Cholesterol Solubilization in Aqueous Micellar Solutions of Quillaja Saponin, Bile Salts, or Nonionic Surfactants

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Quillaja saponin in aqueous solution enhanced cholesterol solubility by as much as a factor of 10^3 at room temperature. Increased temperature and [NaCl] increased cholesterol solubility, whereas solubility was greatest at an aqueous pH of 4.6 at 298 K. Although various saponin sources were observed to differ in their abilities to solubilize cholesterol, trends in their solubilization properties with changing aqueous phase parameters were consistent. Surfactant molecules containing fused-ring structures as their hydrophobic portion, such as sodium cholate, sodium deoxycholate, and quillaja saponin, solubilized cholesterol significantly better than the linear hydrocarbon chain surfactants Tween 20 and Triton X-100. Mixtures of surfactants studied were found to exhibit synergistic effects: they formed micelles at lower concentrations than did those formed by the individual surfactants themselves, and they had a better ability to solubilize cholesterol. The knowledge obtained from these studies improves our understanding of cholesterol association with saponin and other types of surfactants and enhances the potential for using saponins for the solubilization and extraction of hydrophobic solutes in various pharmacological and industrial applications.

Keywords: *Micellar solutions; Triton X-100; Tween 20; surfactant mixtures; surface tension*

INTRODUCTION

Quillaja saponins are naturally occurring amphiphilic molecules, consisting of various sugar groups linked by a glycosidic bond to a hydrophobic triterpene ring. Our recent investigations of the self-assembling properties of this substance in aqueous solution (*1*, *2*) have established conditions and attributes associated with saponin micelles, including the micelles' capacity to incorporate a hydrophobic dye solute, dichlorofluorescein. In the present study the objective was to explore the ability of aqueous quillaja saponin solutions to solubilize a biologically important solute, cholesterol, and to compare those solubilization properties with those of other surfactant molecules.

There are several motivations for studying cholesterol solubility in saponin micelles. As naturally derived compounds from the South American tree *Quillaja saponaria*, food grade saponins are attractive surfactants for use in food, cosmetic, and pharmaceutical applications. In many of these applications cholesterol will also be present. Because they form micelles in water, saponins have the potential to enhance aqueous solubilities of hydrophobic substances, such as cholesterol, a capability that has many practical applications. For example, the efficacy of quillaja saponin derivatives to improve insulin delivery has recently been explored (*3*). The ability of quillaja saponins to boost vaccine effectiveness (*4*−*6*) has led to its use in "iscoms" (immune stimulating complexes) (*7*, *8*), in which it is combined with cholesterol, other lipids, and the immunogen. Cholesterol−saponin interactions are also believed to be important in determining the ability of saponin to increase intestinal permeability to drugs (*9*).

Our study of the cholesterol solubilization properties of quillaja saponin micelles is also motivated by human health concerns about cholesterol. It has been shown that consumption of saponins can lower serum cholesterol levels in vivo (*10*, *11*). Furthermore, in view of the negative health implications associated with cholesterol consumption (*12*−*15*), there is a strong incentive to limit dietary cholesterol (*16*−*18*) by extracting cholesterol from foods. The Food and Drug Administration currently recommends a daily upper limit of 300 mg of dietary cholesterol for an average person, and a limit of 200 mg per day for those at high risk of heart attack (*19*). To put these numbers in perspective, a pat (one serving) of butter has 11 mg, one egg has 215 mg, an 8 oz glass of whole milk has 33 mg, and a 1 oz serving of cheddar cheese contains 30 mg of cholesterol. Removing such small amounts of cholesterol from foods requires an extremely selective process, yet one which can be adaptable to a large scale. Once extracted, cholesterol and other sterols have commercial value as additives to skin lotions and cosmetics and as building blocks for pharmaceutical agents (*20*).

The use of micellar solutions for cholesterol removal (*21*, *22*) offers distinct economic and environmental benefits over other methods. For example, the desired amount of solute to be extracted by the micellar solution can be controlled simply by varying the aqueous phase conditions, whereby the capacity of micelles to "capture"

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the solute changes. Other advantages include convenient scale-up employing liquid−liquid extraction-based technologies, for which low temperatures and pressures are often effective for satisfactory extraction. Moreover, such extractions utilize aqueous solvents, which when combined with food grade surfactants alleviate environmental and health concerns. Micellar extractions have been widely researched in the detergent, pharmaceutical, and environmental fields for various industrial applications, but their use has not been well explored in the food industry.

In this study, we investigated cholesterol solubilization in aqueous solutions of a naturally occurring food grade surfactant, quillaja saponin. Previously, we showed that saponin forms micelles in water above a critical micelle concentration (cmc) and found that various aqueous phase parameters (temperature, salt concentration, and solution pH) affect the micellar properties of saponin (*1*). Our research also demonstrated that cholesterol in solution affects the micellar properties of saponin (*2*). In the present work, we explore how those micellar properties play a role in cholesterol solubilization in aqueous saponin solutions.

There is some evidence that saponins as a class of compounds may have unique abilities to interact with cholesterol. Digitonin, a saponin with a steroidal hydrophobic moiety, forms 1:1 complexes with cholesterol that precipitate in aqueous solution (*23*). Saponins similar to digitonin but with fewer sugar groups complex more weakly (*23*). Bile salts, another surfactant type containing a steroidal hydrophobic subunit, are known to interact in micellar form with cholesterol. In both cases it is likely that hydrophobic interactions between cholesterol and digitonin molecules (*23*) and between cholesterol and bile salt micelles (*24*) are important, although there is also speculation on the role of hydrophilic interactions in these systems (*23*−*25*).

Like these other molecules, quillaja saponin differs from most conventional surfactants in that its hydrophobic subunit is a fused-ring structure (a triterpene), rather than an alkane-based chain. To examine the influence of this molecular structure on cholesterol solubilization, we compared cholesterol solubility in solutions of Tween 20 or Triton X-100 with that in solutions of bile salts or quillaja saponin. The ability of mixtures of these surfactants to dissolve cholesterol was evaluated as well. Surfactant mixtures are known often to possess synergistic effects—in that the mixed micellar solutions achieve higher solute concentrations than do solutions of either surfactant component. Our experiments with saponin−bile salt mixtures, in particular, shed light on the hypercholesteremic effect of saponin in vivo.

EXPERIMENTAL PROCEDURES

Materials. Quillaja saponin was obtained from Sigma Chemical Co. (St. Louis, MO), Acros Organics (Fair Lawn, NJ), and Penco of Lyndhurst Inc. (Lyndhurst, NJ). A molecular weight of 1650 was used to represent this molecule (*1*). Quillaja saponin from Sigma was obtained at two levels of purity: the lower grade (S-7900) is extracted from quillaja bark, whereas the higher grade saponin (S-4521) undergoes further refinement to remove low molecular weight tannins. Henceforth, the different purities of these two saponins will be referenced by their catalog numbers. Sodium chloride, used for investigating the effect of salt concentration on cholesterol solubilization in saponin, was purchased from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid at a concentration of 17.4 M, sodium acetate, sodium carbonate, and sodium bicarbonate were used for preparing buffer solutions at low salt concentrations, which were then employed in studying the influence of pH on the cholesterol solubilization in saponin solutions. These components were all obtained from Fisher Scientific. Polyoxyethylene isooctylphenyl ether, also known as Triton X-100, was obtained from LabChem Inc. (Pittsburgh, PA), whereas polyoxyethylene sorbitan monolaurate (Tween 20) and the bile salts sodium cholate and sodium deoxycholate were purchased from Sigma Chemical Co.. The molecular weights of these surfactants are 625 (Triton X-100), 1225 (Tween 20), 430.6 (sodium cholate), and 414.6 (sodium deoxycholate). All chemicals were used as received. Cholesterol (386.66 MW) purchased from Eastman Kodak Co. (Rochester, NY) was recrystallized with absolute ethanol. Radiolabeled [4-^{14}C]cholesterol (specific activity = 56.6 Ci/mol) was obtained from DuPont NEN (Wilmington, DE).

