Thermodynamic Equilibria of Cholesterol–Detergent–Water†

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ABSTRACT: Cholesterol monomer is incorporated into alkyl sulfate micelles with a unitary free energy of −10.3 kcal/mol. This experimental free energy is in good agreement with that predicted by our previous determination of the hydrophobicity of the sterol suggesting that the partitioning is primarily hydrophobic with little or no contribution to the free energy from head group interactions in this system. The intrinsic hydrophobicity of cholesterol is shown to be insufficient for effective partitioning of the sterol between micelles (or bilayers) and its own self-associated form. This finding strongly supports a model of phospholipid–cholesterol interaction involving significant free energy contributions from head group effects such as alterations in hydrogen bonds or hydration. Since these head group contributions are not observed in the cholesterol–alkyl sulfate system, one concludes that there is a high degree of specificity of interaction between the sterol OH and polar moieties of other amphiphilic molecules.

Cholesterol, an important biological amphiphile, is found in mammalian cell membranes and in the various classes of serum lipoproteins. In order to describe rigorously the interactions in these multicomponent systems, it is necessary to define the physicochemical properties of the individual species in aqueous media. We have previously reported detailed studies of the cholesterol–water system, the results of which are summarized as follows. (1) Cholesterol forms large micellar structures at a critical concentration (cmc) of $20–40 \times 10^{-9}$ *M* which corresponds to a unitary free energy of micelle formation of −12.6 kcal/mol (Haberland and Reynolds, 1973). The cholesterol micelle is stabilized by specific attractive forces between monomers in addition to the gain in free energy predicted from hydrophobicity as is evidenced by an anomalously low partial specific volume and a unitary free energy of micelle formation which is 2–4 kcal/mol more negative than would have been predicted from the intrinsic hydrophobicity of cholesterol (Gilbert et al., 1975). (2) The hydrophobic free energy of transfer of cholesterol monomer from water to a hydrocarbon medium is considerably less than one would predict from estimates of the hydrophobic surface area of the solute (Reynolds et al., 1974; Gilbert et al., 1975). The experimental hydrophobic free energy of transfer of cholesterol from water to hydrocarbon is used to calculate the absolute upper limit of the free energy of transfer of this solute from water to a micelle of −11.4 kcal/mol (Gilbert et al., 1975).

These results suggest that the incorporation of cholesterol into other amphiphilic systems such as lipid bilayers or detergent micelles will be unfavorable relative to the self-association of the sterol unless there are strong head group interactions between the OH group on the steroid ring system and the polar regions of other amphiphilic molecules. In other words, the hydrophobic free energy gained by removing the hydrophobic region of cholesterol from water is insufficient in itself to allow a favorable partitioning of this solute between a mixed micelle and its own self-aggregated form.

In this paper we report an investigation of the equilibrium properties of cholesterol in alkyl sulfate solutions (75% sodium dodecyl sulfate and 25% sodium myristyl sulfate) and compare the results with the binary system cholesterol–water.

Experimental Procedure

[4-^{14}C]Cholesterol (67 Ci/mol) or [1α,2α-^{3}H]cholesterol (47 Ci/mmol) was purchased from Amersham-Searle at greater than 97% purity as determined by reversed phase thin-layer chromatography in 90% v/v acetic acid aqueous solution saturated with paraffin, by thin-layer chromatography on silica gel B in cyclohexane–ethyl acetate (6:4), by thin-layer chromatography on silica gel impregnated with $AgNO_3$ in chloroform–acetone (98:2), and by thin-layer chromatography on aluminum oxide G in benzene–ether (73:3). The small amounts of water-soluble contaminants were readily removed by exhaustive washing of cholesterol solutions in benzene as previously described (Gilbert et al., 1975). The purified cholesterol was subjected to further examination by determining the partition coefficient between water and hexane and by determining the critical micelle concentration in water (Haberland and Reynolds, 1973). In all cases the free energy of transfer from water to hydrocarbon and the critical micelle concentration in water agreed with our previously published results. Crystalline cholesterol (99+% pure) was purchased from Applied Science Laboratories and subjected to the same analyses and purification procedures as described for the radioactive compounds. Sodium dodecyl [^{35}S]sulfate was obtained from Amersham-Searle at an initial activity of 35.6 mCi/g. Unlabeled sodium dodecyl sulfate was purchased from Schwarz/Mann as their highest grade but contained 75% dodecyl sulfate and 25% myristyl sulfate by gas chromatographic analysis of the hydrolysate of the alkyl sulfate.

