Rod-like cholesterol micelles in aqueous solution studied using polarized and depolarized dynamic light scattering

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ABSTRACT Micelles of cholesterol in aqueous solution have been investigated using polarized and depolarized dynamic light scattering. They are shown to be highly extended and characterized by a narrow size distribution. It is shown that a rod-like model is applicable with length, L = 580 nm. Determination of the rotational diffusion coefficient by analysis of the autocorrelation function gave a value of Θ = 150 s^{-1}, which is close to the calculated value for the rod with this dimension. Depolarized dynamic light scattering measurements as a function of angle gave a value of 110 s^{-1}.

INTRODUCTION

Cholesterol is a sterol widely known for its biochemical role in biological membranes (for a review see, e.g., Yeagle, 1985), its interaction with serum lipoproteins (see, e.g., Yeagle et al., 1982) and with bile salts (e.g., Mazer et al., 1980). Moreover, studies of cholesterol aggregation in aqueous medium are essential for an understanding of the occurrence of some pathological situations such as the formation of cholesterol gallstones in bile (Small, 1967; Lonsdale, 1968) and atherosclerotic lesions (Small, 1970; Small and Shipley, 1974). Cholesterol gallstones result from the supersaturation of bile (Admirand and Small, 1968) (which is a solution composed mainly of three kinds of lipids: bile salts, lecithins, and cholesterol) with cholesterol. The excess cholesterol will precipitate (Mazer et al., 1977) forming microcrystals that may start, or enlarge, a process of Epitaxy (Lonsdale, 1968) (growth of one crystal on a substrate of another). If this saturation takes place in the blood, cholesterol accumulates in the atherosclerotic lesions forming thermodynamically stable systems (Small and Shipley, 1974) with other lipids (mainly cholesterol esters and phospholipids). The physical state of these lipids in relation to the biochemical environment can be understood on the basis of their interaction with an aqueous system (Small and Shipley, 1974). Knowledge of the structure and physico-chemical properties of cholesterol aggregates in aqueous medium is thus essential for an understanding of these pathologies.

Aqueous solutions of cholesterol have been little investigated in spite of the fact that knowledge of cholesterol-cholesterol and cholesterol-water interactions is essential to a deeper understanding of complex systems as those involving lipids and/or proteins and/or sterols and water.

Haberland and Reynolds (1973) examined cholesterol aggregates in water employing a dialysis technique and demonstrated that there is a reversible monomer-micelle equilibrium, with a critical micelle concentration of 25–40 nM (25°C) and a solubility limit of 4.7 μM using their method of preparation. A rod-like shape for the micelle was advanced to explain the relationship between aggregate dimensions (<1,000 Å in its largest dimension as deduced from dialysis experiments) and its molecular weight ($2 \cdot 10^5$, from ultracentrifugation experiments). A side-by-side stacking of the ring systems was proposed to account for the high interaction energy between the aggregated monomers. Later, Gilbert et al. (1975) continued this type of investigation and concluded that the unique structure of the hydrophobic portion of cholesterol allows a particularly favorable packing of water molecules around the sterol in an aqueous phase and that some repulsion between polar groups is required to account for the existence of micelles of finite size.

An x-ray study on crystals of cholesterol monohydrate grown from acetone-water solutions (Craven, 1976) supported the ability of cholesterol to stack side-by-side in bilayers of 33.9 Å thickness.

In this work, the structure and some physico-chemical properties of cholesterol micelles prepared from acetone-water solutions, followed by ultrasonification, are elucidated using dynamic light scattering measurements. The investigation forms part of a wider program directed to studies of the interaction between cholesterol and polyene antibiotics, which are only effective against cells having sterol-containing membranes (Bolard, 1986). One of the published models for the interaction of filipin, a polyene antibiotic, and cholesterol (Elias et al., 1979) proposes an association of filipin with the sterol in a 1:1 stoichiometry at the membrane surface, in an aqueous environment. The only method developed so far to study the interaction between antibiotics and sterols (Norman et al. 1972) was recently shown to be incorrect (Castanho et al., 1992) and so this remains an open field.

