water. Extensive adsorption of cholesterol to the vessel walls was always observed and addition of excess cholesterol was required to obtain a given concentration.

Benzene and methanol were spectrophotometric grade (Fisher Scientific Co.). Glass-distilled water was used in all experiments. Glassware was exhaustively washed with benzene, methanol, and water and never subjected to detergent cleaning.

Radioactive samples were counted in an LS-100C Scintillation counter (Beckman Instruments). Scintillation fluid containing 2 liters of toluene, 1 liter of Triton X-100, 8.0 g of 2,5-diphenyloxazole, and 0.4 g of 1,4-bis-2-(5 phenyl-oxazolyl)-benzene was used. The aqueous sample was added to 15 ml of scintillation fluid; the mixture was equilibrated for 4 hr in the dark and counted to 1.5% error. The efficiencies of counting over a range of 0–0.1 ml of water were determined by standardization with [^{3}H]toluene of 3.12×10^6 dpm/ml. A sample of 50–100 μl had a counting efficiency of 40%. All counts were corrected for background, efficiency, and quenching before conversion to concentration units.

Critical micelle concentrations were determined by rates of dialysis by use of 10 ml of water in a Visking dialysis bag (Union Carbide Corp.) suspended in a glass vessel containing 15 or 25 ml of cholesterol solution. The apparatus was designed so that a closed system existed except at the time of sampling. The solution was mixed by rotation at 25°. The dialysis tubing was brought to a boil in water, rinsed extensively, and used within 1 day.

Lengths of micellar aggregates were estimated by use of

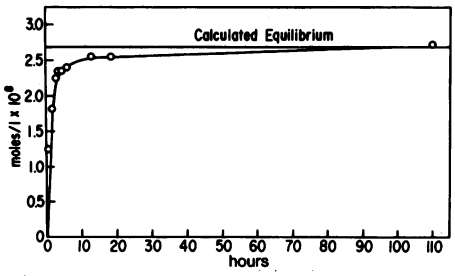

FIG. 1. Dialysis of cholesterol (specific activity = 1.43×10^6 dpm/μg) from a solution of initial concentration 1.5-times the critical micelle concentration into water across a membrane with 24-Å diameter pores. The calculated equilibrium concentration corresponds to an equivalent cholesterol concentration on both sides of the membrane.

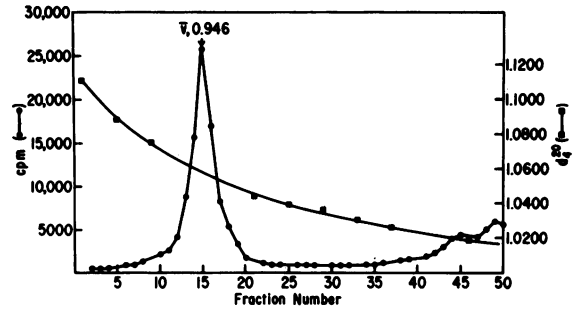

FIG. 3. Equilibrium distribution of cholesterol micelles in a sucrose density gradient centrifuged for 72 hr at 48,000 rpm and 20° in a SW 50.1 rotor of an L2-65 B preparative ultracentrifuge.

membranes of calibrated pore sizes. A lucite equilibrium dialysis cell (Bel Arts Products) was assembled with the appropriate membrane and filled with 1 ml of cholesterol solution on one side and 1 ml of water on the other. The solutions were mixed by rotation at 25°. Nitrocellulose Bac-T-Flex membranes from Schliecher and Schuell of mean pore diameters 100, 200, 450, and 800 nm and regenerated cellulose membrane from Brinkman Instruments of mean pore diameter 20–35 nm were soaked at least 24 hr in water before use.

Sucrose density gradients were prepared according to Martin and Ames (7) either in water or in aqueous solutions of cholesterol and centrifuged in an L2-65B preparative ultracentrifuge (Beckman Instruments) in an SW 50.1 rotor. Fractions from the gradients were collected by gravity directly into scintillation vials. Refractive indices of selected fractions were obtained with a Bausch and Lomb refractometer, and the densities of the sucrose solutions were calculated therefrom. Molecular weights of the cholesterol micelle were determined according to Meselson *et al.* (8).