Methods. Cholesterol detection in aqueous saponin solutions was achieved through radioactive scintillation counting. Cholesterol crystals were washed with cold ethanol and then with doubly distilled water to remove traces of ethanol and then immediately placed in a vacuum desiccator to dry in the absence of oxygen. The purity of the cholesterol was confirmed by thin-layer chromatography (*26*). Cholesterol undergoes oxidation when in contact with oxygen (*27*, *28*), and hence all solubility measurements were performed with degassed doubly deionized distilled water. Radiolabeled [4-^{14}C]cholesterol when purchased had a concentration of 0.71 μmol/mL in ethanol and was stored in the freezer. A known microvolume of this radioactive solution was pipetted into an ethanol solution containing non-radioactive recrystallized cholesterol and stored in the freezer, yielding a solution of known specific activity.

For solubility experiments, an appropriate volume of this solution was pipetted into a plastic vial and the ethanol gently evaporated under a stream of argon. After the ethanol solution was evaporated, 10 mL of aqueous surfactant solution was added to the remaining cholesterol precipitate. The air in the vial headspace was replaced with argon and the vial well-sealed. The mass of cholesterol in the resulting mixture was designed to be well in excess of the aqueous cholesterol solubility of the surfactant solution at that concentration. Aqueous surfactant solutions of different concentrations were prepared in this way. The vials were agitated (shaker speed = 5) in a Gyrotory water bath shaker model G76 (New Brunswick Scientific Co. Inc., Edison, NJ) at a constant temperature for 3 days, a length of time that proved to be sufficient to attain equilibrium. A period of 7 days was required for equilibration of bile salt solutions.

After equilibration, the solutions were filtered with 0.1 μm filters (Millipore, Bedford, MA), and 1.0 mL of the filtrate was pipetted into a scintillation vial (Wheaton Scientific, Millville, NJ) followed by the addition of 9 mL of scintillation fluid (ScintiSafe Econo 1) purchased from Fisher Scientific. Counting was done in a Tri-Carb Packard 1500 liquid scintillation counter (Downers Grove, IL), and quenching corrections were made. To check the possibility of undissolved cholesterol crystals passing through the 0.1 μm filters, we also filtered the solutions with 0.05 μm filters and obtained identical results (<0.2% random deviations). However, when 0.22 μm filters were used, larger cholesterol concentrations (by 16%) were obtained, suggesting the presence of cholesterol aggregates between 0.1 and 0.22 μm in size. Concentration determinations using radioactivity were reproducible within 3%.

Surface tension measurements on solutions of quillaja saponin (S-7900) from Sigma indicated that this mixture contains a highly surface active component not present in some of the other sources of saponin (*1*, *2*). Quillaja saponin (S-7900) was treated by acetone precipitation in methanol solutions (*29*), to extract the more surface active fraction. Approximately 50 g of the saponin was dissolved in 200 mL of water and extracted twice with 200 mL of butanol. The combined butanol was removed using a rotary evaporator and the residue dissolved in 200 mL of hot methanol. Approximately 1 L of acetone was then added to the beaker and allowed to sit overnight. During this time, saponin was observed to precipitate, and the solution was filtered using filter paper. The

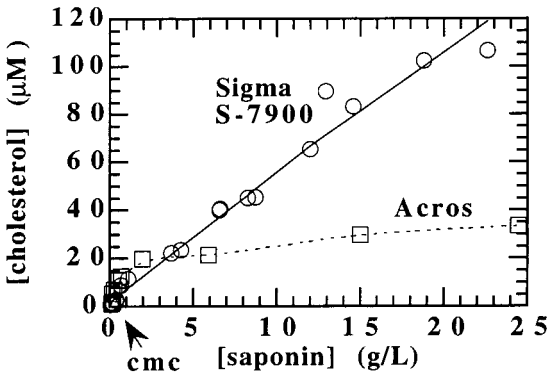

Figure 1. Effect of saponin concentration on cholesterol solubilization at 298 K.

residue was scraped off the filter paper and dried for at least 24 h in a vacuum desiccator to obtain the extracted saponin powder.

Surface tension measurements were made using a Wilhelmy plate Krüss 10 ST (Krüss, Charlotte, NC) equipped with a water bath. The Wilhelmy plate was cleaned by gentle rinsing with doubly distilled water and then flame-heated until just glowing. The glass sample vessel was thoroughly rinsed with ethanol, then repeatedly rinsed with doubly distilled water, and baked to dryness. For the most part, surface tension values equilibrated in an hour, and measurements were always taken after equilibration.

RESULTS AND DISCUSSION

Although there have been numerous studies of cholesterol solubilization in bile salt solutions alone (see, e.g., refs *24* and *30*−*36*), relatively little work has been done to investigate systematically cholesterol solubilization in other surfactants (*26*, *37*−*40*). In the following sections, we present our results obtained from studying cholesterol solubilization in aqueous saponin solutions as a function of solution temperature, salt concentration, and pH. These parameters were found to affect strongly both the tendency of saponin micelles to form as well as the sizes of the micelles thus formed (*1*, *2*). Room temperature, neutral aqueous solutions were a particular focus, because of the importance of these conditions in formulation and extraction applications and in order to facilitate comparison with most surfactant characterization studies. Because of the variations in composition of different sources in saponin (*1*, *2*), we investigated cholesterol solubilization in some of these sources and compared our results with those in bile salt or nonionic surfactant solutions. Finally, we studied cholesterol solubilization in mixed surfactant systems to explore the possibility of synergistic interactions between binary surfactants, which could result in higher cholesterol solubility in mixed micelles.

Effect of Saponin Concentration on Cholesterol Solubilization. Figure 1 presents measured cholesterol solubility in aqueous solution as a function of quillaja saponin concentration. Two different sources of saponin are shown. Cholesterol is effectively solubilized in both saponin solutions, with solubility increasing with increasing saponin concentration. For saponin from Sigma S-7900, there is a linear enhancement in cholesterol solubility with increasing saponin concentration. This linear dependence is consistent with a micellar solubilization mechanism, although the sensitivity of our experiments did not allow us to detect whether a break occurs at the reported cmc (*2*). Because the solubility of cholesterol in pure water is ~10−60 nM (*41*), Figure 1 indicates that a 20 g/L solution of quillaja saponin is able to enhance the solubility of cholesterol by a factor of 10^3−10^4.