The results presented here also have significance for an understanding of the dynamics of cholesterol ex-

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Abbreviations used: ILT, inverse Laplace transformation; DLS, dynamic light scattering.

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change between vesicles (Bruckdorfer and Sherry, 1984), a process commonly used in the preparation of artificial membranes. In addition, the ability of cholesterol to form "pools" in artificial bilayers of phospholipids (Rogers et al., 1979) instead of a random distribution, can only be elucidated if the fundamental cholesterol-cholesterol interactions are clarified.

The results from dynamic light scattering described below, giving the translational and rotational diffusion coefficients, strongly suggest that cholesterol forms long rod-shaped micelles.

EXPERIMENTAL

Materials and preparation

Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received after its purity had been checked by thin layer chromatography on silica plates, using a mixture chloroform:acetone (98:2) as eluent. Tris-HCl was purchased from B. D. H. (London) and used as received to prepare the buffer (Tris-HCl 50 mM, pH 7.4, NaCl 10 mM). NaCl (p. a.) and acetone (p. a.) were from Merck (Darmstadt, Germany).

Micelle preparation

To a given volume of buffer at 85°C, with vigorous but nonturbulent stirring, small volumes (no more than 1/20 of the total volume of buffer) of cholesterol solutions in acetone (0.685 mM) were injected with a syringe. Use of small volumes prevents the effervescence of acetone, so avoiding losses of cholesterol that would otherwise dry and remain on the walls of the container. One minute was the minimum time interval between two additions to prevent acetone accumulating in the solution and boiling locally. This was controlled measuring the temperature of the mixture; as acetone has a boiling point of 56.2°C, accumulation of acetone would decrease the temperature. After complete addition of the cholesterol solution in acetone to the buffer, the aqueous suspensions of cholesterol were left during 15 min at 85°C with vigorous stirring, so that no significant amount of acetone remained in solution. The final concentration of acetone in the aqueous solutions of cholesterol was controlled by UV absorption. Acetone in water has an absorption maximum at 265 nm (ε = 18.5 M^{-1} cm^{-1}) (Hayes and Timmons, 1971). Measurements were carried out in a Perkin-Elmer λ-15 spectrophotometer, using 10-cm path cuvettes, indicating that the molar percentage of acetone in the final solutions was <0.001% (relative to water).

All the suspensions were sonicated for 10 min in a MSE Soniprep 150 sonicator at power 10, and filtered with a Millex-HV Filter Unit (0.45 μm) before use. Unless otherwise mentioned, all measurements were made with freshly sonicated and filtered preparations. With this sonification step, considerably higher concentrations of cholesterol in aqueous solution can be attained than was found by Haberland and Reynolds (1973). It is noted that prolonged ultrasonification (~1 h) does not reduce the micellar size as determined by dynamic light scattering (see below), and we conclude that the particles measured correspond to the minimum dimensions. Furthermore, the solutions of cholesterol prepared as above were stable with time during the experimental period (~1 mo). The material used in contact with cholesterol suspensions was previously washed with chloroform and distilled water and never subject to the action of any detergent. Washing of glassware with concentrated nitric acid (Merck, p. a.) to remove eventual traces of remaining detergent was found not to influence the obtained results.

