Cholesterol Solubilization by Short-Chain Lecithins: Characterization of Mixed Micelles and Cholesterol Oxidase Activity†

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ABSTRACT: The synthetic short-chain lecithins diheptanoylphosphatidylcholine and dioctanoylphosphatidylcholine solubilize cholesterol up to 10 and 18 mol %, respectively. The half-time for diheptanoylphosphatidylcholine solubilization of solid cholesterol is 80 (±30) min. This is much faster than Triton X-100 micelle or egg lecithin vesicle solubilization of solid cholesterol. Both the broadening of lecithin and [4-^{13}C]cholesterol carbon resonances by Mn^{2+} and the observation of surface dilution kinetics for phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) hydrolysis of the lecithins indicate that the cholesterol 3β-hydroxyl group resides at the particle surface exposed to solvent. Analysis of lecithin ^{13}C chemical shifts suggests that cholesterol causes the short-chain lecithin acyl chains to become slightly more trans, although to a lesser extent than it affects egg lecithin chains in liposomes. Lecithin motion as characterized by ^{13}C T_1s and line widths is unaffected by the incorporation of cholesterol. [3,4-$^{13}C_2$]Cholesterol line widths are 5–10-fold narrower in these mixed micelles than in egg lecithin sonicated vesicles, while T_1s in the two systems are comparable. These mixed micelles serve as substrates for cholesterol oxidase (*Nocardia erythropolis*) with a 40-fold rate increase over comparable cholesterol concentrations in egg lecithin vesicles. Part of this rate enhancement can be understood as an increase in interfacial area available to cholesterol oxidase in the micellar systems. These studies suggest that cholesterol oxidase has a weaker affinity for interfaces than other surface active enzymes.

Cholesterol is an important constituent of biomembranes and lipoproteins. Extensive studies of a variety of artificial and natural bilayer structures have led to a consistent picture of the physical effects of cholesterol on bilayers. Cholesterol broadens or disrupts the phospholipid phase transitions (Estep et al., 1978, 1979; Shimshick & McConnell, 1973; Jacobs & Oldfield, 1979; Lippert & Peticolas, 1971), affects phospholipid chain conformation (Mendelsohn, 1972; Godici & Landsberger, 1975; Stockton & Smith, 1976; Oldfield et al., 1978), decreases membrane permeability to a variety of ionic and polar substances (Blok et al., 1977; Gregoriadis & Davis, 1979; Sakanishi et al., 1979), and "condenses" the bilayer surface, causing a decrease in surface area per phospholipid molecule (Joos & Demel, 1969; Marsh & Smith, 1973; De Kruijff et al., 1973). In lipoproteins, free cholesterol is thought to be at the surface in a monolayer with lecithin and protein as well as in a hydrophobic core (Hamilton & Cordes, 1978; Hauser & Kostner, 1979). Interactions of cholesterol with these and other lipoprotein micellar components are not as well-defined as cholesterol bilayer effects. Furthermore, detailed interactions of cholesterol with enzymes involved in steroidogenesis and cholesterol metabolism [cholesterol oxidase, Gottlieb (1977); Moore et al., 1977; cholesterol esterase] can be difficult to discern because of complicated dependencies on lipid substrate aggregate behavior.

The ability of short-chain phosphatidylcholines to solubilize significant quantities of triglycerides (Burns & Roberts, 1981) and cholesterol can be exploited by using these mixed particles as models for lipoproteins and as well-defined matrices to study enzyme–lipid interactions. Mixed micelles are easily and reproducibly formed and yield optically clear solutions. Physical studies such as NMR lead to an extensive analysis of particle structure. The potential use of these particles in the study of cholesterol metabolizing enzymes is explored by an analysis of the activity of cholesterol oxidase against short-chain lecithin mixed micelles and egg lecithin sonicated vesicles.

Experimental Procedures

Materials. Cholesterol (Baker) was recrystallized 3 times from 1,2-dichloroethane, dried thoroughly under vacuum, and stored at −20 °C in benzene. Stock cholesterol concentrations were determined by a ferric chloride cholesterol assay (Courchaine et al., 1959) as modified elsewhere (Estep et al., 1978). Cholesterol concentrations in aqueous solutions were determined by the use of [4-^{14}C]cholesterol (Amersham). [4-^{13}C]Cholesterol (Merck Isotopes) and [3,4-$^{13}C_2$]cholesterol (Kor Isotopes) were used without further purification.

Diheptanoyl-PC[1] and dioctanoyl-PC were synthesized as described elsewhere (Burns & Roberts, 1981). Dihexanoyl-PC was obtained from Calbiochem and further purified by silicic acid chromatography (Burns & Roberts, 1981). Egg lecithin was obtained from Makor Chemicals and used without further purification. Lipid purities were monitored by thin-layer chromatography in $CHCl_3$–CH_3OH–10.5 M NH_4OH (60:35:8) and petroleum ether–diethyl ether–glacial acetic acid (70:30:1). Phospholipid concentrations were determined by phosphate analysis (Eaton & Dennis, 1976).