The cholesterol solubilization behavior in Acros saponin solutions is qualitatively different. The relationship between solubility and surfactant concentration is nonlinear (Figure 1), with solubility approaching a limiting value at high saponin concentrations. This behavior may be a result of greater inhomogeneity of the Acros product, creating a mixed surfactant solution having micellar properties that change as a function of surfactant concentration. A nonlinear relationship between cholesterol and aqueous solubility was also observed by Ueno et al. (*42*) when examining aqueous solutions containing mixtures of sodium cholate and $C_{12}E_8$. Interestingly, the solubility curve shown in Figure 1 is quite different from that exhibited by the hydrophobic dye dichlorofluorescein in Acros saponin solutions (*1*), suggesting a somewhat different interaction between cholesterol and the saponin molecules.

Effect of Temperature. Increasing temperature was found to enhance cholesterol solubilities strongly in aqueous saponin (S-7900 and Acros) solutions, as shown in Figure 2a,b. The dependence of solubility on temperature is nonlinear, with an enhanced effect of temperature at higher values (Figure 2c). With increasing temperature the sizes of the saponin and cholesterol-saturated saponin micelles were also observed to increase (*1*, *2*). Experimentally, it is well-known that micellar size increases and micellar elongation are often associated with increased solubilization capacity for a large number of surfactant/solute systems (*43*). Cholesterol solubility in water alone is also expected to go up at higher temperature, which may also play a role in the results of Figure 2. The influence of temperature on cholesterol solubility in quillaja saponin solutions appears to be significantly stronger than that reported for bile salt and other micellar solutions (*42*).

Interestingly, although temperature had a stronger effect on the sizes of S-7900 micelles compared to those of the Acros saponin micelles, increasing temperature enhanced cholesterol solubilities more dramatically in Acros saponin solutions than in S-7900 saponin solutions. At 315 K the dependence of solubility on saponin concentration is comparable for the two sources, in distinct contrast to results at 298 K. The dependence on Acros saponin concentration also becomes linear at higher temperatures. These observations may point to the importance of other influences in these temperature studies. With mixed surfactants such as quillaja saponin, changes in temperature could alter the role played by various components in micelle formation, thus affecting the solubilization properties of some mixtures more than others.

Salt Effects. The effect of adding sodium chloride is to increase cholesterol solubility in saponin solutions, as shown in Figure 3. This increase was less dramatic for saponin from Acros (Figure 3b) than for saponin Sigma S-7900 (Figure 3a). From previous work, we found that increasing the salt concentration decreased the cmc of the saponin micelles strongly but did not have a discernible effect on micelle size or aggregation number for all sources of saponin studied (*1*). A decreased cmc would shift the cholesterol solubilization curves but have no effect on the slopes of the curves. In contrast, the dependence of cholesterol solubility on saponin concentration increases significantly with added

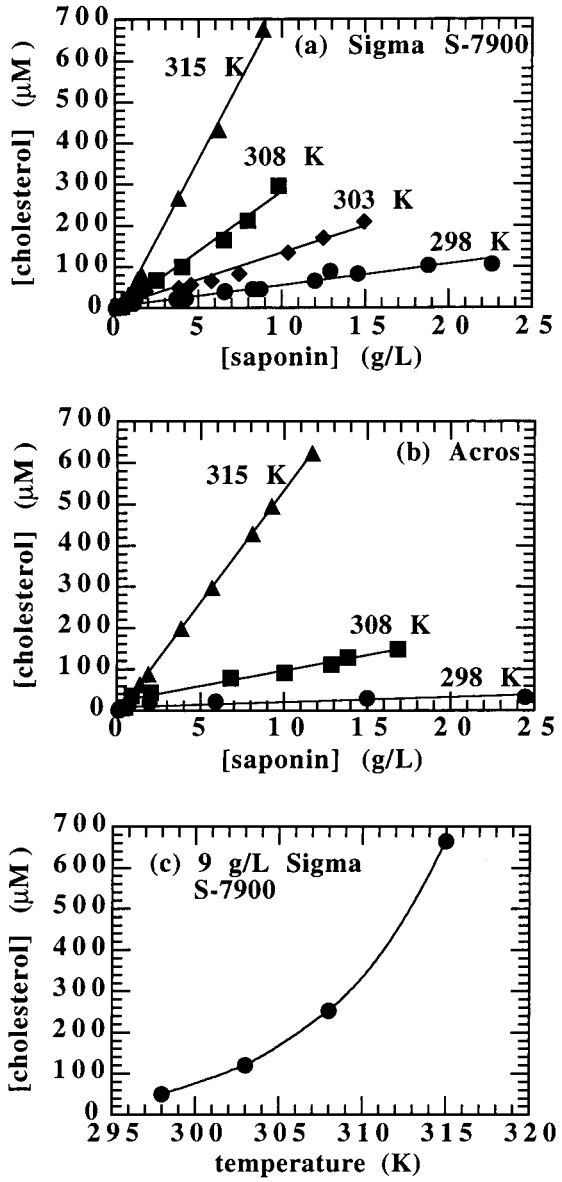

Figure 2. Cholesterol solubility in saponin solutions (a, from Sigma S-7900; b, from Acros) of varying concentrations and at different temperatures. (c) Cholesterol solubility in a 9 g/L solution of saponin from Sigma S-7900 as a function of temperature.

salt. Because the size of the micelles themselves does not appear to be influenced by salt concentration, salt most likely influences the cholesterol/saponin interactions directly. For example, higher salt concentrations may reduce the solubility of cholesterol in pure water due to a salting-out effect, thereby shifting the partitioning of the solute toward the micellar aggregates.

Effect of Solution pH. The quillaja saponin molecule contains glucuronic acid as part of its headgroup, a moiety with a p*K* of 3.18 in water (*44*). Hence, the solubilization properties of saponin solutions can potentially be influenced by varying solution pH values. Solubilization studies with other surfactants containing acid or base groups that exhibit effects of pH on their micellization properties have received significantly less attention in the literature than solubilization studies with surfactant salts.

Figure 4 shows the effect of changing solution pH on cholesterol solubilization in aqueous saponin solutions at 298 K. Our results indicate that, for both sources of

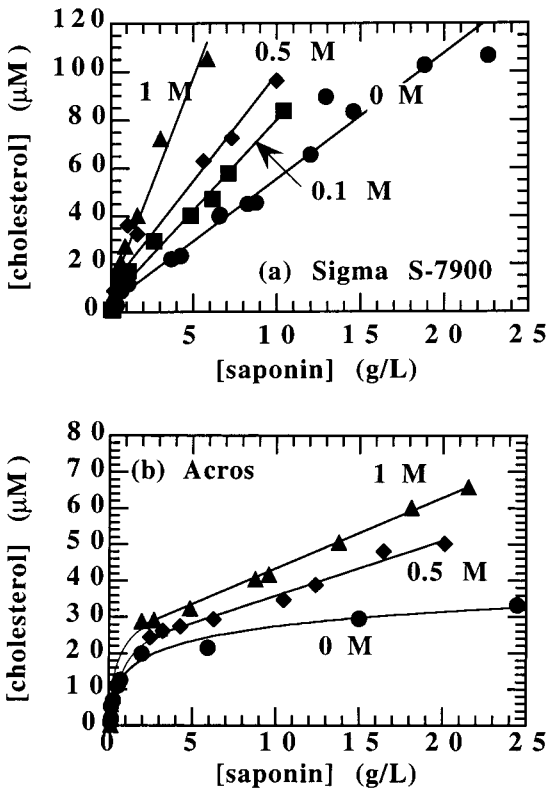

Figure 3. Cholesterol solubility in saponin solutions (a, from Sigma S-7900; b, from Acros) at 298 K at different [NaCl].

