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Cholesterol in Aqueous Solution: Hydrophobicity and Self-Association†

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ABSTRACT: Free energies of transfer of cholesterol monomer from water to organic solvents show that the hydrophobicity of this sterol molecule is significantly less than predicted from hydrophobic surface area considerations. It is suggested that this phenomenon may arise from unusual orientation of water molecules at the surface of the solute. From the direct measurement of the hydrophobic free energy of transfer and comparison with thermodynamic data on micelle formation reported previously we calculate specific attractive interactions between cholesterol monomers in the micelle of 2–4 kcal/mol, which suggests the possibility of self-association (phase separation) in mixed micellar systems such as sterol–lipid complexes.

Cholesterol is a biologically important amphiphile in mammalian systems as a component of cell membranes and serum lipoproteins. However, little is known about the physical properties of cholesterol in aqueous solution despite the fact that this binary system, cholesterol–water, must be well defined in order to describe the more complex systems sterol–lipid–protein–water.

We have previously shown that cholesterol forms a heterogeneous micelle in aqueous solvents at a critical micelle concentration of 20–40 $\times 10^{-9}$ M which corresponds to a unitary free energy of micellization of −12.6 kcal/mol (Haberland and Reynolds, 1973). The cholesterol micelle has a significantly lower partial specific volume than the monomer suggesting specific strong interactions between the monomers in the aggregated state. In order to verify this suggestion we need to know exactly how hydrophobic the cholesterol molecule is, *i.e.*, can the hydrophobic repulsion by water account entirely for the free energy of micellization, and, if not, how much free energy is derived from the specific interactions previously suggested by us? The studies in this paper were designed with this question in mind, but the results are of interest from another point of view as well: the hydrophobicity of the cholesterol molecule in water is significantly less than one would predict from current theory.

The unitary free energy of transfer of a nonpolar solute from water to hydrocarbon is a direct measurement of hydrophobicity (Tanford, 1973) and has been shown to be a regular function of the cavity surface area of the nonpolar

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solute in water (Reynolds *et al.*, 1974). Thus, each Å^2 of surface area of an alkane molecule contributes −20 to −25 cal/mol to the free energy of transfer from water to hydrocarbon. For amphiphilic molecules containing a polar group attached to an alkyl chain, the free energy of transfer is made up of independent contributions from each part. The contribution from the alkyl chain is again proportional to the cavity surface area and of the same magnitude as for the alkanes (Smith and Tanford, 1973; Harris *et al.*, 1973). The relationship between hydrophobicity and surface area holds for linear and branched alkyl chains and for simple saturated cyclic hydrocarbons (Harris *et al.*, 1973; Reynolds *et al.*, 1974), but no compound as complicated as steroids has yet been investigated. We report in this paper the determination of the unitary free energies of transfer of a number of aliphatic alcohols from water to hydrocarbon and have obtained from them the hydrophobic contribution (which depends in the normal way on the alkyl surface area) as well as the polar head group contribution. These results serve as reference data with which similar results for cholesterol are then compared.

Table I: Unitary Free Energies of Transfer of Alcohols and Cholesterol from Water to Hydrocarbon at 25°.

Solute	Solvent	Molar Volume Solute/ Molar Volume Solvent	kcal/mol $\mu^\circ_{HC} - \mu^\circ_W$	kcal/mol $\mu^\circ_{HC} - \mu^\circ_W$ at 1:1 Molar Volume Ratio
Hexanol	Heptane	0.85	−1.9	
	Tridecane	0.51	−2.1	−1.8
Octanol	Heptane	1.07	−3.6	−3.6
Decanol	Heptane	1.30	−4.9	−5.1
Dodecanol	Heptane	1.52	−6.1	−6.4
	Tridecane	0.92	−6.4	
Cholesterol	Heptane	2.67	−5.1	
	Octane	2.41	−5.2	−6.4
	Tridecane	1.61	−5.8	
	Octadecane	1.21	−6.2[a]	

[a] At 39°.

Experimental Procedure

Aliphatic alcohol and sterols were obtained from the following sources: [1-^{14}C]octanol and [1-^{14}C]decanol, International Chemical and Nuclear, Irvine, Calif.; [1-^{14}C]hexanol, American Radiochemical, Sanford, Fla.; [1-^{14}C]dodecanol and [1-^{14}C]cholesterol, Amersham Searle, Arlington Heights, Ill.