Polarized dynamic light scattering

Polarized dynamic light scattering (I_{vv}) measurements were made using the apparatus and techniques described in earlier communications (see, for example, Nicolai et al., 1990). The data were assembled using a wide-band multi-τ autocorrelator (ALV-3000) with 23 simultaneous sampling times allowing characterization of relaxation time distributions extending over eight decades. Average diffusion coefficients were estimated using the method of cumulants, with both second and third order terms. Inverse Laplace transformation was made employing the algorithm REPES (Jakes, 1988), which is similar to Provencher's CONTIN (Provencher, 1979), except that the former directly minimizes the sum of the squared differences between experimental and calculated intensity-intensity $g_2(t)$ functions ($g_2(t) = g_1^2(t)$) using nonlinear programming. For a system exhibiting a distribution of relaxation times, the field correlation function $g_1(t)$ is described by the Laplace transform as a continuous function of the relaxation time τ: $g_1(t) = \int_{-\infty}^{\infty} \tau A(\tau) e^{-t/\tau} \, d \ln \tau$. The range of relaxation times allowed in the fitting was usually between 0.5 μs and 1 s, with a density of 12 points per decade. Relaxation rates are obtained from the moments of the peaks in the relaxation time distribution or, if the peaks overlap, from the peak position. With a broad distribution of relaxation times, both inversion methods can yield multiple peaks in the "unsmoothed" analysis. The "smoothing" parameter (P) was selected as 0.5 in all cases.

Mutual diffusion coefficients (calculated as: $D_m = \Gamma/q^2$) and the relative amplitudes are obtained from the moments of the peaks and are given in the output of the program. Γ is the measured relaxation rate ($\Gamma = \tau^{-1}$) and q is the scattering vector. The rotational diffusion coefficient (Θ) was evaluated from the autocorrelation function by dividing it by the term in Eq. 9 including the translational diffusion coefficient determined at low angles. Θ was then determined by force-fitting the residual function to a single exponential and also by using inverse Laplace transformation.

Depolarized dynamic light scattering

Depolarized dynamic light scattering (I_{VH}) measurements were also used to determine the rotational diffusion coefficient. The depolarized intensity was very low (of the magnitude 0.5 kHz) and these measurements were made for extended duration; the correlation function shown in Fig. 4 *a* was taken up over 15 h.

RESULTS AND DISCUSSION

Dynamic light scattering

Polarized dynamic light scattering (I_{vv}) was used in the homodyne mode to determine the mutual diffusion coefficient ($D_m = \Gamma/q^2$, where Γ is the measured relaxation rate and q the scattering vector). Γ was evaluated as a function of angle using the method of cumulants with both second and third order cumulants terms. Fits were also made by inverse Laplace transformation (ILT) at low angles where the rotational contribution to the autocorrelation function (see below) could be neglected, using the methods described in the Experimental section. Γ is linearly dependent on q^2 establishing a diffusive process.

A typical correlation function obtained at low angle and the corresponding relaxation time distribution from ILT are shown in Fig 1. The diffusion coefficient was concentration independent at the very low concentrations used in this work and gave the mean value 4.3×10^{-12} m^2 s^{-1}. The narrowness of the relaxation time distribution suggests that the micellar solutions prepared in this manner are well defined and rather monodisperse. Due to the extremely low solubility of cholesterol in aqueous media, any method of preparation will tend to