The partial specific volume of cholesterol in benzene was calculated from density measurements determined with an Anton Paar Precision Density Meter.

RESULTS

Table 1 shows the maximum solubility of cholesterol in water determined over a wide concentration range of added crystalline material. The fluctuations observed in the absolute solubility are the result of technical difficulties in sampling a saturated cholesterol solution:

(*i*) The sampling pipette is easily contaminated by the monolayer of cholesterol at the air–water interface and by small crystals of undissolved solute.

(*ii*) The sampling pipette must be washed repeatedly with organic solvent or scintillation fluid to remove the adsorbed cholesterol from the walls. The average value for the solubility of cholesterol in water is 1.8 μg/ml or 4.7 μM. Similar determinations were made in phosphate buffer (pH 8.2), ionic strength 0.033, and agreed within experimental error with the data in Table 1.

The critical micelle concentration of cholesterol in water was determined from rates of dialysis. A cholesterol solution was placed on one side of a membrane with 24-Å diameter pores (Visking dialysis tubing) as described in *Experimental Procedure*, and the concentration on both sides of the membrane was measured as a function of time. Figs. 1 and 2 are typical examples of several such experiments and show the increase in cholesterol concentration on the side of the membrane that initially contained water alone. Fig. 1 indicates the typical rapid equilibration of all samples in which the final equilibrium concentration was $\leq$27 nM. In 20 hr these systems achieved 95% equilibrium. Fig. 2, on the other hand, shows the failure of systems containing higher concentrations of cholesterol to equilibrate beyond a specific critical concentration. Approximate values of this critical concentration can be obtained by construction of tangents corresponding to the initial and final rates. The intersection of these tangents is an approximate measure of the critical micelle concentration of the solution. Three such values are given in Table 2 as a function of total cholesterol concentration.

A small increase in critical micelle concentration with increasing total concentration is predicted for micellar systems (9). The observed increases for cholesterol–water are somewhat larger than those observed for simple detergents (9) and may reflect a broader size distribution of micelles extending to relatively small species.

In several experiments the monomeric solution of cholesterol was removed and replaced by pure water to test the capability of the membrane to transport the solute after prolonged exposure. In all cases transport resumed immediately and the final critical micelle concentration was identical to that observed for a corresponding total cholesterol concentration when a fresh membrane was used. In addition, methylene blue that was added at the conclusion of a dialysis experiment

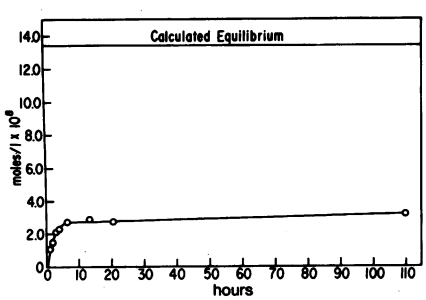

FIG. 2. Dialysis of cholesterol (specific activity = 1.56×10^6 dpm/μg) from a solution of initial concentration 10-times the critical micelle concentration into water across a membrane with 24-Å diameter pores. The calculated equilibrium concentration corresponds to an equivalent cholesterol concentration on both sides of the membrane.

TABLE 1. *Solubility of cholesterol in water**

Cholesterol added, μg/ml	Cholesterol solubilized, μg/ml
9.9	1.3 ± 0.9
19.7	1.1 ± 0.1
39.4	2.7 ± 1.8
98.5	2.6 ± 1.1
172.3	1.1 ± 0.2
	Avg. 1.8 ± 0.8

* [^{3}H]Cholesterol of specific activity 5.3 × 10^4 dpm/μg was used.