Since small amounts of water-soluble impurities would cause serious errors in partition experiments described below, the alcohols and cholesterol dissolved in organic solvent were washed exhaustively with aqueous solution until no change in the distribution coefficient could be observed. The radio and chemical purity of these compounds after partitioning were determined by silica gel chromatography in heptane–ether–acetic acid (70:30:2) (alcohols) or hexane–diethyl ether–acetic acid (80:20:1.5) (cholesterol). Hexanol, dodecanol, and cholesterol were >99% pure. Octanol and decanol were >92% pure and contained small amounts of radioactive contaminants which were not water soluble.

The [^{14}C]cholesterol supplied by Amersham-Searle was >97% pure before aqueous extraction of the organic phase 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 G, 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 2–3% contamination in the original sample was readily detectable and was removed by exhaustive aqueous extraction. The critical micelle concentration of cholesterol in aqueous solution was determined on all sterol samples and agreed with our previously published results (Haberland and Reynolds, 1973).

Partition experiments were performed using varying volume ratios of hydrocarbon to water. Heptane, octane, tridecane, octadecane, and benzene were used as the hydrocarbon phases. Equilibrium was rapidly established (<1 hr) with moderate shaking. Samples were removed from each phase with calibrated micropipettes and counted in either an Intertechnique SL 30 or Beckman LS 100 scintillation counter at 90% efficiency. The scintillation fluid contained 8.0 g of 2,5-diphenyloxazole, 0.4 g of 1,4-bis-[2-(5-phenyloxazole)] in 1 l. of Triton X-100 and 2 l. of toluene. Efficiency was constant for samples ≤0.25 ml.

The unitary free energy of transfer of a solute from water to hydrocarbon is obtained from the partition data by means of the following equation: $\mu^\circ_{HC} - \mu^\circ_W = RT \ln (X_W/X_{HC})$, where μ° is the appropriate standard chemical potential and X_{HC} and X_W are the mole fraction of solute in the organic and water phase, respectively. We have shown in a previous communication (Reynolds *et al.*, 1974) that $\mu^\circ_{HC} - \mu^\circ_W$ for alkanes is a regular function of the cavity surface area in which each square angstrom of hydrophobic surface area contributes 20–25 cal/mol to the free energy of transfer.

The free energies of transfer for alkanes, however, have been determined from water to the self-solvent. In the case of aliphatic alcohols and cholesterol the hydrocarbon phase is not the self-solvent and the ratio of the molar volume of the solute and solvent is not unity. In this latter case small nonideality effects on the free energy are expected due to nonideal entropy terms arising from unequal volumes of solute and solvent (Flory, 1942; Huggins, 1942). We have accordingly carried out distribution measurements using at least two different hydrocarbon solvents of different molar volumes. We have corrected for the nonideality arising from unequal solvent and solute molar volumes by extrapolating $\mu^\circ_{HC} - \mu^\circ_W$ to a hypothetical solvent with the same molar volume as the solute.

Surface areas of the alkyl chains relative to a CH_3 group were measured by the technique of Harris *et al.* (1973). These latter authors as well as Reynolds *et al.* (1974) reported surface areas relative to a *tert*-butyl group rather than a CH_3 group.

Results

The free energies of transfer of four normal aliphatic alcohols from water to hydrocarbon solvents are shown in Table I together with the extrapolated values of $\mu^\circ_{HC} - \mu^\circ_W$ in a hypothetical solvent of the same molar volume as the solute. The nonideality is seen to be very small for these solute molecules.

Figure 1 presents $\mu^\circ_{HC} - \mu^\circ_W$ from the last column in Table I for these alcohols as a function of the cavity surface

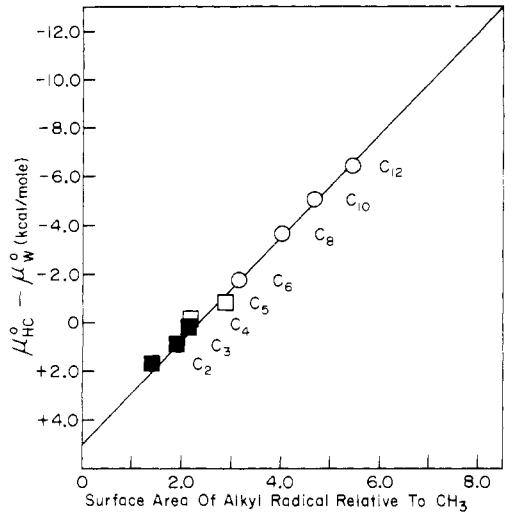

FIGURE 1: Free energy of transfer of aliphatic alcohols from water to hydrocarbon as a function of the cavity surface area formed by the solute in water. (○) Experimental; (□) calculated from Kinoshita *et al.* (1958) (see text); (■) calculated from Hill (1965) (see text).