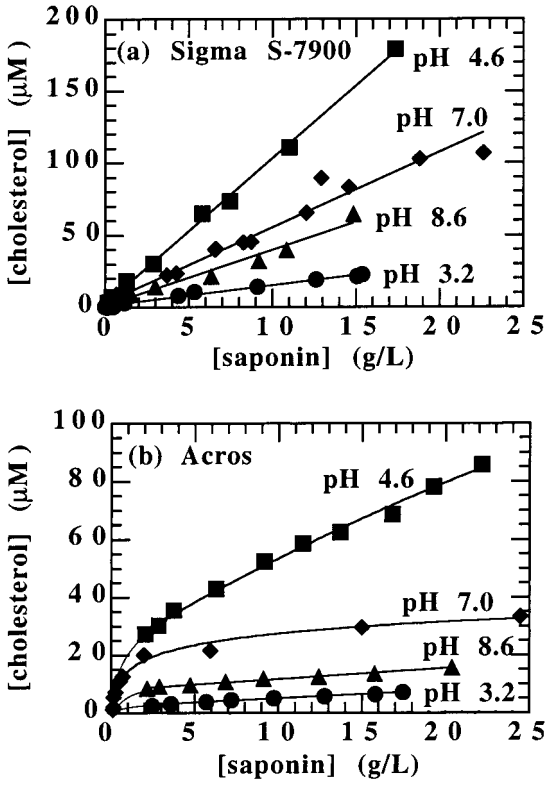

Figure 4. Cholesterol solubility in saponin solutions (a, from Sigma S-7900; b, from Acros) at 298 K at different pH values.

saponin, there exists an optimum solution pH near 4.6 at which cholesterol solubilization is highest. From our earlier work (*1*), we found the effect of increasing pH from 3.2 to 9 was to raise the cmc value of saponin micelles, without significantly influencing the micelle

size or aggregation number. Thus, the clear change in the solubilization capacity of both Sigma S-7900 and Acros saponin with varying pH value cannot be attributed to substantial changes in the micelle size.

The effective p*K* of the saponin micellar solution can differ significantly from the p*K* of functional groups on the quillaja saponin molecule. Shifts in the p*K* can be caused by interactions between acid groups within the micelle and by contributions of minority components within the saponin mixture. Our previous results (*1*) indicated that the cmc of quillaja saponin increased significantly at two pH values, one between a pH of 3 and 4.4 and again at a pH between 7 and 9. Both of these values are above the p*K* for glucuronic acid. The optimum pH of 4.6 observed for cholesterol solubilization in both sources of quillaja saponin is between 1 and 2 units higher than the p*K* for glucuronic acid, consistent with the lower of the two "effective pK values" observed with our cmc measurements. If we assume that there exists an effective p*K* of the saponin micelle between pH 4 and 5, then at a solution pH away from this value the micellar composition will be approximately constant. For example, at a pH of 3.2 the molecules will be predominantly uncharged. However, near the effective p*K*, the ratio of charged and uncharged species varies rapidly, creating mixed micelles (*45*) having solubilization properties that will also vary strongly with pH values. As discussed below, mixtures of surfactants often exhibit synergistic solubilization effects. Thus, a pH of 4.6 may create an optimal mixture of species, causing the enhanced cholesterol solubilization in both Sigma S-7900 and Acros saponin micelles.

Interestingly, an "optimum" solution pH of 8.3 has been reported for cholesterol solubilization in sodium deoxycholate solutions at 303 K (*32*). This optimum differs from the reported p*K* of 6.6 for a bile salt molecule (*46*). Although the cmc values of sodium deoxycholate micelles did not show a clear change in this pH range, they did tend to increase slightly (*32*). Results from experiments with this homogeneous and well-studied bile salt surfactant thus appear to be qualitatively similar to our measurements with quillaja saponin and point to the complex effect of pH on cholesterol solubilization in micelles containing surfactants with acid groups.

Effect of Surfactant Structure. Unlike conventional surfactant molecules such as Tween 20 and Triton X-100, which have a straight-chain hydrocarbon tail and a hydrophilic headgroup, quillaja saponin has a hydrophobic fused-ring (a triterpene) structure to which are attached a number of sugar groups such as galactose, xylose, rhamnose, and fucose. In this respect, its structure somewhat resembles that of bile salt molecules such as sodium cholate (NaC) and sodium deoxycholate (NaDC), which have fused-ring steroidal structures to which are attached hydroxyl groups. The schematic structures of these different surfactants are shown in Figure 5.

Given the structural resemblance between bile salt and quillaja saponin molecules, we compared cholesterol solubilization in the fused-ring surfactants, NaC, NaDC, and saponin, with that in alkane-based nonionic surfactants Tween 20 and Triton X-100 (Figure 5). In Figure 6 is shown cholesterol solubility in solutions of these different surfactants. Due to the large differences in molecular weights of these different surfactants in this figure, we report solubilization data on a mole of

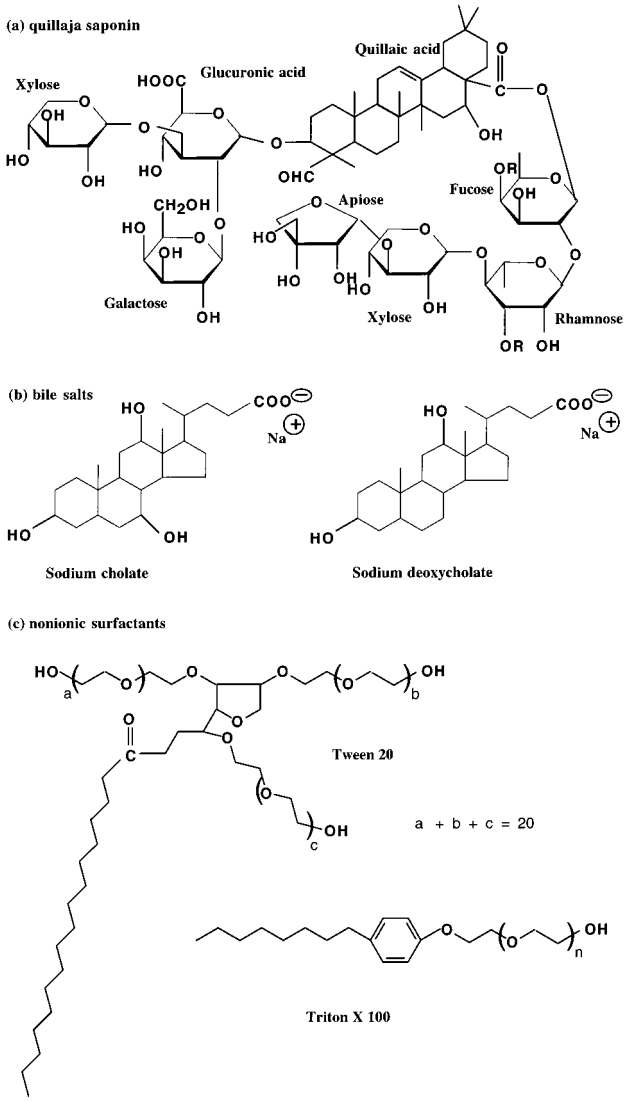

Figure 5. Structure of various surfactants used in this study: (a) quillaja saponin; (b) bile salts; (c) nonionic surfactants.