TABLE 2. *Apparent critical micelle concentration as a function of total concentration**

Total conc. mol/liter × 10^{-8}	CMC,† mol/liter × 10^{-8}
20	2.7 ± 0.5
51	3.7 ± 0.5
61	4.4 ± 0.5

* [^{3}H]Cholesterol of specific activity 1.56 × 10^6 dpm/μg was used.

† Critical micelle concentration.

equilibrated across the membrane within 4 hr, verifying the membrane pores were not blocked by adsorbed cholesterol.

Critical micelle concentrations can also be obtained from data of rate of dialysis by plotting the initial rates as a function of initial concentration. Since the rate of diffusion is directly proportional to the concentration gradient, initial rates should increase linearly with increasing concentration of solute. In micellar systems this phenomenon is observed at concentrations below the critical micelle concentration. At higher concentrations the thermodynamic activity of the monomer increases only slightly with increasing total concentration, and initial rates have a different functional relationship to the total concentration. Analysis of the data for the rate of dialysis of cholesterol in this manner leads to a critical micelle concentration about 25–40 nM, in agreement with the critical micelle concentrations presented in Table 2.

The critical micelle concentration of cholesterol was also determined in 50 mM Tris·HCl (pH 7.2); 50 mM Tris·HCl–0.1 mM Ca^{++} (pH 7.2); 50 mM Tris·HCl–0.1 mM EDTA (pH 7.2). The values obtained were identical within experimental error to the data in Table 2.

Addition of methanol to aqueous cholesterol solutions led to reversible dissociation of the micelle at methanol/water ratios greater than 35/65 (v/v) as evidenced by the total equilibration of cholesterol across dialysis membranes in 48 hr at all solute concentrations.

The weight average molecular weight and partial specific volume of the cholesterol micelle were calculated from sedimentation equilibrium in sucrose density gradients. The equilibrium distribution of the micelle population of a saturated cholesterol solution in water is shown in Fig. 3. The baseline level in this figure represents the critical micelle concentration and has a value of 20–30 nM, in good agreement with the equilibrium dialysis measurements. The increase in radioactivity at the top of the gradient is due to monolayer formation at the air–water interface.

Large adsorptive losses of cholesterol were observed on the gradient-forming apparatus and the walls of the centrifuge tube. However, thin-layer chromatography of the cholesterol micelle band shown in Fig. 3 verified that it was >99% pure.

Meselson *et al.* (8) have shown that a homogeneous population of molecules will be distributed in a Gaussian band in density gradient centrifugation. The logarithm of the concentration at any given point is then a linear function of the square of the band width at that point. Cholesterol micelles do not distribute in a Gaussian function and a plot of log concentration against the square of the band width is concave upward, suggesting molecular weight heterogeneity rather than density heterogeneity. Weight-average molecular weights of the cholesterol micelle were calculated from several density gradient experiments and are given in Table 3 together with the partial specific volume, which is the inverse of the buoyant density at the maximum point of distribution. The range of values obtained for the point-average molecular weights is also presented to emphasize the heterogeneity of the micelle population.

The molecular dimensions of the cholesterol micelle were estimated by dialysis through membranes of specific pore sizes. Fig. 4 presents the results as the percentage equilibrium attained by cholesterol dialyzing across the membranes. A level of 2% in this experiment represents the transport of only the monomer and is the level reached when the membranes have pores <1000 Å. Larger pore sizes allow passage of the cholesterol micelles and eventual equilibration of the system.

The cholesterol micelle, then, has at least one dimension of order 1000 Å. A sphere of this diameter is not consistent with the molecular weight reported in Table 3. However, a rod of 1000-Å length and 20-Å diameter is consistent with a molecular weight of about 200,000. The molecular dimensions of the cholesterol monomer are 5.2 × 6.2 × 18.9 Å (10), so a rod

TABLE 3. *Molecular weights and partial specific volume of cholesterol micelles**