Solubilization of Crystalline Cholesterol. Aliquots of cholesterol (26 μmol) containing [4-^{14}C]cholesterol were evacuated for 22 h at 15 μm pressure. Under these conditions, cholesterol is in the anhydrous crystal form (Igimi & Carey, 1981). Solubilization of these crystals was examined by adding 1.5 mL of solution containing egg PC (ultracentrifuged sonicated vesicles), egg PC/cholesterol (ultracentrifuged son-

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[1] Abbreviations used: diacyl-PC, 1,2-diacyl-*sn*-glycero-3-phosphorylcholine; T_1, spin–lattice relaxation time; EDTA, ethylenediaminetetraacetic acid.

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Table I: Solubilization of Crystalline Cholesterol by Lecithin Aggregates

sample[a]	limiting cholesterol solubilized[b] (mM)	$t_{1/2}$[c] (min)	mole fraction of cholesterol
diheptanoyl-PC	1.4 (0.1)	90	0.074
diheptanoyl-PC	1.7 (0.2)	48	0.090
diheptanoyl-PC + 1 mM cholesterol	1.4 (0.1)	98	0.072
egg PC	0.1 (0.1)	*d*	*d*
egg PC + 8 mM cholesterol	0.03 (0.06)	*d*	*d*
buffer	0.05 (0.06)		

[a] Lecithin concentration was 18 mM in each sample. [b] Limiting cholesterol solubilized at 48 h. [c] Half-time for solubilization of limiting amount of cholesterol. [d] Not measurable on the time scale of 2 days.

ciated vesicles), diheptanoyl-PC micelles, diheptanoyl-PC/cholesterol mixed micelles, or buffer (concentrations are given in Table I) and mixing vigorously. After incubation for a given time, remaining crystals and supernatant were separated by a 10-min centrifugation at 3000*g*. The centrifuge (4 °C) was allowed to stop without the use of the brake; this process took approximately 15 min total. Aliquots were withdrawn for counting on a Packard Tri-Carb 300C. The samples were again vortexed and incubated at 25 °C until the next time point.

Enzymatic Assays. Phospholipase A_2 (*Naja naja naja*) and phospholipase C (*Bacillus cereus*) were purified and assayed by pH stat as described elsewhere (Burns & Roberts, 1981). Assay mixtures contained 10 mM short-chain lecithin and 0.8–1.8 mM cholesterol. Assay mixtures for phospholipase A_2 contained 5 mM $CaCl_2$. Lipid samples were cosolubilized from benzene directly in the assay vessels.

Cholesterol oxidase (*Nocardia erythropolis*) obtained from Boehringer Mannheim was assayed by coupling the production of H_2O_2 to the activity of horseradish peroxidase (Sigma) with guaiacol (Aldrich) as the hydrogen donor (Putter, 1974). Activity was monitored by the optical density at 436 nm. The assays contained 0.4–1.5 mM cholesterol, 2–7 mM egg PC, 5–17 mM short-chain lecithin, 0.3 mM guaiacol, 2×10^{-3} unit/mL cholesterol oxidase, and 400 units/mL peroxidase in 50 mM potassium phosphate–1 mM EDTA, pH 7.0, at 25 °C. Cholesterol oxidase activity was rate limiting as determined by variation of guaiacol and peroxidase concentrations.

Assay samples of cholesterol in egg lecithin sonicated vesicles were made by the method of Newman & Huang (1975) and sized on a 2.5 × 62 cm column of Sepharose 4B run in the ascending direction and/or ultracentrifuged at 100000*g* for 1 h. No difference in cholesterol oxidase activity was detected between these two preparations.

Short-chain lecithins and cholesterol were cosolubilized from benzene, dried under N_2, and evacuated at low pressure for at least 12 h. The appropriate aqueous solution was added with thorough mixing, often by low-power bath sonication for 10 s, and the samples were incubated for at least 1 h at room temperature.

Cholesterol oxidase activities as a function of time were measured by using the "auto zero" feature of the λ 3 spectrophotometer (Perkin-Elmer). Changes in optical density were measured across 0.01 OD full scale; the measured change in optical density per minute was centered in the time interval of the measurement. Base lines in the absence of cholesterol oxidase were stable to ±0.0003 OD units for 5–10 min.

NMR Spectroscopy. ^{13}C NMR spectra were obtained at 67.9 MHz on a Bruker 270 spectrometer equipped with a Nicolet 1080 data system. Buffers, measurements of spin–

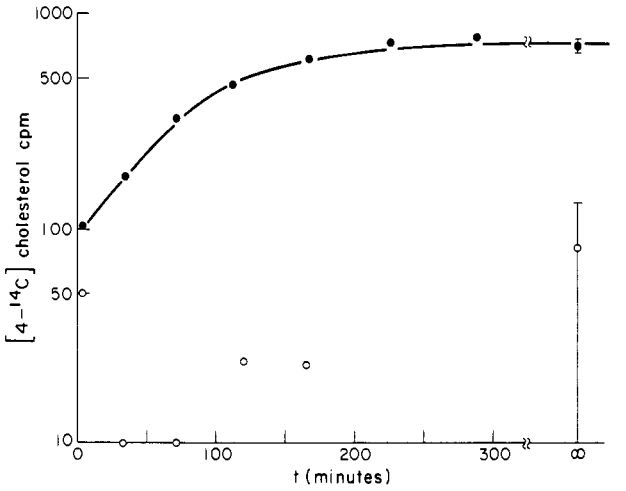

FIGURE 1: Solubilization of solid [4-^{14}C]cholesterol (as indicated by supernatant cpm) by 18 mM diheptanoyl-PC (●) and 18 mM egg lecithin sonicated vesicles (○). Typical errors in the solubilization data are indicated at the "infinity" time point.