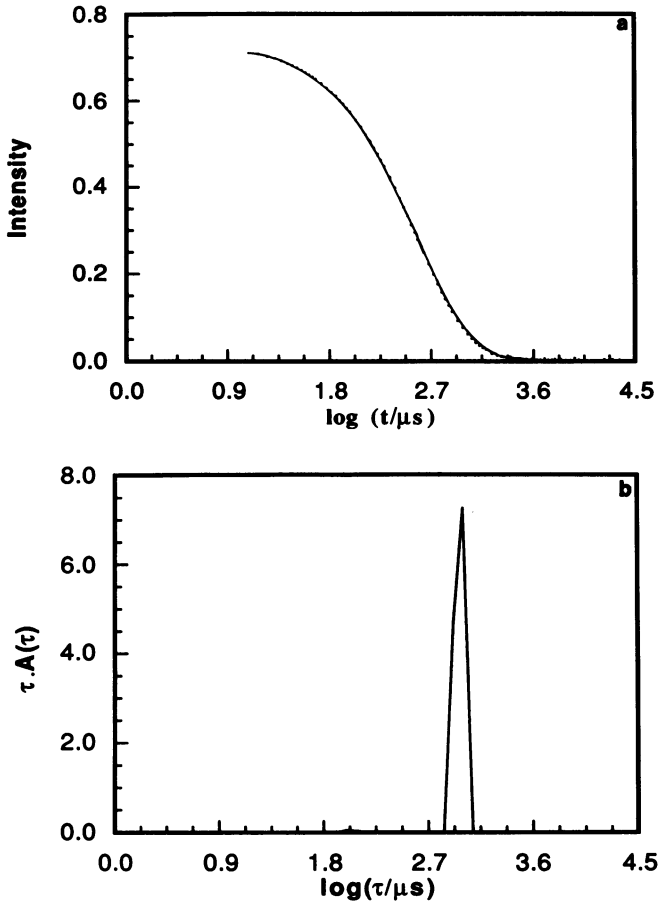

FIGURE 1 Typical correlation function obtained for c = 8.9 μg/ml and at low angle ($\theta = 22°$) (*a*) and the corresponding relaxation time distribution ($\tau A(\tau)$ versus log $\tau/\mu s$) from inverse Laplace transformation (*b*). The vertical axis is $\tau A(\tau)$ providing an equal area representation. The decay is monoexponential, as is confirmed by the monomodal distribution of the function describing the relaxation time distribution (*b*). This observation, together with the narrowness of the peak, points to a well defined and monodisperse system.

influence the size distribution of the suspended particles. However, since prolonged ultrasonification does not significantly reduce the micellar size, we conclude that the particles measured correspond to the minimum dimensions. Because light scattering measurements require optically clean solutions, a filtration step is a prerequisite. The absence of peaks in the distribution corresponding to slowly diffusing material shows that dust has been effectively removed. Furthermore, we have observed that the micelles are stable in size over the time period covered by the measurements and do not have a facile tendency to form larger particles. Precipitation onto the walls of the vessel, a method that has also been used by earlier workers (Renshaw et al., 1983), may lead to the formation of microcrystallites. The relative monodispersity of the preparation and the absence of such large components in the distributions as well as the stability of the solutions, with regard to time and ultrasonification, favor the micellar model over a microcrystalline one.

We follow earlier workers (Haberland and Reynolds, 1973) in assuming that cholesterol forms rod-like micelles, although the extremely low concentrations accessible prohibit characterization of a critical micelle concentration. However, this assumption will also be shown below to be validated by the depolarized dynamic light scattering results.

A sensitive index inferring the relevant model for the particle is also provided by the coefficient C in the relationship (Burchard, 1992):

$$\Gamma_1/q^2 = D_c(1 + Cq^2R_g^2 - \cdots), \quad (1)$$

where C is a structure-dependent coefficient, Γ_1 the first cumulant, and $D_c = D_0(1 + k_DC)$ is the translational diffusion coefficient at concentration C. From measurements over the angular range 22° to 140° on a cholesterol solution with concentration 8.9 μg/ml, we obtain the relation:

$$(\Gamma_1/q^2)/D_c = (1 + 0.028q^2R_g^2). \quad (2)$$

Burchard gives the values $C = 0.042$ for a monodisperse rigid rod and 0.173 for a monodisperse random coil.

Diffusion coefficients may be obtained from Broersma's equation (Broersma, 1960) if the dimensions of the rod-like particle are known:

$$D_m = (kT/6\pi\eta a)\cdot G(\rho), \quad (3)$$

where

$$G(\rho) = \ln(2\rho) - \tfrac{1}{2}[1.46 - 7.4((1/\ln 2\rho) - 0.34)^2 - 4.2((1/\ln 2\rho) - 0.39)^2], \text{ with } a = L/2 \text{ and } \rho = (L/2r). \quad (4)$$

Alternatively, Doi and Edwards (1986) have given an expression for D_m for rigid rods:

$$D_m = \ln(\rho)kT/[6\pi\eta(L/2)]. \quad (5)$$

When these expressions were used in conjunction with experiments on TMV (tobacco mosaic virus) rods (Johnson and Brown, 1992), very close agreement was obtained with the measured diffusion coefficient.