Table 1. Cholesterol Solubilization Ratio *S* (Mass and Molar Bases) for Different Surfactants at 298 K

surfactant	*S* (mole basis)	*S* (mass basis)
quillaja saponin (Sigma S-7900)	8.2×10^{-3}	1.9×10^{-3}
sodium deoxycholate (NaDC)	7.5×10^{-3}	7.0×10^{-3}
sodium cholate (NaC)	5.6×10^{-3}	5.0×10^{-3}
Triton X-100	2.9×10^{-3}	1.8×10^{-3}
Tween 20	8.2×10^{-4}	2.6×10^{-4}
quillaja saponin (Acros)	1.0×10^{-3}	2.4×10^{-4}
quillaja saponin (Penco)	*a*	1.5×10^{-4}

[a] The molecular weight of saponin from Penco is unknown due to the heterogeneity of its composition.

cholesterol per mole of surfactant basis. As shown in Figure 6a, solutions with Sigma S-7900 saponin and the two bile salts all show strong linear increases in cholesterol solubility with increasing micelle concentration, with comparable slopes for the three surfactants. The offset in the curves from each other is a result of the significantly higher cmc for the bile salts (*25*) than for saponin. The lipid-based surfactants shown in Figure 6a are all better solubilizing agents than is Triton X-100 or Tween 20 (Figure 6b).

To analyze further the relative abilities of these different molecules to solubilize cholesterol, we report

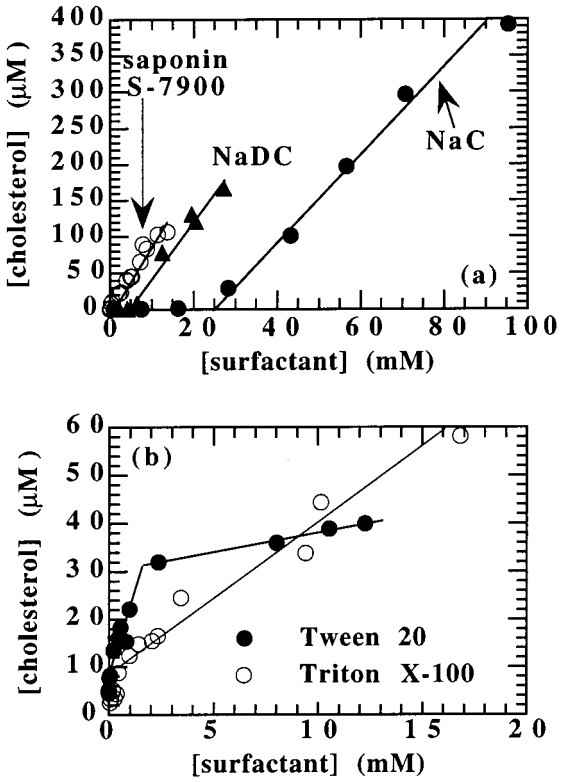

Figure 6. Cholesterol solubility in various surfactants at 298 K: (a) saponin S-7900 and bile salts; (b) nonionic surfactants.

in Table 1 the cholesterol solubilization ratio (S) obtained directly from the slope of the lines in Figure 6 above the cmc values. This ratio is defined as the number of moles of cholesterol solubilized per mole of surfactant, above the cmc. The reciprocal of the molar solubilization ratio indicates the number of surfactant molecules required to solubilize one molecule of cholesterol. From Table 1, we see that it takes considerably fewer saponin or bile salt surfactant molecules to solubilize one molecule of cholesterol than that required by the surfactants with a linear alkane tail. We speculate that the superior solubilization capacity of the fused-ring surfactants may be due to a shared mechanism of interaction of cholesterol with the surfactant aggregate, given the similarity in structure among bile salt, saponin, and cholesterol molecules. However, other properties of saponin micelles may also play a role in their ability to incorporate cholesterol, in particular the likelihood of mixed micelle formation due to the heterogeneity of the saponin mixture.

To appreciate the enhancement of cholesterol solubility achieved by all of the surfactants in Table 1, we define a water−micelle partition coefficient K as

$$K = \frac{\dfrac{\text{mass of cholesterol in micelle solution}}{\text{mass of micellized surfactant}}}{\dfrac{\text{mass of cholesterol in water (no micelles)}}{\text{mass of water}}}$$

The denominator in this equation is obtained from our measured aqueous cholesterol solubility data. Values of K for the surfactants in Table 2 range from 5×10^3 to 1×10^5, indicating that cholesterol strongly prefers the micellar environment over that of the aqueous phase.

Effect of Various Sources of Saponin. Earlier studies with quillaja saponin have shown that the source of saponin has an influence on its micellar (*1*, *2*) and cholesterol extraction properties (*21*, *22*). Because saponin is a complex biological mixture, its composition may differ depending on the method of preparation. Such changes in composition could cause the variations in micellar properties that we have observed previously (*1*, *2*). We also found differences in dichlorofluorescein solubilization among these various saponin preparations (*1*).

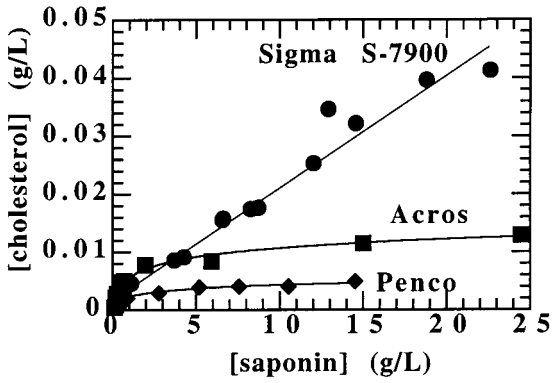

Figure 7. Cholesterol solubility in various sources of aqueous saponin solutions at 298 K.

Table 2. Partition Coefficient K of Cholesterol in Surfactant Micellar Solutions versus in Water at 298 K

surfactant	K
quillaja saponin (Sigma S-7900)	3.8×10^4
sodium deoxycholate (NaDC)	1.4×10^5
sodium cholate (NaC)	1.0×10^5
Triton X-100	3.6×10^4
Tween 20	5.2×10^3
quillaja saponin (Acros)	4.8×10^3
quillaja saponin (Penco)	2.9×10^3

Figure 7 shows cholesterol solubilization in aqueous saponin solutions from the manufacturers Sigma, Acros, and Penco. These results indicate a substantial difference among the abilities of the different sources to incorporate cholesterol, with saponin S-7900 from Sigma solubilizing considerably more cholesterol per weight of surfactant than the other two sources. S values on a mass basis for Acros and Penco are lower than that for Tween 20 and on a molar basis are comparable to Tween 20 (Table 1). Saponin S-7900 from Sigma, on the other hand, has a higher S value than any surfactant in Table 1 on a mole basis.

The differences in cholesterol solubilities in aqueous saponin solutions from Sigma S-7900, Acros, and Penco, shown in Figure 7, are consistent with differences found in dichlorofluorescein solubility in solutions of these various sources of saponin. In fact, the enhancement in solubilization capacity of Sigma S-7900 relative to Acros and Penco is quantitatively comparable for the two different solutes. This observation suggests that the differences among the sources can be traced to differences in the saponin micelles themselves rather than to specific solute/saponin interactions.