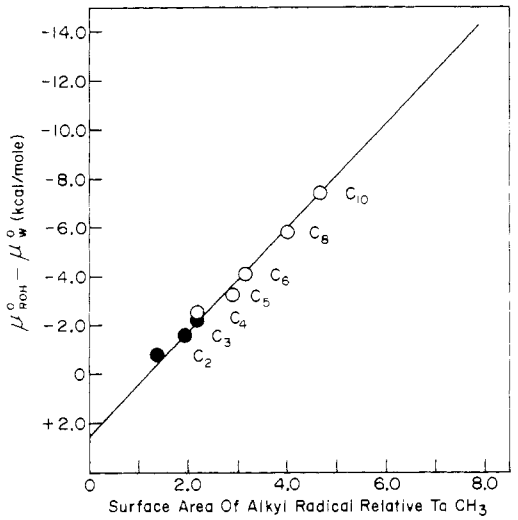

FIGURE 2: Free energy of transfer of aliphatic alcohols from the water to alcohol as a function of the cavity surface area formed by the solute in water. (○) Kinoshita *et al.* (1958); (●) Hill (1965).

area of the alkyl chain here expressed in relative units, with the area of a CH_3 group as unity. The intercept on the ordinate of +5.0 kcal/mol is the unitary free energy of transfer of an OH group from water to hydrocarbon. The slope of the line corresponds to −2.15 kcal/mol per relative surface area unit and can be converted to an absolute value by using the calculated surface area for a methyl group of 100 Å^2 (Reynolds *et al.*, 1974). Thus, each Å^2 of surface area of the alkyl chain contributes −21.5 cal/mol to the free energy of transfer from water to hydrocarbon. This is the same result within experimental error that is obtained from the alkane data if the same assumption is made for conversion of relative to absolute units.

Kinoshita *et al.* (1958) and Hill (1965) have determined the free energy of transfer of shorter chain alcohols from water to the self-solvent. These data are shown in Figure 2 as a function of the relative cavity surface area, and as would be expected each Å^2 of the alkyl chain again contributes −21.5 cal/mol to the hydrophobic free energy. A different intercept is obtained, however, corresponding to a

Table II: Surface Area and Predicted Hydrophobic Free Energy of Cholesterol.

	Relative Surface Area[a]	$\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ (kcal/mol) Predicted[b]	Obsd	$\mu^{\circ}_{ROH} - \mu^{\circ}_{W}$ (kcal/mol) Predicted[c]	Obsd
Fully folded	7.25	−9.4		−12.0	
			−6.4		−7.4
Fully extended	7.85	−10.6		−13.2	

[a] Surface area relative to a CH_3 group. [b] Calculated from Figure 1, corrected by 1 kcal/mol for C=C (Tanford, 1972). [c] Calculated from Figure 2, corrected by 1 kcal/mol for C=C (Tanford, 1972).

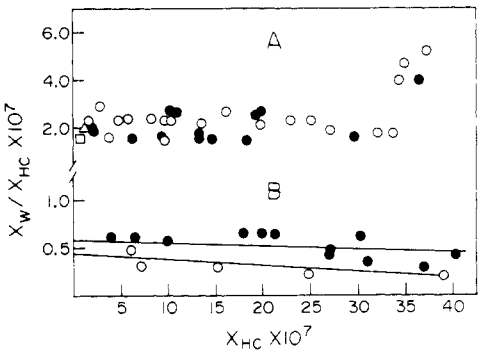

FIGURE 3: Partition ratio of cholesterol distributed between water and hydrocarbon at 25°. (A) (○) Phosphate buffer (pH 8.2, ionic strength 0.033)-heptane; (●) distilled water-heptane; (△) distilled water-octane; (□) 0.01 M HCl-heptane; (B) (○) distilled water-octadecane (39°); (●) distilled water-tridecane.

contribution of +2.5 kcal/mol instead of +5.0 kcal/mol from the OH group. This difference presumably reflects the formation of hydrogen bonds between the alcohol molecules—such bonds are, of course, not formed when alcohols are present at high dilution in pure liquid hydrocarbons. We have calculated $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ from these data for ethyl, propyl, butyl, and pentyl alcohol by correcting $\mu^{\circ}_{ROH} - \mu^{\circ}_{W}$ for these alcohols by +2.5 kcal/mol, and the results are also plotted in Figure 1.