Cholesterol incorporation into alkyl sulfate micelles was determined by layering 0.2-ml samples of cholesterol dissolved in an appropriate concentration of alkyl sulfate in 0.15 *M* NaCl on a 0.9 × 60 cm Sepharose 6B column. The column was equilibrated with an appropriate elution buffer containing varying concentrations of cholesterol and alkyl sulfate as described in Results. Aliquots of the 0.5-ml samples were counted in a scintillation fluid containing 8 g of 2,5-diphenyloxazole and 0.4 g of 1,4-bis[2-(5-phenyloxa-

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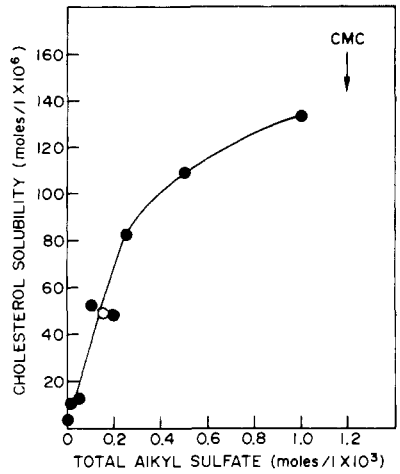

FIGURE 1: Solubility of cholesterol in sodium alkyl sulfate solutions containing monomeric detergent. cmc refers to the critical micelle concentration of the detergent.

zole)] in 2 l. of toluene and 1 l. of Triton X-100. ^{35}S was counted with 42.4% efficiency and ^{3}H with 26.5% efficiency. No spillover of ^{35}S was detected in the ^{3}H window and 6.9% spillover of ^{3}H was observed in the ^{35}S window. Counts were corrected for background, quenching, efficiency, and decay and converted to concentrations by comparison with standard solutions.

Binding of alkyl sulfate to cholesterol micelles was determined by equilibrium dialysis in 2-ml lucite cells. Aliquots from each side of the membrane were taken over a period of 180 hr and analyzed for cholesterol and alkyl sulfate.

Critical micelle concentrations of cholesterol were measured in a sucrose density gradient using equilibrium centrifugation as described previously (Haberland and Reynolds, 1973). Gradients from 2.5 to 20% sucrose were prepared in 5-ml cellulose nitrate tubes. Saturated 0.1-ml solutions containing labeled cholesterol were added to the top of the gradients and centrifuged in a SW 50.1 rotor at 40000 rpm, 25°, for 48–96 hr.

The solubility of cholesterol in alkyl sulfate solutions of varying concentrations was determined as described previously (Haberland and Reynolds, 1973).

Unitary free energies of transfer of a solute from water to a micelle are obtained by means of the following equation: $\mu^\circ_{mic} - \mu^\circ_w = RT \ln (X_w/X_{mic})$ where μ° is the appropriate standard chemical potential and X_{mic} and X_w are the mole fraction of solute in the micelle and in the water phase, respectively. The unitary free energy of self-association of cholesterol is defined as RT ln cmc where cmc is expressed in mole fraction units.

Results

The addition of monomeric sodium alkyl sulfate to solutions of cholesterol in 0.15 M NaCl increases the total solubility of the sterol as shown in Figure 1. In the absence of detergent the maximum solubility is 4.7×10^{-6} M. The increased solubility in the presence of the detergent can result from interaction between the alkyl sulfate and either the cholesterol monomer or the cholesterol micelle or both since the following equilibrium has already been demonstrated (Haberland and Reynolds, 1973).

$$\text{cholesterol (monomer)} \rightleftharpoons \text{cholesterol (micelle)} \quad (1)$$

Figure 2a shows the equilibrium distribution of cholesterol in water in a sucrose density gradient and is identical