lattice relaxation times, and chemical shift analyses have been described previously (Burns & Roberts, 1980). Samples contained 50–100 mM short-chain lecithin or 90 mM egg PC, and up to 16 mM cholesterol. Sonicated vesicles were prepared as described under Enzymatic Assays, with ultracentrifugation to remove multibilayers.

Lipid samples for Mn^{2+} line-broadening experiments were prepared and treated as described previously (Burns & Roberts, 1981). Briefly, this analysis monitors Mn^{2+}-induced line broadening of ^{13}C resonances by peak height changes in successive spectra taken under identical conditions. Selective Mn^{2+} broadening occurs at low metal ion concentrations; at higher Mn^{2+} concentrations, broadening of the internal D_2O lock signal causes all peaks to lose intensity at the same linear rate (15 ± 5% intensity loss/mM Mn^{2+} in these experiments). Extrapolation of these lines to zero Mn^{2+} concentration yields an intensity I_1, which is less than or equal to I_0 (the experimental intensity in the absence of Mn^{2+}). The fractional intensity change, ΔI, is defined as $\Delta I = (I_1 - I_0)/I_0$. ΔI reflects selective broadening due to Mn^{2+} proximity. ΔI equals zero if these is no specific broadening and becomes more negative (but less than −1) with increasing specific Mn^{2+} interaction.

Results

Mixed Particle Formation. Diheptanoyl-PC in the concentration range 10–100 mM will solubilize up to 0.10 mole fraction of cholesterol. Dioctanoyl-PC in the same concentration range will solubilize 0.18 mole fraction of cholesterol in the presence of 0.2 M KSCN. Potassium thiocyanate (or a variety of other "salting-in" salts) is required to form small, optically clear micelles of dioctanoyl-PC (Tausk et al., 1974a,b). In previous studies of the short-chain triglyceride/short-chain lecithin mixed micellar systems (Burns & Roberts, 1981), a 2-h incubation was required for the solutions to become optically clear. Short-chain lecithin/cholesterol mixed micellar solutions are optically clear immediately upon addition of water. Mixed micelles containing high mole fractions of cholesterol are stable for at least 2 days at room temperature, where stability is monitored by the absence of visible phase separated cholesterol in the solution. Mixed micelles with lower mole fraction of cholesterol are stable for greater than 2 weeks at room temperature.

Diheptanoyl-PC micelles are much more effective at solubilizing crystalline cholesterol than are egg PC sonicated vesicles, as Figure 1 demonstrates. Diheptanoyl-PC micelles

Table II: Fractional Intensity Changes of ^{13}C NMR Resonances Induced by Mn^{2+}

carbon atom	ΔI	ΔI_{AVG}
head group and backbone		
$N(CH_3)_3$	−0.23 (0.12)	−0.35 (0.17)
CH_2N	−0.20 (0.29)	
chol-CH_2OP	−0.60 (0.13)	
glyc-CH_2OP	−0.43 (0.15)	
CHO	[a]	
CH_2O	−0.27 (0.11)	
acyl chains		
1	−0.04 (0.16)	0.01 (0.05)
2	−0.03 (0.13)[b]	
3	−0.02 (0.16)[b]	
4	0.02 (0.12)[b]	
5	−0.03 (0.19)	
6	+0.13 (0.26)	
7	−0.02 (0.20)	
cholesterol C-4[c]	−0.30 (0.04)	−0.30 (0.04)
Pipes		
ring	0.07 (0.16)	
NCH_2	0.00 (0.13)	
CH_2SO_4	−0.15 (0.09)	

[a] Peak overlapped by C-3 of cholesterol. [b] Average value for *sn*-1 and *sn*-2 carbon atoms. [c] The C-3 in [3,4-$^{13}C_2$]cholesterol overlaps the lecithin CHO.

solubilize 0.08 (±0.01) mole fraction of cholesterol with a half-time $t_{1/2}$ = 80 (±30) min. These results are independent of the presence of cholesterol in the micelle, as shown in Table I. Egg PC sonicated vesicles with or without cholesterol do not show higher cholesterol solubilization values than buffer alone after 48 h of equilibration. Diheptanoyl-PC micelles reach saturation in several hours. These rates appear to be comparable to those observed with several bile salts (Igimi & Carey, 1981).