Here, we use L as a fitting parameter in Eq. 5 together the measured diffusion coefficient and the value $r = 1.7$ nm, where the latter figure for the micellar radius is that determined crystallographically as the half-thickness of the bilayers in crystals of cholesterol monohydrate (Craven, 1976); we then obtain $L = 580$ nm for the length of the cholesterol micelle.

The hydrodynamic radius was also evaluated using the concentration-independent D_m-value determined at low angles in the Stokes-Einstein equation:

$$R_h = kT/(6\pi\eta D_m). \quad (6)$$

This gives a mean value for the hydrodynamic radius of 59 nm which can be compared with the value calculated using theoretical models.

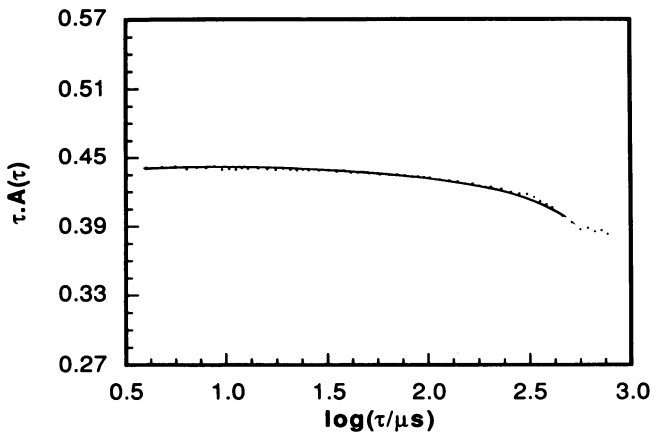

FIGURE 2 Typical reduced correlation function for a solution with $C = 8.9$ μg/ml obtained from the complete correlation function (similar to the one in Fig. 1 *a*) measured at high angle (140°), after division by the diffusional term (see text). The rotational diffusion coefficient, Θ, is evaluated after force-fitting to a single exponential function and also by an inverse Laplace transform analysis.

Broersma's equation for the rigid rod (Broersma, 1960) is:

$$R_h = L/[2\sigma - 0.19 - (8.24/\sigma) + (12/\sigma^2)] \quad \text{with} \quad \sigma = \ln(L/r). \quad (7)$$

This relationship gives $R_h = 56$ nm.

On the other hand, Perrins formula for an ellipsoid (Young et al., 1978):

$$R_h = \frac{b[1 - (a/b)^2]^{1/2}}{\ln\left[\dfrac{1 + (1 - (a/b)^2)^{1/2}}{a/b}\right]}, \quad (8)$$

where (b/a) is the ratio of the semi-major and semi-minor axes of the ellipsoid, gives a value of $R_h = 50$ nm. These values are close to the experimental value (59 nm).

Rotational diffusion coefficient (Θ)

Two possibilities exist for determining Θ using dynamic light scattering. Firstly, one may use the fact that rigid rod rotation is involved in the decay of the autocorrelation function at higher angles. Ignoring coupling between translational and rotational motions, one may write for the measured intensity-intensity correlation function:

$$[g_2(t) - 1]^{1/2} = e^{-Dq^2t}(S_0 + S_1e^{-6\Theta t} + \cdots), \quad (9)$$

where D is the translational diffusion coefficient. The coefficients S_0 and S_1 are the dynamic form factors which are functions of the scattering vector **q** and which may be calculated for a given model structure (Berne and Pecora, 1976). There it is shown that for rods with $qL > 3$, where L is their length, it is possible to estimate Θ with acceptable precision from measurements of DLS on long rods at high angles where S_1 is large. Flamberg and Pecora (1984) have recently made this type of analysis for dynamic light scattering on micellar systems.