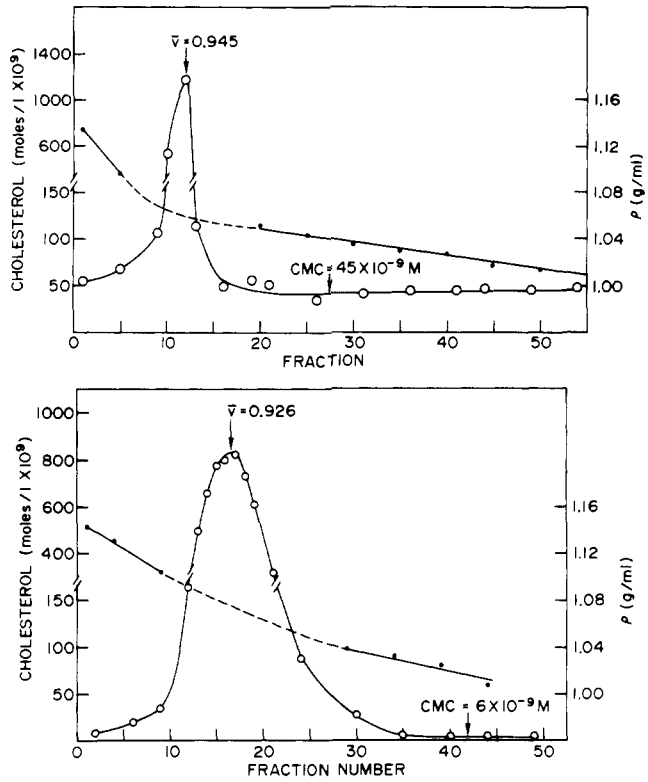

FIGURE 2: (a) Equilibrium centrifugation of cholesterol in a sucrose density gradient in water. Cholesterol micelle has a partial specific volume, $\bar{v}$, of 0.945. The cmc of the cholesterol molecule is 45×10^{-9} M. (b) Equilibrium centrifugation of cholesterol in a sucrose density gradient containing 2×10^{-3} M sodium alkyl sulfate and 0.15 M NaCl. The cholesterol micelle + bound detergent has a $\bar{v} = 0.926$. The cmc of the cholesterol is reduced to 6×10^{-9} M.

Table I: Binding of Alkyl Sulfate to Cholesterol Aggregate.

Unbound Alkyl Sulfate (moles/l. × 10⁴)	$\bar{v}$ [a]
1.2	0.40
2.23	0.47
3.71	0.49
4.67	0.61
8.90	0.63

[a] Moles of alkyl sulfate/mole of cholesterol ± 0.1.

with that previously reported. In contrast, Figure 2b is the distribution observed in the presence of monomeric alkyl sulfate. It is apparent that in this latter case the cmc of cholesterol is reduced to 6×10^{-9} M and the partial specific volume, $\bar{v}$, of the cholesterol micelle is decreased to 0.926 ml/g. Thus, the detergent interacts with the cholesterol aggregate and this complex is more soluble in aqueous solution than the pure cholesterol micelle.

From the following equation the degree of binding of alkyl sulfate to the cholesterol aggregate can be calculated.

$$\frac{[\bar{v}_{\text{cholesterol}} + \delta_1 \bar{v}_{\text{detergent}}]}{(1 + \delta_1)} = \bar{v}_{\text{obsd}} \quad (2)$$

where δ_1 = g of detergent/g of cholesterol. $\delta_1 = 0.34$ g/g or 0.46 mol/mol from the data in Figure 2a and b.

Equilibrium dialysis was also used to estimate the extent of binding and the results of these experiments are shown in Table I. The error in the dialysis measurements is large due

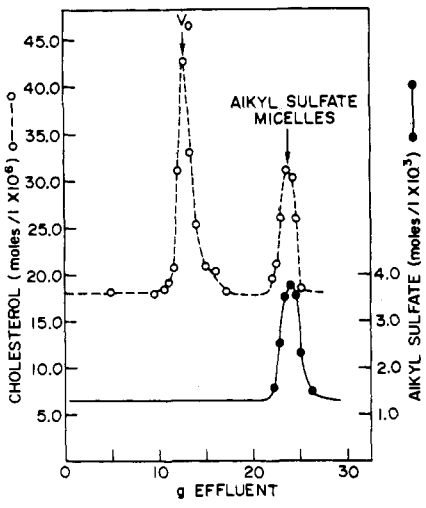

FIGURE 3: Gel filtration of cholesterol in sodium alkyl sulfate micelles. Column elution buffer: 1.3×10^{-3} *M* detergent, 0.15 *M* NaCl, and 18×10^{-6} *M* cholesterol. Sample applied: 0.2 ml of 400×10^{-6} *M* cholesterol, 0.025 *M* sodium alkyl sulfate, and 0.15 *M* NaCl. V_0 = void volume and elution position of the cholesterol micelle containing bound detergent. cmc of the cholesterol = 6×10^{-9} *M*, cmc of the sodium alkyl sulfate = 1×10^{-3} *M*.