One possible difference between saponin S-7900 from Sigma and that from Acros and Penco is the presence of a highly surface active component present in the former (*1*). The presence of this surface active fraction was exhibited by a "dip" in surface tension versus the surfactant concentration curve. To determine whether this fraction played a key role in the superior solubilization properties of S-7900, we extracted this component from Sigma S-7900 saponin and measured the surface tension and cholesterol solubilization properties of the

Table 3. Critical Micelle Concentrations of Surfactants and Their Mixtures at 298 K

surfactant	surfactant: cmc of component (g/L):	saponin S-7900[a] cmc (g/L) 0.40 ± 0.04	saponin S-4521 cmc (g/L) 0.13 ± 0.01	NaC cmc (g/L) 2.98[b]	Triton X-100 cmc (g/L) 0.20−0.60[c]
NaC	2.98	0.3 (multiple breaks)	0.32 ± 0.05		not measured
Tween 20	0.04−0.06[d]	0.02 ± 0.006	0.012 ± 0.001	0.02 ± 0.005	~0.2

[a] Saponin S-7900 with minority surface active component extracted. [b] Asano et al. (*35*). [c] Ray and Némethy (*49*); Streletzky and Phillies (*50*). [d] Pal and Moulik (*40*); Courthaudon et al. (*51*); our measurements.

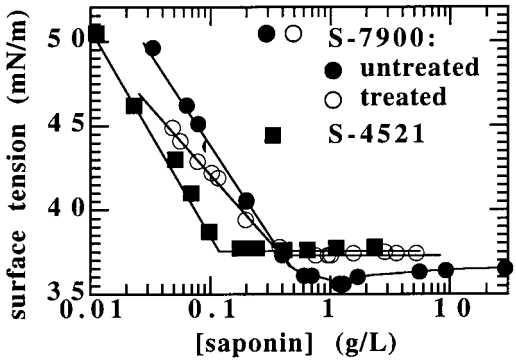

Figure 8. Surface tension of various preparations of Sigma saponin solutions at 298 K.

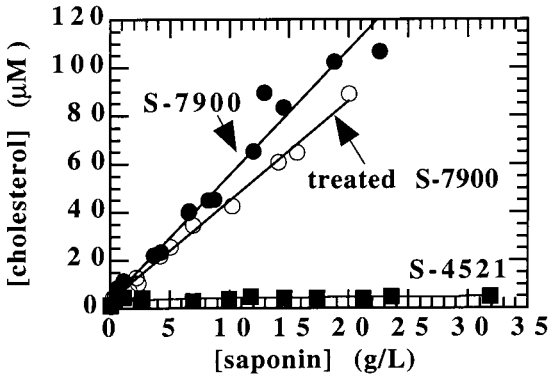

Figure 9. Cholesterol solubility in various preparations of aqueous Sigma saponin solutions at 298 K.

remaining surfactant material. The extraction method is described under Experimental Procedures. We compared this treated and untreated S-7900 saponin with a higher grade saponin (Sigma S-4521).

Figure 8 shows the surface tension of the treated and untreated saponin S-7900. The absence of any dip in the surface tension curve for the treated saponin indicates that the more surface active component(s) has (have) been removed. We found the cmc of the treated saponin to be lower than that of the untreated variety. This result suggests that the extracted component may be responsible for the lower cmc for S-7900 relative to other sources (*1*). We note that the cmc of S-4521 saponin is even lower than that of the S-7900 saponin we treated. This higher grade saponin may contain lower contents of some "impurities" that retard micellization.

Figure 9 shows cholesterol solubilities in the various saponin solutions from Sigma. Surprisingly, removing the more surface active fraction from S-7900 did not reduce substantially cholesterol solubility, whereas the higher grade S-4521 solubilized very little cholesterol. Until the differences in composition of these different saponin samples are known, it is difficult to speculate on the role of different components in the original saponin. However, the results shown here suggest that the mixed nature of quillaja saponin does play an important role in the ability of their micelles to extract hydrophobic solutes.

Effects of Mixed Surfactant Systems on Micellization and Cholesterol Solubilization. Mixtures of surfactants often exhibit synergy in that the combination of the surfactants forms micelles at concentrations lower than those formed by the separate surfactant components. As mentioned above, a mixture of surfactants often also solubilizes solutes in higher amounts than that achieved by the individual surfactants. The synergistic action of certain mixed surfactants is frequently utilized in industrial applications involving surfactants. The tendency of a surfactant mixture to act synergistically, antagonistically, or ideally is controlled by a complex interplay of various factors, including hydrophobic, interfacial, steric, and electrostatic interactions (*47*, *48*). For example, the addition of bile salts to saponin solutions allows saponin micelles to grow into rodlike micellar structures (*29*). It is believed that the bile salt surfactants, with their small polar groups, reduce steric hindrances present between glycose units in the saponin micelles, causing larger and more nonspherical micelles (*29*). Such a mixed micelle could potentially accommodate more cholesterol. In our studies, investigations of cholesterol solubilization in mixtures of saponin and NaC were also motivated by our desire to enhance our understanding of the ability of saponin to lower plasma cholesterol.

We investigated cholesterol solubility in binary solutions of saponin S-4521, Tween 20, NaC, treated saponin S-7900, and Triton X-100 at 298 K. Solutions were always prepared with a 50:50 mixture of two surfactants on a weight basis. We were interested in determining whether these binary combinations of surfactants would result in a lowering of cmc values and/or increased cholesterol solubilization. Figure 10 shows plots of the surface tension versus logarithm of total surfactant concentration of the mixed surfactants. The cholesterol solubilizing capacities of these mixed surfactants are shown in Figures 11−14.

The mixture of treated S-7900 and Tween 20 forms micelles beyond a total concentration of 0.02 ± 0.006 g/L (Figure 10a). This is a lower concentration than the cmc of Tween 20 alone and significantly lower than the cmc of the treated saponin S-7900 (Table 3). The combination of treated S-7900 and NaC shows the possible presence of micelles of various compositions manifested by multiple breaks in the surface tension curve (Figure 10a). The shift in the surface tension curve for the mixture of S-7900 and NaC compared to that of S-7900 and Tween 20 is consistent with the much lower cmc of Tween 20 alone compared to that of NaC (Table 3).

The surface tension curves of mixtures of saponin S-4521 with Tween 20 and NaC are shown in Figure 10b. The mixture of saponin S-4521 with Tween 20 forms micelles beyond a well-defined concentration of 0.012 ± 0.001 g/L, a concentration lower than that formed by the mixture of treated saponin S-7900/Tween