By means of the data in Figures 1 and 2 it is possible to predict the free energy of transfer of cholesterol from water to hydrocarbon or from water to alcohol from a measurement of the relative surface area of the hydrophobic region of the sterol. There is an unknown factor, however, in that the large surface area of the steroid ring might lead to complete or partial folding back of the aliphatic tail of cholesterol in aqueous solution. Table II gives the relative surface areas of cholesterol in its fully extended and fully folded conformation together with the predicted $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ and $\mu^{\circ}_{ROH} - \mu^{\circ}_{W}$.

The partition ratio, X_W/X_{HC}, for cholesterol between water and three different hydrocarbons is shown in Figure 3. No significant concentration effect is observed over the wide range of concentrations investigated, and we interpret the results as referring essentially to the state of infinite dilution. Values of $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ for cholesterol were obtained under a variety of other conditions. It was found that $\mu^{\circ}_{HC} - \mu^{\circ}_{W}$ is not a function of temperature between 4 and 25°, ionic strength between 0 and 0.033, or pH between 2 and 8.3.

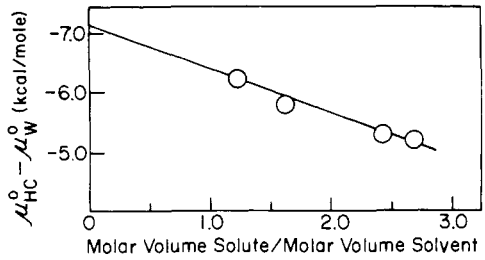

FIGURE 4: The unitary free energy of transfer of cholesterol from water to hydrocarbon as a function of the molar volume of the solvent.

Figure 4 shows the linear extrapolation of $\mu^\circ_{HC} - \mu^\circ_W$ as a function of the ratio of molar volume of cholesterol to molar volume of the solvent, and the ideal value at a molar volume ratio of unity is given in both Tables I and II.

The free energy of transfer of cholesterol from water to octanol was also determined and $\mu^\circ_{ROH} - \mu^\circ_W$ corrected to a solvent with the same molar volume by assuming that the dependence on molar volume is the same as that in Figure 4. This experimental value is also given in Table II together with the predicted $\mu^\circ_{ROH} - \mu^\circ_W$ from surface area measurements.

It is apparent from the data in Table II that $\mu^\circ_{HC} - \mu^\circ_W$ and $\mu^\circ_{ROH} - \mu^\circ_W$ are 3-6 kcal/mol less favorable than predicted by the hydrophobic surface area. Because of the lack of concentration dependence seen in Figure 3 any possible influence of aggregation on these results would seem to be excluded. We have, in addition, used vapor pressure osmometry to determine the molecular weight of cholesterol in a hydrocarbon solvent in order to eliminate the possibility of aggregation. At 5.7×10^{-2} M cholesterol, the molecular weight was 408 in benzene solution which is within experimental error of the monomer weight of 387. Since the concentration employed for these measurements is much higher than the concentration in the organic phase used in the distribution experiments, there is no possibility of cholesterol aggregation in the organic phase in these experiments. Similar measurements for the aqueous phase, to be significant, would have to be made below the cmc and this makes the available concentration too low for any known technique for molecular weight determination. However, the lack of concentration dependence of X_W/X_{HC} in Figure 3 is sufficient proof that no association in the aqueous phase can be present—any difference between the state of aggregation in the two solvents would necessarily lead to a concentration dependent ratio. (The very small concentration effect in tridecane and octadecane seen in Figure 3 is in the wrong direction for association in the aqueous phase). Thus, the results in Table II refer unambiguously to the transfer of monomer.