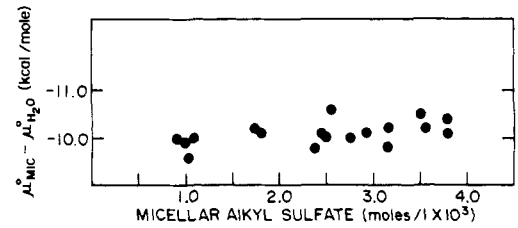

FIGURE 4: Free energy of transfer of cholesterol monomer from 0.15 *M* NaCl to alkyl sulfate micelles at 298°. The detergent concentration is total detergent in micellar form.

Table II: Cholesterol Equilibria in Aqueous Solutions.

	ΔG° (kcal/mol)
1. $\text{cholesterol}_{\text{monomer}} \rightleftharpoons \text{cholesterol}_{\text{micelle}}$	−12.6
2. $\text{cholesterol}_{\text{monomer}} \rightleftharpoons \text{cholesterol}_{\text{micelle}}$ + detergent	−13.5
3. $\text{cholesterol}_{\text{monomer}} \rightleftharpoons \text{cholesterol}_{\text{detergent micelle}}$	−10.3
4.[a] $\text{cholesterol}_{\text{monomer-H}_2\text{O}} \rightleftharpoons \text{cholesterol}_{\text{monomer-HC}}$	−6.4

[a] HC represents a hydrocarbon solvent with the same molar volume as the cholesterol solute (Gilbert et al., 1975).

to the small amounts of cholesterol and the relatively large equilibrium concentration of detergent, but it is apparent from both types of experiments that 0.5 ± 0.1 mol of alkyl sulfate is bound per mol of cholesterol in the micellar state over a wide range of unbound detergent concentration. The unitary free energy of transfer of cholesterol monomer to the self-associated form of the sterol with bound detergent is $RT \ln \text{cmc} = -13.5$ kcal/mol.

When pure detergent micelles are present in the system, cholesterol monomer is partitioned between alkyl sulfate micelles and its own self-associated form. This phenomenon is demonstrated in Figure 3 which is a typical gel-filtration experiment on Sepharose 6B. The cholesterol aggregate with bound detergent appears in the void volume and additional sterol is incorporated in the detergent micelle which is included in the Sepharose pores. If the equilibrium concentration of alkyl sulfate is below the detergent cmc, only the void volume peak is observed. Analysis of cholesterol and alkyl sulfate in the region of the detergent micelle at a variety of total detergent concentrations provides the partition ratio of cholesterol monomer (6×10^{-9} *M*) to the mole fraction of cholesterol in the alkyl sulfate micelles. The unitary free energy of transfer for this equilibrium system was calculated as a function of the concentration of the detergent in micellar form and is shown in Figure 4.

It was not possible to accurately measure the binding of alkyl sulfate to the cholesterol aggregate in the column experiments due to the low concentration of sterol relative to the equilibrium concentrations of alkyl sulfate.

An enumeration of the various equilibria observed in cholesterol–water and cholesterol–detergent–water is presented in Table II together with the appropriate unitary free energies.

Discussion

(*1*) *Interaction of Alkyl Sulfate Monomers with the Cholesterol Micelle.* We have demonstrated binding of monomeric alkyl sulfate to the cholesterol micelle at a level of approximately 0.5 mol/mol over a wide range of detergent concentrations. Since experimental limitations prevent binding measurements at lower unbound alkyl sulfate concentrations, we are unable to obtain the unitary free energy of this equilibrium process. However, detergents are known to form monolayers on many surfaces such as glass and air–water interfaces, so a monolayer adsorption of alkyl sulfate to the cholesterol micelle surface is not an unreasonable model for this complex.