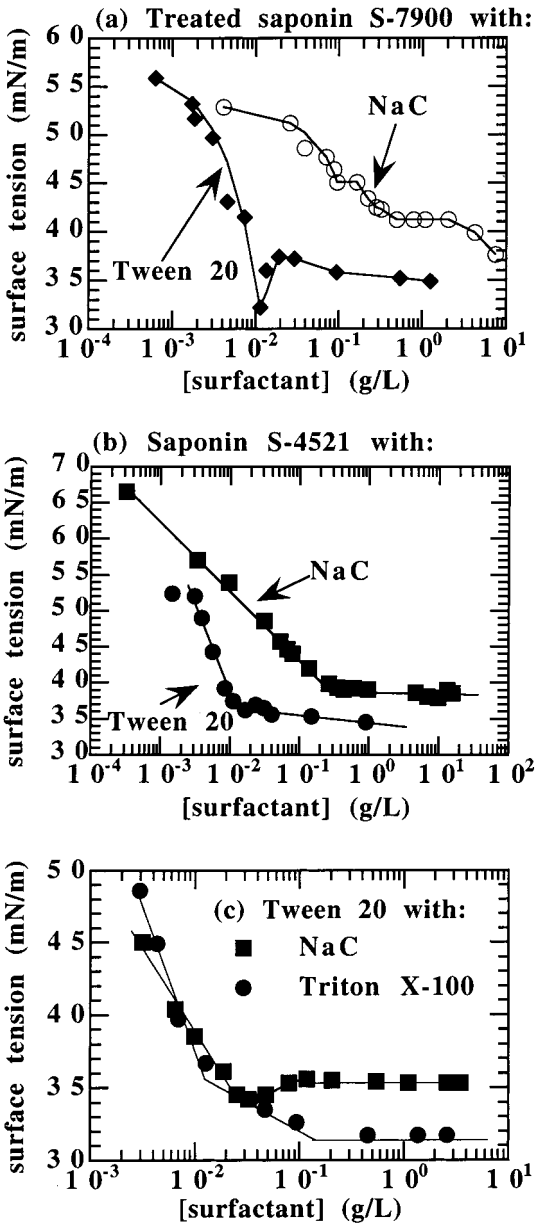

Figure 10. Surface tension at 298 K of 50:50 by weight binary surfactant mixtures of (a) treated saponin S-7900 with NaC or Tween 20, (b) saponin S-4521 with NaC or Tween 20, and (c) Tween 20 with NaC or Triton X-100.

20 (Table 3). This is consistent with the lower cmc of saponin S-4521 alone compared to that of the treated saponin S-7900. There is no evidence of a minimum in the surface tension curve, possibly due to the higher purity of saponin S-4521 grade. The mixture of saponin S-4521 and NaC forms micelles of a fixed composition beyond a distinct concentration of 0.32 ± 0.05 g/L—a concentration well below the cmc of NaC alone but higher than that of saponin S-4521 (Table 3).

When mixed with Tween 20, NaC forms micelles beyond a total concentration of 0.02 ± 0.005 g/L, which is considerably below the cmc of the solutions of NaC with either grade of saponin (Figure 10c). This is likely the consequence of the much lower cmc of Tween 20 alone compared to those of the two grades of saponin. The cmc of the Tween 20/NaC solutions is much lower than that of NaC alone but only slightly lower than that of Tween 20 (Table 3). Tween 20/Triton X-100 mixtures exhibited a gradual break in their surface tension versus

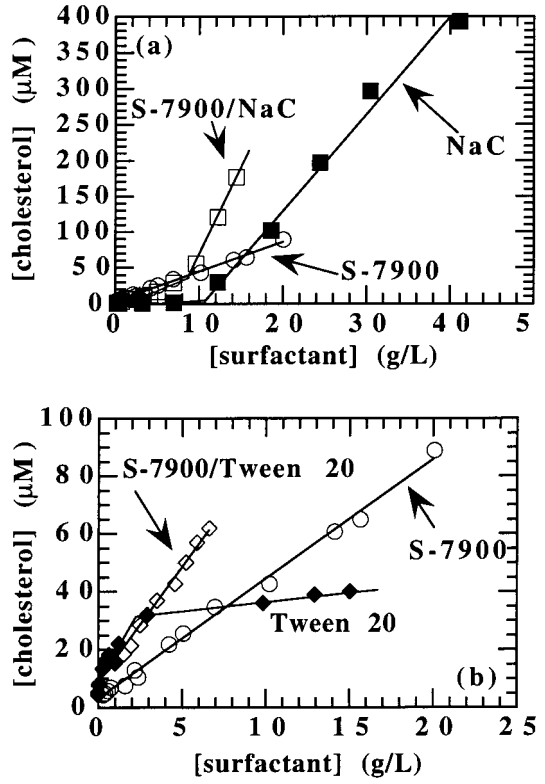

Figure 11. Cholesterol solubility at 298 K in 50:50 by weight binary mixtures of treated saponin S-7900 with (a) NaC or (b) Tween 20. Also shown is cholesterol solubility in solutions of the individual surfactants.

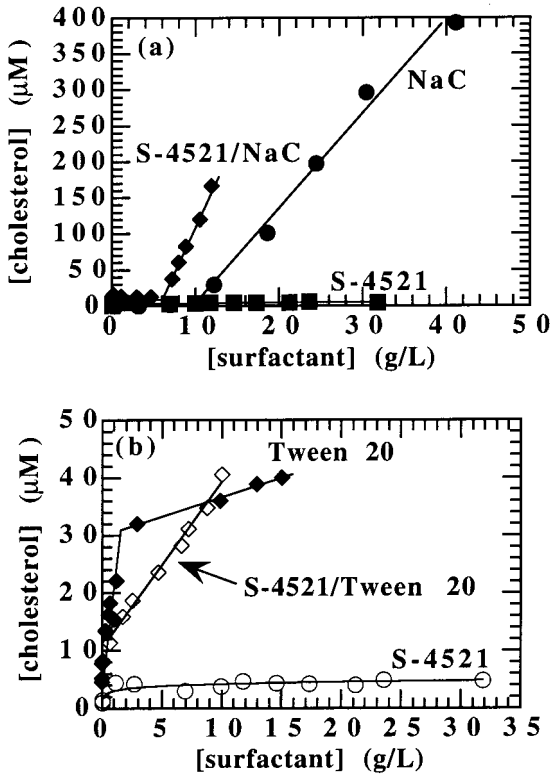

Figure 12. Cholesterol solubility at 298 K in 50:50 by weight binary mixtures of saponin S-4521 with (a) NaC or (b) Tween 20. Also shown is cholesterol solubility in solutions of the individual surfactants.

concentration curve (Figure 10c). The cmc can be estimated to be 0.2 g/L of total surfactant, which is

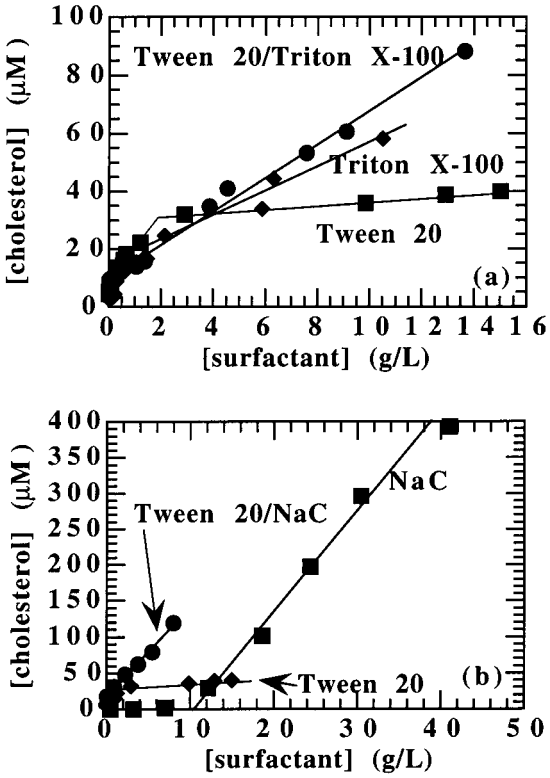

Figure 13. Cholesterol solubility at 298 K in 50:50 by weight binary mixtures of Tween 20 with (a) Triton X-100 or (b) NaC. Also shown is cholesterol solubility in solutions of the individual surfactants.