In this case, we divide out $[g_2(t) - 1]$ by the diffusional term in Eq. 9 using the diffusion coefficient measured at low angles which is not influenced by the rotational term to form a "reduced" correlation function as depicted in Fig. 2. We have used the mean D-value of 4.3×10^{-12} m^2 s^{-1} determined at angles in the range 20–35°. If the correct D-value has been used, the reduced correlation function should reflect only the contribution of the rotational diffusion coefficient. Θ has then been obtained by force-fitting to a single exponential function and by an ILT analysis. From an analysis of DLS data for three cholesterol solutions of different concentration and each at three angles in the range 100° to 140°, we obtain an average value of $\Theta = 150$ s^{-1}. It is shown in Fig. 3 that, in view of the difficulty of making precise measurement of Θ, this value correlates acceptably with theory, and thus the proposed value of the micellar length, using the equation of Broersma (Broersma, 1960):

$$\Theta = [3kT/(16\pi\eta a^3)]F(\rho), \quad (10)$$

in which

$$F(\rho) = 2[(\ln 2\rho) - 1.45 + 7.5((1/\ln 2\rho) - 0.27)^2] \quad (11)$$

and $a = L/2$ and $\rho = L/2r$, employing $r = 1.7$ nm.

Secondly, it may be possible to directly measure the fluctuations of the depolarized light scattering. This approach is, however, particularly demanding since the signal-to-noise ratio in depolarized light scattering is very low (see the discussion of Flamberg and Pecora [Flam-

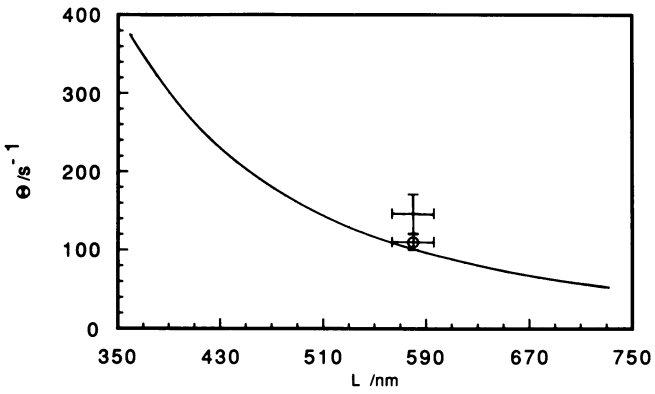

FIGURE 3 Theoretical prediction for the variation of the rotational diffusion coefficient of a rod shaped micelle with its length (solid line) (Broersma, 1960). The experimental data are presented in the form of the average values (over concentration) limited by error bars (standard deviation). The agreement between the measured (+, from polarized DLS; O, from depolarized DLS) and predicted values constitutes further evidence for the proposed shape (rod) and size ($L = 580$ nm) of the micelle.

berg and Pecora, 1984]). Fig. 4 *a* shows the autocorrelation function of the intensity of the depolarized scattered light (I_{VH}) for a solution of cholesterol (23 μg/ml) at angle 135°.

Fig. 4*b* depicts the angular dependence of the inverse decay rate obtained by ILT analysis of such curves. The intercept in Fig. 4*b* is 6Θ. The value of Θ is inserted into Fig. 3 in relation to the theoretical curve for a rigid rod and shows excellent agreement.

Structure of cholesterol micelles

Cholesterol crystals may be mono-hydrated (Craven, 1976) or anhydrous (Shieh, 1977). Loomis et al. (1979) compared the phase behavior of both structures using different techniques (differential scanning calorimetry, polarizing light microscopy, x-ray diffraction). While anhydrous cholesterol undergoes a polymorphic crystalline transition at 39°C, and a crystalline to liquid transition at 151°C, cholesterol monohydrate and water exhibit three transitions at 86,123, and 157°C. At 86°C cholesterol monohydrate looses its water of hydration, forming the high temperature polymorph of anhydrous cholesterol. At 123°C anhydrous crystalline cholesterol in the presence of excess water undergoes a transition to a liquid-crystalline phase, stable over 123–157°C, before melting to a liquid dispersed in water.