Particle Structure. Two lines of evidence support the positioning of the cholesterol 3-β-hydroxyl group at the particle surface: Mn^{2+}-induced line broadening of lecithin and cholesterol ^{13}C NMR resonances and the kinetics of phospholipases A_2 and C hydrolysis of these micelles. Table II shows the Mn^{2+}-induced fractional ^{13}C NMR intensity changes, ΔI, as defined under Experimental Procedures. Also included in this table are average values of this parameter for different parts of the molecules. The largest Mn^{2+} effects are found for head-group and backbone resonances, specifically those carbon atoms surrounding the phosphate moiety. Acyl chain ΔI values are very low and do not vary significantly up and down the chain, as was found for short-chain lecithin/triglyceride micelles (Burns & Roberts, 1981). However, the acyl chain ΔI_{AVG} values are different in these two systems. This may indicate some cholesterol enhanced protection of the acyl chains from solvent exposure. The cholesterol peak is strongly broadened by Mn^{2+}; in contrast, trihexanoin carbons show virtually no specific Mn^{2+} effect (ΔI_{AVG} = −0.05 (±0.10) for eight peaks). The cholesterol ΔI value is comparable to those found for short-chain lecithin backbone and head-group resonances. Thus, cholesterol C-4 appears to be exposed to solvent.

Additional evidence for the presence of cholesterol at the particle surface comes from phosholipase studies. Table III shows the observed specific activities of phospholipase A_2 and phospholipase C against pure short-chain lecithin micelles and mixed micelles with cholesterol. The observed "surface dilution" (Dennis, 1973) and mole fraction of cholesterol in the particles are also indicated. Observed phospholipase A_2 and phospholipase C activities are proportional to the surface concentration of the substrate in the Triton X-100/lecithin

Table III: Specific Activities of Phospholipase A_2 and Phospholipase C toward Short-Chain Lecithin Micelles Containing Cholesterol

	sp act. (μmol min^{-1} mg^{-1})	ratio[a] +CHOL/−CHOL	mole fraction of lecithin
	Phospholipase C		
DiC_7PC[b]	1800 (200)	0.9 (0.1)	0.93
DiC_7PC + 0.8 mM CHOL	1600 (100)		
DiC_8PC	1700 (100)	0.9 (0.1)	0.85
DiC_8PC + 1.8 mM CHOL	1600 (100)		
DiC_8PC (0.2 M KSCN)	240 (10)	0.8 (0.1)	0.85
DiC_8PC (0.2 M KSCN) + 1.8 mM CHOL	200 (20)		
	Phospholipase A_2		
DiC_7PC	1700 (100)	1.0 (0.1)	0.93
DiC_7PC + 0.8 mM CHOL	1700 (100)		
DiC_8PC	1500 (100)	0.9 (0.1)	0.85
DiC_8PC + 1.8 mM CHOL	1400 (100)		
DiC_8PC (0.2 M KSCN)	2300 (300)	0.9 (0.2)	0.85
DiC_8PC (0.2 M KSCN) + 1.8 mM CHOL	2000 (100)		

[a] The ratio of specific activity in the presence of cholesterol compared to pure lecithin micelles. [b] All lecithin concentrations are 10 mM.

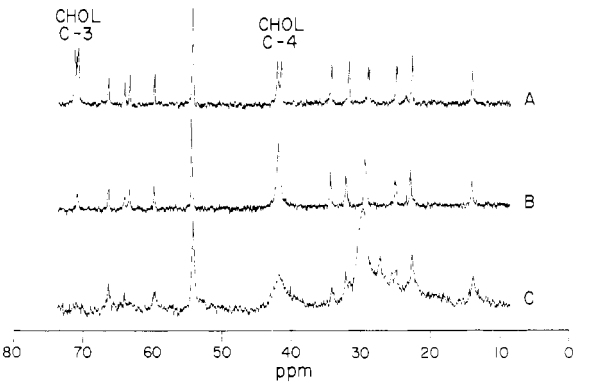

FIGURE 2: ^{13}C NMR spectra at 67.9 MHz (excluding the carbonyl region) of (A) diheptanoyl-PC (60 mM)/[3,4-^{13}C]cholesterol (4.5 mM) mixed micelles, (B) dioctanoyl-PC (60 mM)/[4-^{13}C]cholesterol (9.0 mM) mixed micelles, and (C) egg lecithin (90 mM)/[4-^{13}C]-cholesterol (16 mM) sonicated vesicles. The resonance around 24 ppm is [2-^{13}C]acetate, the internal reference standard.

mixed micelle system (Deems et al., 1975). In that assay system, the Triton molecules are treated as neutral surface diluters. Similar behavior might be expected for diluting short-chain lecithins with cholesterol. Thus, for a given total concentration of lecithin, addition of detergents or other surface dilutants will decrease observed activities. The phospholipase specific activities decrease with increasing cholesterol concentration, although the changes are small and so subject to large uncertainties. In contrast to these findings, phosholipase A_2 and C activities (Burns & Roberts, 1981) against short-chain lecithin/triglyceride mixed micelles demonstrated no change in activity with up to 0.5 mole fraction of triglyceride in the particles.