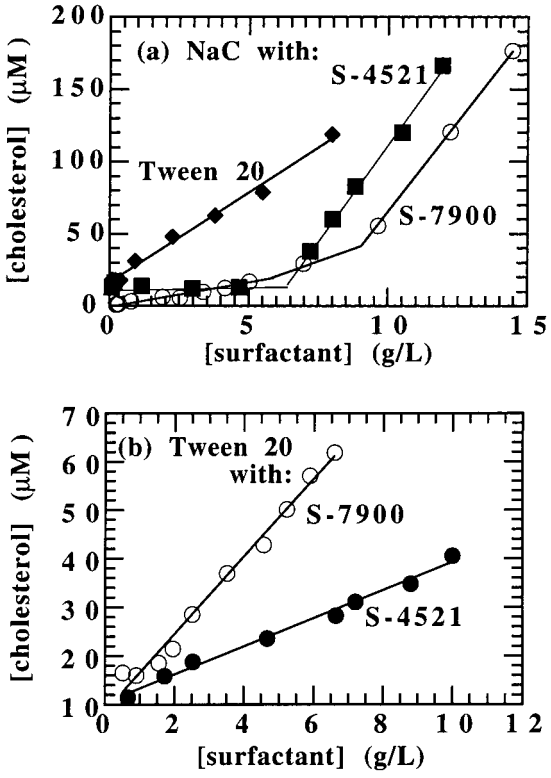

Figure 14. Cholesterol solubility at 298 K in 50:50 by weight binary mixtures of (a) NaC with treated saponin S-7900, saponin S-4521, or Tween 20; and (b) Tween 20 with treated saponin S-7900 or saponin S-4521.

significantly higher than that of Tween 20 alone and comparable to that of Triton X-100 alone. These results are presented in Table 3. There does not appear to be a reduction in the cmc of this mixture compared to that of the individual surfactants. This observation is consistent with the results of Puvvada and Blankschtein (*47*, *48*), which do not indicate synergistic effects with mixtures of hydrocarbon nonionic surfactants.

In Figure 11 is shown the cholesterol solubilizing capacities of binary surfactant mixtures with S-7900. From Figure 11a we see that the mixture of saponin S-7900/NaC has better solubilization capabilities than either surfactant alone. The cholesterol solubilization behavior shows a nonlinear dependence on surfactant concentration, which is likely to be related to varying micellar compositions—this supposition is supported by surface tension measurements. The results suggest that bile salt molecules do little to enhance the capacity of saponin molecules below the self-assembly concentration for the bile salt molecules and that the synergistic interaction is experienced only as the reduced cmc for NaC is approached. The mixture of saponin S-7900/Tween 20 also has better cholesterol solubilization properties compared to those of the individual surfactants, as shown in Figure 11b. This mixture underperforms the saponin S-7900/NaC mixture, reflecting the relative abilities of Tween 20 versus NaC to solubilize cholesterol.

Figure 12a shows cholesterol solubilization in binary solutions containing saponin S-4521 as one component. It is surprising to observe the tremendous enhancement in cholesterol solubilities in saponin S-4521/NaC solutions, given the inability of S-4521 solutions to solubilize cholesterol to any significant extent. As in Figure 11a, solubility in low concentrations of saponin/NaC is comparable to that in the saponin solution alone (in this case, an almost negligible solubility). As the total concentration in the mixture approaches the cmc for the bile salt, cholesterol solubility starts to increase sharply. Cholesterol solubilization in this mixture is higher than that observed in NaC solutions alone. Consistent with these results, the mixture of saponin S-4521/Tween 20 also outperformed Tween 20 alone in terms of its ability to solubilize cholesterol, as seen in Figure 12b. However, as with the treated saponin S-7900, mixtures of saponin S-4521/NaC have substantially greater cholesterol solubilization capabilities as compared to mixtures of saponin S-4521/Tween 20.

Cholesterol solubilization in binary mixtures of Tween 20/Triton X-100 and Tween 20/NaC is shown in panels a and b, respectively, of Figure 13. The mixtures of Tween 20/Triton X-100 enhance cholesterol solubilization, but the enhancement is not substantial compared to that of Triton X-100 alone. Tween 20 when mixed with NaC improves its ability to solubilize cholesterol, but again the combined effect is not much greater than that of NaC alone. Figure 13b indicates that the onset of enhanced solubilization occurs at a much lower concentration than that achieved by NaC alone, consistent with the lower cmc of the mixture. This observation is in contrast to behavior seen with the saponin solutions (Figures 11a and 12a).

We noted the dramatic difference in the abilities of the different grades of saponin S-7900 (treated and untreated) versus S-4521 to solubilize cholesterol. When mixed with NaC, both of these grades of saponin solubilize cholesterol to comparable extents, as shown in Figure 14a. Given the inability of saponin S-4521 to solubilize cholesterol significantly, it is possible that cholesterol solubilization in mixtures of S-4521/NaC is

driven by NaC. However, by comparing the cholesterol solubilization slopes above the cmcs of the saponin mixtures with that of a Tween 20/NaC mixture, we see that the mixtures of NaC/Tween 20 appear to be less effective in solubilizing cholesterol than NaC with either grade of saponin. Thus, quillaja saponins of various sources appear to have superior abilities to enhance cholesterol solubility, at least in combination with bile salts. On the other hand, when mixed with Tween 20, treated saponin S-7900 outperforms saponin S-4521, as seen in Figure 14b. Therefore, when mixed with nonionic surfactants the relative abilities of the two grades of saponin to solubilize cholesterol more closely reflect the capabilities of the saponins alone. Interestingly, we found that Tween 20 improves cholesterol solubilization when mixed with either grade of saponin over that of the individual surfactants. This is particularly striking when we consider the extremely low cholesterol solubilities in saponin S-4521 and in Tween 20 solutions, yet the mixture appears to boost cholesterol solubilization significantly.

Overall, our results indicate that cholesterol solubility is greatly enhanced in quillaja saponin solutions, rivaled only by bile salt solutions. In particular, bile salt/saponin mixed micelles were able to solubilize cholesterol more effectively than any other pure or mixed surfactant solution. This result may provide insight into the hypercholesteremic effect of quillaja saponins. Addition of nonionic surfactants to such mixtures is likely to have little effect on the in vivo solubilizing power of the mixed micelles but could promote micelle formation at a lower concentration (cf. Figure 13b). On the other hand, bile salts cannot be considered a viable commercial source for designing micellar extractions of cholesterol. Factors such as solution temperature, salt concentration, and pH can be manipulated to enhance or decrease cholesterol solubilization in these aqueous saponin solutions.

These results provide a guide for designing cholesterol extractions using aqueous saponin solutions. Our results may also provide useful information for extracting other sterols using quillaja saponins and may pertain to nonsteroidal solutes as well (*1*). Thus, knowledge obtained from this study could be utilized by the detergent, environmental, pharmaceutical, and food industries to develop extractions of a variety of hydrophobic compounds using aqueous saponin solutions or to formulate natural, food grade products containing such compounds.

ABBREVIATIONS USED

cmc, critical micelle concentration; NaC, sodium cholate; NaDC, sodium deoxycholate; *S*, solubilization ratio.

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