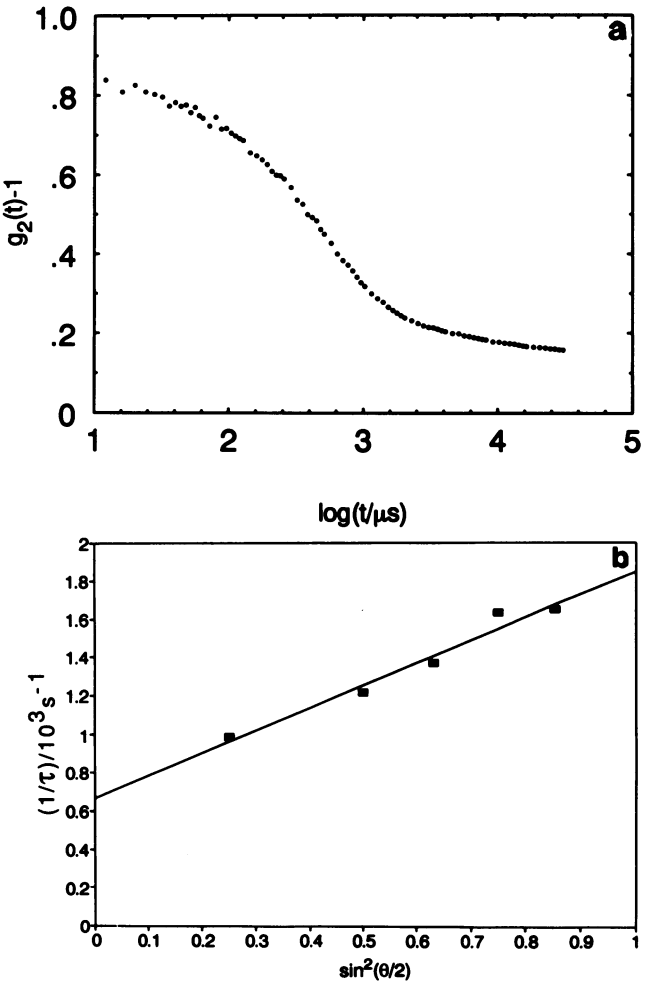

FIGURE 4 (*a*) Autocorrelation function of the intensity of the depolarized scattered light at angle 135° for a 23 μg/ml solution of cholesterol in buffer. (*b*) Angular dependence of the inverse relaxation time derived from the correlation functions from depolarized dynamic light scattering.

TABLE 1 **Parameters characterizing the cholesterol micelle**

Parameter	Experimental	Calculated	
		Value	Equation
$D_0 \cdot 10^{12}/m^2 s^{-1}$	4.30	—	—
L/nm	—	580	5
R_h/nm	59	50	7
	—	56	8
Θ/s^{-1}	150 (DLS)	100	10
Θ/s^{-1}	110 (depolarized DLS)		

Since micelles are prepared using a solution of cholesterol in acetone, the possibility that the cholesterol micelles have a structure related to anhydrous cholesterol (with the hydroxyl groups protected from the solvent, forming OH—OH hydrogen bonding) was considered. However, the method of preparation used in this study includes ultrasonification, a process which is capable of disrupting the structure in the crystalline suspension of anhydrous cholesterol. Upon reassembly of cholesterol molecules after sonication, hydration is complete at room temperature. Loomis et al. (1979) reported the effect of stirring on aqueous solutions of anhydrous cholesterol at room temperature as a complete conversion to the monohydrated form. Being a much stronger process, sonication is expected to emphasize this effect. No changes are detected at 39°C, the temperature at which anhydrous cholesterol undergoes a polymorphic transition. Thus, the micellar structure of cholesterol is suspected to be related to the monohydrate cholesterol crystal, with the hydroxyl groups of cholesterol exposed to water together with hydration water layers.

The formation of cholesterol micelles is at variance with the concept of Renshaw et al. (1983), who suggested the formation of cholesterol microcrystals. Designation of the structures studied in this work as microcrystals would imply a correlation in the position of the molecular units of the crystal in three dimensions. Being a thin rod in dilute solution, the studied structure only has correlation in one dimension, not three, and thus the term "microcrystal" would appear incorrect.