^{13}C NMR Studies. A representative ^{13}C NMR spectrum of [3,4-$^{13}C_2$]cholesterol in mixed micelles with diheptanoyl-PC is shown in Figure 2A. In the diheptanoyl-PC mixed micelles, $J_{^{13}C-^{13}C}$ of 34 Hz is clearly observable, and the cholesterol line width is 10 Hz. For [4-^{13}C]cholesterol solubilized in dioctanoyl-PC (Figure 2B), an intermediate line width is observed

Table IV: [4-^{13}C]Cholesterol T_1 and T_2* Values in Different Lecithin Aggregates

lecithin matrix	T_1 (s)	T_2* (s)	T_1/T_2*
diheptanoyl-PC	0.09 (0.02)	0.033 (0.009)	3 (1)
dioctanoyl-PC	0.12 (0.01)	0.013 (0.002)	9 (2)
egg PC sonicated vesicle	0.09 (0.03)	0.0026 (0.0003)	40 (20)

(40 Hz). For [4-^{13}C]cholesterol in egg lecithin sonicated vesicles (Figure 2C), a line width of 140 Hz is seen. The cholesterol line widths are independent of the mole fraction of cholesterol in the mixed micelles (data not shown). Short-chain lecithin T_1 and T_2* values are not affected by the presence of cholesterol. Table IV summarizes T_1 and T_2* values for [3,4-$^{13}C_2$]cholesterol in these lecithin aggregates. T_1 appears to be independent of the aggregate system; T_2* decreases with increasing aggregate size. Similar values of T_1 and T_2* for cholesterol in vesicles have been found by Yeagle (1981).

If the lecithin ^{13}C chemical shifts are examined as a function of cholesterol concentration, a number of trends appear. Acyl chain carbon atoms show consistently positive (downfield) shift changes with increasing percent of cholesterol in the micelle. Head-group and backbone carbon atoms show smaller and less consistent trends. Figure 3 illustrates the limiting cholesterol-induced shifts for the acyl chains of short-chain lecithins. Chemical shifts changes are smaller for carbon atoms near the ester carbonyl. Larger changes are seen in the middle and at the terminal methyl end of the acyl chain. The changes in chemical shifts are shown for saturating amounts of cholesterol in the lecithin particles. They were derived from linear least-square fits of the slope for ^{13}C chemical shifts vs. mole percent of cholesterol in the particle. Although small, these shifts are significant: for alkyl chain carbons in diheptanoyl-PC, the average shift induced by 8% cholesterol is 3.4 (2.0) Hz; for comparable carbons in dioctanoyl-PC, the average chain shift induced by 15% cholesterol is 5.6 (2.7) Hz. Previous studies of the monomer/micelle transition of dihexanoyl-PC (Burns et al., 1981) and a variety of other studies (Cheney & Grant, 1967) support the interpretation of aliphatic chain ^{13}C chemical shifts in terms of trans/gauche conformational changes in the chain. The chemical shift difference between fully trans and fully gauche conformers has been estimated as 5 ppm (Tonelli et al., 1979; Tonelli, 1979; Cheney & Grant, 1967; Spiescke & Schneider, 1961). Application of this interpretation to the average shift changes for diheptanoyl-PC and dioctanoyl-PC indicates 1.0 (±0.6)% and 1.6 (±0.8)% change in the average P_t (the probability of a trans conformer) between acyl chains in pure lecithin micelles and

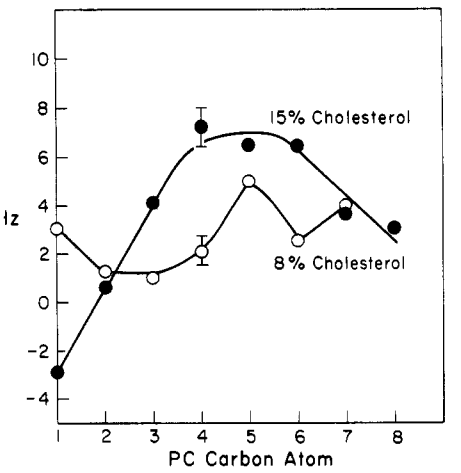

FIGURE 3: Cholesterol-induced ^{13}C chemical shifts of the alkyl chain carbons of short-chain lecithins: (○) diheptanoyl-PC with 8% cholesterol and (●) dioctanoyl-PC with 15% cholesterol. Typical errors are indicated by bars.

in micelles with saturating cholesterol concentrations. For comparison, a previous study (Burns et al., 1981) gave an approximately 6% change in P_t between monomeric and micellar dihexanoyl-PC. The cholesterol-induced changes in P_t, while small, are significant (it should be noted that few techniques could detect such a small change).

Oxidation of Cholesterol by Cholesterol Oxidase. Table V shows the observed activities of cholesterol oxidase from *N. erythropolis* toward egg lecithin sonicated vesicles and short-chain PC micelles containing cholesterol. Specific activities are approximately 25-fold higher in micelles than in sonicated vesicles. At the cholesterol concentrations used in these assays, the rate is only weakly dependent on particle composition (0.1 and 0.3 mole fraction of cholesterol in vesicles) and on the amount of total cholesterol at fixed mole fraction in the particle. The addition of 5 mM dihexanoyl-PC [approximately one-third of the critical micellar concentration (Tausk et al., 1974a)] causes a 30% increase in rate against 30% cholesterol vesicles.