(*2*) *Intrinsic Hydrophobicity of the Cholesterol Molecule.* We have previously shown that cholesterol has a significantly less negative free energy of transfer from water to hydrocarbon than is predicted from the cavity surface area of its hydrophobic component (Gilbert et al., 1975). This experimental determination of the hydrophobicity of cholesterol can be used to predict the free energy of transfer of the solute into a micelle with a fluid hydrocarbon interior, and by comparison of this calculated value with the experimental free energy of transfer the extent of head group interactions can be estimated. In the present system—alkyl sulfate micelles–cholesterol—we have observed an experimental unitary free energy as shown in Figure 4 and Table II of −10.3 kcal/mol. The interior of the alkyl sulfate micelle has a molar volume of approximately 230 ml/mol of alkyl sulfate monomer and cholesterol has a molar volume of 394 ml/mol of sterol monomer. We have previously shown a small dependence of unitary free energy of transfer on the ratio of molar volume solute/molar volume solvent (Gilbert et al., 1975) and using those data we find a free energy of transfer of cholesterol from water to hydrocarbon at a molar volume ratio of 1.7 of −5.8 kcal/mol. The polar OH group on the sterol contributes +5.0 kcal/mol to the unitary free energy of transfer from water to hydrocarbon. Thus, in the transfer of the sterol from water to a micelle in which the OH group is not immersed in the hydrocarbon medium we calculate a unitary free energy of −10.8 kcal/mol based on hydrophobicity alone. This number is in excellent agreement with our experimental value and suggests that the major component of the free energy is hydrophobic with little or no contribution from specific interactions between the sterol OH group and the detergent OSO_3^- polar head.

(*3*) *The Formation of Mixed Micelles between Choles-*

terol and Other Amphiphiles. We are particularly concerned about the interaction of cholesterol with lipids which are normally found in vivo. In light of the data presented in this paper and in the previous publications (Haberland and Reynolds, 1973; Gilbert et al., 1975) it is apparent that the intrinsic hydrophobicity of cholesterol is insufficient to account for the incorporation of 50 mol % into a phospholipid bilayer. Any lipid aggregate must compete with the self-associated form of cholesterol for the monomeric form and it is obvious that the hydrophobic free energy gained by removal of the monomer from water to a fluid hydrocarbon medium is approximately 2–3 kcal/mol too small for effective competition. One must then invoke an additional source of negative free energy for the phospholipid–cholesterol "mixed micelle" since in fact the sterol is incorporated at a 1:1 mole ratio. Self-association or phase separation of the cholesterol within a lipid bilayer is one possible source of such favorable free energy, but we have not observed such a phenomenon in alkyl sulfate micelles which have interiors that are indistinguishable thermodynamically from those of fluid bilayers (Stone, 1975). In the present studies less than 1 mol of cholesterol is always observed per mol of micelle.

The most probable source of an additional favorable free energy in the formation of cholesterol–phospholipid complexes is specific head group interactions between the sterol OH group and the polar moiety of the lipid which in turn may also lead to favorable alterations in the amount of bound water. Brockerhoff (1974) and Yeagle et al. (1975) have presented compelling evidence that the sterol OH is hydrogen bonded to the ester C=O oxygen of phosphatidylcholine. Newman and Huang (1975) have demonstrated complicated alterations in the amount of bound water on a phosphatidylcholine bilayer as a function of incorporated cholesterol and have emphasized the importance of variations in bound water on the stability of the cholesterol–lipid complex.

Our data suggest that there is no significant favorable head group free energy in the interaction between cholesterol and alkyl sulfate micelles. (Head group interactions include both possible hydrogen bonding and alterations in the state of hydration.) It is possible, of course, that the agreement between experimental free energy and calculated free energy based only on hydrophobicity is fortuitous and that, in fact, the hydrophobic free energy of incorporation into the micelle is less negative than predicted due to some steric effect. If this were the case, the head group interaction might be obscured and our apparent agreement between experiment and calculation might result from opposing effects. Further thermodynamic studies on the formation of cholesterol–amphiphile mixed micelles with both varying head groups and hydrocarbon chain lengths should aid in elucidating the exact interactions which occur between cholesterol and naturally occurring amphiphiles. These studies are currently underway in this laboratory with an emphasis on lysophospholipids. Such information is critical to an understanding of the forces involved in formation of both normal complex structures such as serum lipoproteins and membranes and abnormal structures such as atherosclerotic plaques.

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