Since "aggregate" would imply a disordered structure, we consider that "micelle" is the best designation for the studied particles, although the existence of a critical micellar concentration remains an open question.

CONCLUSIONS

Under the conditions used, cholesterol self-associates in aqueous solution to form micelles of a definite size (see

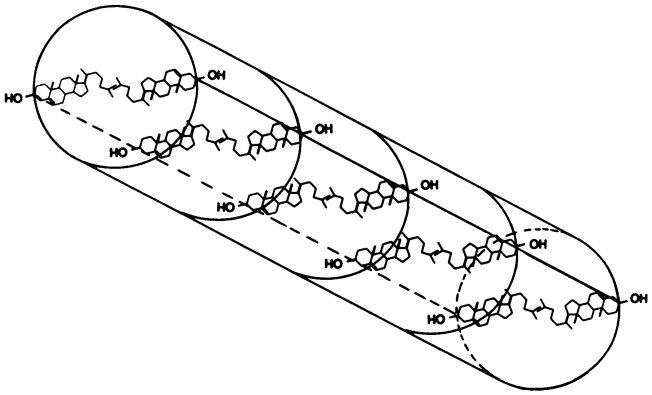

FIGURE 5 Schematic diagram showing some molecules of the total existing in a segment of a cholesterol micelle. This scheme is an oversimplification for the sake of clarity. The units represented in the figure are to be repeated by close-stacking along and around the major axis of the rod. The proposed structure (with the cholesterol molecules stacking "side-by-side", perpendicular to the rod major axis and with the hydroxyl group facing the aqueous environment) is based on the data and conclusions achieved in this work and on the conclusions of the studies performed by Gilbert et al. (1975), Haberland and Reynolds (1973) (both works based on thermodynamic grounds), and by Craven (1976) (work based on x-ray scattering of the cholesterol monohydrate crystal).

Table 1) and rod-like shape. Although a rod-like form cannot be established unequivocally from the light scattering experiments, this conclusion is consistent with the data sets presented: (*a*) The Doi-Edwards equation (5) for the translational diffusion coefficient gives a rod length of $L = 580$ nm. (*b*) The rotational diffusion coefficient obtained directly from depolarized dynamic light scattering measurements ($\Theta = 110\ s^{-1}$) is consistent with the rod-length derived under *a* using Broersma's equation (10). (*c*) The rotational diffusion coefficient obtained from analysis of the autocorrelation function using Eq. 9 gives $\Theta = 150\ s^{-1}$ which is also consistent with *a* and *b*.

The cholesterol micelles are concluded to be long, thin, rigid rods which we speculate to have the structure depicted in the diagram of Fig. 5. In the cross-section of the rod, cholesterol molecules "point" their hydrophobic tails toward the long axis of the micelle, standing perpendicular to it. In this way, the hydroxyl groups of the cholesterol molecules form an hydrophilic layer surrounding the hydrophobic core of the micelle, facilitating its solubilization. According to Gilbert et al. (1975), some repulsion between polar groups is required to account for the existence of cholesterol micelles of finite shape and specific surface effects may occur between the solute and the bulk water phase. In addition, this "tail-to-tail" arrangement accounts for the proposed thickness of the rod (34 Å).

Gilbert et al. (1975) also concluded that there are attractive forces between monomers in the cholesterol micelle, and Haberland and Reynolds (1973) concluded that the forces of interaction are stronger than those usually found in micellar systems, proposing a side-to-side stacking of the ring systems. A parallel stacking of the molecules in the rod micelles is thus proposed (Fig. 5). Besides explaining our results (namely, the rigidity and length of the rod) and those of Gilbert et al. (1975) and of Haberland and Reynolds (1973), a parallel array of cholesterol molecules with the ring systems standing "side-by-side", is similar to that found in the crystal structure of cholesterol monohydrate (Craven, 1976).

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