Figure 4 shows the time course of cholesterol oxidase kinetics against egg PC vesicles, diheptanoyl-PC, and Triton X-100 micelles containing cholesterol. The data can be decomposed into a fixed rate and a first-order enzyme inactivation rate. Values of $t_{1/2}$ (enzyme inactivation) are 2, 20, and 50 min for vesicles, diheptanoyl-PC, and Triton X-100 micelles, respectively. These activity changes do not represent O_2 depletion in the assay or complications with the enzyme couple used. Micellar samples, which show the slowest inactivation, have

Table V: Cholesterol Oxidase Activity toward Cholesterol in Short-Chain Lecithin Micelles, Triton X-100 Micelles, and in Egg Lecithin Sonicated Vesicles

lecithin matrix	mole fraction of cholesterol	cholesterol (mM)	sp act. (units/mg)	surface sp act. [(units/mg)/unit area × 10^6]
diheptanoyl-PC	0.08	1.25	8.2 (0.6)	120
	0.00	0.00	<0.03	
dioctanoyl-PC[a]	0.15	1.25	6.9 (0.6)	203
	0.15	0.40	6.9 (0.6)	406
	0.00	0.00	<0.03	
Triton X-100	0.5	1.25	6.3 (0.6)	51
	0.00	0.00	<0.03	
egg PC	0.3	1.25	0.32 (0.02)	40
	0.3	0.63	0.25 (0.02)	63
	0.1	1.25	0.28 (0.02)	8
+5 mM dihexanoyl-PC	0.3	1.25	0.46 (0.05)	21[b]

[a] KSCN (0.2 M) is present in these samples. [b] For the calculation of surface area, it is assumed that most of the short-chain lecithin is forming a mixed micellar/mixed vesicle system with the egg PC-cholesterol.

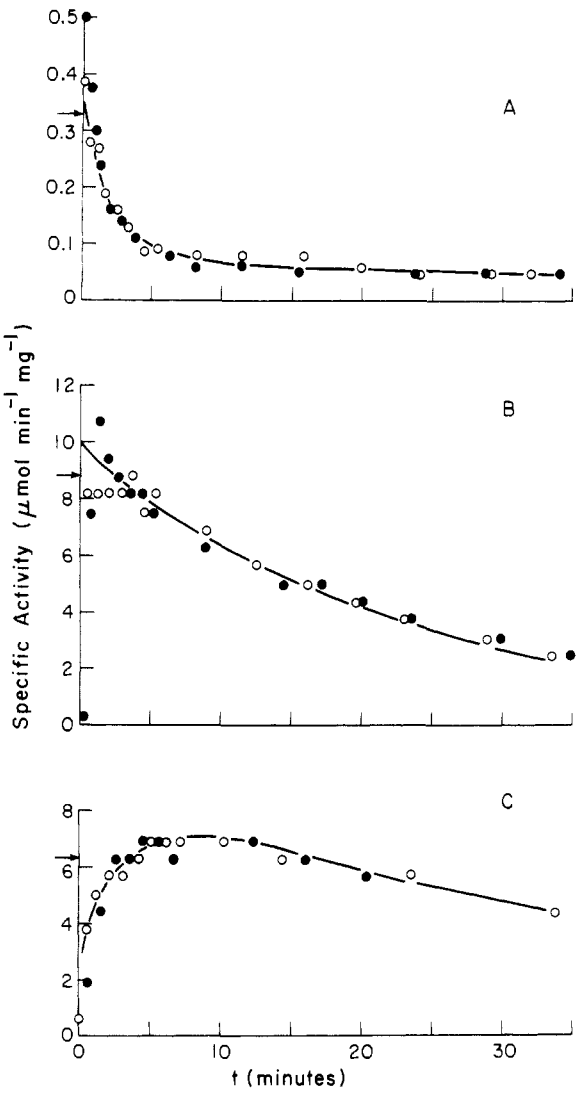

FIGURE 4: Specific activity (μmol min^{-1} mg^{-1}) of cholesterol oxidase as a function of time toward 1.25 mM cholesterol in (A) 17 mM diheptanoyl-PC, (B) 21 mM Triton X-100, and (C) 4.2 mM egg lecithin sonicated vesicles. The arrows indicate the extrapolated specific activities at time zero. The solid and open points are duplicate runs.

consumed twice as much total cholesterol (and therefore O_2) as the vesicle sample in the assays at 20 min. Incubation of the assay system with vesicles or micelles does not seemingly affect the enzyme couple. Addition of an aliquot of H_2O_2 comparable to the total product consumed in the first 5 min of the assay is consumed immediately (only the total OD change can be monitored). This activity profile does not represent trivial substrate depletion; integration of Figure 4A yields a substrate consumption of 0.2% of the available cholesterol in the first 5 min of the assay. The observed rate decrease in these assays is most simply understood as enzyme inactivation by some component of the assay, either products produced by the enzyme couple or the lipid interfaces themselves. This inactivation proceeds fastest with egg lecithin vesicles and slowest with Triton X-100 mixed micelles. This supports surface-generated enzyme denaturation or differential protection from inactivation (for example, by H_2O_2) by each system. In addition, a lag phase is observed for micellar assay samples.