It has been reported (Christian *et al.*, 1970) that 2-3 mol of excess water are transferred to an organic solvent with 1 mol of decanol. We have investigated the solubility of ^3H_2O at unit activity in heptane in the presence and absence of hexanol and cholesterol; 1 mol of ^3H_2O/mol of hexanol appears to be transferred to the hydrocarbon phase in excess of the normal solubility of water, and we attribute this to the exchangeable proton on the OH group; 3 mol of ^3H_2O/mol of cholesterol was found to be transferred to the hydrocarbon phase. Correcting this value for one exchangeable proton, we conclude that a maximum of 2 mol of ^3H_2O is bound to 1 mol of cholesterol in hydrocarbon solvent. Thus, the affinity of cholesterol for water in a hydrophobic medium does not appear to differ markedly from that of normal aliphatic alcohols.

The free energies of transfer of cholestenone and β-sitosterol from water to heptane were also determined and found to be nearly identical to that of cholesterol. Thus, the anomalously low free energy of transfer of cholesterol is also observed in other sterols of similar structure containing only one polar moiety, a fused nonresonating ring system, and a long alkyl chain.

Discussion

The cholesterol monomer has been shown to have a significantly less negative free energy of transfer from water to hydrocarbon than is predicted from the cavity surface area of its hydrophobic component. This is not the result of aggregation at low concentrations or unusual affinity for water in the hydrocarbon phase. Since all other systems thus far investigated—alkanes, alcohols, carboxylic acids, dextromethorphan alkyl sulfates—have free energies of transfer that are identically linearly related to their hydrophobic surface areas (Smith and Tanford, 1973; Tanford, 1972, Harris *et al.*, 1973), we must conclude that the unique structure of the hydrophobic portion of cholesterol allows a particularly favorable packing of water molecules around the sterol in the aqueous phase. The effect of cavity shape on the solution energetics of hydrophobic compounds in water is unknown and special surface effects may occur between the solute and the bulk water phase which have not yet been investigated.

Using the experimental value of $\mu^\circ_{HC} - \mu^\circ_W$ for cholesterol the hydrophobic contribution to the free energy can be calculated. Thus, −6.4 kcal/mol corrected by +5.0 kcal/mol as the polar head group contribution gives −11.4 kcal/mol for the hydrophobic contribution to the free energy of transfer. This represents an absolute upper limit to the free energy of micellization for a normal micelle formed solely as a result of hydrophobic interactions. In most cases, the hydrophobic contribution to the free energy of micellization is less than $\mu^\circ_{HC} - \mu^\circ_W$ because the hydrophobic moiety of an amphiphile in a micelle is more constrained than when free in solution. In addition, some repulsion between polar groups is required to account for the existence of micelles of finite size, and the repulsion makes a positive contribution to the free energy.

Another approach to the estimation of a normal free energy of micellization is to use the fact that normal aliphatic alcohols do not form micelles, but instead form separate phases. If we consider the micellization of cholesterol to be analogous to phase separation, we calculate $\mu^\circ_{mic} - \mu^\circ_W$ = −11.4 kcal/mol (hydrophobic) + 2.5 kcal/mol (polar head group) = −8.9 kcal/mol. From a study of mixed micelle formation between aliphatic alcohols and carboxylic acids, it has been estimated that the theoretical free energy of micellization for alcohols is ~0.4 kcal/mol more positive than the free energy of phase separation (Tanford, 1973). Thus, the calculated value of −8.9 kcal/mol for the free energy of micellization of cholesterol is an upper limit. The experimental value is −12.6 kcal/mol (Haberland and Reynolds, 1972)—not only far greater than −8.9, but also greater than the absolute upper limit of −11.4 kcal/mol. This analysis further supports the view that there are specific attractive forces between monomers in the cholesterol micelle which provide the additional favorable free energy component over and above the hydrophobic free energy.

The hydrophobic component is unique to an aqueous medium, but specific attractive forces should be independent

of solvent and thus cholesterol self-association should exist under a variety of conditions. Several investigators have reported phase separation of cholesterol in mixed monolayer and bilayer systems (Gershfeld and Pagano, 1972; Huang *et al.*, 1974). Although we did not see self-association at mole fractions <0.01 of cholesterol in hydrocarbon solvents, the sterol often is incorporated in monolayers and bilayers at mole fractions as high as 0.5. Studies of the thermodynamics of mixed micelle formation between cholesterol and a variety of other amphiphiles are currently underway in our laboratory in order to clarify the forces involved in biological structures containing cholesterol–lipid complexes.

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