$d\rho/dr$ g/cm^4	$\bar{v}$ cm^3/g	Molecular weight† range	$\overline{M}_w$	Initial sample distribution
0.016‡	0.946	26,000–284,000	155,000	Band
0.020	0.952	38,000–335,000	161,000	Homogeneous
0.022	0.950	15,000–359,000	197,000	Band
0.023§	0.952	39,000–220,000	173,000	Band
0.025	0.946	10,000–478,000	241,000	Homogeneous
0.030	0.946	14,000–327,000	101,000	Homogeneous
0.032	0.949	29,000–164,000	107,000	Band
Avg.	0.949	Avg.	162,000	

* Samples were prepared with 1 μM cholesterol. Specific activities were 10^4–10^6 dpm/μg. Except where noted, the gradients have been centrifuged for 72 hr at 48,000 rpm at 20°.

† Determined from a point-by-point analysis of the distribution according to Meselson *et al.* (8).

‡ 40 hr at 36,800 rpm.

§ 49.5 hr at 48,000 rpm.

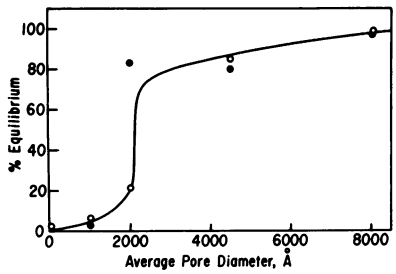

FIG. 4. Percent equilibrium attained by cholesterol (specific activity = 3.17×10^4 dpm/μg) dialyzing into water across membranes of different pore sizes. (Initial concentration = 100-times critical micelle concentration.) ○, 20 hr; ●, 67 hr.

of the dimensions described could be formed by side-to-side stacking of the ring system.

The partial specific volume of cholesterol was determined in benzene. Traube (11) has observed that the partial specific volume in water as compared to organic solvents is normally smaller by 12.7 cm^3/mol for several compounds. Correction of our measured partial specific volume in benzene of 1.021 cm^3/g by 12.7 cm^3/mol gives 0.988 cm^3/g applicable to cholesterol in water. This value is significantly higher than the experimental partial specific volume of the cholesterol micelle of 0.949 cm^3/g and suggests a high interaction energy between monomers in the micellar state.

DISCUSSION

We have shown that cholesterol undergoes a thermodynamically reversible monomer–micelle equilibrium in aqueous solution. The critical micelle concentration is exceptionally low, 25–40 nM at 25°, and several lines of evidence indicate that cholesterol micelles are stabilized by strong intermolecular attractive forces in addition to the hydrophobic repulsion by the solvent. Thus, the partial specific volume of cholesterol in micellar form is lower than that of cholesterol monomers, in contrast to some synthetic detergent systems in which the partial specific volume actually increases slightly as the result of micelle formation (unpublished results from this laboratory by Dr. Y. Nozaki). In addition, preliminary experiments at 10° show that the critical micelle concentration decreases with decreasing temperature and the micellar size increases, whereas the opposite results are observed in polyoxyethylene derivatives (12). Finally, we have shown (unpublished) that hemin dissolves readily in the hydrophobic interior of synthetic detergent micelles, but it cannot be incorporated into the cholesterol micelle, a result indicative of considerable specificity in the force of aggregation.

The implications of these results for incorporation of cholesterol into biological membranes or other lipid–protein complexes may be of great importance. Thus, the binding of any amphiphilic ligand to a protein involves competition with the self-association of the amphiphile (2). Since the free energy gain on self-association is very large, direct binding to a protein can occur only if the protein has a binding site for cholesterol with comparable affinity. For example, cholesterol does not bind to serum albumin, whereas many other steroids do (13). This finding does not necessarily mean that cholesterol has a lower intrinsic affinity for serum albumin than do other steroids, because the large free energy of self-association alone can account for the result.

Since cholesterol is readily incorporated into phospholipid bilayers and monolayers (3), its presence in membranes may result from specific interaction with phospholipid rather than with membrane protein. This hypothesis would also explain why high-density serum lipoprotein cannot bind cholesterol except in the presence of phospholipid (14).

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