The difference in specific activity between vesicles and micelles can be at least partially understood if the specific activity per centimeter squared of interfacial surface per milliliter of solution is calculated. This "surface specific activity" is also shown in Table V. The surface areas were calculated by using 60 Å^2/lecithin molecule [short-chain PC, Tausk et al. (1974a,b), or egg PC, De Bernard (1958)], 36 Å^2/cholesterol molecule (Joos & Demel, 1969), and 80 Å/Triton X-100 molecule (Deems et al., 1975). All micellar surface area was assumed to be available to the enzyme. For vesicles, 0.5–0.7 of the total surface area was used, since the inner surface of the vesicle is not exposed for enzyme interaction. "Surface specific activities" for vesicles and micelles are significantly more similar than the bulk specific activities. This suggests that at least part of the increase in activity observed for micellar assays may be due to the higher available surface area in micellar samples. This also explains the rate increase observed for egg PC vesicles plus 5 mM dihexanoyl-PC. If some of the dihexanoyl-PC is being incorporated into the vesicles, the available surface area would increase, thereby increasing the rate.

Discussion

Solubilization of Cholesterol in Micelles. Studies of the amount of cholesterol solubilized by detergent systems have focused on bile salt/lecithin systems. These micellar systems serve to solubilize various hydrophobic fats and lipids in the small intestine. Bile salt/lecithin systems solubilize up to 7 mol % cholesterol; bile salts alone solubilize approximately 4% cholesterol (Bourges et al., 1967; Tamesue et al., 1973). Bile salt/monoolein/oleate systems (Montet et al., 1979) solubilize 2–7 mol % cholesterol. Other detergents have been tried; 23 mol % cholesterol can be solubilized in sodium dodecyl sulfate micelles (Schmidt et al., 1978) with stabilities around 12 h; 6 mol % cholesterol in Triton X-100 is stable for several weeks (Robson, 1979). In the present study, diheptanoyl-PC was found to solubilize approximately 10 mol % cholesterol if both were cosolubilized in organic solvent, dried, and hydrated. At the highest mole fractions of cholesterol, the samples are not stable indefinitely. Micelles containing somewhat lower mole fractions of cholesterol (6–7%) appear to be extremely stable. The results from studies of solid cholesterol solubilization suggest 8 (±1) mol % cholesterol as the appropriate saturating mole fraction of cholesterol in diheptanoyl-PC micelles. Thus, in a variety of optimized detergent systems at similar total lipid concentrations, approximately 7 mol % cholesterol can be solubilized, implying that the extent of cholesterol solubilized by detergents is not very sensitive to the structure of the detergent, although the rate of solubilization may be very detergent dependent. The rapid rate of solid cholesterol micellization by diheptanoyl-PC compared to Triton X-100 (Robson, 1979) or egg PC vesicles is particularly intriguing and might have clinical value. The common equilibrium value of cholesterol solubilized suggests that solubilization is dominated by simple hydrophobicity. However, bile salts alone only solubilize 4 mol % cholesterol. The most efficient solubilizations apparently require at least some detergent molecules with flexible chains. We have excluded solubilization of cholesterol (up to 18 mol %) by dioctanoyl-PC from this series because of the presence of 0.2 M KSCN, which can be expected to affect solubilization.

Short-Chain Lecithin/Cholesterol Micellar Particles and Comparisons with Bilayers. The short-chain lecithins offer the advantage over other detergent systems of solubilizing cholesterol in an environment which closely simulates interactions in a variety of biological structures. Both Mn^{2+}-induced ^{13}C NMR line intensity changes and phospholipase activities support the conclusion that the cholesterol 3-β-hydroxyl is present at the micellar interface. The ΔI value found in Mn^{2+} titrations is comparable to lecithin head-group and backbone values. Phospholipase A_2 and phospholipase

C kinetics are consistent with the concept of surface dilution (Dennis, 1973) by cholesterol. Neutron and X-ray diffraction studies of liposomal lecithin/cholesterol systems (Franks, 1976; Worcester & Franks, 1976) place the cholesterol hydroxyl at the level of the lecithin ester bonds. In studies of sonicated PC/cholesterol vesicles (De Kruijff, 1978), lanthanide titrations located the [4-^{13}C]cholesterol methylene atom also at the level of the phosholipid ester bond. Therefore, the cholesterol hydroxyl is positioned near the surface in both bilayers and short-chain lecithin micelles. In studies of short-chain lecithin/short-chain triglyceride mixed micelles (Burns & Roberts, 1981), both the Mn^{2+} experiments and phospholipase activities indicate that the triglycerides are predominantly in a core which is surrounded by short-chain lecithin molecules. In these two systems, the triglycerides and cholesterol are found in locations in the lecithin micelle which correspond to the proposed locations of triglycerides and cholesterol in lipoproteins (Hauser & Kostner, 1979; Hamilton & Cordes, 1978). This would suggest that short-chain PC micelles are useful model systems for lipoproteins. Further studies of lecithin/cholesteryl ester, ternary, and quaternary mixed micelles are in progress.

Cholesterol is solubilized in short-chain lecithin micelles with virtually no perturbation of the lecithin molecule as judged by chemical shifts and ^{13}C relaxation times. The lack of cholesterol perturbation of short-chain lecithin molecules is consistent with the absence of a cholesterol condensing effect in monolayer studies for lecithins with acyl chains less than 10 carbon atoms long (Joos & Demel, 1969). The effect on lecithin chemical shifts suggests that the acyl chains become approximately 1% more trans in mixed micelles with saturating concentrations of cholesterol. Lecithin T_1s are similarly unaffected by the presence of short-chain triglycerides in the micelle (Burns & Roberts, 1981). Lecithin T_1 values distinguish between monomeric and micellized lipid (Burns & Roberts, 1980) but indicate that bond isomerizations and other fast motions occur at rates in micellar structures which are not sensitive to particle composition. Recent ^{13}C NMR and Raman studies of short-chain lecithin micelles indicate that the lecithin acyl chains are extremely fluid (Burns et al., 1981). Molecular dynamic calculations (Levy et al., 1979) demonstrate that chain molecule ^{13}C relaxation parameters can be reproduced by using a solvent potential function which extends only 5–10 Å away from the atomic center under study. These calculations attempted on short-chain lecithins would be useful in understanding the difference between monomeric and micellar phospholipid.

In vesicles, ^{13}C line widths but not T_1 values change in the presence of cholesterol (Godici & Landsberger, 1975), and ^{2}H NMR order parameters increase at all chain positions (Stockton et al., 1976; Rice et al., 1979). The increase in order parameter is monotonic with increasing cholesterol (Stockton & Smith, 1976). For egg lecithin/cholesterol, these results were studied by using the acyl chain conformational model of Marcelja (1974) and are consistent with an 11% change in P_t between 0% and 33% cholesterol. If the monotonic increase in order parameter implies a monotonic increase in P_t, a 3% or 4% increase in P_t occurs in bilayers when mole fractions of cholesterol comparable to micellar systems are incorporated. The 1–2% increase in P_t measured for micellar systems is a smaller change.

Surface Behavior of Cholesterol Oxidase. Cholesterol oxidase from *N. erythropolis* exhibits an approximately 25-fold higher activity for cholesterol in micelles than in sonicated vesicles. This difference in observed specific activities for various assay systems is diminished if the specific activity per centimeter squared of interface per milliliter, i.e., the "surface specific activity", is used (see Table V). At least part of the activity increase observed for micellar samples can be accounted for by higher available surface areas in these samples. The Triton X-100 activation of cholesterol oxidase observed by De Martinez & Green (1979) is also consistent with this model. Cholesterol oxidase is 12 times as active against TX-100/cholesterol micelles than against egg PC sonicated vesicles; the "surface specific activity" for vesicles is 4-fold higher than that for the Triton system. The activities observed for a variety of phospholipid vesicle systems give simliar surface specific activities even if a constant surface area/lipid is used for different phospholipids. The relationship between enzyme activity and interfacial surface area suggests that the amount of enzyme *bound* is changing as a function of available surface area. This effect is a binding, and not a kinetic, phenomenon. The true specific activity and V_{max} would be found by extrapolating the observed activity to inifinite surface area. In kinetic analyses of phospholipases or lipases, the affinity of the enzyme for the interface is strong, and so the rate is determined by other factors (such as "surface dilution", Deems et al., 1975) and not the total available surface area. Since the cholesterol oxidase assays were performed at total lipid concentrations where phospholipase rates are independent of surface area, the conclusion is that cholesterol oxidase has a considerably weaker affinity for interfaces.

Cholesterol oxidase has been used to study cholesterol "flip-flop" rates in several bilayer systems (Gottlieb, 1977; Patzer et al., 1978). The strategy in these studies is to demonstrate the ability to oxidize 100% of the assay cholesterol in a given length of time. If the system is a "sealed" bilayer, then the ability to oxidize 100% of the cholesterol suggests that cholesterol "flip-flop" rates must be faster than the oxidation rate. Backer & Davidowicz (1981) have recently demonstrated oxidation of greater than 90% of the cholesterol in 40% cholesterol/egg lecithin vesicles with a half-time of 1 min. Gottlieb (1977) has demonstrated that the half-time of oxidation is dependent on the enzyme/substrate ratio, the higher this ratio, the faster the half-time of oxidation. This observation is consistent with a model where cholesterol oxidase has only weak affinity for interfaces. In order to increase the amount of enzyme–interface complex formed, one could either increase the amount of enzyme in solution or increase the available surface area. In order to increase the surface area, the mass of cholesterol to be oxidized would also increase. In order to oxidize 100% of the assay cholesterol in the fastest amount of time, one would therefore want to (a) use the smallest amount of cholesterol which could be accurately measured and (b) use the highest possible concentration of enzyme. Therefore, according to this model, a high enzyme/substrate ratio would give the fastest percent cholesterol oxidation.

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